



Anais da Academia Brasileira de Ciências

ISSN: 0001-3765

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Academia Brasileira de Ciências
Brasil

FERREIRA, ADÃO S.; ESPÍNDOLA, SUÉLLEN P.; CAMPOS, MARIA RITA C.
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Anais da Academia Brasileira de Ciências, vol. 88, núm. 2, abril-junio, 2016, pp. 1035-1044
Academia Brasileira de Ciências
Rio de Janeiro, Brasil

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Assessment and kinetics of soil phosphatase in Brazilian Savanna systems

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Manuscript received on January 24, 2014; accepted for publication July 17, 2015

ABSTRACT

The activity and kinetics of soil phosphatases are important indicators to evaluate soil quality in specific sites such as the Cerrado (Brazilian Savanna). This study aimed to determine the activity and kinetic parameters of soil phosphatase in Cerrado systems. Soil phosphatase activity was assessed in samples of native Cerrado (NC), no-tillage (NT), conventional tillage (CT) and pasture with *Brachiaria brizantha* (PBb) and evaluated with acetate buffer (AB), tris-HCl buffer (TB), modified universal buffer (MUB) and low MUB. The Michaelis-Menten equation and Eadie-Hofstee model were applied to obtain the kinetic parameters of soil phosphatase using different concentrations of p-nitrophenol phosphate (p-NPP). MUB showed the lowest soil phosphatase activity in all soils whereas AB in NC and NT presented the highest. Low MUB decreased interferences in the assessment of soil phosphatase activity when compared to MUB, suggesting that organic acids interfere on the soil phosphatase activity. In NC and NT, soil phosphatase activity performed with TB was similar to AB and low MUB. K_m values from the Michaelis-Menten equation were higher in NC than in NT, which indicate a lower affinity of phosphatase activity for the substrate in NC. V_{max} values were also higher in NC than in NT. The Eadie-Hofstee model suggests that NC had more phosphatase isoforms than NT. The study showed that buffer type is of fundamental importance when assessing soil phosphatase activity in Cerrado soils.

Key words: soil phosphatase activity, buffer, enzyme kinetics, Michaelis-Menten equation, Eadie-Hofstee model, Cerrado.

INTRODUCTION

Phosphorus (P) availability in soils depends on phosphorus recycling from soil organic matter (Tabatabai and Bremner 1969, Albrecht et al. 2010, Brooks et al. 2013). This availability is also regulated by adsorption of phosphate anions to aluminum and iron oxide compounds present

in soils (Cleveland et al. 2002, Novais and Smith 1999, Sylvia et al. 1999). Nevertheless, soil organic phosphorus mineralization is an enzymatic process carried out by a group of phosphatases that catalyze hydrolytic reactions of phosphate groups (mono or diesters), thereby providing inorganic P to the soil solution (Neble et al. 2007, Criquet and Braud 2008, Tabatabai 1994). Acid phosphatase activity in particular plays an important role in hydrolyzing organic phosphorus to orthophosphate ions in acid

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soils (Tabatabai 1994, Huang and Shindo 2000), including soils of the Cerrado (Vinhai-Freitas et al. 2012).

Soil phosphatase is a primary indicator of soil quality in various land use systems (Dick 1997, Vinhai-Freitas et al. 2013). Although many studies have examined enzymes in Brazilian soils (Vinhai-Freitas et al. 2010, 2012, Pavanelli and Araújo 2010), they have not explored Michaelis-Menten constants. Kinetic parameters can provide important information on the enzymatic mechanisms and soil functions in ecosystems (Nannipieri et al. 1982, Marx et al. 2001, Vinhai-Freitas et al. 2013). Enzyme kinetic parameters are affected by specific soil conditions such as metabolite concentration, microbial community, salinity, metals, and soil pH (Nannipieri and Gianfreda 1998, Marx et al. 2001, Nannipieri et al. 1982). These conditions are greatly affected by the land use, including soil management practices (Nannipieri et al. 1982). Soil enzyme analysis also varies with the pH, reaction buffer and substrate concentration (Malcom 1983, Nannipieri et al. 1982).

In most studies, soil phosphatase activity is determined by using the substrate p-nitrophenol phosphate (p-NPP) in modified universal buffer (MUB) as described by Tabatabai and Bremer (1969). However, in Cerrado soils this methodology tends to underestimate the phosphatase activity in colorimetric assays. Reports have shown that high levels of soil organic matter, iron and aluminum can interfere in colorimetric assays (Trasar-Cepeda and Gil-Sotres 1988, Malcom 1983). Cerrado soils are acidic, highly weathered, contain high levels of iron and aluminum (Ferreira et al. 2008, Vinhai-Freitas et al. 2012), and may possess organic content as high as of native forest soils. Thus, the existing procedures for determining phosphatase activity in Cerrado soils should be reviewed. Due to the properties described above, it is hypothesized that, in spite of its use in many studies, MUB is inadequate for assessing the phosphatase activity

in Cerrado soils when sodium hydroxide is used to extract p-nitrophenol (p-NP).

The objective of this study was to determine the activity and kinetic parameters of soil phosphatase in native and agricultural soils of the Brazilian Savanna. Additionally, four different buffers were applied in order to evaluate sensitivity of soil phosphatase activity measurement in colorimetric assays.

MATERIALS AND METHODS

The study was carried out in natural and agricultural sites in the Cerrado ecoregion near Uberlândia (Minas Gerais state, Brazil). Based on precipitation and temperature, the region's climate was classified as "Cwa" (Köppen 1923). Specifically, the climate is "hot moderate" with a very dry winter and a warm, rainy summer. The soils were classified as clayey Oxisol (Typic Acrustox) according to Soil Taxonomy (USDA 1992). In the summer of 2012, soil samples of 600 cm² (20 cm x 30 cm) were taken at a depth of 5 cm. The sample points were randomly selected within a 1 ha area following a W sampling scheme. After removing organic litter, five soil samples were collected at each site. Three subsamples were collected at each sampling point and mixed into one sample. Samples were transported to the laboratory in isothermic bags (4 °C). Each sample was sieved (~ 3 mm) and stored at 4 °C until the day of analysis (within a maximum of two weeks). Moisture content was measured after oven drying for 48 h at 105 °C. Additional physico-chemical characteristics of the air-dried soil were also analyzed (Table I). Microbial biomass carbon (Ferreira et al. 1999) and microbial respiration (Stotzky 1965) were determined using moist soil and the results were adjusted to dry weight (Table I).

Soil phosphatase activity was determined in a spectrophotometric assay (405 nm) by quantifying the p-NP (p-nitrophenol) released from p-NPP (p-Nitrophenol phosphate) substrate using differ-

TABLE I
Physicochemical characteristics of soils under different land-use systems.

Characterization	System			
	NC	NT	CT	PBb
Sand (g kg ⁻¹)	135	140	120	550
Clay + Silte (g kg ⁻¹)	865	860	880	450
Carbon (g C kg ⁻¹)	48.2	30.3	23.5	26.8
Nitrogen (mg N kg ⁻¹)	3.24	1.93	0.98	0.70
Phosphorus (mg P kg ⁻¹)	1.89	8.29	3.26	2.06
Potassium (mg K kg ⁻¹)	37	106	70	60
pH H ₂ O	4.0	6.5	6.3	6.0
MBC (mg C kg ⁻¹ dry soil)	559	297	186	249
BRS (mg C day ⁻¹ kg ⁻¹ dry soil)	89	13.3	6.8	24.8

MBC, microbial biomass carbon. BRS, basal respiration of soil. NC, native Cerrado; NT, no-tillage; CT, conventional tillage. PBb, Pasture with *Brachiaria brizantha*.

ent buffers (pH 6.5). A 1 g portion of wet soil was transferred into a Falcon tube (50 mL) and 4 mL of buffer and 1 mL of p-NPP substrate (7.5 mM of 4-Nitrophenyl phosphate disodium salt hexahydrate diluted in buffer) were added. Controls without p-NPP were assayed for each soil sample in order to determine a baseline for the buffer's effect. Reaction mixtures were incubated at 37 °C for 1 hour and then calcium chloride (0.5 M CaCl₂) was added. The reaction was stopped by adding 4 mL of sodium hydroxide (0.5 M). An aliquot of the extract (2 mL) was centrifuged at 10,000 x g for 2 minutes and then analyzed in a spectrophotometer (Biomate 3, Thermo Scientific).

Four different buffers were used to assess phosphatase activity in order to evaluate the effect of buffer type on the results. The four buffers used were prepared as follows:

(AB) - Acetate buffer (0.5 M): 41 g of sodium acetate were transferred to 800 mL MilliQ water and then adjusted to pH 6.5 with hydrochloric acid (10 M). The mixture was transferred into a volumetric flask (1 L) and the volume completed with MilliQ water.

(TB) - Tris-Buffer (0.5 M): 60.5 g of tris (hydroxymethylaminomethane) were transferred to 800 mL MilliQ water and then adjusted to pH 6.5 with hydrochloric acid (10 M). The mixture was

transferred into a volumetric flask (1 L) and the volume completed with MilliQ water.

(MUB) - Modified Universal Buffer: 2.42 g of tris (hydroxymethylaminomethane), 2.3 g of maleic acid, 2.8 g of citric acid and 1.26 g of boric acid were transferred to 800 mL MilliQ water and then adjusted to pH 6.5 with sodium hydroxide (10 M). The mixture was transferred into a volumetric flask (1 L) and the volume completed with MilliQ water.

(low MUB) - low Modified Universal Buffer: 0.2 g of tris (hydroxymethylaminomethane), 1.15 g of maleic acid, 1.4 g of citric acid and 0.618 g of boric acid were transferred to 800 mL MilliQ water and then adjusted to pH 6.5 with sodium hydroxide (10 M). The mixture was transferred into a volumetric flask (1 L) and the volume completed with MilliQ water.

The standard curves of each buffer was performed. An extraction solution containing 40 mL NaOH (0.5 M) and 50 mL of each buffer was prepared under the same conditions of the soil sample. A portion of p-NP (0.2 g) was transferred into a volumetric flask (50 mL) and the volume completed with extraction solution. This solution (5 mL) was diluted in another volumetric flask (50 mL) and completed with the extraction solution. The final solution was diluted another time with the same

procedure. After, aliquots of 125, 250, 500, 1,000 and 2,000 mL were transferred to a volumetric flask (10 mL) and the volume was completed with the extraction solution. The final concentrations ranged from 0.5 to 8 $\mu\text{g p-NP mL}^{-1}$. Each point, including the control, was measured in the spectrophotometer.

After the extraction with NaOH (0.5 M), the assay tubes were photographed (Canon digital camera) in order to show the sample staining with the use of different buffers. The results were analyzed by determining the confidence intervals of samples ($p < 0.005$). Michaelis-Menten (1) and Eadie-Hofstee (2) equations were used in the modelling of data and determination of kinetic parameters of the phosphatase activity:

$$V = (V_{\max} \times [S]) / (K_m + [S]) \quad (1)$$

$$V = -K_m V/[S] + V_{\max} \quad (2)$$

where V is the reaction rate of release p-NP, V_{\max} is the maximum phosphatase activity, $[S]$ is the substrate concentration (p-NPP) and K_m is the Michaelis-Menten constant. Equation parameters and statistical significance were determined using the software SigmaPlot 12.0.

RESULTS

The results show that the phosphatase activity measured depends on buffer type. The buffer also

affected the coloring of the control samples (without substrate). MUB caused the greatest change in the absorbance values of the controls (Table II). These values were high, but especially for the NC samples. Figure 1 (a) shows the color of the extract in the control and the substrate (p-NPP) samples. Notably, MUB strongly interfered the measure of phosphatase activity, whereas low MUB minimized this interference. The coloring of the MUB samples remained high also after centrifugation (Fig. 1b).

Buffer resolutions were estimated using a standard curve with p-NP (Table III). The regression coefficients (R^2) for all buffers were high and significant. However, TB resolution was higher than for other buffers and 15% higher than AB. MUB and low MUB resolutions were similar. The sensitivity quantifications were 7.6 and 8.9 ng p-NP mL^{-1} for the standard curves of AB and TB, respectively.

In the assays, the phosphatase activity of all soils ranged from 87 to 795 $\mu\text{g p-NP g}^{-1}$ dry soil. The highest phosphatase activity in NC was observed with AB while the lowest with MUB (Table IV). MUB had the greatest standard deviation in soil phosphatase activity. The NT system had the second highest average phosphatase activity and showed with MUB the lowest activity in this system. Compared to MUB, low MUB decreased the variation in the phosphatase activity but still produced lower activity values than TB and AB. Finally, when the phosphatase activity was low, differences between buffers were not significant.

TABLE II
Absorbance values of control samples (without substrate) in each buffer¹ and agricultural systems².

System	Buffer			
	AB	TB	MUB	low MUB
NC	0.034 \pm 0.013	0.016 \pm 0.008	0.365 \pm 0.053	0.040 \pm 0.007
NT	0.005 \pm 0.004	0.003 \pm 0.007	0.065 \pm 0.007	0.018 \pm 0.004
CT	0.010 \pm 0.004	0.002 \pm 0.016	0.040 \pm 0.016	0.017 \pm 0.009
PBb	0.012 \pm 0.005	0.003 \pm 0.009	0.043 \pm 0.009	0.023 \pm 0.010

¹Buffers used in assays: AB, acetate buffer; TB, Tris-HCl buffer; MUB, modified universal buffer (MUB); low MUB, low modified universal buffer.

²NC, native Cerrado; NT, no-tillage; CT, conventional tillage. PBb, Pasture with *Brachiaria brizantha*.

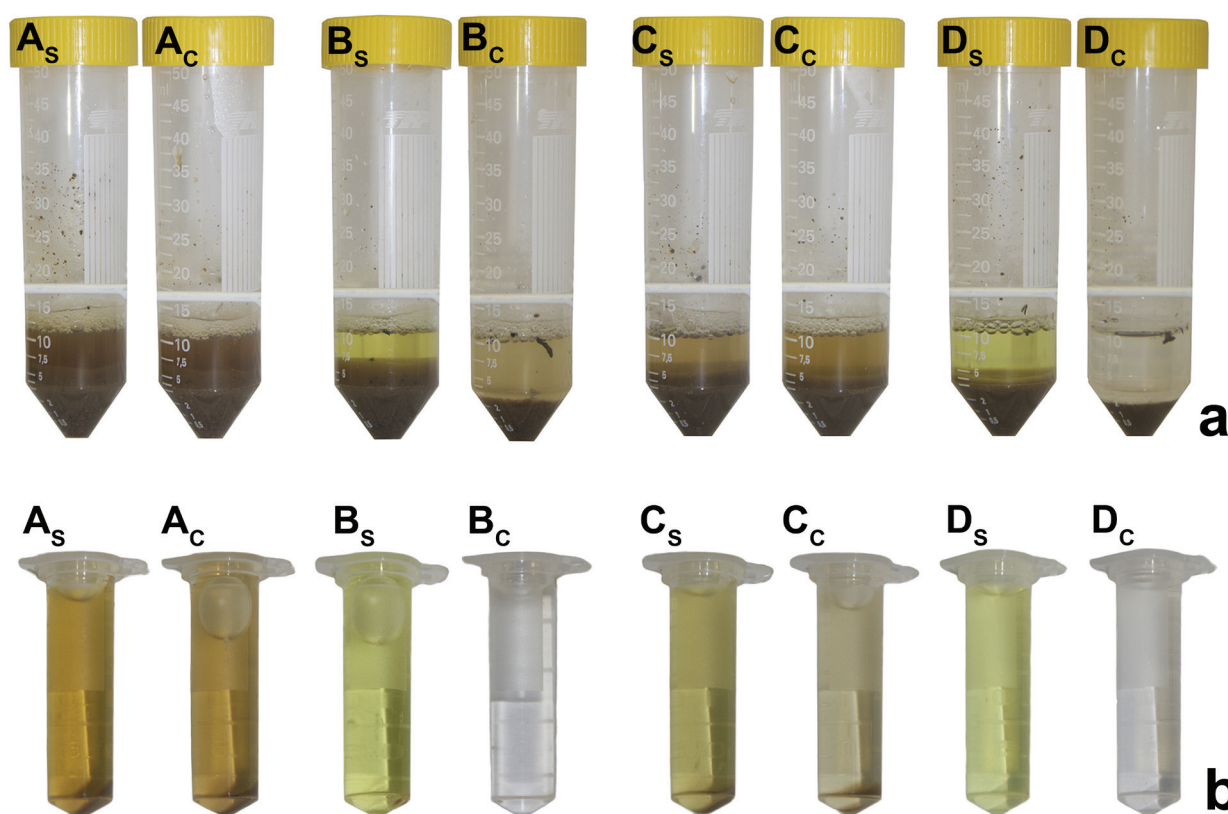


Figure 1 - Interference MUB and low MUB in the staining of control (without) samples and substrate samples in the enzyme extract before (a) and after centrifugation (b). Substrate samples showed in A_s, B_s, C_s and D_s and control samples showed in A_c, B_c, C_c and D_c. Letters A_s, A_c, B_s and B_c shows the staining in the native Cerrado (NC) and C_s, C_c, D_s and D_c in no-tillage (NT). MUB is identified as A_s, A_c, C_s and C_c and low MUB is identified as B_s, B_c, D_s and D_c. (See the colors in the online version).

TABLE III
Parameters of equation used to estimate the p-NP of standard curves in each buffer¹.

Buffer	Equation	R ²	p
AB	y = 0.1897x - 0.0044	0.9999	<0.001
TB	y = 0.1614x - 0.0043	0.9998	<0.001
MUB	y = 0.1726x - 0.0024	0.9999	<0.001
low MUB	y = 0.1735x - 0.0038	0.9999	<0.001

¹Buffers used in assays: AB, acetate buffer; TB, Tris-HCl buffer; MUB, modified universal buffer (MUB); low MUB, low modified universal buffer.

The kinetic parameters of soil phosphatase were evaluated in two systems (NC and NT) because they belong to the same soil class and presented the highest enzymatic activity. The V_{max} of soil phosphatase in NC was 3.59 times higher than in NT, and K_m was 2.45 times greater than in NC (Fig. 2). The regression coefficients (R²) of both systems were high and significant (p<0.0001)

what indicates that the Michaelis-Menten equation is appropriate for soil data from the Cerrado. The Eadie-Hofstee equation also showed significant differences between the systems with a regression coefficient (R²) of 0.72 for NC and 0.88 for NT (Fig. 3a, b). The variation coefficient (%) was higher in NC than in the NT system, which justifying the R² values.

TABLE IV
Soil phosphatase activity^{1,2} (ug p-NP g⁻¹ dry soil) obtained with different buffers³ and under different land-use systems (n=5).

System	Buffer			
	AB	TB	MUB	low MUB
NC	519 ± 85 (16)	305 ± 80 (26)	207 ± 103 (49)	225 ± 63 (28)
NT	185 ± 8.3 (4.5)	179 ± 7.5 (4.1)	151 ± 22 (14)	170 ± 19 (11)
CT	120 ± 6.1 (5.1)	142 ± 13 (9.1)	133 ± 20 (15)	128 ± 6.9 (5.4)
PBb	103 ± 9.2 (8.9)	117 ± 9.2 (7.8)	112 ± 10 (8.9)	95 ± 3.2 (3.3)

¹Mean the soil phosphatase activity with standard deviation (± SD).

²Values inside brackets indicate the percentage (%) of sampling variation (standard deviation) in relation to the average of soil phosphatase activity of each buffer. NC, native Cerrado; NT, no-tillage; CT, conventional tillage; PBb, Pasture with *Brachiaria brizantha*.

³Buffers used in assays: AB, acetate buffer; TB, tris-HCl buffer; MUB, modified universal buffer (MUB); low MUB, low modified universal buffer.

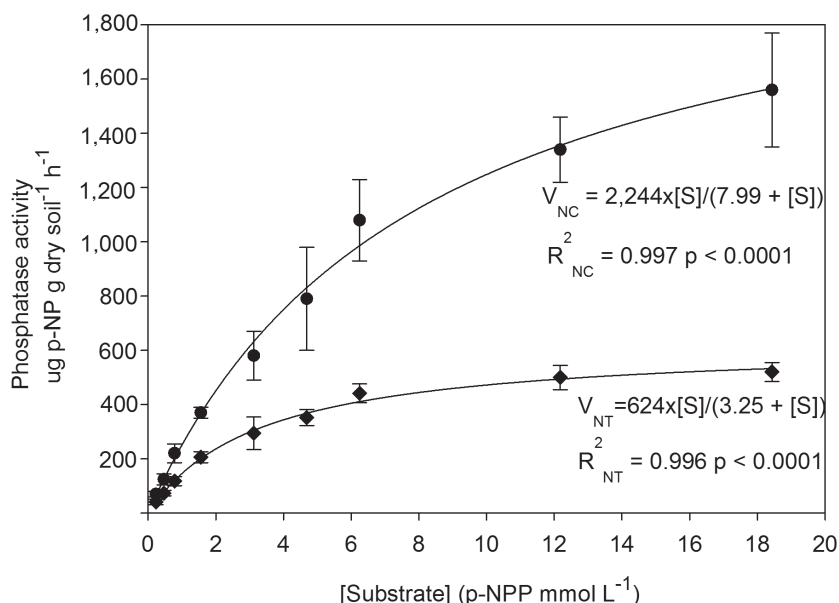


Figure 2 - Regression analysis of soil phosphatase activity using the Michaelis-Menten equation in the native Cerrado (NC) and no-tillage (NT). Where, V_{NC} shows soil phosphatase activity in the native Cerrado (Circle) and V_{NT} shows soil phosphatase activity in no-tillage (Diamond) as a function of substrate (S) concentration. Values of determination coefficient (R^2) and significant level of each equation ($p < 0.001$) are also shown.

DISCUSSION

This study shows that phosphatase activity in Cerrado soil samples differs with the buffer applied in measurements. Phosphatase activity in NC soil samples cannot be estimated with the buffer used by Tabatabai and Bremner (1969) because the staining

of the control (without substrate) and substrate samples is similar (Fig. 1). In all soil samples, phosphatase activity with MUB was lower than the other buffers.

Many factors influence the measurement of the soil phosphatase activity when using p-NPP as substrate (Brooks et al. 2013, Tabatabai and

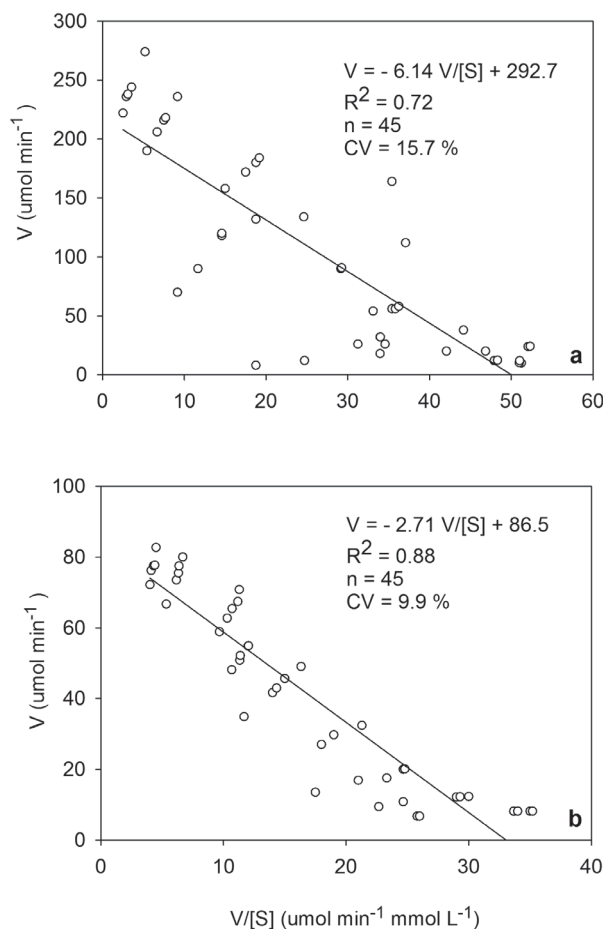


Figure 3 - Regression analysis of soil phosphatase activity using the Eadie-Hofstee model in the native Cerrado (a) and no-tillage (b). Where, values of determination coefficient (R^2), Coefficient of variation (CV %) and number of sampling (n) are shown. Parameters of equations of Eadie-Hofstee model are significantly fitted at a probability level of 1 %.

Bremner 1969, Pettit et al. 1977, Tabatabai 1994). However, the clay content and level of humic acids are considered the most important because they interfere with the coloring extract containing p-NP (Pettit et al. 1977, Malcom 1983, Trasar-Cepeda and Gil-Sotres 1988). The addition of CaCl_2 reduces the dispersion of clay and mitigates the interference in the extraction of p-NP with NaOH. Thus, CaCl_2 was added to all buffers used to assess soil phosphatase activity to eliminate the potential interference from clay. All four soil types evaluated in this study were characterized as

clayey. Nevertheless, interference from clay was only observed in two of the soils (soil from the NC and NT systems). It is believed that, in this study's assays, soil organic matter content was the main cause of interference in NC and NT when MUB was used. This may be because MUB contains acids (citric, boric and maleic), which can extract and disperse great amounts of humic acids from the soil organic matter. Furthermore, the addition of NaOH could also cause humic acid extraction under MUB conditions.

In the assays of this study, low MUB significantly reduced variations in soil phosphatase activity when compared to MUB, and resulted in higher enzyme activity in NC and NT. This reduction in variation is believed to occur because low MUB contains lower acid levels (citric, maleic and boric acids) than MUB. Low MUB also reduced tris levels in comparison to MUB. Nevertheless, tris-HCl is not believed to interfere in the assessment of soil phosphatase activity. Additionally, soil phosphatase activity was higher with TB than with low MUB. In fact, the results show that TB has a higher resolution to quantify p-NP in the final extract. Tris-HCl buffer has commonly been used to assess soil enzymes, including acid and alkaline soil phosphatase activity (Gerritse and van Duk 1978, Geller and Ginzburg 1979, Hui et al. 2013).

The results show that use of AB resulted in higher phosphatase activity measurements in NC and NT soils than the other buffers (Table III). However, AB produced results similar to other buffers when all measurements of soil phosphatase activity were low. Thus, it is concluded that AB may produce more accurate assessments of soil phosphatase activity when enzyme activity is high. Several factors can affect soil phosphatase activity during the assays, including organic matter, microbial biomass and root density (Fioretto et al. 2009, Brooks et al. 2013). This study did not determine the factors that cause differences in phosphatase activity. However, the data showed

that soils from NC and NT systems have higher microbial biomass carbon. Higher soil phosphatase activity with AB could also be explained by specific factors, such as the extraction of intracellular phosphatase, isoenzymes and conditions affecting the enzyme activity in NC and NT systems.

The Michaelis-Menten equation showed a significant difference between NC and NT (Fig. 2). Kinetic parameters (K_m and V_{max}) of soil phosphatase activity were higher in NC than in NT. Low values of K_m , in particular, indicate that the substrate has a higher affinity for the soil phosphates. Soil heterogeneity and multiple enzymes catalyzing the same reaction are factors that reduce enzyme-substrate affinity due to enzyme immobilization in soil constituents (e.g., organic matter and clay) and isoenzyme production (mainly by microorganisms). The results of this study are consistent with those reported in the literature, which show that more complex soils have greater K_m and V_{max} values. On the other hand, high V_{max} indicates that a soil has a greater capacity to provide P for plants and soil microorganisms by transforming various organic-P sources into inorganic-P.

The Eadie-Hofstee model shows specific parameters for enzymatic reactions of soil phosphatase activity in NC and NT systems (Fig. 3). The results show that the NT system has less variation and greater specificity between phosphatase activity and substrate than the NC system. This model uses the most sensitive parameters to assess kinetic variation in the Michaelis-Menten equation (Irving and Cosgrove 1976, Segel 1975). In addition to a specific enzyme-substrate, the Eadie-Hofstee model is also appropriate for enzyme isoforms (e.g., soil phosphatase). The model suggests that enzyme isoforms lead to a greater variation in the equation due to specificities of isoenzymes for the substrate. This study emphasizes the importance of conducting comparative studies on soil phosphatase activity in various land use systems using kinetic models to assess soil enzyme activity.

The Brazilian Cerrado has a semi-humid tropical climate and is therefore one of the most humid Savanna regions. Tillage in the Cerrado has increased since the 1970s. However, since the 1990s, the alternative practice of no-tillage (NT) has been incorporated into the Brazilian agriculture. NT currently accounts for approximately 90% of the area cultivated with annual crops (an estimated 31 million ha). Most soils in this region are deep, weathered, porous Oxisols with clay content greater than 15% (Vinhel-Freitas et al. 2012). Standardizing enzyme activity in Cerrado soils is fundamental to further comparative studies. Moreover, the kinetic parameters of enzymes enlighten over the agroecosystem function. Determining the kinetic parameters, beyond simple measurements of enzyme activity, is important for understanding enzyme mechanisms, enzyme roles in specific soil conditions, enzyme responses to changes in metabolite concentration and also enzyme control mechanisms (Marinari et al. 2008, Henry 2012).

CONCLUSIONS

The study showed that the buffer type is of fundamental importance in order to assess the soil phosphatase activity in Cerrado soils. MUB showed the lowest soil phosphatase activity whereas AB in NC and NT the highest. Low MUB caused less interference in the measurement of soil phosphatase activity when compared to MUB. In NC and NT, soil phosphatase activity with TB was similar to AB and low MUB. There was no difference of buffer type for assessment the soil phosphatase activity in CT and PBb. K_m values from Michaelis-Menten equation were higher in NC than in NT, showing a lower affinity of phosphatase activity for the substrate in NC. V_{max} values were also higher in NC than in NT. The Eadie-Hofstee model suggests that NC had more phosphatase isoforms than NT. Thus, for the sites considered in this study, soil phosphatase activity depends on the buffer type and the kinetic parameters depend on the land use.

ACKNOWLEDGMENTS

The study was financially supported by the Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG).

RESUMO

A atividade e cinética das fosfatases do solo são importantes indicadores para avaliar a qualidade do solo em sítios específicos tais como Cerrado (Savana brasileira). Este estudo objetivou determinar a atividade e os parâmetros cinéticos da fosfatase do solo em sistemas do Cerrado. A atividade de fosfatase do solo foi avaliada em amostras de Cerrado nativo (CN), plantio direto (PD), plantio convencional (PC) e pastagem com *Brachiaria brizantha* (PBb) e avaliada com tampão de acetato (TA), tampão tris-HCl (TT), tampão modificado universal (TMU) e baixo-TMU. A equação de Michaelis-Menten e o modelo de Eadie-Hofstee foram usados para avaliar os parâmetros cinéticos da fosfatase do solo usando diferentes concentrações de p-nitrofenol fosfato (p-NFF). O TMU indicou a menor atividade de fosfatase do solo, enquanto que TA em CN e PC, a maior atividade. O baixo-TMU diminuiu as interferências na avaliação da atividade de fosfatase do solo quando comparado à TMU, sugerindo que ácidos orgânicos interferem na atividade da fosfatase do solo. Em CN e PD, a atividade de fosfatase do solo com TT foi similar à TA e baixo-TMU. Os valores de K_m da equação de Michaelis-Menten foram maiores em CN do que em PD, indicando menor afinidade da atividade de fosfatase para o substrato em CN. Os valores de V_{max} foram também maiores em CN quando comparados com PD. O modelo de Eadie-Hofstee indicou que CN tem mais isoformas de fosfatase no solo quando comparado com PD. O estudo mostrou que o tipo de tampão é de fundamental importância para avaliar a atividade de fosfatase em solos de Cerrado.

Palavras-chave: atividade de fosfatase do solo, tampão, cinética de enzima, equação de Michaelis-Menten equation, modelo de Eadie-Hofstee, Cerrado.

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