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# Histopathology of the initial stages of the interaction between rose flowers and Botrytis cinerea

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**Abstract**

The early stages of the interaction between flowers of the cut rose cv. Sonia and *Botrytis cinerea* was investigated by scanning electron microscopy and light microscopy. Infection of petals by conidial germ tubes evoked a susceptible reaction. In contrast to general findings nutrient addition to the inoculum was not a prerequisite for this phenomenon. At the lower side of germ tube tips the cuticle was penetrated by infection pegs. Already at this early stage of the infection process, the infection sites were macroscopically visible as scattered white spots. After penetration, pegs enlarged to form infection hyphae, which invaded the periclinal wall of outer epidermal cells. At those sites, the petals formed outgrowths of variable appearance at their abaxial side. These outgrowths consisted of remnants of collapsed epidermal cells and of infection hyphae. Subsequent intra- and intercellular growth of hyphae led to a collapse of epidermal and mesophyll cells. The symptoms described generally developed within 24 h. After subsequent incubation the lesions became necrotic. Eventually, the necrosis would spread leading to the death of whole petals.

*Additional keywords: Rosa hybrida*, infection process, susceptible reaction, exogenous nutrients.

## Introduction

*Botrytis cinerea* is a serious pathogen of cut rose flowers. The disease has several names, including grey mould, Botrytis blight and Botrytis rot. In the Netherlands the disease is called 'pokken'. Symptoms may not be visible at the time of harvest but develop rapidly under moist conditions found in storage and during shipment (Jarvis, 1980; Hammer and Marois, 1989). Incipient infections appear as small spots or blister-like patches, scattered over the surface of petals. They are also defined as 'pimples' which consist of infected and raised tissues from which restricted brown lesions develop (Redmond et al., 1987; Elad, 1988). Subsequently a second type of symptom becomes dominant, which is accompanied with the formation of spreading necrosis,

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and results in petal rot (Phillips et al., 1985; Elad, 1988). *B. cinerea* infections cause major losses in postharvest quality and consequently in economic value of roses and other cut flowers. For that reason the pathogen is considered a limiting factor in the storage and shipment of ornamentals (Cline and Bardsley, 1984; Barendse, 1986).

*B. cinerea* is often regarded as a 'weak' pathogen, invading plant tissues through wounds, or via senescent or dead parts. Prior to penetration of the cuticle of undamaged, healthy, non-floral tissues, various structures may be formed: conidial germ tubes, appressoria, and infection cushions (Van den Heuvel and Waterreus, 1983; Backhouse and Willetts, 1987). Often a slight appressorium-like swelling is formed at the tip of germ tubes. From the lower side of the prepenetration structures an infection peg is produced that penetrates the epidermal cell wall (McKeen, 1974; Clark and Lorbeer, 1976). After penetration by conidial germ tubes, the pathogen produces lesions which are usually confined to flecks at the inoculation site. Once inside host tissues, the pathogen grows necrotrophically (Verhoeff, 1980).

In the present study the initial stages of the interaction between rose flowers and *B. cinerea* were investigated by scanning electron microscopy (SEM) and light microscopy (LM). The objective was to deepen insight into the early processes in symptom formation on rose petals in relation to the development of the pathogen. Histological data on fungal growth at the time of appearence of incipient lesions may indicate the stage at which fungal growth should be arrested in order to avoid symptom formation.

## Materials and methods

*Flower and pathogen.* *Rosa hybrida* L. cut rose flowers cv. Sonia were obtained from a commercial grower. Isolate Bc-33 of *Botrytis cinerea* Pers.: Fr. was originally obtained from a naturally infected flower of this cultivar.

*Inoculation and incubation.* Petals on the outer side of flower buds used in the LM study were inoculated on their abaxial side either by dusting 0.4 mg of dry conidia in an inoculation box (Salinas et al., 1989) or by applying 1 $\mu$l droplets containing about 100 conidia in ultrapure (UP) water. Petals on the outer side of flower buds used for SEM were inoculated by spraying a suspension containing $7 \cdot 10^4$ conidia $ml^{-1}$ in UP water. In the last case, individual flowers were sprayed six times with a chromist atomizer from a distance of approximately 15 cm, resulting in a uniform coverage of the petals with small droplets. Flowers inoculated with droplets or sprays of conidial suspensions were air-dried for about 1 h. All inoculated flowers were placed separately in jars filled with tap water in incubation chambers, at room temperature for up to three days. Relative humidity (rh) was high; condensation was observed on the petals throughout the incubation period.

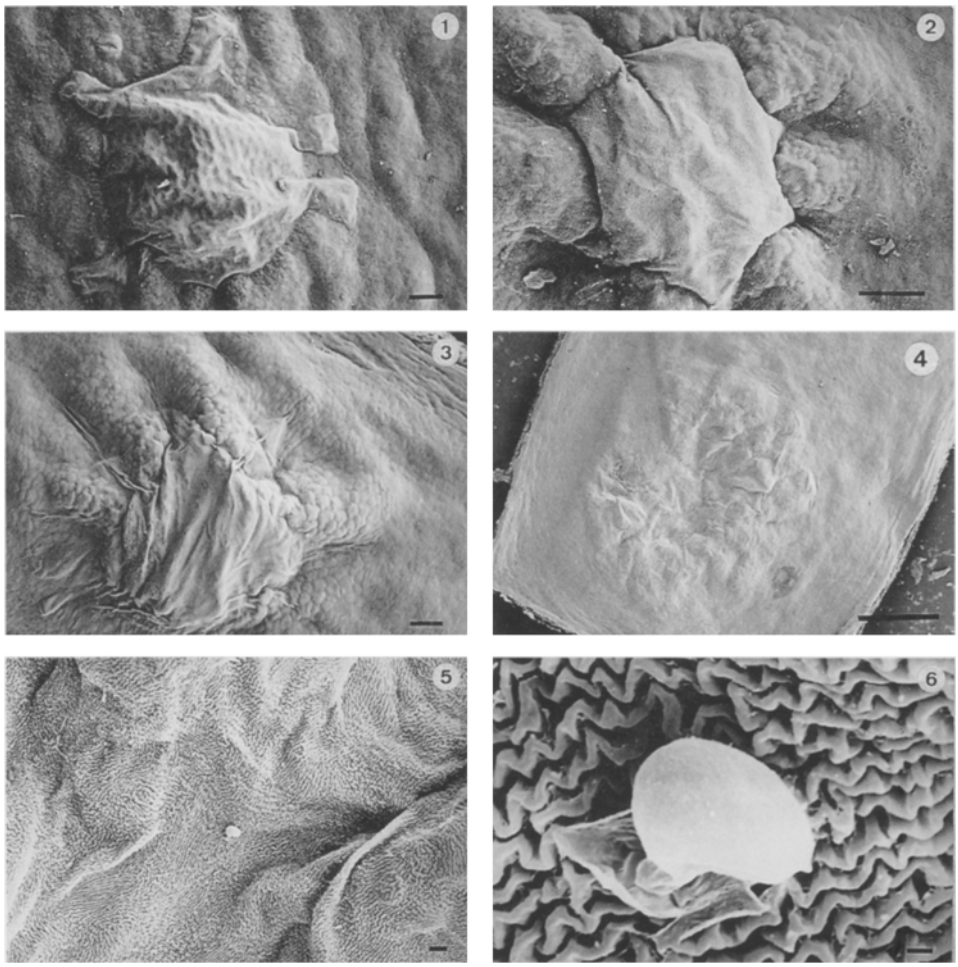
*Scanning electron microscopy (SEM).* Lesions on the petals, which were visible with the naked eye and which resulted from one to six conidia, were cut out and fixed overnight in 6% aqueous glutaraldehyde. Fixed tissues were dehydrated and cleared twice for 30 min in methyl cellosolve and twice for 30 min in acetone. Subsequently, the tissues were dried with liquid $CO_2$ in a Balzers critical point drying apparatus and dusted with gold in a Polaron sputter coater (Samson et al., 1979). Surfaces were scanned with a Jeol JSM-840A electron microscope at an accelerating voltage of 10 kV.

*Light microscopy (LM).* The early stages in the process of symptom formation on petals in relation to the development of the pathogen were followed by LM. Tissues were prepared at 4 h intervals during the first 24 h and at 48 and 72 h after inoculation. The infection process was studied in whole-mount preparations as well as in cross-sections of inoculated petals embedded in plastic. Tissues to be used in whole-mount preparations were fixed in 96% ethanol : 96% acetic acid (1 : 1, v/v) for 24 h, cleared in 75% aqueous lactic acid for 48 h, stained with 0.06% aniline blue in lactophenol and mounted on microscope slides (Erb et al., 1973). These preparations were used to determine the levels of germination and penetration for a total of 100 conidia at ten inoculation sites on five flowers. Fungal development in the petals was investigated in cross-sections of tissues embedded in plastic. Inoculation sites were identified by LM or by the naked eye. Discs of 3 mm diameter, bearing one inoculation site each, were punched from the petals, fixed in 2.5% glutaraldehyde in 25 mM phosphate buffer (pH 6.8) and placed under vacuum to replace air within the discs by fixative. Subsequently, they were washed three times for 5 min in distilled water, dehydrated and cleared twice for 15 min in 2-methoxyethanol (methyl cellosolve) and three times for 5 min in ethanol absolute, and embedded in polyethylene glycol methacrylate (Technovit 7100). Cross-sections of 3 $\mu$m thickness were made using a Reichert-Jung 2040 microtome and placed separately on drops of 10% aqeous acetone on microscope slides. After drying, the sections were stained with 0.05% toluidine blue in 50 mM phosphate buffer (pH 6.8) and rinsed with water (De Leeuw and Kastelein, 1987). Dried preparations were mounted in Euparal. Micrographs were made with a Leitz Orthoplan photomicroscope on Agfapan 25 film (15 DIN).

## Results

*Ultrastructure of the surface of developing lesions.* The ultrastructure of the surface of lesions on rose petals of cv. Sonia caused by *B. cinerea* infections was recorded by SEM. In lesion development, a few stages could be differentiated. Incipient lesions, which were macroscopically visible, appeared as white spots of about 0.5 mm in diameter, each consisting of a small number of white discolored epidermal cells. They were slightly elevated from the abaxial side of the petals, thus giving them a blister-like shape (Fig. 1). An outgrowth of up to 2 mm in diameter was formed at those sites after proceeded elevation of these lesions. Extensive folding of the petal surface effectuated this process (Figs 2 and 3). When the centre of a lesion started to sink, the outgrowth sometimes had a vulcano-like shape (Fig. 4). At this stage of lesion development, the adaxial side of the petal at the infection site was also white discolored. Usually the described symptoms were seen within 24 h of incubation. Even penetration of the epidermal cell wall by a germ tube of a single conidium (Figs 5 and 6) sufficed to induce these symptoms. After prolonged incubation (about 24 h) lesions became necrotic and spreading necrosis developed.

*Histopathology of lesion development.* An investigation into the histopathology of developing lesions was performed by LM in a time-sequence study on the early stages of the infection process. Prior to penetration there was no difference between tissues of healthy inoculated petals and tissues of healthy non-inoculated petals (Figs 7 and 8). Macroscopically, the first symptoms of lesion formation were visible as small white



Figs 1-6. Scanning electron micrographs of the abaxial side of petals of rose cv. Sonia, showing *B. cinerea* lesions at different stages of development, after an incubation period of 24 h at high rh and at room temperature. Flower buds were inoculated on their abaxial side by spraying a suspension containing $7 \cdot 10^4$ conidia $ml^{-1}$ in UP water.

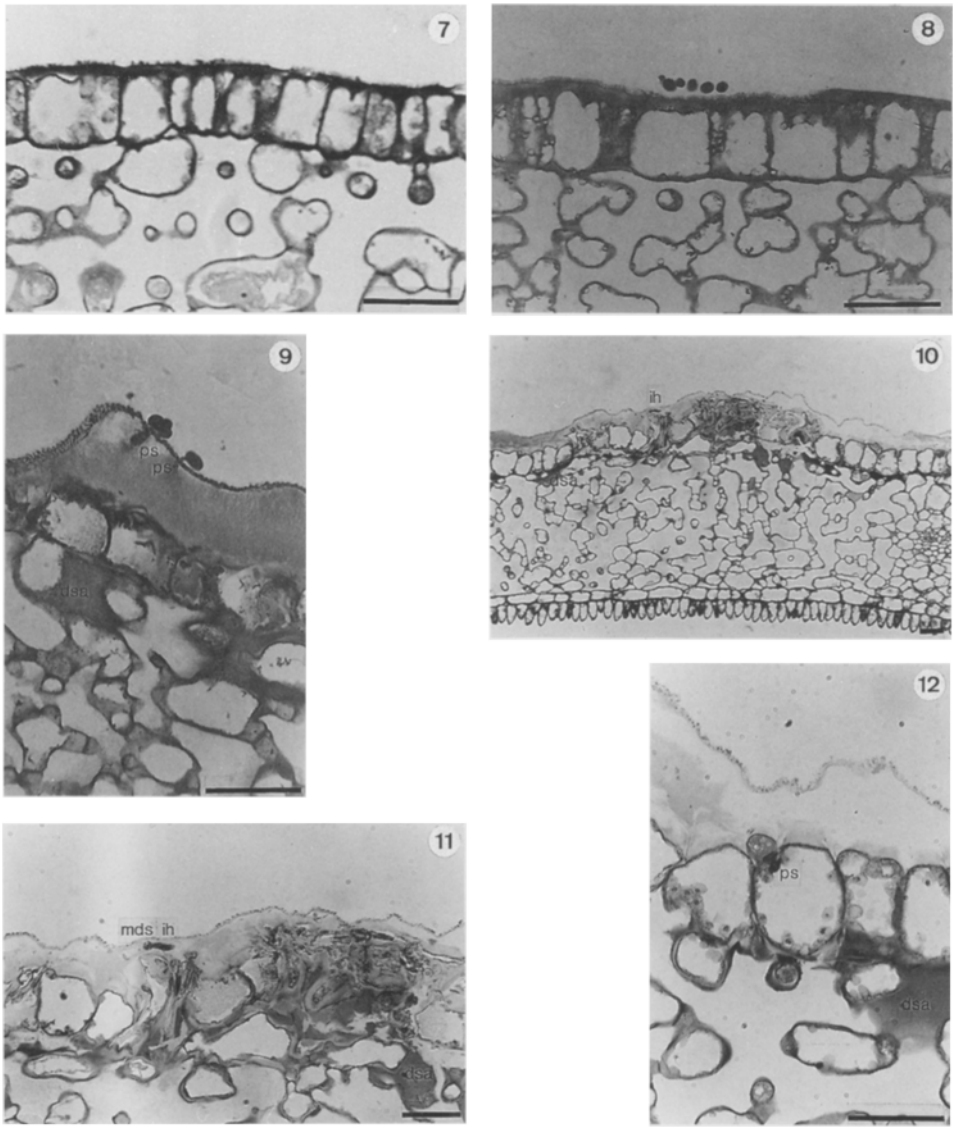
Fig. 1. Blister-like shape of an incipient lesion, consisting of white discolored epidermal cells. Bar: 100 $\mu$m.

Fig. 2. Extensive folding of the petal surface in a lesion. Bar: 100 $\mu$m.

Fig. 3. Outgrowth in a lesion after proceeded development. Bar: 100 $\mu$m.

Fig. 4. Vulcano-like outgrowth after the centre of a lesion has started to sink. Bar: 0.5 mm.

Figs 5 and 6. Germinated conidium on top of an outgrowth. The petal area around the germ tube is covered by unknown material, which might be mucilage. Fig. 6 is a detail of Fig. 5. Bar: 1 and 10 $\mu$m, respectively.




Figs 7-12. Light micrographs of cross-sections of petals of rose cv. Sonia, showing successive stages of *B. cinerea* lesion development, after increasing periods of incubation at high rh and at room temperature. Flower buds were inoculated on their abaxial side with 1 $\mu$l droplets containing about 100 conidia in UP water. Differently staining area - dsa, infection hypha - ih, multiform distended structure - mds, penetration site - ps. Bars: 55 $\mu$m.

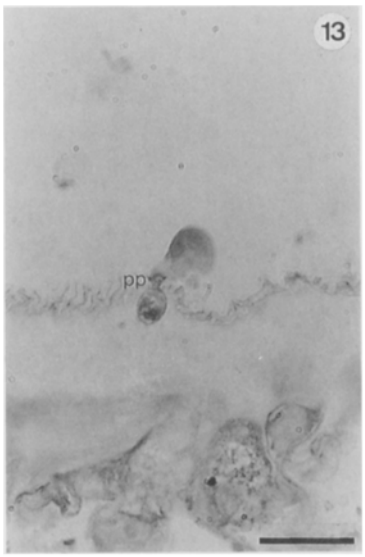
Fig. 7. Healthy non-inoculated tissue, at 0 h.

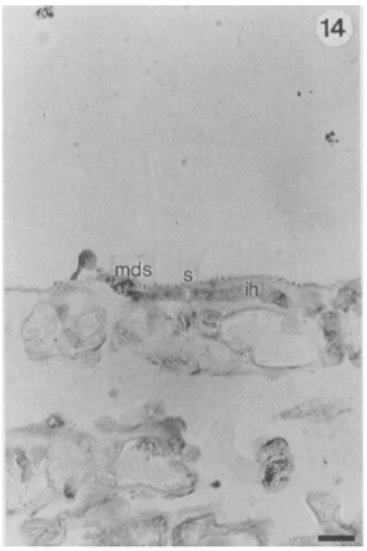
Fig. 8. Non-germinated conidia on the petal surface, at 4 h.

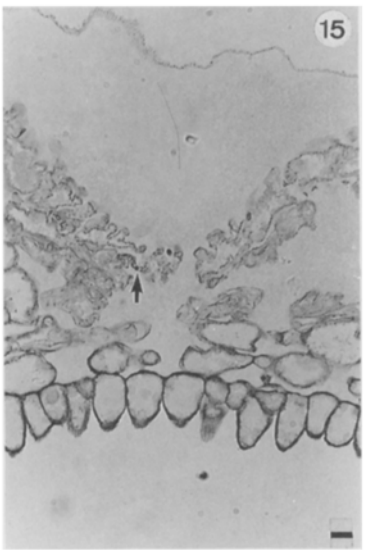
Fig. 9. Penetration of the cuticle leading to an extreme increase in thickness of the epidermal cell wall and to elevation of the cuticle, at 16 h.

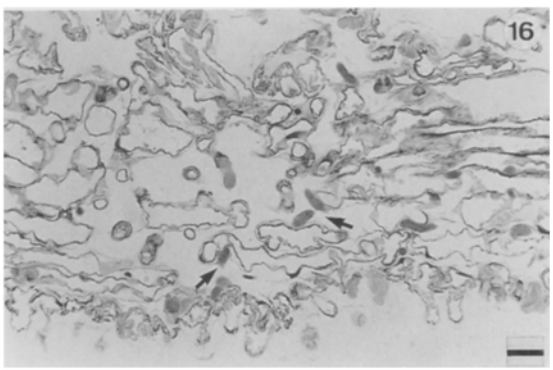
Figs 10 and 11. Outgrowth containing collapsed epidermal cells and infection hyphae, at 20 h. Fig. 11 is a detail of Fig. 10.

Fig. 12. Penetration of an infection hypha into an epidermal cell lumen, at 20 h.




Figs 13-16. Light micrographs of cross-sections of petals of rose cv. Sonia after an incubation period of 48 h (Figs 13-15) or 72 h (Fig. 16) at high rh and at room temperature. Flower buds were inoculated on their abaxial side with 1 $\mu$l droplets containing about 100 *B. cinerea* conidia in UP water (Figs 13-15) or with 0.4 mg of dry conidia dusted in an inoculation box (Fig. 16). Differently staining area - dsa, infection hypha - ih, multiform distended structure - mds, penetration peg - pp, penetration site - ps, septum - s. Bars: 18.5 $\mu$m.

Fig. 13. Penetration of the cuticle by a penetration peg.

Fig. 14. Development of an infection hypha from a distended structure beneath the cuticle, invading the outer periclinal epidermal cell wall.

Fig. 15. Collapse of epidermal and mesophyll cell layers, resulting in a crater covered by the cuticle. Hypha visible between cell remnants (arrow).

Fig. 16. Heavy colonization of a whole rose petal by fungal hyphae (arrows).

Table 1. Progressive development of fungal structures on rose cv. Sonia flowers after inoculation with *B. cinerea*[1].

| Incubation period in h | % conidial germination[2] | % conidial penetration[2] | % multiform distended structures formed[2] |
|---|---|---|---|
| 8 | 21 | 71 | 7 |
| 12 | 18 | 100 | 5 |
| 16 | 54 | 94 | 6 |
| 20 | 51 | 94 | 52 |
| 24 | 62 | 92 | 81 |

[1] Flower buds were inoculated on their abaxial side by applying 1 μl droplets containing about 100 conidia in ultrapure (UP) water, and incubated at room temperature and at high rh for the specified periods.

[2] Percentages were determined from a total of 100 conidia in whole-mount preparations of ten inoculation sites obtained from five flowers. Percentages conidial penetration and multiform distended structures were determined from the germinated conidia only.

spots 8-12 h after the onset of the incubation. Microscopical observation revealed that conidial germination and penetration of the host tissue preceded white spot formation. The conidia produced a short germ tube of up to 30 μm. The tip of a germ tube formed a small peg which penetrated through the cuticle into the outer epidermal cell wall. At this stage, about 20% of the conidia had germinated, whereas over 71% of the germ tubes which had developed had penetrated the cuticle (Table 1). Although little fungal development had occurred up to 16 hours after the onset of the incubation, the response of the tissue to penetration was extensive. The outer wall of cells adjacent to the penetration site began to break down and increased in thickness as a result of which the cuticle became separated from the cell walls (Fig. 9). At the infection sites, some areas stained differently from the surrounding tissue. Sixteen to twenty hours after the onset of the incubation an outgrowth was formed at the abaxial petal side. An outgrowth consisted of remnants of collapsed epidermal cells and infection hyphae (Figs 10 and 11). Also, the first penetrations of epidermal cell lumina (Fig. 12) and some fungal growth underneath the epidermal cell layers were observed. Almost all of the germ tubes formed up to 24 hours after the onset of the incubation had penetrated the cuticle. Penetration pegs were about 1.5 μm in diameter (Fig. 13) and enlarged after passing the cuticle. Half of the pegs had produced a multiform distended structure. Starting from these structures, one or more infection hyphae were formed, which grew into periclinal epidermal cell walls and destroyed them (Fig. 14). The diameter of the structures often was larger than the diameter of the infection hyphae which developed from them. At this point, the centre of about half of the lesions started to sink. This was due to inter- and intracellular hyphal growth which led to a collapse of epidermal and mesophyll cells. As a result, a crater covered by the cuticle was formed (Fig. 15). At a later stage, craters became necrotic and the pathogen gave rise to the development of a spreading necrosis. In necrotic areas the whole tissue was found to be colonized by fungal hyphae (Fig. 16).

## Discussion

The study of the interaction of cut rose petals and *B. cinerea* showed that from the lower side of a germ tube tip a small infection peg was produced, which penetrated through the cuticle. Penetration of the outer epidermal cell wall occurred while the germ tube was short, up to 30 $\mu$m, without the formation of an appressorium. Similar observations were made for the penetration of the epidermal cell wall of broad bean leaves (McKeen, 1974). Infection pegs produced from the lower side of appressoria were found to penetrate epidermal cell walls of onion (Clark and Lorbeer, 1976) and broad bean leaves (Mansfield and Richardson, 1981) and those from the lower side of infection cushions did so on mung bean hypocotyls (Backhouse and Willetts, 1987).

After the penetration peg had passed the cuticle of rose petals, the swelling of the epidermal cell walls and their degradation proceeded very rapidly. The increase in the thickness of the epidermal cell walls was extensive. Swelling of cell walls and disruption of host tissues prior to the presence of infection hyphae has been reported in various histological studies of other plant-*B. cinerea* interactions. These changes have been attributed to the ability of the pathogen to produce a multiplicity of cell wall-degrading enzymes together with toxins which may diffuse through the tissues (Verhoeff, 1980). Swelling of plant cell walls is a well documented symptom caused by the activities of pectic enzymes. Cell walls prepared from cell suspension cultures of kidney bean root tissue have been found to be severely degraded by pectic enzymes and many walls exhibited swelling, separation of wall layers and a different staining intensity in the presence of purified endopectate lyase in vitro (Baker et al., 1980). In vivo, applying commercial pectinase on onion caused effects similar to Botrytis leaf blight infection (Clark and Lorbeer, 1976). Conceivably, the increase in the thickness of the epidermal cell wall and the pushing up of the cuticle observed here in rose petals may have been caused by the hygroscopical nature of pectine degeneration products of the host cell walls.

Starting from the multiform distended structures formed after penetration, one or more infection hyphae were formed, which grew into and destroyed the periclinal epidermal cell wall. Ramification of infection hyphae of *B. cinerea* within the outer periclinal cell wall has been reported for leaves of onion (Clark and Lorbeer, 1976) and broad bean (Mansfield and Richardson, 1981) and for tomato fruits (Rijkenberg et al., 1980). The growth of hyphae in and between epidermal and mesophyll cells of rose petals resulted in a local collapse of these cells and formation of a crater covered by the cuticle. Similar observations were made in leaves of onion (Clark and Lorbeer, 1976) and in tomato fruits (Rijkenberg et al., 1980), although in those cases infection hyphae remained localized at the subcuticle-epidermal level. It has been suggested that the cuticle is not destroyed by infection hyphae, either because there is no direct contact between hyphae and cuticle, which is necessary to induce cutinolytic activity, or because cutinolytic activity is depressed by certain nutrients, e.g. glucose, present in the invading tissue (Rijkenberg et al., 1980).

The cause of white spot formation at the initial stage of lesion development after penetration is unknown. The white halos of ghost spots on tomato fruits have been suggested to contain some air related with fungal enzyme action on epidermal cells (Verhoeff, 1970). The white discoloration of cells in rose petals during the course of lesion development may be caused by cell collapse and/or by oxidation and poly-

merisation of anthocyans.

Addition of exogenous nutrients to an inoculum is a prerequisite for the formation of appressoria and for penetration of onion leaves (Clark and Lorbeer, 1976), strawberry leaves, and cucumber cotyledons (Shirane and Watanabe, 1985). Contrary to these findings, it was found here that *B. cinerea* infections by conidial germ tubes evoked a susceptible reaction in rose petals without the addition of exogenous nutrients to the inoculum. Similar observations were made on floral parts of gerbera (Salinas et al., 1989) and strawberry (Shirane and Watanabe, 1985). With gerbera, small and large necrotic lesions and partial rotting of ray florets or of the whole flower have been reported to occur only after inoculation with conidial suspensions. After inoculation with dry conidia, only small necrotic lesions appeared as a result of a hypersensitive reaction in the ray florets (Salinas et al., 1989). In the case of rose petals, symptom formation apparently is independent of the method of inoculation. So, on flowers *B. cinerea* seems to be a real pathogen, especially on rose flowers.

Since the germination of a single conidium can lead to the observed dramatic susceptible reaction, rose flowers are highly vulnerable to *B. cinerea* infections. To avoid symptom formation on petals, conidial germination and/or penetration of germ tubes into the epidermis must be prevented. The infection of pea stems by *Fusarium solani* f. sp. *pisi* can be prevented by inhibitors of the fungal cutinase (Maiti and Kolattukudy, 1979). Penetration of gerbera ray florets by germ tubes of *B. cinerea* can also be prevented by antibodies against cutinase (Salinas, 1990).

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## References

Backhouse, D. & Willetts, H. J., 1987. Development and structure of infection cushions of *Botrytis cinerea*. Transactions of the British Mycological Society 89: 89-95.

Baker, C. J., Aist, J. R. & Bateman, D. F., 1980. Ultrastructural and biochemical effects of endopectate lyase on cell walls from cell suspension cultures of bean and rice. Canadian Journal of Botany 58: 867-880.

Barendse, L., 1986. Hoeveel geld kost pokken? Vakblad voor de Bloemisterij 41 (10): 32-33.

Clark, C. A. & Lorbeer, J. W., 1976. Comparative histopathology of *Botrytis squamosa* and *B. cinerea* on onion leaves. Phytopathology 66: 1279-1289.

Cline, M. N. & Bardsley, C. E., 1984. Postharvest treatment of cut flowers for Botrytis blight. Proceedings of the British Crop Protection Conference on Pests and Diseases 3: 1169-1173.

Elad, Y., 1988. Latent infection of *Botrytis cinerea* in rose flowers and combined chemical and physiological control of the disease. Crop Protection 7: 361-366.

Erb, K., Gallegly, M. E. & Leach, J. G., 1973. Longevity of mycelium of *Colletotrichum lindemuthianum* in hypocotyl tissue of resistant and susceptible bean cultivars. Phytopathology 63: 1334-1335.

Hammer, P. E. & Marois, J. J., 1989. Nonchemical methods for postharvest control of *Botrytis cinerea* on cut roses. Journal of the American Society for Horticultural Science 114: 100-106.

Heuvel, J. van den & Waterreus, L. P., 1983. Conidial concentration as an important factor

determining the type of prepenetration structures formed by *Botrytis cinerea* on leaves of French bean (*Phaseolus vulgaris*). Plant Pathology 32: 263-272.
Jarvis, W.R., 1980. Epidemiology. In: Coley-Smith, J. R., Verhoeff, K. & Jarvis, W. R. (Eds), The biology of *Botrytis*. Academic Press, London, p. 219-250.
Leeuw, G. T. N. de & Kastelein, P., 1987. Observation of flagellate protozoa (*Phytomonas* sp.) in *Cocos nucifera, Cecropia palmata* and *Euphorbia hirta.* Journal of Phytopathology 120: 25-30.
Maiti, I. & Kolattukudy, P. E., 1979. Prevention of fungal infection of plants by specific inhibition of cutinase. Science 205: 507-508.
Mansfield, J. W. & Richardson, A., 1981. The ultrastructure of interactions between *Botrytis* species and broad bean leaves. Physiological Plant Pathology 19: 41-48.
McKeen, W. E., 1974. Mode of penetration of epidermal cell walls of *Vicia faba* by *Botrytis cinerea*. Phytopathology 64: 461-467.
Phillips, D. J., Margosan, D.A. & Fouse, D.C., 1985. Postharvest control of Botrytis rot of roses with carbon dioxide. Plant Disease 69: 789-790.
Redmond, J. C., Marois, J. J. & MacDonald, J. D., 1987. Biological control of *Botrytis cinerea* on roses with epiphytic microorganisms. Plant Disease 71: 799-802.
Rijkenberg, F. H. J., Leeuw, G. T. N. de & Verhoeff, K., 1980. Light and electron microscopy studies on the infection of tomato fruits by *Botrytis cinerea*. Canadian Journal of Botany 58: 1394-1404.
Salinas, J., 1990. Protection of gerbera flowers against infection of *Botrytis cinerea* with anti-cutinase monoclonal antibodies. Acta Botanica Neerlandica 39: 313-314 (Abstract).
Salinas, J., Glandorf, D. C. M., Picavet, F. D. & Verhoeff, K., 1989. Effects of temperature, relative humidity and age of conidia on the incidence of spotting on gerbera flowers caused by *Botrytis cinerea*. Netherlands Journal of Plant Pathology 95: 51-64.
Samson, R. A., Stalpers, J. A. & Verkerke, W., 1979. A simplified technique to prepare fungal specimens for scanning electron microscopy. Cytobios 24: 7-12.
Shirane, N. & Watanabe, Y., 1985. Comparison of infection process of *Botrytis cinerea* on cucumber cotyledon and strawberry petal. Annals of the Phytopathological Society of Japan 51: 501-505.
Verhoeff, K., 1970. Spotting of tomato fruits caused by *Botrytis cinerea*. Netherlands Journal of Plant Pathology 76: 219-226.
Verhoeff, K., 1980. The infection process and host-pathogen interactions. In: Coley-Smith, J. R., Verhoeff, K. & Jarvis, W. R. (Eds), The biology of *Botrytis*. Academic Press, London, p. 153-180.