Mancozeb rainfastness and residue thresholds for control of *Venturia inaequalis*

by

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SUMMARY

An important apple disease world-wide is *Venturia inaequalis* that causes scab-like lesions on fruit and defoliation of trees. In South Africa, the disease is mainly managed through fungicide sprays that mostly consist of the contact fungicide mancozeb. The volume and intensity of rainfall can influence the efficacy and persistence of mancozeb depositions. Mancozeb spray deposition can be assessed through expensive quantification of Manganese-ion residues, or a cost effective fluorescent pigment of which the coverage is accessed using photomacrography image analyses. The biological efficacy of quantitative mancozeb depositions can be determined through the development of benchmark models. For *V. inaequalis,* this is challenging since *in vitro* production of inoculum is difficult, and leaf lesions are slow to develop and not amendable to quantification through image analyses.

The study showed that a yellow fluorescent pigment was a suitable tracer for five different mancozeb formulations (Dithane M-45 800 WP NT, Mancozeb 800 WP, Pennfluid, Ventum 800 WP, Vondozeb) since a good Pearson's correlation (r = 0.779) existed between fluorescent particle coverage (FPC%) and mancozeb residues (Mn-ion (mg/kg_{DW})).The particle size ranges of Dithane M-45 800 WP NT and Ventum 800 WP were significantly smaller than those off the other formulations, but this did not result in differences in Mn-ion residues realized on apple leaves for WP formulations. The Pennfluid SC formulation deposited markedly less mancozeb than the WP formulations, due to a lower active ingredient label rate used. Rainfastness was evaluated for the Dithane M-45 800 WP NT and Ventum 800 WP formulations, and Ventum 800 WP combined with a sticker-spreader adjuvant Nu-Film P. Simulated rain applied to apple seedlings at a constant rainfall intensity of 5 mm/h at five different rainfall volumes (0, 1, 5, 10 and 15 mm) resulted in no significant differences between the three treatments, based on FPC% and Mn-ion concentrations. Although a good correlation (r = 0.726 to 0.783) existed between FPC% and Mn-ions, the response of FPC% and Mn-ion differed somewhat as was evident from the slopes of exponential regression models, showing slower initial loss in FPC% than for Mn-ions and a markedly larger predicted loss by the model's asymptotic value (61.4% and 32.2%, respectively). A significant loss (11.95%) in mancozeb residue occurred after applying 1mm of rain, but no significant differences in losses (26.32 to 31.67%) occurred after applying 5 to 15mm of rain.

Benchmark development for mancozeb deposition for the control of *V. inaequalis* involved apple seedling leaves being treated with a concentration range of mancozeb and fluorescent pigment (0, 0.15 ×, 0.3 ×, 0.45 ×, 0.6 × and 1.0 ×), followed by inoculation with conidia harvested from naturally infected orchard leaves. *Venturia inaequalis* control was

assessed using a basic fuschin based staining technique and visual assessment. The staining technique was useful for quantifying infection within 6 days, but underestimated percentage control relative to the visual assessment of lesions after 3-4 weeks. Complete control was observed for all mancozeb concentrations based on visual lesion assessment. No function could thus be fitted to deposition quantity data (0.29 to 8.28 FPC% values) versus disease control (staining or visual assessment). The cellophane agar plate technique was optimized for the *in vitro* production of *V. inaequalis* conidium inoculum that can be used in future infection studies. After 1 week, optimum spore production (1.59×10^6 conidia/ml) and viability (± 85%) were observed that were significantly higher than at weeks 2 to 4.

The study provided valuable information for the assessment of mancozeb deposition, mancozeb rainfastness, and for benchmark model development with regards to the rapid quantification of lesions on leaves and the *in vitro* production of *V. inaequalis* inoculum. The yellow fluorescent pigment can now be used as an excellent cost effective tracer for mancozeb deposition of various formulations on apple seedling leaves, and will also be helpful in identifying trends on the effect of rain on the persistence of mancozeb formulations. This has increased research capacity towards evaluating the rainfastness of fungicides using simulated rain, and orchard trials for accessing the effect of spray volumes and machines through fluorescent pigment deposition. Although a benchmark model could not be developed, the development of a model in future will be more feasible since a rapid staining technique for quantification of *V. inaequalis* disease severity was identified that will just have to be optimized further. Secondly, a cellophane agar plate technique, and isolates with high spore production capacity for axenic conidial production will further facilitate model development.

OPSOMMING

Venturia inaequalis is ‘n belangrike appelsiekte wêreldwyd, wat skurfagtige letsels op vrugte en die afval van blare veroorsaak. In Suid-Afrika word die siekte hoofsaaklik deur die spuit van fungisiedes bestuur, wat grootliks uit die kontakfungisied, mankozeb, bestaan. Die volume en intensiteit van reënval kan die doeltreffendheid en werkingsduur van mankozeb deponerings beïnvloed. Mankozeb spuitdeponerings kan deur duur kwantifisering van mangaan-ioon residue bepaal word, of deur ‘n koste-effektiewe fluoresserende pigment waarvan die bedekking bepaal word deur die gebruik van fotomakrografie beeld-analise. Die biologiese doeltreffendheid van kwantitatiewe mankozebdeponerings kan bepaal word deur die ontwikkeling van basisvlak modelle. Vir *V. inaequalis* is dit ‘n uitdaging aangesien *in vitro* produksie van inokulum moeilik is, en blaarletsels stadig ontwikkel, en nie geskik is vir kwantifisering deur beeld-analise nie.

Die studie het getoon dat ‘n geel fluoresserende pigment ‘n geskikte aanwyser van vyf verskillende mankozeb formulasies was (Dithane M-45 800 WP NT, Mancozeb 800 WP, Pennfluid, Ventum 800 WP, Vondozeb), aangesien ‘n goeie Pearson korrelasie (r = 0.779) tussen fluoresserende partikel bedekking (FPC%) en mankozeb residue (Mn-ioon (mg/kg_{DW})) bestaan het. Die partikel grootte reeks van Dithane M-45 800 WP NT en Ventum 800 WP was betekenisvol kleiner as dié van die ander formulasies, maar dit het nie tot verskille in Mn-ioon residue, soos verkry op appelblare vir WP formulasies, gelei nie. Die Pennfluid SC formulasie het merkbaar minder mankozeb as die WP formulasies gedeponeer, weens ‘n laer aktiewe bestanddeel etiket toedienings dosis wat gebruik is. Reënvastheid is vir die Dithane M-45 800 WP NT en Ventum 800 WP formulasies, en Ventum 800 WP, gekombineer met ‘n kleefmiddel-verspreider adjuvant, Nu-Film P, geëvalueer. Gesimuleerde reën wat tot appelsaailinge teen ‘n konstante reënval-intensiteit van 5 mm/h by vyf verskillende reënvalvolumes (0, 1, 5, 10 en 15 mm) toegedien is, het tot geen betekenisvolle verskille tussen die drie behandelings gelei nie, gebaseer op FPC% en Mn-ioon konsentrasies. Hoewel ‘n goeie korrelasie (r = 0.726 tot 0.783) tussen FPC% en Mn-ione bestaan het, het die reaksie van FPC% en Mn-ioon ietwat verskil, soos wat duidelik was vanuit die kurwes van eksponensiële regressie modelle, wat stadiger aanvanklike verlies in FPC% as vir Mn-ione getoon het, en ‘n merkbaar groter voorspelbare verlies deur die model se asimptotiese waarde (onderskeidelik 61.4% en 32.2%). ‘n Betekenisvolle verlies (11.95%) in mankozeb residue het ná die toedien van 1mm reën voorgekom, maar geen betekenisvolle verskille in verliese (26.32 tot 31.67%) het voorgekom ná die toedien van 5 tot 15mm reën nie.

Basisvlak ontwikkeling vir mankozeb deponering vir die beheer van *V. inaequalis* het behels dat appelsaailingblare met ‘n konsentrasiereeks van mankozeb en fluoresserende pigment (0, 0.15 ×, 0.3 ×, 0.45 ×, 0.6 × and 1.0 ×) behandel is, gevolg deur inokulasie met

konidia wat vanaf natuurlik geïnfekteerde boordblare geoes is. *Venturia inaequalis* beheer is bepaal deur die gebruik van ‘n basiese ‘fuschin’-gebaseerde kleurtegniek en visuele bepaling. Die kleurtegniek was bruikbaar vir die kwantifisering van infeksie binne 6 dae, maar het die persentasie beheer relatief tot die visuele bepaling van letsels ná 3-4 weke onderskat. Volledige beheer is vir alle mankozeb konsentrasies waargeneem, gebaseer op visuele letselbepaling. Geen funksie kon dus gepas word op deponering kwantiteit data (0.29 tot 8.28 FPC% waardes) teenoor siektebeheer (kleuring of visuele bepaling) nie. Die ‘cellophane’ agarplaattegniek is geoptimaliseer vir die *in vitro* produksie van *V. inaequalis* konidium-inokulum wat in toekomstige infeksie-studies gebruik kan word. Ná 1 week, is optimum spoorproduksie (1.59×10^6 konidia/ml) en lewensvatbaarheid (± 85%) waargeneem, wat betekenisvol hoër was as by weke 2 tot 4.

Die studie het waardevolle inligting verskaf vir die bepaling van mankozeb deponering, mankozeb reënvastheid, en vir basisvlak model-ontwikkeling met betrekking tot die vinnige kwantifisering van letsels op blare en die *in vitro* produksie van *V. inaequalis* inokulum. Die geel fluoresserende pigment kan nou as ‘n uitstekende koste-effektiewe aanwyser vir mankozeb, vir verskeie formulasies, op appelsaailingblare gebruik word, en sal ook handig wees in die identifisering van tendense van die effek van reën op die volhoubaarheid van mankozeb formulasies. Dit het navorsingskapasiteit verhoog in terme van die evaluering van reënvastheid van fungisiedes deur die gebruik van gesimuleerde reën, en boordproewe vir die bepaling van die effek van spuitvolumes en masjiene deur fluoresserende pigmentdeponering. Hoewel ‘n basisvlak model nie ontwikkel kon word nie, sal die ontwikkeling van ‘n model in die toekoms meer haalbaar wees, aangesien ‘n vinnige kleurtegniek vir die kwantifisering van die hoeveelheid *V. inaequalis* siekte, geïdentifiseer is, wat net verder geoptimaliseer moet word. Tweedens sal ‘n ‘cellophane’ agar plaattegniek, en isolate met hoë spoorproduksie kapasiteit vir *in vitro* konidia produksie, verdere model ontwikkeling aanhelp.

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CONTENTS

CHAPTER 1

Apple Scab and its management: a review

INTRODUCTION

One of the most important deciduous fruit crops produced in South Africa is apples, with more than 40% of the total produce designated for export (National Agricultural Marketing Council, 2013). Apples are farmed throughout South Africa, with the largest apple production region (>50%) being located in the Western Cape, followed by the Eastern Cape (Langkloof 21%), Free State (2%) and the Southern Cape (1%). Many apple cultivars are produced in South Africa with the main cultivars being Granny Smith, Golden Delicious, Royal Gala, Pink Lady, Fuji and Top Red (Department Agriculture: Forestry and Fisheries Republic of South Africa, 2011, 2012).

Apple scab is an important disease of apples in South Africa and other apple producing countries world-wide. It is caused by the fungal pathogen *Venturia inaequalis* (Cke.) Wint. (MacHardy, 1996). The pathogen causes scab-like lesions on fruit that cause the fruit to deform and/or crack. Infection can also cause defoliation of trees that can lead to subsequent yield and tree vigour loss. This can subsequently lead to great economic losses for the producer (Aylor, 1998, Carisse and Rolland, 2004).

Control of apple scab can be achieved using different strategies that include chemical and biological control, cultural practices and combinations of these. However, control mainly relies on chemical control in South Africa. In some countries, cultural practices that consist of orchard sanitation methods that target the primary inoculum (fallen leaves) are also important (Gomez *et al.,* 2006). In general, the application of fungicides is seen as the most economical and feasible control measure (Carisse and Rolland, 2004; Gomez *et al.,* 2006). In commercial orchards, contact fungicides containing the active ingredient mancozeb are frequently used for the control of apple scab. Contact fungicides do not readily redistribute as the target organism or surface grow, and if new target surfaces are formed (flush, twigs and fruit) (Schutte *et al*., 2012), thus requiring frequent applications during active tree growth. Furthermore, rain and wind can increase the weathering of contact fungicides, influencing the efficacy of the fungicide (Hunsche *et al.,* 2006b). The ability of a contact fungicide including mancozeb, to withstand wash-off and weathering is thus important. If fungicide deposition on the target surface is compromised due to weathering and rain wash-off, it can lead to a loss in disease control. It will also influence the frequency of reapplication, and therefore application cost.

The efficacy of fungicide spray applications and deposition of the active ingredient directly affect the bio-efficacy of a product (Van Zyl *et al*., 2010b). Deposition parameters can be determined using various methods that make use of fluorescent dyes on leaves or Water Sensitive Paper (WSP) that can be assessed visually or through digital image analyses (Graham, 2010). This method has been used effectively on citrus leaves to determine spray deposition parameters and for the development of deposition benchmark models that allow for better interpretation of deposition parameter results in relation to disease control (Van Zyl *et al*., 2013). It has also been used to study the retention of copper fungicides on orange fruit and leaves (Schutte *et al*., 2012). Alternatively, residue analyses can be conducted on sprayed leaves to determine the amount of active ingredient present. Limited work has been conducted on spray deposition parameters of mancozeb on apple fruit and leaves as influenced by spray application technology and methodology, and the rainfastness of mancozeb products.

The following literature review will focus on important aspects of apple scab, specifically the life cycle of the pathogen and factors that influence disease development, management strategies for apple scab with the fungicide mancozeb and its characteristics, and an overview of methods used for assessing fungicide spray deposition and the importance of developing spray deposition benchmark models for interpreting fungicide spray deposition data.

APPLE SCAB

Life cycle

The life cycle of *V. inaequalis* starts with the pathogen overwintering in leaf litter in apple orchards, mainly as pseudothecia. The pseudothecia is formed through a sexual reproduction process, just before leaf abscission during autumn (MacHardy, 1996). As spring approaches, ascospores are released from mature pseudothecia following rain events, and are carried to developing fruit and shoot buds by wind (Charest *et al.,* 2002; Gadoury *et al.,* 2004). Ascospores are thus the primary inoculum source that causes the first infections on developing fruit and leaves, which lead to lesion formation and the production of conidia (Gadoury *et al.,* 1984; Charest *et al.,* 2002; Gadoury *et al.,* 2004). In South Africa, an additional primary inoculum source is conidia if these are formed on pygmy fruit late in the season, which then carry over to the next season (Von Diest, 2014). Secondary infections are caused by conidia (secondary inoculum) formed on leaves during the season during wet weather conditions that then spread to new leaves, fruit, and shoots. The continuous reproduction of conidia on infected leaves and fruit forms the polycyclic phase of the pathogen, which can cause severe epidemics if not controlled (MacHardy, 1996; Carisse and Jobin, 2006). As autumn approaches the life cycle is repeated.

The infection process of ascospores on susceptible young leaves is characterized by the ascospore forming germtubes that penetrate directly or it can form an appressorium through which penetration is achieved. The hyphae grow between the cuticle and epidermal cell wall and produce what is known as stroma. Finally the conidiophores and conidia are formed from the stroma. When this occurs, the cuticle ruptures and the conidia producing scab lesions are formed (MacHardy, 1996; Bolar *et al.,* 2000). The released conidia can cause new infections by germinating and penetrating leaves through the formation of appressoria (MacHardy, 1996).

Factors influencing disease development

Tree phenology and environmental factors determine the rate of apple scab development in apple orchards. Wind, relative humidity (RH), temperature, sunlight (UV), leaf wetness and leaf age are factors that play a role in the development of apple scab. Ultimately, these factors, together with primary inoculum load, determine the severity of the epidemic in orchards.

The distribution of ascospores and conidia are influenced by environmental factors such as rain and wind. Rain is required for the release of ascospores from pseudothecia, whereas wind is required to carry the spores to susceptible leaves and developing apples (Gadoury *et al.,* 1984; Aylor, 1998; Charest *et al.,* 2002). Conidia that cause secondary infections are spread by rain and wind resulting in increased disease incidence (Sutton *et al.,* 1976). Studies conducted by Aylor (1998) indicated that the distance travelled by ascopsores can be between 2 to 5 km, depending on the source of spores, wind speed and rainfall rate.

Disease severity is further influenced by inoculum concentration, temperature and leaf wetness duration. Hartman *et al.* (1999) found that an increase in disease incidence can be promoted by an increase in inoculum (1.5 – 81.2 × 10^3 conidia/ml) at four different temperatures (6, 11, 16 and 22°C), providing that the leaf stays wet under favourable conditions. Hartman *et al.* (1999) indicated that lower disease incidence occurred at 22°C than at lower temperatures of 6, 11 and 16°C. Increasing the wetness duration of the leaf leads to increased disease incidence. Optimal infection occurs at wetting periods of 20 to 30 h at temperatures between 17°C and 23°C (Schwabe, 1980). The importance of temperature and leaf wetness has been used to develop disease models that predict the severity of infections (Infection index), for example the Mills table that was later modified by several authors (Schwabe, 1980; MacHardy and Gadoury, 1989). A leaf infection index is determined by multiplying the wetting period (hours) by the temperature prevalent during the wetting period (Schwabe, 1980). Similarly, an index for fruit infection can be determined (Schwabe *et al*., 1984).

The germinability of conidia can be affected substantially by sunlight. Conidia that were exposed to sunlight caused the germinability of the conidia to decline after 9 hours of sunlight. Therefore, the survival of asexual inoculum is negatively affected by prolonged exposure to sunlight (Aylor and Sanogo, 1997).

A phenological factor that plays an important role in disease development is the age of leaves and fruit. Leaves that were inoculated with ascospores showed the greatest number of lesions when leaves were between 3 to 5 days old, while leaves inoculated with conidia showed the greatest number of lesions at 1 to 3 days old (Schwabe, 1979). As the age of leaves increased after unfolding, the number of lesions decreased following inoculation. Thus, leaves became more resistant over time (Aylor and Kiyomoto, 1993; Li and Xu, 2002). Schwabe *et al.* (1984) also investigated the effect of fruit age on infection and indicated that a longer wetting period is required to initiate infection as fruit age increased when inoculated with conidia.

Venturia inaequalis **inoculum production and inoculation methods**

Laboratory and glasshouse experiments investigating the biology of *V. ineaqualis* require the production of inoculum (conidia or ascospores). These inoculum sources can be produced on artificial media (*in vitro*) or on plant material (*in vivo*).The production of inoculum *in vitro* is convenient, since it does not require plants with susceptible leaves. However, *V. ineaqualis* is recalcitrant to the production of conidia on artificial media, and also grows slowly *in vitro* (Roberts and Crute, 1994).

Conidial inoculum can be produced *in vitro* using the wick method, which was first published by Keitt and Palmiter (1938). Mycelium is produced by growing isolates on 2% malt extract agar. A layer of muslin is positioned on the broad side of a sterilized glass medicine flat, which is then lightly smeared with mycelium from the malt agar plate. After the muslin is inoculated with the mycelium, sterile malt extract broth is added to the bottle, so that the muslin acts as a wick drawing up the medium. The wick is removed from the bottle after a growth period of 3 to 5 weeks (Roberts and Crute, 1994). The conidia is then harvested by scraping it from the muslin and placing it in sterile distilled water (Keitt and Palmiter, 1938; Roberts and Crute, 1994; Cronin *et al.,* 1996). Conidia can also be removed from the muslin by pouring the nutrient medium from the bottle and then adding a small amount of distilled water to the wick bottle. Shaking the bottle then removes the conidia from the muslin cloth (Keitt and Palmiter, 1938). The method of Keitt and Palmiter (1938) was modified by Cronin *et al.* (1996), who replaced the malt extract broth in the bottle with weak potato dextrose broth (Cronin *et al.,* 1996). Another modification to this method was published by Roberts and Crute (1994). They found that conidia production can be increased

by placing absorbent cotton wool at the bottom of a glass jar, onto which a double layer of muslin is placed (Roberts and Crute, 1994). The aforementioned published reports are not specific regarding the volume of spores that can be produced by the wick methods, and only specified the concentration of spores that can be obtained that ranged from 2×10^5 to 2×10^8 /ml (Robert and Crute (1994) and 5×10^4 /ml (Cronin *et al.,* 1996).

Venturia inaequalis conidia can also be produced using the cellophane technique. Cellophane membrane disks are soaked in water and then autoclaved, followed by placement on a solid medium (V8 juice, PDA or MEA) in a petri plate. The cellophane containing plates are inoculated by first blending an agar culture of *V. inaequalis* with water in a blender, where after the blended mycelium is distributed onto the cellophane-covered agar surfaces. After an incubation period of 1 to 4 weeks, the cellophane disk is removed and placed into a beaker with water and stirred to release conidia. The conidial suspension is poured through glass wool to remove mycelium and then centrifuged to concentrate the conidia for counting (Parker *et al.,* 1994, Gau *et al.,* 2004; Bus *et al.,* 2005). Similar to the wick method, published reports do not specify the volume of spores that can be obtained, but only the concentration of spores, which can vary from 1×10^5 to 3.75×10^7 /ml (Parker *et al*., 1994; Gau *et al*., 2004; Bus *et al*., 2005).

Another source of *V. inaequalis* conidia that can be used for inoculum is infected apple scab leaves collected from apple orchards. Leaves collected in orchards can be dried and stored at low temperatures (-18°C to -20°C) (Stensvand, 1997; Steiner and Oerke, 2007). This allows for a continual source of inoculum that can be stored for a period of up to 2 years until use (Robert and Crute, 1994). The conidia can be extracted from the sporulating lesions by rinsing in water followed by filtration through layers of cloth (mira-cloth, cheesecloth, muslin) to remove the leaf debris (Stensvand, 1997; Hartman *et al.,* 1999).

Ascospores can also be used as an inoculum source for experimental use. Infected leaves are collected from the orchard floor at the start of the ascospore release season, and are then rinsed to remove surface dirt. The rinsed leaves are placed in a plastic pan with distilled water at room temperature for at least 45 min. The pan can be agitated every few minutes so that the ascospores can be released from the leaves, resulting in their accumulation in the pan with water. The ascospore containing suspension can be filtered through layers of cloth and the spores concentrated through centrifugation (Sanogo and Aylor, 1997).

Another method is host inoculation. Leaves with mature pseudothecia and/or ascospores are used to inoculate potted apple trees. The apple leaves are first wetted by spraying with water on the lower and upper sides, where after they are placed on a wire screen over the potted trees. The wire screen is supported by a steel frame that is covered

by a clear polyethylene sheet, which maintains the surface water on both the potted trees and the leaves containing the pseudothecia (Schwabe, 1980; Stensvand, 1997).

Venturia inaequalis inoculum produced *in vitro* or those that are recovered from leaves can be used to inoculate detached leaves (Yepes and Aldwinkle, 1993; Percival and Boyle, 2009) or seedling leaves (Sanogo and Aylor, 1997; Hartman *et al.,* 1999; Steiner and Oerke, 2007). When seedling leaves are inoculated they must be incubated at 100% relative humidity (RH) at a temperature between 17°C and 21°C for 48 h (Schwabe, 1977). The leaves can be kept wet with a fine spray at a run-time of 20 s at a frequency of 30 to 60 min., since the pathogen requires free water for infection. Evaluation of symptoms can be conducted after a 2 to 3 week period (Schwabe, 1977; Steiner and Oerke, 2007). When detached apple leaves are spray inoculated they must be placed in Petri-dishes with moist Whatman filter paper or water agar. The plates are sealed with parafilm and placed in a growth chamber at 19±1°C and 16 h light/8 h dark cycle (Yepes and Aldwinkle, 1993; Percival and Boyle, 2009).

Lesions occurring on inoculated leaves and seedling leaves can be quantified and scored using different methods. A method that is often used with cultivar screenings, groups the leaf symptoms into five classes: pint point symptoms as class 1, chlorotic lesions without sporulation symptoms as class 2, few restricted sporulating lesions symptoms as class 3, class M can be seen as intermediate between class 2 and class 3, and abundant sporulation symptoms as a class 4 (Chevalier *et al.,* 1991). A method used by Preece (1959) consists of staining the leaves with decolourized basic fuchsin to detect fungus infection on the leaves. Infection points and colonised areas are visible as pink to purple areas on the leaves. This method can be used to detect field infections at a very early stage before sporulation starts.

Virulence of *Venturia inaequalis* and resistance of cultivars

Monogenic resistance in plants against plant pathogens (e.g. fungi) depends on the gene-for-gene interaction between the host plant and plant pathogen. Resistance occur when the avirulence (*avr*) gene of the pathogen is recognized by the resistance (*R*) gene in the plant (Dangl and Jones, 2001; de Wit, 2002; Guérin *et al.,* 2007). A mutation in the pathogen that changes the functionality of the *R* gene, leads to the gene-for-gene resistance interaction breaking down and infection occuring. This will thus change the pathogen from avirulent to virulent (Guérin *et al.,* 2007).

Currently, there are 17 known resistance (R) and avirulence (Avr) gene pairings for *V. inaequalis* and its *Malus* host *spp.* (Bowen *et al.,* 2011). The first resistance gene identified against *V. inaequalis*, was the *Vf* (*Venturia* resistance from *floribund*a) gene, originating from the clone *Malus floribunda* 821. This gene has been used in resistance breeding programs in

apple for many years (Chevalier *et al*., 1991, Crosby *et al.,* 1992; Parisi *et al.,* 1993). However, the gene has been overcome by two new races of *V. inaequalis* (races 6 and 7) (Durel *et al.,* 2003, Calenge *et al.,* 2004). Due to the *Vf* gene being overcome, *V. inaequalis* is capable of causing apple scab on all commercial apple cultivars in the field in most regions of the world (Koch *et al.,* 2000).

MANAGEMENT OF APPLE SCAB

Chemical control

The correct timing of application of fungicides is a major component of managing apple scab. The frequency of fungicide applications will depend on the weather conditions, cultivar susceptibility, tree phenology and disease pressure (Holb *et al.,* 2005; Jamar *et al.,* 2010). Applying fungicides every 7-10 days during active tree growth can prevent infections from occurring, since new growth or flush continually forms on the tree that makes it susceptible to infection. Fungicide applications are also required immediately after a certain amount (25 mm) of rain has fallen (Ellis *et al*., 1984), since fungicides only have a certain level of rainfastness, after which reapplications are required.

There are two broad groups of fungicides available for the control of apple scab that includes protective and curative fungicides. Curative fungicides are absorbed by the plant and affect fungal growth inside the plant tissue, whereas protective fungicides affect spore germination only on the surface of plants (Carisse and Jobin, 2006). Protective fungicides that can be used against apple scab include captan, dithianon and mancozeb, and for curative control fenarimol, etaconazole, flusilazole and difenoconazole can be used, to name just a few (Schwabe *et al.,* 1984; Sharda International Africa cc. 2006, Arysta LifeScience, 2013b). All these active ingredients are sold under different product names by different companies in different formulations. In South Africa, there are approximately 22 products (Table 1) registered with the active ingredient mancozeb for the control of apple scab alone (Agritel, www.agritel.co.za). An example is Dithane M-45 800 WP NT that contains 800 g/kg mancozeb as active ingredient in a wettable powder (WP) formulation (Dow AgroSciences, 2013a).

Mancozeb: characteristics, rainfastness and residues

Mancozeb has a broad-spectrum preventative activity that can control ascomycetes, oomycetes, basidiomycetes and imperfect fungi. This has led to its registration on more than 70 crops and 400 diseases over a period of nearly five decades. The dicarbamate fungicides, to which mancozeb belongs, can be divided into three sub-groups, depending on

their carbon skeleton: dimethyldithiocarbamates (DMDs), ethylenebis (dithiocarbamates) (EBDs), and propylenebis (dithiocarbamates) (PBDs). Mancozeb specifically belongs to the EBDs group (Crnogorac and Schwack, 2009). In South Africa, mancozeb is the key fungicide used for the control of apple scab (Schwabe, 1980).

The active ingredient of mancozeb consists of a mixture of manganese and zinc (zinc ion and manganese ethylene bis-dithio-carbamate). When applied to plants, mancozeb is mixed with water in spray tanks, which results in its breakdown releasing ethylene bisisothiocyanate sulfide (EBIS). Once applied to the plant surface, EBIS is converted into ethylene bisisothiocyanate (EBI) through UV light exposure. EBIS and EBI act as active toxicants against fungi, since they are believed to interfere with enzymes containing sulphydryl groups, and can therefore inhibit different biochemical processes within the cells of fungi, i.e. it is a multi-site inhibitor. Mancozeb is therefore classified as a group M (Multi Site Action) fungicide according to the Fungicide Resistance Action Committee (FRAC), having a low probability for resistance development (Gullino *et al.,* 2010).

Rainfastness of mancozeb

Different crops, cultivars and leave ages can have different leaf surface characteristics that affect fungicide wash off (Kudsk *et al.,* 1991). The surface of the leaf plays a role in fungicide rainfastness (fungicide retained on plant surface after rain events). Wagner *et al.* (2003) stated that studies done over the past 30 years by Baker and Parsons (1971), Barthlott (1981,1990), Barthlott and Ehler (1977), Barthlott and Wollenweber (1981), Bukovac *et al* (1981), Holloway and Baker (1974) and Jeffree (1986) have shown that the leaf can be composed of a number of structures that can be identified at three general levels: cell shape (primary-), cuticular folds (secondary-) and epicuticular wax crystals (tertiary-structure). The cuticle on the leaf is a natural hydrophobic composite that is the interface between plants and their environment (Kolattukudy, 2001). According to Bargel *et al.* (2006), Barthlott and Neinhuis (1997) documented that water formed spherical droplets on the outermost tips of the wax crystals that cause water to be repelled.

The rainfastness of mancozeb can be influenced by the volume and intensity of rain, whereas drying time is less important. As the volume and intensity of rain increases, so does the amount of mancozeb washed off from the leaf. Studies done by Hunsche *et al.* (2006b) on apple seedlings found that mancozeb was readily washed off from apple seedling leaves by simulated rain events. They observed that at a volume of 1 mm of light rain (0.5 mm/h) a 9% loss (i.e. 91% rainfastness) occurred, 55% (45% rainfastness) at heavy rain (5 mm/h) and 80% (20% rainfastness) at torrential rain (48 mm/h). At a rain volume of 5 mm, larger losses occurred with a 50% loss (i.e. 50% rainfastness) for light rain, and 90% (10% rainfastness) for heavy and torrential rain (Hunsche *et al*., 2006b). The time that mancozeb

sprays were left to dry prior to the application of rain (drying time) did not influence the rainfastness, since removal was high irrespective of the drying time (2, 4 and 24 h) applied (Hunsche *et al.,* 2006b).

The rainfastness of mancozeb was also tested on a few other crops including grapes, potato and pea. On grapevine leaves and fruits, it was found that mancozeb had a moderate rainfastness. An amount of 45 mm of rain applied to vineyards in different amounts (45 mm; 30 mm + 15 mm; 15 mm + 15 mm + 15 mm) at a consistent intensity of 60 mm/h, resulted in a comparable amounts of mancozeb being washed off from leaves (80% rainfastness) and grape berries (62% rainfastness). It is important to note that mancozeb was significantly less rain fast on berries than on leaves (Cabras *et al.,* 2001). The type of mancozeb formulation applied to pea and potato foliage influenced the rainfastness of mancozeb (Kudsk *et al.,* 1991). A suspension concentrate (SC) formulation of mancozeb had a significantly higher rainfastness than wettable powder (WP) formulations tested at 3 mm and 9 mm of rain at a high rain intensity of 27 mm/h. The percentage WP formulation retained on the pea and potato plants at 3 mm ranged between 40 and 70%, and at 9 mm between 30 and 50%. The SC formulation retained between 70 and 80% at 3 mm and 60 to 75% at 9 mm on pea and potato plants (Kudsk *et al.,* 1991).

Adjuvants that are added to spray mixes can influence the rainfastness of mancozeb. These products are used to reduce weathering, extend the efficacy of pesticides and can also act as a sticker or spreader to improve the agrochemical distribution and adherence when sprayed (Percival and Boyle, 2009). Hunsche *et al*. (2006a) evaluated the effect of different adjuvants on the rainfastness of mancozeb, and were able to show that adjuvants can increase the rainfastness on apple, bean and kohlrabi seedlings. After applying 5 mm of heavy rain (5 mm/h) on apple seedlings, bean seedlings and kohlrabi, a 6.1%, 64.8% and 15.9% rainfastness was obtained respectively. These rainfastness values were increased on the crops when rapeseed oil ethoxylates containing an average of 5 or 60 ethylen oxide units (RSO) were added to the sprays. On apple seedlings, the rainfastness increased from 6.1% to 21.6% with the addition of 5 RSO and to 12.5% with 60 RSO (Hunsche *et al.,* 2006a). Kudsk *et al.* (1991) found that on potato, at 3, 9 and 27 mm of rain at intensities of 3, 9 and 27 mm/h, the rainfastness of mancozeb was increased with the addition of a sticker adjuvant Spraymate Bond (Newbrook Agric. Products, UK).

Mancozeb spray application and dosages in pome fruit orchards

Application of fungicides to fruit and leaves for controlling apple scab in orchards is mainly done using axial fan ‘mist blower’ sprayers (Cross *et al.,* 2000). In South Africa, various types of applicators (low and high profile, axial and centrifugal fan-types) are used. The Unrath’s formula is used to determine the spray volume that is required for applications to

pome fruit orchards in South Africa. Application volume is calculated at a High Volume Requirement (HVR) using the Tree Row Volume (TRV) formula where TRV $= \frac{Tree\ Height\ (TH) x\ Tree\ Width\ (TW) x\ 937}{Distance\ between\ Rows\ (RW)}$ that calculates the volume of spray in litre that must be applied per hectare (Herrera-Aguirre and Unrath, 1980; Sutton and Unrath, 1984; Dow AgroSciences, 2013a). Using this formula, the height and width of the tree should be measured throughout the season, since these variables will vary for different tree growth stages. The application volume (AV) is adjusted according to tree growth stage. The AV is 60% of the HVR when applying mancozeb from bud break to full blossom. After 75% petal fall to about mid-season (one month later), the AV should be 80% of the HVR, and from mid-season to post-harvest and then just before leaf drop the AV should be 100% of the HVR (Dow AgroSciences, 2013a, b). AV adjustments vary worldwide.

The application volume used in apple orchards can be at a high volume (HV) or a low volume (LV) application. The amount of active ingredient of mancozeb applied per hectare (150 g/100 L) will be between 3 – 5.25 kg/ha at HV (2000 – 3500 L spray mixture/ha) and at LV between 2 – 4 kg/ha (500-900 L water/ha) [Ag-Chem Africa (Edms) Bpk., 2013; Arysta LifeScience, 2013a; Dow AgroSciences, 2013a, b]. The concentration at LV should not exceed the HV concentration by four when applying Dithane M-45 800 WP NT or other products containing mancozeb as the active ingredient (Dow AgroSciences, 2013a, b).

Biological and cultural control

Biological and cultural practices are important in the management of apple scab. An important cultural practice is the managing and removal of leaf litter containing primary inoculum, mechanically through shredding or ploughing of apple leaves into the ground (Vincet *et al.,* 2004; Gomez *et al.,* 2006). Some extent of control has also been achieved biologically through the application of microorganisms (Carrise and Rolland, 2004). These methods are important since they target the primary inoculum of the pathogen, which slows down or reduce epidemic development (Carrise and Rolland, 2004; Vincet *et al,* 2004; Gomez *et al.,* 2006).

Several approaches of leaf removal or destruction have been evaluated due to the importance of this primary inoculum source removal. Leaf shredding and leaf removal has been shown to be effective in United States (Sutton *et al*., 2000), Canada (Vincet *et al.,* 2004) and France (Gomez *et al.,* 2006). Sutton *et al.* (2000) also mentioned similar findings by Louw (1948) in South Africa. Leaf removal, as expected, has been shown to improve control relative to leaf shredding (Gomez *et al.,* 2006).

In organic orchards, post-harvest leaf eradication methods that can be used consist of a combination of leaf sweeping and leaf ploughing. Biological control agents also showed

potential. Leaves are swept from the alleys with a lawn sweeper and then ploughed into the row. Studies by Gomez *et al.* (2006) found that there was a decrease in fruit scab incidence (82.5% and 54.6%) and fruit scab severity in orchards (74% and 67.7%) when these methods were applied (Gomez *et al.,* 2006). Biological agents such as *Microsphaeropsis ochracea* can also be used to control the fungus. Studies by Carrise and Rolland (2004) showed that *M. ochracea* application to where-foliar or fallen leaves in autumn resulted in an ascospore production reduction of 61% to 91%.

Urea sprays can be effective when applied either to leaves that are on the tree or fallen leaves. The mechanism of action for this approach is not well understood, but could include more rapid breakdown of leaves by microbes (Sutton *et al*., 2000; Vincet *et al.,* 2004). Vincet *et al.* (2004) obtained a reduction in ascospore production of up to 90% when urea applications were combined with leaf shredding.

Some studies have evaluated a combination of cultural and biocontrol mechanisms such as leaf shredding and the application of microorganism that include *M. ochracea* or *Athelia bombacina*. Vincet *et al.* (2004) only obtained 80.6% to 85.2% reduction in the production of ascospores when applying single cultural or biological management approaches for the control of apple scab. However, the reduction in ascospore production could be improved to 93.9% when leaf shredding and *M. ochracea* was combined (Vincent *et al*., 2004). If biological and cultural control options are applied in orchards, delayed-fungicide spray programs and ascospore maturation models can possibly be used to achieve effective economic and environmentally sound control of apple scab (Carrise and Rolland, 2004).

METHODS FOR ASSESSMENT OF FUNGICIDE SPRAY DEPOSITION

There are several options for determining the quantity and quality of fungicide spray deposition on real and artificial targets. The simplest method is using Water Sensitive Paper (WSP) that can be placed on frames throughout the tree canopy, but it can also be attached to the leaves. More accurate estimations can be obtained using fluorescent dyes (Holownicki *et al.,* 2002; Palladini, 2005; Koch and Knewitz, 2008) and fungicide residues (Hunsche *et al.,* 2006a, b). Problems that occur with artificial targets such as filter paper are that the deposits are misleading since the deposition, coverage, retention capacity and run-off on the artificial targets do not represent the retention and spray deposition characteristics on plant surfaces (Koch and Knewitz, 2008).

Adequate fungicide deposition quantity can be defined as the amount of active ingredient needed to protect the plant surface from infection or cure infection, whereas deposition quality can be defined as uniformity of active ingredient deposition on a target site (leaf, twig or fruit), as well as between target sites in a tree. The quantity of deposition on

leaves can also be determined using fungicide residue analyses (Sutton and Unrath, 1984; Van Bruggen *et al.,* 1986; Woodrow *et al.,* 1995; Hwang *et al.,* 2001, 2002, 2003; Hamm *et al.,* 2006; Hunsche *et al.,* 2006a, b; Bringe *et al.,* 2007, Paramasivam and Chandrasekaran, 2013), although this is expensive. Fluorescent dyes are often used under field conditions to determine the quantity of deposition since it is more economical than residue analyses (Palladini, 2005). Fluorescent dyes can also have the added advantage in that the deposition quantity and quality on leaves or artificial targets can be assessed using improved methods such as photomacrographic imaging and digital image analysis (Brink *et al.,* 2006, Fourie *et al.,* 2009; Van Zyl *et al.,* 2010a, b, Van Zyl *et al.,* 2013).

Fluorescent dyes

Several different fluorescent dyes have been used to analyse spray deposition on leaves including Fluorescein, Eosine, Tinopal, Rhodamine B and Brilliant Sulfaflavine (Salyani and Whitney, 1988; Cai and Stark, 1997; Barber and Parkin, 2003; Gil and Sinfort, 2005; Gil *et al.,* 2007; Koch and Knewitz, 2008). Fluorescent dyes have a high sensitivity, selectivity and can be analysed rapidly using analytical methods. The disadvantage of some dyes are that when exposed to sunlight or powerful UV or even heat sources they start to degrade faster than other dyes (Cai and Stark, 1997, Gil and Sinfort, 2005). The most ideal fluorescent dyes must be stable and have a particle size range that is representative of the fungicide being evaluated. A good example of such a dye is the SARDI yellow fluorescent pigment (400 g/L, EC; South Australian Research and Development Institute, Loxton, SA, Australia) that has a particle size range from 0.5 to 10 µm (Van Zyl *et al*., 2013), which is similar to copper oxychloride particles.

Visual and Image analyses

Artificial targets or leaves can be sprayed with fungicide mixed with a fluorescent dye, followed by illumination with the correct light source in order to obtain an image of the distribution (quality) and amount (quantity) of fungicide deposited. The fluorescent dye thus act as a tracer of the fungicide and is an indicator of spray distribution on the target, which can be visually assed by eye (Holowinicki *et al*., 2002; Koch and Knewitz, 2008) or using image analyses (Van Zyl *et al.,*2013).

Deposition assessment of fluorescent dyes sprayed onto targets can be classified visually. Palladini (2005) and Furness (2006) each developed visual grading scales. However, these subjective assessment scales or grading systems and the accuracy of it is usually influenced by user experience and is not very accurate.

In more recent years the assessment of the quantity and quality of fluorescent dye deposition has been improved using digital photomicrographic imaging and digital image analysis (Brink *et al.,* 2006; Van Zyl *et al.*, 2010a, b). Subsequently, this technology has been improved by using photomacrographic imaging that has the advantage of determining deposition on whole targets such as leaves and fruit (Van Zyl *et al*., 2013). These methods can measure the quantity and quality of spray deposition on leaves (Brink *et al.,* 2006; Fourie *et al.,* 2009, Van Zyl *et al.,* 2010a, b; Van Zyl *et al*., 2013, 2014), which is a great improvement over the previously used subjective visual assessments.

Fluorometry, colorimetry and spectrophotometry

Fluorescent dye deposition quantity can also be evaluated through absolute fluorescent quantification using flourometry, colorimetry and spectrophotometry. In these methods, that cannot determine the quality, the fluorescent dye deposited onto leaves is washed of the leaves, and then quantified by determining the concentration of the dye present (Salyani and Whitney, 1988; Hayden *et al.*, 1990). Fluorescent dyes that can be used in this approach include Rhodamine-B, brilliant sulphoflavine, Flolene FD&C Blue No.1 and Flolene FD&C Yellow No. 6 (Salyani and Whitney, 1988, Hayden *et al.*, 1990; Gil *et al.,* 2007). The advantage of this technique is that it is less subjective than visual quantity assessment of fluorescent dye deposition by eye since a specific fluorescent value is obtained (Glover, 1956).

Fungicide residue

Scanning Electron Microscope (SEM)

Residues of some fungicides, such as mancozeb, can be determined qualitatively (distribution) and quantitatively with the use of a scanning electron microscope (SEM). This method can also be used to determine the surface characteristics of the leaf that the fungicide is sprayed on, which is an important characteristic influencing fungicide deposition (Hess and Falk, 1990; Hunsche *et al.,* 2006a). However, preparation procedures of the leaf can be problematic due to the methods being used that can consist of critical point drying, freeze-drying and air-drying. This can lead to removal or redistribution of the product on the leaf or even altering the morphology of the leaf surface (Hess and Falk, 1990).

Analytical chemistry for mancozeb residues

Two of the main methods used for measuring mancozeb residues are through carbon disulphide (CS_2) emissions or measuring of manganese ions (Hunsche *et al.,* 2006b; Crnogorac and Schwack, 2009). The measurement of manganese ions involves measuring

the concentration of manganese atoms through atomic absorption spectrometry. A mathematical calculation is then used to determine the mancozeb residue, because the manganese ions constitute 17% of the mancozeb molecular weight. Several scientific studies have used this approach for quantification of mancozeb residues including studies on apple, potato, bean and kohlrabi leaves (Sutton and Unrath, 1984; Van Bruggen *et al.,* 1986; Hamm *et al.,* 2006; Hunsche *et al.,* 2006a, b, Bringe *et al.,* 2007).

Determining mancozeb residue using carbon disulphide (CS_2) emissions can be conducted using different techniques, and is often also used by commercial laboratories. Techniques that can be used to determine carbon disulphide bonds include the use of sulfur-mode flame photometric gas chromatography, spectrophotometry, gas-liquid chromatography (GC) or gas-chromatography-mass-spectrometry (GC-MS). Scientific publications that have used disulphide bond determination for investigating mancozeb residues included studies on apples, gherkins and glass fibre filters (Woodrow *et al.,* 1995; Hwang *et al.,* 2001, 2002, 2003; Paramasivam and Chandrasekaran, 2013).

DEVELOPMENT OF BIOLOGICAL SPRAY DEPOSITION MODELS

Developing a fungicide spray deposition benchmark model for specific crops can assist in interpreting spray deposition parameters in terms of the potential control of the disease present on leaves and fruit. This model can be used to determine the biological efficacy of different spray volumes, adjuvants, spray machines and techniques for disease control (Brink *et al.,* 2006, Van Zyl *et al.,* 2010a; Van Zyl *et al.,* 2013). Very few studies have attempted to establish spray deposition assessment benchmarks for leaves and fruits within orchards. Studies that have developed these benchmarks for citrus and grape vineyard leaves (Brink *et al*., 2012; Van Zyl *et al*., 2013), have set their benchmark levels for disease control at 50% or 75%, since under field conditions 100% control is an unrealistic goal. Recently, deposition benchmarks have also been developed for fungicide deposition on dipped oranges, lemons and soft citrus in packhouses for controlling *Penicillium digitatum* and *Penicillium italicum* (Erasmus *et al*., 2013, 2015; Njombolwana *et al*., 2013).

On grapes, Brink *et al.* (2006) attempted to develop a benchmark for management of *Botrytis* on grape bunches with a fenhexamid fungicide. They, however, were unsuccessful, since the spray deposition levels were too low for obtaining adequate control of *Botrytis* infections. This was most likely due to too low spray volumes, and the highly variable deposition of conidia and fungicides on the grape bunches that has a challenging three dimensional structure. The development of a spray deposition model on grape leaves was more feasible and Brink *et al.* (2012) were able to successfully developed biological efficacy curves at 75% control levels that can be used to interpret spray application and depositions

in vineyards (Brink *et al*., 2012). The grape leaf spray deposition model was used to further show that the addition of certain adjuvants to fungicides can result in a reduction in *Botrytis* infections on leaves (cv. Chardonnay) of *B. cinerea* (Van Zyl *et al.,* 2010a).

Van Zyl *et al.* (2013) developed a spray deposition benchmark model on mandarin leaves for the control of Alternaria brown spot caused by *Alternaria alternata* (Fr: Fr) Keissl., tangerine pathotype, using a fluorescent pigment as a tracer for copper oxychloride. The percentage fluorescent particle coverage (FPC%) that covered the leave area measured was used to predict the level of control expected; 50% and 75% of disease control was predicted at 2.07 FPC% and 4.14 FPC%, respectively (Van Zyl *et al.,* 2013).

CONCLUSION

Apple scab is a world-wide problem wherever apples are grown and can lead to great economic losses, which makes it an important disease for which effective control must be optimized (MacHardy, 1996, Aylor, 1998, Carisse and Rolland, 2004). Since fungicide applications play a pivotal role in scab control, the effective deposition of fungicides and methods for evaluating this are important. The deposition of mancozeb is specifically important since this fungicide forms the backbone of apple scab control programs in South Africa. Recent studies conducted by Brink *et al.* (2006), Fourie *et al.* (2009) and Van Zyl *et al.* (2010a, b) developed a technique using photomicrographic imaging and fluorescent pigment to determine the deposition quantity and quality on leaves of grapes and citrus. This method was later improved by Van Zyl *et al*. (2013) by replacing the photomicrographic imaging with photomacrographic imaging and through the development of benchmarks to better interpret deposition parameters in terms of disease control. Altogether these assessment and interpretation techniques can be used as effective tools for evaluating spray volume and spray machines in orchards (Van Zyl *et al.,* 2013).

Factors that can influence the efficacy of mancozeb applications for controlling scab include rainfall and the addition of adjuvants to scab fungicides. Ellis *et al.* (1984) stated that a new application of mancozeb is applied following 25 mm of rain, whereas in South Africa reapplications are done after 12 mm of rain (personal communication, J.P.B. Wessels, ProCrop Trust, Ceres, South Africa).The ability of a contact fungicide such as mancozeb to withstand wash-off and weathering is thus important. The rainfastness of mancozeb products will influence the frequency of reapplication and therefore application cost. The addition of certain adjuvants can also enhance the rainfastness of products, as shown by Hunche *et al.* (2006a).

The overall aim of this study was to develop tools that can be used to improve the management of apple scab using fungicides. The first aim was to determine the rainfastness

of two commercial mancozeb products on apple seedlings. The effect of the addition of an adjuvant on the rainfastness of one of the products was also investigated. These investigations involved the construction of a rainfall simulator, and determining whether a fluorescent pigment, quantified through photomacrographic imaging, was a suitable tracer for the rainfastness of mancozeb residues. The second aim was to investigate the development of a mancozeb spray deposition benchmark model for apple scab control on apple leaves. To aid model development, a disease severity quantification protocol was developed that enabled the detection of apple scab infections as pin point lesions on leaves using staining techniques and photomacrographic imaging. The further development of apple scab benchmark models will benefit from the optimization of various techniques in the current study.

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Table 1. Mancozeb products registered in South Africa

Active Ingredient	Trade names	Formulation	Registration Numbers (L)	Company
Mancozeb	Vondozeb	800g/kg	L2177	Tsunami Plant Protection, Heidelberg
	Sancozeb 800 WP	800g/kg	L3459	DOW Agroscience Southern Africa, Bryanston
	Ciplazeb	800g/kg	L4754	Cipla Agricare, Cape Town
	Cozeb 800 WP	800g/kg	L7098	Universal Crop Protection, Rustenburg
	Mancozeb 800 WP	800g/kg	L7381	Agchem-Africa (Pty)Ltd, Pretoria
	Dithane M 45 800 WP	800g/kg	L2914	DOW Agroscience Southern Africa, Bryanston
	Dithane 750 WG Neotec	750g/kg	L4213	DOW Agroscience Southern Africa, Bryanston
	Tsumeb	800g/kg	L4797	Tsunami Plant Protection, Heidelberg
	Mancozeb 800 WP	800g/kg	L7352	Volcano Agroscience, Mt. Edgecombe
	Dithane M-45 800 WP NT	800g/kg	L7484	DOW Agroscience Southern Africa, Bryanston
	Villa Unizeb WP	800g/kg	L8056	Villa Crop Protection, Kempton Park
	Tridex	800g/kg	L5323	Total South Africa, Rosebank
	Penncozeb WG	750g/kg	L4655	Total South Africa, Rosebank
	Pennfluid	420g/L	L7733	Total South Africa, Rosebank
	Newzeb 750 WDG	750g/kg	L8811	Universal Crop Protection, Rustenburg
	Mancozeb 800 WP	800g/kg	L9169	Meridian Agrochemical Company, Germiston
	Ventum 800 WP	800g/kg	L8691	Gouws& Scheepers, The Woodlands
	Villa Unizeb 750 WDG	750g/kg	L8812	Villa Crop Protection, Kempton Park
	Multizeb 455 SC	455g/L	L9174	Villa Crop Protection, Kempton Park
	Newzeb 455 SC	455g/L	L9175	Universal Crop Protection, Rustenburg
	Unizeb 480 SC	480g/L	L9179	Villa Crop Protection, Kempton Park
	Newzeb 480 SC	480g/L	L9178	Universal Crop Protection, Rustenburg

CHAPTER 2

Evaluating the deposition of mancozeb formulations through fluorescent pigment quantification, and the rainfastness of mancozeb on apple seedlings

ABSTRACT

Mancozeb spray deposition and the persistence thereof to rainfall are important factors influencing the control efficacy of apple scab, caused by *Venturia inaequalis*. Fungicide deposition can be assessed through quantification of fungicide residues, or a fluorescent pigment of which the coverage is accessed using photomacrography image analyses. The latter method is more cost effective and allows for quantitative and qualitative deposition assessment. The study showed that a yellow fluorescent pigment was a suitable tracer for five different mancozeb formulations (Dithane M-45 800 WP NT, Mancozeb, Pennfluid, Ventum 800 WP, Vondozeb) since a good Pearson's correlation (r = 0.779) existed between fluorescent particle coverage (FPC%) and mancozeb residues (Manganese-ion (mg/kg_{DW})) for all five formulations, when evaluated at different concentrations (0.5×, 1.0×, 1.5× and 2.0×). The particle size ranges of the Dithane M-45 800 WP NT and Ventum 800 WP formulations were significantly smaller than those off the other formulations, but this did not result in differences in Mn-ion residues realized on apple leaves for WP mancozeb formulations; Pennfluid SC formulation deposited markedly less mancozeb than the WP formulations due to a lower active ingredient label rate used. The persistence of mancozeb to different rain volumes was determined for three treatments: Dithane M-45 800 WP NT, Ventum 800 WP, and Ventum 800 WP combined with the sticker-spreader adjuvant Nu-Film P. Simulated rain was applied to apple seedlings at a constant rainfall intensity of 5 mm/h at five different rainfall volumes (0, 1, 5, 10 and 15 mm). There were no significant differences between the three treatments based on FPC% and Mn-ion concentrations. Although a good correlation (r = 0.726 to 0.783) existed between FPC% and Mn-ions, the response of FPC% and Mn-ion differed somewhat as was evident from the slopes of exponential regression models, showing slower initial loss in FPC% than for Mn-ions and a markedly larger predicted loss by the model's asymptotic value (61.4% and 32.2%, respectively). Mn-ion analyses showed that a significant loss (11.95%) in residue occurred after applying 1mm rain, but that no significant differences in losses (26.32% to 31.67%) occurred after applying 5 to 15mm of rain.

INTRODUCTION

In South Africa, regular fungicide applications are used to control apple scab caused by *Venturia inaequalis,* an economically important disease of apples world-wide. A key fungicide used in apple scab management is mancozeb (Schwabe, 1980), a broad spectrum contact fungicide containing a mixture of manganese (manganese ethylene bis-dithio-carbamate) and zinc ions (Crnogorac and Schwack, 2009; Gullino *et al.,* 2010). A typical scab spray program consists of weekly mancozeb sprays starting at green tip. Depending on cultivar differences, green tip can be as early as the first week in August, but seldom later than the second week in September. Weekly sprays continue until petal fall, and then bi-weekly sprays are applied until the beginning of December. From December onwards, sprays are applied every 4 weeks, until 3 weeks before harvest. This can results in a total of 12 to 14 mancozeb sprays applied per season in South Africa (personal communication, J.P.B. Wessels, ProCrop Trust, Ceres, South Africa). Regular weekly mancozeb applications at the start of the season is essential since new shoots and flowers develop continuously, which is highly susceptible to scab (Schwabe, 1979; Aylor and Kiyomoto, 1993; MacHardy, 1996; Li and Xu, 2002).

Rainfall can influence the persistence and efficacy of contact fungicide sprays (Kudsk *et al.,* 1991; Hunsche *et al.,* 2006b). On apple seedlings, the amount of mancozeb washed off from leaves increased as the volume and intensity of simulated rain increased. Mancozeb was reported to have relative poor rainfastness on apple seedling leaves, with 55-80% loss occurring after 1 to 5 mm of heavy rain (5 mm/h) (Hunsche *et al.*, 2006b). However, on grape, pea and potato, mancozeb was shown to be more persistent, with less residue loss at the same volumes and higher rain intensities (Kudsk *et al.*, 1991; Carbas *et al*., 2001) than those used on apple seedlings by Hunsche *et al.* (2006b). In the South African apple industry, mancozeb is perceived as having relative good rainfastness based on unpublished data (personal communication W.F.S. Schwabe, The Fruit Doctor, Somerset West, South Africa). Growers will often only reapply mancozeb after 5-10 mm heavy rain has fallen, or 20 mm of soft rain (personal communication J.P.B. Wessels). In other countries such as the United States of America, reapplications are reported to be made after 25 mm of rain has fallen (Ellis *et al*., 1984).

In South Africa, the Western Cape region is the most important apple producing region (Department Agriculture: Forestry and Fisheries Republic of South Africa, 2011, 2012) and it is classified as a winter rainfall area. Frequent rains and temperatures conducive to scab development continue as late as November in this region, with rainfall during the mancozeb spray window ranging from ± 100 mm - 200 mm in Ceres and Grabouw/Elgin respectively (www.capefarmmapper.com; sdwebx.worldbank.org/climateportal). Rain will sometimes fall

twice within the same week early in the season, causing growers to reapply mancozeb based on the amount of rain fallen, in order to protect new shoots and flowers.

Adjuvants can be combined with fungicide sprays to reduce weathering, extend the efficacy and improve the distribution, adherence and rainfastness of pesticides (Hunsche *et al.,* 2006a; Percival and Boyle, 2009). Only two studies have investigated the effect of adjuvants on the rainfastness of mancozeb. Rapeseed oil ethoxolates improved the rainfastness of mancozeb on apple, bean and kohlrabi seedlings (Hunsche *et al*., 2006a). The addition of various sticker adjuvants also improved mancozeb rainfastness on pea and potato (Kudsk *et al.,* 1991).

The effect of rainfall on the persistence of fungicides can be determined using natural rainfall occurring in orchards during the growing season (Schutte *et al*., 2012), or simulated rain (Cabras *et al*., 2001; Hunsche *et al*., 2006b; Iserloh *et al*., 2012, 2013). Evaluating the effect of simulated rain has the advantage that a range of specific rain volumes and intensities can be investigated. Over the past 62 years various different rain simulators have been built and used to evaluate rainfastness (Iserloh *et al.,* 2012, 2013). These simulators are also essential tools in the assessment of soil erosion, soil water infiltration and chemical wash-off (Grierson and Oades, 1977; Cabras *et al*., 2001; Hunsche *et al*., 2006a, b; Arnaez *et al*., 2007). Although there are several publications that have used rain simulators for various purposes, there is no standard rainfall simulator design, and therefore simulators differ in rainfall intensities and distribution, drop sizes and drop velocities (Iserloh *et al*., 2012, 2013).

The quantification of fungicide deposition is important in rainfastness experiments. Disease control is directly dependent on effective fungicide deposition (Holownicki *et al*., 2002). Both the quantity (the amount of active ingredient on a target surface) and quality (the distribution of the *a.i* on the target surface) of contact fungicide deposition realised on target surfaces (leaves, twigs and fruit) are important (Fourie *et al*., 2009, Van Zyl *et al*., 2010a, b; Van Zyl *et al*., 2013). Fungicide deposition quantity and quality can be investigated cost effectively using a yellow fluorescent pigment in combination with photomicrographic (small target area) or photomacrographic (larger target area) image analyses (Graham, 2010; Van Zyl *et al*., 2010a,b; Van Zyl *et al.,* 2013, 2014).

Fungicide deposition can also be determined through quantification of fungicide residues, although this is expensive and in general only provides information on quantity but not quality of deposition (Sutton and Unrath, 1984; Van Bruggen *et al.,* 1986; Woodrow *et al.,* 1995; Hwang *et al.,* 2001, 2002, 2003; Hamm *et al.,* 2006; Hunsche *et al.,* 2006a, b; Bringe *et al.,* 2007, Paramasivam and Chandrasekaran, 2013). For mancozeb, the main methods used for quantification of residues are the measurement of carbon disulphide (CS_2) (Woodrow *et al.,* 1995; Hwang *et al.,* 2001, 2002, 2003; Crnogorac and Schwack, 2009) or

manganese (Mn)-ion concentrations (Sutton and Unrath, 1984; Van Bruggen *et al*., 1986; Hamm *et al.,* 2006; Hunsche, 2006a, b; Bringe *et al.,* 2007). Mancozeb deposition can also be determined qualitatively and quantitatively using scanning electron microscope approaches (Hess and Falk, 1990; Hunsche *et al.,* 2006a). However, these methods are not amendable for fast and relative high throughput analyses of deposition, since the preparation procedures are time consuming as well as the scanning process, which all add to the relative high cost of the method.

In South Africa, approximately 22 different mancozeb formulations are registered on apple (Agritel, www.agritel.co.za). It is unknown whether these formulations that mostly include cheaper generic formulations, differ in rainfastness and whether the addition of adjuvants can improve rainfastness on apple leaves and fruit. Mancozeb rainfastness is especially important in the later part of the growth season where bi-weekly sprays are applied, and where maximum residue values must not be exceeded. It is also important early in the season since rain often occurs over a 7-day period. If the current perception of mancozeb rainfastness in South Africa is underestimated, it can lead to reduced control following rain events, or if it is overestimated it can result in over application and increased costs (water use, product quantity, labour and diesel consumption). Superior rainfastness of specific mancozeb formulations or improvement of persistence through the addition of adjuvants can improve scab control on leaves early in the season when continues rain occur over 7-day periods, where wet orchards conditions or limitations in available sprayers on a farm prevent re-applications.

Different fungicide formulations and particle size can have an effect on the rainfastness and tenacity of fungicide sprays. The effect of particle size on the tenacity of different fungicides may vary and is somewhat controversial for example copper fungicides. The tenacity of copper oxychloride and copper carbonate have been reported by Somers and Thomas (1956) and Hyre (1942) respectively to increase with decreasing particle size. However, Somers and Thomas (1956) reported that the tenacity of copper carbonate was not influenced by particle size, nor that of cuprous oxide (Somers and Thomas, 1956). Schutte *et al.* (2012) compared copper hydroxide, copper oxychloride and cuprous oxide with each other and found that particle size differences between these products did not affect rainfastness or weathering. For mancozeb, only Kudsk *et al.* (1991) have reported that the rainfastness of mancozeb WP formulations improved with a decrease in particle size (Kudsk *et al*., 1991). The controversial reports on the effect of particle size on the tenacity of copper may in part be due to the fact that in commercial fungicides, surface active compounds such as wetting and sticking agents are added that also influence tenacity (Somers and Thomas, 1956), aside from particle size. This has been reported for mancozeb

where mancozeb SC formulations had a higher rainfastness than WP formulations on pea and potato (Kudsk *et al.*, 1991).

The first aims of the study was to determine the particle size distribution of five different mancozeb formulations (Dithane M-45 800 WP NT, Ventum 800 WP, Mancozeb 800 WP, Pennfluid and Vondozeb) and point of run-off using a yellow fluorescent pigment. Subsequently, the deposition of all five formulations was compared through assessing fluorescent pigment and Mn-residue quantities. The third aim was to design and build a rainfall simulator for evaluating the rainfastness (persistence) of two mancozeb formulations with or without an adjuvant on apple seedling leaves. The persistence of the formulations to five different rain volumes at a moderate rain intensity (5 mm/h) was assessed through manganese ion and fluorescent pigment quantification.

MATERIALS AND MEATHODS

Plant material

Golden Delicious apple seedlings (*Malus domestica* Bork h.) (18-24 month old) were used in all experiments. The seedlings were produced by first extracting apple seeds from fruit and placing it in water containing 2 g/L captan (Captab WP, Universal Crop Protection (Pty) Ltd., South Africa) for one day. After drying, the seeds were stratified in perlite moistened with captan water (2 g/L) at 4°C for 3 months until germination. Germinated seeds were placed in crates with perlite and incubated at 25°C. After 2-3 weeks when seedlings emerged, the seedlings were planted into seedling trays. The seedlings were transplanted after 3 weeks into 1 L plastic bags containing a sterile sand and bark (2:1 v/v) growth medium. Seedlings were grown in a glasshouse at ± 26°C and irrigated every second day for 8 min. Each seedling bag was fertilized with slow release composted poultry manure pellets (2:2:3) (Master-organics, Cape Town, South Africa). After each trial seedlings were cut back to stimulate new flush formation.

Deposition assessment

Point of run-off

The point of spray run-off was determined for five different mancozeb formulations (Table 1) based on yellow fluorescent pigment deposition. Apple seedling leaves were cut top to bottom from randomly selected seedling shoots. The upper leaf surfaces [smallest: ± length 25 mm; largest: ± length 89 mm) were sprayed at different spray volumes (0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 1.75 and 2.00 ml per leaf) for each of the five mancozeb formulations separately, together with a yellow fluorescent pigment [South Australian Research and Development Institute (SARDI) Yellow Fluorescent Pigment, 40% EC (SARDI, Loxton, South

Australia) 1 ml/L] and deionised water (dH_2O). A spray treatment containing only fluorescent pigment was also included. Sprays were applied with a gravity fed spray mist gun (ITW DEVILBISS Spray Equipment Products, USA) with a fluid nozzle tip of 1.5 mm in diameter at a pressure of ± 210 kPa. For spraying, each leaf was placed on a wired mesh tray that was angled at 30° on a steel frame (800 x 1410 x 660 mm). Suspensions were agitated frequently using a magnetic stirrer. The leaves were left to dry on trays at room temperature (± 23°C) until deposition analyses were conducted. The spray gun was cleaned by flushing with a 70% ethanol solution, followed by air-drying after each treatment. The experiment was repeated four times with 10 replicates per treatment; replicates consisted of ten leaves taken from a single shoot.

The sprayed leaves were transported to a dark room. A spray deposition assessment protocol was used to determine spray deposition quantity and uniformity on leaves as described by Van Zyl *et al.* (2013) with slight modifications. Each leaf was positioned separately in the middle of a black-illuminated red Perspex box (300 x 210 x 110 mm) and was covered with a glass pane (200 x 200 x 2 mm) making sure that the edges of leaves did not fold. The centred leaf was illuminated using two ultra-violet light sources (UV-A at ≈ 365 nm; Labino Mid-Light; www.labino.com) in stereo. Digital photos were taken of the upper leaf surfaces using a Canon EOS 40D camera equipped with a 60 mm macro lens attached to a tripod directly above the leaf. Using Digital Photo Professional version 3.1.0.0 RAW image files were converted to 8-bit Exif-TIFF files (CANON INC.; www.canon.com). Image Pro Plus software (Image Pro Plus software version 7.2; Media Cybernetics, www.mediacy.com) was used to determine the deposition quantity of fluorescent particles per leaf, measured as percentage fluorescent particle coverage (FPC%) (Van Zyl *et al*., 2013).

Comparison of manganese ion and fluorescent pigment quantification

The upper leaf surfaces of seedling leaves picked top to bottom from randomly selected seedling shoots were sprayed to the point of run-off (as determined previously). Sprays were applied in the same manner as described above. Spray treatments consisted of the five different mancozeb formulations (Table 1) sprayed separately together with a yellow fluorescent pigment (1 ml/L) and dH_2O at 0 (control), 0.5, 1, 1.5, and 2.0 times the recommended concentrations (Table 1). After spray application, leaves were left to dry at room temperature (± 23°C) until fluorescent pigment and Mn-ion deposition analyses were conducted. The experiment was repeated three times. In each experiment all treatments were replicated 15 times, with a replicate consisting of leaves taken from a single shoot.

Deposition assessment of fluorescent pigment was conducted as described above. After leaf images were captured, leaves were frozen at -80°C and subsequently freeze-dried in a freeze drier (Christ Beta 1-8 LD plus; www.martinchrist.de). The dried leaf samples were

ground into a fine powder and sent for mancozeb residue (manganese ion) analysis at the Central Analytical Facility (CAF) at Stellenbosch University. A sample of 0.5 g dried leaves was acid digested in a Mars Microwave Digestor (CEM) with 7 ml of Merck Suprapure HNO_3 (www.Merck.com). The samples were left to cool and were then made up to 50 ml with deionised water before quantifying the Mn-ion concentrations on a Thermo Icap 6200 ICP-AES machine (Thermo Scientific, Cambridge, England.). Mn-ion concentrations were expressed per dry weight.

Particle size analysis

Yellow fluorescent pigment and the five contact mancozeb formulations (Table 1) were subjected to particle size analysis. Twenty grams of each formulation was sent to a commercial laboratory (Absolute Science, Pretoria, South Africa; www.absolutescience.co.za). A 0.2 g subsample was diluted in 20 ml of deionised water. Five hundred microliters of the suspension was injected into the dispersion chamber of a laser diffraction particle size analyser (Shimadzu SALD-201 V, Nakagyo-ku, Kyoto, Japan). Each of the suspensions was further diluted until an absorbance reading between 0.09 and 0.1 absorbance units (AU) were obtained. Particle size (diameter - µm) was measured and reported as a particle distribution curve.

Rainfall simulation, distribution and mancozeb persistence

Rainfall simulator

A rainfall simulator was built that consisted of a stationary 5.6 wide angle spray full cone nozzle (W) (Spraying Systems Co., www.spray.co.za) mounted on the centre horizontal rod of a collapsible steel frame gazebo (3 m × 2.7 m × 2.3 m). The structure was covered with a rain resistant tarp to prevent outside climatic disturbances. The stationary nozzle was positioned 2.2 m above the total rainfall area of 8.1 m^2. Apple seedlings were only placed within a predetermined rainfall area of 1 m^2 under the nozzle, which was shown to have the most uniform rain distribution (Fig. 4 A). Although in the Western Cape region in South Africa, rainfall varies from light, heavy to torrential (www.capefarmmapper.com, sdwebx.worldbank.org/climateportal), only a heavy rainfall (5 mm/h) intensity, also used by Hunsche *et al.* (2006 a, b), was selected since this often occurs during the apple growing season. The rain intensity was created using a pressure regulator that was calibrated at 200 kPa. This resulted in a consistent flow rate of ± 2.7 L/min, which was monitored with a flow meter (Gardena Water Flow Meter; www.gardena.com) and pressure gauge. A pulsar unit (Watering Seconds Timer; www.turf-ag.co.za) was used to regulate the simulated rain intensity applied, and a timer (Orbit Timer; www.orbitonline.com) to regulate the volume rain applied. The pulsar applied rain at a run time of 1 min every 8 min for 1 h that yielded a rain

intensity of 5 mm/h. Rain was applied at five different volumes (0 [Control], 1, 5, 10, 15 mm), which represented rain volumes used by Hunche *et al*. (2006b).

The simulated rainfall distribution and the optimal settings for achieving an even distribution of ± 5 mm/h across the rainfall area 1 m^2 where seedlings were placed in the final experiments were investigated by first laying out a 8.1 m^2 plot area on the centre of the simulator floor. Twenty five tin cans were placed at 50 × 50 cm distances from each other within this area. Different pressures (200, 250 and 300 kPa) were tested with three different wide angle spray full cone nozzles (W) (2.8 W, 4.3 W and 5.6 W) (Spraying Systems Co., www.spray.co.za) to evaluate the rainfall distribution and intensities inside the simulator. The pulsar was left to run for 2 min, where after the volume of each can was measured (ml). The experiment was repeated 3 times. The volume was subsequently converted to rain intensity (mm/h) using the formula:

$$mm/h = \frac{Volume\ (ml)}{\pi r^2 h} * 30$$

The range of rain volumes used in the mancozeb persistence experiments was obtained by regulating application times using the 5.6W nozzle and 200 kPa pressure. Application times of 15, 60, 120 and 180 min yielded the rain volumes of 1, 5, 10 and 15 mm, respectively. The volume (mm) of rainfall was monitored throughout experiments using five rain gauges located within the 1 m^2 rainfall area.

Mancozeb persistence

Mancozeb persistence to rainfall was evaluated for two of the mancozeb formulations evaluated in the particle size experiments and included formulation Dithane M-45 800 WP NT and Ventum 800 WP (Table 1). These two formulations had similar particle sizes (see Results section). The effect of a sticker-spreader adjuvant at 0.3 ml/L (Nu-film P, Miller Chemical and Fertilizer co., USA) when combined with Ventum 800 WP was also investigated. Yellow fluorescent pigment (1 ml/L) was added to each formulation and spray treatments were applied to apple seedlings placed under a tripod structure holding a gravity fed spray mist gun (ITW DEVILBISS Spray Equipment Products, USA) with a fluid nozzle tip of 1.5 mm in diameter at a pressure of ± 210 kPa, at a height of 121 cm from the nozzle to floor; distance from the nozzle to the target leaves ranged from ± 35 to 50 cm. Each seedling was sprayed at a pre-determined volume of 5 ml per seedling too ensure proper spray deposition and to minimise variation in deposition on the first ten leaves. Suspensions were kept agitated using a magnetic stirrer. Seedlings were left to dry for 24 hours at room temperature (± 23°C) before allocating seedlings to the plot area of the rain simulator. The

seedlings were placed in a specific block design pattern within the seedling plot area (1 m^2), and blocks were rotated sequentially for each trial repeat.

Rainfastness of the three treatments was evaluated at different rainfall volumes 1, 5, 10 and 15 mm at a consistent rainfall intensity of 5 mm/h (heavy rain). After rain volumes were simulated, the seedlings were left to dry at room temperature (± 23°C) for 24 h. After drying, ten leaves were sampled from top to bottom of each seedling and fluorescent pigment quantity (FPC%) and Mn-ion residue analyses were conducted as previously described. The experiment was repeated four times, with five replicate trees per treatment. Pigment and Mn-ion was quantified from 10 leaves per tree.

Statistical analysis

Data were subjected to analysis of variance and suitable regression statistics using XLSTAT Version 2015.5.01.23305 (www.xlstat.com). Hoerl regression analyses ($y = Ax^Be^{Cx}$) (Daniel and Wood, 1971) were performed on mean pigment deposition values of the ten leaves per replicate, but with repeats kept separate. Likewise, replicate means of Mn-ion residue and pigment deposition data following sprays with the five mancozeb products were subjected to linear regression statistics against the concentration range sprayed, as well as to Pearson's correlation analyses to demonstrate the linear relation between pigment deposition quantity and mancozeb residue. For pigment quantity regressions, the Y-intercept was fixed at 0, whereas for Mn-ion the Y-intercept was set as the mean of the Mn-ion concentration (46.02 mg/kg_{DW}) quantified in the control leaves.

Particle size (means for 25%, 50% and 90% distribution range) data and the mancozeb persistence data including mancozeb residue (Mn-ion concentration mg/kg_{DW} medians) and pigment deposition quantity (FPC%) data were subjected to appropriate analysis of variance (ANOVA). Fisher's LSD was calculated to identify significant differences between treatments at a 95% confidence interval. The analyses were conducted using SAS statistical software version 8.2 (SAS institute Inc., 1999). For mancozeb persistence, non-linear regression using the means of replicate leaves, but with experimental repeats kept separate, were conducted, using exponential regression models. The models fitted Mn-ion concentration means and percentage Mn-ion loss, whereas medians were used for FPC%. The medians were used for FPC% in order to ignore outliers according to Van Zyl *et al*. (2013).

RESULTS

Deposition assessment

The yellow fluorescent pigment, illuminated with black light, made droplet distribution visible on leaves. With an increase in volume, an increase in droplet size occurred (Fig. 1 A – D), which subsequently resulted in run-off with increasing volumes being applied (Fig. 1 E -

H).The droplet pattern increased in droplet size from the petiole down towards the leaf tip. This can be ascribed to the angle (30°) at which leaves where sprayed that enabled droplets to connect and form larger or elongated droplets towards the leaf tip. Elongated droplets also formed in the midrib of the leaf (Fig. 1).

Point of run-off

Analysis of variance of deposition quantity (FPC%) data indicated a significant treatment × volume interaction ($P < 0.0001$). Hoerl regression analyses for quantitative yellow fluorescent pigment deposition values (FPC%) for the five mancozeb formulations showed good fits on convex curves that revealed deposition trends on apple seedling leaves (R^2: Dithane M-45 800 WP NT = 0.614; Mancozeb 800 WP = 0.640; Pennfluid = 0.760; Ventum 800 WP = 0.563; Vondozeb = 0.787). The FPC% results indicated that deposition quantity increased as the spray volume applied increased until the apex of the curve was reached for most formulations, except for Ventum 800 WP where an increase was predicted up and till 2 ml. Maximum deposition at the apex of the curve for Dithane M-45 800 WP NT and Penfluid was at 1.5 ml, and for Mancozeb 800 WP and Vondozeb it was at 1.75 ml. With further increase in spray volumes, the deposition quantity for these products decreased due to run-off. Therefore, a 1.5 ml run-off point was selected for the comparison of Mn-ion concentration and fluorescent pigment deposition of the five formulations (Fig. 2).

Comparison of manganese ion and fluorescent pigment quantification

Analysis of variance of deposition quantity (FPC%) data indicated a significant treatment × concentration interaction ($P = 0.0102$). Linear regression analyses of the FPC% data of each treatment separately indicated that deposition quantity increased with increase in treatment concentrations with very good fits (R^2: Dithane M-45 800 WP NT = 0.935; Mancozeb 800 WP = 0.893; Pennfluid = 0.921; Ventum 800 WP =0.907; Vondozeb = 0.874). All WP formulations yielded similar slopes, which differed from the slopes of the Pennfluid SC formulation and the fluorescent pigment lines that were less steep (Fig. 3 B).

Analysis of variance of Mn-ion residue data (mg/kg_{DW}) indicated a significant treatment × concentration interaction ($P < 0.0001$). Similar to the linear regression analyses of the FPC% data, the Mn-ion concentration of each formulation increased at increasing concentrations applied. The Mn residue regression lines of all formulations measured with good linear fits (R^2: Dithane M-45 800 WP NT = 0.850; Mancozeb 800 WP= 0.876; Pennfluid = 0.841; Ventum 800 WP = 0.908; Vondozeb = 0.782) (Fig. 3 A). All WP formulations again yielded similar slopes with the Pennfluid SC formulation having a less steep slope (Fig. 3 A).

Pearson's correlation indicated a good linear relationship between FPC% and Mn-ion residue measured ($r = 0.799$).

Particle size analysis

Analysis of variance of particle size (µm) indicated a significant main effect for treatment at each of the evaluated quartiles (25%, 50% and 90%) (P = 0.0001). At 25, 50 and 90% of particle size distribution, Dithane M-45 800 WP NT and Ventum 800 WP had similar mean particle sizes (25%: 0.96 and 0.97 µm; 50%: 1.54 and 1.56 µm; 90%: 2.99 and 2.86 µm respectively), the smallest of all formulations measured. Mancozeb 800 WP and Vondozeb had similar particle sizes, but were significantly larger than those of Ventum 800 WP and Dithane M-45 800 WP NT at 25, 50 and 90% size distribution. Of all the mancozeb formulations, Pennfluid had the largest particle sizes (25%: 2.14 µm; 50%: 3.53 µm; 90%: 5.62 µm). The yellow fluorescent pigment had the largest particle size (25%: 1.67 µm; 50%: 3.99 µm; 90%: 7.12 µm) (Table 2).

Rainfall simulation, distribution and mancozeb wash-off

Rainfall distribution

The three nozzles evaluated (2.8 W, 4.3 W and 5.6 W) at different pressures (200, 250 and 300 kPa) realised different rainfall intensity distributions over the measured plot area. Overall, rainfall intensity decreased from the centre (10-15 mm/h) to the outer range of the rainfall simulator (0-2 mm/h) for all three nozzles at the different pressures (Fig. 4). Furthermore, distribution uniformity varied substantially between the different nozzles at the different application pressures. Nozzle 5.6 W realised the most uniform distribution at 200 kPa at the desired 5 mm/h intensity (Fig. 4 A) of all nozzle/pressure combinations tested. Nozzle 4.3 W and 2.8 W also realised an intensity of 5 mm/h at 200 kPa, but varied more in rainfall distribution over the plot area (Fig. 4 D and G). As pressure was increased, rainfall intensity at the centre of the simulator increased and decreased distribution uniformity. Nozzle 5.6 W and 4.3 W realised the desired 5 mm/h intensity at 250 kPa (Fig. 4 B and E), but varied in rainfall intensity distribution over the plot area. This was also found with nozzle 4.3 W and 2.8 W at 300 kPa (Fig. 4 F and I). Therefore, nozzle 5.6 W was used in all experiments at 200 kPa.

Mancozeb persistence

The effect of rain volume on the persistence of mancozeb on apple seedling leaves were investigated by evaluating (i) deposition quantity (FPC%), (ii) mancozeb residue (Mn-ion concentration), (iii) percentage loss in FPC% and (iv) percentage loss in Mn-ions. Analyses of variance showed that for all four parameters there were no significant interactions for treatment x volume (P > 0.7414). For all four parameters there were significant effects (P ≤

0.0001) for volume of rain applied, but there were no significant effects ($P > 0.2771$) for treatment (Dithane M-45 800 WP NT, Ventum 800 WP, Ventum 800 WP + Nu-Film P).

Exponential regression models were fitted to the means of five replicates for each of the four experiment repeats (Fig. 5). The FPC% and Mn-ion concentration wash-off from apple seedling leaves was accurately explained by the exponential curves (R^2 = 0.651 – 0.849). The initial phases of the lines for FPC% were less steep than those for Mn-ion concentration, indicating somewhat slower wash-off of FPC% than for Mn-ion. In fact, for Mn-ion a significant difference was observed between means at 0 and 1 mm rain, whereas this difference was not significant for FPC% (results not shown). However, the percentage loss in FPC% was higher (39.97-50.01%) at 5 to 15 mm of rain compared to percentage Mn-ion loss (26.32-31.67%) (Fig. 5 A and C), with a maximum loss of 61.4% and 32.2% predicted for FPC% and Mn-ion, respectively.

Acceptable Pearson's correlations were observed between the persistence of pigment quantity (FPC%) and Mn-ion residue measured on leaves (*r*: Dithane M-45 800 WP NT = 0.729; Ventum 800 WP = 0.726; Ventum 800 WP + Nu-Film P = 0.783).

DISCUSSION

Obtaining sufficient fungicide deposition (quantity and quality) is essential for effective disease control of plant pathogenic fungi. The assessment of fungicide deposition is expensive in research projects if fungicide residues must be quantified, whereas fluorescent dyes combined with macrophotography and image analyses offers an affordable solution to this problem (Van Zyl *et al.*, 2013). Therefore, in this study the suitability of a yellow fluorescent pigment as tracer for mancozeb residues (Mn-ion concentration) was investigated. The fluorescent pigment was shown to be a very good tracer for five different mancozeb formulations, since Pearson's correlation analyses showed a very good linear relationship (r = 0.779) between FPC% and Mn-ion concentration. Therefore, orchard spray trials for the evaluation of the effect of spray volumes and machines can be assessed using yellow fluorescent pigment deposition (FPC%). As pigment deposition was indicative of mancozeb deposition, further experimentation to develop benchmarks indicative of the biological efficacy of deposition quantity and applications will be important.

Particle size has been shown to influence the retention of fungicides such as copper (Schutte *et al.,* 2012), but this was not evident in the current mancozeb study. A comparison of the particle size ranges of five mancozeb formulations showed that the WP formulations Dithane M-45 800 WP NT and Ventum 800 WP had significantly smaller particle sizes than Mancozeb 800 WP and Vondozeb as well as the SC formulation Pennfluid. The slopes of linear regression lines for Mn-ion residues against concentration, however, showed no differences between the depositions and retention of the WP formulations. The slope of the

Pennfluid line had a markedly less steep line, indicating a slower increase in mancozeb deposition than the other products. However, this can be expected for Mn-ion concentration since the amount of active ingredient that must be applied according to the label of the product is less than that of the other products, which resulted in only 0.00084 g *a.i*./ml being applied for Pennfluid as opposed to 0.0012 g *a.i*. /ml for the WP formulations. It is interesting to note that the active ingredient label rates of all SC formulations (Pennfluid, Multizeb, Newzeb and Unizeb) registered in South Africa are also less than that of WP formulations, and in a similar range than that of Pennfluid. Whilst a lower active ingredient content will explain the lower Mn-ion residues measured in the Pennfluid treatments, the correspondingly flatter slope for this treatment's FPC% line is anomalous, as the same pigment concentrations were used as for the other treatments. Since the Penfluid's pigment FPC% line was also markedly flatter than those of the WP formulations, this anomaly might be attributed to differences in FPC% deposition assessment resulting from WP+SC, SC+SC and SC formulations; keeping in mind the pigment is an SC formulation. In research on adjuvant formulations (J.G. Van Zyl, unpublished research), similar anomalous findings were observed, and it was hypothesized that these might be attributed to variable effects of adjuvants on deposition quality and indicative of insensitivity of the deposition assessment particularly of very small pigment particles.

The rainfastness of mancozeb formulations in the current study was evaluated using simulated rain. This had the advantage that specific rain volumes (mm) and intensities (mm/h) could be evaluated. The rain volumes evaluated in the current study were similar to those evaluated by Hunch *et al.* (2006 a, b), but only one rain intensity (5 mm/h) was evaluated since torrential rain (48 mm/h) does not often occur in the Western Cape region during the apple growing season. Although light rain intensity (0.5 mm/h) is also important in the Western Cape region, this was not evaluated in the current study due to limitations in space and time.

A review of rain simulation literature was required to provide information for the development of a feasible rain simulator, since there is no standard available of the numerous rain simulators built over the past 62 years (Iserloh *et al*., 2012, 2013). The rainfall simulator was thus designed and built using available resources, and considering similar rainfall simulators designed and build over the years (Tossell *et al.,* 1987; Pérez-Latorrefor *et al.,* 2010; Iserloh *et al*., 2012, 2013). Distribution of simulated rainfall was not uniform within the seedling testing area (1 m^2) of the rainfall simulator for several of the tested nozzle sizes that were evaluated at different pressures, but with a 5.6 W nozzle at a pressure of 200 kPa a large and uniform distribution at the required rainfall intensity (5 mm/h) was obtained. The variability in rainfall volume and intensity that was identified through measurements within

the experimental area of the simulator was compensated for by using a rotating block design of treatment replicates in the four experiments repeated over time.

The persistence of mancozeb to different rain volumes (0, 1, 5, 0 and 15 mm) showed a good Pearson's correlation between deposition quantity (FPC%) and Mn-ion concentration (r = 0.726– 0.783) for all three formulation treatments (Dithane M-45 800 WP NT, Ventum 800 WP and Ventum 800 WP + Nu-Film P). Schutte *et al.* (2012) observed similar correlation values between FPC% and copper residues on mature orange leaves when evaluating copper persistence for a prolonged period under natural orchard conditions. Although a good correlation existed between FPC% and Mn-ion concentrations, conclusions on the persistence of mancozeb at different rain volumes applied was somewhat different between FPC% and Mn-ion loss. This was due to no significant loss occurring for FPC% after the application of 1 mm of rain, whereas for Mn-ions, significant loss did occur at this rain volume. Furthermore, as the volume of rain applied increased from 1 mm to 15 mm, the FPC% overestimated the percentage loss (9.47% - 50.01%) compared to Mn-ion percentage loss (11.95 – 31.67). Nonetheless, aside for differences in the initial wash-off levels and different asymptotes indicating maximum loss levels, similar behaviour of FPC% and Mn-ion residue to different rain volumes was evident from exponential regression models. Therefore, deposition quantity of the fluorescent pigment can be used as a fair measurement for investigating the persistence of mancozeb to different rain volumes, but for detailed information, it is more appropriate to consider mancozeb residues. Due to the low cost associated with deposition quantification with the fluorescent pigment, it will be valuable for observing trends in rainfastness in future studies, also under orchard conditions. The fluorescent pigment used in this study is photo-stable and has the advantage that plant tissue can still be evaluated for deposition quantity after a 56 day period (Schutte *et al.*, 2012).

The rainfastness of Dithane M-45 800 WP NT and Ventum 800 WP were evaluated at a 5 mm/h rain intensity and five different rain volumes (0, 1, 5, 10 and 15 mm). Dithane M-45 800 WP NT was of particular interest since it is marketed as having superior rainfastness, which sometimes is also associated with increased product cost that is an important consideration for growers. Ventum 800 WP, which is not marketed as having superior rainfastness was selected as a comparable formulation of Dithane M-45 800 WP NT, since the particle size of the two formulations did not differ significantly. The persistence of mancozeb residue (Mn-ions) and deposition quantity (FPC%) of all three treatments did not differ significantly from each other at any of the rain volumes that were applied. Therefore, the effect of rain volume on persistence of mancozeb could be evaluated as the average of all three treatments. The percentage loss in mancozeb residue in the current study at 1 mm and 5 mm rain volume applied at 5 mm/h rain intensity was less (5% and 19%, respectively)

than the respective 55% and 80% reported by Hunsche *et al.* (2006b) at the same rain volume and intensity on apple seedling leaves. The current experiments did not find a significant difference in percentage Mn-ion loss as rain volume increased from 1 mm to 15 mm. This agrees with results of Hunsche *et al.* (2006b) in that higher rain volumes cause little additional removal of mancozeb, although our percentage loss (27 – 30% at 10 -15 mm rainfall) at these rain volumes were less than that reported (80 – 90% at 10 – 15 mm rainfall) by Hunsche *et al.* (2006b).This can be due to the particle size of formulations used in the current study being smaller than that of formulations used by Hunsche *et al.* (2006b), since Kudsk *et al.* (1991) have found that smaller mancozeb particles had increased rainfastness. Particle size differences could also explain why the Hunsche *et al.* (2006b) model differed from the current in that a high percentage of loss was already observed after 1 mm, which is evident in the sharp decline in the graphs after 1 mm of rain. Our results on the persistence of mancozeb to rain rather agrees with that of Carbas *et al.* (2001) on vineyard leaves. Carbas *et al.* (2001) found that mancozeb residue loss was only 20% after 45 mm of torrential rain (60 mm/h). It is, however, difficult to compare rainfastness between crops, since it might be crop specific. Kudsk *et al.* (1991) investigated mancozeb rainfastness on pea and potato in the same study and found that 60 and 30% loss occurred respectively after 3 mm of rainfall at an intensity of 27 mm/h. Similarly at 9 mm of rainfall at intensity of 27 mm/h a loss of 70 and 50% was observed for pea and potato, respectively (Kudsk *et al.,* 1991).

Although adjuvants have been shown to increase the persistence of mancozeb to rain on apple seedlings (Hunsche *et al*., 2006a), this was not observed in the current study. The addition of Nu-Film P to Ventum 800 WP did not result in significantly higher Mn-ion residues after application of any of the rain volumes. Nu-Film P is a sticker and spreader type of adjuvant compared to the hydrophobic (Rapeseed Oil Ethoxylates 5) and hydrophilic (Rapeseed Oil Ethoxylates 60) types of adjuvants evaluated by Hunsche *et al.* (2006a), which increased the rainfastness of mancozeb on apple from 6% to 22% (Rapeseed Oil Ethoxylates 5). Future studies should evaluate this type of adjuvant on apple seedlings for improving mancozeb rainfastness. Kudsk *et al*. (1991) specifically evaluated Nu-Film P and found that it did increase the rainfastness of maneb WP, which is also a dithiocarbamate fungicide like mancozeb, on pea and potato at a rain volume of 9 mm and rain intensity of 27 mm/h. This could be due to differences in the rate of Nu-Film P applied between their (2.5 ml/L) and the current study (0.3 ml/L).

To conclude, the yellow fluorescent pigment is an excellent tracer for mancozeb deposition on apple seedling leaves, and will be helpful in identifying trends on the effect of rain on the persistence of mancozeb. The protocol developed for evaluation of rainfastness for mancozeb using the yellow fluorescent pigment, can be a more cost effective and faster

way to evaluate the rainfastness of mancozeb formulations on apple leaves in orchard experiments. We observed no significant differences in the rainfastness of two mancozeb formulations with similar particle sizes, nor following the addition of a sticker-spreader adjuvant. However, since significant differences were shown in particle size of five mancozeb formulations, future studies should investigate formulations containing different particle sizes to access if particle size will influence persistence. Particle size of fungicides may influence fungicide persistence (Kudsk *et al*., 1991; Schutte *et al*., 2012). It will also be important to evaluate torrential rain intensities to ensure that the rainfastness of different formulations do not differ. Based on results from Hunsche *et al.* (2006b) this can be expected since high and torrential rain intensities showed similar Mn-ion loss patterns. In future studies, the evaluation of mancozeb persistence on apple fruit to rain will also be important, since fruit lesions cause significant economic losses. Mancozeb persistence on fruit may differ from that on leaves since Cabras *et al.* (2001) found that in vineyards mancozeb rainfastness was lower (62%) on grapes than on leaves (89%).

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Table 1. Properties of mancozeb formulations evaluated.

Treatment	**Formulation**	**Registration holder**	***a.i.*[a] g/kg or ml/L formulation (Mancozeb)**	**Label recommended dosage (ml or g/100 L)**	**Amount of a.i.[a] applied per 1 ml spray volume (g/ml)**
Dithane M-45 800 WP NT	Wettable Powder	Dow AgroScience, Bryanston, South Africa	800	150	0.0012
Mancozeb 800 WP	Wettable Powder	ARYSTA LifeScience La Lucia Ridge, South Africa	800	150	0.0012
Pennfluid	Suspension Concentrate	TOTAL South Africa(Pty)Ltd, Rosebank, South Africa	420	200	0.00084
Ventum 800 WP	Wettable Powder	Plaaskem(Pty)Ltd, Witfield, South Africa	800	150	0.0012
Vondozeb	Wettable Powder	ARYSTA LifeScience La Lucia Ridge, South Africa	800	150	0.0012

[a]Active ingredient.

Table 2. Mean particle size (µm) at 25%, 50% and 90% distribution of different mancozeb formulations and yellow fluorescent pigment.

	Particle size (µm)[a]		
Treatment	**D 25%**	**D 50%**	**D 90%**
Yellow fluorescent pigment	1.67 b	3.99 a	7.12 a
Dithane M-45 800 WP NT	0.96 d	1.54 d	2.99 d
Mancozeb 800 WP	1.14 c	1.84 c	3.63 c
Pennfluid SC	2.14 a	3.53 b	5.62 b
Ventum 800 WP	0.97 d	1.56 d	2.86 d
Vondozeb	1.16 c	1.92 c	3.79 c

[a] For each parameter separately, values in each column followed by the same letter do not differ significantly ($P > 0.05$) according to Fisher's least significant difference test.

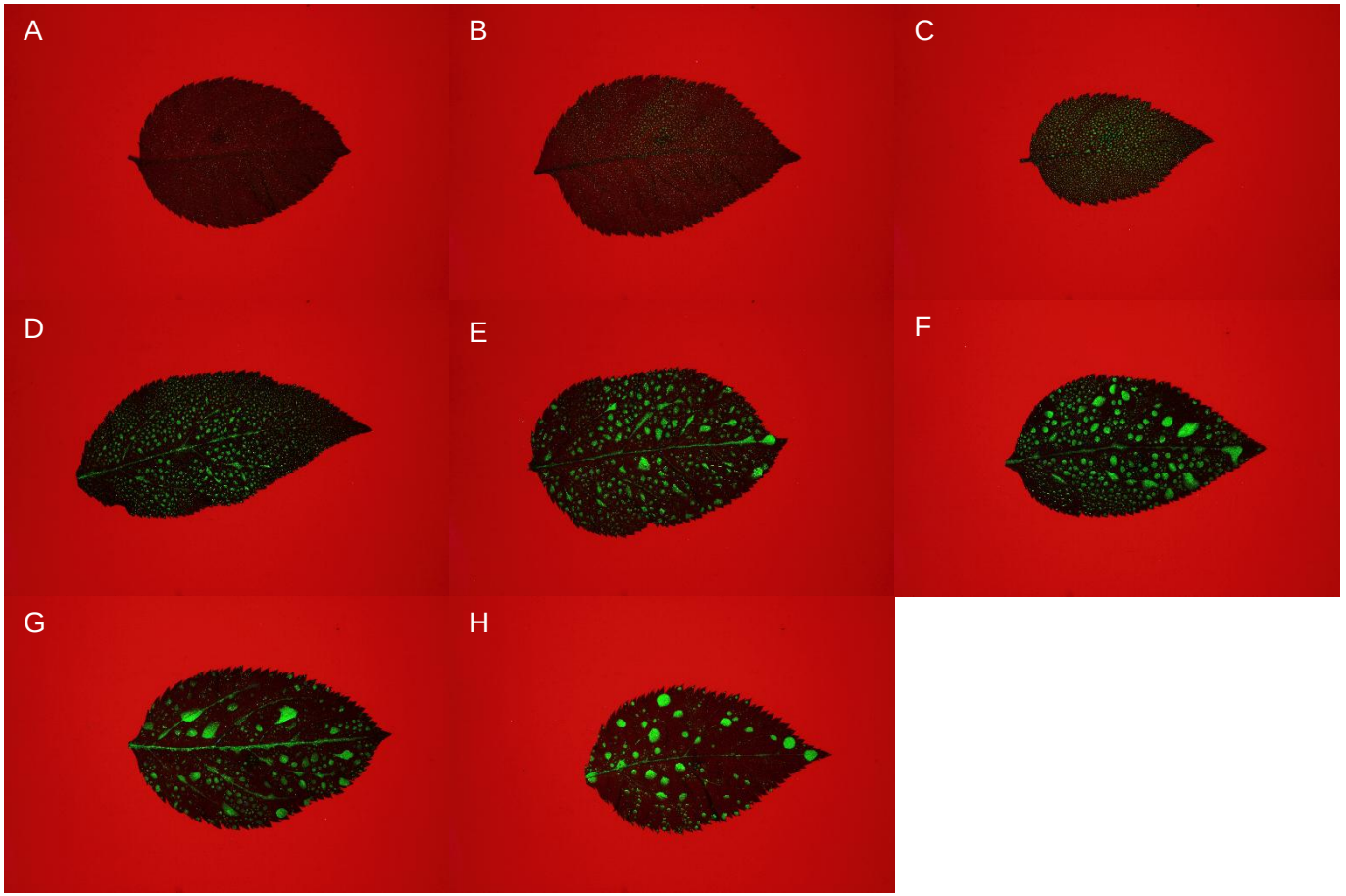


Figure 1. Digital images of the upper leaf surfaces of Golden Delicious apple seedlings sprayed with different volumes of mancozeb formulations combined with a yellow fluorescent pigment, followed by illumination with a black light. The occurrence of increasing droplet size and run-off is evident as spray volume increased from 0.25 (A) to 2.00 ml (H). Other volumes that are shown include 0.50 (B), 0.75 (C), 1.00 (D), 1.25 (E), 1.50 (F) and 1.75 ml (G).

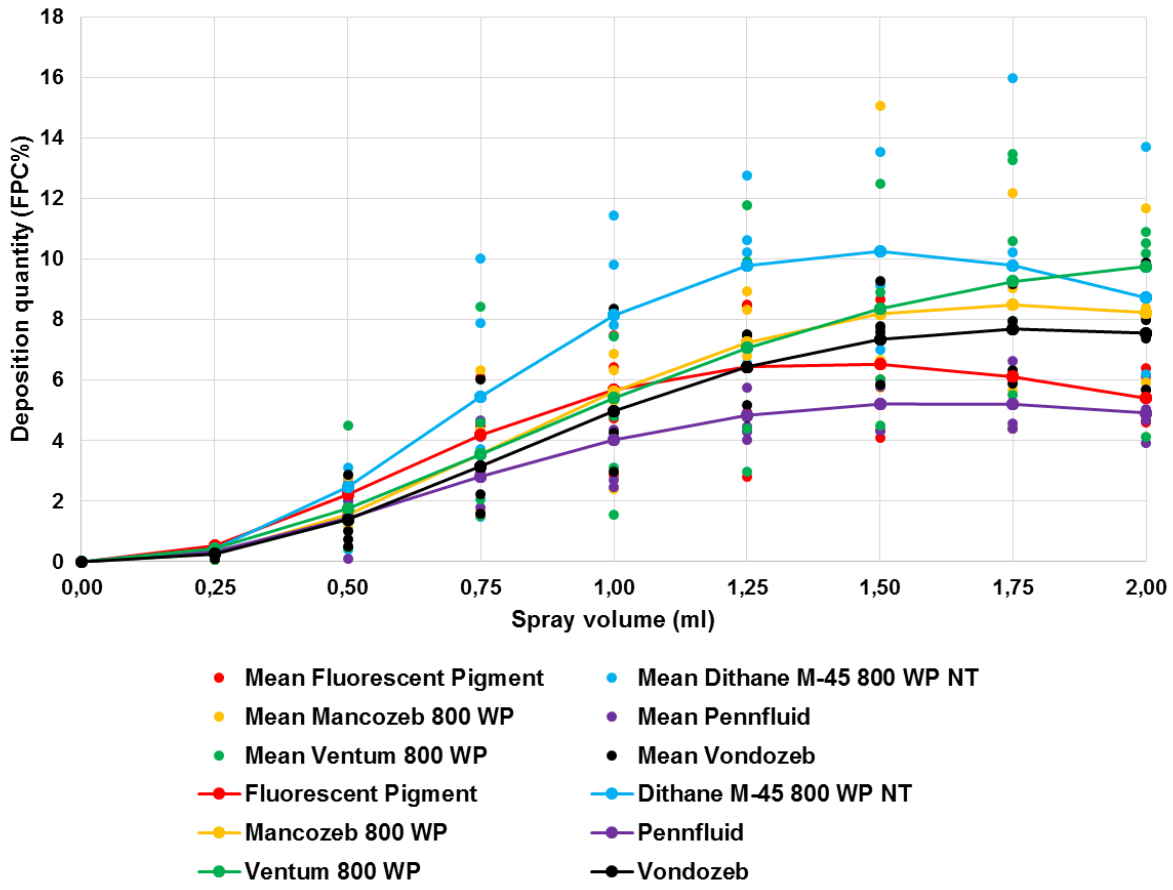


Figure 2. Deposition quantity (FPC%) as predicted by Hoerl regression curves ($y = Ax^Be^{Cx}$) on means (10 leaves per treatment replicate) realised after different spray volumes of mancozeb formulations at 1× concentrations were sprayed to determine point of run-off on apple seedling leaves. Parameters and goodness of fit for treatments were Yellow Flourescent Pigment (A = 40.956; B = 2.778; C = -1.975; R^2 = 0.665); Dithane M-45 800 WP NT (A = 76.799; B = 3.338; C = - 2.245; R^2 = 0.614); Mancozeb 800 WP (A =34.143; B = 3.153; C = -1.804; R^2 = 0.640); Pennfluid (A = 21.080; B = 2.677; C = -1.655; R^2 = 0.760); Ventum 800 WP (A = 15.672; B = 2.388; C = -1.065; R^2 = 0.563); and Vondozeb(A = 27.459; B = 3.063; C = -1.707; R^2 = 0.787).

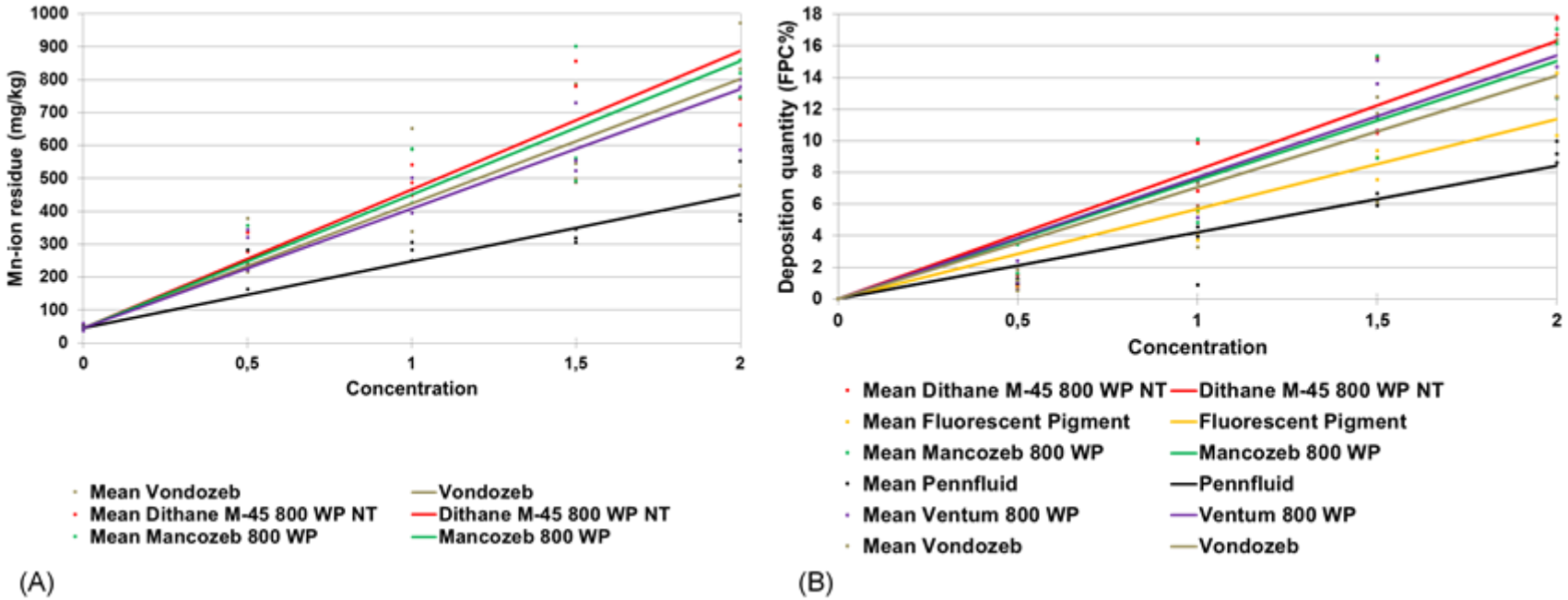


Figure 3. Linear regression of the deposition quantity between a concentration range of (A) mancozeb residue (Mn-ion (mg/kg_{DW})) of five mancozeb formulations (R^2: Dithane M-45 800 WP NT = 0.850, Ventum 800 WP = 0.908, Vondozeb = 0.782, Mancozeb 800 WP = 0.876 and Pennfluid = 0.841) and (B) the deposition quantity of a fluorescent pigment (FPC%) combined with the five formulations (R^2: Dithane M-45 800 WP NT = 0.935, Fluorescent Pigment = 0.929, Ventum 800 WP = 0.907, Vondozeb = 0.874, Mancozeb 800 WP = 0.893 and Pennfluid = 0.921).

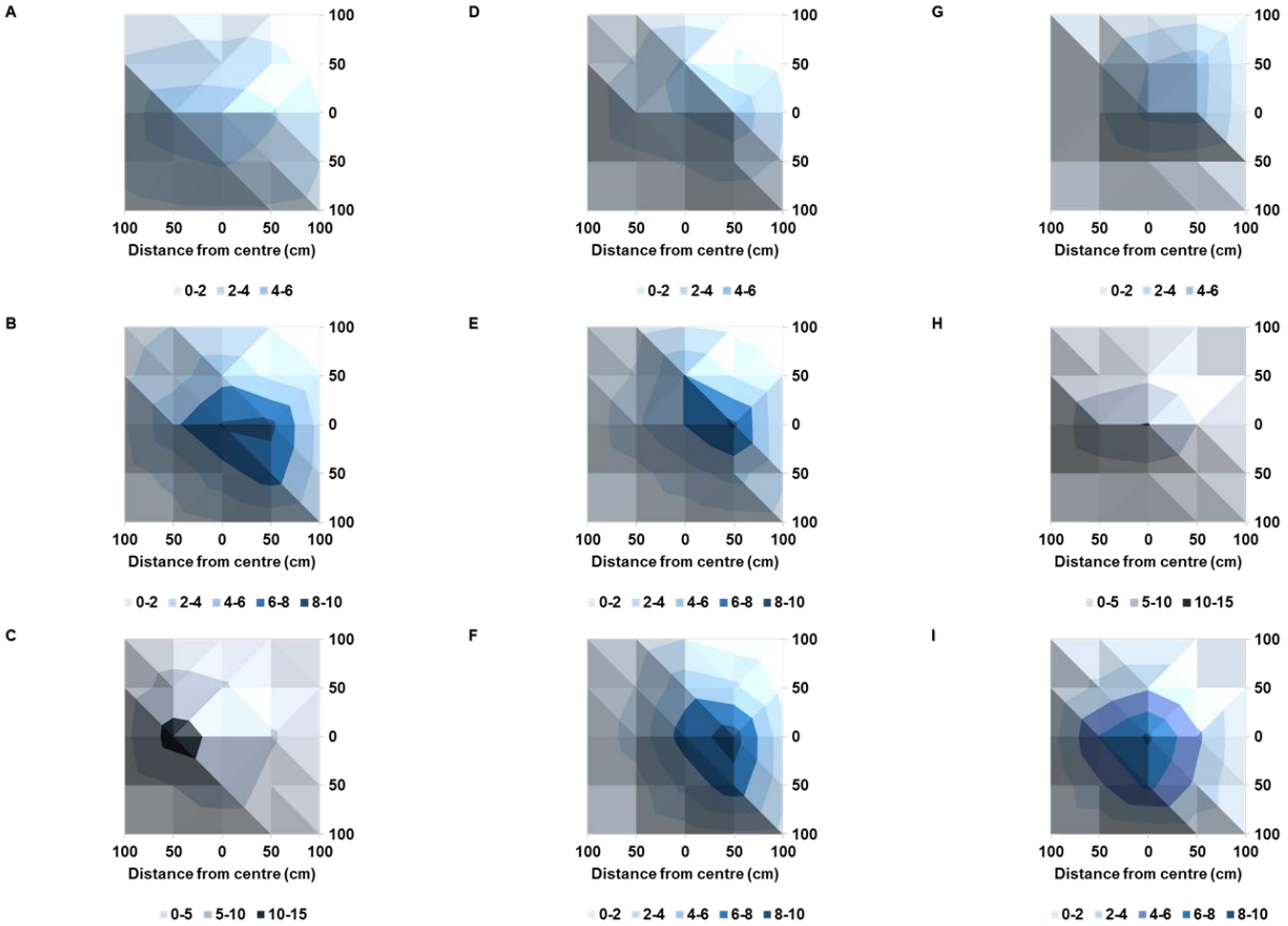
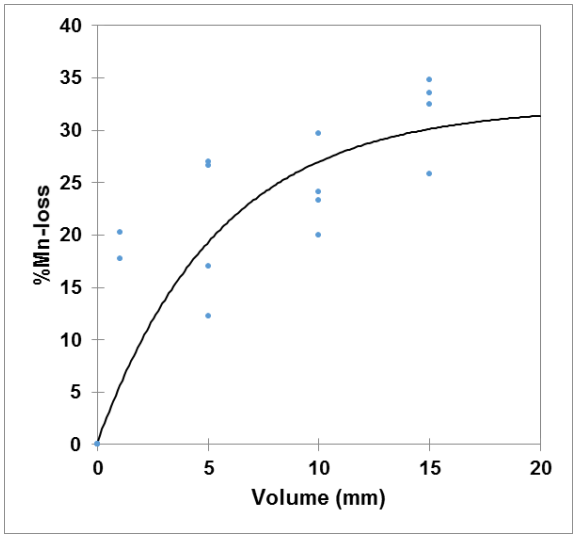
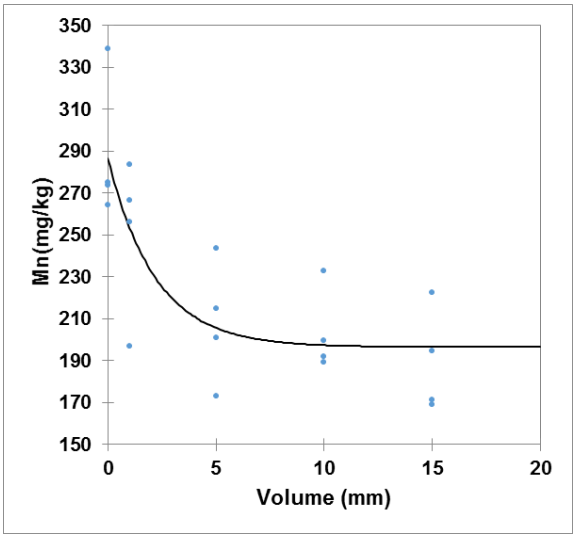


Figure 4. Distribution of rainfall (mm/h) in a rainfall simulator area of 1m^2, as influenced by nozzle size and pressure. The distributions are shown for (A-C) nozzle 5.6 W at 200, 250 and 300 kPa, (D-F) nozzle 4.3 W at 200, 250 and 300 kPa, and (G-I) nozzle 2.8 W at 200, 250, 300 kPa.



(A)



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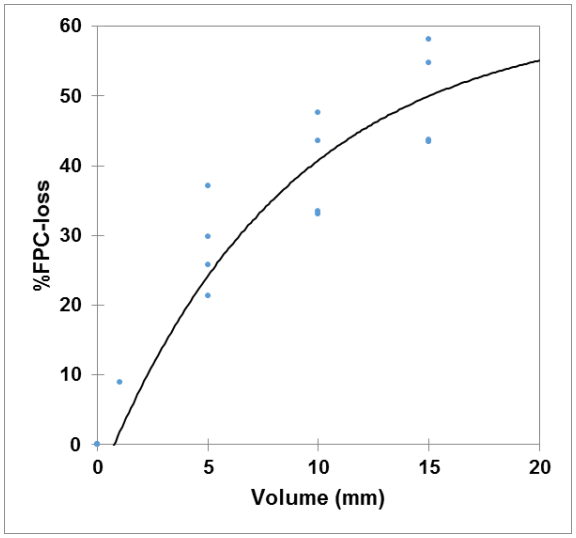
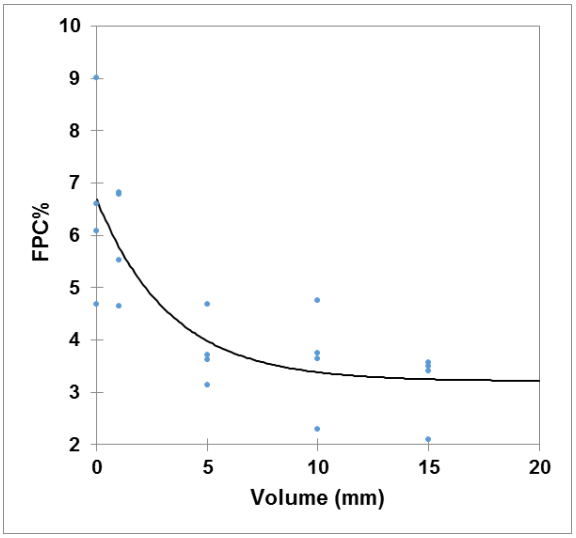


(C)



(D)

Figure 5. Means and predicted mancozeb residue (Mn-ion concentration (g/kg_{DW})) loss (A) and percentage fluorescent particle coverage (FPC%) loss (C), as well as Mn-ion concentration (B) and FPC% quantity (D) following rain volumes (0, 1, 5, 10 and 15 mm) applied at a high rain intensity (5 mm/h) on apple seedling leaves. Parameters and goodness of fit coeffiecients for exponential regression models ($y = Ae^{Bx}+C$) of treatments were: %Mn-loss (A = -31.886, B = -0.181,C = 32.236, R^2 = 0.722); Mn (mg/kg) (A = 90.095, B = -0.456,C = 196.521, R^2 = 0.651); FPC%(A = 3.472, B = -0.301,C = 3.211, R^2 = 0.660) and %FPC-loss (A = -67.073, B = -0.118,C = 61.401, R^2 = 0.849).

CHAPTER 3

Towards the development of mancozeb spray deposition benchmarks for apple scab control on apple leaves

ABSTRACT

Venturia inaequalis is an important disease of apples world-wide and causes scab-like lesions on fruit and defoliation of trees. The disease is managed through fungicide sprays that mainly consist of the contact fungicide mancozeb that is used in a preventative control strategy, with 12 to 14 sprays being applied per season in South Africa. This study investigated the development of a deposition benchmark model indicative of the biological efficacy of mancozeb spray deposition quantity for controlling *V. inaequalis* infections on apple seedling leaves. A yellow fluorescent pigment that is known as a good tracer of mancozeb was used along with photomacrography and digital image analysis to determine mancozeb deposition quantity on apple seedling leaves treated with a concentration range of mancozeb (0, 0.15 ×, 0.3 ×, 0.45 ×, 0.6 × and 1.0 ×). *Venturia inaequalis* control at the range of deposition values obtained (0.29 to 8.28 percentage fluorescent pigment coverage (FPC%) was investigated using a basic fuschin based staining technique that yielded distinct pin-point lesions 6-days after inoculation; these lesions were quantified through digital image analysis. Additionally, disease control was also assessed visually by allowing natural lesion development for 3-4 weeks. Based on staining and image analyses, 58.9% control was achieved at the lowest mancozeb concentration (0.15 ×), which did not differ significantly from control obtained with the other mancozeb concentrations (0.3 ×, 0.45 ×, 0.6 × and 1.0 ×). However, disease control assessed through natural visual lesion development showed that the staining technique was underestimating control since only the unsprayed control treatment developed lesions after 3-4 weeks, and none of the mancozeb concentration yielded any lesions, i.e. 100% control was achieved. Consequently, no function could be fitted to deposition quantity data (FPC% values) versus disease control (staining or visual assessment). The amount of inoculum collected from naturally infected orchard leaves was a limitation in the number of experiments that could be conducted during benchmark model investigations. Therefore, a cellophane agar plate technique was optimized for the *in vitro* production of *V. inaequalis* conidia that can be used in future infection studies. It was shown that the optimal time for conidium production on cellophane agar plates was 1 week after inoculation of plates, since spore concentrations and spore viability was significantly higher at this time point than at 2 to 4 weeks after inoculation for both of the investigated isolates. The optimized technique yielded 2 ml of approximately 1.59 $\times 10^6$ conidia/ml suspension per 90 mm dia. petri plate.

INTRODUCTION

Apple scab, caused by the fungus *Venturia inaequalis,* is an economically important disease of apples world-wide. The pathogen reduces the quality of fruit due to the production of scab-like lesions, whereas lesions on leaves reduce the photosynthetic ability of the tree and can result in tree defoliation in severe cases (MacHardy, 1996; Aylor, 1998; Carisse and Rolland, 2004). The pathogen produces sexual- (ascospores) and asexual (conidia) spores (MacHardy, 1996). Ascospores are the primary inoculum that are released from leaf litter in spring when weather conditions permit (Gadoury *et al*., 1984, 2004; MacHardy, 1996). Ascospores are only produced on leaf litter, and can infect fruit and leaves. Conidia consitute the secondary inoculum source and are produced on leaves and fruit throughout the season, thus contributing to the epidemic nature of the disease (MacHardy, 1996; Aylor, 1998; Charest *et al*., 2002; Carisse and Jobin, 2006). In South Africa, pygmy fruit formed late in the previous season can contain lesions with conidia that can also be a primary source of inoculum for the next season (Von Diest, 2014).

Management of apple scab is mainly based on regular fungicide applications (MacHardy, 1996). The main fungicide used in South Africa, is the contact fungicide mancozeb. Approximately 22 different mancozeb products [suspension concentrate (SC), water dispersible granule (WG) and wettable powder (WP) formulations] are registered on apples in South Africa (Agritel, www.agritel.co.za). A large number of mancozeb applications, ranging from 12 to 24, are made each season in orchards to control apple scab infections (personal communication J.P.B Wessels, ProCrop Trust, Ceres, South Africa). This is due to new susceptible flush being produced continually, which must be protected against the pathogen. Re-application is also often required after a certain amount of rain has fallen (Ellis *et al.*, 1984; Jamar *et al.*, 2010). The quantity of mancozeb deposition required to protect leaves and fruit against apple scab infection is unknown.

Effective disease control in orchards is highly dependent on the quantity and quality of fungicide deposition on leaves, which can be assessed using various methods. Using fluorescent dyes, fungicide deposition can be determined with “visual grading” techniques that has been improved over the years (Palladini, 2005; Brink *et al*., 2006; Fourie *et al*., 2009). Photomacrographic imaging of yellow fluorescent dye deposition combined with image analyses is one of the more recently developed methods that has been used effectively to study spray deposition on biological targets (Van Zyl *et al*., 2013). On apple seedling leaves, it has been shown that the yellow fluorescent pigment dye is a good tracer of mancozeb and that it can be used to effectively quantify mancozeb deposition (Chapter 2). Data on the quantity and quality of fungicide deposition can be used in a more

meaningful manner if it is linked to biological efficacy. Fungicide spray deposition bench mark models have been developed following laboratory spray trials and can assist in interpreting the potential bio-efficacy of different spray volumes, adjuvants and spray machines (Brink *et al*., 2006; Van Zyl *et al*., 2010; Van Zyl *et al*., 2013).

Artificial inoculation studies with *V. inaequalis* are difficult since visible apple scab symptoms on leaves are slow to develop, and an ontogenic resistance exists for leaves (MacHardy, 1996). Optimal infection stages for ascospores and conidia on leaves are when the leaves are between 3 to 5 days old and 1 to 3 days old, respectively (Schwabe, 1979). With an increase in leaf age, a decrease in colony size can also occur (Aylor and Kiyomoto, 1993; Li and Xu, 2002). Visual symptoms can occur on apple leaves between 7 to 36 days after infection, depending on the susceptability of the cultivar. Visual symptoms occur faster on more susceptible cultivars than on more resistant cultivars (Yepes and Aldwinckle, 1993b; Bénaouf and Parisi, 1998; Li and Xu, 2002). Other factors that can have an effect on apple scab development and severity include wind, rain (Sutton *et al.,* 1976; Gadoury *et al.,* 1984; Aylor, 1998; Charest *et al.,* 2002), relative humidity (RH) (Schwabe, 1977), sunlight (UV) (Aylor and Sanogo, 1997), temperature and leaf wetness (Schwabe, 1980; MacHardy and Gadoury, 1989; Hartman *et al.,* 1999).

Venturia inaequalis is a hemibiotrophic fungus that grows very slow on artificial media and does not produce conidia readily on media (Steiner and Oerke, 2007). Therefore, using axenic conidial solutions for inoculation of plant material for developing benchmark models can be challenging. Two *in vitro* methods that have been used for conidium production include a cellophane- and wick method. The cellophane technique consists of conidia being produced on cellophane membrane disks that are overlain onto artificial agar media in petri plates (Parker *et al*., 1994, Gau *et al*., 2004; Bus *et al*., 2005). The wick technique consists of a bottle with a small amount of medium containing a piece of muslin or cheese cloth within it, so that the cloth acts as a wick onto which conidia are produced (Keitt and Palmiter, 1938; Roberts and Crute, 1994; Cronin *et al*., 1996). Studies that have used these techniques most often do not report the viability of the spores or the volume of inoculum that can be produced; only the concentration of spores is reported. These aspects are all important to investigate when large amounts of viable inoculum are required for infection studies.

An alternative to using axenic conidial solutions, is the use of conidia washed from symptomatic apple leaves. Symptomatic leaves can be collected in orchards, and these can be stored for prolonged periods between paper towels at -18 to -20°C (Stensvand *et al.,* 1997; Steiner and Oerke, 2007). The disadvantage of the method is that leaves must often be collected from fungicide sprayed orchards, which may reduce the viability of conidia. Furthermore, a mixture of unknown *V. inaequalis* genotypes is used, which in some instances may affect the consistency of experimental outcomes. Alternatively, conidia can be

produced on apple seedlings where a conidial suspension is spray inoculated onto apple leaves followed by incubation at high humidity for 48 h (Steiner and Oerke, 2007). However, this method has limitations since extra glasshouse space and a room with high humidity is required to ensure consistent infections. Due to the ontogenic resistance of leaves, where only a small fraction of the available leaves are susceptible, several seedlings or larger trees must be inoculated to produce adequate amounts of inoculum. Furthermore, the success rate can be variable if attempted by inexperienced researchers.

The main aim of the study was to develop a spray deposition benchmark model for mancozeb that predicts control of *V. inaequalis* on apple leaves from spray deposition parameters. For model development, the source of inoculum for inoculation of apple seedlings consisted of conidia harvested from symptomatic orchards leaves. This approach, however, limited the number of inoculations that could be made from the same batch of leaves, and the number of experiments that could be conducted. Therefore, to facilitate further model development, the *in vitro* production of conidia using a cellophane agar plate method was optimized.

MATERIALS AND METHODS

Deposition benchmarks indicative of apple scab control

Plant material

Golden Delicious apple seedlings (*Malus domestica* Bork h.) (12-18 months old) were used in all experiments. The seedlings were produced and grown in a glasshouse as described in Chapter 2. The youngest flush of seedlings were used in experiments, after which the seedlings were pruned back to stimulate new flush formation.

Spray application

Apple seedlings were sprayed with a gravity fed spray mist gun (ITW DEVILBISS Spray Equipment Products, USA) with a fluid nozzle of 1.5 mm in diameter at a pressure of ± 160 kPa. Seedlings were sprayed with a mixture of yellow fluorescent pigment [South Australian Research and Development Institute (SARDI) Yellow Fluorescent Pigment, 40% EC (SARDI, Loxton, South Australia) 1 ml/L] and mancozeb (Dithane M-45 800 WP NT; Dow AgroScience, Bryanston, South Africa; 1.5 g/L) at different concentrations (0× [Control], 0.15×, 0.3×, 0.45×, 0.6×, 1.0×). Each seedling was sprayed at a pre-determined volume of 5 ml per seedling to ensure proper spray deposition and minimise variation in deposition on marked leaves. Suspensions were kept agitated using a magnetic stirrer. The spray gun was cleaned with 70% ethanol and air-dried after each treatment. Seedlings were left to dry for 24 hours at room temperature (± 23°C). For each treatment, there were nine replicates, with each replicate consisting of one seedling. Three replicates of each treatment were used for

spray deposition analysis, whereas the remaining six replicates were used for inoculation with *V. inaequalis.* For inoculations, three of the six replicates were used for evaluating pathogen infection using a staining technique (as described below), and the other three replicates were used for visual assessment (lesion formation and sporulation) of pathogen infection. For each replicate, only the leaves that flushed in 5 days, i.e. 1 to 5 day old leaves, were evaluated for spray deposition and infection. The number of leaves used for each replicate consisted of the number of leaves that flushed in a 5 day period, which was used as block replicates in data analyses. The 1 to 5 day old leaves were marked on apple seedlings using small multi-coloured rubber bands. With each new flush that occurred, the leaf petioles of completely unfolded leaves were marked with a specific coloured rubber band. The exact age of each leaf was therefore recorded on the day of inoculation. The experiment was repeated four times.

Deposition analysis

Sprayed leaves from selected seedlings for deposition assessment were detached and transported to a dark room on trays. A spray deposition analysis protocol described by Van Zyl *et al.* (2013) with slight modifications was used to determine spray deposition parameters on leaves. Each leaf was positioned individually in the middle of a back-illuminated red Perspex box (300 × 210 × 110 mm) and covered with a glass pane (200 × 200 × 2 mm), ensuring that the leaf edges did not fold. The leaf was illuminated in stereo using two ultra-violet light sources (UV-A at ≈ 365 nm; Labino Mid-Light; www.labino.com). Digital photos were taken of the upper leaf surfaces using a Canon EOS 40D camera equipped with a 60 mm macro lens attached to a tripod directly above the leaf. Raw image files were converted to 8-bit Exif TIFF files using Digital Photo Professional version 3.1.0.0 (CANON INC.; www.canon.com). Image Pro Plus software (Image Pro Plus software version 7.2; Media Cybernetics, www.mediacy.com) was used to determine the deposition quantity of fluorescent particles per leaf, measured as percentage fluorescent particle coverage (FPC%) (Van Zyl *et al.*, 2013).

Spray inoculation of apple seedlings with V. inaequalis *conidia*

Conidia washed from symptomatic orchard leaves were used to inoculate seedlings. The leaves were stored between moist paper towels at -80°C prior to use. After thawing the leaves, conidia were washed from the leaves using a 1 ml pipet. A droplet of distilled water was pipetted onto a lesion and repeatedly pipetted up and down to dislodge the conidia. The conidia were pipetted into a glass container and quantified using a haemocytometer, and diluted to 2×10^5 spores/ml. Viability of conidia was evaluated for each trial by spraying the

spore suspension onto water agar petri dishes, followed by incubation of the plates at room temperature (± 23°C) for 24 h. The number of germinated conidia from the total conidia was calculated. For each trial the viability of the conidia was in the range of 80%. *Venturia inaequalis* spores suspensions were sprayed onto apple seedling leaves (1 to 5 days old) at ± 0.4 ml per leaf using a gravity fed spray mist gun to obtain a uniform and consistent drop distribution just before run-off (± 200 kPa, fluid nozzle diameter 0.8 mm).

Inoculated seedlings were placed in a humidity chamber at a temperature of 17°C for 48 h at a relative humidity of 99 – 100%. The seedlings were then transferred to a glasshouse at ± 23°C, and infection and disease severity were evaluated using a staining or visual assessment method.

Staining of inoculated leaves for disease severity quantification

The leaves of seedlings were evaluated for infection 6 days after inoculation (2 day wetting period and 4 day infection period) to determine the number of infection points. Inoculated leaves were detached from the seedlings and rinsed in deionised water for 5 min. Staining was done as described by Preece (1959). This consisted of leaves first being placed in a 1% sodium periodate ($NaIO_4$) solution for 5 min, followed by rinsing in deionised water for 5 min. Subsequently, the leaves where placed for 5 min in decolorized basic fuchsin ($C_{20}H_{20}ClN_3$). The decolorized basic fuchsin solution was prepared by dissolving 1 g basic fuchsin in 200 ml deionised water, which was autoclaved. Next, 20 ml of 1 N hydrogen chloride (HCl) was added and mixed, followed by the addition of 1 g of potassium disulphide ($K_2O_5S_2$) that was dissolved and left to stand overnight. The solution developed a straw-colour. After the decolorized basic fuschin solution step, leaves were placed for 5 minutes in a sulphurous acid solution [10 ml 10% potassium disulfite ($K_2O_5S_2$) solution plus 10 ml of 1 N HCl dissolved in 200 ml distilled water]. In the final step, leaves were rinsed for 5 minutes in deionised water and placed in Petri-dishes with deionised water for examination (Preece, 1959). Care was taken to regularly replace each of the solutions during staining, which ensured consistent staining results. Infection points were visible as pink to purple pinpoints on the leaves (Fig. 1).

The stained leaves were photographed to quantify the number of infection points on each leaf. A disease severity protocol was used as described by Van Zyl *et al.* (2013), with slight modifications. Each leaf was placed on a white Perspex box (300 × 210 × 110 mm) under a glass pane and illuminated with white light and photographed. Image Pro Plus software was used to manually analyse each photograph and calculate the severity of infection on each leaf. The percent control was then calculated using the formula

$$\%\ Control = \frac{\%infection\ (control\ 0\times) - \%infection\ (concentration\times)}{\%infection\ (control\ 0\times)} * 100.$$

Visual assessment of infection

The inoculated seedlings were left in the glasshouse for 3-4 weeks to allow lesion development on leaves. The development of sporulation on leaves were encouraged by detaching infected leaves from the seedlings, 3 weeks after inoculation, and incubating it in a moist petri dish chamber at 19°C for 5-7 days. The visual assessment was only conducted in three of the four trials.

***In vitro* production of conidia with a cellophane agar plate method**

The cellophane agar plate method was used to produce *V. inaequalis* conidia *in vitro* (Parker *et al.,* 1994). Conidium production and viability was evaluated for two *V. inaequalis* isolates, isolates CGL 227-1 and CGL 228-1 that were both isolated from Golden Delicious apple leaves in Ceres. The cultures were supplied by T. Koopman (Agricultural Research Council, Bien Donne, Paarl, South Africa).

Cellophane membrane disks (85 mm dia.) were soaked in 3 L sterilised deionised water for 24 h. The soaked disks were packed between 90 mm filter papers (www.sigmaaldrich.com) and placed horizontally within a 90 mm dia. glass beaker containing deionised water, covered with aluminium foil and subsequently autoclaved. The sterilized cellophane disks were overlaid onto potato dextrose agar (PDA) plates (90 mm). Care was taken to avoid the formation of air bubbles underneath the cellophane. Mycelial growth of 3-4 week old *V. inaequalis* cultures grown on malt extract agar (MEA) plates under continuous light at ± 23°C was sliced into small pieces using a scalpel, and placed into sterile 50 ml Falcon tubes containing 20 ml of sterilised deionised water. The tubes were vigorously shaken. 2 ml of the suspension was pipetted onto each of the cellophane PDA plates, three plates per isolate, which each served as a replicate. The cultures where incubated at ± 23°C under continuous light. Conidia were washed from the colonized cellophane circles using 15 ml sterilized deionised water per plate on a weekly basis for 4 weeks. The conidial suspension was strained through a double layer of muslin cloth into 15 ml Falcon tubes that were centrifuged at 2000 rpm for 5 min to concentrate the spore suspensions for quantification. The pelleted conidia were re-suspended in 2 ml of deionised water and amount of conidia produced per petri plate was determined using a haemocytometer. Additionally, the viability of the conidia were determined by spreading 20 µl of the conidial suspension onto water agar using an L-shaped sterile plastic rod. The number of germinated conidia was counted after 24 h by cutting an area (± 3 mm × 3 mm) into the water agar and counting the number of germinated conidia among the total number of conidia present. The experiment was repeated four times.

Statistical analyses

Median deposition quantity (FPC%) and percent disease control (%) data were subjected to appropriate analysis of variance (ANOVA). Fisher's LSD was conducted at a 95% confidence level to determine differences between treatments. Statistical analyses were done using SAS statistical software version 8.2 (SAS institute Inc., 1999).

Venturia inaequalis spore count and viability data from the cellophane agar plate technique were subjected to appropriate ANOVA using STATISTICA 12 (Dell software 2013). Normality of data was tested with the Shapiro-Wilk test, and variance within experiments was evaluated using Leven's Test for Homogeneity of Variances. The Students LSD test at a 95% confidence level was used to determine difference between treatments. In cases where weighted means were analyzed due to Leven's test being significant ($P < 0.01$), the Games-Howell *post hoc* test was conducted at a 95% confidence level.

RESULTS

Deposition benchmarks indicative of apple scab control

Spray application

The yellow fluorescent pigment, illuminated with black light, made droplet distribution visible on the leaf. The illuminated spray deposit varied in visibility between concentrations. Fluorescent pigment deposition was clearly more visible at the higher concentrations (Fig. 1 C, D and E), and as distinct patterns indicating the dried remnants of droplets. The droplet pattern that formed showed an increase in droplet size towards the petiole from the leaf tip. This can be ascribed to the upwards angle of the leaf that is attached to the stem when sprayed from above with the gravity fed spray mist gun. This enabled droplets to connect and form larger or elongated droplets (Fig. 1). At the lower concentrations, pigment particles were visible, but not as distinct droplet patterns (Fig. 1 A and B).

Deposition analysis

Analysis of variance of deposition quantity data (FPC%) indicated a significant main effect for mancozeb concentration ($P < 0.0001$). As mancozeb concentration increased, so did deposition quantity (FPC%) with an excellent linear correlation ($R^2 = 0.973$) $Y = 8.67X - 0.80$. Deposition quantity increased from 0.29 FPC% at the 0.15%× (lowest) to 8.28 FPC% at the 1× (highest) concentration (Table 1).

Staining of inoculated leaves for disease severity quantification

Infection points were not visible after a 6 day infection period. After staining, lesions were visible as purple to pink pin point lesions on leaves (Fig. 2). Analysis of variance of disease

control (%) data indicated significant treatment effect ($P < 0.0001$). All concentrations evaluated realised similar control levels (58.90 to 72.49%) except for the 0× concentration which realised 0% disease control. Pearson's correlation indicated a very poor relationship ($r = 0.217$) between deposition quantity and disease control indicating no linear relationship. Various non-linear models were evaluated on deposition quantity *vs*. control data but no relationship could be found.

Visual assessment of infection

Visual assessment of leaves showed that *V. inaequalis* lesions developed on all leaves of the control treatment (0×). No visible lesions could be identified on any of the leaves that were treated with the different mancozeb concentrations (0.15, 0.30, 0.45, 0.60 and 1.0×) (Fig. 3).

In vitro production of conidia using the cellophane agar plate method

Analyses of variance indicated that there were no significant isolate × week interactions for the quantity of spores ($P = 0.1532$) or percentage viable spores ($P = 0.4316$) (Table 2). Therefore, both isolates behaved similar in their spore production over the 4 weeks. The two isolates did not differ significantly from each other in the amount of spores produced ($P = 0.2524$) or the percentage viable spores ($P = 0.0999$). For both parameters, there were significant differences between the four weeks analysed. A significantly higher number of spores were produced at week 1 (1.56×10^6/ml), compared to week 2 (9.49×10^5/ml) to 4 (3.27×10^5/ml) (Fig. 4 A). Thus, if spores were harvested after 1 week, 2 ml of a 1.56×10^6/ml solution could be obtained from one cellophone agar plate. Spore viability differed significantly ($P < 0.001$) with week 1 (85%) significantly higher than at weeks 2 (72%) to 4 (36%) (Fig. 4 B). Trial also had a significant effect on spore viability ($P < 0.001$), but not for quantity ($P = 0.63$). Trials 1 and 2 (72% and 62%, respectively) yielded more viable spores than trials 3 and 4 (50% and 56%, respectively), with significantly differences between trial 1 and 3.

DISCUSSION

The study investigated the development of a mancozeb benchmark model for the control of *V. inaequalis* on apple leaves, which requires accurate quantification of pathogen infection (disease severity or number of infections). This is a difficult aim in this host-pathogen system since scab lesions takes 3-4 weeks to become visible, and the lesions can then not be accurately quantified due to variation in lesion size. The lesions are not discrete and form expanding lesions that can be quantified but is inaccurate for identifying pin-point lesions.

The identification of a staining protocol to identify early infections before lesions expand is thus an important step towards the development of a working benchmark model. In our study, we used a staining protocol that was first published by Preece (1959). Ayesu-Offei and Clare (1970) used this method with slight modifications to evaluate the infection process of barley leaves by *Rhynchosporium secalis*. Yepes and Aldwinckle (1993a) evaluated the pathogenesis of *V. inaequalis* on shoot-tips and on greenhouse-grown apple cultivars, where the staining allowed the use of light microscopy evaluation of conidium germination and formation of appressoria. Although this is a very old technique that has not been used often in literature, it was very effective. It allowed the quantification of *V. inaequalis* infection within 6 days after inoculation. Distinct pin-point lesions could be quantified using the image analysis methods described by Van Zyl *et al.* (2013). To ensure that the staining technique was accurately revealing the presence of infections, visual assessment of infections were also conducted by allowing lesion development in a subset of the treated seedlings for 3-4 weeks. The latter technique showed that the > 58% control observed in the mancozeb treatments using the staining technique was underestimated since no lesions developed on the leaves that were assessed visually after 3-4 weeks. It therefore appears than some false identification of infection points by the staining technique occurred, probably as a result of slight bruising of leaves during the staining process. This highlights the importance of inclusion of an untreated, uninoculated control to establish a baseline error factor to be subtracted from all inoculated treatments.

The yellow fluorescent pigment used in this study proved to be an ideal tracer for mancozeb in determining deposition quantity (FPC%) on apple leaves in combination with macrophotography (Chapter 2) and very good correlations were again observed between FPC% and mancozeb concentrations. However, very high levels of *V. inaequalis* control were observed and it was not possible to fit any function to the deposition quantity (FPC%) and disease control data. There were also no significant difference between the highest (1.0×) and lowest (0.15×) mancozeb concentrations in providing control. Future studies will thus have to investigate lower mancozeb concentrations in order to develop a benchmark model.

Obtaining complete control at only 15% (22.5 g/100 L) of the registered mancozeb dosage (150 g/100 L) was unexpected. However, other studies have also found that the deposition quantity of fungicides required for control can be much lower than registered dosages. Vicent *et al.* (2009) obtained efficient control of Alternaria brown spot on ‘Fortune’ when copper fungicides where applied at 0.5 × (0.5 g/l) of the recommended concentration (1.0 g/l). Van Zyl *et al.* (2013) found that reduced rates of 0.34× and 0.68× of copper oxychloride still resulted in 50 and 75% control of Alternaria brown spot on ‘Nova’ mandarin leaves. Although in the current study complete control was obtained at 0.15× concentration,

this was 24 h after sprays were applied to leaves. Under orchard conditions a higher concentration may be required, to compensate for rainfall and consequent residue loss. For example, it has been shown that 1 mm of simulated rain can result in 11.95% residue loss on apple leaves (Chapter 2), which would have reduced the 0.15× to 0.13×. Under orchard conditions residue loss can even be higher if weathering (time) and tissue growth are also considered. Schutte *et al.* (2012) found a 48-60% decrease in copper residues after a 14 day period due to weathering, rainfall and fruit growth. Another important factor to also consider is that the 0.15× concentration gives complete control on leaves, but fruits must also be protected. It will thus also be important to determine if the same amount of residue is landed on fruit than on leaves under orchard spray conditions, and what the efficacy of residue is on fruit. In citrus orchards it was found that much less residue is landed on fruit than on leaves in spray trials (Schutte *et al.*, 2012).

In the current study, natural orchard inoculum was used. This was not as reliable an inoculum source for laboratory studies as sufficient quantities was often difficult to obtain. The cellophane agar plate method was shown to be a useful method for producing axenic conidial solutions. Results from the present study showed that the highest yield of conidia was obtained 1 week after inoculation: 2 ml of a 1.56 $\times 10^6$ conidia/ml spore suspension could be obtained per petri dish, whereas only 3.27 $\times$ 10^5 conidia/ml was obtained 4 weeks after inoculation. The conidial suspension obtained after 1 week also had a higher viability (± 85%) than at 4 weeks (± 36%), which is vitally important for inoculation studies. The cellophane agar plate technique was robust and similar conidium quantities and quality could be obtained in four independent experiments. Although the wick method has been published as a method for producing high conidium quantities in *V. inaequalis* (Keitt and Palmiter, 1938; Roberts and Crute, 1994; Cronin *et al.,* 1996), the current study did not find it as a feasible method due to the method being prone to contamination with fungi and bacteria, especially when attempting large scale experiments (unpublished results).

The cellophane agar plate technique has been used by several studies, but not for rearing inoculum for inoculation of plant material. The reported range of conidial concentrations by these studies ranged between 1 $\times$ 10^5 to 1 $\times$ 10^7 conidia/ml (Parker *et al*., 1994; Gau *et al*., 2004; Bus *et al*., 2005), but the volume of spore suspensions obtained per petri dish was not reported, nor spore viability. Parker *et al*. (1994) is the only other study that investigated the effect of culture age on spore concentration, but no other study has investigated the effect of culture age on conidia viability. The other studies that have used the cellophane technique just stated that they harvested spores after 1 week (Gau *et al.*, 2004) or 3 weeks (Kucheryava *et al*., 2008) or the time of harvesting was not mentioned (Bus *et al.*, 2005). In the study of Parker *et al.* (1994), it was found that at week 1 and 2 after inoculation, significantly higher spore concentrations were obtained than at week 3, but an

increase in spore production followed in week 4. This was ascribed to a second mycelial growth cycle and development of new spores (Parker *et al.*, 1994). This differs from the finding in the current study where a continued decrease in spore production was observed from week 1 to 4. It is possible that the growth conditions in the current study was less optimal than those of Parker *et al.* (1994), and that an increase in spore production might have occurred in week 5 if a second mycelial growth cycle occurred. However, since it was shown in the current study that a significant decrease in spore viability occurs from week 1 to week 4, using 5 week old cultures would not be optimal. The spore suspension would inevitably contain conidia with a lower viability due to the presence of conidia produced in the first mycelial cycle. From our findings, it was also demonstrated that harvesting spores after 1 week rather than week 4 was very important for obtaining significantly higher quantities of conidia with high viability. The two isolates used in the current study showed no significant difference in conidial production quantity and the viability of the conidia. However, these isolates were pre-selected in a pilot trial among five different isolates, amongst which two of the isolates were very poor spore producers (results not shown). Similar observations were made by Parker *et al.* (1994). Selecting the correct isolate is therefore important for optimal spore production since there can be variability in the spore producing capacity in *V. inaequalis* isolates (Parker *et al*., 1994).

In conclusion, the current study was unable to develop a benchmark model for control of *V. inaequalis* by mancozeb. Complete control was achieved at 0.15× mancozeb concentration (0.29 FPC%). However, whether this rate could be used as a testing benchmark for evaluating the efficacy of spray machines, spray volumes and fungicide concentrations in orchard trials needs to be evaluated further through comparison with deposition quantity levels obtained following best-practice orchard spraying. Further field studies will also have to be conducted to demonstrate whether the control levels obtained with very low mancozeb levels will provide sustained control throughout the spraying interval periods of up to 3 weeks at the end of the growing season.

Valuable techniques were investigated and optimized in the study that can be used in future benchmark model development in this host/pathogen system. First of all, a staining technique for quantification of *V. inaequalis* disease severity through image analysis was developed. The staining technique will have to be optimized further to confirm that the percentage control can be determined accurately if the baseline staining of an uninoculated mancozeb treatment is subtracted from the inoculated mancozeb treatments and inoculated control. Secondly, a cellophane agar plate technique for axenic conidial production was optimized. Isolates were also identified with high spore producing capacity that can be used in future benchmark model development.

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Table 1. Deposition quantity of fluorescent pigment (FPC%) and percentage *Venturia inaequalis* disease control (%) determined through basic fuschin acid staining on Golden Delicious apple leaves sprayed at different mancozeb concentrations.

Concentration[a]	Deposition quantity[b] (FPC%)	Percentage disease control[b]
1.00	8.28 a	63.75 a
0.60	4.28 b	64.86 a
0.45	2.82 bc	66.17 a
0.30	1.21 cd	72.49 a
0.15	0.29 d	58.90 a
0.00	0.00 d	0.00 b

[a]Concentration factor of recommended registered application rate of mancozeb (1.5 g/Land yellow fluorescent pigment (1 ml/L).

[b]Values in each column followed by the same letter do not differ significantly ($P > 0.05$) according to Fisher's least significant difference test. Values are the mean of four experiments.

Table 2. Analysis of variance of the quantity and viability data of *Venturia inaequalis* spores produced using the cellophane technique over a 4-week period.

	Quantity of spores				Percentage viable spores		
Source of variation	**DF**	**MS**	**F**	***P***	**MS**	**F**	**P**
Trial	3	1.41×10^{11}	0.59	0.63	2160.7	7.18	< 0.001
Isolate	1	3.21×10^{11}	1.33	0.25	836.0	2.78	0.10
Week	3	5.56×10^{12}	23.09	< 0.001	10395.6	34.53	< 0.001
Isolate × week	3	4.35×10^{11}	1.81	0.15	279.4	0.93	0.43
Error	73	2.41×10^{11}			301.1		

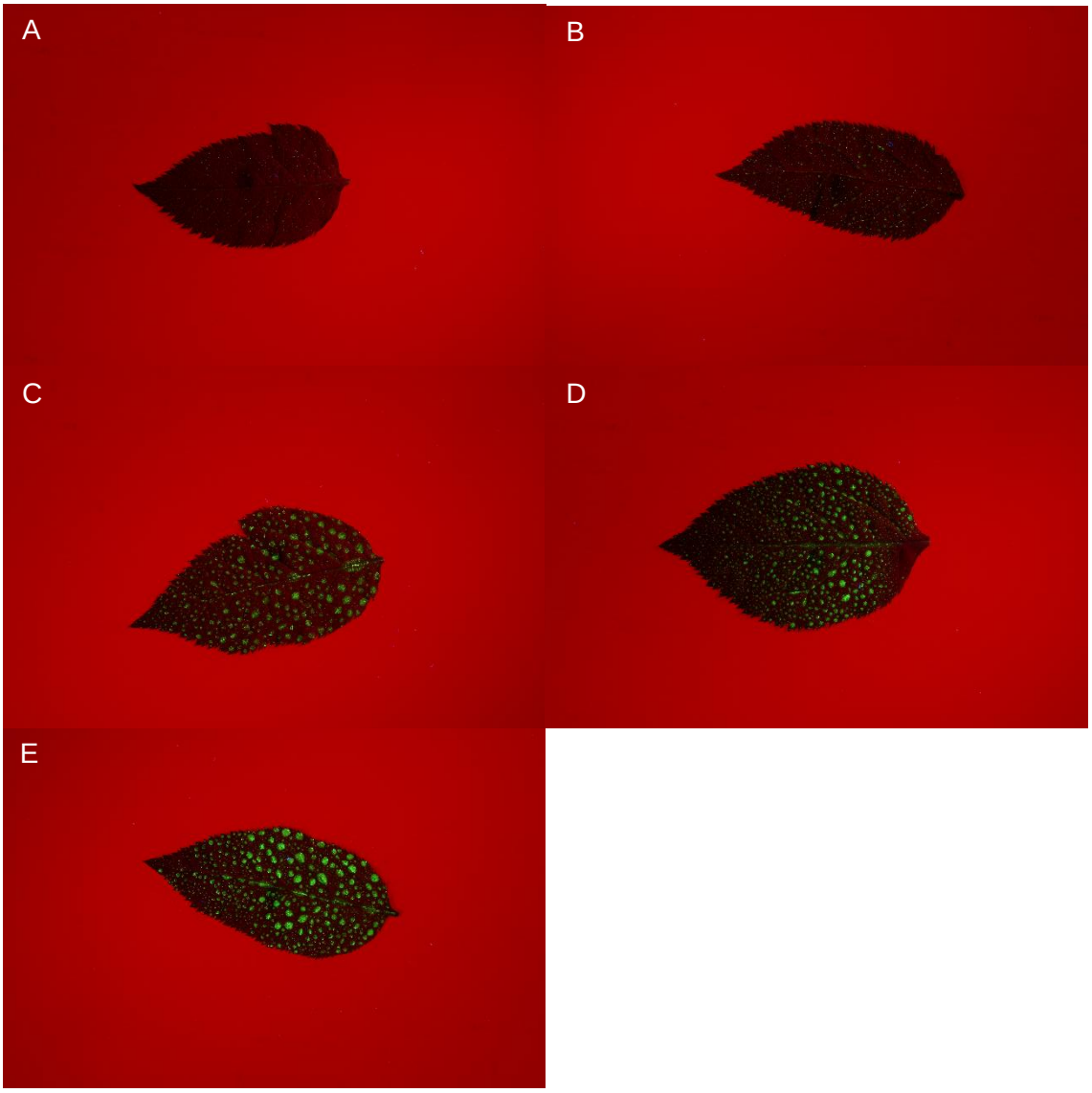

Figure 1. Digital images of the upper leaf surfaces of Golden Delicious apple seedling leaves illuminated with a black light (UV-A at 365 nm) illustrating the increase of concentration of yellow fluorescent pigment from 0.15× (A), 0.30×(B), 0.45×(C), 0.60× (D) and 1.00×(E) concentration (1 ml/L)

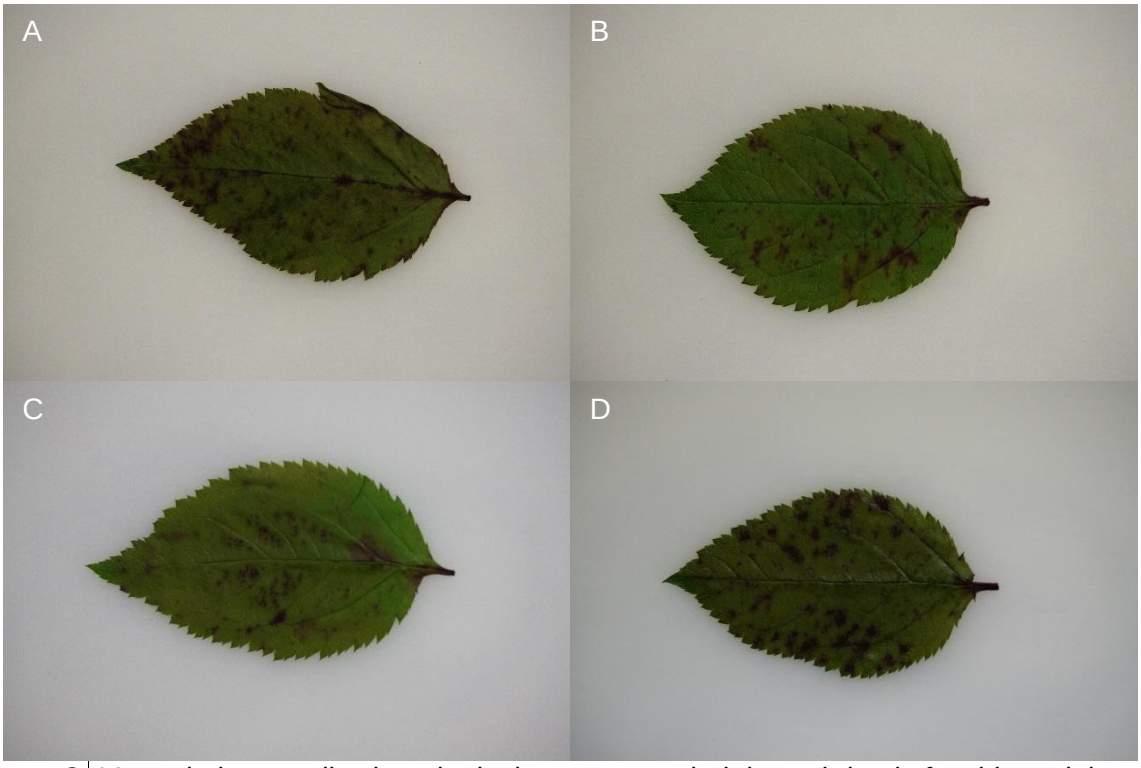

Figure 2. *Venturia inaequalis* pin point lesions as revealed through basic fuschin staining on 2- (A), 3- (B), 4- (C) and 5-day (D) old leaves.

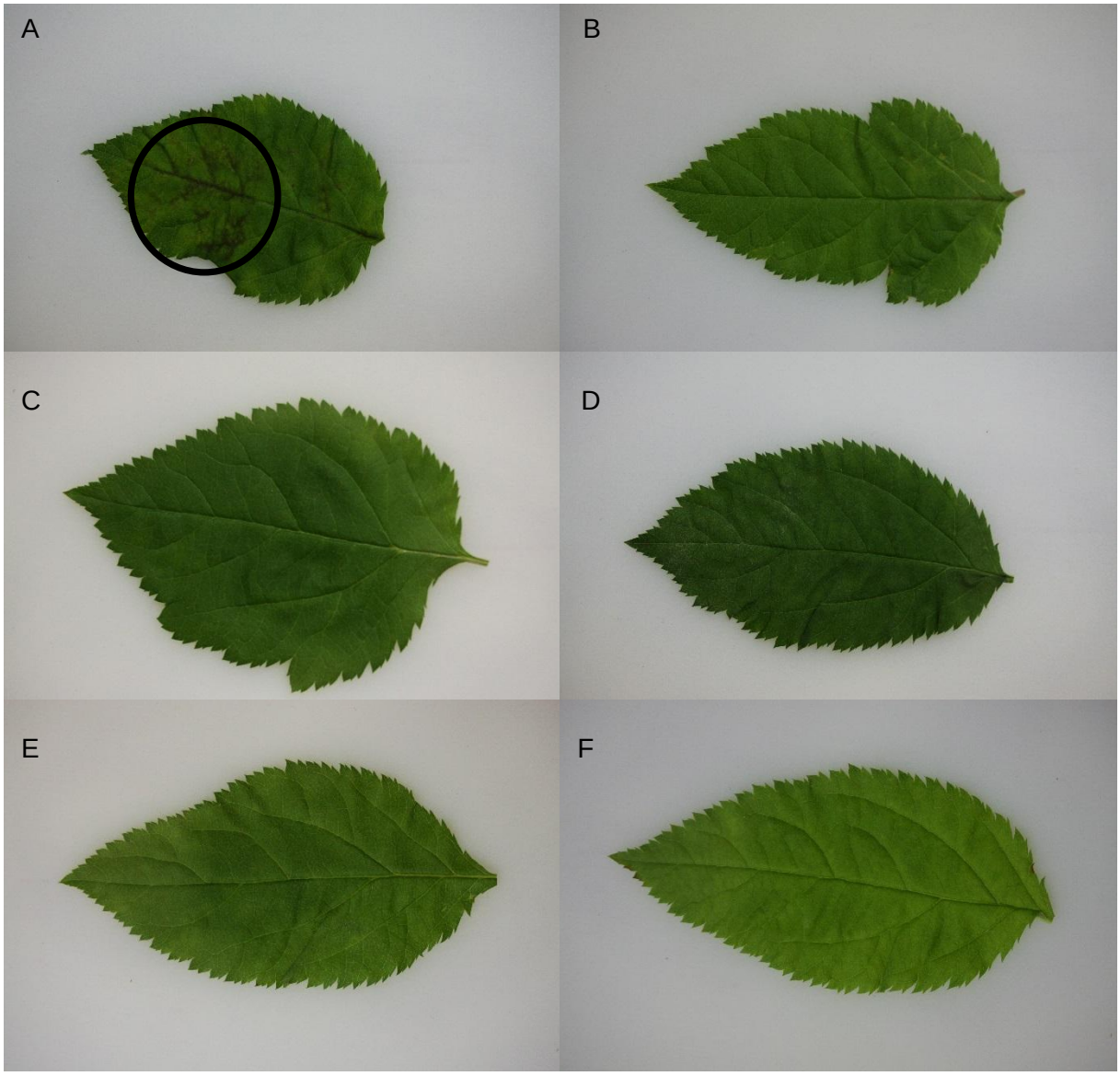

Figure 3. Visual assessment of disease control on apple leaves inoculated with *Venturia inaequalis* 4 weeks after inoculation. The leaves were sprayed with different mancozeb concentrations including 0 for the control (A), 0.15× (B), 0.30× (C), 0.45× (D), 0.60× (E) and 1.00× (F).

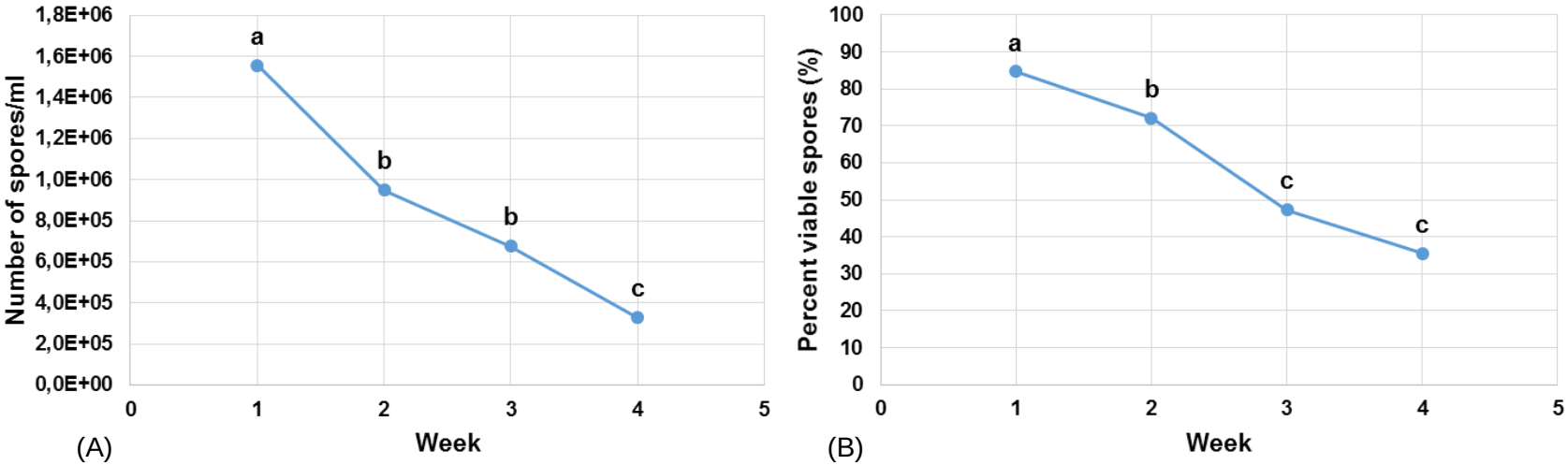

Figure 4. The mean quantity (A) of *Venturia inaequalis* spores of two isolates (CGL 227-1 and CGL 228-1) produced using a cellophane technique, and their (B) viability over a 4 week period. Two isolates (CGL 227-1 and CGL 228-1) were evaluated. Line markers followed by the same letter do not differ significantly ($P \geq 0.05$) from each other according to the Students Least Significant Difference tests. Vertical bars denote 0.95 confidence intervals.