QUANTIFICATION OF SPRAY COVERAGE ON GRAPE BUNCH PARTS AND THE INCIDENCE OF *BOTRYTIS CINEREA*

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and has not previously in its entirety or in part been submitted at any university for a degree.

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SUMMARY

Various studies revealed that *Botrytis cinerea*, the causal pathogen of *Botrytis* bunch rot, is mostly associated with pedicels, rachises, laterals and berry bases, and not with berry skins as previously understood. Provided that sufficient coverage of inner bunch parts was achieved, laboratory studies have shown that fungicides can effectively reduce the amount of *B. cinerea* at the various positions in bunches, and prevent infection and symptom expression at all growth stages. The same efficacy was, however, not achieved with the same fungicides when using conventional spraying methods in vineyards. Poor disease control on fruit and leaves in vineyards is attributed to inappropriate timing of fungicide applications and/or insufficient coverage of susceptible tissue. Previously, spray coverage evaluations in South Africa were based on the use of water-sensitive cards. A variety of other methods have been used to assess spray coverage in vineyards, but none of these methods could assess spray deposits on a very small, three-dimensional area of interest such as the susceptible grape bunch parts. The methods were furthermore dependent on human objectivity, which lacks quantitative measuring and speed of measurement. Suitable technology to determine spray coverage on susceptible bunch parts is, therefore, not available.

The aim of this study was to develop a protocol to visualise and quantify spray deposits in grape bunches, specifically on the inner bunch parts and to use the protocol to determine the effect of different levels of spray cover on artificially inoculated *B. cinerea* grape bunches, in order to facilitate future determination of minimum effective coverage levels for effective *B. cinerea* control.

A spray coverage assessment protocol using fluorometry, photomicrography and digital image analyses was developed to measure spray coverage on susceptible grape bunch parts. Among several fluorescent pigments tested, a yellow fluorescent pigment (SARDI Fluorescent Pigment) from Australia was selected on the basis of its small particle size (2.45 - 4.90 μm). Bunches were sprayed at pea size and bunch closure with different volumes of a mixture of fenhexamid and the yellow fluorescent pigment. Sprayed parts from bunches were illuminated under black light (UV-A light in the 365 nm region) and visualised under a stereo microscope at 20 x magnification. Photos of the berry skin, pedicel and rachis were taken

with a digital camera (Nikon DMX 1200). Image analysis of photos was done with Image-Pro Discovery version 4.5 for Windows (Media Cybernetics) software. The total area of deposited pigment in selected areas of interest (AOI) was calculated. The percentage area covered was subsequently calculated for each AOI. Good correlation was evident between the parameters, sum of objects and percentage area covered. Bunch parts at pea size generally had higher coverage values than at bunch closure. Spray applications earlier in the season would therefore result in higher and more effective spray coverage of the susceptible bunch parts. Similar deposition trends were observed on the inner bunch parts (pedicel and rachis). These were, however, significantly different from berry skins, which had significantly higher levels of spray deposits than the inner bunch parts. The variance component analysis indicated that the highest variance was observed for berries and bunches, and substantially less for image readings. For the same accuracy, means for percentage coverage values of at least 10 bunches per treatment (1 part per bunch and 3 readings per part) will be sufficient.

In order to determine the biological efficacy of different levels of spray coverage on *B.* cinerea incidence on grape bunches, bunches were sprayed at pea size and bunch closure with different volumes of a mixture of fenhexamid and a yellow fluorescent pigment and the percentage fluorescent pigment coverage on pedicels was determine. Bunches were subsequently dusted with dry airborne conidia of *B. cinerea* in a settling tower and incubated for 24 h at high relative humidity (98%). Infection was determined by estimating the amount of *B. cinerea* infections occurring on sprayed bunch parts with isolations on to paraquat and Kerssies mediums. Linear regressions for the part x stage combinations of percentage *B. cinerea* incidence on different bunch parts were fitted on mean coverage levels. An increase in spray cover caused linear reductions in levels of *B. cinerea* on susceptible bunch parts. Higher *B. cinerea* incidences were recorded at pea size. Furthermore, higher B. cinerea incidences were found on paraquat medium for both stages, than on Kerrsies medium. The information gathered from this study will be used to facilitate future determination of minimum effective coverage levels for effective *B. cinerea* control in grape bunches.

In these validation experiments, the results clearly showed that the protocol can be used to determine the effect of different levels of spray coverage on *B. cinerea* incidence and that an increase in spray coverage will decrease *B. cinerea* incidence. The information gathered from this study will be used to facilitate future determination of minimum effective coverage levels for effective *B. cinerea* control in grape bunches and subsequently be used as benchmarks to evaluate spray application in vineyards.

KWANTIFISERING VAN SPUITBEDEKKING IN DRUIWETROSSE EN *BOTRYTIS CINEREA* INFEKSIE

OPSOMMING

Vaalvrot by wingerde word veroorsaak deur *Botrytis cinerea*. Verskeie studies het getoon/gewys dat die oorsaaklike patogeen meestal geassosieer word met die pedisel, ragis, laterale en die korrelbasis, en nie met die korrelskil soos voorheen beweer nie. Laboratorium studies het getoon dat swamdoders wel effektief is om *B. cinerea* by alle trosdele te verminder en simptoomontwikkeling te voorkom tydens alle groeistadia, mits die binne-trosdele voldoende spuit bedekking ontvang het. Dieselfde effektiwiteit is egter nie gevind in wingerde met konvensionele spuittegnieke nie. Onvoldoende siektebeheer van vrugte en blare van wingerde kan toegeskryf word aan verkeerde spuit skedulering en/of swak spuitbedekking van vatbare gasheerweefsel. Evaluering van spuitbedekking is voorheen in Suid Afrika deur middel van water-sensitiewe papier gedoen. Verskeie ander metodes is al gebruik om spuitbedekking te evalueer in wingerde, maar nie een van hierdie metodes kan gebruik word om spuitbedekking op ’n baie klein, drie-dimensionele oppervlak, soos die vatbare trosdele, te evalueer nie. Verder was die tegnieke afhanklik van menslike objektiwiteit, en gevolglik ontbreek kwantitatiewe meting en metingspoed. Daar is dus nie geskikte tegnologie vir die evaluering van spuitbedekking op vatbare trosdele nie.

Die doel van hierdie studie was die ontwikkeling van ‘n protokol vir die visualisering en kwantifisering van spuitbedekking op spesifiek die binne-tros dele en om die protokol dan te gebruik om die effek van verskillende vlakke van spuitbedekking op *B. cinerea*-geinokuleerde druiwetrosse te bepaal,

Protokol vir evaluasie van spuitbedekking op vatbare druifdele is ontwikkel deur gebruik te maak van fluorometrie, fotomikrografie en digitale beeldanalise. Van die verskillende fluoresensie pigmente wat getoets is, is ‘n geel flouresensie pigment (SARDI Flourescent Pigment) van Australië gekies op grond van sy klein partikelgrootte (2.45 - 4.90 µm). Druiwetrosse is gespuit tydens ertjie- en trostoemaakstadia met verskillende volumes van ’n mengsel van fenheksamied en die geel fluorosensie pigment. Die gespuite druifdele is dan verlig onder swartlig buise (UV-A lig in die 365 nm spektrum) en gevisualiseer deur ’n stereo mikroskoop by 20x vergroting. Foto’s van die korrelskil, pedisel en ragis is met ‘n digitale kamera (Nikon DMX 1200) geneem. Beeldanalise is gedoen met ImagePro Discovery weergawe 4.5 vir Windows (Media Cybernetics) sagteware. Die totale area

neerslag van die pigment is in geselekteerde areas bereken. Die presentasie area bedek is bereken vir elkeen van hierdie areas. Goeie korrelasie is gevind tussen die parameters aantal fluoresserende partikels en die persentasie bedekte area. Trosdele tydens ertjie-stadium het in die algemeen hoër waardes gehad as by trostoemaak. Dit blyk dus dat spuittoediening vroeg in die seisoen meer effektief sal wees vir die bedekking van vatbare trosdele. Soortgelyke bedekkings patrone is gevind by die binne trosdele (pedisel en ragis). Dit het egter betekenisvol verskil van die korrelskil, wat betekenisvol meer spuitbedekking as die binne trosdele gehad het. 'n Variasie komponent analise het getoon dat die meeste variasie gevind is tussen korrels en trosse, en heelwat minder vir die beeld analise lesings. Om dieselfde akkuraatheid te behou, is ten minste 10 trosse per behandeling (1 deel per tros en 3 lesings per deel) nodig.

Vir die bepaling van biologiese effektiwiteit van verskillende vlakke van spuitbedekking op *B. cinerea* voorkoms op druiwe, is druiwe gespuit tydens ertjie- en trostoemaak-stadia met verskillende volumes van 'n mengsel van fenheksamied en die geel fluorosensie pigment. Die persentasie fluoresensie pigment is bepaal op die pedisels. Trosse is vervolgens geinokuleer met droë luggedraagde konidia van *B. cinerea* in 'n inokulasie-toring en geïnkubeer vir 24 h by hoë relatiewe humiditeit (98%). Die voorkoms van *B. cinerea* infeksie op gespuite tros dele is bepaal deur middel van isolasies op paraquat en Kerssies medium. Liniêre regressies vir trosdeel x stadium kombinasies van persentasie *B. cinerea* voorkoms op verskillende trosdele is gepas vir gemiddelde bedekkings waardes. 'n Verhoging in spuit bedekking het 'n liniêre vermindering van *B. cinerea* voorkoms op vatbare trosdele veroorsaak. Verder is hoër vlakke van *B. cinerea* op paraquat medium as op Kerssies medium vir beide die groeistadia gevind. Die kennis wat verkry is uit hierdie studie sal gebruik word om minimum effektiewe spuitbedekkingsvlakke vir die beheer van *B. cinerea* op druiwetrosse te bepaal.

CONTENTS

1. INTEGRATED MANAGEMENT OF *BOTRYTIS CINEREA* IN GRAPE VINEYARDS

INTRODUCTION

Botrytis cinerea Pers.:Fr causes grey mould (Nair & Hill, 1992) on grapevine (*Vitis vinifera* L.) in all vineyards of the world and can severely reduce crop quality and yeild. In table grape production, it can cause serious losses during pre-harvest and storage stages (Bulit & Dubos, 1994). Symptoms become more eminent in transit, because fruit is usually subjected to periods of raised temperatures in cold storage, which leads to long periods (>65 h) of continuous fruit wetness (Fourie, 1992). In wine grape production, juice from *Botrytis* infected grapes is darker, higher in volatile acids, higher in pectin and mucins and more bitter than the must from healthy grapes (Bulit & Dubos, 1994). Low amino nitrogen and high sugar levels can lead to slow fermentation (Somers, 1984). Wines from such grapes have off-flavours and are sensitive to oxidation and secondary contamination by bacteria making the wine unsuitable for aging (Bulit & Dubos, 1994).

EPIDEMIOLOGY

Most studies on host resistance, timing of fungicide applications, biological control, control by cultural practices and disease prediction models of *B. cinerea* on grapevines were based on assumptions and conclusions made on mature berries (Avissar & Pesis, 1991; Broome *et al.*, 1995; Chardonet *et al.,* 1997; De Kock & Holz, 1991; Marios *et al.,* 1986b, 1987; Nair & Nadtotchei, 1987; Nair *et al.,* 1988), the reasoning being that the most evident phase of the disease is found on the berries (Nair & Nadtotchei, 1987, McClellan & Hewitt, 1973; Nair, 1985; Nair & Hill, 1992; Nair & Parker, 1985). In most studies, where grapes were artificially inoculated, mature berries were atomised with (De Kock & Holz, 1991; Nair, 1985; Nair *et al*., 1988), dipped in (Broome *et al*., 1995), or injected with (Avissar & Pesis, 1991; Marios *et al*., 1986a; Thomas *et al*., 1988) conidium suspensions, or suspension droplets were placed onto the berry cheek (Chardonnet *et al*., 1997; Marios *et al*., 1987; Marios *et al*., 1986b). These methods allowed for depositions of groups of conidia on berries, and differ from primary natural infection in vineyards where single conidia may be deposited at several sites on bunch surfaces. Data from these studies may not give a true indication of

the natural occurrence of *B. cinerea* and its epidemiology in vineyards, which is needed for improved control strategies. Nonetheless, these research findings have resulted in the recommendation of four window periods (at the end of flowering, at bunch closure, at véraison and three weeks prior to harvest) for control (Pearson & Riegel, 1983; Nair, *et al*., 1988; De Kock & Holz, 1991, 1994).

For any disease to occur, inoculum and susceptible host material are needed. This chapter will focus on recent insights into *B. cinerea*'s natural inoculum in South African vineyards at different phenological stages, the susceptibility of grape bunch parts and how it can assist in control strategies.

Inoculum sources

The inoculum source within a crop comes from the crop itself, in particular from necrotic host plant tissue that is colonised by the fungus (Nair *et al*., 1995; Walter *et al*., 2001). The pathogen overwinters as sclerotia or mycelia on infected plant material. These structures are considered to be the most important structures involved in the survival of *B. cinerea* (Bulit & Dubos, 1994; Nair *et al*., 1995). Sclerotia can survive adverse environmental conditions and have a considerable capacity for producing successive crops of conidia (Coley-Smith, 1980). Thomas *et al*. (1981) showed that a vast amount of sclerotia recovered from vineyard soils in the Western Cape Province developed on grapevine leaves and shredded prunings. Seyb (2003) showed that the most important source of primary inoculum is the rachides on the ground. Louis *et al*. (1996) showed that sclerotia can also occur in insects and might germinate in the crop of the vinegar fly, *Drosophila melanogaster*, and form microsclerotia which can over-winter in the adult fly and could play a role in the winter conservation of *B. cinerea*.

Conidia are in general regarded as short-lived propagules in the field and their survival will largely be determined by temperature extremes, moisture availability, microbial activity and sunlight exposure (Holz *et al*., 2004a). Symptomatic leaves and colonised senescent floral debris and aborted berries retained in developing fruit clusters can contribute to the inoculum levels occurring in the bunch, especially early in the season in the bunch itself (Gessler & Jermini, 1985; Jermini *et al*., 1986, Northover, 1987; Wolf *et al*., 1997; Seyb, 2003). Necrotic leaves in the canopy (which are commonly generated from trimming) were identified by Seyb (2003) as an important source of inoculum.

Inoculum dispersal

The fungus moves in vineyards as conidia in air currents (Jarvis, 1962a), through insects (Fermaud & Le Menn, 1992; Gessler & Jermini, 1985; Louis *et al*., 1996; Engelbrecht, 2002) and to a lesser degree, through rain droplets (Jarvis, 1962b). Hardly any of the *B. cinerea* conidia become wet enough to enter a rain droplet and are rather carried on the droplet surface (Jarvis, 1962b). In air currents, conidia were only transported over a short range (Fitt *et al*., 1985). The rate of deposition, and therefore the steepness of deposition gradients, was futhermore affected by whether the spores are dispersed singly or in clusters. The higher the number of spores clumped together, the faster the settling speed (Ferrandino & Aylor, 1984). In New Zealand vineyards, 95% of *B. cinerea* are deposited within 1 m from the ground source (Seyb, 2003). Spore trapping has shown that the amount of spores vary in air currents throughout the year (Seyb, 2003; Van Schoor, 2004). In South African vineyards, Van Schoor (2004) found a high amount of propagules from bloom to bunch closure, thereafter the amount of spores decreased rapidly to a level where there were only a few individually spores after bunch closure in air currents. The same pattern was found in the various positions within bunches (Van Schoor, 2004). Coertze *et al*. (2001) showed that spores are dispersed individual in the air and not in clusters. This is important when studying the mode of infection in vineyards, as the infection with spore clusters gives a misleading indication of disease development (Coertze *et al*., 2001).

The ecology of Botrytis on grapevines

Leaf surface. Grape leaf blades carry high amounts of *B. cinerea* and this suggests that leaf infection is an important primary infection event that plays an important role in the epidemiology of the pathogen on grapevines (Holz *et al.*, 2003). Young leaves are highly susceptible and are infected at especially the leave base, which remains asymptomatic (Holz *et al.,* 2003). As the leaves mature, they get increasingly resistant to infection due to a thicker cuticle layer and the presence of inhibitory compounds (Langcake & Pryce, 1976).

Inflorescences. *B. cinerea* grows on senescent tissues, penetrates the stamens and invades their bases situated on the receptacle and the localised necrotic areas around the abscission layers of the shredded calyptra (Holz *et al*., 2004b). It also readily colonises the rachises, laterals, pedicels, ovaries and developing berries. Host resistance actions to the fungus are not uniform in various bunch parts.

Inner bunch inoculum. Early in the season from the prebloom stage, conidia dosages are high (Van Schoor, 2004) and the structural bunch parts are susceptible and dusted with pollen, which sustains the pathogen's growth on their surfaces (Holz *et al.,* 2003). Structural bunch parts are susceptible to infection during the younger stages, but their resistance increases moderately as the season progresses (Gütschow, 2001). Conidia and germlings can also survive for extended periods on structural bunch parts (Coertze & Holz, 2002). Various workers (Coertze *et al.,* 2001; Coertze & Holz 2002; Holz *et al.,* 2003; Holz *et al.,* 2004b) have found that *B. cinerea* symptom expression was predominantly associated with the bases of the berry and the pedicel. The next prominent position occupied was the rachises and laterals and not the berry cheek.

Berry surface. The amount of *B. cinerea* on berry surfaces is low throughout the season, and *B. cinerea* occurs as single colony-forming units (Coertze & Holz, 2002). The pathogen does not live for extended periods on immature and mature berry surfaces (Coertze *et al*., 2001; Coertze & Holz, 2002). *Botrytis cinerea* always attempts to penetrate sound berries directly through the skin, but these penetrations are mostly unsuccessful. The grape skin therefore is an effective barrier to penetration (Holz *et al*., 2004b). Coertze and Holz (2002) showed that when berries were inoculated with dry air borne conidia at bunch closure and at harvest, incubated for 4 days and subsequently wounded, wounds were not infected. Conidia or germlings adhering to the cuticle are not easily dislodged or easily redistributed from grape berry surfaces to fresh wounds, therefore infections of wounds could only take place if the newly deposited conidia were to land in or very near to the wound and grow into the wound under prevailing conditions (Coertze & Holz, 2002; Spotts & Holz, 1996). Therefore, in the event of wounding (wind, birds, insects), a combination of fresh wounds, freshly dispersed conidia, and free water on the berry surface is a necessity for successful wound infection (Coertze & Holz, 2002). Due to the necrotrophic ability and berry-to-berry contact, severe bunch rot can develop from a single berry that becomes symptomatic from such a wound, or at the base of the berry or pedicel (Holz *et al*., 2003).

Latent infection

Despite the high amount of inoculum occurring in bunches early in the season, *B. cinerea* will remain latent and grey mould symptoms are generally only prominent in vineyards after bunch closure (Holz & Volkmann, 2002). Latent mycelium was found to decline slowly during the season, but can still play an important role in latent infection. On the fruit of nectarine, plum and pear, germlings produced from dry airborne *B. cinerea* conidia

formed chlamydospores on short germ tubes when fruits were subjected to intermittent dry periods, or were kept for 48h at 5^{o}C (Holz, 1999). Chlamydospores can therefore serve as short term survival structures which can assist the fungus to overcome short unfavourable periods encountered on plant surfaces (Urbasch, 1983). Chlamydospores can therefore play an important role in latent infection (Holz *et al.*, 2003) and might be underestimated in disease expression.

Disease expression

Holz *et al*. (2003) showed that disease expression in bunches displayed the same pattern showed by the inoculum ecology, and that disease expression consistently developed first at the berry-pedicel attachment zone. Coertze *et al.* (2001) demonstrated through inoculations with single dry airborne conidia that berries at different phenological stages remained asymptomatic after extended periods (3 to 96 h) of moist or wet incubation and therefore provided an effective barrier to penetration. Symptom expression only developed when host resistance was terminated by applying stress factors (Coertze *et al*., 2001). These factors can lead to stress at the pedicel and the berry-base that lead to the seeping of berry juice, which plays a prominent role in disease development (Nair *et al.,* 1988). Bunch density, turgor, berry rupture, wind and insect damage are all stress factors on the susceptible parts (Holz *et al*., 2004a). Insects play an important role in disease development as they can carry masses of inoculum, depositing them individually or in groups on plant parts (Holz *et al.*, 2003; Engelbrecht, 2002). Fruit flies drastically increased the occurrence of *B. cinerea* at the pedicel (due to the seeping berry juice that is a food source for fruit flies) and infections on undamaged ripe berries. In these cases, symptoms developed in the absence of humid conditions or free water (Holz *et al.*, 2003; Engelbrecht, 2002).

MANAGEMENT

Cultural control

Inoculum reduction. Overwintering crop debris on the vineyard floor, the current season's floral debris, fruit and leaf debris within a bunch and trapped within the canopy are an abundant source of *B. cinerea* (Thomas *et al.,* 1981; Savage & Sall, 1983; Seyb, 2003; Van Rooi & Holz, 2003).

Hedging, shoot removal and leaf plucking, is often carried out during the season to reduce the risk of *B. cinerea* infection. This may also provide substrates for *B. cinerea* colonisation and inoculum production (Seyb, 2003). Leaves removed from vines during pre-bloom and during the early stages of bunch development should therefore be removed from the vineyard to reduce conidial and mycelium infection of susceptible bunch parts (Van Rooi & Holz, 2003). Removing inoculum sources has proved an efficient cultural control strategy for *B. cinerea* in other cropping systems, such as strawberries (Braun & Sutton, 1987).

Limiting factors that assist B. cinerea. Management and cultural practices are important factors in integrated control that can lower disease incidence, severity and consequently results in fewer fungicide applications. These practices must be focused to limit factors assisting *B. cinerea* and should improve air circulation, spray penetration and reduce humidity and wounds to bunches.

Management decisions prior to planting. A choice of a suitable site (De Kock & Holz, 1991), cultivar, row direction (De Kock & Holz, 1991) rootstock, irrigation system trellis (Thomas, 1983) and training type (Seyb, 2003) are some of the decisions that have to be taken before planting. The climate of a region is a good indication of the *B. cinerea* disease potential within a vineyard (De Kock & Holz, 1991). It is known that less symptom expression is found when row direction corresponds with the prevailing wind direction in the summer. If the row direction is correct, the bunches and leaves will dry faster after rain and heavy dew (De Kock & Holz, 1991). The factor considered when planting a specific grape cultivar is usually not its susceptibility to *B. cinerea*, though there is variation between cultivars, such as bunch architecture (Vail & Marios, 1991). Bunch rot is most prominent in cultivars that develop dense canopies and compact fruit clusters (Savage & Sall, 1983). Rootstocks can not directly influence the susceptibility of the vines to *B. cinerea*, but by selecting rootstocks that influence the vigor of the vine it will influence the canopy microclimate. Any irrigation system that wets the bunches can hamper for *B. cinerea* control (Thomas, 1983).

Prophylactic measures. Measures such as limitation of nitrogen fertiliser to avoid excessive vigour (Leroux, 1995, Chambers *et al*., 1993), removal of leaves around bunches (Leroux, 1995), effective control of downy mildew (*Plasmopara viticola*) and powdery mildew (*Uncinula necator*) by using compounds that have a secondary effect on *B. cinerea* (Leroux & Clerjean, 1985; Leroux, 1995), insects (Leroux, 1995) and weed control (De Kock

& Holz, 1991) should be applied to assist *B. cinerea* control. Cultural practices that influence microclimate of the canopy can be used for reduction of disease incidence and severity (Gubler *et al*., 1987). The removal of basal leaves alters the microclimate within the canopy (English *et al*., 1989) and reduces the development of *Botrytis* bunch rot (Gubler *et al*., 1987). Canopy and bunch manipulations can be used to reduce *B. cinerea*. Canopy density can be manipulated by shoot thinning, leaf plucking and leaf trimming, or by reducing vine vigor through inter-row planting and water management. Weeds in grapevines create a favourable microclimate for *B. cinerea*. It is also a barrier for the penetration of fungicides. A good weed control strategy is important to prevent weeds like black radish (*Raphanus raphanistrum*) and sorrel that can act as secondary hosts in the winter (De Kock & Holz, 1991). Gubler *et al*. (1987) reported that leaf removal (leaves and laterals located opposite, one node above, and one node below each flower cluster were removed by hand at late bloom) resulted in excellent disease control even under conditions suitable for severe rot. To prevent sunburn under South African conditions it is advisable to minimise the removal of leaves in the northern and western sides of the canopy (De Kock & Holz, 1991). Leaf removal is done in table grapes to prevent leaves from damaging the bunches, at the same time conditions unfavourable for *B. cinerea* are created (De Kock & Holz, 1991).

Chemical and biological control

Knowledge of the ecology of *B. cinerea* on leaves and in bunches, latency, and the relationship between the incidence of *B. cinerea* and disease expression at various positions on leaves and in bunches is needed to plan effective disease control strategies. This includes devising disease prediction models, timing of fungicide applications, biological control, and resistance breeding (Holz *et al.,* 2003). Identification of target sites naturally revolves around the susceptibility of various plant parts at different phenological stages, but also requires an in-depth knowledge of the pathogen's ecology in vineyards as well as its infection pathways at the various stages. Different workers (Coertze *et al.,* 2001; Coertze & Holz, 2002; Holz *et al*., 2003) found that under South African conditions, *B. cinerea* symptom expression was predominantly associated with the base of berries and the pedicel. The next prominent position occupied was the rachises and laterals and not the berry cheek. Berry surfaces are thus, contrary to the structural bunch parts, not covered by conidia or germlings between bunch closure and harvest (Van Schoor, 2004). The importance of *B. cinerea* occurring at the berry base (Pezet & Pont, 1986; Coertze *et al*., 2001; Gütschow, 2001; Holz *et al*., 2003; Holz *et al*., 2004b) might have been previously underestimated in the epidemiology of *B. cinerea* in

control strategies. This is an indication of where control is needed, namely on structural bunch parts.

Studies on the ecology of *B. cinerea* in South African vineyards have furthermore shown that conidium levels in air currents and within bunches are high from bloom to late pea size, after which it declines to very low levels (Van Schoor, 2004). At these stages, weather conditions are favourable for infection in a time when the structural bunch parts are susceptible and dusted with pollen which sustains the pathogen's growth on their surfaces. On the basis of these findings, the timing of fungicide application should be reconsidered (Van Rooi & Holz, 2003; Van Schoor, 2004). Thus, to effectively reduce *B. cinerea* in grapevines, three preventative applications are recommended to reduce primary infection events: firstly, between budding and pre-bloom stages to counteract primary leaf infection; secondly, during bloom to pea-size stage to reduce the amount of the pathogen in clusters and to prevent colonisation of floral debris; and thirdly, at bunch closure to reduce the amount of *B. cinerea* at various positions of the inner bunch, especially for cultivars with tight bunches .

Biological control

Biological control of plant diseases can involve several methods. These include the release of microbes as biological control agents and the application of plant extracts, such as thyme oil (Seyb, 2003). In cultivated plants, the currently known mechanisms of antagonism include mycoparasitism, competition for space and nutrients, direct antibiosis, induction of host resistance, and/or indirect toxicity (Cippolini & Styles, 1993). The greatest potential for the control of *B. cinerea* is where organisms are used with more than one mode of action (Elad, 1996). Biocontrol agents should be selected for their specific qualities. Holz and Volkmann (2002) demonstrated this principle by using *Ulocladium atrum, Gliocladium roseum, Trichoderma harzinum* and *Trichosporon pullulans*. The antagonists had the ability to survive on susceptible bunch parts, but were found to be inconsistent between seasons (Holz & Volkman, 2002). *T. harzianum* controlled infections in the pedicel and berry bases more effectively than any other treatment (Holz & Volkmann, 2002). The success of biocontrol agents are often affected by relatively small changes in environmental factors, such as humidity and temperature, which can greatly affect the degree of disease suppression (Hannusch & Boland, 1996). Climate variation and poor dispersal in the inner bunches by conventional spray apparatus after bunch closure, might be reasons why the efficacy of biocontrol agents fluctuates drastically in a season (Holz & Volkmann, 2002).

Chemical control

Chemical control remains the only way to reduce the incidence of grey mould in grapevine (Leroux, 1995). The use of most fungicides is restricted because of the risk of exceeding the maximum residue level (MRL) values (Leroux, 1995). Laboratory studies with several fungicides showed that fungicides, if applied properly to bunches under controlled conditions, effectively penetrated and covered the inner bunch parts, reduced the amount of *B. cinerea* at the various positions in bunches and prevented infection and symptom expression at all growth stages (Van Rooi, 2001). The same results were not achieved in vineyards with conventional spraying methods (Fourie, 1996). Poor control can be attributed to three factors: 1) improper spray timing, 2) improper spray techniques resulting in poor spray coverage and 3) reduced sensitivity to fungicides in the pathogen populations. (P.H. Fourie, pers.com). Research has clearly indicated the critical periods for control (Van Rooi & Holz, 2003) and strategies for the management of fungicide resistance (Fourie, 1996). However, research regarding spray coverage on grape bunches is lacking.

Coverage

Without good coverage on the specific spray targets, spraying at the right time with the best fungicides will not give sufficient control. The target to which fungicides are applied constantly changes as the shape and form of the grape bunches vary while maturing (Thwaites, 2001).

Spray coverage assessment. Previously, coverage evaluations in South Africa were based on the usage of water-sensitive cards. This method does not give a good indication of the spray coverage on certain critical positions grape bunches. Residue recovery techniques provide an overall assessment of the quantity of spray deposits, but spray deposits alone do not give a good indication of application quality such as uniformity or spray distribution on the leaves and bunch parts (Holownicki *et al*., 2002). Visual assessment gives an indication of the quality of the application, but the human eye lacks quantitative measuring and speed of measurement (Derkson & Jiang, 1995). Water-sensitive papers are widely used for visual assessment as well as for image analyses in spray application experiments (Holownicki *et al*., 2002). However, to give a true indication of spray deposits and penetration, cards need to be the same size and orientation as the target. Therefore this method does not give a good indication of the spray coverage on critical positions in bunches. The target to which fungicides are applied also changes constantly, because of the transformation of grape

bunches during the growth season (Barry & Weber, 2002). Three stages of growth can be distinguished within a season, namely flowering/set, pea-size and bunch closure. Each stage differs in how open or closed the bunch is and it therefore influences the spray application. With an open bunch, for example, increased air velocity blows droplets on the grapes, particularly at the front. In a medium packed bunch, extra air velocity blows drops off the front but helps the liquid filter through the bunch to the grapes at the back. In a closely packed bunch, extra blowing makes no difference since filtration is negligible (Barry & Weber, 2002). Research regarding spray application to ensure efficient spray coverage is desperately needed to ensure more effective disease management.

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2. DEVELOPMENT OF A PROTOCOL TO QUANTIFY SPRAY DEPOSITS ON GRAPE BUNCHES

ABSTRACT

Optimisation of spray deposition on target sites (i.e. susceptible grapevine tissue) is an essential requirement for effective disease management. In South Africa, *Botrytis cinerea*, the causal pathogen of *Botrytis* bunch rot, is mostly associated with pedicels, rachises, laterals and berry bases, and not with berry skins as previously understood. Laboratory studies showed that, provided sufficient coverage of inner bunch parts was achieved, fungicides effectively controlled *B. cinerea* at all growth stages. Suitable technology to determine spray deposits on inner bunch parts is, however, not available. The aim of this study was to develop a protocol to visualise and quantify spray deposits in grape bunches. Bunches were sprayed with different volumes of a mixture of fenhexamid and a yellow fluorescent pigment, illuminated under black light, visualised under a stereo microscope, and inner bunch parts were digitally photographed at 20 x magnification. Several image contrasting and filtering processes were performed and area of deposited pigment in selected areas was quantified. Fluorescent pigment coverage had a significant linear fit on spray volume. Coverage levels at pea-size were significantly higher than at bunch closure, while levels on berry skins were significantly higher than pedicels and rachises. Variance component analysis revealed that variation could be reduced by increasing the number of bunches, rather than the samples per bunch or measurements per image. The described protocol provides an essential tool that can be used to optimise spray application of agro-chemicals and/or biological control agents.

INTRODUCTION

Grapevine downy mildew (*Plasmopara viticola*), powdery mildew (*Uncinula necator*) and *Botrytis* bunch rot (*Botrytis cinerea*) are economically the most important diseases on grapevines. Given favourable environmental conditions and poor management strategies, these diseases can cause severe crop losses. Practical management of these diseases relies

almost exclusively on well-timed and/or routine fungicide applications and producers invest heavily in chemical products and spray equipment. However, improper spray application results in poor spray coverage and insufficient control of these diseases. As much as 40-50% of foliar spray is lost to the ground, especially when high volumes are applied to run-off (Matthews, 1997). Optimisation of spray deposition on target sites (i.e. susceptible grapevine tissue) is therefore an essential requirement for effective disease management.

Identification of target sites naturally revolves around the susceptibility of various plant parts at different phenological stages, but also requires an in-depth knowledge of the pathogen's ecology in vineyards and its infection pathways at the various stages. Substantial research was conducted on these aspects of *Botrytis cinerea* in South African vineyards (Coertze *et al.,* 2001; Van Rooi, 2001; Coertze & Holz, 2002; Holz *et al.,* 2003; Van Schoor, 2004). Collectively, these studies found that *B. cinerea* was most frequently found in the air and on/in plant parts during the pre-bloom stage until bunch closure. The pathogen frequently occurred asymptomatically in young leaves and on structural bunch parts (rachis, laterals and pedicel). It infrequently occurred on berry skins or stylar ends of berries. In fact, the berry skin was found to be resistant to *B. cinerea* infection. From bunch closure onward, the amount of *B. cinerea* in the air and in bunches declined rapidly. Bunch rot, which occurred shortly before harvest and post-harvest, can thus be attributed to latent inocula (mycelia, conidia, chlamydospores or microsclerotia) in the inner bunch parts that facilitate decay (G. Holz, pers. comm.). Several factors, such as bunch density, turgor, berry rupture, insect damage (fruit flies) and wind (Holz *et al.,* 2003), lead to stress and seeping of berry juice at the pedicel of the berry base, which plays a prominent role in disease development (Nair *et al.,* 1995). In these cases, symptoms developed in the absence of humid conditions or free water (Holz *et al.,* 2003). On the basis of these research findings, three preventative fungicide applications were recommended to reduce primary infection events of *B. cinerea* in South African vineyards: firstly, between budding and pre-bloom to counteract primary leaf infection; secondly, during bloom to pea-size stage to reduce the amount of the pathogen in clusters and to prevent colonisation of floral debris; and thirdly, at bunch closure to reduce the amount of *B. cinerea* at various positions of inner bunch, especially for cultivars with tight bunches (Van Rooi & Holz, 2003). Furthermore, laboratory studies showed that fungicides, if applied properly to bunches under controlled conditions, effectively penetrated and covered the inner bunch parts, thereby reducing the amount of *B. cinerea* at various positions in bunches, preventing infection and symptom expression at all growth stages (Van Rooi, 2001).

The same efficacy was, however, not achieved with the same fungicides using conventional spraying methods in vineyards (Holz *et al.*, 2003). This can largely be attributed to improper deposition of fungicides on the susceptible target sites. Research regarding the optimisation of spray deposition in vineyards is therefore of utmost importance.

A variety of methods have been used to assess spray coverage in vineyards. These methods include visual assessment on water-sensitive paper, bioassay and chemical residue recovery techniques (Holownicki *et al.*, 2002). Visual assessment was greatly improved by image analyses of stained water-sensitive paper or by adding fluorescent dyes to spray mixtures, followed by visualisation under black light (Furness, 2000a, 2000b). Visual assessments are, however, dependant on human discretion and the human eye lacks quantitative measuring and speed of measurement (Derkson & Jiang, 1995) and cannot assess spray deposit on a very small, three-dimensional area of interest such as grape bunches and more specifically the susceptible bunch parts.

The aim of this study was therefore to develop and validate a user-friendly spray assessment protocol that would enable the quantitative and qualitative measurement of spray deposits on the critical target sites in grape bunches.

MATERIALS AND METHODS

Experimental design

Grape bunches from the table grape cultivar Dauphine, selected at pea-size and bunch closure from two vineyards in the Paarl and Worcester regions were used for the development of the spray assessment protocol. The experimental design was a randomised complete block design with 5 spray volumes (1, 3, 6, 9, 15 ml and an unsprayed control), replicated in 5 blocks with a randomly selected bunch as experimental unit. For each spray treatment, 3 samples, each consisting of a berry and pedicel, were taken from the sprayed side of a selected bunch. At bunch closure, 2 rachis samples were also taken. This experiment was repeated once for each of the 2 stages.

Spray application

Sprays consisted of a mixture of fenhexamid (Teldor® 500 SC, Bayer) at the recommended dose (75 ml/100 l) (Nel *et al.,* 2003) and Yellow Fluorescent Pigment® (400 g/L, EC) (South Australian Research and Development Institute, Loxton SA 5333 Australia) at 2L/100L (Furness, 2000a). Spray volumes ranging from 1 to 15 ml were applied by means of a gravity feed mist spray gun (ITW DEVILBISS Spray Equipment Products, 195 Internationale Blvd, Glendale Heights IL 60139 USA) in a spray chamber [660 x 1410 x 800 mm (h/l/w)]. These spray volumes equate to 111.11 – 1666.67 l/ha in vineyard conditions (Furness *et al.,* 1998). Water sensitive cards (Syngenta SA, Halfway House, 1685) were included in each treatment to visually assess droplet dispersal and dispersal of fluorescent pigment in droplets.

Image processing and analysis

The fluorescence excitation light source included six BLB T5/6W fluorescent tubes (Lohuis, Kruisweg 18, Netherlands), which were installed in a custom-made hexagonal illumination box that fits closely around the P-Plan 1 X lens (10.0-63.0 x magnification) of a Nikon SMZ 800 stereoscopic zoom microscope. Images were digitally captured through the stereoscopic microscope at 20 x magnification (Fig. 1A) using a high-quality photomicrographic digital camera (Nikon DXM 1200). Image sizes of 1280 x 1024 (1.3 million pixels) for berry skin and 3840 x 3072 (12 million pixels) for pedicels and rachises were selected using the "Fine mode" option (microstep photomicrography) provided by the capturing software (NikonACT-1 Version 2.00, www.microscopyu.com). A Dell Intel Pentium 4, 1.70 GHz computer was used for capturing and analysis.

Image analysis was done with Image-Pro Discovery version 4.5 for Windows (Media Cybernetics, www.mediacy.com) software. In order to reduce background noise and enhance fluorescent pigment, brightness, contrast and gamma settings ("Contrast Enhancement" command) and Luminance, Red and Blue colour channel settings were adjusted (Fig. 1B). By using the measurement tools, pigments within a selected Area of Interest (AOI) box (berry skin = 0.259 mm^2, pedicel = 0.465 mm^2 and rachis = 0.623 mm^2) were automatically counted and measured. The total area of deposited pigment was automatically calculated in five AOI's for each photo (Fig. 1C). Fluorescent pigment coverage was calculated as the percentage area covered by fluorescent pigment in each AOI.

Statistical procedure

Fluorescent pigment coverage data were subjected to the appropriate analysis of variance, linear regression analysis and variance component analysis using SAS v 8.2 statistical software (SAS Institute, 1999). The Shapiro-Wilk test was performed to test for normality (Shapiro & Wilk, 1965). Student's t-Least Significant Difference were calculated at the 5% significance level to compare treatment means of significant effects (Snedecor & Cochran, 1967).

RESULTS

Visual assessments of water-sensitive cards indicated that fluorescent pigments were deposited in all water droplet stains. However, fluorescent pigments were also observed on parts without water stains. This might be attributed to droplets smaller than 50 µm that evaporated before staining the paper (Anonymous, 1999). Furthermore, initial calculations revealed that percentage area covered by fluorescent pigment was substantially lower than that of percentage area stained on water-sensitive paper (data not included).

Significant 3-factor (part x stage x volume) interaction was observed in the analysis of variance ($P = 0.03$). More than 85% of the variation for volume was explained by a linear trend (data not included) and linear regressions for part x stage combinations were therefore fitted and compared (Table 1). Intercepts did not differ significantly ($P = 0.94$), nor did it differ from zero ($P < 0.01$). Significantly higher slopes were recorded for berry skins compared to pedicels and rachises, which in turn did not differ significantly ($P > 0.05$). Furthermore, higher slopes were recorded for berry skins at pea size compared to bunch closure (Fig. 2).

Variance component analyses revealed that more variation occurred between samples within bunches than between bunches and images within bunches (Table 2). Variation can be reduced by increasing the number of bunches to 9 or more, with 1 sample per bunch and 1 measurement per image.

DISCUSSION

Observations on water-sensitive paper have indicated that fluorescent pigments most likely occurred in all droplets. However, fluorescent pigment coverage as measured by image analysis was substantially lower than droplet coverage observed on water-sensitive paper. Therefore, coverage values analysed by the described protocol accurately revealed the quantitative and qualitative fluorescent pigment coverage, which is a good indication of, but not equal to, the area where the spray mixture contacted the plant surface.

In the validation experiments, the results clearly showed that the described protocol could be used to accurately determine coverage on the susceptible bunch parts in grape bunches. An increase in spray volume generally led to an increase in coverage. Coverage was significantly influenced by growth stage and bunch parts. The highest mean fluorescent pigment coverage was measured at pea size on berry skins, while the lowest mean fluorescent pigment coverage was measured at bunch closure on rachises. In general, pea size bunches had a higher mean percentage area coverage on the different bunch parts than bunches sprayed at bunch closure. This can be explained by higher porosity of bunches at pea size compared with more compact bunches at bunch closure (Barry & Weber, 2002). These results clearly showed that spray applications earlier in the season will result in higher and more effective spray deposition on the susceptible bunch parts. Moreover, berry skins consistently had the highest, and pedicels and rachises the lowest mean fluorescent pigment coverage. This effect, which was consistent among all treatments and phenological stages, can be attributed to a higher air flow retention on the front of bunches (berry skins) than on the inner bunch parts (Barry & Weber, 2002). This shows the need to improve and accurately compare spray parameters such as droplet size, density, velocity, impaction, adhesion and different adjuvants to efficiently cover these susceptible parts of the grape bunch. These parameters would have a significant effect on the efficiency of spray deposition. Murphy *et al.* (2000) showed that the amount of spray volume retained on/in a grape bunch is a function of air velocity and that retention on the front side was approximately twice that of berries on the back side. With the inclusion of a surfactant, this ratio could be reduced from 2:1 to 1:1 (Murphy *et al.,* 2000). In a medium-packed bunch, extra air velocity blew drops off the front but helped filter liquid through the bunch to the berries at the back. In a close-packed bunch

extra blowing made no difference since filtration was negligible (Barry & Weber, 2002). The retention ratio (front:back) in compact bunches changed from 4:1 to 2:1 when a surfactant was added to the spray (Murphy *et al.*,2000). Therefore, it is important to evaluate the effects of spray adjuvants on the development of *B. cinerea* in grape bunches. Marois *et al.* (1987) showed that some adjuvants can significantly increase or decrease the overall disease incidence through the disturbance of the epicuticular waxes on grape berries.

The described protocol provides an essential tool that can be used to optimise spray application of agro-chemicals and/or biological control agents at various phenological stages and on different trellising systems. Hence, adequate deposition of active ingredient on the susceptible vegetative and reproductive parts of grapevines for effective pathogen or pest control can be facilitated. The technology developed in the grey mould/grapevine model will directly benefit the management of other foliar and fruit diseases of grapevine, such as powdery and downy mildew as well as diseases or pests in other cropping systems.

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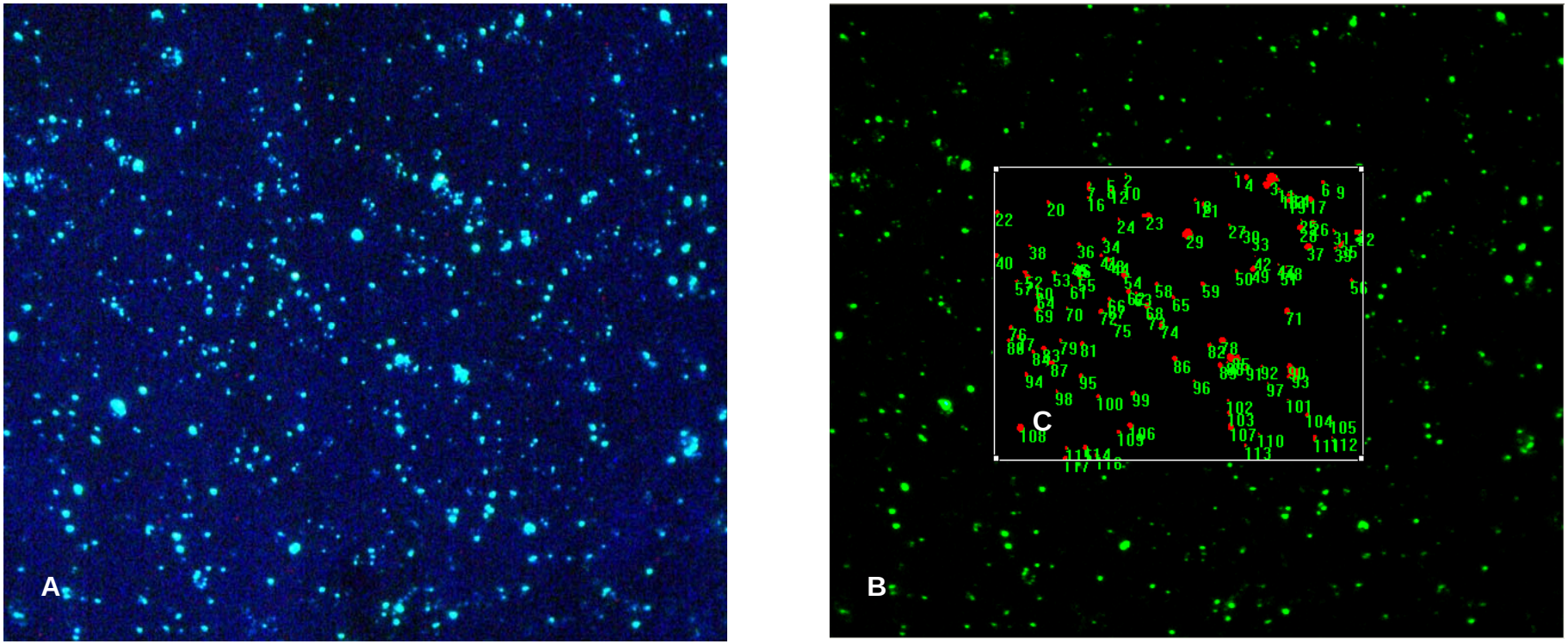

Figure 1. Image processing and analysis of a digital photo taken of a berry skin on a grape bunch at pea size stage that was sprayed with a mixture of fenhexamid and Yellow Fluorescent Pigment. (A) Selected objects are UV-illuminated and digitally photographed at 20x magnification, (B) subjected to several image contrasting and filtering processes, (C) an AOI selected and the total area of deposited pigment calculated for each AOI using Image-Pro Discovery image analysis software.

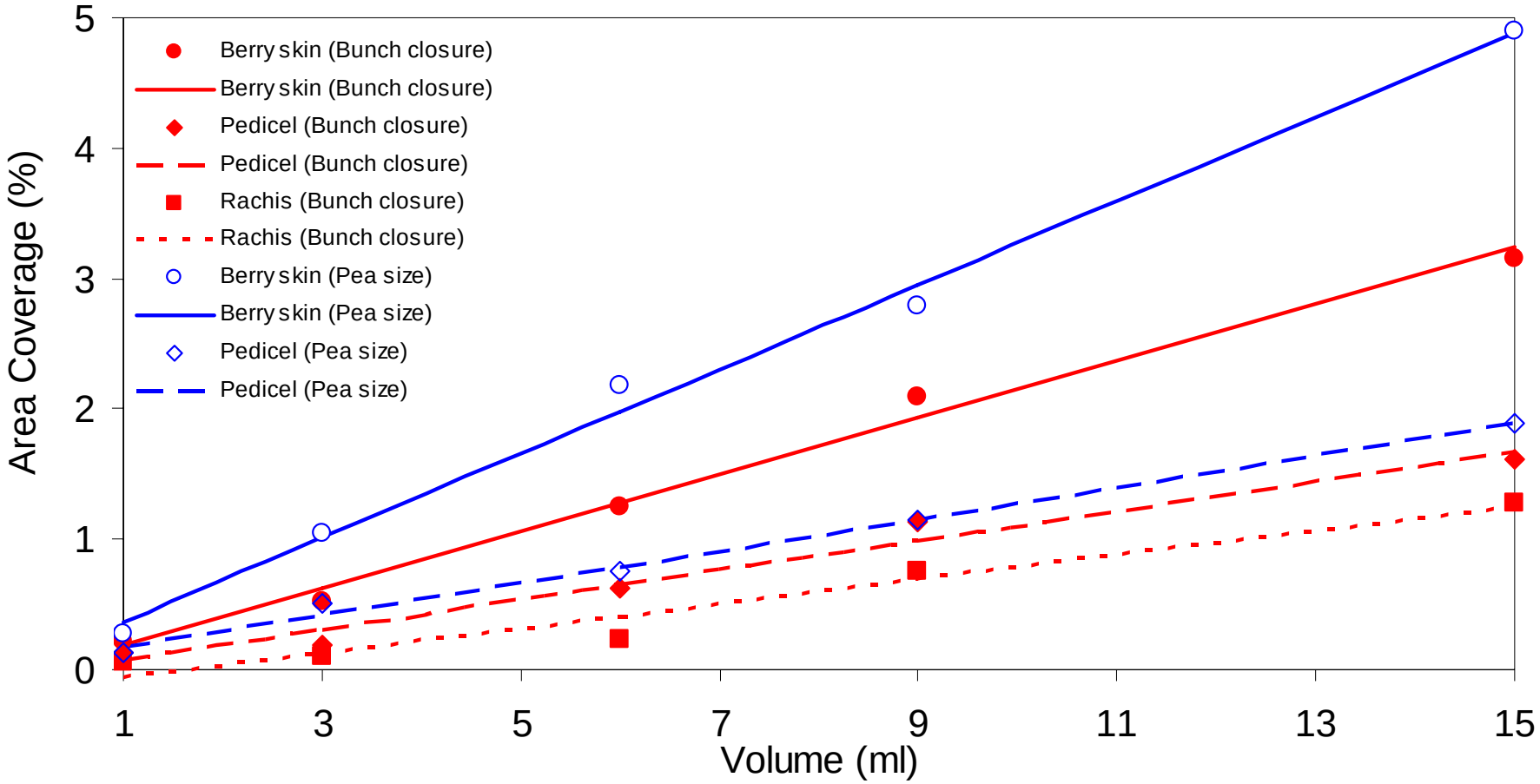

Figure 2. Mean fluorescent pigment coverage (% area) on berry skin, pedicel and rachis (bunch closure stage only) at pea size and bunch closure stages and linear regression lines fitted on spray volume for part x stage combinations.

Table 1. Equations of linear regression lines of fluorescent pigment coverage (y) on pedicel, berry skin and rachis (bunch closure stage only) at pea size and bunch closure stages that were fitted on spray volume (x) for different parts and stages

Stage	Part	Linear equation (±Standard error)*	Std Error (48 df)	R^2
Pea size	Pedicel	$y = 0.06(\pm0.139) + 0.12(\pm0.017)x$ [c]	0.3299	53.2%
Pea size	Berry skin	$y = 0.05(\pm0.292) + 0.32(\pm0.035)x$ [a]	1.4649	64.2%
Bunch closure	Pedicel	$y = -0.03(\pm0.113) + 0.11(\pm0.014)x$ [c]	0.2203	59.6%
Bunch closure	Berry skin	$y = -0.03(\pm0.195) + 0.22(\pm0.023)x$ [b]	0.6544	65.1%
Bunch closure	Rachis	$y = -0.15(\pm0.126) + 0.09(\pm0.015)x$ [c]	0.2710	44.6%

*t-LSD comparison of slopes: Value followed by the same letter do not differ significantly ($P = 0.05$).

Table 2. Variance component analyses of fluorescent pigment coverage on pedicel, berry skin and rachis for variance between bunches, between samples within bunches and between measurements within samples

Variation Source	**Pedicel**	**Berry skin**	**Rachis**
Between bunches	0.10898	0.71284	0.09622
Between samples within bunches	0.29801	0.78001	0.34013
Between measurements within samples	0.13940	0.15133	0.04172

3. EFFECT OF FUNGICIDE SPRAY COVER ON BOTRYTIS CINEREA INFECTION IN GRAPE BUNCHES

ABSTRACT

Poor control of fruit and foliar diseases in vineyards is attributed to inappropriate timing of fungicide applications and/or insufficient coverage of susceptible tissue. Studies revealed that the pedicels, rachises, laterals and berry bases and not the berry skins, as previously understood, are most susceptible to *Botrytis cinerea*, the causal pathogen of *Botrytis* bunch rot. A spray cover assessment protocol using fluorometry, photomicrography and digital image analyses was developed to measure spray coverage on susceptible grape bunch parts. The aim of this study was to determine the effect of fungicide spray cover on *Botrytis cinerea* infection in grape bunches. Bunches were sprayed at pea size and bunch closure with different volumes of a mixture of fenhexamid and a yellow fluorescent pigment and the percentage fluorescent pigment coverage on pedicels was determine. Bunches were subsequently dusted with dry airborne conidia of *B. cinerea* in a settling tower and incubated for 24 h at high relative humidity (98%). Infection was determined by estimating the amount of *B. cinerea* infections occurring on sprayed bunch parts with isolations on to paraquat and Kerssies mediums. Linear regressions for the part x stage combinations of percentage *B. cinerea* incidence on different bunch parts were fitted on mean coverage levels. An increase in spray cover caused linear reductions in levels of *B. cinerea* on susceptible bunch parts. Higher *B. cinerea* incidences were recorded at pea size. Furthermore, higher B. cinerea incidences were found on paraquat medium for both stages, than on Kerrsies medium. The information gathered from this study will be used to facilitate future determination of minimum effective coverage levels for effective *B. cinerea* control in grape bunches.

INTRODUCTION

Disease management strategies are based on the knowledge of epidemiology, infection pathways and inoculum ecology of *Botrytis cinerea* in vineyards. In South Africa, weather conditions are usually favourable for colonisation of inflorescences and young bunches from bloom to late pea-size stage. Conidium dosages in air and in the various positions in bunches are high until bunch closure, and then rapidly decrease to very low levels (Van Schoor, 2004). Furthermore, bunch parts are often dusted with pollen, which sustains the pathogen's growth on their surfaces. *B. cinerea* grows on senescent tissues, penetrates the stamens and invades their bases situated on the receptacle and the localised necrotic areas around the abscission layer of the shredded calyptra on the receptacle (Holz *et al*., 2004). It also readily colonises the rachises, laterals, pedicels, ovaries and developing berries.

Host resistance varies in the different bunch parts. Structural bunch parts are susceptible to infection during the younger stages, but their resistance increase moderately as the season progressed. Conidia and germlings can survive for extended periods on structural bunch parts but do not survive for long on the surface of immature berry skins or are of short duration when latent infection occurs in the skin (Holz *et al*., 2004). *B. cinerea* symptom expression was furthermore predominantly associated with pedicels and the bases of berries under South African conditions (Coertze *et al*., 2001; Coertze & Holz, 2002; Holz *et al*., 2003). This is also true for other fungi (*Penicilium, Aspergillus, Alternaria, Mucor* and *Rhizopus* spp.) commonly associated with bunch rot (Hewitt, 1974; Holz *et al.,* 2003). The next prominent positions occupied by *B. cinerea* were rachises and laterals and not berry cheeks. *Alternaria alternata* also penetrated rachises and pedicels through stomata and lenticels, and caused rot of cold-stored table grapes when subjected to stress conditions (Swart & Holz, 1994; Thomas *et al*., 1988). Bunch density, turgor, berry rupture, wind and insect damage (Holz & Volkmann, 2002) are factors that lead to stress at the pedicel and the berry-base. These factors can also lead to the seeping of berry juice, which plays a prominent role in disease development (Nair *et al*., 1988). In these cases, symptoms developed in the absence of humid conditions or free water (Holz *et al*., 2003). Berry surfaces are thus, contrary to the structural bunch parts, not covered by conidia or germlings between bunch closure and harvest (Van Schoor, 2004). These findings imply that the berry-pedicel

attachment zone is underestimated in the development of bunch rot epihytotics in grapevine (Holz *et al*., 2003).

The control of *B. cinerea* infection by chemical, cultural and biological means can only be achieved by reducing inoculum on susceptible bunch parts at the appropriate growth stage when propagules are present (Van Rooi & Holz, 2003). Chemical control remains the most effective strategy to reduce the incidence of grey mould in grapevine. In order to reduce primary infection events of *B. cinerea* in South African vineyards, three preventative fungicide applications are recommended according to these findings: firstly, between budding and pre-bloom to protect susceptible inflorescences; secondly, during bloom to pea-size stage to reduce the amount of the pathogen in clusters and to prevent colonisation of floral debris; and thirdly, at bunch closure to reduce the amount of *B. cinerea* at various positions of the inner bunch, especially for cultivars with tight bunches (Van Rooi & Holz, 2003; Van Schoor, 2004).

Laboratory studies (Van Rooi, 2001) showed that when fungicides were properly applied to the susceptible target sites in bunches, the amount of *B. cinerea* at the various sites within the bunches was reduced, and infection and symptom expression were prevented at all growth stages. The same efficacy was, however, not achieved with the same fungicides when using conventional spraying methods in vineyards (Holz *et al*., 2003). This can largely be attributed to improper deposition of fungicides on the susceptible target sites. Higher volumes will generally lead to better coverage (Chapter 2), but might lead to higher risks of exceeding MRL (Maximum Residue Level) values (Leroux, 1995) or to reduced coverage because of run-off. The optimisation of fungicide application in order to facilitate sufficient spray cover for effective disease management is therefore of utmost importance.

A variety of methods have been used to assess spray cover in vineyards, but none of these methods could quantify spray deposits on a very small, three-dimensional area of interest such as the susceptible grape bunch parts (Holownicki *et al*., 2002). Furthermore, methods that are dependant on human discretion that lacks quantitative measuring and speed of measurement (Derkson & Jiang, 1995). A spray coverage assessment protocol using fluorometry, photomicrography and digital image analyses was developed to accurately measure spray cover on susceptible grape bunch parts (Chapter 2). The aim of this study was to use the protocol to determine the effect of different levels of spray cover in grape bunches on *B. cinerea* infection on different bunch parts. Data obtained from this study would

facilitate future determination of minimum effective coverage levels for effective *B. cinerea* control.

MATERIALS AND METHODS

Grape bunches from the table grape cultivar Dauphine were selected at pea size and bunch closure stages from two vineyards in the Paarl and Worcester regions. Bunches were surface-sterilised before use (30 s in 70% ethanol, 2 min in 0.35% sodium hypochlorite, and 30 s in 70% ethanol) and air-dried.

Experimental design

The experimental design was a randomised complete block design with 6 spray volumes, replicated in 5 blocks. Each block was divided in 4 groups (treatment combinations) of 6 bunches each. Each bunch was sprayed with a different spray volume (1, 3, 6, 9, 15 ml and an unsprayed control). Two groups of bunches within each block was inoculated with *B. cinerea*, while the remaining two were left uninoculated. In order to determine *B. cinerea* incidence after the incubation period, isolations from these bunches were made onto Kerssies medium and paraquat medium. From each bunch, 4 samples each from of pedicels, rachises and receptacles for each spray treatment were used to assess *B. cinerea* incidence. For each block and spray volume an extra bunch was included for spray cover assessment.

Spray application and spray coverage assessment

Sprays consisted of a mixture of fenhexamid (Teldor® 500 SC, Bayer) at the recommended dose (75 ml/100 l) (Nel *et al.,* 2003) and Yellow Fluorescent Pigment® (400 g/l, EC) (South Australian Research and Development Institute, Loxton SA 5333 Australia) at 2 l/100 l (Furness, 2000). *In vitro* tests with the fluorescent pigment did not influence *B. cinerea* growth on PDA medium (data not included). Spray volumes ranging from 1 to 15 ml were applied by means of a gravity feed mist spray gun (ITW DEVILBISS Spray Equipment Products, 195 Internationale Blvd, Glendale Heights IL 60139 USA) in a spray chamber [660 x 1410 x 800 mm (h/l/w)]. These spray volumes equate to 111.11 – 1666.67 l/ha in vineyard conditions (Furness *et al.,* 1998). After each spray, the chamber was ventilated for 5 minutes

before the next application. Following spraying, the peduncles of bunches were inserted into containers with water and kept for 24 h at 22°C before inoculation.

Spray cover assessment was done by means of the protocol described in Chapter 2. Three pedicel samples for each spray treatment were taken from the sprayed side of a selected bunch. Images were digitally captured through the stereoscopic microscope at 20 x magnification using a high-quality photomicrographic digital camera (Nikon DXM 1200). Image analysis was done with Image-Pro Discovery version 4.5 for Windows (Media Cybernetics, www.mediacy.com) software. By using the measurement tools, pigments within a selected Area of Interest (AOI) box (0.465 mm^2) were automatically counted and measured. The total area of deposited pigment was automatically calculated in five AOI's for each photo. Fluorescent pigment coverage was calculated as a mean of the totals percentage area covered by fluorescent pigment in each AOI.

Inoculation and incubation

A virulent isolate of *B. cinerea,* obtained from a naturally infected grape berry, was maintained on potato dextrose agar (PDA) at 5°C. For the preparation of inoculum, the isolate was first grown on canned apricot halves. Conidiophores from the colonised fruit were transferred to PDA in Petri dishes and incubated at 22°C. Dry conidia were harvested with suction-type collector and stored at 5°C until use. Storage time did not affect germination of dry conidia (Spotts & Holz, 1996). Bunches were inoculated with 3 mg dry conidia, which were dispersed by air pressure into the top of an inoculation tower (Plexiglass, 3 x 1 x 1 m [height x depth x width]) according to the method of Salinas *et al*. (1989). The conidia were allowed 20 minutes to settle onto the bunches that were positioned on two screens on the floor of the inoculation tower. By using this inoculation technique, approximately three conidia were evenly deposited as single cells on each mm^2 of bunch surface (Coertze & Holz, 1999). Petri dishes with water agar (WA) were placed next to the bunches and the percentage germination was determined after 6 h post inoculation (100 conidia per Petri dish, two replicates).

Following inoculation, the groups of bunches were placed on sterile epoxy-coated steel mesh screens (53 x 28 x 2 cm) in ethanol-disinfected perspex chambers (Cape Plastics, Cape Town, South Africa) lined with a sheet of chromatography paper with the base placed in water to establish high relative humidity (≥93% RH). The chambers were incubated for 24 h at 22°C. These conditions are similar to what the pathogen encounters on grape bunches in

nature, namely dry conidia on dry bunch parts under high relative humidity. Non-inoculated bunches were used to determine the natural infection levels of *B. cinerea.*

Assessment of *B. cinerea*

From each cluster five receptacles, pedicels and rachis sections (5 mm each) were isolated on Petri dishes containing Kerssies' *B. cinerea* selective medium (Kerssies, 1990), or on water agar medium supplemented with paraquat (Grindrat & Pezet, 1994). The plates were incubated at 22°C under diurnal light and the sections were daily monitored for symptom expression and the development of *B. cinerea*. After 11 days the number of sections yielding sporulating *B. cinerea* colonies were recorded, and the numbers used to determine the percentage incidence of *B. cinerea* occurring at the various positions in the bunches. The different treatments provided conditions that facilitated the development of *B. cinerea* by conidia on the surface of bunch tissue, or by mycelia in the tissue, during the period of incubation. Previous studies (Coertze & Holz, 1999; Coertze *et al*, 2001; Gütschow, 2001) with grape bunch tissue on Kerssies' medium showed that segments retained their active defense abilities and no superficial mycelial growth developed on the segments during the first 5 days of incubation. Hyphal growth usually occurred from cells underlying the cuticle into the medium after 5 days, which indicated direct penetration by conidia on the surface, and the development of mycelia from the host tissue during the incubation period. *B. cinerea* development and colony formation on segments therefore gave an indication of infection at that site as influenced by the amount of surface conidia and mycelia confronted by active defense. Paraquat terminated host resistance in the cells of the cuticular membrane without damaging host tissue (Grindrat & Pezet, 1994), and allowed the development of conidia and mycelia on the surface, and mycelia in the tissue in the absence of active defense (Coertze & Holz, 1999; Coertze *et al*, 2001).

Statistical procedure

Means of percentages of cover and *Botrytis* incidence on pedicels, receptacles and rachises were calculated for volume, medium and stage combinations. Linear regressions were fitted on percentage cover using SAS v. 8.2 statistical software (SAS Institute, 1999). Slopes were compared by using the standard error of means.

RESULTS

Germination of the inoculated *B. cinerea* conidia on water agar plates was between 86 and 96%. Incidence of natural *B. cinerea* infection on bunch parts were very low (< 1%) and the data were therefore not considered for statistical analyses.

Pea size stage

Mean fluorescent pigment coverage obtained by spray volumes 0 to 15 ml was 0%, 0.13%, 0.51%, 0.75%, 1.15% and 1.89% respectively (Fig. 1). This resulted in a linear reduction in *B. cinerea* incidence from 46.54% - 67.5% to 29.37 - 47.85% for the various bunch parts (Fig. 1). Good linear fits (R^2 = 44.35% - 90.60%) were obtained (Table 1). The decrease of infection levels showed no significant difference in slopes between parts and mediums at pea size nor was a consistent difference in susceptibility among bunch parts observed (Table 1). Furthermore, significantly more *B. cinerea* occurred on unsprayed bunches (x = 0) was recorded on paraquat medium (52.69% - 65.46%) compared with the Kerssies medium (40.74% - 46.54%) on all parts (Table 1).

Bunch closure

Botrytis cinerea incidence on the pea size stage was markedly higher (40.74% - 63.58%) compared with bunch closure stage (16.59% -34.60%) [(Table 1, 2); (Fig. 1, 2)]. Mean fluorescent pigment coverage affected by spray volumes 0 to 15 ml was also markedly lower at bunch closure than at pea size [(0%, 0.13%, 0.19%, 0.62%, 1.13% and 1.61%, respectively) (Fig. 2)]. A general reduction in *B. cinerea* from 13.5% - 39% to 8.5 - 32% was also observed for the various bunch parts (Fig. 2). However, most combinations gave poor linear fits at bunch closure [R^2 = 3.71% - 36.86% (Table 2)]. At this stage, only the receptacle gave a favourable fit (R^2 = 80.92%). Despite the poor linear fits, similar trends and slopes were observed as those observed for pea size stage. Markedly higher *B. cinerea* incidence on unsprayed bunches (x = 0) was also recorded on paraquat medium (28.31% - 34.60%) compared with the Kerssies medium (16.59% - 25.00%) on all parts (Table 2).

DISCUSSION

This study showed that an increase in percentage cover reduced the infection levels of *B. cinerea* on susceptible bunch parts. This shows the importance of adequate spray cover on susceptible bunch parts for control of *B. cinerea* in grapevines. The linear relation between spray cover and *B. cinerea* incidence was, however, less evident at bunch closure. This can be explained by the significant influence of growth stage and different bunch parts on deposition of fungicides. The highest deposition was measured early in the season and it decreased as bunches became more compact (Chapter 2). This is largely attributed to the higher porosity of bunches at pea size compared to the more compact bunches at bunch closure and higher air flow retention on the front of bunches than on inner bunch parts (Barry & Weber, 2002). It can furthermore be hypostasised that the bunches at pea size would receive higher dosages of evenly dispersed dry conidia than at bunch closure. This would explain the varying levels of deposition of spray coverage and *B. cinerea* incidence at bunch closure.

Markedly higher infection levels were recorded at pea size compared with bunch closure. This can be ascribed to differences in host resistance (Holz *et al.*, 2004). No consistent difference in susceptibility among bunch parts was observed. This is contrary to the findings of Gütschow (2001) who found pedicels to be significantly more susceptible than rachises (cultivar Dauphine). In our study, rachises would have lower levels of fungicide cover than pedicels (Chapter 2), and therefore a slightly higher incidence of *B. cinerea*.

Markedly better linear fits and higher infection levels were recorded when isolations were made on paraquat medium. Since *B. cinerea* incidence on paraquat medium was the result of infection after the termination of host resistance by the herbicide (Grindat & Pezet, 1994), infection levels would therefore provide a clear indication of the fungicide action in the absence of active host resistance. When studying *B. cinerea* infection on Kerssies medium, fungicidal action was masked by host resistance (Coertze & Holz, 1999; Coertze *et al*., 2001), and incidence data were highly variable.

In this study the lowest infection level (39.83%) was achieved with the highest spray volume at pea size on the paraquat medium, compared to 0% measured by Van Rooi (2001) with the same inoculation and isolation technique, but with higher spray volumes. The spray

cover levels found in this study were therefore not high enough to result in adequate control of *B. cinerea* infections.

From these findings, we conclude that an increase in spray coverage will result in decreased infection. However, several factors, [i.e. too low spray volumes (i.e. spray coverage), three dimensional bunches, and bunch compactness] led to inconsistent conidium and fungicide spray deposition on the structural bunch parts. Future studies aimed at determining minimum effective coverage values should use higher coverage levels to ensure adequate control of *Botrytis* infection. Isolations onto paraquat medium would furthermore lead to higher and less variable assessment of *B. cinerea* infection levels. Moreover, variation in conidium and spray deposition can also be minimised by cutting bunches into a two dimensional shape, which would subsequently lead to better linear relations between spray cover and *B. cinerea* infection levels.

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Table 1. Equations of linear regressions lines of *B. cinerea* infection levels (y) on pedicel, receptacle and rachis at pea size stage, as determine by isolations onto Kerssies and paraquat medium, that were fitted on percentage fluorescent pigment coverage (x) for different parts x growth mediums combinations

Part	Medium	Linear equation (±Standard error)*	R^2
Pedicel	Paraquat	$y = 63.58\ (\pm 4.544)^{a} - 11.73(\pm 4.647)x^{a}$	61.45%
Receptacle	Paraquat	$y = 52.69\ (\pm 3.351)^{b} - 6.80(\pm 3.426)x^{a}$	49.60%
Rachis	Paraquat	$y = 65.46\ (\pm 1.467)^{a} - 9.31(\pm 1.500)x^{a}$	90.60%
Pedicel	Kerssies	$y = 40.79\ (\pm 2.556)^{d} - 6.04(\pm 2.613)x^{a}$	57.17%
Receptacle	Kerssies	$y = 40.74\ (\pm 3.392)^{d} - 6.19(\pm 3.469)x^{a}$	44.35%
Rachis	Kerssies	$y = 46.54\ (\pm 2.078)^{c} - 8.40(\pm 2.114)x^{a}$	79.79%

*Comparison of slopes using standard error of means

Table 2. Equations of linear regressions lines of *B. cinerea* infection levels (y) on pedicel, receptacle and rachis at bunch closure stage, as determine by isolations onto Kerssies and paraquat medium, that were fitted on percentage fluorescent pigment coverage (x) for different parts x growth mediums combinations

Part	**Medium**	**Linear equation (±Standard error)***	**R^2**
Pedicel	Paraquat	$y = 34.60\ (\pm 2.495)^{a} - 3.54(\pm 2.938)x^{\ ab}$	26.64%
Receptacle	Paraquat	$y = 28.38\ (\pm 2.304)^{b} - 4.24(\pm 2.713)x^{\ ab}$	36.86%
Rachis	Paraquat	$y = 28.51\ (\pm 1.061)^{b} + 1.06(\pm 2.702)x^{\ b}$	3.71%
Pedicel	Kerssies	$y = 20.48\ (\pm 3.015)^{c} - 3.35(\pm 3.551)x^{\ ab}$	18.21%
Receptacle	Kerssies	$y = 25.00\ (\pm 1.283)^{b} - 6.22(\pm 1.511)x^{\ a}$	80.92%
Rachis	Kerssies	$y = 16.59\ (\pm 2.476)^{c} - 2.31(\pm 2.916)x^{\ ab}$	13.53%

*Comparison of slopes using standard error of means

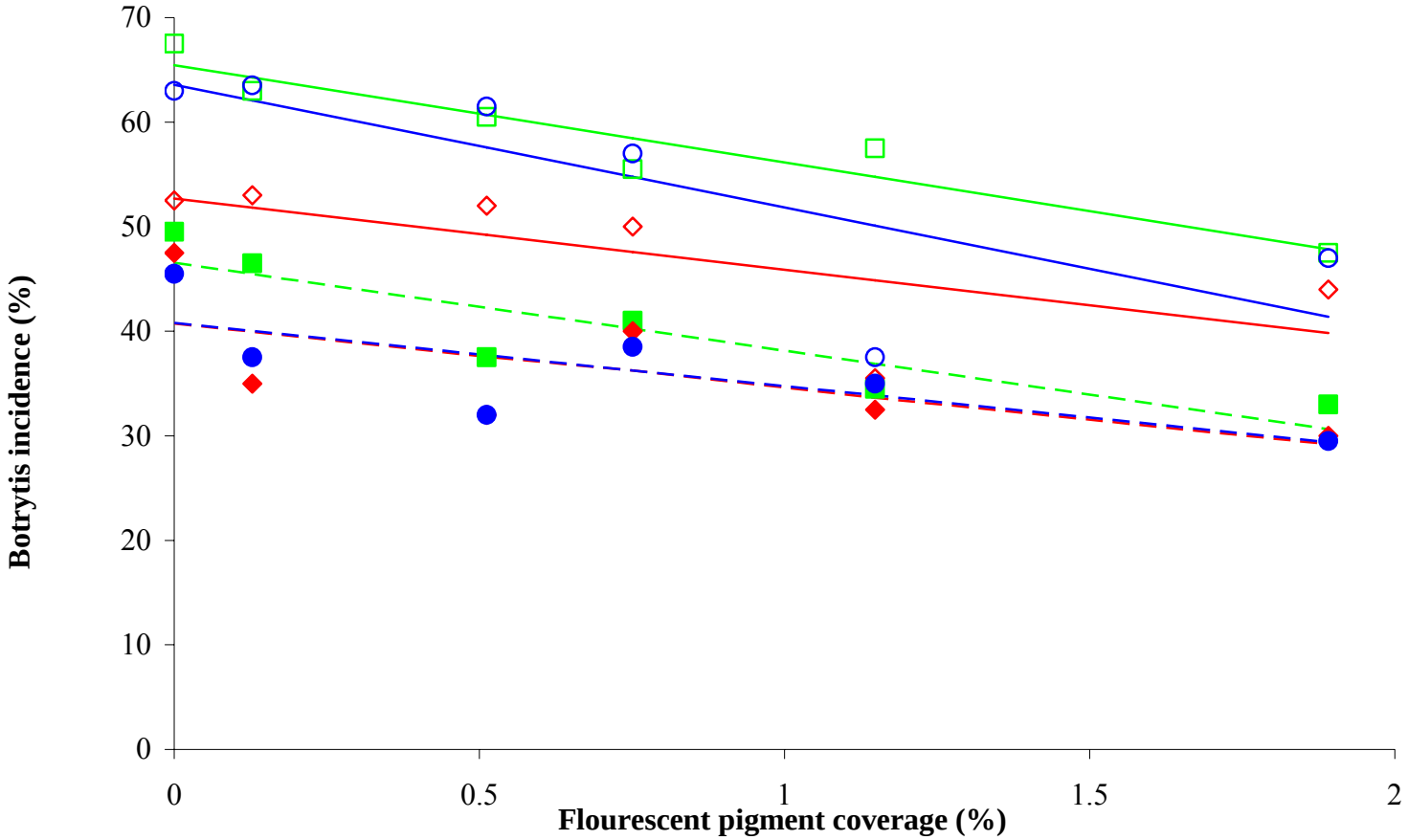

Figure 1. Mean percentage *B. cinerea* incidence on rachises (Kerssies ■, paraquat □), pedicels (Kerssies ●, paraquat ○) and receptacles (Kerssies ♦, paraquat ◊) of inoculated bunches with different coverage levels resulting from fenhexamid / fluorescent pigment mixture sprayed with volumes ranging from 0 to 15 ml at pea size. Linear regression lines were fitted to the data: Kerssies medium (-----) and paraquat medium (——).

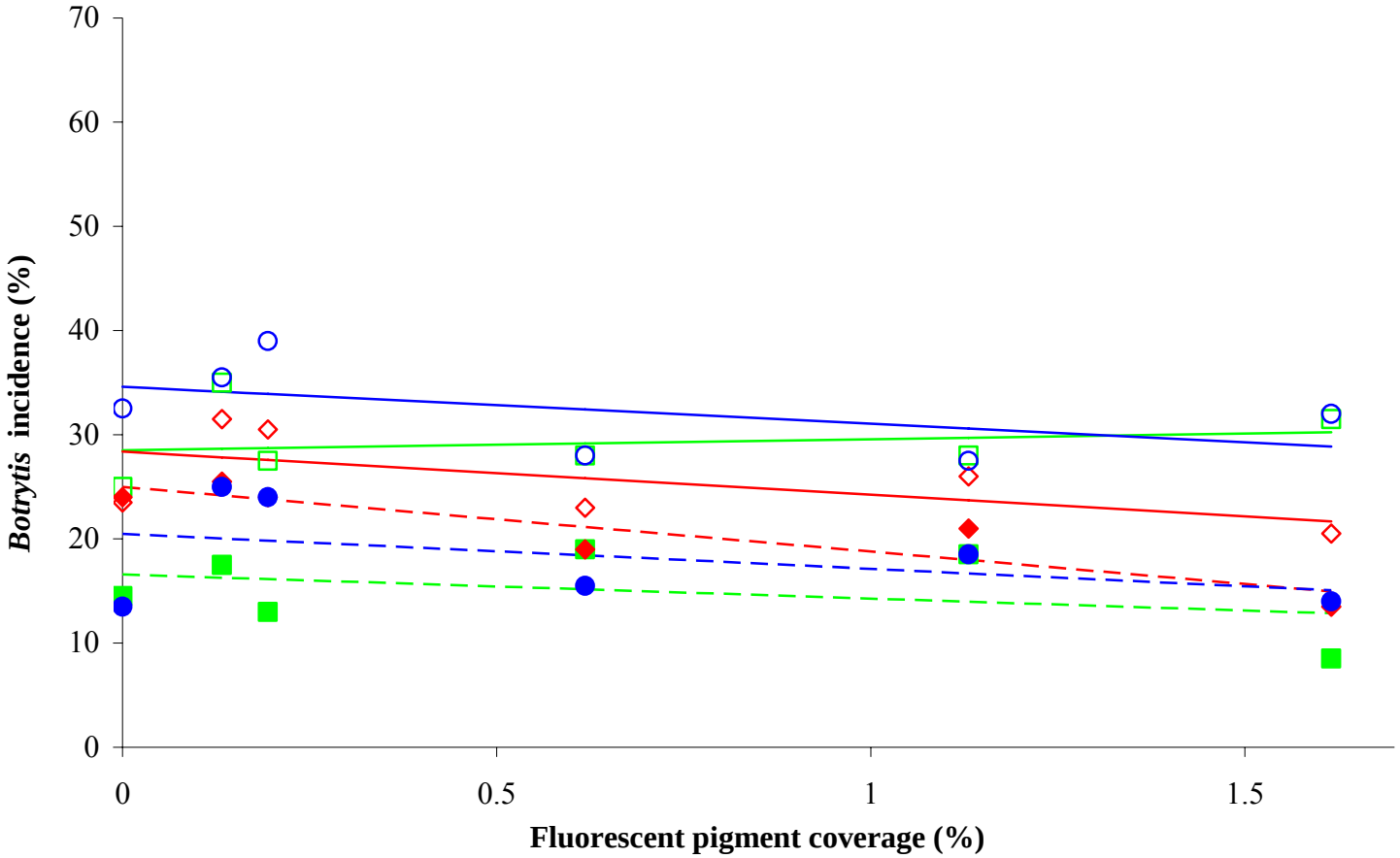

Figure 2. Mean percentage *B. cinerea* incidence on rachises (Kerssies ■, paraquat □), pedicels (Kerssies ●, paraquat ○) and receptacles (Kerssies ♦, paraquat ◊) of inoculated bunches with different coverage levels resulting from fenhexamid / fluorescent pigment mixture sprayed with volumes ranging from 0 to 15 ml at bunch closure. Linear regression lines were fitted to the data: Kerssies medium (-----) and paraquat medium (——).

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