Applications of KOH-Aniline Blue Fluorescence in the Study of Plant-Fungal Interactions

M. E. Hood and H. D. Shew

Department of Plant Pathology, North Carolina State University, Raleigh 27695.

We thank the following people for contributions of specimens or organisms: M. Starrett (Department of Horticulture, NCSU) for ericoid mycorrhizae of mountain andromeda; W. Gutierrez (Department of Plant Pathology, NCSU) for ascospores of *Sclerotinia sclerotiorum*; S. Leath (Department of Plant Pathology, NCSU) for powdery mildew of wheat; C. E. Main (Department of Plant Pathology, NCSU) for blue mold of tobacco; J. Antonovics (Department of Botany, Duke University, Durham, NC) for *Ustilago violacea* inoculum and *Silene alba* seed; L. F. Grand (Department of Plant Pathology, NCSU) for samples of *Monilia linhartiana* on *Prunus virginiana* and *Oidium monilioides* on *Agropyrum repens* from the Mycological Herbarium, Department of Plant Pathology, NCSU; and T. Creswell (Plant Disease and Insect Clinic, NCSU) for *Fusarium* sp. on *Solanum melongena*. The remaining specimens were prepared or collected by the authors.

Accepted for publication 8 April 1996.

ABSTRACT

Hood, M. E., and Shew, H. D. 1996. Applications of KOH-aniline blue fluorescence in the study of plant-fungal interactions. Phytopathology 86:704-708.

A KOH-aniline blue technique for fluorescent staining of fungi in association with plant tissues was developed. The technique provided rapid, simple, and effective documentation of plant-fungal interactions with specimens representing the Deuteromycotina, Ascomycotina, Basidiomycotina, and Mastigomycotina. Applications of the technique were investigated, including documentation of host-penetration events, characteristics of host colonization, fungal reproduction, and detection of inoculum. In the standard KOH-aniline blue procedure, fresh specimens were autoclaved for 15 min at 121°C in 50 ml of 1 M KOH, rinsed in deionized water, mounted in the stain solution, and examined with ultraviolet fluorescence. The stain solution was prepared as 0.05% aniline blue dye (CI #42755 or CI #42780) in 0.067 M K_2HPO_4 at pH 9.0. Modifications of the standard procedure also were tested, including use of variously preserved specimens, alteration of the KOH treatment, and moderate variation of the stain solution. The technique produced a high degree of resolution and contrast between hyphae and host-plant tissues. The resulting documentation supported previous observations and, in some cases, provided new information about the nature of specific host-pathogen interactions.

Additional keywords: histology, histopathology.

Plant health can be greatly influenced by interactions with pathogenic or mutualistic fungi, and microscopic examination of these interactions provides information about the biology of host-parasite relationships and serves as a useful tool for diagnosticians. Staining techniques that aid in microscopic examination by differentiating hyphae from host tissue are fundamental to plant pathology, mycology, and related disciplines. Such techniques have been compiled in recent publications (4,6), and their applications to observational and experimental research include examination of infection and colonization processes, identification of fungal inoculum or hyphal presence in asymptomatic tissue, and other instances in which visualizing a fungus has advantages over less descriptive observational methods. However, staining techniques generally are not categorized by their appropriateness to these types of applications, because staining characteristics of fungi and plant tissues can vary substantially with the particular interaction.

Aniline blue fluorescence has been widely used in botanical histochemistry, particularly to stain callose plugs in phloem (14). Although the common name of this stain varies with the author or source (i.e., aniline blue, methyl blue, water blue, Poirrier's blue, cotton blue, and others), the dyes indicated in the current study are aniline blue color index (CI) #42755 and CI #42780 (11). The fluorochrome, which comprises a small portion of these aniline blue dyes, was critically analyzed by Smith and McCully (17). These authors demonstrated binding of the fluorochrome to various glucans and plant polysaccharides, and they suggested that the strong association of aniline blue fluorescence with β-1,3-glucans, such as callose, is attributable to loose packing of these polymers, providing greater accessibility to them by the fluorochrome. Other investigators have utilized aniline blue fluorescence to examine glucan composition of fungal cell walls (13,21) and to detect yeast cells in industrial applications (10). A widely recognized use of aniline blue fluorescence in research of plant-fungal interactions is the identification of host-produced callose depositions upon intracellular infection by fungi (9,18). In addition, a recent article by Williamson et al. (23) described treatment of *Peronospora*-infected leaves with Carnoy solution (ethanol/chloroform/glacial acetic acid) and NaOH prior to aniline blue fluorescent staining. For these applications, aniline blue stain commonly was prepared at concentrations from 0.005 to 1% in 0.067 M K_2HPO_4 at pH 8.5 to 10.

Other fluorescent dyes and brighteners also have been applied in the study of plant-fungal interactions. These compounds include 4′,6-diamidino-2-phenylindole, ethidium bromide, diethanol, and Calcofluor, as well as the more recent developments of Fungalase-F (Anomeric, Baton Rouge, LA) and Fungi-Fluor (Polysciences, Warrington, PA). Some problems commonly encountered with fluorescent stains in researching plant-fungal interactions are the autofluorescence of plant tissues and nonspecific binding of the fluorochrome. The resulting background fluorescence may be sufficiently intense to hinder differentiation of hyphae from host-plant tissues. In general, the usefulness of fluorescent stains is determined by (i) the visual contrast and resolution provided be-

Corresponding author: H. D. Shew; E-mail address: d_shew@ncsu.edu

Publication no. P-1996-0425-01R

tween hyphae and host tissue, (ii) cost, and (iii) ease of use, including rapidity of staining procedures and hazards associated with reagents.

In the current study, a rapid procedure requiring 30 to 40 min and utilizing aniline blue fluorescence is described for staining of fungi in association with host-plant tissues. Our objective was to develop a simple, effective technique to enhance visualization of associations between a broad range of fungal taxa and their respective host-plant tissues. We investigated use of the technique with fresh and variously preserved specimens and tested several modifications to the standard procedure that may have additional benefits under certain experimental conditions. The technique was used to examine various stages in host-parasite interactions, including penetration events, characteristics of host colonization, fungal reproduction, and detection of inoculum.

MATERIALS AND METHODS

Staining procedures. For the standard procedure of KOH-aniline blue fluorescence, fresh specimens were autoclaved for 15 min at 121°C in 50 ml of 1 M KOH, followed by three rinses in deionized water. From deionized water, specimens were mounted on glass slides in several drops of the stain solution and examined with an Axiophot microscope (Carl Zeiss, Thornwood, NY). The microscope was equipped for epifluorescence microscopy with an HBO 100-W/2 mercury burner and G365 nm exciter:LP 420 nm barrier fluorescence filters. The stain solution was prepared at least 2 h prior to use as 0.05% aniline blue dye in 0.067 M K_2HPO_4 at pH 9.0 (pH adjustment normally was not needed). Stain solutions were stored at room temperature in brown-glass bottles for not more than several months. Aniline blue dyes were obtained commercially: CI #42755 (CAS 28631-66-5; F.W. 737.8; $C_{32}H_{25}N_3O_9S_3Na_2$) from Fisher Scientific (Pittsburgh) and CI #42780 (CAS 28983-56-4; F.W. 799.8; $C_{37}H_{27}N_3O_9S_3Na_2$) from Sigma Chemical Company (St. Louis). From preliminary results, differences were not detected between aniline blue dyes of the two CI numbers, and throughout subsequent investigations, aniline blue CI #42755 was used predominantly.

The following augmentations to the standard procedure were tested: (i) use of specimens that were glutaraldehyde-fixed/ethyl alcohol-dehydrated, KOH-preserved, agar-embedded, or air-dried in an herbarium for approximately 100 years; (ii) variation of the time, temperature, and strength of KOH treatments; (iii) moderate variation of the concentrations of stain solution components, including aniline blue dye, K_2HPO_4, and pH (adjusted with KOH or HCl). These augmentations are described where used with specific interactions.

Preparation of plant-fungal interaction specimens. The specimens examined included *Ustilago violacea* (Pers.) Roussel (Basidiomycotina; anther smut) on *Silene alba* (Mill.) E.H.L. Krause; *Thielaviopsis basicola* (Berk. & Broome) Ferraris (Deuteromycotina; black root rot) on *Nicotiana tabacum* L. (tobacco); *Hymenoscyphus ericae* (Read) Korf & Kernan (synamorph *Pezizella ericae* Read) (Ascomycotina; ericoid mycorrhizae) on *Pieris floribunda* (Pursh ex Sims) Benth & Hook (mountain andromeda); *Blumeria graminis* (DC.) E.O. Speer (Ascomycotina; powdery mildew) on *Triticum aestivum* L. (wheat); *Peronospora tabacina* D.B. Adam (Mastigomycotina; blue mold) on *N. tabacum*; *Fusarium* sp. (Deuteromycotina; vascular wilt) on *Solanum melongena* L. (eggplant); *Erysiphe lagerstroemiae* E. West (3) (Ascomycotina; powdery mildew) on *Lagerstroemiae indica* L. (crape-myrtle); *Gymnosporangium juniperi-virginianae* Schwein (Basidiomycotina; cedar apple rust) on *Juniperus virginiana* L. (eastern red cedar); *Coleosporium ipomoeae* (Schwein) Burrill (rust) on *Ipomoea purpurea* (L.) Roth. (morning-glory); *Oidium monilioides* Lk. (teleomorph *B. graminis*) (Ascomycotina; powdery mildew) on *Agropyrum repens* (L.) P. Beauv. (quackgrass); *Monilia linhartiana* Sacc. (? *M. angustior* (Sacc.) Reade [5,19]) (Ascomycotina) on *Prunus virginiana* L. (chokecherry); seedborne inoculum on flue-cured tobacco seed (*N. tabacum*); and airborne inoculum of *Sclerotinia sclerotiorum* (Lib.) de Bary (Ascomycotina).

Inoculations of *U. violacea* on *Silene alba* and *Thielaviopsis basicola* on *N. tabacum* were conducted under aseptic conditions. Surface-sterilized seeds were placed on Gamborg's B-5 basal salt medium with minimal organics (Sigma), and seedlings were grown under continuous fluorescent light, *Silene alba* at 18°C and *N. tabacum* at 22 to 25°C. Drops (50 µl) of a *U. violacae* sporidial suspension (1,000,000 sporidia per ml) were applied to leaves of 3-week-old *Silene alba* seedlings, and leaf infections were observed as whole mounts 5 days after inoculation following the standard procedure. Agar plugs containing hyphae of *Thielaviopsis basicola* were placed 5 to 10 mm from roots of 10-day-old *N. tabacum* seedlings, so hyphae of *Thielaviopsis basicola* grew through the agar and contacted root tissue. Infected roots were observed 24 h after inoculation by excising the specimen as whole mounts embedded in agar. Agar-embedded specimens were treated at room temperature for 4 h in 10 M KOH, rinsed, and stained following the standard procedure.

In addition, 2-week-old greenhouse-grown *N. tabacum* seedlings were inoculated by drenching the soil with 50 ml of *Thielaviopsis basicola* endoconidial suspension (2×10^6 endoconidia per ml), and roots were excised and rinsed in deionized water 72 h after inoculation. After rinsing, greenhouse-grown, infected *N. tabacum* roots were preserved in 1 M KOH for 5 months at room temperature. KOH-preserved specimens were autoclaved, rinsed, and stained following the standard procedure and observed as whole mounts. Other greenhouse-grown, infected *N. tabacum* roots were fixed upon excision with 2% glutaraldehyde in 0.05 M potassium phosphate buffer (pH 7.2) for 12 h at 4°C. After fixation, roots were washed (3 × 20 min) in the same buffer and dehydrated to 70% ethyl alcohol. Fixed/dehydrated roots were autoclaved, rinsed, and stained following the standard procedure and observed as whole mounts. Fresh specimens of greenhouse- and in vitro-produced *Thielaviopsis basicola* on *N. tabacum* also were observed as whole mounts (i) without KOH treatment and with standard staining, (ii) with standard KOH treatment and without staining, and (iii) without either KOH treatment or staining.

H. ericae on *Pieris floribunda* and *B. graminis* on *Triticum aestivum* were allowed to develop under greenhouse conditions. Nine-month-old *Pieris floribunda* seedlings were inoculated by drenching soil with a slurry of hyphae in deionized water. Roots were excised 2 years after inoculation and examined as whole mounts following the standard procedure. Four- to six-week-old *Triticum aestivum* seedlings were inoculated by incubation adjacent to diseased (powdery mildew) *Triticum aestivum* plants. *Triticum aestivum* leaves were sampled and examined as whole mounts following the standard procedure when powdery mildew symptoms and signs developed.

Peronospora tabacina on *N. tabacum* was obtained from plants in controlled-environment chambers of The Southeastern Plant Environment Laboratories of North Carolina State University (NCSU), Raleigh. Although the Mastigomycotina are not considered true fungi, their growth forms, ecology, and agricultural impact are similar to true fungi, and they are important plant pathogens. Plants were grown under 9 h of fluorescent light with a 1-h incandescent light-interruption period at 22°C day and 18°C night temperatures. *N. tabacum* plants were inoculated with *Peronospora tabacina* by atomizing an inoculum suspension (10,000 sporangia per ml of deionized water) onto leaves, followed by a 24-h dark period. Leaves with symptoms of blue mold were sampled between 4:00 and 6:00 a.m. and were observed as hand-made cross-sections following the standard procedure. Specimens also were observed with 1 M KOH treatments, including (i) room temperature for 5 days, (ii) 80°C for 30 min, and (iii) autoclaved for 15 min at 121°C and stored for 1 month at room temperature; each treatment was followed by rinsing and staining following the standard procedure.

A specimen of *Fusarium* sp. on *Solanum melongena* was obtained from a sample submitted to the NCSU Plant Disease and Insect Clinic. Hand-made longitudinal sections of infected tap root tissue were fixed with 2% glutaraldehyde in 0.05 M potassium phosphate buffer (pH 7.2) for 12 h at 4°C. After fixation, sections were washed (3 times for 20 min each) in the same buffer and dehydrated in up to 70% ethyl alcohol. Fixed/dehydrated sections were autoclaved, rinsed, and stained following the standard procedure.

E. lagerstroemiae on *L. indica*, *G. juniperi-virginianae* on *J. virginiana*, *C. ipomoeae* on *I. purpurea*, and seed of flue-cured tobacco were collected locally. Powdery mildew of *L. indica* and flue-cured tobacco seed was observed as whole mounts following the standard procedure. Galls of cedar apple rust specimens (*G. juniperi-virginianae* on *J. virginiana*) and symptomatic tissue of *I. purpurea* infected by *C. ipomoeae* were observed as hand-made cross-sections following the standard procedure. Sections of cedar apple rust galls also were observed (i) without KOH treatment and with standard staining, (ii) with standard KOH treatment and without staining, and (iii) without either KOH treatment or staining.

Ascospores of *Sclerotinia sclerotiorum* were vacuum collected on nylon filter disks after release from apothecia. Filter disks were treated following the standard procedure. Ascospores were observed as whole mounts by being scraped from the disks into stain solution on glass slides. Ascospores were additionally mounted in variations of the standard stain solution, including solutions prepared at 0.02 to 1% aniline blue, 0.02 to 5 M K_2HPO_4, and pH 8 to 13.

Herbarium specimens were obtained from the Fungi Columbiani Collection (2) at the Mycological Herbarium, Department of Plant Pathology, NCSU. Specimens were observed as whole mounts following the standard procedure and included *O. monilioides* on *A. repens* collected in 1890 and *M. linhartiana* on *Prunus virginiana* collected in 1897.

RESULTS

The KOH-aniline blue technique was effective at providing descriptive observation and documentation of the plant-fungal interactions with specimens representing the Deuteromycotina, Ascomycotina, Basidiomycotina, and Mastigomycotina (Fig. 1A through P). Substantial hyphal fluorescence was not observed without both KOH treatment and staining. Staining of specimens without KOH treatment resulted in only faint fluorescence of hyphae, strong autofluorescence of foliar plant tissues, and conventional staining of plant-produced callose deposits. For example, in root hairs of *N. tabacum* infected by *Thielaviopsis basicola*, callose depositions around the penetrating hyphae (8) fluoresced distinctly with only aniline blue staining (Fig. 1M). However, when the specimens were treated with KOH prior to staining, hyphae fluoresced brightly with substantial resolution of fungal structures and excellent contrast to host tissue (Fig. 1N). Melanized fungal structures did not fluoresce with any treatment tested, and plant tissues fluoresced in all cases but not such that differentiation of fungi was hindered.

Modifications to the standard procedure also were successful in examining the *Thielaviopsis basicola-N. tabacum* system. Agar-embedded specimens that were treated in 10 M KOH for 4 h and then stained maintained the orientation of penetrating hyphae on root hair cells (Fig. 1N). In addition, *Thielaviopsis basicola* hyphae in greenhouse-grown, infected *N. tabacum* roots, whether stored for 5 month in 1 M KOH or glutaraldehyde-fixed and dehydrated, fluoresced with equal quality to fresh specimens after treatment following the standard procedure. Infections by *Thielaviopsis basicola* were characterized by thin penetration pegs with terminal swellings that advanced to colonize the host cell (Fig. 1N). Host-produced callose deposits often were not observed around penetration structures after KOH-aniline blue treatment.

Penetration of foliar tissue was observed with *U. violacea* on *Silene alba* specimens. With epifluorescence, the resolution and contrast provided by the KOH-aniline blue technique allowed use of whole mounts and resulted in pseudo-3D representation of infection. Infection hyphae penetrated leaf tissue through stomata (Fig. 1A).

Characteristics of leaf-tissue colonization were observed with *M. linhartiana* on *Prunus virginiana*, *Peronospora tabacina* on *N. tabacum*, *B. graminis* on *Triticum aestivum*, and *O. monilioides* on *A. repens*. The 100-year-old herbarium specimens were restored with the standard procedure (Fig. 1B and E). The extent of colony expansion (Fig. 1B) and even the fine morphology of haustoria could be observed in the herbarium specimens as easily as in the fresh specimens (Figs. 1D and E). Intercellular hyphae of *Peronospora tabacina* colonizing *N. tabacum* (12) were observed in cross-sections of leaf veins at the edge of lesions. Specimens of *Peronospora tabacina* on *N. tabacum*, whether treated in 1 M KOH for 5 days at room temperature, for 30 min at 80°C, or autoclaved and stored for 1 month at room temperature, fluoresced with equal quality to fresh specimens after being rinsed and stained following the standard procedure. Hyphae grew almost exclusively in intercellular spaces at the junction of three adjacent leaf-vein parenchyma cells and among leaf-vein vasculature (Fig. 1C). In these leaf-vein cross-sections, hyphae also were observed only in cross-section, indicating directional growth rather than random ramification of hyphae through this tissue.

Intercellular hyphae of rust fungi were observed in leaf tissue of *I. purpurea* infected by *C. ipomoeae* and in gall tissue of cedar apple rust specimens. Fluorescence of these specimens by the standard KOH-aniline blue procedure was exceptional among the specimens examined. Intercellular hyphae of the rust fungi predominantly fluoresced a diffuse blue (Fig. 1H), whereas hyphae in other specimens treated following the standard procedure fluoresced yellow-green. Haustorial mother cells of rust fungi, however, fluoresced yellow-green like hyphae in other specimens (Fig. 1I and J). Without both KOH treatment and staining, intercellular-rust hyphae and haustorial mother cells did not fluoresce, and standard staining without KOH treatment resulted in conventional fluorescence only of host-deposited callose around haustoria. As observed with *Thielaviopsis basicola* infection of *N. tabacum*, host-produced callose deposits were not observed around rust penetration structures after KOH-aniline blue treatment.

Root tissue colonization was observed with *H. ericae* on *Pieris floribunda* and *Fusarium* sp. on *Solanum melongena*. Hyphae of *H. ericae* colonized cells of *Pieris floribunda* hair roots (7) by penetration of the adjacent host cell through the contiguous cell walls (Fig. 1O). Glutaraldehyde-fixed and dehydrated specimens of *Fusarium* sp. on *Solanum melongena* fluoresced with equal quality to fresh specimens after treatment following the standard procedure. *Fusarium* sp. hyphae colonized host xylem elements, which also significantly fluoresced (Fig. 1P).

Fungal sporulation was observed with specimens of *E. Lagerstroemiae* on *L. indica* and *Peronospora tabacina* on *N. tabacum*. Taxonomically important morphology of *E. lagerstroemiae* conidia and conidiophores was observed in detail, and septations within conidiophores could be distinguished easily (Fig. 1F). Ellipsoidal conidia were observed singly or in short chains on cylindrical conidiophores. Conidia of *E. lagerstroemiae* fluoresced white to blue-white compared to conidiophores and hyphae. Stages of *Peronospora tabacina* sporangiophore development were observed with specimens collected from 4:00 to 6:00 a.m. The most mature of these specimens were characterized by dichotomously branching stalks that terminated in spherical sporangia (Fig. 1G).

Observation of airborne inoculum and investigation into limitations of stain solution composition were performed with ascospores of *Sclerotinia sclerotiorum*. KOH-aniline blue treatment of filters disks on which ascospores had been vacuum-collected allowed manipulation of ascospores prior to observation (Fig. 1K). Modifications to the staining solution did not appreciably alter the intensity of ascospore fluorescence from that achieved with the standard stain composition. Flue-cured tobacco seed was examined

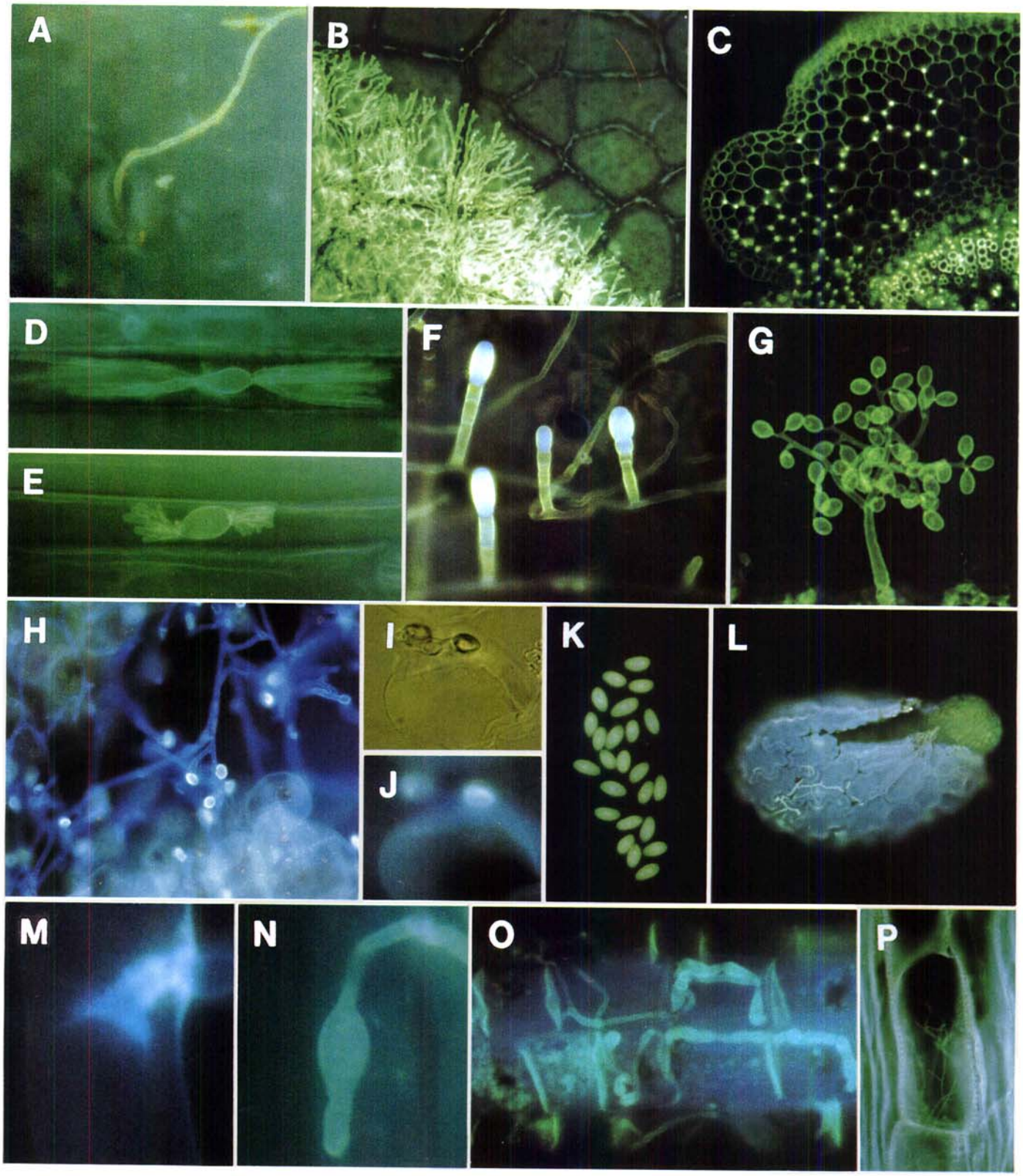

Fig. 1. Observations of plant-fungal interactions by the KOH-aniline blue technique. **A,** Stomatal penetration of a *Silene alba* leaf by *Ustilago violacea*. **B,** *Monilia linhartiana* hyphal growth on a *Prunus virginiana* leaf (prepared from an 1897 herbarium specimen). **C,** Cross-section of a *Nicotiana tabacum* leaf vein showing intercellular colonization by *Peronospora tabacina*. **D,** Haustorium of *Blumeria graminis* in a *Triticum aestivum* leaf cell (prepared from a fresh specimen). **E,** Haustorium of *Oidium monilioides* in a *Agropyrum repens* leaf cell (prepared from an 1890 herbarium specimen). **F,** Conidiophores of *Erysiphe lagerstroemiae* on a *Lagerstroemiae indica* leaf. **G,** Sporangiophore of *Peronospora tabacina* on a *N. tabacum* leaf. **H,** Intercellular hyphae of *Gymnosporangium juniperi-virginianae* in a cedar-apple rust gall on *Juniperus virginiana*. **I and J,** Bright-field illumination and KOH-aniline blue fluorescence of a haustorial mother cell and a haustorium of *G. juniperi-virginianae*, respectively. **K,** Airborne inoculum (ascospores) of *Sclerotinia sclerotiorum*. **L,** Fungal contamination of flue-cured tobacco seed. **M and N,** Haustorium-like penetration structures of *Thielaviopsis basicola* formed upon infection of *N. tabacum* root hair. **M,** In an agar-embedded specimen stained without KOH treatment, conventional aniline blue fluorescence of plant-deposited callose around a *Thielaviopsis basicola* penetration structure is shown. **N,** In agar-embedded specimens treated in 10 M KOH for 4 h at 25°C, hyphae fluoresced brightly with contrast to host tissue. **O,** Ericoid mycorrhizae (*Hymenoscyphus ericae*) of *Pieris floribunda*. **P,** A fixed (2% glutaraldehyde) and dehydrated section of *Fusarium* sp. colonizing a xylem element of *Solanum melongena*.

for the presence of fungal contamination. Hyphae and spores were observed on the seedcoat surface of the majority of seeds examined (Fig. 1L).

DISCUSSION

The KOH-aniline blue technique that was developed in this study provided simple and effective documentation of plant-fungal interactions in a broad range of applications. The technique enhanced observation of infection and colonization of host tissue, fungal reproduction, and detection of fungal inoculum. Excellent resolution and contrast of diverse fungal taxa were provided by the KOH-aniline blue technique compared to other fluorescent methods of staining hyphae in plant tissue ([15,20,23], Fungalase-F, and Fungi-Fluor). Although fluorescence of plant tissues was common, the color and intensity of fungal fluorescence permitted easy differentiation of fungi and host tissues. In fact, the ability to visualize the morphology of host tissue often aided the interpretation of fungal interactions with the host.

In addition to being highly effective in staining fungi, this technique offers many advantages. For example, the chemicals required for the standard procedure (KOH, K_2HPO_4, and aniline blue dye) are inexpensive and readily obtainable through commercial sources, and use of these chemicals as described is not associated with significant health risks or precautions. The rapidity (30 to 40 min) and plasticity of the technique are of particular benefit. Frequently, other protocols for fluorescent staining describe incubation in multiple reagents and require significantly longer time to complete than the KOH-aniline blue technique ([15,16,20,23] and Fungalase-F). The plasticity of the standard procedure was investigated with regard to common limitations of research. Long-term storage of specimens in KOH, completion of treatments without autoclaving or heating, and the use of variously preserved and agar-embedded specimens will accommodate many specific experimental needs. For example, the successful use of the KOH-aniline blue technique with extremely old herbarium specimens suggests a valuable means of investigating plant-fungal interactions for which fresh samples are not currently or locally available. Further augmentations or manipulation of the standard procedure may prove beneficial to applications not investigated in this study.

Observations of fungal morphology supported previous reports in some of the interactions observed in this study (1,3,7,8,12,22); however, with other specimens, the KOH-aniline blue technique afforded original observations on the nature of specific host-pathogen interactions. Examples of results supporting previous observations include xylem colonization by *Fusarium* sp. (1), hair root colonization by *H. ericae* (7), the morphology of penetrating hyphae of *Thielaviopsis basicola* (8), and sporulating structures of *E. lagerstroemiae* (3,22) and *Peronospora tabacina* (12). Previously unreported events of specific interactions include stomatal penetration of *Silene alba* by *U. violacea*, the nature of leaf-vein colonization by *Peronospora tabacina*, and differential staining of rust intercellular hyphae and haustorial mother cells. The results obtained with rust specimens warrant further investigation. It is likely that the differential staining of intercellular hyphae and haustorial mother cells indicates differences in cell wall composition. With rust specimens, as in other studies (13,21), aniline blue fluorescence may be employed to investigate fungal cell-wall architecture.

LITERATURE CITED

1. Agrios, G. N. 1988. Vascular wilts caused by ascomycetes and imperfect fungi. Pages 408-422 in: Plant Pathology, 3rd ed. Academic Press, San Diego.
2. Bartholomew, E. 1910. Index to Fungi Columbiani, Centuries 1 to 30. Record Print, Stockton, KS.
3. Braun, U. 1987. Monograph of the Erysiphales (powdery mildews). Beih. Nova Hedwigia 89:622.
4. Dhingra, O. D., and Sinclair, J. B. 1995. Basic Plant Pathology Methods. 2nd ed. CRC Press, Boca Raton, FL.
5. Farr, D. F., Bills, G. F., Chamuris, G. P., and Rossman, A. Y. 1989. Fungus list. Pages 555-1028 in: Fungi on Plants and Plant Products in the United States. APS Press, St. Paul, MN.
6. Fox, R. T. V. 1993. Principles of Diagnostic Techniques in Plant Pathology. CAB International, Oxford.
7. Harley, J. L., and Smith, S. E. 1983. Ericiod mycorrhizas. Pages 237-256 in: Mycorrhizal Symbiosis. Academic Press, New York.
8. Hood, M. E., and Shew, H. D. 1996. Pathogenesis of *Thielaviopsis basicola* on a susceptible and a resistant cultivar of burley tobacco. Phytopathology 86:38-44.
9. Jordan, C. M., Endo, R. M., and Jordan, L. S. 1988. Penetration and colonization of resistant and susceptible *Apium graveolens* by *Fusarium oxysporum* f. sp. *apii* race 2: Callose as a structural response. Can. J. Bot. 66:2385-2391.
10. Kock, H. A., Bandler, R., and Gibbson, R. R. 1986. Fluorescence microscopy procedure for quantitation of yeasts in beverages. Appl. Environ. Microbiol. 52:599-601.
11. Lillie, R. D. 1969. H. J. Conn's Biological Stains: A Handbook on the Nature and Uses of the Dyes Employed in the Biological Laboratory. Waverly Press, Baltimore.
12. McKeen, W. E., and Svircev, A. M. 1990. Growth of *Peronospora* in tobaccos. Pages 19-34 in: Blue Mold Disease of Tobacco. C. E. Main and H. W. Spurr, eds. Delmar Co., Charlotte, NC.
13. Nicholas, R. O., Williams, D. W., and Hunter, P. A. 1994. Investigation of the value of β-glucan-specific fluorochromes for predicting the β-glucan content of the cell walls of zoopathogenic fungi. Mycol. Res. 98:694-698.
14. O'Brien, T. P., and McCully, M. E. 1981. The Study of Plant Structure Principles and Selected Methods. Termacarrphi PTY, Melbourne, Australia.
15. Rohringer, R., Kim, W. K., Samborski, D. J., and Howes, N. K. 1977. Calcofluor: An optical brightener for fluorescence microscopy of fungal plant parasites in leaves. Phytopathology 67:808-810.
16. Schots, A., Dewey, F. M., and Oliver, R. 1994. Modern Assays for Plant Pathogenic Fungi: Identification, Detection and Quantification. CAB International, Oxford.
17. Smith, M. M., and McCully, M. E. 1978. A critical evaluation of the specificity of aniline blue induced fluorescence. Protoplasma 95:229-254.
18. Stanghellini, M. E., Rasmussen, S. L., and Vandemark, G. J. 1993. Relationship of callose deposition to resistance of lettuce to *Plasmopara lactucae-radicis*. Phytopathology 83:1498-1501.
19. Sumstine, D. R. 1913. Studies in North American Hyphomycetes-II. Mycologia 5:45-61.
20. Trese, A. T., and Loschke, D. C. 1990. High contrast resolution of the mycelia of pathogenic fungi in corn tissue after staining with Calcofluor and destaining with cellulase. Phytopathology 80:196-200.
21. van Sengbusch, P., Hechler, J., and Muller, U. 1983. Molecular architecture of fungal cell walls. An approach by use of fluorescent markers. Eur. J. Cell. Biol. 30:305-312.
22. von Arx, J. A. 1987. Plant Pathogenic Fungi. Beih. Nova Hedwigia 87: 71.
23. Williamson, B., Breese, W. A., and Shattock, R. C. 1995. A histological study of downy mildew *Peronospora rubi* infection of leaves, flowers and developing fruits of Tummelberry and other *Rubus* spp. Mycol. Res. 99:1311-1316.