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Automated Robotic Assay of Phosphomonoesterase Activity in Soils

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ABSTRACT

Phosphorus cycling in most ecosystems is dependent on plant and microbiologically derived phosphatase enzymes, and available P limits both microbial and plant growth. Measurements of enzymes in soil systems are often time-consuming and labor intensive. In this study, we examined phosphomonoesterase activity in soils using a Zymark XP laboratory robotic system for soil handling, solvent addition and exchange, filtration, incubation, reagent addition, and final sample preparation. Phosphomonoesterase activity was measured using phenyl phosphate as substrate, and samples were analyzed using a 96-well microplate reader. Results indicated that our robotic system was capable of effectively measuring phosphomonoesterase activity in soils differing in physical and chemical characteristics. The results obtained using manual and robotic procedures were comparable in accuracy and precision. The robotic system decreased labor associated with this assay by about a factor of 4.5 relative to the manual system, with considerable savings on reagent costs and labor.

MANY KEY BIOCHEMICAL REACTIONS in soils have been shown to be due to microbial enzymes that catalyze the transformation of both organic and inorganic compounds. These enzymes are of up-most importance for the biogeochemical cycling of elements and the mineralization and immobilization of biogenic materials in soils. Analyses of soil enzymes have received recent attention as a means to examine small-scale distribution of soil microbial processes in different microhabitats (e.g., soil–litter interface, rhizosphere, or particle-size fractions) (Tscherko et al., 2004; Marx et al., 2005; Sessitsch et al., 2004).

The phosphatases represent a broad group of enzymes catalyzing the hydrolysis of esters and anhydrides of phosphoric acid, and include phosphoric acid monoesterases (E.C. 3.1.3.), phosphoric acid diester hydrolases (E.C. 3.1.4.), triphosphoric monoesterhydrolases (E.C. 3.1.5.), phosphoryl-containing anhydrides hydrolases (E.C. 3.6.1.), phosphatases hydrolyzing P–N bonds (E.C. 3.9.), and phosphoamidases (E.C. 3.9.1.1.) (Tabatabai, 1994; Criquet et al., 2004). The phosphomonoesterase enzymes, often called "phosphatases," comprise a large and important group of biocatalysts involved in the hydrolysis of ester-linked organophosphorus compounds to orthophosphate, and are thought to be the major phosphatases in most soils and litter (Turner et al., 2002). The activities of both acid and alkaline phosphatases are influenced by various soil properties, soil organism interactions, vegetation cover, leachate inputs, the presence of inhibitors, activators, and heavy metals (Juma and Tabatabai, 1977; Kandeler et al., 1996; Hysek and Sarapatka, 1998; Belyaeva et al., 2005) and organic matter content (Dick et al., 1994; Jordan et al., 1995), and as such, have been used to provide estimates of changes in soil quality (Gil-Sotres et al., 2005).

Determination of phosphatase activity in soils often relies on the use of colorimetric substrates, including phenylphosphate, *p*-nitrophenyl phosphate, and fluorescent substrates (4-methylumbelliferyl phosphate, fluorescein diphosphate) (Tabatabai 1994; Schinner et al., 1996; Marx et al., 2001). Reactions are usually quantitative, follow Michaelis-Menton Kinetics, and are relatively easy to perform. However, some assays require long incubation times, contain multiple steps, or use substrates whose products require immediate analysis, thereby limiting throughput.

Robotic-based high-throughput technologies have revolutionized biology, research methods, and opened the door for large-scale genomic analyses of macro- and microorganisms. Such technologies will undoubtedly have an impact on the examination of soils and soil processes. To analyze the large number of samples generated in large field and laboratory experiments with high precision and accuracy, it is often necessary to automate analytical procedures (Koskinen et al., 1991). This is especially true of soil monitoring and landscape-level studies where large numbers of samples are often collected. While robotic analysis systems have been developed for routine analysis of soil characteristics (Quigley and Reid, 1998; Hill et al., 2002), pesticides in soil and water (Koskinen et al., 1991; Kraemer 1997), and microbial and enzymatic activity in dairy and food products (Richardson et al., 1988), there have been no reports on the use of robotic systems to analyze microbial enzyme activities in soils.

In this study we developed and evaluated an automated procedure to quantify alkaline phosphomonoesterase activity in two Midwestern soils using phenylphosphate as substrate. Enzyme reactions with this substrate release phenol, which is subsequently quantified spectrophotometrically using the color reagent 2,6-dibromoquinone-4-chloroimide. The procedure utilized a commercially available laboratory robotic system and results were compared to those obtained using manual procedures.

MATERIALS AND METHODS

Soils and Chemicals

Two soils with different physicochemical properties were obtained from Minnesota and Iowa and sieved through a 1.18-mm

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pore sieve before use. The Minnesota soils was a Zimmerman fine sand (Alfic Udipsamment), collected in Princeton MN, with a particles-size distribution of 94.5, 2.7, and 3.8% sand, silt, and clay, respectively, pH 6.0 ($CaCl_2$) and 0.9% soil organic matter. The Iowa soil was a Nicollet loam (Aquic Hapludol) soil, collected approximately 6.4 km south of Ames, IA, with a particles-size distribution of 35, 45, and 30% sand, silt, and clay, respectively, pH 6.3 ($CaCl_2$) and 4.7% soil organic matter. Soils were stored at 4°C until used. Phenylphosphate disodium salt (95% purity) and 2,6-dibromoquinone-4-chloroimide were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). All other reagents were obtained from Fisher Chemicals (Fisher Chemicals, Fairlawn, NJ).

Manual Phosphomonoesterase Assay

Alkaline phosphomonoesterase activity in 5 g of moist soils was determined essentially as described by Hoffmann (1968), and later modified by Öhlinger (1996), using 0.2 *M* borate buffer (pH 10) and 0.1 *M* disodium phenylphosphate as a substrate. Samples were incubated at 37°C for 3 h, and color development was quantified using a Beckman DU-70 spectrophotometer at 630 nm, after a further 75-min incubation with the color reagent 2,6-dibromoquinone-4-chloroimide. Calibration blanks used contained no soil, and controls had substrate solution replaced by water. Forty samples were analyzed, in two separate runs, and values (μg phenol g^{-1} soil h^{-1}) are presented as means ± standard error on means.

Automated Phosphomonoesterase Assay

A Zymark XP robot core system (Zymark Corp., Hopkinton, Ma) was used for the automated assay of phosphomonoesterase activity in soils. For these studies, the robotic system required the use of the following hardware: 9- to 16-mm tube hand, 30- to 50-mm tube hand, 1- to 4-mL pipetting hand, 1-mL tip rack, 16 by 100 mm tube racks, 50-mL centrifuge tube rack, filling station, vortex mixing station, filtering station, water bath, and microtiter plate station. The layout of the robotic system is shown in Fig. 1. The automated assay for phosphomonoesterase activity in soils was adapted from the procedure described by Hoffmann (1968), and later modified by Öhlinger (1996), however many modifications were required to adapt this procedure to the robotic platform used. Briefly, field-moist soil (1.25 g) was manually weighed into 39 of the 16 by 100 mm test tubes and 2.5 mL of 0.2 *M* borate buffer (pH 10), and 1.25 mL substrate solution (0.1 *M* phenylphosphate disodium salt) was added. Tubes were mixed for 20 s, placed in a water bath incubator at 37°C for 3 h, mixed for an additional 15 s, and 1 mL of 0.5 *M* $CaCl_2$ and 4 mL of distilled water was added. Tubes were mixed for 15 s, 2.75 mL of distilled water was added, and the contents filtered through an empty Varian Bond Elut LRC cartridge (Varian, Inc., Palo Alto, CA) packed with a #1 Whatman filter paper disc and 0.8-μm glass wool. A 0.5-mL aliquot of the filtered solution was added to a 50-mL centrifuge tube, and 1.25 mL of 0.2 *M* borate buffer (pH 10), 23 mL of distilled water, and 0.25 mL of color reagent (2 mg of 2,6-dibromoquinone-4-chloroimide) per milliliter in 36% ethanol (v/v) were added. The solution was mixed for 15 s, incubated for 75 min at 25°C, and 300-μL aliquots of each reaction tube were transferred to a 96 well microtiter plate. Absorbance of samples was measured at 630 nm using a Bio-Tek Model ELx808 Microplate Reader (Winooski, VT). This wavelength was chosen due to availability of filters in the microplate reader, and analyses done using a spectrophotometer at both 630 and 614 nm produced similar response curves, with a mean absorbance unit difference of 0.009, when tested against 1 to 200 μg of phenol. Method precision and variability was evaluated by analyzing forty replicate samples, in two separate runs of the experiment. Values are presented as means of μg phenol g^{-1} soil h^{-1} ± standard error on means. Linear regression analysis was done using Microsoft Excel 2002 (Microsoft Inc., Redmond, WA).

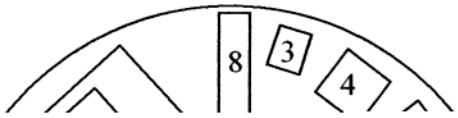

Fig. 1. Schematic diagram of the automated robotic soil analysis system: (1) Zymate II core system; consisting of arm, computer, and controller; (2) 16 by 100 mm tube rack (initial soil samples); (3) Filling station (buffer, substrate, distilled water, calcium chloride, and color reagent); (4) Vortex station; (5) Water bath for incubation with 16 by 100 test tube rack inside; (6) Zymark custom hand; (7) Filtering station and column holder; (8) column disposal slide; (9) 16 by 100 mm filtrate test tube rack; (10) custom hand; (11) 50-mL test tube rack; (12) Zymark pipetting hand and pipette tip rack; (13) Microtiter plate rack.

RESULTS AND DISCUSSION

The robotic system we developed allowed for the serial processing of 40 soil samples in the batch mode without intervention. If run in a fed-batch mode, where finished and analyzed samples are removed from the input and output racks at fixed intervals, samples can be processed on a continual basis, allowing analysis of about 80 samples per day with limited intervention by personnel. Since each assay tube can theoretically contain replicates of different soils, many soil samples can be processed simultaneously. In addition, adoption of this system to methods requiring shorter incubation times (Tabatabai, 1994) will further result in increased time savings for high throughput applications. Results in Table 1 show that the automated procedure reduced the amount of labor hours required for analysis by a factor of 4.5 relative to that required for the manual method. One of the main time saving steps was in the filtering part of the method, labor hours were reduced from 2 to 0 h using the robotic apparatus as all steps were handled automatically. There were also many other time constraints in the manual assay, which were eliminated or reduced using the robotic system. For example, quantification of the color produced from hydrolysis of the

Table 1. Labor hours involved in manual versus automated analyses of phosphomonoesterase activity in soils.†

Procedure	Manual Analysis	Automated Robotic Analysis
	h:min	h:min
Soil Prep	0:05	0:05
Weighing Samples	0:30	0:30
Borate Addition	0:05	0:00
Substrate Addition	0:05	0:00
Water Addition	0:10	0:00
Filter Prep	0:05	0:20
Filtration	2:00	0:00
Borate Addition	0:05	0:00
Color Reagent Addition	0:05	0:00
Water Addition	0:10	0:00
Mixing	0:05	0:00
Spectrophotometric Analysis	1:00	0:05
Approximate Total Time	4:25	1:00

† For both methods, reaction mixtures were incubated for 3 h, and color development was quantified after an additional 75-min incubation with the color reagent.

substrate to phenol, in the presence of 2,6-dibromoquinone-4-chloroimide, was facilitated by use of the microplate reader, which could read 96 samples in <2 min.

The automated procedure we adopted was essentially as that described by Öhlinger (1996). However, the automated procedure was proportionately scaled down by a factor of four from the original assay without loss of accuracy. This allowed us to use less reagents, resulting in overall cost reduction for analyses. We also modified the original procedure by reducing the amount of water addition before filtration by 12%, and by using a 0.5 *M* $CaCl_2$ solution to facilitate flocculation of soil particle before automated filtration. The use of $CaCl_2$ in phosphatase assays has previously been reported (Tabatabai, 1994) to enhance *p*-nitrophenol measurements in soil. In this study the use of $CaCl_2$ resulted in an increased filtration rate, and also reduced variance in final absorbance values obtained by about fourfold. For example, phosphomonoesterase activity in quadruplicate samples of Nicollet loam soil was 1063.9 ± 32.2 and 1016.9 ± 8.3 μg phenol g^{-1} soil h^{-1}, respectively, without or with flocculation. These values are in the range of previously reported results for agricultural soils (Ebersberger et al., 2003; Marschner et al., 2003). Lastly, due to the common filter set used in our automated microplate reader, we also modified the procedure by quantifying the amount of phenol produced at 630 nm, rather than 614 nm, and allowing color to stabilize in microplates for 75 min before measurement. A standard curve of A_{630} versus phenol concentration was linear from 1 to 200 μg of phenol using this wavelength ($R^2 = 0.999$), which was applicable to absorbance values obtained in soils of disparate fertility. This was similar to what was found at 614 nm (data not presented). The 75-min stabilization period was empirically found to be optimal to obtain consistency in maximum absorbance readings. In this study, we found that this period can be extended to 36 h, without significant changes in absorbance. This is in contrast to what was found when phosphatase assays were done using *p*-nitrophenyl phosphate as the substrate, absorbance values changed over time after filtration and in 96 well microplates. However, since programming of the robot steps is fairly simple, it will allow easy adoption to other phosphatase assays, both alkaline and acid, some of which have shorter incubation periods and produce stable products (Tabatabai, 1994). This will allow further increases in throughput that will facilitate large-scale analyses of soils.

Results in Table 2 show that the robotic procedure was capable of determining alkaline phosphatase activity in both the Nicollet loam and Zimmerman fine sand soils. In addition, the accuracy of results obtained by manual and automated methods was similar, and in a separate experiment a mean of 774.0 ± 40.9 μg phenol was produced using the manual method on 40 replicates of the Nicollet loam soil. Moreover, repeated automated runs using the same soil produced similar results over time. This is most likely due to the fact that the robotic system allows for accuracy during every step of the procedure, by performing each step exactly the same each time. This is similar to what has been reported for the robotic analysis of atrazine and alachlor from soils (Koskinen et al., 1991).

As expected, the Zimmerman fine sand soil had consistently less phosphomonoesterase activity than did the Nicollet loam soil, most likely due to lower overall microbial activity and biomass, owing in part to differences in organic matter and clay content (Dick et al., 1994; Jordan et al., 1995; Gil-Sotres et al., 2005). The Nicollet soil is characterized by around 4.7% soil organic matter and in contrast to agricultural soils with lower soil organic matter content (around 1.5%), we expect higher variation of soil microbiological properties since organic matter is not uniformly distributed in soils. Moreover, the Zimmerman sand soil was much drier than the Nicollet soil, also possibly reducing overall enzyme activity. While it has been argued that air drying soil before analyses will allow better comparison of the relative differences between soils and treatments, air drying also creates unexpected changes in enzyme activities, depending on soil chemical and physical properties. In a separate experiment, air drying of the low organic matter Zimmerman soil before manual analyses reduced overall phosphomonesterase activity approximately eight-fold relative to the soil stored at 4°C. Thus, our initial studies were done using moist soils. However, overall variation in results would have been improved if air drying was done before analysis. Nevertheless, the robotic procedure was clearly capable of measuring and

Table 2. Phosphomonoesterase activity in two Midwest soils using manual versus automated robotic analyses.

		Run 1		Run 2	
Soil	Method	Mean μg phenol	Std. error	Mean μg phenol	Std. error
		g^{-1} soil h^{-1}			
Nicollet loam	Robotic	952.1†	6.3	763.6	5.8
Nicollet loam	Manual	954.6‡	20.7	797.5	13.8
Zimmerman sand	Robotic	132.1	4.6	138.4	3.4
Zimmerman sand	Manual	140.5	16.6	146.1	13.4

† Values obtained from the robotic analyses are the means of 40 replicate samples.

‡ Values obtained from manual analyses are the means of four replicate samples.

differentiating phosphomomoesterase activity in soils differing in physicochemical characteristics.

In summary, our results indicate that a robotic system can be adapted and used to automate the analysis of enzyme activities in soils. The automated method we used produced similar results of phosphomonoesterase activity as compared with the manual method. This system will most likely have great utility in long-term monitoring programs of soil microbiological properties for characterization of soil quality and soil health, as well as in landscape-level studies where large numbers of samples are often collected. The robotic system we used can clearly be adapted for use to examine other enzymes in soils. The reduction of sample size will also allow the use of the robot system in studies where only small amounts of sample are available, such as those examining spatial variability in microbial processes and soil properties.

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