Effect of Fungicide Spray Cover on *Botrytis Cinerea* Infection in Grape Bunches

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The effect of fungicide spray cover on *Botrytis cinerea* infection in grape bunches was determined. 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 determined. 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 on susceptible bunch parts (pedicel, receptacle and rachis) with isolations onto paraquat (herbicide) and Kerssies (*B. cinerea* selective) mediums. Linear regressions for the part $\times$ 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 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 Kerssies 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.

Botrytis bunch rot management strategies are based on knowledge of epidemiology, infection pathways and inoculum ecology of the causal pathogen *Botrytis cinerea* in vineyards (Holz *et al*., 2003). In South Africa, weather conditions are usually favourable for colonisation of inflorescences and young bunches from bloom to late pea-size stage. Conidium numbers in air and at 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*., 2003). 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 resistance increases moderately as the season progresses. 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 when latent infection occurs in the skin (Holz *et al*., 2003). *B. cinerea* symptom expression is 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 (*Penicillium*, *Aspergillus*, *Alternaria*, *Mucor* and *Rhizopus* spp.)

that are commonly associated with bunch rot (Hewitt, 1974; Holz *et al.,* 2003). The next prominent positions occupied by *B. cinerea* are rachises and laterals, and not berry cheeks. *Alternaria alternata* also penetrates rachises and pedicels through stomata and lenticels, and causes 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 develop 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 epiphytotics in grapevines (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 (Van Rooi & Holz, 2003). Chemical control remains the most effective strategy to reduce the incidence of grey mould in grapevines**.** Three preventative fungicide applications are recommended: firstly, between budding and pre-bloom, to protect susceptible inflorescences; secondly, during bloom to pea-size stage, to reduce inoculum in clusters and to prevent colonisation of floral debris;

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and thirdly, at bunch closure, to reduce inoculum 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) have shown that when fungicides are properly applied to the susceptible target sites in bunches, the amount of *B. cinerea* at the various sites within bunches is reduced, and infection and symptom expression are prevented at all growth stages. The same efficacy is, however, not achieved with the same fungicides when using conventional spraying methods in vineyards (Holz *et al*., 2003). This can largely be attributed to incorrect deposition of fungicides on the susceptible target sites. Higher volumes will generally lead to better coverage (Brink *et al.*, 2004), but may lead to a higher risk of exceeding permitted maximum residue levels (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 can 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 dependent on human discretion lack 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 (Brink *et al.*, 2004). 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.

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 block design with six spray volumes, replicated in five blocks. Each block was divided in four groups (treatment combinations) of six 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 were 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 and paraquat mediums. From each bunch, four samples each of pedicels, rachises and receptacles 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 showed that the fluorescent pigment did not influence *B. cinerea* growth on PDA medium (unpublished results). Bunches were sprayed from two sides, with spray volumes ranging from 1 to 15 mL, 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 [$660 \times 1410 \times 800$ mm (h/l/w)]. These spray volumes equate to 111.11 to 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 developed and validated by Brink *et al.* (2004). Three pedicel samples for each spray treatment were taken from the sprayed side of a selected bunch. Images (12 megapixels) were digitally captured through the stereoscopic microscope at 20× 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. Using the measurement tools, pigments within a selected area of interest (AOI), with a size of 0.465 mm², were automatically counted and measured. The total area of deposited pigment was automatically calculated in five AOIs for each photo. Fluorescent pigment coverage was calculated as a mean of the percentage area covered by fluorescent pigment in each AOI.

Inoculation and incubation

Dry conidia from a virulent isolate of *B. cinerea* were prepared as described by Coertze and Holz (1999). Bunches were inoculated with 3 mg dry conidia, which were dispersed by air pressure into the top of an inoculation tower (Plexiglass, $3 \times 1 \times 1$ m [height × depth × 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 mesh screens on the floor of the inoculation tower. By using this inoculation technique, approximately three conidia were evenly deposited as single cells on each mm² of bunch surface (Coertze & Holz, 1999). Petri dishes with water agar (WA) were placed next to the bunches in the inoculation tower and the percentage germination was determined 6 h after inoculation (100 conidia per Petri dish, two replicates).

Following inoculation, the groups of bunches were placed on sterile epoxy-coated steel mesh screens ($53 \times 28 \times 2$ 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 bunches were incubated for 24 h at 22°C. These conditions are similar to what the pathogen might encounter in nature, namely dry conidia on dry bunch parts under high relative humidity. Uninoculated bunches were used to determine the natural infection levels of *B. cinerea*.

Assessment of *B. cinerea*

From each bunch, five receptacles, pedicels and rachis sections (5 mm each) were placed on Petri dishes containing Kerssies *B. cinerea* selective medium (Kerssies, 1990), or on water agar medium supplemented with paraquat (Grindrat & Pezet, 1994), for isolations. The plates were incubated at 22°C under diurnal

light and the sections were monitored daily for symptom expression and the development of *B. cinerea*. After 11 days the number of sections yielding sporulating *B. cinerea* colonies were recorded, and these were used to determine the percentage incidence of *B. cinerea* at the various positions in the bunches. The different isolation 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 five days of incubation. Hyphal growth usually occurred from cells underlying the cuticle into the medium after five 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 fluorescent pigment 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, SAS Campus Drive, Cary, North Carolina 27513). 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 from uninoculated bunch parts was 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, 1, 3, 6, 9 and 15 mL was 0%, 0.13%, 0.51%, 0.75%, 1.15% and 1.89% respectively (Fig. 1). This was associated with a linear reduction in *B. cinerea* incidence from 46.54% – 67.50% 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). Regression lines for part × medium combinations at pea size showed no significant difference between slopes, and a consistent difference in susceptibility among bunch parts was not observed (Table 1). Furthermore, significantly higher *B. cinerea* levels were recorded

TABLE 1

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

Part	Medium	Linear equation (±Standard error)*	R^2
PPedicel	Paraquat	y = 63.58 (±4.544)[a] – 11.73(±4.647)x [a]	**61.45%**
Receptacle	Paraquat	y = 52.69 (±3.351)[b] – 6.80(±3.426)x [a]	**49.60%**
Rachis	Paraquat	y = 65.46 (±1.467)[a] – 9.31(±1.500)x [a]	**90.60%**
Pedicel	Kerssies	y = 40.79 (±2.556)[d] – 6.04(±2.613)x [a]	**57.17%**
Receptacle	Kerssies	y = 40.74 (±3.392)[d] – 6.19(±3.469)x [a]	**44.35%**
Rachis	Kerssies	y = 46.54 (±2.078)[c] – 8.40(±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 determined by isolations onto Kerssies and paraquat mediums, that were fitted on percentage fluorescent pigment coverage (x) for different part × growth medium combinations.

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

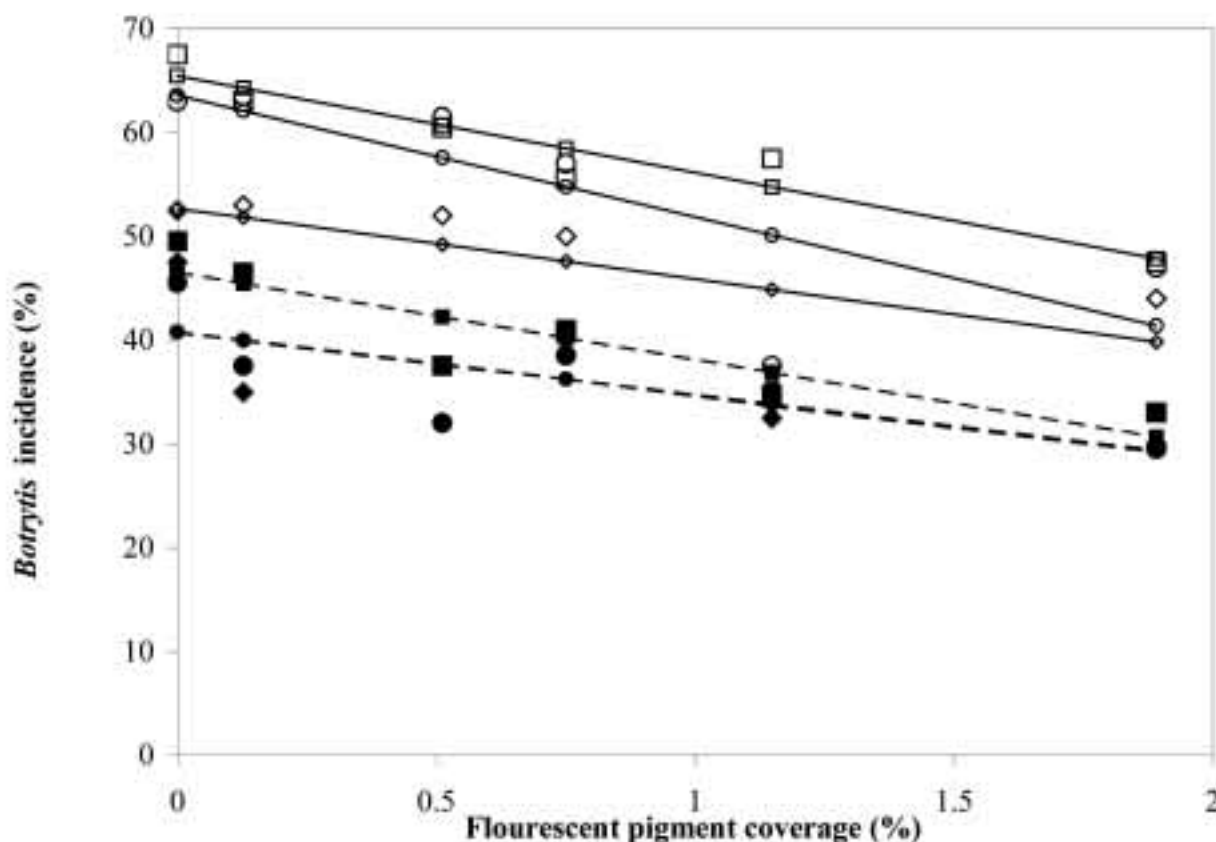*Comparison of slopes using standard error of means

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 (———).

on paraquat medium (52.69% – 65.46%) compared to on the Kerssies medium (40.74% – 46.54%) on all parts of unsprayed bunches (x = 0) (Table 1).

Bunch closure

Botrytis cinerea incidence recorded at bunch closure stage (16.59% – 34.60%) was markedly lower than that recorded at pea size stage [40.74% – 63.58% (Tables 1 and 2; Figs. 1 and 2)]. Markedly higher *B. cinerea* incidence on unsprayed bunches was recorded on paraquat medium (28.31% – 34.60%) compared with the Kerssies medium (16.59% – 25.00%) on all parts (Table 2). Mean fluorescent pigment coverage effected by spray volumes 0, 1, 3, 6, 9 and 15 mL was markedly lower at bunch closure [0%, 0.13%, 0.19%, 0.62%, 1.13% and 1.61%, respectively (Fig. 2)] than at pea size (Fig. 1). A general reduction in *B. cinerea* from 13.5% – 39% to 8.5 – 32% was observed for the various bunch parts with increased spray cover (Fig. 2). However, most combinations gave poor linear fits at bunch closure (R^2 = 3.71% – 36.86%), with omissible slopes for the rachis × paraquat, pedicel × kerssies and rachis × kerssies combinations (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.

DISCUSSION

This study showed that an increase in fungicide spray cover reduced the infection levels of *B. cinerea* on susceptible bunch parts. This indicates the importance of adequate spray cover on susceptible bunch parts for the control of *B. cinerea* in grapevines. The linear relation between spray cover and *B. cinerea* incidence was, however, less evident at bunch closure. This might be explained by the influence of the growth stage and different bunch parts on the deposition of fungicides. The highest deposition was measured early in the season and it decreased as bunches became more compact (Brink *et al.*, 2004). This was largely attributed to the higher porosity of bunches at pea size compared to the more compact bunches at bunch closure, as well as higher air flow retention on the front of bunches than on inner bunch parts (Barry & Weber, 2002). It can furthermore be hypothesised that bunches at pea-size stage would have received higher dosages of evenly dispersed dry conidia than bunches 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
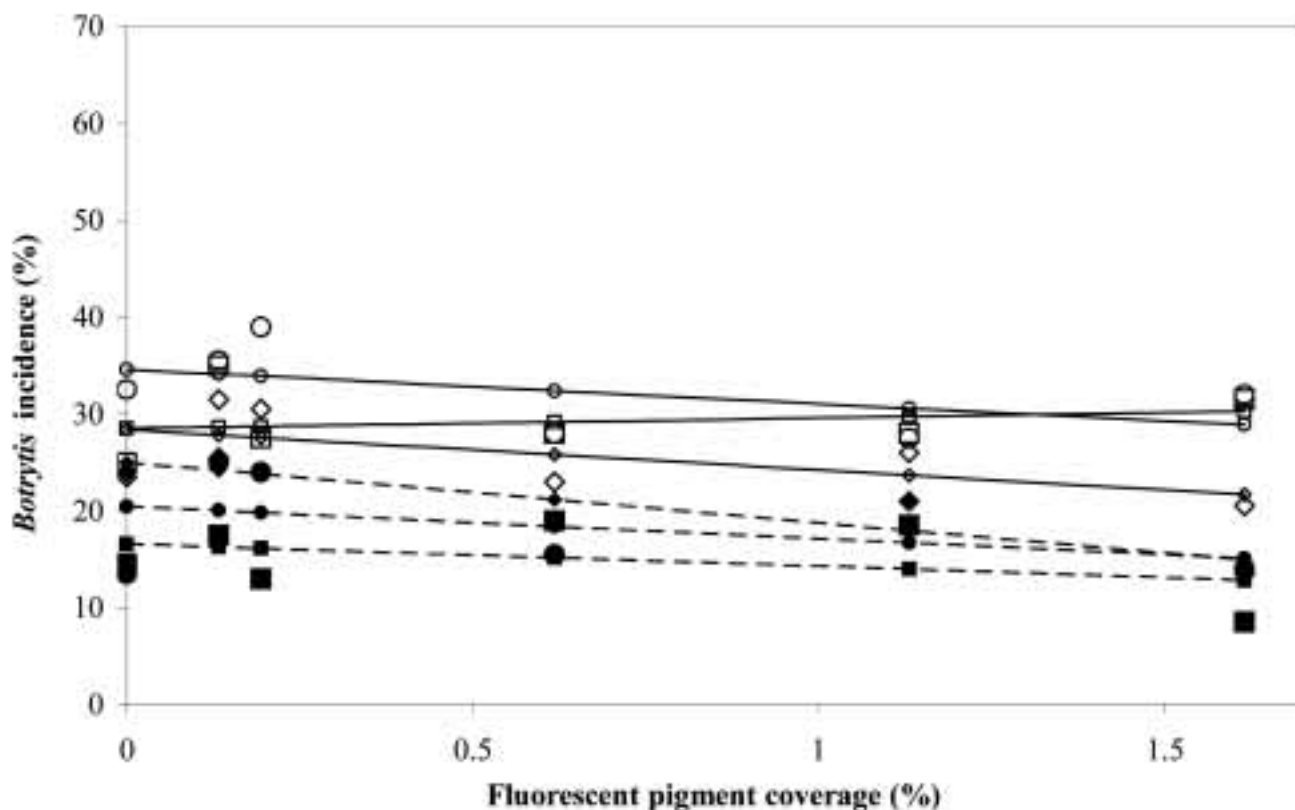
FIGURE 2

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 (———).

our study, rachises might have had lower levels of fungicide cover than pedicels (Brink *et al.*, 2004), 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), which might have contributed to the variability of incidence data.

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 cover will result in decreased infection. However, several factors, [i.e. too low spray volumes (i.e. spray cover), 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 a 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 two-dimensional shapes, which might lead to better linear relations between spray cover and *B. cinerea* infection levels.

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