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The importance of water availability in the reaction equilibrium of hydrolases in forest litters from a Mediterranean area: a study on lipases

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Summary

Water is one of the main variables affecting the carbon cycle (carbon storage or assimilation) in soils or litters from the Mediterranean area, though in most studies it has been considered *via* soil moisture measurements only. Here, we investigated the effect of water availability as characterized by water activity, a_w , on the equilibrium of enzyme reactions (hydrolysis/synthesis) for litters from three species characteristic of the Mediterranean area (*Quercus pubescens* L., *Q. ilex* L. and *Pinus halepensis* L.). Lipases were used as models and an organic phase was used as the reaction medium to adjust water amount. We found that the activities of hydrolysis/transesterification increased with a_w , showing that both can occur at the same a_w in litter. Furthermore, these lipase activities in litter decreased for a_w close to 1, which has been described with purified lipases in organic media, indicating potential enzyme aggregation. Variations in a_w with moisture were studied with sorption isotherms, which were found to be similar (isotherm type 2) for all the studied litters. Water activity is a crucial indicator for soils under hydric stress at small water contents, which should be considered for describing more precisely enzyme functioning and giving valuable information about carbon dynamics in soils or litters.

Introduction

Mediterranean ecosystems are subjected to severe climatic conditions, mainly characterized by long summer droughts. Moisture contents in soils or litters can thus vary strongly according to spatio-temporal patterns (Fioretto *et al.*, 2001). Water influences the biological processes of organic matter transformation at different scales: microbial growth, enzyme production by microorganisms, protein conformation (Wehtje *et al.*, 1997) and/or the equilibrium of certain enzymatic reactions such as hydrolases, for which water is a substrate of the reaction. For instance, lipases (EC 3.1.1.3) can catalyse either the hydrolysis or the synthesis of esters depending on the amount of water available. Summer drought may favour the synthesis pathway of lipase activity by limiting water availability, leading to carbon storage in litters through the esters produced. On the other hand, when amounts of water are greater (in spring or autumn under the Mediterranean climate), the hydrolytic activity of lipases may be enhanced and favour carbon assimilation (Smyth *et al.*, 2011).

While studies on enzyme activities in litters or soils have focused mostly on hydrolysis to assess carbon assimilation by microorganisms (Fioretto *et al.*, 2000; Qasemian *et al.*, 2012), considering the balance between synthesis and hydrolysis and its dependence on available water can be of major importance because it gives valuable information about the carbon cycle (carbon storage or assimilation). Moreover, water in environmental samples is, most of the time, characterized only as humidity, though mass loss after drying can result from volatile compounds and very tightly-bound water molecules can still be sorbed on soil particles. Taking into account the quantity of water actually available is possible using thermodynamic water activity, a_w , which is used widely in the food industry (Mathlouthi & Rogé, 2003). Thermodynamic water activity can be defined as the ratio of water vapour pressure in the system over pure water pressure at constant pressure and temperature.

We suggest that a_w may be a useful concept to apply to soils and the aim of this study was to investigate how a_w may influence the reaction equilibrium of hydrolases in litter, using lipases as model enzymes. To do so, a reaction medium with organic solvent was used; this was of crucial importance because, under these

conditions, the amount of water was controlled and thus, the influence of the water actually available in the litter on hydrolysis or transesterification can be described. Furthermore, by describing the relationship between humidity and a_w , we examined whether the chemical composition of litters of different plant species, *Quercus pubescens* L., *Q. ilex* L. and *Pinus halepensis* L., may affect water availability.

Materials and methods

Litter sampling: litters of *Quercus pubescens* (QP), *Q. ilex* (QI) and *Pinus halepensis* (PH) were collected as three composite samples for each species at Petit Sambuc (Var, France, 43°37'17.868"N, 5°39'1.835"E) in February 2012. For each litter, horizon L was sieved (2 mm diameter). All the experiments described below were performed for each type of litter.

Water activities were measured in an incubator at 25°C with the Hygropalm probe (Rotronic, Fisher Scientific, Illkirch, France) using 2 g litter (fresh mass). In order to obtain different a_w values, litter was gently dried at room temperature (around 22°C) with a fan. Lipase activities (both hydrolysis and transesterification) were then measured from litters with the different a_w values found. The litter moisture was measured after 72 hours of incubation at 80°C using 1 g fresh litter and was expressed as the percentage of water in fresh litter. The experiment was performed three times for each sample.

Lipase hydrolysis activities were measured as described by Farnet *et al.* (2010); this assay was adapted for litters from the method of Pencreac'h & Baratti (1996) and using MTBE (methyl-tertbutylether) as the organic solvent. The reaction mixture was 10 mM *p*-nitrophenyl laurate dissolved in 2 ml MTBE and 1 g litter. The reaction mixture was incubated at 30°C under magnetic stirring at 500 rpm for 2 hours. Then, 500 ml of the organic phase was added to 4 ml 0.1 M NaOH and *p*-nitrophenol was measured at 412 nm with a spectrophotometer (Biomate; Fisher Scientific).

Lipase transesterification activities were based on an assay adapted for litters by Goujard *et al.* (2009) from the method described by Teng & Xu (2007) and using also MTBE as the organic solvent. The reaction mixture was 10 mM of *p*-nitrophenyl laurate solubilized in MTBE, 400 µl pentanol, 1 g of litter in 3.6 ml MTBE. The protocol was the same as described earlier. Three replicates were performed for each experiment. Activities were expressed as µmoles of *p*-nitrophenol released per hour per g dry mass. A calibration curve of *p*-nitrophenol dissolved in MTBE was made with and without litter under the same experimental conditions.

Chemical characterization of litter was carried out by solid-state NMR of ¹³C; CP-MAS 13C NMR spectra were obtained on a Bruker DSX 400 MHz (Bruker, Milano, Italy) spectrometer operating at 100.7 MHz. Samples (600 mg) were spun at 10 KHz at the magic angle. Contact times of 2 ms were applied with a pulse width of 2.8 µs and a recycle delay of 3 s. Chemical shift values were referenced to glycine signal (carbonyl C at 176.03 ppm). The ¹³C NMR spectra were divided into seven chemical-shift regions

according to Ziarelli (2004). Dmfit 2003 software was used to determine the intensity of each chemical-shift region (Massiot *et al.*, 2002). An aromaticity ratio was also calculated to estimate the proportion of aromatic compounds in each litter (Baldock *et al.*, 1997).

One-way ANOVA was performed to compare variations in hydrolysis and transesterification activities taking into account the type of litter (*Quercus pubescens*, *Q. ilex* and *Pinus halepensis*). Data were transformed as necessary to respect the normality and homogeneity of variance. Then pairwise comparison of means using Student's test was made. The peaks assigned to each chemical region of the NMR spectrum (COOH-C, aromatic-C, O-alkyl-C and alkyl-C) were compared taking into account the type of litter by using this procedure. Statistica Vs 6 (StatSoft, Maison-Alfort, France) was used for statistical analysis and *P* values < 0.05 were considered as being significant.

Results

Chemical characterization of litters from three Mediterranean species

Relative percentages of COOH-C, aromatic-C, O-alkyl-C and alkyl-C were calculated from the solid-state NMR data obtained from the three types of litter under study (Table 1). One-way ANOVA showed that the type of litter strongly influenced the amount of apolar molecules assigned to aromatic compounds (phenols) and lipids. More precisely, aromatic-C and alkyl-C signals were significantly larger in the *Pinus halepensis* and *Quercus ilex* litters, respectively. On the other hand, the O-alkyl signal, assigned to polysaccharides, was similar in the three types of litter.

Sorption isotherms in litters

The relationship between humidity and a_w can be described using sorption isotherms. Figure 1 shows the isotherms found for the three litters and demonstrates that a similar pattern was obtained whatever the litter studied. These isotherms are characteristic of type-2 sigmoidal isotherms as described by Brunauer *et al.* (1940). This type of isotherm is characterized by the presence of multi-layers of water molecules at the surface of a material. Here, and according to Brunauer *et al.* (1940), the sorption isotherms found can be described as follows: for a_w ranging from 0 to 0.2, water is strongly bound to the material, from 0.2 to 0.6, molecules of water are organized in multi-layers, and from 0.6 to 1, molecules of water are free or loosely bound to the external surface of the multilayer.

Variations in lipase activities dependent on a_w and moisture content

It is noteworthy that both hydrolysis and transesterification activities were detected at the same time for a_w ranging from 0.2 to 1 and that the type of litter did not strongly influence

Figure 1 Sorption isotherms for litters of *Pinus halepensis* (◇), *Quercus pubescens* (▲) and *Q. ilex* (□). Each point is a mean of five values; standard deviations were < 3%.

Table 1 Chemical characterization of litters of *Pinus halepensis*, *Quercus pubescens* and *Q. ilex*

(a)	COOH-C	Aromatic-C	O-Alkyl-C	Alkyl-C
<i>Pinus halepensis</i>	4.53 ± 0.07	11.17 ± 0.21	58.92 ± 1.02	25.72 ± 0.85
<i>Quercus ilex</i>	8.21 ± 0.32	14.15 ± 0.75	61.79 ± 2.01	15.85 ± 0.98
<i>Quercus pubescens</i>	5.98 ± 0.58	9.2 ± 0.90	65.58 ± 1.87	19.26 ± 0.74

(b)	d.f.	Sum of squares	Mean square	F	P
Between controls	2	0.954	0.460	3.56	< 0.05
Within controls	30	10.618	0.253		
Total	32	11.538			

(a) Averages of relative percentages and standard deviations of each chemical regions were calculated from solid-state NMR data (10 peaks for each chemical regions, $n = 3$). (b) One-way ANOVA on each chemical region as variables was performed.

the variations observed for both hydrolysis and transesterification activities (Figure 2a,b). These activities followed the same pattern, increasing with a_w , more strongly at values greater than 0.6 when the moisture content became more than 15% (Figure 2a,b).

The results of comparisons of means using ANOVA on both hydrolysis and transesterification activities showed that litter significantly affected both enzyme activities (Table 2). Moreover, pairwise comparison of means showed that both activities differed significantly between *Quercus pubescens* litters on the

one hand and both *Q. ilex* and *Pinus halepensis* litters on the other (with $P < 0.01$ in each case). The lipase activity patterns (both hydrolysis and transesterification) obtained from *Q. pubescens* litter were different from those observed in the *P. halepensis* or *Q. ilex* litters. These activities seemed to be less influenced by the increase in free water molecules from $a_w = 0.6$. For all the litters studied, a decrease in hydrolysis and transesterification activities was observed for water activities near, or equal to, 1 (Figure 3a,b). Here, quantitative differences in lipase activities between the different litters cannot be taken into account because variations in microbial communities resulting from the type of the litter may influence the enzymatic activity level.

Discussion

Water availability in litters from different vegetal species

We first investigated how the amount of available water, characterized by a_w , varied with moisture content depending on the type of litter considered (from *Quercus pubescens*, *Q. ilex* or *Pinus halepensis*). We hypothesized that various percentages of polar molecules (such as polysaccharides and proteins) and apolar molecules (mainly aromatics and lipids) could be found in the structure of three different species typical of Mediterranean areas and thus could influence water sorption on the vegetal polymers. Here, a greater quantity of alkyl-C and aromatic-C was obtained for *P. halepensis* and *Q. ilex* litters respectively, which

Table 2 (a) Means of hydrolysis and transesterification activities and standard deviations ($n = 10$) calculated for the three litters. (b) One-way ANOVA on both hydrolysis and transesterification activities of lipases as variables measured in litters of *Pinus halepensis*, *Quercus pubescens* and *Q. ilex*

(a)	Means of hydrolysis activities / g^{-1} DW		Means of transesterification activities / g^{-1} DW		
<i>Quercus ilex</i>	1.280 ± 0.293		1.364 ± 0.314		
<i>Quercus pubescens</i>	3.899 ± 1.200		3.927 ± 1.098		
<i>Pinus halepensis</i>	0.950 ± 0.240		0.906 ± 0.196		

(b)	d.f.	Sum of squares	Mean square	F	p
Hydrolysis activities					
Between groups	2	45.122	22.561	49.599	< 0.0001
Within groups	25	11.372	0.455	—	—
Total	27	56.494	—	—	—
Transesterification activities					
Between groups	2	45.572	22.786	58.934	< 0.0001
Within groups	25	9.666	0.387	—	—
Total	27	55.238	—	—	—

Figure 2 Hydrolysis (a) and transesterification (b) activities in litters of *Pinus halepensis* (◆), *Quercus pubescens* (▲) and *Q. ilex* (■) depending on a_w . Each point is a mean of three values; standard deviations were < 3%.

has been reported before, although not demonstrated using solid-state NMR of ^{13}C (Coulis *et al.*, 2009). However, these differences in chemical structure did not influence water sorption because a similar isotherm was obtained. Type-2 isotherms (Brunauer *et al.*, 1940) are frequently found in food products but, to our knowledge, have never been described before for litters. This demonstrates that

Figure 3 Hydrolysis (a) and transesterification (b) activities in litters of *Pinus halepensis* (◆), *Quercus pubescens* (▲) and *Q. ilex* (■) depending on moisture content. Each point is a mean of three values; standard deviations were < 3%.

free water molecules are available in the three litters studied from a_w values greater than 0.6, because 0.6 is a point of inflexion of the curve, from which the shape of the sorption isotherm becomes concave (Mathlouthi & Rogé, 2003). This can be linked with the results found considering variations in lipase activities, which increased from a_w of 0.6. Free water is thus available for microbial activities from 15% of water content (a_w 0.6) in litters from three typical Mediterranean species.

Influence of a_w on the balance between hydrolysis and transesterification activities of lipases

We hypothesized that the dynamics of hydrolysis and transesterification activities depend on the amount of water available and that a succession of activities (hydrolysis for larger a_w values and transesterification for smaller a_w) may occur in litters. However, we found that both activities were taking place at the same time. This trend had also been observed by Chowdary & Prapulla (2002) and Ma *et al.* (2002) with purified lipases (purchased from either Sigma Chemicals, St Louis, MI, USA, or Amano Enzyme Co. Ltd., Nagoya, Japan) from *Candida rugosa* and *Rhizopus oryzae*. We also found that both activities increased with a_w and more precisely from $a_w = 0.6$; as noted earlier, sorption isotherms showed that free molecules of water were available from $a_w = 0.6$, which may favour both enzyme conformation and interaction with substrate. However, lipase activities in *Q. pubescens* litter were less influenced by the increase in free water molecules from $a_w = 0.6$; the chemical composition of this litter, with a smaller amount of apolar molecules than *Q. ilex* and *Pinus halepensis* litters, may have modified interactions between water, enzymes and the substrate. Physico-chemical characteristics of the material, such as porosity and hydrophobic molecule contents, strongly influence the effective moisture diffusivity (Roca *et al.*, 2006). Thus here water mobility probably differs between the litter of *Q. pubescens* and that of *Q. ilex* and *Pinus halepensis* leading to a different pattern in lipase activities.

Chowdary & Prapulla (2002) noted that the optimum a_w for synthetic activities of lipases is strongly and positively correlated with the quantity of substrate. Thus, substrate concentration has a large effect on the optimum water activity controlled by any limitation of water's mass transfer. Here, a large concentration of *p*-nitrophenyl laurate was used (to reach concentrations for enzyme saturation), which explains a large optimum a_w for the synthetic activities of lipases. In fact, the activities were measured here with a large amount of *p*-nitrophenyl laurate; under natural conditions, and for the same quantity of water, variations in lipase activities (both hydrolysis and transesterification) greatly depend on the relative proportion of available substrates, such as alcohols, acids or esters. From our results, we can conclude that both types of lipase activities potentially occur even for very small water activities (ranging from 0.2 to 0.4). Thus, carbon assimilation can still be realized *via* hydrolytic activities (though to a lesser extent) under drastic drought conditions (with moisture contents ranging from 10 to 20%).

Under our experimental conditions, both lipase activities decrease for large water activity values. In organic media, the hydration level of enzymes strongly influences both hydrolysis and transesterification activities, which is probably linked to the effect of mass transfer as previously explained. Moreover, Yamane *et al.* (1989) have suggested that aggregation of lipases may occur for large water activities, leading to a decrease in enzyme reaction yields. In our previous study (Farnet *et al.*, 2010), 2 ml of water were added for hydrolysis quantification (a_w more than 1), which obviously optimized the hydrolysis

reaction yields, the huge quantity of water favouring both enzyme conformation and reaction. Here, the reaction mixture with organic solvent was not complemented with water to measure hydrolysis activity; this allowed us to quantify the level of lipase hydrolysis activity obtained with water present only in the litter. Thus these experimental conditions may be more representative of enzyme activity levels potentially expressed in the field.

Conclusion

Thermodynamic water activity, a_w , should be taken into account to understand enzyme functioning under hydric stress better, as it implies experimental conditions that are more representative of *in natura* conditions by using an organic phase instead of the usual buffered aqueous-phase conditions. This study also demonstrated that certain enzymes can still be active at small water contents in litters. This has major relevance for understanding carbon dynamics in soils or litters and defining which conditions favour either carbon storage or assimilation. This balance in enzyme activities could be a useful indicator for soil management because it would determine how certain landscape uses (for both agrosystems and forests) could mitigate the effects of the climatic changes predicted through carbon storage in soils. This type of study should be extended to other enzymes such as glycosidases, which can catalyse both transglycosylation and hydrolysis.

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