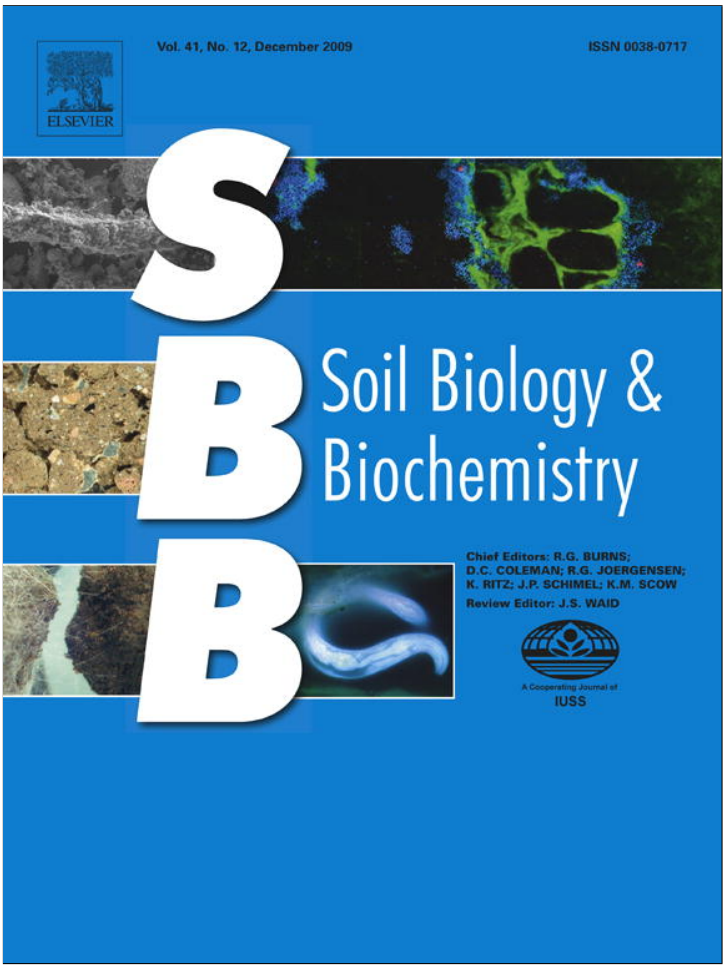

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Determination of arylsulphatase and phosphatase enzyme activities in soil using screen-printed electrodes modified with multi-walled carbon nanotubes

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ABSTRACT

Sustainability of agricultural systems has become an important issue all over the world. The activity of enzymes is potentially an important quality bioindicator in soils. The aim of the present study was to develop a simple and convenient assay to determine the activity of arylsulphatase (AS), acid (ACP) and alkaline phosphatase (ALP) in agricultural soil. The activities of these enzymes were detected using a non-electroactive substrate, which produces an electroactive product. To this end, p-aminophenyl phosphate (pAPP) was used as a substrate which is converted to p-aminophenol (pAP) after enzymatic dephosphorylation; and 4-nitrocatechol sulphate (4-NCS) was used as a substrate for AS activity based on its catalytic effect on the hydrolysis of 4-NCS into 4-nitrocatechol (4-NC). The products of both enzymatic reactions were quantified on carbon-based screen-printed electrodes (SPCEs) modified with carbon nanotubes (CNTs), using Osteryoung square-wave voltammetry (OSWV). The determination of the reaction products allowed more sensitive determination of ALP, ACP and AS activities in soil than that obtained with a spectrophotometric method. This assay also diminishes the generation of waste, which is desirable in green analytical chemistry. The optimization of the analytical procedure in terms of the nature of electrode type, applied potential, pH of solution, and precision of measurements is reported.

1. Introduction

In soil ecosystems, phosphatase (PS) and arylsulphatase (AS) play crucial roles in phosphorus (P) and sulphur (S) cycles, respectively (Speir and Ross, 1978). The enzymes regenerate inorganic nutrients from organic materials and have been reported as the rate-limiting step in the nutrient cycling process (Chróst, 1991). Enzymatic activities have several important functions in soil. They are intimately involved in the cycling of nutrients, in the efficiency of fertilizer utilisation, reflect the microbiological activity in soil and can play a role as indicators to monitor soil change (Dick et al., 2000).

Organic P present in soil must be mineralized into inorganic orthophosphate (PO_4^{3-}) ions to be assimilated by many organisms, particularly plants. Only enzymes produced by plants and/or microorganisms are able to hydrolyze organic P into phosphates. These enzymes can be located in soil microorganisms, in root cells and in extracellular forms in soils. The general term phosphatases describes a broad group of enzymes that catalyze the hydrolysis of both esters and anhydrides of phosphoric acid (Schmidt and Laskowski, 1961). PS activity is expected to be enhanced by the application of various organic manures, which often results in enhanced P availability in soil. Among PS enzymes, acid (ACP) and alkaline phosphatase (ALP) (E.C. 3.1.3.) and phosphodiesterases (E.C. 3.1.4.) are considered as the predominant PS in most types of soil and litter (Tabatabai, 1994; Criquet et al., 2004). The activities of these phosphatases are influenced by various soil properties, soil organism interactions, vegetation cover, leachate inputs and the presence of inhibitors or activators (Juma and Tabatabai, 1977). The understanding of these factors has remained unclear, despite numerous attempts to relate phosphatase activities to P pools in soils (Turner and Haygarth, 2005). The method used to measure this enzyme activity was proposed by Tabatabai and Bremner (1969). This method is based on conversion of the synthetic substrate p-nitrophenyl phosphate to p-nitrophenol, which can be quantified by a spectrophotometric method.

AS activity is widespread in soil (Cooper, 1972; Gupta et al., 1993; Ganeshamurthy et al., 1995), and is typically measured according to the method of Tabatabai and Bremner (1971). This method is very similar to the one used for PS, but in this case the substrate is p-nitrophenyl sulphate which is converted to p-nitrophenol and quantified by a spectrophotometric method.

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The AS catalyses the hydrolysis of aromatic sulphate esters to phenols and sulphate. In soil, sulphate esters represent a large fraction (25.3–93.1%) of the total S and, therefore, arylsulphatases are important for mobilisation of inorganic SO_4^{2-} for plant nutrition (Fitzgerald, 1976).

With regards to the detection system, the formation of the product is generally followed by spectrophotometric, fluorescence or chemiluminescence detection (Girotti et al., 1994; Tillyer et al., 1994; Kawakami and Igarashi, 1995; Withold et al., 1996; Lin et al., 1997). However, in recent years, analyses via electrochemical detection constitute a methodology used extensively (Lasalle et al., 1994; Della Ciana et al., 1995; Jiao et al., 2002; Gyurcsanyi et al., 2002). Some advantages of this method include speed, accuracy, and precision. Square-wave voltammetry (SWV) is one of the electrochemical techniques more widely applied to quantitative analysis, especially due to its high sensibility, which is a consequence of the rejection of most of the capacitive currents (Garay and Lovrić, 2002; O'Dea et al., 1981). A variety of substrates were used in electrochemical assays for the quantification of ACP and ALP activities, such as phenylphosphate, p-aminophenyl phosphate (pAPP), p-nitrophenyl phosphate (pNPP), and 3-indoxyl phosphate (Wehmeyer et al., 1986; Tang et al., 1988; Abad-Villar et al., 2000; Fanjul-Bolado et al., 2004).

Carbon nanotubes (CNTs) are a novel type of carbon material and can be considered as the result of folding graphite layers into carbon cylinders. There are two groups of carbon nanotubes, multi-walled carbon nanotubes (MWCNT) and single-walled carbon nanotubes (SWCNT) (Zhao et al., 2002). The CNTs have generated great interest in future applications based on their field emission and electronic transport properties (Murakami et al., 2000), their high mechanical strength and their chemical properties (Treacy et al., 1996). The research has been focused on their electrocatalytic behaviour toward the oxidation of biomolecules and their performance has been found to be much superior to those of other carbon electrodes in terms of reaction rate, reversibility and detection limit (Li et al., 2005).

The advantages of carbon-based screen-printed electrodes (SPCEs), such as simple and low-cost fabrication and conveniently practical application in detection of biomolecules, have been extensively illustrated (Gilmartin et al., 1995; Hart and Wring, 1997; Wang et al., 1998; O'Halloran et al., 2001). The uses of CNTs for preparation of CNT-modified carbon-based screen-printed electrodes (CNTs-modified SPCEs) have been reported previously (Fanjul-Bolado et al., 2007; Sato and Okuma, 2008; Lee et al., 2007; Ye and Ju, 2005).

The aims of the present study were test and use a simplified and convenient procedure for the assay of AS, ACP and ALP activities in agricultural soil. The activities of these enzymes were detected electrochemically using a non-electroactive substrate, which produces an electroactive product. This method allows improve the sensitivity of the determination of the reaction product produced by ALP, ACP and AS activities in soil compared to the spectrophotometric method. This assay was performed in order to reduce the amount of solvents required and also to diminish the generation of wastes, which is an important requisite in green analytical chemistry (Armenta et al., 2008). The optimization of the analytical procedure in terms of the nature of electrode type, applied potential, pH of solution, and precision of measurements was studied.

2. Materials and methods

2.1. Reagents and solutions

All reagents used were of analytical reagent grade. The pNPP disodium salt hexahydrate were purchased from Fluka Chemie (Steinheim, Switzerland). pAP, pNC and pNCS were obtained from Sigma Chemical Co. (St. Louis, MO). The SPCEs was purchased from EcoBioServices & Researches s.r.l. (Fienze, Italy). All other reagents employed were of analytical grade and used without further purifications. Aqueous solutions were prepared using purified water from a Milli-Q system.

2.2. Apparatus

Electrochemical detection was performed using a BAS 100B/W electrochemical analyzer (Bioanalytical Systems, West Lafayette, IN, USA) which was used for cyclic voltammetric analysis and OSWV.

All pH measurements were made with an Orion Expandable Ion Analyzer (model EA 940, Orion Research, Cambridge, MA, USA) equipped with a glass combination electrode (Orion Research). The absorbencies were detected by a Beckman DU 520 general UV/Vis spectrophotometer (Fullerton, CA, USA).

2.3. Soil samples

The samples were obtained from the upper horizon (0–15 cm) of four Entisols soils of San Luis and two Mollisols soils of Santa Fé. The samples were from Argentinean soils used for agricultural activities. The coordinates of the obtained samples were soil 1 (33°19′ S, 66°20′ O), soil 2 (33°74′ S, 65°55′ O), soil 3 (32°32′ S, 65°14′ O), soil 4 (34°06′ S, 66°44′ O), soil 5 (32°53′ S, 60°56′ O), soil 6 (31°37′ S, 61°01′ O).

The moist soil sample was sieved ($\leq$2 mm) after removing the plant material and roots. Soil samples were kept at 4 °C in plastic bags for a few days to stabilize the microbiological activity disturbed during soil sampling, handling. The analyses were achieved within two weeks after the sample collection. The physical and chemical characteristics of the soil are given in Table 1.

2.4. Synthesis of pAPP

Synthesis of pAPP by catalytic hydrogenation of pNPP was performed using the procedure (Gehring et al., 1996) with the following modifications. In a 100 mL glass hydrogenation vessel, 2 g of pNPP was dissolved in 30 mL of 50% ethanol containing 0.11 g of 10% palladium on charcoal catalyst. The hydrogenation reaction was conducted overnight at room temperature at an initial pressure of 1.3 atm. The resultant mixture was filtered on a Buchner funnel to remove the catalyst and the volume of solvent was reduced to 10 mL using a rotary evaporator. The oily residue was diluted to 20 mL with distilled deionised water and clarified by filtration. Cold ethanol (20 mL, 4 °C) was added to the filtrate and the precipitated product was recovered by filtration, dried under vacuum and stored at −10 °C. The pAPP product was greater than 98% pure as determined by NMR and electrochemical methods.

2.5. Preparation of the CNTs-modified SPCEs

An electrode pretreatment was carried out before each voltammetric experiment in order to oxidize the graphite impurities and to obtain a more hydrophilic surface (Wang et al., 1996), with

Table 1
Physical and chemical characteristics of soils.

Characteristic	Soil 1	Soil 2	Soil 3	Soil 4	Soil 5	Soil 6
pH	8.1	8.0	8.3	6.9	5.3	6.0
Clay (%)	5.3	6.5	8.2	9.0	29.2	35.0
Sand (%)	80	75	63	64	20	13.4
Organic matter (%)	0.63	0.84	0.97	0.59	1.63	2.07
Total N mg kg^{-1}	842	1070	1208	798	206	317

the aim of improving the sensitivity and reproducibility of the results. The graphite electrode surface is pretreated applying a potential +1.6 V (versus Ag-SPE) for 120 s and +1.8 V (versus Ag-SPE) for 60 s in 5 mL of 0.25 M acetate buffer, containing 10 mM KCl (pH 4.75), under stirred conditions. Then, the electrodes were washed using 0.01 M phosphate buffered saline (PBS), pH 7.2 and stored in the same buffer at 4 °C.

One milligram of MWCNT was dispersed with the aid of ultrasonic stirring for 45 min in methanol/water (50:50 v/v) in an aqueous 0.1% nafion solution. A 5 μl aliquot of this dispersion was dropped on the screen-printed graphite working electrode surface and then the solvent was evaporated under an infrared heat lamp (Vega et al., 2007).

2.6. Electrochemical determination of phosphatase and arylsulphatase enzymes activities in soil

The assay for the determination of the enzyme activities consisted in placing 0.1 g of soil (<2 mm) in a 1.5 mL Eppendorf flask with 1 mL of substrate solution. The pAPP was used as a substrate and it was converted to p-aminophenol (pAP) after the enzymatic dephosphorylation (Tang et al., 1988; Pemberton et al., 1998). The 4-nitrocatechol sulphate (4-NCS) was used like a substrate for AS based on its catalytic effect on the hydrolysis of 4-NCS into 4-nitrocatechol (4-NC). These products were quantified by electrochemical detection. The generation of both products contains phenolic functional group which can be oxidized at potentials significantly lower than that required to oxidize the corresponding sulphate or phosphate substrates and can be quantified on CNTs-modified SPCEs using Osteryoung square-wave voltammetry (OSWV).

For the ALP assay, diethanolamine buffer (DEA) (100 mM diethanolamine, 50 mM KCl, 1 mM $MgCl_2$, pH 9) was used to prepare the pAPP substrate solution. For the AC assay acetate buffer 20 mM (pH = 6) was used to prepare the pAPP substrate solution. The flask was placed in a shaker at 37 °C. After 1 h, the entire content was directly put into an electrochemical cell and the products of the enzymatic reactions were quantified using OSWV (−200 to +300 mV, 30 mV sweep width amplitude, 15 Hz frequency, 10 mV step potential, 10^{-5} A/V, sensitivity samples per point: 256).

The assay for AS determination consisted in placing 0.1 g of soil (<2 mm) in a 1.5 mL Eppendorf flask along with 1 mL of 4-NCS in acetate buffer 0.1 M (pH 5.8) substrate solution. The flask was placed in a shaker at 37 °C. After 1 h, the entire content was directly put into an electrochemical cell and the products of the enzymatic reactions were quantified using OSWV (−200 to +300 mV, 30 mV sweep width amplitude, 15 Hz frequency, 10 mV step potential, 10^{-5} A/V, sensitivity samples per point: 256).

The peak currents were determined by drawing a tangent line across the base of the peak using the BAS 100 W software. A standard curve for the electrochemical determination of pAP in DEA buffer (pH 9) and acetate buffer 20 mM (pH 6) and 4-NC in acetate buffer 0.1 M (pH 5.8) were obtained. The enzymes activities were calculated from the standards curves correcting for blanks.

The stock solutions of pAPP and 4-NCS were prepared freshly before the experiment and stored under the exclusion of light for the duration of the experiment. All data points were measured in triplicate ($n = 3$) unless otherwise stated.

Increasing concentration of pAPP was studied in the range of 0.5–25 mM of pAPP in DEA buffer (pH 9.6) and pAPP in acetate buffer 20 mM (pH 6) for ALP and ACP respectively. The temperature dependence of the enzymatic reaction was also investigated in the range 10–60 °C. Furthermore, the pH profile at 37 °C under similar conditions showed a maximum reaction rate over the pH range 9.1–10 for ALP, 5.7–6.3 for ACP and 5.4–6.3 for AS. Moreover, we studied the relationship between activity and quantity of soil (i.e. amount of enzyme) from 0.01 to 0.5 g using 18 mM of pAPP in DEA buffer (pH 9.6) and acetate buffer 20 mM (pH 6) for ALP and ACP respectively.

2.7. Acid and alkaline phosphatase and arylsulphatase activity measurements by classical methods

Soil phosphatase activity was determined using p-nitrophenyl phosphate (pNPP) as an orthophosphate monoester analogue substrate (Tabatabai, 1994). Briefly, 0.1 g of each soil sample (<2 mm) was placed in a 1.5 mL Eppendorf flask, 1 mL of THAM solution (Tris-hydroxymethyl-aminomethane, with citric, maleic and boric acids), buffer (pH 6.5 for acid phosphatase assay or pH 11 for alkaline phosphatase assay) and 0.25 mL of pNPP solution prepared in the same buffer, were added and swirled the flask for a few seconds to mix the contents. After 1 h of incubation at 37 °C, 0.1 mL of 0.5 M $CaCl_2$ and 0.4 mL of 0.5 M NaOH were added, and swirled the flask for a few seconds to stop the reaction. The soil suspension was filtered (0.45 mm HA nitrocellulose, Millipore) to prevent the interference of possible precipitates. Absorbance was measured with a spectrophotometer at 405 nm against the reagent blank and p-nitrophenol content determined by reference to a calibration curve.

For arylsulphatase activity, 0.1 g of soil (<2 mm) of soil was placed in a 1.5 mL Eppendorf flask, and 1 mL of acetate buffer, 0.25 mL of toluene and 0.25 mL of p-nitrophenyl sulphate (p-NPS) solution added, mixed and incubated at 37 °C for 1 h. Then 0.1 mL of 0.5 M $CaCl_2$ and 0.4 mL of 0.5 M NaOH were added, swirled for a few seconds, and filtered (0.45 mm HA nitrocellulose, Millipore). Absorbance at 405 nm was determined against the reagent blank and p-nitrophenol content calculated by referring to a calibration curve.

3. Results

3.1. Electrochemical study of pAP and 4-NC with CNTs-modified SPCEs

The substrates of both phosphatases and AS should not produce electrochemical signals at the same potential as their own dephosphorylated or desulphated forms. The electrochemical behaviour of the hydrolysis products (pAP and 4-NC) of the enzyme substrates was examined by cyclic voltammetry at CNTs-modified SPCEs A cyclic voltammetric study of 1.0 mM of pAP in DEA buffer (pH 9) and acetate buffer 20 mM (pH 6) were performed by scanning the potential from −300 to 500 mV versus Ag/AgCl. CV showed well-defined anodic and corresponding cathodic peak, which corresponds to the transformation of pAP to *p*-benzoquinoneimine (QI) and vice versa within a quasi-reversible two-electron process (Fig. 1a and b).

A OSWV study of 1.0 mM of pAP in DEA buffer (pH 9) and acetate buffer 20 mM (pH 6) were performed by scanning the potential from −250 to 400 mV, 30 mV sweep width amplitude, 15 Hz frequency, 10 mV step potential (10^{-5} A/V) the peak current was determined by drawing a tangent line across the base of the peak using the BAS 100 W software. Typical responses are shown in Fig. 1d and e, whilst Fig. 1c and f shows CV and OSWV of 3.0 mM of 4-NC in acetate buffer 0.1 M (pH 5.8) at a scan rate (v) of 100 mV s^{-1}. The 4-NC shows an irreversible oxidation process.

A linear calibration curve for the detection of pAP was produced over the range of 1.07–0.09 × 10^{-3} mM in DEA buffer (pH 9.6), the linear regression equation was $i = 0.449 + 110.01^{*}C_{pAP}$ with the linear relation coefficient $r = 0.998$ and the limit of detection (DL) of

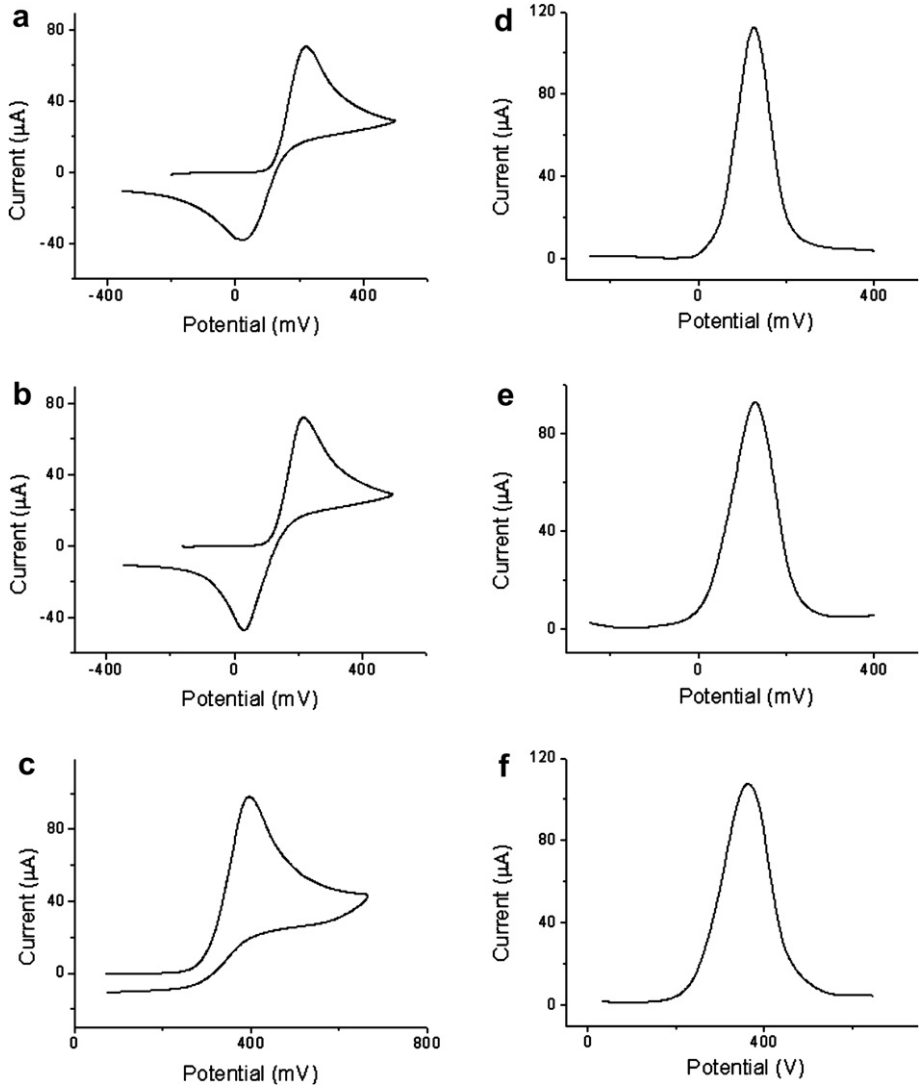

Fig. 1. Electrochemical study of pAP and 4-NC with the CNT-modified SPCEs. Cyclic voltammogram in aqueous solution containing 1.0 mM of pAP in a) DEA buffer (pH 9.6) and b) acetate buffer 20 mM (pH 6). Scan rate: 100 mV s^{-1}. c) Cyclic voltammogram of 4-NC 3.0 mM in acetate buffer 0.1 M (pH 5.8). A OSWV of 1.0 mM of pAP in d) DEA buffer (pH 9.6) and e) acetate buffer 20 mM (pH 6). f) A OSWV of 4-NC 3.0 mM in acetate buffer 0.1 M (pH 5.8). 30 mV sweep width amplitude, 15 Hz frequency, 10 mV step potential, 10^{-5} A/V.

0.04×10^{-3} mM (calculated as the concentration corresponding to three times the standard deviation of the estimate). A linear calibration curve for pAP was produced over the range of $0.88–0.63 \times 10^{-3}$ mM in acetate buffer 20 mM (pH 6), the linear regression equation was $i = 0.352 + 72.32^{*}C_{pAP}$ with the linear correlation coefficient $r = 0.996$ and the limit of detection (DL) of 0.34×10^{-3} mM. For 4-NC the linear range was from 0.75 to 5.1×10^{-3} mM in acetate buffer 0.1 M (pH 5.8) with a DL of 3.1×10^{-3} mM, the linear regression equation was $i = 0.435 + 39.74^{*}C_{4\text{-NC}}$ with the linear correlation coefficient $r = 0.996$.

3.2. Study of the enzymatic process

The enzymatic rates depend on the buffer conditions and the substrates concentration. Enzyme activities are commonly determined by methods involving saturation of the enzyme with the substrate (Hassan et al., 1995).

In order to study the activity of ALP, ACP and AS, the substrates were added in excess and the reactions were independent of the substrate concentration. For this reason, the first step in this study was the determination of the concentration at which the enzyme activity was not modified due to the substrate concentration. Thus, increasing concentration of pAPP was studied in the range of 0.5–25 mM of pAPP in DEA buffer (pH 9.6) and pAPP in acetate buffer 20 mM (pH 6) for ALP and ACP respectively. In the case of AS, the same range was evaluated for 4-NCS in acetate buffer 0.1 M (pH 5.8). Fig. 2 shows the curve of the enzymatic activity in response to the increasing substrate concentrations. As a result, we decided to perform the following studies with 18 mM and 5 mM for AP and AS respectively.

The timecourse of the reactions were studied for ALP, ACP and AS, with this purpose various reaction mixture were incubated at increasing time in the range 0–360 min using 18 mM of pAPP for ALP and ACP and for AS, 5.0 mM of 4-NCS. Fig. 3 confirms a linear relationship between time of incubation and the amount of product released.

3.3. Effects of temperature and pH on the rate of reaction

The temperature dependence of the enzymatic reaction was also investigated in the range 10–60 °C. The results obtained are shown

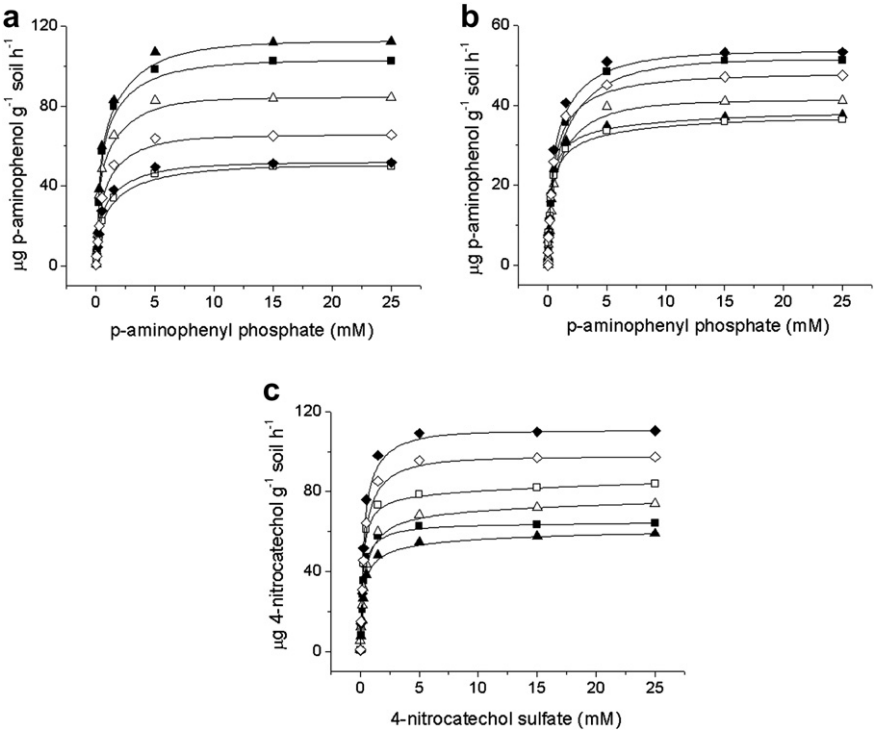

Fig. 2. Effect of substrate concentration ($n = 6$ for each analysis): a) pAPP in DEA buffer (pH 9.6) and b) pAPP in acetate buffer 20 mM (pH 6). c) 4-NCS in acetate buffer 0.1 M (pH 5.8). The flasks were placed in a shaker at 37 °C for 1 h. Soil 1 (■); soil 2 (△); soil 3 (▲); soil 4 (□); soil 5 (◆); soil 6 (◇).

in Fig. 4. The reaction rate reached maximum at 35–42 °C in DEA buffer (pH 9) for ALP, 30–42 °C in acetate buffer 20 mM (pH 6) for ACP and 32–45 °C in acetate buffer 0.1 M (pH 5.8) for AS. All subsequent measurements of the enzymatic reactions rate were carried out at 37 °C for 1 h.

The pH profile at 37 °C under similar conditions showed a maximum reaction rate over the pH range 9.1–10 for ALP, 5.7–6.3 for ACP and 5.4–6.3 for AS (Fig. 5). All subsequent measurements of the enzymatic reaction rate were carried out in DEA buffer (pH 9) for ALP and acetate buffer 20 mM (pH 6) for ACP using pAPP 18 mM.

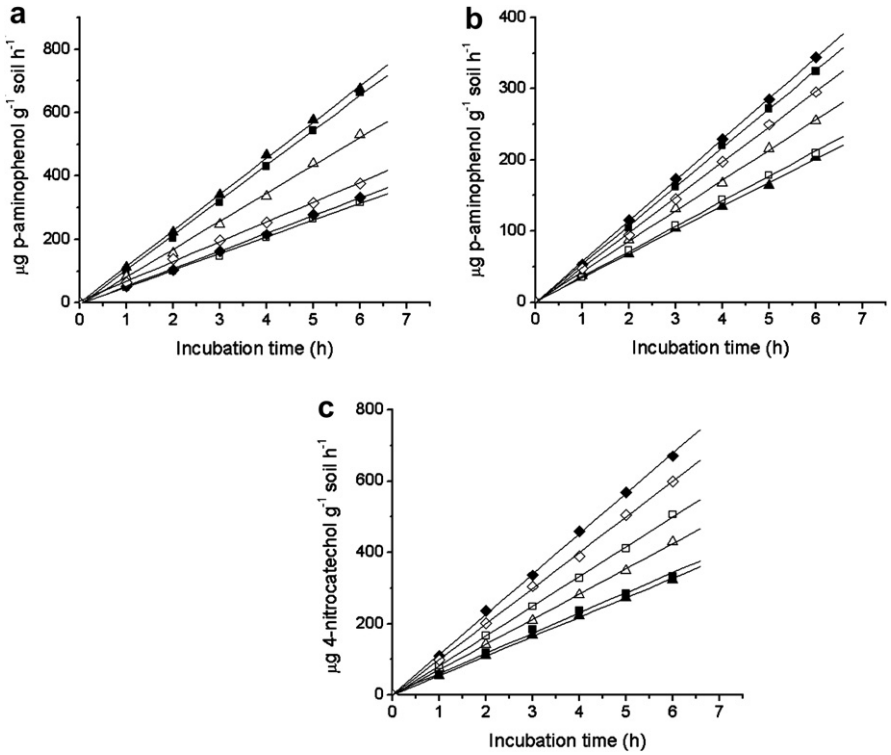

Fig. 3. Effect of time of incubation on release of enzyme products ($n = 6$ for each analysis). a) ALP activity in DEA buffer (pH 9.6) and b) ACP activity in acetate buffer 20 mM (pH 6). c) AS activity in acetate buffer 0.1 M (pH 5.8). Soil 1 (■); soil 2 (△); soil 3 (▲); soil 4 (□); soil 5 (◆); soil 6 (◇).

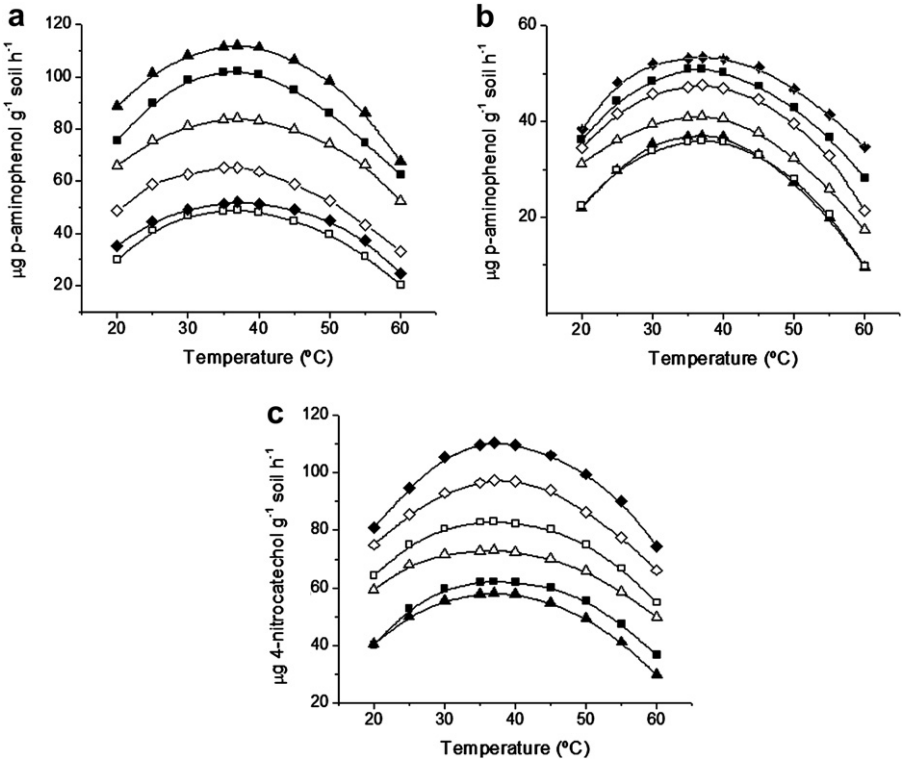

Fig. 4. Effect of temperature of a) ALP activity in DEA buffer (pH 9.6), b) ACP activity in acetate buffer 20 mM (pH 6) and c) AS activity in acetate buffer 0.1 M (pH 5.8). Soil 1 (■); soil 2 (△); soil 3 (▲); soil 4 (□); soil 5 (◆); soil 6 (◇) ($n = 6$ for each analysis).

In the case of AS, 5.0 mM of 4-NCS in acetate buffer 0.1 M (pH 5.8) was used and the flasks were placed in a shaker during 1 h.

We also studied the relationship between activity and quantity of soil (i.e. amount of enzyme) from 0.01 to 0.5 g using 18 mM of pAPP in DEA buffer (pH 9.6) and acetate buffer 20 mM (pH 6) for ALP and ACP respectively. In the case of AS, 5.0 mM of 4-NCS in acetate buffer 0.1 M (pH 5.8) was used and the flasks were placed in a shaker at 37 °C for 1 h (Fig. 6).

The high precision of the method described is illustrated by the results of replicated analyses of ALP, ACP and AS enzymes in one soil

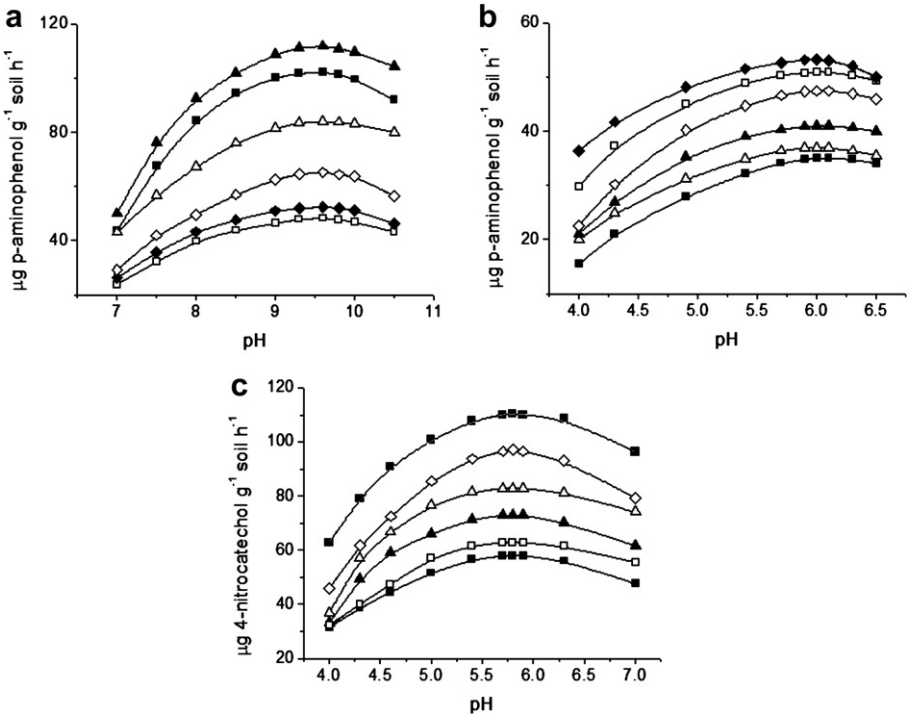

Fig. 5. Effect of pH on the activity of a) ALP, b) ACP and c) AS. Soil 1 (■); soil 2 (△); soil 3 (▲); soil 4 (□); soil 5 (◆); soil 6 (◇) ($n = 6$ for each analysis).

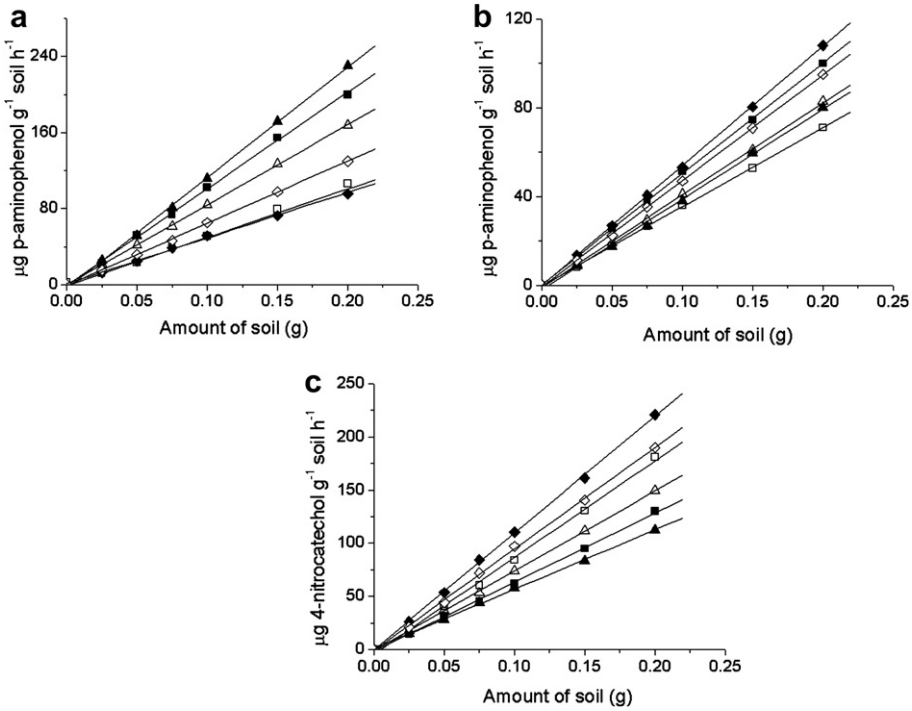

Fig. 6. Effect of amount of soil on release ($n = 6$ for each analysis) a) pAP for ALP in DEA buffer (pH 9.6), b) pAP for ACP in acetate buffer 20 mM (pH 6) and c) 4-NC for AS using in acetate buffer 0.1 M (pH 5.8). Soil 1 (■); soil 2 (△); soil 3 (▲); soil 4 (□); soil 5 (◆); soil 6 (◇).

(Table 2). The within-assay precision was tested with five measurements in the same run for each sample. These series of analyses were repeated for three consecutive days in order to estimate the between-assay precision. The results obtained are presented in Table 2. The proposed assay showed good precision; indicating that the analysis of enzyme activities in soil by the proposed method is highly reproducible.

With the purpose of determinate the sensitive of our method we developed an assay in which we compared the proposed method with the classical methods (Tabatabai and Bremner, 1969, 1971). Table 4 shows the results of this comparison.

4. Discussion

The most used methodology to determine the phosphatase and arylsulphatase activity in soil was proposed by Tabatabai and Bremner (1969, 1971), respectively. These methods have some disadvantages: the suggested concentration of the p-nitrophenol phosphate for the reaction is extremely high and this solution has an intense yellow coloration because the substrate solution already has some p-nitrophenol concentration. Consequently it is difficult to obtain a suitable reproducibility in the measurement using this method. Moreover, these methods are hindered by the use of toluene (a hazardous chemical) and filtration step of the assay mixture prior to analysis.

Table 2
Within-assay precision (five measurements in the same run for each soil sample) and between-assay precision (five measurements for each soil sample, repeated for three consecutive days) for soil 1.

	Within-assay		Between-assay	
	Mean ± SD	CV %	Mean ± SD	CV %
Alkaline[a] phosphatase	102.34 ± 2.36	2.30	103.27 ± 3.74	3.62
Acid[a] phosphatase	51.22 ± 1.66	3.24	52.41 ± 2.58	4.92
Arylsulfatase[b]	62.64 ± 1.86	2.97	63.74 ± 2.07	3.25

[a] µg p-aminophenol g^{-1} soil h^{-1}.
[b] µg 4-nitrocatechol g^{-1} soil h^{-1}.

The method developed in this study demonstrates to be more sensitive and precise than the methods which were described above. The high precision of the method proposed is probably due largely to the fact that the technique used to determine release of pAP and 4-NC are quantitative and that the entire assay procedure is simple and readily standardized. Furthermore, comparing the calculated LD of the methods (electrochemical method: ALP 40 nM of pAP, ACP 0.34 µM of pAP and AS 3.1 µM of 4-NC; spectrophotometric method: ALP, ACP and AS 8.1 µM of p-nitrophenol), we can establish that our method is more sensitive than the spectrophotometric one. Also, we can support this information with the results presented in Table 4.

Since the discovery of carbon nanotubes (CNT) in 1991, they have been the subject of much attention due to their unique properties (Iijima, 1991; Valentini et al., 2004). Because of their novel structural and electronic properties and their high chemical strength, CNT have been considered for a wide range of applications from structural materials to nanoelectronic components. Recently, significant progress has been made in achieving direct electron transfer via CNT. In particular, the ability of CNT-modified electrodes to promote electron-transfer reactions in biomolecules has been documented (Qi et al., 2006; Liu et al., 2005). The improved amperometric response at the CNT-modified electrode is attributed to the increased surface area upon electrode modification. Other relevant advantages of the amperometric detection at CNTs–GCE are the high repeatability and very low and stable background currents of the measurements achieved over the whole potential range (Agüí et al., 2007). The ability of carbon nanotubes to circumvent electrode surface fouling during amperometric sensing has been recognized for molecules such as NADH (Musameh et al., 2002) and can be attributed to the unique spatial structure of CNTs. The usefulness of CNT-modified screen-printed electrodes to

Table 3
The values for the activities of ALP, ACP and AS in soil ($n = 6$).

Soil n°	Enzyme activity					
	Alkaline[a] phosphatase		Acid[a] phosphatase		Arylsulfatase[b]	
	Mean ± SD	CV%	Mean ± SD	CV%	Mean ± SD	CV%
1	102.34 ± 2.36	2.30	51.22 ± 1.66	3.24	62.64 ± 1.86	2.97
2	84.37 ± 3.12	3.69	41.31 ± 1.43	3.46	73.6 ± 2.44	3.31
3	112.37 ± 2.98	2.65	37.64 ± 1.23	3.26	58.02 ± 1.48	2.55
4	49.20 ± 1.59	3.23	35.97 ± 0.98	2.72	83.80 ± 1.76	2.10
5	51.7 ± 1.41	2.72	53.3 ± 1.52	2.85	110.5 ± 2.78	2.51
6	65.3 ± 2.09	3.20	47.5 ± 1.18	2.48	97.3 ± 2.17	2.23

[a] μg p-aminophenol g^{-1} soil h^{-1}.
[b] μg 4-nitrocatechol g^{-1} soil h^{-1}.

determine ALP, ACP and AS activity in soil was demonstrated. The development of the proposed method showed to be reliable and easy, representing a suitable and convenient alternative to the spectrophotometric method. This is the first method able to be applied directly to the soil samples, without centrifugation or filtration steps.

Related to Table 3 and the data show in Table 1 there are a clear relation between the pH, organic matter and the activity of the enzymes. In soils in which the pH was high and the organic matter was low, the ALP activity seems to be lower. In the case of ACP there were not important changes with the physics parameters. Surprisingly, the results associated with AS were very different to those related to the ALP. The AS activity showed an increase in soils with low pH and high amount of organic matter. The values of the activities of ALP, ACP and AS in the different soils are showed in Table 3.

Fig. 2 shows the effect of varying the time of incubation in the method described. The linear relationship between the amount of soil and the amount of product released is supplementary evidence that this method measures enzymatic activity and that the substrate concentration is not a limiting factor in this method. These results agree with published data (Tabatabai and Bremner, 1969, 1971; Elsgaard et al., 2002), also confirmed by or data (Table 4) which demonstrate a linear relationship between the active soil and the product released.

Taking into account current public concern on environmental matters, environmental analytical studies and the consequent use of toxic reagents and solvents have increased to a point at which they became unsustainable to continue without an environmentally friendly perspective. Miniaturization is one way to avoid side effects of analytical methods, thus we developed a method which reduce considerably the amounts of reagents consumed and wastes generated (Armenta et al., 2008).

This method does not require skilled technicians or expensive and complex equipment. The results of this study clearly demonstrate the potentiality and versatility of this method, which could be applied to routine soil enzyme activity monitoring.

We present and discuss for the first time the electrochemical determination of AS, ACP and ALP activity in soil, resulting in a simple, fast and sensitive method among other analytical methods. This method could be used for the screening of microbial activities in real matrixes and also could have a significant application in agricultural industry as a monitoring method.

Table 4
Comparison of the result obtained for soil 1 between proposed and classic method ($n = 6$).

Active soil[a] (%)	ALP		ACP		AS[b]	
	Proposed method[b]	Classical method[c]	Proposed method[b]	Classical method[c]	Proposed method[d]	Classical method[c]
0.1	0.246	–	–	–	–	–
1	1.245	–	0.481	–	–	–
10	11.37	8.215	4.742	–	6.251	–
50	52.24	35.69	25,72	17.47	31.98	25.98
100	102.34	70.99	51.22	37.03	62.64	53.95

[a] All the assays were performed with 0.1 g of soil. The percentages in the table correspond to the amount of active soil. The rest of soil added to complete the 0.1 g of soil was heated at 200 °C during a day. This process produces a total destruction of the biological reactions.
[b] μg p-aminophenol g^{-1} soil h^{-1}.
[c] μg p-nitrophenol g^{-1} soil h^{-1}.
[d] μg 4-nitrocatechol g^{-1} soil h^{-1}.

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Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.soilbio.2009.08.024.

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