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Short communication

A protocol for the assay of arylesterase activity in soil

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ABSTRACT

We set up a protocol for the assay of the arylesterase activity, using *p*-nitrophenyl acetate (*p*-NPA) as substrate, dimethylsulfoxide as solvent, modified universal buffer at pH 7.5, and determination of the reaction product (*p*-nitrophenol) after separation of non-hydrolysed *p*-NPA after reaction, and tested it using eight soils with a wide range of characteristics. Various incubation temperatures and times, pH values and substrate concentrations were also used to find the optimal conditions for the enzyme activity and to determine characteristics and kinetic parameters of soil arylesterase. Arylesterase activity was significantly correlated with total organic C, total N, and soil ATP content. Soil arylesterase activity showed a pH optimum at 7.5, optimal temperature between 55 and 65 °C and linear increase with incubation time. The K_m values ranged from 4.3 to 8.5 mM, the V_{max} values from 326 to 803 μmol *p*-NP g^{-1} h^{-1}, with higher K_m values observed in soils with higher organic matter content. We conclude that the proposed assay protocol is suitable to determine the arylesterase activity in a wide range of soils.

Arylesterase (EC 3.1.1.2) is a carboxylic ester hydrolase catalysing the hydrolysis of phenolic esters such as phenyl acetate to phenol and acetate. It occurs in microorganisms (Toshimitsu et al., 1986; Chang et al., 1995; Sakai et al., 1998), plants (Lourmina et al., 1968; Antoun and Roberts, 1975) and other living organisms including humans (Metcalf et al., 1972; Yawetz et al., 1979; Reid and Dunnill, 1969; Stephen and Cheldelin, 1970). This enzyme catalyses the hydrolysis of toxic metabolites (e.g. paraoxon) and is also involved in the degradation of plastics and hydrolysis of organophosphates (Emmelot et al., 1964; Wilde and Kekwick, 1964; Primo-Parmo et al., 1996). Therefore, determination of this enzyme activity in soil might be important for evaluating the response of soil microbial communities to organic contamination and remediation measures, assessing the fate of pesticides and other aromatic compounds present in pharmaceuticals or as organic contaminants potentially toxic to microorganisms and soil organisms (Singh and Jain, 2003; Moon and Smith, 2005). Few studies have focused on the determination of soil arylesterase activity, and the only two available protocols have not been tested with different soils (Satyanarayana and Getzin, 1973; Nakamura et al., 1990).

The aim of our work was to set up a suitable protocol for the assay of the arylesterase activity in soils with a wide range of properties, using *p*-nitrophenyl acetate as substrate. We propose a modification of the method of Nakamura et al. (1990) based on the use of 2.6 mM *p*-nitrophenyl acetate as substrate, 1 g of soil suspension in modified universal buffer (MUB) at pH 7 and incubation at 30 °C. To achieve our aim, we selected eight surface soils with a wide range of properties and under different long-term management (Table 1). All soils were sieved (<2 mm) at field moisture, moistened to 50% water holding capacity and pre-incubated at 25 °C in the dark for 7 days prior to analysis. Although preincubation is recommended, it is not strictly necessary and therefore recently collected samples should be used and these should be processed in the usual way for the analysis of microbiological and biochemical properties. In the optimized protocol the MUB buffer (Skujiņš et al., 1962) adjusted to pH 7.5 was selected. We also tested other buffers commonly used for the assay of arylesterase activity in biological samples (e.g. Tris–HCl), but they were not able to maintain soil pH at the optimal value in acidic soils. The *p*-nitrophenyl acetate (p-NPA) stock solution (200 mM) was prepared by dissolving 0.906 g of *p*-nitrophenyl acetate in 25 ml of dimethylsulfoxide (DMSO). Assays were conducted in six replicates for each soil, using 0.5 g plus 2 ml of MUB, and 0.5 ml of *p*-NPA solution, therefore the DMSO concentration in the final reaction mixture was 20%. Samples were vortexed and immediately incubated in a shaking water bath for 1 h at 37 °C. After incubation, the reaction can be stopped by placing the tubes in ice or in a water bath at 4 °C, followed by centrifugation at 4 °C (6000×*g* for 5 min). One millilitre of the supernatant was pipetted into a new tube, added with 2 ml of *n*-hexane and the mixture was shaken for 7 min, to remove non-hydrolysed *p*-NPA. The upper layer was discarded, and the phase separation was repeated in the same way. After

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Table 1
Main properties of soils.

Soil	Management	Soil type	$pH_{(H_2O)}$[a]	TOC[b] (%)	N_{tot}[c] (%)	CEC[d] (cmol kg^{-1})	Clay (%)	Silt (%)	Sand (%)	ATP[e] content (μg kg^{-1})	Respiration[f] (mg CO_2-C kg^{-1} d^{-1})	Arylesterase activity (μmol *p*-NP g^{-1} h^{-1})	
												Mean (SD)	CV (%)
Bordeaux 1 (Bor 1)	Fallow	Arenic Udifluvent	7.6	0.7	0.08	14.6	17	15	68	669.2	7.1	302 (11)	3.5
Bordeaux 2 (Bor 2)	Fallow	Arenic Udifluvent	6.5	0.3	0.03	14.1	17	15	68	312.8	8.6	164 (5)	3.2
Vallombrosa (Vall)	Woodland	Fragic Dystrudept	5.0	3.7	0.21	44.5	2	20	78	1805.1	44.6	798 (13)	1.6
Romola (Rom)	Woodland	Eutric Cambisol	7.2	0.7	0.07	12.2	11	7	82	556.7	8.9	281 (9)	3.4
Vicarello (Vic F)	Forest	Vertic Xerochrept	8.1	2.2	0.22	132.9	40	29	31	3269.2	51.6	776 (17)	2.2
Vicarello (Vic G)	Grassland	Vertic Xerochrept	8.1	2.1	0.22	122.2	40	29	31	2154.6	47.2	612 (14)	2.3
Vicarello (Vic L)	Lucerne crop	Vertic Xerochrept	8.2	1.1	0.1	138.6	40	29	31	1463.8	38.2	317 (17)	5.3
Vicarello (Vic W)	Wheat crop	Vertic Xerochrept	8.2	1.0	0.1	132.5	41	29	30	1202.5	29.9	272 (7)	2.7
LSD[g]												14	

[a] $pH_{(H_2O)}$ was measured by using a soil to water ratio of 1:2.5.
[b] Total Organic C was measured according to Walkley and Black (1938).
[c] Total N by a CHN analyser (Perkin Elmer NA 2400 II).
[d] EC is the cation exchange capacity measured according to Federico-Goldberg and Farini (1994).
[e] ATP content was measured with the method by Ciardi and Nannipieri (1990).
[f] Soil respiration estimated by gas-chromatography according to Blackmer and Bremner (1977).
[g] LSD is the least significant difference ($P < 0.05$) value calculated by the Tukey–Kramer test.

phase separation, a 0.5 ml aliquot of the aqueous phase was transferred into new tubes, and added with 0.5 ml of 1 M NaOH and 4 ml of distilled H_2O. Removal of residual *p*-NPA was required because of non-enzymatic hydrolysis at pH > 8 occurs. Removal efficiency using *n*-hexane was tested independently using *p*-NPA solutions of known concentrations and accounted for 98.5%. Concentration of *p*-nitrophenol (*p*-NP) was determined spectophotometrically at 400 nm against a standard *p*-NP curve, which should be prepared in the presence of soils, because variable adsorption of *p*-nitrophenol onto soil colloids may occur, depending on the soil organic matter content and other experimental conditions such as temperature, pH and used buffer (Vuorinen, 1993). Controls were performed with the substrate being added after the 1 h incubation, immediately before centrifugation. Different MUB pH values (5.5–8.0), temperatures (30–65 °C), and reaction times (30–120 min) were tested to find the optimal assay conditions and various substrate concentration (0.5–40 mM) ranges were used to determine the kinetic parameters of arylesterase in four out of the eight soils, selected because of their low and high enzyme activities (Table 1). The K_m and V_{max} were estimated with the EnzFitter (Biosoft, UK) software. Because two of the studied soils (Vall and Vic F) deviated from Michaelis–Menten kinetics, the equation of a sigmoidal model was fitted using an iterative least-squares procedure. In this case, the V_{max} value was estimated as the plateau of the sigmoid and the apparent K_m was estimated as the substrate concentration which yields an activity of $V_{max}/2$. Regression analyses were performed with the curve-fitting software SigmaPlot 9.0.

The studied soils showed different arylesterase activities (Table 1), significantly correlated with total organic C ($r^2 = 0.87$), total N ($r^2 = 0.92$) and ATP content ($r^2 = 0.75$). Arylesterase activity was detected within the tested pH range (Fig. 1a), and all soils showed the same pH optimum (7.5), in agreement with what has been reported either for soils or biological samples (Satyanarayana and Getzin, 1973; Bell and Van Petten, 1977; Nakamura et al., 1990). Carboxylic ester hydrolases acting on phenolic esters can also be measured using 4-methylumbelliferyl acetate as substrate (Vepsäläinen et al., 2001; Niemi and Vepsäläinen, 2005); nevertheless, this enzyme activity is not strictly classified as arylesterase, but as methylumbelliferyl acetate deacetylase, with optimal pH between 4.0 and 5.5 (Niemi and Vepsäläinen, 2005). Arylesterase activity increased linearly with incubation time (Fig. 1b) and increased with increasing temperature, peaking between 55 and 65 °C (Fig. 1c); however, 37 °C was selected as assay temperature to avoid chemical hydrolysis of *p*-NPA, which is significant at temperature higher than 45 °C (data not shown). Soil arylesterase activity increased with increasing substrate concentration up to 15 mM in Rom and Vic W, and 20–25 mM in Vall and Vic F (Fig. 1d). The K_m values ranged from 4.3 and 4.5 (Rom and Vic W) to 7.0 and 8.5 mM (Vic F and Vall); the V_{max} values ranged from 326 and 331 of Vic W and Rom soils, respectively, to 802 and 803 μmol *p*-NP g^{-1} h^{-1} of Vall and Vic F soils, respectively. Nakamura et al. (1990) reported K_m and V_{max} values of 1.6 mM and 720 mU g^{-1} (43 μmol *p*-NP g^{-1} h^{-1}), respectively, values being lower than those obtained in this study, possibly because the soil used had lower organic matter content than soils used in our study; however, this is an hypothesis as no soil characterization was given by Nakamura et al. (1990). The higher K_m values observed in soils with higher organic matter content could be due to the partial adsorption of the substrate by soil organic constituents, as well as by changes in the protein conformation of enzymes adsorbed by surface-reactive particles resulting in loss of their affinity towards the substrate (Trasar-Cepeda and Gil-Sotres, 1987). However, as all soils had relatively low organic C content, even if they differed for one order of magnitude (Bor 2 0.3% – Vall 3.7%), differences in K_m and V_{max} values could be also due to the action of different enzyme isoforms. These factors may also explain the deviation of the Vall and Vic F soils from the typical Michaelis–Menten kinetic. Nakamura et al. (1990) used a 2.6 mM final concentration of *p*-NPA in the reaction mixture, and this concentration being only 1.6 times the calculated K_m probably did not ensure the enzyme saturation and the approach to zero order kinetics (Fig. 1d). The *p*-NPA substrate can be dissolved in several organic solvents such as methanol, ethanol, acetone, propanol or DMSO, but the latter was chosen because it maintained the *p*-NPA solution stable for at least five days, it was not toxic at the concentration used in the assay as assessed by the Biotox™ test (Aboatox Oy, Turku, Finland), and it is unlikely to cause protein denaturation at the used concentration (Arakawa et al., 2007). Concentrations of *p*-NPA higher than 40 mM in the reaction solutions are not stable and precipitate in aqueous solutions. The *p*-NPA can also be hydrolysed by other acetyl esterases capable of hydrolysing acetic esters (Panda and Gowrishankar, 2005).

We conclude that the proposed optimized protocol is sufficiently sensitive and precise to determine the arylesterase activity in a wide range of soils. Main innovations with respect to the protocol by Nakamura et al. (1990) were to test the proposed

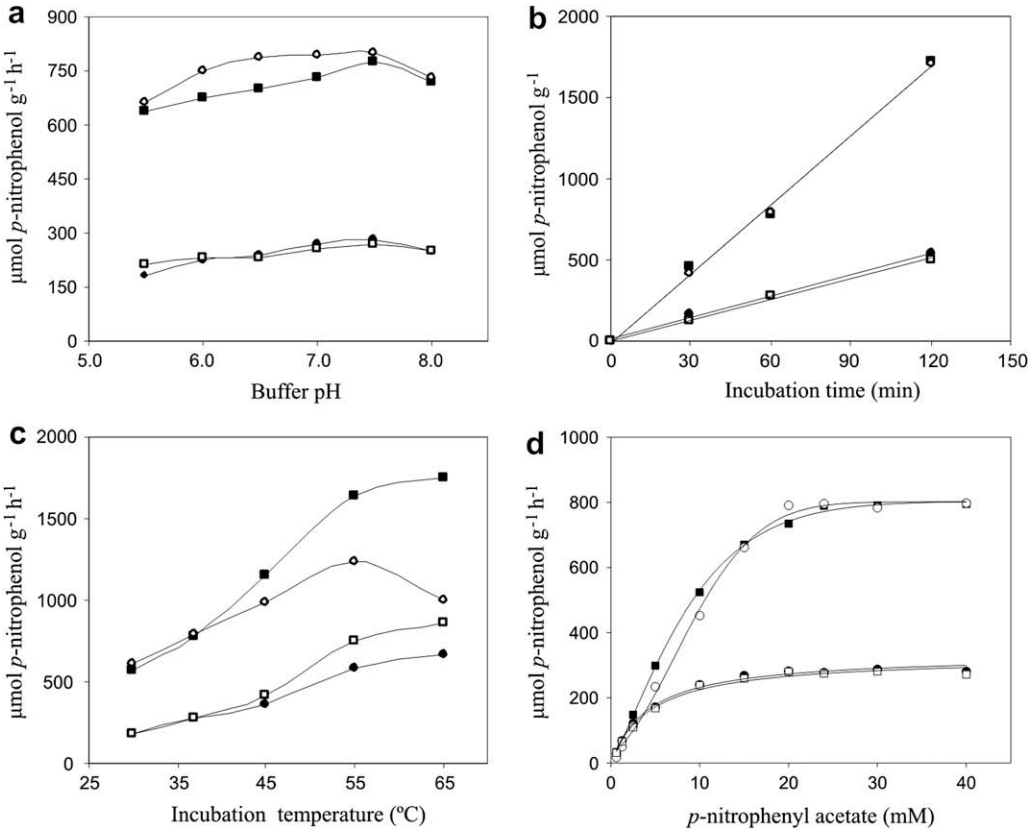

Fig. 1. Effect of buffer pH (a: 0.5 g soil, 40 mM *p*-NPA, 1 h of incubation at 37 °C), incubation time (b: 0.5 g soil, 40 mM *p*-NPA, pH 7.5, incubation at 37 °C), incubation temperature (c: 0.5 g soil, 40 mM *p*-NPA, pH 7.5, 1 h of incubation) and substrate concentration (d: 0.5 g soil, pH 7.5, 1 h of incubation at 37 °C) on arylesterase activity in Vall (○), Vic F (■), Rom (●) and Vic W (□).

protocols with various soil types, to find the optimal conditions of enzyme activity and to measure the relative kinetic parameters. However this method needs to be tested in soils with high organic matter content and strongly acidic pH.

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