Extension AP05002: Alternaria Fruit Spot: New Directions

Nick Macleod
The Department of Agriculture, Fisheries and Forestry, Qld

Project Number: AP06007

AP06007

This report is published by Horticulture Australia Ltd to pass on information concerning horticultural research and development undertaken for the apple and pear industry.

The research contained in this report was funded by Horticulture Australia Ltd with the financial support of the apple and pear industry.

All expressions of opinion are not to be regarded as expressing the opinion of Horticulture Australia Ltd or any authority of the Australian Government.

The Company and the Australian Government accept no responsibility for any of the opinions or the accuracy of the information contained in this report and readers should rely upon their own enquiries in making decisions concerning their own interests.

ISBN 0 7341 3269 7

Published and distributed by:
Horticulture Australia Ltd
Level 7
179 Elizabeth Street
Sydney NSW 2000
Telephone: (02) 8295 2300
Fax: (02) 8295 2399

Horticulture Australia

AP06007- Extension AP05002

Alternaria Fruit Spot: New Directions

Final Report

Dr. Femi Akinsanmi

Assoc. Prof. André Drenth

Mr. Nick Macleod (Project Leader)

December 2013

HAL Horticulture Australia

Apple & Pear Australia Ltd.

Queensland Government

NSW Government Department of Primary Industries

The University Of Queensland Australia | QAAFI Queensland Alliance for Agriculture and Food Innovation

Project Details

Project Title: **Extension AP05002: Alternaria Fruit Spot: New Directions**

HAL Project Number: AP06007

Project Leader(s): Department of Agriculture, Fisheries and Forestry, Qld. (DAFF)

- Mr. Nick Macleod (2011- 2013)
- Mr. Allan McWaters (2010 -2 011)
- Dr. Shane Dullahide (2008 - 2010)
- Ms Christine Horlock (2006 - 2008)

Applethorpe Research Station
PO Box 501, Stanthorpe, Qld 4380
Phone: 07 46816128; Fax: 07 46811769;
E-mail: nick.macleod@daff.qld.gov.au

Key Personnel: DAFF Queensland

- Mr. Allan McWaters (Experimentalist)
- Mr. Clinton McGrath (Extension officer)
- Dr. Roger Shivas (Herbarium curator and Mycologist)
- Ms. Lynette Haselgrove (Technical staff- 2006-2010)

The University of Queensland, QAAFI, Brisbane.

- Assoc. Prof. André Drenth (Team leader & PhD advisor)
- Dr. Femi Akinsanmi (Team member & PhD advisor)
- Ms Dalphy Harteveld (PhD student)

Ecosciences Precinct 2C West,
GPO Box 267, Brisbane, Qld 4001.
Phone: 07 3255 4391; Fax: 07 3846 2387
E-mail: a.drenth@uq.edu.au

Funding sources: This project has been funded by Horticulture Australia Limited (HAL) using the Australian apple and pear industry levy and matched funds from the Australian Government

Date of Report: 23 December, 2013

Statement of purpose of the project:
This project determined the identity of causal pathogens, epidemiology and disease cycle of Alternaria leaf blotch and fruit spot in Australian apples and provided a management strategy for both diseases for inclusion in the integrated fruit production manual.

Project start date: 01/09/2006 **Project end date:** 31/12/2013

Media Summary

This project was established to provide a better understanding of two diseases of apple caused by similar pathogens in Australia. Our research has resolved the identity of the species of *Alternaria* pathogen(s) that cause leaf blotch and fruit spot of apples in Australia. We have identified the risk of more widespread impact of fruit spot in all apple producing regions of Australia. The potential exists for these diseases to become a significant contributor to production losses in the Australian apple industry.

Outcomes from field trials performed on commercial orchards have provided a better understanding of the lifecycle of the pathogen, the sources of inoculum and timing of infection for both diseases. This information has enabled the development of a disease management strategy for the apple industry. When implemented, it is estimated to save the Queensland apple industry over $2.25 million in lost production annually. In the short term it will save the Australian apple industry between 15-25% in lost production of the high value varieties. The long-term benefit of the research outcomes will lead to reduction in reliance on chemicals for controlling the diseases. These findings of our research have been published in scientific journals and apple industry magazines and communicated to growers at several meetings.

This project highlights the importance of securing long-term plant pathology capability for all endemic apple diseases, screening for disease resistance, managing endemic diseases in a cost effective manner as well as preparedness for possible incursion of exotic pathogens in the Australia apple industry, to safeguard production and local markets. A PhD student was successfully trained in this project.

This project has identified future research priorities for diseases caused by *Alternaria* in apple including associated risks for fruit quality and food safety from contamination of fruit infected with *Alternaria* as fruit spot or mouldy core rot. It has highlighted the need to monitor development of fungicide resistance in the apple industry. Essential research priorities for diseases caused by *Alternaria*, other endemic diseases and the need to maintain local expertise for these diseases both endemic and exotic diseases of apple in Australia are emphasized in this report.

Technical Summary

Alternaria leaf blotch and fruit spot diseases cause significant fruit losses in Qld and NSW apple production areas. Occasional, but noteworthy, severe leaf losses have been observed in Western Australia and South Australia. These problems have been ongoing for several seasons, and while fungicide applications have lessened effects in some areas, a lack of understanding of the identity of the pathogen, timing of infection and disease cycle has hindered implementation of reliable disease management strategies. Therefore, the main focus of this project was to determine why Alternaria diseases of apple leaves and fruit are not being effectively controlled by fungicide applications. We approached this problem in two ways; firstly by increasing our understanding of the pathogen biology including the identity of *Alternaria* species associated with the disease symptoms in different apple production areas in Australia, by providing a description of the disease cycle and the conditions required for infection and disease development. Secondly, we developed and evaluated a disease management strategy for the effective control of both Alternaria diseases in Australia.

This project used a combination of DNA sequencing of two nuclear gene regions and morphological characters of 51 isolates obtained from apple leaves and fruits in all Australian growing regions to resolve the identity of *Alternaria* species causing leaf and fruit spot of apples in Australia. Our results showed that multiple *Alternaria* species are associated with leaf blotch and fruit spot on apple. Four species of *Alternaria*; *A. arborescens, A. tenuissima, A. alternata* and *A. longipes* were identified. Unlike most reports from overseas studies where *A. mali* is identified as the causal agent of both diseases (Filajdic & Sutton, 1991, Sawamura, 1972), different species are mostly associated with the diseases in Australia. This is the first report of occurrence of these four different Alternaria species on apples in Australia, however, three of the species have previously been associated with these two apple diseases in Italy (Rotondo et al., 2012).

Although the distribution of these four different Alternaria species varies among apple growing regions in Australia, *A. arborescens* was the most prevalent species in all regions, mostly on leaf blotch. The occurrence of multiple species in all regions suggests that an *Alternaria* species complex is responsible for leaf blotch and fruit spot of apple in Australia.

Irrespective of the origin of each isolate, whether from leaf or fruit symptoms, isolates of all the four *Alternaria* species were pathogenic to both apple leaf and fruit. This indicates that in regions where only leaf blotch but not fruit spot currently occur, the risk of fruit infection is high when disease-conducive environmental conditions prevail. Therefore, all apple growing regions, rather than only Qld and NSW, are at risk of severe yield losses.

This study has elucidated the disease cycle for both leaf blotch and fruit spot of apple in Australia. The timing of infection, source of inoculum and spore production dynamics and optimum climatic conditions that favour infection and disease development were established. Any of the four putative sources of inoculum identified in our study can serve as a significant source of spores for leaf blotch infection. However, leaf residue contributed over one thousand-fold more spores than other possible sources from plant parts within the orchard. This finding is similar to previous reports from the USA (Filajdic & Sutton, 1995) and Japan (Miyagawa & Sakuma, 1984, Sawamura, 1972). The information obtained from our disease epidemiological studies was used to develop and validate a disease management strategy for leaf blotch and fruit spot. Applications of control measures which include orchard hygiene at postharvest or preseason period, monitoring leaf blotch development from 40 days after bloom and fungicide application when leaf blotch disease thresholds exceeds approximately 15% incidence in the summer months will significantly reduce the risk of fruit spot infections that usually occur about 2-3 weeks before harvest.

Multivariate analyses of the relationships among disease incidence and development, spore production dynamics and climatic factors showed that warm temperatures, high relative humidity and rainfall influenced infection and disease development for leaf blotch and fruit spot. These findings suggest that warm and wet conditions favour development of both diseases.

In conclusion, this project has significantly enhanced our understanding of the aetiology of Alternaria leaf blotch and fruit spot of apple in Australia. The outcomes of this research project have provided insights into why some fungicide applications are not effective for the diseases in some regions in Australia. Further studies and essential research priorities for diseases caused by *Alternaria* including mouldy core rot, screening for disease resistance and the need to maintain local expertise for exotic diseases of apple in Australia are emphasized in this report.

Table of contents

1 General Introduction

1.1 Project history

Previous Alternaria project AP02011 "Managing Alternaria leaf and fruit spot in apples", demonstrated that fungicides known to be effective against *Alternaria* species affecting apples overseas did not work in Australia. Furthermore, a literature review completed for the same project, revealed that no two countries reported the same fungicide to be the most effective in managing Alternaria diseases in apples. Field trials conducted in 2002 – 2005 in Queensland (Qld) and 2001 – 2003 in New South Wales (NSW) showed a lack of adequate information on the disease cycles in the Australian production systems and the lack of an effective disease management strategy.

Subsequent project AP05002 "Alternaria fruit spot: New Directions" showed that a range of *Alternaria* species may be responsible for apple leaf and fruit infections in NSW and Qld orchards. Attempts to determine the identity of the Australian *Alternaria* isolates, tentatively identified few of the isolates as *Alternaria mali*, the most commonly cited species causing Alternaria leaf blotch of apple overseas. The project suggested that most of the Australian *Alternaria* isolates appear to be similar or more closely related to *A. alternata* but somewhat different among regions. Exactly what this means is unclear, but a complex of *Alternaria* species causing similar symptoms in Australian apple orchards was considered likely. These results suggested that a good understanding of the disease cycle, epidemiology and pathogen biology was needed to underpin the development of an effective disease management strategy.

In 2006, a new project AP06007 "Alternaria Fruit Spot: New Directions" was established to resolve the issues identified in AP05002 and to develop a management strategy for Alternaria leaf blotch and fruit spot diseases. However, with the departure of the Plant Pathologist leading the project, and due to lack of pathology capability and expertise required to achieve and deliver the project outcomes in the Department of Agriculture, Fisheries and Forestry (DAFF) at the Applethorpe research station, the project was re-developed in 2009 as project AP06007 "Extension AP05002: Alternaria Fruit Spot: New Directions" with the aid Plant Pathologists at the University of Queensland to increase the scientific capability and manage the project. Therefore, this report mostly consists of the outcomes and outputs achieved through

the revised 2009 project version. However, summaries of trials performed from 2006-2008 on the efficacy of a range of fungicide against Alternaria leaf blotch by DAFF, Qld employees is provided in Chapter 2.

Overall, this project sought to address the following issues with regards to Alternaria leaf blotch and fruit spot:

1. Poor disease control, despite fungicide application and the variability of fungicide efficacy in different production areas.
2. Lack of understanding of the pathogen – which *Alternaria* species are causing problems in different production areas, and what stage of their life cycle is the most effective time to use control measures.
3. Conditions for disease development – where is the pathogen coming from / over wintering, when does infection occur and what conditions are required for spore germination and pathogen growth.
4. Development of an effective management strategy that effectively, and economically, reduces the impact of this disease and minimizes the potential for chemical resistance development.
5. Postgraduate training in plant pathology to maintain and enhance the skills base in pathology for the apple industry.

1.2 Project rationale

Alternaria leaf blotch and fruit spot diseases annually cause significant fruit losses to growers in Qld and NSW production areas and occasional, severe leaf losses in Western Australia and South Australia. These problems have been ongoing for several seasons and while fungicide applications have lessened effects in some areas; a method for sustainable and economically viable management of the diseases is needed to reduce the risk of disease losses.

Of particular concern to apple growers from other states in Australia is the fact that *Alternaria* species similar to those causing fruit disease and limiting production in Qld and NSW occur on leaves in orchards in all Australian states. So if the same fungi appear to be present everywhere, why is there a significant fruit problem only in Qld and NSW? The interactions between new varieties, reduced use of broad spectrum

fungicides and an uneven distribution of pathogenic isolates may be responsible for the disparity in fruit spot incidence among the states.

1.2.1 Alternaria leaf blotch

In Australia, Alternaria leaf blotch was first observed in Stanthorpe, Qld in the early 1990s (Horlock, 2006). Alternaria leaf blotch symptoms are small circular to irregular brown or blackish brown spots on leaves that enlarge to about 2-5 mm in diameter with dark brown to purple margin (Fig. 1.1a). Diseased leaves defoliate more readily than healthy leaves from January/February onwards at an early stage in the season. Severe defoliation stresses the apple trees resulting in flowering problems, reduced tree vigour and a reduced yield in the following seasons. There is a considerable variation among apple varieties and between seasons. In Australia, Royal Gala, Fuji, Cripps Pink (Pink Lady™) and Red Delicious are often severely affected (Dullahide, 2009, Horlock, 2006).

Worldwide, Alternaria leaf blotch has been reported from all major apple production nations including Japan (Sawamura, 1962), India (Kishore & Sharma, 2005), China (Wang et al., 1997), Korea (Hwang et al., 1987), Iran (Soleimani & Esmailzadeh, 2007), Turkey (Ozgonen & Karaca, 2006), Yugoslavia (Bulajic et al., 1996), Russia (Gagkaeva & Levitin, 2000), North America (Filajdic & Sutton, 1991) and Brazil (Rollemberg et al., 2011), and in all states of Australia (Horlock, 2006).

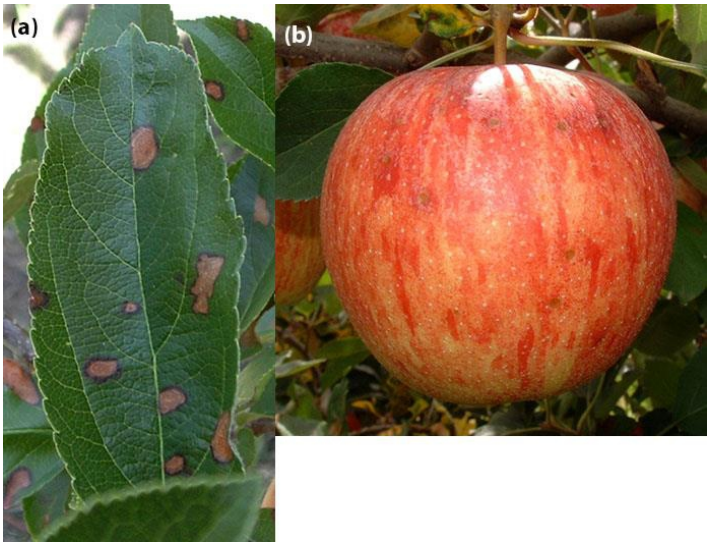


Fig. 1.1: Symptoms of (a) Alternaria leaf blotch and (b) Alternaria fruit spot of apple.

1.2.2 Alternaria fruit spot

Alternaria fruit spot symptoms are characterized by small slightly sunken, light to medium brown spots on mature fruit (Fig. 1.1b) (Persley & Horlock, 2009). In Australia, the disease occurs regularly in the Granite Belt in Qld, the Sydney basin and Orange in NSW. There are anecdotal reports that Alternaria fruit spot occurs in other apple producing states in Australia. Fruit losses in Royal Gala, Pink Lady and Fuji to individual growers in Qld and NSW are commonly between 15-25% when diseased fruits are downgraded for juicing resulting in over 90% financial penalty. Generally, little information is available about Alternaria fruit spot (Wu et al., 2003, Sawamura, 1972). Alternaria fruit spot has only been reported in few countries including Australia (Horlock, 2006), Japan (Sawamura, 1962) and Italy (Rotondo et al., 2012).

1.2.3 Project objectives

The main focus of this project was to determine why Alternaria diseases of apple leaves and fruit are not being effectively controlled by fungicide applications. We approached this problem in two ways; firstly by increasing our understanding of the pathogen biology including the identity of *Alternaria* species responsible for disease symptoms in different apple production areas in Australia, secondly, we developed and evaluated a disease management strategy for the Alternaria diseases for inclusion in the Apple Fruit Production Manual. Specifically, the aims of this project were to:

1. Determine the identity of *Alternaria* species associated with fruit spot and leaf blotch symptoms on Australian apples.
2. Elucidate the disease cycle of Alternaria diseases on Australian apples, determine the sources of inoculum, environmental conditions needed for infection, the infection process and disease development from infection to sporulation.
3. Refine methods for accurate assessment of disease incidence, severity and economic impact of Alternaria diseases in apple.
4. Assess efficacy of fungicides for Alternaria disease management.
5. Determine the most effective timing of fungicide applications, based on an understanding of the conditions needed for infection.

6. Develop an integrated approach to Alternaria management in Australian apple orchards, combining chemical, physical and cultural control methods.

2 Summary of field trials from 2006-2008

A range of field trials were performed between 2006 and 2008 at the start of this project but the results were inconclusive. These include trials involving the application of fungicides (Appendix 1a and 1b); cultural control methods for reducing inoculum (Appendix 1c); observation of disease onset in commercial apple varieties and various field trials in NSW (Appendix 1d). A brief summary of each field trial is provided below (section 2.1-2.4).

2.1 The use of early, late and whole-of-season fungicide applications to manage Alternaria leaf blotch and fruit spot in apple

Christine Horlock - Full report in Appendix 1a

In order to determine the most effective fungicides for the reduction of leaf blotches and fruit spots caused by *Alternaria* species on apple and the time of the season during which fungicide application is most effective in Qld, 18 treatments as single and a mixture of fungicide spray applications were examined during the 2006-07 season. Results showed that Dithane® was the most effective at reducing *Alternaria* symptoms, resulting in incidence levels 10 times lower than the untreated control when applied late in the season or throughout the whole season. In general, fungicides applied late in the season or throughout the whole season appeared to give better control of *Alternaria* symptoms, although this may simply be due to the late onset of symptoms associated with late summer rainfall.

2.2 Late season fungicide applications to manage Alternaria leaf blotch and fruit spot in apple

Duncan Cameron and Christine Horlock – full report in Appendix 1b

Efficacy of different fungicides to control Alternaria leaf blotch and fruit spot as late season fungicide spray applications on Royal Gala was examined in field trials at the Granite Belt in Qld in the 2007-08 season. The fungicides were sprayed to about 3 L/tree at fortnightly intervals from mid-December until harvest (31-01-08) and after harvest, the trees were sprayed once every 4 weeks until April. Five mature leaves closest to the growing point were rated for leaf blotch symptoms on twelve shoots on

the eastern aspect of the tree at the onset of symptoms, at harvest approx. 10 days later, 3 weeks after harvest and prior to leaf fall. Attempts to assess Alternaria fruit spots symptoms accessed on 25 fruits per tree were unsuccessful. Overall, the percent of leaves with incidence of *Alternaria* leaf blotch was significantly reduced by the application of mancozeb and metiram but not with diathianon (Fig. 2.1).

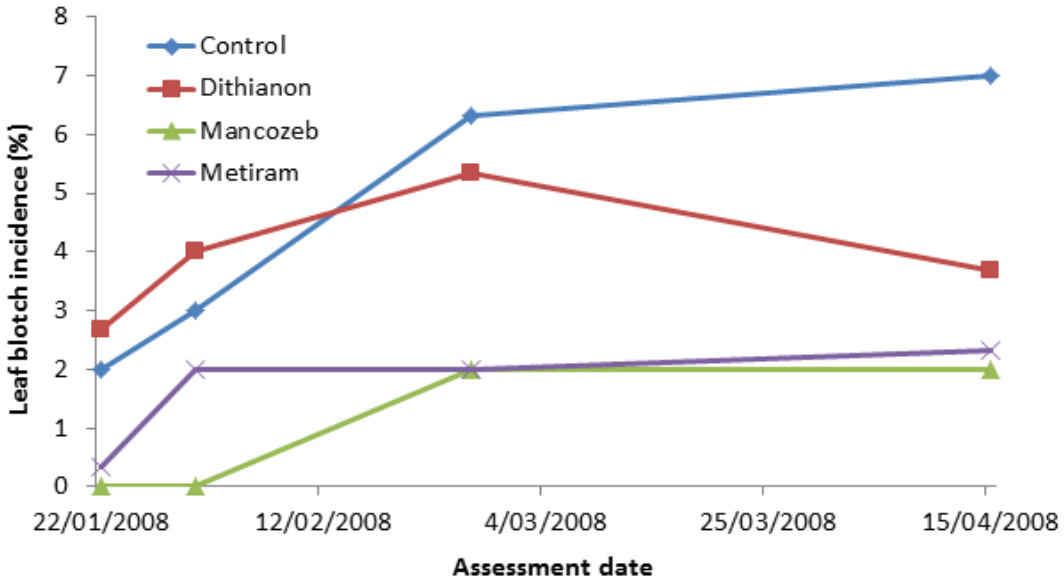


Fig. 2.1 Percent of apple leaves of cv. Royal Gala infected with Alternaria leaf blotch at Stanthorpe in 2008 after spray application of different fungicides (Source: adapted from Table in Appendix 1b).

2.3 Managing inoculum sources for Alternaria leaf blotch and fruit spot in apples. Effect of bud removal on Alternaria leaf blotch in apples

Dean Beasley, Christine Horlock and Duncan Cameron – full report in Appendix 1c

In order to determine the effect of dormant lime sulphur sprays, leaf removal and mulching, or debudding on overwintering inoculum sources in apple trees, field trials with Royal Gala at Applethorpe Research Station were established. Results of the initial trials to assess the effects of lime sulphur as an over-winter spray to reduce inoculum levels of *Alternaria* in apple orchards were inconclusive (Fig. 2.2). The application of lime sulphur during winter dormancy had no significant impact on the incidence of *Alternaria* leaf blotch or fruit spot on the variety Royal Gala. However, it

appeared that mulching under the drip line of the tree reduced leaf blotch incidence but not fruit spot incidence.

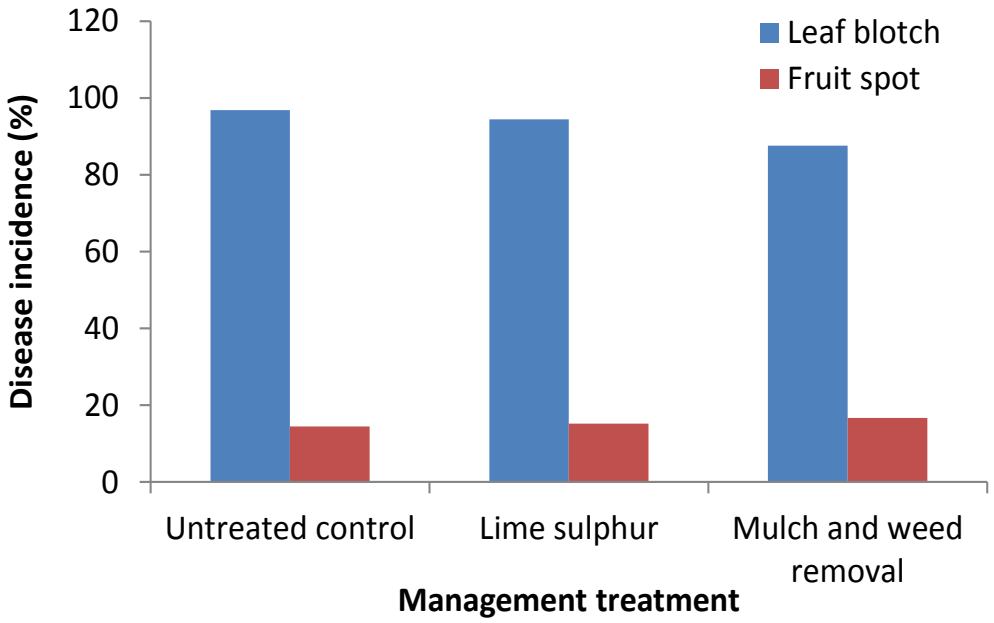


Fig. 2.2 Incidence (%) of *Alternaria* on fruit and leaves of cv. Royal Gala following application of putative control treatments. (Source: adapted from Table in Appendix 1c)

2.4 Managing Alternaria leaf spot – report on NSW DPI trial 2006/07

Shane Hetherington and Deirdre Gunning –full report in Appendix 1d

Work in Qld has identified late-season application of the fungicides dithianon, metiram and mancozeb as a potential management strategy. However late season applications are limited because of withholding periods. This situation is exacerbated with varieties such as Royal Gala that require multiple harvests. In the 2006-07 NSW trials, mixtures of pyrimethanil and fluquinconazole and trifloxystrobin were examined as early-mid season spray application for Alternaria leaf blotch control. Results showed that application of trifloxystrobin early in the season (without late season metiram, dithianon and mancozeb applications) does not provide sufficient disease control but there were statistically significant differences between other treatments at harvest and very late in the season.

3 Identity of *Alternaria* species causing leaf blotch and fruit spot of apple in Australia

Dalphy Harteveld, Femi Akinsanmi and André Drenth

3.1 Introduction

Worldwide, *Alternaria mali* is associated with Alternaria leaf blotch and fruit spot of apple (Filajdic & Sutton, 1991, Bulajic et al., 1996), but recently in Italy, three *Alternaria* species *A. arborescens*, *A. tenuissima* and *A. alternata* have been reported to cause leaf blotch and fruit spot of apple (Rotondo et al., 2012). Many reports, in a broad sense, sometimes identified the causal agent of leaf blotch of apple as *A. alternata* (Kusaba & Tsuge, 1994). In Australia, previous project AP05002 (Alternaria fruit spot: New Directions) using 11 isolates collected from apple leaves and fruit in Australia showed that the Australian isolates were different from *A. mali* and suggested that seven of the isolates tested were most similar to *A. arborescens*, and three were most similar to *A. tenuissima* and one was most similar to *A. alternata*. However, the majority of the Australian isolates collected from a survey as part of project AP05002 suggested that 60% of the isolates were *A. alternata* and possibly one isolate resembled *A. mali*. Inconsistency of identification based on morphological characteristics confounds precise identification of the isolates and the use of DNA sequencing of commonly used conserved nuclear gene regions did not distinctly differentiate the species. Therefore, the lack of proper identification of the pathogen(s) and the inability to confirm if multiple *Alternaria* species are involved in causing either or both diseases prevented development of effective disease management practices.

Although morphological characteristics, in particular, conidia of the genus *Alternaria* are distinct and easy to recognize, it is often difficult to distinguish the various species within the genus (Simmons, 1992). Molecular techniques offer an additional tool for identification of species within the genus *Alternaria* and have been used to separate some species in the *A. alternata*-complex (Peever et al., 2000, Pryor & Michailides, 2002, Hong et al., 2006, Laich et al., 2008).

In order to identify the Australian *Alternaria* isolates obtained from leaf blotch and fruit spot symptoms in apple growing regions, we used a combination of morphological and molecular tools. Specifically, we determined the identity and the distribution of the Australian *Alternaria* isolates associated with Alternaria leaf blotch and fruit spot in Australian apple orchards.

3.2 Materials and methods

3.2.1 Source of *Alternaria* isolates and culture preparation

The identity of 51 isolates selected from a collection of about 400 Alternaria isolates obtained from previous project AP05002 and this project (AP06007) from apple leaves with leaf blotch symptoms and fruit with fruit spot symptoms was determined in this study (Table 3.1).

Each isolate was purified to and single spored to produce monoconidial cultures as described by Harteveld et al. (2013b). In brief, the isolates were grown on ½-strength potato dextrose agar (PDA) (Difco Laboratories Incorporated) at 25ºC in the dark for 2 weeks, then spores were harvested in 500 µl of sterile water, streaked on to a water agar plate and thereafter a germinated spore was excised under aseptic conditions unto a fresh PDA plate. Monoconidial cultures were stored in 15% glycerol at -80 ºC for further morphological and molecular analyses

Isolates were selected to represent different apple producing regions in each state and sourced from leaf and fruit. Reference strains of *A. alternata* (EGS 34-016), *A. arborescens* (EGS 39-128), *A. tenuissima* (EGS 34-015), *A. mali* (EGS 38-029) and *A. longipes* (EGS 30-033) from Genbank accessions in the National Centre for Biotechnology Information (NCBI) were included for comparison. Each isolate was purified to and single spored to produce monoconidial cultures as described by Harteveld et al. (2013b). In brief, the isolates were grown on ½-strength potato dextrose agar (PDA) (Difco Laboratories Incorporated) at 25ºC in the dark for 2 weeks, then spores were harvested in 500 µl of sterile water, streaked on to a water agar plate and thereafter a germinated spore was excised under aseptic conditions unto a fresh PDA plate. Monoconidial cultures were stored in 15% glycerol at -80 ºC for further morphological and molecular analyses.

Table 3.1 Location and sources of representative *Alternaria* isolates obtained from apple in Australia used in this study.

Location	Fruit	Leaf	Total
New South Wales	6	7	13
Queensland	5	8	13
South Australia	0	5	5
Tasmania	0	7	7
Victoria	0	5	5
Western Australia	3	5	8
Total	**14**	**37**	**51**

3.2.2 Genomic DNA extraction and sequencing

To accurately determine the species of Alternaria present and compare them to type specimens DNA extractions were performed with 40 mg of mycelia of each monoconidial isolate using the Promega Wizard® Genomic DNA Purification Kit (Promega Corporation) as described by Harteveld et al. (2013b). DNA obtained from the isolates was used as template for further Polymerase Chain Reaction (PCR) amplifications. Initially three nuclear gene regions commonly used in fungal systematics were targeted for PCR amplification and subsequent sequencing. These regions included the Internal Transcribed Spacer regions ITS-4 and ITS5, elongation factor-1-alpha (EF-1α) and Actin (ACT) genes. The results showed the ITS, ACT and EF-1α were not sufficiently variable to separate species within the genus *Alternaria* (Neilsen, 2010). Additional two nuclear gene regions, endopolygalacturonase (*endoPG*) and Alternaria major allergen (*Alta1*) used to delineate the closely related species within the *A. alternata*-complex (Hong et al., 2005, Andrew et al., 2009, Peever et al., 2004) were used.

The PCR conditions were optimised and performed in a Bio-Rad c1000 Thermal Cycler (BioRad Laboratories Incorporated) as described by Harteveld et al. (2013b) using corresponding primers (Table 3.2). The PCR fragments were sequenced at the Australian Genome Research Facility (Brisbane, Australia) with the same primers as used for amplification. Identity of the nucleotide sequences were determined with BLAST searches (Altschul et al., 1990) of NCBI in Genbank. Phylogenetic analysis of the aligned sequences and reference strains were performed in MEGA 5 software

(Tamura et al., 2011) using a maximum likelihood phylogeny of the best-fit of 21 models of evolution. Tree stability was tested by using 1000 bootstrap replications.

Table 3.2 Detail of primers used in this study.

Primer name	Sequencing direction	Sequences (5` - 3`)	Reference
ITS4	Forward	TCCTCCGCTTATTGATATGC	White et al. (1990)
ITS5	Reverse	GGAAGTAAAAGTCGTAACAAGG	
AC512	Forward	ATGTGCAAGGCCGGTTTCGC	Carbone & Kohn (1999)
AC783	Reverse	TACGAGTCCTTCTGGCCCAT	
EF1-728F	Forward	CATCGAGAAGTTCGAGAAGG	Carbone & Kohn (1999)
EF1-986R	Reverse	TACTTGAAGGAACCCTTACC	
PG3	Forward	TACCATGGTTCTTTCCGA	Andrew et al. (2009), Peever et al. (2004)
PG2b	Reverse	GAGAATTCRCARTCRTCYTGRTT	
Alt-for	Forward	ATGCAGTTCACCACCATCGC	Hong et al. (2005)
Alt-rev	Reverse	ACGAGGGTGAYGTAGGCGTC	

3.2.3 Morphological and cultural characterisation

Morphological and cultural characteristics including colony shape, colour and texture, conidiation and sporulation patterns of the isolates were examined. These characters were examined for 2-4 isolates for alignment with each clade from the results of the molecular analysis. A modified method as described by Simmons (2007) was used to examine the morphological and cultural characters of the isolates on low strength PDA (Hong et al., 2006, Pryor & Michailides, 2002). Sporulation patterns were examined under a compound microscope (Leica DM5500B, Leica Microsystems) after 9 days of incubation of a drop of spore suspension on a water agar plate and compared to the description of representative species in the *Alternaria* Identification Manual (Simmons, 2007).

3.3 Results and discussion

3.3.1 Identity of the Australian *Alternaria* isolates based on DNA sequencing

DNA sequencing of the ITS, ACT and EF-1α showed very limited variation between the isolates and were not sufficiently variable to separate most isolates into species,

but indicated the presence of an *A. alternata* species complex (Neilsen, 2010). Whereas, amplification of the *Alta1* and *endoPG* differentiated the *A. alternata* species complex and reference strains with the Australian *Alternaria* isolates. The identity of the Australian isolates was confirmed as *A. arborescens*, *A. longipes*, *A. tenuissima* and *A. alternata*. Overall, 51% of the Australian isolates were *A. arborescens*, 26% were similar to *A. alternata*, 16% were similar to *A. tenuissima* and 8% were related to *A. longipes*.

All the species were obtained from both fruit and leaf, but most (60%) of the isolates obtained from leaf were *A. arborescens* whereas, 50% of the isolates obtained from fruit were *A. alternata* (Fig. 3.1). *A. arborescens* occurred in all apple producing states in Australia, but only two *Alternaria* species were obtained from Western Australia, South Australia and Victoria, whereas, all the four species were isolated from Qld (Fig. 3.2)

A. arborescens is the most prevalent species and were mostly associated with leaf blotch symptoms. This may explain the reason leaf blotch occurs in all apple growing states of Australia. The majority (50%) of the isolates obtained from fruit were identified as *A. alternata* predominantly from NSW and Qld, thus, may explain the frequent occurrence of Alternaria fruit spot in these states in Australia.

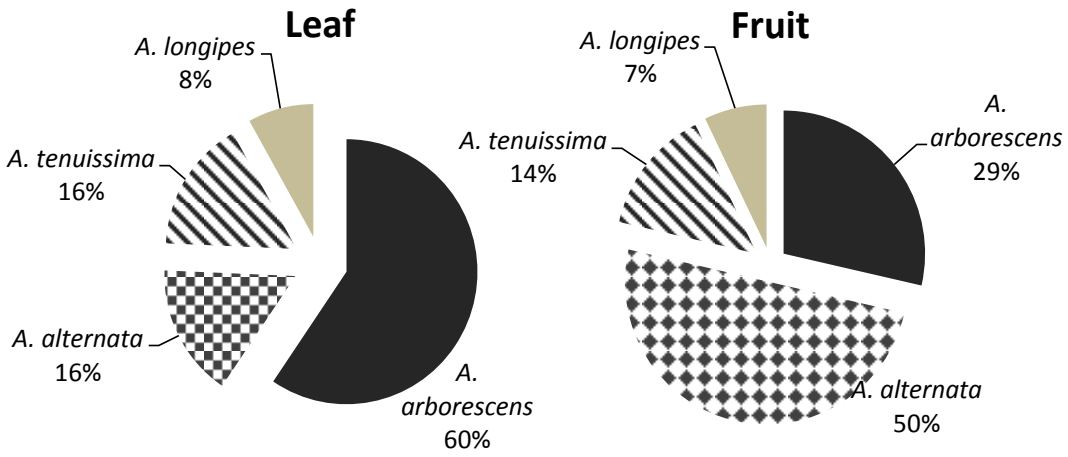


Fig. 3.1 Identity and proportion of *Alternaria* species from leaf blotch and fruit spot of apple in Australia.

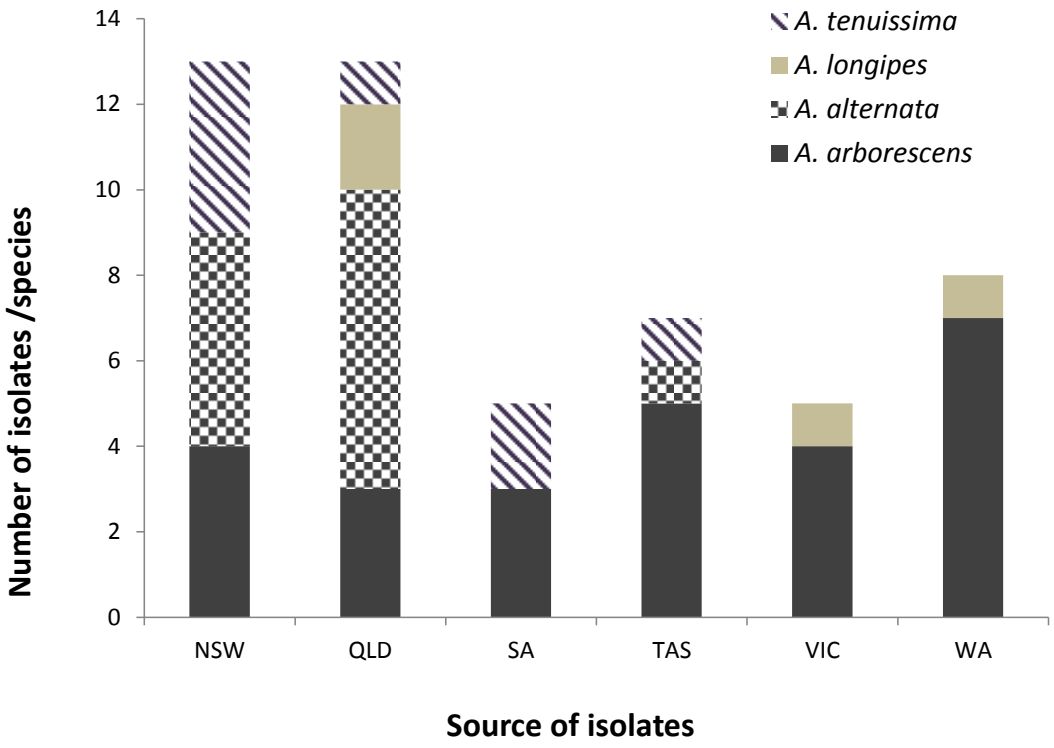


Fig. 3.2. Identity and proportion distribution of *Alternaria* species associated with leaf blotch and fruit spot of apples in the Australian apple producing states - New South Wales (NSW), Queensland (QLD), Western Australia (WA), Victoria (VIC), South Australia (SA) and Tasmania (TAS).

3.3.2 Identity of the Australian *Alternaria* isolates based on morphological and cultural characteristics

The morphological and cultural characteristics of the selected isolates to represent each of the four species identified using DNA sequencing were consistent for all the triplicate plates.

- The *A. arborescens* isolates produced colonies on culture media plates that were irregular in shape, dark green to black, felty with a 5-10 mm white margin and distinct 6-8 concentric rings and colony diameter of 40-70 mm (Fig. 3.3a).

- Whereas, *A. tenuissima* isolates produced grey to brown-greenish colonies with woolly whitish mycelial on top of the colony and 3 - 5 vague concentric rings to form 42-60 mm in colony diameter (Fig. 3.3b).

- *A. alternata* isolates were olive green to dark green, felty and a bit woolly with a 5 mm margin with 4 - 7 concentric rings and 57 - 66 mm in colony diameter (Fig. 3.3c). Colonies of *A. longipes* isolates were grey-green with a light woolly texture, 5 concentric rings and 65-70 mm in diameter (Fig. 3.3d).

- The sporulation patterns of the species were different, varied from long conidiophores with extensive terminal branching and the primary conidial chains contained 2-7 conidia in length and the secondary and tertiary branches contained 1 to 6 conidia (Fig. 3.3e) to long primary chains of 5 - 13 conidia in length with few occasional secondary branches of 1-4 conidia in length (Fig. 3.3f) (Harteveld et al., 2013b).

The combination of DNA sequencing and morphological tools clearly revealed that four *Alternaria* species are associated with both Alternaria leaf blotch and Alternaria fruit spot disease on apple in Australia. Although the similarity of the Australian *Alternaria* isolates to the overseas isolates of *A. mali* has not been well established in this study, results of the phylogenetic analysis with the reference strains suggest that the Australian *A. tenuissima* isolates may be related to the *A. mali* reference isolate used in this study. Similar to the findings of this study, multiple *Alternaria* species have been reported to be associated with both diseases in Italy (Rotondo et al., 2012).

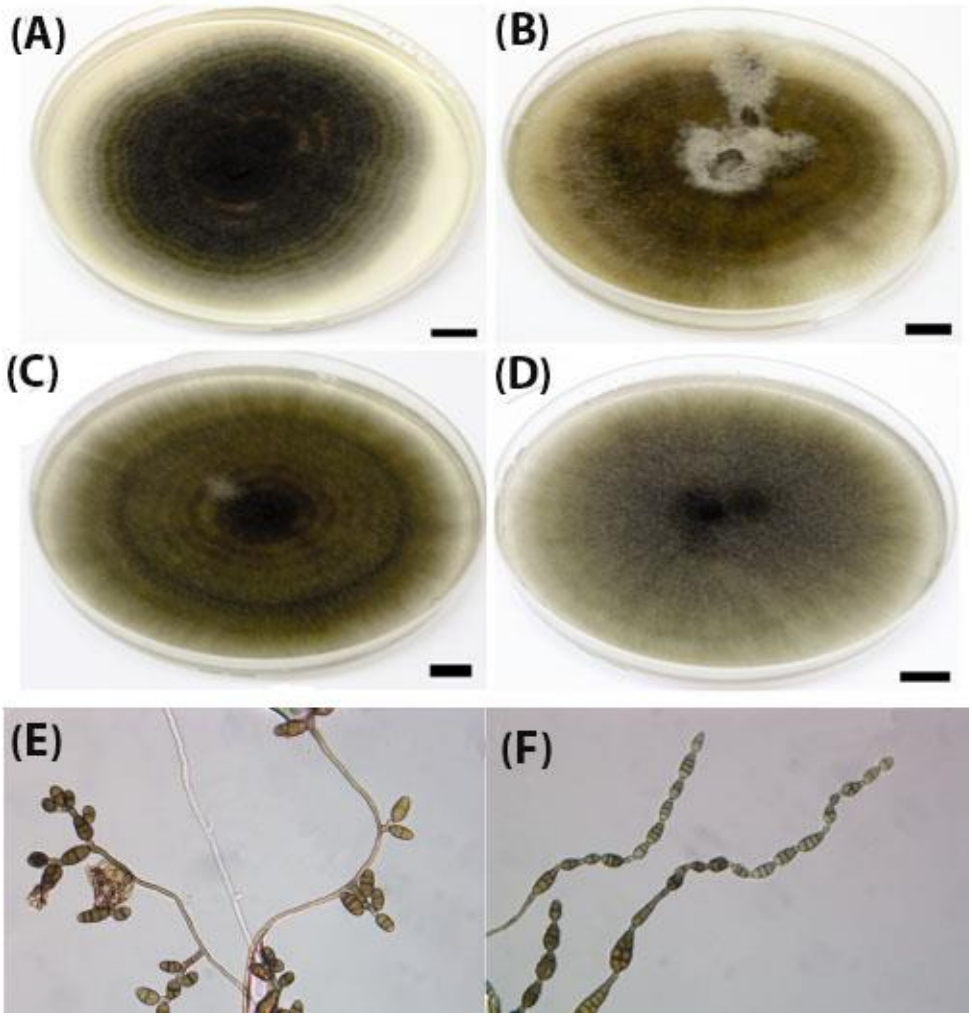


Fig. 3.3 Colonies and representative sporulation patterns of four *Alternaria* species obtained from Alternaria leaf blotch and fruit spot of apple in Australia after 14 days of incubation. (A) *A. arborescens*, (B) *A. tenuissima*, (C) *A. alternata*, (D) *A. longipes*, (E) extensive terminal branching sporulation pattern and (F) long primary chains sporulation pattern. Bar = 1 cm.

3.4 Conclusions

Four different Alternaria species were obtained from both fruit and leaf samples and identified as *A. arborescens*, *A. alternata*, *A. tenuissima* and *A. longipes*. *A. arborescens* occurred in all apple producing states in Australia, but only two *Alternaria* species were obtained from Western Australia, South Australia and Victoria, whereas, all the four species were isolated from Qld. The involvement of multiple *Alternaria* species causing both leaf blotch and fruit spot of apple in Australia and Italy has been reported in diseases in other tree crops. For instance, as black spot disease of Japanese pear caused by *A. gaisen* and *A. alternata* and four

other unnamed *Alternaria* species groups (Simmons & Roberts, 1993); Alternaria late blight of pistachio caused by *A. alternata, A. tenuissima, A. arborescens* and *A. infectoria* (Pryor & Michailides, 2002); Alternaria leaf spot of almond caused by *A. alternata, A. tenuissima* and *A. arborescens* (Teviotdale et al., 2001); and leaf spot of rough lemon caused by *A. limoniasperae* and *A. citrimacularis* (Simmons, 1999). Whether isolates of the species from these crops can infect apples to cause Alternaria leaf blotch and fruit is not known.

Similar pathogens belonging to the *A. tenuissima*-species group, *A. alternata* and *A. arborescens* have also been reported to cause mouldy core (Gao et al., 2013, Serdani et al., 2002), however, the association and relationship of isolates of the four Australian *Alternaria* species involved in leaf blotch and fruit spot with mouldy core rot in Australia is not known and require further investigations. Whether these pathogens independently cause fruit spot and mouldy core rot is not known.

This study shows that in Australia the *Alternaria* species were not specific to Alternaria leaf blotch or Alternaria fruit spot or a geographical region. The occurrence of common species obtained from leaf and fruit in Qld and NSW in all other states suggests that both Alternaria leaf blotch and fruit spot may become more widespread in Australia than has been reported. Therefore, similar management strategies may need to be adopted in all the apple growing regions of Australia. These findings significantly increase our understanding of the Australia's situation regarding these diseases and should underpin future work including assays for selection of resistant cultivars and the development of improved control options.

3.5 Acknowledgements

Most of the isolates used were from collection from previous project AP05002 - Alternaria fruit spot: New Directions by Ms. Christine Horlock (DAFF, Qld) in the Queensland Plant Pathology Herbarium Brisbane. J.H.C Woudenberg and Professor P.W. Crous of the Centraal Bureau voor Schimmelculturen, The Netherlands provided sequences of reference strains.

4 Pathogenicity of isolates of *Alternaria* species for leaf blotch and fruit spot of apple

Dalphy Harteveld, Femi Akinsanmi and André Drenth

4.1 Introduction

The issues arising from the preceding chapter that multiple *Alternaria* species are associated with both leaf blotch and fruit spot diseases in Australia require that the pathogenicity of the *Alternaria* isolates of different species are examined. In addition, cross-pathogenicity of isolates obtained from leaf and fruit should be examined on different varieties. There is no information concerning the pathogenicity, defined as the ability to cause apple leaf blotch and/or fruit spot, of the four *Alternaria* species in Australia. It is not known if pathogenicity of the each isolate is specific to the host tissue where it was originally obtained from. The reasons why *A. arborescens* is the most prevalent species associated with leaf blotch in all Australian apple growing regions, and *A. tenuissima* and *A. alternata* species were mainly obtained from fruit in Qld and NSW are not fully understood. Anecdotal reports show that different apple varieties are affected in different states and regions. Whether this is due to variation in species in the regions or other factors is not known.

Therefore, the overall aim of this study was to determine if there is variation in pathogenicity among and within the *Alternaria* species causing leaf blotch and fruit spot of apple in Australia. Specifically, to determine if all four *Alternaria* species can cause both leaf and fruit diseases or if the isolates are specific to its tissue of origin, and to examine if the four *Alternaria* species groups are pathogenic on leaf and fruit of different apple cultivars. This information will provide better understanding of the epidemiology of both diseases in Australian apple production systems.

4.2 Materials and methods

4.2.1 Development and evaluation of pathogenicity assays

4.2.1.1 Development processes for fruit inoculation

In order to determine the stage at which fruit is most susceptible to infection unsprayed fruits of cv. apple varieties Royal Gala and FB22-47 at five different fruit stages was used (Table 4.1). The infection experiments were conducted both with detached fruit and *in planta* in the field between November – February in the 2010-11 season, at the Applethorpe Research Station, DAFF, Qld. At each stage, three fruit per variety were examined for size, sugar content and starch level. At each stage a total of 24 fruits of each variety were used. Fruit diameter was determined using a calliper (Carba-Tec®, Australia) and the sugar content was measured using a refractometer (Atago Co. Ltd. Tokyo, Japan). A starch test (Watkins, 2003, Srum, 1985) was performed by cutting the fruit in half, horizontally through the seed cavity, and sprayed with iodine solution. Iodine stained the starch into a blue-black colour. Maturity can be determined since in mature fruit, the starch clears from the core area outwards, whereas in immature fruit, starch is found throughout the flesh. Two inoculation protocols with three *Alternaria* isolates (BRIP 46550, BRIP 46361 and BRIP 46492) in three replicates per variety were evaluated. Fruit inoculated with sterile water served as a control.

Apples were routinely collected at 3 week intervals from November – February and used in detached fruit assays. Each fruit was inoculated using a pressure sprayer containing a 10^5 spores/ml suspension amended with a drop of Tween80 (Sigma-Aldrich) and placed in a humidity box which contained wet paper towels. Initially, boxes were placed on a bench at room temperature, but the fruit rotted before assessment, thereafter, the boxes were maintained at 26ºC. The apples were assessed for fruit spot symptoms every 2 weeks after inoculation for 2 months. To confirm infection of *Alternaria* spp., pieces of any observed fruit spot symptoms were isolated, washed with 1% bleach, dried on a sterile filter paper and placed on ½-strength PDA. To validate our detached fruit inoculation assay, a second inoculation assay was conducted *in planta*. Fruits on the trees were inoculated on the same day and stage as the detached fruits was collected, Fruits at stages 1-4 were used while fruit at stage 5 was harvested.

At each fruit stage, the three *Alternaria* isolates and sterile water as control were used to inoculate three replicate fruit per variety on the tree. The fruit were inoculated using a pressure sprayer containing a 10^5 spores/ml suspension amended with a drop of Tween80. Resealable plastic bags were used to cover and incubate the fruit overnight where after a white paper bag was placed over the fruit to cover the fruit from natural infections. All fruit were assessed for fruit symptoms every 2 weeks post inoculation for 2 months. Positive infection was confirmed as described above. This protocol was later refined using inoculum soaked filter paper as described below (section 4.2.3).

Table 4.1 Fruit development stages used in this study.

Stages	Developmental stage [a]	Days after anthesis	Month	Size (mm)[b]	Sugar (ºBx)[b]
Stage 1	Just after cell division period	35-50	November	28-30	3.0 – 6.0
Stage 2	Peak rate of cell expansion, starch accumulation	50-70	December	29-35	6.6 - 7.8
Stage 3	Cell expansion, starch accumulation	70-90	January	51-65	6.8 - 8.7
Stage 4	Starch decline, ripening	90-120	February	65-72	5.5 - 11.2
Stage 5	Ripening	120-150	Late February	65-83	9.3 -10.3

[a] Based on fruit development description by Janssen *et al*.(2008)
[b] The fruit size and sugar content is presented as the range of 6 fruit (two varieties).

4.2.1.2 Assessment of Alternaria fruit spot development after long term storage

In order to investigate if long term storage has an effect on Alternaria fruit spot development, 90 Royal Gala and FB22-47 fruits each at different developmental stages were collected, inoculated and placed in a cold room at 3 ±1ºC. After 2 months, the fruits were incubated in a closed box containing wet paper towels at 26ºC in darkness for 48 hours to induce infection. After incubation the fruit was placed at room temperature and assessed for disease development every two weeks for 2 months.

4.2.1.3 Development processes for leaf inoculation assay

In order to evaluate the leaf stage and/or size, at which the leaves are most susceptible to infection, leaves at different stages (young to senescence) and from the top, middle and bottom of trees in glasshouse-grown potted trees of Royal Gala, FB22-47 and Galaxy were used. Six leaves from the glasshouse potted trees were collected per stage and position and inoculated separately with two *Alternaria* isolates (BRIP 46550 and BRIP 46492), two leaves per variety per isolate were used. Inoculation was performed using a pressure sprayer containing a 10^5 spores/ml amended with a drop of Tween80 suspension. Leaves were placed in a box containing wet paper towels and another set of wet paper towels was placed on top to maintain high (near 100%) humidity in the box. The box was sealed and placed at 26ºC under 8h/16h light/dark cycle. Leaf blotch incidence was assessed at 7 and 14 days post inoculation. Positive infection was confirmed from isolation of pieces of symptomatic plant tissue as described above.

4.2.2 Pathogenicity to leaf using detached leaf inoculation assay

A total of 16 isolates representing the four *Alternaria* species were used in this study (Table 4.2). Conidial suspensions were prepared as described by Harteveld *et al.* (2013b) and conidial concentration was adjusted to 10^5 spores/mL as the inoculum. The first five fully expanded leaves on the terminal shoots used in the inoculation assays were obtained from glasshouse-grown potted trees of cv. Royal Gala. Prior to inoculation the leavers were rinsed with deionised water to remove dust particles and any contaminants and then air-dried. The experimental design consisted of 3 replicates (leaf) per isolate, two 0.5 cm^2 square inoculum soaked filter paper discs and a repeat. The inoculum filter paper discs were placed on the abaxial side of each leaf close to the midrib (Fig. 4.1). Leaves inoculated with sterile water-soaked discs served as control. Each inoculated leaf was placed in a moistened plastic bag containing wet cotton wool and incubated at 25ºC in darkness for 5 days. Thereafter, occurrence of leaf blotch symptoms and severity (proportion of leaf area diseased) were recorded.

Table 4.2 Source of *Alternaria* isolates used for pathogenicity assays for leaf blotch and fruit spot on apple on cultivar Royal Gala.

BRIP accession[a]	*Alternaria* species [b]	Host tissue	Cultivar	Location
46452	*A. arborescens*	Fruit	Pink Lady	Western Australia
46571	*A. arborescens*	Leaf	Fuji	South Australia
46872	*A. arborescens*	Leaf	Fuji	Victoria
46512	*A. arborescens*	Leaf	Pink Lady	Western Australia
46398	*A. alternata*	Fruit	unknown	New South Wales
46550	*A. alternata*	Fruit	Fuji	Queensland
46590	*A. alternata*	Leaf	Fuji	Tasmania
46545	*A. alternata*	Leaf	Royal Gala	Queensland
46361	*A. tenuissima*	Leaf	Royal Gala	New South Wales
46574	*A. tenuissima*	Leaf	Royal Gala	South Australia
46414	*A. tenuissima*	Leaf	Braeburn	New South Wales
54639	*A. tenuissima*	Fruit	Pink lady	New South Wales
46356	*A. longipes*	Fruit	Fuji	Queensland
46455	*A. longipes*	Leaf	Pink Lady	Western Australia
47966	*A. longipes*	Leaf	unknown	Queensland
46899	*A. longipes*	Leaf	unknown	Victoria

[a]Accession numbers represent the BRIP codes of the isolates as coded by the Queensland Plant Pathology Herbarium, Brisbane, Australia.
[b]Identity of the isolates as described by Harteveld *et al.* (2013b).

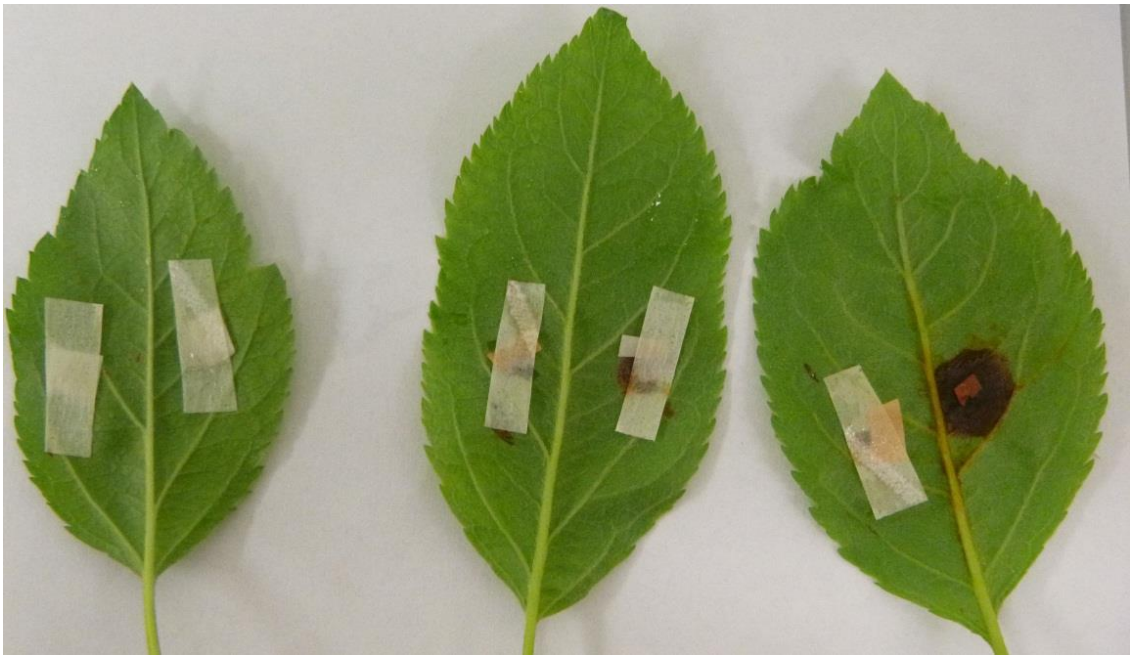


Fig. 4.1 Detached leaf inoculation assay using inoculum-soaked filter paper discs attached to the adaxial side of the leaf and attached in place with sterile tape. Paper discs on the left were soaked in sterile water, discs in the middle and right side were soaked in spore suspension of *Alternaria* sp.

4.2.3 Pathogenicity to fruit using *in planta* fruit inoculation assay

The same set if 16 *Alternaria* isolates was used for *in planta* fruit inoculation assays (Table 4.2). The assay consisted of unwounded apples on trees of cv. Royal Gala on trees at the Applethorpe Research Station, DAFF, Qld. Prior to inoculation, fruits selected for the trial were covered with white waterproof T20 paper bags (Palmwoods Farm and Garden Supplies, Qld) for 2 months. Fruit maturity stage of the trees was monitored using starch and sugar contents tests as described by Chennell *et al.* (2002). Fruits were inoculated at near maturity at about 2 to 3 weeks before harvest. Experimental design included 16 isolates, 3 replicate (fruits) per isolate, two 0.5 cm^2 inoculum soaked filter paper discs per fruit and tree per isolate. The trial was performed twice in the 2011-12 and the 2012-13 production seasons. Inoculum soaked discs were attached with sterile tape to the surface of each selected fruit, and kept under high humid conditions in a moistened plastic seal bag containing wet cotton wool for 24 h, before replacing the plastic bag with new white waterproof T20

paper bag. Fruit inoculated with sterile water served as control. Incidence of fruit spot symptoms was recorded at 2 weeks after inoculation.

4.2.4 Assessment of isolates of the four *Alternaria* species for their ability to cause leaf blotch and fruit spot

In order to examine if isolates from fruit spot cause leaf blotch and vis-à-vis, cross-pathogenicity of the isolates were analysed in relation to the original source host tissue of the isolates. The reason for the high prevalence of *A. arborescens* in all the apple growing states was evaluated by comparing the disease severity of the four *Alternaria* species on leaf and fruit.

4.2.5 Pathogenicity on different apple varieties

In order to determine if all the four *Alternaria* species are pathogenic on different varieties, both detached leaf *in planta* fruit inoculation assays were performed on different varieties. The leaf inoculation assay was performed on Royal Gala, FB22-47, Galaxy, Red Delicious and Pink lady™, while the fruit inoculation assay was performed on Royal Gala and FB22-47 in the 2011-13 seasons. Disease severity was measured as described above.

4.2.6 Data analysis

Disease severity data for each experiment was analysed using a general analysis of variance (ANOVA) procedure in GenStat 14th ed. (VSN International, Hertfordshire, UK). Differences among isolates, isolates nested within species for each trial, cultivar and their interactions were examined. Significant treatment means were compared using Fisher's protected Least Significant Difference (LSD), P =0.05. Differences among the five varieties for leaf blotch severity were explored Principal Component Analysis (PCA) procedure in XLSTAT software version 2013.4 (Addinsoft, Paris, France), where isolates were termed as observations and the disease severity data was treated as variables to produce the eigenvalues and the eigenvectors of the first three components that explained over 80% of the relationship and variance. Correlation biplot of the observations and variables was constructed using the first two components. Putative association between the disease severity and the varieties was evaluated using the Pearson χ^2 statistic test in XLSTAT.

4.3 Results and discussion

4.3.1 Development and evaluation of pathogenicity assays

4.3.1.1 Detached fruit inoculations with Alternaria isolates

There were no significant (P>0.10) differences in the fruit sizes and sugar content of fruits of the two varieties at the developmental stages. Fruit inoculation using the incubation conditions of the first protocol resulted in fruit rot and other diseases before fruit spot assessment, whereas, the second protocol maintained the fruit for a longer period of time than the first protocol. Fruit spot symptoms were not observed at the stage 1-4 in Royal Gala, but were visible at stage 3 and 4 in FB22-47. Unlike fruit inoculated at near maturity at stage 5, timing and incidence of fruit spot symptoms was inconsistent in the stages 1-4. The three *Alternaria* isolates were pathogenic to fruit while fruits inoculated with water did not show fruit spot symptoms. Even when the fruit was sprayed in both detached and *in planta* assays, often few fruit spots developed on the inoculated fruit. Comparison of the detached fruit and the *in planta* fruit spray inoculations showed that the *in planta* spray inoculations developed symptoms more consistently then the detached fruit spray assay. Comparison of the *in planta* spray inoculation and the inoculum-soaked filter paper discs inoculation methods revealed that the later method produced more consistent and reliable infection at the point of inoculation than the spray inoculation method. Therefore, this was adopted and refined for further trials.

4.3.1.2 Assessment of Alternaria fruit spot development after long term storage

No symptoms were observed in any fruits of stages 1 and 2, but fruits at stage 3 -5 were infected and symptoms observed from 30 days after cold storage.

4.3.1.3 Detached leaf inoculation with Alternaria isolates

The leaves from the top section of the young trees of both varieties used were most susceptible to infection to *Alternaria* sp. and showed Alternaria leaf blotch symptoms at 7 days post-inoculation. Disease incidence increased over time along with senescence of the leaves which resulted in completely dark brown leaves which could not be scored for leaf blotch symptoms at 14 days post inoculation.

4.3.2 Pathogenicity to leaf using detached leaf inoculation assay

All four *Alternaria* species caused leaf blotch on Royal Gala in the detached leaf assay. No disease symptoms appeared in the control inoculations. The amount of fruit spot produced (disease severity) varied between isolates. Some isolates, particular, *A. alternata* isolate 46590 and *A. tenuissima* isolate 46414 were more aggressive and caused a mean disease severity of 75% higher than other isolates, while *A. arborescens* isolate 46571 was the least aggressive isolate. This indicates that multiple isolates should be used in tests for susceptibility to disease.

4.3.3 Pathogenicity to fruit using *in planta* fruit inoculation assay

In the *in planta* fruit inoculation assay with inoculum-soaked discs on Royal Gala, at least one isolate of *A. alternata*, *A. tenuissima* and *A. longipes* caused fruit spot. Out of the four isolates of each species used, three isolates of *A. tenuissima*, two isolates of *A. alternata* and one isolate of *A. longipes* isolate caused distinct fruit spots. None of the four isolates of *A. arborescens* and control inoculations caused fruit spot. Overall, the mean fruit spot disease severity was highest in *A. tenuissima* in both seasons followed by *A. alternata*. Between the seasons, fruit spot incidence and severity in the 2011-12 season inoculations was more than that of the 2012-13 season and isolates within species groups showed significant ($P<0.001$) variation in fruit spot severity in both seasons, which indicates the effect of seasonal variations on disease development.

4.3.4 Assessment of isolates of the four *Alternaria* species for their ability to cause leaf blotch and fruit spot

Since all the four *Alternaria* species caused leaf blotch, the severity of the disease was not dependent on the source of the isolate or the host tissue where the isolates were obtained from (Fig. 4.2). This indicates that isolates from either leaf blotch or fruit spot symptoms may be used in testing for disease resistance among varieties. However, it is not known if isolates of the same *Alternaria* species obtained from different host are pathogenic to apple leaves. Although all the isolates obtained from fruit spot and leaf blotch symptoms caused leaf blotch, only 40% of the isolates obtained from leaf blotch or fruit spot caused fruit spot symptoms in the pathogenicity assays. This indicates that a certain level of host tissue specificity exists in fruit spot

infection. This may contribute to why fruit spot disease is less prevalent compared to leaf blotch disease.

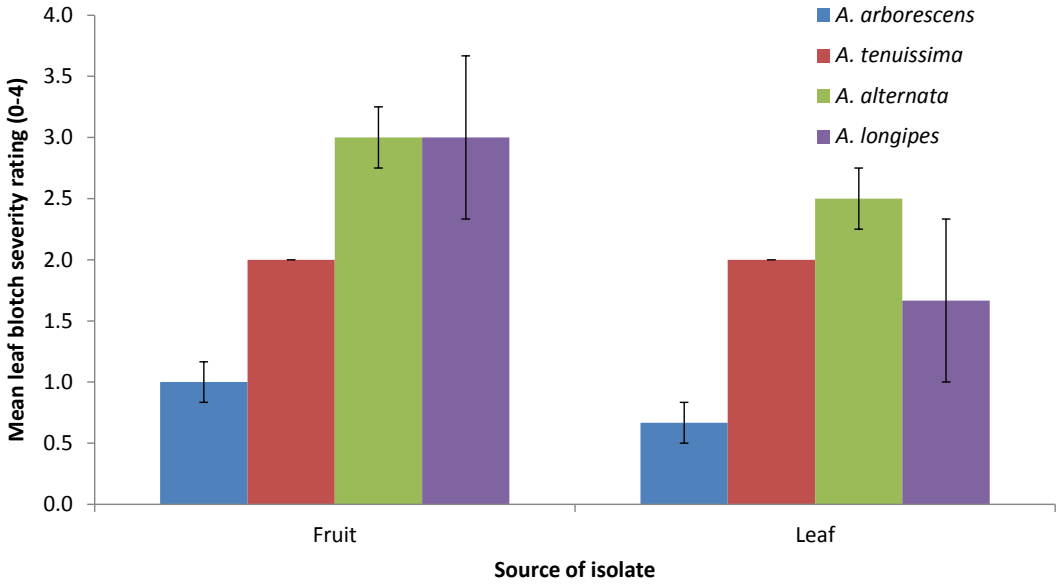


Fig. 4.2 Mean severity Alternaria leaf blotch on Royal Gala caused by isolates of four *Alternaria* species obtained from fruit spot and leaf blotch symptoms on apples in Australia. Severity rating is based on detached leaf inoculation assay using paper discs, which correspond percentage of inoculated area diseased; 0 = <1%; 1 = 1-25%; 2 = 25-50%; 3 = 51-75% and 4 = >75%.

4.3.5 Pathogenicity on different apple varieties

All the four *Alternaria* species were pathogenic on the leaves of the five apple varieties tested. However, there were variations in severity and incidence of leaf blotch caused by each isolate among the varieties (Fig. 4.3). *A. tenuissima* isolate 46414, *A. alternata* isolate 46590 and *A. longipes* isolate 46899 were the most pathogenic isolates showing very severe disease symptoms on all the five cultivars in both trials. The exploratory analysis of the leaf blotch severity for isolate × cultivar interaction showed that the first three PCA scores explained 84% of the total variance. The PCA1 (48%) component reflected the variance caused by susceptibility of the varieties to the isolates and PCA2 (22%) reflected the variation in the amount of disease caused by the isolates on the varieties. This showed that the variation in susceptibility to leaf blotch among the cultivars is somewhat associated with isolate or species. The correlation biplot of PCA1 and PCA2 scores showed that the isolates

were partitioned based on the leaf blotch severity levels on the most susceptible varieties (Fig. 4.4).

Comparison of the pathogenicity of the isolates on inoculated fruits of cultivars Royal Gala and FB22-47 showed that 38% (6) of the isolates of *A. alternata, A. tenuissima* and *A. longipes* were pathogenic on Royal Gala and only 13% (2) of the isolates of *A. tenuissima* were pathogenic on fruit of the FB22-47 only in the 2011-12 season. The reason for the disparity in fruit infection between the seasons may be due to variations in the environmental conditions between the 2011-12 season and the 2012-13 season.

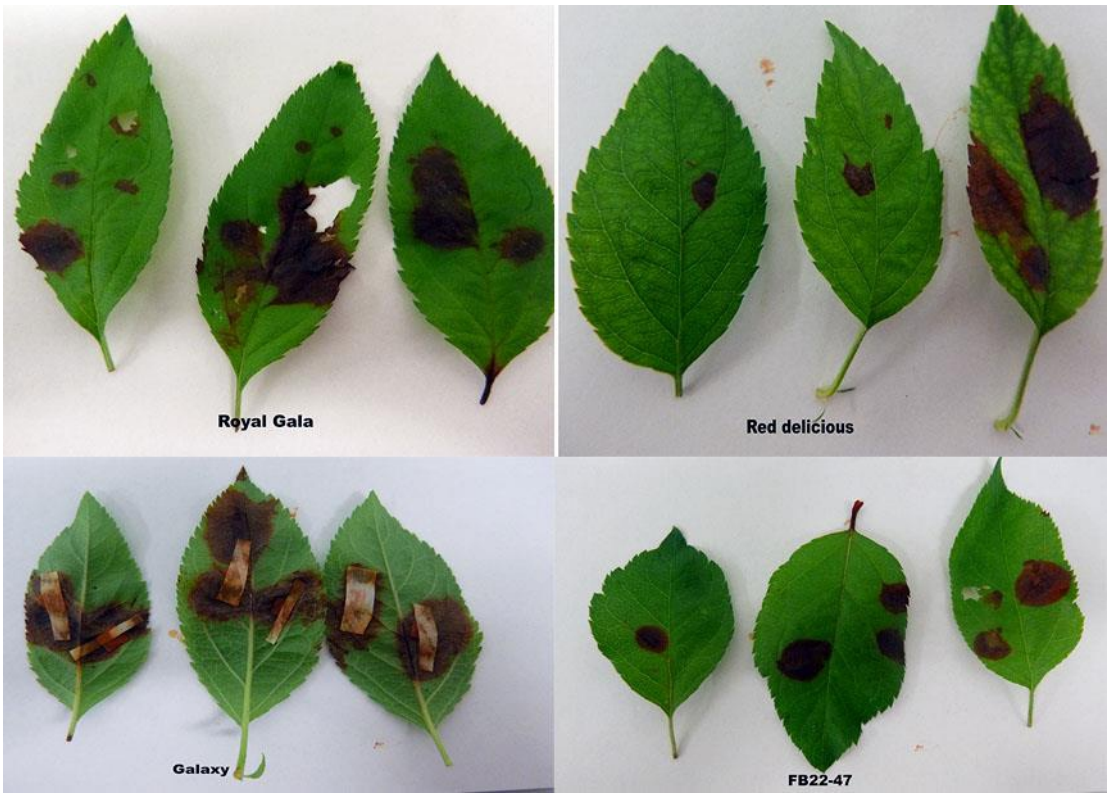


Fig. 4.3 Leaf blotch symptoms on four apple varieties from detached leaf assay using inoculum-soaked filter paper discs attached to the adaxial side of the leaf and attached in place with sterile tape.

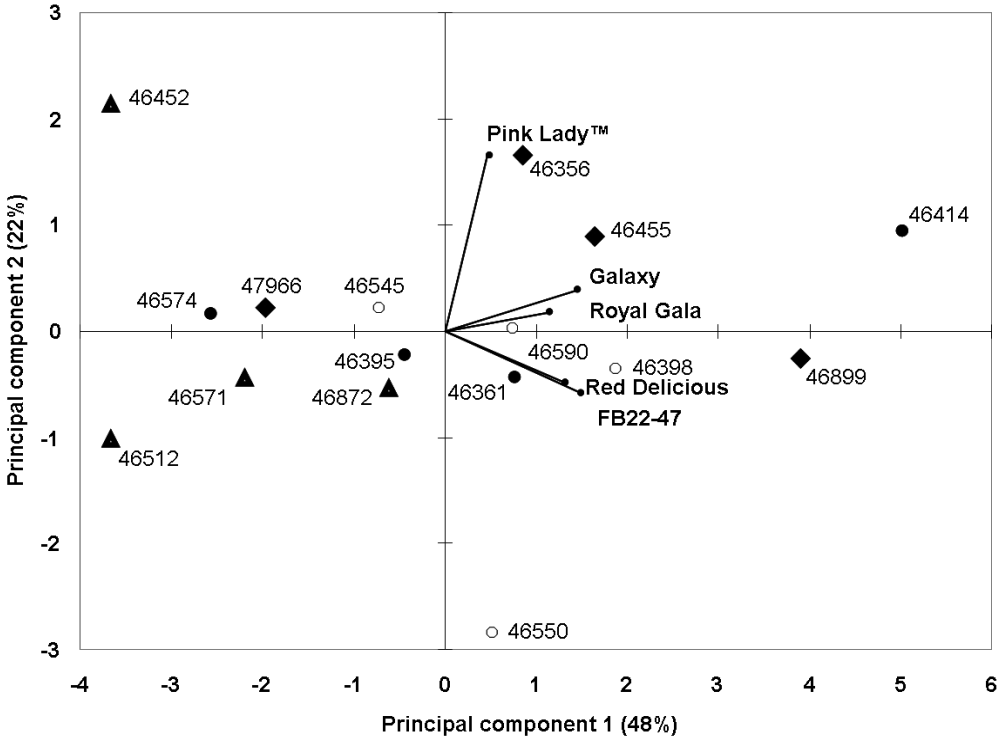


Fig. 4.4 Correlation biplot of the first two principal components that explained a total of 70% of the variance showing the pathogenic variation of leaf blotch of isolates of four *Alternaria* species; ▲ *A. arborescens*, ● *A. tenuissima*, ○ *A. alternata* and ♦ *A. longipes* on Royal Gala, Galaxy, FB22-47, Red Delicious and Pink Lady™.

4.4 Conclusions

This study clearly demonstrated that all the four *Alternaria* species; *A. arborescens, A. longipes, A. tenuissima* and *A. alternata* obtained from leaf blotch and fruit spot symptoms of apple in Australia can cause leaf blotch on the five varieties (Royal Gala, Galaxy, FB22-47, Red Delicious and Pink Lady) used in this study. In contrast, only three of the *Alternaria* species, except *A. arborescens* caused fruit spot on the two varieties (Royal Gala and FB22-47) used in the study. The reasons for the involvement of several *Alternaria* species in both leaf blotch and fruit spot disease of apple is still not known.

The *Alternaria* isolates within each species showed considerable variation in their pathogenicity and severity of the diseases they cause on leaves and fruits. This

observation is similar to report on leaf blotch and fruit spot of apple in Italy (Rotondo *et al*. (2012).

This study has revealed that even though leaf blotch is more widespread than fruit spot, the likelihood of fruit spot developing in areas where it has not been observed is very high. Thus, meaningful attention to Alternaria disease management is necessary in regions where fruit spot is not severe or not currently evident. Since isolates of *A. tenuissima, A. alternata* and *A. longipes* were obtained from other apple orchards in Tasmania, Western Australia, Victoria and South Australia (Harteveld et al., 2013b), these areas are similarly at risk, under favourable conditions, to Alternaria fruit spot as Qld and NSW. Differences in environmental conditions between these regions may influence disease severity and need to be considered.

This study has developed assays for testing pathogenicity of *Alternaria* on leaf and fruit of apple. Unlike leaf assays where detached leaves may be used, pathogenicity of fruit is more reliable when fruits are inoculated whilst still on the tree. It appears, fruit infection occur more readily when fruit is near maturity than at early fruit developmental stages, whereas, leaf infection may occur at any leaf stage irrespective of the age and size. Due to the variability among *Alternaria* species and isolates within the species, care must be taken when selecting isolates to evaluate apple germplasm for disease resistance.

4.5 Acknowledgements

Technical assistance provided by Mr. Allan McWaters and Dr. Shane Dullahide for field trials and maintenance of the potted apple trees at the Applethorpe Research Station, DAFF Qld are acknowledged.

5 Timing of infection and development of Alternaria leaf blotch and fruit spot under field conditions

Dalphy Harteveld, Femi Akinsanmi and André Drenth

5.1 Introduction

The five distinct seasonal physiological stages of the apple season including the green tip, flowering or bloom, fruit development, harvest and dormancy stages (Mooney et al., 2012), potentially could be the most susceptible period for *Alternaria* infection. Information of the timing of these stages in the orchard has been used to time control of other disease in apple (Mooney et al., 2012). Knowledge of the susceptible stage for Alternaria diseases will easily slot into the current apple diseases management systems.

Preceding chapters have shown that multiple *Alternaria* species occur on apple in all growing regions in Australia and can cause both leaf blotch and fruit spot. Although it is not known if different species infect apple at different stages or time or whether occurrence of a particular *Alternaria* species is dependent on certain conditions in the orchard, timing of application of control may be similar. Few studies (Filajdic & Sutton, 1992, Sawamura, 1972) have investigated features of the disease cycle of Alternaria leaf blotch and fruit spot, but there is disparity in the reports, mainly because different environmental factors are attributed to infection and disease development (Filajdic & Sutton, 1991, Sawamura, 1962, Thayer, 2005, Filajdic & Sutton, 1995, Tanaka et al., 1989). In some instances, in the USA, hours of leaf wetness and a temperature of about 23°C were considered important for leaf infection and disease development by *A. mali* (Filajdic & Sutton, 1992), whereas, in Japan, temperatures between 28°C and 30°C were considered as optimum conditions for leaf infection (Sawamura, 1972). Involvement of rainfall and relative humidity to leaf blotch disease development has been suggested (Filajdic & Sutton, 1992, Kim et al., 1986, Sawamura, 1972, Thakur & Nirupma, 2010, Yoon et al., 1989).

Generally in Australia, Alternaria leaf blotch and fruit spot symptoms become visible between December and February, this period coincides with fruit development in the summer months. However, many critical factors that are needed for infection and

disease development are not known. Such as, how many days or months before disease incidence did infection occur? What conditions are needed for infection and disease development? Are the spur or shoot leaves both important for fruit infection? What role does canopy architecture play in disease incidence and development? In Australia, both diseases occur in warm, humid summer months, but there is little information on the optimum conditions and climatic factors critical for infection and disease development in the orchards.

In the USA and Japan, the expression of initial symptoms of Alternaria leaf blotch has been reported to occur between the flowering stage to one month after bloom in (Sawamura, 1962, Filajdic & Sutton, 1991). Symptoms of fruit spot were mainly observed at harvest and after storage (Rotondo et al., 2012, Horlock, 2006). Anecdotal observations in the Australian orchards suggest leaf blotch incidence is higher in the lower part of the tree canopy than the top of the tree and fruit spots occur 6 to 8 weeks before harvest (Horlock, 2006). However, there is no systematic study on the timing of *Alternaria* infection on leaf and fruit. Therefore, the aim of this study was to determine the timing of infection and the associated climatic factors for Alternaria leaf blotch and fruit spot of apple needed for infection in Australia. The position of leaves and fruit play in the canopy with regards to in Alternaria leaf blotch and fruit spot infection was also examined.

5.2 Materials and methods

5.2.1 Field site and trial design.

Field trials were conducted on Royal Gala and FB22-47 trees at the Applethorpe Research Station, DAFF, Qld. Experimental design included a total of nine sample trees in three replicates per variety. The trees were separated with 10 to15 buffer trees between sample trees. Field trials were established in the 2010-11 and the 2011-12 seasons.

5.2.2 Timing of infection of Alternaria leaf blotch

5.2.2.1 Observation of leaf blotch incidence in orchard trees

Alternaria leaf blotch incidence was monitored on each sample tree at 2 to 3 weeks interval beginning at bloom stage until the tree dormancy stage. On each data collection date, recorded as the days after bloom (DAB) for each cultivar, a total of 60 shoots were randomly selected on all aspects and levels of the tree canopy. Ten sampling units were examined for leaf blotch symptoms. A sampling unit consisted of 10 spur leaves and 10 shoot leaves. Disease incidence was recorded as percentage of leaves showing symptoms.

5.2.2.2 Exposure and observation leaf blotch development in potted trees

In order to assess the timing of infection, three pots of glasshouse-produced trees each of FB22-47 and Royal Gala with 4-week-old leaves were routinely placed on the apple orchard floor to expose to natural *Alternaria* inoculum, at the different five developmental stages of the tree starting from 30 DAB to 130 DAB. Each pot was exposed for 3 weeks before returning to the glasshouse, initially kept in a chamber at above 90% relative humidity and 25°C conditions for 48 h before placing on the bench in the glasshouse (at about 23°C ± 4°C). Percentage of the leaves with symptoms on each tree was first recorded before (LB_0) incubating in the high humid conditions in the glasshouse, then, after incubation (LB_1) and at fortnightly intervals for 8 weeks ($LB_2 – LB_5$). Pieces of symptoms were excised and tested to confirm that the symptoms were caused by *Alternaria* sp. as described by Harteveld et al. (2013b).

5.2.3 Timing of infection of Alternaria fruit spot

5.2.3.1 *Observation of fruit spot incidence in natural conditions in apple orchard*

Fruits on three trees each of FB22-47 and Royal Gala were monitored for fruit spot at fortnightly intervals from 40 DAB until harvest (~135 DAB). Fruits randomly selected in the tree for all aspects and positions in the tree canopy. On each observation date, 150 fruits were arbitrarily selected in the canopy of each tree and examined for fruit spot symptoms. Disease incidence was recorded as the percentage of total fruit examined.

5.2.3.2 Observation of fruit spot incidence in inoculated fruits in tree canopy

In planta fruit inoculation was performed, fortnightly during fruit development stage starting at approximately 40 DAB until harvest, on fruits on three trees each of FB22-47 and Royal Gala using four isolates of *Alternaria* spp. (BRIP 46550; BRIP 46492; BRIP 46361 and BRIP 46455). In order to mimic natural infection, a spray inoculation method and procedure as previously described (section 4.2.1.1) was used. Fruit spot incidence was recorded fortnightly for 8 weeks as the percentage of total fruit examined.

5.2.4 Development and distribution of Alternaria leaf blotch in the tree canopy

Development and distribution of leaf blotch in the tree canopy was monitored on each sample tree as described above in sub section 5.2.2.1 at three sections of the tree canopy. Each section represents about ⅓ of the tree canopy height corresponding to low, middle and upper canopy heights. At each canopy height, 10 sampling units were examined for leaf blotch. Disease incidence was recorded as the percentage of total leaves infected per leaf type.

5.2.5 Development and distribution of Alternaria fruit spot in the tree canopy

Development and distribution of fruit spot in the tree canopy was monitored at fortnightly intervals from 40 DAB until 135 DAB at the three canopy heights (low, middle and upper) on 50 arbitrarily selected fruits. Disease incidence was recorded as the percentage of fruit with fruit spot symptoms at each observation date as described above.

5.2.6 The influence of climatic factors on leaf blotch and fruit spot development

Influence of weather conditions including daily minimum and maximum temperature, daily mean relative humidity and daily rainfall on disease incidence and development was explored using the data collected from the trials from July 2010 - July 2012. The weather data were obtained from the Bureau of Meteorology, Australia, Applethorpe station 041175 at the Applethorpe Research Station, DAFF, Qld.

5.2.7 Data analysis

Alternaria leaf blotch and fruit spot incidence data were analysed separately using GenStat statistical software. In order to examine if any significant differences exist

between the variables (DAB, canopy heights and leaf types), a repeated measures analysis of variance (RM-ANOVA) was performed with varieties and seasons as the main factors. Canopy height and leaf type were treated as fixed effects, whereas block effects included canopy height and leaf type within canopy height per tree. The disease incidence measured at different DAB in both seasons was used as the time factor. Relationships between the variables were examined by pair wise comparisons using Fisher's protected least significant differences test. Disease incidence data per season were further analysed as area under disease progress curve (AUDPC) (Madden et al., 2007), square root-transformed to stabilize variance with ANOVA. Effects of each and combination of climatic factors on leaf blotch and fruit spot incidence were explored using the average of each climatic factor between the periods of data collection dates including the 2 weeks preceding observation for the first period. All subset regression analysis based on a generalized linear model with normal distribution was performed to determine the best fit model of the climatic factors on each disease. The regression model was evaluated based on the adjusted coefficient of determination (R^2).

5.3 Results and discussion

5.3.1 Timing of infection of Alternaria leaf blotch

5.3.1.1 Observation of leaf blotch incidence in orchard trees

In the 2010-11 season, Alternaria leaf blotch symptoms were first observed at approximately 40 DAB on both spur and shoot leaves in both cultivars (Fig. 5.1). Overall, leaf blotch incidence increased from the initial 5% to 41% at 125 DAB in FB22-47, whereas, it increased from initial incidence of 2% to 25% at 110 DAB in Royal Gala. In contrast, in the 2011-12 season, initial leaf blotch incidence was <0.2% in both cultivars and the highest incidence was 44% in FB22-47 and 16% in Royal Gala at 150 DAB (after harvest) (Fig. 5.2). In both seasons and cultivars, extensive defoliation started after the highest disease incidence and continued until the tree dormancy stage.

Leaf blotch incidence occurred at similar DAB in both seasons, but further disease development was influenced by weather conditions. Delay in disease progression was

attributed to low (55 to 65%) relative humidity and rapid disease progression followed rain storm events which caused significant defoliation of diseased leaves from the canopy.

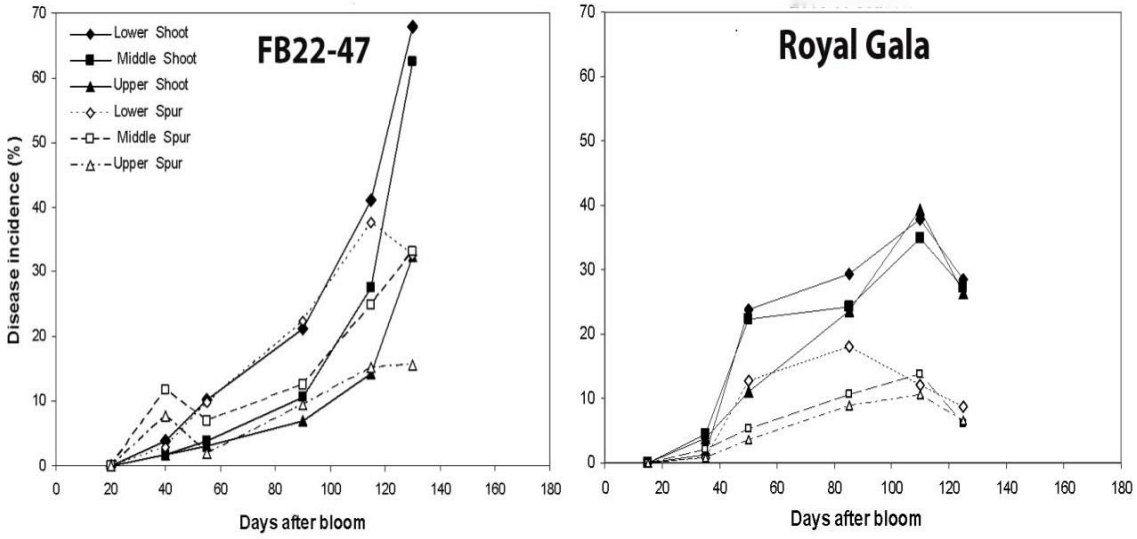


Fig. 5.1 Incidence of Alternaria leaf blotch in spur and shoot leaves in the lower, middle and upper canopy height of apple trees of the cultivars FB22-47 and Royal Gala in the 2010-11 production season.

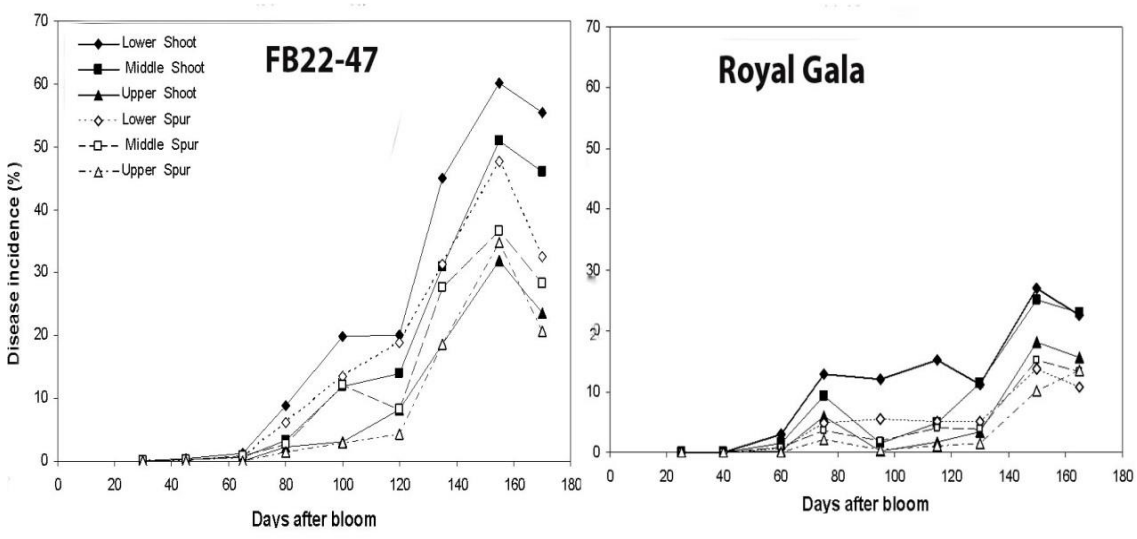


Fig. 5.2 Incidence of Alternaria leaf blotch in spur and shoot leaves in the lower, middle and upper canopy height of apple trees of the cultivars FB22-47 and Royal Gala in the 2011-12 production season.

5.3.1.2 Exposure and observation leaf blotch development in potted trees

Results of the sequential exposure of potted trees to natural infection showed that the most significant infections occurred 70 - 90 DAB and 90 -110 DAB (Fig. 5.3). At 30 - 50 DAB and 50 - 70 DAB, leaf blotch symptoms were consistently absent in both seasons and cultivars (Fig. 5.3). Comparison of the two varieties showed that leaf blotch incidence was significantly higher in Royal Gala with LB_0 of 30% and 7% than in FB22-47 with LB_0 of 7% and 3% in the 2010-11 and 2011-12 seasons, respectively. Although leaf blotch continued to develop 2 weeks after incubation in the glasshouse at LB_2 but was only significantly (P = 0.05) higher in FB22-47 in trees exposed at the 90 -110 DAB stage in the 2010-11 season (Fig. 5.3).

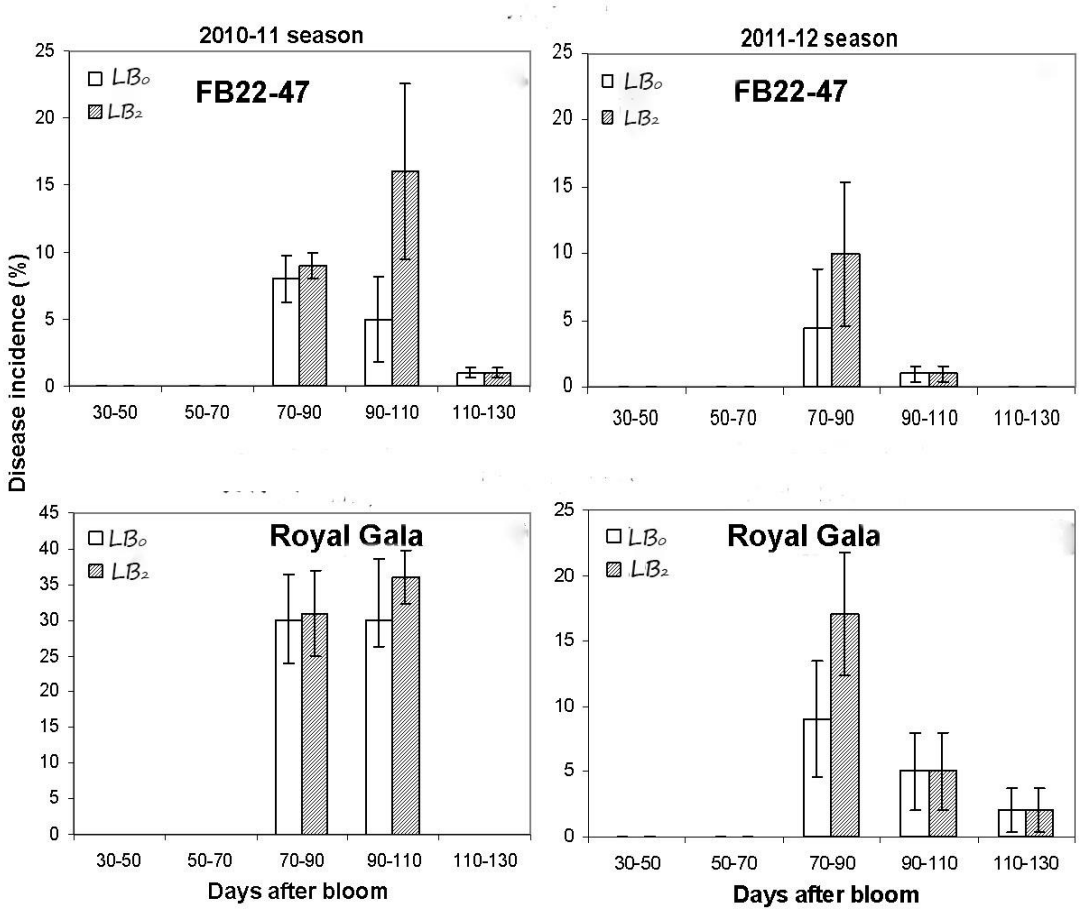


Fig. 5.3 Incidence of Alternaria leaf blotch in potted trees of the cultivars FB22-47 and Royal Gala) that were routinely exposed to natural infection in apple orchard in the 2010-11 season (left graphs) and the 2011-12 season (right graphs). LB_0 indicates leaf blotch incidence 20 days after exposure and LB_2 indicates leaf blotch incidence 2

weeks after incubation (or 34 days after exposure) in the glasshouse. Lines on bars indicate standard error.

5.3.2 Timing of infection of Alternaria fruit spot

5.3.2.1 Observation of fruit spot incidence in natural conditions in apple orchard

In the 2010-11 season, Alternaria fruit spot (0.4%) was first observed at 110 DAB in FB22-47 and at 115 DAB in Royal Gala. Disease incidence increased from 0.7% to 1.7% at 130 DAB just before harvest in Royal Gala but remained at 0.4% in FB22-47. In the 2011-12 season, there was no Alternaria fruit spot in both varieties.

5.3.2.2 *Observation of fruit spot incidence in inoculated fruits in tree canopy*

The results of the *in planta* fruit inoculations showed that among the four isolates, only two isolates (BRIP 46361 and BRIP 46550) caused fruit spot on Royal Gala but no infection developed in FB22-47. In Royal Gala, fruit spot occurred at all growth stages 2 weeks after inoculation. There was no significant correlation of fruit size and sugar content and/or growth stage with infections on the inoculated fruit. No fruit symptoms were observed in the detached fruit inoculations at all fruit stages.

5.3.3 Development and distribution of Alternaria leaf blotch in the tree canopy

Leaf blotch incidence was significantly higher in the lower canopy than in the upper canopy level (Fig. 5.4). This indicates that the primary source of inoculum may be from the orchard floor. This may be due to shading and closeness to moist grass early in the day, resulting in a prolonged higher humidity conditions and favourable microclimatic conditions at the lower canopy heights for leaf infection and disease development. Comparison of the shoot and spur leaves showed that leaf blotch incidence mainly higher in shoot leaves than spur leaves in both cultivars. In Royal Gala, leaf blotch incidence was almost twice as much in the shoot leaves than in the spur leaves (Fig. 5.4). At each canopy height, no significant effect of leaf type was observed. In the 2010-11 season, defoliation was first observed in spur leaves in the lower canopy in both varieties at about 20 days earlier (90-115 DAB) compared to higher canopy heights.

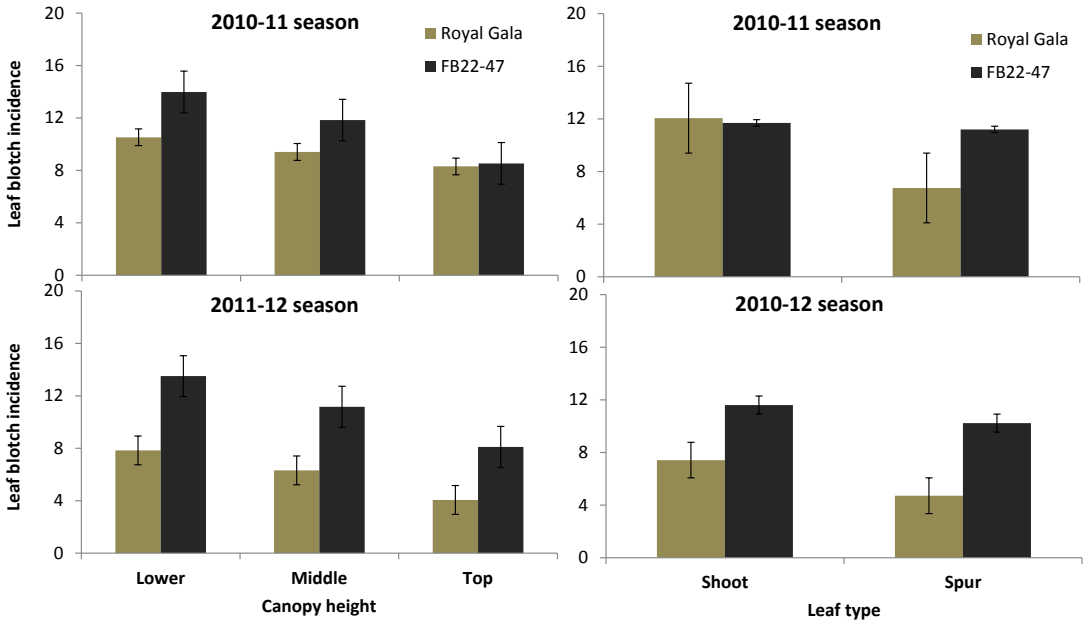


Fig. 5.4 AUDPC values of Alternaria leaf blotch incidence at different canopy heights and leaf types (shoot and spur) of two apple varieties in the 2010-11 and 2011-12 production seasons. Lines on the bars indicate standard errors.

5.3.4 Development and distribution of Alternaria fruit spot in the tree canopy

In the 2010-11 season, fruit spot mostly occurred at the lower canopy height at 115 DAB in Royal Gala from mean initial incidence of 2.2% to final incidence of 3.3% at 130 DAB. The final fruit spot incidence at the middle height was 1.6% and 0.2% at the upper canopy. In FB22-47, fruit spot was observed at 110 DAB with mean incidence of 0.6% at the lower height and 0.6% at the middle canopy. No disease was observed in the trees in the 2011-12 season. The higher occurrence of fruit spot at the lower height followed the early occurrence of leaf blotch at the same height. This suggests that fruit infection may have arisen from inoculum from the diseased leaves in close proximity to the fruit. The results also suggest that the optimum climatic conditions for leaf and fruit infection may be similar.

5.3.5 The influence of climatic factors on Alternaria leaf blotch and fruit spot disease in the field

Results of the exploratory analyses of the association of climatic factors to leaf blotch and fruit spot infection and disease development showed that the prevailing temperature, especially the minimum temperature, significantly influenced infection

and disease incidence. The contribution of high relative humidity and amount of rainfall in conjunction with temperature significantly increased leaf blotch incidence and disease development, whereas, temperature and daily rainfall influenced fruit spot development in the orchards. Studies from overseas have shown that the optimal temperatures for *Alternaria* infection in apple range between 25-31ºC (Filajdic & Sutton, 1992, Rotem, 1994, Sawamura, 1972) and infection is enhanced by the presence of moisture on the leaf and fruit surface (Rotem, 1994) and increases in relative humidity and rainfall (Harteveld et al., 2013a, Rotem, 1994, Yoon et al., 1989, Kim et al., 1986).

5.4 Conclusions

This study has clearly demonstrated the timing of infection of both Alternaria leaf blotch and fruit spot in Australian growing conditions and established the influence of temperature and moisture on disease incidence and development. Generally, Alternaria leaf blotch infections occurred from 40 DAB and continue throughout the season, resulting in premature defoliation in January-February, whereas, Alternaria fruit spot infections followed leaf blotch from about 100 DAB or about 2 - 3 weeks before harvest. All leaf stages and types are susceptible to infection and infection generally starts from the lower canopy height close to the orchard floor most likely due to a higher inoculum level resulting from rain splash and increased humidity lower in the canopy providing more opportunities for infection.

This study confirmed the anecdotal observation in the orchards that severities of both diseases tend to increase when warm and rainy conditions prevail in the summer months of Australia. In particular, warm days coupled with storms in the evenings between December and January significantly influenced Alternaria leaf blotch and fruit spot development.

Results of this study indicate that dense canopies especially close to the orchard floor should be avoided to reduce risk of infection. Timing of spray application fungicide for disease control should focus on reducing leaf blotch development from initial incidence and fruit spot from 100 DAB, especially when warm and wet conditions prevail.

5.5 Acknowledgements

Mr. Allan McWaters and Dr. Shane Dullahide for their assistance at the Applethorpe Research Station, DAFF Qld are acknowledged. Kerri Chandra (née Dawson) is acknowledged for assistance with the statistical analyses.

6 Sources and availability of *Alternaria* inoculum associated with leaf blotch and fruit spot of apples in the orchards

Dalphy Harteveld, Femi Akinsanmi and André Drenth

6.1 Introduction

Effectiveness of fungicide applications to control Alternaria leaf blotch and fruit spot in Australia is often erratic and choice of effective fungicide varies among regions (Horlock, 2006). At present an effective disease management strategy is urgently needed for both diseases. A lack of understanding of the disease cycle of the pathogen hinders development of effective control strategies. A critical aspect of disease control is good understanding of the source of inoculum, critical period when inoculum is available for infection and the factors that influence both systems in the orchard.

Overseas reports on the epidemiology of Alternaria leaf blotch have shown that spores of *A. mali* overwinter on various plant parts of apple trees. In North America, *A. mali* was found to overwinter in leaf residue on the orchard floor (Filajdic & Sutton, 1995), whereas, in Southern Japan the bark of nodes, internodes and on scaly leaves were reported to inhabit *A. mali* spores over winter months (Tanaka et al., 1989) and in Northern Japan the fungus resided in lenticels of diseased fruit, winter bud scales and diseased leaf residue (Sawamura & Yanase, 1964). Although these studies identified sources of inoculum, none indicated or quantified the relative importance of each source of inoculum in the orchard and the production dynamics during the season. A better understanding of the dynamics of the amount of spores produced from different potential sources of inoculum during the seasons could aid in better timing of disease control. Eradication of primary sources of inoculum has been shown to be a successful management option for plant diseases (Kim et al., 2010, Gomez et al., 2007).

Therefore, the aims of this study were to determine the relative role of leaf residue, canopy leaves, twigs and buds to *Alternaria* inoculum in the orchard and establish the main source of inoculum through the tree dormancy stage in the orchard. The influence of climatic factors on inoculum production was examined.

6.2 Materials and methods

6.2.1 Experimental design and sampling

Two commercial orchards at Applethorpe, Qld and trees at the Applethorpe Research Station, DAFF, Qld were selected for the trials. A total of nine trees were selected per orchard where samples of leaf residue, canopy leaves, buds and twigs were collected every 3-4 weeks from July 2010 until August 2012. At each sample collection date, the tree phenological stages as described in section 4.1 were recorded. Each commercial orchard was managed by the grower according to their established management practices. At each orchard and sampling date, four sampling units were obtained. These included 10 leaves (leaf residue) on the orchard floor under the tree, 10 leaves attached to the tree in the canopy, 20 buds on the tree and 8 pieces of >1 year old twigs obtained at random from each tree.

6.2.2 Assessment of amount of *Alternaria* spores

The samples were kept in cool conditions until assessed following a modified method as described by Scherm *et al.* (2008). Briefly, each sample unit was divided into two and incubated in moist conditions at 25°C in the dark for 72 h, thereafter, the surface area of each sample was recorded and each subsample was rinsed in 80 ml sterile distilled water containing 0.05% Tween80 (Sigma-Aldrich) to dislodge the spores. The solution was filtered through two layers of cheesecloth and 10 ml of the suspension was centrifuged at 6000 rpm for 10 min. and the resultant pellet was resuspended in 1 ml of sterile water. The concentrations of the *Alternaria* spores were determined in triplicates using a haemocytometer.

6.2.3 Confirmation that the *Alternaria* spores from the samples can cause disease

In order to determine if the *Alternaria* spores from the samples can cause Alternaria leaf blotch, detached leaf inoculations as described above in section 4.2.2 were performed on cv. Galaxy using spore suspensions from at least four different samples of leaf residue. Leaves were examined for Alternaria leaf blotch symptom development after 7 days. Single spore cultures were obtained from each spore

suspension and the identity of the *Alternaria* isolates was determined by morphology (Harteveld et al., 2013b).

6.2.4 Influence of climatic factors on inoculum production in the orchard

The influence of daily minimum and maximum temperature, relative humidity and rainfall on production of *Alternaria* inoculum in the orchard was examined as previously described in section 5.2.6.

6.2.5 Data analysis

In order to compare the plant parts, the relative amount of spores produced per sample was calculated as conidia/cm² sample surface area. Average number of spores/cm^2 was plotted per period between sampling dates. In order to compare spore production patterns during the different stages between years and sample units, the area under spore production curve (AUSPC) as described by Scherm *et al.* (2008) was calculated. The relationship among the climatic factors (mean temperature, mean relative humidity, mean rainfall, cumulative amount of rain and number of rainy days) and amount of spores produced from the samples was examined using correlation and model selection analyses using all-subsets regression in a generalized linear model procedure with Poisson distribution (log e) in GenStat. The most significant climatic factor and optimal subset selection for the model were determined using the adjusted R^2 values.

6.3 Results and discussion

6.3.1 Assessment of amount of *Alternaria* spores

Alternaria spores were obtained from all sample units (leaf residue, canopy leaves, buds and twigs) at all the three orchards and years. Overall, the amount of spores obtained from leaf residue was over one thousand-fold higher than those obtained from other sample units (Fig. 6.1). The number of spores obtained from canopy leaves was higher than twigs followed by buds. The amount of spores obtained from the sample units varied between collection dates throughout the seasons (Fig. 6.1). All sample units - leaf residue, canopy leaves, twigs and buds are potential sources of inoculum in Australian apple orchards, this finding confirms previous reports from

Japan (Sawamura & Yanase, 1964, Tanaka et al., 1989) and North Carolina, USA (Filajdic et al., 1995). However, this study established the relative importance of the different plant parts and their contribution to available inoculum in apple orchards. Overwintering of inoculum in twigs and buds has been shown to play significant roles in causing *Alternaria* diseases in peach and apricot (Yousefi & Shahri, 2009) and overwintering of *A. alternata* in buds has been reported to cause dead flower buds of pears (Wenneker et al., 2011).

Timing of spore dispersal is critical for infection. Peak numbers of spores were recorded from leaf residue in winter in late July in the 2010-11 season and early spring in August-September in the 2011-12 season (Fig. 6.1). This period corresponds to bloom stage when leaves are prone to infection. Generally, spores were recorded on the canopy leaves during the summer and autumn season (Fig. 6.1). The timing of peak numbers of spores in canopy leaves varied between the orchards and the seasons. Although spores were obtained from the twigs all year round, the amount was generally low (Fig. 6.1), while spores were recorded from buds at two distinct periods corresponding with spring and winter (Fig. 6.1). The most significant rate of increase in spore production during the production season occurred in leaf residue between dormancy stage and bloom stages, which signifies that leaf residue is a major source of inoculum in the orchards.

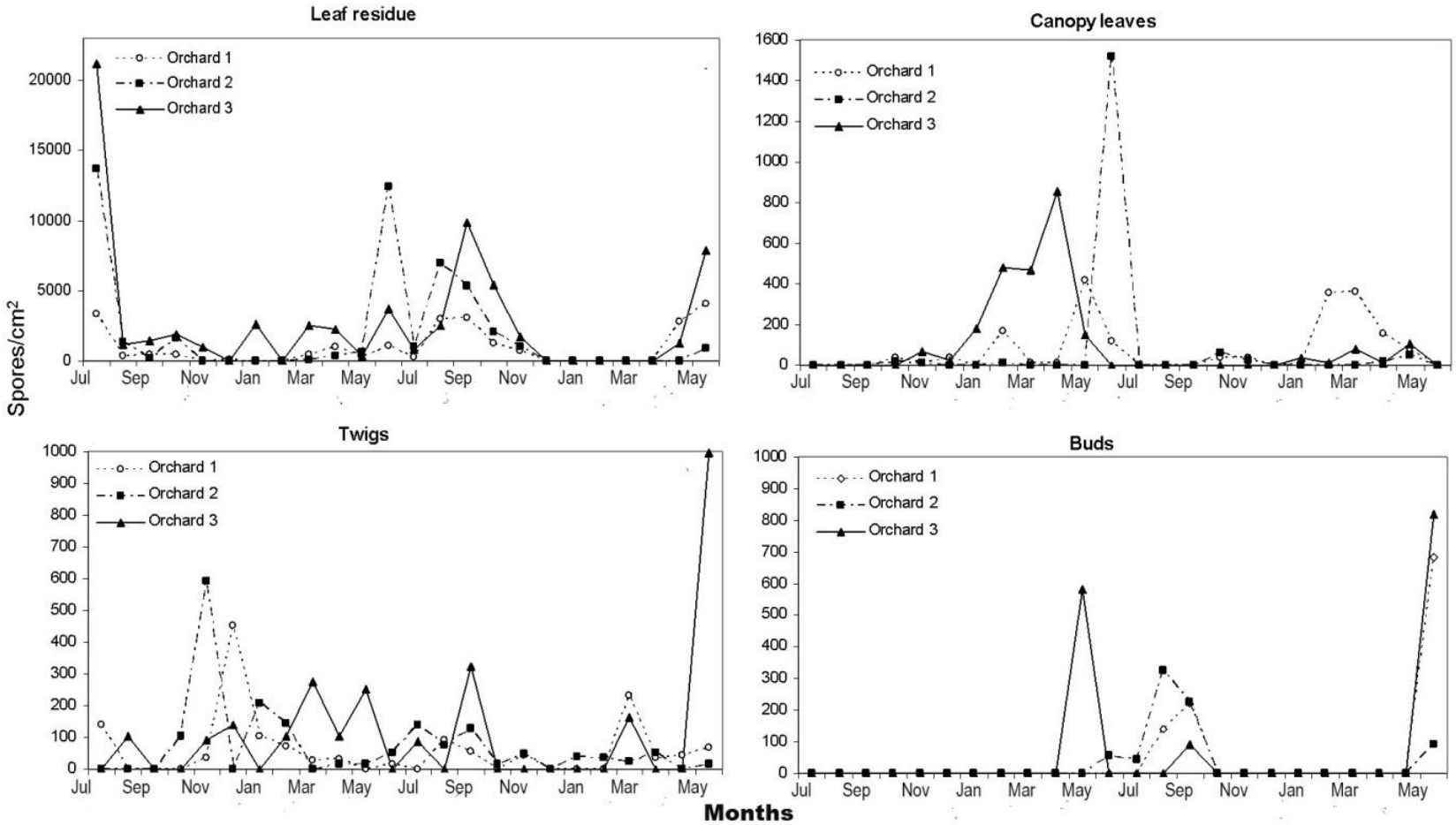


Fig. 6.1 Amount of Alternaria spores obtained on Royal Gala from different sample units (leaf residue, canopy leaves, twigs and buds) in three apple orchards at Applethorpe, Qld from July 2010 to July 2012.

6.3.2 Confirmation that the *Alternaria* spores from the samples can cause disease

The representative samples tested confirmed the identity of the spores obtained from the leaf residue as *A. tenuissima* and *A. alternata*. Results of the detached leaf inoculation assay showed that the spores from the leaf residue caused leaf blotch on the inoculated apple leaves, but no leaf blotch developed in the water control inoculations.

6.3.3 Influence of climatic factors on inoculum production in the orchard

The amount of spores produced throughout the season and between years was mostly influenced by temperature and rainfall. Environmental parameters that best describe spore production dynamics include mean temperature, mean rainfall and cumulative rainfall.

6.4 Conclusions

This study has substantially improved our understanding of the disease cycle of Alternaria leaf blotch and fruit spot of apple in Australian orchards. It showed that leaf residue, canopy leaves, twigs and buds are sources of inoculum and overwintering sites for *Alternaria* species causing leaf blotch and fruit spot of apple. Timing of spore dynamics indicated leaf residue as the major source of inoculum starting from bud break through fruit development period to dormancy stages. Climatic factors that influence spore production and seasonal variations were identified. Insight into factors which influence spore production may underpin the development of a disease forecasting system for both leaf blotch and fruit spot in Australia.

The fact that leaf residue serves as a major source of inoculum in the orchards may provide options for control such as the removal of leaf litter to be considered in disease control practices. Practical implications of our findings include the eradication of leaf residue from the orchard floor. This may be achieved by application of urea (Gomez et al., 2007, Sutton et al., 2000), mulching (Gomez et al., 2007, Holb, 2006), covering by plastic foil (Holb, 2006), the application of lime sulphur (Holb, 2006), shredding of the leaf litter (Sutton et al., 2000), propane flamers (Desilets et al., 1997)

and physical removal of the leaf residue (Gomez et al., 2007, Holb, 2006). The efficacy of these methods to reduce Alternaria leaf blotch and fruit spot development should be tested.

In addition, the occurrence of the spores in the canopy in twigs signifies the importance for sanitation after harvest to clean out the tree. The best timing for control and prevention of spore accumulation in twigs is at green tip stage or as postharvest fungicide spray application. Spray application of urea (Norton et al., 2012) may be used to remove remnant leaves in the canopy. Further research is needed to establish the most efficient practice to reduce *Alternaria* inoculum in the Australian apple orchards.

6.5 Acknowledgements

We acknowledge the assistance of the growers and staff of Applethorpe Research Station who provided access to the orchards for sample collection. Thanks to Kerri Chandra (née Dawson) for assistance with the statistical analyses.

7 Disease cycle of Alternaria leaf blotch and fruit spot of apple

Dalphy Harteveld, Femi Akinsanmi and André Drenth

7.1 Disease cycle

The research findings contained in this report provide a fundamental understanding of the disease cycle of Alternaria leaf blotch and fruit spot of apple in Australian orchards (Fig. 7.1). The disease cycle shows that the production of spores from the overwintering sites found within the orchards and tree canopy starts in spring and continues throughout the production season. *Alternaria* spores are most likely dispersed by rain-splash from leaf residue, twigs, and diseased leaves in the canopy from green tip stage throughout the season, when warm and wet conditions prevail.

First appearance of leaf blotch may be expected from 40 DAB and fruit spot from 100 DAB at spring and summer seasons. Fruit infection is expected to occur at near maturity, and most likely at high levels of leaf blotch in the tree canopy near fruit maturity stage, in particular 2 -3 weeks prior to harvest. Leaf blotch epidemics occur when warm temperatures coincide with incessant rainfall. Fruit infection is also favoured at ambient temperatures close to 26°C in the presence of free water and/or high relative humidity.

Diseased leaves defoliate more readily near fruit maturity in the summer months and may remain on the orchard floor as residue through the dormancy stage. This will serve as reservoir for the spores for the following seasons.

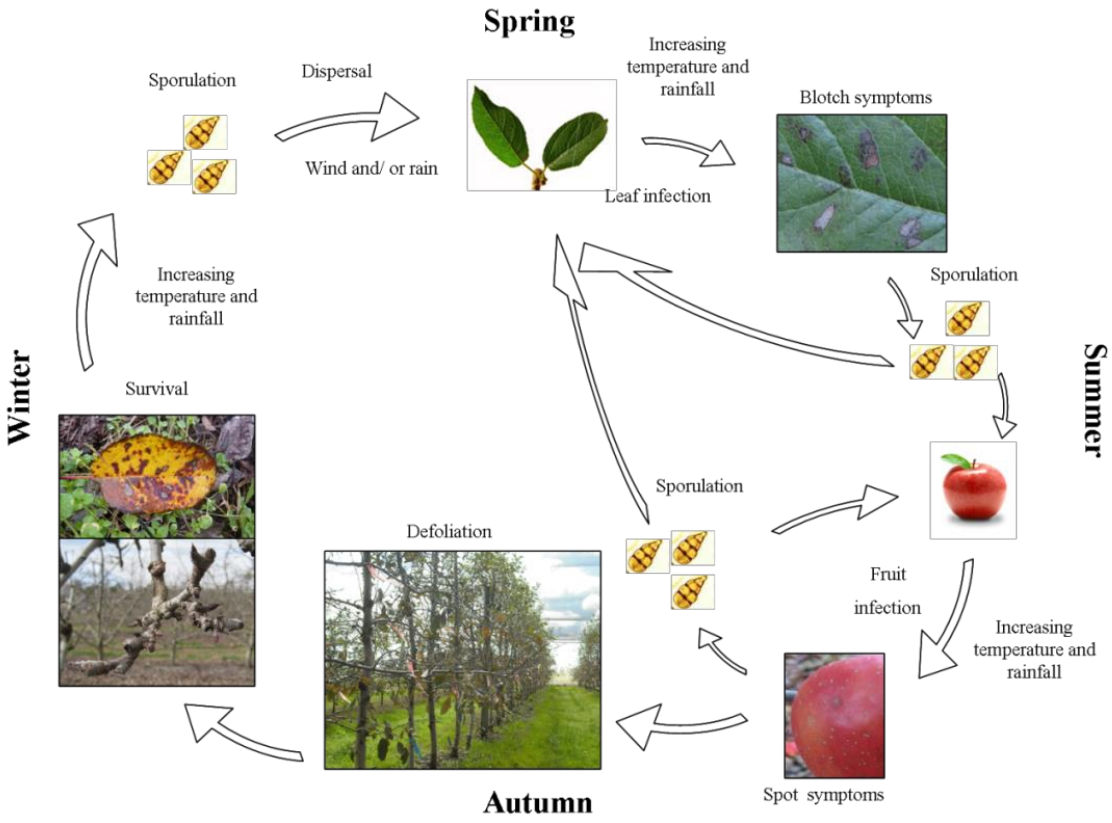


Fig. 7.1 Disease cycle of Alternaria leaf blotch and fruit spot of apple in Australia.

8 General discussion and recommendations

8.1 General discussion

8.1.1 Economic analysis of impact of Alternaria leaf blotch and fruit spot

The impact of Alternaria on apple production has two components; direct impact on fruit quality due to fruit spot and reduction of productive capacity of the tree in the following seasons due to leaf blotch as a result of premature defoliation.

An analysis of fruit rejects at a commercial apple orchard and packing shed at the Granite Belt, Qld revealed that out of every 10 bins (380kg fruit/bin), at least one bin is full of fruit rejects. About 42% of the total rejects is due to Alternaria fruit spot. Therefore, loss due to fruit spot is approximately 4.5% of the total yield.

In Qld, apple occupies about 1500 ha with an estimated value of $50 million. Thus, the impact of Alternaria fruit spot in Qld alone is estimated at $2.25 million per annum without factoring in the costs of control using fungicides, harvesting and sorting costs. This is a significant impact on productivity. This analysis was performed in the 2011-12 seasons when climatic conditions were unfavourable for Alternaria fruit spot incidence. Therefore, more significant yield losses may occur when disease-conducive conditions prevail and adequate control measures are not employed.

In addition to lowering of fruit quality, severe premature leaf defoliation leads to long term reductions in tree vigour and yield when after harvest carbohydrate levels are not restored prior to winter. The exact impact of this component of the disease is much harder to quantify and has not been measured as part of this project. Alternaria leaf blotch and fruit spot are particularly significant because they affect high value apple varieties such as Royal Gala, Pink Lady, Fuji and Red Delicious.

8.1.2 Key findings that underpin development of disease management strategy

In this project we have addressed the following issues on disease cycle of Alternaria leaf blotch and fruit spot of apple:

1. Identity and distribution of the causal pathogens in Australian apple orchards.

2. The existence of differences in levels of pathogenicity among the four different *Alternaria* species groups.
3. Environmental conditions needed for infection and disease development.
4. Stages when leaf and fruit are most susceptible to infection.
5. Source and timing of inoculum dispersal in the orchard
6. Comparison of disease severity and incidence on different varieties.
7. The role leaf litter plays in carrying over the disease between seasons and identification other sources of inoculum.

8.2 Recommendations for disease management

8.2.1 Integrated management strategy

Based on the findings achieved in this project as described in the preceding chapters, an integrated disease management strategy was developed. The proposed management strategy for Alternaria leaf blotch and fruit spot is a four-step approach;

1. Removal of leaf residue using enhanced leaf decomposition agents e.g. urea. Rationale: removal of a main source of inoculum.
2. Post-harvest cleanup of tree canopy with fungicide spray application using any registered protectant fungicide. Rationale: reduce residual inoculum.
3. Timing of spray applications (Fig. 8.1) to reduce Alternaria leaf blotch disease progression above the critical limit in October-December. Rationale: prevent extensive leaf defoliation and source of inoculum for fruit infection (Fig. 8.1).
4. Timing of fungicide applications to prevent fruit infection in mid-January-February. Rationale: prevent fruit infection.

8.2.2 Critical issues for effective implementation of recommendations

- The critical control strategy for Alternaria leaf blotch and fruit spot is orchard hygiene. This targets the source and amount of inoculum available to cause infection during the growing season.
- Spray applications of fungicides (using recommended fungicides in apples such as Delan®, Pristine®, or Polyram®) at the bud burst period, to clean the tree canopy which is a common practice in the industry, may invariably reduce incidence of Alternaria leaf blotch.

- Currently in Australia there are about 18 different fungicides registered for use on apples. The routine fungicide spray applications to control other diseases such as scab are most likely to reduce Alternaria leaf blotch disease severity without the need for any additional spray applications if the principles of orchard hygiene described above are adopted.
- Leaf blotch incidence increases rapidly during periods with high relative humidity (above 65%) and warm temperatures (26 - 33°C). An initial incidence of less than 5% of leaves infected can result in over 20-45% of leaves infected at 110 days after bloom, causing severe leaf defoliation.
- Risk of Alternaria fruit spot incidence is high when Alternaria leaf blotch is severe between December and January.
- The key disease management strategy for fruit spot is to monitor incidence and severity of leaf blotch.
- If necessary, fungicide spray applications (using the recommended fungicides for apples such as Delan® or Polyram®) should be applied from January-February at 90 DAB if the leaf blotch incidence is more than 15%, which practically equates to about one out of every six leaves expressing leaf blotch symptoms in January (3-4 weeks before fruit maturity or harvest).
- Fruit infections may occur at about 110 DAB if leaf infections are not controlled.

8.2.3 Validation of disease management strategy

The proposed disease management strategy was communicated to growers in 2011. Reports of results in reduction of diseases in orchards that adopted the control strategy have demonstrated its effectiveness. Results of the field trials in the 2011-13 seasons showed that incidence of Alternaria leaf blotch was significantly reduced when leaf residue was removed from under the tree canopy.

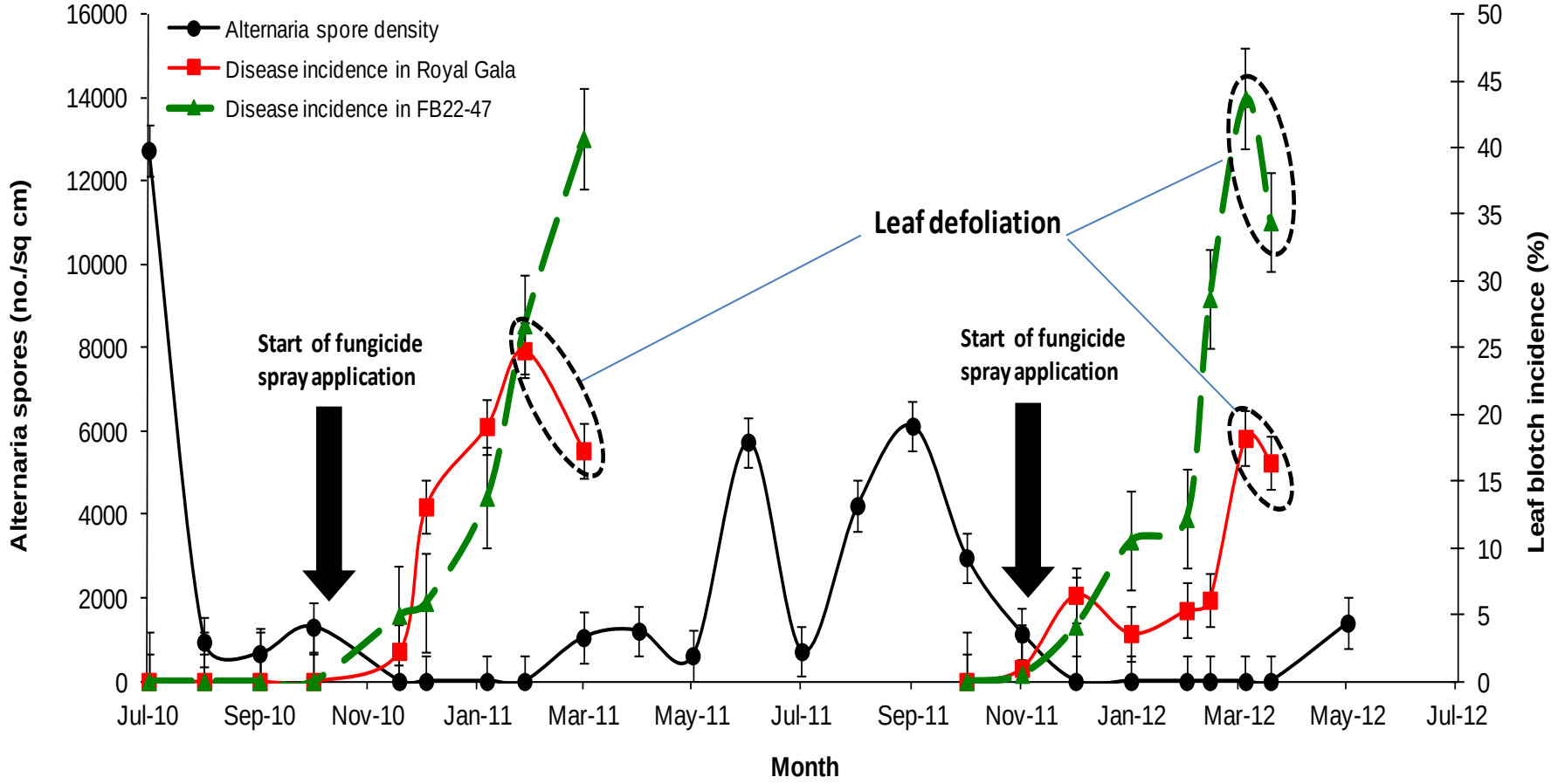


Fig. 8.1 Progress of Alternaria leaf blotch on two varieties of apple and the mean amount of spores of *Alternaria* spp. obtained from leaf residue in apple orchards at Applethorpe, Qld. Black arrows indicate proposed timing of fungicide spray application for leaf blotch control; circles around disease progress indicate timing of extensive defoliation of diseased leaves.

9 Proposed future research priorities

9.1 Further research studies on Alternaria diseases

9.1.1 Establish the impact of Alternaria leaf blotch and fruit spot at a national scale

The concern of apple growers in other states than QLD and NSW in Australia where similar *Alternaria* species have been isolated from apple leaves in their orchards, but severe fruit spot has not been reported to cause production limiting levels of loss as in Qld and NSW are reasonable. This project has shown that under favourable climatic conditions, significant production-limiting losses due to Alternaria may be expected in all states. Australian apple orchardists currently affected by production limiting levels of Alternaria leaf blotch and fruit spot infection are in the Granite Belt in Qld, Bilpin/Berambing and Orange in NSW as well as Manjimup and the Perth Hills in Western Australia. Other Australian apple growers, especially those in high spring/summer rainfall production areas are at high risk of being severely affected. Anecdotal fruit spot outbreaks have also been reported in the Adelaide Hills in South Australia and the Yarra Valley/Dandenong Ranges in Victoria. Under favourable climatic conditions and presence of adequate inoculum in the orchard, significant production-limiting losses could be more widespread in all apple producing regions in Australia. However detailed information is still lacking concerning;

- How widespread Alternaria fruit spot is in Australia?
- The extent of the overall economic impact of the occurrence of fruit spot to the Australian apple industry?

9.1.2 Effect on fruit quality

Species of *Alternaria* are known to produce many toxic metabolites, some of which help them to invade host plants. Some isolates are pathogenic and cause disease symptoms but some Alternaria species may also be present as saprophytic strains causing fruit spoilage such as mould core rot of apple when the fruit ripens. Unlike the mycotoxins produced by other fungal species such as *Aspergillus*, *Fusarium* and *Penicillium*, the effects of the *Alternaria* toxins have not received the same attention. Consumers generally avoid fresh apple fruits that are visibly mouldy, and therefore escape mycotoxin exposure through the consumption of fresh fruit. However, the risk

associated with contamination of the apple due to Alternaria toxins is twofold. First, from fruit infected with fruit spot sold as fresh fruits and second due to infected fruits with fruit spot and mouldy-core rot used in the production of apple juice giving rise to the presence of toxins in processed apple products (Logrieco et al., 2009). Alternaria mycotoxins should not be underestimated and the following three areas may need further attention:

- Since we have established the identity of *Alternaria* species causing fruit spot in Australia, including *A. alternata,* wider surveys on the potentially contaminable fruits, is an important key for establishing the toxicological risk due to *Alternaria* contamination due to fruit infection.
- Do all the four Australian *Alternaria* species causing leaf blotch and fruit spot produce similar metabolites?
- If yes, are the metabolites of biological and food quality significance?

9.1.3 Disease resistance

Although certain varieties are deemed to be susceptible, there is no information whether other varieties are indeed resistant. These varieties may be equally susceptible to *Alternaria* infection and may be severely affected when conditions are favourable for disease development and epidemics. Therefore, future research questions and issues include:

- Are there resistant commercial varieties?
- How susceptible are the new apple varieties to fruit spot and leaf blotch under different environmental conditions? Development and adoption of cultivars with specific resistance to a particular pathogen such as for example scab may inadvertently increase their susceptibility to other pathogens.
- Further research is necessary to screen and determine the levels of susceptibility of major and new apple varieties to Alternaria in Australia.

9.1.4 Effect of rootstock on Alternaria diseases

During field visits to growers' properties, we observed significant differences in initiation of natural leaf senescence after harvest in the same variety but on different rootstocks. The importance of prolonged 'greening' of trees after harvest on disease

development and levels of carryover inoculum to disease development on late maturing varieties and between seasons needs to be investigated.

- The effect of rootstock and scion interaction on expression of Alternaria disease symptoms is not known.
- Whether certain rootstocks predispose the scions more readily to leaf and fruit infection may need to be investigated.

9.1.5 Fungicide resistance and efficacy

A lot of previous research effort prior to this project has been directed towards screening fungicides for their efficacy against Alternaria and other apple diseases. At present in Australia there are about 18 different fungicides registered for use on apples. These fungicides fall into different classes of active ingredients. Reduced use of broad spectrum fungicides, variable weather conditions and an uneven distribution of pathogenic Alternaria species may account for the variation in efficacy of fungicides in different areas. Many fungicides and especially ones with a more specific mode of action have given variable results in the control of Alternaria. A lot of this variability may be due to differences in composition of the dominant *Alternaria* species and variable environmental conditions creating a scenario for high risk of development of fungicide resistance resulting in poor disease control. Therefore,

- Routine monitoring of pathogen sensitivity to fungicides in commercial orchards is necessary.
- Future research project should include proactive fungicide resistant management strategies to prolong the lifespan of currently registered Agrochemicals in the apple industry.

9.1.6 Alternative hosts and cross-infectivity

An important part of a pathogens lifecycle can occur on alternative host plants. Similar pathogens belonging to the *A. tenuissima*-species group, *A. alternata* and *A. arborescens* have also been reported to cause mouldy core of apple and diseases in other fruit and tree crops in Australia. The association and relationship of isolates of the four Australian *Alternaria* species involved in leaf blotch and fruit spot with

mouldy core rot in Australia is not known and further investigations may need to address;

- Whether these Alternaria pathogens simultaneously cause fruit spot and mouldy core.
- Whether these species from other hosts can also cross infect apples to cause Alternaria leaf blotch and fruit spot.

In some apple growing areas, numerous abandoned orchards and many rogue apple trees exist in close proximity to commercial orchards. These abandoned orchards and rogue trees are potential sources of *Alternaria* inoculum for commercial orchards, therefore, making achieving effective disease control very challenging.

- Future research should investigate the importance of this scenario in leaf blotch and fruit spot development in managed commercial orchards.

9.2 Endemic and exotic apple pathogens

All crop plants are under continuous attack from pests and diseases. In addition to endemic diseases a great many exotic pests and diseases exists which can adversely impact on the production of apples. In addition to incursion of exotic pathogens, endemic ones also have the ability to evolve to overcome disease resistance in the host plant or develop resistance to fungicides.

In addition the registration of existing and new Agrochemicals is under constant review giving rise to withdrawal of previous effective chemicals due to environmental or health issues. Hence, effective control of plant diseases requires constant research and regular changes to control strategies and application of different Agrochemicals to respond to these changes. In order to do this a flexible research capability is needed which can respond to short and long term pest and disease issues and provide the industry with cost effective ways to control plant diseases. Essential research priorities for endemic diseases and the need to maintain local expertise for both endemic and exotic diseases of apple in Australia are needed to protect the productivity and profitability of the Australian apple industry.

10 Technology transfer and information dissemination

10.1 Research outputs and industry communications

Research findings were communicated to the industry on a routine basis. Several modes of extension and communication were used throughout the project. These include

- visits to meet growers in their orchards;
- discussions at industry meetings including orchard walk' events;
- oral presentations at industry meetings;
- routine communication with extension officers;
- interaction with APAL industry development officer; and
- published articles in growers' journals and newsletters.

10.2 List of research outputs (2009-2013)

10.2.1 Scientific journal publications

1. Harteveld DOC, Akinsanmi OA, and Drenth A, 2013. Multiple *Alternaria* species groups are associated with leaf blotch and fruit spot diseases of apple in Australia. *Plant Pathology* 62, 289-297.
2. Harteveld, DOC, Akinsanmi OA, Chandra, K and Drenth A. 2013. Timing of infection and development of Alternaria diseases in the canopy of apple trees. *Plant Disease*. In Press.
3. Harteveld DOC, Akinsanmi OA, Dullahide S and Drenth A 2013. Sources and seasonal dynamics of *Alternaria* inoculum associated with leaf blotch and fruit spot of apples. *Crop Protection.* In press.
4. Harteveld DOC, Akinsanmi OA, and Drenth A, 2013. Pathogenic variation of *Alternaria* species associated with leaf blotch and fruit spot of apple in Australia. *European Journal of Plant Pathology (under review*)

10.2.2 Scientific conference presentations

1. Harteveld DOC, Akinsanmi OA, Drenth A, 2013. The disease cycle of Alternaria leaf blotch and fruit spot of apple. In *19th Biennial Australasian Plant Pathology Conference*, 25 – 28 November, Auckland, New Zealand.
2. Harteveld DOC, Akinsanmi OA, Drenth A, 2013. Alternaria on apples. In *QAAFI Annual Research Meeting*, 6-7 August, Redcliff, Australia.
3. Harteveld DOC, Akinsanmi OA, Drenth A, 2013. Alternaria fruit spot new directions. In *Innovate or Real-estate Fruit Industry Conference*, 17-19 July, Gold Coast, Australia.
4. Harteveld DOC, Akinsanmi OA, Drenth A, 2012. Aetiology and epidemiology of Alternaria leaf blotch and fruit spot of apples in Australia. In *American Phytopathology Society Annual Meeting*, 4-8 August, Providence, Rhode Island, USA.
5. Harteveld DOC, Akinsanmi OA, Drenth A, 2012. Epidemiology of Alternaria leaf blotch and fruit spot of apples in Australia. In *QAAFI Annual Research Meeting*, 11 July, Gold Coast, Australia.
6. Harteveld DOC, Akinsanmi OA and Drenth A, 2011. Disease cycle of Alternaria in apples. In *Proceedings of the 4th Asian Conference on Plant Pathology* and *18th Biennial Australasian Plant Pathology Society conference*, 26-29 April 2011, Darwin, Australia.
7. Neilsen M, Drenth A and Akinsanmi OA. Characterisation of *Alternaria* species causing leaf blotch and fruits spot in apples in Australia. In *Proceedings of the 4th Asian Conference on Plant Pathology* and *18th Biennial Australasian Plant Pathology Society conference*, 26-29 April 2011, Darwin, Australia.

10.2.3 Industry magazine articles

1. Around the Orchard' - Newsletter of the Apple and Pear Growers Association of South Australia Inc. July 2013 Vol. 5; Issue 6; page 3.
2. Orchard Plant Protection Guide for deciduous fruit in NSW 2012-13 pages 138-140. http://www.dpi.nsw.gov.au/data/assets/pdf_file/0006/249729/Orchard-plant-protection-guide-2012-13.pdf

3. Harteveld DOC, Akinsanmi OA, Drenth A, 2012. Alternaria leaf blotch and fruit spot of apples in Australia. *Fruit West Magazine*. Perth, Australia: Agricultural Produce Commission, Vol. 3.4, 30-32.
4. Harteveld DOC, Akinsanmi OA, Drenth A, 2011. Alternaria leaf blotch and fruit spot of apples in Australia. *Australian Fruit grower*. Melbourne, Australia: Apple and Pear Australia Ltd. Vol. 5, 16-17.

10.2.4 Study travel report

1. Harteveld DOC, 2012. Summary on Alternaria and apple related research. Apple and Pear Australia Limited. http://apal.org.au/

10.2.5 Industry presentations and growers meetings

1. Dalphy Harteveld, Femi Akinsanmi, André Drenth, 2013. Alternaria fruit spot new directions. In *Innovate or Real-estate Fruit Industry Conference*, 17-19 July, Gold Coast, Australia.
2. Future orchards presentations November 2012 oral presentation at Stanthorpe Orchard walk 8-11-12; and distributed the presentation to all apple regions.
3. Field visits Bilpin 30 May- 1 Jun 2012
4. Dalphy Harteveld and André Drenth - Research update on Alternaria leaf blotch and fruit spot of apples in Australia. Oral presentation at Stanthorpe, Qld, November 2012.
5. Field visits to apple growers in Bilpin, New South Wales; 19-20 May 2011.
6. Future orchards presentations November 2011 oral presentation at Stanthorpe, Qld Orchard walk.
7. Grower Day Presentation and field visits. Bilpin 24-25 October 2011.
8. Future orchards and orchard walks, Stanthorpe, Qld (2009-2013).

11 Bibliography

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ, 1990. Basic local alignment search tool. *Journal of Molecular Biology* **215**, 403-410.

Andrew M, Peever TL, Pryor BM, 2009. An expanded multilocus phylogeny does not resolve morphological species within the small-spored *Alternaria* species complex. *Mycologia* **101**, 95-109.

Bulajic A, Filajdic N, Babovic M, Sutton TB, 1996. First report of *Alternaria mali* on apples in Yugoslavia. *Plant Disease* **80**, 709.

Carbone I, Kohn LM, 1999. A method for designing primer sets for speciation studies in filamentous ascomycetes. *Mycologia* **91**, 553-556.

Chennell A, Bates V, Williams D, 2002. Apple maturity management. In. Melbourne, Victoria, Australia, 38. (Environment DONRA, ed.)

Desilets H, Rochefort S, Coulombe J, Yelle S, Brodeur J, 1997. Potential of propane flamers for reduction of apple scab primary inoculum on orchard ground. *Hortscience* **32**, 267-270.

Dullahide S, 2009. Better understanding of fruit spot will cut fruit losses *Apple and pear industry report 2008-2009*, 4.

Filajdic N, Sutton TB, 1991. Identification and distribution of *Alternaria mali* on apples in North Carolina and susceptibility of different varieties of apples to Alternaria blotch. *Plant Disease* **75**, 1045-1048.

Filajdic N, Sutton TB, 1992. Influence of temperature and wetness duration on infection of apple leaves and virulence of different isolates of *Alternaria mali*. *Phytopathology* **82**, 1279-1283.

Filajdic N, Sutton TB, 1995. Overwintering of *Alternaria mali*, the causal agent of Alternaria blotch of apple. *Plant Disease* **79**, 695-698.

Filajdic N, Sutton TB, Walgenbach JF, Unrath CR, 1995. The influence of European red mites on intensity of Alternaria blotch of apple and fruit quality and yield. *Plant Disease* **79**, 683-690.

Gagkaeva TY, Levitin MM, 2000. Identification of the pathogen causing leaf spots on apple trees in Krasnodar province orchards. *Mikologiya i Fitopatologiya* **34**, 58.

Gao LL, Zhang Q, Sun XY, *et al.*, 2013. Etiology of moldy core, core browning and core rot of Fuji apple in China. *Plant Disease* **97**, 510-516.

Gomez C, Brun L, Chauffour D, Le Vallee DD, 2007. Effect of leaf litter management on scab development in an organic apple orchard. *Agriculture Ecosystems & Environment* **118**, 249-255.

Harteveld DOC, Akinsanmi OA, Chandra K, Drenth A, 2013a. Timing of infection and development of Alternaria diseases in the canopy of apple trees. *Plant Disease* **In Press.**

Harteveld DOC, Akinsanmi OA, Drenth A, 2013b. Multiple *Alternaria* species groups are associated with leaf blotch and fruit spot diseases of apple in Australia. *Plant Pathology* **62**, 289-297.

Holb IJ, 2006. Effect of six sanitation treatments on leaf litter density, ascospore production of *Venturia inaequalis* and scab incidence in integrated and organic apple orchards. *European Journal of Plant Pathology* **115**, 293-307.

Hong SG, Cramer RA, Lawrence CB, Pryor BM, 2005. Alt a 1 allergen homologs from *Alternaria* and related taxa: analysis of phylogenetic content and secondary structure. *Fungal Genetics and Biology* **42**, 119-129.

Hong SG, Maccaroni M, Figuli PJ, Pryor BM, Belisario A, 2006. Polyphasic classification of *Alternaria* isolated from hazelnut and walnut fruit in Europe. *Mycological Research* **110**, 1290-1300.

Horlock CM, 2006. Management of Alternaria leaf and fruit spot in apples. In. Sydney, Australia: Horticulture Australia Limited, 76.

Hwang BK, Yun JH, Kim ZS, 1987. Geographic variation of esterase isozymes in populations of *Alternaria mali* on apple leaves. *Journal of Phytopathology-Phytopathologische Zeitschrift* **119**, 225-231.

Janssen BJ, Thodey K, Schaffer RJ, *et al.*, 2008. Global gene expression analysis of apple fruit development from the floral bud to ripe fruit. *Bmc Plant Biology* **8**.

Kim CH, Cho WD, Kim SC, 1986. An empirical model for forecasting Alternaria leaf spot in apple. *Korean Journal of Plant Protection* **25**, 221-228.

Kim YC, Lee JH, Bae Y-S, Sohn B-K, Park SK, 2010. Development of effective environmentally-friendly approaches to control Alternaria blight and anthracnose diseases of Korean ginseng. *European Journal of Plant Pathology* **127**, 443-450.

Kishore K, Sharma IM, 2005. Nursery management of leaf blotch disease of apple seedlings. *Plant Disease Research* **20**, 29-31.

Kusaba M, Tsuge T, 1994. Nuclear ribosomal DNA variation and pathogenic specialization in *Alternaria* fungi known to produce host specific toxins. *Applied and Environmental Microbiology* **60**, 3055-3062.

Laich FS, Alcoba-Florez J, Perez-Roth E, Bahaya Y, Delgado JL, Mendez-Alvarez S, 2008. Molecular characterization of *Alternaria alternata* causing ocular infection: detection of IGS-RFLP intraspecific polymorphism. *Medical Mycology* **46**, 615-619.

Logrieco A, Moretti A, Solfrizzo M, 2009. Alternaria toxins and plant diseases: an overview of origin, occurrence and risks. *World Mycotoxin Journal* **2**, 129-140.

Madden LV, Hughes G, Van Den Bosch F, 2007. Temporal analysis I: Quantifying and comparing epidemics. In. *The study of plant disease epidemics.* St. Paul: The American phytopathology society, 106-108.

Miyagawa H, Sakuma T, 1984. Studies on alternaria leaf blotch of apples 1. Some characterisitcs of *Alternaria mali* in the apple orchard *Bulletin of the Fruit Tree Research Station Series C (Morioka)*, 63-74.

Mooney A, Wilk P, Dodds K, 2012. Apples - Pests and Growing Periods. In. *Orchard Plant Protection Guide for Deciduous Fruits in NSW 2012-13.* Orange, NSW, Australia: Department of Primary Industries, 28. (22.)

Neilsen M, 2010. *Characterisation of Alternaria isolates obtained from apple fruit and leaf in Australia*. Brisbane, Australia: University of Queensland, Honours thesis.

Norton R, Smith CA, Machardy W, Lord W, 2012. The effect of post-harvest foliar urea applications on tree health and ascospore production of *Venturia inaequalis*. *International Organisation for Biological and Integrated Control/ West Palaearctic Regional Section Bulletin* **84**, 91-97.

Ozgonen H, Karaca G, 2006. First report of *Alternaria mali* causing necrotic leaf spot of apples in Turkey. *Plant Pathology* **55**, 578.

Peever TL, Olsen L, Ibanez A, Timmer LW, 2000. Genetic differentiation and host specificity among populations of *Alternaria* spp. causing brown spot of grapefruit and tangerine x grapefruit hybrids in Florida. *Phytopathology* **90**, 407-414.

Peever TL, Su G, Carpenter-Boggs L, Timmer LW, 2004. Molecular systematics of citrus-associated *Alternaria* species. *Mycologia* **96**, 119-134.

Persley D, Horlock CM, 2009. Apple and Pear. In: Cooke T, Persley D, House S, eds. *Diseases of fruit crops in Australia.* Collingwood, Australia: CSIRO Publishing, 31-32.

Pryor BM, Michailides TJ, 2002. Morphological, pathogenic, and molecular characterization of *Alternaria* isolates associated with Alternaria late blight of pistachio. *Phytopathology* **92**, 406-416.

Rollemberg CL, Fayad F, Hamada NA, De Mio LLM, 2011. First report of *Alternaria mali* on apples in Brazil. *Phytopathology* **101**, S155-S156.

Rotem J, 1994. *The genus Alternaria: Biology, epidemiology, and pathogenicity*. American Phytopathological Society (APS) Press.

Rotondo F, Collina M, Brunelli A, Pryor BM, 2012. Comparison of *Alternaria* spp. collected in Italy from apple with *A. mali* and other AM-toxin producing strains. *Phytopathology* **102**, 1130-1142.

Sawamura K, 1962. Studies on spotted disease of apple 1. Causal agent of Alternaria blotch. *Bulletin of the Tohoku National Agricultural Experiment Station* **23**, 163-175.

Sawamura K, 1972. Studies on apple Alternaria blotch caused by *Alternaria mali* Roberts. *Bulletin of the Faculty of Agriculture, Hirosaki University*, 152-235.

Sawamura K, Yanase H, 1964. Studies on spotted diseases of apples. IV. On host-penetration of *Alternaria mali* Roberts. *Bulletin Horticultural Research Station* **2**, 69-74.

Scherm H, Savelle AT, Boozer RT, Foshee WG, 2008. Seasonal dynamics of conidial production potential of *Fusicladium carpophilum* on twig lesions in southeastern peach orchards. *Plant Disease* **92**, 47-50.

Serdani M, Kang JC, Andersen B, Crous PW, 2002. Characterisation of *Alternaria* species-groups associated with core rot of apples in South Africa. *Mycological Research* **106**, 561-569.

Simmons EG, 1992. *Alternaria* taxonomy: Current status, viewpoint, challenge. In: Chelkowski J, Visconti A, eds. *Topics in Secondary Metabolism; Alternaria: Biology, plant diseases and metabolites.* Elsevier Science Publishers B.V.; Elsevier Science Publishing Co., Inc., 1-35.

Simmons EG, 1999. Alternaria themes and variations (226-235) - Classification of citrus pathogens. *Mycotaxon* **70**, 263-323.

Simmons EG, 2007. *Alternaria. An identification manual.* Utrecht: CBS fungal biodiversity centre.

Simmons EG, Roberts RG, 1993. *Alternaria* themes and variations (73). *Mycotaxon* **48**, 109-140.

Soleimani MJ, Esmailzadeh M, 2007. First report of *Alternaria mali* causing apple leaf blotch disease in Iran. *Australasian Plant Disease Notes* **2**, 57-58.

Srum O, 1985. The iodine test to judge readiness of apples and pears for harvesting. *Gartneryrket* **75**, 568...570.

Sutton DK, Machardy WE, Lord WG, 2000. Effects of shredding or treating apple leaf litter with urea on ascospore dose of *Venturia inaequalis* and disease buildup. *Plant Disease* **84**, 1319-1326.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S, 2011. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance and maximum parsimony methods. *Molecular Biology and Evolution* **28**, 2731-2739.

Tanaka S, Shimomura S, Takashima E, Katumoto K, Nishi Y, 1989. Occurence of fungicide-resistant strains of *Alternaria mali* in Tokusa Ato-Cho Yamaguchi Prefecture Japan. *Bulletin of the Faculty of Agriculture Yamaguchi University*, 49-60.

Teviotdale BL, Viveros M, Pryor B, Adaskaveg JE, 2001. First report of Alternaria leaf spot of almond caused by species in the *Alternaria alternata* complex in California. *Plant Disease* **85**, 558.

Thakur VS, Nirupma S, 2010. Epidemic outbreak of apple blotch disease: epidemiology and management in Himachal Pradesh. *Indian Phytopathology* **63**, 141-144.

Thayer CL, 2005. *Plant disease forecasting and model validation: classic and modern approaches*. Raleigh: North Carolina State University, Master of Science.

Wang J, Li M, Qiao Z, 1997. Experiment on suitable timing of applying special germicide for control of apple leaf spot disease. *China Fruits*, 29-30.

Watkins CB, 2003. Principles and practices of postharvest handling and stress. In: Ferree DC, Warrington IJ, eds. *Apples: Botany, production and uses.* CABI Publishing, i-660.

Wenneker M, Joosten N, Anbergen R, Vink P, Bruggen ASV, 2011. Epidemiology and effective control of *Alternaria alternata,* causal agent of dead (dormant)

flower bud disease of pear. In: Sanchez EE, Sugar D, Webster AD, eds. *Acta Horticulturae, XI International Pear Symposium.* International Society for Horticultural Science 485-490.

White TJ, Bruns T, Lee S, Taylor J, 1990. *Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics*.

Wu G, Wang Y, Wang P, Wang J, Liu C, Gong B, 2003. Spot diseases of bagged grown Red Fuji fruit and the pathogens identification. *China Fruits*, 6-7.

Yoon JT, Lee JT, Park SD, Park DO, 1989. Effects of meteorological factors on the occurrence of Alternaria leaf blotch caused by *Alternaria alternata* f.sp. *mali*. *Korean Journal of Plant Pathology* **5**, 312-316.

Yousefi A, Shahri MH, 2009. Brown spot disease of peach and apricot trees, pathogenicity and overwinter. *Asian Journal of Plant Pathology* **3**, 61-69.

12 APPENDIX 1A

Alternaria Fruit Spot: New Directions

HAL Reference No: AP06007

12.1 The use of early, late and whole-of-season fungicide applications to manage *Alternaria* leaf blotch and fruit spot in apple.

13 APPENDIX 1B

13.1 Late season fungicide applications to manage Alternaria leaf blotch and fruit spot in apple

Duncan Cameron and Christine Horlock.

14 APPENDIX 1C

14.1 Managing inoculum sources for *Alternaria* leaf blotch and fruit spot in apples

Effect of bud removal on *Alternaria* leaf blotch in apples

Dr Dean Beasley, Christine Horlock and Duncan Cameron

15 APPENDIX 1D

15.1 Managing Alternaria leaf spot – Report on NSW DPI Trial 2006/07

Dr Shane Hetherington

Deirdre Gunning

Orange Agricultural Institute, Forest Road, Orange, NSW 2800

Appendix 1A
Alternaria Fruit Spot: New Directions
HAL Reference No: APO6007

The use of early, late and whole-of-season fungicide applications to manage *Alternaria* leaf blotch and fruit spot in apple.

Aim

To determine the most effective fungicides for the reduction of leaf blotches and fruit spots caused by *Alternaria* species on apple in Queensland.

To determine the time of the season during which fungicide application is most effective at reducing Alternaria leaf blotch and fruit spot of apple in Queensland.

Materials and Methods

This fungicide spray trial was undertaken in a commercial orchard of nine year old apple trees (cv. 'Royal Gala'), which had been severely affected by *Alternaria* leaf blotch and fruit spot in previous seasons. The trial consisted of four adjacent blocks of trees with 75 m long rows of 49 trees, oriented roughly north to south. Treatment trees were selected on the basis of similar levels of size and vigour, with buffer (untreated) trees in between each treated tree. Treatments were applied in a randomised complete block design over the four rows.

Treatments

The fungicides screened against *Alternaria* included: Chorus® (cyprodinil), Delan® (diathianon); Dithane® (mancozeb), Flint® (trifloxystrobin), Polyram® (metiram) and Vision® (pyrimethanil + fluquinconazole). Treatments were: unsprayed (control), Delan, Dithane, Polyram and Flint, along with alternating applications of Vision / Chorus and Flint / Delan. Treatments were applied under three different timing regimes; early season only, late season only or whole of season. Early season sprays were applied fortnightly from petal fall (beginning of October) to end of November. Late season sprays were applied fortnightly from January until harvest (mid February), after harvest, trees were sprayed once every four weeks until March. Whole of season sprays were applied approximately every four weeks from petal fall (beginning of October) through to March. The fungicides were prepared as per the manufacturers instructions and applied using a petrol driven (Honda 2.5 hp pump) sprayer through an adjustable hollow cone nozzle (20 bar). For each treatment the fungicide was sprayed until runoff (ca. 5 L/tree).

Assessment of disease

Leaf assessments were made during April and May, approximately 14-21 days after the onset of symptoms. The number of spotted leaves were counted for the 5 terminal leaves on 20 branches (10 on the north and 10 on the south side) of each tree. Fruit were harvested randomly (independent of maturity) on the 12 February 2007, the number of spots per fruit, and the number of spotted fruit was recorded from 20 fruit (10 from the north and 10 from the south side) of each tree. All disease lesions, insect and physical damage were marked and fruit were then cold stored at 2-4^{o}C for six months before being rated again.

Results and Discussion

All of the fungicides tested during the 2006/2007 season significantly reduced the incidence of *Alternaria* symptoms on cv. 'Royal Gala' leaves (Table 1). Dithane® was the most effective at reducing *Alternaria* symptoms, resulting in incidence levels 10 times lower than the untreated control when applied late in the season or throughout the whole season (Table 1). In general, fungicides applied late in the season or throughout the whole season appeared to give better control of *Alternaria* symptoms, although this may simply be due to the late onset of symptoms associated with late summer rainfall. The lower incidence levels recorded in the second assessment (May 2007) is thought to be related to leaf defoliation.

Table 1. Incidence (%) of *Alternaria* on cv. 'Royal Gala' fruit assessed in April (pre-harvest) and May 2007 (post-harvest).

	Incidence (%)	
Treatment [1]	**April 2007**	**May 2007**
Control	35.58 a[2]	22.08 a[A]
Vision/Chorus (E)	13.25 b	8.75 b
Delan (E)	12.50 b	8.25 bc
Flint/Delan (E)	12.00 b	7.50 bc
Flint (E)	11.00 b	7.00 bc
Polyram (E)	11.00 b	6.75 bc
Vision/Chorus (L)	11.00 b	6.25 bc
Delan (W)	10.00 b	5.50 bc
Vision/Chorus (W)	9.00 b	5.00 bc
Flint/Delan (W)	8.75 b	4.75 bc
Dithane (E)	7.00 b	4.50 bc
Flint (L)	6.25 b	4.25 bc
Flint (W)	4.75 b	3.75 bc
Delan (L)	4.50 b	3.75 bc
Polyram (L)	4.50 b	3.00 bc
Flint/Delan (L)	4.25 b	3.00 bc
Polyram (W)	3.50 b	2.50 bc
Dithane (L)	3.00 b	2.00 bc
Dithane (W)	2.25 b	1.50 c

[1]E = early season; L = Late season; W = whole season spray applications

[2]Means followed by the same letter are not significantly different at P=0.05.

There was no significant difference in either the incidence or severity of *Alternaria* on cv. 'Royal Gala' fruit assessed immediately after harvest (Table 2). The incidence and severity of *Alternaria* was very low for all treatments, less than 5% of fruit were affected and a maximum of 5 spots per 80 fruit were found (Table 2).

Table 2. Incidence (%) and severity (no. of spots per fruit) of *Alternaria* on cv. 'Royal Gala' fruit assessed at harvest in February 2007.

Treatment[1]	Incidence (%)	No. spots per fruit
Vision/Chorus (W)	3.75 a[2]	0.063 a[A]
Vision/Chorus (E)	3.75 a	0.038 a
Dithane (E)	2.50 a	0.038 a
Dithane (L)	2.50 a	0.025 a
Flint (W)	2.50 a	0.025 a
Flint/Delan (L)	2.50 a	0.025 a
Flint/Delan (W)	2.50 a	0.025 a
Polyram (W)	2.50 a	0.025 a
Delan (L)	1.25 a	0.013 a
Flint (E)	1.25 a	0.013 a
Flint (L)	1.25 a	0.013 a
Polyram (E)	1.25 a	0.013 a
Control	0.83 a	0.008 a
Delan (E)	0.00 a	0.000 a
Delan (W)	0.00 a	0.000 a
Dithane (W)	0.00 a	0.000 a
Flint/Delan (E)	0.00 a	0.000 a
Polyram (L)	0.00 a	0.000 a
Vision/Chorus (L)	0.00 a	0.000 a

[1]E = early season; L = Late season; W = whole season spray applications
[2]Means followed by the same letter are not significantly different at P=0.05.

Conclusions

- Low levels of disease in general reduces the significance of the results, but provides some useful indications of the relative effectiveness of the chemicals trialled.
- Indicates that for both leaf blotches and fruit spots chemical application might be more critical at the time of symptom development – i.e. an integrated approach of monitoring environmental conditions and inoculum levels for effective disease is likely to be highly effective.

Appendix 1B

Late season fungicide applications to manage Alternaria *leaf blotch and fruit spot in apple*

Duncan Cameron and Christine Horlock.

Summary

Previous trials on the Granite Belt in Queensland, have shown mancozeb, metiram and dithianon to be most efficacious in reducing Alternaria leaf blotch and fruit spot symptoms. Late season treatments, after the first signs of disease in the orchard, were found to be most effective.

Aim

To determine whether fungicide applications late in the season are an effective means of managing *Alternaria* leaf blotch and fruit spot in Queensland.

Materials and methods

This spray trial was undertaken on a commercial orchard in the Pozieres district of the Granite Belt in southern Queensland. Two rows of five year old 'Royal Gala' apple trees with a history of *Alternaria* infection were used. The trees were protected by hail net and with rows running north to south. Treatments were applied in a one way randomised block design, with guard trees between treatment trees.

The grower applied his customary fungicide program, primarily for the control of apple scab (caused by *Venturia inaequalis*) during spring. The last spray applied by the grower was a combination of mancozeb and phosphorous acid on 28th November 2007. Parathion-methyl was applied with these fungicides for codling moth control.

The trees were sprayed as soon after rainfall as conditions allowed, with a minimum spraying interval of a fortnight, from mid-December until Harvest on 31 January 2008 (two sprays). After harvest, trees were sprayed once every four weeks (starting four weeks after the late preharvest spray) until April (three sprays). Pre-harvest, thiacloprid was applied to all trees (including control and guard trees) for codling moth control.

The fungicides were prepared as per the manufacturers instructions and applied using a petrol-driven (Honda 2.5 hp pump) sprayer through an adjustable hollow cone nozzle (20 bar). For each treatment the fungicide was sprayed until runoff (ca. 3 L/tree).

Fungicide treatments

Treatment	Rate
Unsprayed Control	Unsprayed*
dithianon	18g/100L
mancozeb	175g/100L
metiram	175g/100L

*Thiacloprid was applied to all trees (including control and guard trees) with the pre-harvest sprays.

Assessment of disease

Leaf infection assessments were made at the onset of symptoms, at harvest (ca. 10 days later), three weeks after harvest and prior to leaf fall. Five mature leaves closest to the growing point

were rated on twelve shoots on the eastern aspect of the tree. The number of leaves with *Alternaria* symptoms was recorded.

Fruit spots were assessed at the onset of leaf symptoms and ten days later at harvest. Fruit were assessed *in situ* with 25 pieces of fruit examined per tree for symptoms of *Alternaria.*

Results and Discussions

The incidence of *Alternaria* leaf blotch in this trial was significantly reduced by the application of mancozeb and metiram. In contrast to previous experiments diathianon was much less effective in reducing symptoms.

Percent of leaves infected with Alternaria leaf blotch in cv. 'Royal Gala'.

Treatment	22/01/08	31/01/08	26/02/08	15/04/08
Control	2.0 a	3.0 c	6.33 e	7.0 g
Dithianon	2.67 a	4.0 c	5.33 e	3.67 gh
Mancozeb	0.0 a	0.0 d	2.0 f	2.0 h
Metiram	0.33 a	2.0 cd	2.0 f	2.33 h

There were no symptoms of *Alternaria* detected on the fruit of any treatment.

Conclusions

- The most effective treatments for leaf blotch reduction in this trial were metiram and mancozeb.
- In previous trials dithianon had been shown to be as effective as metiram, so this result was quite unexpected. Potential reasons for this difference include: applications after rainfall or the 4 weekly spray applications after harvest, This emphasises the need for a greater understanding of the disease cycle and the patterns of inoculum spread.
- It is unfortunate that no fruit spot data was able to be collected.

Appendix 1C

Managing inoculum sources for *Alternaria* leaf blotch and fruit spot in apples
Effect of bud removal on *Alternaria* leaf blotch in apples
Dr Dean Beasley, Christine Horlock and Duncan Cameron

Introduction

Previous experiments at Applethorpe Research Station on the effects of lime sulphur as an over-winter spray to reduce inoculum levels of *Alternaria* in apple orchards were inconclusive, with surrounding sources of inoculum a possible overwhelming influence.
The experiment was repeated in a more isolated orchard, and expanded to include other cultural practices to reduce the source of inoculum within the orchard.

Lateral buds have been recorded as a substantial source of *Alternaria* inoculum in several crops (Rotem 1994). Previous experiments (unpublished) had indicated that *Alternaria* could be easily isolated from dormant apple buds. The role of visible buds as a source of inoculum for leaf blotch infections in apples could significantly affect the development of any management strategies for *Alternaria* disease management. A preliminary experiment at Applethorpe Research Station showed that bud removal tended to lower the incidence of *Alternaria* leaf blotch, although low numbers of replicates and high variability meant no significant results were obtained.

Aim

To determine the effect of dormant lime sulphur sprays, leaf removal and mulching, or debudding on overwintering inoculum sources in apple trees.

Materials and Methods – leaf litter removal and mulching, leaf litter removal and lime-sulphur

Trees
Aging Royal Gala trees in an isolated orchard at Applethorpe Research Station were rated for tree health and butt girth. They were divided into five blocks of three similar trees and treatments were randomly allocated within blocks. Guard trees were maintained between datum trees.

Fallen leaves and other debris were raked out from directly underneath all trees in the orchard into the inter-row spaces. The resulting mix of leaves and twigs was then broken up using a tractor drawn flail mower.

Mulching
Pasture hay (predominantly Rhodes grass) was placed under the drip-lines of the trees to a depth of approximately 150mm. Low branches were removed to a minimum of 300mm above the mulch. Throughout the season, any weeds that encroached within the mulch were removed by hand.

Lime Sulphur
Lime sulphur (present as calcium polysulphide sulphur) was prepared as per the manufacturers instructions (5 L/100 L) and applied using a backpack sprayer to the dormant apples during July 2008.

No fungicides with any known effect on *Alternaria* were applied to these trees over the course of the summer. Insecticides were applied sparingly to ensure an assessable crop.

Assessment of disease

Fruit were harvested in one operation when the earliest were ready for picking. All harvested fruit were assessed for symptoms of *Alternaria*, with representative samples used for isolation in the laboratory to verify disease presence.

Leaf assessments were made approximately a fortnight after the harvest of fruit, with the number of leaves with spots counted for the five terminal leaves on 10 branches around the tree.

Materials and Methods - debudding

Trees

Susceptible five year old apple trees (cv. 'Galaxy') growing in an isolated orchard at Applethorpe Research Station were used in this trial. Trees were in two rows and two meters apart. Treatments were allocated in a completely randomised design, with guard trees between datum trees.

Treatment

All visible fruit and leaf buds were removed (by hand) from nine dormant apple trees. Another nine (control) trees retained all visible buds. Guard trees between datum trees also had all visible fruit and flower buds removed. No fungicides were applied to these trees over the course of the summer.

Assessment of disease

Leaf assessments were made approximately one month after the onset of symptoms. The number of spotted leaves was counted for the five terminal leaves on 10 branches of each tree.

Results - leaf litter removal and mulching, leaf litter removal and lime-sulphur

The application of lime sulphur during winter dormancy had no significant impact on the incidence of *Alternaria* leaf blotch or fruit spot on the variety Royal Gala. Mulching under the drip line of the tree did reduce the incidence of disease on leaves but not on fruit (Table 1.)

Table 1. Incidence (% affected) of *Alternaria* on fruit and leaves of the apple variety 'Royal Gala' when sources of inoculum are reduced by spraying with lime sulphur or under-tree mulch is applied.

Treatment	Leaf blotch	Fruit spot
Untreated control	96.8 a*	14.5 c
Lime sulphur	94.4 a	15.2 c
Mulch and weed removal	87.6 b	16.7 c

* Means followed by the same letter are not significantly different at P=0.05.

Results - debudding

The removal of apple leaf and flower buds during dormancy had no significant effect on the incidence of *Alternaria* leaf blotch. Eighty percent of leaves surveyed on the control trees were infected, with a slight though not significant (p=0.143) reduction in the levels of disease in the trees where the buds were removed.

Discussion

The importance of different sources of *Alternaria* inoculum within the orchard is not yet known. These experiments attempted to differentiate between sources in the canopy and under the tree on the orchard floor. While there was some reduction in the incidence of leaf blotch in trees where mulching was used to negate infection from beneath the tree, the levels of disease recorded suggest cultural practices alone are not sufficient to control this disease. There was no significant

reduction in disease incidence in trees where the overwintering buds were removed. This would suggest the overwintering inoculum load is more wide spread than merely the buds, or that once infection is initiated in the growing season, disease levels increase to overcome any lower initial inoculum levels.

An integrated approach, combining these practices with a careful chemical program would be needed to combat this disease.

References

Rotem J (1994) 'The genus *Alternaria*: biology, epidemiology, and pathogenicity.' (American Phytopathological Society: St Paul, Minnesota, USA.)

APPENDIX 1D

NSW DEPARTMENT OF
PRIMARY INDUSTRIES

MANAGING ALTERNARIA LEAF SPOT – REPORT ON NSW DPI TRIAL 2006/07

Dr Shane Hetherington
Deirdre Gunning
Orange Agricultural Institute, Forest Road, Orange, NSW 2800

CONTENTS

MANAGING ALTERNARIA LEAF SPOT

Introduction

Alternaria leaf spot is a disease of high value apple varieties (e.g. Gala and Pink Lady) caused by the fungal pathogen Alternaria mali. In Australia commercially limiting disease outbreaks have been restricted to Granite Belt (Qld) and Sydney Basin (NSW) regions. The disease is characterised by large red-brown lesions on leaves, premature defoliation and sunken brown lesions on fruit. Disease control has proven difficult and repeated cycles of premature defoliation can seriously limit production.

Work in Queensland has identified late-season application of the fungicides dithianon, metiram and mancozeb as a potential management strategy. However late season applications are limited because of withholding periods. Mancozeb – the latest allowed application – cannot be applied within two weeks of harvest. Orchardists are concerned that this leaves leaves unprotected at the time of harvest. This situation is exacerbated with varieties that require multiple picks and have a consequently extended harvest period (e.g. Gala)

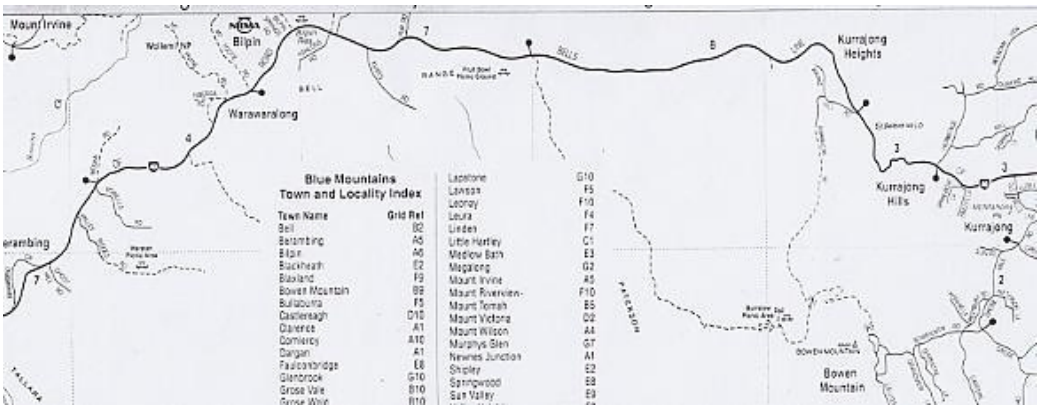
In NSW trials in previous seasons shown that a commercial mixture of the fungicides pyrimethanil and fluquinconazole have activity against Alternaria leaf spot in commercial orchards. Additionally, overseas data and domestic circumstantial evidence suggests that the fungicide Trifloxystrobin may provide useful levels of disease control. However, because of longer withholding periods these fungicides must be applied during early-mid season.

The work carried out by NSW DPI during 2006/07 aimed to establish a fungicide application schedule which involved these fungicides and was effective against Alternaria leaf and fruit spot of apples. The question underlying this research was:

'*Can early-mid season application of pyrimethanil and fluquinconazole or Trifloxystrobin reduce disease pressure sufficiently to allow late season metiram, dithianon and mancozeb applications to be effective to the point of harvest?'*

Collaborator details and trial site

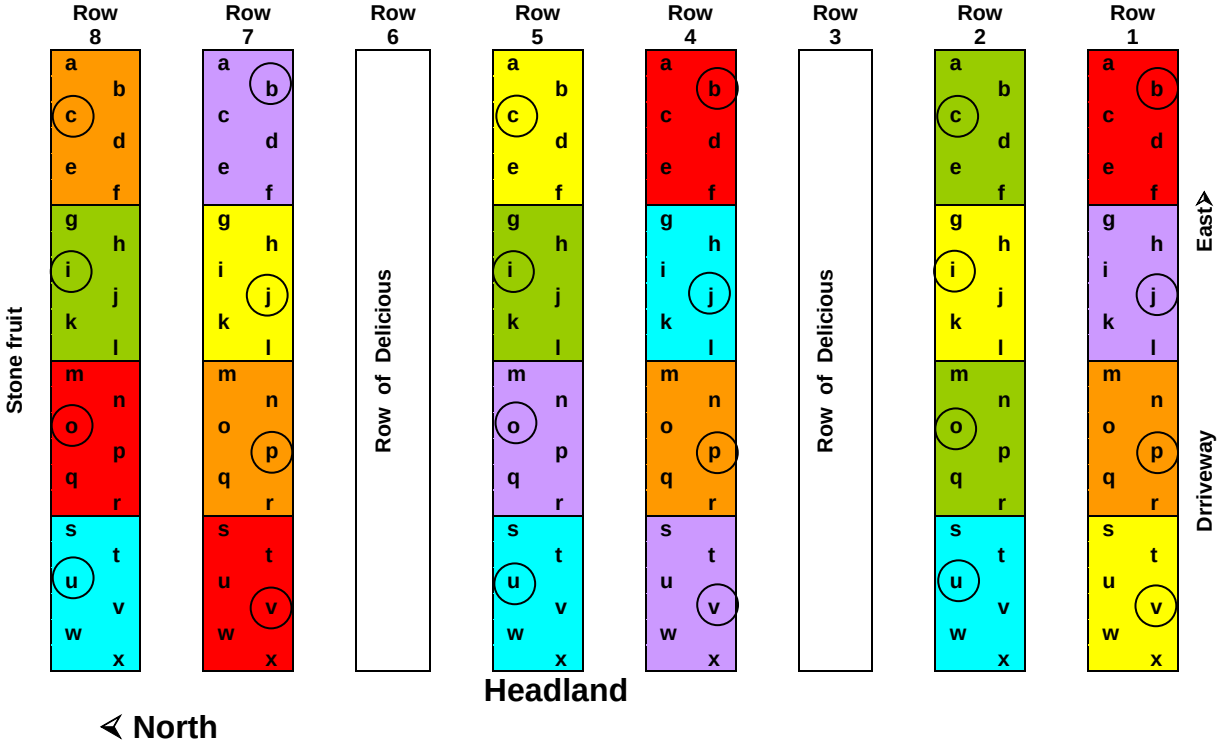
This trial was conducted on the property of:
Mr Joe Tadrosse
Bilpin Fruit Bowl
Bell's Line of Road
Bilpin



The trial block is a portion of a larger block of apples which had been severely infected by *A. mali* during previous seasons. The block was not irrigated and

contained 6 double-planted rows of Gala and 2 rows of red delicious which had been grown from seedlings. This experiment used the first (orientated from the headland in an easterly direction) 24 Gala trees. The trees were planted at high density. The orchard was hail-netted and surrounded by mature stone fruit.

Experimental Design



Block co-ordinates

Corner A. (NW): S33°30.734' E150°33.464
Corner B. (NE): S33°30.748' E150°33.446'

Corner C. (SW): S33°30.747' E150°33.451'
Corner D. (SE): S33°30.765' E150°33.469'

Treatment 1 Treatment 2 Treatment 3

Treatment 4 Treatment 5 Treatment 6

The experiment had a randomised block design with four replicates per treatment. The experiment had single tree replicates with each replicate (datum) tree buffered from neighbouring trees by at least two neighbouring trees. Datum tree position was also manipulated to take advantage of the buffering inter-row effect provided by the rows of red delicious trees.
With the exception of treatment application and disease assessment all normal orchard operations (e.g. weed control, fruit thinning, harvest) were conducted by our orchardist collaborator.

Treatments

Six treatments were applied to the trial block:

<table>
<tr><th rowspan="2">Trt</th><th rowspan="2">Green tip</th><th rowspan="2">Petal fall</th><th rowspan="2">Mid</th><th colspan="7">Weeks pre-harvest</th></tr>
<tr><th colspan="2">7 6</th><th colspan="3">5 4 3</th><th>2</th><th>1</th></tr>
<tr><td>1</td><td>Flint x3 (weekly)</td><td>Syllit x 3 (weekly)</td><td rowspan="5">Growers Choice*</td><td colspan="2">Metiram x 2</td><td colspan="3">Delan x 3</td><td rowspan="4">Dithane</td><td rowspan="5">Nil</td></tr>
<tr><td>2</td><td>Vision x3 (weekly)</td><td>Syllit x 3 (weekly)</td></tr>
<tr><td>3</td><td>Syllit x 3 (weekly)</td><td>Flint x3 (weekly)</td></tr>
<tr><td>4</td><td>Syllit x 3 (weekly)</td><td>Vision x3 (weekly)</td></tr>
<tr><td>5</td><td>Syllit x 3 (weekly)</td><td>Flint x3 (weekly)</td><td colspan="6">Triforine</td></tr>
<tr><td>6</td><td colspan="10">No fungicides</td></tr>
</table>

* Grower's choice included fungicides which have no recorded activity against A. mali. They were applied at the discretion of the orchardist using a conventional air blast sprayer. Details of all spray applications were recorded by the orchardist.

Treatments 1 and 2 tested the efficacy of late season mancozeb, dithianon and metiram applications (with the last fungicide application 2-weeks preharvest) when accompanied by very early season applications of pyrimethanil + fluquinconazole or Trifloxystrobin.

Treatments 3 and 4 tested the efficacy of late season mancozeb, dithianon and metiram applications (with the last fungicide application 2-weeks preharvest) when accompanied by applications of pyrimethanil + fluquinconazole or Trifloxystrobin as late as allowed by their prescribed withholding periods.

Treatment 5 tested the efficacy of Trifloxystrobin as late as allowed by its prescribed withholding periods.

Syllit and triforine were used in treatments 1-5 as 'buffer' sprays to protect the trial from the confounding effect of apple black spot. Neither syllit or triforine have any know effect against Alternaria leaf spot.

No fungicide of any kind was applied to treatment 6.

Application details.

All fungicides were applied to the point of run off (approximately 2000 L/Ha)using a hand lance spray rig and Davey pump.

The fungicide concentrations used were the same as those used for apple scab control

Flint: (500g/kg Trifloxystrobin) 10g / 100L
Vision: (200g/L Pyrimethanil + 50g/L Fluquinconazole) 75mL / 100L
Dodine: (400g/L Dodine) 80mL / 100L
Delan (700g/kg Dithianon) 18 g / 100 L
Dithane Rainshield (750g/kg Mancozeb) 200g/ 100L
Polyram: (700g/kg Metiram) 200g / 100L
Triforine

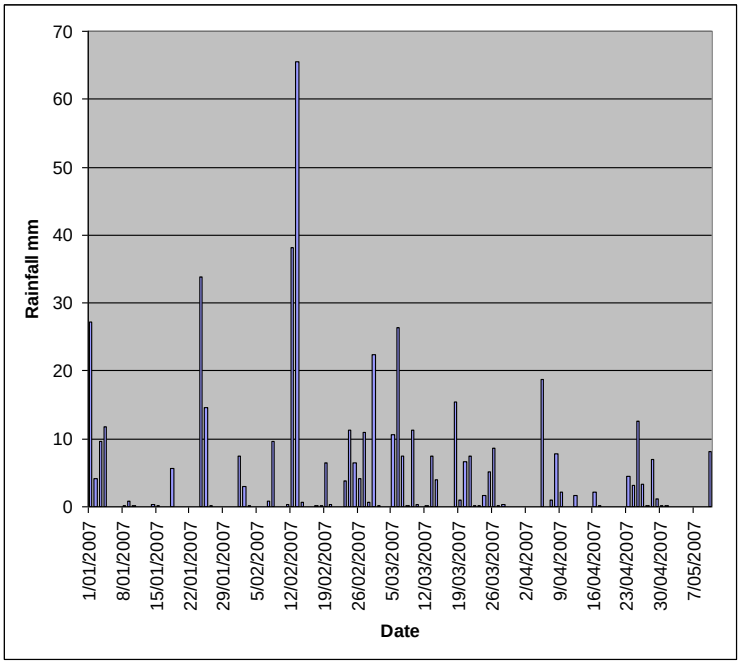
A complete index of batch numbers and other data relating the QA of these fungicides (production date, opening date) are available upon request.

Fungicides were dissolved in Bilpin town water and agitated during application to maintain an even suspension. No surfactants were added to any fungicide.

The first fungicide application occurred on the 15th of September 2006, corresponding with green tip. Fungicide applications were then made at 7 day intervals until the 18th of January 2007 (2 weeks prior to harvest). Full application details are available.

Weather

A tiny tag temperature and humidity sensor was placed within the canopy of trees involved in this trial before the first fungicide application. Rainfall was monitored.



Disease assessment

Leaf fall and disease severity were measured fortnightly from the time that the first lesions appeared (4th January 2007). Assessments finished when natural leaf fall started to distort results (10th May 2007).

One tree was assessed for each replicate (the datum tree). This tree was separated from other treatments by at least two other trees to reduce the risk of overspray.

Twenty branches were assessed on each datum tree. Branches for assessment were evenly distributed around the tree and were between 50 cm and 2m above the ground.

Each branch which was assessed was numbered and marked with two pieces of tape. There were approximately 20 leaves between these two pieces of tape at the time of the first assessment. At each assessment the number of leaves and the number of diseased leaves between these pieces of tape were counted.

Descriptive Results

The first observation of leaf infection occurred on the 4th of January 2007. The trial received a large rainfall event on the 12th and 13th of February and disease severity increased quickly. Almost all of the leaves on the untreated control trees (98.6%) had lesions by the end of March. At this time 73.5% of leaves on treatment 5 trees had lesions and other treatments had lower numbers with the minimum number of lesions being 18.5% of leaves with lesions for treatment 3.

The last assessment was conducted on the 10th of May 2007. It was decided that at this assessment time, natural leaf fall was confounding leaf fall due to the disease and this assessment was not included in the trials analysis.

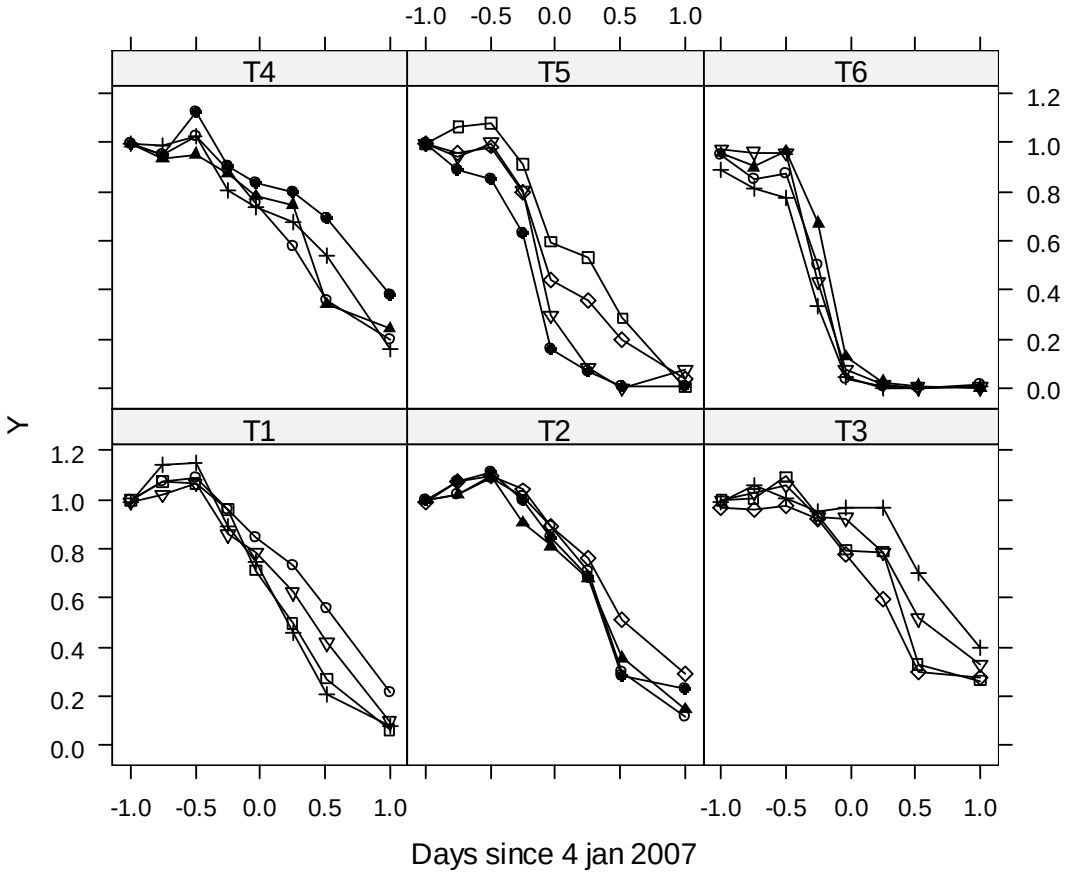
On the 13th of April 2007 99.3% of leaves on the control trees had lesions and 57.6% of leaves had fallen. For treatment 5, 87.4% of leaves bore lesions and 33.6% of leaves had fallen. The minimum disease severity was recorded on Treatment 4 where 42.5% of leaves bore lesions and 15.3% of leaves had fallen.

Harvest occurred during the first week of March

Analytical method

Because the two parameters being assessed (leaf fall and leaf infection) are not independent, they were combined into a single parameter (healthy leaves).
The variate analysed from this trial, denoted by Y and determined for each tree at each sampling time, is the mean (over all branches of the tree) of relative number or healthy leaves at time T to the number of leaves at 18 Jan 2007. Hence for a healthy plant with no disease we have Y equal to 1 at 18 Jan 2007 and expect this value to increase (with acquisition of new leaves) or at least not decrease. However for a diseased plant Y will eventually decline as leaves become diseased and as they fall off. A quicker decline in Y is assumed to indicate a more severe impact of the disease.

A plot of the data Y against Days for each Tree, grouping on treatments, is given below.



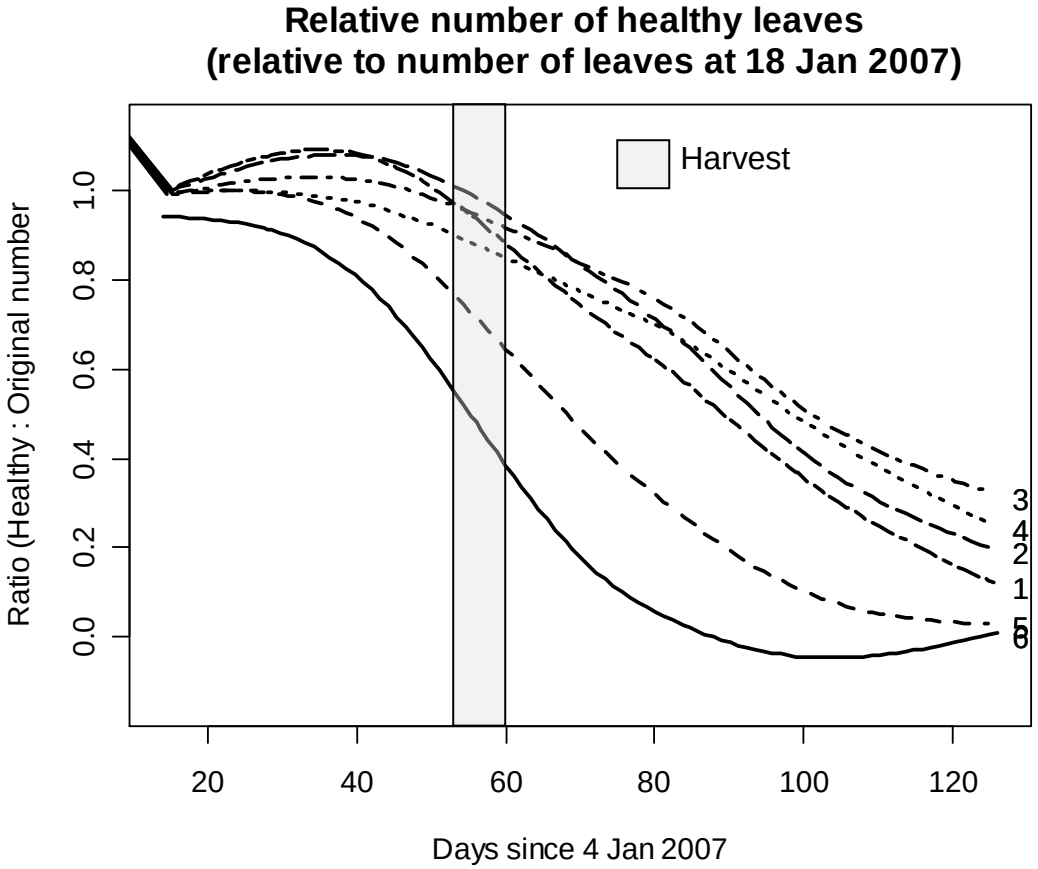
To model the above longitudinal data we use spline models, allowing for an overall spline model with time plus a separate spline model for each treatment, In addition, to model the correlations in the data we include separate spline models for each Tree plus random Row and Column effects. Finally the random error variances at sampling date (18/01/2007, 1/02/2007, 15/02/2007, 1/03/2007, 13/03/2007, 29/03/2007, 13/04/2007 and 10/05/2007) are allowed to differ.

Analysis

For the regression model all spline terms are significant. The modelled predicted values for each Treatment against time are given on the plot below. Care must be

exercised not to over interrupt the curves, in particular the dip below zero for Treatment T6 after Days since 4 Jan 2007 equals 85. This is a consequence of the spline not accommodating adequately the rapid change in gradient at this time. Here the predicted values should be interpreted as zero.

In the table immediately below the figure are the predicted means for each Treatment at each sampling date, together with standard errors and finally, within each sampling date, an LSD Ranking. In the LSD.Rank column predicted values (Pred.Val) with a letter in common are not significantly different at the approximate 0.05 level.



Day 14

Trt	JDay	Pred.Val	Std.Err	LSD.Rank
T1	14	0.9969	0.0093	b
T2	14	0.9944	0.0093	b
T3	14	0.9897	0.0093	b
T4	14	0.9964	0.0093	b
T5	14	0.9902	0.0096	b
T6	14	0.9416	0.0093	a

Day 28

Trt	JDay	Pred.Val	Std.Err	LSD.Rank
T1	28	1.0773	0.0192	d
T2	28	1.0637	0.0192	cd
T3	28	1.0250	0.0192	bc
T4	28	0.9974	0.0192	b
T5	28	0.9953	0.0194	b
T6	28	0.9132	0.0192	a

Day 42

Trt	JDay	Pred.Val	Std.Err	LSD.Rank
T1	42	1.0740	0.0251	d
T2	42	1.0737	0.0251	d
T3	42	1.0193	0.0251	cd
T4	42	0.9653	0.0251	bc
T5	42	0.9199	0.0253	b
T6	42	0.7775	0.0251	a

Day 56

Trt	JDay	Pred.Val	Std.Err	LSD.Rank
T1	56	0.9361	0.0284	cd
T2	56	0.9846	0.0284	d
T3	56	0.9462	0.0284	cd
T4	56	0.8799	0.0284	c
T5	56	0.7164	0.0285	b
T6	56	0.4802	0.0284	a

Day 68

Trt	JDay	Pred.Val	Std.Err	LSD.Rank
T1	68	0.7701	0.0358	c
T2	68	0.8572	0.0358	c
T3	68	0.8540	0.0358	c
T4	68	0.7880	0.0358	c
T5	68	0.5021	0.0359	b
T6	68	0.2145	0.0358	a

Day 84

Trt	JDay	Pred.Val	Std.Err	LSD.Rank
T1	84	0.5711	0.0435	c
T2	84	0.6563	0.0435	cd
T3	84	0.7145	0.0435	d
T4	84	0.6631	0.0435	cd
T5	84	0.2668	0.0436	b
T6	84	0.0255	0.0435	a

Day 99

Trt	JDay	Pred.Val	Std.Err	LSD.Rank
T1	99	0.3683	0.0491	c
T2	99	0.4248	0.0491	cd
T3	99	0.5218	0.0491	d
T4	99	0.4929	0.0491	cd
T5	99	0.1096	0.0492	b
T6	99	-0.0431	0.0491	a

Day 126

Trt	JDay	Pred.Val	Std.Err	LSD.Rank
T1	126	0.1160	0.0310	b
T2	126	0.1941	0.0310	bc
T3	126	0.3176	0.0310	d
T4	126	0.2461	0.0310	cd
T5	126	0.0276	0.0311	a
T6	126	0.0091	0.0310	a

Discussion

The weather provided good conditions for this trial. A relatively dry spring and early summer allowed us to apply all fungicides according to our experimental schedule. The trial block then received heavy rain during February which initiated a disease outbreak. All treatments were subject to sufficient disease pressure to validate the trial.

Treatment 5 provided the lowest level of disease control. Disease was consistently more severe on this treatment that any other except the untreated control. At the time of the experiments conclusion there was no statistically significant difference between infection recorded on treatment 5 and the untreated control. This means that application of Trifloxystrobin early in the season (without late season metiram, dithianon and mancozeb applications) does not provide sufficient disease control.

There were statistically significant differences between treatments 1-4 at most assessment times including harvest and very late in the season. Treatments 1-4 included identical applications of metiram, dithianon and mancozeb. They varied in their timing (green tip or late flowering) and also in their fungicide applied (Trifloxystrobin or pyrimethanil + fluquinconazole).

Results of the assessment conducted 126 days after the first assessment indicate that application of Trifloxystrobin as late as allowed will improve the efficacy of late season applications of metiram, dithianon and mancozeb.

Future Directions

Should further funding become available it would be worthwhile repeating this trial with the inclusion of a treatment with late season application of metiram, dithianon and mancozeb without Trifloxystrobin or any other fungicide with activity against *Alternaria mali*. This would enable us to quantify the enhancement of late season fungicide applications which is provided by Trifloxystrobin.
Consideration is also being given to shortening the duration of the withholding period of the strobilurin fungicides. A treatment including a late season block of three Trifloxystrobin applications is likely to provide worthwhile Alternaria management.

Acknowledgements

We would like to acknowledge the contribution of Mr Joe Tadrosse, owner of the Fruit Bowl orchard, Bilpin. Joe agreed to leave a proportion of his trees unsprayed allowing us to make valid assessments of our treatments efficacies. On a more practical level he was always there to un-bog us.
We also acknowledge the contribution of Trifloxystrobin and pyrimehtanil + fluquinconazole by BayerCropsciences.