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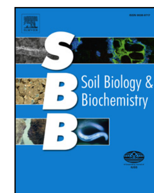

Cross-laboratory comparison of fluorimetric microplate and colorimetric bench-scale soil enzyme assays

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ABSTRACT

There has been growing interest in fluorescence-based microplate methods to measure enzyme activities due to the sensitivity of fluorimetric detection and the potential for simultaneous and rapid assaying of multiple enzyme activities in the same soil suspension. However, micro-scale methods could introduce considerable operator error such as: 1) the requirement to put soil samples into a suspension; 2) the very small amounts of soil placed in each microplate well; 3) pipetting error because μL volumes are required; and 4) the need for standard curve calibration with every sample to account for quenching. For valid data comparison and interpretation, there is clearly a need to have a strict and agreed-upon enzyme assay protocol to standardize the microplate-based method. Therefore, the objectives were to: 1) determine the reproducibility and comparability of the standard *p*-nitrophenol bench-scale and 4-methylumbelliferone microplate enzyme assays measured by five laboratories for β-glucosidase (EC 3.2.1.21) and acid phosphomonoesterase (EC 3.1.3.2) on the same soil samples; and 2) determine the degree and the sources of variability associated with the assays within and among the laboratories. The results showed that overall variability was highest for replication on the microplate (n = 4), whereas suspension replication had low CVs. This suggests an important source of variation is from pipetting not variability from soil suspensions. A major effort was made to control for methodological differences by using air-dried soils (therefore more stable over varying storage periods) and operator consistency for each task across the labs (e.g. preheated reagents, microplate reader sensitivity set to the highest standard, readings taken within an hour of reaction termination, and controls for substrate autohydrolysis). As a result, the differences among labs were much smaller than differences due to soil type for the microplate method, indicating operator error can be minimized by following the same strict protocol. At the molar level, enzyme activity rates measured across the five labs were not the same between bench and MUF microplate methods (although they were within an order of magnitude), but were quite similar in terms of ranking of soil management treatments and soil types (Table 2). Correlations between bench and microplate assays were strong for both enzymes, although slightly stronger for acid phosphomonoesterase (r = 0.93) than β-glucosidase (r = 0.81). Additionally, for both acid phosphomonoesterase and β-glucosidase, correlation r values were mostly similar for MUF microplate and PNP bench method correlation with EL-FAME biomarkers, suggesting both methods were measuring activity originating from the same microbial groups. We conclude that different labs using the same MUF microplate protocol tested, gives reasonably similar absolute activity values, variability, and ranking of treatments (highest to lowest). We propose that the MUF microplate method described in this study be considered as a standard protocol for assaying soil enzyme activities, providing that the buffer pH for the incubation be adjusted to the optimal pH according to the enzyme of interest.

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1. Introduction

Soil enzyme assays have traditionally been performed at the bench scale using spectroscopic methods. There has been growing interest in fluorescence-based microplate methods to measure enzyme activities because fluorimetric detection is sensitive, and the microplate format has potential for simultaneous and rapid assaying of multiple enzyme activities from the same soil suspension. In contrast, bench-scale methodology has lower throughput and sensitivity. Microplate-format enzyme protocols have already been incorporated in many research laboratories; however, the lack of a standardized method for performing the assays and the potential for methodological problems (German et al., 2011) limits their usefulness for cross-study data comparisons and meaningful data interpretation. Several validation steps are recommended prior to standardization and widespread adaptation of new microbiological methods (AOAC, 2006; FEM, 2009; Green, 1996). Preliminary validation involves establishing performance characteristics such as specificity, sensitivity, reproducibility, and accuracy based on comparative testing with a reference method. Additionally, a collaborative study may be done to compare the assay's performance across laboratories.

Evaluative studies of various fluorimetric microplate assays using 4-methylumbelliferone (MUF) based substrates have been done in several laboratories (Deng et al., 2013; Drouillon and Merckx, 2005; Marx et al., 2001; Pritsch et al., 2004; Trap et al., 2012), and most were done in the same laboratories that developed the methods. However, the resulting data have not consistently had the same outcomes between the fluorescent microplate and the colorimetric *p*-nitrophenol (PNP) bench-scale methods (Dick et al., 2013; Marx et al., 2001). Therefore, further cross-comparison between these methods is required to determine the sources of variation.

Methodological discrepancies may account for some differences in assay performance. Notable in the above studies were the different pHs under which the assays were performed. In some, the buffer pH was adjusted to the pH of the soil (Drouillon and Merckx, 2005; German et al., 2011; Trap et al., 2012), and in others, to the optimal pH for the enzyme (DeForest, 2009; Deng et al., 2013; Dick et al., 2013; Marx et al., 2001).

It has long been established that MUF fluorescence intensity is pH dependent, with the highest fluorescence signal around pH 10. Moreover, when NaOH is used to increase MUF fluorescence at the end of a fluorimetric enzyme assay, MUF fluorescence signal decreases over time after NaOH addition, making it challenging for obtaining quantitative fluorescence readings and for valid data comparison (DeForest, 2009; Drouillon and Merckx, 2005). German et al. (2011) found NaOH addition to be a significant source of variation and recommended against the NaOH addition for samples at pH 4.5 and higher, as they had no difficulty detecting the accumulation of MUF over time at pH ≥ 4.5, even without increasing the sensitivity setting on their microplate fluorimeter. THAM pH 10–12 is the optimal solution to add after the enzyme assay because the fluorescence intensity highest due to the high pH and the signal remains stable up to three hours because of the THAM buffer (Deng et al., 2013).

One advantage of fluorimetric over colorimetric microplate methods is that fluorescence, unlike absorbance, is not increased by the presence of soil particles (Deng et al., 2013). However, fluorescence chemistry presents its own complexities because of quenching effects and the potential for chemical hydrolysis; both influence reproducibility. The quenching effect, due primarily to the presence of soil particles and dissolved organic matter in the assay mixtures, has been shown to vary temporally and spatially (Freeman et al., 1995), and thus requires a calibration curve be developed for every sample assayed.

Thus, microplate enzyme assay methods introduce several sources of variation that have the potential to substantially affect assay reproducibility, and require careful execution of certain steps by the operator to minimize the variation. In part, this is due to the very small amounts of soil that must be used (0.83 µg in a 250 µL reaction volume in the current study) to minimize the quenching effect and to accommodate the small-volume microplate wells. Furthermore, dispensing µL volumes of soil/buffer slurry with a pipette results in wide variations in the amount of soil added to each assay well. Because of this, the analytical error intrinsic to microplate enzyme assays is expected to be considerably larger than that of the conventional bench method. To minimize error, more replication is required compared to traditional bench-scale methods.

Thus, there are legitimate concerns about reproducibility and whether different labs can obtain comparable data from of the same samples. For valid data comparison and interpretation, there is clearly a need to have a strict and agreed-upon enzyme assay protocol to standardize the microplate-based method where different operators who follow these procedures obtain the same results. This is not case for the MUF microplate method as there has been considerable variation in the protocols used in the literature. Therefore, a cross-lab study was done on an optimized MUF procedure that has evolved from a number of labs (Marx et al., 2001; DeForest, 2009; German et al., 2011; Deng et al., 2011, 2013; Dick et al., 2013).

Since PNP bench-scale enzymes assays are widely used, largely vetted for standardization, and accepted, the objectives of this study were to determine the reproducibility by different laboratories for assaying activity of β-glucosidase (EC 3.2.1.21) and acid phosphomonoesterase (EC 3.1.3.2) in the same soil samples using MUF microplate methods in comparison with standard PNP bench-scale assays; and to determine the degree and the sources of variability associated with these two assays within and among five laboratories.

2. Materials and methods

2.1. Soils, sampling and storage

The four soils selected were: 1) a sandy soil from the Ottokee fine sand series (sandy, mixed, mesic Aquic Udipsamments) under soybean (*Glycine max*) near Napoleon, Ohio (OTB); 2) a soil with higher clay content from the Miamian silt loam series (fine, mixed, active, mesic Oxyaquic Hapludalfs) under mixed grasses at the Ohio State University Waterman Farm, Columbus, Ohio (MMN); 3) a Jory silty clay loam (fine, mixed, active, mesic Xeric Palehumults) under Christmas tree (*Pseudotsuga menziesii* at time of sampling) management (32 yrs) and vegetation-free except for the trees, near Corvallis, Oregon (JMN); and 4) a Jory silty clay loam under > 90 yrs unmanaged Douglas Fir (*Pseudotsuga menziessi*) forest (JOG). The two Jory soil sites are side by side.

Approximately 3 kg of soil were collected at each sampling site along a transect at three spatially separated points (field replications) approximately 50 m apart. At each sampling site a 2 m diameter area was sampled by taking about 30 0–15 cm depth cores with a probe (2.54 cm dia.). Field moist soils were passed through a 2 mm sieve, which resulted in a thoroughly homogenized sample. A large portion was air-dried; while a small portion was left field-moist. Air drying was done by spreading soil on butcher block paper, spread at about 0.5 cm thick for 24 h. The air-dried soils were separated into 500 g samples and placed in sealed zip lock bags for shipment on ice in Styrofoam containers to the collaborating laboratories. The field level replication was maintained throughout the research, with each laboratory receiving three separate replicates of each soil, for a total of 12 soil samples. The field-moist samples were either stored at 4 °C for pH and soil texture analyses, or stored at − 20 °C for Ester-Linked Fatty Acid Methyl Ester (EL FAME) analysis as an index microbial community composition.

2.2. Soil chemical and biological properties

Total C and N contents were measured by dry combustion (950 °C) with a Vario Max CN Analyzer (Elementar; Hanau, Germany). Particle

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