# Application of Interference Microscopy to the Study of Fungal Penetration of Epidermal Cells

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ABSTRACT

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Penetration of outer epidermal cells of susceptible white onion leaf bases by *Botrytis allii* was accompanied by halo formation. Halos were visible using interference microscopy due to dry mass loss in the cell wall around the penetration site. Halos also were visible using cell wall stains. Reaction mass (dry mass deposition) was observed below penetration sites in resistant cells of red onions.

*Additional key words*: onion bulb, *Botrytis allii*, penetration halo, reaction mass.

A halo in an infected plant cell is recognized as an area in the host cell wall around the penetration point of a fungal pathogen which differs in stainability from that of the normal cell wall as a result of degradation of cell wall constituents caused by enzymes secreted from the pathogen during penetration (2, 6, 8). There is no published report of halo observation using interference microscopy, and there are very few reports about halo formation in diseases incited by facultative saprophytes (1, 5). The purpose of this study was to use interference microscopy to observe and compare the effects of penetration by *Botrytis allii* Munn. into cells of susceptible and resistant onion epidermal tissue.

## MATERIALS AND METHODS

Two techniques were used to study cell wall penetration:pieces of stripped, outer epidermis of white onion (*Allium cepa* L.) bulb scale (equatorial region, turgid bulb scale) were floated on water or kinetin solution ($10^{-4}$M); and, square pieces of red and white onion bulb scales were placed in petri dish incubators. Both types of onions were obtained locally and the source and cultivar names were not known. The host tissues were inoculated with spore suspensions of *B. allii* (isolated from onions and grown on Czapek-Dox agar for seven days at 25 C) and incubated in the dark at 25 C for 24 hr. Floated epidermal strips or those removed from incubated pieces were observed using a Leitz interference microscope with polychromatic light from a tungsten lamp, and the dry mass of the halos was compared to that of normal cell walls using the primary and secondary interference fringes. Technical aspects of interference microscopy have been described by Ross (9). The infected epidermal cells also were observed after staining with safranin or methylene blue. Pyronin B was used to stain the epidermal cells of the incubated pieces of red onion.



## RESULTS

Disk-shaped halos around penetration sites were observed as areas of distinct color contrast to parts of the cell wall not penetrated using polychromatic light and interference microscopy (Fig. 1). Although halos were observed in both water and kinetin treatments, more penetrations and larger halos were observed in the cell wall of epidermal strips floated on kinetin solution. Using an interference fringe, the dry mass in the halo area was found to be less than that in the cell wall more distant from the penetration site and the anticlinal walls (Fig. 2). The interference fringe appears to be refracted to a less extent in the halo than in the cell wall proper; that is, the entire fringe appears to bend in an opposite direction to that seen passing through anticlinal cell walls which represent areas of high dry mass concentration per unit area of wall being observed. When the outer surface of the same halo area was stained with methylene blue and the lower surface with safranin, a negative reaction was noted for safranin in the penetrated cell wall in the halo area (Fig. 3). The halo appeared to be stained only faintly blue compared to the deep blue staining of spores and germ tubes. The halos averaged $13 \times 10^{-6}$cm$^2$ in area 24 hr after spore inoculation. The halos found in the epidermal cell wall of the incubated pieces from white onion bulbs were mostly of the same size.

When strips from resistant red onion bulb scales were studied, large areas with higher dry mass per unit area (like that in anticlinal walls) were observed (Fig. 4). We call this area "reaction mass." This was not observed in white onion. The increase in dry mass was inferred from the displacement of the interference fringes like that observed in anticlinal walls (Fig. 5). The size of the reaction mass associated with the epidermal cell wall of the red onion that became stained by pyronin B was equal

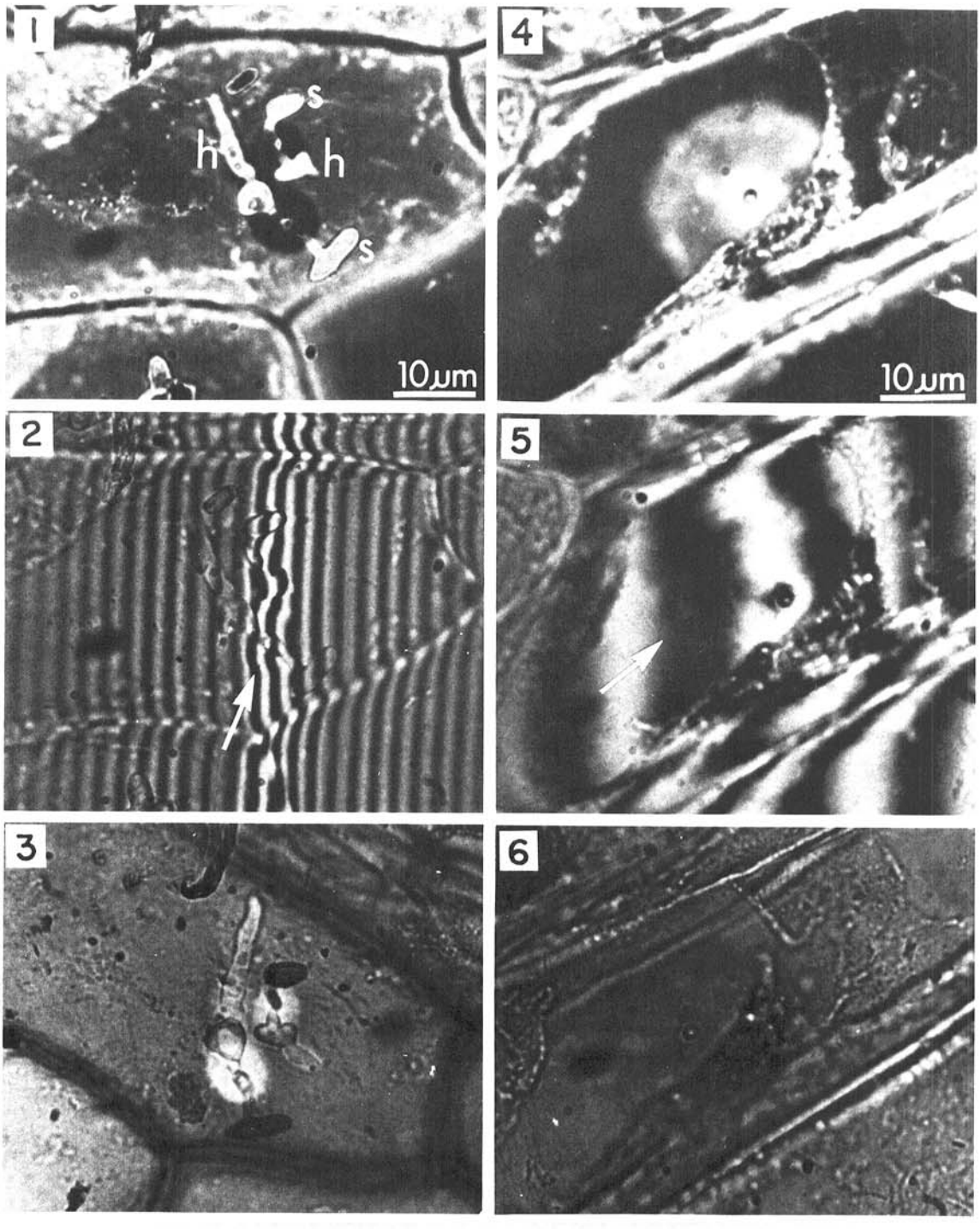


**Fig. 1-6.** Two modes of polychromatic interferometry and two types of staining procedures to study the penetration sites of *Botrytis allii* in susceptible white onion epidermis with halo formation (1-3) and in resistant, red onion epidermis with "reaction mass" (4-6). All cells were photographed at ×500 and enlarged as indicated (S = spore; H = internal hyphae). In Fig. 1-3 the same cell is presented in each photo. **1)** The primary fringe is spread to create a uniform color background. The halo appears as a dark area around the penetration site. **2)** The primary and secondary fringes are collapsed and observed as they pass through the cell and halo. The arrow shows where the fringe appears to be refracted as it passes through the halo. **3)** Methylene blue was used to stain the spore and germ tube on the outer surface of the cell wall and safranin was used to stain the inner surface of the cell wall and the hyphal tip that has penetrated the cell wall. The halo is seen around the penetration site. In Fig. 4-6 the same cell is presented in each photo. **4)** The primary fringe is spread to create a uniform color background. The reaction mass appears as a brighter area around the penetration site. **5)** The primary and secondary fringes are collapsed and observed as they pass through the cell and reaction mass. The arrow shows where the fringe appears to be refracted as it passes through the reaction mass. **6)** The reaction mass can be stained with pyronin B.

to that of reaction mass found by interference microscopy (Fig. 6). No halo was observed around the penetration site following methylene blue and safranin staining.

## DISCUSSION

The present study is the first report that interference microscopy can be used in research regarding the mechanism of penetration and halo or reaction mass formation in living cells. The technique with living cells has a great advantage over the normal fixing and staining procedures which kill the cells and prevent sequential studies. The formation of halos on white onion epidermal cell walls was caused by a decrease in cell wall mass. We infer that cell wall penetration involved decomposition by enzymes secreted from the pathogen. Fungal pathogens have been shown to produce cuticle and cellulose-degrading enzymes (4, 10). The negative reaction of safranin with the cell wall in the halo area also suggests that enzymes were acting locally at the penetration site. Methylene blue did not stain the halo in the host cell wall penetrated by *B. allii* as deeply as it stained the spores and germ tubes. McKeen et al. (8) reported that a blue disk-shaped halo was observed in powdery mildew-infected plant tissues stained with methylene blue. Lupton (7) has discussed the mechanism of halo formation in host-parasite interactions in obligate parasites, and found the size of halo varied according to the species inoculated.

In our studies, variation in the size of halos was observed. The largest halos were associated with penetration into walls of susceptible tissue floated on kinetin solutions. Halos were not observed in walls of cells of resistant tissue but penetration induced a structure which we call the reaction mass. Halos were smaller in size than reaction mass. Based on qualitative interference microscopy, we conclude that halos in white onion cells lost dry mass whereas reaction mass in resistant tissue gained dry mass relative to normal walls. Reaction mass may be a result of an increase in cytoplasm around the penetration site, an active deposition of dry mass between the cytoplasmic membrane and the wall like pappilae or calosities (1,3), or both. Reaction mass could be formed as part of a resistance reaction since certain phenolic substances in the outer epidermis of colored scales have been shown to be toxic to this fungus (11). Since penetration is initiated in resistant cells, we anticipate that small halos form in walls but cannot be detected because reaction mass material fills the halo. The distinction between halo and reaction mass will require further study using several cytological methods including quantitative and qualitative interference microscopy over the entire penetration period to reveal details of earlier phases of both halo and reaction mass formation.

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