Acridine Orange as a Lysosome Marker in Fungal Spores

Charles L. Wilson, Gene A. Jumper, and David L. Mason

Research Plant Pathologist, Science and Education Administration, U.S. Department of Agriculture, and also Adjunct Professor, Department of Plant Pathology, Ohio Agricultural Research and Development Center, Wooster, OH 44691; Graduate Research Associate, Department of Plant Pathology, The Ohio State University, Columbus, OH 43210; and Associate Professor, Biology Department, Wittenburg University, Springfield, OH 45507.

The research was partially supported by NIH Grant AI 11556-01 from the National Institute of Allergy and Infectious Diseases.

Approved for publication as Journal Article 47-78 of the Ohio Agricultural Research and Development Center, Wooster.

Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture, and does not imply approval of it to the exclusion of other products that also may be suitable.

Accepted for publication 1 June 1978.

ABSTRACT

WILSON, C. L., G. A. JUMPER, and D. L. MASON. 1978. Acridine orange as a lysosome marker in fungal spores. Phytopathology 68:1564-1567.

Acridine orange fluoresces red-orange in the vacuoles of spores of *Ceratocystis ulmi, Botrytis cinerea,* and *Cryptococcus neoformans* placed under UV light. To compare animal and fungal lysosomes, mouse macrophages that had engulfed spores of *C. neoformans* were treated with acridine orange. Similar red-orange fluorescence under UV was observed in the animal macrophage and in the vacuoles of the fungal spore. Acid phosphatase (a marker for lysosomes) was concentrated primarily in the vacuole of the spores of *B. cinerea*. These results indicate that the vacuoles of *C. ulmi, B. cinerea,* and *C. neoformans* probably are lysosomes and that acridine orange can be used as a selective stain for these lysosomes in fungi just as it is used to detect lysosomes in animal cells.

Additional key words: vacuoles, *Ceratocystis ulmi, Botrytis cinerea*.

Acridine orange is a vital dye that has been used for a variety of cytological determinations. In fixed tissue, its fluorescence under ultraviolet (UV) light can be used to characterize DNA and RNA in the cell (1). Animal cytologists also use acridine orange to stain lysosomes in living cells (1). Lysosomes fluoresce red-orange under UV light when cells are allowed to take up acridine orange. Pitt (6) illustrated acridine orange fluorescence under UV in *Botrytis cinerea* hyphae, but he did not investigate this phenomenon in detail. The purpose of this investigation was to determine whether acridine orange could be used as a marker for fungal lysosomes.

MATERIALS AND METHODS

For light microscopic observation of the uptake of acridine orange, spores from 4-day-old cultures of *Ceratocystis ulmi* (Buism.) C. Moreau, *Botrytis cinerea* Pers. ex Fr., and *Cryptococcus neoformans* (Sanfetice) Vuillemin grown on PDA were mounted in a solution of acridine orange (1: 20,000, w/v) on a microscope slide and a coverslip was attached to the slide with melted paraffin. The preparations were examined by fluorescence microscopy with a Zeiss epifluorescence system. An HBO 200-W lamp with strong emission in the blue-violet region (400-500 nm) was used as a light source. A BG-12 excitation filter was employed and a yellow minus blue suppression filter was used for viewing. Exposures of 2-12 sec with Kodak Ektachrome X film were necessary to record the color fluorescence of acridine orange (Fig. 1).

To stain acid phosphatase (a lysosomal marker) in *B. cinerea,* spores were fixed in 6% glutaraldehyde, pH 7.2, in 0.1 M potassium-sodium phosphate buffer for 3 hr. Then the spores were recovered from the fixative by filtration and washed in three 5-min buffer washes. Spores were treated for the Gomori reaction (2) for which the following served as controls: (i) Gomori medium minus substrate and (ii) Gomori medium with 1 mM NaF. All preparations were incubated for 2 hr at 37 C, after which spores were washed in 1% acetic acid for 5 min and then washed in potassium-sodium phosphate buffer. Dehydration, embedding, and sectioning were done according to standard procedures for Epon resin.

For observation of macrophages engulfing spores of *C. neoformans,* mice first were inoculated subcuticularly behind the left front shoulder with 1-1.5 ml of tetanus toxoid to promote macrophage production (7). One wk later a similar amount (1-1.5 ml) of tetanus toxoid was injected intraperitoneally. One day after the second dose, spores of *C. neoformans* were injected intraperitoneally into the mouse. The next day, the peritoneal cavity was opened and the accumulated macrophages were extracted with sterile saline solution and concentrated by low-speed centrifugation. A drop of the concentrated saline solution containing macrophages and spores of *C. neoformans*

This article is in the public domain and not copyrightable. It may be freely reprinted with customary crediting of the source. The American Phytopathological Society, 1978.

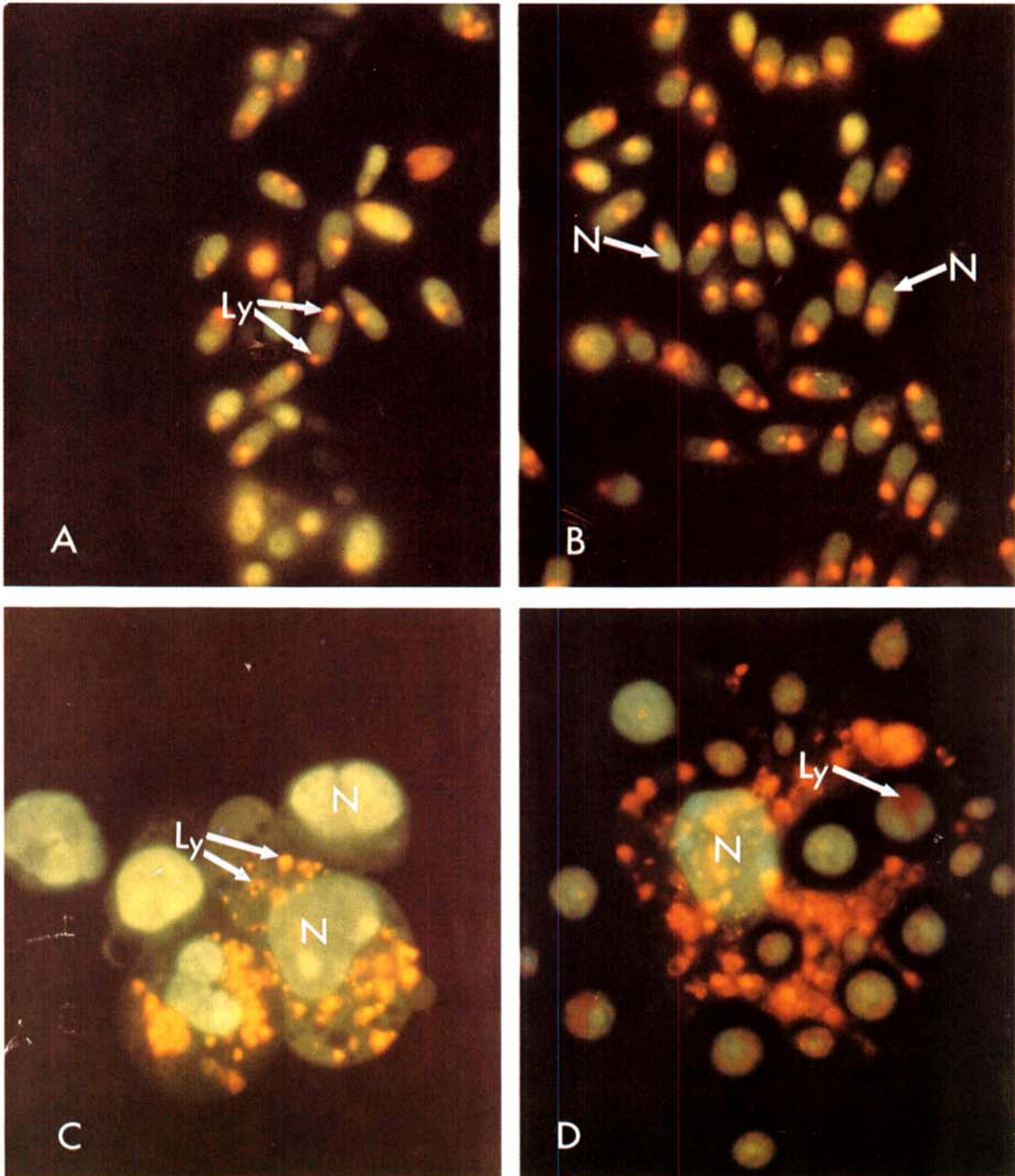

Fig. 1-(A to D). Acridine orange fluorescence of spores of *Ceratocystis ulmi* (A, B) and *Cryptococcus neoformans* (D) and mouse macrophages (C, D). **A)** Spores of *C. ulmi.* Acridine orange fluoresces red-orange in vacuoles indicating that they are lysosomes (Ly). (~× 5,000). **B)** Spores of *C. ulmi.* Acridine orange fluoresces green in nuclei (N). (~× 5,000). **C)** Mouse macrophages. Acridine orange fluoresces red-orange in particulate bodies (lysosomes) (Ly). Nuclei (N) fluoresce green. (~× 5,000). **D)** Mouse macrophage engulfing spores (phagocytosis) of *C. neoformans.* Orange fluoresence of lysosomes (Ly) in macrophage cell and vacuoles of *C. neoformans* spores. Macrophage nucleus (N) fluoresces green (~× 5,000).

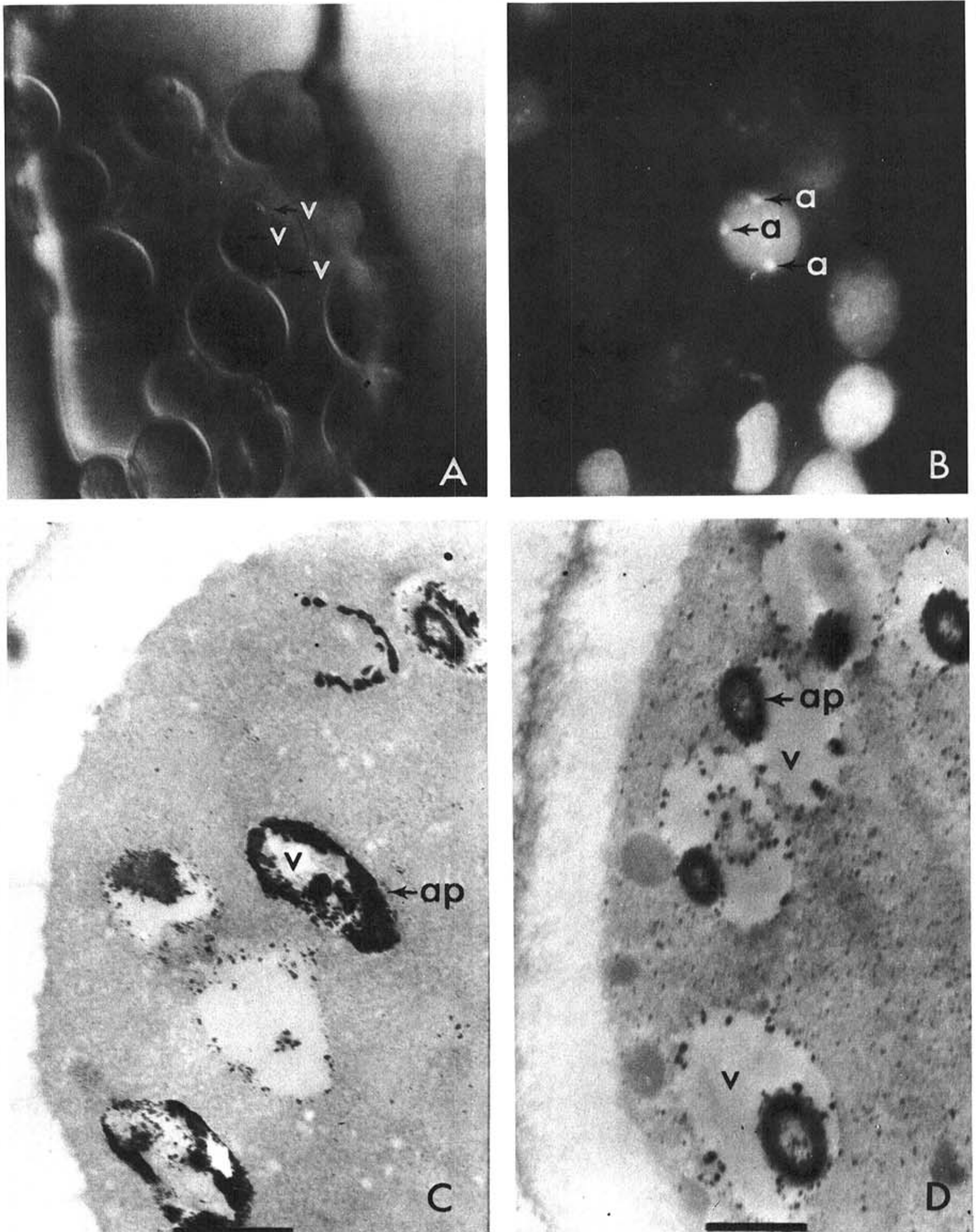

Fig. 2-(A to D). Vacuoles (lysosomes) in spores of *Botrytis cinerea*. **A**) Nomarski interference micrograph of spores of *B. cinerea* treated with acridine orange. Vacuoles appear as raised inclusions (×3,500). **B**) Same as A except viewed with fluorescence microscopy. Vacuoles have particulate inclusions (a) that fluoresce red-orange (×3,500). **C**) Electron micrographs of spore of *B. cinerea* treated for Gomori reaction. Dark-staining acid phosphatase (ap) is concentrated in vacuoles (V). Bar represents 1 μm. **D**) Electron micrograph of spore of *B. cinerea* treated for Gomori reaction. Dark-staining acid phosphatase (ap) in particulate bodies within the vacuole (V). Bar represents 1 μm.

was placed on a microscope slide with a drop of acridine orange solution (1:20, 000, w/v). A coverslip was attached as indicated for spores of *B. cinerea* and examinations were made under UV light.

RESULTS

Mouse macrophages treated with acridine orange exhibited red-orange fluorescence of particulate bodies (Fig. 1-C). These bodies are considered by animal cytologists to be lysosomes. The similar fluorescence observed in the vacuoles of *C. neoformans* (Fig. 1-D) is presumed likewise to indicate a lysosomal area.

After 3 min of exposure to acridine orange, discrete red-orange fluorescing bodies appeared in vacuoles of spores of *C. ulmi, B. cinerea,* and *C. neoformans* [Fig. 1-(A,B,D); Fig. 2-(A,B)]. Often these bodies exhibited Brownian movement within the vacuole. Sometimes after 10-15 min of exposure to the acridine orange there was a general staining of the vacuolar sap with the loss of identity of the discrete acridine orange-containing bodies in the vacuole (Fig. 1-D). The nuclei in acridine orange-treated fungal spores and mouse macrophages fluoresced green under UV light (Fig. 1).

In *B. cinerea* spores treated for the Gomori reaction, most of the localized acid phosphate activity was associated with vacuoles (Fig. 2-C,D). In some cases vacuoles showed acid phosphatase staining (Fig. 2-C). Other vacuoles had discrete acid phosphatase-staining bodies within them (Fig. 2-D). Only nonspecific lead staining was observed in the controls for the Gomori reaction experiment.

DISCUSSION

Since fungal vacuoles are single-membrane-bounded and contain hydrolytic enzymes (3,4,5), they may be called lysosomes. The concentration of acridine orange in the vacuoles of *C. ulmi, B. cinerea,* and *C. neoformans* indicates that this vital dye may serve as a lysosomal marker in fungal cells as it does in animal cells (1).

The pathway by which acridine orange was taken up through the cell wall, plasmalemma, and ground plasm into the vacuole could not be followed. Apparently the fluorescence in any pinocytic or phagocytic vesicles that might be involved was not intense enough to allow its detection. It seems reasonable, however, that acridine orange may enter spores through pinocytic vesicles that are autophagocytized by the vacuole as described for the uptake of neutral red (8).

LITERATURE CITED

1. DINGLE, J. L., and H. B. FELL (eds). 1969. Lysosomes in biology and pathology: Frontiers of biology (monogr. ser.). North Holland, Amsterdam and London. 668 p.
2. JENSON, W. A. 1962. Enzymes: microscopic histochemistry. Pages 328-386 *in* G. W. Beadle, R. Emerson, and D. M. Whitaker, eds. Botanical Histochemistry. W. H. Freeman and Co., San Francisco and London. 408 p.
3. MATILE, Ph. 1971. Vacuoles, lysosomes of Neurospora. Cytobiologie 3:324-330.
4. MATILE, Ph. 1975. The lytic compartment of plant cells. Cell Biol. Monographs Vol. I. Springer-Verlag, New York, Wein. 183 p.
5. MATILE, Ph., and G. WIEMAKER. 1967. The vacuole as the lysosome of the yeast cell. Arch. Mikrobiol. 56:148-155.
6. PITT, D. 1975. Lysosomes and cell function. Longman, New York. 165 p.
7. UNANUE, E. R. 1970. Thymus dependency of the immune response to hemocyanin: an evaluation of the role of macrophages in thymectomized mice. J. Immun. 105:1339-1343.
8. WILSON, C. L. 1973. A lysosomal concept for plant pathology. Annu. Rev. Phytopathol. 11:247-272.