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Microscopical studies of the infection of gerbera flowers by *Botrytis cinerea*

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Abstract

The formation of lesions on ray florets of gerbera flowers caused by single conidia of *Botrytis cinerea* was studied in two cultivars infected by two isolates of the pathogen. No differences in reaction after inoculation with conidia of either isolate were seen on either cultivar. The conidia produced usually one germ tube not longer than 10 μm, but conidia with five germ tubes were also seen. Direct penetration of germ tubes through the upper cuticle of ray florets was observed. No appressoria or other specialised structures were observed before penetration, and degradation of the cuticle did not occur. Germination of conidia and subsequent flower infection was dependent on the availability of free water, but not on the addition of external nutrients.

Between 18 to 25 °C, fungal development usually stopped after cuticle penetration, two to four cells around the site of penetration becoming necrotic. This number did not increase when inoculated flowers were subsequently placed at 4 °C, conditions conducive for the formation of spreading lesions. When flowers were incubated constantly at 4 °C, lesions became visible 3 days after inoculation as a group of 10 to 14 cells. Initially from a vesicle-like structure, mycelium spread subcuticularly or in the lumen of epidermal cells resulting in the death of 40 to 50 cells at 18 days after inoculation. Ungerminated conidia and conidial germlings which has not yet penetrated the cuticle did not cause any visible symptoms in underlying epidermal cells.

Introduction

Infection of flowers by *Botrytis cinerea* has become an important limiting factor for the production of gerbera flowers in the Netherlands. Although conidia, mycelia and sclerotia are important for dispersal of the fungus in the field [Jarvis, 1980], the conidia are probably the only source of inoculum in glasshouses [Kerssies, 1993]. Conidia produced in large numbers on infected dead plant parts are easily spread through the air. After landing on the flowers no symptoms of infection are visible until water, for instance through condensation, is available for the fungus. Flowers subsequently become infected within a few hours [Salinas *et al.*, 1989].

Especially in autumn and spring, infections caused by the fungus lead to serious economic losses. Symptoms occur in greenhouses during the growth of the flowers, but can also develop later during storage or transport and shipment when temperature changes lead to conditions of high humidity and formation of condensation droplets on the flowers. Infections can usually be observed as small necrotic fleck lesions 'spots' on ray florets. In the Netherlands, the disease is therefore called 'smet' [Bakker, 1986; Verberkt, 1986]. Partial or total rotting of flowers can also occur, particularly when flowers are stored in cold rooms [Salinas *et al.*, 1989].

In the laboratory, small necrotic fleck lesions can be produced by inoculation of flowers with dry conidia of *B. cinerea* and storing the flowers under moist conditions. Infection develops between 4 and 25 °C, while at 30 °C symptoms do not develop at all. Under conditions of low humidity, conidia remain ungerminated on the ray florets and symptoms do not appear [Salinas *et al.*, 1989].

Histological data on the initial interaction and colonisation of ray florets of gerberas after inoculation with conidia of *B. cinerea* is largely lacking. Infections of *B. cinerea* on other hosts have been the object of several studies [Verhoeff, 1980]. The fungus usually invades plant tissues through wounds, or attacks dying or dead plant parts. Penetration of healthy intact plant tissue by conidial germ tubes of *B. cinerea* has been reported, but the mechanism of this direct penetration is still unknown. Rijkenberg *et al.* [1980] described degradation of the cuticle in tomato fruits around the penetrating germ tubes of *B. cinerea*. McKeen [1974] detected esterase activity at the tip of germ tubes during the penetration of the cuticle of *Vicia faba* leaves. These two reports suggest an enzymatic breakdown of the cuticle by the penetrating fungus.

During storage, beside gerberas, most of the cut flowers, fresh fruits, vegetables and bulbs are attacked by *Botrytis* spp. resulting in substantial losses. With the available fungicides, chemical control of *B. cinerea* is often difficult and incomplete, especially due to the development of fungal resistance to most of the chemicals in use [Gullino, 1992]. Due to increasing concerns about health and the environment, the development of new fungicides having different modes of action is difficult. Biological control may provide a promising alternative, but it is only in its infancy [Dubos, 1992]. Moreover, the independence of *B. cinerea* of external nutrients for infection of gerberas and roses, and the rapid penetration into the host makes the selection of effective antagonists difficult. Storage of harvested crops in controlled atmospheres can only solve part of the problem [Reyes, 1990].

A thorough knowledge of the histology and biochemistry of the interaction between *B. cinerea* and gerbera can provide useful information for breeding programmes as well as for biological and chemical disease control.

This paper describes the initial stages of the interaction of *B. cinerea* and gerbera flowers. In a time-course study, lesion development under different conditions, as well as microscopical studies of the infection process, by means of light and scanning electron microscopy are described.

Materials and methods

Inoculum and flowers. All experiments were performed with two isolates of *Botrytis cinerea* Pers.: Fr., isolate Bc-7 obtained from an infected field-grown tomato plant and isolate Bc-12 obtained from a gerbera flower in a glasshouse. Both isolates were kept at 4 °C in darkness. For production of conidia, the isolates were subcultured to PDA plates and incubated for 3 days at 21 °C in darkness. Pieces of agar bearing mycelium were then placed on tomato agar (see below) and incubated for 3 days in darkness, followed by 9 to 11 days under continuous light and 21 °C. Conidia were collected dry and used directly, or stored at −20 °C until used [Salinas *et al.*, 1989]. Tomato agar was prepared by grinding 300 g of tomato leaves in 1 l distilled water.The mixture was kept at 50 °C in a water bath during 2 h, thereafter 20 g agar was added before sterilisation. Flowers of two susceptible gerbera cultivars were used, cvs Rebecca and Joyce [obtained from P. Schreurs BV, Amsterdam and from Florist de Kwakel at Aalsmeer, the Netherlands, respectively]. Flowers were prepared for inoculation as described previously [Salinas *et al.*, 1989].

Inoculation. For inoculation, 0.1 mg of dry conidia of *B. cinerea* were used, unless stated otherwise and therefore no exogenous nutrients were added. With the application of 0.1 mg dry conidia, the inoculum was spread on the surface of ray florets such that lesions developed separately. An inoculation box was used for all experiments [Salinas *et al.*, 1989] and inoculated flowers were kept either at low relative humidity (70% rh) or at high relative humidity (100% rh) and at different temperatures, according to the experiment to be carried out.

Lesion formation. In these experiments, each lesion resulted from the penetration of an epidermal cell initially by a germ tube from a single conidium. To follow lesion formation under different conditions, experiments were carried out as shown in the following scheme (Fig. 1). The experiments always included both gerbera cultivars and both isolates of *B. cinerea*. For measuring the diameter of lesions, 30 lesions per treatment were randomly selected and the largest diameter was measured using a magnifier with a micrometer. Measurements were done at 4 and 20 °C under high humidity to prevent changes in the incubation conditions. In addition, ray florets from other flowers were used to determine the number of necrotic cells per lesion. In all cases data were obtained from lesions which were the result of the penetration of a germ tube originating from a single conidium, with no other conidia in the vicinity.

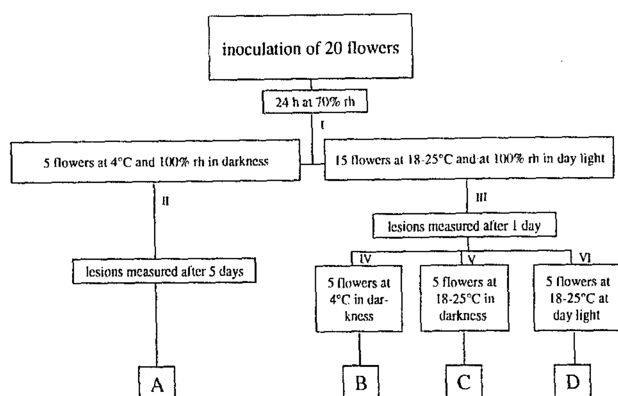


Fig. 1. Outline of the various experiments carried out to follow infection and lesion formation on gerbera flowers. Inoculation of flowers with 0.1 mg dry conidia of *B. cinerea* was performed using an inoculation box. The flowers were incubated under the conditions indicated. Per treatment [A, B, C and D], 2 lesions from 3 ray florets each per flower were then numbered and measured.

Light microscopy. For determining the number of necrotic cells per lesion, ray florets were fixed, cleared and stained with lactophenol aniline blue, according to the method described by Erb *et al.* [1973]. Ray florets were then mounted on microscope slides and the number of epidermal necrotic cells was counted at 400× magnification. To obtain transverse sections, pieces of ray florets, before and after appearance of lesions, were fixed in 2.5% glutaraldehyde in phosphate buffer saline (PBS). To improve fixation of the plant material, fixation was carried out under reduced air pressure. Fixation took place at 4 °C during approximately 6 h. Specimens were then washed three times in phosphate buffer saline (PBS) and dehydrated as described by Feder and O'Brien [1968]. Specimens were embedded in glycol methacrylate using a JB-4 embedding kit [Polysciences Inc. Warrington, PA, USA]. Sections 2 to 3 μm thick were made with a Leitz rotary microtome equipped with a glass knife. Sections were stained with 0.05% toluidine blue in 50 mM sodium phosphate buffer (pH 6.8) for 1 min and mounted in Euparal after drying at room temperature.

Scanning electron microscopy. Samples of inoculated ray florets, before and after appearance of symptoms, were fixed and dehydrated according to the method described by Samson *et al.* [1979] and specimens were examined with a Jeol 840A SEM at 7 to 10 kV accelerating voltage.

Results

Reactions of gerbera flowers inoculated with B. cinerea. Under the conditions of these experiments, no differences in reaction were seen between either of the cultivars after inoculation with either isolate of *B. cinerea.* Therefore, unless stated otherwise, only the results obtained with cv Rebecca and isolate Bc-7 are presented.

Based upon previous work [J. Salinas, unpubl.] and results presented earlier [Salinas *et al.*, 1989], the following was expected (see Fig. 1). In step I, conidia settled on the adaxial epidermal surface of ray florets, but lesion development did not occur. In step II, lesions were first measurable after 3 days. In step III, lesions could be measured after 24 h. Step IV was included to test whether flecks formed at 18 to 25 °C (step III) could develop into spreading lesions. Steps V and VI were included to study the effect of the presence or absence of light.

Inoculations with 0.1 mg of dry conidia resulted in an inoculum density per surface area of 0.5 to 1 $\times$ 10^2 conidia cm^{-2}.

Symptoms produced on ray florets after infection by *B. cinerea* were small necrotic fleck lesions 'spots' (Fig. 2) and spreading lesions. Complete rotting of flowers occurred both at high inoculum densities (Fig. 3A) and in the case of spreading lesions that were formed close to each other. Such symptoms were visible only in flowers incubated at 100% rh where an aqueous film was formed on the surface of the ray florets. Inoculated flowers kept constantly at 70% rh did not develop any symptoms (Fig. 3B). The diameter of lesions produced at 4 °C increased from 0.75 mm at five days, to 1.5 mm at 18 days of incubation. Lesions formed at 18 to 25 °C were smaller and usually did not increase in diameter under conditions conducive for formation of spreading lesions (Fig. 4, left).

Microscopical studies. The germination of conidia, structural changes on the surface of ray florets, fungal colonisation and tissue necrosis were recorded by SEM and light microscopy. The adaxial surfaces of ray florets are free of stomata, trichomes or other epidermal hair structures and glands. The top of every epidermal cell is covered with cutin arranged in small transverse ridges. SEM studies showed that under conditions of low rh even after 2 weeks of incubation conidia did not germinate on the surface of the ray florets (Fig. 5) and under these conditions no symptoms were seen (Fig. 3B). At 100% rh germination occurred and pen-

Figs. 2–3. Symptoms in ray florets of cv. Rebecca caused by *B. cinerea* at 18 to 25 °C. Flowers were inoculated in an inoculation box with 0.1 mg (*Fig. 2*) and 20 mg (*Fig. 3*) dry conidia. Figure 2. Small necrotic fleck lesions 'spots' (arrows) after 2 days incubation at 100% rh. Figure 3. Rotting symptoms after 12 days at 70% rh followed by a further 2 days at 100% rh (A), but not after 2 weeks at 70% rh (B).

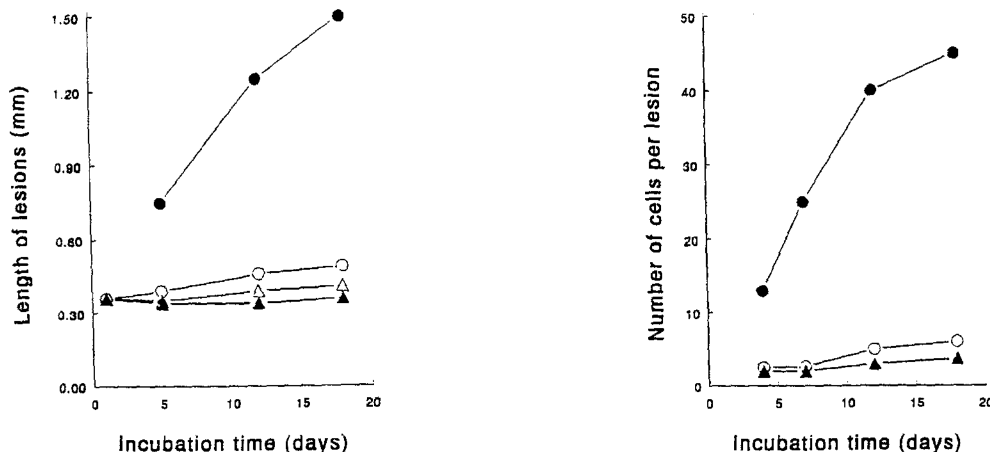
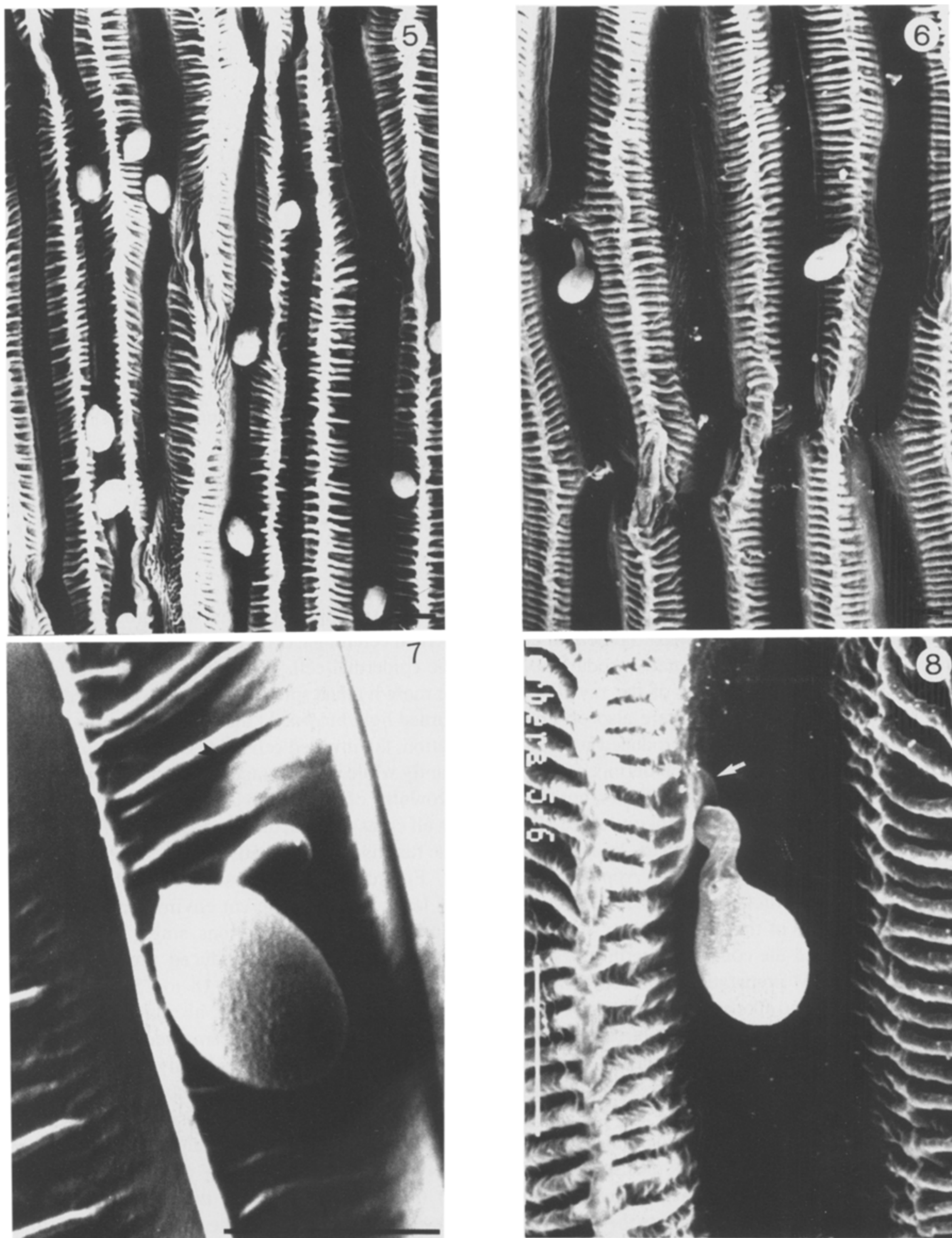


Fig. 4. Lesion development at 100% rh on ray florets of cv. Rebecca, inoculated with 0.1 mg dry conidia of isolate Bc-7. Lesions were caused by the penetration of a germ tube from a single conidium. Each point is the average of 30 lesions per treatment A, B, C and D (treatment are explained in Fig. 1). Left: length of lesions (mm). Measurements were made 5, 12 and 18 days in treatment A (●) or 1, 5, 12 and 18 days in treatments B (○), C (△) and D (▲). Right: number of necrotic epidermal cells per lesion. Necrotic cells were counted 3, 7, 12 and 18 days after starting treatment A (●), B (○) or D (▲).

etration of germ tubes either through the top of an epidermal cell, or close to an adjacent epidermal cell occurred (Fig. 6). The conidia generally produced one to two germ tubes on the surface of the ray florets, but conidia with five germ tubes were also observed (Figs. 6–8, 12 and 13). Germ tubes produced at the side of the conidia in contact with the epidermis could be seen by light microscopy as dark blue flecks, but those located under the conidia, were difficult to see (Fig. 13). Before penetration of the cuticle, both isolates produced germ tubes up to 10 μm long. Appressoria or other pre-penetration structures did not develop (Figs. 7 and 8). Degradation of the cuticle on the surface of the ray florets around the conidia or germ tubes was never observed. SEM preparations showed lifting of the cuticle and volcano-like structures around fungal penetration sites (Figs. 7 and 8); such structures were produced at both incubation temperatures.

Light microscopy and SEM revealed the first signs of germination of conidia within 4 h at 18 to 25 °C, while within 7 h, the first necrotic epidermal cells were observed (Fig. 12). Penetration was mostly restricted to the cuticle, after which hyphal growth ceased (Figs. 9 and 10). Occasionally penetration into the epidermal cell could also be seen but in these cases, maximally a single cell was invaded by the fungus. Studies of lesions formed at 4 °C showed that epidermal vesicles and thick fungal hyphae were produced by the fungus after passing through the cuticle (Figs. 9, 11, 14 and 15). These fungal structures were produced in the epidermal cell lumen (Fig. 11) or under the cuticle outside the epidermal cell. From these fungal structures, one or more hyphae spread into neighbouring cells, which turned light brown. At the beginning of fungal colonisation, the invaded cells did not chance colour significantly while in later stages it was not clear whether the browning of cells began before or after hyphal contact. In all cases studied, direct penetration of the cuticle by the fungus was observed.

Figure 4 (right) shows results of the development of lesions under different environmental conditions. Under conductive conditions, single germ tubes from single conidia already induced the formation of a lesion. Lesions formed at 18 to 25 °C consisted of approximately two to four necrotic epidermal cells; mesophyll cells rarely became necrotic. The number of necrotic epidermal cells hardly increased when the flowers were subsequently incubated at 4 °C. After 4 days incubation at 4 °C, the lesions formed consisted of already 10 to 14 epidermal cells, and the number increased in the experimental period to 40 or 50 cells. At the latter temperature, the epidermis as well as the mesophyll cells became light brown resulting in spreading lesions.



Figs. 5–8. Scanning electron micrographs of the adaxial surface of ray florets inoculated with *B. cinerea*. Flowers were inoculated with dry conidia and incubated at 70% rh (Fig. 5) or 100% rh (Figs. 6—8). The top of every epidermal cell is covered with cutin, arranged in small transverse ridges in a corrugated pattern. Volcano-like structure (arrow), cuticle elevation (arrowhead). Bar = 10 μm. *Fig. 5.* Ungerminated conidia on a ray floret of cv. Rebecca after 2 weeks at 18 to 25 °C. *Fig. 6.* Conidial germ tubes penetrating the top and the side of external faces of epidermal cells. Flowers of cv. Rebecca incubated for 2 days at 18 to 25 °C. *Fig. 7.* cv. Joyce infected by *B. cinerea* isolate Bc-12 after 3 days at 18 to 25 °C. *Fig. 8.* cv. Rebecca infected by *B. cinerea* isolate Bc-7 after 5 days at 4 °C.

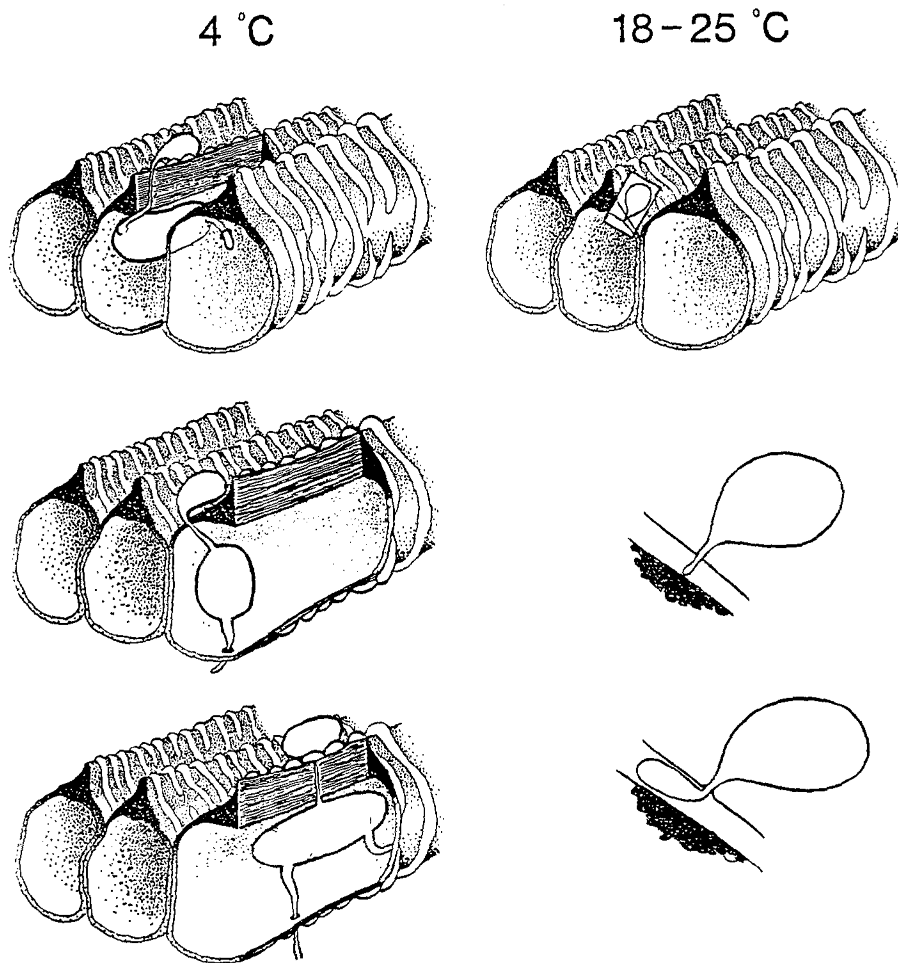
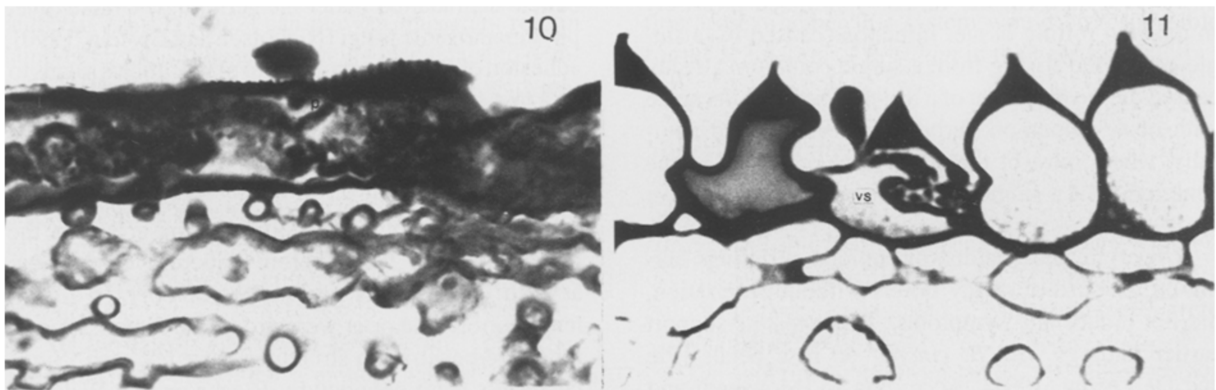
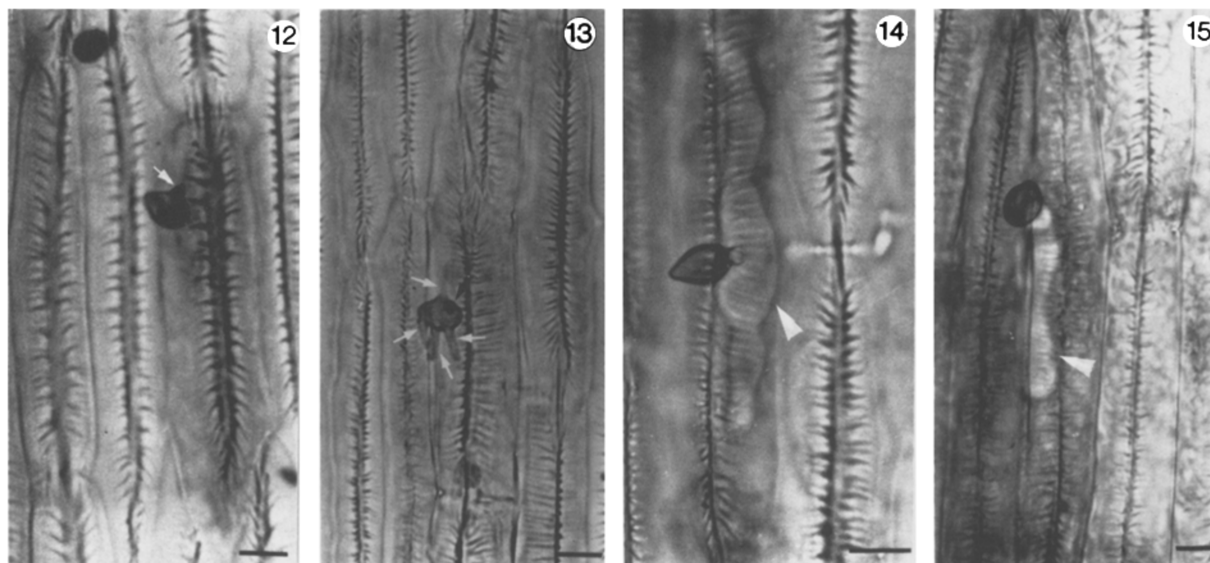


Fig. 9. Schematic representation of typical epidermal vesicles produced by *B. cinerea* after infection of gerbera ray florets. Flowers were incubated at 100% rh at 4 °C (left) or at 18–25 °C (right).



Figs. 10–11. Cross-sections through *B. cinerea*-infected gerbera ray florets. Penetration through the adaxial cuticle by conidial germ tubes. Penetration peg (p), vesicle formation in an epidermal cell (vs). Bar = 10 μm. *Fig. 10.* Penetration peg formed at 18–25 °C. *Fig. 11.* Vesicle formed at 4 °C.



Figs. 12–15. Light micrographs of cleared ray florets infected by *B. cinerea*. Flowers were inoculated with 0.1 mg dry conidia of *B. cinerea* and incubated at 100% rh. Conidial germ tubes (arrows), thick fungal hyphae and vesicle structures (arrowheads). Bar = 10 μm. *Fig. 12.* Necrotic epidermal cell produced after penetration by a germ tube. The inoculated flower was kept for 7 h at 18–25 °C and 100% rh. *Fig. 13.* Germinated conidium with five germ tubes (arrows) on the surface of a ray floret. Germ tube located under the conidium is indicated by a dark arrow. *Figs. 14–15.* Vesicle-like structures formed by the fungus after penetration of the cuticle. Inoculated flowers were incubated at 4 °C and 100% rh.

Discussion

Inoculation of gerbera flowers with conidia of *B. cinerea* lead to infection, but only when free water was available for the conidia. In ray florets, the most typical symptoms were small necrotic fleck lesions 'spots' while, depending on storage conditions and inoculum density, spreading lesions and total rotting of flowers would also occur. While sporulation was not observed on necrotic lesions, the fungus sporulated profusely in infected rotting tissue. Infections caused by a single germ tube arising from a single conidium already caused the development of a lesion. These results agree with observations on gerbera flowers infected naturally, where most of the spots were produced by the penetration of a single conidial germ tube [Salinas *et al.*, 1989].

Ungerminated conidia, or conidial germlings laying on the surface of ray florets without penetration, did not induce any symptoms. These results support earlier findings with *B. cinerea* on rose petals [Pie and De Leeuw, 1991]. According to [Movahedi and Heale, 1990; Verhoeff and Liem, 1978], conidia and conidial germlings release different products, e.g., enzymes involved in cell death and tissue maceration. Thus the fact that no symptoms were visible before cuticle penetration suggest a non-permeability of ray florets cuticles to fungal products, e.g., proteases and pectinases, as was reported by Rijkenberg *et al.* [1980] for tomato cuticles. Before penetration, the fungus was firmly attached to the ray floret, because conidia remained on the ray floret surface even after preparation for microscopical studies. Contrary to Garcia Arenal and Sagasta [1980], we did not observe cuticle degradation under the germlings. Unlike other phytopathogenic fungi [Nicholson and Epstein, 1990], adhesion of *B. cinerea* conidia to plant cuticles seems to be a less specific process [Doss *et al.*, 1993]. This may be advantageous for a polyphagous fungus such as *B. cinerea* which attacks different plant parts in numerous plant families.

The incubation temperature had no effect on the length of germ tubes before cuticular penetration; at both temperatures germ tubes of only 10 μm in length or often shorter were produced. Appressoria or other pre-penetration structures were not seen. Similar results were obtained by Blackman and Welsford [1916], McKeen [1974] and Rijkenberg *et al.* [1980] working with *B. cinerea* on bean leaves and tomato fruits. Formation of long germ tubes before penetra-

tion was only observed when pollen was spread on the ray florets [J. Salinas, unpubl.]. Addition of nutrients to the inoculum leads to the formation of long germ tubes and different penetration structures [Garcia Arenal and Sagasta, 1980; Van den Heuvel and Waterreus, 1983]. Therefore, the absence of long germ tubes and pre-penetration structures indicates that nutrients available for the fungus were scarce on the surface of the ray florets.

Penetration took place equally frequently on the top, as well as on the side of epidermal cells. An optimal location for penetration did not seem to exist, as described by Rijkenberg *et al.* [1980] for infection of tomato fruits. Although the fungus always penetrated the cuticle directly, evidence for cuticle degradation around the penetrating germ tube was not found. This is remarkable, as *B. cinerea* produces cutinase [Salinas *et al.*, 1986] and infection of ray florets by *B. cinerea* can be prevented using specific cutinase inhibitors [Salinas, 1992]. Probably, cutinase is secreted in small quantities only during penetration and therefore cuticle degradation can not be seen. McKeen [1974] demonstrated esterase activity only at the tip of germ tubes of conidia of *B. cinerea* and degradation of the cuticle was shown only around the penetrated germ tube.

The volcano-like structures and cuticle elevations observed in these studies are probably caused by swelling of epidermal cell walls and outwards pushing of the cuticle after enzymatic degradation of the epidermal cell wall. These structures were not observed before penetration of the cuticle. McKeen, [1974] described similar structures formed after the fungus reached the epidermal cell wall of *Vicia faba,* and he suggested that raising of the cuticle may have been caused by degradation products from the host wall. In fact, different pectic enzymes were identified during early infections of French bean and tomato leaves by *B. cinerea* [Leone, 1990]. A more active role for these enzymes can be postulated in the formation of blister-like structures described in infected rose petals by Pie and De Leeuw [1991].

The epidermal vesicles resemble dilated penetration tubes and multiform distended structures reported by Rijkenberg *et al.* [1980] and Pie and De Leeuw [1991] in, respectively, epidermal cell walls of infected tomato fruits and rose flowers. Why these structures were only located in epidermal cell walls is not clear. Perhaps the rapid collapse of epidermal cells made it difficult to visualise these distended hyphae in the cell luming of tomatoes and roses. We suggest that the vesicles serve to take up nutrients needed for further tissue colonisation. Moreover, the layered cell wall of the vesicles, as shown by Rijkenberg *et al.* [1980], may protect the invading fungus against constitutive or induced fungitoxic plant products and assist the establishment of infection.

The different reactions of flowers to fungal invasion at various temperatures and relative humidities are difficult to explain. At 18–25 °C, fungal spread was stopped rapidly by a hypersensitive reaction-like necrosis of epidermal cells of the ray floret. This reaction was a barrier to further colonisation but the fungus remained alive and could be isolated readily from the lesions [Salinas *et al.*, 1989]. The fungus was incapable of overcoming this barrier when conditions became conducive for formation of spreading lesions. The formation of physical barriers, the presence of preformed fungitoxic products and the accumulation of phytoalexins are possible mechanisms implicated in resistance against *B. cinerea* [Mansfield, 1980]. These mechanisms are the result of complex biochemical processes [Doke *et al.*, 1987; Nicholson and Hammerschmidt, 1992] but under storage conditions at low temperatures (2 to 4 °C) such processes occur slowly and/or are incomplete [Jarvis, 1980], thus allowing *B. cinerea* to cause spreading lesions on the ray florets. In stored carrots, the development of spreading lesions caused by *B. cinerea* was directly related to the slow accumulation of phytoalexins [Mercier *et al.*, 1993].

Since flowers bearing fleck lesions cannot be sold, effective control of *B. cinerea* on gerberas must be achieved before fungal penetration, by prevention of either conidial germination or cuticular penetration. Germination of conidia can be prevented by keeping the relative humidity low. Specific inactivation of the enzyme cutinase with monoclonal antibodies also prevented cuticle penetration and subsequent infection [Salinas, 1992]. These two findings can be very useful for flower producers in the selection of gerberas for the production of export flowers.

Acknowledgements

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