Induced Autofluorescence in Fungi and Its Correlation with Viability: Potential Application of Fluorescence Microscopy

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ABSTRACT

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A microscopic test based on cytoplasmic autofluorescence induced by specific stresses was developed. Conidia of 15 species of fungi were subjected to heat stress that resulted in death of the conidia. Conidia of seven of the 15 heat-stressed species were induced to autofluoresce brightly. As with natural autofluorescence, the artifically induced autofluorescence was inversely related to viability in these seven species. Also an increase in percentage of fluorescent conidia and a reciprocal decrease in percentage of germination was observed when conidia of *Colletotrichum graminicola* were incubated in Tween-80 (0.0–2.0%) or different relative humidities (0, 53, and 100%). The correlation between natural or induced autofluorescence and conidial germination of *C. graminicola* was highly significant ($r = -0.982$). Thus, fluorescence microscopy may complement or replace germination assays or vital stain tests to determine viability of fungal propagules. The procedure is precise, rapid, and easy, and has potential applications in mycological, pathological, and ecological studies of fungi.

Additional key words: dormancy, germination.

In a previous paper (13) we described natural autofluorescence in fungi. Natural autofluorescence was observed consistently in 12 fungal species over wide ranges of conditions, indicating that it is a common phenomenon in fungi. Studies of the relationship between fluorescence and viability have helped us to refine previous concepts of dormancy and viability and have contributed to a better understanding of survival mechanisms. A current working hypothesis is that autofluorescence in fungi indicates cell death and that fluorescence microscopy can be used to distinguish death from dormancy.

The purpose of this study was to determine if autofluorescence could be induced in fungi by exposing conidia to different stresses and to determine if induced fluorescence shows an inverse relationship with viability, similar to that found with natural autofluorescence. This hypothesis was tested by subjecting conidia to lethal stress conditions and observing them for fluorescence and germinability.

MATERIALS AND METHODS

Fluorescence microscopy and relationship of fluorescence to viability. A Leitz Wetzlar epifluorescence system (Scientific Supply Co., Chicago, IL 60660) consisting of a Leitz Dialux microscope with a Ploemopak fluorescence vertical illuminator was used for bright-field and autofluorescence studies. The light source was a 50-W ultra-high pressure mercury lamp (HBO 50). A Leitz Wetzlar Fluota lens was used to enhance the brightness of fluorescence at wave lengths of 390–490 μm (Leitz narrow-band filter H2). Photomicrographs were made with a manual 35-mm Leitz camera system using ASA 400 fast film and 20–40 sec exposure times.

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The fluorescence of conidia was compared with germination to determine the relationship between fluorescence and viability (13). For the fluorescence assay, one drop of each conidial suspension was pipetted onto a microscope slide, and for the germination assay another drop was pipetted onto a 1.5-cm-diameter disk of acidified potato-dextrose agar (APDA). This provided a final concentration of about 50 conidia per field under ×200 magnification. The APDA disk was incubated at 22 ±2 C for 12–24 hr.

Qualitative observations were made by examining conidia on an APDA disk under the fluorescence microscope for autofluorescence and under bright-field for germination. Quantitative assays were made by comparing the percentage of fluorescent conidia with percentage of germination of random samples (13). The quantitative assay for each treatment contained three to four replicates, with at least 400 spores per replicate. Each experiment was repeated at least twice. Correlation coefficients (r) between fluorescence and germination were calculated from quantitative experimental data.

All fungal cultures were incubated under constant fluorescent light (3,200 lux) at 22±2 C. Isolates of *C. graminicola* and *C. trifolii* were maintained on oatmeal agar, *Bipolaris maydis* on lactose casein hydrolysate agar, and all other fungi (Table 1) on potato-dextrose agar.

Autofluorescence under chemical stress of Tween-80. Conidia of *C. graminicola* were scraped from 10-day-old cultures; suspended in 2.0, 0.5, 0.1, 0.05, or 0.01% (v/v) solutions of Tween-80 or in sterile distilled water (control) at a concentration of 4×10^5 conidia per milliliter; and 10-ml suspensions of each treatment were incubated in 50-ml flasks at 22 ±2 C. Random samples were removed from the conidial suspensions at 3-day intervals for 30 days, qualitatively observed, and quantitatively assayed. In quantitative assays, in addition to determining the percentage of conidia that fluoresced in each suspension as previously described (13), fluorescence on the APDA germination disk was also determined. This is because Tween-80 that was not washed from conidia before the germination assays may have continued to kill viable conidia after they were transferred from suspensions to germination disks.

Autofluorescence under heat stress. Conidia of 15 species of fungi (Table 1) were heated to induce autofluorescence. Direct heating was done by placing a few drops of conidial suspension on a glass microscope slide over the flame of a Bunsen burner until all visible water had evaporated (about 10–15 sec). Indirect heating was done by placing a few milliliters of conidial suspension in test tube in a water bath at 55 C for 15 min. In another test, suspensions of conidia of *C. graminicola* were heated at 40, 55, 65, and 75 C for 15 min. Heat-treated spores were examined for natural or induced autofluorescence, and the brightly fluorescent conidia were placed on germination agar disks to determine if fluorescent conidia would germinate.

Autofluorescence under desiccation stress. Conidia of *C. graminicola* were desiccated by air-drying a few drops of a conidial suspension on a clean glass slide for 1 hr at 22 C. Conidia were resuspended in water, examined for induced autofluorescence, and assayed for germination. Also, conidia in the mucilaginous conidial matrix were subjected to relative humidities of 53 and 0% by the method of Nicholson and Moraes (7). The 53% RH was obtained with a saturated $Mg(NO_3)_2 \cdot 6\ H_2O$ solution (12), and 0% RH by using $CaCl_2$ crystals. A control of 100% RH was obtained by using sterile distilled water.

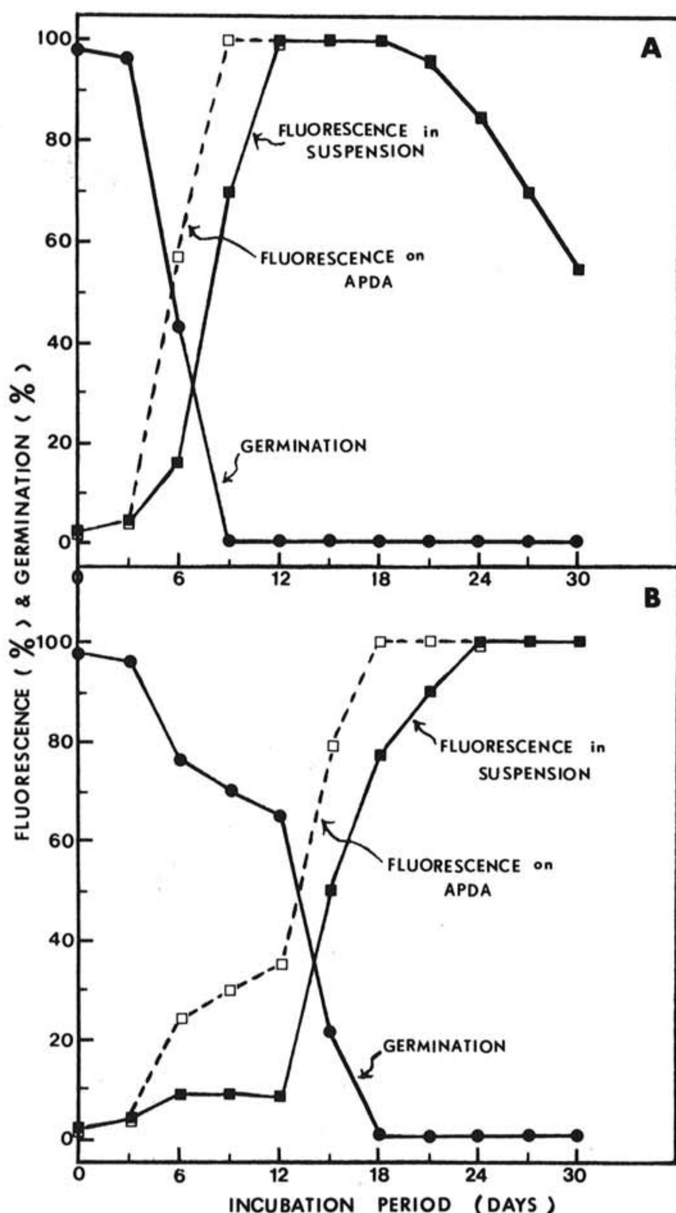

Fig. 1. Inverse relationship of induced autofluorescence to germination of stressed conidia of *Colletotrichum graminicola* incubated in **A,** 2.0% and **B,** 0.5% Tween-80 solutions.

Conidial samples were recovered by soaking a small piece of Millipore filter with conidia in a few drops of distilled water on a glass slide. The conidial suspension was assayed for germination and fluorescence. Four random samples were taken from each treatment after incubation at 22 C for 10, 20, 30, and 60 days.

RESULTS

Autofluorescence under stress of Tween-80. An increase in percentage of fluorescent conidia (5 to 100%) and a reciprocal decrease in percentage of germination (95 to 0%) with time of incubation was observed when conidia of *C. graminicola* were incubated in 2.0 or 0.5% Tween-80 solutions for 3–9 days, or 3–18 days, respectively (Fig. 1A and B). Some nonfluorescent conidia in the Tween-80 solutions did not germinate on APDA disks, probably because continuous exposure to Tween-80 may have caused the cytoplasm to leak from the cell. However, a perfect inverse relationship between germination and fluorescence on the APDA disks by quantitative countings (Fig. 1A and B) and qualitative observations (Fig. 2A and B) was obtained. The frequency of fluorescent conidia gradually declined after it reached 100% (Fig. 1A) because of leakage of the cytoplasm (Fig. 2A) which was the cell component that fluoresced (Fig. 2B).

Conidia in Tween-80 solutions at 0.1, 0.05, and 0.01% as well as those in distilled water (control) survived up to 30 days and less than 10% of them fluoresced.

Autofluorescence under heat stress. The effects of direct and indirect heating on conidia of *C. graminicola* are shown in Table 2. After the conidia were heated for 10 (Fig. 3A and B) or 15 sec over the flame, 100% of the conidia exhibited a bright orange fluorescence, and none germinated. However, after 20 sec the conidia no longer fluoresced. With indirect heating in a water bath at 40 C for 15 min, only 2% of the conidia fluoresced, similar to the control (Table 2). The low percentage of fluorescent conidia in the control and the 40 C treatment represents natural rather than induced autofluorescence. All conidia fluoresced brightly when heated for 15 min at 55 C (Fig. 3C and D) and none germinated (Table 2). With increasing temperature, the intensity of fluorescence decreased.

Heat stress caused fluorescence of all seven species with hyaline conidia and four of eight species with pigmented conidia (Table 1, and Fig. 3). Even the conidiophore of *Penicillium* sp. fluoresced (Fig. 3E and F). Among isolates with pigmented conidia, *Bipolaris maydis* (Fig. 3G and H), *Exserohilum turcicum*, and *Stenocarpella macrospora* (Fig. 3I and J) fluoresced, whereas no fluorescence was detected in *Alternaria* sp., *Curvularia* sp., *Stenocarpella maydis*, and *Periconia macrospinosa* (Table 1).

Based on qualitative observations, there was an inverse relationship between fluorescence and germination for all fungi with bright, induced autofluorescence (Table 1). This relationship was not studied in dimly fluorescent conidia because of difficulty in determining fluorescence in individual conidia. Morphological changes such as agglutination and plasmolysis of cytoplasm were observed in fluorescent, heat-stressed conidia.

Autofluorescence under desiccation stress. After air-drying for 1 hr, all conidia of *C. graminicola* fluoresced and none germinated. Conidia in the mucilaginous matrix also were induced to autofluoresce under low relative humidities but at a slower rate because the matrix protected the conidia from desiccation (7). As shown in Fig. 4, for these desiccation-stressed conidia there was an inverse relationship of fluorescence and germination ($r = -0.977$). The only substantial deviation from this relationship was observed for conidia that were stored at 100% RH for 60 days (Fig. 4). Twelve percent of the conidia were devoid of cytoplasm, probably as a result of aging, and they neither fluoresced nor germinated.

In all studies with induced autofluorescence of fungal propagules, a perfect negative correlation between fluorescence and germination was observed qualitatively; ie, fluorescing conidia did not germinate, and germinating conidia did not fluoresce. This inverse relationship was confirmed by quantitative assays on autofluorescence of *C. graminicola*. The correlation coefficient was -0.982, and the regression equation was $Y = 0.988X + 97.8$ (Fig. 5).

DISCUSSION

Results of this study demonstrate that autofluorescence can be induced by stressing the conidia of many fungi. Similarly to natural autofluorescence (13), induced autofluorescence of fungal propagules is inversely related to germination and viability. Fluorescence has also been observed in UV-irradiated yeast cells (5) and desiccated bacterial cells (10), however, no explanation was reported for its appearance. We suggest that the fluorescence observed in yeast and bacterial cells may be induced by lethal stress. These reports support our view that autofluorescence is a common phenomenon in microorganisms (13).

We observed previously that cytoplasm is the cell component of fungal propagules that emits natural autofluorescence (13). This is also true for stress-induced autofluorescence based on the observations that cytoplasm outside of the cell wall of conidia killed with Tween-80 fluoresced brightly (Fig. 2A and B), and that the cytoplasm, but not the cell wall or septa, of heat-killed conidia fluoresced (Fig. 3G to J). Likewise, the cytoplasm of the UV-irradiated yeast cells was reported to autofluoresce (5).

Conidia devoid of cytoplasm neither fluoresced nor germinated. This accounts for the deviation from the inverse relationship of fluorescence and germination (but not fluorescence and viability) seen in data obtained from Tween-80 treatments in which the

TABLE 1. Induced autofluorescence of conidia of several fungi under heat stress and its relationship to germination

Fungal isolates	Pigmentation of conidia	Intensity of fluorescence[a]	Inverse relationship between fluorescence and germination[b]
Alternaria sp.	pigmented	−	...[c]
Bipolaris maydis (Nisik.) Shoemaker	pigmented	+++	Yes
Cercospora kikuchii (Matsu and Tomoyasu) Chupp.	hyaline	+	...
Colletotrichum graminicola (Ces.) G. W. Wils.	hyaline	+++	Yes
Colletotrichum trifolii Bain	hyaline	+++	Yes
Curvularia sp.	pigmented	−	...
Exserohilum turcicum (Pass.) Leonard and Suggs	pigmented	+++	Yes
Fusarium moniliforme Sheld.	hyaline	++	Yes
Fusarium roseum Lk. ex Fr.	hyaline	+++	Yes
Penicillium sp.	intermediate	+	...
Periconia macrospinosa Lefebvre et A. G. Johnson	pigmented	−	...
Pyricularia grisea (Cooke) Sacc.	hyaline	+	...
Stenocarpella macrospora (Earle) Sutton	pigmented	+	...
Stenocarpella maydis (Berk.) Sutton	pigmented	−	...
Verticillium sp.	hyaline	++	Yes

[a] Random samples (conidial suspensions) were heated in a water bath at 55 C for 15 min, then examined under a fluorescence microscope. Intensity was ranked as: +++ = very bright, ++ = moderately bright, + = dim, and − = no autofluorescence.
[b] The relationship was determined by qualitative observations.
[c] Germination of nonfluorescent and dimly autofluorescent conidia was not tested.

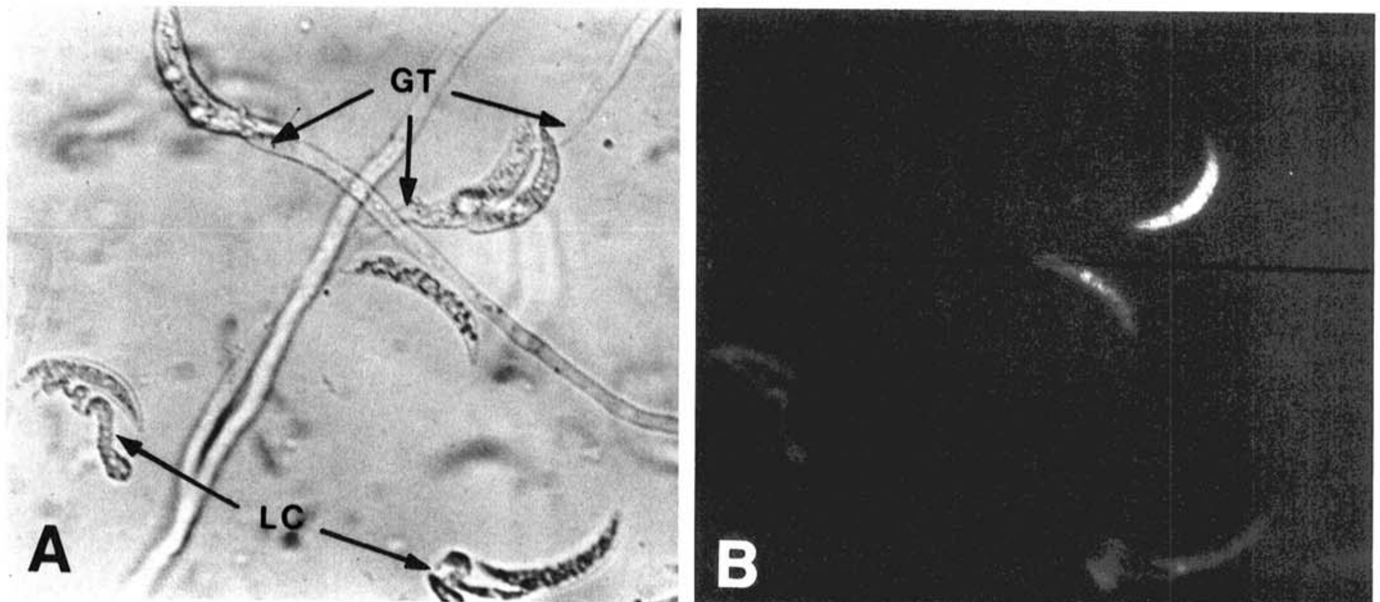

Fig. 2. Leakage and induced autofluorescence of cytoplasm of stressed conidia of *Colletotrichum graminicola* incubated in a 2.0% Tween-80 solution and nonfluorescence of germinating conidia. GT = germ tube, LC = leakage of cytoplasm. Pictures were taken of same field under **A,** bright-field and **B,** fluorescence microscopy (×2,000).

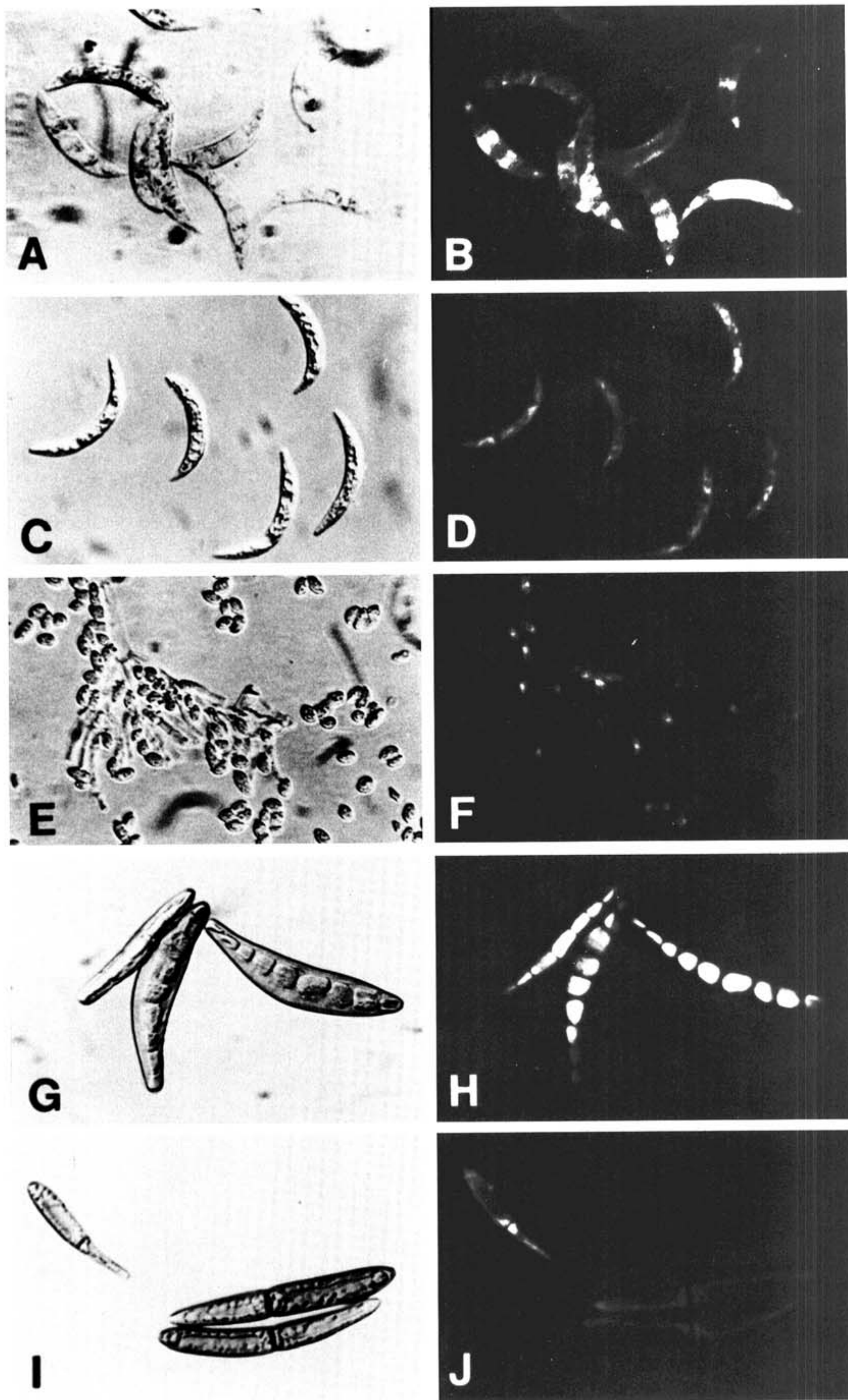

Fig. 3. Induced autofluorescence of heat-killed fungi. **A and B,** Conidia of *Colletotrichum graminicola* directly heated over a flame for 10 sec (×1,000). **C and D,** Conidia of *C. graminicola* indirectly heated in a water bath at 55 C for 15 min (×1,000). **E to J,** Heat-killed (by direct heating over a flame for 10–15 sec): **E and F,** conidia and conidiophores of *Penicillium* sp. (×1,000); **G and H,** conidia of *Bipolaris maydis*, (×750); and **I and J,** conidia of *Stenocarpella macrospora* (×1,000). Pictures were taken of the same field under bright-field/phase-contrast **(A, C, E, G, and I)** and fluorescence **(B, D, F, H, and J)** microscopy.

percentage of fluorescence declined after reaching 100% (Fig. 1A), and from the desiccation treatment in which conidia were incubated at 100% RH for 60 days (Fig. 4). Leakage of cytoplasm from conidia of *C. graminicola* was observed during the conidial aging process (*unpublished*), and for antagonized conidia (13) as well as those stressed by exposure to Tween-80 (Fig. 2A).

Although autofluorescence in fungi is an indication of cell death, the accumulation of fluorescent compounds may be a secondary effect rather than the cause of death. This view is supported by our data which show that fluorescence of conidia of *C. graminicola* occurs within 15 sec of direct heating or 2–5 min of indirect heating. The time span probably is too short to have a cause-effect relationship between formation of new compounds and death. In addition, fluorescent materials do not shorten the life span of animals (8) nor aid pathogen penetration into host plants (6).

The cause of autofluorescence in fungi is still uncertain. The fluorescent compounds may either be preformed and masked through conjugation or some other mechanism or may be synthesized de novo and/or converted from nonfluorescent to fluorescent compounds. It is possible that the fluorescent compounds exist endogenously but are masked by cell walls in viable propagules. After fungal propagules die, the cell walls may be degraded thus exposing the fluorescent material as has been suggested for plant cell autofluorescence (6).

The more probable mechanism is the production of fluorescent compounds by de novo synthesis or transformation of nonfluorescent precursors in living cells. Because autofluorescence can be induced within 10–15 sec of heating, fluorescent compounds may be geometrical (*cis-trans*) or optical stereoisomers of nonfluorescent compounds. A similar mechanism has been reported for a self-inhibitor of *Puccinia graminis* f. sp. *tritici* (1).

From our study, it is known that heat stress can induce autofluorescence, however, four of the 15 fungal species tested did not fluoresce after being heated, and all four had pigmented conidia. It is possible that these fungi emitted autofluorescence at a wavelength beyond the range of detection of our fluorescence microscope, or more likely that the fluorescence was absorbed or masked by the pigmented, and usually thickened cell wall. High temperature (⩾65 C) in a water bath or prolonged heating over a flame diminished or totally quenched the fluorescence emission, presumably because of the destruction of cell contents and fluorescent substances under harsh conditions.

The advantages of fluorescence microscopy as a tool are accuracy, efficiency, and qualitative and quantitative results. Because fluorescence microscopy can distinguish dormancy from death (13) it is more precise than germination tests in which accuracy is influenced by incubation time and temperatures, media, and self-inhibitors (2,4). Fluorescence microscopy gives an immediate measure of viability of fungal propagules. Germination is a retrospective method for detecting death (9), because spores may die during incubation on the germination medium. We found (*unpublished*) that immature conidia of *C. graminicola* or ascospores of *Leptosphaerulina briosiana* did not germinate when transferred onto germination media because they die if they are

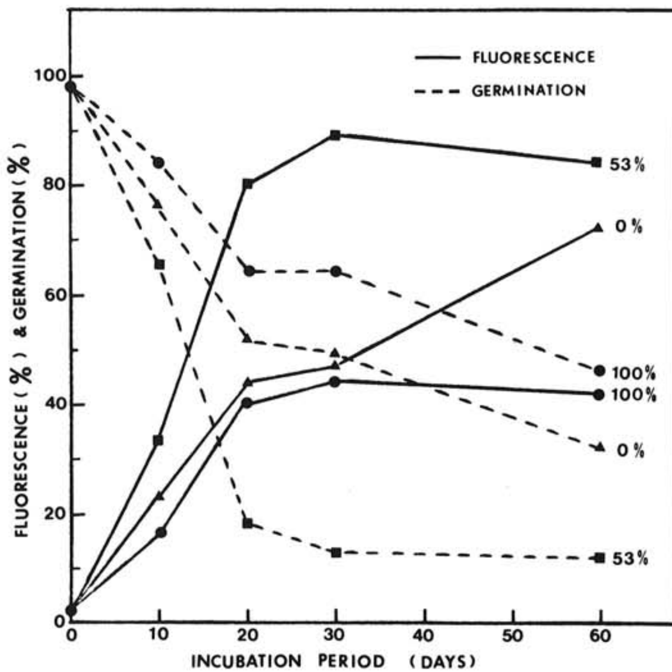

Fig. 4. Inverse relationship of autofluorescence to germination of conidia of *Colletotrichum graminicola* incubated under low relative humidities of 0 and 53%, or at a control of 100% for the indicated incubation periods.

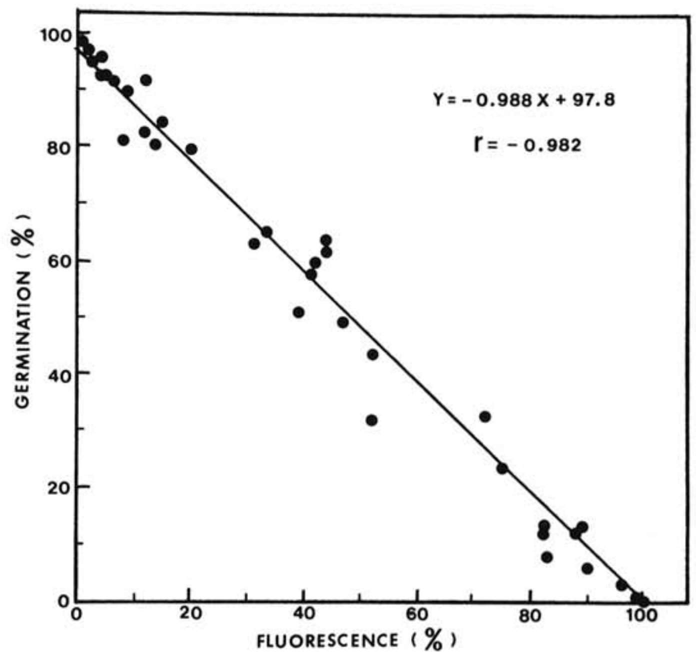

Fig. 5. Inverse relationship of autofluorescence to germination of conidia of *Colletotrichum graminicola* from all studies.

TABLE 2. Inverse relationship of induced autofluorescence to germination of heat-stressed conidia of *Colletotrichum graminicola* and the effect of heating on the intensity of autofluorescence

	Nonheated control	Direct heating[a] (sec)			Indirect heating[b] (C)			
		10	15	20	40	55	65	75
Germination (%)	98	0	0	0	98	0	0	0
Fluorescence (%)	2	100	100	0[d]	2	100	100	100
Intensity of autofluorescence[c]	+++	+++	+++	−	+++	+++	++	+

[a] Conidial suspensions were heated on a slide placed over the flame of a Bunsen burner until visible moisture was gone.
[b] Conidial suspensions were heated in test tubes in a water bath for 15 min at indicated temperatures.
[c] Same designation as Table 1 footnote *a*.
[d] Conidia turned brown and no longer fluoresced.

detached before maturity (3). They did not fluoresce immediately after detachment, but fluoresced later, indicating that they died on the germination disks. Thus, the fluorescence microscopic technique may assess more accurately the time of death than the germination test. The fluorescence technique is quick and easy to perform because preparatory work is simple, no chemicals such as vital stains are required for its use, and no incubation time is necessary. Finally, fluorescence microscopy can be qualitative and quantitative. A direct comparison of morphology of conidia can be made under light- and fluorescence microscopes. Usually, fluorescent, dead spores and nonfluorescent, viable spores are morphologically different. Thus, fluorescence microscopy can be used to relate spore morphology to death and may be useful for basic mycological studies. Also the percentage of viable spores of a population can be determined by quantifying the fluorescent spores.

There are perhaps several studies that can benefit from the fluorescence microscopic technique. It can be applied to complement or replace germination tests in experiments dealing with spore viability, and complement vital stain methods (11) in distinguishing living from dead propagules (eg, mycelia) in situ where the germination test is not feasible. Thus, this technique has potential application in mycological, pathological, and ecological studies of fungi.

LITERATURE CITED

1. Allen, P. J. 1972. Specificity of the *cis*-isomer of inhibitors of uredospore germination in the rust fungi. Proc. Nat. Acad. Sci. USA 69:3497-3500.
2. Cochrane, V. W. 1963. Physiology of Fungi. John Wiley & Sons, Inc., New York. 524 pp.
3. Gottlieb, D. 1950. The physiology of spore germination in fungi. Bot. Rev. 16:229-257.
4. Hawker, L. E. 1950. Factors influencing the survival and germination of spores. Pages 209-233 in: Physiology of Fungi. L. E. Hawker, ed. University of London Press Ltd., London. 360 pp.
5. Johnson, B. F., Yoo, B. Y., and Calleja, G. B. 1974. Cell division in yeasts. II. Template control of cell plate biogenesis in *Schizosaccharomyces pombe*. Pages 153-166 in: Cell Cycle Controls. E. M. Padilla, I. L. Cameron, and A. Zimmerman, eds. Academic Press, New York. 370 pp.
6. Kidger, A. L., and Carver, T. L. W. 1981. Autofluorescence in oats infected by powdery mildew. Trans. Br. Mycol. Soc. 76:405-409.
7. Nicholson, R. L., and Moraes, W. B. C. 1980. Survival of *Colletotrichum graminicola*: Importance of the spore matrix. Phytopathology 70:255-261.
8. Petrakis, P. L. 1975. The older you grow, the more you glow. Study of fluorescent tissue pigments yields clues on aging. Aminco Lab. News 31(3):1-5.
9. Postgate, J. R. 1976. Death in macrobes and microbes. Pages 1-18 in: The Survival of Vegetative Microbes. Proc. 26th Symp. of the Society for General Microbiology. T. R. G. Gray and J. R. Postgate, eds. Cambridge University Press, London. 432 pp.
10. Repentigny, J. de., and Sonea, S. 1958. Detection microscopique de la fluorescence primaire chez les bacteries. Can. J. Microbiol. 4:17-23.
11. Soderstrom, B. E. 1977. Vital staining of fungi in pure cultures and in soil with fluorescein diacetate. Soil Biol. Biochem. 9:59-63.
12. Tuite, J. 1969. Plant Pathological Methods. Fungi and Bacteria. Burgess Publishing Company, Minneapolis, MN. 239 pp.
13. Wu, C. H., and Warren, H. L. 1984. Natural autofluorescence in fungi and correlation with viability. Mycologia 76:(In press).