Chemical Pollutant Toxicity Determined with a Fungal Assay

H. Babich K.D. Fox

There is much concern and interest in environmental pollution and toxicology. Yet, there are few commercially available kits for classroom use that demonstrate environmental toxicology. Most general biology laboratory manuals lack a specific exercise to illustrate the response of a biological system to chemical stress. In order for students to more fully understand and appreciate the impact of chemical pollution on biota, hands-on involvement is needed in the laboratory.

This manuscript describes a microbial bioassay, using filamentous fungi ("molds") as test organisms, which clearly demonstrates toxicities of environmental pollutants. The assay is performed by inoculating a fungus to the center of Petri dishes containing a nutrient agar medium as the control and to a nutrient agar medium into which varying amounts of toxicants have been introduced. At appropriate time intervals, the diameters of mycelial growth extension are measured with a ruler; toxicity is quantified by comparing mycelial extensions on experimental media with that on the control medium. This assay is visually dynamic: The students can clearly see and evaluate the effect of progressively increasing concentrations of test agent on fungal growth. In addition, the use of solidified agar medium avoids accidental spills, optimizing student safety.

Test Organisms

Fungi are a diverse group of eukaryotic, heterotrophic organisms that range in complexity from unicellular yeasts to multicellular, filamentous molds. A mold consists of a mycelium, which is a mat of thread-like filaments, termed hyphae, that function both in nutrition and in reproduction. When inoculated on a nutrient agar medium, some hyphae grow on and in the substrate, releasing digestive enzymes. Extracellularly digested nutrients are then absorbed through the plasma (cell) membranes of the hyphae. If the fungal inoculum is placed in the center of a nutrient agar-containing Petri dish, the mycelial mat will spread, as a progressively increasing circular colony, over the entire culture plate. Some fungal hyphae grow upwards and are involved in the production of reproductive units, or spores. Such reproductive hyphae, with the spores, may be brightly colored.

H. Babich, associate professor, and **K. D. Fox,** laboratory technician/instructor, teach at Stern College, Yeshiva University, department of biological sciences, 245 Lexington Ave., New York, NY 10016-4699.

Methods

Stock cultures of filamentous fungi are grown and maintained at room temperature on slants of Sabouraud dextrose agar (SDA) until abundant spores develop. For several fungi sporulation is readily evident because the mycelia develop colored spores upon maturation. The tubes are stored at 4°C until needed. Using sterile technique and a microbiological inoculating loop, samples of the fungus are transferred from the tube to the center of a Petri dish containing 15 to 20 ml of SDA. This transferred sample usually contains abundant spores with some hyphae. Care should be taken so that the fungal inoculum is introduced onto the center of the Petri dish only, and that no spores inadvertently fall and contaminate other portions of the agar plate. After a few days of incubation at room temperature, a circular fungal colony will fill the Petri dish. Such fungal plates can now be distributed to the class for use in the bioassay.

Using a sterilized metal cork borer (either wrapped in foil and autoclaved or dipped in 70 percent alcohol and flamed), circular plugs (4 to 6 mm in diameter) are punched into the periphery of the fungal colony. Using the tapered end of a sterile microspatula, fungal plugs, with the fungal growth upwards, are transferred to new Petri dishes containing SDA, unamended (control) and amended with varied concentrations of the test chemical. The key to success in this experiment is to punch the plugs from the periphery of the fungal colony only, as this portion of the organism is youngest and lacks spores. When present, spores tend to fall during the transfer and contaminate multiple regions of the test agar plate.

The Petri dishes with their fungal plugs are incubated at room temperature for the desired number of days. Figure 1 shows the mycelial growth of a fungus on control and experimental media. Fungal mycelial extensions are measured in millimeters (mm) with a ruler. The dimensions of the colony are defined by taking measurements in three directions and then averaging the values. Growth rates, in mm/day, can be computed by dividing the mycelial extension by the number of days of growth. Mycelial growth extensions, or growth rates, on experimental media are compared to those on the control medium. These calculations can be used to construct a concentration-response toxicity curve, by plotting either the mycelial extensions or the growth rates on the Y-axis and the concentrations of test agent on the X-axis. Experiments can be designed to compare the response of a specific fungus to several test agents (Figure 2) or the response of several fungi to a specific test agent (Figure 3). Furthermore, the student can then estimate the midpoint toxicity value (i.e.

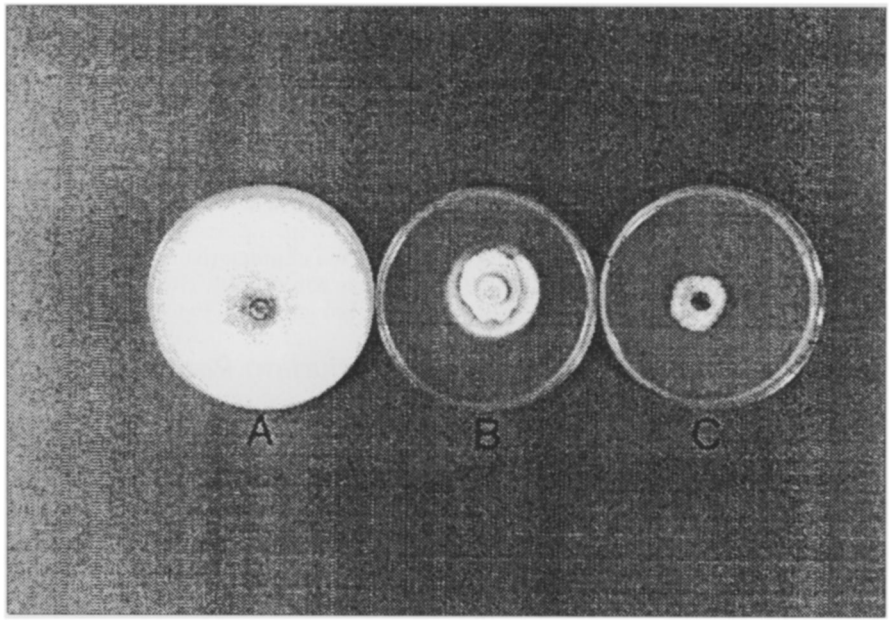

Figure 1. Growth of *Tricoderma viride* after three days of incubation at room temperature on control medium (A) and on medium amended with 2.5×10^{-3} M mercury ion, as $HgCl_2$ (B), and 5×10^{-4} M nickel ion, as $NiCl_2$ (C).

the concentration of test agent that inhibited either the mycelial growth extension or the growth rate by 50 percent). The environmental toxicologist typically uses such midpoint toxicity values to rank chemicals according to their potencies towards a specific test organism and to determine the relative sensitivities of different organisms to a specific test agent.

This bioassay is suitable for use with most filamentous fungi. The selection of the specific test organism can be made to conform with the scheduling of the laboratory sessions. For example, a relatively slow growing fungus can be used for a class that meets once a week. Inoculations are made during the first week and measurements of mycelial extension are made the following week. Fungi suitable for a seven-day reading are *Fusarium solani, Aspergillus fischeri, Aspergillus nidulans* and *Penicillium vermiculatum*. For a class that meets twice a week (e.g. Monday and Wednesday; Tuesday and Thursday), the experiment can be completed within a two-day span if fast growing fungi are used. Such fast-growing fungi include *Rhizopus stolonifer, Phycomyces blakesleeanus* and species of *Achyla* and *Saprolegnia. Cunninghamella echinulata, Trichoderma viride* and *Botrytis cinerea* are suitable for a three-day assay, and *Aspergillus niger, Aspergillus giganteus* and *Fusarium solani* are suitable for a five-day assay.

This bioassay has been used primarily with heavy metals, such as cadmium, chromium, lead, manganese, mercury, nickel and zinc added as salts (Babich & Stotzky 1983), although it is also applicable for use with organic test agents (Babich & Stotzky 1985). Furthermore, if noninocuous test agents are desired, the bioassay can be used to demonstrate how each of the following variables affects mycelial growth extension: acid precipitation [by adjusting the test media to different pH values (Babich & Stotzky 1982)]; food additives (such as nitrate and bisulfite); and osmotic pressure (by adjusting the test media with varied levels of NaCl).

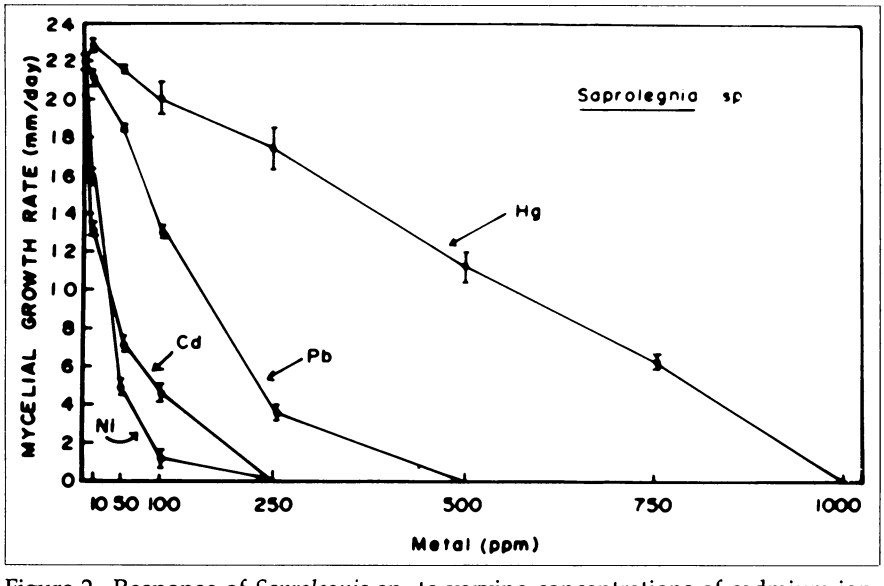

Figure 2. Response of *Saprolegnia* sp. to varying concentrations of cadmium ion, as $CdCl_2$, mercury ion, as $HgCl_2$, nickel ion, as $NiCl_2$, and lead ion, as $Pb(NO_3)_2$. Metal concentrations are expressed in parts per million (ppm), i.e. milligrams of metal ion per liter.

Other Applications

The above discussion has been geared to the use of this bioassay in a general biology laboratory. An important concept in environmental toxicology which can easily be shown with this bioassay is that the toxicity of a chemical pollutant is dependent on the abiotic (or physicochemical) parameters of the recipient environment. Well studied examples include the influence of temperature, pH and particulates on the toxicity of metals (Babich & Stotzky 1983). These abiotic factors can be studied by appropriately adjusting the medium or the exposure conditions. For example, *Aspergillus niger* grows at room temperature and at 37°C; thus, chemical toxicity can be studied at various temperatures. The influence of particulate matter on metal toxicity can be shown by incorporating inorganic clay mineral particles, such as kaolinite (kaolin, Fisher Scientific Co.) and montmorillonite (bentonite, Fisher Scientific Co.), or organic humic acid particulates (Aldrich Chemical Co.) into the growth medium (Babich & Stotzky 1977, 1979). The influence of acid precipitation on metal toxicity can be shown by adjusting metal-amended media to different pH values (Babich & Stotzky 1982).

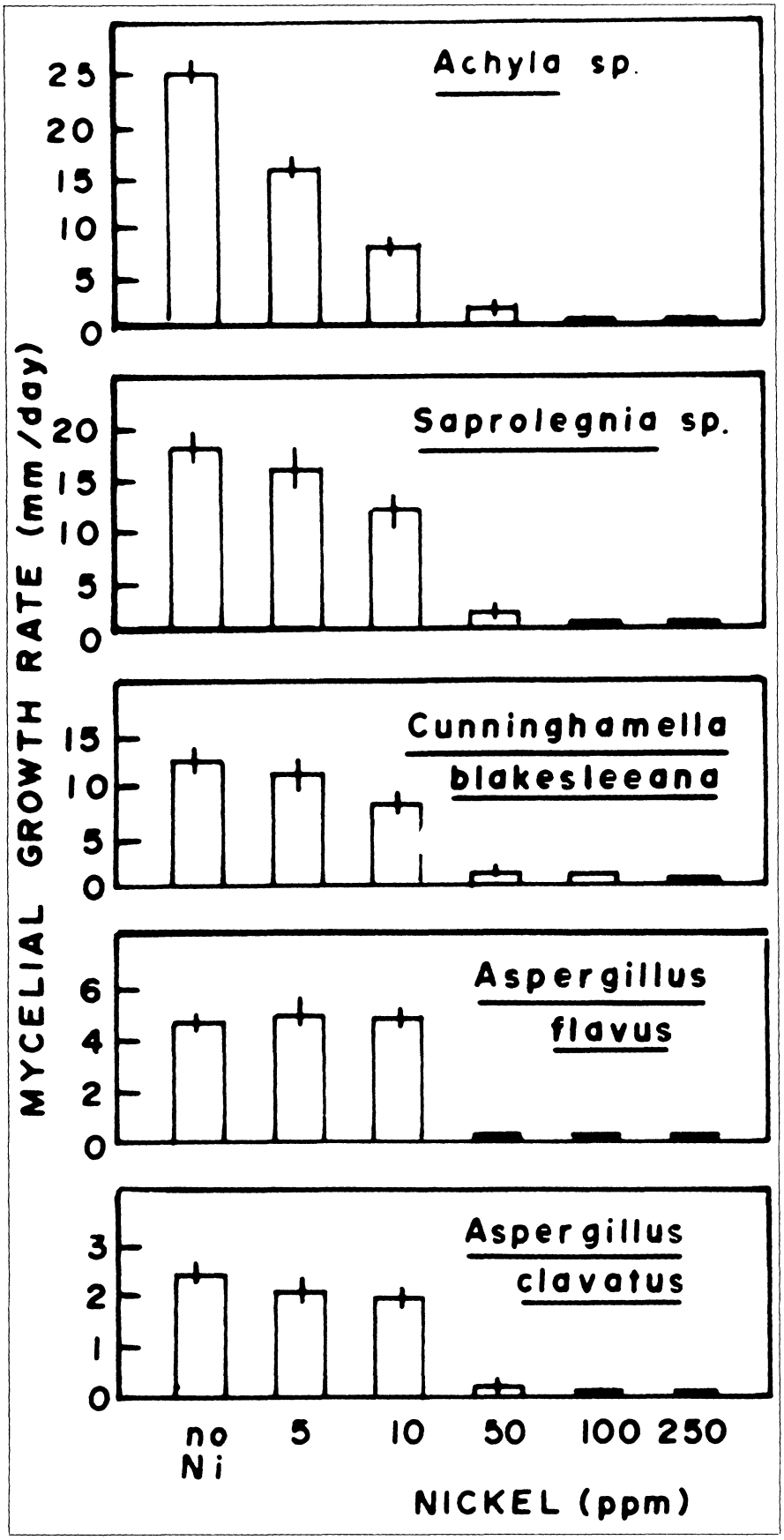

Figure 3. Response of several filamentous fungi to nickel ion, as $NiCl_2$. Metal concentration is expressed in parts per million (ppm), i.e. milligrams of nickel ion per liter.

With a more detailed analysis of the effects of the chemical agent on the test organism, this bioassay can be used in a microbiology laboratory. Thus, in addition to affecting mycelial growth extension, the test agent also may be used to study its adverse effects on fungal pigmentation, morphology and spore production. The latter two characteristics can be examined microscopically (Babich & Stotzky 1982, Babich et al. 1982).

Concluding Remarks

The preparation for this bioassay is relatively uncomplicated, although it involves the availability of an autoclave to prepare the medium. This fungal bioassay is economical, provides reproducible data, can easily be performed by students without requiring extensive laboratory skills and optimizes safe exposure to the test agents. Furthermore, activities can be added to increase the complexity of the exercise (i.e. to include an analysis of abiotic environmental factors as mediators of pollutant toxicity and to include microscopic analysis of fungi exposed to inhibitory levels of test agents).

Acknowledgments

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