Benchmarks

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Microscale Assay Monitors Algal Growth Characteristics

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Today's agriculture and landscaping require effective and environmentally responsible herbicides. A detailed knowledge of the herbicide's molecular mechanism of action would promote the design of new, safer, and more effective herbicides, but often their mode of action is unknown. As in other areas of research, a microorganism model provides advantages over the long generation time of many crop plants. The algae *Chlamydomonas reinhardtii* and higher plants share conserved photosynthetic machinery, and both organisms show similar responses to herbicides (6). *C. reinhardtii* presents unique opportunities to study photosynthesis-inhibiting

herbicides since the addition of a carbon source allows the algae to grow without photosynthesis (4). Their facultative heterotrophism allows the identification and characterization of non-photosynthetic mutant strains with altered herbicide sensitivity. Further advantages lie in the fact that *Chlamydomonas* are unicellular, haploid eukaryotes with generation times measured in hours rather than weeks or months. Well-defined genetics and molecular tools such as the ability to transform the nuclear, mitochondrial, and chloroplast genomes make *Chlamydomonas* a very effective model in studies that require a photosynthetic organism.

The assay we describe overcomes previous technical hurdles that hinder growth analyses and provides an easy and efficient means to test multiple replicates of many herbicides simultaneously. Standard growth assays employ manual cell counts, making them quite labor intensive (2). As the combinations of time points, conditions, and/or replicates are reduced to manageable numbers, the statistical significance of the results decreases. Also, in addition to the universal premium on research space, assays for studying photosynthesis are best performed in small areas to protect against fluctuations in light intensity.

The assay employs samples in 96-well plates monitored by spectrophotometry. To protect against evaporation

over an eight-day time course, sterile, covered microplates (FisherBrand®, 0.45-mL, flat-bottom; Fisher Scientific, Pittsburgh, PA, USA) are used, and sterile water is placed in the outermost rows and columns. The 60 remaining wells are conveniently divided into six replicates of 10 different experimental conditions. Under these conditions, light variations are effectively eliminated, as the entire plate is only 8.5 × 13 cm.

Cultures for *Chlamydomonas* growth assays are usually grown in cultures greater than or equal to 6 mL, and cell counts determined using a hemocytometer. Unlike bacteria and yeast, the literature reports no standard wavelength for measuring *Chlamydomonas* growth. A spectrophotometric growth assay was tested by comparing the cell count versus A_{655} or A_{750} of wild-type cells (cc-125) grown in "large" (6 mL) cultures (measurements used Bio-Rad® 3550-UV Microplate Reader/Microplate Manager 2.20; Bio-Rad Laboratories, Hercules, CA, USA). The readings at A_{655} and A_{750} measure chlorophyll content and biomass, respectively (1,2). Growth curves show similar correlation between manual cell count and absorbance readings at either wavelength (Figure 1). Readings at 750 nm are particularly valuable for herbicide sensitivity studies. They provide a longer period for data collection before readings plateau and, therefore, may allow detection of more subtle changes in growth.

Figure 1. Comparison of growth curves based on absorbance with growth curves based on manual cell counts. Standard (6-mL) cultures of wild-type *C. reinhardtii* were grown in the presence of acetate for eight days. Data were collected at 750 nm (squares), 655 nm (triangles), or by manual cell counts (diamonds). Data points shown are the average of two separate trials.

Benchmarks

Also, since 750-nm readings measure biomass, the assay is not dependent on chlorophyll production, a biosynthetic pathway that many herbicide affect. At 750 nm, *Chlamydomonas* mutants with abnormal levels of chlorophyll can be evaluated and changes in growth rate and morbidity can be separated from an herbicide-induced decrease in chlorophyll found in an otherwise viable cell.

Growth trends of microscale cultures are comparable to those of standard-size cultures. The 6-mL and 0.35-mL cultures of wild-type *C. reinhardtii* show different maximal growth levels but similar trends for entrance into and duration of log phase growth (Figure 2). To ensure that experiments were performed on cells in similar growth phases, large-scale (6 mL) and microscale (0.35 mL) cultures were prepared from the same 24°C, 24–35 h overnight cultures diluted to 5×10^4 cells/mL. During the assay, the microplate cover was taped to the plate in two places to assure that it would not be dislodged during shaking. It was not sealed to assure that the samples have adequate gas exchange. Aeration was provided by growing large cultures in slanted racks (20°) shaking at 180 rpm and 96-well plates agitated in a microplate shaker at 1000 rpm (Titer Plate Shaker; Lab-Line Instruments, Melrose Park, IL, USA). The high rate was necessary to combat surface tension across the small culture volume in the microscale assay. The 96-well plate's higher agitation rate may have allowed more gas exchange and account for the increased maximum growth and absorbance reached in the microscale assay versus the large-scale one (Figure 2, 750-nm samples).

The utility of the spectrophotometry-based microscale assay is demonstrated by the ease of determining wild-type *C. reinhardtii*'s sensitivity to a protoporphyrin oxidase (PPO) inhibitor herbicide. Previous reports showing the algae's sensitivity to the PPO inhibitor, S-23142, lead to the isolation of resistant strain, rs-3 (3) and the identification of the mutation in PPO that confer resistance while allowing normal chlorophyll biosynthesis (5). With this approach in mind, we characterized the sensitivity of a wild-type strain to a different PPO inhibitor, flumichlorac pentyl ester (FPE; Valent, Walnut

Creek, CA, USA) (7). The growth of cells challenged with from 0 to 5 μM FPE was monitored over 195 h. To provide conditions that require photosynthesis and most closely mimic those found in higher plants, the herbicide-challenged cells were grown in high light (196 $\mu\text{mol}/\text{m}^2/\text{s}$) and "HS" acetate-free media (2), and all plates were agitated at 1000 rpm. Preparing replicate trials in sterile troughs and applying six replicates of 0.35 mL to the 96-

well plate with a multichannel pipettor simplified sample preparation. Every 8–12 h, growth was measured as the absorbance at 750 nm. The plate reader agitated the samples for 10 s before each reading to assure the culture was well mixed. *C. reinhardtii* is flagellated, but settling of dead cells might affect spectrophotometry.

As expected, *C. reinhardtii* is sensitive to the herbicide (Figure 3). Comparison of the growth curves reveals the

Figure 2. Comparison of growth in standard and microscale cultures. Standard cultures (6-mL, open symbol) and microscale cultures (0.35-mL, shaded symbols) of wild-type *C. reinhardtii* were grown in the presence of acetate for eight days. Data were collected at 750 nm (squares) and 655 nm (triangles). Data shown include the 95% confidence interval for two standard cultures or six microscale ones.

Figure 3. Greater than 2 μM FPE causes the death of wild-type *C. reinhardtii*. Growth curves were prepared for wild-type cells grown in the presence of 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, and 5.0 μM FPE under conditions requiring photosynthesis (HS, high light). All data were collected at 750 nm. The 4.5 and 5.0 μM trials are not included in the graph, as no growth was seen. Data shown include the 95% confidence interval for six wells/conditions.

sensitivity of this assay. Increases of 0.5 μM FPE affect the entrance into log phase first, resulting in almost parallel curves between 50 and 100 h for samples with 0, 0.5, and 1 μM FPE (Figure 3, top three growth curves). A 6-fold decrease in growth rate ($\Delta\text{Abs/h}$) is seen during log phase when herbicide concentrations are increased from 1 to 2 μM , and the strain shows no significant growth in higher concentrations of herbicide (Figure 3).

The results indicate that the spectrophotometric microscale assay is an effective way to analyze growth characteristics of multiple samples. The savings afforded by this spectrophotometric microscale assay are easily quantified. Sixty of the traditional large (6-mL) cultures would require 360 mL media and greater than 15 360 cm^2 shaker surface area. In microscale, the same tests could be completed with 21 mL media and 110 cm^2 shaker space. The small size ensures that light levels remain constant across all samples and do not provide a confounding variable within the experiment. The savings in handling time per assay is even more dramatic. Each culture is monitored 17 times during the eight-day duration of a growth assay (t_0 and two times/day). Using a hemocytometer, counting the cells in one culture takes approximately 3 min. Given this rate, just determining the cell count for 60 cultures requires 6 h/day for a total of 51 h over the course of the assay. By contrast, the spectrophotometric reading of all 60 microscale samples takes only 20 s/day, which is less than 5 min for the entire growth assay. The 50 h 53 min saved could be easily devoted to data analysis rather than data collection. Also, unlike the standard assay, the microscale assay can be scaled up with little increase in time investment. Since microplate shakers can accommodate at least four plates, 240 tests can be run simultaneously on one shaker, and readings for all 240 samples still take less time than is required for the manual cell count of one culture.

Although, as presented, the assay measures *C. reinhardtii* herbicide resistance, the approach could easily be modified to accommodate other growth conditions, screening for mutants, or characterizing other microorganisms. The results indicate that the spectro-

photometric microscale assay is very promising for characterizing the herbicide sensitivity of strains with known defects, screening for new mutants with altered sensitivities, and testing the efficacy of new herbicide formulations.

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