Interference Microscopy of Onion Epidermal Nuclei in Response to Botrytis allii Infection

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ABSTRACT

The nuclear area (NA), nuclear dry mass/cm^2 (DM/A), and the total nuclear dry mass (NDM) of host cells (inner epidermis of the equatorial region of onion bulb scale) were determined in three locations (0 through 5 mm, 6 through 10 mm, and 16 through 20 mm) adjacent to a cork-borer wound (sterile or inoculated with *Botrytis allii*). Inoculation with *B. allii* resulted in a decrease in NA (72%), DM/A (82%), and NDM (60%) of cells 0-5 mm from the site of inoculation when compared with those 16-20 mm from this site (48-hr incubation) within the same bulbs. It also resulted in decreases of NA (71%), DM/A (96%), and NDM (69%) in cells from inoculated bulbs 0-5 mm from the site of inoculation when compared with these characteristics for nuclei of cells in the same location in noninoculated sites. Wounding induced an NA increase (104%) and DM/A and NDM decreases (87 and 93%, respectively) in nuclei of cells 0-5 mm away from the wounding site when compared with those 16-20 mm away. We conclude that *B. allii* secretes substances which induce nuclear pycnosis in host cells. Phytopathology 61:724-727.

Nuclear size in plant and animal cells changes in response to various environmental conditions. Bessis (6) indicated that nuclear pycnosis is one symptom in the "death agony" of a cell, and characterized pycnosis as a combination of decline in nuclear size, increase in nuclear density, and increase in the affinity for nuclear stains. Eckert & Cooper (10) reported that response of animal tissues to radiation included: first, enlargement of nuclei; then pycnosis. Kasten (12) reported that nuclei of kidney cells undergoing autolysis increased in size to 180% of the controls in 1 hr, decreased to approx 70% of the controls by 2 hr, and continued to decrease reaching 50% of the controls by 28 hr. Berenbom et al. (5) determined that nuclei lost 96% of their RNA in 48 hr in autolyzing liver tissue, whereas DNA and protein decreased more gradually. Goodman et al. (11) indicated that first swelling and then degradation of host nuclei are common symptoms in fungus infections. Nuclear volume increases have specifically been shown in wheat rust (1, 7, 8, 21) and in club root of cabbage (22). Decreases in nuclear size generally follow the initial increases which occur under degradative conditions such as radiation (10, 19), autolysis (12, 15), aging (6), and fungal infections (11, 21). Langley (14) suggests that there is greater permeability in damaged (removed) nuclei, while Lai et al. (13) describe several membrane permeability altering factors in mung bean tissues infected by *Rhizoctonia solani.*

Although the mechanism of pycnosis and nuclear swelling is not known, it is interesting to note that positively charged macromolecules such as polylysine and protamine caused a marked contraction of free nuclei of *Amoeba proteus,* while negatively charged macromolecules such as polyglutamate and heparin resulted in swelling (2). The induced contraction and swelling were found to be reversible. Wolpert & Gingell (23) stated that ribonuclease is a positively charged macromolecule like polylysine, and found that both induced contraction of the surface membrane of the egg of *Xenopus laevis.* It may be that ribonuclease could induce both pycnotic and autolytic changes in injured cells.

Almost all cytochemical quantitative investigations present formidable difficulties and are subject to appreciable errors. Recent advances in quantitative interference microscopy and microdensitometry can overcome some errors by allowing the measurement of the dry mass distribution in living or fixed cells or in any cellular object that can be clearly viewed using light microscopy. These measurements are based on the retardation of light passing through the object being viewed in comparison with a reference beam of light. The accuracy for mass determination is very great (1×10^{-14} g/square μ), and permits the mass determination of objects as small as nucleoli or the comparison of mass distribution in the cytoplasm. Both visual and densitometric measurements have been used to determine phase change. Much of this work has been reviewed by Ross (17). Once total mass has been determined, the mass of other specific components can be measured by preferential digestion and extraction followed by reweighing. Using this type of quantitative interference microscopy, Bassleer (3) measured the protein and DNA content in nuclei of fixed and living chick, rat, and mouse fibroblasts during mitosis. He concluded that protein and DNA content doubled during interphase just before cell division, but that the two syntheses were not synchronous. The effect of a number of antimitotic agents, as well as ribonuclease and deoxyribonuclease on rat fibroblasts, was studied.

Several others have attempted to measure the amount of DNA per nucleus using quantitative interference microscopy (4, 9, 16, 20). In some studies, the nuclear

dry mass was determined before and after trichloroacetic acid hydrolysis and before and after deoxyribonuclease treatment. In each, the loss of nuclear dry mass was considered to correspond to DNA content. Good agreement was obtained with chemical methods to determine average DNA content for nuclei. Bibbiani et al. (9), using the deoxyribonuclease digestion method, consistantly found that 5 to 8% of the DNA remained in the nucleus following enzyme digestion. He concluded that this method was better than chemical methods for determining DNA in nuclei. Ross & Jans (18) studied nucleolar dry mass and density changes associated with dystrophied cells of mice. Myoblasts from animals with muscular dystrophy had nucleoli of abnormally low dry mass. As the disease progressed, the number of nuclei with this condition expressed increased. We have found no previous studies in plant pathology utilizing quantitative interferometry.

The present study was designed to measure changes induced in nuclear area (NA), nuclear dry mass/cm^2 (DM/A), and nuclear dry mass (NDM) in inner epidermis cells of onion wounded and infected with *Botrytis allii,* a neck rot pathogen of onion.

MATERIALS AND METHODS.—Eight white onions (*Allium cepa* L.) which were approx 9 cm in diam were each bored with a sterile 5-mm diam cork-borer to a depth of 2 cm at each end of a diam at the equator of the bulb, and the discs of bulb scale tissue were removed. One boring was left as a control; the other on the opposite side of the bulb was inoculated with *Botrytis allii* Munn. The onions were incubated in a covered, humid chamber in the dark at a temperature of 25 C for 48 hr. After incubation, the onions were removed from the chamber and four consecutive 5-mm^2 inner epidermis samples to the left of each hole in the equatorial plane were removed. The first, second, and fourth such samples were studied. Five to 10 nuclei were photographed in each sample, the photographic negatives were projected, the nuclear images traced, and the area of each image was determined by means of a planimeter. In each case of wounding and inoculation, the fungal mycelium was confined to the first mm adjacent to the site of inoculation, but watery lesions extended for greater distances. None of the tissue around noninoculated wounds contained any visible pathogen.

Since control and infected tissue were 180 degrees apart around the onion's equator, and it was not known whether a uniformity of cell and nuclear characteristics existed around the bulb, it was decided that nuclei of locations 4 (assumed to be least influenced by either wounding or infection) would be used as a reference for determining the responses of nuclei of locations 1 and 2. In addition, treatment means for each location would be compared.

A min of 5 nuclei and a max of 10 were measured/tissue sample. In the control, 54, 55, and 54 nuclei were measured in locations 1, 2, and 4, respectively. The corresponding numbers measured from infected tissues were 51, 49, and 56. Average phase retardation per nucleus was measured using light of 546 nm wavelength. An α value of 0.0018 was used in converting phase retardation to DM/A. NA was multiplied by DM/A to yield NDM, according to the method given by Beneke (4).

RESULTS.—Locations 1, 2, and 4 adjacent to *B. allii*-infected borings will be referred to as B1, B2, and B4. These locations around noninoculated borings (control) will be referred to as C1, C2, and C4.

The data (Table 1) indicate that in B1 the decline in NA due to infection was 28% of the NA in B4 and 29% of the NA of C1. The decline in NA in B2 was only 7% with respect to B4, indicating that the major effects of infection were in B1. C1 and C2 exhibited NA increases of 4 and 3%, respectively, with reference to nuclei in C4. These are interpreted to be wounding responses. In B4, the nuclei were 3% greater in NA than C4. The NA response in onion to *B. allii* infection can be summarized as a sharp decline with proximity to the infection site and a slight increase at a distance from it.

The DM/A in B1 declined by 4% due to infection (compared to C1) and increased by 2% in B4 (compared to C4). DM/A declined due to wounding (C1 is 87% of C4) and wounding plus infection (B1 is 82% of B4). DM/A in B2 (compared to B4) and C2 (compared to C4) decreased 3 and 2%, respectively. Again, locations 2 were little different from locations 4, with

TABLE 1. Comparison of mean values for nuclear area (NA), dry mass per unit area (DM/A), and nuclear dry mass (NDM) of control (C) and *Botrytis allii* infected (B) epidermal tissue of onion

	Nuclear characteristic								
	NA			NDM			DM/A		
	Location[a]			Location[a]			Location[a]		
Treatment	1	2	4	1	2	4	1	2	4
		$\times 10^{-6}$ cm^2			$\times 10^{-10}$ g			$\times 10^{-5}$ g/cm^2	
Botrytis-infected	6.16	7.95	8.55	9.2	14.0	15.3	15.1	17.8	18.4
Control	8.62	8.51	8.27	13.4	14.3	14.4	15.8	17.8	18.1
		%			%			%	
B as % C, each location	71	93	103	69	98	106	96	100	102
B1 and B2 as % B4	72	93	100	60	92	100	82	97	100
C1 and C2 as % C4	104	103	100	93	99	100	87	98	100

[a] Locations 1, 2, and 4 represent first, second, and fourth 5-mm square tissue samples away from the site of inoculation and/or wounding. Each number represents the mean of 8 replications averaging 7 subsamples each.

most of the influence of wounding and infection being felt in locations 1. Decreases due to wounding and to wounding plus infection were similar, with the latter being slightly greater.

The NDM in C1 (compared to C4) declined by 7%, and that in B1 (compared to B4) by 40%. The NDM in B1 was 31% less than that in C1. In locations 2, there was a 1% decline in NDM due to wounding (C2 compared to C4) and an 8% decline due to infection (B2 compared to B4). The NDM in B4 exhibited a 6% increase when compared to C4.

Correlation coefficients (r) were calculated between pairs of nuclear characteristics (Table 2). Correlation was negative between NA and DM/A, with the latter decreasing as the former increased in all cases. In the wounded but noninoculated tissue, correlation between NA and DM/A decreased, NA and NDM increased, and DM/A and NDM remained nearly the same with proximity to the wound. In infected tissue, correlation between NA and NDM increased, while that between NA and DM/A and between DM/A and NDM decreased with increasing proximity to the infection. In C4, the most normal, NDM was more closely correlated to DM/A ($r = 0.61$) than to NA ($r = 0.28$).

Discussion.—Several bases for comparison of the data are possible, and two of these have been employed in Table 1. The first relates wounded and infected (B) to wounded noninoculated (C) as a percentage within each location. In this manner, wounding effects can be subtracted, leaving infection effects only, assuming such effects are simply additive. The second comparison relates data for locations 1 and 2 to their respective location 4. This assumes that location 4 was affected little or none, which is indicated by the similarities of location 4 (infected vs. noninfected) means, with differences ranging from 2% in concentration to 6% in total dry mass. There is no certainty, however, that infected and control locations 4 would continue to be as similar as they were here, given slightly different conditions or pathogens. Furthermore, should wounding and infection produce common effects in locations 4, then the assumption that locations 4 have not changed would be invalid, and comparisons of locations 1 and 2 to locations 4 would be erroneous estimates of infection effects. Comparisons to location 4 would tend to minimize differences which might develop as a result of nuclei differing in properties around the bulb (e.g., if C and B were initially different in nuclear properties, then it would be more appropriate to relate C1 to C4 and B1 to B4 rather than C1 and B1 to C4). More importantly, however, this comparison permits an analysis of effects of wounding and infection with distance from the wounding site.

Table 2. Correlation coefficients (r) between nuclear area (NA), dry mass per unit area (DM/A), and nuclear dry mass (NDM) of control (C) and *Botrytis allii* infected (B) epidermal tissue of onion

Treatment	Location[a]	r values		
		NA & DM/A	NA & NDM	DM/A & NDM
C	1	−0.25	0.59	0.62
C	2	−0.59	0.38	0.50
C	4	−0.56	0.28	0.61
B	1	−0.11	0.86	0.40
B	2	−0.22	0.48	0.65
B	4	−0.55	0.58	0.33

[a] Locations 1, 2, and 4 represent first, second, and fourth 5-mm square tissue samples away from the site of inoculation and/or wounding.

It must be stressed that *B. allii* entered only the first mm of epidermal tissue (B1), and was entirely absent elsewhere. The nuclei in contact with mycelium were disintegrated, making their measurement impossible with the techniques employed in this study. The data from location 1, whether in response to wounding or to infection, represent an average of nuclei adjacent to mycelium or as much as 5 mm away from it, whereas the cells adjacent to the mycelium (or wound) might exhibit greater treatment effects. In either case, wounding or wounding plus infection, the response of nuclei at a distance from the wounding-inoculation site suggests that biologically active chemicals are being released and are eliciting responses away from their site of production or release.

The results indicate that NA, DM/A, and NDM declined by 29, 4, and 31%, respectively, due to fungus infection (B1 to C1 in each case). Wounding increased NA by 4% and decreased DM/A and NDM by 13 and 7%, respectively (C1 to C4). Locations 2 responded very little compared to locations 1, although infection produced slight decreases in NA and NDM.

Location 4, at considerable distance from the inoculation site, exhibited 3, 2, and 6% increases in NA, DM/A, and NDM (B4 compared to C4), respectively. The latter changes, while small in degree, are comparable in trend to those reported in a number of plant diseases. The magnitude of the increases suggests that they may not be real ones. It is not known whether the increases or decreases in nuclear measurements discussed herein are due directly to fungal products or indirectly to products formed or released by the host in response to stimuli initiated by the fungus (or wounding).

While the average decrease in NDM was one of 40% (all replicates) due to *B. allii* (B1 compared to B4), the greatest decrease observed was one of 78% (one replicate), and, as has been suggested, the decreases were probably greater in the immediate proximity of mycelium. The conclusion follows that major biological components of host nuclei are being lost, with the implications from the work of Berenbom et al. (5) and Kasten (12) that these are RNA and nonhistone protein, initially, followed by a gradual loss of DNA-histone.

It is interesting to note that plant and animal tissues respond in the same general ways to deleterious factors on the subcellular level, making it probable that cytological studies will become as important in plant pathology as they have in animal pathology. A similar relationship may exist in cellular and nuclear studies of aging in plants and animals. Diagnosis of damage, identification of pathogens, and understanding of disease physiology may all be enhanced by an emphasis on

quantitative interferometric and cytochemical studies in plant pathology.

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