CHAPTER 2

THE ECOLOGY OF *BOTRYTIS* ON PLANT SURFACES

Gustav Holz[1], Sonja Coertze[1] and Brian Williamson[2]

[1]Department of Plant Pathology, University of Stellenbosch, Private Bag X1, Matieland (Stellenbosch), South Africa; [2]Scottish Crop Research Insitute, Invergowrie, Dundee DD2 5DA, UK

Abstract. The initiation of disease by members of *Botrytis* species depends on a complex sequence of biological events involving host and environment sensing, chemical and physical interactions between the fungal propagules and the host surface and the microbial interactions on the surface of the host. The pathogen's inoculum is central to the understanding of this interaction. This chapter describes the inoculum ecology of *Botrytis* species on plant surfaces and relates this information to an understanding of disease initiation. *Botrytis* species deploy several propagules and survival structures. A knowledge of the precise behaviour of these propagules, especially the hydrophobic conidia, when dispersed and deposited on the host at high relative humidity in the presence or absence of water droplets is important for disease initiation and control. The responsiveness of propagules to the environment, and the diversity shown in attack strategies by these pathogens are discussed with examples of the infection pathways used. Special comment is made about suitable inoculation procedures to study grey mould in leaves and fruits.

1. Introduction

Botrytis species have a necrotrophic life style occurring as pathogens infecting a single specific host or closely related host, or as the broad spectrum pathogen *B. cinerea* infecting numerous host plants: after infection and death of host tissues all these fungi can survive and sporulate as saprophytes on the necrotic tissue, or produce long-term survival structures, such as sclerotia. These survival structures can be associated with living plants or with plant debris lying on or buried in soil. For species more specialized in their parasitism (*B. aclada*, *B. byssoidea*, *B. squamosa*, *B. gladiolorum*, *B. tulipae*, *B. elliptica*, *B. fabae*), the inoculum source will inevitably be within the crop, or debris from a previous crop in the vicinity. For *B. cinerea*, for which host range is extremely wide, the primary inoculum also is most likely generated within the crop (Johnson and Powelson, 1983), but the potential for incoming primary inoculum from a different crop or weed host is greater than for the host-specific pathogens, and will be affected by the phasing of crop growth and harvest within a district or region.

The fungus exists in the different habitats as mycelia, micro- and macro-conidia, chlamydospores, sclerotia, apothecia and ascospores and these are dispersed by

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diverse means (Jarvis, 1980b). Although *B. cinerea* releases its macroconidia mainly in dry air currents, it is surprising that the majority of published work describes infection arising from suspensions of conidia in water droplets. This chapter summarises the new information available about the behaviour of *B. cinerea* and other *Botrytis* spp. and their responsiveness to different micro-environments, especially the effects of relative humidity (RH). It is particularly difficult to measure and maintain the RH of a host when inoculations are made and the host is incubated for periods to determine the outcome of the interaction. Harrison et al. (1994) reviewed these technical difficulties and devised specialised equipment that provides the best regulation of RH known to the authors. Results of work performed with dry-conidial inoculations, as well as the most recent achievements in inoculation with water droplets, are discussed.

2. Survival

The disease cycles of *Botrytis* species and the growth habit and phenologies of their host plants are often inextricably linked. Dormant or metabolically inactive fungal structures play a central role in each of these disease cycles. Each part of the fungus thallus can serve as a survival structure.

2.1. Sclerotia

All species of *Botrytis* form sclerotia which may, depending on isolate and cultural conditions, differ in size and shape. Sclerotia are generally considered to be the most important structures involved in the survival of *Botrytis* species. Sclerotia can survive adverse environmental conditions, can produce apothecia after a sexual process and possess a considerable capacity for producing successive crops of conidia in many *Botrytis* species (Coley-Smith, 1980). Under laboratory conditions, *B. cinerea* sclerotia continue to sporulate for about 12 weeks after the production of the first crop of conidia (Nair and Nadtotchei, 1987). Suppression of sporulation when the conidia were left on sclerotia and resumption of sporulation when the conidia were removed from the surface could extend the period of conidial production. Under natural conditions, rainfall would be expected to dislodge conidia from germinating sclerotia and initiate conidial production by removing the suppression in sporulation.

The internal structure and histochemistry of sclerotia of *B. cinerea* and *B. fabae* are similar; the rind walls contain melanic pigments, the medullary hyphae are surrounded by a continuous matrix of β-glucans, and the intracellular nutrient reserves are protein, glycogen, polyphosphate and lipid (Backhouse and Willets, 1984). The genetic control of the switch from rapid vegetative growth to production of sclerotia is not known. Recent work with the closely related species *Sclerotinia minor* suggests that β-carotene may be important for protection against oxidative stress when sugars and other nutrients decline in presence of light (Zervoudakis et al., 2003).

Formation of sclerotia in the field is generally associated with plant tissue. However, it also occurs in insects. Louis et al. (1996) demonstrated the ability of the vinegar fly, *Drosophila melanogaster,* to serve as vector for *B. cinerea*. Long-term *D. melanogaster*/*B. cinerea* relationships were found during the life of the insect. Conidia germinated in the insect foregut, developed into mycelium, and differentiated into microsclerotia, which can be carried by the flies during their entire life. Since the fly overwinters as an adult, it was concluded that it could play a role in winter conservation of *B. cinerea* inoculum.

2.2. Chlamydospores

Chlamydospores have been found in *B. cinerea*, *B. anthophila* and *B. fabae* (Coley-Smith, 1980). The chlamydospores of *B. cinerea* are hyaline cells of extremely variable form and size (Urbasch, 1983, 1986). They are generally found in ageing cultures and commonly occur in the stromatic sectors of cultures of the fungus which are contaminated by other organisms, and in association with sclerotia. Chlamydospores are formed as terminal or intercalary cells by transformation of vegetative mycelium parts and are liberated by hyphal disintegration. They were observed on and in tissue of naturally and artificially infected tomato and *Fuchsia hybrida* leaves and their numbers increased in older lesions (Urbasch, 1983, 1986). Under moist conditions and without added nutrients, the chlamydospores germinated on the leaves by microconidia which remained dormant. When fresh nutrients were supplied to the chlamydospores, they germinated with hyphae penetrating the host, or they produced a new crop of macroconidia. Histological studies of the infection process by *B. elliptica* show the formation of corresponding structures after conidium germination on oriental lily leaves (Hsieh et al., 2001). On tomato fruit, unsuccessful penetration was often associated with germ tubes which, after attachment to the host, differentiate into several cells (chlamydospores) at the point of attachment (Rijkenberg et al., 1980). On fruit of nectarine, plum and pear, germlings produced from dry airborne *B. cinerea* conidia formed chlamydospores on short germ tubes when fruits were subjected to intermittent dry periods, or were kept for 48 h at 5°C (Holz, 1999). Chlamydospores can therefore serve as short term survival structures which may help the fungus to overcome short unfavourable periods encountered on plant surfaces (Urbasch, 1983, 1986).

2.3. Conidia

Conidia of *Botrytis* are generally regarded as short-lived propagules in the field and their survival will largely be determined by temperature extremes, moisture availability, microbial activity and sunlight exposure. In the soil, *Botrytis* species are not particularly effective competitors and their conidia are subjected to fungistasis (Coley-Smith, 1980). Conidia of *B. cinerea* were able to survive on fruit surfaces of kiwifruit, remaining viable and infectious throughout the growing season (Walter et al., 1999b). Salinas et al. (1989) reported that conidia stored dry were able to survive at room temperature for up to 14 months, when some conidia were capable of

germinating *in vitro* and on ray florets of gerbera flowers to cause lesions. However, on the surface of Anjou pears, the viability of *B. cinerea* conidia after 7 weeks had declined to 10% germination (Spotts, 1985). In Scotland, conidia of *B. fabae* placed out of doors on cobwebs gradually lose their infectivity; only 15% of conidia were infective after 10 days exposure to ambient weather during summer (Harrison, 1983). When *B. cinerea* conidia were exposed to direct sunlight at midday in an Israeli summer, survival was only minutes (Rotem and Aust, 1991). In a New Zealand vineyard, mean percentages of conidia germinating after exposure to 4 h of sunlight ranged between 81 and 91% and between 49 and 50% after 8 h of sunlight exposure. Upon re-exposure on the second day, just 10 min of exposure to sunlight caused germination to drop between 26 and 27% for all isolates tested (Seyb, 2003). The UV spectrum of sunlight appeared to be the most important environmental factor influencing mortality of conidia (Rotem and Aust, 1991; Seyb, 2003).

Microconidia, which occur in all *Botrytis* species, provide an alternative microscopic propagule for these fungi when subjected to adverse conditions. In general they are found in ageing cultures of the fungus or those which are contaminated by other organisms, and in association with sclerotia. Microconidia develop from germ tubes produced by macroconidia, more mature hyphae, inside empty hyphal cells, and from appressoria and sclerotia (Jarvis, 1980a; Lorenz and Eichhorn, 1983). Germlings of *B. cinerea* form microconidia and chlamydospores in a corresponding manner on plant surfaces. On tomato plants, the dedifferentiation of *B. cinerea* appressoria proceeded by production of microconidia directly on appressoria, or by terminally and laterally outgrowing hyphae and their subsequent formation of microconidia (Urbasch, 1985a). The appressoria lost their function and the infection process at the site of interaction was interrupted. A similar process was infrequently observed on fruit surfaces of nectarine and plum that were subjected to intermittent dry periods, or were kept at 5°C after inoculation with dry, airborne *B. cinerea* conidia (Holz, 1999). Although their sole function is believed to be one of spermatization, they may also help the fungus to survive adverse conditions. The unicellular structures are generally produced in chains, but Urbasch (1984a) noted that after prolonged adverse conditions, *B. cinerea* formed clusters of microconidia bearing phialides and then embedded aggregates of these conidia in mucilage, which is then enclosed within a protective covering (hülle). Due to protection by this covering, the enclosed microconidial aggregates survived on dry agar plates without degeneration for up to 6 months and formed new mycelia when placed on fresh media. Urbasch (1984b) described a microcycle induced by nutritional deficiency that leads to production of microconidia and the oxygen concentration determined whether macro- or microconidia resulted, the latter being favoured by low O_2 concentrations. She also provides a good ultrastructural analysis of the differentiation of microconidia and comments on their rather thick outer wall (0.2 μm) suggestive of long-term survival (Urbasch, 1985b).

Macroconidia of *B. fabae* on agar films, buried in moist soil, germinated within a few days to produce short germ tubes which bore phialides and microconidia (Harrison and Hargreaves, 1977). After 29 days in moist soil, the macroconidia were dead and ruptured whereas the microconidia appeared to be quite healthy. Some

germination was observed amongst microconidia which had been left outside for 25-27 days during winter, suggesting that exposure to cold may be a factor in breaking the dormancy of microconidia. The ability of the microconidia to remain dormant under adverse conditions suggests that they may be important in the survival of *B. fabae* from one season to the next.

2.4. Mycelium

The survival of mycelium of *Botrytis* species under natural conditions has hardly been investigated and, unless particular care is taken, it is often difficult in practice to decide whether survival is by mycelium or whether microsclerotia or chlamydospores are involved. There is some evidence that the mycelium of certain *Botrytis* species, and especially those more specialized in their parasitism, can survive for considerable periods in bulbs, seeds and other vegetative plant parts (Coley-Smith, 1980). *B. cinerea* is considered to be a characteristic component of aerial surfaces of some species of plants whilst being absent or infrequently isolated from others. The frequency of isolation of the fungus tends to increase as the season progresses, reflecting an increasing ability to enter plant tissue as a weak parasite or as a saprophyte during senescence (Blakeman, 1980). Kobayashi (1984) observed that *B. cinerea* conidial masses developed throughout the year from mycelium in the fallen petals of 28 plant species belonging to 19 genera of 14 families.

3. Inoculum production and dispersal

It is generally assumed that for *B. cinerea*, inoculum is always present in the field and that production, liberation and dispersal of inoculum is an ongoing process (Jarvis, 1980b). This is clearly not always the case in all crops (Sosa-Alvarez et al., 1995; Seyb, 2003). There are various factors essential for high propagule numbers in the air: a viable, productive inoculum source, conditions favourable for propagule production, and for their dispersal at the source site. Correlations have been found between dispersal and conditions favourable for sporulation (usually surface wetness with moderate temperature) in many *Botrytis* species (Jarvis, 1980b). The frequency and duration of wetness events, and temperature, vary greatly during a growing season. It is anticipated that interrupted wetness periods, and temperature fluctuations, will affect the number of propagules produced (Rotem et al., 1978). A complicated relationship thus exists in the field between environmental conditions and propagule production and dispersal.

3.1. Dispersal and deposition

If it is to infect, the pathogen must conquer space (Zadoks and Schein, 1979), that is to move from the primary source and land on susceptible tissue. Each part of the fungus thallus can serve as a dispersal unit. These propagules are dispersed by wind, rain and insects.

3.1.1. Conidial dispersal by wind and rain

Conidia, which are dry and predominantly wind-dispersed, are generally considered to be the most important dispersal propagule of *Botrytis* species. Wind dispersal of conidia has three highly interdependent, yet distinct phases; liberation, transport and deposition (Aylor, 1990). Release of conidia in *Botrytis* species is caused by a twisting of the conidiophore which is brought about by changes in the relative humidity (Fitt et al., 1985) and their ejection by a mechanical shock. This mechanical shock has been considered to be caused by two forces, wind and splash (Fitt et al., 1985). Conidia of *Botrytis* species are typically found in highest concentration in the atmosphere during the daytime, often reaching a peak concentration near or shortly after mid-day (Jarvis, 1962a; Fitt et al., 1985) when wind speed and the level of turbulence near the ground are usually highest. A threshold wind speed has been demonstrated for their removal, and conidia of a given species are generally removed over a range of wind speeds. For these, the cumulative percentages of conidia removed increases rapidly with increasing speed. These curves tend to level off because a certain percentage of the conidia are difficult to remove from the source at any reasonable speed (Harrison and Lowe, 1987). Conidia of *Botrytis* species are released at different patterns from colonies, which can be ascribed to differences in spore size affecting the drying rate. Conidia of *B. cinerea* were consistently released at a faster rate from naturally infected broad bean leaflets than those of *B. fabae* (Harrison and Lowe, 1987). Conidia of *B. cinerea*, being smaller than those of *B. fabae*, may dry faster and consequently become more loosely attached than those of *B. fabae*.

Although average wind speeds in the lower part of closed crop canopies are typically a fraction of the speed above the canopy, gusts of wind with speeds several times faster than the local mean speed occur well inside plant canopies. These occur frequently enough to be important in the removal of conidia (Harrison and Lowe, 1987; Aylor 1990). After conidia are liberated from the source, some are transported within the canopy air space and some escape the canopy into the more freely moving air above. The number of conidia that escape the canopy depends largely on the balance between two competing forces, deposition and turbulent transport, and the vertical position of the inoculum source. In general, conidia produced on a source on the ground and lower in the canopy are exposed to slower wind speeds, less turbulence and rapid rates of sedimentation. They are thus transported over a short range (Fitt et al., 1985). In vineyards, 95% of *B. cinerea* conidia are deposited within 1 m from the ground source (Seyb, 2003). A similar pattern has been reported for *B. cinerea* dispersal in snap bean fields in which few conidia were detected beyond 2.5 m from the source (Johnson and Powelson, 1983).

The last phase in the dispersal of wind-borne conidia, deposition, is composed of two main processes, sedimentation and impaction (Aylor, 1978), both of which are influenced by wind strength. Sedimentation occurs in still air and is the process during which conidia descend under the influence of gravity with a certain terminal velocity. Air is rarely ever still; deposition is therefore a continuum of sedimentation and impaction (McCartney, 1994). Impaction is the process by which a conidium, because it is heavier than the surrounding air molecules, does not exactly follow the

fluctuations in the air and strikes an object even when air flows around it (McCartney, 1994). However, the effect of wind on deposition can be modified by attributes of the conidia. The rate of deposition, and therefore the steepness of deposition gradients, has been shown to be affected by whether the conidia are dispersed singly or in clusters. The greater the number of conidia clumped together, the faster the settling speed (Ferrandino and Aylor, 1984). Under simulated wind conditions, conidia of *Botrytis* species are released from different sources singly, and in clumps, consisting of c. three and five conidia per clump, for *B. cinerea* and *B. fabae*, respectively (Harrison and Lowe, 1987). Because similar proportions of conidia fell as clumps from undisturbed inverted cultures as from those blown by a strong wind and because the mean numbers of conidia per clump were similar, wind appears to have little effect on clumping (Harrison and Lowe, 1987).

Little is known about the deposition of airborne conidia under field conditions on different plant surfaces such as leaves, shoots and fruits. Fluorescence microscopy of leaves, berries and the inner bunch parts of grape (Coertze and Holz, 1999; Holz, 1999) and fruits of nectarine, plum and pear (Holz, 1999) dusted with dry *B. cinerea* conidia in settling chambers revealed that conidia were consistently deposited singly, and not in clumps or clusters. In these studies conidia were released from cultures or fruit with sporulating lesions in vacuum-operated settling chambers, or dispersed by air pressure into the top of the settling chamber.

Rain has been associated with large concentrations of airborne *Botrytis* conidia in both raspberry (Jarvis, 1962a) and grape (Vercesi and Bisiach, 1982), suggesting that it may be important in the release of conidia which are subsequently rain-dispersed. Investigations on the role of wind and rain in dispersal of *B. fabae* conidia in field bean plots, however, suggest that the majority of conidia are dispersed dry by wind, even during rain. Raindrops hitting leaves dislodge dry conidia from infected leaves, and experiments with simulated raindrops show puffs of dry conidia when the drops first hit dry leaves with sporulating lesions (Fitt et al., 1985). Laboratory experiments have shown that simulated raindrops can carry conidia within, or on the outside of splash droplets (Jarvis, 1962b; Hislop, 1969). Very few of the *B. cinerea* conidia dispersed by raindrops become wet enough to enter the droplets, and the majority are carried on the droplet surface as a dry coating (Jarvis, 1962b). Conidia of *B. cinerea* attach in two distinct stages to hydrophobic surfaces (Doss et al., 1993, 1995). The first stage, immediate adhesion, occurs upon hydration of freshly deposited conidia. Conidia of *B. cinerea* adhere to tomato fruit cuticle, grape berry epidermis, and leaves and petals of other hosts immediately upon hydration. Dry conidia of *B. cinerea* applied to wet fruit surfaces adhered to the same degree as conidia from liquid suspensions to the surface of plum and grape. The conidia adhere more strongly when applied in water suspension or to the wet surface of grape berries than when dry conidia are applied to a dry surface (Spotts and Holz, 1996). Raindrops may therefore deposit conidia carried on their surfaces as single cells on to plant surfaces during run-off. Data on washings made from grape berries in Californian (Duncan et al., 1995) and South African vineyards (G. Holz, University of Stellenbosch, South Africa, unpubl.) indicated that the number of *B. cinerea* conidia on berry surfaces was very low throughout the season, and *B. cinerea* occurred as single colony-forming units. These findings imply that infection

by solitary conidia, and not by conidial clusters, should play a prominent role in the epidemiology of *Botrytis* diseases.

3.1.2. Conidial dispersal by insects

Conidia of *Botrytis* species are also insect-dispersed. The conidia of *B. cinerea* are trapped in the ornamentations of segments, cuticle, body hairs and sculptured areas of the vinegar fly (*D. melanogaster*) (Louis et al., 1996), the grape berry moth (*Lobesia botrana*) (Fermaud and Le Menn, 1989), the New Zealand flower thrips (*Thrips obscuratus*) (Fermaud and Gaunt, 1995) and the Mediterranean fruit fly (*Ceratitis capitata*) (Engelbrecht, 2002). Ingested conidia also remain viable inside faeces of these insects. In the case of the Mediterranean fruit fly, digital photography and visual observations (Engelbrecht, 2002) of grape berries showed that the flies initially preferred to feed on the macerated tissue of the lesions that served as inoculum. However, 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. Fluorescence microscopy revealed (Engelbrecht, 2002) that flies deposited conidia singularly, in feeding packages and in faeces on the surface of unblemished grape berries. Conidia in feeding packages were ensheathed by saliva and occurred in clusters of 10 to 50 conidia. An average of 60% of the conidia in feeding packages germinated under dry conditions (c. 56% RH).

3.1.3. Dispersal of other propagules

In some diseases, particularly those caused by *B. cinerea*, conidia seem of less importance than saprophytically-based mycelial inocula in establishing infections. It may well be that ascospores are more important than generally assumed; apothecia are easily overlooked in the field. Due to the ability of chlamydospores to germinate, they also represent dispersal units which can function as structures of infection. Urbasch (1984a) noted that in moist conditions the protective covering around microconidia aggregates became sticky and speculated that this may aid microconidia to adhere to surfaces of plants and insect vectors, which is indicative of their potential role in the survival and dispersal of the fungus.

B. cinerea can infect pollen grains and petals of strawberry (Bristow et al., 1986) and such floral organs can then be dispersed by wind, attach to other tissues for mycelial spread and infection and serve as a site for production of another generation of conidia. Colonised senescent blossoms of *Phaseolus vulgaris* lying on the moist soil surface beneath a bean crop produce large quantities of secondary inoculum (Johnson and Powelson, 1983).

4. Growth on plant surfaces

Germination and germ tube growth of *Botrytis* conidia on plant surfaces, host penetration, and duration of the incubation period are important stages in the process

of infection that can be used to investigate various aspects of host resistance, fungicide action and biological control. Current knowledge on the behaviour of conidia in most of these studies is based primarily on interpreting germling growth during artificial inoculation. For artificial inoculation, plant parts are sprayed with, dipped in, or injected with conidial suspensions, or suspension droplets are placed on to plant parts. Infection studies with conidial suspensions of *Botrytis* on different hosts have shown generally that the more conidia in the infection drop the more likely is aggressive infection. Therefore, to achieve symptom expression during inoculation, conidial suspensions usually contain a high number of conidia, $1x10^3$ to $1x10^5$ per millilitre of carrier, which is mostly sterile distilled water. In some cases, conidial suspensions are supplemented with nutrients to increase the possibility of penetration of tissue otherwise resistant to infection.

Microscopic observation of the sequence of events accompanying germination in conidia-bearing droplets on susceptible hosts revealed rapid germination with germ tubes protruding within 1-3 h after inoculation. Various penetration stuctures, ranging from simple to compound appressoria (see Emmet and Parbery (1975) for details) are formed prior to penetration of the cuticle. These structures form within 6 h after germination when germ tubes reach lengths of 10-15 µm. In *B. cinerea,* germtubes commonly form protoappressoria (slightly swollen, hyaline germ tube apices adhering to the host and giving rise to an infection peg) and simple appressoria after 6 h (Clark and Lorbeer, 1976; Fourie and Holz, 1994, 1995). When exogenous nutrients are available, multicellular, lobate appressoria are formed after 12 h (Garcia-Arenal and Sagasta, 1980; Van der Heuvel and Waterreus, 1983). Continued growth in the presence of exogenous nutrients often leads to the formation of infection cushions (Backhouse and Willetts, 1987). In inocula with high conidial concentrations, a high proportion of germ tubes produce protoappressoria, whereas with lower conidial concentrations germ tubes produced predominantly multicellular, lobate appressoria and infection cushions (Van der Heuvel and Waterreus, 1983). Addition of exogenous nutrients to inoculum is a prerequisite for the formation of multicellular, lobate appressoria and for infection cushions on cucumber leaves (Akutsu et al., 1981), strawberry leaves and cucumber cotyledons (Shirane and Watanabe, 1985). However, these structures are all formed by *B. cinerea* conidia without the addition of exogenous nutrients on floral tubes, fragile petals and fruits of nectarine and plum (Fourie and Holz, 1994, 1995) and leaves and berries of grape (Holz, 1999; Coertze et al., 2001).

Infection studies suggest that conidial density and nutrient supplements may not only influence the pre-penetration activities in conidia-bearing droplets on plant surfaces, but also subsequent symptom expression. In water, *B. squamosa* induced symptoms on onion leaves. Addition of exogenous nutrients to inoculum increased the frequency of lesion formation (Clark and Lorbeer, 1976). Leaves of onion (Clark and Lorbeer, 1976), cucumber (Akutsu et al., 1981), strawberry (Shirane and Watanabe, 1985), broad bean (Harper et al., 1981), cucumber cotyledons (Shirane and Watanabe, 1985) and fruits of plum and nectarine (Fourie and Holz, 1998) remained asymptomatic when *B. cinerea* was inoculated in water. Addition of exogenous nutrients to inoculum was requisite for symptom induction by *B. cinerea* on these hosts. The pathogen could not induce symptoms on cucumber leaves when

conidial density in glucose or sucrose suspensions were low, but enhancing the conidial density caused rapid spreading lesions (Akutsu et al., 1981). Inoculations of primary leaves of French bean with conidia of *B. cinerea* suspended in glucose supplemented with KH_2PO_4 or Na-ATP as infection stimulants, yielded mostly spreading lesions (Van der Heuvel and Waterreus, 1983). Decreasing concentrations of conidia caused a delay of 1-4 days in the formation of spreading lesions. Although in most of these studies conidia suspended in nutrients allowed more extensive germ tube and hyphal growth and the development of a range of appressoria, only a small proportion of germlings of such inocula gave rise to penetrations (Williamson et al., 1995). The number of visible penetrations produced by inocula containing high conidial concentrations amounted to only c. 5-10% of all conidial germlings. These percentages were higher (20-80%) with lower conidial concentrations (Van der Heuvel and Waterreus, 1983). This agrees with Hill et al. (1981) studying unsupplemented conidial suspensions where from a total of 3500 conidia per 15.9 mm^2 cuticle surface, only 1-2 conidia were able to penetrate the cuticle layer of grape berries.

Use of glucose and phosphate supplements in small droplets (5 µl) as to ensure an adequate oxygen supply to conidia is now a standard method for host inoculation with *B. cinerea* in gene knock-out studies (e.g. Klimpel et al., 2002; Schouten et al., 2002) and for chemical studies on lesions (Muckenschnabel et al., 2002). Another significant factor affecting the success of inoculations made with conidial suspensions is the spectrum of light to which the host and pathogen is exposed. Islam et al. (1998) showed that near-UV and blue light (300-520 nm) induced negative phototropism in *B. cinerea* inoculated on to leaves of *V. faba*, and that red light (600-700 nm) induced positive phototropism and reduced the number of successful infections substantially.

Aggressive infection due to the addition of exogenous nutrients to inoculum may be ascribed to factors other than an increase in surface colonisation and successful penetration. Stimulation of infection by *B. cinerea* after addition of certain sugars to artificial inoculation is probably due to the active forms of oxygen formed (see Chapters 7 and 8), rather than to a nutritional effect (Edlich et al., 1989). Sugars act as substrates for the production of hydrogen peroxide and other forms of superoxide and hydroxyl radicals, which are highly toxic and may be capable of destroying relatively inert materials, such as cutin (see Chapter 5 for details). The addition of sugars also enables *B. cinerea* to overcome the inhibitory action of wyerone acid, an important phytoalexin produced by *Vicia faba* (Mansfield and Deverall, 1974). The mode of action of KH_2PO_4 or ATP in aggressive infection is unknown. Although phosphates might act by predisposing leaves to infection by *B. cinerea* (Van den Heuvel, 1981), they might also influence fungal metabolism, e.g. activity of cell wall-degrading enzymes, more directly.

The sequence of events accompanying germination of natural inoculum on plant surfaces, and how conidial density and substances occurring on these surfaces relate to infection and subsequent symptom expression has rarely been studied. The data from artificial inoculation studies mostly relate to detached plant parts kept in moist chambers, a situation that could lead to greater susceptibilty to the pathogen than

that of parts attached to the host plant. The single-drop inoculation of plant parts with a high number of conidia in the laboratory also differs from inoculation in the field, where single, airborne conidia may be deposited intermittently at several sites on a fruit surface. In the event of rain, the frequent run-off of inoculum-containing raindrops would promote faster drying of host surfaces and a lower incidence of infection than might be expected from laboratory-inoculated material. In the latter instance, drops deposited on host surfaces remain undisturbed for longer periods, which could create highly localised zones of disease pressure and the collapse of host resistance (Fourie and Holz, 1995; Coertze and Holz, 1999; Coertze et al., 2001).

Dispersal by airborne conidia is an important mechanism by which a disease epidemic is perpetuated; its investigation requires a dry inoculation technique to simulate the natural dispersion pathway. Uncontrolled clouds of dry *B. cinerea* inoculum have been discharged over target hosts (Rijkenberg et al., 1980; Walter et al., 1999a). Alternatively, amounts of dry *B. cinerea* conidia have been directly brushed on to the host (Williamson et al., 1987). Settling chambers have been constructed to provide more controlled delivery of dry conidia (Salinas et al., 1989; Reifschneider and Boiteux, 1998). With this method, dry conidia are dusted in a settling chamber on to plant surfaces. The conidia can be subjected to conditions commonly encountered by the pathogen on plant surfaces: dry conidia on a dry surface under dry conditions, dry conidia on a dry surface under high relative humidity, and dry conidia exposed to a film of water on the host surface. Working with gerbera flowers, Salinas et al. (1989) observed that germ tubes of dry-inoculated conidia were mostly short; less than 1% of the germ tubes were longer than 20 µm. Dry-inoculated conidia of *B. cinerea* germinated in a similar fashion on fruits of tomato (Rijkenberg et al., 1980), plum and nectarine (Fourie and Holz, 1994), grape (Coertze et al., 2001), grape leaves (Holz, 1999) and rose petals (Pie and de Leeuw, 1991) held at high humidity. In fact, some germlings formed a protoappressorium underneath the conidium (Holz, 1999; Coertze et al., 2001). Although dry conidia were used in these studies, the plant material was held at high humidity in conditions where surface moisture may have formed. Williamson et al. (1995) describes the behaviour of dry and wet conidia of *B. cinerea* on the surface of rose petals held at precisely controlled humidities. Although conidia in all cases germinated with one or more germ tubes, the subsequent growth and behaviour of developing germ tubes varied considerably according to the mode of inoculation. Dry conidia germinated in the absence of surface water under humidities ranging from 94 to 100% RH, but germ tubes mostly remained shorter than the conidia. No extracellular material was visible on germ tubes arising from these conidia. On spray inoculated petals, conidia in water droplets germinated to produce long germ tubes. The morphogenesis of *B. cinerea* and *B. fabae* germ tubes was similarly affected whether conidia were inoculated dry or in the presence of aqueous glucose on to *Vicia faba* leaves (Cole et al., 1996). In the latter study, conidia and germ tubes grown in the presence of glucose were often encased by a sheath of fibrillar-like matrix material. Transmission electron microscopy revealed that a distinct amorphous pad of matrix material surrounded the short germ tubes on the bean leaf surface. The matrix material probably acts as an adhesive pad and thus serves to

secure the position of the germ tube at the site of penetration (see Chapter 5 for ultrastructural studies).

Studies with dry and wet *Botrytis* conidia provide evidence that the mode of inoculation may not only influence conidial growth on plant surfaces, but also subsequent symptom expression. On gerbera flowers, only inoculation with dry *B. cinerea* conidia led to the development of the typical necrotic lesions, as found in practice (Salinas et al., 1989). Inoculation with conidial suspensions led to the appearance of different types of symptoms: smaller and larger necrotic lesions, partial rotting of ray floret or of the whole flower, or even no symptoms at all. Working with conidial suspensions of *B. cinerea* in distilled water on mature berries, Nair and Allen (1993) showed that a 14-h wetness period is needed to give 63% symptomatic berries at 23°C. Berries at different phenological stages inoculated with single airborne conidia remained asymptomatic after extended periods (3-96 h) of moist, or wet incubation (Coertze and Holz, 1999; Coertze et al., 2001). This finding suggests that when high humidity (c. 93% RH) prevails in nature, airborne conidia will have an equal potential to infect dry and wet berry surfaces. This finding can have a major impact on the validation of disease prediction models.

5. Infection pathways on diverse plant organs

Botrytis pathogens are well known for their ability to form either spreading lesions in host tissues, or latent infections in young fruit and seeds. The route used by the pathogen to enter the host usually plays an important role in the establishment of the two types of infection.

5.1. Penetration through specialised host structures

Different routes have been described for the penetration and establishment of quiescent or latent infections by *B. cinerea* in flowers and developing fruit. In blackcurrant, the pathogen can grow through the style to the carpels (McNicol and Williamson, 1989). In pear (De Kock and Holz, 1992), as in strawberry (Bristow et al., 1986), styles might not be an important source of latent infection. On the other hand, infected stamens are important penetration sites in pear. Unlike the styles, hyphae in pear filaments grew without restriction and progressed, via vascular tissue, through sepals into tissues of the upper end of the flower receptacle, or of the mesocarp adjoining the sepals. *B. cinerea* has been associated with transmitting tissue of styles specialised to guide and nourish pollen tubes as they grow rapidly to the ovules in raspberry (McNicol et al., 1985), strawberry (Bristow et al., 1986) and blackcurrant (McNicol and Williamson, 1989).

Besides the specialised stigmatic fluids secreted by hosts for pollen germination, *B. cinerea* seems to have a remarkable ability to utilise other host fluids secreted for defence against insects. For example in chickpea (*Cicer arietinum*), dry-inoculated conidia of *B. cinerea* germinated in the malic acid-rich exudate released by stalked multicellular glands studied by low temperature scanning electron microscopy, and penetrated the gland cells to grow basipetally into the leaf lamina, as seen by

fluorescence microscopy; it is not clear if this is a major infection pathway but further studies are required (G. Senthil, G.H. Duncan and B. Williamson, Scottish Crop Research Insitute, Dundee UK, pers. comm.)

Inoculation of immature grape berries with *B. cinerea* showed that the pathogen can enter styles and then become latent in the necrotic stylar tissue (McClellan and Hewitt, 1973). However, studies conducted on the occurrence of natural *B. cinerea* inoculum at various positions in grape bunches showed that styles might not be an important source of latent infection on grape. By use of a differential set of paraquat and freezing treatments on untreated and surface-sterilized berries, it was found that at all phenological stages the stylar end was virtually free of *B. cinerea* (Holz et al., 2003). The isolation studies showed that the pathogen seldom occurred on the surface or in the skin tissue near the proximal end, 'cheek' (equator) or stylar end of the berry. These findings indicate that *Botrytis* bunch rot was unlikely to be caused by colonisation of the pistil, and subsequent latency in the stylar end, as was observed elsewhere. Instead, berry rot consistently developed from the berry-pedicel attachment zone where micro-fissures in the epidermis may lead to exudation of nutrients.

5.2. Penetration through undamaged host tissue and natural openings

Direct penetration of the undamaged cuticle and natural openings by germlings in conidial suspensions has been observed in many *Botrytis*–host combinations (see Chapter 5). *B. elliptica* conidia germinated on both adaxial and abaxial surfaces of Oriental lily, but germ tubes failed to invade epidermal cells on the adaxial surface. On abaxial surfaces, germ tubes penetrated through stomatal openings, through the epidermis near guard cells, or directly through epidermal cells (Hsieh et al., 2001). *B. cinerea* penetrated fruits of plum and nectarine directly in the centre of the epidermal cells at the indentation above the anticlinal wall, at the indentation in the fruit surface adjacent to guard cells, through the guard cells, and through stomatal openings (Fourie and Holz, 1995). Sometimes more than one penetration occurred from the same or different conidia. Nelson (1951) found that *B. cinerea* penetrated grape berries directly through the cuticle. Others (Pucheu-Planté and Mercier, 1983) found the primary sites for penetration to be stomata and micro-fissures in the grape berry skin. The fungus entered French bean leaves directly and also through trichomes (Van der Heuvel and Waterreus, 1983). For *B. squamosa* on onion leaves, penetration occurred through stomata or the cuticle (Clark and Lorbeer, 1976). Histological studies with *B. cinerea* on fruit of plum, nectarine (Fourie and Holz, 1995), pear and grape (Holz, 1999) revealed that conidia suspended in droplets were inclined to settle in the centre of the droplet which caused an agglomeration of conidia. This action forced conidia to settle around or on stomata, and to enter these sites. They germinated and hyphal mats formed on the host surface in most droplets. It was also noted that micro-fissures, which acted as avenues for penetration by hyphal mats, developed with time in the cuticle under the droplet. Simulation of natural infection by dusting surfaces of these hosts with dry conidia in settling chambers indicated that conidia seldom landed on stomata or lenticels. In such an

event, the germ tubes formed by conidia on moist surfaces were too short to enter these structures. On wet surfaces, germ tubes or hyphae usually grew around the raised stoma or lenticel. Furthermore, attempted penetration was always direct, irrespective of germ tube length, number, or branching (Holz, 1999; Coertze et al., 2002).

5.3. Penetration through wounds

Wound infection occurs when conidia enter a wound in the host tissue. In nearly all experiments with *Botrytis* species, especially *B. cinerea*, inoculations of fresh wounds with varying numbers of conidia in water suspensions result in establishment of infection. Little is known about the relationship between the inoculum dosage in air and incidence of wound infection, and how the relationship is influenced by environmental, wound and host factors. To better understand this relationship, information is needed on the period over which conidia have accumulated, the time they are able to survive and remain infectious, time of wounding in relation to conidium arrival at the infection court and host surface wetness. Different patterns of conidium and germling dieback were observed by microscopic observation amongst individuals on fruit and leaf surfaces (Holz, 1999; Coertze et al., 2001). On moist fruit, some conidia or germlings died, or only the conidium or short germ tube died on some germlings. A similar pattern of germling dieback was observed on wet fruit. Sections of long germ tubes, or branched germ tubes of some germlings, died, whereas on some germlings the conidium remained viable and the extended germ tube succumbed. Complete dieback was most pronounced in germlings without appressoria. Dieback of conidia and germlings occurred at a significantly higher rate on wet than on moist surfaces, and was more pronounced on immature than on mature fruits.

B. cinerea conidia or germlings adhering to the cuticle are not easily dislodged from fruit surfaces (Spotts and Holz, 1996). Therefore, to infect a wound in the host tissue, newly arrived conidia should alight in or near the wound and grow into the wound under the prevailing conditions. On the other hand, in the event of wounding, propagules of *B. cinerea* may occur in various growth stages at the wound site. Firstly, there may be conidia in a dormant state adhering to the skin. Secondly, there may be germlings that had penetrated the skin, but were localised by host defence. In the case of dormant conidia adhering on a dry surface, wounding should be near a conidium thereby breaking the cuticle and supplying the conidium with necessary moisture and nutrients to germinate and to infect. In the case of a germling that had penetrated the skin, but was localised by host defence, wounding should be near the germling, an action that should overcome the host resistance and supply the established pathogen with the necessary nutrients to escape the host defence barrier and cause the tissue to rot.

Coertze and Holz (2002) described infection of wounds on grape berries exposed to freshly deposited airborne conidia, and of wounds on berries carrying previously deposited conidia and germlings (latent infections). Fresh (immature and mature), and cold-stored grapes (mature), which are respectively highly resistant and highly

susceptible to infection and symptom expression by single airborne conidia of *B. cinerea* (Coertze and Holz, 1999; Coertze et al., 2001), were included in their study. In the case of berries inoculated at bunch closure and harvest stage, wounds were not infected by conidia deposited on berries 4 days prior to wounding. This finding indicated that, following adhesion and the first stages of growth, the pathogen did not survive for extended periods on surfaces of immature and mature grape berries. Freshly deposited dry conidia were needed to infect the wounds. The freshly deposited conidia furthermore needed free water, and not high humidity or wound exudates, to infect the fresh wounds. Proportions of wounds infected were extremely low. Fluorescence microscopy explained the inability of the conidia to infect the wounds by the phenomenon that conidia seldom landed at the wound periphery, or in the wound cavity. Nearly half the number of wounds on berries at bunch closure did not produce an exudate. When exudates were formed, it was produced on to a narrow margin of the surrounding skin. Germlings in the vicinity of wounds seldom had the capacity to reach the wound periphery and to enter the wound cavity.

5.4. The role of insects in wound infection

A synchronisation of a combination of events necessary for successful wound infection (Coertze and Holz, 2002), fresh wounds, freshly dispersed conidia and free water on the host surface, may not commonly occur in the field. Insects may play a very prominent role in this context and can be considered primary role players in disease outbreaks in the field. They can act both as suppliers of inoculum to wounds, and as initiators of the disease cycle under conditions generally unfavourable for disease development (Engelbrecht, 2002). Inocula packages consisting of clusters of conidia or/and mycelia, which might also be deposited into wounds, should be more aggressive than single conidia that land near the wound and that should grow into the wound under the prevailing conditions (Coertze and Holz, 2002; Engelbrecht, 2002). In both kiwifruit (Fermaud and Gaunt, 1995) and grape (Marroni et al., 2003), latent *B. cinerea* infection has been ascribed to the activities of the New Zealand flower thrips (*T. obscuratus*). The studies (Marroni et al., 2003) with thrips infested with a wild *B. cinerea* strain, and a marker strain deficient in nitrate reductase, showed that asymptomatic latent infections at flowering were always sited at the receptacle of the berry pedicel, the primary position for *B. cinerea* infection in grape bunches (Holz et al., 2003). Engelbrecht (2002) investigated the transport of *B. cinerea* by the Mediterranean fruit fly (*C. capitata*) on grape, and found a peculiar interaction between the pathogen, the fly and symptom expression at the berry-pedicel attachment zone. She monitored the activities on grape berries of flies using digital photography, and observed that visitations to the berry-pedicel attachment zone increased substantially from véraison to harvest, indicating the possibility of nutrient leakages at this site. The systematic explorative behaviour of the vinegar fly (*D. melanogaster*) on fruits and grapes at various maturity stages makes the fly a self-guided agent responsible for *B. cinerea* dispersal in vineyards and orchards (Louis et al., 1996). Vinegar flies were observed to emerge from

unblemished, surface disinfested grapes, nectarine and plum fruit incubated in moist chambers after 1 h freezing at –12°C (Holz, 1999).

6. Conclusions

It is important to recognize the distinction between the growth of *Botrytis* species and penetration of host tissue in artificial infection studies under ideal laboratory conditions and natural infection in the field. In the field, each part of the host plant is a potential target for deposition, growth and penetration of *Botrytis* inoculum. The inoculum consists of macro- and microconidia, ascospores, macro- and microsclerotia, chlamydospores and mycelia. The deposition site on the host will be determined by factors such as the position of the primary inoculum source, wind, rain, insect activity and feeding preferences. Growth on the host surface, and the pathway used to enter host tissue, will depend on factors such as inoculum type, the availability of free water and nutrients, cuticle characteristics, host natural exudates at floral organs or other glands, the abundance of natural openings and the size and age of wounds. Current knowledge on the options taken by different *Botrytis* species to enter a host is based primarily on interpreting germling growth during artificial inoculation of specific plant parts or organs with large numbers of macroconidia. There is little information about the behaviour of microconidia, ascospores, macro- and microsclerotia, chlamydospores and mycelia. It is therefore important to gain further knowledge on the ecology and behaviour of natural inoculum, and to simulate its behaviour in infection studies, as well as on host resistance, disease prediction models, timing of fungicide application and biological control.

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