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Spotting of tomato fruits caused by Botrytis cinerea

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Abstract

Botrytis spot or ghost spot on tomato fruits occurs after penetration of germ tubes of *B. cinerea* into epidermal cells. A few days after the penetration a halo appears around the infected necrotic cells. These symptoms can be reproduced by inoculating young fruits with a few dry conidia. When many conidia alight on the epidermis of the fruit, scab-like symptoms develop, while under conditions of high humidity, blisters can be formed on the fruit surface before the fungus spreads through the fruit parenchyma. Under conditions of low humidity, necrotic areas are formed.

In the necrotic cells, developed after inoculation with many or with a few conidia, no mycelium could be found by the histological methods so far used. However, *B. cinera* can be reisolated, implying that the *Botrytis* spot is a latent infection by the fungus. No renewed growth takes place when the fruit is fully ripe.

Introduction

An important economic aspect of infection by *Botrytis cinerea* Pers. ex Fr. on tomatoes is the spotting of the fruits, usually referred to as ghost spot or *Botrytis* spot. Neither keeping quality nor taste is affected, but fruits with spots can not be sold for export and consequently command a lower price.

Only a few publications deal with this type of infection by *B. cinera*. Read (1937) described *B. cinerea* as the causal organism of what he called water spot. Ainsworth et al. (1938) gave the following hypothesis for the development of spots. Conidia of the fungus alight on a fruit and germinate under conditions of high humidity. The germ tubes penetrate through the cuticule and the outer epidermis cell wall, where some pectinase is produced. When the humidity of the air decreases, the water on the fruit evaporates and the fungus is evidently killed since it cannot be isolated from mature spots. However, a few epidermal cells are already dead, causing the small necrotic spots on the fruit. This paper deals with a more detailed study of the infection process and the development of symptoms on the fruit.

Material and methods

In all experiments, plants of the variety 'Moneymaker' were used. For inoculation of fruits, spore suspensions and "dry" conidia were used. Spore suspensions were obtained from 7–14 days old cultures, growing on modified Richard's agar, with 5% sucrose as the only carbon source, at 22 °C. Spores were collected by pouring distilled water over a culture. After centrifuging at 3000 r/m for 2 min, the spores were resuspended in distilled water to a concentration of approximately 15.000 conidia per ml. When necessary, lower concentrations were obtained by dilution. Drops of approxi-

mately 0.025 ml were placed on the fruits. Spraying of a spore suspension on the fruits was rarely used, as the results of this type of inoculation were comparatively poor. Dry spores were taken from cultures, 14–21 days old, growing on modified Richard's agar or on autoclaved tomato leaves. By gently brushing the surface of the culture with a camel hair brush, clouds of spores were obtained. By varying the distance between the culture and the fruits, many or few spores alight on the fruit surface. For 16–24 h after the inoculation, the fruits were wrapped in polyethylene bags when attached to plants; when detached from the plants, they were placed in polyethylene boxes so as to maintain high relative humidities.

To determine the time necessary for the fungus to penetrate, fruits were surface sterilized with mercuric chloride (0.1 %) or with calcium hypochlorite (0.1 %) and washed with water, these treatments being carried out at various times after the inoculation. When no symptoms developed it was assumed that no penetration had occurred.

To locate conidia rapidly under the microscope, optical brighteners were used, as described by Wilson (1966). Conidia were labeled with Tinopal BOP (Geigy) or Calcofluor white RW (Am. Cyanamid) by incubating them in a glycerol solution of the brightener for 16–18 h at 5 °C. After washing three times with buffered NaCl (pH 7) and twice with distilled water, the conidia were dried by pouring the suspensions on to filter paper. About 50 % of the conidia remained alive after the treatment.

For reisolation of the fungus from inoculated or infected fruits, fruits were surface sterilized with 0.1 % calcium hypochlorite and washed with water. Small pieces of the fruit wall were than aseptically placed on cherry agar.

For microscopical examination, epidermis strips and microtome sections of 10 μ were used. In the latter case, parts of inoculated fruit walls were fixed in a mixture of formalin-propionic acid/ethyl alcohol 50 %; 5, 5 and 90 parts v/v, respectively, and afterwards dehydrated with tertiary butyl alcohol. Sections were stained in cotton blue and safranin (Lepik, 1928).

Development of symptoms

On mature fruits, each spot consists of a small necrotic lesion, usually 1 mm or less in diameter, surrounded by a white, silvery shining ring (Fig. 1). This ring is not always immediately around the necrotic centre; there can be an annulus of apparently healthy fruit wall tissue of 1–3 mm wide between the necrotic centre and the white ring. Sometimes the whole spot has a silvery appearance. In some cases, there is more than one necrotic spot within a white-coloured ring, while on fruits with many spots these rings coalesce. Many necrotic centres of spots seem to be rised above the surrounding tissue.

Inoculating fruits with conidia of *B. cinerea* does not necessarily lead to the development of typical spotting. The symptoms depend upon the type of inoculum used, viz. many or a few dry spores or drops of a spore suspension. After inoculation of fruits with various diameters, symptoms only developed on young green fruits, with a diameter below about 30 mm, regardless the method of inoculation used. Consequently all the inoculation experiments were carried out on fruits with diameters of 15–25 mm.

When a few dry conidia alight on the epidermis, small necrotic lesions can be seen within 30 h after the inoculation. One or two days later, a white ring appears around nearly all the necrotic spots. The tissue immediately around the necrotic centre seems

Fig. 1. *Botrytis* spot on a ripe tomato.

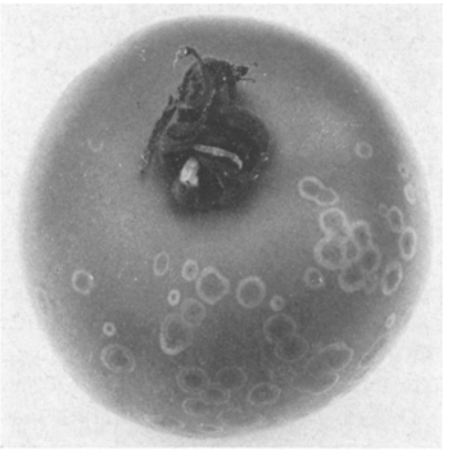


Fig. 1. Botrytis-stip op een rijpe tomaat.

Fig. 2. Blister development on a young green fruit, 30 h after inoculation with many conidia, under continuous high humidity.

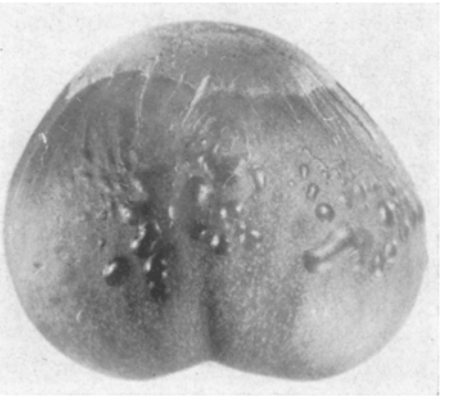


Fig. 2. Blaasvorming op een jonge groene vrucht, 30 uren na inoculatie met veel conidiën, bij voortdurend hoge luchtvochtigheid.

to be swollen, giving the whole spot a volcano-like appearance. These symptoms remain during the ripening process of the fruit, though the necrotic centre as well as the white ring may increase in size slightly, while the volcano-like appearance gradually decreases and even disappears in many cases. The relative humidity of the air has no effect on the development of symptoms once the humidity has been high during 16–24 h after the inoculation. The fruits do not rot from these lesions.

When many conidia alight on the fruit surface, the epidermis shows scab-like symptoms within 24 h after the inoculation. Under conditions of high humidity, the epidermis can become blistered and sometimes a great part of the fruitwall develops into one great blister, partly filled with air and on which mycelium can be seen (Fig. 2). After some days the blisters usually break; in the meantime, the fungus has grown through the fruit parenchyma and the fruit begins to rot. Blister formation occurs mainly on fruits which developed on plants early in spring.

When the humidity of the air is decreased about 16 h after inoculation hardly any blistering develope. Within 24 h the surface of the fruit is covered with many small, dark brown lesions which are not surrounded by white rings. During fruit ripening, these lesions change only slightly and fruits so affected, do not rot.

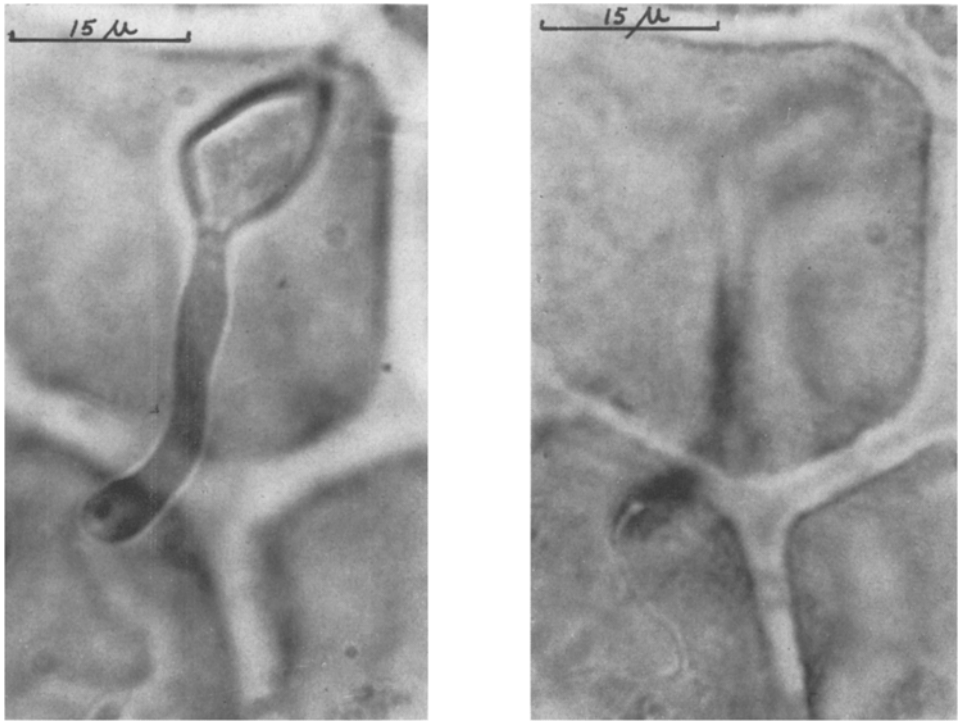
When small drops of a spore suspension are used for inoculation, different symptoms develop. After about 24 h, the epidermis under the infection drop turns brown and sometimes a blister develops there. Under continuous high humidity, lesions develop and the whole fruit rots. When the suspension dries about 24 h after inoculation, there is only a brown colouration of the epidermis and the fungus does not grow through the fruit. When a dilute spore suspension is used for inoculation, the browning of the epidermis is less dark, while with approximately 150 spores per ml (about 4 conidia per droplet) no symptoms develop.

Symptom development in fruits inoculated and incubated at room temperature can be prevented by surface sterilants only when they are used within 5 or 6 h of inoculation.

Morbid anatomy

Germinated conidia can be seen on epidermal strips taken from inoculated fruits 24 h after inoculation with a few dry spores. The length of the germ tubes varies, but is usually less than the length of the conidium. Near the top, the germ tube narrows and penetrates through the cuticule into an epidermal cell. In cross sections, a small "canal" through the cuticule and the outer epidermis cell wall can be seen. In the epidermal cell, the penetrating hypha enlarges again. The infected cell and some surrounding epidermal cells turn dark brown, while the wall of the infected cell seems to be swollen (Fig. 3, 4 and 5). One or two days later, meristematic activity can be seen in the underlying parenchyma cells; the necrotic cells become separated from the healthy tissue and the oldest are often raised above the surrounding epidermal cells (Fig. 6). During the growth of the fruit this elevation is leveled off by a widening area of meristematic cells and by squashing the necrotic cells flat between the cuticule and developing parenchyma. It can only occasionally be seen in cross sections through spots on mature fruits. Sometimes penetration through a hair on the epidermis was observed.

Fig. 3. Germinated conidium of *B. cinerea* on a tomato. Near the top of the germ tube, penetration into the underlying epidermal cell has taken place. A: focussed on the germtube; B: focussed on the penetrated hypha.



A B

Fig. 3. Gekiemde spore van B. cinerea op een tomaat. Nabij de top van de kiembuis heeft binnendringen in de onderliggende epidermis cel plaats gevonden. A: scherp gesteld op de kiembuis; B: scherp gesteld op de binnengedrongen hyfe.

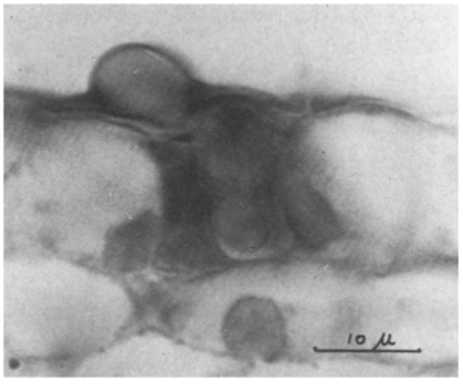


Fig. 4. Cross section through the epidermis of a young tomato, showing an infected epidermal cell, 16 h after inoculation.

Fig. 4. Dwarsdoorsnede door de epidermis van een jonge tomaat met een geïnfecteerde epidermiscel, 16 uren na de inoculatie.

Botrytis spots on ripe fruit consist of a number of necrotic cells, containing no mycelium, both from the epidermis and from the underlying parenchyma, separated from the underlying tissue by some layers of small cells (Fig. 7). Nothing could be seen of the silvery white ring around the necrotic centre.

When many dry conidia are used for the inoculation, a great number of epidermal and parenchymal cells contribute to the necrotic areas, which usually coalesce into larger areas. On many sites, penetration by germ tubes can be observed. After the development of a blister, the epidermis or the epidermis and some layers of the underlying parenchyma cells appear to be separated from the underlying tissue. When this type of symptoms develops, mycelium can be easily seen in cross sections below the cuticule and throughout the surrounding cells. This is not the case, when necrotic areas developed, then mycelium cannot be seen at all.

After the inoculation of fruits with drops of a spore suspension containing approximately 15,000 conidia per ml, the same type of infection can take place as described above, though germ tubes can become very long before penetration occurs, usually

Fig. 5. Cross section through the epidermis of a tomato with a diameter of 16 mm, showing the way of penetration through the cuticule.

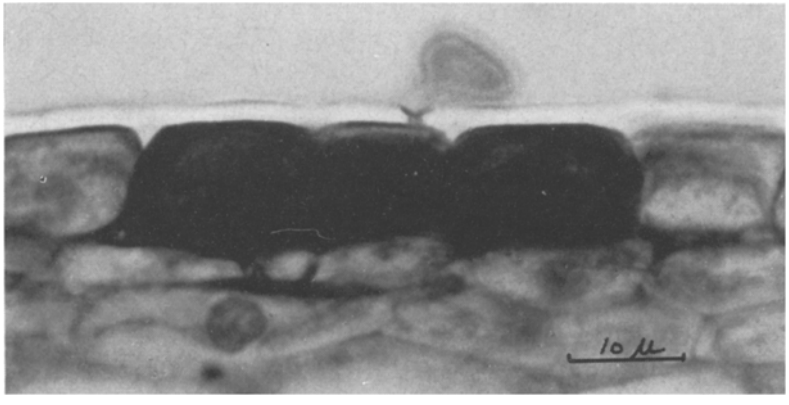


Fig. 5. Dwarsdoorsnede door de epidermis van een tomaat met een diameter van 16 mm, tonend de penetratie door de cuticula.

Fig. 6. Cross section through a *Botrytis* spot 5 days after inoculation.

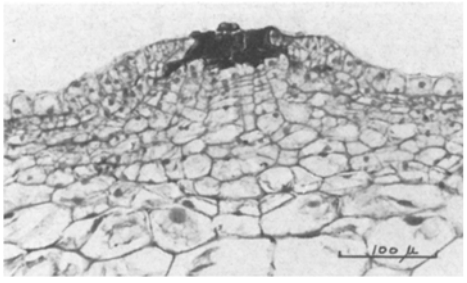


Fig. 6. Dwarsdoorsnede door een Botrytis-stip, 5 dagen na inoculatie.

Fig. 7. Cross section through a *Botrytis* spot on a ripe tomato.

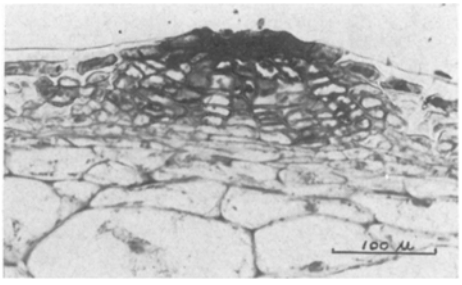


Fig. 7. Dwarsdoorsnede door een Botrytis-stip op een rijpe tomaat.

with the formation of appressoria-like swellings. Occasionally, finger-shaped appressoria are seen. Many germ tubes just grow over the epidermis without penetrating. Under the growing germ tubes, the epidermis cells turn brown. When a dilute spore suspension is used for inoculation, germ tubes can be seen growing over the epidermis, though they do not penetrate. Under them a few cells turn brown.

Reisolation of B. cinerea

Although occasionally hyphae could be found in necrotic cells only in the first days after inoculation, reisolation of *B. cinerea* was very easy, both from necrotic centres of spots and necrotic areas. Reisolation was possible immediately after inoculation and throughout ripening. From *Botrytis* spots on commercially grown tomatoes, the fungus could also be isolated very easily.

Discussion

The description of the symptoms, as far as this was done by Read (1937) and by Ainsworth et al. (1938) were confirmed in our experiments. They do not mention the effect of many conidia together, though this does not occur under commercial conditions where only typical spots are found. The effect of many conidia together seems to be a cumulative effect of the reaction by one or a few. It is quite possible, that many conidia produce enough enzymes to kill sufficient cells to provide a large enough substrate of moribund tissue to spread in the fruit, when conditions are otherwise favourable. This effect can be seen after inoculation with many dry conidia as well as with spore suspensions. If penetration is mainly an effect of enzym production, this might explain the fact, as preliminary experiments showed, that other *Botrytis* species e.g. *B. paeoniae* Oudem. and *B. tulipae* Lind., are capable of producing similar necrotic lesions on tomato fruits, though typical spots have not yet been observed as was found by Ainsworth and Oyler (1938) with *B. tulipae*. On the other hand, the cells in the wall of a growing fruit must be very active, because of the rapid increase in size of the fruits. This might explain the meristematic activity, which takes place in the parenchyma cells just under the epidermis so soon after penetration, regardless the number of conidia

used for inoculation. Only under conditions of high humidity, the spread of the fungus is apparently so fast, that the cells are killed before any division can take place.

As was found by Ainsworth et al. (1938) and by Ferrer and Owen (1959), fruits larger than about 30 mm in diameter are not infected. This might indeed be related to the development of the cuticule. Together with the outer epidermal cell wall, this layer is about 4 μ thick when the fruits have a diameter of 30 mm.

Preliminary experiments showed a stimulating effect of fruit washings on spore germination; this stimulation may play a role in the penetration of the fruit epidermis. No explanation can be given for the development of blisters, nor for the fact, that they were mainly observed on fruits developed in the spring. Here again, physiological processes of the host may be involved.

In cross sections through spots no halo was observed in the surrounding tissue. It has been suggested (Ainsworth et al., 1938; Ferrer and Owen, 1959) that some air has come under the epidermal cells due to the action of enzymes produced by the penetrating hypha. In view of the results with many conidia, where small and large blisters are developed before the fungus spreads in the underlying tissue, it might well be the case that the ring contains some air. Naturally this disappears during the dehydration and staining process.

From *Botrytis* spots, *B. cinerea* can be reisolated. This is in contrast to the statement of Ainsworth et al. (1938), that the fungus can not be isolated after desiccation of germ tubes, though they gave no details of their surface-sterilization technique, which may be a critical point. In fact, after penetrating into the epidermal cells the fungus remains latent but does not grow out again however. In the case of latent infections in strawberry and raspberry fruits, the renewed growth of the fungus might be correlated with the increase of carbohydrates in the ripening fruits (Jarvis, personal communication). Though also in tomato fruits an increase in carbohydrates takes place during ripening (Winsor, 1966), the total concentration might be too low. This might explain the fact, that latency is maintained in mature fruits.

Acknowledgments

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Samenvatting

Botrytis-stip op tomatevruchten

Botrytis-stip op tomatevruchten ontstaat na binnendringen van kiembuizen van *B. cinerea* in epidermiscellen van een jonge vrucht. Enkele dagen na de infectie treden celdelingen op in het onderliggende parenchymweefsel. De geïnfecteerde cel en enkele aangrenzende cellen sterven af, terwijl rondom dit necrotische plekje een zilverwit gekleurde ring ontstaat. Worden veel conidiën bijeen op een vruchtwand gebracht, dan krijgt de epidermis binnen 24 uur een schurftig uiterlijk. Blijft de luchtvochtigheid na de inoculatie hoog, dan kunnen daarentegen kleinere en grotere blaasjes in de vruchtwand ontstaan. Deze barsten na enkele dagen open, terwijl mycelium zich door de

vrucht verbreidt. Daalt de luchtvochtigheid circa 16 uur na de inoculatie, dan blijft de aantasting beperkt tot de epidermis. Hoewel in het necrotische weefsel geen mycelium van *B. cinerea* kon worden aangetoond, blijkt herisolatie van de schimmel gemakkelijk te zijn. Ook uit *Botrytis*-stippen van uit de praktijk afkomstige vruchten kan *B. cinerea* geïsoleerd worden. Dit betekent, dat het hier om een latente infectie gaat, waarbij evenwel geen hernieuwde groei plaats vindt als de vrucht geheel rijp is.

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