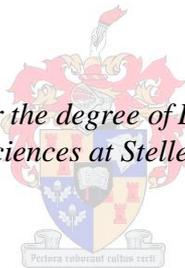


# **Responses of *Venturia inaequalis* to sanitation and regional climate differences in South Africa**

by  
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# **RESPONSES OF *VENTURIA INAEQUALIS* TO SANITATION AND REGIONAL CLIMATE DIFFERENCES IN SOUTH AFRICA**

## **SUMMARY**

The apple industry in South Africa currently relies entirely on chemical fungicides to control apple scab, caused by *Venturia inaequalis*. In this dissertation, alternative management strategies against *V. inaequalis* were tested for the first time in South Africa. New information on the behaviour of the sexual winter phase of *V. inaequalis* in different climatic conditions was found and sources of asexual inoculum overwintering in apple orchards were identified.

The effect of leaf shredding on fruit and leaf scab incidence and severity was tested against a non-shredded, non-sprayed negative control, a positive control that followed a commercial fungicide programme and a combined treatment of a commercial fungicide programme with leaf shredding, from 2010 to 2013. Reductions in fruit and leaf scab incidence and severity in the leaf shredding treatment were significantly lower compared to the negative control. Quantitative real-time polymerase chain reaction (qPCR) of airborne ascospores trapped using volumetric spore traps was used to measure the reduction in airborne ascospores in the shredded plots, and confirmed the efficacy of shredding found by comparing scab incidence and severity on fruit and leaves. Shredding twice during leaf-drop increased the efficacy of the treatment. Results indicate that leaf shredding should be integrated into scab management strategies in future. However, practical considerations unique to South African orchards, e.g. timing of leaf shredding relative to leaf-drop and orchard layouts, need to be addressed.

Pseudothecial densities (PD, number of pseudothecia per fertile lesion) and ascus densities (AD, number of asci per pseudothecium) were compared between in Koue Bokkeveld (KB), a cold winter region, and Elgin (EL), a warm winter region experiencing climate warming, in 2012 and 2013. Scabbed leaves were detached during leaf-drop and overwintered in their region of origin and in the other region. The PD in leaves collected in KB and overwintered in KB was significantly higher than for leaves collected in EL and overwintered in EL, and leaves collected in KB and overwintered in EL. These results agreed with what was expected, as temperature during pseudothecial formation (*i.e.* the first four weeks after leaf-drop) was significantly lower in KB than in EL. However, the PD for leaves collected in EL and overwintered in EL did not differ significantly from EL leaves overwintered in KB. AD values in all treatments did not differ significantly from one another. Results suggest that factors other than temperature may be involved in controlling PD, e.g. the EL population may include strains not present in the KB population, with higher optimal temperatures for pseudothecial formation.

Apple buds and pygmy apples were collected and tested for presence, number and viability of conidia in 2010, 2011 and 2012. Pygmy apples are small, late season fruit that remain attached to the tree throughout winter, especially in regions with warmer winters where trees do not experience sufficient chilling to complete dormancy. High conidial numbers were found on outer bud tissue and low numbers on inner bud tissue, but viable conidia were only found on inner bud tissue, using microscopy, and generally in orchards with high scab levels in the previous season. Molecular methods using PCR-RFLP and qPCR confirmed the presence of high amounts of *V. inaequalis* DNA in outer bud tissues, although calculated conidial amounts were higher than data obtained when using microscopy, which could indicate presence of mycelia not detected during microscopic examination. Higher numbers of conidia with higher percentage viability were found on pygmy apples, which are a more likely source of asexual inoculum in South African apple orchards than the low number of viable conidia on inner bud tissue.

# **RESPONSES OF *VENTURIA INAEQUALIS* TO SANITATION AND REGIONAL CLIMATE DIFFERENCES IN SOUTH AFRICA**

## **OPSOMMING**

Die Suid-Afrikaanse appelbedryf is tans afhanklik van chemiese swamdoders vir die beheer van die appelskurf patogeen, *Venturia inaequalis*. In hierdie proefskrif is alternatiewe bestuurstrategieë vir die eerste keer in Suid-Afrika ondersoek. Nuwe inligting te opsigte van die gedrag van die geslagtelike winterfase van *V. inaequalis*, is onder verskillende klimaatstoestande ingewin en bronne van die oorwinterende ongeslagtelike inokulum in appelboorde, is identifiseer.

Die invloed van blaarversnippering op die voorkoms en erns van appelskurf op vrugte en blare, is vanaf 2010 tot 2013 ondersoek en met 'n negatiewe kontrole (onversnipperde blare sonder spuitprogram), 'n positiewe kontrole ('n kommersiële swamdodersspuitprogram is gevolg) en gekombineerde behandelings (kommersiële swamdodersspuitprogram en blaarversnippering) vergelyk. Daar was 'n betekenisvolle verskil in die voorkoms en erns van skurf op vrugte en blare met blaarversnippering teenoor die negatiewe kontrole. Kwantitatiewe intydse polimerase kettingvermeerderingsreaksie (kPKR) van luggedraagde askospore, vasgevang in volumetriese lokvalle, is gebruik om die afname van luggedraagde askospore in versnipperde behandelings te meet. Die doeltreffendheid van versnippering as behandeling, is bevestig deur die voorkoms van appelskurf te vergelyk met die ernstigheidsgraad daarvan op vrugte en blare. Die uitvoer van blaarversnippering twee keer gedurende die blaarvalperiode het die effektiwiteit van hierdie behandeling verhoog. Hiervan kan dus afgelei word dat blaarversnippering voordelig sal wees vir die bestuur van appelskurf en in toekomstige bestuurspraktyke ingesluit moet word. Praktiese oorwegings, uniek aan Suid-Afrikaanse boorde, soos boorduitleg en die tydsberekening van blaarversnippering teenoor blaarval, moet egter in ag geneem word.

Pseudothesiale digtheid (PD; die aantal pseudothesia per vrugbare letsel) en askale digtheid (AD; die aantal aski per pseudothesium) is gedurende 2012 en 2013 vir die Koue Bokkeveld (KB), 'n koue winterstreek, en warm winterstreek Elgin (EL), 'n winterstreek wat klimaatsverwarming ervaar, vergelyk. Blare, met skurf, is gedurende blaarval gepluk en oorwinter in hul gebied van oorsprong, asook in die ander klimaatstreek. Blare wat in KB versamel is en in KB oorwinter het, se PD was aansienlik hoër as dié wat in EL versamel is en in EL oorwinter het, sowel as dié wat in KB versamel is en in EL oorwinter het. Hierdie resultate stem ooreen met wat verwag is, om rede die temperatuur gedurende pseudothesiale vorming, d.w.s. die eerste vier weke na blaarval, aansienlik laer in KB as in EL was. Die PD van blare wat in EL versamel en daar oorwinter het, het egter nie betekenisvol verskil van blare wat in KB oorwinter het nie. Die AD-waardes tussen behandelings

verskil nie noemenswaardig nie en word as onbeduidend beskou. Die verkrygte resultate dui aan dat daar ander faktore as temperatuur betrokke is by die beheer van PD, bv. die EL-skurfpopulasie, waar die warmer klimaat meer optimaal is vir pseudotesiale vorming, rasse wat nie in die KB-bevolking teenwoordig is nie, mag insluit.

Appelknoppe en dwerg-appels is gedurende 2010, 2011 en 2012 versamel en vir die teenwoordigheid, aantal en lewensvatbaarheid van konidiospore getoets. Dwergappels is klein laatseisoen appeltjies wat reg deur die winter aan die boom bly hang; veral in die streke met warmer winters waar die bome nie die nodige koue ervaar om dormansie te voltooi nie. Met behulp van mikroskopie is 'n hoë aantal spore op die buitenste knopweefsel en lae getalle in die binneweefsel bespeur; maar lewensvatbare spore is net in die binneweefsel van knoppe waargeneem, wat hoofsaaklik afkomstig is van boorde wat hoë vlakke van appelskurf in die vorige seisoen ervaar het. Molekulêre tegnieke, PKR-RFLP en kPKR, is gebruik vir bepaling van *V. inequalis* DNA hoeveelhede op die buitenste knopweefsel. Hoër getalle konidiospore is met die molekulêre analise gevind, as dié verkry met mikroskopiese ondersoek en dui op die moontlike teenwoordigheid van miselium wat nie met visuele waarneming sigbaar was nie. Meer konidiospore met 'n hoër vlak van lewensvatbaarheid is op dwerg-apples gevind en dit is moontlik 'n meer waarskynlike bron van ongeslagtelike inokulum in Suid-Afrikaanse appelboorde, as die lae getalle van lewensvatbare konidiospore op die binneweefsel van die appelknoppe.

## **DECLARATION**

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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## PREFACE

Apple scab, caused by *Venturia inaequalis*, is considered the most important apple disease worldwide. Superficial damage caused on fruit significantly reduces exportability of fruit, which, together with the cost of fungicidal control, greatly impacts on the economic profit of apple producers and the export-driven South African apple industry. The increasing risk of fungicide resistance development and the increasing demand for lower or no residues on fruit in markets, due to concerns of fungicide effect on human health and the environment, have prompted the need for alternative management strategies for scab. However, effective management of *V. inaequalis* relies on accurate information of the pathogen's epidemiology and how its behaviour may have changed, given the warming climate in some regions in the Western Cape.

In **Chapter 1**, a comprehensive literature review was conducted on the economic impact, etiology, epidemiology and management of apple scab, with particular reference to South Africa. Aspects of the biology, dissemination and epidemiology of *V. inaequalis* are summarized, focusing on existing knowledge of the pathogen's behaviour in South Africa. Existing methods to manage the disease locally, as well as alternative strategies tested and used elsewhere are discussed.

In **Chapter 2**, sanitation treatments that have been proven to be effective in studies overseas were tested under South Africa orchard conditions. The effect of leaf shredding, in particular, on number of airborne ascospores in shredded and non-shredded areas and on leaf-litter density was investigated to further our understanding of the mode of action of leaf shredding. Implementation of sanitation treatments under local conditions is also discussed.

In **Chapter 3**, the influence of temperature on certain stages in the sexual winter phase of the life-cycle of *V. inaequalis*, was investigated under natural conditions in warm and cold winter regions, as was the effect of moving isolates from one climatic winter region to another to ascertain whether populations in these regions have adapted to prevailing climatic conditions.

In **Chapter 4**, the occurrence of asexual inoculum overwintering in South African apple orchards was determined, as viable asexual inoculum sources pose a threat to current chemical control measure focused on preventing infections by ascospores.

Research chapters aimed to increase our understanding of the biology of *V. inaequalis* in South Africa. This knowledge will aid the South African apple industry in developing and implementing alternative management strategies to reduce the reliance on fungicides and minimize the economic impact of the pathogen.

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## CHAPTER 1

# ***A REVIEW OF THE ETIOLOGY, ECONOMIC IMPACT, EPIDEMIOLOGY AND MANAGEMENT OF APPLE SCAB WITH REFERENCE TO SOUTH AFRICA***

### **INTRODUCTION: ETIOLOGY, ECONOMIC IMPACT AND ORIGIN OF APPLE SCAB**

Apple scab is caused by the fungus *Venturia inaequalis* (Cooke) Winter amend. Aderhold, anamorph *Spilocaea pomi* Fries, previously *Fusicladium dendriticum* (Wallroth) Fuckel (MacHardy, 1996). Continuous research on *V. inaequalis* over more than one hundred years (Holb *et al.*, 2005b) has given rise to a large and comprehensive knowledge base for apple scab, and published information until the 1990's is summarised and discussed in MacHardy (1996). Apple scab is known by various common names, including 'black spot' in Australasia (Louw, 1947a; MacHardy, 1996) and 'fusi' (from *Fusicladium*) in South Africa. *Venturia inaequalis* is classified in the family *Venturiaceae*, order *Pleosporales* and sub-class *Loculoascomycetidae*. This ascomycetous fungus produces bitunicate (double-walled) asci in locules, inside an ascostroma pseudothecia (ascocarps). It is a hemibiotrophic pathogen, *i.e.* the fungus infects the host tissue in the same manner as a biotroph, but further development and sporulation only occurs after an incubation period and after the host tissue has died (Luttrell, 1974). *Venturia inaequalis* infection is host-specific to members of the genus *Malus*, including cultivated varieties of apples and both wild and common crab apple varieties (MacHardy, 1996).

Of the more than 200 reported diseases on apple, only 10-15 routinely approach economic importance (Sutton, 1996). Apple scab, which affects all apple-growing areas, is considered to be the most important disease in apple production world-wide (Schwabe, 1980; MacHardy, 1996; Gladieux *et al.*, 2008; Schumacher *et al.*, 2008; Bowen *et al.*, 2010). The fungus causes superficial dark, velvety lesions on fruit, and the resulting economic loss is due to both the loss in marketable crop harvested and the cost of control measures implemented (Schwabe, 1980). No information is available on the total economic cost of scab due to control measures and losses in South Africa. In South Africa, scabbed fruit are either sold on low-grade local markets, at low or no profit, or juiced, which producers often choose to do in order to avoid sorting the severities of scabbed fruit. Severely infected orchards can have a considerably lower yield than healthy orchards (Clinton, 1901). If no control measures are implemented and if weather conditions favour disease development, the entire harvest may be lost (Vincent *et al.*, 2004).

World apple production in 2012 was 75 635 283 tons, of which South Africa produces approximately 1%. The South African apple industry ranks fourth largest in the Southern Hemisphere and is primarily export-driven (42% of total production), with an industry value of R5.5 billion. Apple production covered approximately 22 166 ha in South Africa in 2012, which accounted for 29% of the total area of deciduous fruit trees planted, and the majority of production areas lie in the Western Cape. Although the total area planted with apples has not changed much over the last few years (8% increase from 2007 to 2012), the area planted per cultivar has seen many changes. The most widely planted cultivars, ‘Granny Smith’ and ‘Golden Delicious’, covered 50% of the area under apple production in 2008, but decreased to 45% in 2012. Cultivars such as ‘Kanzi’, ‘Pink Lady®’/‘Cripps Pink’ and ‘Fuji’ are steadily increasing in area in South Africa as their popularity due to overseas market demand increases. The price for export quality apples is increasing steadily and, therefore, the pressure to minimize losses due to disease and insect damage is increasing (Key Deciduous Fruit Statistics, DFPT, 2012).

Most commercially grown apple cultivars worldwide (Gessler *et al.*, 2006; Holb, 2007), and all commercially grown cultivars in South Africa, are susceptible to scab. The history of the apple scab host, the apple tree (*Malus x domestica* Borkh), has been well-researched. The apple tree’s centre of origin is in the mountains of Central Asia, in present day Kazakhstan. Apples were transported along the Silk Roads, spanning from Rome, through Samarkand in Uzbekistan, to Luoyang in China, as far back as 5000 to 8000 years ago. The Romans introduced and spread the apple across Europe and the Mediterranean areas. European settlers transported apple trees to new-found lands approximately 500 years ago and today it is grown in all temperate regions around the world today.

*Venturia inaequalis* emerged in Central Asia and followed its host to wherever apple production expanded (Gladieux *et al.*, 2008). Apple scab symptoms on apple trees were reported in South Africa for the first time in 1888 (Louw, 1947b), although the sexual phase (pseudothecia) of the fungus was first reported in South Africa by Louw in 1947 (Louw, 1947b). The South African and New Zealand populations of *V. inaequalis* act as sinks for gene flow, *i.e.* genetic material is transported into, but rarely out of, these areas, and the main source of genetic variation in South Africa arises from plant material imported from Europe (Gladieux *et al.*, 2008). The pathogen’s infection process suits most apple-growing regions world-wide, leading to a broad geographic distribution with well-established populations. These populations display a high genetic variability and regular sexual reproduction provides genetic diversity that is the basis for selection to effectively increase in fitness as conditions change and become less suitable for disease development (MacHardy *et al.*, 2001; Gladieux *et al.*, 2008).

## EPIDEMIOLOGY, LIFE-CYCLE AND SYMPTOMS OF *VENTURIA INAEQUALIS*

The presence of all three components of the disease triangle is required for apple scab development: susceptible host plant parts (leaves and reproductive parts), the pathogen (dissemination organs, *i.e.* conidia and ascospores) and suitable weather conditions (temperature and water being the most important factors). The life-cycle of *V. inaequalis* is depicted in Fig. 1.

### Current knowledge of epidemiology of apple scab

The epidemiology of apple scab in South Africa has been thoroughly researched and reported by Louw (1951) and Schwabe (1980). *Venturia inaequalis* survives the winter mainly in infected, fallen apple leaves, on which pseudothecia are formed after fertile lesions reproduce sexually (anastomosis) within four weeks after leaf-drop (Gadoury and MacHardy, 1982). Asci, formed inside pseudothecia in late winter, contain ascospores that ripen in spring and early summer and are projected after the fallen leaves and fruit from the previous season have been thoroughly wetted. Air currents carry ascospores to unprotected, susceptible plant parts and primary infection occurs if conditions are suitable (MacHardy, 1996). Leaves are most susceptible during the 2 to 5 days (Louw, 1947a; Aylor, 1998; Schwabe, 1980) after unfurling and develop ontogenetic resistance to scab after 13 days after unfurling (Schwabe, 1979). The most susceptible leaves are those on vegetative shoots that emerge from flower buds (Sanogo and Aylor, 1997). Fruit are susceptible at any stage of development, but the requirements for light infection (and therefore resistance) of fruit increases with fruit age (Schwabe, 1979), *i.e.* fruit show an increase in ontogenetic resistance similar to that of leaves (Sanogo and Aylor, 1997). The risk of disease in a season is directly proportional to the amount of ascospores that are present during the infection period and the movement and deposition of discharged, airborne ascospores are influenced mainly by wind and precipitation (Gadoury and MacHardy, 1986; Aylor, 1998). The development of the disease is highly dependent on prevailing weather conditions: disease incidence and severity are significantly higher in apple-growing areas with high moisture (rain and relative humidity) and can vary greatly between seasons (Schwabe, 1980). Free water is necessary for the formation, release and infection of ascospores, whereas disease development increases as relative humidity increase. Ascospore germination occurs between 0.5 and 26°C, with 17°C being optimal, and 30°C or higher will inhibit infection (Louw, 1947b; Schwabe, 1980).

Asexual lesions with conidia (summer spores) are formed from ascospore infections after an incubation period of 7 to 21 days (depending on cultivar, leaf age and weather conditions) and become visible when conidiophores rupture the cuticle of infected apple tissue (Louw, 1947a,

MacHardy, 1996; MacHardy *et al.*, 2001). Conidial production (infectious period) in a lesion stops after *c.* 30 days, although availability of viable conidia in a lesion (infective period) can be as long as 3 months (MacHardy, 1996). These spores are detached and disseminated by wind and/or water to other unprotected, susceptible host tissue, where secondary infection occurs if conditions are suitable (Louw, 1947a; Sutton *et al.*, 1976; Gladieux *et al.*, 2008). Conidial sporulation and germination occurs at temperatures between 0 and 30°C, although temperatures between 16 and 20°C are optimal and 30°C or higher will greatly inhibit germination (Louw, 1947b; Louw, 1948b; Schwabe, 1980; MacHardy, 1996). Schwabe (1980) found that conidial infection requires a longer wetting period than infection by ascospores at any temperature between 4 and 25°C, whereas Stensvand *et al.* (1997) found that conidia and ascospores require the same period of wetting between 2 and 8°C. The disease is polycyclic, producing new generations of conidia during the season to re-infect susceptible tissue, if the correlation between temperature and moisture are suitable for disease development (Sutton *et al.*, 1976).

The duration of exposure of susceptible plant parts to free water (wetting period), which can be precipitation, overhead irrigation or dew, is determined by the prevailing temperature. However, dew alone is not sufficient to create an adequate wetting period, and is often only supplementary to other forms of free water. Intermittent wetting periods reduce infection on mature apple fruit and fruit infection differs from leaf infection, since infection on leaves is not reduced by intermittent wet periods if they are interrupted by dry intervals of shorter than 32 hrs (Schwabe, 1980). Findings for leaf infections by Schwabe (1980) were confirmed by Becker and Burr (1994) and Stensvand *et al.* (1997). The degree of infection on apples increases as the duration of the continuous wetting period increases (Schwabe, 1980). Foliar lesions initially appear chlorotic and water-soaked, spread in a dendritic pattern and later turn olive-green and then dark-brown. These lesions may coalesce, covering large areas of canopy in total, which lowers the photosynthetic capacity of the tree and lowers the ability of the leaves to export photosynthates to fruit, for adequate fruit growth and development. This may also affect the amount of photosynthates exported to reserves in the tree later in the season after harvest, for bud development and maintenance of metabolism during winter. Infection of buds or blossoms leads to blossom drop or severe infection of immature fruit. Fruit scab lesions are velvety, round, olive-green under wet conditions and grey or yellow-brown under dry conditions, that later also turn dark-grey to black, and may also merge to cover a larger area of the fruit. If young fruit are infected, they are disfigured and often have cracks (Schwabe, 1974). Mature fruit with invisible, latent infections develop storage scab during pack-house storage. These lesions are often smaller, rounder, smoother and more numerous per fruit than field scab lesions and have an intense black colour. If the skin of the fruit is damaged, the fruit becomes more susceptible to secondary rot organisms like *Penicillium expansum* and *Botrytis cinerea* (Schwabe, 1974).

## **Current knowledge of the effect of climatic conditions in the Western Cape on the sexual overwintering stage of *Venturia inaequalis***

Louw (1951) and Schwabe (1980) investigated the effect of Western Cape winter conditions on the certain developmental phases of the sexual stage of *V. inaequalis*. After leaf fall and during winter, pseudothecial production and development are dependent on environmental conditions (Louw, 1951; Schwabe, 1980; Gadoury and MacHardy, 1982a). Gadoury and MacHardy (1982a) reported that the number of pseudothecia produced per fertile lesion increased at a rate inversely proportional to temperature between 4 and 20°C, but pseudothecial diameter increased most rapidly at 10°C and hardly developed above 20°C under both field and laboratory conditions. Similar results were reported by Louw (1951) and James and Sutton (1982). However, Louw (1951) found that pseudothecia could form at temperatures as low as 1°C and were most abundant at 13°C in the Western Cape in South Africa. Louw (1951) also suggested that different strains of *V. inaequalis* may exist between Elgin and Koue Bokkeveld.

Over the past four decades, the Western Cape has experienced a warming trend in its weather patterns (increase in 0.8°C) in all seasons for most regions, and a further increase of 1 to 2°C is predicted over the next 30 years (Midgley and Lötze, 2011). The Koue Bokkeveld is regarded as a cold winter apple-growing region (Midgley and Lötze, 2011) and apple trees still experience adequate chilling units annually and have normal growth patterns (Cook, 2010). Events in the sexual phase of the *V. inaequalis* life-cycle (*i.e.* ascospore discharge) are also synchronized with apple tree phenological events in this region in spring (Louw, 1951; Schwabe, 1980). However, Elgin is regarded as a warm winter region (Midgley and Lötze, 2011) and apple trees in this region are experiencing increasingly insufficient chilling units. Trees respond to this with atypical growth patterns, such as delayed or incomplete leaf-drop, not fully entering dormancy or incomplete dormancy, and delayed foliation (uneven bud-break and bloom) (Louw, 1951, Gladieux *et al.*, 2008; Cook, 2010). Louw (1951) reported that most leaves in Elgin fell in late May; however, in recent years leaf-drop has always occurred over a long time period, starting in May and ending only in July, depending on the cultivar. In some years, leaves fail to enter dormancy completely and leaves have been observed to remain attached to trees until the following spring. The practice of applying hydrogen cyanamide and oil in early spring (e.g. Dormex<sup>TM</sup>) to force trees out of incomplete dormancy for uniform foliation began in warmer winter regions. Changes in apple tree phenology due to climate change have also been reported in Germany (Kunz and Blanke, 2011) and Lithuania (Romanovskaja and Bakšiene, 2009).

In 1942, significant ascospore discharge in Elgin started in August and mid-August and peaked in late August and late August (Louw, 1951). In 1977, significant ascospore discharge in the

same region started in mid-August and peaked in late September (Schwabe, 1980). If this highly important seasonal event in the life cycle of *V. inaequalis* shifted by several weeks over a period of 35 years in the Western Cape, more information is needed for whether or not other changes have occurred in the life-cycle of *V. inaequalis* in the Western Cape, within and/or between regions and within regions, since the studies by Louw (1951) and Schwabe (1980). Additionally, findings by Louw (1951) and Schwabe (1980) would need to be verified. Such information would be important in helping to predict how *V. inaequalis* might behave over the next few decades and how these changes would affect scab management strategies in the Western Cape.

### **Monitoring and quantifying ascospore maturity and discharge**

The maturity and discharge of ascospores during spring is probably the most important part of the life-cycle of *Venturia inaequalis* that needs to be understood for epidemiological studies on apple scab, as they constitute the primary scab inoculum in spring (Keitt and Jones, 1926; Aylor, 1993). The presence and discharge of the first and last mature ascospores, as well as when peak maturity and discharge concentration of ascospores trapped using spore-trapping instruments can be used to estimate and monitor the primary inoculum in spring (Rotem, 1988; Gadoury *et al.*, 2004).

Hutton and Burchill (1965) developed a “water extraction” method for discharging mature ascospores from leaf discs. Since mature ascospores remain inside pseudothecia until sufficient wetting has occurred, this method is useful in distinguishing mature ascospores from immature ascospores as soon as they had reached maturity for discharge. This method collects all mature, discharged ascospores in the solution that leaves are agitated in, allowing a higher total number of ascospores to be counted than methods that rely on trapping airborne ascospores. Schwabe and Heyns (1974) and Schwabe and Mathee (1976) utilized this method to evaluate differences between treatments for postharvest sanitation of scab inoculum. However, agitation of leaves in water is an unnatural manner for ascospores to be discharged, and a disparity exists between the morphological maturity of ascospores and the physiological maturity of asci (Gadoury *et al.*, 1992), and so the vibrations experienced by ascospores could be a factor affecting mature ascospore concentrations determined in this manner. Crushed pseudothecia can be examined under microscopes (squash mounts) to monitor relative ascospore maturity and discharge throughout the primary season (Gadoury and MacHardy; 1982). The degree-day model has been proven to be highly correlated with observed cumulative ascospore discharge, and therefore reliable in estimating the depletion of total discharged ascospores. This method was shown to be more reliable in determining the relative quantity of primary inoculum than discharge tests or squash mounts (Gadoury *et al.*, 2004).

Some of the first studies to effectively trap airborne ascospores used wind tunnels (Hirst and Stedman, 1962; Gadoury *et al.*, 1996), and rotating arm impaction air samplers, or rotorods, and suction or volumetric spore traps, based on the Hirst-type spore traps, as described in Rotem (1988). Each type of spore trap has its own advantages and disadvantages, making the selection of the type of spore trap for a specific study important, and often a combination of spore trap types can be used to complement each other to obtain more useable information (Aylor, 1993). Wind tunnels are used for controlled-environment studies, while suction traps and rotorods are used more often in field studies. Suction or volumetric spore traps, where a constant volume of air is sucked through an orifice and material is deposited onto a sticky surface, is more accurate for quantitative purposes and has a higher trapping efficiency than rotorods, where small glass or plastic rods or slides are rotated at a constant speed in open air (Rotem, 1988). However, rotorods are simpler, less expensive, more convenient and can be used in greater numbers than suction traps and their efficiency is less sensitive to wind speed than suction traps (Rotem, 1988; Aylor, 1993).

Due to their relative accuracy in quantifying airborne inoculum concentrations, volumetric spores traps have been used in many epidemiological studies (Giosué *et al.*, 2000; Rossi *et al.*, 2001; Rossi *et al.*, 2003; Gadoury *et al.*, 2004; Stensvand *et al.*, 2005). However, spore traps are expensive and labour-intensive (Giosué *et al.*, 2000; Gadoury and MacHardy, 2004), mainly due to cost of the equipment and the time required to examine samples microscopically. Various studies have used quantitative real-time polymerase chain reaction (qPCR) methods to measure DNA of fungal species in air samples (Zeng *et al.*, 2006; Carisse *et al.*, 2009; van Wyk, 2011). Carisse *et al.* (2009) developed a highly sensitive, reliable qPCR assay for quantifying airborne *Botrytis squamosa* conidia trapped with rotorods in onion fields. In the study by van Wyk (2011), *Fusarium circinatum* spores that had been trapped on filter paper in pine forests were quantified using qPCR. Zeng *et al.* (2005) developed a qPCR method to detect airborne *Cladosporium* spores indoors and outdoors, which were trapped using a volumetric spore trap. Sholberg *et al.* (2005) developed a DNA macro-array to accurately detect *V. inaequalis* from rotorod samples in apple orchards, as well as being able to identify *V. inaequalis* and four other apple pathogens in the same test. The methods developed by Carisse *et al.* (2009), van Wyk (2011) and Sholberg *et al.* (2005) didn't combine volumetric spore traps and molecular quantification methods, but Zeng *et al.* (2005) showed that highly sensitive methods could be developed. Volumetric spore traps could be useful in measuring accurate treatment differences, and if the time and cost of using them could be reduced and the accuracy increased further, then they would be a useful tool in disease management studies. To date, qPCR assays in combination with spore traps to more accurately quantify *V. inaequalis* inoculum has not yet been used in any published studies.

## MANAGEMENT OF APPLE SCAB

Due to the susceptibility of apples to infection by *V. inaequalis*, management strategies are required to minimize losses to scab damage and ensure that a profitable crop can be harvested. Scab management strategies include the use of tolerant or resistance cultivars, disease risk forecasting models, fungicide sprays applied to prevent or stop infections, and alternative strategies that aim to reduce inoculum before the primary season. No tolerant or resistant cultivars are available yet for commercial use in South Africa, but such cultivars are being developed in ongoing research programmes. Therefore, only the latter three types of management strategies will be discussed.

### Disease forecasting models

Scab development is greatly dependent on prevailing weather conditions and the phenological stage of the tree. Disease incidence and severity are significantly higher in areas with high moisture levels (rain and relative humidity) and can vary greatly between seasons (Schwabe, 1980). For these reasons, the timing of fungicide spray applications in spring is critical for effective management of this disease.

An infection index, using the Mills-Table (Mills, 1944) which was originally published for the timing of sulphur dusting for scab control, can be used to quantitatively calculate the incidence and severity of infection of leaves and fruit (Schwabe, 1980). Modified by Jones *et al.* in 1980 (referred to as a “modified Mills”) and then revised by MacHardy and Gadoury in 1989 (referred to as “revised Mills”) (Sutton, 1996), the infection index can be used as a guide for determining when the first fungicide sprays need to be applied in spring based on when peak ascospore discharge is most likely to occur. Schwabe (1980) developed the ‘fusi’ infection index for South Africa, which is still currently in use and is based on the Mills-table. An infection index in this risk prediction system is calculated using forecasted weather data: the length of time in hours that apples or leaves are wetted by rain is multiplied by the mean temperature in degrees Celsius during that period. A low (100-150), moderate (150-224) or high (>225) infection mean index allows producers to decide whether or not to apply curative sprays in addition to a standard protective fungicide programme.

Gadoury and MacHardy (1986) developed the potential ascospore dose (PAD) system to estimate the total number of ascospores that could be released per square metre of orchard floor during the whole primary scab season. The PAD of an orchard is the forecasted risk of disease in the following season for that orchard. This was done because most disease forecasting models for fruit orchards did not differentiate between orchards with high and low inoculum densities, and both were considered to be at the same risk. The PAD model provides a threshold for spray application

decisions based on the measured incidence and severity of scab just before leaf fall, and leaf litter density at bud-break. However, PAD differs between cultivars and is not uniform within an orchard of the same cultivar, which may affect the accuracy of using this system (Charest *et al.*, 2002). This, together with the time required for scab assessment in autumn and the need for trained personnel may explain why PAD is not used widely by apple producers in the USA (MacHardy, 1996). Nevertheless, PAD is still widely used in research experiments. Wilcox *et al.* (1992) developed an integrated, reduced-spray program that combined delaying the first fungicide spray in spring in orchards with a low inoculum level (determined using local weather data to calculate Mill's infection indices and by monitoring scab incidence and severity), with post-infection DMI fungicide applications. This reduced the need for calculating PAD and sprays against scab could then be applied when insecticides and miticides were applied (Sutton, 1996).

RIMpro, a dynamic scab simulation programme, was developed in the 1990's by Marc Trapman and was built to encompass many previous scab risk models (Trapman, 1994; Trapman and Polfliet, 1997). This model utilizes a large number of factors, including weather data, PAD, ascospore biofix, susceptibility of apple cultivars to scab and residual activity of preceding fungicide treatments, to determine an "absolute infection risk" for apple scab and other apple pests and diseases. This system also takes into account the percentage of ascospores that have matured and have been discharged, as well as the percentage of ascospores that have yet to be discharged, to aid in determining whether a rain event would be relatively more or less important than the previous rain event (Phillion *et al.*, 2009; Trapman, 1994). The absolute infection risk calculated by RIMpro aids producers in determining whether or not certain fungicide sprays in a spray programme need to be applied. One requirement, however, is for orchards to receive annual sanitation treatments to maintain low inoculum levels. Although not many scientific studies have used this forecasting system, it has been validated extensively in practice and is constantly being updated in collaboration with other internationally renowned scab researchers (Phillion *et al.*, 2009; Trapman *et al.*, 2004). It has been one of the most widely used throughout Europe for the past 20 years, has been implemented for years in Quebec in Canada, Chile and Australia, and has recently begun to be adopted by growers in USA (Marc Trapman, personal communication).

RIMpro appears to be the most comprehensive scab risk forecasting system and has the potential to be used in integrated management of apple scab in South Africa, although many of the factors or components in the RIMpro system (and PAD) would need to be adapted for South African conditions.

## Chemical control

Typically, producers apply between 6 and 15 fungicide treatments per season, depending on weather conditions and disease levels (MacHardy, 1996; MacHardy *et al.*, 2001; Ellis *et al.*, 1998; Carisse and Dewdney, 2002) amounting to more than 50% of the total pesticide use (Creemers and van Laer, 2006), 90% of the yearly cost of fungicides in apple production (Holb, 2009) and 10% of the total cost of apple production (Vincent *et al.*, 2004). No figures exist for the cost of fungicide application for scab control in South Africa. If the inoculum pressure in an orchard is high enough, just one spray missed during the primary season can spell economic disaster, despite the high total number of fungicide sprays applied per season (Creemers *et al.*, 2006).

Until the 1930s, lime-sulphur and Bordeaux mixture were used to control apple diseases, including apple scab (Louw, 1943; Roberts, 1946; Sutton, 1996). Organic fungicides were first introduced in 1934 through the use of the dithiocarbamate class of fungicides (Kommendahl, 2000). Dodine, a guanidine fungicide, was introduced in 1959, but ten years later *V. inaequalis* resistance was reported in New York, in the USA (Sutton, 1996). This was also the first report of field resistance to fungicides. Systemic fungicides were first released with the development of oxanthins and benzamidazoles in the 1960s. Benomyl was introduced in the early 1970s, but just three years later resistance in the *V. inaequalis* population was reported in the United States (Sutton, 1996). Tolerance to benzamidazoles and dodine in South Africa was reported by Schwabe (1977). In the 1980s (ergo)sterol-biosynthesis inhibiting (EBI or SBI) fungicides, also known as demethylation-inhibiting fungicides (DMIs), were introduced as effective chemical control of apple scab and *V. inaequalis* resistance to EBI's in South Africa was reported by Schwabe *et al.* (1988). Resistance to DMI fungicides has expanded to become a worldwide problem over the past 20 years. Resistance to fungicides such as dodine, benzamidazoles and strobilurins (Q<sub>0</sub>I) is stable (Sutton, 1996; Ma and Michailides, 2005), so even decades later these chemicals cannot be used in orchards where the fungal populations are resistant to these chemicals.

Today, there are numerous fungicides, from different chemical classes and with different modes of action, available for the control of apple scab in South Africa, which include Captab, Dithane (multisite activity fungicides), ) DMIs such as Nustar (fluzilazole), Score (difenoconazole), Rally (myclobutanil), Dorado and Topaz (propiconazole), Chorus (anilinopyrimidine) and Strobly (strobilurin). The risk of resistance development differs between chemical classes of fungicides and their specific modes of action, and is highest when a fungicide is used at high rates and short intervals over a long period of time. Selection pressure on the pathogen population increases the frequency of resistant individuals that survive in a population. When a pathogen population becomes resistant to a fungicide belonging to one class of fungicide, other fungicides in that group

may become ineffective in controlling the pathogen if there is cross-resistance (Kunz *et al.*, 1997). The Fungicide Resistance Action Committee (FRAC) was developed in 1981 to provide guidelines for fungicide resistance management in order to prolong the effectiveness of "at risk" fungicides. FRAC guidelines are integrated into most fungicide programmes in South Africa today. A resistance management program is the most important concept in fungicide use that needs to be applied by apple growers (Sutton, 1996). According to Creemers and Vanmechelen (2000), an anti-resistance management strategy includes limiting the number of fungicide applications from one fungicide family to three times per season and alternating and combining different fungicide classes used. This involves, for example, using a single mode of action fungicide once per season, but combining its use with other fungicides with different modes of action, preferably one with a broad-spectrum of activity. Creemers and Vanmechelen (2000) emphasized the importance of timing fungicide applications based on their mode of action (relative to biological and climatic factors) and assessing the "complementarity" between two fungicide classes used together. This will ensure that the risk of resistance is lowered significantly and that those fungicides that are still highly effective in controlling scab will remain effective for much longer. Another important recommendation for producers is the timely use of protective multisite fungicides to prevent infection. If however, infection does occur, the kick-back time of curative (DMI) fungicides should be adhered to, since their activity will not be effective if they are applied too late. Curative postharvest sprays are commonly used in an attempt to stop fungal growth of *V. inaequalis* in leaves so that the pseudothecial development does not occur and the risk of disease in the following season is reduced. However, multiple applications of fungicides from the same chemical group during a season greatly increase the risk of resistance development to that group of fungicides.

Due to the tolerance by *V. inaequalis* to certain chemical classes of fungicides (e.g. tolerance of EBIs by a *V. inaequalis* population in Elgin (Wolf Schwabe, unpublished data)) that are used by producers during the apple growing season, postharvest fungicide applications are not recommended as often as they were two decades ago in South Africa. The focus of current fungicide programmes is rather on preventative sprays being applied regularly and timeously in spring. It is essential that producers adhere to the customised, recommended spray programme compiled for them by consultants. These programmes are designed to combat scab with effective chemicals for controlling scab and will prevent scab infections from becoming epidemic, while limiting the risk of resistance.

## **Alternative control measures and integrated pest management**

Apple scab is generally managed with intensive fungicide applications. However, the cost of fungicide application, development of resistance in fungal populations to fungicides, the high cost of developing new fungicides and the market's demand for residue-free fruit greatly impacts the economic profit of apple production. This led to the introduction of integrated pest/pathogen management (IPM) into apple production in the latter part of the twentieth century (Holb, 2009). Over the past 20 years there has been an increasing trend in apple scab research on alternative control strategies and IPM. IPM aims to integrate disease management strategies in order to reduce the use of fungicides and combines the use of alternative methods of control, including disease forecasting methods, for scab with fungicide sprays (Holb, 2009). The most effective alternative control methods to date have been cultural control methods, such as orchard sanitation and the use of organic chemicals, which are often also used as nutrient amendments.

The drive for apple producers to lower their reliance on fungicides is fuelled mainly by public concern about the effect of fungicides on human health and the environment, especially in export markets in Europe (Schneider and Dickert, 1994; Ellis *et al.*, 1998; Creemers and van Laer, 2006; Jamar *et al.*, 2010). Public concern about the use of pesticides in agriculture and the effect of pesticides on human and animal health and on the environment are one of the main factors which has fuelled the “organic” farming movement. Schneider and Dickert (1994) compiled a comprehensive literature review on the benefits and costs to human and animal health of fungicide use in agriculture. The authors state that “the acute toxicity of fungicides to humans tends to be low, but most can cause chronic effects with prolonged exposure”. Prolonged exposure to fungicides in or on fruit may occur for South African consumers, especially since maximum residue levels (MRLs) have been established for all fungicides and are rigorously tested for European and other export markets, but are hardly ever tested for South African markets. Schneider and Dickert (1994) presented many cases in which fungicides from various chemical classes caused several acute toxic effects in human and animal health, the most common of which are allergy and irritation. The most common acute effects of dicarboximides and chlorthalonil are skin rashes and sensitization, which can range from itching and erythema to vesiculation, as well as acute renal toxicity and respiratory effects. However, although the actual mutagenic and carcinogenic effects of these and other chemical classes of fungicides have not been scientifically established for humans, the extrapolations from various animal studies are used to project the potential effects (Schneider and Dickert, 1994). Such information is enough to cause consumers to become more aware of what they eat and from where and how the food they eat was produced. The authors also justify the benefit of agricultural fungicides where mycotoxigenic fungi are controlled with effective fungicides.

However, *V. inaequalis* does not produce any known mycotoxins that are harmful to humans, so the only benefit of fungicidal control of scab is the increase in the quality and quantity of apple crops. It would seem that, if effective alternative measures to control scab were available, then producers would be obliged to reduce their reliance on fungicides to produce a profitable crop. In conclusion, Schneider and Dickert (1994) state that consumers have “valid concerns regarding the use of agricultural fungicides”. The likelihood that the movement towards “organic” farming will decrease is also very small, especially in lucrative export markets. Therefore, the pressure for the South African agricultural industry to decrease any unnecessary use of fungicides will only increase.

### ***Orchard sanitation***

Sanitation is defined by van der Plank (1963) as “a process that reduces, or completely eliminates, or completely excludes the initial inoculum from which epidemics start”. Scientists have known about control of apple scab through treating the leaves on which they overwinter for over a century. Clinton (1901) is one of the first authors to describe sanitation and integrated management practices of scab. He mentioned that properly pruning orchard trees leads to reduced scab infections in orchards, since increased airflow means that fungicide sprays are more easily and evenly applied and microclimatic conditions in the canopy of the tree are drier and, thus, less favourable for scab infection and development. Proper soil cultivation, as well as proper control of grass and weeds, is needed to enhance scab management (Clinton, 1901), because neglected grass and weeds on orchard floors allows leaves to be trapped and protected over winter, which is optimal for pseudothecial development and subsequent infection in the following season. Uneven and waterlogged orchard floors lead to pools of water and ditches, which also offer protection to fallen leaves in winter and prevent consistent maintenance of orchards, since tractors cannot move between rows for spraying and workers cannot access trees to prune trees properly. Waterlogged trees are under more stress than trees with well-drained root-systems. Not only does the tree suffer from anaerobic conditions and a reduced ability to absorb nutrients, but this may also lead to a reduced defence against pathogen infections. Both of these could result in a reduced crop quantity and quality.

Hesler (1917) wrote “The destruction of fallen leaves would appear to lessen the primary infection. But in actual practice that method alone is not reliable and at best is only to supplement spraying or dusting”. Studies on the summer epidemics of scab have shown that there is a direct, positive correlation between the amount of ascospore inoculum in spring and the incidence and severity of scab in summer (Holb, 2009). Assuming that each airborne ascospore represents a possible infection and foliar lesion, the percentage reduction of spring ascospore inoculum achieved

through a non-chemical strategy can be a measure of the percentage reduction of disease that these ascospores could cause (Sutton *et al.*, 2000). Therefore, the percentage reduction of ascospores can be used as a measure of how effective a non-chemical sanitation measure is.

Louw (1948a) tested ploughing leaf litter into the ground in South Africa in the 1940's and reported a significant reduction in foliar and fruit scab, respectively, but the author stated that such a practice would not be recommended as a control strategy alone. At the time of the study, the technology to be able to apply ploughing widely for scab control was not sufficiently advanced. However, ploughing in modern times is considered detrimental to soil structure and the roots of orchard trees, as well as being highly energy-intensive, so it is unlikely that such a practice would be adopted by South African producers in future. Until now, no further studies on treating the leaves mechanically to reduce ascospore inoculum have been published.

Studies in Europe and the USA have shown that one of the most effective methods of reducing ascospore inoculum is to apply surface sanitation treatments in autumn and/or early spring. There are a number of non-chemical practices that have been proven to reduce ascospore inoculum in spring; however, leaf shredding has been shown to be one of the most effective non-chemical sanitation practices (Holb, 2006; MacHardy, 1996; Sutton *et al.*, 2000; Vincent *et al.*, 2004). Removal of fallen leaves or leaf sweep was shown to be more effective than leaf shredding in reducing primary scab inoculum sources in the orchard, because the inoculum source is removed from the orchard, rather than being only shredded (Holb, 2006; Gomez *et al.*, 2007). Mulching with straw in late winter was not significantly effective in reducing ascospore reduction (Holb 2006). Additionally, South African producers already apply straw mulches underneath trees to increase soil carbon in carbon-poor soils (typical of South African soils), although not annually, but have not reported any reduction in scab. The increasing cost of straw mulches means that mulching for scab management would likely not be economically feasible.

Sanogo and Aylor (1997) stated that “a cornerstone of apple scab management is the minimization of primary infection of apple tissue by ascospores of *V. inaequalis*”. The best method of reducing and controlling the disease is to remove the overwintered ascospores that cause primary infections in spring (Holb, 2009). Since ascospores overwinter on fallen leaves from the apple trees, the highest reduction of spring ascospore inoculum would be achieved by removing the fallen leaves in autumn (Holb, 2009), assuming that the primary inoculum in spring only arises from these overwintered ascospores. Another option would be to apply sanitation treatments to the fallen leaves in autumn or early spring. Although methods of control that remove inoculum sources from the ground would be highly effective, they would not be able to eliminate the ascospore inoculum from wind-blown ascospores from neighbouring orchards or from those parts of an orchard where disease control has not been sufficiently effective. Aylor (1998) found that airborne ascospores can

be transported over distances of 5 km between orchards, and Holb *et al.* (2004) found that ascospores within an orchard can be transported by wind up to 70 m. These ascospores can contribute a significant amount of inoculum in orchards where sanitation treatments have effectively removed the majority of ascospores discharged from the orchard floor, to cause primary spring infections and a scab epidemic. However, Gomez *et al.* (2007) conclusively proved, with ascospore gradient measures, that ascospore spreading more than 20 m from the source was not of importance to experimental trials.

Holb (2006) conducted a study implementing different combinations of sanitation treatments on cultivars with varying susceptibility. The six treatments included lime-sulphur (lime-S) sprays in autumn on both canopy and orchard floor; lime-S sprays followed by mulch cover; mulch cover in late winter; leaf collection in autumn; leaf collection followed by mulch cover; leaf collection followed by plastic foil; non-sanitized control. In the treatments involving mulching, the entire experimental area was covered with a 10 cm-layer of wheat straw mulch, after the other treatments were applied where mulching was combined with another sanitation measure. The authors found that leaf collection alone caused a decrease in ascospore production of 56 to 79%. In a study by Gomez *et al.* (2007), leaf collection reduced the number of ascospores trapped by 95%, provided the leaves between the rows, as well as within the rows, were removed. The most effective treatment in the study by Holb (2006) was leaf collection followed by a plastic cover, which reduced ascospore production by over 95%. However, this treatment would not be economically or environmentally justifiable, as the plastic used for this treatment is expensive to obtain and lay down and the discarding of the plastic afterwards makes it environmentally unfriendly. The second most efficient treatment was leaf collection followed by mulch cover, where ascospore production was reduced by between 72 and 92%. This method removes the inoculum source and utilizes the numerous benefits of a mulch cover for the apple orchard under question (stabilizing soil structure, balancing micro-organism populations, buffering soil temperature and pH, maintaining an optimal soil water status), which would aid in lowering scab populations. Mulching alone and lime-S sprays followed by mulching reduced ascospore production by between 24 and 38% and between 27 and 46%, respectively, whereas lime-S sprays had no significant effect on apple scab. The latter three treatments had no significant effect in reducing leaf and/or fruit scab incidence, whereas leaf collection or leaf collection with mulching or plastic foil cover reduced leaf and/or fruit scab incidence by between 18 and 57% when compared to non-sanitized plots.

The challenge that South African apple producers face with leaf collection or shredding is the acquisition of the equipment needed to carry out the task in an efficient manner. Holb (2009) discusses options of incorporating leaf collection into general production technology: previous articles describe how a leaf collection adaptor could be used alone or in combination with a disc-

tiller, behind commercial tractors, which would reduce scab between 65 and 75%. These machines would only remove the inoculum between rows, since it is difficult to remove leaves within rows (Gomez *et al.*, 2007). Even though the leaves within rows do contribute to scab inoculum, it is not known to what extent this occurs in comparison to the leaves between rows. It will not be economically viable to address the leaves within rows of South African commercial apple orchards, unless a leaf-blower, to blow the leaves to the middle of the inter-rows or a machine which reaches between the trees, is built or imported.

In a five-year study in the north-eastern United States, Sutton *et al.* (2000) reported that leaf shredding applied in autumn reduced the amount of leaf litter by 52%, the number of foliar lesions on trees by 65% and the scab incidence on fruit by 46%. However, the reduction in leaf litter was variable, which the authors attributed to the percentage of the orchard floor that was shredded, which was affected by ground cover. The reduction in percentage ascospores trapped did not increase if leaves shredded in autumn were shredded in spring again. If the leaf litter was not properly shredded (*i.e.* between 10 and 35% leaves in total not shredded), then the risk of scab infection was only reduced by between 50 and 65%, which would be not significant enough for reducing scab risk for the following season. This would most likely be due to the limited offset of the flail mower used to shred the leaves, or the spread of the tree canopy under which the flail mower was used. In this study, leaves were shredded most effectively when orchard floors were well-managed *e.g.* tall grass and weeds were cut and/or sprayed and the orchard floor was even enough for the flail mower to manoeuvre efficiently. In general, the authors found that shredding the leaf litter in autumn or spring (Northern Hemisphere) reduced the risk of scab infection by 80 to 90%, but that more than 80% of leaves need to be shredded for the reduction in risk to be significant. Additionally, the authors state that leaf shredding in orchards where leaf-drop occurs earlier should lead to an increased efficacy of leaf shredding as a sanitation treatment.

Vincent *et al.* (2004) conducted a study to evaluate the effect of five non-chemical treatments on loose, naturally infected apple leaves of ‘McIntosh’, a highly susceptible cultivar. The treatments included leaf shredding, 5% urea, two fungal biological antagonists (*Microsphaeopsis ochracea* and *Athelia bombacina*) and an untreated control. Ascospore ejection patterns were found to be similar on treated and untreated leaves, although shredding was found to reduce ascospore production by 85.2%. However, the authors recommended that shredding be used in conjunction with urea or a fungal antagonist, as these combined treatments reduced ascospore production by 90.5 and 93.9% respectively. It was also recommended that leaf shredding be used as part of a sustainable, long-term, integrated pest management program for apple orchards.

According to Carisse and Dewdney (2002) most apple producers are reluctant to apply sanitation strategies to control scab, even though many of the methods have been proven to be

highly effective in controlling scab. The inability of researchers to distinguish between the high ascospore inoculum in experimental orchards and the low ascospore inoculum found in commercial orchards was stated as a possible reason (Sutton *et al.*, 2000). This changed when researchers began using the method to determine PAD described by Gadoury and MacHardy (1986). Another reason why sanitation strategies are not more commonly used in commercial apple production is that sanitation strategies were previously not economically justified when compared to the efficacy of fungicide sprays. However, fungicide sprays have more recently been used in conjunction with sanitation strategies to reduce the number of sprays per season. Holb (2009) states that only those sanitation strategies that reduce scab inoculum pressure by more than 50% can be used to successfully control apple scab. However, if a producer wants to reduce scab inoculum to a level that would put minimum or no disease pressure on an apple orchard, one could argue that the reduction in scab inoculum should be greater. Sutton *et al.* (2000) questioned whether a sanitation treatment is economically feasible or not if scab risk is reduced by 50%, as well as discussed the relation of reducing scab inoculum to a sanitation action threshold to reduce fungicide use, even in orchards with moderate foliar scab incidence. MacHardy (2000) described how the PAD of an orchard can be used to determine whether or not an orchard requires sanitation: in an orchard with  $n$  trees, the number of trees that would need to be assessed is  $n/60$  and if the number of scabbed leaves on 600 extension shoots is 50, the orchard is “low risk” and fungicide use can be reduced using several tactics. If the number of scabbed leaves out of 600 shoots is 50 to 100, then the orchard is “moderate risk” and the producer should apply a sanitation strategy (leaf shredding or urea are recommended) before considering reducing fungicide use. These action thresholds aid producers in integrating sanitation with their existing scab management strategies and offer tactics to reduce their reliance on fungicides.

### ***Chemicals accepted in organic apple production and fungal antagonists***

The application of urea after harvest prevents the formation of pseudothecia by increasing the pH in leaves and increases the microbial activity in and on fallen leaves, also promoting leaf degradation (Burchill *et al.*, 1965; Burchill, 1968; Schwabe and Matthee, 1972; Schwabe and Heyns, 1974). This increases the competition for nutrients and space between *V. inaequalis* and micro-organisms which are antagonistic towards the apple scab fungus. Burchill *et al.* (1965) reported a 55 and 97% reduction in ascospores discharged from leaves sprayed urea at 2 and 5%, respectively, in the previous autumn. The authors concluded that pseudothecial formation on leaves had been inhibited, which may have been due to the higher nitrogen content and increased microbial activity measured in treated leaves compared to non-treated leaves. This was confirmed by Burchill and Cook (1971),

who showed that 5% urea delayed the early ascus developmental stage of pseudothecia and prevented the development of later stages.

Burchill (1968) reported that dipping leaves in 2 and 5% urea reduced ascospore productivity by 55 and 97%, respectively, which confirmed results by Burchill *et al.* (1965). In the study by Burchill (1968), apple plants were sprayed with 5% urea and leaves from treated and non-treated plants were detached 2 h, 48 h and one week afterwards and then at weekly intervals until all the leaves had fallen naturally from plants. The study was done over two seasons. No ascospores were discharged in the following spring from leaves detached 2 h and 48 h after urea sprays, and leaves detached one week and two weeks after treatment had an average ascospore reduction over both seasons of 86 and 32% respectively. The author concluded that the inhibitive effect on pseudothecial production by urea sprays was lost if leaves were detached from trees one week or more after urea application. Urea treatments accelerated leaf-drop in both seasons, but leaf-drop did not occur fast enough to allow urea to cause the desired effect of ascospore inhibition. Leaves that had been sprayed with 5% urea during leaf-drop and were dipped in 3, 4 or 5% urea again shortly before bud-break inhibited ascospore discharge by 100%. However, these results do not accurately represent what would occur if leaves had been sprayed on the ground, because the same coverage would then not be achieved as the coverage by dipping. In another experiment by Burchill (1968), in an orchard that had high scab levels, 5% urea was hand-sprayed on the trees and ground during leaf-drop. Leaves detached from the trees one week after treatment showed 96% ascospore reduction in the following spring, whereas leaves collected from the orchard floor in the following spring had an average ascospore reduction over both seasons of 43%. Scab incidence on leaves during bloom, on trees treated with 5% urea sprays during leaf-drop, was 55% less than on trees that had not received sprays. Urea-treated leaves decomposed faster than non-treated leaves.

Schwabe and Matthee (1972) reported that, in a laboratory study where leaves were immersed in a 5% urea solution and then left in open orchard conditions over winter, more than 90% of the primary inoculum was eradicated. In field tests done, a single 5% urea spray applied to leaves in late autumn eradicated primary infection by between 20 and 70%. Schwabe and Heyns (1974) dipped detached leaves in 3 and 5% urea solutions, of both spray-grade and ground fertilization grade, for one minute each, and then leaves were overwintered in a laboratory and outside under natural conditions in Stellenbosch. In all four treatments, 3 to 11% of ascospores survived in the laboratory experiments and 40 to 79% of ascospores survived in the field experiments. According to the author, the reduction in ascospore survival in the field experiments was not sufficient, especially since the synthetic chemicals tested were 100% effective. Schwabe and Matthee (1976) again tested the efficacy of urea as a postharvest eradicator. Detached leaves were dipped in 2.5, 3 and 5% urea solutions for laboratory tests and the same concentrations were

used for the field experiment in the same layout as done by Schwabe and Matthee (1972). The 2.5, 3 and 5% urea solutions reduced mean ascospore discharge in the laboratory by 99, 99 and 92%, respectively, and in the field by 56, 70 and 92%, respectively.

According to the Schwabe and Matthee (1972; 1976) and Schwabe and Heyns (1974), urea is not recommended as a post-harvest control treatment for scab to eradicate primary infection source and that the promising results obtained in laboratory or glasshouse trials cannot be obtained in field trials. The authors suggest that the urea applied on leaves in the orchard is incorporated into the plant or soil or washed off before it can exert a significant eradicated effect on apple scab. If urea is applied before leaf drop in test areas with a shorter leaf drop period, most of the compound is still present on the leaves by the time they abscise and can be more effective than if applied in areas with longer leaf drop periods. In laboratory tests, the leaves were detached from the tree and had received complete coverage with urea. The same effective coverage can never be achieved for all the leaves on a tree in an orchard (Schwabe and Matthee, 1972).

Sutton *et al.* (2000) conducted a comprehensive study that tested the effect of leaf shredding, urea applications and a combination of leaf shredding with urea in autumn on ascospore production, as well as on scab incidence and severity. They reported that urea reduced the number of ascospores by 50% when applied to leaf litter after 95% of the leaves had fallen in autumn, in comparison with the 66% reduction if applied at the end of winter, before bud break. Urea applied in autumn to the leaf litter of a single orchard site did not have a significant effect on the severity of foliar lesions, nor did it reduce the amount of leaf litter on the orchard floor by the beginning of spring. Urea applied in spring to the leaf litter of a different orchard two years later reduced the fruit scab incidence of that season by 41% and the number of foliar lesions by 80%. These treatment differences could be attributed to seasonal and/or climatic variations in scab incidence and severity, or to differences in trial sites, such as slope, aspect or microclimate. Over a period of 5 years, urea applied in spring to the leaf litter reduced ascospore production by an average of 70%. The leaf litter in the combined treatment (urea with leaf shredding) was not reduced more than in the treatment where only leaf shredding was applied in autumn. Fruit scab incidence and leaf scab severity in the combined treatment was reduced by 65 and 42%, respectively, whereas in the leaf shredding treatment it was reduced by 75 and 29%, respectively. However, whether or not the differences between treatments are significant from one another is not clear, but the authors conclude that the combined treatment did not improve on the performance of leaf shredding alone. The combined treatment was not repeated, due to the low efficacy of this treatment for the one season that it was tested. However, the author states that this treatment should be repeated, since it was only tested in one season.

Carisse *et al.* (2000) tested the effect of 5% urea, *Athelia bombacina*, *Microsphaeropsis* sp., *Microsphaeropsis arundinis*, *Ophiostoma* sp., *Diplodia* sp., and *Trichoderma* sp. on ascospore productivity on artificially and naturally infected detached leaves under both controlled and natural winter conditions. With the exception of the treatment with *Ophiostoma* sp., treatments with *Trichoderma* sp., *A. bombacina*, urea, and *Microsphaeropsis* sp. significantly reduced ascospore production by of 83.7, 84.2, 87.7, and 90.4%, respectively. Large-scale trials were also conducted with both *Microsphaeropsis* sp. applied at 90% leaf fall either as a postharvest spray to leaves or as a ground application, which significantly reduced ascospore production by 75%.

In the study by Vincent *et al.* (2004), urea, applied in a 5% solution (46% N) at a rate of 1 ml per leaf as a treatment, was found to be the most efficient treatment in reducing ascospore production (92.1%). This was done in comparison with leaf shredding, two fungal biological antagonists (*Microsphaeropsis ochracea* and *Athelia bombacina*) and an untreated control.

The theory behind the efficacy of lime-sulphur (lime-S) or dolomitic lime as a method to control scab and other pathogens, is that microbial activity is enhanced and soil pH is increased, leaf litter decomposes more rapidly, which suppresses ascospore production (Spotts *et al.*, 1997; Holb *et al.*, 2003).

Louw (1951a) tested the effect on scab incidence and severity of various concentrations of lime-S applied at various stages on trees. Results indicated that the effect of lime-S concentrations of 1:100 at pre-bloom was significant (98% reduction) and did not differ significantly from higher concentrations, but foliar injury was less at this concentration. Wettable sulphur caused more damage to trees, but post-bloom applications were as comparably effective as lime-S. Holb *et al.* (2003) reported that lime-S applied during the season lowered fruit scab damage significantly when applied either preventatively (76%) or curatively (67%), as opposed to wettable sulphur (45%), when compared to the negative control. However, lime-S treatments showed high phytotoxicity values and caused a reduction in leaf size and yield quality, which agrees with results reported by Louw (1951a). In another study, Holb (2006) found that lime-S did not significantly reduce leaf-litter density or ascospore production compared to non-treated plots.

In the experiment by Spotts *et al.* (1997), dolomitic lime (22.7% calcium and 11.8% magnesium), applied with a flour sifter on loose apple leaves before being placed between two layers of nylon mesh, reduced the ascospore dose in the following season by 55 to 92%. Each of the six plots, each covering 0.35 m<sup>2</sup>, received different concentrations of dolomitic lime, the highest of which was 5.08 tons per hectare. Ascospore dose was determined by collecting five to ten leaves at bi-weekly intervals for 3 months from the time of bud-swell. Pseudothecial development and area covered by pseudothecia was assessed, but no disease assessment in the orchard was done. The dolomitic lime application only reduced leaf area by 18%, and so the author suggested that factors

other than leaf decomposition were more involved in ascospore reduction. Dolomitic lime was applied in higher concentrations and more evenly than typically found on commercial apple orchard floors. Additionally, the authors report counting very few pseudothecia per leaf, in treated and non-treated leaves, and so the reduction in ascospores due to lime may be more pronounced than if the leaves had had more pseudothecia per leaf. Therefore, it is difficult to gauge whether or not the results from this trial would aid in determining if dolomitic lime is effective in controlling apple scab in commercial orchards.

Apple producers worldwide do not readily use lime-S as a treatment for scab control as the application of lime-S is costly and causes corrosive damage to spray machines, especially if used at relatively high concentrations (Bekker Wessels, personal communication). This, together with the results of the above studies, indicates that dolomitic lime and lime-S would not be a suitable alternative control against apple scab in South African orchards.

Fungal antagonists show promising results, although production and use of these antagonists is not yet available for commercial orchards in South Africa. Urea applications to the ground in spring may not achieve the desired coverage, although if applied in autumn, may be more beneficial to apple producers, if not absorbed by, or washed off, leaves during long leaf-drop periods.

### ***Reducing number of fungicide sprays per season***

Schwabe (1980) advocated a reduction in the number of fungicide applications per season in South African apple orchards. The author described various ways in which this could be done, including autumn assessment of scab levels in each orchard for decision-making on scab management practices post-harvest and in the following season; monitoring of weather in each climatic region for the infection risk index model, and ongoing improvement of the scab warning system that he developed; and continuous research on apple scab in South Africa.

Local apple producers would need evidence over many seasons and for various regions in the Western Cape that scab fungicide applications can be omitted during spring or summer before they would consider doing so on their own farms, and even then producers may not be convinced, mainly due to their own negative experiences with scab. Due to the high risk of scab epidemics arising from improper management of scab in spring, attention should be given to possibly reducing or eliminating fungicide applications later in the season. According to Creemers and van Laer (2006) leaf shredding used as a sanitation method to reduce primary inoculum does not merit the reduction of fungicide applications early in the season. Rather, reductions could happen later in the season to controlling infections by secondary inoculum, which would be less since the primary inoculum would have been reduced through shredding. This approach fits in well with an anti-

resistance management approach, as this would benefit producers in avoiding the persistence of insensitive subpopulations of *V. inaequalis* in problem orchards that are intensively sprayed with single-site fungicides. It would also aid producers in reducing residue levels on fruit, since the need to spray shortly before the fungicide withholding period starts would be reduced, which would mean that producers would be able to market their produce as low residue fruit in more profitable markets. Using risk forecasting methods, such as PAD and RIMpro, would aid producers in determining which orchards require sanitation and/or which orchards could receive a reduced spray programme. Carisse and Dewdney (2002) stated that “Ultimately, sustainability will depend on the cost effectiveness of integrated approaches as compared to total dependence on fungicides to control apple scab”.

### ***Integrated management systems for apple scab***

Apple producers worldwide currently use between 6 and 15 sprays per season (MacHardy, 1996; Ellis *et al.*, 1998; Carisse and Dewdney, 2002; Cuthbertson and Murchie, 2003). The number of applications can be greatly reduced through selecting less resistant, but still desirable, cultivars; using effective, low resistance risk fungicides; using sanitation to reduce inoculum; using effective, organic chemicals; and adjusting spray programmes and timing (Jamar *et al.*, 2010; Creemers and van Laer, 2006). This is what constitutes integrated pest/pathogen management (IPM). Reducing ascospore inoculum even by as much as 99% in an orchard with a high inoculum level in the previous season through sanitation does not necessarily mean that the number of sprays in the following season can be reduced. However, in orchards with low to moderate inoculum levels in the previous season, significantly reducing ascospore inoculum through sanitation could eliminate the need for one or more fungicide sprays (Sutton *et al.*, 2000).

Fig. 2, a schematic representation of how various practices can be integrated for scab management, is redrawn and modified from MacHardy *et al.* (2001). Autumn scab assessments are essential in determining whether or not an orchard will have a low, medium or high risk of scab infections in the following season. High and medium risk orchards require sanitation to reduce the ascospore inoculum, slow a scab epidemic and prevent the build-up of resistant *V. inaequalis* strains. The use of tolerant cultivars further slows a scab epidemic, and cultivars resistant to all races do not require fungicide applications. Cultivars with minor gene resistance, whether planted in monoculture or in a mixed cultivar orchard, require that a scab warning system is followed and appropriate fungicides are applied. Low risk orchards fall outside of the scab risk action threshold, and so they do not require sanitation and can be monitored using the scab warning system. Developing such a comprehensive integrated management system would most likely require

optimization for each climatic region where apples are grown, and would be highly time-consuming. However, the overall benefit of maintaining consistently low scab levels may well be worth the time and cost input. If such a system were utilized in South Africa, apple scab epidemics would very likely never occur, as there would be numerous factors checking the disease.

There are no commercial apple cultivars in South Africa that are resistant to apple scab with all being susceptible to a greater or lesser degree. However, some resistant commercial cultivars are grown overseas, although the production rights for these cultivars are protected. Also, producers are not likely to plant new cultivars, which have not been proven to be accepted and bought by consumers in well-established markets. According to Carisse and Dewdney (2002), “apples are one of the few horticultural crops that are purchased on the basis of recognition of the cultivar name”; therefore, a cultivar with an unfamiliar name and with an unfamiliar appearance tends not to sell as well as a cultivar to which a consumer is accustomed. Although breeding for resistance to apple scab in apples is a daunting task that few scientists have taken on, resistance genes have been identified in certain apple cultivars and IPM can be used to manage apple scab and utilize and exploit what little resistance there is in apple trees (Bus *et al.*, 2009; Patocchi *et al.*, 2009).

Mundt *et al.* (2002) describes three aspects of IPM that need to be considered when discussing the effect of IPM on resistance durability. The first is the positive epidemiological synergism that can be obtained through combining disease management practices, each of which may only provide less than adequate disease reduction when used alone, and through disease management on a regional scale. Here, the author focuses on the effect of spatial scale on epidemiological impacts and on how resistance should be incorporated into regional-sized production areas, such as using cultivar mixtures, gene pyramiding and combining host resistance with other management practices. The second aspect is the potential that IPM has for increasing resistance durability, by reducing pathogen population sizes and decreasing the probability for resistance breakdown. The authors point out that, though this theory may seem logical, no empirical data exists to justify this, due to the immense difficulty that conducting such a trial would involve. They also state that almost all models that compare the impact of deploying race-specific resistance genes in mixtures, pyramids or in rotation over time assume a genetic cost associated with virulence; however, such costs are difficult to quantify empirically. The third aspect is integrating resistance into overall crop management, especially when breeding for agronomic characteristics. Although factors such as yield quantity and quality, plant size and growth habit, and adaptability to environmental conditions need to be taken into account, breeding programmes never realistically incorporate all desired characteristics. Those characteristics that cannot be incorporated can be amended for as far as possible with cultural practices (e.g. peach trees with a low tolerance to wetness can be planted on a slope where they grow in well-drained soil).

Fungicides affect not only fungal pathogens, but insect pests and their natural enemies as well. Cuthbertson and Murchie (2003) found that mancozeb sprays used to control apple scab have a significantly detrimental effect on both pest and their predator species of mites. The authors also mention studies that show that mancozeb is highly toxic to other predatory mites. Other fungicides, such as captan and dithianone, were not as toxic, although the authors mention other studies which had variable results. The authors also state that, although it is not economically feasible to produce apples, “without the aid of a fungicide programme, the choice of such a programme should also consider effects on predatory mites and insects”. This is because a large percentage of fungicides are sprayed very early in the season, coinciding with elevated predatory mite populations, which effectively reduce pest mite populations. In a two-year study done on four different apple cultivars, Bower *et al.* (1995) reported that a fungicide spray programme of six applications of mancozeb and benomyl significantly decreased the population of predacious mites when compared to a non-sprayed control. The authors also state, in their abstract, that this study shows “the potential benefit of eliminating fungicide applications”. Soil fauna, primarily earthworms, feed on decomposing organic material and convert it into fertile soil. MacHardy *et al.* (2001) mentions that soil fauna that bury and decompose leaves quicker can be used to reduce ascospore inoculum during winter, but only if chemical control of scab is applied appropriately. This could be an especially viable aspect of scab management in integrated and organic orchards, but also in orchards where less toxic fungicides are used. The health of micro-organisms in soil is also beneficial to the overall health of the orchard. The authors mention that benzimidazole fungicides, which are highly harmful to soil fauna, primarily earthworms, should be avoided if integrated management using soil fauna is to be effective (MacHardy *et al.*, 2001).

As our knowledge on the integration of resistance to current and IPM practices increases, the likelihood that farmers will use these practices and durable resistance may also increase.

## **OVERWINTERED CONIDIAL INVOLVEMENT IN PRIMARY SPRING INFECTIONS**

It has long been known that ascospores may not be solely responsible for primary infections in spring and various authors have considered that asexual conidia formed in summer could remain viable throughout winter in apple orchards and cause infection in the following season (Clinton, 1901; Louw, 1951b) before ascospore maturation (Becker *et al.*, 1992). Sepals are of the first green tissues that unfurl from buds and remain attached to developing fruit throughout the growing season (Keitt and Jones, 1926; Becker, 1990). They are highly susceptible to infection during the green-tip phenological stage and if these tissues become infected, they could provide an important conidial inoculum source for secondary infections (Becker, 1990).

Clinton (1901) rarely found conidia on twigs, although the few that were found before scab infection in the orchard in spring were not believed to be viable. In an orchard with a severe infection in that season, leaves that had not yet fully unfolded from apple buds in spring had infections on them. Although the degree of infection on these immature leaves is not stated, the infections were most abundant on the lower surface of the leaves in the vicinity of the midrib. This may be because it is the area closest the inside of the bud in early spring, and if these infections originated from the inside of the bud, then viable conidia that had survived the winter may be the cause of these infections (Clinton, 1901).

In a seven year investigation by Gloyer (1937) in New York, dormant branches that were believe to be infested with conidia produced lesions on scale leaves when buds opened and the author concluded that the role of conidia in the initiation of primary infections is of greater importance than is generally accepted. Loewel and Friedrich (1938) enclosed apple shoots in waterproof bags before ascospore discharge began and infections were observed on them, even though no scab lesions or pustules were visible on the shoots before being enclosed. These authors concluded that conidia or mycelia were responsible for these infections and that those conidia in the previous season may have been deposited on buds and crevices on shoots with condensation water. The authors also noted that the oldest infections were seen on leaf petioles and bases, which they concluded was the source of conidia for later infections. Louw (1951b) discussed studies done by McKay (1938, 1939), who reported that scab infections developed in the absence of both scab-infected wood and overwintered leaves and the author traced the source of infection to buds, where viable scab stromata (mycelia) were found on the bud-scales, yet buds exhibited no external symptoms or signs of the disease. It seems that these four studies (Gloyer, 1937; Loewel and Friedrich, 1938; McKay, 1938, 1939) did not consider that inner tissue in apple buds could harbour viable conidia. In studies done on apple scab in the winter-rainfall area of the Western Cape, Louw (1951b) reported that *Spilocaea (Fusicladium)* mycelia on scab-infected apple leaves and fruit did not produce conidia in the following spring. The author most likely looked at fruit that had fallen onto the ground, which would be senescent fruit, instead of overwintered fruit still attached on the tree. Becker (1990) suggested that the senescence of host tissue and the presence of competitive micro-organisms could be some of the factors that contribute to the decreased survival and persistence of viable conidia within lesions on detached fruit and leaves.

Louw (1951b) enclosed branches in an orchard that had been severely infected in the previous season with waterproof paper bags. Half of these branches were uncovered during a rainy period during ascospore discharge (confirmed with spore-trapping) and the other half kept closed and only wetted with distilled water on a dry day before being closed again. The branches that had been exposed to ascospores developed scab on one or more leaves per shoot, whereas the branches

that had only been wetted with distilled water did not develop scab. In another experiment, Louw (1951b) grafted marked shoots from an orchard in Ceres that had been severely infected in the previous season onto potted, scab-free seedlings in the following season and kept outdoors under conditions extremely favourable for scab to develop. Scab did not develop and no conidia or mycelia were found on the bud-scales on any of the 150 shoots that ultimately grew from these grafts. The author concluded that this indicates that no viable spores were present on the shoot surfaces or between the bud-scales of the grafted buds. In another test, Louw (1951) dissected and examined approximately one quarter of the buds from a number of severely-infected twigs sampled in spring from Ceres, Elgin and Somerset West. Germination tests were done on all scab mycelia found, but no mycelium were found to remain viable on the bud-scales. Although the author dissected many buds, inner bud tissue was not examined for scab mycelium. In another test done, buds were thoroughly washed in distilled water and the spore suspensions were microscopically examined for conidia. Although small numbers of conidia were found, none were found to be viable. The author states these conidia were the remains of the previous season's spore load which had adhered to the outer surface of the buds or had become embedded between the bud-scales. Attempts to isolate the scab fungus from the bud-scales of buds from severely infected shoots by incubating the scales on agar plates produced no *V. inaequalis* mycelium. Louw (1951b) concluded that scab infections in spring in apple orchards in the winter rainfall region of the Western Cape were caused by ascospores and not by conidia. However, in apple-growing areas such as Elgin, Witzenberg Valley and the Koue Bokkeveld, there have been reports of apple scab infections before ascospore release (Bekker Wessels, private consultant, Ceres region and Frikkie van Schalkwyk, private consultant, Grabouw region, personal communication). Louw (1951b) investigated the surfaces of apple twigs and the outer surfaces of buds (bud-scales) for overwintering conidia only, but did not investigate inner bud tissues as sources of viable conidia.

The first studies to address whether viable *V. inaequalis* conidia could overwinter inside apple buds, and the first report that they do indeed do so, were completed by Becker (1990). This study was conducted to determine whether *V. inaequalis* conidia overwinter in lesions on shoots, leaves or fruit or in dormant apple buds and, if so, whether they contribute to primary infections in spring. Conidial distribution within dormant buds was also determined. Samples were analyzed from a commercially sprayed orchard, two unsprayed orchards and one which received irregular sprays, over two years in New York. Viable conidia were seldom found on lesions on infected shoots or on the outer surfaces of buds, and never on the surfaces of lesions of leaves, shoots and fruit removed from the trees and left on the orchard floor during summer. However, up to 142 viable conidia that had overwintered in the inner tissues of flower buds were found, and apple scab lesions developed before ascospores had matured and dispersed to infect buds and shoots. These

conidia were mainly found in orchards where the scab incidence in the previous orchard was moderate to high.

In order to determine when conidia were most likely to infest and/or infect apple buds, Holb *et al.* (2005b) conducted a complex analysis on the progression of apple scab epidemics in integrated and organic orchards in the Netherlands and in Hungary. According to the author, a rise in leaf scab incidence occurred together with shoot growth during summer, during which most bud scales were open. This is when conidia could be deposited between bud scales by wind and rain and may infect inner bud tissue. Even if overwintered conidia did not infect inner bud tissues, they could remain viable due to their protection against unfavourable conditions and cause infections in the next season before ascospore discharge when conditions became more favourable (Holb *et al.*, 2004, 2005a). Holb *et al.* (2005b) found that the apple phenological period with the highest risk of conidial entrapment between bud scales was when shoot growth stopped in autumn. The authors concluded that, in order to prevent conidial entrapment, producers would need to spray regularly until the end of shoot growth, but after that fungicides are ineffective against conidia that may have already become trapped.

The numbers of viable conidia that overwinter vary between seasons as the climatic conditions vary and the detection of these conidia is likely to be affected by the sampling and detection methods (Becker, 1990). As the distribution of scab within an orchard is variable, a small percentage of buds within an orchard may harbour a high percentage of viable conidia over winter. No viable conidia were found on the exterior of dormant buds or in lesions on infected fruit, leaves and shoots, as these exposed areas are not suitable for the survival of conidia (Becker *et al.*, 1992). This is supported by a study by Becker and Burr (1994), who found that a significant number of conidia, especially those that had already germinated, do not remain viable after being subjected to extreme and fluctuating relative humidity for longer than 96 hours. Such conditions would typically be found on the outer surfaces of buds and shoots during winter. However, the inside of a bud is protected and provides an environment that may be more favourable to apple scab conidia and other micro-organisms.

Holb *et al.* (2004) reported that 65% of dormant shoot samples had superficial black mycelia or conidia in densities of 581 to 1033 conidia per 1-cm shoot length in apple orchards in the Netherlands over three consecutive seasons. These conidia were insufficient to contribute to scab incidence as their viability was less than 1.5%. Microscopic examination of bud tissues revealed that, of the >3000 conidia per 100 buds counted, only 3.7 to 10.5% in the inner tissues and 0.7 to 1.9% in the outer tissues were viable. The overwintered conidia contributed to 0.3 to 3.8% of scab infection in spring during tight-cluster phenological stage, particularly in orchards with a high scab incidence. However, the differences in numbers of conidia and the differences in viability of conidia

between inner and outer bud tissues were statistically significant at  $P \leq 0.0001$ . Therefore, it is more likely that this percentage of infection was caused by conidia from the inner bud tissues, since fluctuating conditions on outer bud tissues and on shoots was not conducive to conidial survival. The authors suggest that, in orchards with a high scab incidence but a reduced ascospore inoculum, trees should be protected with fungicide treatments at green-tip in early spring.

The number of conidia on 1-cm shoot pieces was far higher in organic orchards (90 to 1000 conidia) that had a significantly higher foliar scab incidence in the previous season (>60%) than in integrated orchards (1 to 6 conidia) with less than 20% scab incidence (Holb *et al.*, 2005a). The percentage of viable conidia that survived in outer bud tissue and shoots was less than 2% for all orchards, but in inner bud tissue it was approximately twice as high in heavily diseased organic orchards (2 to 11%) as in integrated orchards (0 to 6%). The number of conidia that survived the winter increased exponentially if the incidence of scab was higher than 40% in autumn. The authors concluded that integrated orchards may not necessarily require treatments to reduce overwintering conidia if the incidence of scab is low in the preceding autumn season. However, the potential early infection risk posed by overwintering conidia in organic orchards is higher than in integrated orchards. Organic orchards require preventative control measures in the previous season as well as in early spring; if not, the chances of asexual inoculum remaining viable during winter are greatly increased (Holb *et al.*, 2005a).

The findings in the above studies suggest that viable mycelia and conidia do overwinter, but do not remain viable, on bud and shoot surfaces in South Africa. However, viable conidia do overwinter inside apple buds in orchards overseas, but this has yet to be investigated in South Africa. Conidia also do not need to be deposited on live, wet plant tissue before germination is initiated, unlike ascospores, which do require rain for discharge and germination (Schumacher *et al.*, 2008; Steiner and Oerke, 2007). Therefore, viable conidia that have overwintered inside apple buds could cause infection before buds open and leaves are exposed to rain, but also before leaves are exposed to protective fungicides. This would have major impacts on the timing of fungicide applications and efficiency, which is crucial for minimizing primary inoculum in spring and disease incidence and severity for the rest of the season.

## CONCLUSION

Apple scab is the most important disease of apples worldwide and causes superficial lesions on leaves and fruit. Scabbed fruit cannot be exported and are sold on local markets or are juiced, bringing in a fraction of the income compared to export quality fruit. The cost of fungicides required to control scab infections and the reduced income from scabbed fruit impacts on the profit of apple production in South Africa. Apple producers in South Africa rely solely on chemicals to control scab, as this is still the easiest and most economic option available to them. However, increased problems with fungicide resistance and the market demand for low-residue or residue-free fruit is increasing the need for alternative control strategies for apple scab. Producers generally do not change disease control strategies unless forced to do so by law or regulations, or unless the market that they supply and profit from applies pressure. The selection pressure placed on local *V. inaequalis* populations by the numerous fungicide applications in some apple-growing regions has led to tolerant sub-populations. Due to the consistently high number of fungicide applications currently used, newer strains of this pathogen that are resistant to certain chemical classes may appear in these and other regions in the future. This emphasizes the need for alternative control measures. Ultimately, a lower risk of resistance of scab to certain fungicide classes, brought about by integrated management, and thereby reducing the use of high-risk fungicides per season, would prolong the efficacy of these chemical classes. Such a strategy would also serve to benefit producers, consumers and the environment.

Integrated pest/pathogen management (IPM) of scab can benefit apple producers by reducing the number of fungicide sprays per season and the cost associated with it. This also lessens the impact that chemical control has on humans, animals and the surrounding environment. The shift towards apple producers voluntarily implementing an IPM program in orchards requires the collaboration between scientists and industry to provide tangible proof to producers that IPM programs are effective in controlling scab under the conditions in their region or country. However, to date, there are no published data on alternative control strategies that are used and are effective in controlling scab in South Africa.

Of all the studies done on the efficacy of sanitation treatments for control of apple scab, leaf removal and leaf shredding were generally found to be the most effective. Removing the sheer volume of leaves from an orchard is highly impractical on a commercial scale and detrimental to the orchard long-term, since a vital source of carbon for the following season (in the form of decomposed leaves) is removed from the soil. Leaf shredding only requires leaves to be shredded, but not removed and should be evaluated under South African orchard conditions to establish whether the results from such a study confirm results from previous studies. However, more than

80% of leaves in an orchard need to be shredded for the reduction in scab to be significant in the following season. The efficacy of leaf shredding under South African conditions needs to be evaluated before an economically viable method of leaf shredding for commercial-scale orchards is investigated. Urea applications of 5% to trees in autumn increase nitrogen content and increase the microbial activity in and on fallen leaves, inhibiting the early developmental stages of pseudothecia and reducing the number of ascospores discharged in the following season significantly. However, if leaves remain attached to trees for one week or longer, the inhibitive effect of urea decreases dramatically. In South Africa, laboratory studies showed that 5% urea greatly reduced the number of ascospores discharged from leaves in the following season, while field studies showed that the reduction in number of ascospores was less. The results of the studies in South Africa should be verified under modern orchard conditions.

Various disease risk prediction models have been developed over the past few decades, the most comprehensive of which is RIMpro, which takes into account potential ascospore dose (PAD). However, factors that contribute to this system would need to be verified under local conditions before it can be used in South Africa, such as the pseudothecial and ascospore density constants.

*Venturia inaequalis* is reported to behave differently in the Western Cape compared to populations overseas, e.g. in ascospore discharge patterns and temperature optima for sexual stages of its life-cycle. However, more information is needed on the development of the various sexual sub-stages of this pathogen's life-cycle. The historical warming trend in the Western Cape and further predicted increases has already caused significant changes in apple tree phenology in warm winter regions, which may also mean that this pathogen, which adapts to its host phenology, may evolve to exhibit even more differences to populations overseas. Viable conidia do not overwinter on the surfaces of shoots on bud-scales in apple orchards in the Western Cape. However, viable conidia do overwinter in inner bud tissues in orchards overseas where the previous season's scab levels were high and particularly where winters are relatively mild. Numbers and viability of conidia overwintering in inner bud tissue in South African apple orchards has yet to be determined.

Only when scientists, producers and other specialists in the apple industry work together, in South Africa and internationally, can there be consistent management of apple scab and a significant reduction in the use of fungicides. This requires more published research on alternative control strategies for apple scab in South Africa, the presentation of this research to the industry and to producers, as well as producer education in how and why to apply IPM programs. This means that more people who are trained in researching and controlling apple scab are needed. Also needed are tools for the quantitative and/or qualitative detection of resistance in regional *V. inaequalis* populations to specific fungicide groups. This will aid in determining, before fungicides are sprayed, which fungicides are still effective in controlling scab in specific areas.

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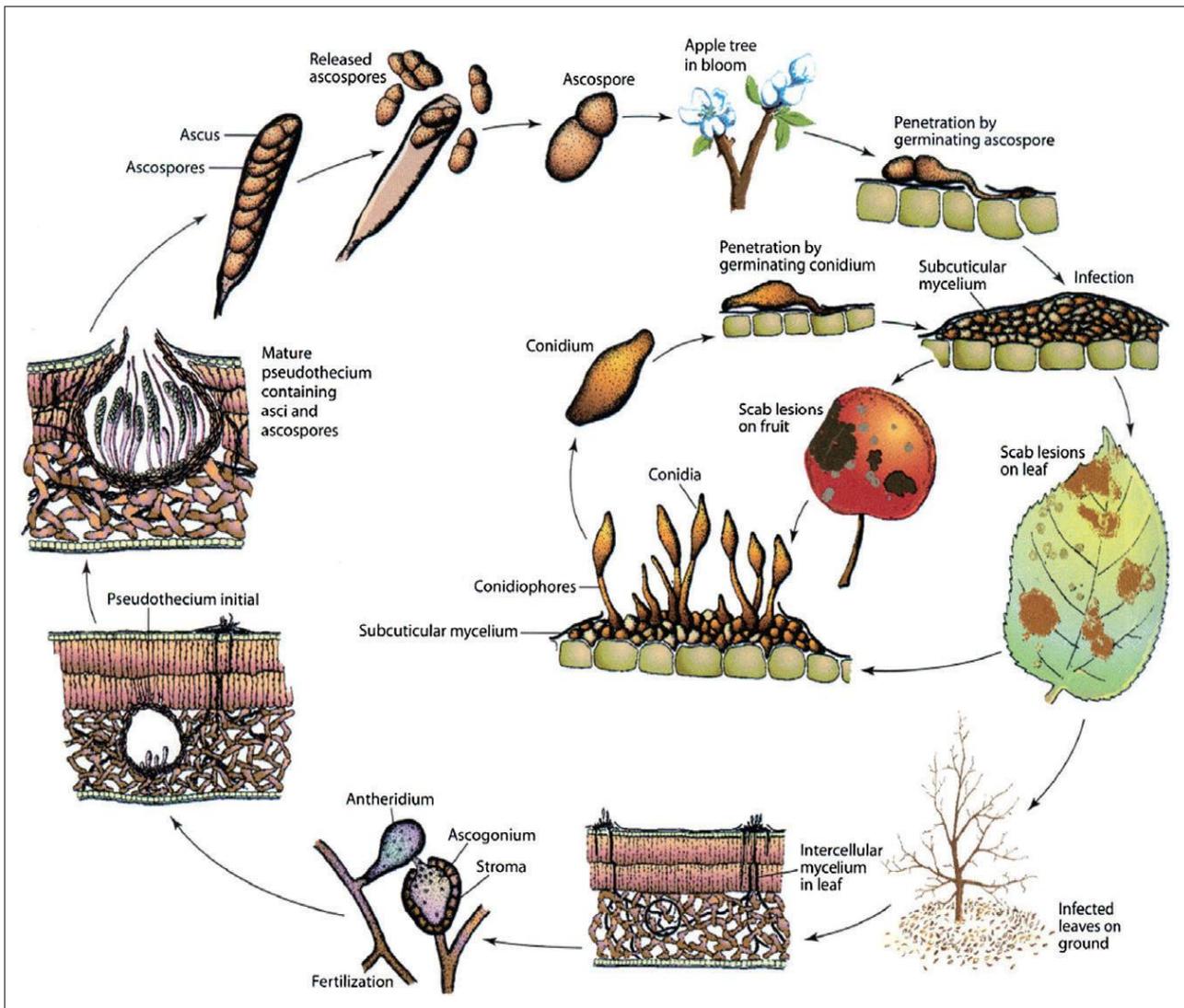
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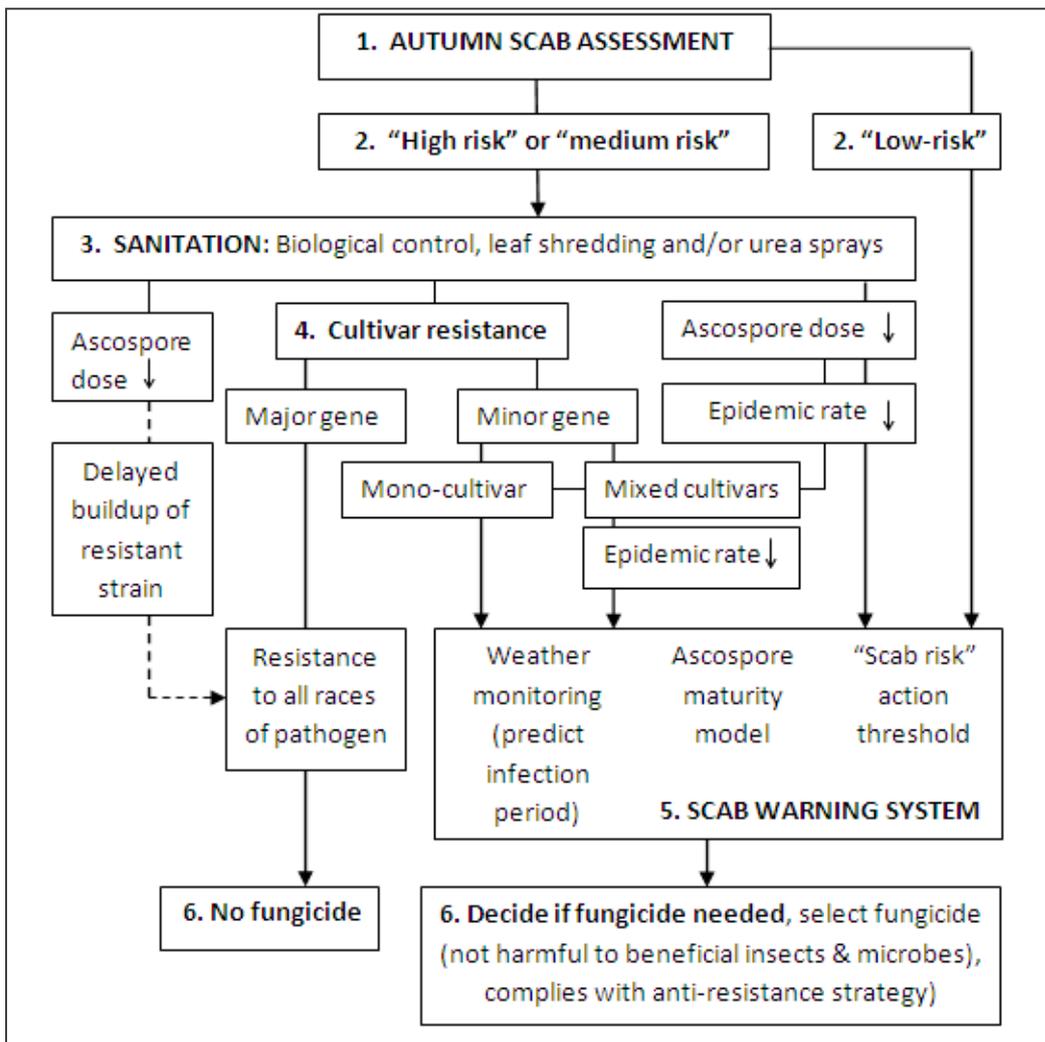
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**Figure 1.** *Venturia inaequalis* life cycle, from Agrios (2005).



**Figure 2.** Schematic representation of apple scab management practices, redrawn from MacHardy *et al.* (2001).

## CHAPTER 2

### ***THE APPLICATION AND EFFECT OF ORCHARD SANITATION FOR CONTROL OF APPLE SCAB IN SOUTH AFRICAN APPLE ORCHARDS***

#### **ABSTRACT**

The South African apple industry currently relies entirely on chemical fungicides to control apple scab (*Venturia inaequalis*). The rising cost of fungicide applications, the risk of fungicide resistance development, the increasing demand for lower or no residues on fruit in markets has prompted the need for alternative management strategies for scab. The objective of this study was to evaluate the effectiveness of sanitation strategies in reducing apple scab incidence and severity, airborne ascospores and leaf litter density in South African apple orchards. Leaf shredding with no fungicide sprays was tested against a non-sprayed, non-shredded negative control and a positive control that followed a commercial fungicide programme over four years, and also against a combined treatment of a commercial fungicide programme with leaf shredding over three years. Two treatment replicates were applied in a randomized block design in each of two orchards. Scab incidence and severity on fruit and leaves were assessed weekly from green-tip until fruit-set in the following spring. Pooled data from three years revealed that fruit scab incidence and severity and leaf scab severity (51, 55, and 39%, respectively,  $P < 0.05$ ) and leaf scab incidence (33%,  $P < 0.1$ ) were significantly lower in the leaf shredding than in the negative control. In 2012 and 2013, volumetric spore traps were placed in the two replicates of the leaf shredding and negative control plots in one orchard and a species-specific real-time quantitative polymerase chain reaction (qPCR) method was optimized to quantify DNA in samples of airborne ascospores between these treatments. Ascospore discharge in these two treatments' plots coincided with leaf wetness, rain and fusi (scab) infection risk indices. Reduction in ascospores in the shredded plots was 36 and 76% in 2012 and 2013, respectively, with an average of 56%, although these reductions were not statistically different from the negative control. This is the first study to evaluate the effect of leaf shredding in South African orchards, and results indicate that this treatment is highly effective and should be integrated into scab management strategies in future. This could potentially have major impacts for apple production in South Africa.

## INTRODUCTION

The South African apple industry is primarily export-driven and reducing losses to apple scab, caused by *Venturia inaequalis* (Cooke) Winter, is crucial for ensuring profitable harvests, as cosmetic damage caused by scab reduces marketability of fruit. Various chemical fungicides have been tested against apple scab in South Africa over the past few decades in published (Schwabe and Heyns, 1974; Schwabe and Matthee, 1972) and unpublished studies. Tolerance or a decrease in sensitivity to certain chemical classes in local *V. inaequalis* populations has been found (Schwabe, 1977; Schwabe, 1979; Schwabe and Shabi, 1994; Schwabe and van de Rijst, 1997). Although judicious use of fungicides against scab is recommended and is applied on most farms, the potentially harmful effect on human and animal health and the environment, especially when fungicides are not used according to recommendations, as well as residues on fruit of certain chemical classes, warrants the search for alternative strategies for scab control.

There is a direct, positive correlation between the amount of ascospore inoculum in spring and the incidence and severity of scab in summer (Holb, 2009); therefore, by treating the leaf litter to reduce ascospore inoculum, the risk of disease (Gadoury and MacHardy, 1986) is reduced and, subsequently, scab incidence and severity. Removing the leaves from an orchard after leaf-drop removes the potential source of primary inoculum for the following spring and has been shown to be an effective sanitation strategy against scab (Holb, 2006; Gomez *et al.*, 2007). Shredding the leaf litter has been reported to be comparably effective and more practical (Holb, 2006; Sutton *et al.*, 2000; Vincent *et al.*, 2004). Urea has been tested as a post-harvest chemical and sanitation strategy for scab control, and in overseas studies, 5% urea was effective in reducing ascospore discharge by 92 to 100% when leaves were dipped in urea, and by 32 to 97% when urea was sprayed onto trees in the previous autumn (Burchill *et al.*, 1965; Burchill, 1968; Sutton *et al.*, 2000; Carisse *et al.*, 2000; Vincent *et al.*, 2004). In South Africa, urea was effective in reducing ascospore production significantly in the laboratory (89 to 99%), but authors concluded that the percentage reduction of ascospores in the field trials (20 to 92%) was inconsistent and too low to recommend using urea as a post-harvest treatment for scab (Schwabe and Heyns, 1972; Schwabe and Heyns, 1974; Schwabe and Matthee, 1976). Schwabe and Matthee (1976) suggested that two applications of 5% urea may have the desired eradicant effect, as opposed to a single application.

Potential ascospore dose (PAD) is the total seasonal production of *V. inaequalis* ascospores per square metre orchard floor (Gadoury and MacHardy, 1986). PAD can be used as an indicator of the level of risk for a specific apple orchard for primary scab infection in spring, to aid producers in making informed decisions about which orchards require more immediate preventative scab management in spring (Gadoury and MacHardy, 1986). The reduction in PAD can also be used to

express the effectiveness of sanitation in applied research on reduction of primary ascospore dose in orchards. However, for both of these purposes, PAD would need to be determined in treated and non-treated plots or orchards.

PAD is calculated using the equation  $PAD = LD \times PD \times AD \times LLD \times n$ , where LD = lesion density, PD = pseudothecial density, AD = ascus density, LLD = leaf litter density and  $n$  = number of ascospores per ascus (constant). LD is not affected by sanitation treatments or by winter conditions, as it is fixed by scab lesions that developed before leaf drop (Gadoury and MacHardy, 1986; Sutton *et al.*, 2000). Therefore, LD would need to be determined for each orchard every season shortly before leaf-drop, to allow producers to decide which orchards are high risk and would require autumn sanitation, and to obtain accurate PAD measurements for the following season to determine whether or not a full spray programme needs to be applied in low-risk orchards (Gadoury and MacHardy, 1986; Sutton *et al.*, 2000). PD and AD constants can be determined, as was done by Gadoury and MacHardy (1986) for New Hampshire; however, the authors also suggest that PD and AD constants need to be determined for different climatic regions, as these parameters are directly dependent on temperature and other weather conditions during winter. LLD is usually determined at bud-break using the point-intercept method (Gadoury and MacHardy, 1986). LLD, which is affected by winter conditions, biological activities and orchard characteristics that are unique for each orchard, is the only density component that can be reduced by sanitation practices. Thus, determining reduction in LLD due to sanitation would provide useful information on the efficacy of a sanitation treatment in reducing PAD. Holb (2006) measured the reduction in LLD due to various sanitation treatments, but leaf shredding was not included in his treatments. Sutton *et al.* (2000) reported a 52% reduction in LLD due to leaf shredding compared to non-shredded plots; however, leaf-pieces in this study were large enough for the point-intercept method to be used.

Assuming that each airborne ascospore represents a possible infection and foliar lesion, the percentage reduction of spring ascospore inoculum achieved through a non-chemical strategy can be a measure of the percentage reduction of disease that these ascospores could cause (Sutton *et al.*, 2000). Sutton *et al.* (2000) used microscopic analysis of trapped ascospores as a measure of efficacy of sanitation strategies on number of airborne ascospores and found that leaf shredding at 95% leaf-drop reduced number of ascospores by an average of 72%. Various studies have used quantitative real-time polymerase chain reaction (qPCR) methods to measure DNA of fungal species in air samples (Zeng *et al.*, 2006; Carisse *et al.*, 2009; van Wyk, 2011). Holb *et al.* (2004) used volumetric spore traps (VSTs) to determine the association between dispersal of *V. inaequalis* ascospores and disease gradients from defined inoculum sources. However, in a parallel study in previous years (Meitz-Hopkins *et al.*, Stellenbosch University, unpublished data), microscopic measurements of trapped airborne ascospores samples between sanitation treatments proved to be too time-

consuming and potentially inaccurate. No published studies to date have been found that use VSTs in combination with qPCR to measure sanitation treatment efficacy.

The objective of this study was to evaluate the effectiveness of sanitation strategies, specifically leaf-shredding, in reducing apple scab incidence and severity, airborne ascospores and leaf litter density in South African apple orchards.

## **MATERIALS AND METHODS**

The objective of Experiment 1 was to determine the effect of orchard sanitation strategies on the incidence and severity of apple scab primary infections. The objective of Experiment 2 was to further our understanding of the mode of action of leaf shredding on *Venturia inaequalis* by measuring its effect on airborne inoculum in spring and on leaf litter density during winter.

### **Experimental orchard details and initial scab assessment of trial orchards**

Four orchards were used for Experiments 1 and 2 of this research chapter. Orchards were selected based on their history of high scab incidence, despite the high number of fungicide applications, their layout, and maintenance: weeds, grass and pruning material on orchard floors are cleared seasonally and trees are pruned to keep tractor lanes clear, all of which allowed for easier application of sanitation treatments. Orchards A and B, in the Koue Bokkeveld (33°12'21'' S, 19°19'28'' E), were planted next to each other in 1996 with cultivar 'Fuji' on M793 rootstocks, with a tree spacing of 4 x 1.25m and total areas of 2.17 ha and 2.99 ha, respectively. Orchard C, also in the Koue Bokkeveld (33°12'46'' S, 19°19'39'' E), was planted in 1989 with 'Early Red One' on M793 rootstocks with a tree spacing of 4.5 x 2m and total area of 3.05 ha. Orchard D, in Elgin (34 °10'.0.03'' S, 19°02'47'' E), was planted in 1994 with 'Braeburn' on seedling rootstocks with a tree spacing of 4 x 1.2m and total area of 2.32 ha.

In March 2010, scab incidence in orchards A and B was assessed using a simple field method adapted from Pringle and Giliomee (1992), and similar to the method used by Schwabe (1980). Lesions on 20 fruit and the leaves on six shoots on 25 trees per 2 ha were counted. Scab incidence for each orchard was determined by dividing the percentage of fruit or leaves with at least one scab lesion by the percentage of fruit or leaves with no lesions, and finally the average of all trees assessed was calculated. Orchard C was included in the trial after harvest and during leaf-drop in April 2010 and, so could not be assessed for fruit using the field method described above. Autumn fruit scab incidence, but not severity, was obtained from the producer for orchard C after harvest (percentage scabbed fruit of total harvest). Foliar scab incidence was assessed in orchard C,

despite leaf-drop having begun. Therefore, scab incidence data for orchard C may not be representative of the actual scab level in the field in the 2009/10 season. In April 2011, when Experiment 1 was changed to reduce the number of sanitation treatments, orchard D was assessed using the method described for orchards A and B, as described below.

### **Treatment description and application**

For the trial in 2010, three post-harvest sanitation practices: leaf removal, leaf shredding and urea sprays were selected, based on their efficacy in published studies, and applied once each in orchards A, B and C. Leaf removal and leaf shredding treatments were applied after 100% leaf-drop in July 2010 due to delayed leaf-drop in South Africa. For leaf removal, the leaves were manually raked from under the trees to the middle of the tractor lane, collected in 100 kg fertilizer bags and driven approximately 1 km away from any apple orchard, where the bags were emptied. For leaf shredding, leaves were raked as for the leaf removal treatment and shredded to a fine mulch in the tractor lane with a Nobili RM 155 triturator mulcher (Southtrade, Cape Town, South Africa), fitted with 012 universal composter blades. For the urea treatment, two separate applications of 5% urea were applied at 70 and 100% leaf-drop to leaf litter within the tree rows. Urea sprays were applied at 1000 L ha<sup>-1</sup> with a tractor-drawn Hardi herbicide sprayer (Hardi Crop Protection SA Ltd., Cape Town). A combined treatment of leaf shredding and urea was included to determine if the combined effect of these treatments exceeded the effect of each treatment. Sanitation treatments were compared to a non-sanitized, non-sprayed negative control and to a non-sanitized positive control sprayed according to a commercial scab fungicide programme. The negative control did not receive any scab fungicides from postharvest in 2010 until December 2010, but other normal practices were applied, e.g. insect control, weed management and pruning. The six treatments were replicated once in each trial orchard using a randomized block design, with each orchard treated as a block. Each treatment was applied over a minimum of five rows of trees, with the outer two rows of each treatment acting as a buffer zone of minimum 20 m to minimize ascospore movement and interference between treatments.

In 2011, due to practical constraints, only the leaf shredding treatment was repeated based on the high potential it showed in reducing scab incidence and severity in the first season of the experiment. The size of the plots and number of orchards used for the trial were also reduced, with the trial only being conducted in orchards C (in the Koue Bokkeveld) and D (in Elgin), but the number of replicates per treatment per orchard was increased to two. There were four treatments: a positive control, leaf shredding treatment and negative control as in 2010, and an additional combined treatment of leaf shredding with a standard scab fungicide spray program. Treatments

with leaf shredding were applied in July 2011 after 100% leaf-drop with a Nobili BNU 160 triturator mulcher, fitted with 01 universal blades, as detailed above. Raked leaves were shredded twice on the same day, to ensure that all leaves were shredded and shredded leaf pieces were sufficiently small, and shredded leaf litter left in the work row. Each treatment was applied over five rows of trees, with the outer two rows of each treatment acting as a buffer zone of minimum 20 m to minimize ascospore interference between treatments.

The 2011 trial was repeated in 2012 and 2013, with the difference of leaf shredding being applied using a Kverneland chopper (Kverneland South Africa, Pietermaritzburg, South Africa) in 2012. These machines are available on the farms where the experiment was being conducted in 2012, while Nobili mulchers were not. In 2013, a Nobili BNU 160 mulcher was used in orchard C and a Sicma mulcher (Rovic Leers, Cape Town, South Africa) with hammers in orchard D, and shredding was done twice on the same day and twice during the leaf-drop period, as opposed to twice on the same day and once during the leaf-drop period in 2011 and 2012.

### **Treatment evaluation using scab incidence and severity in spring**

For the trial in 2010, treatment effect on scab incidence and severity on fruit and leaves in the subsequent season was evaluated in late November 2010 and February 2011. Scab lesions on 20 fruit and all leaves on six shoots on 10 trees in the middle row of 5 rows were counted. In spring of 2011, 2012 and 2013, scab incidence and severity was assessed weekly from bud-break until physiological fruit drop, regardless of ascospore discharge, and number of shoots assessed per tree was increased to ten. Fruit and leaves were also assessed within two weeks of harvest in 2011, 2012 and 2013. Scab incidence was calculated by dividing the number of fruit or leaves with at least one scab lesion by the number of fruit or leaves with no lesions and multiplying by 100. Scab severity was determined by counting all the lesions on each fruit or leaf and assigning each fruit or leaf to a category. The equation from Kremer and Unterstenhöfer (1967) was used to calculate scab severity from these categories. Treatments were compared using a method modified from Holb (2006) that assessed scab incidence and severity in the subsequent season.

### **Ascospore maturity monitoring in 2010 and 2011**

A method developed by Schwabe and Heyns (1974) was used to determine when matured ascospores were first discharged in Koue Bokkeveld in spring 2010 and 2011. In May, 15 mm diameter leaf discs with scab lesions were bored out of scabbed leaves collected from randomly selected trees throughout the negative control plots in the sanitation trials in 2010 and 2011. Gauze

bags, each containing a set of 50 leaf discs, were placed in a wooden framed 1 m x 1m x 8 cm box with shade net spanned over it, and secured to the ground, approximately 1 km from trial orchards (orchards A, B and C in 2010, orchards C and D in 2011), where it remained throughout winter.

From early September, one bag was removed every two weeks and leaf discs were stored at 2°C until ascospore extraction. Ascospores were extracted from leaf discs using a method modified from Hutton and Burchill (1965), and counted on a Neubauer haemocytometer. The following equation was used to determine the number of ascospores discharged cm<sup>-2</sup> leaf surface area for a specific sample: (no. ascospores ml<sup>-1</sup>) / (50 x 1.5 cm<sup>2</sup>) = no. ascospores discharged by 1 cm<sup>2</sup> leaf surface area.

### **Airborne ascospore detection and quantification in 2012 and 2013**

For approximately two months, from week 35 (end August) until week 43 (end October) in 2012 and 2013, one volumetric spore traps (VSTs) (Interlock Systems, Pretoria, South Africa) was placed on a wooden pallet in the centre of the plots in each of the two replicates of the negative control and leaf shredding treatment plots in orchard C, with the orifice approximately 50 cm from the soil surface. A thin layer of aerosol petroleum jelly (Vaseline) was sprayed on the eight-day rotating discs that sampled 20 L min<sup>-1</sup>, rotating every hour to sample 4.32 m<sup>-3</sup> day<sup>-1</sup>. Discs were collected every 7 days in spring and the petroleum jelly from each day's section on the disc was collected separately with cotton buds dipped in 0.1% Ipegal 630 (Sigma-Aldrich, St. Louis, MO, USA).

A standard Phenol-Chloroform extraction protocol described by Ma and Michailides (2007) was used for DNA extraction from all samples, but the extraction buffer was modified to include 2% polyvinylpyrrolidone (PVP, Sigma cat no. PVP40), 50 mM EDTA pH 8, 100 mM Tris-HCl pH 8, 0.5 M NaCl, 0.25% sodium dodecyl sulphate, 0.7% β-mercaptoethanol). A RNase A (Sigma, 0.6 U per sample) digest step for 30 min at 23°C and a proteinase K (Biolone, final concentration at 50 µg ml<sup>-1</sup>) digest step for 60 min at 37°C were included before phenol-chloroform purification and ethanol precipitation. DNA pellets were resuspended in 20 µl dH<sub>2</sub>O and stored at -20°C. The repeatability of this extraction method was proven in a parallel study (Meitz-Hopkins *et al.*, unpublished data). Daily samples were labelled as treatment-treatment replicate (rep), e.g. negative control-rep 1 = NC-1, leaf shredding-rep 2 = LS-2.

*Venturia inaequalis* DNA present in VST samples was quantified using real-time PCR, which was optimised using species-specific primers of the 14α-demethylase gene (*CYP51A1*) and Bio-Rad iTaq SYBR® Green Supermix (Bio-Rad Laboratories). Species-specific forward primer AJ250 (3'-GAGGCTACAACAGAT-3') used with reverse primer AJ244 (5'-TGAGAGCTTCGGTGGTGAGAC-3') (Schnabel and Jones, 2001) produces an amplicon of 135

bp in length. These primers were used instead of the ITS primers, because although they were less sensitive, they proved to be more specific, when tested in a parallel study (Meitz-Hopkins *et al.*, unpublished data). Using the recommended thermal cycling protocol provided by the manufacturers for the enzyme mix used, a thermal gradient from 52°C to 64°C was used to determine the optimal annealing temperature. Optimal concentrations for both forward and reverse primers were also optimized in a gradient matrix from 50 nM to 900 nM. The annealing temperature and primer concentrations at which the highest sensitivity and specificity was obtained, based also on melt curve analysis, was then used. Specificity of primers was again tested against *A. alternata*. Each reaction contained 1x KAPA SYBR® FAST, 500 nM of each primer and 2 µl of template DNA in a total reaction volume of 20 µl. Each DNA sample was analyzed in triplicate. Runs were performed using clear 96-well plates (Lasec, Pretoria, South Africa) and in a Bio-Rad Real-Time PCR System machine (Bio-Rad Laboratories, Rosebank, South Africa), using CFX 96 software. The thermal cycle consisted of initial denaturation at 95°C for 5 min, followed by 40 reaction cycles of 95°C for 10 s, 56°C for 15 s, and 72°C for 15 s and a melt from 72-95°C with 0.5°C increments.

Repeatability (intra-assay variance) and reproducibility (inter-assay variance) of the qPCR method was tested as follows: three samples of known concentration into three subsamples (quantified using the Qubit 2.0 fluorometer), to test accuracy of pipetting aliquots of a DNA solution, and running each subsample in triplicate in the same qPCR run (intra-assay variance). This was then repeated to test inter-assay variance. Results were analyzed to determine whether the  $\Delta C_q$  between the measured and calculated  $C_q$  of the undiluted sample was  $<0.5$  (Boutigny *et al.*, 2011).

To determine the detection limit of the qPCR method, 10-fold dilution series of genomic *V. inaequalis* DNA extracted from conidia ranging from 10 to  $1.69 \times 10^{-6}$  ng  $\mu\text{L}^{-1}$  (quantified using the Qubit 2.0 fluorometer) was subjected to qPCR analysis. Standard curves based on quantification cycles ( $C_q$ ) were constructed using an 8-fold dilution series of *V. inaequalis* DNA. The double-stranded DNA (dsDNA) concentration of the first standard dilution was quantified using the Qubit 2.0 fluorometer (Invitrogen, LifeTechnologies) using the fluorescent-based High-Sensitivity dsDNA Assay; DNA was quantified in a final volume of 200µl and the HS-DNA module and the protocol was followed according to the manufacturer. A 2 µl aliquot of each standard dilution in triplicate was used in qPCR reactions. After amplification, a standard curve was automatically generated by the CFX 96 software. Criteria for acceptable qPCR runs are  $M = -3.6$  to  $-3.1$  for slope and  $R^2 > 0.98$  for linearity. The  $C_q$  values of amplified products were plotted against the logarithm of DNA concentration to determine  $S_q$  (DNA concentration of samples in ng  $\mu\text{l}^{-1}$ ) and melt analysis was used to verify *V. inaequalis* template identity.

To calculate the ascospore number per daily VST sample, the mean DNA amount measured during qPCR was divided by twice the calculated DNA amount per conidial spore ( $3.2 \times 10^{-2}$  pg)

from a parallel ongoing study (Meitz-Hopkins *et al.*, unpublished data), since ascospores are 2-celled as opposed to unicellular conidia, and this amount was corrected for volume of air sampled ( $4.32 \text{ m}^3$ ), to represent the airborne concentration of ascospores  $\text{m}^{-3} \text{ day}^{-1}$  in that treatment replicate. Samples of days that amplified within the reliable detection range are reported and the mean number of ascospores  $\text{m}^{-3} \text{ day}^{-1}$  in each treatment was calculated, but if a biological rep of a treatment on any of these days did not amplify within the reliable detection range, the result for the other biological rep was divided in two. Only those days where all treatment replicates amplified above the lower limit of the detection range were used to calculate treatment differences. This is because treatment differences would have been skewed if the number of spores measured at the lower detection limit was allocated to those samples which did not amplify within the reliable detection range. Treatment differences were calculated by calculating the percentage difference between the means of treatments.

### **Determining leaf-litter density**

In June 2012 and 2013 after 100% leaf-drop, six plots for non-shredded leaves and six plots of shredded leaves, each with an area of  $1 \text{ m}^2$  under hardware netting secured to the ground, were laid out in orchards C and D. Plots were laid out at the edge of the orchard rows in the tractor lanes, to prevent shadowing from trees when digital photographs were taken. In 2013, shade-cloth was also placed under the leaves and hardware netting to prevent interference from earthworms and grass growth. Photographs were taken within two weeks after 100% leaf-drop and at bud-break, to determine differences in leaf litter density between those time points. The percentage ground covered by leaf litter (leaf litter density, LLD) in the plots was determined by analysing the photographs with Assess2.0 Image Analysis Software for Plant Disease Quantification (APS Press). Results for LLD in the non-shredded negative control plots in Experiment 2.2 were compared with LLD determined in the negative control plots used in Experiment 1 using the point-intercept method (Gadoury and MacHardy, 1986). Leaf pieces in the shredded plots were too small for the point-intercept method to be used. At three points in the non-shredded plots, a measuring tape was spanned diagonally between adjacent rows of four trees. Starting at 0, each 30 cm interval along these diagonals was marked as either having a leaf directly under, or being directly in contact with, the measuring tape at that point or not. The percentage of points that had leaves present was used as the percentage of orchard floor covered by leaves (LLD).

## Weather data

Mean daily temperatures (°C) and total precipitation (mm) values were obtained from iLeaf Integrated Weather Data Interpretation Software ([www.ileaf.co.za](http://www.ileaf.co.za)) from weather stations near the experimental plots in each region for September, October and November in 2010 to 2013. Weather stations were located at 33°12'27.15"S, 19°19'50.68"E on Nooitgedacht farm in Koue Bokkeveld and at 34°9'57.5"S, 19°1'31.9"E on Beaulieu farm in Elgin.

In September 2013, technical problems were encountered with the leaf wetness sensor until 29 September at the Nooitgedacht weather station, so weather data from the next closest weather station, located at 33°9'59.1"S, 19°20'9.4"E on Esperanto farm in the Koue Bokkeveld, was used. Thereafter, the Nooitgedacht weather data was again used. Nooitgedacht and Beaulieu weather stations are approximately one kilometre from the respective experimental sites in each region, and Esperanto weather station is approximately five kilometres from the experimental site in Koue Bokkeveld. Fusi infection indices were calculated by software on the iLeaf website from weather data using the equations developed by Schwabe (1980).

## Statistical analysis

A two-way analysis of variance (ANOVA) was used to compare sanitation treatments in different weeks in Experiment 1 using STATISTICA 11 data analysis software system (StatSoft, Inc. 2012, [www.statsoft.com](http://www.statsoft.com)). A mixed-model repeated measure ANOVA was done to compare sanitation treatments in individual years, and also over 2011, 2012 and 2013, where weeks and years were included as main effects. A Pearson correlation was used to determine whether there was a relationship between scab incidence and severity on fruit and leaves for data pooled from 2011 to 2013. A mixed-model repeated measure ANOVA was done to compare sanitation treatments on airborne ascospores in Experiment 2.1. Fisher's Least Significance Difference (LSD) t-test was used for post-hoc testing. Significant differences were all evaluated on a 5% significance level ( $P < 0.05$ ).

## RESULTS

### Initial assessment of the trial orchards

Initial incidence of fruit scab in March 2010 in orchards A, B, and C was 80, 78 and 28%, respectively, and leaf scab incidences were 42, 65 and 32%, respectively. Fruit scab and leaf scab incidence in orchard D in April 2011 were 55 and 35%, respectively.

### Treatment effect

Analysis of variance of the three-way interaction orchard x time of assessment x treatment indicated that the effects of time of assessment for the 2010/11 season and the orchards were not significant ( $P > 0.05$ ; data not shown), so the data from the three orchards (A, B and C) in December 2010 and February 2011 were pooled. All measurements were significantly lower in the positive control than in the other five treatments. In the leaf shredding treatment, fruit scab incidence and severity were significantly lower (11.5 and 26.6% respectively) when compared to the negative control, while leaf scab incidence and severity did not differ significantly to the negative control. Leaf removal, urea sprays and the combined treatment (leaf shredding with urea sprays) did not differ significantly from the negative control in any of the parameters measured ( $P < 0.05$ ) (Table 1).

When data for weeks 42 to 45 for fruit, and weeks 41 to 45 for leaves, in spring from 2011 to 2013 were pooled, analysis of variance indicated a non-significant interaction: orchard x treatment for fruit scab incidence, fruit scab severity, leaf scab incidence and leaf scab severity ( $P = 0.25$ ,  $P = 0.44$ ,  $P = 0.64$  and  $P = 0.75$ , respectively); therefore, data for the two separate orchards C and D could be pooled together for each of these parameters. Years 2011 and 2012, and 2011 and 2013 did not differ significantly from one another, but 2012 and 2013 differed significantly from one another ( $P = 0.21$ ,  $P = 0.34$  and  $P = 0.03$ , respectively). Leaf shredding significantly reduced fruit scab incidence and severity by 50.5 and 55.0% respectively and leaf scab severity by 40.0% ( $P > 0.05$ ), compared to the negative control, but not leaf scab incidence (33.3%,  $P = 0.06$ ) (Table 2). There was a strong positive correlation between scab incidence and severity on fruit and leaves ( $r = 0.96$  for both relationships).

The analysis of variance for separate weeks in 2011, 2012 and 2013 revealed a highly significant interaction: orchard x treatment ( $P < 0.05$ ; data not shown), except for week 45 in 2012 and weeks 44 and 45 in 2013 ( $P > 0.05$ ; data not shown). Scab incidence and severity data pooled from all weeks in each year are presented in Tables 3 and 4, and scab incidence and severity data

for each week in each orchard are presented separately in Tables 5 and 6 for 2011, Tables 7 and 8 for 2012 and in Tables 9 and 10 for 2013.

In spring 2011, scab was first detected in week 39 in orchard D on leaves and in week 40 in orchard C (100% full-bloom) and D (beginning of petal-drop) on leaves and flowers. Fungicide sprays to control scab in the leaf shredding and negative control treatments were withheld until week 43 in both orchards and the normal spray programme from that week onwards was applied. Data for flower/fruit and leaf scab incidence in weeks 39 to 45 in spring 2011 are shown in Table 5 and severity data are shown in Table 6. Scab incidences and severities on fruit and leaves in the positive control and combined treatment (full spray programme with leaf shredding) did not differ significantly from each other in any week. When data for the 2011 spring season are pooled, fruit scab incidence in the leaf shredding treatment was reduced by 65.8% in fruit scab incidence and severity orchard C and by 45.7 and 43.5% in orchard D, respectively, when compared to the negative control ( $P < 0.05$ ) (Tables 3 and 4). Fruit scab incidence and severity in orchards C (38.2 and 45.8%, respectively) and D (32 and 36.4, respectively) were significantly lower in the leaf shredding treatment than in the negative control shortly before harvest in March 2012 ( $P < 0.05$ ). Leaf scab incidence and severity was also significantly lower in orchard D (44.4 and 49.4%, respectively) in the leaf shredding treatment than in the negative control shortly before harvest in March 2012 ( $P < 0.05$ ) (Tables 5 and 6).

In spring 2012, scab was first detected in week 41 in orchard C on leaves and in week 42 on leaves and flowers in orchards C (100% full-bloom) and D (beginning of petal-drop). The normal spray programme for the negative control and leaf shredding treatments was followed from week 42 in orchard D and week 43 in orchard C. Data for flower/fruit and leaf scab incidence in weeks 41 to 45 in spring 2012, and shortly before harvest in February 2013, are shown in Table 9 and severity data are shown in Table 10. When data for weeks 42 to 45 for this season are pooled, fruit scab incidence and severity in the leaf shredding treatment was reduced significantly by 49.1 and 59.8%, respectively, when compared to the negative control ( $P < 0.05$ ) (Tables 3 and 4). Shortly before harvest in February 2013, leaf scab incidence and severity was significantly lower in orchards C (44.9 and 55.6% respectively) and D (17.4 and 22.2% respectively), as well as fruit scab incidence and severity in orchard C (13.5 and 32.8%), in the leaf shredding treatment than in the negative control ( $P < 0.05$ ). However, fruit scab incidence and severity in orchard D in the leaf shredding treatment did not differ significantly from the negative control shortly before harvest (Tables 7 and 8).

In spring 2013, scab was first detected in week 41 in orchard C on leaves and in week 42 on leaves and flowers in orchards C and D (75% full-bloom). Fungicide sprays to control scab in the leaf shredding and negative control treatments were withheld until week 44 in both orchards, after

which the normal spray programme from that week onwards was applied. Data for flower/fruit and leaf scab incidence in weeks 41 to 45 in spring 2013 are shown in Table 10 and severity data are shown in Table 11. When data for weeks 42 to 45 for this season are pooled, fruit scab severity in orchard C (84.5%) and fruit incidence and severity in orchard D (78.1 and 50.6 %, respectively) in the leaf shredding treatment was reduced significantly, compared to the negative control ( $P < 0.05$ ) (Tables 3 and 4). Scab incidences and severity on fruit and leaves in the positive control and combined treatment of a full spray programme with leaf shredding did not differ significantly from each other ( $P > 0.05$ ) (Tables 9 and 10).

### **Ascospore maturity monitoring in 2010 and 2011**

Ascospores were first found in weeks 39 and 40 in 2010 and 2011, respectively. Ascospores  $\text{cm}^{-2}$  leaf area ranged from 0 to 1138 and 0 to 125 in 2010 and 2011, respectively (Table 11).

### **Airborne ascospore detection and quantification in 2012 and 2013**

The qPCR method proved to be reproducible and repeatable: triplicate qPCR on the same sample within a single run showed a low variation in  $C_q$  values (mean SD = 0.01,  $n = 81$ ) (intra-assay variance), and low variation was observed between  $S_q$  of independent runs (mean SD = 0.23,  $n = 3$ ) (inter-assay variance). Non-template and negative controls did not amplify or amplified at  $C_q \geq 38$ . Melt analysis showed that amplified products of *V. inaequalis* (positive control) and *A. alternata* (negative control) DNA had differential melt peaks at  $80 \pm 0.5$  and  $86 \pm 0.5^\circ\text{C}$  respectively. *Venturia inaequalis* DNA was detected reliably within a range of  $1 \times 10^{-3}$  to 3.33 ng per reaction, with the lower detection limit at  $C_q = 30 \pm 0.1$ . Amplification of DNA samples from week 39 in 2013 (23 to 29 September) are shown Figure 1, to illustrate that  $M$  (slope) and  $R^2$  (linearity) for qPCR runs were acceptable. When the lower detection limit (equivalent to 27 ascospores per reaction, or  $62 \text{ ascospores m}^{-3}\text{day}^{-1}$ ) was used to identify which samples amplified within the detection range, ascospore discharge was detected in 2012 on 30 August (NC-2), 2 September (LS-1 and LS-2), 3 September (NC-1 and NC-2), 4 September (NC-1), 8 and 9 September (NC-1 and NC-2), 18 and 21 September (all reps), 25 and 26 September (NC-1 and NC-2, respectively), 27 September (all reps), 28, 29 and 30 September (NC-2, LS-2 and NC-2, respectively), 1 October (NC-2, LS-1 and LS-2), 3-6 October (all reps), 7 October (NC-2 and LS-1), 8 October, (NC-1, NC-2 and LS-2), 9 and 10 October (NC-1, LS-1), 11 October (NC-1), 13 October (all reps) and 14 October (NC-1, NC-2 and LS-2) (Fig. 2, Table 12).

In 2012, number of airborne ascospores  $\text{m}^{-3} \text{day}^{-1}$  on those days when all treatments had reliably detectable data (18, 21 and 27 September and 3-6 and 13 October) was 0 to 79% lower in the leaf shredding treatments than in the negative control, with an average reduction of 34.3%, with the highest reductions on 18, 21 and 27 September (63, 79 and 72.3%, respectively) (Table 12). Mean number of ascospores  $\text{m}^{-3} \text{day}^{-1}$  for the days analyzed was  $348 \pm 385$  and  $622 \pm 509$  in the leaf shredding and in the negative control treatments, respectively. Analysis of variance indicated that the leaf shredding treatment did not have a significant effect in reducing number of airborne ascospores on the days analyzed ( $P = 0.38$ ).

In 2013, ascospore discharge was detected reliably on 15 September (all reps), 20 September (NC-1 and NC-2), 24 September (NC-1) and 27 September (NC-2 and LS-2) (Fig. 3). The number of replicates per treatment was too low and variation between the replicates of the treatments was too high between them to conduct accurate statistical analysis data from 15 September 2013. However, reduction in ascospores in the leaf shredding treatment on 15 September was 76%, compared to the negative control.

### **Leaf-litter density**

In 2012, the growth of the grass under the hardware netting was vigorous enough to push the netting up and cover the leaves in those plots completely. Leaves were also too degraded and eaten, presumably by earthworms, so photographs of the leaves in the plots could not be taken and no comparison could be made between autumn and spring photographs.

It was not possible to determine an accurate percentage LLD for most of the photos in 2013, so the percent reduction in LLD between photographs in winter and in the following spring could not be determined. The beige shade netting used as a backdrop in winter had been stained brown by spring for most of the photographs, which did not provide enough colour difference for the Assess2.0 program to sufficiently differentiate between the brown leaf pieces and the brown background (Fig. 4). Results of leaf-litter density determined using the point-intercept method in 2012 and 2013 are shown in Table 13. Loss of leaf litter from after 100% leaf-drop until bud-break ranged from 70.5 to 82.8%, with an average of 76.1%.

### **Weather data**

Daily leaf wetness, rain and scab (*fusi*) infection risk indices in spring 2012 and 2013 are shown in Figures 2 and 3, respectively. The number of rainfall events and infections indices in 2012 was higher in 2012 than in 2013.

## DISCUSSION

The South African apple industry currently relies solely on fungicides to control apple scab and urgently needs to improve its scab management strategies and reduce its reliance on fungicides. This study provided evidence of a sanitation strategy that might be effective in reducing scab inoculum after harvest for the following season.

In 2010, leaf shredding was the only sanitation treatment that significantly lowered fruit scab incidence and severity when compared to the negative control (no fungicides or sanitation applied): *i.e.* 11.5% lower incidence and 26.6% lower severity on scabbed fruit, respectively. Combining urea sprays with leaf shredding did not improve on the effect of leaf shredding alone on fruit or leaf scab incidence, which is similar to results reported by Sutton *et al.* (2000).

Scab incidence and severity in the urea treatment was higher than, but did not differ significantly from, the negative control. These results are not consistent with published studies that have been conducted on urea as a postharvest scab eradicator (Burchill, 1968; Sutton *et al.*, 2000; Vincent *et al.*, 2004), but are in agreement with previous studies in the Western Cape (Schwabe and Heyns, 1974; Schwabe and Matthee, 1972; Schwabe and Matthee, 1976; Mac an tSaoir *et al.*, 2010). Leben and Keitt (1948) found that urea was one of the four most favourable nitrogen sources for *V. inaequalis*. Urea applied as a scab sanitation treatment aims to stimulate microbial growth on leaves to compete with, and inhibit, *V. inaequalis* pseudothecial formation (Sutton *et al.*, 2000; Carisse and Dewdney, 2002). It may be possible that the double application of 5% urea to leaf-litter in this study stimulated, rather than inhibited, the sexual stage of *V. inaequalis* in the urea treatment plots. This may have caused a higher pseudothecial density per fertile lesion and ascospore dose, and may explain the higher scab levels found in those treatment plots. Urea sprays applied to the canopy shortly before leaf-drop should be tested in South Africa. However, Burchill (1968) found that urea applications more than a week before leaf-drop significantly reduced the efficacy of the treatment. During long leaf-drop periods that are typical under South African conditions, the use of defoliant to shorten leaf-drop periods should be investigated in combination with urea sprays. Additionally, Sutton *et al.* (2000) reported that ascospore productivity was lower (66%) when 5% urea was applied shortly before bud-break and this should also be tested in future studies in South Africa.

Leaf removal equates to inoculum removal, since *V. inaequalis* uses only fallen apple leaves as substrate for winter survival in regions where asexual inoculum of *V. inaequalis* does not remain viable throughout winter on the tree. The scab levels in the leaf-removal treatments in 2010 did not correspond with what was expected, considering that a high percentage of leaves were removed (estimated 99%) in this study. Very few scab infections were seen in the treatment plots during late

October 2010 and scab assessments did not begin until late November 2010, when trees were at physiological fruit drop stage. However, the sanitation treatment plots were severely infected, as was the case in 2011 and 2012. Louw (1951) observed the first scab lesions (three lesions per 100 leaves) in early October in the Koue Bokkeveld, and the next scab observation was only in late October on the calyx leaves of recently set fruit, when lesions were “only just macroscopically discernible”. Louw (1951) also reported that the first conidia were found on slides hung in tree canopies for the first time on 20 October, so scab lesions that were observable in November would have included lesions caused by ascospores and conidia. Therefore, timing of observation of the first scab lesions in 2010 in this study corresponds with results reported by Louw (1951). Thus, although the data in this study indicate that the effect of leaf shredding was effective until late spring, the data are not representative of the effect the sanitation treatments had on scab incidences and severity caused by early spring primary infections, as they would have included lesions caused by conidial infections. Since sanitation of leaf litter aims to reduce ascospores for the following season, the scab lesions that develop from the earliest primary infection is the best indicator of sanitation efficacy, before lesions produce conidia that cause secondary infections. The effect of sanitation treatments becomes less evident as scab builds up from each secondary infection period due to the addition of conidial inoculum. The late timing of the scab assessment in 2010 obscured the true effect of all treatments (including leaf removal) in reducing primary infections in spring. Leaf removal in commercial orchards in South Africa would be impractical, due to the sheer volume of leaf material that drops in autumn and the large size and number of South African orchards, so it is unlikely that this treatment would be appropriate for use in South African orchards.

In 2010 and 2011, the low numbers and timing of ascospores discharged (Table 11) may be due to mature ascospores having been discharged during infection periods between leaf sample collections. The ascospore extraction method to monitor ascospore maturation, or perhaps the application of the method, used in 2010 and 2011 to monitor ascospore discharge was not reliable, which is why disease assessments were done weekly in 2011, 2012 and 2013 and volumetric spore traps were used to monitor ascospore discharge in 2012 and 2013.

Data pooled from 2011, 2012 and 2013 indicated that leaf shredding significantly reduced fruit scab incidence and severity and leaf scab severity (50.5, 55.0 and 40.0%, respectively), compared to the negative control, but not leaf scab incidence (33.3%) (Table 2). However, the *P*-value of the treatment difference in leaf scab incidence indicates a trend in lower leaf scab incidence in the leaf shredding treatment compared to the negative control. These results are similar to the reductions of 46 and 65% in fruit and leaf scab incidence, respectively, reported by Sutton *et al.* (2000).

There are several reasons why the efficacy of shredding leaves may vary. Poorly managed weeds and tall grass in the tractor drive row can be associated with fewer leaves being shredded and pieces of shredded leaves being larger, thus increasing the variability of effects of leaf shredding between orchards, lowering the percentage of leaf shredding (which directly affects the efficacy of the treatment) and decreasing the overall effect of leaf shredding (Sutton *et al.*, 2000). Pruning material on the orchard floor made raking the leaf litter very difficult in 2010 and 2011; consequently, not all leaf-litter may have been raked to the middle of the tractor lane and shredded as intended. There were also a limited number of dry periods in winter long enough for leaves to dry and be raked efficiently for shredding, since both regions are winter rainfall regions (Mediterranean climate) of the Western Cape. For these two reasons, it is highly likely that pseudothecia formed on leaves that fell earlier and that the late timing of leaf shredding in both regions could not prevent pseudothecial formation.

The strong positive correlation ( $r = 0.96$ ) between incidence and severity on both fruit and leaves agrees with previous studies (Jeger, 1981; Holb, 2003). Considering that assessing leaf severity is considerably more time-consuming and less accurate than assessing scab incidence (Holb, 2003) and the strong positive correlation between incidence and severity and scab development in spring, this study strongly supports the recommendation to assess fruit and leaf scab incidence in autumn, as is done for PAD (MacHardy, 2001).

Jeger (1984) reported that scab severity did not affect number or maturity of pseudothecia that developed on scabbed leaves, but he did state that “late infections on abaxial surfaces are important in that they are much more difficult to observe in conventional disease assessments and the grower or advisor may be lulled into a false sense of security by an apparent absence of scab at leaf-fall”. Thus, shredding the leaf litter has the added feature of reducing pseudothecial development from lesions on the abaxial surface. This reduces the risk of an unexpected scab build-up due to infections that may occur from ascospores produced on the under-surface of the leaf.

Fruit scab incidence and severity in the leaf shredding treatment was significantly lower than the negative control shortly before harvest for the first three seasons (2010, 2011 and 2012) of Experiment 1, except for orchard D for the third season (February 2013). Apple scab is a polycyclic disease, and usually, as soon as conidia are dispersed in spring, the disease increases at an exponential rate if unchecked (Sutton *et al.*, 1976). However, van der Plank (1963) describes how the onset of disease in a sanitized area is later and final disease levels at the end of a season are often less than in non-sanitized areas, provided that a sufficient reduction in inoculum occurred before the season began. Therefore, the significant reduction in initial inoculum in leaf shredding plots may have prevented scab levels from increasing as quickly as in negative control plots throughout the season, and may explain the reductions in scabbed fruit shortly before harvest.

Scab lesions were observed earlier in orchard D Elgin than in orchard C in Koue Bokkeveld, which agrees with reports by Louw (1951). The higher rainfall in Elgin in September and October in 2011, 2012 and 2013 (which is more conducive for *V. inaequalis* infections (Schwabe, 1980)) may have contributed to a more noticeable earlier build-up of scab.

Personal observation indicated that although 012 universal composter blades are not commercially available in South Africa they did shred leaves to a powder. If these blades were to be used in future in practice, this would increase the efficacy of shredding, provided leaves were shredded within four weeks after they dropped (two shredding applications may be needed per season) to prevent pseudothecial formation and to allow advanced leaf degradation. Other blades systems used in 2011, 2012 and 2013, required treating the same piece of ground twice or three times to achieve a similar effect although shredded leaves were still not as finely shredded as leaves shredded with the 012 composter blades. Due to the variation in performance between blades, different blades should be tested for efficacy before being used for leaf-shredding in commercial orchards. The 012 universal composter blades can be fitted to any machine that currently uses 01 universal blades (Marius Ras, Rovic Leers, personal communication), so blades could be tested on the same machine.

According to the qPCR method used, peak ascospore discharge occurred on 21 September in 2012 and on 15 September in 2013. Ascospore detection above the lower detection limit that occurred in all treatment reps on 18, 21 and 27 September and 3-6, 13 and 14 October in 2012 and on 15 September 2013 corresponded with leaf wetness data, rainfall and fusi infection indices, although ascospores were not necessarily detected on all days with rain, which agrees with Louw (1951). This confirms that the fusi infection risk model (Schwabe, 1980) is still accurate and useful. However, the model does not take into account PAD, ascospore biofix, maturity and discharge in relation to weather, or susceptibility of apple cultivars to scab and residual activity of preceding fungicide treatments. The model RIMpro, a dynamic scab simulation programme, does take these factors into account (Phillion *et al.*, 2009; Trapman, 1994) and the integration of the fusi infection risk model with this model should be investigated.

The low statistical power of the experimental set-up for Experiment 2.1, in which each treatment was only replicated twice due to practical constraints, did not allow for robust statistical analysis. Although the difference between treatments was not statistically significant, the reduction in airborne ascospores due to leaf shredding was considerable, with the largest reductions occurring earlier in the season in 2012 and 2013, as expected (Fig. 2 and Table 12). The range of reduction in airborne ascospores in this study (e.g. 0 to 79% in 2012) is similar to the 34 to 91% (average 72%) reduction in airborne ascospores when leaves were shredded when 95% of the leaves had fallen in the previous autumn reported by Sutton *et al.* (2000). The average reductions in airborne ascospores

of 34.3 and 76% in 2012 and 2013, respectively, indicates that shredding leaves twice during leaf-drop will greatly increase treatment efficacy and associated risk of infection. This supports the recommendation to shred leaves more than once during and/or after leaf-drop to reduce inoculum load for the following season.

The qPCR method selected for this study was not as sensitive or as accurate as it should have been. In a parallel study, insufficient amounts of ascospores were obtained from discharge tests to compile a standard curve using ascospores; thus, conidia were used. The presence of inhibitors in the material, most likely due to the DNA extraction method used, together with the use of conidia to compile a standard curve for ascospores, may have contributed to the inability of the method to detect *V. inaequalis* DNA at sufficiently low detection levels. Alternative methods, such as the immune-detection of *V. inaequalis* ascospores using phage antibodies developed by Ribbert *et al.* (2007) may have been faster and more accurate. In addition, the immune-detection method can distinguish between ascospores and conidia of *V. inaequalis*, so that ascospore discharge patterns on VST samples could still be monitored without interference from conidial DNA presence.

The reduction in leaf litter between winter and spring using the point-intercept method, was between 71 and 83%, with an average of 76.1%, over the two years and in both orchards. These reductions were greater than the 20 to 48% reduction in leaf litter from leaf-drop until bud-break reported by Gadoury and MacHardy (1986). This could be due to the relatively warmer winter conditions in South Africa, especially in Elgin, which is more conducive to accelerated leaf degradation. Nevertheless, such a large loss of leaf litter is not enough to reduce the ascospore dose enough to allow leaf shredding to replace fungicide in a scab management programme, as shown by the high incidence of foliar scab found in the research orchards.

The method using image analysis software (Assess2.0) to determine effect of leaf shredding on loss of leaf litter density did not work, but the experiment should be repeated and the experimental set-up changed to take into account the problems encountered in this study. The information gained from such a study would further our understanding of how leaf shredding affects leaf-litter degradation, compared to non-shredded leaves, as LLD is a component of PAD and a reduction in LLD would result in a reduction in PAD.

## CONCLUSION

Leaf shredding was significantly effective as a post-harvest sanitation treatment in reducing fruit and leaf scab over four years. However, efficacy of leaf shredding in reducing scab inoculum and disease levels in orchards varies and relies on a number of factors, including timing, since the

longer the delay in shredding after leaf-drop, the greater the opportunity for fertilization and pseudothecial formation, *i.e.* PD, which directly impacts on the efficacy of the treatment. Delayed leaf-drop, especially in warm winter regions (e.g. Elgin), means that leaf-drop occurs over up to two months, and results suggest that shredding more than once over the period of leaf-drop will increase efficacy, as was shown in the airborne ascospore trapping experiment. Ridges in orchards and rocky soils, wet winters affecting when leaf shredding can be applied and lack of equipment suited to South African orchards are also important factors that must be addressed as they have been shown to lower shredding efficacy. Developing shredding machines better suited for South Africa orchard characteristics and conditions would increase the efficacy of this sanitation practice.

Scab incidence and severity was assessed too late in spring in 2010, after conidial infections had contributed to scab build-up. This may explain why two applications of 5% urea, leaf shredding with urea and leaf-removal did not differ significantly from non-treated plots and why these results do not correspond with previous studies. Nevertheless, leaf removal is far too time-consuming and labour-intensive, and given the huge area of apple orchards planted in South Africa, it is impractical for this treatment to be implemented in South African orchards and will likely not be tested in future studies. The use of defoliant sprays should be tested with urea sprays to determine whether the amount of time between urea spray application and leaf abscission can be reduced to less than a week, which would increase efficacy of urea as a sanitation treatment.

The considerable reduction in airborne ascospores by as much as 79% in the leaf shredding plots agrees with results reported in previous studies and confirms the efficacy of leaf shredding in reducing risk of scab infections. The days on which ascospores were detected with the reliable detection range in all treatment replicates corresponded with weather data and fusi infection risk indices confirms that the infection risk model developed by Schwabe (1980) is still valid. However, integration of the fusi model with other risk prediction models used overseas should be explored.

The average 76% leaf-litter density loss for non-shredded leaves, higher than reported in overseas studies, may have been due to warmer winter conditions in South Africa being more favourable to leaf degradation. Using the Assess2.0 software to determine the effect of leaf shredding on leaf-litter density loss over winter was not successful. However, it was obvious from visual observation (see examples in Fig. 4) that leaf-litter density in shredded leaf plots was lower at bud-break than in plots with non-shredded leaves, and this points to the efficacy of leaf shredding in reducing substrate for pseudothecia to develop and survive on until the following season.

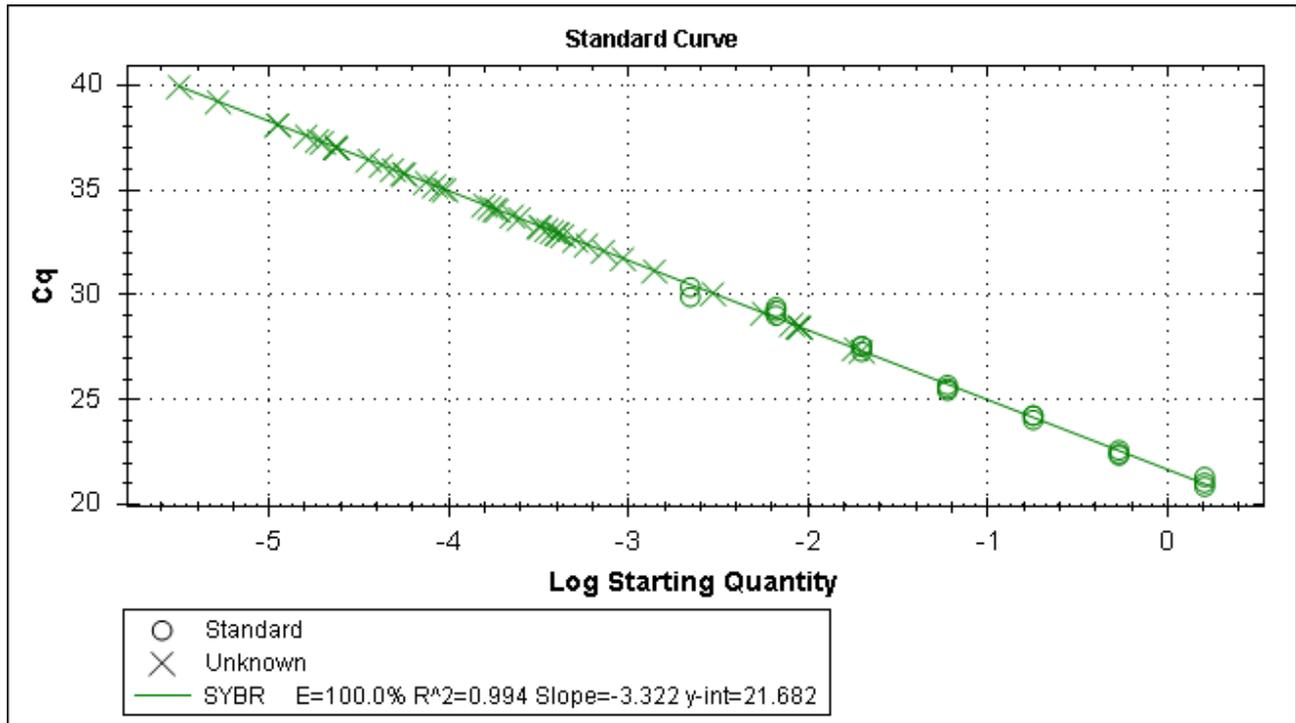
Leaf shredding could become widely adopted in the South African apple industry, if it were optimized for local orchard conditions. However, more studies are needed, specifically to compare urea sprays shortly before bud-break with shredding twice during the leaf-drop period. This could aid our industry in remaining competitive for export markets and remaining highly profitable.

## LITERATURE CITED

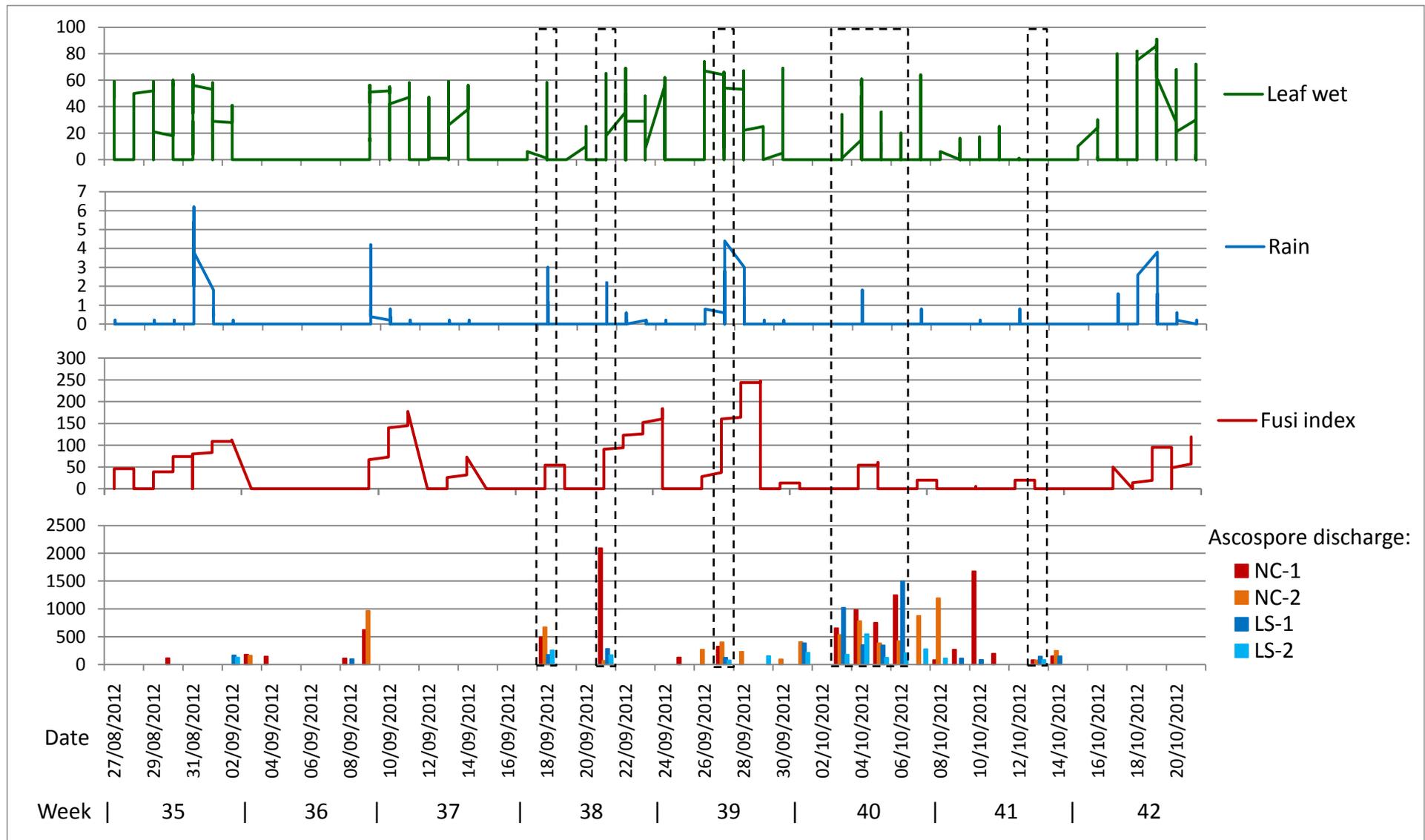
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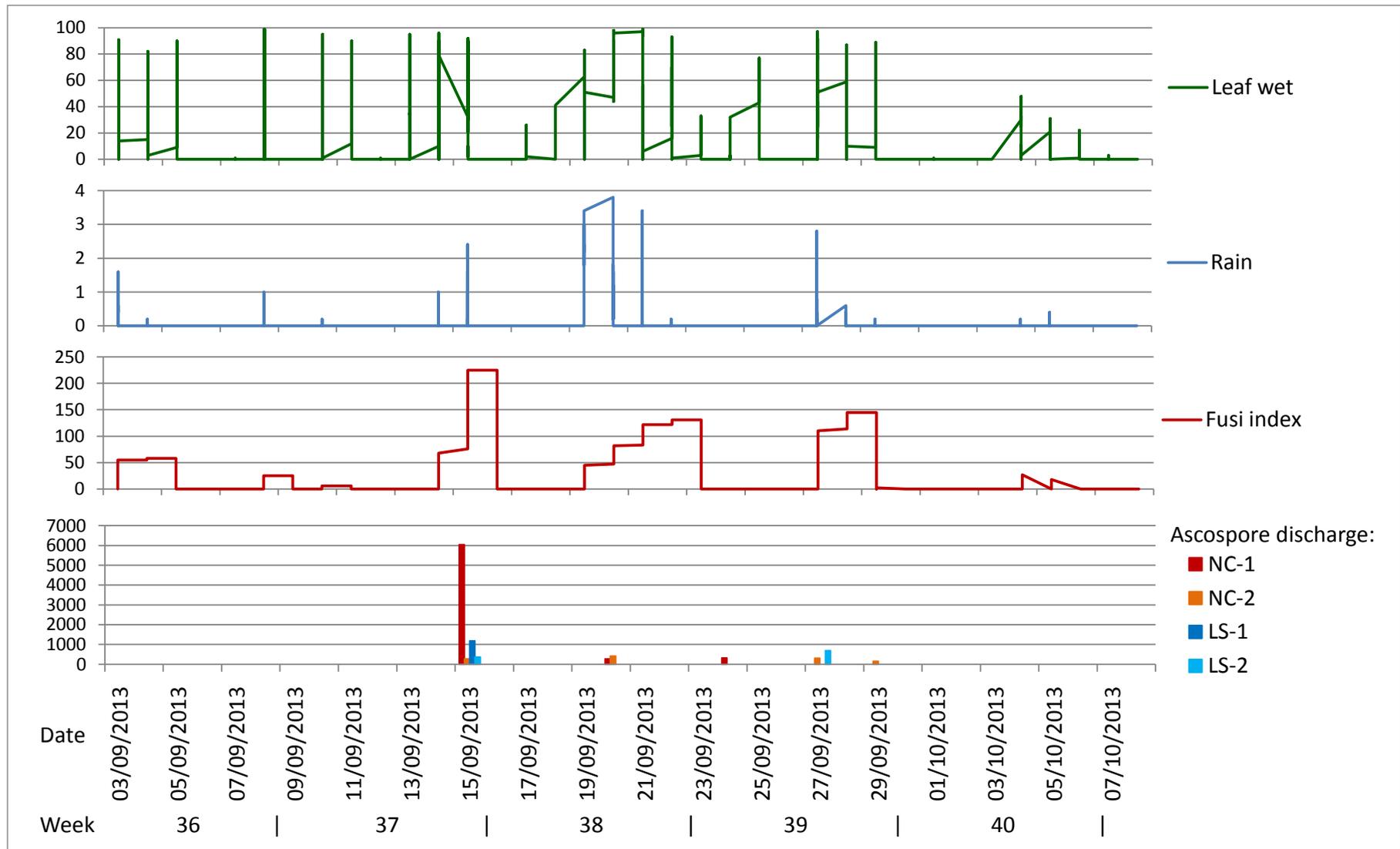
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**Figure 1.** Amplified qPCR products of DNA samples from week 39 (23 to 29 September) in 2013, collected using volumetric spore traps, plotted on the standard curve. Slope (M) values are within the acceptable ranges of -3.6 to -3.1 (optimal -3.32) and linearity ( $R^2$ ) is >98%. The reliable lower detection limit is at  $C_q = 30 \pm 0.1$ .



**Figure 2.** Leaf wetness (wet) (%), rain (mm), fusi infection risk index (from iLeaf) and number of airborne ascospores  $\text{m}^{-3}\text{day}^{-1}$  detected above the lower detection limit ( $62 \text{ ascospores } \text{m}^{-3}\text{day}^{-1}$ ) in the non-shredded negative control (NC) and leaf shredding (LS) treatments, from 27 August until 29 October 2012 in orchard C in the Koue Bokkeveld, using volumetric spore traps and qPCR. Numbers after treatment abbreviations indicate treatment replicates. Dashed rectangles indicate days on which ascospores released in all treatment replicates could be correlated with leaf wetness, rain and fusi indices.



**Figure 3.** Leaf wet (wetness) (%), rain (mm), fusi infection risk index (from iLeaf) and number of airborne ascospores  $\text{m}^{-3}\text{day}^{-1}$  detected above the lower detection limit ( $62 \text{ ascospores m}^{-3}\text{day}^{-1}$ ) in the non-shredded negative control (NC) and leaf shredding (LS) treatments, using volumetric spore traps and qPCR, from 27 August until 28 October 2013 (only 3 September to 7 October shown on graph below) in orchard C in the Koue Bokkeveld. Numbers after treatment abbreviations indicate treatment replicates.



**Figure 4.** Photographs taken of shredded leaves (top) and non-shredded leaves (bottom) in Elgin, on 11 July 2013 at 100% leaf-drop and of the same plots on 1 October 2013 at bud-break, in an attempt to determine the effect of leaf shredding in apple orchards on leaf litter density (a component of PAD).

**Table 1.** Pooled data of scab incidence and severity on fruit and leaves, in orchards A, B and C in Koue Bokkeveld, assessed in early summer (December 2010) and shortly before harvest (February 2011).

Treatment	Fruit		Leaf	
	Incidence	Severity	Incidence	Severity
Positive control <sup>1</sup>	5.2 a <sup>2,3</sup>	0.6 a <sup>4</sup>	1.7 a	0.2 a
Leaf removal	94 bc	56 bc	40 bc	16 b
Leaf shredding	85 b	47 b	38 b	15 b
2 x urea sprays	98 c	68 c	45 c	18 b
Leaf shredding & urea	88 bc	52 bc	40 bc	15 b
Negative control <sup>1</sup>	96 c	64 c	44 bc	18 b

<sup>1</sup> Positive control=full scab fungicide programme, negative control=no scab fungicides or sanitation

<sup>2</sup> Incidence is defined as percentage of fruit or leaves with at least one lesion.

<sup>3</sup> Severity is defined as percentage of fruit or leaf area visibly affected by scab.

<sup>4</sup> Means with the same letter in a column are not significantly different ( $P < 0.05$ ).

**Table 2.** Pooled data for mean fruit and leaf scab incidence and severity in October and early November in 2011, 2012 and 2013 in orchards C in Koue Bokkeveld and orchard D in Elgin.

Treatment	Fruit <sup>1</sup>		Leaf <sup>2</sup>	
	Incidence <sup>3</sup>	Severity <sup>4</sup>	Incidence	Severity
Positive control <sup>5</sup>	1.9 a <sup>6</sup>	0.5 a	1.2 a	0.3 a
LS+PC	2.4 a	0.5 a	1.0 a	0.3 a
Leaf shredding	15.2 b	4.5 b	8.4 b	3.6 a
Negative control	30.7 c	10 c	12.6 b	6.0 b

<sup>1</sup> Fruit data from week 42 (during bloom) until week 45 (physiological fruit thinning).

<sup>2</sup> Leaf data from week 41 (bud-break) until week 45 (physiological fruit thinning)

<sup>3</sup> Incidence is defined as percentage of fruit or leaves with at least one lesion.

<sup>4</sup> Severity is defined as percentage of fruit or leaf area visibly affected by scab.

<sup>5</sup> Positive control = full scab fungicide programme applied, LS+PC = full scab fungicide programme and leaf shredding applied, LS = only leaf shredding applied, Negative control = no scab fungicides or sanitation applied.

<sup>6</sup> Means with the same letter in a column are not significantly different ( $P < 0.05$ ).

**Table 3.** Mean scab incidence on fruit from weeks 42-45 (October and early November) in 2011, 2012 and 2013 in orchards C (Koue Bokkeveld) and D (Elgin).

Year	2011		2012		2013	
Orchard	C	D	C	D	C	D
PC <sup>1</sup>	0.1 a <sup>2,3</sup>	11 a	0.3 a	0.7 a	2.1 a	0 a
LS+PC	0 a	9.7 a	0.3 a	0.8 a	1.6 a	0 a
LS	5.2 b	16.9 b	35.6 b	11.9 b	14 ab	6.1 b
NC	15.2 c	31.1 c	70 c	14.8 b	22.2 b	27.8 c

<sup>1</sup> PC (positive control) = full scab fungicide programme applied, LS+PC = full scab fungicide programme and leaf shredding applied, LS (leaf shredding) = only leaf shredding applied, and NC (Negative control) = no scab fungicides or sanitation applied.

<sup>2</sup> Incidence is defined as percentage of fruit or leaves with at least one lesion.

<sup>3</sup> Means with the same letter in a column are not significantly different ( $P < 0.05$ ).

**Table 4.** Mean scab severity on fruit from weeks 42-45 (October and early November) in 2011, 2012 and 2013 in orchards C (Koue Bokkeveld) and D (Elgin).

Year	2011		2012		2013	
Orchard	C	D	C	D	C	D
PC <sup>1</sup>	0.1 a <sup>2,3</sup>	3.2 a	0.1 a	0.3 a	0 a	0.5 a
LS+PC	0 a	3 a	0.1 a	0.2 a	0 a	0.4 a
LS	1.9 ab	5.2 b	10.1 b	4.7 b	1.3 a	4 b
NC	3.9 b	9.2 c	25.1 c	5 b	8.4 b	8.1 c

<sup>1</sup> PC (positive control) = full scab fungicide programme applied, LS+PC = full scab fungicide programme and leaf shredding applied, LS (leaf shredding) = only leaf shredding applied, and NC (Negative control) = no scab fungicides or sanitation applied.

<sup>2</sup> Severity is defined as percentage of fruit or leaf area visibly affected by scab.

<sup>3</sup> Means with the same letter in a column are not significantly different ( $P < 0.05$ ).

**Table 5.** Mean scab incidence on fruit and leaves in spring 2011 and March 2012 in orchards C (Koue Bokkeveld) and D (Elgin). Fungicide sprays were delayed until week 43 in leaf shredding and negative control plots.

Week	39 <sup>1</sup>				40				41				42			
Orchard	C		D		C		D		C		D		C		D	
Treatment	fruit	leaf	fruit	leaf	fruit	leaf	fruit	leaf	fruit	leaf	fruit	leaf	fruit	leaf	fruit	leaf
PC <sup>2</sup>	~	0	~	0a <sup>3,4</sup>	~	0a	~	2.4a	~	0a	~	6.4a	0a	0a	7a	6a
LS + PC	~	0	~	0a	~	0a	~	2.5a	~	0a	~	6.7a	0a	0a	7a	5a
LS	~	0	~	0.6a	~	0.3a	~	6ab	~	1.3a	~	10.3a	2.5a	2.8a	10ab	9a
NC	~	0	~	1.3a	~	0.7a	~	10b	~	2.1a	~	15.9a	10a	3.9a	33b	11a

Week	43				44				45				Mar-12			
Orchard	C		D		C		D		C		D		C		D	
Treatment	fruit	leaf	fruit	leaf	fruit	leaf										
PC	0.5a	0.1a	15ab	5a	0a	0.1a	12a	3.6a	0.5a	0a	12a	3a	1a	0.1a	14a	2.6a
LS + PC	0a	0a	9a	4a	0a	0a	9.5a	3.3a	0a	0a	11.5a	2a	0.5a	0a	11a	2.1a
LS	8ab	3a	15ab	5a	6ab	3ab	15a	5.5a	5a	3a	35ab	11.3b	34b	15b	55b	20b
NC	17b	4a	26b	9a	18b	6b	36b	20b	16a	6a	46b	12.4b	55c	17b	81c	36c

<sup>1</sup> Week 41 = full bloom, week 45 = physiological fruit drop, March 2012 = pre-harvest.

<sup>2</sup> PC (positive control) = full scab fungicide programme applied, LS+PC = full scab fungicide programme and leaf shredding applied, LS (leaf shredding) = only leaf shredding applied, and NC (Negative control) = no scab fungicides or sanitation applied.

<sup>3</sup> Incidence is defined as percentage of fruit or leaves with at least one lesion.

<sup>4</sup> Means with the same letter in a column are not significantly different ( $P < 0.05$ ).

**Table 6.** Mean scab severity on fruit and leaves in spring 2011 and March 2012 in orchards C (Koue Bokkeveld) and D (Elgin). Fungicide sprays were delayed until week 43 in leaf shredding and negative control plots.

Week	39 <sup>1</sup>				40				41				42			
Orchard	C		D		C		D		C		D		C		D	
Treatment	fruit	leaf	fruit	leaf	fruit	leaf	fruit	leaf	fruit	leaf	fruit	leaf	fruit	leaf	fruit	leaf
PC <sup>2</sup>	~	0	~	0a <sup>3,4</sup>	~	0a	~	0.5a	~	0a	~	1.7ab	0a	0a	1.4a	1.7a
LS + PC	~	0	~	0a	~	0a	~	0.6a	~	0a	~	1.9ab	0a	0a	1.3a	1.4a
LS	~	0	~	0.1a	~	0.1a	~	1.7ab	~	0.3a	~	3.3ab	0.5a	0.5a	1.4ab	3.0a
NC	~	0	~	0.2a	~	0.1a	~	3.5b	~	0.5a	~	6.1b	1.9a	0.9a	6.5b	3.6a

Week	43				44				45				Mar-12			
Orchard	C		D		C		D		C		D		C		D	
Treatment	fruit	leaf	fruit	leaf	fruit	leaf	fruit	leaf	fruit	leaf	fruit	leaf	fruit	leaf	fruit	leaf
PC	0a	0a	2.8ab	1.5ab	0a	0a	2.4a	0.8a	0a	0a	3.2a	0.6a	0.2a	0a	3.5a	0.8a
LS + PC	0a	0a	1.8a	0.9a	0a	0a	1.8a	1.0a	0a	0a	3.7a	0.7a	0.2a	0a	3.5a	0.7a
LS	1.5ab	0.7a	3ab	1.4ab	1.1a	0.6a	3.4a	1.5ab	1a	0.8a	16b	5.9b	13b	6.5b	21b	8.6b
NC	3.4b	0.9a	5b	3.2b	3.7a	1.3a	9b	3.5b	3.1a	1.4a	22b	5.9b	24c	6.5b	33c	17c

<sup>1</sup> Week 41 = full bloom week, 45 = physiological fruit drop, March 2012 = pre-harvest.

<sup>2</sup> PC (positive control) = full scab fungicide programme applied, LS+PC = full scab fungicide programme and leaf shredding applied, LS (leaf shredding) = only leaf shredding applied, and NC (Negative control) = no scab fungicides or sanitation applied.

<sup>3</sup> Severity is defined as percentage of fruit or leaf area visibly affected by scab.

<sup>4</sup> Means with the same letter in a column are not significantly different ( $P < 0.05$ ).

**Table 7.** Mean scab incidence on fruit and leaves in spring 2012 and February 2013 in orchards C (Koue Bokkeveld) and D (Elgin). Fungicide sprays were delayed in leaf shredding and negative control plots until week 42 in orchard C and week 43 in orchard D.

Week	41 <sup>1</sup>				42				43			
Orchard	C		D		C		D		C		D	
Treatment	fruit	leaf	fruit	leaf	fruit	leaf	fruit	leaf	fruit	leaf	fruit	leaf
PC <sup>2</sup>	~	0a <sup>3,4</sup>	~	0	0a	0a	0a	0a	0a	0.4a	1a	1a
LS + PC	~	0a	~	0	0a	0a	0a	0a	1a	0.5a	0.5a	0.5a
LS	~	6b	~	0	14b	16b	3a	1.4a	62b	20b	9b	5b
NC	~	11c	~	0	75c	32c	7.5b	3.1a	94c	42c	15c	6b
Week	44				45				Feb-13			
Orchard	C		D		C		D		C		D	
Treatment	fruit	leaf	fruit	leaf	fruit	leaf	fruit	leaf	fruit	leaf	fruit	leaf
PC	0.5a	0.1a	0a	0.6a	2a	3a	2a	4a	3a	0.2a	3.5a	1.4a
LS + PC	0.5a	0a	0.5a	0.1a	0.5a	0.3a	3a	2a	3.5a	0.5a	4.5a	0.6a
LS	38b	27b	8b	8b	30b	19b	29b	35b	83b	27b	61b	38b
NC	62c	28c	14c	10c	50c	26b	25b	30b	96c	49c	62b	46c

<sup>1</sup> Week 42 = full bloom, week 45 = physiological fruit drop, February 2013 = pre-harvest.

<sup>2</sup> PC (positive control) = full scab fungicide programme applied, LS+PC = full scab fungicide programme and leaf shredding applied, LS (leaf shredding) = only leaf shredding applied, and NC (Negative control) = no scab fungicides or sanitation applied.

<sup>3</sup> Incidence is defined as percentage of fruit or leaves with at least one lesion.

<sup>4</sup> Means with the same letter in a column are not significantly different ( $P < 0.05$ ).

**Table 8.** Mean scab severity on fruit and leaves in spring 2012 and February 2013 in orchards C (Koue Bokkeveld) and D (Elgin). Fungicide sprays were delayed in leaf shredding and negative control plots until week 42 in orchard C and week 43 in orchard D.

Week	41 <sup>1</sup>				42				43			
Orchard	C		D		C		D		C		D	
Treatment	fruit	leaf	fruit	leaf	fruit	leaf	fruit	leaf	fruit	leaf	fruit	leaf
PC <sup>2</sup>	~	0a <sup>3,4</sup>	~	0	0a	0a	0a	0a	0a	0a	0.4a	0.2a
LS + PC	~	0a	~	0	0a	0a	0a	0a	0.1a	0.1a	0.1a	0.1a
LS	~	1.7b	~	0	3b	5b	1.8ab	0.3a	18b	6.3b	2.1ab	1.2a
NC	~	4.6c	~	0	21c	13c	1.5b	0.7a	36c	16.4c	3.6b	1.4a
Week	44				45				Feb 2013			
Orchard	C		D		C		D		C		D	
Treatment	fruit	leaf	fruit	leaf	fruit	leaf	fruit	leaf	fruit	leaf	fruit	leaf
PC	0.1a	0a	0a	0.2a	0.2a	0.1a	0.8a	1.1a	0.6a	0a	0.6a	0.6a
LS + PC	0.1a	0a	0a	0a	0.1a	0.1a	0.7a	0.4a	0.6a	0.1a	0.8a	0.3a
LS	10.4b	11b	1.8a	2.7ab	9b	10b	14b	22b	45b	12b	24b	21b
NC	22.5c	17c	2.7a	3.9b	22c	13b	12b	19b	67c	27c	22b	27c

<sup>1</sup> Week 42 = full bloom, week 45 = physiological fruit drop, February 2013 = pre-harvest.

<sup>2</sup> PC (positive control) = full scab fungicide programme applied, LS+PC = full scab fungicide programme and leaf shredding applied, LS (leaf shredding) = only leaf shredding applied, and NC (Negative control) = no scab fungicides or sanitation applied.

<sup>3</sup> Severity is defined as percentage of fruit or leaf area visibly affected by scab.

<sup>4</sup> Means with the same letter in a column are not significantly different ( $P < 0.05$ ).

**Table 9.** Mean scab incidence on fruit and leaves in spring 2013 in orchards C (Koue Bokkeveld) and D (Elgin). Fungicide sprays were delayed in leaf shredding and negative control plots until weeks 44 in both orchards.

Week	40 <sup>1</sup>				41				42			
Orchard	C		D		C		D		C		D	
Treatment	fruit	leaf	fruit	leaf	fruit	leaf	fruit	leaf	fruit	leaf	fruit	leaf
PC <sup>2</sup>	~	0	~	0	~	0a <sup>3,4</sup>	~	0a	0a	0a	0a	0a
LS + PC	~	0	~	0	~	0a	~	0a	0a	0a	0a	0a
LS	~	0	~	0	~	0a	~	0a	0a	0.5a	0a	0.5a
NC	~	0	~	0	~	0.2a	~	0a	1a	1a	0a	1a
Week	43				44				45			
Orchard	C		D		C		D		C		D	
Treatment	fruit	leaf	fruit	leaf	fruit	leaf	fruit	leaf	fruit	leaf	fruit	leaf
PC	0a	0a	0a	1.3a	0a	0a	0.5a	1.1a	0a	0.1a	5.5a	3.2a
LS + PC	0a	0a	0.3a	1.6a	0a	0a	2.3a	1.9a	0a	0a	5.8a	4.2a
LS	0.8a	0.4a	6 ab	4.1ab	2.3a	0.9a	8ab	7.9b	22b	10b	42b	36b
NC	7.3a	2.2a	9.3b	5.1b	23b	8.8b	17b	11b	81c	30c	62c	43b

<sup>1</sup> Week 42 = full bloom, week 45 = physiological fruit drop.

<sup>2</sup> PC (positive control) = full scab fungicide programme applied, LS+PC = full scab fungicide programme and leaf shredding applied, LS (leaf shredding) = only leaf shredding applied, and NC (Negative control) = no scab fungicides or sanitation applied.

<sup>3</sup> Incidence is defined as percentage of fruit or leaves with at least one lesion.

<sup>4</sup> Means with the same letter in a column are not significantly different ( $P < 0.05$ ).

**Table 10.** Mean scab severity on fruit and leaves in spring 2013 in orchards C (Koue Bokkeveld) and D (Elgin). Fungicide sprays were delayed in leaf shredding and negative control plots until weeks 44 in both orchards.

Week	40 <sup>1</sup>				41				42			
Orchard	C		D		C		D		C		D	
Treatment	fruit	leaf	fruit	leaf	fruit	leaf	fruit	leaf	fruit	leaf	fruit	leaf
PC <sup>2</sup>	~	0	~	0	~	0a <sup>3,4</sup>	~	0a	0a	0a	0a	0a
LS + PC	~	0	~	0	~	0a	~	0a	0a	0a	0a	0a
LS	~	0	~	0	~	0a	~	0a	0a	0.1a	0a	0.1a
NC	~	0	~	0	~	0a	~	0a	0.2a	0.2a	0a	0.2a
Week	43				44				45			
Orchard	C		D		C		D		C		D	
Treatment	fruit	leaf	fruit	leaf	fruit	leaf	fruit	leaf	fruit	leaf	fruit	leaf
PC	0a	0a	0.1a	0.4a	0a	0a	0.1a	0.2ab	0a	0a	1.3a	1a
LS + PC	0a	0a	0.1a	0.4a	0a	0a	0.5a	0.4ab	0a	0a	1.3a	1a
LS	1.2a	0.1a	1.4ab	1.1b	0.5a	0.3a	1.8ab	2.6b	4.5a	3.5a	13b	23b
NC	1.7a	0.5a	2.3b	1.4b	6b	3.9b	4.6a	4.6c	26b	20b	26c	31c

<sup>1</sup> Week 42 = full bloom, week 45 = physiological fruit drop.

<sup>2</sup> PC (positive control) = full scab fungicide programme applied, LS+PC = full scab fungicide programme and leaf shredding applied, LS (leaf shredding) = only leaf shredding applied, and NC (Negative control) = no scab fungicides or sanitation applied.

<sup>3</sup> Severity is defined as percentage of fruit or leaf area visibly affected by scab.

<sup>4</sup> Means with the same letter in a column are not significantly different ( $P < 0.05$ ).

**Table 11.** Pooled data from Koue Bokkeveld and Elgin for ascospores trapped  $\text{cm}^{-2}$  leaf area at 14-day intervals in spring 2010 and 2011.

Week	Representative of	Ascospores $\text{cm}^{-2}$ leaf area	
		2010	2011
35	end Aug/beg Sept	0	0
37	mid Sept	0	125
39	end Sept/beg Oct	71	71
41	mid Oct	9	0
43	end Oct/ beg Nov	40	0
45	mid-Nov	18	0
47	end-Nov/beg Dec	1138	0

**Table 12.** Days in 2012 on which airborne ascospores were detected in non-shredded negative control (NC) and leaf shredding (LS) treatments, from 27 August until 29 October, in orchard C (Koue Bokkeveld).

Week	Date	Trt-rep. <sup>1</sup>	No. ascospores <sup>2</sup>	Week	Date	Trt-rep.	No. ascospores
35	30 August	NC-1	116	39	30 September	NC-1	~
35	30 August	NC-2	~	39	30 September	NC-2	91
35	30 August	LS-1	~	39	30 September	LS-1	~
35	30 August	LS-2	~	39	30 September	LS-2	~
35	2 September	NC-1	~	39	1 October	NC-1	~
35	2 September	NC-2	~	39	1 October	NC-2	406
35	2 September	LS-1	167	39	1 October	LS-1	382
35	2 September	LS-2	126	39	1 October	LS-2	213
35	3 September	NC-1	178	40	<b>3 October</b>	<b>NC-1</b>	<b>654</b>
35	3 September	NC-2	160	40	<b>3 October</b>	<b>NC-2</b>	<b>535</b>
35	3 September	LS-1	~	40	<b>3 October</b>	<b>LS-1</b>	<b>1020</b>
35	3 September	LS-2	~	40	<b>3 October</b>	<b>LS-2</b>	<b>178</b>
36	4 September	NC-1	144	40	<b>4 October</b>	<b>NC-1</b>	<b>984</b>
36	4 September	NC-2	~	40	<b>4 October</b>	<b>NC-2</b>	<b>781</b>
36	4 September	LS-1	~	40	<b>4 October</b>	<b>LS-1</b>	<b>348</b>
36	4 September	LS-2	~	40	<b>4 October</b>	<b>LS-2</b>	<b>538</b>
36	8 September	NC-1	108	40	<b>5 October</b>	<b>NC-1</b>	<b>754</b>
36	8 September	NC-2	~	40	<b>5 October</b>	<b>NC-2</b>	<b>384</b>
36	8 September	LS-1	95	40	<b>5 October</b>	<b>LS-1</b>	<b>345</b>
36	8 September	LS-2	~	40	<b>5 October</b>	<b>LS-2</b>	<b>121</b>
36	9 September	NC-1	624	40	<b>6 October</b>	<b>NC-1</b>	<b>1248</b>
36	9 September	NC-2	966	40	<b>6 October</b>	<b>NC-2</b>	<b>422</b>
36	9 September	LS-1	~	40	<b>6 October</b>	<b>LS-1</b>	<b>1497</b>
36	9 September	LS-2	~	40	<b>6 October</b>	<b>LS-2</b>	<b>203</b>
<b>38</b>	<b>18 September</b>	<b>NC-1</b>	<b>490<sup>3</sup></b>	40	7 October	NC-1	~
<b>38</b>	<b>18 September</b>	<b>NC-2</b>	<b>671</b>	40	7 October	NC-2	877
<b>38</b>	<b>18 September</b>	<b>LS-1</b>	<b>176</b>	40	7 October	LS-1	279
<b>38</b>	<b>18 September</b>	<b>LS-2</b>	<b>254</b>	40	7 October	LS-2	~
<b>38</b>	<b>21 September</b>	<b>NC-1</b>	<b>2094</b>	40	8 October	NC-1	81
<b>38</b>	<b>21 September</b>	<b>NC-2</b>	<b>66</b>	40	8 October	NC-2	1191
<b>38</b>	<b>21 September</b>	<b>LS-1</b>	<b>282</b>	40	8 October	LS-1	~
<b>38</b>	<b>21 September</b>	<b>LS-2</b>	<b>171</b>	40	8 October	LS-2	111
39	25 September	NC-1	126	41	9 October	NC-1	270
39	25 September	NC-2	~	41	9 October	NC-2	~
39	25 September	LS-1	~	41	9 October	LS-1	112
39	25 September	LS-2	~	41	9 October	LS-2	~
39	26 September	NC-1	~	41	10 October	NC-1	1675
39	26 September	NC-2	270	41	10 October	NC-2	~
39	26 September	LS-1	~	41	10 October	LS-1	83
39	26 September	LS-2	~	41	10 October	LS-2	~
<b>39</b>	<b>27 September</b>	<b>NC-1</b>	<b>232</b>	41	11 October	NC-1	196
<b>39</b>	<b>27 September</b>	<b>NC-2</b>	<b>400</b>	41	11 October	NC-2	~
<b>39</b>	<b>27 September</b>	<b>LS-1</b>	<b>124</b>	41	11 October	LS-1	~
<b>39</b>	<b>27 September</b>	<b>LS-2</b>	<b>76</b>	41	11 October	LS-2	~
39	28 September	NC-1	~	41	<b>13 October</b>	<b>NC-1</b>	<b>80</b>
39	28 September	NC-2	231	41	<b>13 October</b>	<b>NC-2</b>	<b>76</b>
39	28 September	LS-1	~	41	<b>13 October</b>	<b>LS-1</b>	<b>143</b>
39	28 September	LS-2	~	41	<b>13 October</b>	<b>LS-2</b>	<b>84</b>
39	29 September	NC-1	~	41	14 October	NC-1	149
39	29 September	NC-2	~	41	14 October	NC-2	248
39	29 September	LS-1	~	41	14 October	LS-1	~
39	29 September	LS-2	151	41	14 October	LS-2	149

<sup>1</sup> Numbers after treatment (trt) abbreviations indicate treatment replicates (rep.).

<sup>2</sup> Number (no.) airborne ascospores  $\text{m}^{-3}\text{day}^{-1}$  was determined using volumetric spore traps and qPCR. Days on which detection occurred below the reliable lower detection limit (62 ascospores  $\text{m}^{-3}\text{day}^{-1}$ ) are indicated with a tilde (~).

<sup>3</sup> Days on which detection occurred above the reliable detection limit in all treatment replicates are shown in bold.

**Table 13.** Leaf-litter density, determined using the point-intercept method<sup>1</sup>, in two orchards after 100% leaf-drop and at bud-break, and the percentage loss in leaf-litter between the two observation times.

Region	Koue Bokkeveld			Elgin		
	After 100% leaf-drop	Bud-break	% loss	After 100% leaf-drop	Bud-break	% loss
2012	59.6 <sup>2</sup>	17.6	70.5	32.8	8.3	74.7
2013	57.6	13.5	76.6	55.8	9.6	82.8

<sup>1</sup> Point intercept method as described by Gadoury and MacHardy (1986).

<sup>2</sup> Leaf-litter density is defined as the proportion of orchard floor covered by fallen leaves.

## CHAPTER 3

### ***THE EFFECT OF CLIMATIC CONDITIONS IN RELATIVELY WARM AND COLD WINTER REGIONS ON THE SEXUAL OVERWINTERING STAGE OF VENTURIA INAEQUALIS***

#### **ABSTRACT**

Pseudothecial density (PD) and ascus density (AD) are important components for potential ascospore dose (PAD), which is a measure of the risk of primary apple scab (*Venturia inaequalis*) infection in an orchard for the following season. No information exists on these components under field conditions in the Western Cape to date. The objective of this study was to compare PD and AD between the Elgin (EL) region, a warm winter region experiencing climate warming, and the Koue Bokkeveld (KB) region, a cold winter region not experiencing climate change, to determine the potential influence of long-term climate warming on PD and AD under field conditions. In 2012 and 2013, scabbed apple leaves were collected from trees during leaf-drop in KB and EL. In a randomized experimental design, leaves were overwintered in their region of origin (KB-KB or EL-EL) or in the other region (KB-EL or EL-KB) to investigate the effect of temperature on number of pseudothecia per fertile lesion (PD) and average number of asci per pseudothecium (AD). In 2012 and 2013, the PD for leaves collected in KB and overwintered in KB (24.7 and 24.3, respectively) was significantly higher than leaves collected in KB and overwintered in EL (15.2 and 19.4, respectively), as well as leaves collected in EL and overwintered in EL (12.7 and 18.4, respectively). The temperature during pseudothecial formation (*i.e.* the first four weeks after leaf-drop) was significantly lower in KB than in EL. However, in 2012 and 2013, the PD for leaves collected in EL and overwintered in EL did not differ significantly from EL leaves that overwintered in KB (13.8 and 19.9, respectively) ( $P < 0.05$ ). In 2012 and 2013, AD values in all treatments did not differ from one another (average 151.6 and 146.3, respectively). Although temperatures differed significantly between regions, these differences may not have been sufficient to cause a detectable difference in AD. One reason for PD, but not AD, differing between treatments EL-EL and EL-KB may be a fitness factor resulting from an adaptation by the Elgin *V. inaequalis* population used in this study to shorter, warmer winters over a number of decades, so that fewer pseudothecia are formed to compete with each other after leaf-drop, but asci in these pseudothecia still develop normally and produce mature ascospores to be discharged around bud-break. Additional seasons for this experiment would be required to verify these results before PD and AD constants can be determined for application of PAD in South Africa.

## INTRODUCTION

Apple scab, caused by *Venturia inaequalis* (Cooke) Winter, is the most important apple disease in South Africa. The South African apple industry is primarily export-driven and effective management of apple scab relies on accurate information on development of the pathogen (Gadoury and MacHardy, 1982b). The sexual phase of the life-cycle of *V. inaequalis* begins after leaf-drop, when different mating types on the same fallen leaf anastomose to produce sexual fruiting bodies (pseudothecia) in autumn and early winter. Asci form within these pseudothecia in late winter, and eight ascospores form within each ascus in early spring (Gadoury and MacHardy, 1982a). The number of ascospores produced and discharged during late winter and early spring is directly proportional to the risk of infection and resulting disease development in the following season (Gadoury and MacHardy, 1986).

Changing weather patterns over the past 40 years are characterized by a warming trend in all seasons for most regions in the Western Cape in South Africa, and an increase in air temperatures of 1 to 2°C is predicted over the next 30 years (Midgley and Lötze, 2011). In Elgin, regarded as a warm winter apple-growing region (Midgley and Lötze, 2011), apple trees have responded to climatic warming with delayed or incomplete leaf-drop, not fully entering dormancy, and delayed foliation (uneven bud-break and bloom) (Cook, 2010). Changes in apple tree phenology due to climate change have also been reported in Germany (Kunz and Blanke, 2011) and Lithuania (Romanovskaja and Bakšiene, 2009). In the Koue Bokkeveld, regarded as a cold winter apple-growing region (Midgley and Lötze, 2011), apple trees do not experience the phenological disruption experienced in Elgin. Temperatures in the Koue Bokkeveld are within the range that apple (*Malus x domestica*) Borkh. is thought to have evolved, as evidenced by the adequate chilling units and normal growth pattern experienced by apple trees in that area, while temperatures in Elgin are outside this range, as evidenced by the increasingly insufficient chilling units experienced by an atypical growth pattern of apple trees in Elgin (Gladieux *et al.*, 2008; Cook, 2010). *Venturia inaequalis* sexual events are synchronized with tree phenological events in the Koue Bokkeveld region (Louw, 1951; Schwabe, 1980), but ascospore discharge often occurs before delayed foliation in Elgin and so susceptible host tissue is not always available for scab infection (Louw, 1951).

Seasonal events in the life cycle of *Venturia inaequalis* have also shifted over time in the Western Cape. In 1942 (Louw, 1951) and 1977 (Schwabe, 1980), significant ascospore discharge in Elgin started in August and mid-August and peaked in late August and late September, respectively, whereas in spring 2011 (Chapter 2), ascospore discharge in Elgin started in early September and peaked by late September, which was similar to results reported by Schwabe (1980), except that the exact date of when peak ascospore discharge occurred in 2011 is not known. Louw (1951) did not

find any viable overwintered conidia on apple bud outer scales or on shoot surfaces in the Ceres, Elgin or Stellenbosch areas, nor did the author report any presence of ‘pygmy’ apples (late season apples that remain on trees throughout winter). In Chapter 4 of this dissertation, no mycelia were observed on bud tissues; however, viable conidia were observed to overwinter on inner apple bud tissues in Elgin, Koue Bokkeveld and Witzenberg Valley in 2010, 2011 and 2012, and high numbers of viable conidia were present on pygmy apples that remained on trees throughout winter in 2011 and 2012.

Louw (1951) and Schwabe (1980) investigated the development of the sexual stage of *V. inaequalis* under Western Cape winter conditions. These findings need to be verified in light of increasing evidence of climate changes in the Western Cape and their influence on apple tree phenology. However, regular high incidences of scab, especially when control strategies are not applied optimally, are evidence that the winter climate in all apple production regions of the Western Cape are conducive to pseudothecial production and development, and ascospore maturation in late winter and early spring.

Potential ascospore dose (PAD) is the total seasonal production of *V. inaequalis* ascospores per square metre orchard floor (Gadoury and MacHardy, 1986), and is calculated using the equation  $PAD = LD \times PD \times AD \times LLD \times n$ . PAD can be used to determine the risk of primary scab infection in spring for a specific apple orchard, or blocks within an orchard, and this aids producers in making informed decisions about which orchards require more immediate preventative scab management in spring (Gadoury and MacHardy, 1986; Sutton *et al.*, 2000). Lesion density (LD) and leaf litter density (LLD) are not sexual stages in the PAD equation, so are addressed in terms of integrated scab management in Chapter 2. The factor  $n$ , the number of ascospores per ascus, is a constant of eight. Pseudothecial density (PD) is the number of mature pseudothecia per visible lesion and ascal density (AD) is the number of asci per pseudothecium. Constants for PD (21.6) and AD (122) have been determined for *V. inaequalis* populations in New Hampshire in the north-eastern USA (Gadoury and MacHardy, 1986), but these constants may not apply in South Africa. To date, determinations of PD and AD have not been reported for South Africa, and so PAD cannot be accurately determined in South Africa until constants for these factors have been determined.

PD and AD are dependent on environmental conditions after leaf fall and during winter (Louw, 1951; Gadoury and MacHardy, 1982a, 1982b). Many studies have investigated the effect of temperature on pseudothecial development and temperature, humidity and light on the production, development and maturation of ascospores (Wilson, 1928; Brook, 1969; Szkolnik, 1969; James and Sutton, 1982; Gadoury and MacHardy, 1982a; Gadoury *et al.*, 1998). Most pseudothecia are formed within the first four weeks after leaf-drop (Gadoury and MacHardy, 1982a); therefore, temperatures during the first four weeks after leaf fall also directly affect PD. Gadoury and MacHardy (1982a)

reported that number of pseudothecia increased at a rate inversely proportional to temperature between 4 and 20°C, but pseudothecial diameter increased most rapidly at 10°C and hardly developed above 20°C under both field and laboratory conditions. Similar results were reported by Louw (1951) and James and Sutton (1982). However, Louw (1951) found that pseudothecia could form at temperatures as low as 1°C and were most abundant at 13°C in the Western Cape in South Africa. Relatively high temperatures (above 13°C) may retard the initial formation of pseudothecia, which may affect later developmental stages of pseudothecia and ascospores (Louw, 1951). Pseudothecial maturation in the Western Cape winter rainfall region was most rapid between 13 and 23°C (Louw, 1951). Louw (1948) noted differences in temperature ranges and optima for pseudothecial formation between different mono-conidial isolates of *V. inaequalis* in the Western Cape, with the optimal temperature being higher than the optimum recorded in earlier American studies that the author mentioned. Louw (1948) also considered it likely that *V. inaequalis* may adapt to different climates and that strains with higher temperature optima could adapt and be dominant in warmer regions, and *vice versa*. The number of asci per pseudothecium increases at a rate inversely proportional to temperature between 6 and 20°C before ascospore maturation starts, after which temperature has no significant effect on ascus production (Gadoury and MacHardy, 1984). As winter progresses towards spring, the thermal requirement for optimal ascospore maturation shifts to a higher temperature range and has been reported to be 20°C by Wilson (1928) in USA, Louw (1951) in the Western Cape, South Africa and Gadoury and MacHardy (1982a) in New Hampshire, USA. However, this optimum was reported to be 16°C to 18°C by James and Sutton (1982) in North Carolina, USA. Gadoury and MacHardy (1986) state that based on the direct effect of spring temperatures on AD, AD values should be determined for each climatic region when using PAD to accurately determine risk of disease for an orchard in the following season; therefore, a similar argument could be made for the need to determine PD in different regions.

The objective of this study were (1) to compare pseudothecial and ascus densities, and ascospore discharge, between the Elgin region, that is experiencing climate warming, and the Koue Bokkeveld region, that is not experiencing climate change, (2) to determine the potential influence of long-term climate warming on PD and AD, and (3) to determine the effect of moving leaves from one climatic winter region to a different climatic winter region to ascertain whether populations in these regions have adapted to their prevailing climatic conditions.

## MATERIALS AND METHODS

### Leaf collection and experimental set-up

'Braeburn' orchards in this study are orchard D in Elgin (34 °10'.003" S, 19°02'47" E), planted in 1994 on seedling rootstocks, in an east-west row direction, with 4 x 1.2m planting distances and a total area of 2.32 ha, and orchard E in the Koue Bokkeveld (33°07'49.15"S, 19°19'32.24"E), planted in 1995 on M25 rootstocks, in a north-south row direction, with 4.5 x 1.5 m planting distances and a total area of 5.25 ha.

According to Gadoury and MacHardy (1986), disease incidence in an orchard should be above 10% to allow the accurate and significant measure of *V. inaequalis* pseudothecial density on leaves and to eliminate the error involved with this measurement. Foliar scab incidence was assessed in autumn 2012 and 2013 using the disease assessment method described in Chapter 2, except that 25 trees per 2 ha were randomly selected for disease assessment.

Approximately 1000 senescent, scabbed leaves from trees across each orchard were detached during leaf-fall in May and June, in 2012 and 2013. Detached leaves were transferred to the overwintering sites within two days to ensure that temperature influence on PD occurred almost entirely when the leaf-sets were at the overwintering sites. Leaves originating from Koue Bokkeveld were collected by mid-May 2012 (week 20) and in May 2013 (week 21). Leaf-drop in Elgin is usually delayed, due to warm winter conditions in that region, so leaves originating from Elgin were collected in early May (week 19) and mid-June 2012 (week 24), but only once in mid-June 2013 (week 25). In 2013, leaves in both regions were collected only once to prevent staggering of pseudothecial formation stages that would have occurred on leaves collected at different times, as occurred in 2012. Leaves were laid out in experimental plots for overwintering on the same or within the following two days after being collected to limit storage time and potential effect that storage may have on pseudothecial formation and/or development. For determining pseudothecial and ascus densities, scabbed leaves with only two, three or four lesions were collected in an attempt to limit the number of visibly distinct fertile lesions that would form on the leaves.

Two treatments were laid out in each region in a randomised experimental design, with one hundred leaves per treatment plot for determining pseudothecial and ascus densities, and two hundred leaves per plot for monitoring ascospore discharge. Treatment KB-KB and EL-EL comprised leaves from the region of origin (Koue Bokkeveld and Elgin, respectively) that were overwintered in the same region, to use as the negative control treatments. Treatment KB-EL and EL-KB comprised leaves from the region of origin overwintered in the other region. GPS coordinates for overwintering trial sites are 33°12'34.10"S, 19°20'16.26"E in Koue Bokkeveld and

34°9'36.78"S, 19°4'26.24"E in Elgin. In each region, plots for both experiments were spaced minimum 20 m from each other to eliminate any possibility of treatments interfering with each other. Leaves were overwintered with the adaxial (top) side facing upwards, on top of two layers of beige netting (20% shade) and underneath 1 m<sup>2</sup> of dark-green hardware netting (4 x 4 cm) secured to the ground. In mid-July 2012, the overwintering plots in Elgin were disturbed by the vigorous growth of grass from underneath the leaves. The grass was removed and leaves had to be rearranged with their adaxial side facing upwards, since the grass had caused some leaves to turn sideways or to turn over so that their abaxial (bottom) side faced upwards. In 2013, a layer of shade-netting was placed underneath the leaves, to prevent potential disruption by grass growth.

### **Determining pseudothecial densities**

Autumn foliar scab in orchards D and E were 30 and 10% in 2012 and 49 and 12% in 2013, respectively. In mid-August 2012 and 2013, 30 scabbed leaves from each treatment were cleared using a modified protocol from Shobe and Lersten (1967). Leaves were placed in 25 ml aq. 2 M NaOH (8 g/ 100 ml, Merck, South Africa) in Petri dishes and allowed to discolour in the NaOH solution for three days. The solution was then discarded and another 25 ml NaOH was added to each Petri dish containing the leaves for one day, to allow further discolouration. Petri dishes with leaves were placed on a Bellydancer Shaker (Stoval, Life Science inc., Greensboro USA) for two hours, at a low speed, to allow further discolouration. The NaOH was then discarded and leaves were rinsed three times with distilled water. Four ml aqueous chloral hydrate (200 g/ 100 ml, Sigma-Aldrich) was added to each Petri dish. After one day, the leaves were rinsed with distilled water and dehydrated with 95% ethanol. In 2013, an alternative method, using 2.5% sodium hypochlorite in place of chloral hydrate (McCauley and Evert, 1988), was tested as well on 3 additional leaves. This was done because chloral hydrate is a regulated narcotic that is becoming increasingly difficult and expensive to obtain (Jürschik *et al.*, 2012).

Leaves were covered with a glass slide in the Petri dishes and viewed under a Nikon SMZ 1500 stereomicroscope. Digital photographs were taken of 100 fertile lesions (Nikon Digital Camera DXM1200C and NIS-Elements F 3.0 software), and the number of pseudothecia associated with each fertile lesion was counted. A requirement for pseudothecia to be included in the counts was that they had to have a subcutaneous hyphal connection and be of a suitable size with a visible ostiole and the correct brown colour. Fruiting structures of small size, without the brown colour, with no visible ostiole or were covered by hyphae and were not distinguishable, were not counted.

## **Determining ascal densities**

In early August 2012 and late July 2013, leaves in each plot were moved to an outdoor shelter near the treatment plots, to keep the leaves dry to prevent ascospore discharge, while maintaining them at the same environmental temperature as non-protected leaves. Leaves were wetted every day that it rained, but only before sunrise or after sunset, to minimize ascospore discharge, since ascospores are rarely discharged in darkness (Gadoury *et al.*, 1998). In late August 2012 and early September 2013, overwintered leaves from each treatment were collected, and randomly selected pseudothecia were picked off using a scalpel and transferred to a drop of lactic acid on a microscope slide. Squash mounts were prepared using the methods described in Gadoury and MacHardy (1982b). A coverslip was placed over the pseudothecium and pressure applied to break the pseudothecial wall and allow the asci to spread out evenly on the slide. The number of asci inside each pseudothecia was determined by viewing the squash mounts at 40 x magnification on a Nikon Eclipse E600 light microscope.

## **Monitoring ascospore discharge**

In order to monitor ascospore discharge in each treatment's leaf set for determining pseudothecial densities, a Rotorod rotation impaction sampler (model 20, Sampling Technologies, Inc., Los Altos, California, USA) was placed in the centre of each treatment plot, each containing 200 leaves, using the same experimental set-up as for determining pseudothecial and ascal densities, with the exception that each treatment was replicated twice in each region. Rotorods were suspended on metal poles so that the collecting rods were 40 cm above the leaves and the two collecting rods of each Rotorod collected subsamples from each plots. The Rotorods were connected in parallel to a battery, with the battery connected to a leaf-wetness sensor and a custom-designed timer. From the end of August (week 35) until beginning of November (week 44), when precipitation occurred, the leaf wetness sensor activated the batteries to activate the Rotorods, which were programmed to run only from 10:00 until 16:00, since ascospore discharge increases as light intensity and precipitation increases (Gadoury *et al.*, 1998). In 2013, windbreaks were erected around the treatment plots using iron "droppers" or Y-poles (Kaap Agri, Stellenbosch) and the same shade netting used under the overwintered leaf sets used for determining pseudothecial and ascal densities.

## Temperature and precipitation data

Hourly dry-bulb temperatures (°C) and precipitation (mm) in 2012 and 2013 were collected from permanent weather stations near the experimental plots in each region, located at 34°9'57.5"S, 19°1'31.9"E in Elgin and at 33°12'27.15"S, 19°19'50.68"E in the Koue Bokkeveld, maximum one kilometre from the respective experimental sites in each region. Weather data was obtained from iLeaf Integrated Weather Data Interpretation Software (<http://www.ileaf.co.za/>). Mean daily temperatures and precipitation for the period 1 May until 31 August in both 2012 and in 2013 were compared.

The prevailing temperatures during the main pseudothecial formation stage in each region, *i.e.* first four weeks after overwintering began (Gadoury and MacHardy, 1986), was also compared. Ascal formation begins from four weeks after overwintering (Burchill and Cook, 1971). The first ascospores usually mature and are ready to be discharged in the Western Cape from mid-August (Louw, 1951; Schwabe, 1980), after which temperature has no significant effect on AD (Gadoury and MacHardy, 1984). Therefore, the temperatures from the fifth week after overwintering began until week 33 (mid-August) in the respective regions were compared with the AD in each treatment. Scab, or fusi, infection indices were calculated by iLeaf using the equations described in Schwabe (1980). Scab infection indices were compared to the data obtained for ascospore discharge monitoring, to determine whether ascospore discharge coincided with infection indices.

## Statistical analysis

A two-way analysis of variance (ANOVA) was used to compare pseudothecial and ascal densities in the different leaf-sets using STATISTICA 11 data analysis software system (StatSoft, Inc. 2012, [www.statsoft.com](http://www.statsoft.com)). Fisher's Least Significance Difference (LSD) t-test was used for post-hoc testing. Due to problems encountered in 2012, it was decided not to pool data from 2012 and 2013. The t-test was used to determine whether the regions Koue Bokkeveld and Elgin differed in mean temperature and precipitation for the period 1 May to 31 August in 2012 and 2013, as well as in the mean temperature during pseudothecial and ascal formation. Significant differences were all evaluated at the 5% significance level ( $P < 0.05$ ).

## RESULTS

### Pseudothecial densities

The leaf-clearing method was highly effective in clearing leaf tissue, while leaving fungal tissue naturally coloured. The alternative leaf-clearing method that used sodium hypochlorite in place of chloral hydrate also bleached fungal tissue, which made it impossible to distinguish between plant and fungal material.

Analysis of variance of PD in 2012 indicated that the PD for leaves collected in KB and overwintered in KB ( $24.7 \pm 1.2$ ) was significantly higher ( $P < 0.05$ ) than for KB leaves that were overwintered in EL ( $15.2 \pm 1.2$ ). The PD for leaves collected in EL and overwintered in EL ( $12.7 \pm 1.3$ ) did not differ significantly from EL leaves that overwintered in KB ( $13.8 \pm 1.3$ ) ( $P = 0.44$ ), but the PD in these two treatments was significantly lower than the PD for leaves collected in KB, regardless of where the leaves had overwintered ( $P < 0.05$ ) (Table 1).

In 2013, the PD for leaves collected in KB and overwintered in KB ( $24.3 \pm 1.3$ ) was significantly higher than for leaves collected in EL and overwintered in EL ( $18.5 \pm 1.2$ ) and for leaves that were overwintered in EL ( $19.4 \pm 1.3$ ) ( $P < 0.01$ ). The PD for leaves collected in EL and overwintered in EL did not differ significantly from EL leaves that overwintered in KB ( $19.9 \pm 1.3$ ) ( $P = 0.81$ ), and PD in these treatments did not differ from leaves collected in KB and overwintered in EL ( $P = 0.48$  and  $P = 0.64$ , respectively) (Table 1).

### Ascal densities

In 2012, the AD in KB-KB, KB-EL, EL-EL and KB-EL were  $159.3 \pm 13.3$ ,  $128.8 \pm 14.7$ ,  $156.1 \pm 13.7$  and  $162.1 \pm 13.3$ , respectively. In 2013, the AD in KB-KB, KB-EL, EL-EL and KB-EL was  $144.4 \pm 9.0$ ,  $142.1 \pm 8.6$ ,  $149.2 \pm 8.9$  and  $147.6 \pm 8.8$ , respectively. Analysis of variance indicated that in 2012 and 2013, the mean AD of leaf sets that remained in the region where they originated (KB-KB or EL-EL) were not significantly different from leaf sets that originated in one region and were overwintered in the other region (KB-EL or EL-KB) ( $P > 0.05$ ) (Table 2).

### Ascospore discharge

The first ascospores trapped using Rotorods was in weeks 37 and 36 in 2012 and 2013, respectively. Ascospores trapped per week ranged from 0 to 49 in 2012 and 0 to 66 in 2013. Ascospore discharge

data per week, compared to the sum of fusi infection risk indices per week, are shown in Tables 3 and 4. Data were not adequate to conduct statistical analyses.

### Temperature and precipitation

Mean dry-bulb temperatures for the period 1 May to 31 August in both years were significantly higher in Elgin than in the Koue Bokkeveld ( $P < 0.05$ ), although precipitation during this period between the two regions did not differ significantly in 2012 ( $P = 0.51$ ) or in 2013 ( $P = 0.18$ ) (data not shown). Mean temperatures during the respective pseudothecial and ascus formation stages were significantly lower in the Koue Bokkeveld than in Elgin in both years ( $P < 0.05$ ) (Tables 1 and 2). Fusi infection risk indices per week were added together and were compared with the ascospore discharge data (Tables 3 and 4). Fusi indices were higher in Elgin than in Koue Bokkeveld in both years, although they did not correspond with ascospore discharge data.

## DISCUSSION

Apple scab management decisions rely on accurate information about the risk of infection before a season begins. The risk of primary scab infection for a specific orchard in spring can be determined using PAD, (Gadoury and MacHardy, 1986). Constants for components PD and AD in PAD can be calculated for a specific climatic region, but have not yet been reported for South Africa, and until these constants have been determined, PAD cannot be accurately determined in South African orchards.

The PD for leaves collected in KB in 2012 and 2013 and overwintered in KB was significantly higher than for KB leaves overwintered in EL. Also, in 2012 and 2013, the temperature in KB during the first four weeks of overwintering was significantly lower than in EL. These results are consistent with previous studies (Gadoury and MacHardy, 1982a; Louw, 1951) that reported an inverse relationship between temperature during the first four weeks after leaf drop and pseudothecium production.

In 2012, the PD for leaves in EL-EL and KB-EL was lower than in 2013 (Table 1). In 2012, the orientation of the leaves overwintered in Elgin was disrupted by grass growing underneath the leaves, and *Venturia inaequalis* pseudothecia are negatively geotropic (Gadoury and MacHardy, 1985). Therefore, before the leaves' orientation was adjusted, some of the pseudothecia may have already started developing on the under- (abaxial) side of the leaves that were then facing upwards, but further development of these pseudothecia could have been aborted and only fewer, newly

formed pseudothecia continued to develop to the size that was a prerequisite for pseudothecia to be included in measurements.

In 2012 and in 2013, the PD in EL-EL and EL-KB did not differ significantly from one another, even though the changed leaf orientation in 2012 may have influenced the results. Louw (1948) observed consistent, distinct differences in various features (size, colour, culture topography, colony margin type, type of submerged growth, amount of aerial mycelia, abundance of conidia) between cultured colonies of different mono-conidial *V. inaequalis* isolates in the Western Cape, which agreed with reports by earlier studies that the author mentioned. More importantly, Louw (1948) reported differences in temperature ranges and optima for, and abundance of, pseudothecial initials between some isolates, with one isolate not producing any pseudothecia at all on any of the growth media tested while another isolate produced pseudothecia on various media of the same kind. However, the author stated the “the most divergent types of cultures may be isolated from the same habitat”, and even “many isolations from individual trees revealed a considerable heterogeneity in their scab populations. On the other hand identical cultures were obtained from widely separated regions and from apple varieties with very different phenological characters”. Unfortunately, however, the author does not state from where the individual isolates originated. Louw (1951) also found that one isolate in the Western Cape produced pseudothecia between 1 and 23°C, with an optimal range of 10 to 20°C, while another isolate only produced pseudothecia between 1 and 10°C. A more recent ongoing study by Koopman *et al.* (2013) to characterize *V. inaequalis* isolates from various regions in South Africa, including Elgin and Ceres, using genotyping, indicated that high variation and sexual out-crossing exists in local populations. It may be that the isolate with the 1 to 10°C optimal range for pseudothecial production predominates in the Koue Bokkeveld population whereas the isolate that produces pseudothecia between 10 and 20°C predominates in the Elgin population. If so, it can be speculated that the PD for the EL-KB leaf-sets, although slightly higher in both years, did not differ significantly from that of the EL-EL leaf-sets because of the influence of the genotype of these isolates in each region (Table 1). However, since only a single population from each orchard was used from each region, and due to the heterogeneity in genotypes within regions (Koopman *et al.*, 2013), this should be confirmed with more populations in both regions in future studies.

PD values for 2012 and 2013 ranged from 12.7 to 24.7 (Table 1), which are similar to ranges reported by Jeger *et al.* (1982) (9 to 33), Jeger (1984) (13.6 to 20.4) and Gadoury and MacHardy (1986) (8 to 57). Meszka *et al.* (2008) and Jeger *et al.* (1982) found significant differences between cultivars in pseudothecial densities, while James and Sutton (1982), Smith and MacHardy (1992) and Cruz (1998) did not. Whether PD constants need to be calculated for various cultivars in the different climatic apple production regions in South Africa remains to be determined. However,

information on PD in each region is important for accurately determining the PAD in autumn and associated scab infection risk in spring (Gadoury and MacHardy, 1986). Two seasons is not sufficient to determine a PD constant for a region and a minimum of two more seasons would be required to verify the findings to date before any PD values could be used in practice in South Africa. Since 2.5% sodium hypochlorite did not replace chloral hydrate well in the leaf-clearing method, alternative chemicals or leaf-clearing methods will need to be investigated for future work on PD in the Western Cape, such as the method described by Percival and Boyle (2009).

In 2012 and 2013, significant temperature differences between the region of origin and region of overwintering did not significantly influence the mean AD in leaf sets that originated in one region and overwintered in the other region. This suggests that factors other than winter conditions may have influenced the ascus densities in leaf-sets in 2012 and 2013. The procedure described by Gadoury and MacHardy (1982b) was not followed in this study: weekly squash mounts were not made to determine at what time in spring AD would be highest, and AD was only determined once. However, AD increases only until the first ascospores start to mature (Gadoury and MacHardy, 1984), and mature ascospores were found in asci counted in both years (average 51% of asci across all treatments contained mature asci in 2012). Disintegration of empty asci was not taken into account, but since so few asci had been discharged (average 0.5% of asci across all treatments had discharged their asci in 2012) and had therefore disintegrated, results for AD in 2012 and 2013 should still be sufficiently accurate to determine whether differences exist between treatments.

In 2012 and 2013, the AD for KB-KB and EL-EL leaf sets did not differ significantly (Table 2). This could be because, although the temperatures between the two regions differed significantly, these differences were not great enough to have a significant effect on AD. Temperatures in 2012 and 2013 did not differ greatly, but both regions had unusually low or no rainfall in week 31 in 2013. Since moisture can be a limiting factor in ascus formation (James and Sutton, 1982) this may explain why AD in treatments KB-KB, EL-EL and KB-EL were lower in 2013 compared to 2012.

Mean AD in 2012 and 2013 ranged from 128.8 to 162.1 (Table 2), which are higher than the mean AD reported by Gadoury and MacHardy (1986). Plotting temperatures during the ascus formation periods and ascus densities at these temperatures in this study onto Fig. 8a (effect of temperature on ascus development) in Gadoury and MacHardy (1986), indicate that the ascus densities in this study do follow the expected trend. However, the higher AD values in this study could support theories such as the South African population including a different strain, or multiple strains, not found in the north-eastern US. As mentioned above, two previous studies have provided evidence for this theory (Louw, 1951; Koopman *et al.*, 2013). As with the PD results, a minimum of

two more seasons would be required to verify these results before an AD constant could be determined and used in practice.

The reason for PD, but not AD, differing between treatments EL-EL and EL-KB could perhaps be a fitness factor that has resulted from an adaptation of the Elgin population to warmer winter temperatures over a number of decades, which has been caused by climate change (Midgley and Lötze, 2011). One possible explanation is that the *V. inaequalis* population in Elgin has adapted to form consistently fewer pseudothecia at higher temperature, since PD did not differ significantly when leaves from Elgin were moved to Koue Bokkeveld. It could be that the EL population has also adapted to form fewer pseudothecia in a shorter winter period, since the region is warmer and leaf-drop is delayed by up to two months compared to other apple-growing regions. This would allow the fewer pseudothecia to mature faster in a shorter time period, since competition for resources is lessened, and the fungus could instead spend energy on ascus formation and maturation sooner, so that ascospore discharge is closer to bud-break. In future, if the climate in the Western Cape continues to change as predicted (Midgley and Lötze, 2011), this shift may become more significant. However, this is purely speculation and would need to be tested.

In 2012, the total ascospores trapped were much lower than expected and did not correspond with rain events, fusi infection risk indices, or historical ascospore discharge patterns, and consequently data collection was stopped in mid-October. This experiment was set-up in very exposed, open fields, since these were the only available areas in the regions that were sufficiently far from apple orchards. This was done to reduce the risk of introduction of isolates from one region into apple orchards of another region, since reports of *V. inaequalis* isolates that are tolerant to certain fungicides have been reported (Schwabe, 1977; Schwabe, 1979; Schwabe and Shabi, 1994; Schwabe and van der Rijst, 1997). It is very likely that strong winds in both regions carried significant numbers of airborne ascospores away from the traps.

In 2013, the number of ascospores trapped, although higher than in 2012, was still lower than expected, and as in 2012, the trappings again did not correspond with rain events or fusi infection risk indices, even though windbreaks had been erected around the treatment plots. The Vaseline layer applied to the trapping rods was spread on the rods instead of sprayed, but possibly the Vaseline layer was too thin, because other material captured (e.g. pollen, dust and other fungal spores) was also less on the rods in those weeks that no ascospores were seen.

In the Koue Bokkeveld, the leaf-wetness sensor could only be placed on the south side of the irrigation pump house where the battery and timer were set up. At the beginning of the experiment in 2013, it was ensured that the leaf-wetness sensor's circuit closed during rain; however, most of the rain in the region comes from the north and if the precipitation was light, ascospores may have been discharged, but it is possible that the leaf-wetness sensor did not receive

enough wetness for the circuit to close and to turn the Rotorods on to collect ascospores. In Elgin, a herd of cattle that was moved every few days between pastures past the experimental set-up disrupted the experiment, despite requests to the livestock manager to avoid moving the cattle past the experiment. This caused the shade-nets of the windbreaks to lean on the Rotorods suspended on the metal poles, which may have interfered with data collection. Additionally, grasses still grew from underneath the shade-net layers, and although grass stalks were pulled out or cut weekly, in some weeks, grass stalks grew enough between data collection to become entangled in the spinning parts of the Rotorods in some treatment plots, which may also have interfered with data collection. Aylor (1998) found that airborne ascospore concentrations decreased rapidly as distance from source increased, due to atmospheric turbulence and rain, and that the distance that ascospores are forcibly projected into the air is between 3 and 7 mm. Since the distance from the leaves on the ground to the rotating arms was 40 cm, it is possible that air movements between these two points greatly affected data obtained.

For these reasons, the data for discharged ascospores in this experiment was not sufficient to meet the objective of the experiment and no conclusions could be drawn from the data. The study would need to be repeated and various factors adjusted to meet the objective of determining whether there are differences in onset, peaking and ending of ascospore discharge between isolates of warm and cold winter regions, and whether or not ascospore discharge patterns change when isolates are moved from one climatic region to another. For future work, it is recommended to use an anemometer to compare wind movement inside and outside windbreaks. The optimal height for capturing ascospores from leaf litter should also be determined and leaves used for such an experiment should be more numerous per plot. If possible, a method of capturing ascospores that does not require Vaseline should be developed.

## CONCLUSION

This is the first study to determine PD and AD under natural winter conditions in the Western Cape. It is also the first study to evaluate differences in PD and AD between regions with different winter climates, including one that is experiencing climate warming, *i.e.* Elgin. In 2012 and 2013, the PD for leaves collected in KB and overwintered in KB was significantly higher than for leaves collected in EL and overwintered in EL. The lower PD in leaves overwintered in Elgin in 2012 could have been caused by the changed orientation of the leaves which may have affected the formation of negatively geotropic pseudothecia. However, results in 2013 confirmed that PD in leaves collected in Elgin did not differ when overwintered in Elgin or in Koue Bokkeveld. This suggests that the *V. inaequalis* population in Elgin may include strains not present in the Koue

Bokkeveld population which may have a higher optimal temperature range for pseudothecial formation, which has been suggested in a previous study. This may be the result of warmer winters in Elgin that are caused by climate change. Results from a parallel ongoing study in South Africa provide evidence for the existence of different strains between these two regions.

In 2012 and 2013, AD values in all treatments did not differ from one another and were higher than those reported in previous studies, but followed the expected trend of AD increasing when temperature decreases. Temperatures were highly significantly different between regions, although differences may not have been sufficient to cause a detectable difference in AD.

Ascospore discharge results were much lower than expected and were inconclusive. However, a number of practical problems associated with the experimental set-up were most likely responsible for the failure of the experiment. Changes would need to be made to the set-up before the experiment can be repeated.

Additional seasons for this experiment would be required to verify the results in this trial, since the PD and AD in 2012 may not have been influenced by weather conditions alone, before constants could be determined for application of PAD in the South African apple industry. This study will aid in determining the PD and AD constants for the Koue Bokkeveld and Elgin regions in future. Such information will be useful in developing a more accurate risk prediction system to determine which orchards carry the highest risk for scab in the following season, so that inoculum levels in those orchards could be addressed first and preventative spray applications in spring could be applied to those orchards first in spring.

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**Table 1.** Mean pseudothecial densities per treatment in leaves from Koue Bokkeveld (KB) or Elgin (EL) (region of origin) and overwintered in KB or EL (region of overwintering) in 2012 and 2013.

Year		2012		2013	
Region of origin	Region of over-wintering	PD <sup>1</sup>	Temp. <sup>2</sup>	PD	Temp.
KB	KB	24.7 a <sup>3</sup>	9.2 a	24.3 a	9.4 b
KB	EL	15.2 b	10.7 b	19.4 b	11.2 a
EL	EL	12.7 c	10.5 b	18.5 b	10.9 a
EL	KB	13.8 c	8.6 a	19.9 b	9.4 b

<sup>1</sup> PD (pseudothecial density) = number of mature pseudothecia per visible lesion

<sup>2</sup> Temp = mean dry-bulb temperatures (°C) during the first four weeks that the leaves were overwintered in the respective treatments, when most pseudothecia were expected to form.

<sup>3</sup> Means with same letters do not differ significantly ( $P < 0.05$ ).

**Table 2.** Mean ascus densities per treatment in leaves from Koue Bokkeveld (KB) or Elgin (EL) (region of origin) and overwintered in KB or EL (region of overwintering) in 2012 and 2013.

Year		2012		2013	
Region of origin	Region of over-wintering	AD <sup>1</sup>	Temp. <sup>2</sup>	AD	Temp.
KB	KB	159.3 ab	7.2 a	144.4 a	8.5 a
KB	EL	128.8 a	10 b	142.1 a	10.8 b
EL	EL	156.1 ab	9.7 b	149.2 a	7.7 a
EL	KB	162.1 b	6.7 a	147.6 a	10.8 b

<sup>1</sup> AD (ascus density) = number of asci per pseudothecia.

<sup>2</sup> Temp = mean dry-bulb temperatures (°C) from the fifth week after the leaves were overwintered until the middle of August, in the respective treatments, when ascus formation was expected to be the most influenced by temperature.

<sup>3</sup> Means with same letters do not differ significantly ( $P < 0.05$ ).

**Table 3.** Sum of scab (fusi) infection risk indices (II) (Schwabe, 1980) per week in Koue Bokkeveld (KB) and Elgin (EL) in weeks 35 to 41 in 2012, and the number of ascospores trapped in treatments per week.

Week	Sum of II in KB	No. ascospores trapped		Sum of II in EL	No. ascospores trapped	
		KB-KB <sup>1</sup>	EL-KB		EL-EL	KB-EL
35	120	0	0	28	0	0
36	112	0	0	272	0	0
37	251	49	0	375	0	11
38	54	0	0	345	0	0
39	431	1	0	735	0	0
40	73	8	1	0	0	0
41	45	2	4	788	20	0

<sup>1</sup> Treatments: Leaves from KB or EL were either kept in their region of origin (KB-KB and EL-EL) or moved to the other region (KB-EL and EL-KB).

**Table 4.** Sum of scab (fusi) infection risk indices (II) (Schwabe, 1980) per week in Koue Bokkeveld (KB) and Elgin (EL) in weeks 35 to 44 in 2013, and the number of ascospores trapped in treatments per week.

Week	Sum of II in KB	No. ascospores trapped		Sum of II in EL	No. ascospores trapped	
		KB-KB <sup>1</sup>	EL-KB		EL-EL	KB-EL
35	0	0	0	125	0	0
36	59	0	0	428	2	0
37	31	14	0	356	0	4
38	225	0	0	253	0	0
39	276	0	0	391	0	0
40	45	0	0	0	28	5
41	0	1	66	1487	10	0
42	118	0	0	419	5	2
43	365	0	0	314	2	0
44	368	0	0	730	0	0

<sup>1</sup> Treatments: Leaves from KB or EL were either kept in their region of origin (KB-KB and EL-EL) or moved to the other region (KB-EL and EL-KB).

## CHAPTER 4

### ***OVERWINTERING OF ASEXUAL SCAB INOCULUM IN SOUTH AFRICAN APPLE ORCHARDS***

#### **ABSTRACT**

Studies done in Europe and North America show that, in milder climates, *Venturia inaequalis* (apple scab) may overwinter in orchards in both the asexual (conidial and mycelial) and sexual (pseudothecial and ascospore) forms. The presence of viable asexual inoculum before ascospore discharge poses a threat to current chemical control measures, which focus on preventing infections by ascospores early in the season. Objectives of this study were to (1) determine the occurrence of overwintering of asexual inoculum of *V. inaequalis* in various apple production areas of the Western Cape in South Africa, and (2) clarify their role in inciting early spring infections. In July 2010, 2011 and 2012, thirty buds were randomly sampled from six orchards in Koue Bokkeveld, Witzenberg Valley and Elgin regions of the Western Cape for *V. inaequalis* DNA detection and/or quantification, and in August for conidial viability testing. Buds were dissected to separate inner and outer bud tissue, which were analyzed separately. In August 2011 and 2012, scabbed pygmy apples, if present, were also collected from each orchard and tested for conidial viability testing. High numbers of conidia per orchard were found in bud-washes, ranging from 0 to 677. However, viable conidia were only found in inner bud tissues of orchards with high scab levels in the previous season, with a range of 0 to 29% viability (average viability 4%). Conidial numbers on pygmy apples were high, and conidial viability ranged from 0 to 80% (average 29%). Molecular results using PCR-RFLP indicated the presence of *V. inaequalis* DNA in 90% of all bud-washes in 2010. Real-time (quantitative) polymerase chain reaction (qPCR) of bud-washes in 2011 and 2012 confirmed the presence of high amounts of *V. inaequalis* DNA in outer bud-washes and low amounts in inner bud-washes. However, number of conidia calculated per orchard using qPCR was significantly higher than results obtained with microscopy. The qPCR method was not a suitable method for this study and may not have been accurate, which may also explain the discrepancy in the results. Results suggest that orchards with high scab levels in the previous season have a higher risk of developing pygmy apples and harbouring viable conidia on lesions and on inner bud tissues. However, the higher number and higher percentage viability of conidia on pygmy apples suggest that scabbed pygmies are a more likely source of infection before ascospore discharge. This is the first report of viable conidia overwintering on pygmy apples.

## INTRODUCTION

Apple scab (*Venturia inaequalis* (Cooke) Winter) occurs in an asexual (conidial) form in summer and a sexual (ascosporic) form in winter (MacHardy, 1996). However, there have been reports of infection before ascospore release in spring in some apple production areas in South Africa. If viable asexual inoculum overwinters in apple orchards, they could cause infection earlier than expected, i.e. before ascospore release and fungicide sprays in spring (Holb *et al.*, 2004). Due to the polycyclic nature of scab during the season, these infections could develop into an epidemic if the correct measures are not applied (Sutton *et al.*, 1976). This could render protective fungicide applications useless and could greatly reduce the efficacy of curative fungicides, which could have major implications for the control of this disease during the season, for the cost of control for producers and for sales of scab fungicides.

In studies done overseas, conidia and mycelia were found to overwinter on shoots and on buds in orchards, and viable conidia remained protected over winter mainly under bud scales and inside buds in New York (Becker *et al.*, 1992) and in the Netherlands (Holb *et al.*, 2004, 2005). In South Africa, only one study has previously been conducted on this phenomenon and no viable overwintered conidia were found on the surfaces of twigs and bud-scales in the orchards tested in the Western Cape winter regions (Louw, 1951). Superficial mycelia were observed on apple twigs in the study by Louw (1951), although the author did not remain viable until the following spring. Inner bud tissues and other possible overwintering sites were not examined in the study by Louw (1951). However, Becker (1990), Holb *et al.* (2004) and Holb (2005) reported that conidial numbers on inner bud tissue were significantly lower than on outer bud tissue, or on shoot surfaces, but viability of conidia on inner bud tissue was higher and the risk posed by these viable conidia was significant only in orchards with high scab incidences (>40%) in the previous season. Conidia that overwinter on outer surfaces of apple buds and shoots are exposed to extreme and fluctuating relative humidity for long periods, which renders these exposed areas not suitable for conidia survival (Becker *et al.*, 1992; Becker and Burr, 1994). Therefore, conidia that may overwinter in inner bud tissue in apple orchards in South Africa would pose a higher risk of infection in the following season than conidia that overwinter on outer bud and shoot surfaces.

There have been reports of small, late season apples (called ‘pygmies’ by producers) that remain on trees throughout winter in most seasons in certain apple production regions of the Western Cape. In Elgin, which is considered a warm winter region (Midgley and Lötze, 2011), producers report that pygmies occur nearly every year, especially on cultivars such as ‘Braeburn’. Fresh, asexual scab lesions on pygmies were observed in apple orchards in Elgin and Witzenberg Valley in spring 2010 (personal observation, Fig. 1). However, Witzenberg Valley has not been

classified as a warm or a cold winter region in published reports. This phenomenon is possibly due to the milder winters and sometimes irregular and delayed flowering and fruiting which is common in some Western Cape areas (Cook, 2010), which does not occur as often in overseas apple production areas. No published studies have been found that report pygmy apples occurring in growing regions outside South Africa.

Objectives of this study were to (1) determine the occurrence of overwintering of asexual inoculum of *V. inaequalis* in the various apple production areas of the Western Cape in South Africa and (2) clarify their role in inciting early spring infections.

## **MATERIALS AND METHODS**

### **Orchards and apple scab incidence assessment**

Six orchards in three of the main apple growing regions of the Western Cape were selected for this trial, based on a history of scab incidence and cultivar susceptibility to scab (which influences scab incidence and may be linked to amount and/or viability of overwintered inoculum). Two orchards are located in each of the Koue Bokkeveld (orchards A and B), Witzenberg Valley (orchards C and D) and Elgin (orchards E and F). Orchard A is 'Fuji', orchard B is 'Early Red One' and orchards C to G are 'Braeburn'. Foliar scab incidence was assessed in autumn 2010, 2011 and 2012 using the disease assessment method described in Chapter 2, except that 25 trees per 2 ha were randomly selected for disease assessment.

### **Sample collection and dissection**

Thirty reproductive (flower) apple buds, distinguished from vegetative buds by their larger size and rounder shape, were randomly sampled in July from orchards A, B, D and E in 2010, orchards A to F in 2011 and orchards B to G in 2012, for DNA detection (2010) or quantification (2011 and 2012). Another 30 buds were sampled in August for conidial germination (viability) testing. In 2012, orchard G ('Braeburn') was sampled instead of orchard A ('Fuji') because 'Braeburn' is believed to be more susceptible and scab incidence in orchard G was higher than orchard A in autumn 2012. Buds were stored at 4°C until dissection, which was done using a modified method from Becker *et al.* (1992). Bud scales were examined using a stereo microscope for presence or absence of black mycelia and/or lesions, and were then dissected by removing outer scales and teasing inner tissue apart. Outer and inner bud tissues were placed in separate 2 ml Eppendorf tubes, each with 1 ml sterilized dH<sub>2</sub>O, labelled and separately examined. Eppendorf tubes were sonicated in an ultrasonic water bath (model UMC2, Ultrasonic manufacturing Company, Krugersdorp, South

Africa) for 10 min. Buds were not incubated for 1 hour at 20°C, as described by Becker *et al.* (1992), so that the amount of time conidia spent in the water at ambient temperature was reduced in order to prevent conidia from germinating before the bud processing was complete. The time it took for buds taken from storage to warm naturally in the lab while being dissected was enough to soften tissues for dissection and sonication. After sonication, bud tissue was removed with a sterilized tweezer and the supernatant in each tube was centrifuged at 13 000 rpm for 10 min in an Eppendorf centrifuge. The supernatant was removed carefully and the pellets were either stored at -20°C until DNA extraction or re-suspended by vortexing in 100 µL sterilized dH<sub>2</sub>O for viability testing. In August 2011 and 2012, thirty pygmy apples with visible lesions (if present in the orchard) were collected and prepared for germination testing on the day of sampling. Pygmy apples had been present in orchard F in 2011, but they had been removed before sampling - this is common practice on the farm where orchards E and F are located, as this producer suspects that these pygmies cause conidial infections on early leaf tissue in spring.

### **Microscopic conidial detection and germination testing**

Since molecular methods only provide information about the type and/or amount of DNA present in a sample, microscopy is required to determine what types of fungal tissue (reproductive or vegetative) are present in the sample and, if spores are present, whether or not they are viable. A method described by Becker *et al.* (1992) was modified and used for microscopic conidial detection and viability testing. In 2010, the full supernatant of 100 µl was placed on 1% water agar in Petri dishes and incubated at 100% RH for 24 to 48 hrs at 20°C. The conidia on each plate were morphologically identified and counted under a light microscope and the number of germinated conidia recorded. In 2011 and 2012, a different method was used for microscopic detection and germination testing of conidia: three droplets of 33 µl each (each representing a subsample of each 100 µl biological sample) of the suspension were pipetted onto a slide. The slides were incubated in a closed moisture chamber at 20°C at 100% RH. After 48 hrs, one drop of 100% lactic acid was added to the droplet and covered with a glass cover slip. This prevented evaporation of the bud-wash water and inhibited further microbial growth, since yeasts and other fungi overgrowth on slides obstructed conidial viewing. The conidia in each droplet were morphologically identified and counted under a light microscope and the number of germinated conidia recorded. Germination was only recorded if the germ tube was 1.5 times the length of the conidium. For germination testing of scab lesions on pygmy apples, each lesion on each apple was washed with 100 µl deionised, autoclaved water (dH<sub>2</sub>O) and the wash prepared for germination testing as described above. Percentage viability of 50 conidia per wash was determined.

## **Positive control, negative control and standard dilution series sample preparation for molecular detection**

For negative controls for PCR-RFLP and for qPCR, *Alternaria alternata* and *Cladosporium cladosporioides* were isolated on potato dextrose agar from apple orchards in the same regions as the orchards used for this study. Pure isolates were obtained by single-sporing and mycelia from these pure isolates was scraped and stored at -20°C until DNA extraction. For positive controls, sporulating scab lesions on apple leaves from a glasshouse inoculated with mixed isolates of *V. inaequalis* were agitated with dH<sub>2</sub>O in a 15 ml Falcon tube to dislodge conidia. The conidial suspension was spun down, the supernatant removed and the remaining pellet stored at -20°C until DNA extraction. Conidial counts were done with a Neubauer haemocytometer.

## **DNA extraction**

A standard Phenol-Chloroform extraction protocol described by Ma and Michailides (2007) was used for DNA extraction from all samples, but the extraction buffer was modified to include 2% polyvinylpyrrolidone (PVP, Sigma cat no. PVP40), 50 mM EDTA pH 8, 100 mM Tris-HCl pH 8, 0.5 M NaCl, 0.25% sodium dodecyl sulphate, 0.7% β-mercaptoethanol). A RNase A (Sigma cat. no. R4875, 0.6 U per sample) digest step for 30 min at 23°C and a proteinase K (Bioline cat. no. BIO-37037, final concentration at 50 µg ml<sup>-1</sup>) digest step for 60 min at 37°C were included before phenol-chloroform purification and ethanol precipitation. DNA pellets were resuspended in 20 µl dH<sub>2</sub>O and stored at -20°C. The repeatability of this extraction method was proven in a parallel study (Meitz-Hopkins *et al.*, unpublished data).

## **Molecular *V. inaequalis* DNA detection in 2010 bud-washes**

Bud-washes from 2010 were run through species specific PCR amplification, followed by restriction fragment length polymorphism (PCR-RFLP) analysis, to determine whether or not *V. inaequalis* DNA was present in the samples. *Venturia inaequalis* DNA was used as positive control and *A. alternata* and *C. cladosporioides* DNA were used as negative controls, the concentrations of which were quantified using a Nanodrop<sup>TM</sup> spectrophotometer and diluted to 5 ng µl<sup>-1</sup>, and dH<sub>2</sub>O was used as non-template control. Amplification of the internal transcribed spacer regions of fungal rRNA genes was conducted using forward primer ITS1 (3'-TCCGTAGGTGAACCTGCGG-5') and reverse primer ITS4 (5'-TCCTCCGCTTATTGATATGC-3') to amplify a 550 bp DNA fragment (Schnabel *et al.*, 1999; Le Cam *et al.*, 2001). The PCR was done using the optimized conditions for

the primers (10x buffer, 1.5 mM MgCl<sub>2</sub>, 0.2 mM deoxynucleoside triphosphates (dNTPs), 0.2 mM of each primer, 1.25 U Bioline *Taq* polymerase, 1 mg ml<sup>-1</sup> BSA) in a total volume of 40 µl. Amplifications were performed in an ABI2700 PCR machine (Applied Biosystems, Foster City, CA). The thermal cycle consisted of initial denaturation at 94°C for 2 min, followed by 30 reaction cycles of 94°C for 30 s, 54°C for 1 min, 72°C for 1 min, and a final extension of 72°C for 7 min. PCR products were stored at 4°C. PCR reaction products were analyzed by electrophoresis through a 1% agarose gel in 1X Tris-EDTA buffer (0.02 M Tris, 0.001 M EDTA, pH 8.0). Ethidium bromide-stained DNA fragments were visualized and photographed under an ultraviolet light.

To confirm presence of *V. inaequalis* in bud-wash DNA samples that had amplified during PCR, an ITS-Taq1 digest (10 x buffer, 10 µl PCR-RFLP product, 1 µl enzyme) in a total volume of 20 µl, was performed on these samples to produce two products of 277 bp and 220 bp in length. ITS-Taq1 digest products were analyzed by electrophoresis on a 3% agarose gel as above.

### **Quantification of *V. inaequalis* DNA in 2011 and 2012 bud-washes using qPCR**

*Venturia inaequalis* DNA present in the washes of the 2011 and 2012 bud tissue samples was quantified using real-time PCR, which was optimised using species-specific primers of the 14 $\alpha$ -demethylase (*CYP51A1*) gene and KAPA SYBR® FAST Universal Mastermix (Kapa Biosystems). Species-specific forward primer AJ250 (3'-GAGGCTACAACAGAT-3') used with reverse primer AJ244 (5'-TGAGAGCTTCGGTGGTGAGAC-3') (Schnabel and Jones, 2001) produces an amplicon of 135 bp in length. These primers were used instead of the ITS primers, because although they were less sensitive, they proved to be more specific, when tested in a parallel study (Meitz-Hopkins *et al.*, unpublished data). Using the recommended thermal cycling protocol provided by the manufacturers for the enzyme mix used, a thermal gradient from 52°C to 64°C was used to determine the optimal annealing temperature. Optimal concentrations for both forward and reverse primers were also optimized in a gradient matrix from 50 nM to 900 nM. The annealing temperature and primer concentrations at which the highest sensitivity and specificity was obtained, based also on melt curve analysis, was then used. Specificity of primers was again tested against *A. alternata*. Each reaction contained 1x KAPA SYBR® FAST, 500 nM of each primer and 2 µl of template DNA in a total reaction volume of 20 µl. Each DNA sample was analyzed in triplicate. Runs were performed using clear 96-well plates (Lasec) and in a Bio-Rad Real-Time PCR System machine (Bio-Rad Laboratories), using CFX 96 software. The thermal cycle consisted of initial denaturation at 95°C for 10 min, followed by 40 reaction cycles of 95°C for 10 s, 56 °C for 10 s, and 72°C for 15 s and a melt from 72-95 °C with 0.5 °C increments.

To determine the detection limit of the qPCR method, 10-fold dilution series of genomic *V. inaequalis* DNA extracted from conidia ranging from 10 to  $1.69 \times 10^{-6}$  ng  $\mu\text{L}^{-1}$  (quantified using the Qubit 2.0 fluorometer) was subjected to qPCR analysis. Standard curves based on quantification cycles ( $C_q$ ) were constructed using an 8-fold dilution series of *V. inaequalis* DNA. The double-stranded DNA (dsDNA) concentration of the first standard dilution was quantified using the Qubit 2.0 fluorometer (Invitrogen, LifeTechnologies) using the fluorescent-based High-Sensitivity dsDNA Assay; DNA was quantified in a final volume of 200  $\mu\text{L}$  and the HS-DNA module and the protocol was followed according to the manufacturer. A 2  $\mu\text{L}$  aliquot of each standard dilution in triplicate was used in qPCR reactions. After amplification, a standard curve was automatically generated by the CFX 96 software. Criteria for acceptable qPCR runs are  $M = -3.6$  to  $-3.1$  for slope and  $R^2 > 0.98$  for linearity. The  $C_q$  values of amplified products were plotted against the logarithm of DNA concentration to determine  $S_q$  (DNA concentration of samples in ng  $\mu\text{L}^{-1}$ ) and melt analysis was used to verify *V. inaequalis* template identity.

Too few of the bud-wash DNA samples fell within the detection range and then only at the lower end of the detection range. For each orchard's samples, the remaining volume of the bud-wash samples was recorded and ten bud-washes were then pooled, keeping washes of inner and outer tissues separate, so that each orchard had three biological replicates for each of the inner and outer tissues. In addition, the qPCR method was changed to be used with Bio-Rad iTaq SYBR® Green Supermix (Bio-Rad Laboratories), which was more sensitive than KAPA SYBR® FAST when compared following the respective manufacturer's guidelines for reaction conditions on two known concentrations of *V. inaequalis* DNA. The reaction conditions were optimized to use Bio-Rad SYBR®Green, and the following changes were made to the cycling conditions: initial denaturation at 95°C for 5 min, instead of 10 min, and extension at 56°C for 15s, instead of 10s.

Repeatability (intra-assay variance) and reproducibility (inter-assay variance) of the qPCR method was tested as follows: three samples of known concentration into three subsamples (quantified using the Qubit 2.0 fluorometer), to test accuracy of pipetting aliquots of a DNA solution, and running each subsample in triplicate in the same qPCR run (intra-assay variance). This was then repeated to test inter-assay variance. Results were analyzed to determine whether the  $\Delta C_q$  between the measured and calculated  $C_q$  of the undiluted sample was  $<0.5$  (Boutigny *et al.*, 2011). The amount of DNA measured during qPCR was divided by the calculated DNA amount per spore ( $3.2 \times 10^{-2}$  pg) from a parallel ongoing study (Meitz-Hopkins *et al.*, unpublished data) to calculate the conidial number per pooled sample. Total conidial numbers per orchard determined using qPCR were compared with microscopically analysed samples in two ways: in one method, those samples which did not have enough *V. inaequalis* DNA to amplify within the reliable detection range using

qPCR were assigned 0 conidia, while in the second method, the same samples were assigned the number of spores calculated for the lower detection limit of the qPCR method.

## Statistical analysis

A variance components analysis was done on the  $C_q$  and  $S_q$  of samples for qPCR repeatability (intra-assay) and reproducibility (inter-assay) respectively, to determine the breakdown of total variance in data into sources of variance, namely biological samples, subsamples, independent qPCR runs and technical replicates (error). A two-way mixed model repeated measures analysis of variance (ANOVA), with region and year as fixed effects and orchard as random effect, was used to compare total number of conidia determined using qPCR and total number of conidia determined using microscopy between regions, years and tissue types, respectively. Fisher's Least Significance Difference (LSD) t-test was used for post-hoc testing. A Spearman correlation was calculated to determine the relationships between (i) total number of conidia per orchard determined using microscopy and total number of conidia per orchard determined using qPCR, (ii) total number of conidia per orchard determined using qPCR and percentage viability of total conidia per orchard, and (iii) total number of conidia per orchard determined or microscopy and percentage viability of total conidia per orchard. All statistical analyses were done using STATISTICA 11 data analysis software system (StatSoft, Inc. 2012, [www.statsoft.com](http://www.statsoft.com)) and significant differences were all evaluated on a 5% significance level ( $P < 0.05$ ).

## RESULTS

### Microscopic conidial detection, germination testing and DNA detection in 2010

An average of 3 conidia per orchard, none of which were viable, was found in washes of inner bud tissues in 2010, using microscopic methods. Washes from outer bud tissues could not be analysed due to problems associated with visualization of washes under the microscope. Qualitative molecular detection of bud-washes in 2010 indicated that *V. inaequalis* DNA was detected in 90% of all inner bud-washes.

Number of conidia per orchard found in bud-washes ranged between 0 and 677 in 2011 and 0 and 35 in 2012. However, only conidia in inner bud tissues were viable, with a range of 0 to 29% (average 6.7%) in 2011 and 0 to 12% (average 2%) viability in 2012, with the exception of 1 germinated conidium in outer bud tissue in orchard B in 2011 (Table 1). Total number of conidia per orchard did not differ significantly between inner and outer bud tissue ( $P = 0.08$ ) or between

years ( $P = 0.07$ ). However, trends were significant at 10% ( $P < 0.1$ ), for total number of conidia per orchard being less in washes of inner bud tissues vs. outer bud tissues and in bud-washes in 2012 vs. 2011. There was a very weak positive correlation between total conidial number per orchard and mean percent viability of conidia per orchard ( $r_s = 0.23$ ).

Number of conidia per wash of scabbed pygmies was too high to be counted. Mean viability of conidia from washes of scab lesions on pygmy apples was 10, 11 and 30% in orchards A, B and E in 2011 and 28 and 27% in orchards E and F in 2012. Viability of conidia in ranged from 0 to 80%, with an average of 30% in 2011 and 27.5% in 2012.

### **Quantification of *V. inaequalis* DNA in 2011 and 2012 bud-washes using qPCR**

The qPCR method proved to be reproducible and repeatable: triplicate qPCR on the same sample within a single run showed a low variation in  $C_q$  values (mean SD = 0.01,  $n = 81$ ) (intra-assay variance), and low variation was observed between  $S_q$  of independent runs (mean SD = 0.23,  $n = 3$ ) (inter-assay variance). Non-template and negative controls did not amplify or amplified at  $C_q \geq 38$ . Melt analysis showed that amplified products of *V. inaequalis* (positive control) and *A. alternata* (negative control) DNA had differential melt peaks at  $80 \pm 0.5$  and  $86 \pm 0.5^\circ\text{C}$  respectively. *Venturia inaequalis* DNA was detected reliably within a range of  $1 \times 10^{-3}$  to 3.33 ng per reaction, with the lower detection limit at  $C_q = 30 \pm 0.1$  (equivalent to 54 spores) using Bio-Rad SYBR® Green. Slope (M) and  $R^2$  (linearity) values were acceptable for all runs (Fig. 3). *Venturia inaequalis* DNA was detected reliably in the washes of outer bud tissue from all orchards in 2011 and from orchard C in 2012, and in washes of inner bud tissues from orchard A in 2011 and orchard B in 2011 and 2012 (Table 1), with SD < 0.1 in  $S_q$  for each these samples.

There was no difference in statistical analysis results when those samples that did not contain enough *V. inaequalis* DNA to amplify within the reliable detection range were assigned 0 spores were compared to using the lower detection limit of 54 spores. For both data sets, there was a non-significant interaction: region x bud tissue type for the total number of conidia per orchard determined using both microscopy ( $P = 0.76$ ) and qPCR ( $P = 0.62$ ) and total number of conidia per orchard did not differ between regions or years for the microscopic method ( $P = 0.44$  and  $P = 0.07$ , respectively) or for the qPCR method ( $P = 0.47$  and  $P = 0.2$ , respectively). Total number of conidia in inner and outer bud tissue differed significantly from each other when determined using the qPCR method ( $P = 0.02$ ), and showed a trend in higher counts of conidia in outer bud tissues than inner bud tissues when using the microscopic method ( $P = 0.08$ ). The only difference between the data sets was the total number of conidia per orchard detected in inner and outer bud tissue, *i.e.* for below-detection samples assigned 0 spores, total conidia in inner and outer tissues were 6104 and

52010, respectively, whereas for below-detection samples (assigned 54 spores), total conidia in inner and outer tissues were 6590 and 52230, respectively. Total conidia per orchard in inner and outer tissues using microscopy were 18 and 139, respectively.

For both data sets, there was a strong positive correlation between the microscopic and qPCR method for determining total number of conidia per orchard ( $r_s = 0.88$ ), but a very weak positive correlation between total conidial number per orchard determined using qPCR respectively, and mean percent viability of conidia per orchard ( $r_s = 0.18$ ).

## DISCUSSION

The overwintering of asexual inoculum in South African orchards could render ineffective control strategies against primary scab infections that currently rely exclusively on fungicides against ascospores. This study provided evidence that two potential sources of overwintered asexual inoculum exist in South African orchards, *i.e.* viable conidia on inner bud tissues and pygmy apples.

No scab lesions were found on bud tissues, which corresponds with what Holb *et al.* (2004) found. Low numbers of conidia were found in 2010, but higher numbers were found in washes of bud tissues in 2011 and 2012, using microscopy, despite bud-washes not being filtered. This is likely due to the change in preparation of samples for conidial counts and germination testing. In 2010, bud-washes were incubated on 1% water agar. This was different to the method described by Becker *et al.* (1992), but was done to minimize movement in the bud-washes during incubation and possible disruption of conidial germination tubes. However, the time required to scan each Petri dish microscopically was longer than expected and fungal and yeast growth may have interfered with visualizing and counting conidia. In 2011 and 2012, the method more similar to Becker *et al.* (1992) was used and bud-wash droplets were incubated on glass slides. This made microscopic examination over a smaller area easier and the time required per slide was shortened, and fewer other organisms, such as other fungi and yeasts, grew in the shortened time period that could interfere with visualizing and counting conidia. However, germination tubes of conidia may have still been disrupted when a glass slide was placed on the bud-wash droplet, and non-germinated conidia may still have moved to the edge of the glass slide, which may explain the low numbers and viability of conidia found in some orchards.

Numbers of conidia that overwinter varies greatly between buds within an orchard and between seasons (Becker, 1990). Lower numbers of conidia were found in bud-washes in 2012 that coincided with the scab incidence on shoots in autumn in 2012 being generally lower than in 2011, which may explain the lower conidial numbers found in 2012 compared to 2011. Fewer conidia

present in an orchard during late summer means that relatively fewer conidia may be trapped between bud scales and remain there throughout winter (Holb *et al.*, 2005).

Only one conidium out of 1605 conidia found in outer bud tissues in all orchards in 2011 was viable. This is in agreement with Becker *et al.* (1992) and with Louw (1951), who reported that small numbers of conidia were found on bud and shoots taken from orchards that had high scab levels in the previous season in the Western Cape, and that none germinated. Viable conidia in inner bud tissues were generally found in orchards where shoot scab incidence in the previous season was exceptionally high, which is similar to previous studies (Holb, 2004; Becker, 1990, 1992), although one orchard with viable conidia only had 12% autumn scab incidence in 2012. The total conidial number per orchard was very weakly correlated with mean percent viability of conidia per orchard, but it suggests that as number of conidia that overwinter inside buds increases, the proportion of viable conidia also increases. Percentage viability of conidia in 2011 (0 to 29%) was higher, but similar in 2012 (0 to 12%) to the percentage viability of conidia in inner bud tissues reported by Holb *et al.* (2004) and Holb *et al.* (2005) (3.7 to 10.5% and 0 to 11%, respectively), and both studies concluded that conidia harboured in inner bud tissues were more likely responsible for the scab infections during tight cluster stage in the following season. The orchards which contained high conidial numbers and high numbers of viable conidia were used in a parallel study and did not receive the full spray programme early in the previous season, which contributed to the high scab levels in those orchards later in the season. The number of conidia that survive winter increases exponentially if the incidence of scab was higher than 40% in the previous autumn (Holb *et al.*, 2005).

The problems encountered with outer bud-washes in 2010 were likely due to the method used to prepare bud-washes for microscopic analysis: bud-washes were not filtered through Myra cloth, as described by Becker (1990), as it was believed that conidia may have been trapped with other material and number of conidia per bud-wash affected. The surface area of the 1% water agar in Petri dishes was very large and scanning across this area under a microscope took longer than expected. Yeast cells multiplied and other fungal species' spores germinated and grew hyphae so quickly on the agar within 24 hrs that any conidia present were not easily visible. Consequently, few conidia were counted. The method used by Becker *et al.* (1992) to wash conidia from buds proved to be effective, as numbers of conidia found in this study (up to 677 conidia per 30 buds) were comparable with numbers reported by Louw (1951) (small numbers, exact number not reported) and Holb *et al.* (2004) (up to 5000 conidia per 100 buds, or 1500 per 30 buds calculated), depending on scab incidence of orchards in the previous season, e.g. Holb *et al.* (2004) sampled organic and integrated orchards, which had autumn scab incidences of >90%, so higher conidial numbers were expected. It is possible that bud tissues with very low numbers of conidia did not

provide measurable amounts of conidia for microscopic detection. This could explain the large difference between the microscopic and molecular method used in 2010 to determine number of bud-washes that contained conidia.

There are other problems associated with microscopic detection of asexual inoculum in bud-washes. Non-viable or desiccated conidia are difficult to identify with certainty, as their shape and colour change. When determining the percentage of germinated conidia, it is possible that pipetting the concentrated bud-wash onto a glass slide for incubation may have broken off germination tubes from conidia. Alternatively, when a glass slide is placed on top of the droplet, the movement may have detached the conidia from the germination tubes that had adhered to the glass slide. This was observed with *Alternaria* spores retrieved in the same study and *Alternaria* germination tubes are larger and sturdier than those of *V. inaequalis*. If the total volume of water plus lactic acid is greater than the volume of liquid that is able to fit under a glass slide, the mixture overflows. Streaming in this film of liquid causes material from the bud-washes to dislodge, also potentially damaging germination tubes. It is also difficult to view material that is in this film, which may have affected numbers of conidia counted if they streamed into this film and if the number of conidia in the bud-washes is already low.

Viability of conidia from washes of scabbed pygmy apples was very high, with an average of 29% of all conidia tested over 2011 and 2012. Pygmy apples were only seen in orchards with a high incidence in the previous season in 2011. Stress from high inoculum could contribute to late flowering and, therefore, to the presence of late apples. Pygmies were only seen in orchards E and F in 2012, but scab incidence in the previous season was not high. However, according to the production manager of these orchards, 'Braeburn' has a tendency to form pygmies, especially in the Elgin region, and the producer had noticed that by removing these pygmies before green-tip the following season, scab incidences were lower than in seasons where pygmies were not removed (Ernst Heydenrych, personal communication). However, pygmies are not removed from orchards, but are left on the ground. If green-tip occurs with a few days after scabbed pygmies are removed, it is possible that conidia from scab lesions on pygmies transported by the air currents (that also transport ascospores from leaf litter (Aylor, 1998) to susceptible plant tissue could pose a higher risk of infection, rather than viable conidia that had overwintered inside buds, due to their higher viability. This is because conidia can survive fluctuating relative humidity for up to 96 hrs (Becker and Burr, 1994). Therefore, it is recommended that pygmy apples be removed from orchards and not just from trees. The high number of conidia and high percentage of viability of conidia on scabbed pygmy apples suggest that these conidia are more likely responsible for the reports of infections before ascospore discharge, than conidia that overwinter in or on buds (Fig. 2). However, pygmy apples have been observed in apple orchards as late as October in Elgin and Witzenberg

Valley, during bloom time when far more susceptible plant issue is available, which suggests that, even if ascospore discharge is low during that time or during that season, conidia from pygmies still provides sufficient inoculum for an epidemic if fungicide sprays are not able to protect susceptible tissues from infection.

The lower detection limit (54 to  $10 \times 10^5$  conidia) for the qPCR method was similar to the range reported by van Wyk (2011) for quantification of airborne *Fusarium circinatum* spores from filter paper using qPCR ( $272$  to  $2.72 \times 10^7$  spores). However, the detection range in this study was narrower and the lower detection limit was five times more sensitive than the lower detection limit reported by van Wyk (2011). This could be due to the difference in fungal genus and species, DNA extraction method used and qPCR method used.

The strong positive relationship between the microscopic and qPCR method for determining total number of conidia per orchard indicates that both methods can quantify relatively low or high numbers of conidia in bud-wash samples. No superficial black mycelia or lesions were observed on outer or inner bud surfaces during dissection in 2010, 2011 or 2012, but it is possible that thin mycelia strands present were not visible during microscopic investigation before tissue was washed, since hairs on bud tissue may have obscured the visibility of their presence. However, short mycelia strands seen in the bud-washes may have been mistaken for, or may not have been distinguished from, early mycelia growth that had germinated from other fungal spores, and were not noted. Mycelia are also multi-cellular, and since calculation of conidial numbers during molecular quantification of *V. inaequalis* DNA in bud-washes yielded unexpectedly high results, this may explain the high DNA yield quantified in samples. Louw (1951) conducted several experiments to determine presence and viability of asexual inoculum on apple trees in winter and observed mycelia on shoot and bud surfaces in various experiments, but in none of the experiments did mycelia that were found remain viable until the following spring. The author concluded that mycelia do not remain viable until spring on bud surfaces and that scab infections in spring in apple orchards in the winter rainfall region of the Western Cape were caused by ascospores and not by conidia. The weak positive correlation between the total number of conidia per orchard determined using qPCR (higher than when microscopy was used) and mean percent viability of conidia per orchard could also be explained by the presence of non-visible mycelia in bud-washes processed for qPCR in July.

In retrospect, the qPCR method used was not suitable for this experiment, partly because the method could not distinguish between viable and non-viable inoculum, and could not distinguish between conidia and mycelia, and so microscopically obtained results could not be confirmed. Developing a quantification method where viable and non-viable conidia could be distinguished between and viable conidia measured, would have been more appropriate, such as the method developed by Bentsink *et al.* (2002) or by Wang and Levin (2006).

Pygmy apples seem to be a relatively new phenomenon, as there are no published reports to date. This is possibly due to the advent of different cultivars being planted and climate change causing winters in some regions to become warmer over the last six decades (Midgley and Lötze, 2011). The warming trend over the past 40 years in the winter-rainfall regions of the Western Cape is predicted to continue and could result in winters becoming drier and warmer, with spring and summer months experiencing more rain (Cook, 2010; Midgley and Lötze, 2011). To date, this has led to the altered growth response and delayed defoliation of trees in Elgin due to increasingly insufficient chilling units (Cook, 2010). This could mean that more pygmies may form and overwinter in future. The results of this study suggest that conidia on scabbed pygmy apples may significantly increase the risk of scab infection before ascospores begin to mature. Holb *et al.* (2005) showed that the threat of viable asexual inoculum only becomes significant in orchards where the scab incidence in the previous season was high (>40%). However, airborne inoculum in an orchard can still be high even if scab lesion incidence in orchards is low due to successful preventative spray programmes. Therefore, airborne conidial load in summer may still be high, which increases the risk of conidia becoming trapped in apple buds or infecting pygmy apples late in the season, and increases the number of conidia (and viable conidia) that overwinter. This justifies the use of integrated management techniques in winter to reduce the ascospore inoculum in spring and, consequently, throughout the rest of the season (Holb *et al.*, 2004). Additionally, conventional apple production practices do not include applying scab fungicides to leaves after harvest, which may further contribute to the build-up of inoculum in orchards and on pygmies, which further increases the risk of infection before ascospore discharge in the following season.

## CONCLUSION

Conidia do overwinter on and in apple buds in the Western Cape region of South Africa, but viable conidia were found only in orchards with a high scab incidence in the previous season. However, few conidia remained viable until August in the following season, approximately one month before bud-break, and effectively all viable conidia overwintered on inner bud tissue surfaces. These results agree with previous studies. Molecular quantification of *V. inaequalis* DNA in bud-washes did not agree with results obtained by microscopy, as numbers of conidia determined using qPCR were higher. This may be due to the presence of mycelia that may not have been visible on buds during dissection or in bud-washes, or the qPCR method not yielding accurate results. Previous studies have proven that mycelia found on buds do not remain viable until the following spring, and it is unlikely that mycelia pose a risk of infection before ascospore release in the following season. High numbers of viable conidia were found on scabbed pygmy apples, and they represent an

inoculum source in the orchard before and after release of ascospores. These conidia may be more likely responsible for scab infection before ascospore discharge than conidia overwintering in and/or on buds. It is recommended that pygmies, which may provide an inoculum source even if they were removed from trees but left on the ground, be removed from orchards. It is also recommended that sexual inoculum be reduced in orchards with high scab incidences in the previous season and that a recommended fungicide programme be followed early in the season so that asexual inoculum does not build up later in the season and overwinter until the following season.

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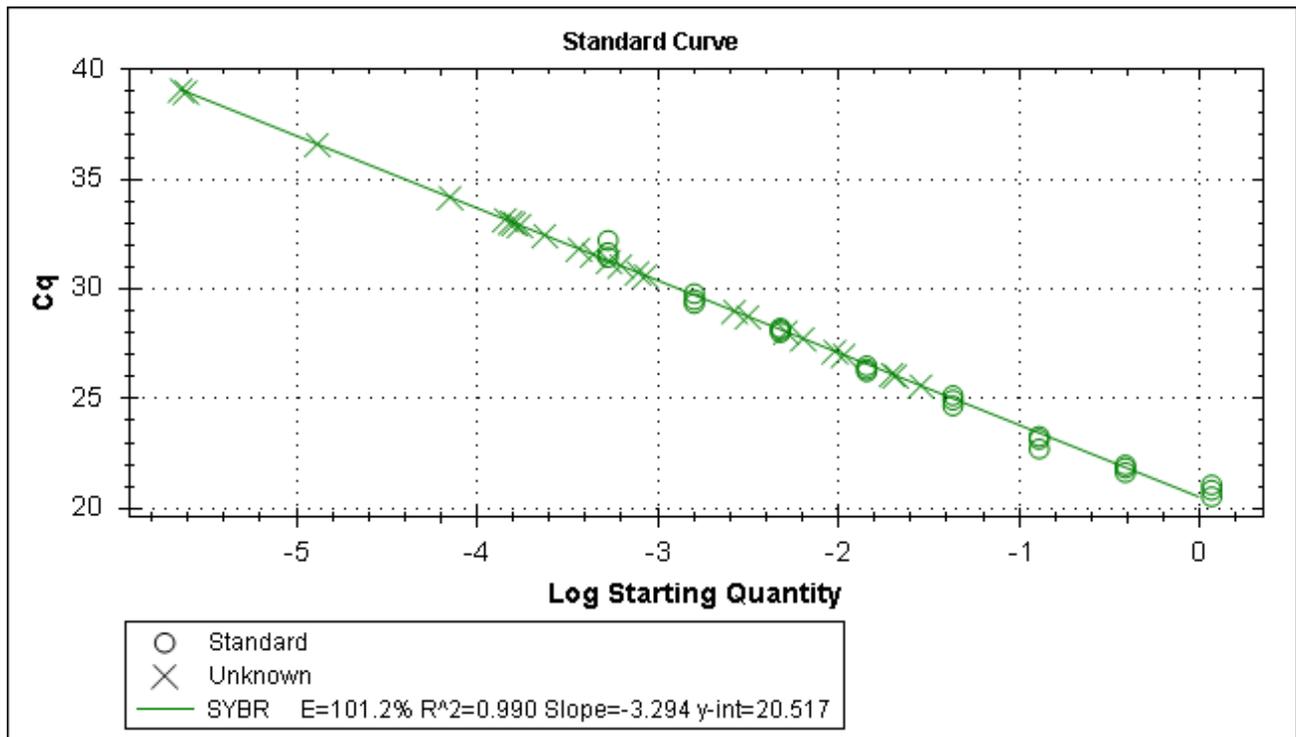
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**Figure 1.** Scabbed pygmy apples in orchard C in October 2010. Note the proximity of sporulating lesions to susceptible host tissue.



**Figure 2.** Scabbed pygmy apples in orchard E in July 2012.



**Figure 3.** Amplified qPCR products of DNA samples of inner and outer bud-washes of orchard E, in 2011 and 2012, plotted on the standard curve. Slope (M) values are within the acceptable ranges of -3.6 to -3.1 (optimal -3.32) and linearity (R<sup>2</sup>) is >98%. The reliable lower detection limit is at C<sub>q</sub> = 30 ± 0.1.

**Table 1.** Total number of conidia on inner and outer bud tissues per orchard in six orchards over 2011 and 2012, quantified using microscopy and qPCR, and total number of viable conidia in bud-washes and on pygmy apples, compared with leaf scab incidence near the end of the previous season.

Region	Orchard	Year	Full scab spray programme applied	Autumn leaf scab incidence (%)	Bud tissue type	Total conidial number per 30 buds per orchard		Total number viable conidia in bud-washes (microscopic) in August	Mean % viability of conidia in bud-washes	Mean % viability of conidia on pygmies in August
						Molecular	Microscopic			
Koue Bokkeveld	A	2011	No	50	inner	≤ 54*	5	11	6	10
	A	2011	No	50	outer	19164	177	0	0	10
	G	2012	Yes	11.6	inner	≤ 54	0	0	0	no pygmies
	G	2012	Yes	11.6	outer	≤ 54	0	0	0	no pygmies
	B	2011	No	49	inner	73253	154	45	29	11
	B	2011	No	49	outer	412805	677	1	0	11
	B	2012	No	12	inner	≤ 54	33	4	12	no pygmies
	B	2012	No	12	outer	≤ 54	34	0	0	no pygmies
Witzenberg Valley	C	2011	Yes	0.1	inner	≤ 54	3	0	0	no pygmies
	C	2011	Yes	0.1	outer	43239	315	0	0	no pygmies
	C	2012	Yes	1	inner	≤ 54	0	0	0	no pygmies
	C	2012	Yes	1	outer	18466	0	0	0	no pygmies
	D	2011	Yes	0.3	inner	≤ 54	0	0	0	no pygmies
	D	2011	Yes	0.3	outer	68118	4	0	0	no pygmies
	D	2012	Yes	2	inner	≤ 54	0	0	0	no pygmies
	D	2012	Yes	2	outer	≤ 54	0	0	0	no pygmies
Elgin	E	2011	No	38	inner	≤ 54	17	12	5.2	30
	E	2011	No	38	outer	47107	215	0	0	30
	E	2012	Yes	2.6	inner	≤ 54	0	0	0	28
	E	2012	Yes	2.6	outer	≤ 54	10	0	0	28
	F	2011	Yes	2	inner	≤ 54	0	1	0	no pygmies collected
	F	2011	Yes	2	outer	15222	217	0	0	no pygmies collected
	F	2012	Yes	0.9	inner	≤ 54	0	0	0	27
	F	2012	Yes	0.9	outer	≤ 54	15	0	0	27

\* For samples with too little *V. inaequalis* DNA to be amplified within the reliable detection range, total conidial number per orchard is labelled as “≤ 54”.