THE ROLE OF THE MEDITERRANEAN FRUIT FLY, *CERATITIS CAPITATA*, IN BOTRYTIS BUNCH ROT OF GRAPE

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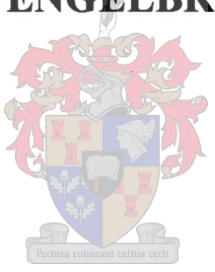

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DECLARATION

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.

SUMMARY

THE ROLE OF THE MEDITERRANEAN FRUIT FLY, *CERATITIS CAPITATA*, IN *BOTRYTIS* BUNCH ROT OF GRAPES

Botrytis bunch rot of grape is caused by *Botrytis cinerea* Pers.:Fr. Conidia of the pathogen, which is dispersed by wind, water droplets and by insects, can penetrate the intact grape berry cuticle, but disease expression occurs only under predisposing conditions. Since relatively high infection rates often occur in vineyards, predisposing factors must play a fundamental role in primary infection and subsequent disease occurrence. Insects can play a very important role in this regard by depositing inocula at wound sites during feeding and by providing fresh wounds during their oviposition and feeding activities. The aim of this study was (i) to determine the potential of the Mediterranean fruit fly to transfer *B. cinerea* and other bunch and fruit rot fungi *in natura*, (ii) to investigate the transport, deposition and subsequent disease expression on grape berries *in vitro*, and (iii) to investigate fruit fly activities and the nature of deposited conidia and mycelia of *B. cinerea* by aid of digital photography and epifluorescence microscopy, respectively.

Two Sensus fruit fly traps containing the para-pheromone, Capilure, were installed in orchards and five neighboring vineyards on four farms in the Stellenbosch region. *Ceratitis* fruit flies were collected weekly, identified and counted to determine the fluctuations in fruit fly population. Following field collection, the fruit flies were plated on Kerssies' *B. cinerea* selective medium and the number of flies yielding the pathogen was recorded. Two fruit fly species, *C. capitata* and *C. rosa*, were captured during the study period. *Ceratitis rosa* numbers comprised only 1% of the total number of fruit flies captured. *Ceratitis capitata* numbers, and the percentage *B. cinerea* contaminated flies generally increased after harvest in the different orchards and vineyards. Following harvest, the percentage flies yielding *B. cinerea* was higher in vineyards compared to orchards. Furthermore, in each vineyard an increase in percentage *B. cinerea* contaminated fruit flies was preceded by a corresponding increase in its neighboring orchard. The levels of both *Penicillium* and *Alternaria* contaminated fruit flies stayed high throughout the investigation period, especially after harvest of the orchard cultivars. Low incidence of *Aspergillus*, *Mucor* and *Rhizopus* spp. were recorded on *C. capitata*. These findings suggest that the Mediterranean fruit fly may

play an important role in the dispersal of inocula of fungi associated with postharvest decay from early-maturing stone fruit orchards to mid- and late-maturing wine grape vineyards, and in disease induction under conditions unfavourable for natural infection.

Three experiments were conducted to determine the potential of fruit flies in provoking *B. cinerea* decay. In the first experiment, transport of conidia and disease expression were investigated on rachis segments bearing unwounded berries only. In the second experiment, the effect of wounding on disease expression was investigated. In the third experiment, the effect of inoculum type (mycelia and conidia) on transportation and disease expression was investigated on rachis segments bearing unwounded berries, and on segments with wounded berries. The table grape cultivar, Dauphine, and the wine grape cultivar, Shiraz, were used at véraison, two weeks before harvest and harvest, and the transport studies were conducted in ethanol-disinfected perspex cages. Disease expression was studied in dry (≥56% RH), ethanol-disinfected perspex chambers incubated at 22°C. The isolations from berries revealed that the flies deposited, without preference, high amounts of *B. cinerea* at various positions on the grape berry's surface. The freezing studies showed that the deposited conidia germinated and penetrated the berry skin at various positions. However, *B. cinerea* developed more often at the pedicel end than on the cheek or style end, which indicated a peculiar interaction between *B. cinerea,* the fruit fly and host tissue at this part of the berry. This phenomenon was substantiated by the finding that *B. cinerea* also developed more often at the pedicel end of berries that were not frozen. Further evidence for this interaction was found on intact berries exposed to flies that carried mycelia after feeding on berries without sporulating colonies of the pathogen, but showing symptoms of slippery skin. Significantly more decay developed on wounded berries compared to the unwounded berries and more so at the wound site. In addition, female fruit flies were responsible for significantly more decay development than male fruit flies. The study thus proved that the Mediterranean fruit fly can promote *B. cinerea* disease development under conditions unfavorable to natural infection.

The activities of the Mediterranean fruit fly, *Ceratitis capitata*, on grape berries were monitored by aid of digital photography. In addition, the deposition of conidia and mycelia of *Botrytis cinerea* at three sites (pedicel end, cheek and style end) on the grape berry, germination of the fungal structures after dry (±56% RH) and moist (±93% RH) incubation and wounds inflicted during ovipositioning were examined with an epifluorescence microscope. The observations revealed that the fruit fly's activities were generally restricted

to the grape berry. They visited the grape berry cheek more often, but visitations to the pedicel end of berries increased substantially from véraison to harvest, indicating the possibility of nutrient leakages at this site. Microscopy revealed that the flies deposited conidia singular, in feeding packages and in faecal excrements on the berry surface. The conidia in feeding packages were ensheathed by salivical fluids and occurred in clusters of 10 to 50 conidia. An average of 60% of the conidia in feeding packages germinated under dry conditions (±56% RH). Conidia that passed through the intestinal tract of the fruit fly and that were deposited in faecal excrements were deformed and low in viability. These conidia did not occur in cluster format, but were proportionally spread with the faeces on the surface of the grape berry. Conidia that were deposited singular and in faecal excrements did not germinate unless incubated under moist conditions (± 93% RH). Wounds inflicted by female fruit flies during ovipositioning were most frequently observed on the cheek. This predisposition to *B. cinerea* infection of grape berries by the activities of fruit flies, suggested an important role for the flies in the initiation of *Botrytis* bunch rot epidemics in vineyards.

OPSOMMING

DIE ROL VAN DIE MEDITERREENSE VRUGTEVLIEG, *CERATITIS CAPITATA*, IN *BOTRYTIS CINEREA* TROSVERROTTING VAN DRUIWE

Botrytis-trosverrotting van druiwe word deur *Botrytis cinerea* Pers.:Fr. veroorsaak. Konidia van die patogeen wat deur wind, waterdruppels en insekte versprei word, kan die intakte druiweskil binnedring, maar siekte-uitdrukking vind slegs onder spesiale omstandighede plaas. Aangesien relatief hoë infeksie vlakke algemeen in wingerde voorkom, moet predisponerende faktore 'n fundamentele rol in die primêre infeksie, en die daaruit voortspruitende siektetoestand speel. Insekte kan 'n baie belangrike bydrae lewer deur inokula tydens voeding by wonde te deponeer. Nuwe wonde kan ook tydens oviposisionering en voeding ontstaan. Die doel van hierdie studie was om (i) die potensiaal van die Mediterreense vrugtevlieg om *B. cinerea* en ander tros- en vrugverrottingswamme *in natura* oor te dra, te bepaal; om (ii) die verspreiding, deponering en daaropvolgende siekte-uitdrukking op druiwekorrels *in vitro* te ondersoek; en om (iii) die aktiwiteite en aard van die gedeponeerde konidia en miselia met behulp van digitale fotografie sowel as epifluoressensie-mikroskopie waar te neem.

Twee Sensus-vrugtelokvalle met die paraferomoon, Capilure, is in vrugteboorde en aangrensende wingerde in die Stellenbosch-omgewing aangebring. *Ceratitis*-vrugtevlieë is weekliks versamel, geïdentifiseer en getel om fluktuasies in die vrugtevliegpopulasie te bepaal. Na die veldversameling is die vrugtevlieë op Kerssies se *B. cinerea*-selektiewe medium uitgeplaat. Gedurende die studie is twee spesies vrugtevlieë, *C. capitata* en *C. rosa*, gevang. Na oesstyd het die aantal *Ceratitis*-vrugtevlieë en die persentasie vrugtevlieë, besmet met *B. cinerea*, in die verskillende boorde en wingerde toegeneem. Na oestyd was die persentasie vrugtevlieë wat *B. cinerea* gedra het, hoër in die wingerde as in die boorde. Elke toename in die persentasie *B. cinerea*-besmette vrugtevlieë in 'n wingerd is voorafgegaan deur 'n ooreenkomstige toename in die aangrensende vrugteboord. Die aantal vrugtevlieë besmet met *Penicillium* en *Alternaria* spp. het tydens die navorsingstydperk deurgaans hoog gebly, veral nadat die vrugteboord-kultivars geoes is. Die voorkoms van *Aspergillus*-, *Mucor*- en *Rhizopus* spp. op *Ceratitis*-vrugtevlieë was deurgaans laag. Hierdie bevinding wys daarop dat vrugtevlieë 'n belangrike rol speel in die verspreiding van swaminokula, wat met

na-oes verrotting geassosieer word, van vroegrypwordende steenvrugteboorde na mid- en laatrypwordende wyndruifwingerde.

Drie eksperimente is *in vitro* onderneem om vrugtevlieë se potensiaal om *B. cinerea*-verrotting te veroorsaak te bepaal. In die eerste eksperiment is ragi met slegs ongewonde korrels gebruik om die oordrag van konidia en siekte-ontwikkeling te ondersoek. In die tweede eksperiment is die effek van verwonding op siekte-ontwikkeling ondersoek. In die derde eksperiment is die effek van inokulumtipe (miselia en konidia) op verspreiding en siekte-ontwikkeling ondersoek deur ragis-segmente met gewonde korrels sowel as ragis-segmente met ongeskonde korrels te gebruik. Die tafeldruif-kultivar Dauphine en die wyndruif-kultivar Shiraz, by kleurbreuk, twee weke voor oes en by oestyd, is in die eksperimente gebruik. Die oordragstudies is in etanol-ontsmette perspex-hokke uitgevoer. Siekte-ontwikkeling is bestudeer in droeë (±56% RH), etanol-ontsmette perspex-kamers en geinkubeer by 22°C. By ondersoek is gevind dat vlieë, sonder voorkeur, groot hoeveelhede *B. cinerea* op verskeie dele op die druiwekorrel-oppervlak deponeer. Bevriesingstudies het aangetoon dat die gedeponeerde konidia op verskeie dele van die korrel ontkiem en die skil binnedring. *Botrytis cinerea* het egter meer dikwels by die korrelsteelkant as by die stempelkant, of op die wang, ontwikkel. Hierdie bevinding het 'n eiesoortige interaksie tussen *B. cinerea*, die vrugtevlieg en gasheerweefsel by die korrelsteelkant van die korrel aangetoon. Die verskynsel is gestaaf deur die bevinding dat *B. cinerea* ook meer dikwels by die korrelsteelkant van die korrels wat nie gevries is nie, ontwikkel het. Verdere bewys van hierdie interaksie is gevind by ongeskonde korrels wat aan die vlieë wat miselia gedra het blootgestel is. Die siekte het beduidend meer dikwels op gewonde as ongewonde korrels en verder aansienlik meer dikwels op die wondoppervlakte ontwikkel. Dit was ook duidelik dat vroulike vrugtevlieë baie meer vir verrotting verantwoordelik was as manlike vrugtevlieë. Die studie bewys dus dat Mediterreense vrugtevlieë die ontwikkeling van *B. cinerea* kan bevorder in omstandighede wat ongunstig is vir natuurlike infeksie.

Die aktiwiteite van die Mediterreense vrugtevlieg *C. capitata* op die druiwekorrels is met behulp van digitale fotografie waargeneem. Verder is die deponering van konidia en miselia van *B. cinerea* op die verskillende dele (korrelsteelkant, wang en stempelkant) van die korrel, ontkieming van die swamstrukture na droeë (±56% RH) en nat (±93% RH) inkubasie en wonde wat tydens oviposisionering veroorsaak is, met epifluoressensie-mikroskopie ondersoek. Die waarnemings het onthul dat die vrugtevlieg se aktiwiteite gewoonlik tot die

druiwekorrel beperk is. Hulle het korrelwange meer dikwels besoek. Besoek aan die korrelsteelkant het aansienlik toegeneem van kleurbreuk tot oestyd, wat op die moontlikheid van voedingstof-lekkasie by die deel aandui. Mikroskoopstudies het aangedui dat vlieë konidia enkel, in voedingspakkies en in fekale uitskeidings op die korreloppervlakte deponeer. Die konidia in die voedingspakkies is deur speekselvloeistof omhul en het in groepe van 10 tot 50 konidia voorgekom. Gemiddeld 60% van die konidia in voedingspakkies het in droeë omstandighede (±56% RH) ontkiem. Konidia wat deur die spysverteringskanaal van die vrugtevlieg gegaan het en in die fekale ekskresie gedeponeer is, was misvorm en het lae lewensvatbaarheid gehad. Laasgenoemde konidia was nie in groepe gedeponeer nie, maar is proporsioneel met die feces op die oppervlak van die druiwekorrel versprei. Konidia wat enkel en in feces gedeponeer is, het nie ontkiem nie, tensy toestande vogtig (±56% RH) was. Wonde wat deur die vroulike vrugtevlieë tydens oviposisionering veroorsaak is, is meer dikwels op die wang van die korrel opgemerk. Hierdie predisposisie van druiwekorrels tot *B. cinerea*-infeksie, meegebring deur die aktiwiteit van die vrugtevlieg, dui daarop dat die rol wat die vrugtevlieg in die inisiëring van *Botrytis* trosverrotting-epidemies in wingerde speel, van beduidende belang is.

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1. THE ROLE OF INSECTS IN *BOTRYTIS* BUNCH ROT, WITH REFERENCE TO *CERATITIS* FRUIT FLIES

INTRODUCTION

Botrytis cinerea Pers.:Fr., the asexual stage of the teleomorph *Botryotinia fuckeliana* (de Bary) Whetzel, is a polyphagous and ubiquitous fungus, which causes grey mold on a wide variety of crop plants (Agrios, 1997). Botrytis bunch rot is one of the most important diseases of grapevine (*Vitis vinefera* L.) (Hewitt, 1974; Nair & Hill, 1992). Conidia of *B. cinerea*, responsible for primary infection, are almost always present in vineyards (Corbaz, 1972; Bulit & Verdu, 1973) and are dispersed by wind (Jarvis, 1980), water droplets (Jarvis, 1962) and insects (Fermaud & Le Menn, 1989, 1992; Michailides & Spotts, 1990; Fermaud & Giboulot, 1992; Fermaud *et al.*, 1994; Fermaud & Gaunt 1995; Louis *et al.*, 1996; Michailides & Morgan, 1996; Mondy *et al.*, 1998; Mondy & Corio-Costet, 2000). Conidia dispersed by air currents are deposited as single cells on spore traps (G. Holz, *unpublished data*) and occur as single colony forming units in nature (Duncan *et al.*, 1995). In addition, very few conidia, dispersed in water droplets, become wet enough to enter the droplets, and are subsequently deposited as single cells during run-off (Jarvis, 1962). Coertze and Holz (1999) and Coertze *et al.* (2001) confirmed that the intact grape berry skin provides an effective barrier to penetration of solitary conidia. These findings suggest that only two pathways exist for primary infection by *B. cinerea* in nature. The first is through the flower parts, leading to latent infections and the second is through predisposed tissue (e.g. maturity, weather conditions / rain, high relative humidity, wounding). Since relatively high infection rates often occur in vineyards (Nair & Nadtotchei, 1987), predisposing factors such as wounding must play a fundamental role in primary infection and subsequent disease occurrence (Du Plessis, 1934; Hill *et al.*, 1981; Nair *et al.*, 1988; Coertze & Holz, 1999). A combination of fresh wounds and new inoculum is, however, needed for successful wound infection (Mercier & Wilson, 1994; Spotts *et al.*, 1998; Coertze & Holz, 1999). Insects can play a very important role in this regard by ensuring the timely placement of fungal conidia on the susceptible tissue of the host plant.

BOTRYTIS CINEREA

Overwintering and dispersal

Botrytis spp. overwinter as dormant structures including sclerotia (Nair & Nadtotchei, 1987), mycelia (Pearson & Goheen, 1994) and microconidia (Coley-Smith, 1980). Sclerotia are hard structures that are resistant to extremes in temperature (Coley-Smith, 1980; Nair & Martin, 1987). A common source of sclerotia is mummified grapes formed during previous seasons (Hewitt, 1974). Sclerotia and mycelia are sources of primary inoculum by formation of conidia (Nair & Nadtotchei, 1987), but also infect host tissue directly (Nair & Hill, 1992; Pearson & Goheen, 1994). Conidia are responsible for primary infection (Doss *et al.*, 1995) and are dispersed by wind (Jarvis, 1980), water droplets (Jarvis, 1962) and insects (Fermaud & Le Menn, 1989, 1992; Michailides & Spotts, 1990; Fermaud & Giboulot, 1992; Fermaud *et al.*, 1994; Fermaud & Gaunt 1995; Louis *et al.*, 1996; Michailides & Morgan, 1996; Mondy *et al.*, 1998; Mondy & Corio-Costet, 2000).

Germination

Germination is dependent on a specific set of environmental conditions. High humidity or wetness (Nelson, 1951; Duncan *et al.*, 1995), and low wind speed (Thomas *et al.*, 1988), temperature (Kerssies, 1994) and light infiltration (Kerssies, 1992, 1993; Duncan *et al.*, 1995) promote *B. cinerea* germination. Germination occurs at temperatures between 1 and 30°C (optimum 20°C) (Nair & Nadtotchei 1987; Kerssies, 1994; Pearson & Goheen, 1994) and germ tube elongation is optimal at a temperature of 30°C (Hennebert and Gilles in: Jarvis, 1980).

Conidial germination and growth outside the cuticle are stimulated by different nutrient sources, such as pollen, floral parts or exudates from the berry. Simple sugars, of which fructose is the most effective, promote conidial germination (Blakeman, 1975). Kosuge and Hewitt (1964) found that sugar concentrations as high as 5×10^{-4} M are taken up by free water on the surface of mature grape berries. Conidia that germinate in the presence of these sugars produce long germ tubes, and are ensheathed by a fibrillar-like matrix. Dry inoculated conidia produce

short germ tubes and attempt to penetration directly (Cole *et al.*, 1996; Coertze *et al.*, 2001). Other nutrients found in exudates, such as amino acids and pollen, are also found to promote conidial germination (Chou and Preece, 1968; McClellan & Hewitt, 1973).

Infection

Infection of host plants by *B. cinerea* arises from either conidial germ tubes or mycelium growing on dead plant tissues or on extraneous organic material (Verhoeff, 1980). Infection, as for germination, is subjected to specific environmental conditions. High relative humidity (Nelson, 1951; Kerssies, 1992, 1993; Harrison *et al.*, 1994; Eden *et al.*, 1996) and/or wetness (Nelson, 1951; Corbaz, 1972; Hewitt, 1974; Gessler & Jermini, 1985; Bulger *et al.*, 1987; Broome *et al.*, 1995) are conducive to infection. Primary infection occurs predominantly after the first spring rains (Jarvis, 1980).

Coertze and Holz (1999) and Coertze *et al.* (2001) demonstrated that single conidia are unable to induce disease symptoms on sound grape berries under moist or wet conditions. Ripe grapes are, however, considered to be susceptible to decay by clusters of conidia (Nelson, 1956; Hill *et al.*, 1981; Nair & Allen, 1993; Broome *et al.*, 1995). Penetration by clusters of conidia at a single site could alter the host response to infection and hence the estimate of the level of susceptibility (Fourie & Holz, 1995; Holz & Coertze, 1996; Holz *et al.*, 1997, 1998). Van den Heuvel and Waterreus (1983) and Eden *et al.* (1996) ascribed the increased infectivity associated with higher inoculum concentrations to more germ tubes produced. The increased germ tube numbers are responsible for increased enzymatic activity and thereby facilitate direct penetration of the host surface.

Botrytis bunch rot is associated with two infection modes, early-season latent infections through flowers and immature tissue (McClellan & Hewitt, 1973; Hewitt, 1974; Gessler & Jermini, 1985; Marais, 1985; Nair & Parker, 1985; Nair *et al.*, 1988) and late-season infections of mature or predisposed tissue (McClellan & Hewitt, 1973; Marais, 1985; Nair & Parker 1985, Nair *et al.*, 1988). McClellan and Hewitt (1973) found that *B. cinerea* invaded the stigma and style during bloom after which it became latent. Pollen stimulated germination of conidia and

germ-tube growth and thus enhanced colonization of flower parts by *B. cinerea*. It remained in the necrotic stigma and style tissue until véraison when latency was negated and *B. cinerea* resumed growth. Latency in immature stages was ascribed to lack of moisture and the inhibitory effect of exudates from the immature berry skin (McClellan & Hewitt, 1973). Nair and Parker (1985) assigned resistance in immature Shiraz berries to lower sugar levels. Defense systems operating in the fruit skin also generated latency (Hewitt, 1974). Late-season infections occurred predominantly on predisposed tissue. Maturation, wounding, bunch architecture, moribund tissue and weather conditions played a significant role in predisposing fruit to infection.

Host resistance

Passive defense, proanthocyanidins in skins (Hill *et al.*, 1985) and substances in berry exudates (Kosuge & Hewitt, 1964; McClellan & Hewitt, 1973; Pezet & Pont, 1986; Padgett & Morrison, 1990; Vercesi *et al.*, 1997), play a significant role in the resistance of grape to infection by *B. cinerea*. Passive defense occurs mainly in preformed morphological barriers in the outer layers (epidermal cell wall and cuticle) of the fruit skin. The structure and thickness of the cuticle are regarded as the major factor of resistance against *B. cinerea* (Hill, 1985a,b; Marois *et al.*, 1986). The removal of the epicuticular wax of mature berries enhances infection by *B. cinerea* (Hill, 1985b). Kerssies and Frinking (1996) however found no relation between thickness in cuticular wax and host resistance. Phenols are one of several groups of compounds, found in the grape berry skin, that inhibit the penetration of germ tubes (Padgett & Morrison, 1990). Proanthocyanidins, tannin-like substances, in immature grape berry skins, also play a significant role in inhibiting macerating enzymes of *B. cinerea* (Hill *et al.*, 1981). The pH of grape berry exudates inhibits germination and growth of *B. cinerea*. Pucheu-Planté and Mercier (1983) reported an optimum at pH 4.0, with depressed growth at either higher or lower values, and total inhibition at pH extremes. Germ tube growth and colonization by *B. cinerea* are inhibited during the early stages of the grape berry when organic acids are the main carbon source (Vercesi *et al.*, 1997).

Immature fruit, lacking the protection of the cuticle, however maintain resistance to *B. cinerea* infection. The phenomenon that green berries are resistant to infection, is largely due to built-in

defense mechanisms. Active defense mechanisms include lignification-like reactions (Hill, 1985b), phytoalexins (Langcake, 1981; Creasy & Coffee, 1988; Hoos & Blaich, 1988) and suberin (Hill, 1985b). During penetration, these active defense systems are triggered around the infection site in response to injury. Hill (1985b) found that immature berries lose their ability to resist *B. cinerea* infection when wounded and concluded that active defense mechanisms are localized in the grape berry skin. It was however observed that the induction of stilbene and suberin are inhibited from véraison onwards (Coertze *et al.*, 2001).

Predisposition

Wounds. Wounds are regarded as a very important route of entry for *B. cinerea* (Nair *et al.*, 1988; Edlich *et al.*, 1989; Brook, 1991; Sharrock & Hallett, 1991; Elad & Evensen 1995). Elad and Evensen (1995) attributed this to the presence of substrates found on injured berry cuticles, which are necessary for the synthesis of cell wall-degrading enzymes. The success of wound infection is, however, largely dependent to the age of the wound. Histochemical tests done on wounded mature pear fruit by Spotts *et al.* (1998) showed the formation of callose, suberin, tannins and pectic substances, as well as gums and starch within four days after wounding. Mercier and Wilson (1994) reported that one-day-old wounds were less susceptible to infection then fresh wounds. Coertze and Holz (2001) found that conidia and germlings never infected dry wounds, but only fresh wounds by fresh conidia under humid or wet conditions.

The wounding of fruit can take place in a number of ways including mechanical activities (pruning and harvesting), weather conditions (sun, hail, frost, rain and wind) and biological factors (bunch architecture, other pathogens and insects) (Jarvis, 1980; Verhoeff, 1980; Savage & Sall, 1983; Gessler & Jermini, 1985; Nair *et al.*, 1988; Spotts *et al.*, 1998). The role of insects in wounding and subsequently enhancing disease development has been extensively reported reported. A study done by Nair *et al.* (1988) showed that grape berry bunches infested with light-brown apple moth (*Epiphas postvittana* Walker) are more susceptible to infection by *B. cinerea* due to wounds inflicted by their feeding behavior. This was also demonstrated for kiwifruit (*Actinidia deliciosa*) infested by the snails (*Helix aspersa* Mueller) and thrips (*Thrips obscuratus* Crawford). Wounds caused by snails eating the sepals around the receptacle area (Michailides &

Morgan, 1996) and thrips feeding on the flower petals (Fermaud & Gaunt, 1995) promote *B. cinerea* infection. In a study done to evaluate the influence of mite and aphid infestations on the biological control of *B. cinerea* by *Clonostachys rosea* (Link: Fr.) on rose (*Rosa hybrida*) leaves, it was found that conidial germination and growth were enhanced by the wounds made by these insects (Morandi *et al.*, 2000), thus enhancing the efficacy of this biological control method.

Maturation. A general increase in susceptibility to infection was observed as the grape berry matures (Stalder, 1953; Hill *et al.*, 1981; Northover, 1987; De Kock & Holz, 1991; Chardonnet *et al.*, 1997; Coertze *et al.*, 2001). This phenomenon was attributed to crack formation and a decrease in cutin content during maturation (Commenil *et al.*, 1997). Sucrose, glucose and fructose tended to increase in exudates from véraison onwards (Brown & Coombe, 1985; Nair *et al.*, 1988; Padgett & Morrison, 1990; Nair & Hill, 1992). In addition to increased sugar levels, a decrease in malate levels in berry exudates was also found as the berry matured (Coombe, 1987). This decrease in malate occurredfirstly at the pedicel end, but levels remained constant at the style end.

Bunch architecture. Tight bunch clusters, found in most wine cultivars, were associated with development of severe *Botrytis* bunch rot (Savage & Sall, 1983; Marois *et al.*, 1986; Vail & Marois, 1991). Tight clusters did not dry as fast as loose clusters and the increased pressure during maturation lead to grape berry rupture (Nair & Parker, 1985). The pedicel-berry attachment area was especially prone to rupture. Compression between berries in the bunch causing splitting and partial severance of the grape from its pedicel lead to increased exudates, thus enhancing germination and infection by *B. cinerea*. Development of cuticle and epicuticular wax was also inhibited at sites of berry contact (Marois *et al.*, 1986; Percival *et al.*, 1993). Epicuticular wax played an important role in resistance. Thus, an inhibition of its formation predisposed the berry to infection.

Moribund tissue. *Botrytis cinerea* rapidly colonized senescing or moribund tissue including stamens, loose or adhering calyptra, shed pollen and pistils, immature aborted berries, dead flowers and miscellaneous leaf, stem and tendril pieces (Jarvis, 1980; Northover, 1987; Nair *et al.*, 1988). Infected dead floral parts that remained in grape clusters were highly infectious and served as potent inocula for cluster infection prominent during grape berry ripening (Northover,

1987). This senescing or moribund plant tissue could also serve as latent infections (Nair & Parker, 1985; Nair *et al.*, 1988; Kerssies, 1992, 1993; Nair & Hill, 1992; Sirjusingh *et al.*, 1996). Infected floral debris trapped within grape bunch clusters or attached to ripening berries, which served as infection foci could even infect resistant, immature grape berries (Marais, 1985).

Weather conditions. Epidemics caused by *B. cinerea* occurred in cool, wet and humid weather conditions. These conditions favoured infection and sporulation of *B. cinerea* (Jarvis, 1980; Kerssies, 1993). Cool, wet weather can also predispose the host to infection. High rainfall can put the host under oxygen stress and can cause berry rupture. Excessive uptake of water leads to increased turgor pressure in grape berries and results in splitting, cracking and subsequent infection (Nair *et al.*, 1988). Host tissue damaged by frost is altered susceptible to infection (Jarvis, 1980).

THE BUNCH ROT COMPLEX

Microorganisms associated with bunch rot include bacteria, fungi and yeasts. Fungi, other then *B. cinerea*, commonly associated with bunch rot include *Aspergillus niger, Alternaria tenius, Cladosporium herbarum, Rhizopus stolonifer, R. arrhizus* and green spored *Penicillium* sp. (Hewitt, 1974; Nair & Parker, 1985; Duncan *et al.*, 1995). *Acetobacter* bacteria are also commonly associated with the bunch rot complex (Hewitt, 1974; Flaherty *et al.*, 1992). Of these, only *B. cinerea* was able to penetrate the intact grape berry cuticle (Nelson, 1952), and more so under predisposing conditions (Coertze & Holz, 1999). *Alternaria alternata* can cause rot of sound, mature berries when inoculated in a water suspension. Under very favourable conditions, in water or grape juice, *A. niger* and *R. arrhizus* can cause infection of sound, mature berries. They are, however, generally regarded as secondary rot fungi. Other secondary invaders are *A. flavus*, *Penicillium* sp. and *R. stolonifer* (Hewitt, 1974).

Etiology

An over-all increase in population densities of bunch rot fungi occurs at or after véraison (Nair & Parker, 1985). Duncan *et al.* (1995) ascribed this increment in fungal populations to a steady build-up of secondary inoculum throughout summer, as well as to changes in exudate

composition. *Alternaria alternata* and *Cladosporium* spp. are the most abundant of all fungi isolated from grape berry surfaces (Hewitt, 1974; Duncan *et al.*, 1995). Large numbers of *Penicillium* and *Rhizopus* spp. are associated with vineyards bordering nectarine and plum orchards (Hewitt, 1974). Bunch rot is mainly associated with late-season rains and tight clustered cultivars as a result of berry rupture. Northover (1987) found wounded berries to be colonized rapidly by fungi, thus providing foci from which the pathogen spreads to adjacent berries. Activities associated with bunch rot lead to water loss and subsequent formation of aromatic features that are highly attractive to fruit flies (Pucheu-Plante & Mercier, 1990).

CERATITIS FRUIT FLIES

Fruit fly species in the genus *Ceratitis* (Diptera: Tephritidae) (Drew, 1989) are major pests in countries with subtropical and moderate climates. Species in this genus are known for their polyphagous feeding habits and are pests of a wide range of fruit types, especially stone fruit species (Christenson & Foote, 1960; Bateman, 1972). In the Western Cape, *Ceratitis* fruit flies also infest grape, which are usually regarded as a non-host. Two types of *Ceratitis* fruit fly occur in the Western Cape, the Natal fruit fly, *Ceratitis* (*Pterandrus*) *rosa* Karsch and the Mediterranean fruit fly, *Ceratitis capitata* Wiedemann (Myburgh, 1962, 1964; White, 1992, Schwartz, 1993).

Distribution and host range

The genus *Ceratitis* originated in Africa (Fletcher, 1989). The seven species of *Ceratitis* include one widespread species, the Mediterranean fruit fly, *C. capitata*, and six very localized species restricted to East Africa, Madagascar and nearby islands. The Mediterranean fruit fly is believed to have originated from one of two localities, namely Eastern and Southern Africa and Madagascar, or the Mediterranean region. Since the other six *Ceratitis* species all originated in Southern Africa and Madagascar it seems reasonable that the origin of *C. capitata* lies with its congeners (Maddison & Bartlett, 1989). During the last hundred years *C. capitata* has spread to a number of countries throughout the world, including parts of South and Central America and Australia. The genus *Ceratitis* contains some of the most significant pest species of deciduous

fruit, of which *C. capitata* and *C. rosa* are regarded as the most important (Hancock, 1989). *Ceratitis capitata* has been recorded from about 300 species of fruits, nuts and vegetables, of which the majority are of tropical origin (Liquido *et al.*, 1990). Based on the existing host records, *C. capitata* seems to be the most adaptable and polyphagous of the tephritid fruit flies. The Natal fruit fly, *C. rosa*, although restricted to southern parts of Africa and islands in the Indian Ocean, appears to have fairly similar life history characteristics to *C. capitata*. Although *C. rosa* is slightly larger than *C. capitata*; their life cycles, habits and seasonal occurrence are very similar (Fletcher, 1989, Grové *et al.*, 1997).

The Mediterranean fruit fly, *C. capitata*. Studies done in the Western Cape show that *C. capitata* populations vary depending on host availability. Numbers increase in spring in orchards with early ripening fruit such as apricots, then move to peach orchards in midsummer, then to vineyards in early autumn and finally, in late autumn and winter, to citrus orchards (Myburgh, 1964). *Ceratitis capitata* has been recorded from the following hosts in South Africa, apple (*Malus domestica*), apricot (*Prunus ameriaca*), coffee (*Coffea* sp.), common guava (*Psidium guajava*), fig (*Ficus* sp.), grenadilla (*Passiflora* sp.), lychee (*Litchi chinensis*), mango (*Mangifera indica*), mulberry (*Morus* sp.), navel and Valencia oranges (*Citrus sinensis*), peach (*Prunus persica*), pear (*Pyrus communis*), plum (*Prunus domestica*), quince (*Cydonia oblanga*) and grape (*Vitis vinifera*) (White, 1992).

The Natal fruit fly, *C. rosa*. In Southern Africa the Natal fruit fly is primarily a pest of the northern and eastern parts, extending as far as the Eastern Cape (Hancock, 1989). The Natal fruit fly has been recorded from the following hosts in South Africa, apricot (*P. ameriaca*), avocado (*Persea occurredo*), common fig (*F. arica*), common guava (*P. guajava*), lychee (*L. chinensis*), mango (*M. indica*), navel and Valencia oranges (*C. sinensis*), papaya (*Carica papaya*), peach (*P. persica*), pear (*P. communis*), plum (*P. domestica*), quince (*C. oblanga*) and grape (*V. vinifera*) (White, 1992).

Life cycle

Ceratitis capitata and *C. rosa* are multivoltine, with the number of generations per year being determined by temperature. In the tropics and subtropics, reproductive activity is continuous throughout the year. In the more temperate parts, the coldest months of the year are passed in the pupal stage (Fletcher, 1989). Peak densities occur during dry months (Borge & Basedow, 1997). Life cycles range from a few weeks to several months depending on temperature. Survival up to a year has been reported under *in vitro* conditions, but *in vivo* survival does not exceed two to three months. *In vivo* mating activities start approximately during the 15th day after emergence, whereas for laboratory flies, reproduction starts as early as two days after emergence (Bravo & Zucoloto, 1997). Mature females lay up to 14 eggs per fruit depending on its size and can produce 300-1000 eggs throughout its life (McDonald & McInns, 1985). Larval development varies significantly in different hosts (Carey, 1984). High mortality can occur in certain fruit types, such as grapes, as a result of the high juice content (Hendrichs & Hendrichs, 1990). Pupation occurs in the ground, and peak adult emergence takes place in the morning (Myburgh, 1962). Long distance flights have been recorded when fruit is unavailable. Movement to more favourable conditions is however restricted to a few hundred metres per week (Christenson & Foote, 1960).

Behavioural activities

Observations on fruit flies in controlled environments (Prokopy & Hendrichs, 1979; Prokopy *et al.*, 1987) or in the field (Hendrichs & Hendrichs, 1990) have shown that male and female fruit flies have different agendas. Females must nourish themselves and their developing eggs by feeding on carbohydrate and protein, copulate with the best male and locate an oviposition site. Males must also feed on carbohydrate and protein, and engage in one of two extremely time-consuming mating tactics to realize their reproductive potential. These tactics include lekking, fruit guarding, or both (Prokopy & Hendrichs, 1979; Whittier *et al.*, 1992). The synchronized behaviour of the population indicates daily periodic visitations to feeding, courtship, mating, oviposition and resting sites. Warburg and Yuval (1997) found that male activities were

regimented and discrete, with a clear separation between feeding and reproductive activities. Conversely, females engage in different activities in an opportunistic manner throughout the day.

Feeding behaviour. Fruit fly adults need carbohydrate, lipid and protein in order to perform the biological activities necessary for survival and reproduction (Bateman, 1972). Adult fruit flies feed predominantly on ripe and wounded fruits (Hendrichs & Hendrichs, 1990; Warburg & Yuval, 1997), because they acquire carbohydrates from feeding on fruit juices and honeydew. Protein sources include decomposing fruit and bird faeces (Hendrichs & Hendrichs, 1990). Microorganisms that colonize rotting fruit supply insects with additional nutrients. These microorganisms can either be contaminants of honeydew, symbionts or plant pathogens (Boush *et al.*, 1969). The nutritional benefit gained from rotting fruit was first observed by Malavasi *et al.* (1983) who studied the feeding behaviour of the American fruit fly, *Anastrepha fraterculus* (Wiedemann). This was also demonstrated in the mutualistic relationship between the grape berry moth, *Lobesia botrana* (Den. & Schiff.) and *B. cinerea.* It was observed that *L. botrana*, reared on a diet including fungal material, has a better growth and survival rate and increased fecundity (Savopoulou-Soultani & Tzanakakis, 1988 in: Fermaud & Le Menn, 1989; Mondy & Corio-Costet, 2000) than flies fed on a diet deficient in fungal material. Other substances also ingested by tephritid adults are floral nectar, bacteria and yeast (Prokopy & Roitberg, 1989). Lipids, essential for oogenesis and male pheromone production can be synthesized *de novo* if not acquired externally (Cangussu & Zucoloto, 1992; Warburg & Yuval, 1996).

Protein plays a significant role in the switch to reproductive mode in female fruit flies. Carey *et al.* (1998) found that the addition of protein to female diets was responsible for the switch from a waiting mode (in which mortality and reproduction were low) to a reproductive mode (in which mortality was very low at the onset of ovipositioning, but increased during ovipositioning). During the adult phase protein deprivation reduced longevity, diet ingestion and egg production, increased ingestion of new protein sources and reduced sexual acceptance of males by deprived females (Cangussu & Zucoloto, 1997; Müller *et al.*, 1997). Cangussu and Zucoloto (1992, 1995), however, confirmed that sugars are the most important nutrients for adult females since they somehow succeed in producing eggs without ingesting a protein source, although production increases with protein ingestion. Feeding during the larval phase has a greater influence on egg production during the pre-ovipositioning phase than feeding during the adult phase. However,

after the pre-ovipositioning phase has passed, feeding during the adult phase plays a more important role in egg production (Fernandes-da-Silva & Zucoloto, 1997).

Changes in light intensity have a strong influence on fruit fly foraging behaviour. Females feed primarily in the morning hours (Prokopy & Roitberg, 1989). During the rest of the day active searching for food resources does not occur. Males, however, feed only during the evening (Warburg & Yuval, 1997). Females tend to only feed opportunistically upon encountering food (Hendrichs & Hendrichs, 1990). Bateman (1972) found that mature tephritid females to depart rapidly from host trees that no longer bear acceptable fruit in search for hosts with a higher nutritional value.

Courtship behaviour. The mating system of *C. capitata* is based primarily on leks, where males perform a complex courtship display. Lek formation is characterised by an initial release of pheromone by a male, the male is subsequently joined and challenged by other males (Hendrichs & Hendrichs, 1990). This pheromone calling behaviour begins on the third day after emergence, when the male is sexually mature (Warburg & Yuval, 1996). Courtship involves male calling, female approach, male wing vibrating, female standing, male wing fanning and copulation (Liimatainen *et al.*, 1997). A switch in location of sexual activities occurs during the day (Prokopy and Hendrichs, 1979). During the morning and afternoon males court females by wing waving actions. During the midday males gather in leks where pheromone release and calling are primary activities (Warburg & Yuval, 1997).

Male and female fruit flies secrete pheromones that have a distinct difference in function, namely mating and host marking pheromones. During courtship, sexually mature males deposit pheromone secretions on the leaf surface and thereafter remain within the treated area in order to lure virgin females (Prokopy & Hendrichs, 1979). Host-marking pheromone, secreted by females after ovipositioning, mediates population dispersion of individuals among available resources (Averill & Prokopy, 1989).

Mating behaviour. Temperature and light intensity are regarded as the most important regulators of mating activity in *C. capitata* and *C. r*osa (Hancock, 1989; Hendrichs & Hendrichs, 1990). In *C. capitata* mating occurs during the day (Myburgh, 1962; Whittier *et al.*, 1992).

Causse and Fèron (in: Smith, 1989) found that mating activity reached a peak during the first seven hours of the photophase, thereafter it declines to low levels. Mating pairs have been observed to occur within lek sites and nowhere else (Warburg and Yuval, 1997). Mating by *C. rosa* occurs in the evening (Myburgh, 1962; Hancock, 1989).

Jang (1995) reported a change in olfactory-mediated behaviour of female *C. capitata* that occurred as a result of mating. It involved a switch from attraction to male-produced pheromone to host fruit odour for oviposition. The switch was mediated by accessory gland fluid transferred from male to female during copulation.

Ovipositioning behaviour. Ovipositioning takes place during the day and peaks during midday in the case of *C. capitata* (Prokopy & Hendrichs, 1979; Smith, 1989). The oviposition site is of critical importance, because larvae of these insects have little mobility and depend for survival on the nutritive value of the chosen host. The mortality rate of larvae in certain fruit types such as grapes (a non-host) is very high as a result of the high juice content. Fruit fly populations in vineyards thus originate from other fruiting hosts in the vicinity, where sexual and oviposition activities predominantly occur (Hendrichs & Hendrichs, 1990). Studies have shown that, under conditions of no choice, females will oviposit on hosts in which the chances of survival are low or absent (Carey, 1984). Fruit fly females lay their eggs underneath the skin of the host fruit. Direct damage to fruit, caused by females during oviposition when the fruit skin is pierced in order to lay eggs, can lead to indirect damage such as decay by fungal pathogens (Grovè *et al.*, 1997).

VECTOR RELATIONSHIPS

One of the first records of microorganism dissemination by insects was by Louis Pasteur at the end of the 19th century. Pasteur's work showed the involvement of *Drosophila* spp. in dissemination of yeasts and bacteria involved in wine fermentation and alcohol acidification (Louis *et al.* 1996). Observations on this phenomenon were extended to fruit production when Waite (1891) demonstrated the transmittance of fire blight (*Erwinia amylovora* (Burrill) Winslow) of pears by bees (Leach, 1940). Insects promote infection by plant pathogens in three

main ways. Firstly, by providing infection sites as a result of wounding the plant by feeding and oviposition activities, secondly, by distributing spores and, thirdly, by increasing the supply of nutrients on the surface of the plant by increased leakage from areas where they wounded the cuticle or by secretion of honeydew by the insect (Blakeman, 1980). Plant pathogens can be disseminated externally on the insect's body or internally through the digestive tract and deposited with faeces. The habit of fruit flies of regurgitating the contents of the crop when feeding is also an effective way by which they can disseminate plant pathogens (Leach, 1940). Insects are especially attracted to fruits infected by soft and fermentative rots. This attraction can be attributed to the fragrance emitted by rotting plant parts. In addition to fragrance, infected plant parts also excrete sugars and reflect ultraviolet light. This evidently causes insects to perceive the conidia-bearing leaves as flowers. This form of aggressive mimicry ensures the timely placement, by insects, of fungal conidia by insects on the susceptible tissue of the host plant (Batra, 1991).

Insect vectors of fungal pathogens

Ceratitis capitata. In a study done by Cayol *et al.* (1994) the external and internal mode of transmission of the pathogen *Rhizopus stolonifer* by the Mediterranean fruit fly, *C. capitata*, was proved *in vitro*. The external mode of transmission involved the mechanical transfer of conidia on the fruit fly's body. Scanning electron microscopy showed that the spores of *R. stolonifer* were carried on the proboscis, head, tarsus and legs of the fruit fly. The internal mode of transmission, involving partial (regurgitation) or total (faeces) transit through the digestive tract, was defined as semi-persistent (spores stay viable after passage through the digestive tract) (Harris, 1980).

Drosophila melanogaster. *Drosophila melanogaster* Meig. has long been known as a microorganism disseminator, particularly in vinification. In a study done by Louis *et al.* (1996), *D. melanogaster* was regarded as a plurimodal vector of *B. cinerea*. *Drosophila melanogaster* was considered as a non-persistent (ability to carry spores externally), semi-persistent and potentially persistent (fungus develops in the crop of the fly and generates resistant forms) transmission agent (Harris & Maramorosch, 1980). Louis *et al.* (1996) found that the spores of

B. cinerea accumulated in the fly crop and develop there into mycelia for at least one month. Since *D. melanogaster* overwinters as an adult, it might play a role in the overwintering and primary infection of *B. cinerea*. Transmission of *Rhizopus stolonifer, Alternaria* spp., *Mucor* spp. and *Geotrichum candidum* by *D. melanogaster* was also demonstrated (Butler, 1960; Butler & Bracker, 1963; Michailides & Spotts, 1990).

Lobesia botrana. Fermaud and Le Menn (1989, 1992) demonstrated that conidia of *B. cinerea* were carried externally or internally on grape berry moth larvae (*Lobesia botrana* Denis & Schiffermeuller). The number of sites for primary infection of *B. cinerea* was positively correlated with the number of larvae per grape cluster (Fermaud & Giboulot, 1992). The disseminated conidia rapidly infected wounds made by the feeding larvae and gave rise to *Botrytis* epidemics. The passage of conidia through the digestive tract of these larvae did not modify the germination ability of the conidia. Mondy *et al.* (1998) regarded the association between *L. botrana* and *B. cinerea* as a mutualistic relationship. *Botrytis cinerea* contained in the diet of *L. botrana* increased survival and fecundity of insects and decreased the total duration of development (Mondy & Corio-Costet, 2000). They concluded that the mutualistic relationship between this phytophagous insect and fungus is based on fungal sterols, having profound physiological effects on the insect.

Amorbia emogratella* and *Cryptoblabes gnidiella. In an attempt to eradicate the noxious weed *Myrica faya* (Aiton) threatening the native ecosystem in Hawaii, *B. cinerea* was investigated as possible biological control agent (Duffy, 1994). Adults of the fruit feeding moth larvae *Amorbia emogratella* Busck and *Cryptoblabes gnidiella* Milliere were heavily infested with *B. cinerea* and were subsequently used to vector the pathogen. Scanning electron microscopy showed the external transmission of *B. cinerea* on the head, around the mouthparts, the base of the body setae, and among the hook like claws of the abdominal legs of larvae from both the moth species. In addition to *B. cinerea*, other fungi such as *Penicillium* spp. were recovered from faecal droppings.

Thrips obscuratus. A study done by Fermaud *et al.* (1994) and Fermaud & Gaunt (1995) showed that the New Zealand flower thrips (*Thrips obscuratus* Crawford) carries conidia of *B. cinerea* externally and suggested that the adhesion was mechanical (non-persistent

dissemination). Kiwifruit (*Actinidae deliciosa*) infested by *T. obscuratus* was more susceptible to *B. cinerea*. They concluded that significant reductions in stem-end rot of kiwifruit might be achieved by controlling populations of *T. obscuratus* in orchards during flowering.

Helix aspersa. Michailides and Morgan (1996) found an increase in *B. cinerea* incidence in kiwifruit (*A. deliciosa*) infested by snails (*Helix aspersa* Mueller). This was attributed to wounds made by snails feeding on the sepals around the receptacle area of the kiwifruit. The increase in *Botrytis* rot was also attributed to stimulation of conidial germination by snail slime.

***Carpophilus* and other beetles.** Tate and Ogawa (1975) found a close association between brown rot of stone fruit caused by *Monilinia fructicola* (Wint.) Honey and nititulid beetles. They regarded the nititulid species, *Carpophilus mutilatus* and *Haptonchus luteolus*, as important vectors of *M. fructicola*. However, the species *C. freemani* and *C. hemipterus* were not regarded as important vectors because they did not visit healthy fruit.

CONCLUSION

Botrytis cinerea is regarded as the major organism responsible for bunch rot of grapes (Nelson, 1951; Hewitt, 1974). The fungi, *A. niger, A. tenius, C. herbarum, R. stolonifer, R. arrhizus* and green spored *Penicillium* sp., commonly associated with *Botrytis* bunch rot, infected the host predominantly as secondary invaders (Hewitt, 1974; Nair & Parker, 1985; Duncan *et al.*, 1995). Thus, *Botrytis* bunch rot commences with the primary infection of *B. cinerea*. Studies done by Coertze and Holz (1999) and Coertze *et al.* (2001) showed that solitary conidia of *B. cinerea* were unable to induce disease on intact berries. However, ripe grapes are considered to be susceptible to decay by clusters of conidia (Nelson, 1956; Hill *et al.*, 1981; Nair & Allen, 1993; Broome *et al.*, 1995). Thus, for successful disease induction, solitary conidia should be deposited on fresh wounds, or in clusters. *Ceratitis* fruit flies can play a very important role in this regard by depositing inocula at wound sites during feeding and by providing fresh wounds during their oviposition activities (non-persistent dissemination). In addition, conidia can be deposited in clusters, during faecal excretion and regurgitation (semi-persistent dissemination). *Ceratitis* fruit flies can act as self-guided missiles and be responsible for rot dissemination as they

are attracted to wounded and rotting fruit in vineyards and orchards. The polyphagous behaviour of *C. capitata* added to plant pathogen vection potential, could considerably increase its economic importance.

Leach (1940) lists four requirements that constitute adequate proof of insect transmission of plant disease: close association of the insect with the diseased plants; regular visitations of the insect to healthy plants under conditions suitable for the transmission of the disease; the presence of the pathogen in or on the insect in nature; the disease must be produced experimentally by insect visitation under controlled conditions. In order to determine if *Ceratitis* fruit flies play a significant role in the dispersal and subsequent disease development of *B. cinerea* on grapevine, these requirements were regarded as the main objectives of this study.

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2. THE MEDITERRANEAN FRUIT FLY, *CERATITIS CAPITATA*, AS A VECTOR OF *BOTRYTIS CINEREA* IN ORCHARDS AND NEIGHBOURING VINEYARDS

ABSTRACT

The occurrence of the Mediterranean fruit fly, *Ceratitis capitata*, and its potential to transmit *Botrytis cinerea* and other fungi (*Penicillium*, *Aspergillus*, *Alternaria*, *Mucor* and *Rhizopus* spp.) associated with post-harvest decay of stone fruit and bunch rot of grape *in natura* from early-season peach and plum orchards to neighbouring mid- and late-season wine grape vineyards, was investigated. Two Sensus fruit fly traps containing the para-pheromone Capilure were installed in five orchards each bordering on a vineyard. They were installed on four farms in the Stellenbosch region. *Ceratitis* fruit flies were collected weekly, identified and counted to determine the fluctuations in fruit fly population levels. Following field collection, the fruit flies were plated on Kerssies' *B. cinerea* selective medium and the number of flies yielding the pathogen was recorded. Two fruit fly species, *C. capitata* and *C. rosa*, were captured during the study period. *Ceratitis rosa* numbers comprised only 1% of the total number of fruit flies captured. *Ceratitis capitata* numbers, and the percentage flies contaminated with *B. cinerea* generally increased after harvest in the orchards and vineyards. Following harvest, the percentage flies yielding *B. cinerea* was higher in vineyards compared to orchards. Furthermore, in each vineyard an increase in percentage *B. cinerea* contaminated fruit flies was preceded by a corresponding increase in its neighbouring orchard. The levels of contaminated fruit flies with *Penicillium* and *Alternaria* remained high throughout the investigation period, especially after harvest of the fruit in the orchards. Low incidences of *Aspergillus*, *Mucor* and *Rhizopus* spp. were recorded on *C. capitata*. These findings suggest that the Mediterranean fruit fly may play an important role in the dispersal of inocula of fungi associated with post-harvest decay from early-maturing stone fruit orchards to mid- and late-maturing wine grape vinyards, and in disease induction under conditions unfavourable for natural infection.

INTRODUCTION

The Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) is one of the major orchard pests in the world. It is probably one of the most adaptable and polyphagous of the tephritid fruit flies (Liquido *et al.*, 1990). In the Western Cape province, the Mediterranean fruit fly does not only infest hosts such as stone fruit, but also grape (*Vitis vinifera* L.), which was widely considered to be a non-host (Myburgh, 1962, 1964; Hendrichs & Hendrichs, 1990; Liquido, *et al.* 1990; White & Elson-Harris, 1992; Schwartz, 1993). The ability of the Mediterranean fruit fly to vector the plant pathogen *Rhizopus stolonifer in vitro* was shown by Cayol *et al.* (1994). The plant pathogen vection potential of the Mediterranean fruit fly, in addition to its polyphagous behaviour, could considerably increase its economic importance.

Ceratitis capitata prefer to feed on ripe and wounded fruit (Hendrichs & Hendrichs, 1990). The American fruit fly, *Anastrepha fraterculus* (Wiedemann), that fed on microorganism-colonised, rotting or wounded fruit, showed enhanced nutrition and performance (Malavasi *et al.*, 1983). The added nutritional value of microorganism-colonised fruits has also been observed for grape berry moth larvae. Savopoulou-Soultani and Tzanakakis (1988) reported enhanced growth in grape berry moth larvae (*Lobesia botrana* Den. & Schiff.), which fed on *Botrytis* infected fruit (cited in: Fermaud & Le Menn, 1989). This was also demonstrated in the mutualistic relationship between the grape berry moth *L. botrana* and *B. cinerea* (Mondy & Corio-Costet, 2000). They found that *L. botrana* reared on a diet including fungal material had better growth, survival rate and increased fecundity.

In the Western Cape Province of South Africa, *C. capitata* populations vary depending on host availability. Numbers increase during spring during orchards with early ripening fruit, such as apricots. The flies move to peach orchards in midsummer, then to vineyards in early autumn and finally, in late autumn and winter, to citrus orchards (Myburgh, 1964). High incidence of *Penicillium* spp. and *Rhizopus* spp. are associated with vineyards bordering nectarine and plum orchards (Hewitt, 1974). In vineyards, an over-all increase in population densities of these and other fungi associated with bunch rot, e.g. *Botrytis cinerea, Aspergillus* spp. and *Alternaria* spp. (Hewitt, 1974; Nair & Parker, 1985; Duncan *et al.*, 1995), was observed after véraison (Nair & Parker, 1985). Thus, whilst the Mediterranean fruit fly

migrates from one fruiting species to another, drawn by mature and rotting fruit, fruit and bunch rot inoculum levels increase.

In South Africa, *Botrytis cinerea* is the major organism associated with postharvest decay of stone fruit (Fourie & Holz, 1985; Fourie, Holz & Calitz, 2001) and bunch rot of grape (De Kock & Holz, 1991, 1994). In a survey of postharvest decay of stone fruit in the Western Cape Province, 73% of decay was attributed to *B. cinerea* (Fourie & Holz, 1985). The pathogen is most prominent on early- and mid-season stone fruit cultivars (Fourie *et al.*, 2001). Other fungi, mainly *Penicillium*, *Aspergillus*, *Alternaria*, *Mucor* and *Rhizopus* spp., were also recorded on stone fruit and grapes, but their incidence was low (Fourie & Holz, 1985; Swart & Holz, 1991). In the case of Botrytis bunch rot of grape, wounding has been regarded as a major entry site for the pathogen (Du Plessis, 1937; Hill *et al.*, 1981; Nair *et al.*, 1988; Coertze & Holz, 1999; Coertze *et al.*, 2001). Insects can deposit inocula at wound sites during feeding and provide fresh wounds during their oviposition and feeding activities. The aim of this study was to determine the occurrence of the Mediterranean fruit fly, and its potential to transfer *B. cinerea* and other fungi associated with post-harvest decay of stone fruit and bunch rot of grape (*Penicillium*, *Aspergillus*, *Alternaria*, *Mucor* and *Rhizopus* spp.) *in natura* from early-season peach and plum orchards to neighbouring mid- and late-season wine grape vineyards.

MATERIALS AND METHODS

Orchards and vineyards. The flies were collected in the Stellenbosch region in five wine grape vineyards, four adjacent to stone fruit orchards and one adjacent to a pear orchard. At the farm Klein Simonsvlei, a Pinotage vineyard was adjacent to a Blacks peach orchard and a Colombard vineyard adjacent to a Santa Rosa plum orchard. At the farm Warwick a Cabernet Sauvignon vineyard was adjacent to a Leatitia plum orchard. At the Simonsig farm a Bukettraube vineyard was bordering on a Packham's Triumph pear orchard, and at the Morgenstêr farm a Chardonnay vineyard was neighboring a Reubennel plum orchard.

Climatic conditions and phenological stage of the grapes. The daily temperature, rainfall and percentage humidity for the 2000-2001 growing season were recorded at a weather station in the Stellenbosch region. *Botrytis cinerea* infection periods were determined on the basis of the infection criteria of Sall *et al.* (1981). A rainy period was

considered conducive to the development of *B. cinerea* if more than 5 mm of rain was recorded during 24 h (relative humidity ±92%; average temperature 15-22°C), or if 1-5 mm rain fell on each of two consecutive days (relative humidity ±92%; average temperature 15-22°C). The date at which distinctive phenological stages occurred and the percentage sugar (°Brix) at each stage for each of the grape cultivars are also recorded (Table 1).

Fruit fly traps. Sensus fruit fly traps containing the para-pheromone, Capilure, were used to trap *Ceratitis* fruit flies (Von Broembsen, 1998). A trap consisted of a capsule-containing a blue lid and a transparent receptacle in which the dead flies were collected. Capilure was applied to a felt ring fitted in the capsule. A 6 g D.D.V.P. tablet (dichlorvos 195 g/kg) was placed in the trap to fumigate the fruit flies. Two traps per vineyard and two per orchard were installed at each locality. Pheromones were changed monthly.

Monitoring of fruit rot organisms. *Ceratitis* fruit flies were collected weekly and counted to determine the fruit fly population fluctuations. Following field collection, fruit fly species were identified (White & Elson-Harris, 1992), plated on Kerssies' *B. cinerea* selective medium (Kerssies, 1990) in Petri dishes and incubated at 22°C under diurnal light. The active ingredients in Kerssies' *B. cinerea* medium initially inhibited the growth of fungi such as *Penicillium*, *Aspergillus*, *Alternaria*, *Mucor* and *Rhizopus* spp. As the effectivity of the medium decreased with time (G. Holz, *personal communication*), *B. cinerea* was recorded within 2 wks, and the other fungi after 3 wks.

Monitoring of bunch rot. Vineyards were monitored weekly for visual symptoms of bunch rot by randomly inspecting 50 bunches per vineyard.

RESULTS

Climatic conditions and phenological stage. The mean daily temperature, rainfall and humidity are given in Figure 1. Climatic conditions that favoured development of *B. cinerea* occurred from the end of March 2001 (week 13) onwards. Before that period conditions were generally unfavorable for the development of the pathogen.

***Ceratitis capitata* numbers and incidence of flies yielding *B. cinerea*.** Fruit flies were trapped from October 2000 (week 43) onwards only. In the 32-wk period, a total of 15 439

fruit flies was collected at the five sites studied. Two fruit fly species were captured namely the Mediterranean fruit fly, *C. capitata* and the Natal fruit fly, *C. rosa* (Karsch). *Ceratitis rosa* numbers were very low, comprising only 1% of the total number of fruit flies captured.

The mean percentage of *C. capitata* trapped in orchards and adjacent vineyards, and the mean percentage flies yielding *B. cinerea*, are given in Fig. 2. The highest fruit fly numbers were recorded during mid February 2001 (week 8) at Klein Simonsvlei in the stone fruit orchards. This coincided with the period of highest average daily temperature and lowest average relative humidity (Fig. 1). *Ceratitis capitata* numbers, and the percentage of *B. cinerea* contaminated flies increased after harvest in the different orchards and vineyards. This phenomenon was observed in the Blacks and Santa Rosa orchards on the farm Klein Simonsvlei. Following harvest, the percentage flies yielding *B. cinerea* was higher in vineyards than in orchards. Furthermore, in each vineyard an increase in percentage *B. cinerea* contaminated fruit flies was preceded by a corresponding increase in its neighbouring orchard. From mid March 2001 (week 13) onwards the percentage *B. cinerea* contamination was substantially enhanced when rainfall started to increase (Fig. 1).

Incidence of flies yielding other pathogens associated with fruit rot. A sudden increase in percentage fruit flies yielding *Mucor* spp. (Fig. 3) and *Rhizopus* spp. (Fig. 4) occurred during and after harvest in all the orchards and vineyards. The percentage flies yielding *Aspergillus* spp. (Fig. 5) increased prior to harvest, then remained at a high level during and after harvest. The occurrence of *Penicillium* and *Alternaria* spp. on fruit flies followed a different pattern. In the orchards and vineyards at Klein Simonsvlei (Fig. 6-7), percentages of flies yielding the organisms were consistently high during the 32-wk period. At Warwick, Simonsig and Morgenstêr (Fig. 6-7), percentages increased prior to harvest. Incidences of flies yielding the latter organisms were higher in the orchards than in the vineyards. Furthermore, the flies yielded these organisms earlier in the orchards than in the vineyards.

Monitoring of bunch rot. Prior to harvest no visual symptoms of bunch rot were observed in any of the vineyards. Following harvest, *Botrytis* bunch rot developed in most of the bunches that were left on the vines.

DISCUSSION

The percentage fruit flies contaminated with *B. cinerea* that was collected in orchards and the adjacent vineyards, increased noticably during and after harvest. This increase in fly incidences yielding the pathogen occurred earlier in the stone fruit orchards than in adjacent vineyards. The stone fruits were early-maturing cultivars and were harvested before the grapes. Since the increases were recorded during harvest, it could be assumed that *B. cinerea* generally developed on fruit during this stage in the orchards. As time passed the grape cultivars matured and more food for the fruit flies was available in the vineyards than in the previously harvested orchards. Transfer of inoculum from the orchards to the vineyards by the fruit flies could thus be expected. A relationship between increased *Botrytis* contaminated fruit flies and favourable weather conditions was evident in the post-harvest period. A noticable increase in *B. cinerea* contamination was accompanied by increased rainfall from the end of March 2001 (week 13) onwards. An increase in wetness (Nelson, 1951; Corbaz, 1971; Hewitt, 1974; Gessler & Jermini, 1985; Bulger *et al.*, 1987; Broome *et al.*, 1995) is known to promote *B. cinerea* infection and subsequent disease development. The favourable weather conditions, after harvest, resulting in a marked increase in *B. cinerea* contaminated fruit flies, indicated high inoculum levels in the vineyards during this period. This suggested that fruit flies could be an important source of primary inoculum for the following season in wine grape vineyards. Louis *et al.* (1996) reported the development of microsclerotia during the internal transit of conidia through the digestive tract of the vinegar fly, *Drosophila melanogaster* Meig, since *D. melanogaster* overwinters as an adult, concluded that it could play a role in winter conservation of inoculum. Since *C. capitata* also overwinters in the adult stage during moderate winters (Fletcher, 1989) and bunch rot were visually observed in the vineyards after harvest, the possibility for inoculum transfer from the previous season to the next is likely.

Species of *Alternaria* and *Penicillium* occur in vineyards as early as the bloom stage and increase gradually (Hewitt, 1974). Hewitt (1974) also found higher *Rhizopus* and *Penicillium* incidence in vineyards adjoining plum orchards. These fungi appeared to increase as plums ripened and began to drop. This explains the high level of fruit flies contaminated with both *Penicillium* and *Alternaria* throughout the study period, especially after harvest of the orchard fruits.

The species of fungi recorded on the fruit flies captured at the four study sites are important in the bunch rot of grapes (Hewitt, 1974). Of these only *B. cinerea* is able to penetrate the intact grape berry cuticle (Nelson 1951), and only under predisposing conditions (Coertze & Holz, 1999). Bunch rots are mostly initiated by an injury. Cracks, punctures, bird pecks, etc., break the grape berry's skin and expose the tissues to infection. Odours from the exposed grape flesh attract fruit flies, beetles and other insects. By feeding at wound sites, insects bring inocula into contact with oozing exudates, thus expediting disease development. Fruit flies also inflict wounds during oviposition and can thus bring inocula to these wound sites.

Fruit flies captured in vineyards yielded organisms associated with bunch rot in a differential pattern throughout the study period. Bunch rot was, however, never visually observed in the vineyards prior to harvest. Thus, the fruit flies could either have contracted the bunch rot organisms from rotting bunches that went unnoticed, or more likely, from fruit that were left to rot in the orchards after harvest. These findings suggest that fruit flies do not only play an important role in the dispersal of inocula from early-maturing stone fruit orchards to mid- and late-maturing wine grape vineyards, but also in disease induction under conditions unfavourable for natural infection.

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Table 1. Week and °Brix for each phenological stage of the grape cultivars

	Week and °Brix			
Cultivar	**Pea-size**	**Bunch closure**	**Véraison**	**Harvest**
Pinotage	Week 47 (5.5°Brix)	Week 51 (7.5°Brix)	Week 1 (11.0°Brix)	Week 8 (23.0°Brix)
Colombard	Week 47 (5.0°Brix)	Week 52 (8.0°Brix)	Week 2 (16.0°Brix)	Week 8 (23.0°Brix)
Cabernet Sauvignon	Week 47 (5.5°Brix)	Week 52 (6.0°Brix)	Week 2 (11.5°Brix)	Week 10 (25.0°Brix)
Bukettraube	Week 47 (4.5°Brix)	Week 51 (5.0°Brix)	Week 1 (11.0°Brix)	Week 10 (28.0°Brix)
Chardonnay	Week 47 (4.0°Brix)	Week 51 (5.0°Brix)	Week 1 (11.5°Brix)	Week 9 (28.0°Brix)

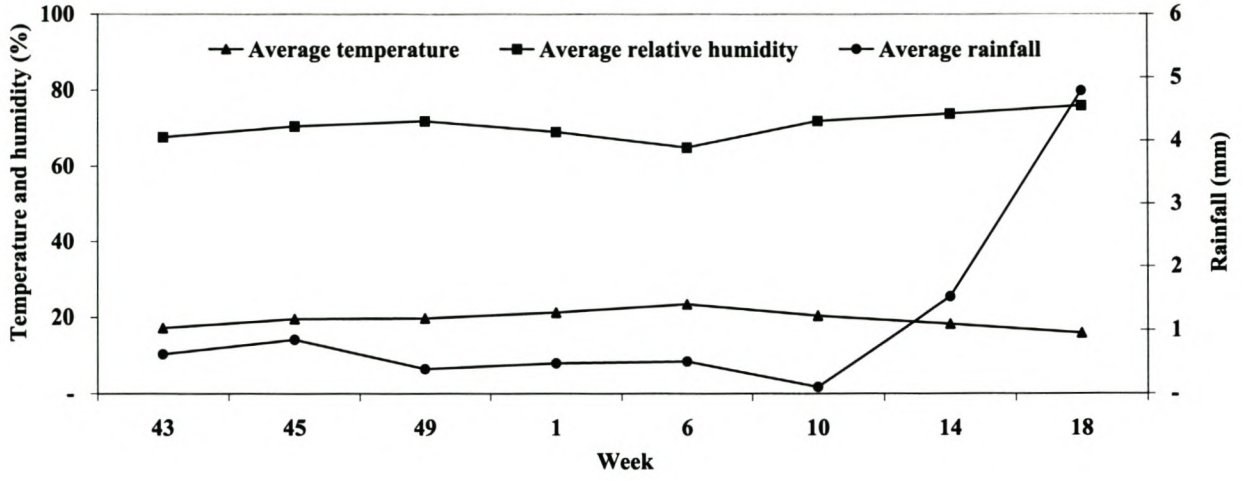

Fig. 1. Average temperature, humidity and rainfall recorded in the Stellenbosch district from October 2000 (week 43) to May 2001 (week 22).

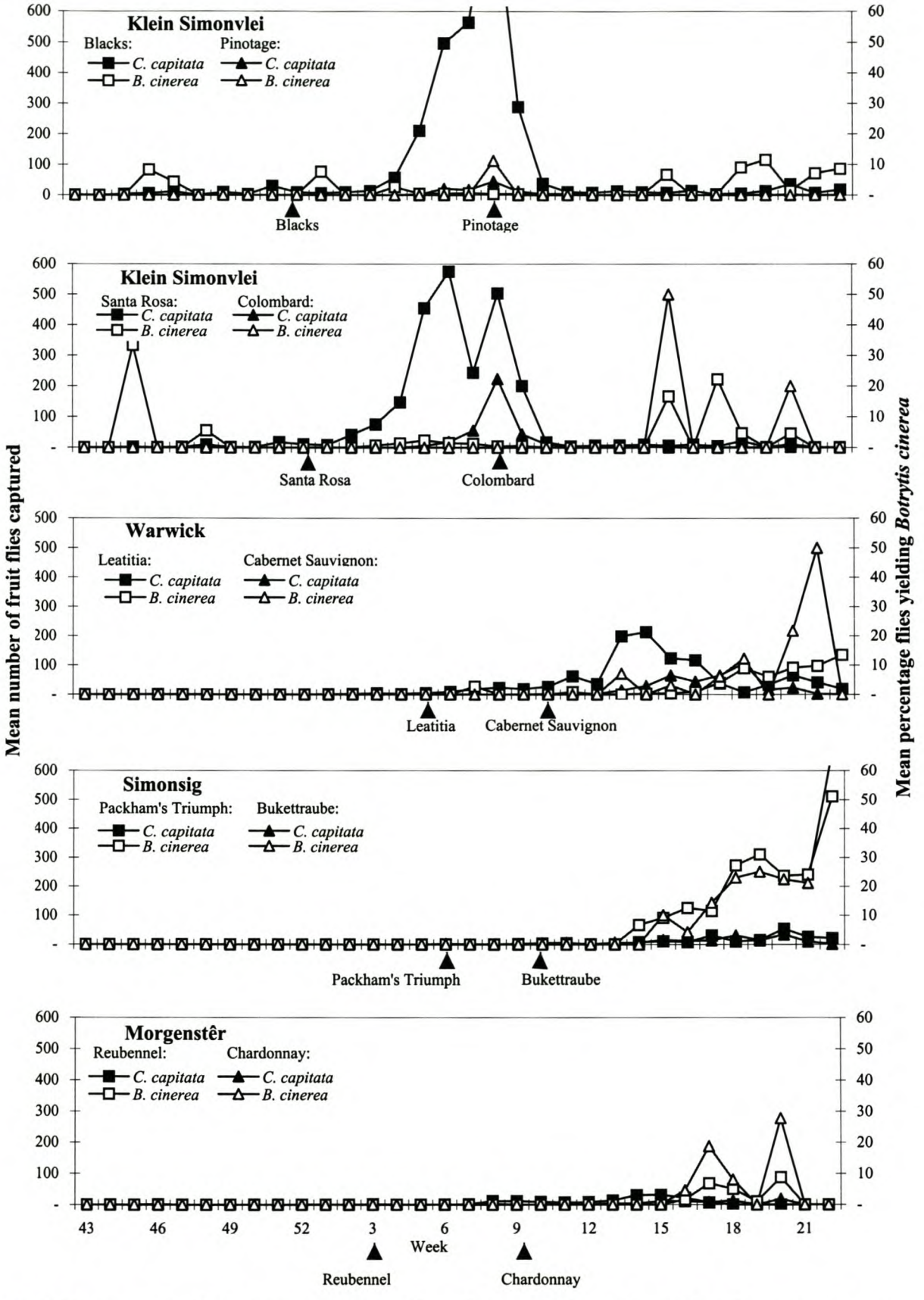

Fig. 2. Mean number of *Ceratitis capitata* trapped in orchards and adjacent vineyards, and mean percentages flies yielding *Botrytis cinerea* on Kerssies' medium. Harvest dates are indicated by an arrow.

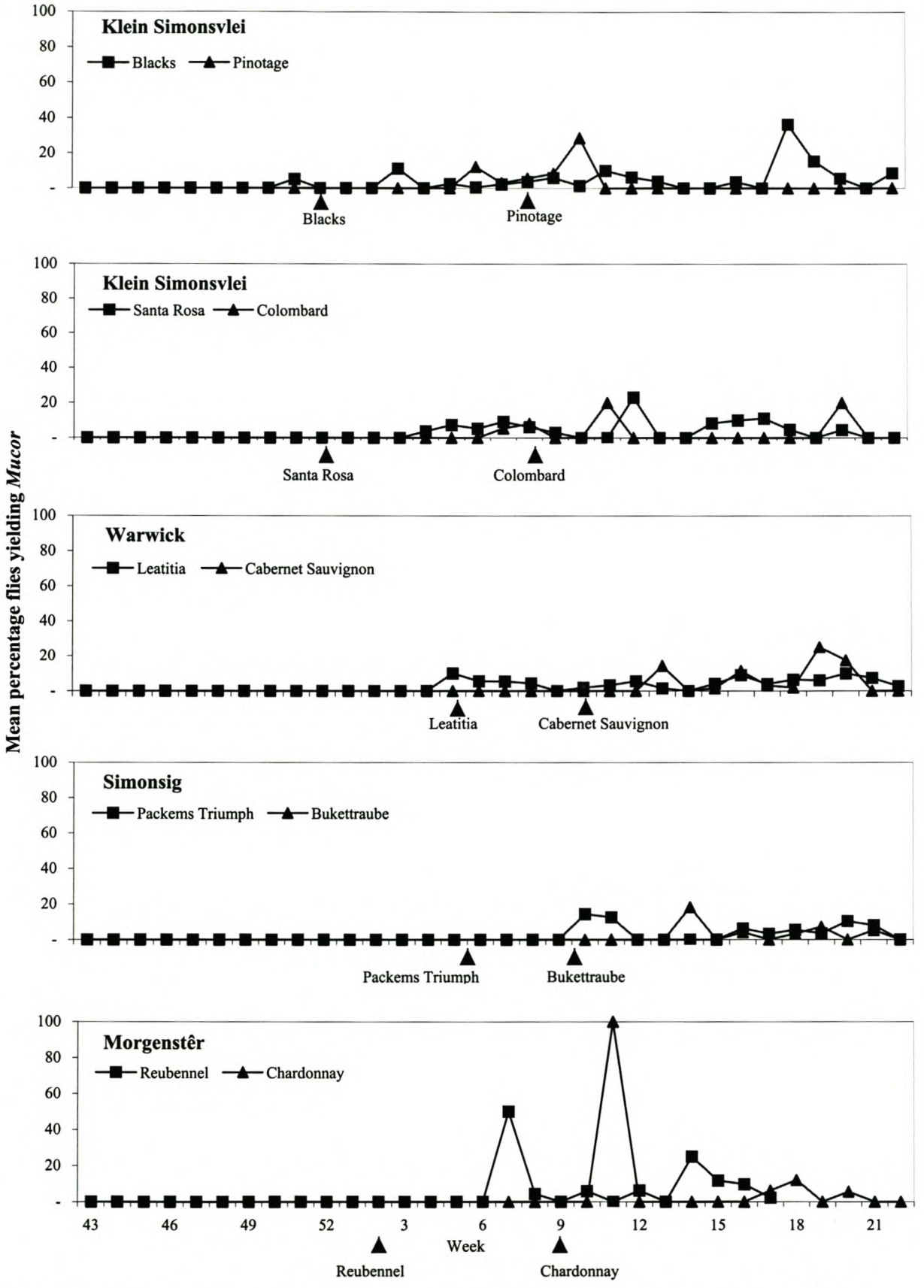

Fig. 3. Mean percentages *Captitata capitata* flies, trapped in different orchards and vineyards, that yielded *Mucor* spp. on Kerssies' medium. Harvest dates are indicated by an arrow.

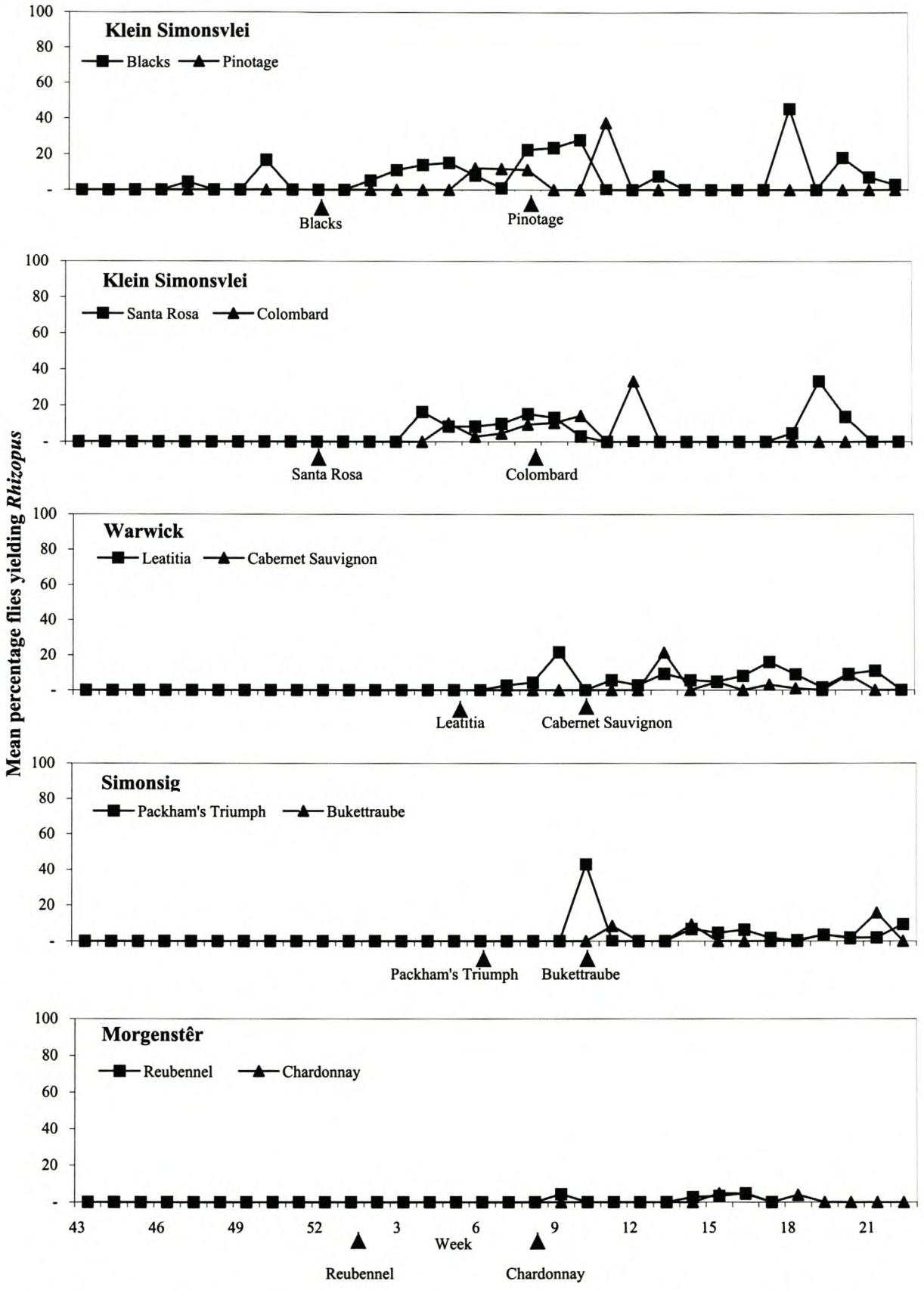

Fig. 4. Mean percentages *Ceratitis capitata* flies, trapped in different orchards and vineyards, that yielded *Rhizopus* spp. on Kerssies' medium. Harvest dates are indicated by an arrow.

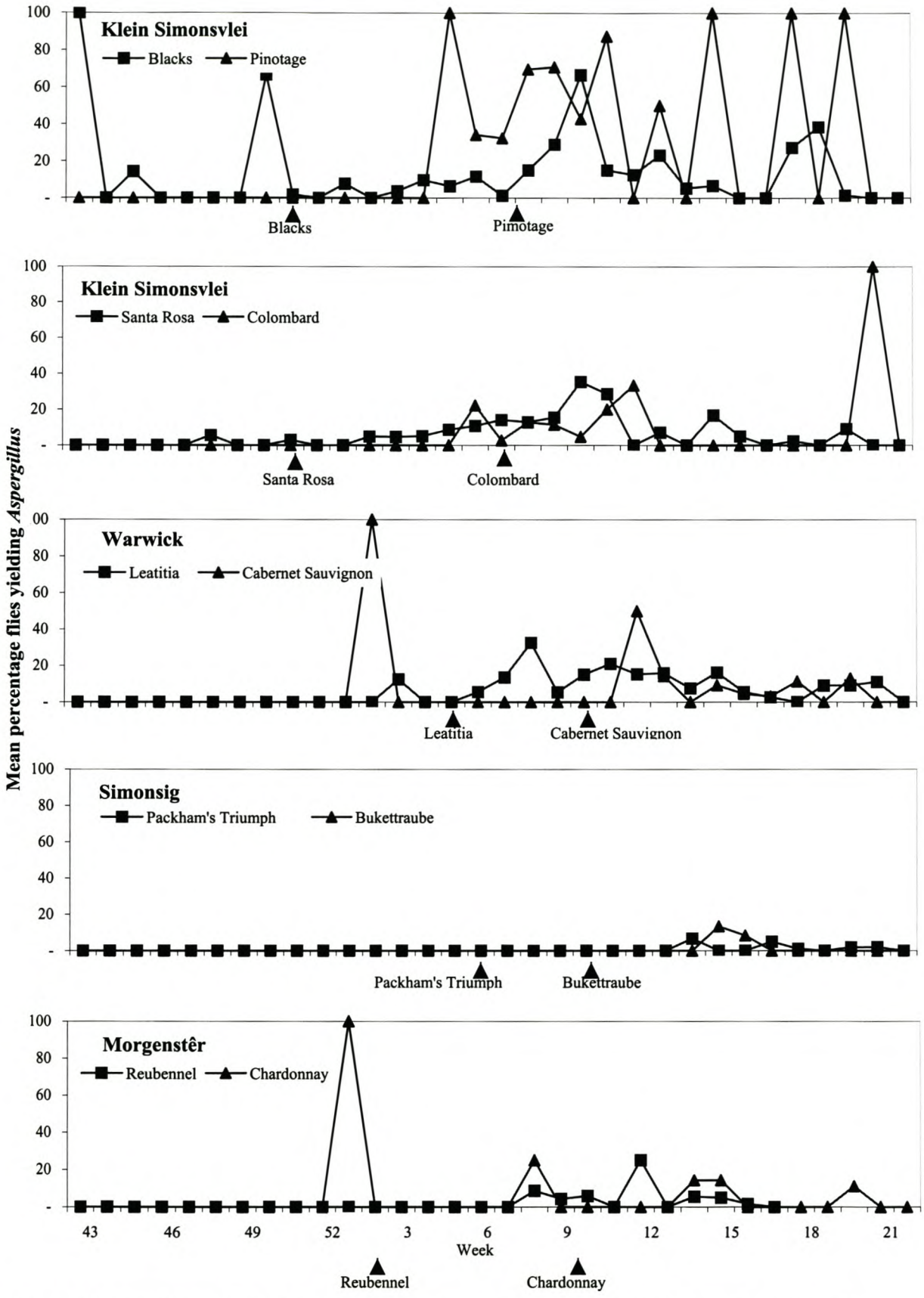

Fig. 5. Mean percentages *Ceratitis capitata* flies, trapped in different orchards and vineyards, that yielded *Aspergillus* spp. on Kerssies' medium. Harvest dates are indicated by an arrow.

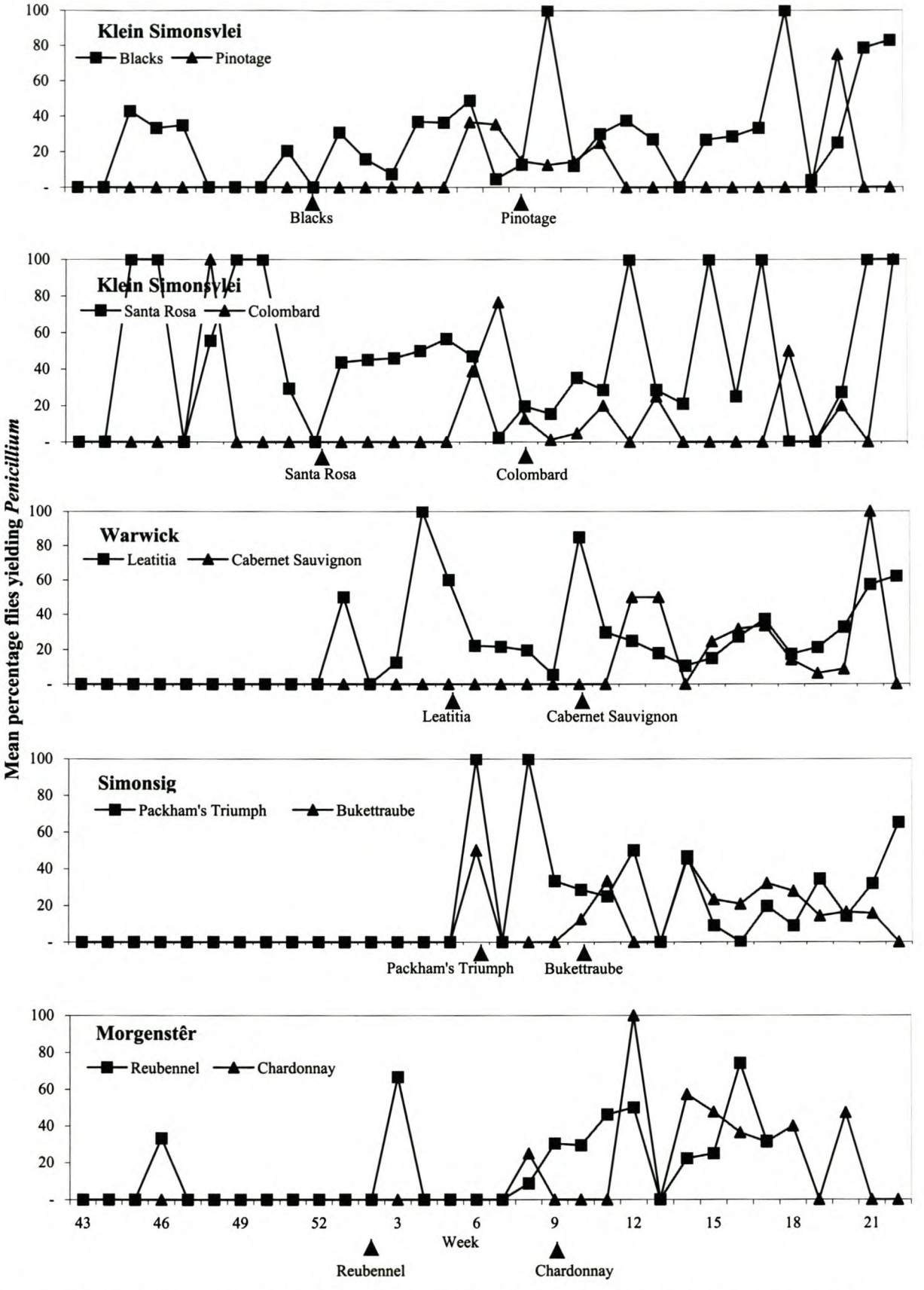

Fig. 6. Mean percentages *Ceratitis capitata* flies, trapped in different orchards and vineyards, that yielded *Penicillium* spp. on Kerssies' medium. Harvest dates are indicated by an arrow.

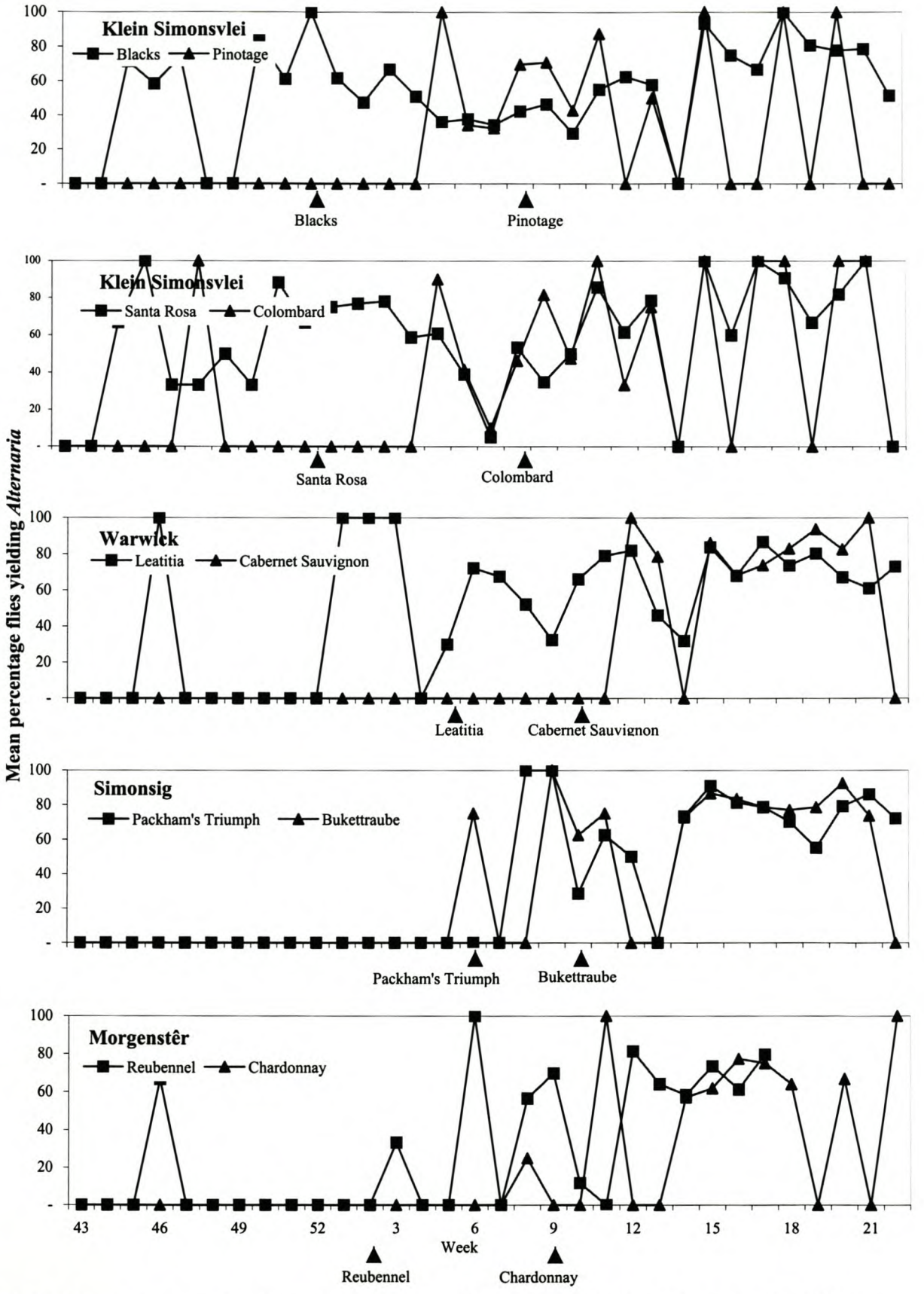

Fig. 7. Mean percentages *Ceratitis capitata* flies, trapped in different orchards and vineyards, that yielded *Alternaria* spp. on Kerssies' medium. Harvest dates are indicated by an arrow.

3. TRANSPORT AND DEPOSITION OF *BOTRYTIS CINEREA* BY THE MEDITERRANEAN FRUIT FLY, *CERATITIS CAPITATA*, AND DISEASE EXPRESSION AT DIFFERENT SITES ON GRAPE BERRIES

ABSTRACT

The transport of *Botrytis cinerea* by the Mediterranean fruit fly, *Ceratitis capitata*, and disease expression on grapes were investigated. The amount of *B. cinerea* deposited at specific sites on berries was estimated using a differential set of isolation and freezing techniques on bunch sections subjected to two sterility regimes. Three experiments were conducted to determine the potential of fruit flies in provoking *B. cinerea* decay. In the first experiment, transport of conidia and disease expression were investigated on rachis segments bearing unwounded berries only. In the second experiment, the effect of wounding on disease expression was investigated. In the third experiment, the effect of inoculum type (mycelia and conidia) on transportation and disease expression was investigated on rachis segments bearing unwounded berries, and on segments with wounded berries. The table grape cultivar Dauphine, and the wine grape cultivar Shiraz were used at véraison, two weeks before harvest and at harvest, and the transport studies were conducted in ethanol-disinfected perspex cages. Disease expression was studied in dry (±56% RH) ethanol-disinfected perspex chambers incubated at 22°C. The isolations from berries revealed that the flies deposited, without preference, high amounts of *B. cinerea* at various positions on the grape berry's surface. The freezing studies showed that the deposited conidia germinated and penetrated the berry skin at various positions. However, *B. cinerea* developed more often at the pedicel end than on the cheek or style end, which indicated a peculiar interaction between *B. cinerea,* the fruit fly and host tissue at this part of the berry. This phenomenon was substantiated by the finding that *B. cinerea* also developed more often at the pedicel end of berries that were not frozen. Further evidence for this interaction was found on intact berries exposed to flies that carried mycelia after feeding on berries without sporulating colonies of the pathogen, but showing symptoms of slippery skin. Significantly more decay developed on wounded berries compared with the unwounded berries and more so at the wound site. In addition, female fruit flies were responsible for significantly more decay development than male fruit flies. The study thus

proved that the Mediterranean fruit fly can promote *B. cinerea* disease development under conditions unfavourable to natural infection.

INTRODUCTION

Botrytis bunch rot, one of the most important diseases of grapevine (*Vitis vinifera* L.), is caused by *Botrytis cinerea*. Different infection pathways have been reported for *B. cinerea* on grape berries. They include the stylar end (McClellan & Hewitt, 1973; Nair & Parker, 1985), the pedicel end (Pezet & Pont, 1986; Holz *et al.*, 1997, 1998), and by direct penetration of the intact berry cuticle (Nelson, 1956). However, Coertze and Holz (1999) found that only a few conidia, usually deposited as single colony forming units in nature (G. Holz, *unpublished data*; Duncan *et al.*, 1995), do penetrate the grape berry cuticle. Single conidia, however, did not induce any disease symptoms on berries. Since relatively high infection rates often occur in vineyards (Nair & Nadtotchei, 1987), these findings suggest that predisposing factors such as wounding may be a very important route of entry for *B. cinerea* (Du Plessis, 1934; Hill *et al.*, 1981; Nair *et al.*, 1988; Coertze & Holz, 1999).

Wounding of fruit can take place in a number of ways including mechanical activities (pruning and harvest), weather conditions (sun, hail, frost, rain and wind) and biological factors (maturation, bunch architecture, other pathogens and insects) (Jarvis, 1980; Verhoeff, 1980; Savage and Sall, 1983; Gessler & Jermini, 1985; Nair *et al.*, 1988; Spotts *et al.*, 1998). The success of wound infections is, however, dependant on two important factors. Firstly, infection only occurs on fresh wounds, and more so under humid conditions (Mercier & Wilson, 1994; Spotts *et al.*, 1998; Coertze & Holz, 1999). Secondly, inocula should be deposited near the wound, or the wound should be inflicted near viable conidia or latent mycelia for infection to occur (Coertze *et al.*, 2001). Wounding of grape berries does not only include injury of the cuticle, but also the rupture of the fragile pedicel end attachment. The latter is especially prominent in the tight clustered, wine grape cultivars (Nair & Parker, 1985). Wounding causes nutrient and moisture leakages and negates host defense mechanisms, thus facilitating infection and subsequent disease development. Insects can play a very important role in this regard by depositing inocula at wound sites during feeding and by providing fresh wounds during their oviposition and feeding activities.

A recent study (Part 2), which confirmed the presence of *B. cinerea* and other bunch rot agents on the Mediterranean fruit fly, *C. capitata*, in orchards and vineyards in the Western Cape Province, suggests an important role for this insect in the dissemination of *B. cinerea*. Fruit flies are inclined to feed on rotting fruit (Hendrichs & Hendrichs, 1990; Warburg & Yuval, 1997). They can, therefore, become contaminated with conidia when they come into contact with sporulating colonies whilst feeding on lesions, or mycelia when lapping-sucking from macerated tissue in lesions without sporulating colonies of the pathogen. The aims of this study were to investigate the transport and deposition of *B. cinerea* conidia and mycelia by the Mediterranean fruit fly *C. capitata*, and disease expression on grape berries under dry conditions.

MATERIALS AND METHODS

Grapes. Fruit fly numbers were found to increase during and after véraison (Part 2), thus studies were conducted on mature grapes only. Sound, unblemished bunches were selected at véraison, two weeks before harvest and at harvest during the 2000-2001 season in a table grape vineyard (cultivar Dauphine; 9°, 14° and 19° Brix, respectively) located in the De Doorns region, and in a wine grape vineyard (cultivar Shiraz; 15°, 20° and 25° Brix, respectively) located in the Wellington-Malmesbury region. Low *B. cinerea* incidences were expected in both vineyards because of the dry climatic conditions associated with these districts. Following harvest, the bunches were surface-sterilised (30 s in 70% ethanol, 2 min in 0.35% sodium hypochlorite, 30 s in 70% ethanol), air-dried and stored at 5°C in polythene bags in carton boxes. Before an experiment, the boxes were removed from cold-storage and kept overnight in a laboratory for the bunches to reach ambient temperature. The bunches were then carefully cut into sections bearing five berries on a short rachis.

***Botrytis cinerea* inoculum.** A virulent isolate of *B. cinerea*, obtained from a naturally infected berry, was maintained on potato-dextrose agar (PDA; 12g Biolab agar, 200g potatoes, 20g sucrose and 1 000 ml of water) at 5°C. Inoculum was prepared by culturing the fungus at 23°C under diurnal light on potato-dextrose agar in Petri dishes. Dry conidia were harvested after 14 days from cultures using a spore cyclone. This harvesting method avoided contamination of spores by nutrients from the medium. The conidia were stored in dry, sterile McCartney bottles at 5°C until use. Storage time did not affect spore germination (Spotts &

Holz, 1996). Spore suspensions were prepared by suspending dry conidia in sterile deionised water and then sonicating them for 3 min in an ultrasonic bath (Branson model B3).

Two weeks before an experiment was conducted, sound grape berries were wounded with an insect needle, 1.5 mm, deep and inoculated by placing a 20 µL droplet of the conidial suspension onto the wound. The droplets were air-dried, and the berries were placed in ethanol-disinfected perspex (Cape Plastics, Cape Town, South Africa) chambers (60 x 30 x 60 cm) lined with a sheet of chromatography paper with the base resting in deionised water to establish high relative humidity (≥93% RH). The chambers were kept at 22°C to promote decay at the wound sites on the berries. In experiments where the transportation of conidia were studied, berries with decay on approximately a third of the cheek, and with sporulating colonies of *B. cinerea* in the center of the lesion, were used as an inoculum source. Berries with a corresponding decay pattern, but without sporulating colonies, were selected as source of mycelial inoculum. These berries were kept at 5°C to restrict the development of sporulating colonies before use.

***Ceratitis capitata* laboratory strain.** Pupae from a wild-type *C. capitata* strain, reared on the Krige diet (3 090 g of bran, 1 350 g of sucrose, 59 g of torula yeast, 20 g of sodium benzoate, 80 ml of HCL, and 4 910 ml of water) under aseptic conditions in the laboratory, were obtained from the Infruitec Pest Management Division (ARC Infruitec/Nietvoorbij, Fruit, Vine and Wine Research Institute, Private Bag X5013, Stellenbosch 7599). The pupae were kept in ethanol-disinfected perspex (Cape Plastics, Cape Town, South Africa) cages (29 x 29 x 38 cm) at 25°C. Preliminary studies showed that the yeast component usually included in the adult diet inhibited *B. cinerea* development. The yeast was thus excluded from the diet of emerging flies, as it was previously shown that larval stages accumulated sufficient protein reserves for development during the early adult stages (Fernandes-da-Silva, 1997). The flies were therefore fed on sucrose and sterile, deionized water only and used when they were 10 days old or sexually mature (Fig. 1).

Dissemination. Six ethanol-disinfected perspex (Cape Plastics, Cape Town, South Africa) cages (30 x 60 x 40 cm) were used. Three cages were allocated to male and three to female flies. Sixty rachis sections were placed in each cage. Two decayed grape berries (with or without sporulating colonies) provided inoculum, and 50 flies per cage served as potential vectors. The fruit flies were provided of water by wetting sterile cotton wool with

50 ml of sterile deionised water and put in the lid of Petri dishes. Rachis sections kept in cages without flies, but with an inoculum source served as the control treatment. At set stages (24, 48 and 72 hr) a third (20) of the rachis sections were removed. In addition a third (±17) of the flies were also removed to keep the ratio between fruit fly numbers to grape berry numbers constant. All experiments were conducted under dry conditions (±56% RH) at 22°C.

Transport and depositing of *B. cinerea* conidia at specific sites on berries. The amount of *B. cinerea* deposited at specific sites on berries was estimated by using a differential set of isolation and freezing techniques on rachis sections subjected to two sterility regimes. Half of the number of rachis sections was left unsterile to determine surface colonisation. The other sections were surface-sterilised (5 s in 70% ethanol) to eliminate the pathogen on the berry surface and to determine penetration (Sarig *et al.*, 1997; Coertze & Holz, 1999). In the isolation studies, epidermal tissue segments (approximately 5 x 7 mm), cut in the proximity of the pedicel end, the cheek and near the style end of the berries, were placed with the cuticle upward on *B. cinerea* selective medium (Kerssies, 1990) and incubated at 22°C. Previous studies (Coertze & Holz, 1999) showed that uninfected skin segments retained their turgidity and remained green for 6 days, whereafter colour changes indicative of natural cell death, appeared. Skin segments, therefore, exuded substances to their surfaces for a considerable period and retained their active defense abilities. Under the sterile regime, immersion in ethanol removed the exudates and killed the fungal structures on the grape berry surface. Therefore only the hyphae that penetrated the skin before sterilisation grew further. After 14 days the number of segments yielding sporulating colonies of the pathogen was recorded and used to quantify the amount of *B. cinerea* deposited at each site on the berries.

In the freezing studies, the rachis sections were placed on sterile epoxy-coated steel mesh screens (53 x 28 x 2 cm), and exposed to -12°C for 1 h. Following freezing, the screens were placed in dry ethanol-disinfected perspex (Cape Plastics, Cape Town, South Africa) chambers (60 x 30 x 60 cm) and incubated under dry conditions (±56% R.H.) at 22°C. Holz *et al.* (1995), using naturally infected and artificially inoculated berries, showed that a 1 h chilling period at -12°C was needed to promote the development of established infections. On these berries, the cuticle remained intact and provided a mechanical barrier to penetration, but the underlying cells lost their ability to actively respond to the pathogen (Coertze & Holz, 1999). Under both the sterility regimes, conidia and mycelia were influenced by passive and active

defense during the period before freezing, but not by active defense thereafter. The berries were examined daily for disease expression at the pedicel end, cheek or style end. After 14 days the number of frozen berries yielding sporulating colonies of the pathogen was recorded and used to quantify the amount of *B. cinerea* deposited at each site on the berries.

Experimental layout for the isolation and freezing studies was a 2 x 2 x 2 x 3 x 3 x 3 factorial design. There were three replications and six main effects. The main effects were cultivar (2), fly gender (2), sterility regime (2), phenological stage (3), exposure period (3) and infection site (3).

***Botrytis cinerea* symptom expression at specific sites on berries.** Three experiments were conducted to determine the potential of fruit flies to provoke *B. cinerea* decay on grape berries. In the first experiment, infection and disease expression from conidial inoculum were investigated on rachis sections bearing unwounded berries only. The experimental layout was a 2 x 2 x 3 x 3 x 3 factorial design. There were three replications and five main effects. The main effects were cultivar (2), fly gender (2), phenological stage (3), exposure period (3) and infection site (3).

In the second experiment, infection and disease expression from conidial inoculum as influenced by wounding were investigated. Five wounds were made on the cheek of each berry of half the number of rachis sections. Wounds were inflicted with a cork stopper through which five insect needles protruded in criss-cross manner. The needles were 5 mm apart and inflicted wounds approximately 0.8 mm in diameter and 1.5 mm deep. Berries on the remainder of the sections were left unwounded. The experimental layout was a 2 x 2 x 2 x 3 x 3 x 3 factorial design. There were three replications and six main effects. The main effects were cultivar (2), fly gender (2), wound treatment (2), phenological stage (3), exposure period (3) and infection site (3).

In the third experiment, the effect of inoculum source (mycelia and conidia) on disease expression was investigated. For these studies a group of cages were provided with rachis sections bearing unwounded berries. Another group of cages were provided with sections bearing unwounded berries, and with sections bearing wounded berries. The experiment was conducted with Dauphine grapes at the harvest stage only. The experimental layout was a 2 x 2 x 1(unwounded) or 2 (wounded versus unwounded) x 3 x 3 factorial design. There were

three replications and five main effects. The main effects were inoculum source (2), fly gender (2), wounding treatment (1 or 2), exposure period (3) and infection site (3).

In all the experiments, following removal from the cages, the rachis sections were placed on sterile epoxy-coated steel mesh screens (53 x 28 x 2 cm), and the screens placed in dry, ethanol-disinfected perspex chambers. The chambers were incubated at 22°C under dry conditions (±56% RH). The berries were examined daily for disease expression at the pedicel end, cheek or style end. After 14 days the number of berries yielding sporulating colonies of the pathogen was recorded and the number of berries infected at each site on the berries calculated. The number of infected wounds out of five on each berry was also recorded.

Infestation of fruit flies with *B. cinerea*. At the completion of each experiment, 20 male and 20 female flies were selected from each cage. From both genders, half the number of flies was left unsterile. Care was taken not to injure the cuticle of the unsterile flies thereby exposing the internal inoculum. The other flies were surface-sterilised (1 min in 0.7% sodium hypochlorite) to eliminate the pathogen on the fly's surface, rinsed in sterile deionised water and air-dried. The internal inoculum in the surface-sterilised flies was exposed by carefully squeezing the flies with a sterile forceps, thereby breaking the cuticle. The flies were plated onto Kerssies' *B. cinerea* selective medium (Kerssies, 1990) in Petri dishes and incubated at 22°C. The number of flies yielding sporulating colonies of *B. cinerea* was recorded after 14 days, and the percentage flies carrying the pathogen externally, or internally, was calculated.

***Botrytis cinerea* in fruit fly faeces.** To determine if inocula remained viable after passage through the digestive tract, faecal droppings deposited on the inside of the cages were collected with moist, sterilised ear buds. The ear buds were rinsed in 5 ml of sterile deionised water in 25 mL McCartney bottles. Aliquots (1 mL) of the suspensions were spread inoculated with sterile glass "hockey sticks" on the *B. cinerea* selective medium in Petri dishes and incubated at 22°C. The mean number of *B. cinerea* colonies that developed on the dishes after 14 days was determined.

Statistical analysis. The factorial analyses were performed using STATISTICA (StatSoft, Inc., USA). The results were examined for interactions. Appropriate comparisons of pairs of means or groups of means for describing the amount of *B. cinerea* on berries were

made using Scheffe's *post-hoc* method for multiple comparisons (Neter & Wasserman, 1974). Significance levels of $P = 0.05$ were used to compare pairs of means or groups of means.

RESULTS

Infestation of fruit flies with *B. cinerea*. Both unsterile (external transport) and surface-sterilised (internal transport) flies yielded the pathogen on Kerssies's medium. However, incidences of flies carrying the pathogen were higher for the unsterile than the sterile regime. For both sterility regimes, significantly more flies exposed to conidial than to mycelial inoculum yielded *B. cinerea* (Fig. 2). Furthermore, slightly more male than female flies carried the pathogen (Fig. 3).

***Botrytis cinerea* in fruit fly faeces.** The pathogen developed from all suspensions made from faecal droppings prepared from cages, where conidia was the inoculum source. Suspensions made from faecal droppings prepared from cages where mycelia was the inoculum source, did not yield the pathogen (data not shown).

Transport and deposition of *B. cinerea* conidia at specific sites on berries. *Berry skin segments.* Table 1 shows the analysis of variance (ANOVA) for the effect of cultivar, phenological stage, fruit fly gender, sterility regime, exposure period and infection site on the amount of *B. cinerea* on berries. Phenological stage, fly gender, sterility regime, exposure period all had a significant effect ($P < 0.02$) on the number of segments yielding *B. cinerea*. The interaction of fly gender x sterility regime ($P = 0.004$) and the interaction of cultivar x phenological stage x sterility regime x exposure period ($P = 0.001$) were also significant. On unsterile berries, male flies were responsible for significantly more skin segments yielding *B. cinerea* than female flies (Fig 4). The significant interaction of cultivar x phenological stage x sterility regime x exposure period was mainly due to the non-significant effect of exposure period on surface-sterilised berries while there were significant differences between exposure time on unsterile berries (Table 2). For each cultivar at each growth stage, berries on surface-sterilised sections constantly yielded low numbers of skin segments with *B. cinerea*, regardless of the period that the rachis sections were exposed to *C. capitata*. However, on unsterile berries, the number of *B. cinerea* yielding skin segments increased with exposure period, and was significantly higher after a 72-h exposure period compared to a 24-h period. More *B. cinerea* yielding skin segments were taken from unsterile berries than from sterile

berries at each sampling period. None of the segments removed from berries unexposed to flies yielded *B. cinerea*.

Frozen berries. The ANOVA of data showed that all the main effects had a significant effect on the number of berries yielding *B. cinerea* ($P < 0.001$), and the interaction of cultivar x phenological stage x fly gender x sterility regime x exposure period x infection site ($P = 0.008$) were significant (Table 3). The interaction of cultivar x phenological stage x fly gender x sterility regime x infection site, using the mean for exposure period, is summarised in Table 4. This interaction of cultivar x phenological stage x fly gender x sterility regime x infection site was mainly due to the different reaction of the two cultivars to infection at the three different berry sites. Under both sterility regimes, *B. cinerea* developed more often at the pedicel end (Fig. 5C) than on the cheek or at the style end of the berry. However, on Shiraz at véraison, *B. cinerea* developed in nearly equal proportions at the three berry sites. Mean percentage berries yielding *B. cinerea* were generally low and were similar in the two sterility regimes. At harvest, mean percentages of infected berries were higher, and more so on unsterile rachis sections. Furthermore, on unsterile sections, significantly more berries yielded the pathogen at the pedicel end than at the other two sites. On Dauphine at véraison and 2 wk before harvest, berries on surface-sterilised rachis sections were virtually free of the pathogen. However, on unsterile sections, the pathogen developed at each growth stage from the pedicel end of a relatively high percentage berries. None of the berries unexposed to flies developed *B. cinerea* decay.

***Botrytis cinerea* symptom expression.** *Conidial infection on unwounded berries*. Table 5 shows the ANOVA for the effect of cultivar, phenological stage, fruit fly gender, exposure period and berry part on symptom expression by *B. cinerea*. Cultivar, phenological stage, fly gender and infection site had a significant effect ($P < 0.05$) on symptom expression by *B. cinerea* (Table 5). Significantly more berries developed decay when they were exposed to the flies at harvest than at the other growth stages (Fig. 6), and significantly more berries decayed at the pedicel end (Fig. 5B) compared to the other sites (Fig. 7). Significantly more berries exposed to female than male fruit flies decayed (Fig. 8). In addition, the effect of the interaction phenological stage x infection site ($P = 0.009$) was also significant (Table 5). This was mainly due to the non-significant effect of infection site on symptom expression at véraison, while 2 wk before harvest and at harvest, significantly more berries developed

symptoms at the pedicel end than at the other sites (Fig. 9). No disease occurred on berries that were not exposed to fruit flies.

Conidial infection of unwounded versus wounded berries. The ANOVA of data including three exposure periods showed that wounding, exposure period and infection site ($P < 0.001$) had highly significant effects on the number of berries yielding *B. cinerea* decay (Table 6), while the interaction of cultivar x phenological stage x wounding x exposure period x infection site was also significant ($P < 0.001$). Several general responses can be derived from the significant ($P = 0.001$) effect of the interaction of cultivar x phenological stage x wounding x infection site (using the mean of exposure period) summarised in Table 7. On unwounded berries of both cultivars, infection was generally low at véraison and similar at all three sites. However, at the other two stages infection was higher and significantly more berries were infected at the pedicel end than at the other sites. On both cultivars at all stages, wounding caused a significant increase in infection at the berry cheek (Fig. 5A). However, the two cultivars differed in their reaction at the pedicel end of the berry. On Shiraz at véraison and at harvest, wounding at the cheek also led to higher infection at the pedicel end. This reaction was not found on Dauphine. No disease occurred on wounded and unwounded berries that were unexposed to fruit flies.

Infection of unwounded berries by conidial and mycelial inoculum. The ANOVA for the effect of inoculum source, fruit fly gender, exposure period and infection site showed that infection site ($P = 0.017$), inoculum source ($P < 0.001$) had a significant effect on symptom expression of *B. cinerea* (Table 8). In addition, the interaction between the two factors was also significant ($P = 0.040$). The significant interaction of infection site x inoculum source was mainly due to a non-significant effect of mycelial inoculum at the three sites, while there were significant differences between sites in the case of conidial infections (Fig. 10). Berries exposed to mycelium-bearing fruit flies developed decay only at the pedicel end. At this site, significantly more berries exposed to conidia-bearing fruit flies than mycelium-bearing fruit flies decayed. No disease occurred on berries that were not exposed to fruit flies.

Infection of unwounded and wounded berries by conidial and mycelial inoculum. The ANOVA of data including three exposure periods showed that inoculum source, wounding, exposure period had significant effect on the number of berries yielding *B. cinerea* decay ($P < 0.001$) (Table 9). The interaction of inoculum source x wounding x infection site ($P < 0.001$)

This is summarised in Table 10. Significantly more wounded than unwounded berries decayed, and significantly more berries exposed to conidia-bearing fruit flies decayed compared to those exposed to mycelia-bearing flies (Table 10). On both unwounded and wounded berries, the percentage infected berries were low when inoculum was provided as mycelia. However, the percentage infection were marginally higher when berries were wounded at the cheek. A different pattern was observed when conidia served as the inoculum source. On unwounded berries significantly more berries developed decay at the pedicel end than at the cheek or style end. On wounded berries, 92% of the berries developed wound infection, whereas the other sites were virtually free from the pathogen. No disease occurred on wounded and unwounded berries that were not exposed to fruit flies.

DISCUSSION

The *in vitro* studies with *C. capitata* proved that external and internal mode of transmission of *B. cinerea* by the Mediterranean fruit fly occurs. The isolations from berries revealed that the flies deposited, without preference, high amounts of *B. cinerea* at various positions on the grape berry surface. The freezing studies showed that the deposited conidia germinated and penetrated the berry skin at various positions. However, *B. cinerea* developed more often at the pedicel end than on the cheek or style end, which indicated a peculiar interaction between *B. cinerea,* the fruit fly and host tissue at this part of the berry. This phenomenon was substantiated by the finding that *B. cinerea* also developed more often at the pedicel end of berries that were not frozen. Further evidence for this interaction was found on intact berries exposed to flies that carried mycelia after feeding on berries without sporulating colonies of the pathogen, but showing symptoms of slippery skin. These findings indicate an important role for the Mediterranean fruit fly in the infection of grape berries by *B. cinerea,* and in symptom expression in grape bunches.

Studies with single, airborne conidia (Coertze & Holz, 1999; Coertze *et al.*, 2001; Gütschow, 2001; Van Rooi, 2001) showed that intact berries remained asymptomatic after extended periods of moist or wet incubation, and that grape berry skins provided an effective barrier to penetration by dry, airborne conidia of the pathogen. In this study, infection and subsequent symptom expression occurred when berries were exposed to conidia bearing fruit flies under dry conditions (±56% RH). Working with *Rhizopus stolonifer*, Cayol *et al.* (1994) proved that there was external and internal transmission of the pathogen by *C. capitata in*

vitro. The external mode of transmission involved the mechanical transfer of conidia on the fruit fly's body and the internal mode involved partial (regurgitation) or total (faeces) transit through the digestive tract of the fruit fly. Thus, fungal inocula could be deposited dry (mechanically) or wet (regurgitated products and faecal excrements) on the host surface. Since adult fruit flies acquire carbohydrates from feeding on fruit juices (Hendrichs & Hendrichs, 1990; Warburg & Yuval, 1997) the nutrient content in the regurgitated products (hence called saliva) must be high. The fact that the conidia deposited by the flies infected the berries under dry conditions, implies that the inoculum consisted of groups of conidia, and that moisture from faeces and saliva, and nutrients in saliva facilitated the germination and growth of the deposited *B. cinerea* conidia.

The finding that higher infection levels were correlated with grape berry maturation, can be ascribed to more conidia deposited at these sites, since adult fruit flies are drawn to areas of nutrient leakage on fruit (Hendrichs & Hendrichs, 1990; Warburg & Yuval, 1997). Sucrose, glucose and fructose increase in the grape berry from véraison onwards (Brown & Coombe, 1985; Nair *et al.*, 1988; Padgett & Morrison, 1990; Nair & Hill, 1992) and promote growth of *B. cinerea* in the presence of grape berry exudates (Kosuge & Hewitt, 1964; Chou & Preece, 1968; McClellan & Hewitt, 1973; Blakeman, 1975). Phenomena such as increased crack formation (Comménil *et al.*, 1997) and decreased cutin content of the curticle (Marois *et al.*, 1986; Percival *et al.*, 1993), that are coupled with the maturation process, lead to nutrient leakages on the berry surface. Furthermore, increased compression between berries in developing bunches can cause partial severance of the grape from its pedicel (Nair & Parker, 1985) resulting in nutrient leakages at the pedicel-berry attachment area. These factors may play a role in the infection of *B. cinerea* on grape berry skins, and may lead to higher infection at the pedicel end of the berry. Different workers (Holz *et al.*, 1988; Gütschow, 2001; Holz & Calitz, 2001; Van Rooi, 2001) reported high natural *B. cinerea* inoculum levels at the pedicel end of the grape berry during bunch development, and ascribed *Botrytis* bunch rot primarily to the colonisation at this site in bunches. During the course of this study feeding wounds at the pedicel end of Shiraz grape berries at the bunch closure stage were observed in vineyards (R. Engelbrecht, *personal observation*). Close observation revealed that the wounds were inflicted by the fruit weevil *Phlyctinus callosus*. In addition, sporulating colonies of *B. cinerea* were also observed at these feeding sites. These findings suggest that insects in general may play an important role in supplying inoculum at the pedicel end of grape berries, and initiating symptom expression at this site.

Wounds are regarded as a very important route of entry for *B. cinerea* (Nair *et al.*, 1988; Edlich *et al.*, 1989; Brook, 1991; Sharrock & Hallett, 1991; Elad & Evensen, 1995). Wounding causes nutrient and moisture leakages and negates host defense mechanisms in the skin, thus facilitating infection and subsequent disease development. The success of wound infections is, however, dependant on two important factors. Firstly, infection only occurs on fresh wounds, and more so under humid conditions (Mercier & Wilson, 1994; Spotts *et al.*, 1998; Coertze & Holz, 1999). Secondly, inocula should be deposited near the wound, or the wound should be inflicted near viable conidia or latent mycelia for infection to occur (Coertze *et al.*, 2001). The proboscis of the fruit fly is unable to penetrate the host skin during the feeding process and fruit flies are thus dependant on, and attracted to, wounded or rotting fruit for nutrients (Hendrichs & Hendrichs, 1990; Warburg & Yuval, 1997). In this study more than 80% of the wounded berries developed decay. This could be attributed to the timely placement of inoculum on fresh wounds by fruit flies during feeding. In addition, the number of wound infections increased with exposure time. Conidia and germlings did not infect dry wounds, but only infected wounds under humid or wet conditions (Coertze & Holz, 1999). One-day-old wounds were also less susceptible to infection than fresh wounds (Mercier & Wilson, 1994). These findings support the opinion that inocula are deposited with moisture and/or nutrients (faeces and saliva). If inocula were deposited dry, an increase in infection with exposure time would not have been possible at the wound site as suberisation starts 20 hr after wounding (Hill, 1985a,b; Coertze *et al.*, 2001). Intact grape berries also developed significantly more decay after exposure to female fruit flies than to males. This could be attributed to the wounds inflicted by the female ovipositor of the female fruit fly. Thus, by breaking the grape berry skin, females flies destroy the barrier capacity thereof, and render the grape berry more susceptible to infection by *B. cinerea*.

Since fruit flies are attracted to, and dependent on, wounded and rotting fruit for their nutrition, they can be responsible for the induction of disease epidemics. In a previous study (Part 2) the transport of *B. cinerea* inoculum from early-season peach and stone fruit orchards to neighbouring mid- and late-season vineyards by fruit flies was confirmed. In this study it was shown that fruit flies are able to induce *B. cinerea* decay on grapes under unfavourable conditions. These findings suggest an important role for fruit flies in the infection of *B. cinerea* by ensuring the timely placement of inocula on the susceptible tissue of grape bunches. Thus, host physiology and climatic, edaphic or cultural factors predisposing grape bunches to infection, must be avoided to prevent *Botrytis* epidemics in vineyards.

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Table 1. Analysis of variance of data for the effect of cultivar, phenological stage, fly gender, sterility regime, exposure period and infection sit on the percentage of skin segments yielding *Botrytis cinerea*

Source of variation	df	MS	*F*-value	*P*
Cultivar (C)	1	0.324	0.126	0.722
Phenological stage (P)	2	10.691	4.177	0.016
Fly gender (G)	1	21.987	8.590	0.004
Sterility regime (SR)	1	1,929.608	753.885	<0.001
Exposure period (T)	2	147.863	57.769	<0.001
Infection site (I)	2	5.714	2.232	0.109
C x P	2	23.149	9.044	<0.001
C x G	1	3.622	1.415	0.235
P x G	2	6.512	2.544	0.080
C x SR	1	1.164	0.455	0.500
P x SR	2	4.684	1.830	0.162
G x SR	1	21.620	8.447	0.004
C x T	2	12.124	4.737	0.009
P x T	4	9.252	3.615	0.007
G x T	2	6.517	2.546	0.080
SR x T	2	113.425	44.314	<0.001
C x I	2	15.331	5.990	0.003
P x I	4	1.934	0.756	0.555
G x I	2	5.208	2.035	0.132
SR x I	2	12.516	4.890	0.008
T x I	4	0.738	0.288	0.885
C x P x G	2	3.802	1.486	0.228
C x P x SR	2	21.763	8.503	<0.001
C x G x SR	1	0.047	0.018	0.893
P x G x SR	2	8.760	3.423	0.034
C x P x T	4	14.339	5.602	<0.001
C x G x T	2	6.254	2.443	0.088
P x G x T	4	3.104	1.213	0.305
C x SR x T	2	4.825	1.885	0.153
P x SR x T	4	9.486	3.706	0.006
G x SR x T	2	6.709	2.621	0.074
C x P x I	4	1.398	0.546	0.702
C x G x I	2	2.378	0.929	0.396
P x G x I	4	0.812	0.317	0.866
C x SR x I	2	14.366	5.613	0.004
P x SR x I	4	4.654	1.818	0.124
G x SR x I	2	4.634	1.810	0.165
C x T x I	4	1.431	0.559	0.692
P x T x I	8	0.885	0.346	0.948
G x T x I	4	2.138	0.835	0.503
SR x T x I	4	0.401	0.157	0.960
C x P x G x SR	2	3.049	1.191	0.305
C x P x G x T	4	3.066	1.198	0.311
C x P x SR x T	4	11.812	4.615	0.001
C x G x SR x T	2	7.595	2.967	0.052
P x G x SR x T	4	4.185	1.635	0.164
C x P x G x I	4	1.200	0.469	0.759
C x P x SR x I	4	1.767	0.690	0.599
C x G x SR x I	2	2.397	0.937	0.393
P x G x SR x I	4	1.546	0.604	0.660
C x P x T x I	8	1.129	0.441	0.896
C x G x T x I	4	2.144	0.837	0.502
P x G x T x I	8	0.588	0.230	0.985
C x SR x T x I	4	1.910	0.746	0.561
P x SR x T x I	8	0.302	0.118	0.999
G x SR x T x I	4	2.279	0.891	0.469
C x P x G x SR x T	4	2.573	1.005	0.404
C x P x G x SR x I	4	1.493	0.583	0.675
C x P x G x T x I	8	0.849	0.332	0.954
C x P x SR x T x I	8	0.633	0.247	0.981
C x G x SR x T x I	4	1.805	0.705	0.589
P x G x SR x T x I	8	0.292	0.114	0.999
C x P x G x SR x T x I	8	1.016	0.390	0.926
Error	431	2.560	~	~

Table 2. Means of the effect of the interaction of cultivar x phenological stage x sterility regime x exposure period on the percentage skin segments, cut from grape berries after exposure periods of 24 to 72 h to *Botrytis cinerea* bearing fruit flies, that yielded the pathogen on Kerssies' medium

	Skin segments[y] on grape berries infected by *B. cinerea* (%)								
Cultivar and	**Véraison**			**2 weeks before harvest**			**Harvest**		
sterility regime[z]	**24 hr**	**48 hr**	**72 hr**	**24 hr**	**48 hr**	**72 hr**	**24 hr**	**48 hr**	**72 hr**
Shiraz									
Sterile	0	2	2	0	4	8	0	2	16
Unsterile	1	100	90	64	82	92	8	66	100
Dauphine									
Sterile	4	0	6	0	0	0	2	8	4
Unsterile	34	82	70	22	82	84	82	84	100

[y] Epidermal segments (5 x 7 mm) were cut from the pedicel end, cheek and style end of each grape berry, placed with the cuticle upward on Kerssies' medium.

[z] Sterile = berries surface-sterilised (5s in 70% ethanol) before isolation; unsterile = berries left unsterile.

Table 3. Analysis of variance of data for the effect of cultivar, phenological stage, fly gender, sterility regime, exposure period and infection s on the percentage frozen berries yielding *Botrytis cinerea*

Source of variation	df	MS	*F*-value	*P*
Cultivar (C)	1	20.056	19.311	<0.001
Phenological stage (P)	2	37.506	36.113	<0.001
Fly gender (G)	1	37.556	36.160	<0.001
Sterility regime (SR)	1	144.500	139.132	<0.001
Exposure period (T)	2	32.710	31.495	<0.001
Infection site (I)	2	52.085	50.150	<0.001
C x P	2	16.463	15.851	<0.001
C x G	1	52.247	50.306	<0.001
P x G	2	7.407	7.132	0.001
C x SR	1	0.056	0.053	0.817
P x SR	2	6.000	5.777	0.003
G x SR	1	0.025	0.024	0.878
C x T	2	7.574	7.293	0.001
P x T	4	10.890	10.486	<0.001
G x T	2	19.241	18.526	<0.001
SR x T	2	3.852	3.709	0.025
C x I	2	3.338	3.214	0.041
P x I	4	4.265	4.107	0.003
G x I	2	0.060	0.058	0.944
SR x I	2	37.042	35.666	<0.001
T x I	4	0.594	0.572	0.683
C x P x G	2	2.599	2.502	0.083
C x P x SR	2	21.056	20.273	<0.001
C x G x SR	1	0.000	0.000	1.000
P x G x SR	2	4.914	4.731	0.009
C x P x T	4	17.620	16.966	<0.001
C x G x T	2	23.728	22.847	<0.001
P x G x T	4	5.676	5.465	<0.001
C x SR x T	2	0.019	0.018	0.982
P x SR x T	4	5.505	5.300	<0.001
G x SR x T	2	0.599	0.577	0.562
C x P x I	4	11.370	10.948	<0.001
C x G x I	2	0.400	0.385	0.681
P x G x I	4	1.537	1.480	0.207
C x SR x I	2	2.671	2.572	0.078
P x SR x I	4	1.472	1.418	0.227
G x SR x I	2	0.066	0.064	0.938
C x T x I	4	3.898	3.753	0.005
P x T x I	8	2.278	2.194	0.027
G x T x I	4	0.343	0.330	0.858
SR x T x I	4	0.282	0.272	0.896
C x P x G x SR	2	4.019	3.869	0.022
C x P x G x T	4	4.900	4.718	0.001
C x P x SR x T	4	3.880	3.736	0.005
C x G x SR x T	2	2.907	2.799	0.062
P x G x SR x T	4	3.154	3.037	0.017
C x P x G x I	4	1.293	1.245	0.291
C x P x SR x I	4	7.296	7.025	<0.001
C x G x SR x I	2	0.042	0.040	0.961
P x G x SR x I	4	2.330	2.244	0.064
C x P x T x I	8	4.997	4.811	<0.001
C x G x T x I	4	0.603	0.581	0.677
P x G x T x I	8	0.705	0.679	0.710
C x SR x T x I	4	1.843	1.774	0.133
P x SR x T x I	8	1.355	1.305	0.239
G x SR x T x I	4	0.043	0.042	0.997
C x P x G x SR x T	4	1.079	1.039	0.387
C x P x G x SR x I	4	2.213	2.131	0.076
C x P x G x T x I	8	0.598	0.575	0.798
C x P x SR x T x I	8	2.714	2.613	0.008
C x G x SR x T x I	4	1.060	1.021	0.396
P x G x SR x T x I	8	0.984	0.948	0.477
C x P x G x SR x T x I	8	0.499	0.480	0.870
Error	432	1.039	~	~

Table 4. Means of the effect of the interaction of cultivar x phenological stage x sterility regime x berry part on the percentage frozen berries[y] that yielded *Botrytis cinerea* at different berry positions upon exposure to fruit flies bearing *B. cinerea* conidia

	Berries infected by *B. cinerea* (%)								
Cultivar and	**Véraison**			**2 weeks before harvest**			**Harvest**		
sterility regime[z]	**Pedicel end**	**Cheek**	**Style end**	**Pedicel end**	**Cheek**	**Style end**	**Pedicel end**	**Cheek**	**Style end**
Shiraz									
Sterile	8.8	10	7.8	12.2	4.4	3.4	16.6	17.8	16.6
Unsterile	10	11.2	8.8	80	21.2	15.6	47.8	37.8	32.2
Dauphine									
Sterile	0	0	0	2.2	0	0	11.2	14.4	3.4
Unsterile	47.8	21.2	1.2	17.8	1.2	0	71.2	41.2	3.4

[y] Berries were frozen for 1 hour at -12°C after exposure to *B. cinerea* bearing fruit flies.

[z] Sterile = berries surface-sterilised (5s in 70% ethanol) before isolation; unsterile = berries left unsterile.

Table 5. Analysis variance of data for the effect of cultivar, phenological stage, fly gender, exposure period and infection site on the percentaj unwounded berries that developed *Botrytis cinerea*

Source of variation	df	MS	*F*-value	*P*
Cultivar (C)	1	2.086	4.537	0.034
Phenological stage (P)	2	9.225	20.060	<0.001
Fly gender (G)	1	3.568	7.758	0.006
Exposure period (T)	2	1.346	2.926	0.056
Infection site (I)	2	7.133	15.510	<0.001
C x P	2	0.151	0.329	0.720
C x G	1	0.012	0.027	0.870
P x G	2	0.466	1.013	0.365
C x T	2	1.938	4.215	0.016
P x T	4	0.461	1.003	0.407
G x T	2	5.790	12.591	<0.001
C x I	2	0.281	0.611	0.544
P x I	4	1.596	3.470	0.009
G x I	2	1.818	3.953	0.021
T x I	4	0.383	0.832	0.506
C x P x G	2	0.281	0.611	0.544
C x P x T	4	3.739	8.131	<0.001
C x G x T	2	1.568	3.409	0.035
P x G x T	4	1.785	3.883	0.005
C x P x I	4	0.068	0.148	0.964
C x G x I	2	0.040	0.087	0.916
P x G x I	4	0.299	0.651	0.627
C x T x I	4	0.160	0.349	0.845
P x T x I	8	0.123	0.268	0.975
G x T x I	4	0.512	1.114	0.351
C x P x G x T	4	1.156	2.513	0.043
C x P x G x I	4	0.142	0.309	0.872
C x P x T x I	8	0.850	1.849	0.070
C x G x T x I	4	0.179	0.389	0.816
P x G x T x I	8	0.133	0.289	0.969
C x P x G x T x I	8	0.184	0.399	0.920
Error	216	0.460	~	~

Table 6. Analysis of variance of data for the effect of cultivar, phenological stage, fly gender, wounding, exposure period and infection site the percentage unwounded and wounded berries that developed *Botrytis cinerea*

Source of variation	df	MS	*F*-value	*P*
Cultivar (C)	1	0.039	0.035	0.852
Phenological stage (P)	2	2.352	2.131	0.120
Fly gender (G)	1	1.890	1.713	0.191
Wounding (W)	1	384.261	348.253	<0.001
Exposure period (T)	2	25.699	23.291	<0.001
Infection site (I)	2	384.222	348.218	<0.001
C x P	2	6.099	5.527	0.004
C x G	1	4.014	3.638	0.057
P x G	2	0.451	0.408	0.665
C x W	1	3.409	3.090	0.080
P x W	2	8.043	7.290	0.001
G x W	1	1.681	1.523	0.218
C x T	2	2.039	1.848	0.159
P x T	4	8.343	7.561	<0.001
G x T	2	7.427	6.731	0.001
W x T	2	11.853	10.743	<0.001
C x I	2	23.302	21.119	<0.001
P x I	4	4.928	4.466	0.002
G x I	2	3.006	2.724	0.067
W x I	2	393.784	356.884	<0.001
T x I	4	12.352	11.194	<0.001
C x P x G	2	0.722	0.655	0.520
C x P x W	2	6.747	6.115	0.002
C x G x W	1	3.409	3.090	0.080
P x G x W	2	0.222	0.201	0.818
C x P x T	4	15.571	14.112	<0.001
C x G x T	2	2.032	1.842	0.160
P x G x T	4	3.752	3.400	0.009
C x W x T	2	0.353	0.320	0.726
P x W x T	4	4.789	4.340	0.002
G x W x T	2	0.977	0.885	0.413
C x P x I	4	6.356	5.760	<0.001
C x G x I	2	7.019	6.361	0.002
P x G x I	4	0.615	0.557	0.694
C x W x I	2	17.877	16.201	<0.001
P x W x I	4	4.004	3.629	0.006
G x W x I	2	0.389	0.352	0.703
C x T x I	4	1.358	1.231	0.297
P x T x I	8	3.433	3.111	0.002
G x T x I	4	0.932	0.845	0.497
W x Ti x I	4	11.654	10.562	<0.001
C x P x G x W	2	0.080	0.073	0.930
C x P x G x T	4	0.727	0.659	0.621
C x P x W x T	4	2.122	1.923	0.106
C x G x W x T	2	0.187	0.169	0.844
P x G x W x T	4	0.963	0.873	0.480
C x P x G x I	4	0.067	0.061	0.993
C x P x W x I	4	8.110	7.350	<0.001
C x G x W x I	2	8.340	7.558	0.001
P x G x W x I	4	0.410	0.371	0.829
C x P x T x I	8	5.342	4.841	<0.001
C x G x T x I	4	1.315	1.192	0.314
P x G x T x I	8	2.034	1.843	0.067
C x W x T x I	4	0.765	0.694	0.597
P x W x T x I	8	2.715	2.460	0.013
G x W x T x I	4	0.352	0.319	0.865
C x P x G x W x T	4	1.247	1.130	0.342
C x P x G x W x I	4	0.171	0.155	0.961
C x P x G x T x I	8	1.204	1.091	0.368
C x P x W x T x I	8	5.492	4.978	<0.001
C x G x W x T x I	4	0.562	0.509	0.729
P x G x W x T x I	8	1.317	1.194	0.301
C x P x G x W x T x I	8	0.615	0.557	0.813
Error	432	0.039	~	~

Table 7. Means of the effect of the interaction of cultivar x phenological stage x wounding x infection site on the percentage *Botrytis cinerea* that developed on unwounded and wounded berries at different berry positions upon exposure to fruit flies bearing *B. cinerea* conidia

	Berries infected by *B. cinerea* (%)								
Cultivar and	**Véraison**			**Two weeks before harvest**			**Harvest**		
wounding	**Pedicel end**	**Cheek**	**Style end**	**Pedicel end**	**Cheek**	**Style end**	**Pedicel end**	**Cheek**	**Style end**
Shiraz									
Unwounded	5.6	4.4	2.2	14.4	1.2	0	24.4	14	6.6
Wounded[z]	15.6	68.8	7.8	7.8	103.4	1.2	35.6	80	4.4
Dauphine									
Unwounded	0	0	0	8.8	2.2	0	18.8	12.2	2.2
Wounded	0	100	1.2	2.2	100	2.2	1.2	48.8	0

[z] Five wounds inflicted on the cheek of the grape berry with a wound maker.

Table 8. Analysis of variance of data for the effect of inoculum, fly gender, exposure period and infection site on the percentage unwounded berries that developed *Botrytis cinerea*

Source of variation	df	MS	*F*-value	*P*
Inoculum (N)	1	7.787	18.689	<0.001
Fly gender (G)	1	1.120	2.689	0.105
Exposure period (T)	2	0.731	1.756	0.180
Infection site (I)	2	1.787	4.289	0.017
N x G	1	0.750	1.800	0.184
N x T	2	0.843	2.022	0.140
G x T	2	0.620	1.489	0.232
N x I	2	1.398	3.356	0.040
G x I	2	0.843	2.022	0.140
Ti x I	4	0.245	0.589	0.672
N x G x T	2	0.361	0.867	0.425
N x G x I	2	0.528	1.267	0.288
N x T x I	4	0.329	0.789	0.536
G x T x I	4	0.218	0.522	0.720
N x G x T x I	4	0.264	0.633	0.640
Error	72	0.417	~	~

Table 9. Analysis of variance of data for the effect of inoculum, fly gender, wounding, exposure period and infection site on the percenta unwounded and wounded berries that developed *Botrytis cinerea*

Source of variation	df	MS	*F*-value	*P*
Inoculum (N)	1	53.005	81.199	<0.001
Fly gender (G)	1	1.671	2.560	0.112
Wounding (W)	1	16.116	24.688	<0.001
Exposure period (T)	2	0.519	0.794	0.454
Infection site (I)	2	36.977	56.645	<0.001
N x G	1	1.338	2.050	0.154
N x W	1	11.116	17.028	<0.001
G x W	1	0.042	0.064	0.801
N x T	2	0.519	0.794	0.454
G x T	2	1.185	1.816	0.166
W x T	2	2.907	4.454	0.013
N x I	2	30.310	46.433	<0.001
G x I	2	0.421	0.645	0.526
W x I	2	34.921	53.496	<0.001
T x I	4	1.713	2.624	0.037
N x G x W	1	0.005	0.007	0.933
N x G x T	2	1.852	2.837	0.062
N x W x T	2	3.907	5.986	0.003
G x W x T	2	0.056	0.085	0.918
N x G x I	2	0.366	0.560	0.572
N x W x I	2	27.421	42.007	<0.001
G x W x I	2	1.014	1.553	0.215
N x T x I	4	2.824	4.326	0.002
G x T x I	4	1.019	1.560	0.188
Tr x T x I	4	0.796	1.220	0.305
N x G x W x T	2	0.352	0.539	0.584
N x G x W x I	2	0.421	0.645	0.526
N x G x T x I	4	1.630	2.496	0.045
N x W x T x I	4	1.296	1.986	0.100
G x W x T x I	4	0.278	0.426	0.790
N x G x W x T x I	4	0.352	0.539	0.707
Error	114	0.653	~	~

Table 10. The means of the interaction of source of *Botrytis cinerea* inoculum x wounding x infection site on the percentage unwounded and wounded berries[z] that developed *B. cinerea* at different berry positions upon exposure to fruit flies bearing *B. cinerea* conidia

	Berries infected by *B. cinerea* (%)					
	Mycelia			Conidia		
Treatment	**Pedicel end**	**Cheek**	**Style end**	**Pedicel end**	**Cheek**	**Style end**
Unwounded	1.2	0	0	18.8	12.2	2.2
Wounded	1.2	5.6	0	1.2	92.2	0

[z] Five wounds inflicted on the cheek of the grape berry with a wound maker.

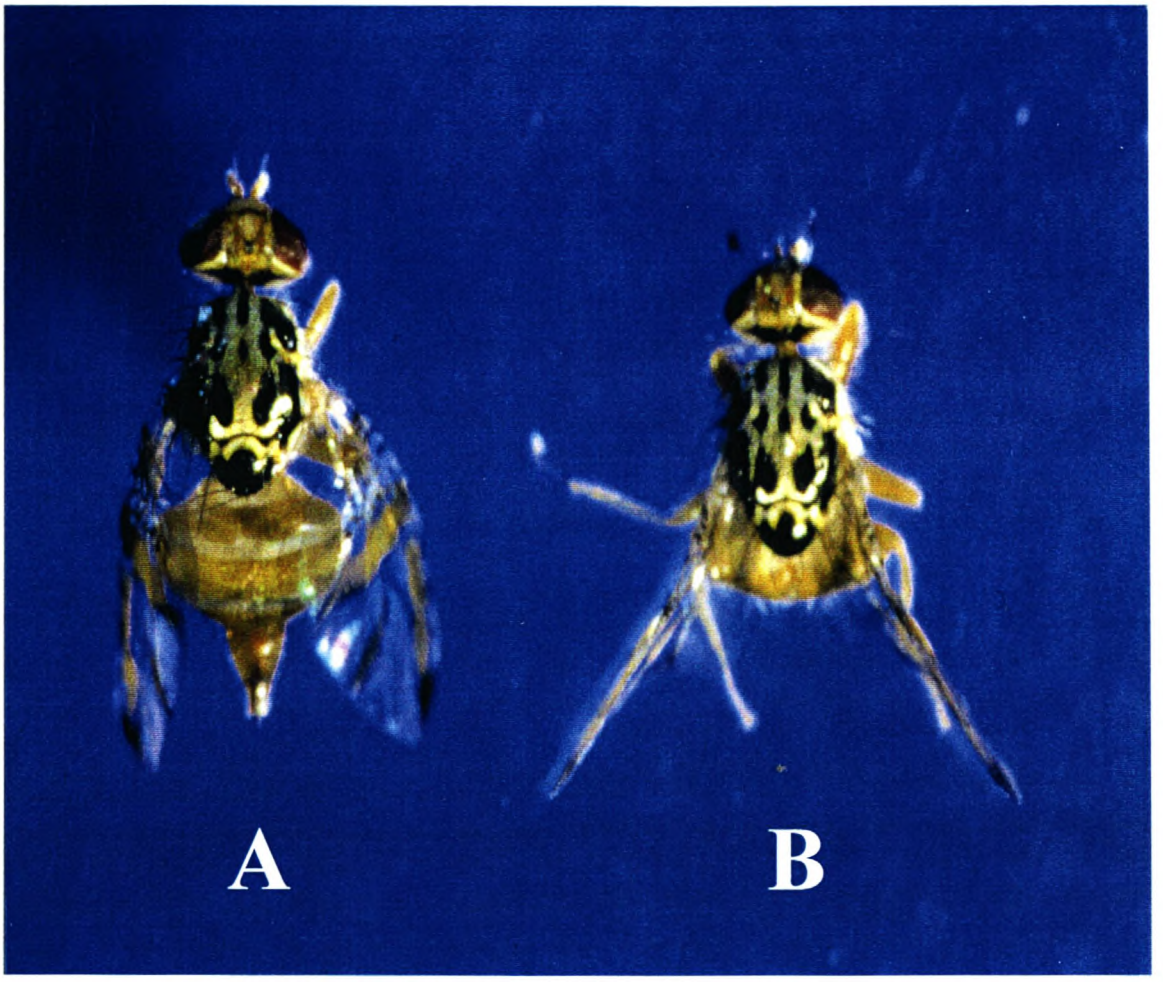

Fig. 1. Sexually mature Mediterranean fruit fly *Ceratitis capitata*. **A.** Female. **B.** Male.

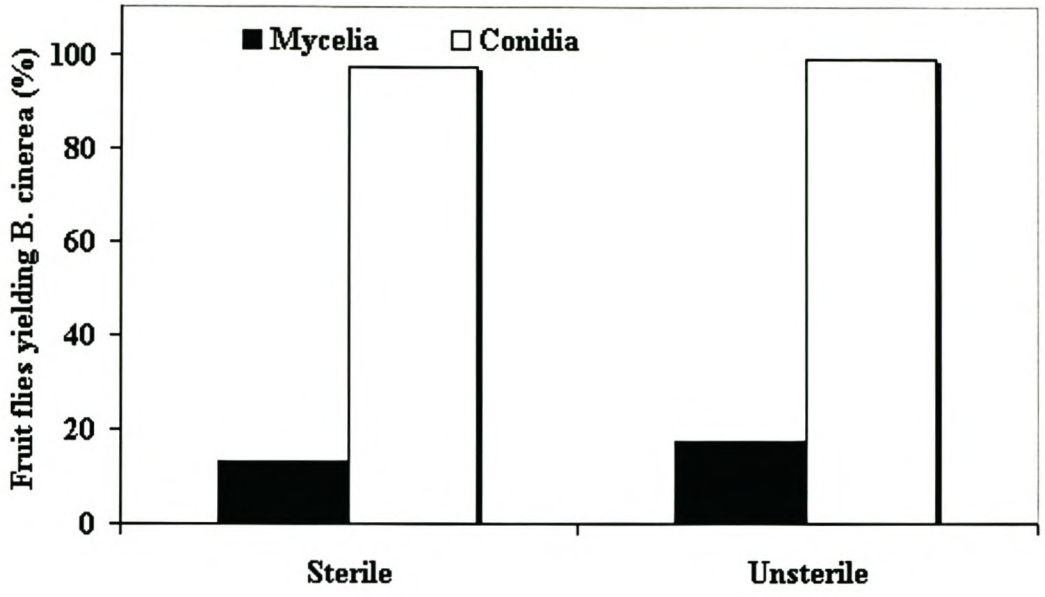

Fig. 2. Mean percentage fruit flies that yielded *Botrytis cinerea* on Kerssies' medium. Conidia = flies exposed to grape berries bearing sporulating colonies of *B. cinerea*. Mycelia = flies exposed to grape berries bearing no conidia, but showing signs of slippery skin. Sterile = flies were surface-sterilised for 1 min in 0.7% sodium hypochlorite before incubation. Unsterile = flies left unsterile.

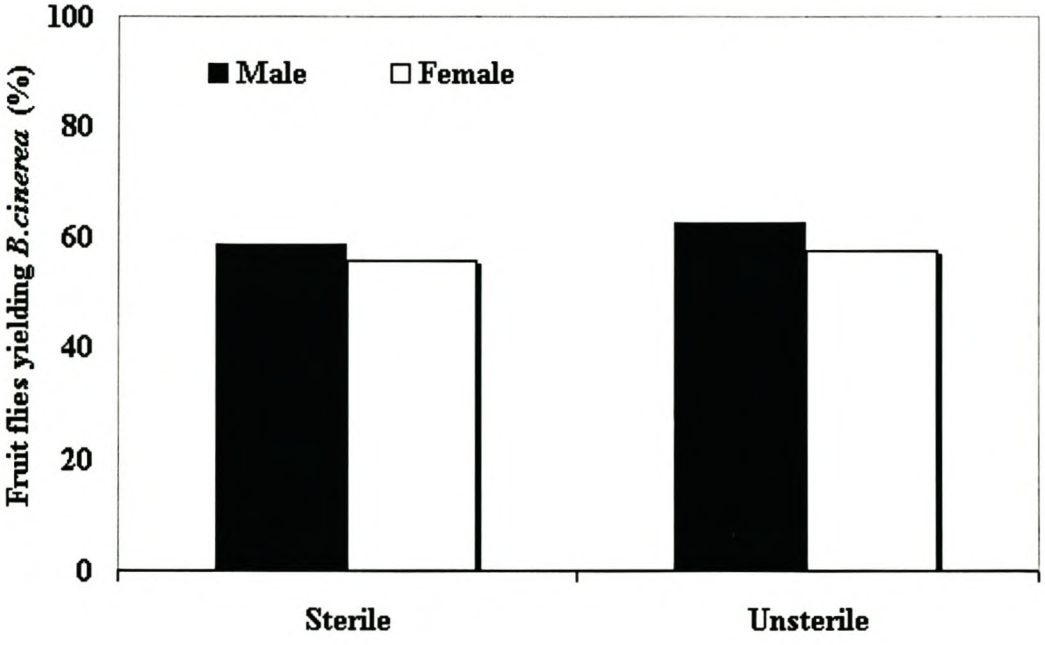

Fig. 3. Mean percentage male and female fruit flies that yielded *Botrytis cinerea* on Kerssies' medium. Sterile = flies were surface-sterilised for 1 min in 0.7% sodium hypochlorite before incubation. Unsterile = flies left unsterile.

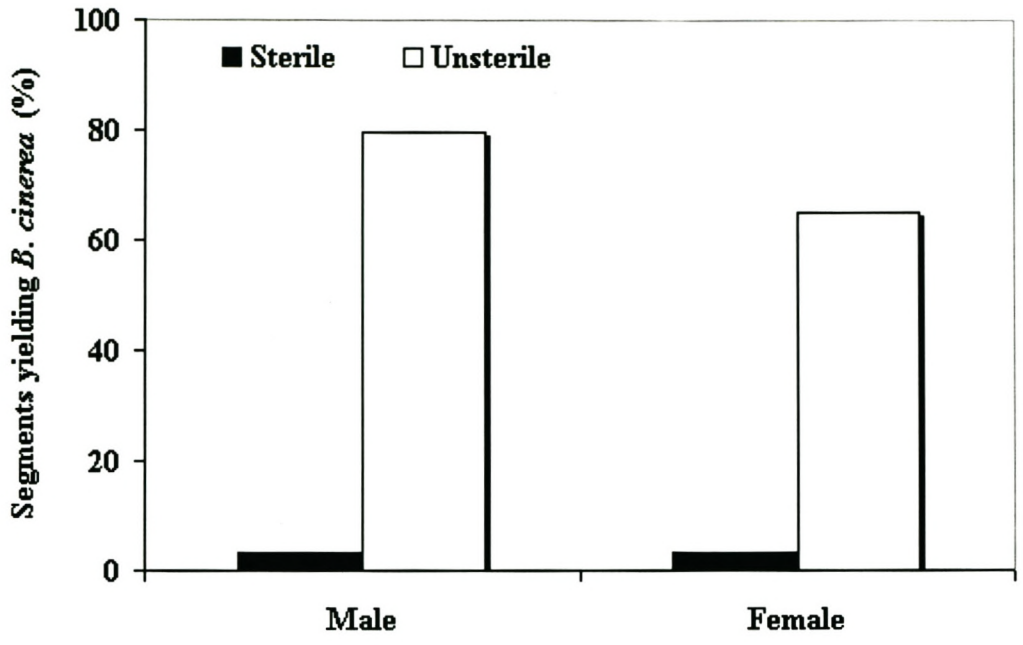

Fig 4. Effect of fly gender x sterility regime interaction on the percentage skin segments (5 x 7 mm) cut from grape berries after exposure to *Botrytis cinerea* bearing fruit flies that yielded the pathogen on Kerssies' medium. Sterile = berries surface-sterilised before removal of skin segments. Unsterile = berries left unsterile.

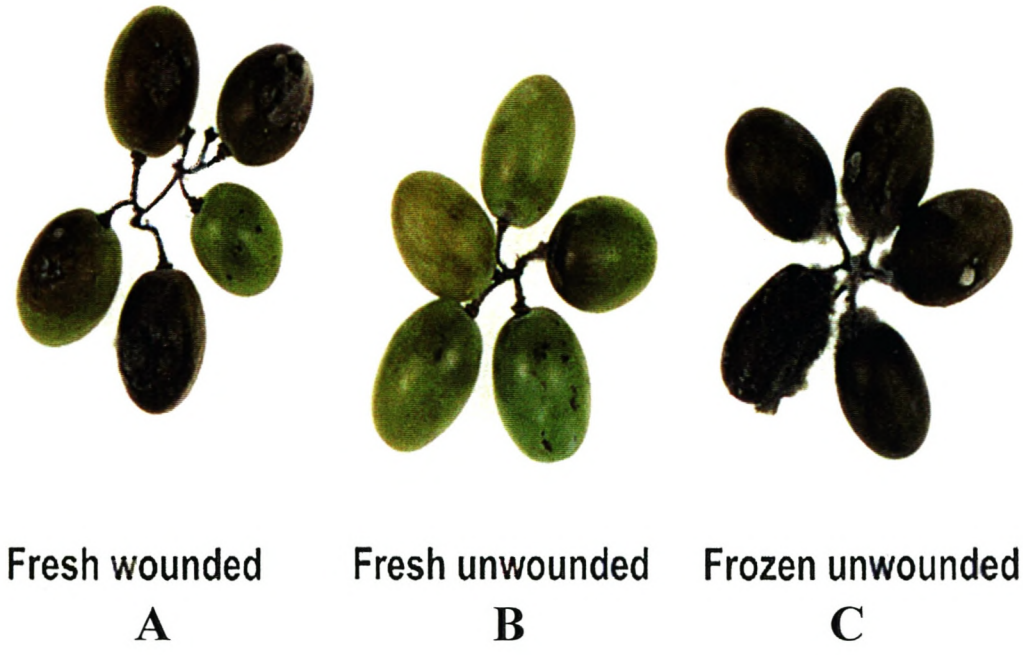

Fig. 5. *Botrytis cinerea* symptom expression on grape berries after exposure to conidia bearing fruit flies. **A**. At wounds on cheeks; **B** and **C**, at the pedicel end.

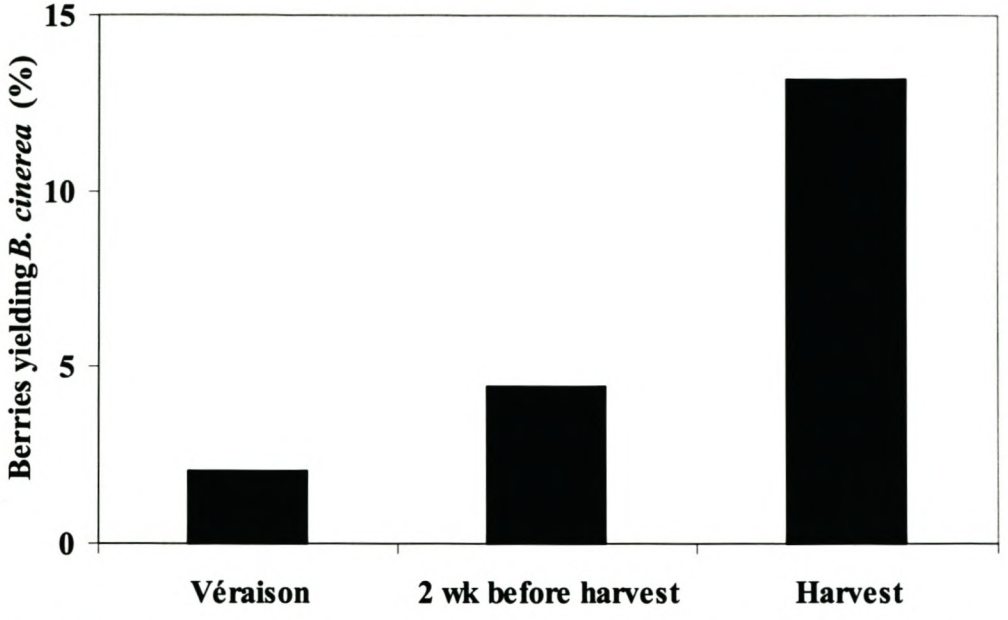

Fig. 6. The effect of phenological stage on percentage grape berries that developed *Botrytis cinerea* symptoms after exposure to fruit flies bearing conidia of the pathogen. Values averaged for cultivars.

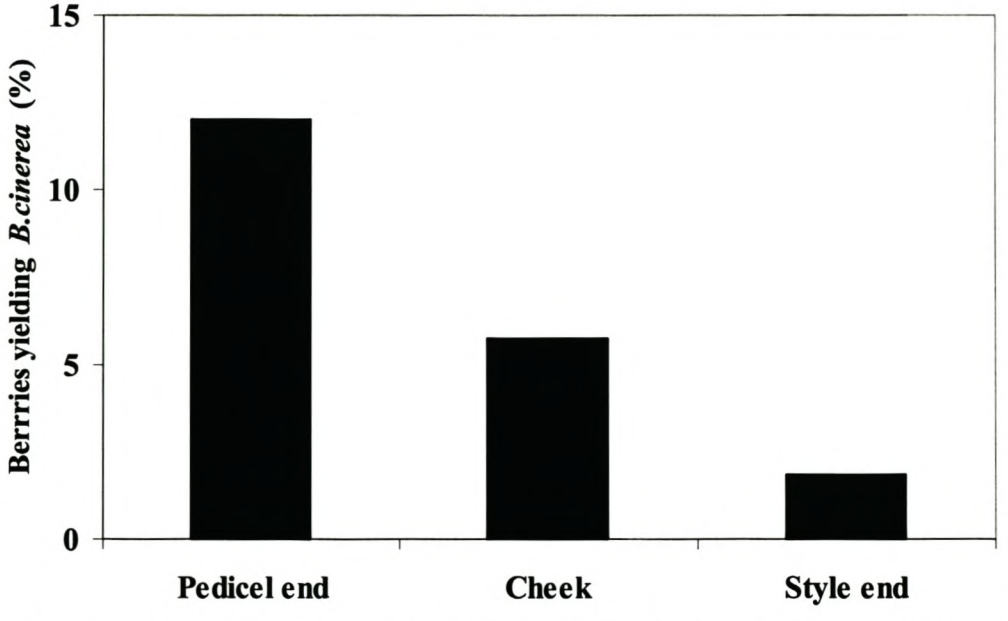

Fig. 7. The effect of infection site on percentage grape berries that developed *Botrytis cinerea* symptoms, at a given berry position, after exposure to fruit flies bearing conidia of the pathogen. Values averaged for cultivars.

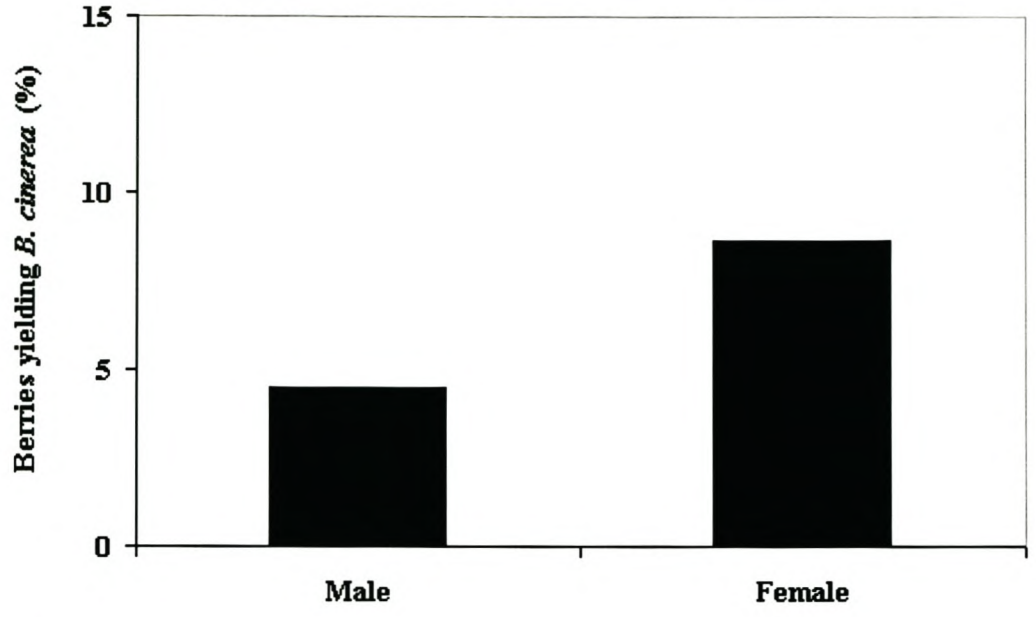

Fig. 8. The effect of fly gender on percentage grape berries that developed *Botrytis cinerea* symptoms after exposure to fruit flies bearing conidia of the pathogen. Values averaged for cultivars.

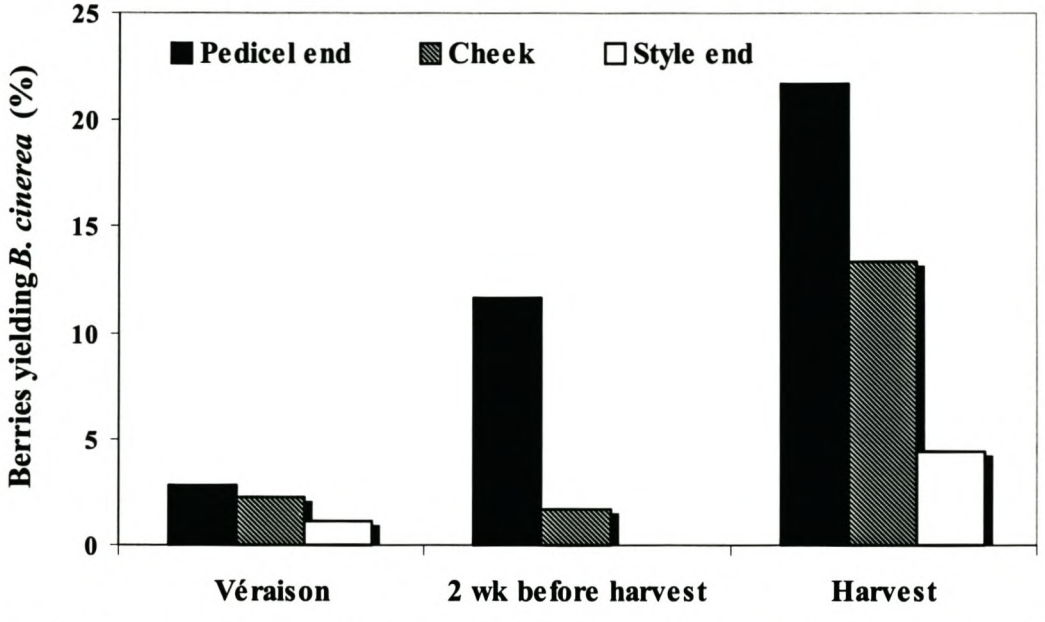

Fig. 9. The effect of phenological stage and infection site on percentage grape berries that developed *Botrytis cinerea* symptoms, at a given berry position, after exposure to fruit flies bearing conidia of the pathogen. Values averaged for cultivars.

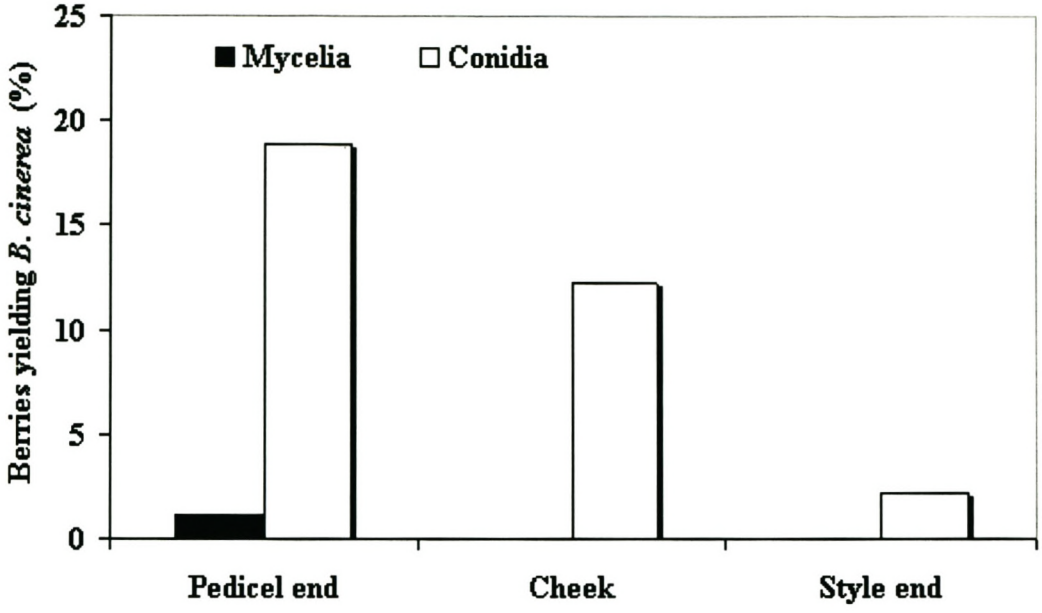

Fig. 10. The effect of source of *Botrytis cinerea* x infection site interaction on percentage grape berries that developed *B. cinerea* symptoms, at a given berry position, after exposure to fruit flies bearing conidia of the pathogen.

4. ACTIVITIES OF THE MEDITERRANEAN FRUIT FLY *CERATITIS CAPITATA* AND THE NATURE OF DEPOSITED *BOTRYTIS CINEREA* CONIDIA AND MYCELIA ON GRAPE BERRY SURFACES

ABSTRACT

The activities on grape berries of the Mediterranean fruit fly *Ceratitis capitata* were monitored using digital photography. In addition, the deposition of conidia and mycelia of *Botrytis cinerea* at three sites (pedicel end, cheek and style end) on the grape berry, germination of the fungal structures after dry (±56% RH) and moist (±93% RH) incubation and wounds inflicted during ovipositioning were examined with an epifluorescence microscope. The observations revealed that the fruit fly's activities were generally restricted to the grape berry. They visited the grape berry cheek more often, but visitations to the pedicel end of berries increased substantially from véraison to harvest, indicating the possibility of nutrient leakages at this site. Microscopy revealed that the flies deposited conidia singular, in feeding packages and in faecal excrements on the berry surface. The conidia in feeding packages were ensheathed by salivical fluids and occurred in clusters of 10 to 50 conidia. An average of 60% of the conidia in feeding packages germinated under dry conditions (±56% RH). Conidia that passed through the intestinal tract of the fruit fly and that were deposited in faecal excrements were deformed and low in viability. These conidia did not occur in cluster format, but were proportionally spread with the faeces on the surface of the grape berry. Conidia that were deposited singular and in faecal excrements did not germinate unless incubated under moist conditions (± 93% RH). Wounds inflicted by female fruit flies during ovipositioning were most frequently observed on the cheek. This predisposition to *B. cinerea* infection of grape berries by the activities of fruit flies, suggested an important role for the flies in the initiation of Botrytis bunch rot epidemics in vineyards.

INTRODUCTION

The Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann), is known for its polyphagous feeding habits and is a pest of a wide range of fruit types, especially stone fruit species (Christenson & Foote, 1960; Bateman, 1972). In the Western Cape province, *C. capitata* is

found to also infest grape cultivars, usually regarded as a non-host (Myburgh, 1962, 1964; Hendrichs & Hendrichs, 1990; Liquido, *et al.* 1990; White, 1992; Schwartz, 1993). Grey mould and *Botrytis* bunch rot, caused by *Botrytis cinerea*, are the most important pre- and postharvest diseases of stone fruit and grapevine in Western Cape orchards and vineyards (Fourie & Holz, 1985; De Kock & Holz, 1991, 1994; Fourie, 2001; Holz & Calitz, 2001). It was recently shown (Part 2) that the percentage of fruit flies contaminated with *B. cinerea* collected in orchards and their neighbouring vineyards increased meaningfully during and after harvest, which point to the potential of the fruit fly to transfer *B. cinerea* inoculum *in natura* from early-season peach and plum orchards to neighbouring mid- and late season wine grape vineyards. A study (Part 3) on the transport and deposition of *B. cinerea* by the Mediterranean fruit fly on grape, proved the external and internal mode of transmission of *B. cinerea* by the Mediterranean fruit fly and showed that the flies deposited, without preference, high amounts of *B. cinerea* at various positions on the grape berry's surface. However, a subsequent investigation (Part 3) on disease expression at different sites on grape berries, showed that decay predominated at the pedicel end of unwounded berries, or at wounds made on the berry cheek. This finding indicates a peculiar interaction between *B. cinerea,* the fruit fly and host tissue at the pedicel end of the berry, and a preference of feeding at wound sites. The behavioural activities of fruit flies include daily periodic visitations to feeding, courtship, mating, oviposition and resting sites on host plants (Warburg & Yuval, 1997). Thus, a better understanding of fruit fly behaviour in grape bunches may help to verify their potential to transport and deposit *B. cinerea* inocula, and to enhance expression of *B. cinerea* bunch rot in vineyards.

A study done by Cayol *et al.* (1994) showed that fruit flies are able to disseminate the fungal pathogen, *Rhizopus stolonifer*, externally and internally. The external mode of transmission involves the mechanical transfer of the pathogen's conidia on the fruit fly's body and the internal mode involves partial (regurgitation) or total (faeces) transit through the digestive tract of the fruit fly. Recent *in vitro* studies with *C. capitata* (Part 3) proved the external and internal mode of transmission of *B. cinerea* by the Mediterranean fruit fly. Many researchers reported the promoted effect of moisture on infection of mature grapes by *B. cinerea* conidia deposited in clusters (Nelson, 1951; Hill *et al.*, 1985; Bulger, *et al.*, 1987; Nair & Allen, 1993; Broome *et al.*, 1995). These findings and the successful decay development of intact berries exposed to inocula-bearing fruit flies under dry conditions (Part 3) suggested that fruit flies deposited inocula in moisture and in clusters on the host surface.

In order to prove these hypotheses, the activities of fruit flies on berries were monitored *in vitro* by the aid of digital photography. Following conidial and mycelial transportation and deposition, the berries were examined by epifluorescence microscopy to determine the nature of the inocula deposited by male and female fruit flies. In addition, by using véraison- and harvest-stage grape berries, the role of growth stage in feeding preference of fruit flies and in germination of deposited inocula was also investigated.

MATERIALS AND METHODS

Grapes. Sound, unblemished bunches were selected at véraison and at harvest in a Dauphine table grape vineyard (9° and 19° Brix respectively) located in the De Doorns region. Low *B. cinerea* incidences were expected in the vineyard because of the dry climatic conditions associated with this district. Following harvest, the bunches were surface-sterilised (30 s in 70% ethanol, 2 min in 0.35% sodium hypochlorite, 30 s in 70% ethanol), air-dried and stored at 5°C in polythene bags in carton boxes. Before an experiment, the boxes were removed from cold-storage and kept overnight in a laboratory for the bunches to reach ambient temperature. The bunches were then carefully cut into sections bearing five berries on a short rachis.

Inoculum. A virulent isolate of *B. cinerea*, obtained from a naturally infected grape berry, was maintained on potato-dextrose agar (PDA) at 5°C. Inoculum was prepared by culturing the fungus at 23°C under diurnal light on potato-dextrose agar in Petri dishes. Dry conidia were harvested after 14 days from cultures using a spore cyclone. This harvesting method avoided contamination of spores by nutrients from the medium. The conidia were stored in dry, sterile McCartney bottles at 5°C until use. Storage time did not affect spore germination (Spotts & Holz, 1996). Spore suspensions were prepared by suspending dry conidia in sterile deionised water and then sonicating them for 3 min in an ultrasonic bath (Branson model B3).

Two weeks before an experiment was conducted, sound grape berries were wounded with an insect needle 1.5 mm deep and inoculated by placing a 20 µL droplet of the conidial suspension onto the wound. The droplets were air-dried, and the berries were placed in ethanol-disinfected perspex (Cape Plastics, Cape Town, South Africa) chambers (60 x 30 x 60 cm) lined with a sheet of chromatography paper with the base resting in deionised water to establish high relative humidity (±93% RH). The chambers were kept at 22ºC to promote

decay at the wound sites on the berries. In experiments where the transportation of conidia were studied, berries with decay on approximately a third of the cheek, and with sporulating colonies of *B. cinerea* in the center of the lesion, were used as inoculum source. Berries with a corresponding decay pattern, but without sporulating colonies, were selected as source of mycelial inoculum. These berries were kept at 5°C to restrict the development of sporulating colonies before usage.

Laboratory strain of *C. capitata*. Pupae from a wild-type *C. capitata* strain, reared on the Krige diet (3 090 g of bran, 1 350 g of sucrose, 59 g of torula yeast, 20 g of sodium benzoate, 80 ml of HCL, and 4 910 ml of water) under aseptic conditions in the laboratory, were obtained from Infruitec Pest Management Division (ARC Infruitec/Nietvoorbij, Fruit, Vine and Wine Research Institute, Private Bag X5013, Stellenbosch 7599). The pupae were kept in ethanol-disinfected perspex (Cape Plastics, Cape Town, South Africa) cages (29 x 29 x 38 cm) at 25°C. Preliminary studies showed that the yeast component usually included in the adult diet inhibited *B. cinerea* development. However, previous studies showed that pupal stages provided of enough protein, accumulated sufficient protein reserves for development during the early mature stages (Fernandes-da-Silva, 1997). Protein was therefore excluded from the diet of emerging fruit flies, which were fed on sucrose and sterile, deionised water only. The flies were used when they were 10 days old or sexually mature.

Dissemination and photography. Two ethanol-disinfected perspex (Cape Plastics, Cape Town, South Africa) cages (29 x 29 x 38 cm) were used. One cage was allocated to male and one to female fruit flies. Each cage was provided with two short rachis sections bearing five berries. Two decayed grape berries, with or without sporulating colonies of *B. cinerea*, provided inoculum, and 50 fruit flies per cage served as vectors. Water was provided to the flies by wetting sterile cotton wool, placed in the lid of a Petri dish, with 50 ml of sterile deionised water. Following placement in the cages, the grape berries on the rachis sections were photographed every half hour for 48 hours (60 half hours of daylight) with a digital camera (Saerim color CCD) with an automatic iris lens (Spacecom). The frequency at which the fruit flies visited the different infection sites on the berries were then calculated. All experiments were conducted under dry conditions (±56% RH) at 22°C.

Experimental design. There were two replications and three main effects. The main effects were fly gender (male and female), fungal form (conidia and mycelia) and

phenological stage (véraison and harvest). The trials were conducted on the cultivar Dauphine using conidial inocula. The experiment conducted with mycelia as inoculum, was only performed on harvest stage Dauphine grape berries.

Histology. Following photography, 10 berries were taken from each perspex cage, divided into two groups of five berries each and packed on sterile epoxy-coated steel mesh screens (53 x 28 x 2 cm). One screen with berries was placed in dry ethanol-disinfected perspex chambers (60 x 60 x 30 cm) and incubated under (±56% RH) conditions. The other screen was placed in an ethanol-disinfected perspex chamber lined with a sheet of chromatography paper with the base resting in deionised water to establish high relative humidity (±93% RH). The chambers were incubated for 24 h at 22°C with a daily 12-h photoperiod. The conditions in the chambers provided circumstances commonly encountered in nature by the pathogen on grape bunches, namely dry conidia on dry bunch parts under dry coniditions, and dry conidia on dry bunch parts under high relative humidity. Studies (Coertze & Holz, 1999; Coertze *et al.*, 2001; Gütschow, 2001) with dry conidia of *B. cinerea* under high humidity showed that germination, surface colonisation and skin penetration reached a maximum during this period. Histology was performed following incubation. Thin hand-sectioned pieces (approximately 5 x 5 mm) were cut with a razor blade from three different infection sites on the grape berry (from the pedicel end, cheek and style end). Skin sections comprised of cuticle, epidermis, and a few cell layers. The sections were stained for 5 min. in a differential stain containing fluorescence diacetate ([FDA] Sigma Chemical Co., St Louis, MO, USA), aniline blue ([AB] B. D. H. Laboratory Chemicals Division, Poole, England) and blankophor ([BP] Bayer, Germany), mounted on a glass slide in 0.1 M KH_2PO_4 buffer (pH 5.0) and covered with a cover slip. FDA (2 mg/ml acetone) and AB (0.1% in KH_2PO_4 buffer, pH 5.0) were prepared as stock solutions and stored at -20C and 5C respectively. Before a histology session, BP (0.5%) was added to the AB solution and a fresh stain prepared by mixing 25 µl of the FDA stock solution with 1 ml of the AB/BP stock solution in a 1.5 ml polypropylene Eppendorf tube, which was then kept on ice. The presence of conidia or mycelia at three different infection sites on the grape berry (pedicel end, cheek and style end), germination of the fungal structures and wounds inflicted during oviposition were examined with the aid of a Zeiss Axioskop microscope equiped with an epifluorescence condensor, a high-pressure mercury lamp, Neofluar objectives and Zeiss filters 02 and 18. These sets include excitation G 365, BP 436/8 and BP 395-425, respectively. With this set-up, protoplasts of viable fungal

structures fluoresced brilliant yellow-green with filter 02 and 18, whereas protoplasts of dead cells were blue-black (filter 18) (O'Brien & McCully, 1981).

RESULTS

Photography. Digital photography and visual observations revealed that the flies initially preferred to feed on the macerated tissue of the lesions on berries that served as inoculum. However, in the course of time they tended to feed on the sporulating colonies on the lesions. This was evident by the distinctive "feeding paths" that appeared in the colonies as a result of their activities, and the disappearance of *B. cinerea* conidia from the colonies. The observations furthermore revealed that the fruit fly's activities were generally restricted to the grape berry fruit, since visitations to the vegetative bunch parts were very low (data not shown). In addition, the flies were not attracted to the wound sites at the cut end of rachis sections, although they prefered to feed at wound sites on the grape berry cheek (Part 3). On berries the flies visited the cheek more frequently than the pedicel end or the style end, regardless of fly gender or phenological stage (Table 1). This was especially prominent during véraison, although the number of visitations to the pedicel end by female flies was relatively high at this stage. At harvest, compared to véraison, visitations to the cheek and style end decreased, but increased at the pedicel end. The mean number of flies visiting the different infection sites per half hour is given in Table 2. The average number recorded per half hour was higher at the cheek than at the other sites. When the mean number of half hour visitations (Table 1) and the mean number of flies per infection site (Table 2) are considered, females visited the pedicel end more frequently and in higher numbers than males. Contrary to this, male flies visited the style end of grape berries more often.

Histology. Histology revealed that flies deposited conidia individually (Fig. 1A), in feeding packages (Fig. 2A-B) and in faecal excrements (Fig. 3). Conidia deposited singular were more frequently found at the cheek, regardless of fly gender or phenological stage. Conidial clusters in feeding packages were found to be ensheathed by salivical liquids. These packages were more frequently deposited at the pedicel end of harvest stage grape berries, whereas at véraison the deposition of these packages was more frequent at the cheek. Conidia that passed through the intestinal tract of the flies and were deposited with faecal excrements were deformed and low in viability. These conidia were not deposited in clusters, but were proportionally spread with the faeces on the surface of the grape berry. Mycelia were most

frequently deposited individually and germinated only under moist conditions (Fig. 1B). There were no recordings of viable mycelia in faecal excrements. Both the mycelia and conidia in faecal excrements were deposited most frequently at the cheek, compared to the other sites.

The mean percentage germination of conidia under dry (±56% RH) and moist (±93% RH) conditions is given in Table 3. Conidia deposited individually and in faecal excrements did not germinate under dry (±56% RH) conditions. The highest germination levels under dry (±56% RH) conditions were recorded in feeding packages deposited at the pedicel end of the grape berry. In addition, a 100% increase in germination at this site was found from véraison to harvest. Contrary to the dry incubated grape berries, berries incubated under moist conditions showed high germination levels of individually deposited conidia. However, this, germination of conidia in feeding packages was substantially higher than single conidia. Again the increased germination from véraison to harvest was evident. Mycelia occurred predominantly as single fragments and germinated only under moist conditions. The number of conidia in feeding packages ranged from 10 to 50, of which an average of 60% and 90% germinated respectively under dry and moist conditions. The number of conidia in faecal excrements was much higher, ranging from 10 to 250 conidia. These conidia were deformed and showed very low germination ability.

If the difference in germination levels under dry conditions (Table 4) between male and female deposited conidia are considered, it is evident that more germination occurred when conidia were deposited by female than male flies. In addition, the only germination of conidia deposited in faecal excrements occurred after deposition by female fruit flies. During microscopic examination of grape berry segments exposed to female flies, wounds (Fig. 4A-B) inflicted during the oviposition behaviour, were observed. These wounds were more frequently observed on segments taken from the cheek of grape berries than the other infection sites. None of the berries exposed to male flies bore similar wounds.

DISCUSSION

According to Warburg & Yuval (1997), the activities of fruit flies included daily periodic visitations to feeding, courtship, mating, oviposition and resting sites on host plants. Adult fruit flies fed predominantly on ripe and wounded fruits (Hendrichs & Hendrichs, 1990; Warburg & Yuval, 1997). Courtship and mating, involving male calling, female approach, male wing vibrating, female standing, male wing fanning and copulation (Liimatainen, *et al.*, 1997), were performed predominantly on the abaxial side of host plant leaves, although these activities were found to also occur on the host fruit. Ovipositioning occurred exclusively on the host fruit and fruit flies were found to rest during warm midday hours on the abaxial side of leaves. The digital photography done in this study on grape rachis sections revealed that the fruit fly's activities were generally restricted to the grape berry fruit, since their were no leaves. In addition, the flies visited the berry cheek more often than the pedicel end or the style end. These findings suggest that courtship, mating and oviposition activities took place on grape berry surfaces, explaining the frequent visitations to the cheek. The microscopic study revealed that higher numbers of conidia, deposited single and in faecal excrements, were found at the cheek than at the other sites on the berry. Since single conidia are deposited mechanically, the higher conidial numbers at the cheek could thus be attributed to the courtship, mating and oviposition activities that took place at this site.

Adult fruit flies need carbohydrate, lipid and protein in order to perform the biological activities necessary for survival and reproduction (Bateman, 1972). Sugars, a source of carbohydrates found in fruit juices, are the most important nutrient for adults, especially female fruit flies (Cangussu & Zucoloto, 1992, 1995). Since the proboscis of the fruit fly is unable to penetrate the host skin, adult fruit flies are forced to feed on ripe, decayed and wounded fruits (Hendrichs & Hendrichs, 1990; Warburg & Yuval, 1997). This was supported by the findings in this study. Fruit flies tended to visit the decayed berries, which served as inoculum, more often than the sound berries. It was previously shown (Part 3), with intact grape berries exposed to *B. cinerea* inocula-bearing fruit flies, that substantially more berries decayed at the pedicel end than at other sites on berries. In the present study an increase in the frequency at which fruit flies visited the pedicel end from véraison to harvest, suggests that the increased sugar levels during harvest and nutrient leakages at the pedicel end are highly attractive to fruit flies. This also may explain the high numbers of feeding packages

and germination at the pedicel end and the increase in germination from véraison to harvest. In addition, the findings that female fruit flies visited the pedicel end more frequently and in higher numbers than males are supported by the higher dependence of female fruit flies on carbohydrates than males.

The microscopic study revealed that fruit flies deposited conidia individually, in feeding packages and with faecal excrements. Conidia deposited with faecal excrements were deformed and low in viability and were proportionally spread with the faeces on the surface of the grape berry. Conidia deposited singular (mechanical and in faecal excrements) occurred in higher numbers at the cheek site. Germination of these individually deposited conidia at the cheek and style end of the grape berry under dry (±56% RH) conditions was not facilitated and, thus, infection could not occur. However, under moist conditions, these conidia were found to germinate readily, but infection was unlikely because of host defense mechanisms located in the grape berry skin (Coertze & Holz, 1999; Coertze *et al.*, 2001). Mycelial fragments were also deposited by the flies on the grape berry surface, although germination of these mycelial fragments occurred after moist incubation only. Infection by these structures was highly unlikely, since they did not occur in clusters and they were confronted by the defense mechanisms located in the grape berry skin, which is regarded to be resistant to infection by single germ tubes (Coertze & Holz, 1999; Coertze *et al.*, 2001). Thus, decay development from single conidia or mycelial fragments was only possible under moist conditions, which facilitated germination, and only if deposited in the vicinity of wounds (pedicel end and ovipositional wounding).

Fruit flies did not only deposit inocula individually, but also in salivical fluids and in clusters. Microscopic investigation revealed that conidia deposited in regurgitated products (feeding packages) were ensheathed by salivical fluids and occurred in clusters. Under dry conditions, ± 60% of the conidia in these clusters, which contained 10 to 50 conidia, germinated successfully. Intact grapes are considered to be resistant to infection by single, airborne *B. cinerea* conidia under both humid and wet conditions (Coertze & Holz, 1999; Coertze *et al.*, 2001). However, they are susceptible to decay by groups of conidia when mature (Nelson, 1951; Hill *et al.*, 1985; Nair & Allen, 1993; Broome *et al.*, 1995) and moisture promotes germination and infection (Nelson, 1951; Corbaz, 1972; Hewitt, 1974; Gessler & Jermini, 1985; Bulger *et al.*, 1987; Broome *et al.*, 1995). From this it can be

concluded that infection by the conidial clusters through the intact grape berry skin was possible under unfavourable conditions for disease development.

Wounds on grapes are regarded as a very important route of entry for *B. cinerea* (Nair *et al.*, 1988; Edlich *et al.*, 1989; Brook, 1991; Sharrock & Hallett, 1991; Elad & Evensen 1995). Wounding causes nutrient and moisture leakages and negates host defense mechanisms in the skin, thus facilitating infection and subsequent disease development. In a previous study (Part 3) decay development was significantly higher on intact grape berries exposed to female fruit flies than male fruit flies. This was attributed to the wounds inflicted by the female fruit flies during ovipositioning. In this study these wounds were observed during the microscopic examinations. Conidia deposited singular and in feeding packages had substantially higher germination levels after deposition by female fruit flies compared to males. In addition, it was observed that conidia deposited in faecal excrements germinated if deposited by female fruit flies only. The enhanced germination might be attributed to nutrient leakages, and infection, because of wounds, of inocula deposited near the ovipositioning site.

Botrytis cinerea conidia are dispersed by wind (Jarvis, 1980), water droplets (Jarvis, 1962) and by insects (Fermaud & Le Menn, 1989, 1992; Michailides & Spotts, 1990; Fermaud & Giboulot, 1992; Fermaud *et al.*, 1994; Fermaud & Gaunt, 1995; Louis *et al.*, 1996; Michailides & Morgan, 1996; Mondy *et al.*, 1998; Mondy & Corio-Costet, 2000). Observations made in vineyards showed that conidia dispersed in wind are deposited as single colony forming units (G. Holz, *unpublished data*; Duncan *et al.*, 1995). Very few of the conidia dispersed in water droplets become wet enough to enter the droplets (Jarvis, 1962) and are thus deposited singular during run-off (Coertze & Holz, 1999). Single conidia are, however, unable to induce disease on intact berries, because of host defenses located in the grape berry skin (Coertze & Holz, 1999; Coertze *et al.*, 2001). Since relatively high infection rates often occur in vineyards (Nair & Nadtotchei, 1987), these findings suggest that factors predisposing the grape berry to infection must play an important role in *Botrytis* bunch rot (Du Plessis, 1934; Hill *et al.*, 1981, 1988; Coertze & Holz, 1999). Findings in this study provide ample evidence that fruit flies play an important role in predisposing the grape berry to infection. Close observation showed that fruit flies were attracted to wounded and rotting fruit. This behaviour enabled them to contract conidia when they came into contact with sporulating colonies whilst feeding on lesions, or mycelia when lapping-sucking from macerated tissue in lesions without sporulating colonies of the pathogen. These inocula were

subsequently deposited on the grape berry surface in clusters and in moisture, thus facilitating disease under unfavourable conditions. In addition to preferential feeding at wound sites (e.g. the pedicel end), female ovipositioning also promoted infection of conidia under unfavourable conditions. This predisposition of grape berries to *B. cinerea* infection by the activities of fruit flies, suggested an important role for the flies in the initiation of *Botrytis* bunch rot epidemics in vineyards.

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Table 1. Mean number of visitations[y] recorded at half-hour intervals at different positions on grape berries by the Mediterranean fruit fly *Ceratitis capitata*

	Berry position[z]					
	Pedicel end		Cheek		Style end	
Fly gender	Véraison	Harvest	Véraison	Harvest	Véraison	Harvest
Male	0	1	55	9	13	4
Female	26	28	50	45	8	1

[y] The fruit flies were photographed with a digital camera, at half-hour intervals over a 30 hr daylight period (total of 60 half hours).

[z] Berry positions were the pedicel end, cheek and style end.

Table 2. Mean number of fruit flies[y] recorded at half-hour intervals at different positions on grape berries

	Berry Position[z]					
	Pedicel end		Cheek		Style end	
Fly gender	Véraison	Harvest	Véraison	Harvest	Véraison	Harvest
Male	0	2	3	3	1	1
Female	2	2	3	2	1	1

[y] The fruit flies were photographed with a digital camera, at half-hour intervals over a 30 hr daylight period (total of 60 half hours).

[z] Berry positions were the pedicel end, cheek and style end.

Table 3. Mean percentage *Botrytis cinerea* conidia deposited in different formats at different positions on grape berries by the Mediterranean fruit fly *Ceratitis capitata*, that germinated after dry or moist incubation[x]

Incubation and conidial format	Berry position[y]					
	Pedicel end		Cheek		Style end	
	Véraison	Harvest	Véraison	Harvest	Véraison	Harvest
Dry						
Single conidia	0.0 (±0.0)[z]	0.0 (±0.0)	0.0 (±0.0)	0.0 (±0.0)	0.0 (±0.0)	0.0 (±0.0)
Faecal excrements	0.0 (±0.0)	0.0 (±0.0)	0.0 (±0.0)	0.6 (±1.3)	0.0 (±0.0)	0.0 (±0.0)
Feeding packages	25.0 (±13.0)	50.3 (±46.8)	10.3 (±15.1)	5.7 (±12.5)	9.3 (±13.0)	15.8 (±13.2)
Moist						
Single conidia	5.0 (±0.0)	80.0 (±75.8)	97.9 (±97.1)	76.0 (±67.7)	71.7 (±67.2)	50.0 (±50.0)
Faecal excrements	0.0 (±0.0)	0.0 (±0.0)	8.3 (±12.8)	0.0 (±0.0)	0.0 (±0.0)	0.0 (±0.0)
Feeding packages	57.7 (±54.2)	91.9 (±77.3)	79.9 (±69.3)	93.3 (±88.7)	68.5 (±58.0)	81.8 (±88.0)

[x] Incubated for 24 hr in perspex chambers under dry (±56% RH) or under moist (±93% RH) conditions.
[y] Skin segments (5 x 5 mm) cut from the pedicel end, cheek and style end of grape berries were observed with fluorescence microscopy.
[z] Numbers in parenthesis are the standard deviation from the mean percentage germination.

Table 4. Mean percentage *Botrytis cinerea* conidia deposited in different formats by male and female fruit flies at different positions on grape berries that germinated after dry incubation[x]

Fly gender and conidial format	Berry position[y]					
	Pedicel end		Cheek		Style end	
	Véraison	Harvest	Véraison	Harvest	Véraison	Harvest
Male						
Single conidia	0.0 (±0.0)[z]	0.0 (±0.0)	0.0 (±0.0)	0.0 (±0.0)	0.0 (±0.0)	0.0 (±0.0)
Faecal excrements	0.0 (±0.0)	0.0 (±0.0)	0.0 (±0.0)	0.0 (±0.0)	0.0 (±0.0)	0.0 (±0.0)
Feeding packages	0.0 (±0.0)	28.8 (±29.6)	2.0 (±3.4)	0.0 (±0.0)	0.0 (±0.0)	3.3 (±5.2)
Female						
Single conidia	0.0 (±0.0)	0.0 (±0.0)	0.0 (±0.0)	0.0 (±0.0)	0.0 (±0.0)	0.0 (±0.0)
Faecal excrements	0.0 (±0.0)	0.0 (±0.0)	0.0 (±0.0)	0.6 (±1.3)	0.0 (±0.0)	0.0 (±0.0)
Feeding packages	25.0 (±13.0)	21.4 (±17.2)	8.3 (±11.7)	5.7 (±12.5)	9.3 (±13.0)	12.5 (±8.1)

[x] Incubated for 24 hr in perspex chambers under dry (±56% RH) conditions.
[y] Skin segments (5 x 5 mm) taken from the pedicel end, cheek and style end of grape berries were observed with fluorescence microscopy.
[z] Numbers in parenthesis are the standard deviation from the mean percentage germination.

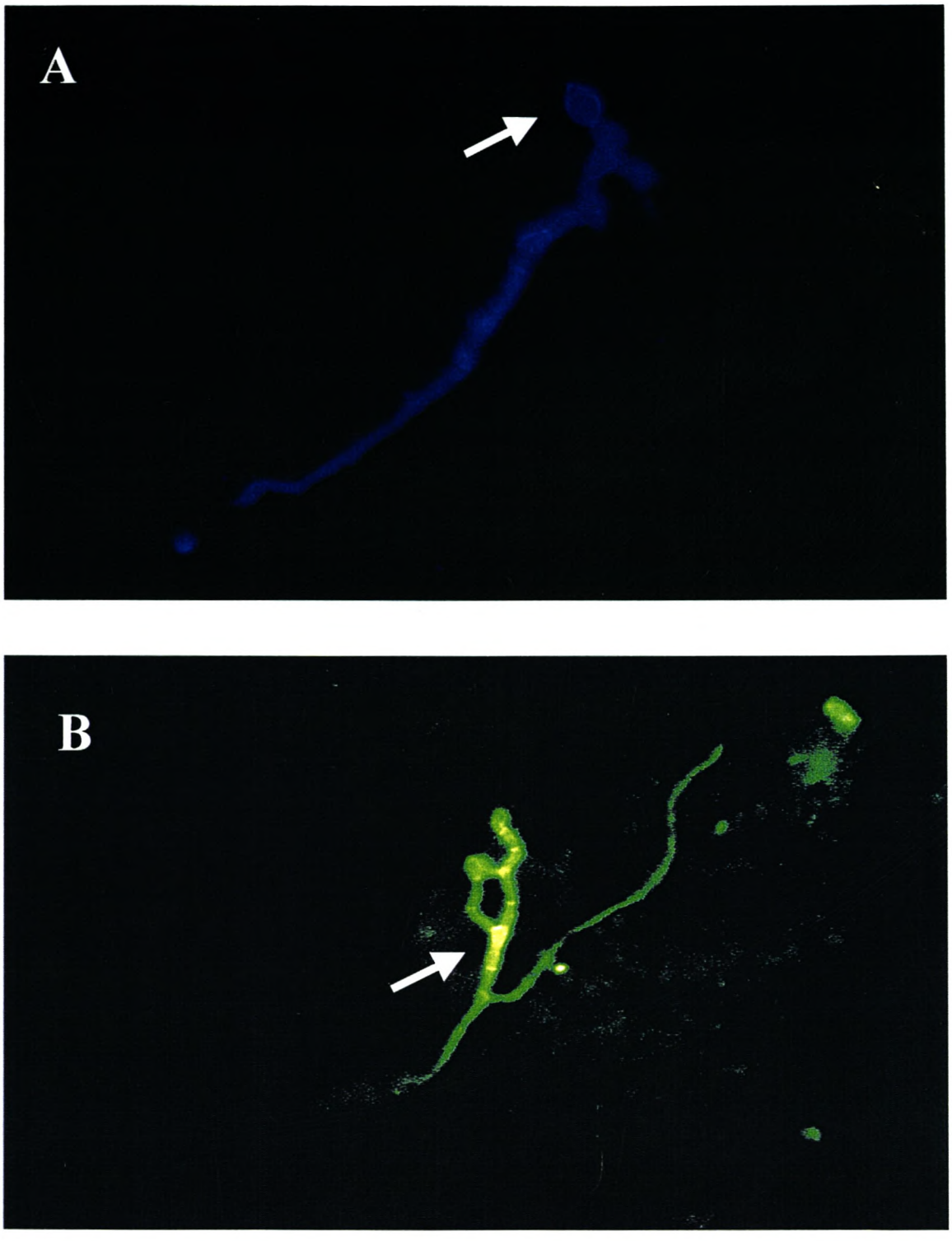

Fig. 1. Germination under high relative humidity of *Botrytis cinerea* deposited by the Mediterranean fruit fly, *Ceratitis capitata*. **A.** Single conidia (arrow) (filter 02), and **B**, mycelial fragment (arrow) (filter 18).

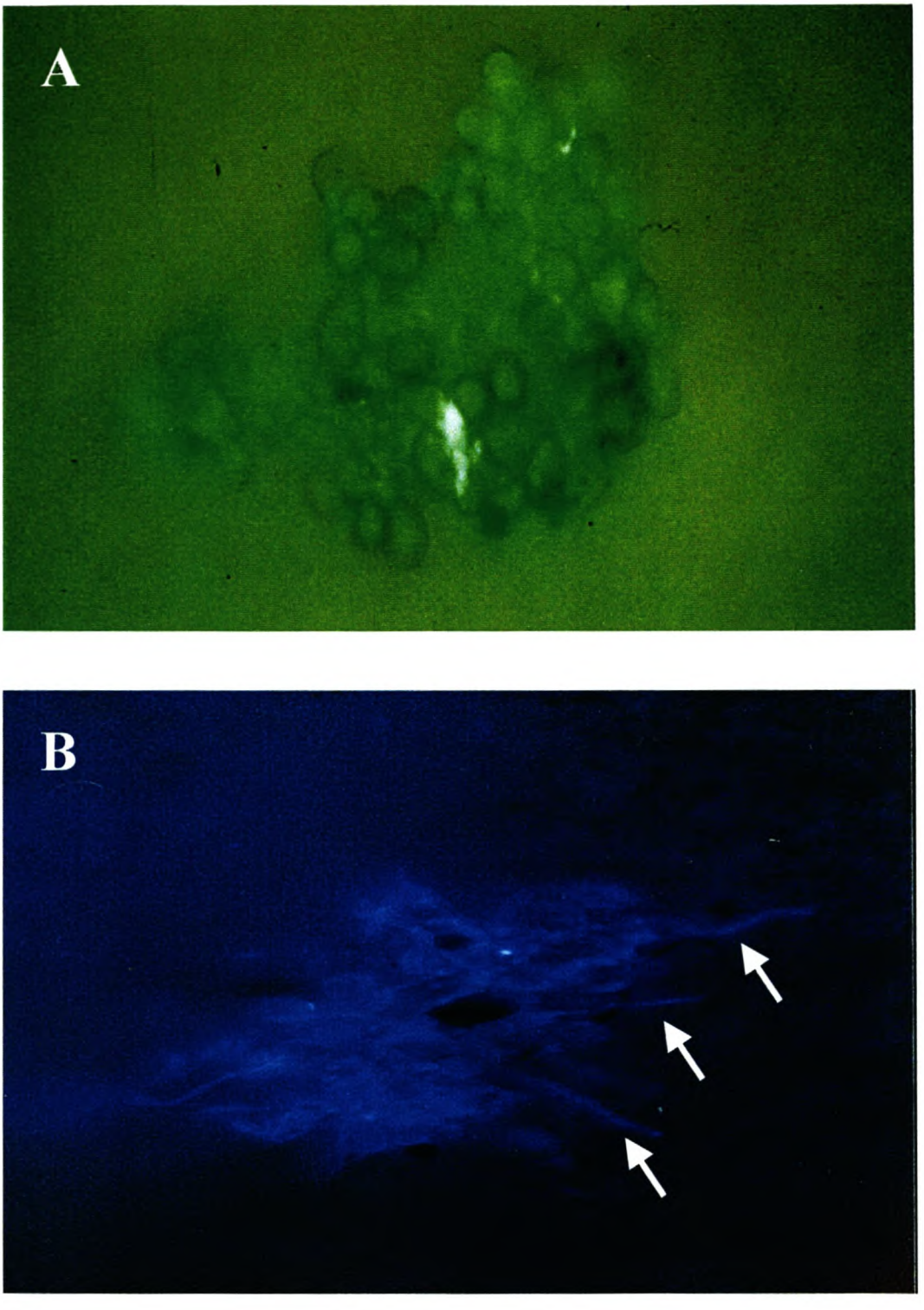

Fig. 2. Conidia of *Botrytis cinerea* deposited in feeding packages on grape berries by the Mediterranean fruit fly, *Ceratitis capitata*. **A.** Non-germinated conidia (filter 18) and **B**, germinated (arrows) conidia (filter 02).

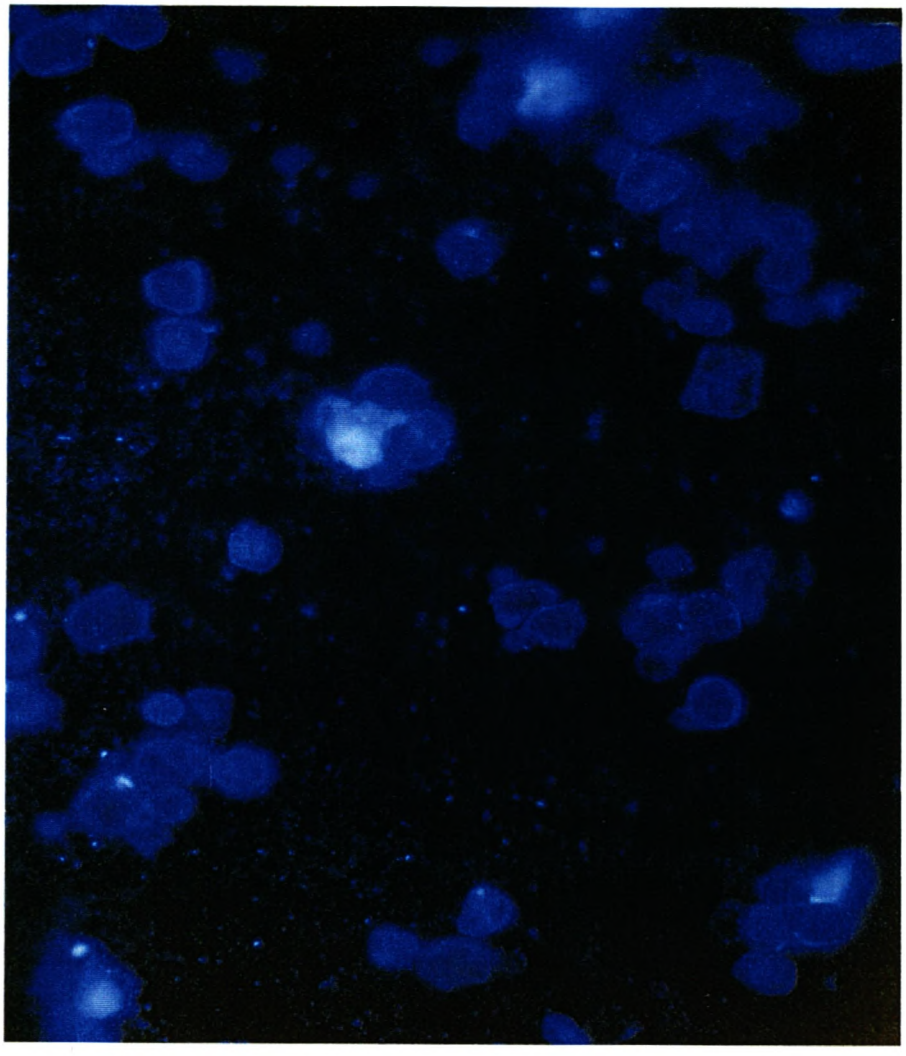

Fig. 3. Conidia of *Botrytis cinerea* deposited in faecal excrements on the grape berry by the Mediterranean fruit fly, *Ceratitis capitata* (filter 02).

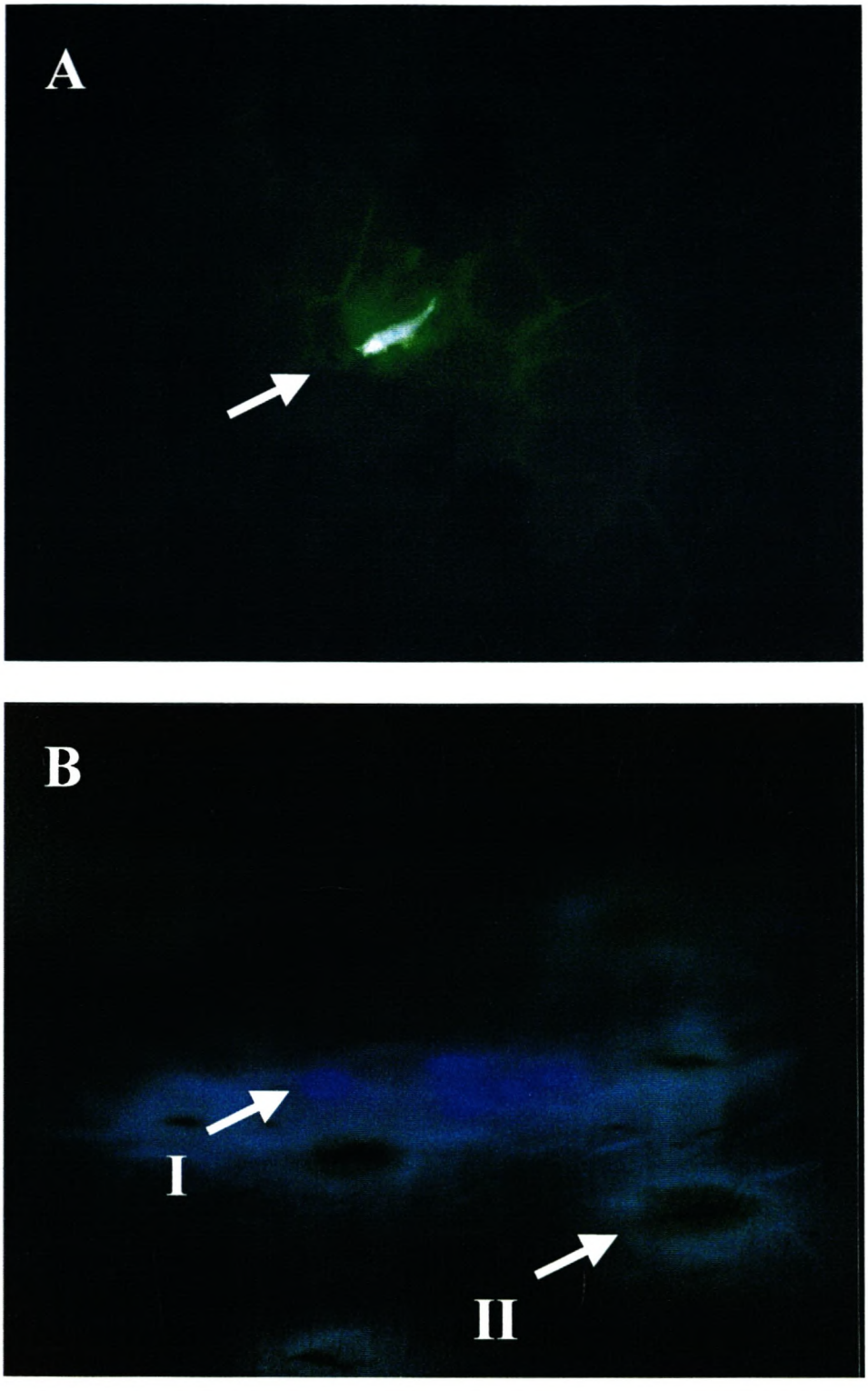

Fig. 4. Wounds (arrows) inflicted on the grape berry surface by the ovipositor of the female fruit fly. **A.** Wound only (filter 18). **B.** *Botrytis cinerea* conidia (I) deposited in the vicinity of wounds (II) (filter 02).