A Note on the Effects of a Product of *Aspergillus niger* on Nuclei of *Allium cepa*

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Received November 21, 1978

An avirulent *Aspergillus niger* van Tiegh. reduced nuclear dry mass (NDM) of cells of *Allium cepa* L. 0-5 mm ahead of mycelium by 11 % in comparison with normal cells 15-20 mm ahead (Kulfinski and Pappelis 1971, 1976, Kulfinski *et al.* 1973). The fungus penetrated host tissue by less than 1 mm, few cells were killed in advance of mycelium and only a very narrow watery lesion was produced. In contrast, virulent *A. niger* isolated from an infected white onion bulb produced wide watery lesions, killed cells in advance of mycelium, and reduced NDM of cells 0-5 mm ahead of mycelium by approximately 40 % (Kulfinski and Pappelis 1976, Kulfinski and Pappelis 1978, Kulfinski *et al.* 1973). We inferred that *A. niger* secreted a toxic substance into host tissue and that, if the amount of this substance varied with the fungal isolate, then this substance might be responsible for differential virulence. The purpose of this study, therefore, was to test the hypothesis that *A. niger* will also produce this toxic substance *in vitro*. We wished to produce the substance in quantity as a prelude to pathogenicity, purification and characterization studies. We recognized the possibility that different toxic substances might accumulate *in vivo* and *in vitro*; however, we proceeded on the premise that the same substance would be produced. Nuclear degeneration and cell death in squares of epidermal tissues 5 mm on a side were used to assay for presence and effectiveness of fungal product.

Materials and methods

Virulent *A. niger* isolated from white onion was cultured in Czapek-Dox broth (500 ml per 1500 ml Ehrlenmeyer flask) on a reciprocating shaker at 90 excursions per minute under ambient cool-white fluorescent lighting for 7 days at 23°C. The spent culture medium was sterilized by filtration through a 0.04 µm porosity filter. Two adjacent squares of tissue 5 mm on a side were removed from the equatorial region of the third turgid bulb scale of each of six white onions measuring 10 cm in diameter. One square was placed in distilled water and the second in culture filtrate. After incubation under ambient laboratory conditions for a period of 15 minutes, each square was recovered and its adaxial epidermis was mounted in distilled water. A cover slip was added and ringed with paraffin oil to prevent evaporation. Average optical path difference per nucleus was determined by means of a Leitz interference microscope using light of 546 nm, and converted into dry

mass per unit area (DM/A) using $\alpha=0.0018$ (Beneke 1966). Nuclei were photographed, negatives projected, and nuclear images traced. The tracings were measured by means of a planimeter and converted to nuclear area (NA). DM/A and NA were multiplied to yield nuclear dry mass (NDM).

A modification of the Luyet (1937) neutral red staining technique was used for determining cell death. Onion epidermis was immersed in a 0.5% aqueous solution of neutral red and then mounted in water and examined microscopically. In our experience with living cells, cytoplasm remained colorless, whereas intact vacuoles stained rose-red. Cells were defined as dead when the vacuolar membrane lost its integrity, at which time the sharp distinction between cytoplasm and vacuole disappeared and both became orange in color. This is consistent with the "moment of cell death" as defined by Bessis (1964). Following cytoplasmic death, the nucleus often became red, whereas it remained colorless in live cells. Neutral red differential stainability correlated with ability to plasmolyze. Epidermal strips were immersed for 15 sec in culture filtrate and the percent viable cells was determined using neutral red stainability. No differences were found in percent viable cells between cells incubated in distilled water or those incubated in fresh nutrient broth as controls.

Results and discussion

Neutral red staining and plasmolysis studies showed that 50% of the live cells in stripped epidermis were killed by a 15 sec immersion in culture filtrate. When pieces of epidermis were incubated in culture filtrate for 15 min, all cells were killed. Very few cells were killed in controls (distilled water or Czapek-Dox broth). The killing effectiveness of the culture filtrate was not diminished after 15 min boiling followed by cooling to 23°C. Epidermal cells incubated in filtrate for 15 min decreased by 31% in NA and 29% in NDM, whereas the DM/A remained essentially unchanged (Table 1). NA and NDM of individual cells were not well correlated

Table 1. Characteristics of nuclei of control and culture filtrate treated onion epidermal cells. NA=nuclear area in $\text{cm}^2 \times 10^{-8}$, DM/A=dry mass per unit area in $\text{g}/\text{cm}^2 \times 10^{-5}$ and NDM=nuclear dry mass in $\text{g} \times 10^{-10}$. Each value is a mean of 60 nuclei among 6 replicates

Treatments	Nuclear characteristics		
	NA	DM/A	NDM
Control	8.7	12.4	10.4
Filtrate	6.0 ^a	12.3	7.4 ^a
F/C Percent	69	99	71

^a Characteristics so marked are significantly different from their respective controls, at the 95% level of probability.

in either control or filtrate-treated tissue, the correlation coefficients being $r=0.38$ and $r=0.26$, respectively.

The rapid killing of stripped host epidermal cells by culture filtrate and the

reduction in NA and NDM were accompanied by the development of a water-soaked appearance of bulb scale tissue and generalized development of minute vacuoles (bubbling) within the cytoplasm of these cells. These symptoms are comparable to those in onions infected with *A. niger*, particularly just ahead of advancing hyphae. We infer that both symptoms are attributable to a toxic substance produced by this pathogen. Narain and Prakash (1968) reported finding that *A. niger* isolated from onion produced a thermostable toxin in culture. Khazaradze (1960) stated that the first morphological changes in onion tissue infected with *Botrytis allii* appear in nuclei and postulated the production of toxic substances by that pathogen. Kulfinski, *et al.* (1973), and Kulfinski and Pappelis (1976) demonstrated that *B. allii* and virulent *A. niger* produce similar nuclear degeneration. *B. allii* reduced NDM by 41% and virulent *A. niger* reduced NDM by 42%.

Our data confirm the presence of a toxic factor in *A. niger* culture filtrate and suggest that it operates at the level of the nucleus. Since similar damage is produced by virulent *A. niger* infection as by its culture filtrate, we cannot reject the hypothesis that the same toxic factor is involved in producing the damage in each case.

Abstract

A heat stable substance was produced by *A. niger*, accumulated in the culture medium, and induced nuclear area and nuclear dry mass decreases in inner epidermal cells of onion bulb scales comparable to decreases observed 0–5 mm ahead of mycelium in infected bulbs. When tissue squares 5 mm on a side were incubated in culture filtrate, half of the cells were dead after 15 seconds incubation and all were dead in 15 minutes. Because the effects of infective mycelium and culture filtrate were comparable in kind of nuclear changes produced and similar in magnitude of these changes, we suggest that the same substance was produced by the fungus *in vitro* as during infection.

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