A Microcalorimetric Technique for Rapid Assessment of Fungicide Activity

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ABSTRACT

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Screening for antifungal metabolites from microbial fermentation broths or extracted plant samples is used to identify compounds with potential novel modes of action. The isolation of fungicidal secondary metabolites from these samples relies on the use of a sensitive detection method that can be evaluated in a timely fashion. This paper describes the use of isothermal microcalorimetry to rapidly assess fungicide activity. An in vivo screening test can require from 1 to 4 weeks and milligram quantities of material. Many in vitro tests require only microgram quantities, with test durations of from 2 to 5 days. Microcalorimetry, with its ability to detect microwatt changes in metabolic heat, extends this range of sensitivity. By this method, fungicidal activities can be assessed in 4 to 8 hours with only nanogram quantities of material. Techniques for the use of isothermal microcalorimetry were developed for the filamentous fungi *Pythium aphanidermatum* and *Pyricularia oryzae*.

Secondary metabolite screening for compounds has been a mainstay of the pharmaceutical industry for many years (4) and has increased in importance in the agricultural industry (11). Screening plants and microbial sources against new targets continues in an effort to identify novel compounds (2,9,10,20,23,24,26). Traditionally, fermentation broths as well as plant extracts are tested against a panel of organisms for activity. If active, the material is then isolated by a series of steps, which ultimately yields a purified product for chemical analysis and structural identification. Every purification step involves the evaluation of the antimicrobial activity of each chemical fraction generated. The utility of the test system is only as efficient as its sensitivity and the length of time necessary to complete the assay. Each subsequent purification step can only be initiated after a full evaluation of the previous step. For in vivo testing, this means a reduction in disease levels (15,21); milligram quantities of material may be needed, and disease development can take from 2 days to 4 weeks. For in vitro testing, such effects are commonly expressed as a reduction in growth rate, changes in hyphal morphology on agar, or spore germination inhibition (3,5,6,12,27). Biological growth assays normally take between 2 and 5 days before rating.

Obtaining biological data for compounds in a timely fashion using a minimal amount of material can be a problem during the isolation of a fungicidal secondary metabolite natural product. The concentration of the active moiety is often at micrograms per milliliter or lower levels in extracts of fermentation broths. Isolating an active compound present at such low levels requires the use of a sensitive small-scale assay system. This is particularly true during the final stages of purification, when the small amount of material left is necessary for both confirmation of fungicidal activity and structural analysis. Microcalorimetry offers the potential to be such a sensitive system.

Microcalorimetry is a very sensitive technique; biological differential scanning calorimeters can measure a heat quantity as small as 1 µW under isothermal conditions. This means that isothermal microcalorimetry can detect heat output from a colony of about 10^5 microorganisms. Indeed, the technique has been used to study growth patterns of bacteria and their response to antibiotics, as well as cultural conditions (1,8,18,25). The basic principle involved is that heat is generated during growth and, therefore, substances that hinder growth will cause a reduction in metabolic heat. Most literature on bacteria is limited to growth under anaerobic conditions. Of the fungi, only the unicellular yeasts (13,19) have been examined by this system. Filamentous fungi and their responses to fungicides have not been tested. The principle defining growth is valid for fungi; however, the aerobic requirement by most fungi and their spherical growth habit in liquid culture make them a difficult system to investigate experimentally.

To evaluate the potential of microcalorimetry for fungal assessment, two fungi were chosen as model systems: the Phycomycete *Pythium aphanidermatum* Edson (Fitzp.), a causal agent of damping off, and the Ascomycete *Pyricularia oryzae* Cavara, the causal agent of rice blast. These two taxonomically diverse species were chosen to show the broad applicability of this technique. Described in this paper are the methods developed to study these fungi and the microcalorimetric results obtained on their growth with and without the presence of a fungicide.

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MATERIALS AND METHODS

Organisms. Stock cultures of *P. aphanidermatum* were maintained on malt extract agar (16,17) in the dark at 24°C. Stock dishes of *Pyricularia oryzae* were grown on V8 juice agar (7) under similar conditions. Cultures for experimental use were prepared using 5 ml of agar in 100 × 15 mm petri dishes. Culture dishes for *P. aphanidermatum* were used 24 h after inoculation with a mycelial plug in the center of the dish. The *Pyricularia oryzae* dishes were used 48 h after the agar surface had been flooded with a heavy spore suspension. At this point, hyphal growth was heavy and sporulation had begun. On two different occasions, in order to assess the effect of culture age on metabolic heat production, cultures at 66-h postinoculation were also used.

Experimental procedures. The calorimetric measurements were carried out at 24°C on a Setaram Bio-Differential Scanning Calorimeter (Bio-DSC) (Setaram, Caluire, France), which can measure heat quantity better than 1 µW under isothermal conditions. To accommodate the sample and to achieve aerobic conditions needed for growth by the fungi, the sample flow-cell supplied with the instrument was modified. A new top consisting of a copper heat exchanger fitted with two 19-gauge stainless steel tubes was machined to the same dimensions as the one on the original flow-cell. This was connected to the stainless steel vessel via a viton O-ring (Fig. 1). The tube at the center, which was slightly shorter than the sample cell, was the inlet for the gas used to purge the cell. The other tube, which formed the exit for the gas, terminated at the conical piece used to compress the viton O-ring.

To maximize the surface area available for sample placement, a stainless steel support with five ledges was fabricated to fit onto the gas inlet tube. The support was held in place with a short length of silicon tubing. Prior to use, the sample vessel and the support were cleaned in soapy water and rinsed in deionized water, followed by a rinse in 70% ethanol, and heated in a 95°C oven overnight.

Samples were cut from the culture dishes with a special cork borer. This tool was made by joining a Swagelok 3.2- to

3.2-mm fractional tube to a fractional tube stub reducer, a 6.4- to 3.2-mm reducing union, and a length of 6.4-mm stainless steel tube. The internal diameter of this sample cutter was approximately 2 mm. The cutter was sterilized during use by flaming over a Bunsen burner.

To treat the fungus with the test solution, 20 disks of *P. aphanidermatum* were aseptically cut 1 to 2 cm behind the leading edge of the colony. The plugs were soaked in 200 μl of sterile test solution in one well of a Costar 24-well cell culture plate (Costar Corp., Cambridge, MA) for 1 h. Solutions used were distilled water (control) and metalaxyl (Ridomil, technical grade, Ciba-Geigy, Basel, Switzerland) at 2 or 4 μg/ml in sterile water. After pipetting off the test solution, we allowed the disks to dry for 10 min. Melted malt extract agar was cooled to 55°C and amended with the same concentration of fungicide used in the initial soaking process. Twenty microliters of the amended agar was pipetted evenly over both surfaces of the five ledges of the stainless steel support described previously. Two fungal disks were placed in contact with the agar on opposite sides of each ledge for a total of 20 disks per trial.

The procedure used for *Pyricularia oryzae* was similar to that described above, except in this case the fungal disks were used without presoaking in the test solutions. Cycloheximide, an antibiotic derived from the actinomycete *Streptomyces griseus* Krainsky (Waksman & Henrici) (Sigma Chemical Co., St. Louis, MO), was evaluated in this system. The 20 disks were cut from the actively growing mycelial culture dishes. Two disks were placed in direct contact with each surface of the metal ledges. V8 agar was melted, cooled, and amended with sterile aqueous cycloheximide solution to final concentrations of 0, 0.1, 0.5, 1.0, and 2.0 μg/ml. Twenty microliters of the amended agar was pipetted to fill the space between the disks.

After the support was prepared, the DSC sample vessel was assembled through the viton O-ring. Filtered oxygen was bubbled through a small water reservoir and then connected to the gas inlet of the sample vessel. This provided a constant purge at a rate of about 0.5 cm^3/min. At this point, the sample vessel was introduced into the calorimeter and allowed to equilibrate for 30 min prior to data collection. An empty flow-cell supplied with the instrument was used as a reference during measurements. This vessel was placed in the instrument in advance of the sample vessel. Duration of the test for all treatments was 5 h.

The effect of cycloheximide against *Pyricularia oryzae* also was evaluated on agar dishes using treated paper disks. Sterile aqueous solutions of cycloheximide were prepared and pipetted onto 5-mm-diameter sterile filter paper disks to final concentrations of 0, 0.1, 1.0, 10.0, and 50 μg per disk. Two hundred microliters of a spore suspension of *Pyricularia oryzae* (1.0×10^6 conidia per milliliter) was evenly spread on the surface of culture dishes of V8 agar. After the free moisture evaporated from the dish, five disks (one each at the above concentrations) were applied in a circle equidistant from each other on a single dish. Growth inhibition was measured after 72 h.

RESULTS AND DISCUSSION

The growth profiles for *P. aphanidermatum* (Fig. 2) and *Pyricularia oryzae* (Fig. 3) both clearly show the antifungal effect of the agent tested within the 5-h time frame. In each case, the individual curves were superimposed on one another by shifting along the y-axis to arrive at the composite plot. The results are both quantitative and reproducible. Although the test duration was 5 h, metalaxyl at either concentration used reduced the rate of heat production from *P. aphanidermatum* after only 2 h (Fig. 2). This reduction reflects a slower growth rate of the fungus in response to metalaxyl. This effect was more pronounced with *Pyricularia oryzae* when cycloheximide was the test compound (Fig. 3). The reduction in heat production was apparent after only 1 h; at 0.1 μg/ml, cycloheximide stopped heat evolution

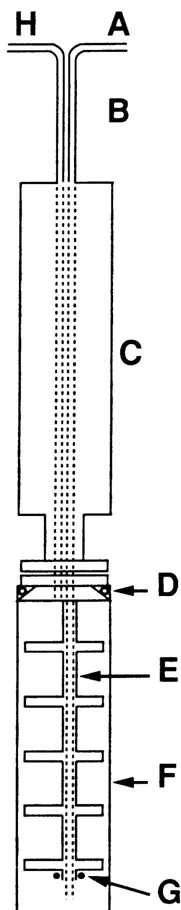

Fig. 1. Sample flow-cell for use with filamentous fungal samples in the Setaram Bio-DSC: A, gas inlet–oxygen (filtered and water saturated at flow rate of about 0.5 cm^3/min); B, 19-gauge stainless steel dip tube; C, copper heat exchanger; D, viton O-ring; E, stainless steel sample support, height 18 mm, distance between ledges 4 mm, ledge diameter 7 mm; F, stainless steel sample vessel; G, silicon tubing; and H, gas exit.

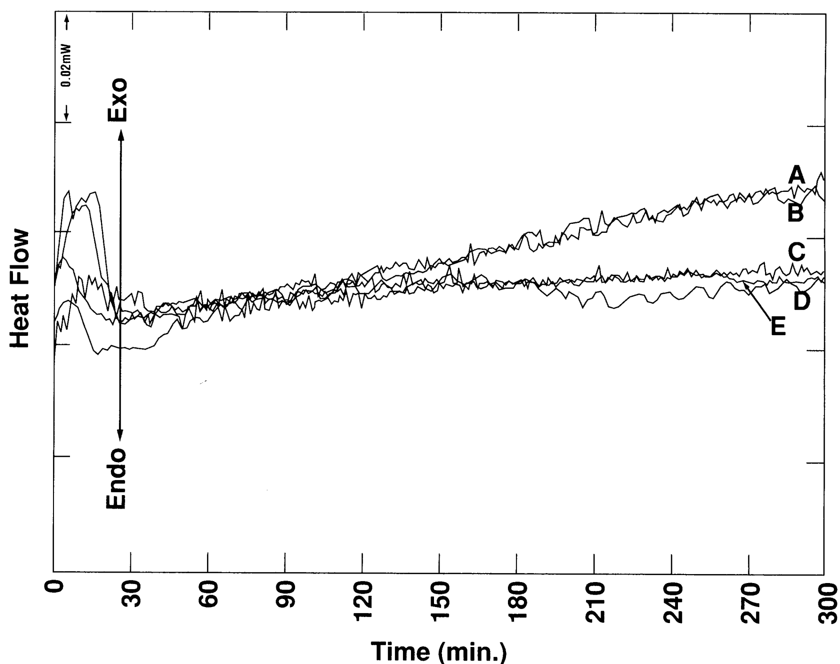

Fig. 2. Effect of different concentrations of metalaxyl (technical grade) tested against *Pythium aphanidermatum* in the Setaram Bio-DSC. Reduction in growth is measured as a decrease in metabolic heat (mW) over time. *P. aphanidermatum* was grown on malt extract agar. Metalaxyl concentrations in agar: Curves A and B, 0 μg/ml; C and D, 2.0 μg/ml; and E, 4.0 μg/ml.

from the fungus completely. At higher concentrations, rather than having positive heat production, the profiles were negative. This indicates a negative growth rate of the fungus; hence, these concentrations may represent lethal doses. The two control curves, B and C, were obtained 1 month apart. In both cases, the culture used was 66 h postinoculation. The curves were almost superimposable, showing the reproducibility of the technique. Additionally, the rates of heat production were less than that in curve A, illustrating the weaker growth with the older culture.

Fungi grow differently under aerobic and anaerobic conditions. The two fungi in this study require oxygen. In his review on yeast, Lamprecht (13) discussed the difficulties involved in achieving aerobic conditions for microcalorimetric studies. Because of the limited solubility of oxygen in water, growth on solid media should be better than in liquid culture. However, only a few calorimetric studies of microbial growth on solid agar have been reported (14,22). Perhaps this was due to the intrinsic experimental difficulties both in sample handling and in the surface area constraints inside the DSC vessel. The design of our sample support was a response to these problems. It provides enough surface for the inocula and growth medium, and also sufficient room for easy sample manipulation. In addition, the oxygen delivery system supplies sufficient amounts for growth. Effects due to sample drying (i.e., water evaporation) or wetting as a result of water condensation from the premoistened oxygen were not observed.

It is important to note that our studies were made with fungi grown on 5 ml of agar in 100 × 15 mm petri dishes. These culture dishes are much thinner than those normally used for in vitro assays. These conditions permit more efficient diffusion of nutrient and antifungal compounds to the fungus. Fungal inocula cut from the surface of normal thickness plates (20 ml of agar per dish) did not function well in the assay system. Examination of a sample under a dissecting microscope indicated that the mycelia grew down into the agar plug rather than into the surrounding amended medium. Thus, for rapid assays, inoculum from thin plates is essential.

Each fungal system used needed some separate optimization prior to the calorimetric measurements. *P. aphanidermatum* required a presoak period in the test solution. Without it, an initial burst of growth was observed, with its concomitant generation of heat, followed by a sudden cessation of heat generation. This might have been due to the diffusion time necessary before this fast-growing fungus came into contact with the toxicant. Presoaking circumvented this problem. For *Pyricularia oryzae*, the use of cultures older than 48 h resulted in a flatter growth curve, i.e., a lower growth rate (Fig. 3). At 48 h, hyphal growth was heavy and sporulation had just begun. Control cultures produced high metabolic heat and displayed great reduction in growth after treatment with cycloheximide. It was at this age that the fungus was most suitable for calorimetric studies.

To address the issue of sensitivity of the microcalorimetric technique, the in vitro effect of cycloheximide against *Pyricularia oryzae* was determined using treated disks on agar dishes. A clear zone of inhibition was not observed until the concentration reached 1 µg per disk. In contrast, the microcalorimetric assay showed complete inhibition at 0.1 µg/ml (a total of 0.02 µg or 20 ng per 200 µl of agar). This represents a 50-fold reduction in the amount of toxicant necessary for detection. In a single trial (data not shown) using cycloheximide at 0.01 µg/ml (a total of 2 ng per 200 µl of agar), inhibition of growth also was observed. This represents a 500-fold decrease in the amount of material necessary for testing.

The high degree of sensitivity offered by the microcalorimetric method makes it an ideal technique for situations where the amount of the antifungal candidate available for testing is low. This is true for both synthetic chemicals and the isolation of natural products from complex mixtures. In natural products, in isolation from either a crude biological sample or fermentation broth, multiple fractions are produced during each step of purification. Fraction quantity often becomes a serious limitation during the final steps. Enough material must be available to allow for the next purification step or structural identification, as well as for biological activity testing. An additional factor is the unavoidable loss of sample due to the necessity of filter sterilization of aqueous samples prior to testing. It is at these latter stages that this microcalorimetric method can make the most valuable contribution to natural products screening. With it, the amount of material needed for activity evaluation is greatly reduced, freeing it for other uses. In addition, the reduction in analysis time is beneficial, since the decision time between steps is shortened. The condensed time frame is important, especially when the active moiety lacks long-term stability. The usefulness of this technique will be further enhanced when a microcalorimeter suitable for high-volume screening becomes commercially available.

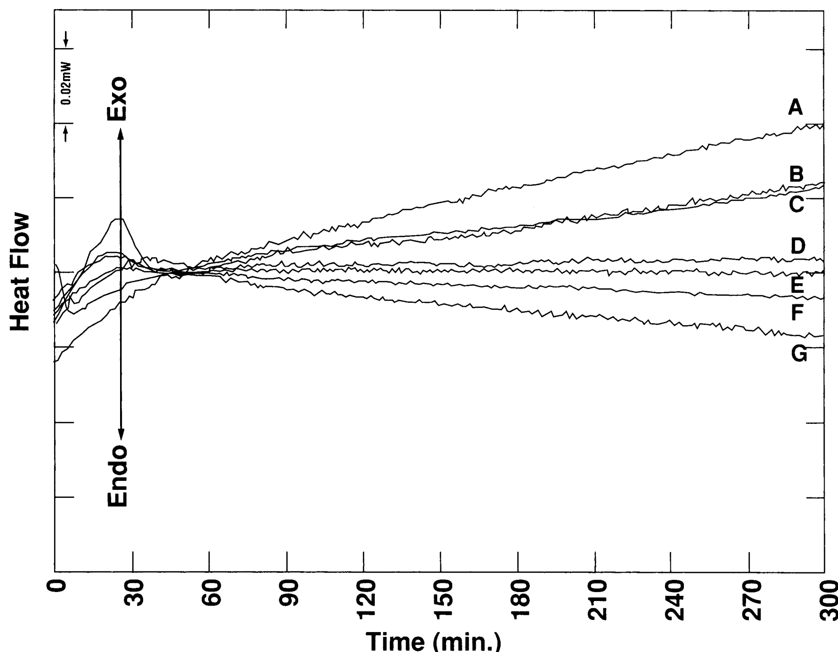

Fig. 3. Effect of different concentrations of cycloheximide tested against *Pyricularia oryzae* in the Setaram Bio-DSC. *Pyricularia oryzae* was grown on V8 agar. Reduction in metabolic heat (mW), and hence fungal growth, is apparent after 60 min in test. Cycloheximide concentrations in agar: Curves A to C, 0 µg/ml; D, 0.1 µg/ml; E, 0.5 µg/ml; F, 1.0 µg/ml; and G, 2.0 µg/ml. Culture age: 48 h except in B and C (66 h).

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