

Infection of Table Grape Bunches by *Alternaria alternata*

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Infection of table grape bunches by *Alternaria alternata* was investigated by light, fluorescence and scanning electron microscopy. Conidia in 20- μ l drops of spore suspension germinated readily on the fruit surface and within 16 h grew extensively on berries, pedicels and rachises of immature and mature bunches. Appressoria were formed within 16 h at the tip of germ tubes and hyphae, and on short side branches on the different bunch parts, but no evidence of direct penetration was found. The pathogen penetrated the host tissue through stomata, lenticels and microcracks in the epidermis. Infection hyphae remained localized in the substomatal cavities or surface cells of lenticels, and were restricted to a few epidermal cells in the case of epidermal microcracks, but caused no cell necrosis. Under conditions of high humidity, the fungus grew out of the stomata, lenticels and microcracks and formed an extensive superficial growth within 168 h. Although the fungus was able to grow into epidermal cells adjacent to those surrounding wounds, it would appear that this process is a slow one. The behaviour of *A. alternata* partly explains the extensive superficial growth of the pathogen that may occur on rachises and pedicels of table grapes at the end of the cold storage period.

Alternaria alternata (Fries) Keissler causes bunch rot of export table grapes which is characterized by firm, superficial, dark-brown to black lesions on berries near the pedicel, and fluffy gray tufts of fungus growing on rachises and pedicels (Swart & Holz, 1991). The disease develops in cold-stored fruit and its sporadic occurrence in some consignments is a serious challenge to the prolonged storage of table grapes at low temperatures (Swart & Holz, 1991, 1994). Previous studies showed that *A. alternata* colonizes berries, pedicels and rachises during the entire period of bunch development (Swart & Holz, 1994), but that stress factors during cold storage might predispose table grape bunches to *A. alternata* decay. Little information pertaining to the infection of table grape bunches by *A. alternata* has been documented. This study aimed at elucidating the infection of table grape bunches by *A. alternata* through the use of light, fluorescence and scanning electron microscopy.

MATERIALS AND METHODS

Inoculation: A culture of *A. alternata* isolated from a naturally infected grape berry was maintained in a lyophilized state. Inoculum was prepared by growing the fungus on potato-dextrose agar (PDA) at 25°C under a mixture of near-ultra violet and cool fluorescent light. Conidia were harvested by flooding 14-day-old cultures with sterile distilled water containing 0,1% Tween 20, dislodging conidia with a sterile glass rod and filtering the suspension through two layers of cheesecloth. Spore counts were made with a haemocytometer and the suspensions diluted to approximately $1,5 \times 10^6$ spores/cm³.

Sound, unblemished bunches (cultivar Dauphine) were obtained at bunch closure and at harvest from a commercial vineyard from the Paarl area. Bunches were surface disinfested in 70% ethanol for 5 s, dipped in sterile distilled water and dried on a laminar flow bench. In order to inoculate the different bunch parts, some of the berries were removed with their pedicels to expose the rachis. Bunches were then placed on top of water-saturated, ster-

ile aluminium-foil covered "oases" (florist's sponge) and their stems plugged into small holes made in the sponge. Sites to be inoculated on the rachises, pedicels or on the berries were circled with a black felt-tipped pen (preliminary studies showed no phytotoxic effect). On some mature bunches berries were wounded before inoculation by puncturing them 2 mm deep with inoculation needles. A 20 μ l-droplet containing approximately 200 spores was placed inside the marked area. Control fruit were treated the same way, but a drop of sterile distilled water was used. Oases with grapes were placed in sterile distilled water in moist chambers and incubated at 25°C. This ensured the high humidity needed for infection (Harvey, 1955; Hewitt, 1974).

Pieces of tissue for microscopic study were removed from the marked areas 16, 24, 48, 72, 120, and 168 h after inoculation.

Light microscopy: Samples were fixed in a formalin-acetic acid-alcohol (FAA) mixture (Berlyn & Miksche, 1976), dehydrated in tertiary butanol (Berlyn & Miksche, 1976) in a Shandon automatic tissue processor and embedded in Paramat extra (BDH Laboratory Supplies, Poole, England). Embedded material was sectioned at 8-12 μ m with a Leitz rotary microtome, the sections affixed to slides with chrome alum and dried on a slide warmer. Sections of the pedicel and rachis were stained with erythrosin and fast green, while berry sections were stained with safranin and fast green (Sass, 1951). Stained sections were examined and photographed with a Zeiss Axioskop photomicroscope.

Fluorescence microscopy: Fresh and FAA-fixed tissue sections were stained in 0,1% (w/v) aniline blue in 0.1M KH₂PO₄. Conidial germination and fungal growth were examined with the aid of a Zeiss Axioskop microscope equipped with an epifluorescence condenser, a high-pressure mercury lamp, Neofluar objectives and Zeiss filters 06 and 18. These sets include excitation filters BP 436/8 and BP 395-425, respectively. With this setup, conidia and fungal hyphae fluoresced yellow.

Specimens embedded in wax were observed for induced fluorescence of fungal tissue by a modification of the technique described by Alexander (1980). Sections ($10\mu\text{m}$) were stained for 2 min with 0,5% aqueous malachite green, then counterstained for 20 min with 0,01% acridine orange in a phosphate buffer at pH 6. After being washed in the buffer, sections were air-dried and mounted in immersion oil. With this setup, hyphae in plant tissue fluoresced orange. Photomicrographs were prepared using 400 ASA Agfachrome film.

Scanning-electron microscopy: Pieces of tissue were fixed in 3% (v/v) glutaraldehyde in 0,05 M sodium cacodylate buffer, pH 6,82-7,24 for 8 h, washed twice in 0,05 M sodium cacodylate buffer, post-fixed for 2 h in 2% osmium tetroxide in 0,05 M sodium cacodylate buffer, washed twice in 0,05 M sodium cacodylate buffer, dehydrated in a graded ethanol series and dried in a critical-point drier under CO_2 . The specimens were mounted on stubs, gold coated in a Giko IB-2 Coater and viewed with an ISI 100-A SEM.

Specimens embedded in wax were sectioned at $10\mu\text{m}$ with a rotary microtome, the sections affixed to slides with chrome alum, dried on a slide warmer and the wax removed from specimens by washing with xylene. The slides were dried in a dust-free area, and prepared for SEM as described above.

RESULTS AND DISCUSSION

Unwounded berries: Fungal behaviour on immature and mature berries was essentially the same. Conidia germinated readily and at least 70% had germinated after 16 h. Germ tube and hyphal growth varied, but was mostly longer than $100\mu\text{m}$. Appressoria occurred at the tips of germ tubes (Fig. 1a), hyphal tips, and on short side branches (Fig. 1b). Adhesion of appressoria was very prominent, and some appressoria were completely embedded in cuticular wax. Hyphae had entered the stomata and microcracks in the lenticels on immature berries, or had grown into microcracks formed in the cuticles on immature and mature berries. After 24 h, hyphal growth was extensive for most conidia (Fig. 1c). After 48 h virtually all the conidia had germinated. Germ tubes and hyphae adhered firmly to the cuticle and lengths of these structures were covered by cuticular wax (Fig. 1d). Wax crystals in the vicinity of some germ tubes and hyphae were completely altered.

Conspicuous hyphal thickenings in the wax layer were observed by fluorescence microscopy after 72 h. Hyphae grew out of the stomata and microcracks in the epidermis of both berry types, and hyphae were seen underneath the cuticle in the first few epidermal cells adjacent to the microcracks on mature berries (Fig. 2a). Superficial fungal growth developed on the inoculated surface after 168 h, but no lesions were formed on either immature or mature berries. In the samples studied there was no indication that the fungus had penetrated the epidermal cells directly.

Wounded berries: Only samples from mature berries were studied. A dense mat of hyphae formed over the injured surface after 16 h. Hyphae grew down into the wound openings, but did not penetrate the surrounding cells. At 48 h after inoculation individual hyphae grew

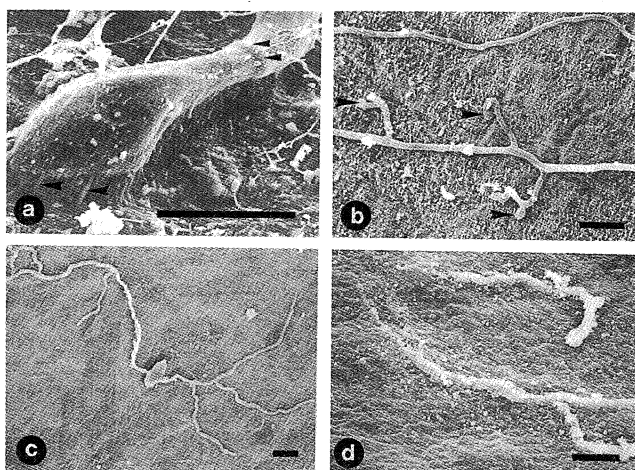


FIGURE 1

Scanning electron micrographs of *Alternaria alternata* on table grape berries. (a) Simple, hyaline appressorium, with a distinct septum (arrows), formed at the tip of a short germ tube on an immature berry 16 h post-inoculation. The appressorium is firmly attached to the cuticle by thread-like adhesive (arrows). (b) Appressoria (arrows) on short side branches of a hypha on an immature berry 16 h post-inoculation. (c) Extensive hyphal growth by a single conidium on an immature berry 24 h post-inoculation. (d) Hyphae embedded in cuticular wax on a mature berry 48 h post-inoculation. Scale bar = $10\mu\text{m}$.

laterally between the epidermal cells. At 72 h, superficial growth was evident and conidia formed on hyphae at the wound opening. Epidermal cells surrounding wounds were heavily colonized, but hyphae had not yet spread into the undamaged parenchymatous cells. Superficial growth at the wound area was extensive after 168 h, and epidermal and parenchyma cells adjacent to the wound were colonized (Fig. 2b). The cuticle was still intact and no indication of direct penetration was found. No visible lesions were observed at the wound site.

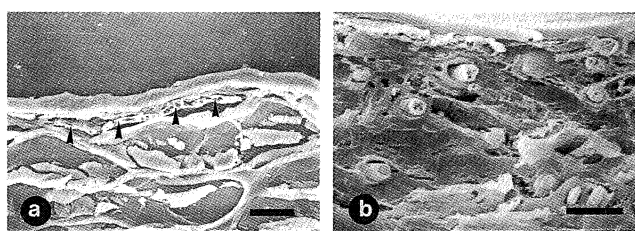


FIGURE 2

Scanning electron micrographs of *Alternaria alternata* in unwounded and wounded table grape berries. (a) Hypha (arrows) growing underneath the cuticle in epidermal cells adjacent to a microcrack in the cuticle of a mature berry 72 h post-inoculation. (b) Hyphae colonising epidermal and parenchyma cells adjacent to a wound in a mature berry 168 h post-inoculation. Scale bar = $10\mu\text{m}$.

Pedicels: Spore germination and hyphal growth were the same as on berries. Distinct appressoria were observed after 16 h. Numerous lenticels occurred on pedicels, and germ tubes and hyphae appeared to grow in the direction of lenticels. Most of the lenticels were covered with a thin cuticle at bunch closure, whereas the cuticle on some was cracked. The fungus entered lenticels through the microcracks (Figs 3a and 3b) and grew into the surface cells. Lenticels on mature bunches were well developed and uncovered. After 48 h hyphae that grew into the lenticels were localized to the surface cells (six to seven cells deep) (Fig. 3c). After 72 h hyphae grew out of lenticels with

superficial fungal growth being evident after 168 h. Direct penetration and colonization of epidermal cells was not observed and the pedicels were asymptomatic.

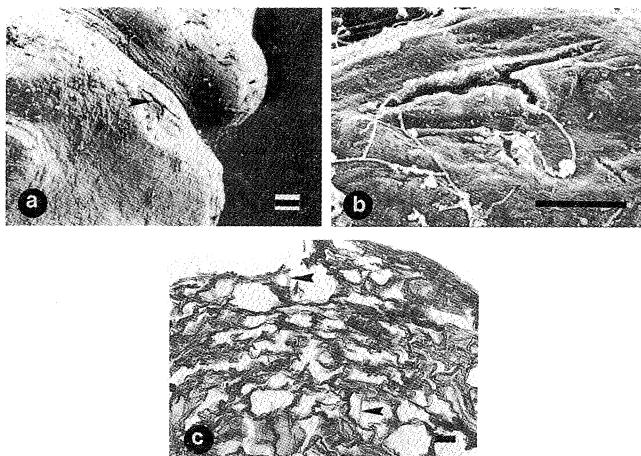


FIGURE 3

Light and scanning electron micrographs of *Alternaria alternata* on table grape pedicels. (a) Pedicel of an immature berry showing a microcrack (arrow) in the cuticle covering the lenticel. (b) Hyphae penetrating a microcrack in the cuticle over a lenticel on an immature berry 16 h post-inoculation. (c) Hyphae (arrows) in the surface cells of a lenticel on an immature berry 48 h post-inoculation. Scale bar = 100µm.

Rachises: Fungal development on rachises of immature and mature table grape bunches was essentially the same. Germ tube and hyphal growth was extensive after 16 h. Germ tubes and hyphae grew in and across the longitudinal grooves (Fig. 4a). Lengths of germ tubes and hyphae were covered by cuticular wax. Appressoria formed at the tips of germ tubes and hyphae, and on short side branches on ridges and in grooves. Some appressoria were embedded in cuticular wax. In some cases germ tubes grew into stomata, failed to penetrate and grew out again (Fig. 4b). After 72 h a few hyphae were seen to colonize microcracks in the cuticle. However, no clear evidence of successful direct penetration of epidermal cells could be found at this stage.

Rachises became flaccid after 120 h and cortex cells were distorted. Hyphae were seen in the epidermal cells, but their presence could not be ascribed to direct penetration. At this stage, hyphae grew out of colonised stomata and microcracks. After 168 h a network of intercellular hyphae had developed in the epidermis of flaccid rachises, with considerable superficial fungal growth over the rachises. No lesions were observed.

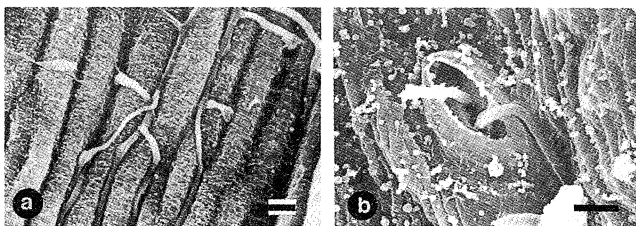


FIGURE 4

Scanning electron micrographs of *Alternaria alternata* on table grape rachises. (a) Hyphae growing in the longitudinal grooves of a rachis of an immature bunch 72 h post-inoculation. (b) Hypha entering and exiting a stoma on the rachis of a mature bunch 16 h post-inoculation. Scale bar = 10µm.

DISCUSSION

A. alternata is generally considered as an opportunistic pathogen that gains entry into plant tissue via wounds (Ben-Arie, 1966; Pearson & Hall, 1975; Cappellini & Ceponis, 1977) or natural openings (O'Donnell & Dickinson, 1980; Prusky *et al.*, 1983). In the case of persimmon *A. alternata* penetrates the fruit directly (Prusky *et al.*, 1981). In the present study no evidence was found for direct penetration of table grape bunches by *A. alternata*. Hyphae entered berries, pedicels and rachises through stomata, lenticels and microcracks in the epidermis. The fungus did not form an extensive network of intercellular hyphae within the living tissue after entry via natural openings, as was seen on bean leaves by O'Donnell & Dickinson (1980) and on wheat leaves by Dickinson (1981). Hyphae remained localized in the substomatal cavities or lenticels and, although the fungus was able to grow into epidermal cells adjacent to microcracks in the epidermis, or those surrounding wounds, it would appear that this process is a slow one. No necrotic tissue formed around wounds after 168 h following inoculation under temperature and humidity conditions conducive to the fungus. In the case of epidermal cracks, hyphae seemed to be restricted to a few epidermal cells and did not cause cell necrosis. The present study therefore substantiates previous reports (Swart & Holz, 1991, 1994) of the weakness of *A. alternata* as a pathogen of table grape bunches.

Hyphae of *A. alternata* can colonize large areas of the surface of green plants without requiring any nutrients other than those contained in the conidia (Dickinson & Bottomley, 1980). Our observations on table grapes substantiate this epiphytic growth habit of *A. alternata*. Conidia of the fungus germinated readily and grew extensively on berries, pedicels and rachises of immature and mature table grape bunches. This behaviour must inevitably increase the ability of the fungus to find sites suitable for penetration. The formation of thick-walled hyphae underneath the wax layer, and colonization of stomata and lenticels, might furthermore help *A. alternata* to survive for long periods under unfavourable conditions and account for the high level of detection of the fungus from triple-sterilized bunch parts during the entire period of bunch development (Swart & Holz, 1994). Survival of *A. alternata* on the phylloplane has previously been ascribed to dark-pigmented thick-walled hyphae (Dickinson, 1981).

Previous studies indicated that export table grapes might be predisposed to *A. alternata* decay by prolonged storage at low temperature and sulphur dioxide (SO₂) treatment (Swart & Holz, 1991, 1994). Our data on the histopathology of the pathogen support this premise. Export table grapes, which are kept at -0,5°C during transit, are fumigated for a short period after package with SO₂ to protect them against new *Botrytis cinerea* infections. This process is regulated by adding a sodium metabisulfite generator inside a polyethylene bag in boxes (László *et al.*, 1981; Nelson, 1983). Several problems are still encountered with SO₂ treatment. The SO₂ can damage the fruit by entering wounds in their surfaces caused by stem tears and cracks (Ballinger & Nesbitt, 1984). SO₂ can also damage berries by bleaching, increase the rate of water loss and thus cause premature browning of stems. These effects are

usually noticed during storage on grapes not properly pre-cooled (László *et al.*, 1981). However, fumigation with SO₂ will also kill *Alternaria* spores or mycelium on the surface of grapes, but will not kill mycelium that has invaded the berry (Harvey, 1955; Couey, 1965; Smilanick *et al.*, 1990). Hyphae in stomatal chambers, lenticels, in epidermal tissue adjacent to microcracks, and probably those protected by epidermal wax, will not be affected by SO₂. They will emerge and develop over the surface to infect damaged tissue under conditions of high humidity prevailing inside polyethylene bags when ambient temperature is raised from -0,5 to 10°C. This might explain the extensive superficial growth of *A. alternata* observed on rachises and pedicels at the end of the cold storage period (Swart & Holz, 1991).

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