



## Biochemically active humic substances in contrasting agricultural managements

Emilio Benitez<sup>1</sup>, Rogelio Nogales<sup>1</sup>, Serena Doni<sup>2</sup>, Grazia Masciandaro<sup>2</sup> and Beatriz Moreno<sup>1</sup>

<sup>1</sup> Consejo Superior de Investigaciones Científicas (CSIC), Estación Experimental del Zaidín (EEZ), Department of Environmental Protection, c/ Profesor Albareda 1, 18008 Granada, Spain. <sup>2</sup> Consiglio Nazionale per la Ricerca (CNR), Institute of Ecosystem Study (ISE), Via Moruzzi 1, 56124 Pisa, Italy.

### Abstract

Because their crucial role in several soil biochemical cycles and their fast response to changes in soil management, extracellular enzymes activities are widely used as sensitive indicators of ecological change and soil quality. The aim of this work was to determine the effects of soil management on the stable pool of soil carbon cycling enzymes as indicators of essential functions. For this, extracellular  $\beta$ -glucosidase enzymes bounded by humic acids (C higher than  $10^4$  Da) were used to compare four long-term contrasting agricultural managements in a rainfed olive orchard representative of semi-arid Mediterranean habitats. The study was conducted for 30 years by designing a random-block of four treatments (nude vs. covered soils) and four replicates. Maintaining cover crops through fall, winter and early spring provoked a more stable and active pool of extracellular  $\beta$ -glucosidase in soils only if spontaneous vegetation was managed with mechanical methods. When herbicides were used during 30 years, the pattern of the molecular composition and activity of humus  $\beta$ -glucosidase complexes were similar in covered and nude soils, although higher activity was retrieved in the former. Tillage management increased carbon mineralization and the level of humic substances and the activity of  $\beta$ -glucosidase humic-bound were quite lower than in the rest of treatments. Given the ecological role of extracellular soil carbon cycling enzymes, the characterization of humus  $\beta$ -glucosidase complexes could be an adequate indicator of sustainability of agricultural management systems.

**Additional key words:** *Olea europaea*;  $\beta$ -glucosidase enzyme; humic substances; sustainability; cover spontaneous vegetation.

**Abbreviations used:** CH (cover crop + herbicides); CM (cover crops + mower); IEF (isoelectric focusing); NC (non-tillage and no-cover); PNG (4-nitro-phenyl- $\beta$ -D-glucanopyranoside); PNP (p-nitrophenol); T (tillage).

**Authors' contributions:** Conceived and designed the experiments: EB, RN and BM. Analyzed and interpreted the data: EB, SD and BM. Critical revised the manuscript for important intellectual content: EB, RN, GM and BM. Contributed reagents/materials/analysis tools: RN, SD, GM and BM. Wrote the paper: EB and BM. Coordinated the research project: EB.

**Citation:** Benitez, E.; Nogales, R.; Doni, S.; Masciandaro, G.; Moreno, B. (2016). Short communication: Biochemically active humic substances in contrasting agricultural managements. Spanish Journal of Agricultural Research, Volume 14, Issue 3, e03SC01. <http://dx.doi.org/10.5424/sjar/2016143-8431>.

**Received:** 07 Aug 2015. **Accepted:** 22 Jun 2016

**Copyright © 2016 INIA.** This is an open access article distributed under the terms of the Creative Commons Attribution-Non Commercial (by-nc) Spain 3.0 Licence, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

**Funding:** Cofinanced ERDF / Spanish Ministry of Economy and Competitiveness (Grant CGL2009-07907).

**Competing interests:** The authors have declared that no competing interests exist.

**Correspondence** should be addressed to Emilio Benitez: [emilio.benitez@eez.csic.es](mailto:emilio.benitez@eez.csic.es)

### Introduction

Microbial extracellular enzymes can remain active in soil for a long time bound and protected by humic substances. The ecological importance of humic-enzyme complexes is that their activity is not affected by microbial stresses, therefore playing an important role in a large number of biochemically mediated processes under adverse conditions for soil microorganisms (Nannipieri *et al.*, 1996; Doni *et al.*, 2012). Humic-bound enzymes extracted from soils or other organic materials can be separated by isoelectric focus-

ing (IEF) on the basis of the isoelectric point of the humic substances. IEF did not modify either the enzyme activity or the molecular structure of the complexes (Benítez *et al.*, 2000; Ceccanti *et al.*, 2008).

Beta-glucosidase activity has been proposed as a good indicator for soil functions because its critical role on soil-carbon cycle (Nannipieri *et al.*, 2012). The importance of extracellular  $\beta$ -glucosidase lies in the capacity of the soil to hydrolyse C substrates too large or insoluble for direct absorption by microbial cells. The characterization of stable humic- $\beta$ -glucosidase compounds could assess the ability to fulfil essential

functions in a highly vulnerable –and economically vital– agroecosystem as rainfed olive farming. Within this framework, the aim of this work was to determine the effects of soil management on the stable pool of soil carbon cycling enzymes as indicators of essential functions.

## Material and methods

Humic- $\beta$ -glucosidase complexes were extracted and then characterized from soils under four agricultural managements in a rainfed olive orchard representative of semi-arid Mediterranean habitats. The main characteristics of the experiment and soils are described in Moreno *et al.* (2009). The soil has been classified as an Anthropic Regosol (FAO, 1998). The study was conducted for 30 years in Jaen (southeastern Spain) by designing a random-block of four treatments and four replicates (plots). The treatments tested were:

- Tillage (T): Bare soils where weeds were eliminated by 3–4 annual passes with a disk harrow (at 30 cm deep) and/or a cultivator in spring, followed by a tine harrow in summer.
- Non-tillage and no-cover (NC): Bare soils where weeds were eliminated by applying the pre-emergence herbicide oxyfluorfen in autumn. Initially, simazine and diuron were used until 12 years ago. In the spring, glyphosate was applied locally.
- Cover crop + herbicides (CH): Covered soils where weeds were left to grow each year to be eliminated in spring with herbicides. Initially, diquat/paraquat was used until prohibition, being replaced by glyphosate at a later date.
- Cover crops + mower (CM): weeds were eliminated with various passes of a chain mower in spring.

Two samples were collected in the centre of each plot by a modified soil-sample ring kit (Eijkelkamp) which includes a cylinder of 20 cm (depth of sampling) specifically manufactured for this purpose and then bulked. The samples were stored at 4°C until analyses were made.

Humic substances were extracted from soil at 37°C for 24 h under shaking, using  $\text{Na}_2\text{P}_4\text{O}_7$  (0.1 M, pH 7.1) in a 1:10 w/v dry soil/extract ratio (Ceccanti *et al.*, 1978). Then, the suspension was centrifuged (12,400×g) and filtered through a 0.22- $\mu\text{m}$  Millipore membrane. The C-derived fraction  $>10^4$  Da was obtained through ultrafiltration on AMICON PM10 membrane of the C extract. The C content was determined by acid digestion with  $\text{K}_2\text{Cr}_2\text{O}_7$  and  $\text{H}_2\text{SO}_4$  at 140°C

for 2 h and then quantified according to Yeomans & Bremner (1988). This C fraction was used to determine extracellular  $\beta$ -glucosidase activity as described by Garcia *et al.* (1993), and it is based on the use of 0.05 M 4-nitro-phenyl- $\beta$ -D-glucanopyranoside (PNG) as a substrate (Hayano & Tubaki, 1985). The p-nitrophenol (PNP) released by  $\beta$ -glucosidase activity was extracted and determined spectrophotometrically at 398 nm (Tabatabai & Bremner, 1969).

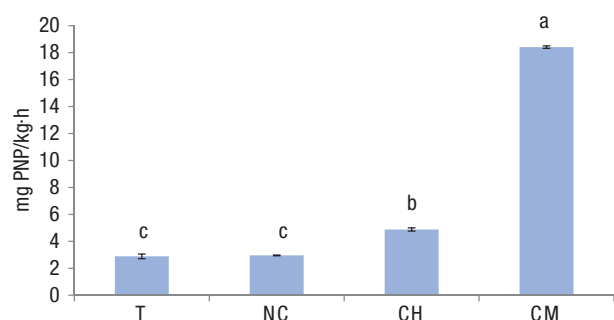
Isoelectric focusing was carried out in cylindrical gel rods (0.5×8 cm) containing polyacrylamide gel (5% w/v) and carrier ampholytes in the pH 4–6 range (Bio-Rad Laboratories, Richmond, CA, USA) at a final concentration of 2% (Ceccanti *et al.*, 1986; 1989). N,N,N',N'-tetramethyl-1,2-diaminomethane and ammoniumperoxy-disulfate were also added in gel solution at a concentration of 0.03%. Organic material (100  $\mu\text{L}$  of pyrophosphate extract derived fraction  $>10^4$  Da) at 4.4% of glycerine was applied at the top of the gel rod (cathode). A little amount (5  $\mu\text{L}$ ) of glycerine at 2.2% was added on the top of the sample to avoid the mixing with the cathodic solution (0.02 N NaOH); 0.01 M  $\text{H}_3\text{PO}_4$  was used for the anodic cell. A pre-run of 1 h at the same current intensity and voltage used for the samples run were carried out for each gel tube (1.5 mA for each tube, 100–800 V); subsequently, the samples run was carried out for 2 h or longer until a stable IEF banding was reached. The electrophoretic bands were scanned by a Bio-Rad GS 80 densitometer, obtaining a typical IEF profile for each soil investigated. The IEF peak area was determined for each soil IEF profile, assuming as 100% the area under the entire IEF profiles (representative of the total loaded C). Gel pH was measured at 0.5 cm intervals with an Orion microprocessor (model 901, Orion research) connected to a microelectrode gel-pHiler (Bio-Rad Laboratories, Richmond, CA, USA). To determine  $\beta$ -glucosidase activity of the humic bands obtained by IEF, the gel was gently removed from the glass tubes. The bands were cut and pre-washed for 1 h with 2 mL of 0.1 M phosphate buffer, pH 6.4, at 37°C (pre-washing removes the carrier ampholytes, salts, and other impurities from the gel, without releasing the gel-trapped humic matter). After removal of the buffer, 2 mL of fresh 0.1 M phosphate buffer, pH 6.4, and 0.5 mL of 0.05 M PNG were added and the mixture was incubated at 37°C under shaking for 17 h.

Results are the means of 12 replicates (three per plot). The results were subjected to a factorial analysis of variance (ANOVA) using the STATISTICA software program (StatSoft Inc., Tulsa, OK, USA). The *post hoc* Tukey HSD test in a one-way ANOVA was used. To be evidence of statistical significance *p*-values of under 0.05 were considered.

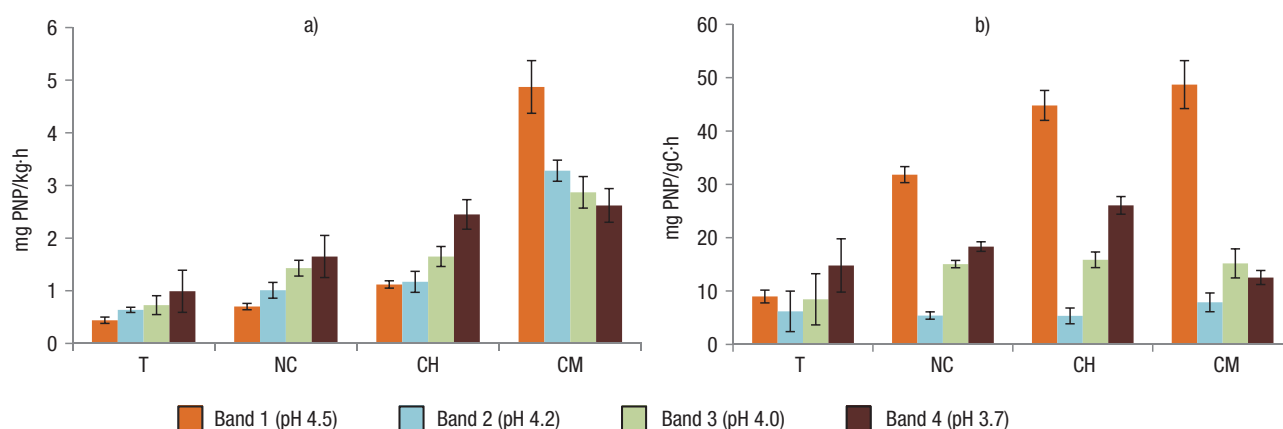
## Results and discussion

Extracellular  $\beta$ -glucosidase was higher in soils under cover vegetation (Fig. 1), especially when these were managed with mechanical methods, evidencing the effect of sustainable managements on the soil biochemical activity. No significant differences were found between soils where spontaneous vegetation was regularly eliminated by tillage (T) or herbicide applications (NC). The above agrees with previous experimental works where extracellular enzyme production has been linked to those agricultural managements increasing the quantity, quality and chemical diversity of cover crops residues (Tiemann *et al.*, 2015).

The humic carbon in the bands after IEF humic carbon calculated from the IEF peak areas focused in the different selected bands (1, 2, 3 and 4) is shown in Table 1. The sum of the humic carbon in the IEF bands represent the  $C > 10^4$  Da fraction of high molecular weight heteropolymers traditionally described as humic acids. CM soils showed the greater levels of humic



**Figure 1.** Extracellular  $\beta$ -glucosidase activity (mg of p-nitrophenol (PNP) released) in tillage (T), non-tillage and no-cover (NC), cover vegetation + herbicides (CH), and cover vegetation + mower (CM) soils (means  $\pm$  SE). Significant differences are indicated by different letters ( $p < 0.05$ , ANOVA, Tukey post-hoc).



**Figure 2.**  $\beta$ -glucosidase (a) and specific  $\beta$ -glucosidase (b) activity (mg of p-nitrophenol (PNP) released) in the bands after isoelectric focusing in tillage (T), non-tillage and no-cover (NC), cover vegetation + herbicides (CH), and cover vegetation + mower (CM) soils (means  $\pm$  SE).

**Table 1.** Humic carbon (mg C/kg) in the pH range 4.5–4.2 after isoelectric focusing in tillage (T), non-tillage and no-cover (NC), cover vegetation + herbicides (CH), and cover vegetation + mower (CM) soils. Significant differences are indicated by different letters ( $p < 0.05$ , ANOVA, Tukey post-hoc).

|                 | T     | NC    | CH    | CM    |
|-----------------|-------|-------|-------|-------|
| Band 1 (pH 4.5) | 49    | 22    | 25    | 100   |
| Band 2 (pH 4.2) | 103   | 187   | 219   | 416   |
| Band 3 (pH 4.0) | 86    | 95    | 104   | 189   |
| Band 4 (pH 3.7) | 67    | 90    | 94    | 209   |
| $C > 10^4$ Da   | 305 c | 393 b | 443 b | 914 a |

substances, both total and those corresponding to each IEF band. Humic C focused at pH 4.2 was higher in all the treatments, confirming the stability of the humic substances isolated (Govi *et al.*, 1994; Doni *et al.*, 2012).

The  $\beta$ -glucosidase activity was also quite higher in all the four humic bands of CM soils (Fig. 2a). For T, NC and CH soils, the higher  $\beta$ -glucosidase activity was measured in the lower pH where band focalized. The trend was just the opposite for CM soils, and the higher activity was found at pH 4.5, pH range where enzymatic proteins generally are stabilized by humus molecules Ceccanti & Masciandaro (2003). However, the specific  $\beta$ -glucosidase activity, *i.e.* the ratio between  $\beta$ -glucosidase activity and  $C > 10^4$  Da, was higher at this pH except for soil where tillage was the main management (Fig. 2b).

In this work, the stable pool of soil  $\beta$ -glucosidase enzymes were used to compared four long-term contrasting agricultural managements. Maintaining cover crops through fall, winter and early spring provoked a more stable and active pool of extracellular  $\beta$ -glucosidase in soils only if spontaneous vegetation was managed with mechanical methods.

When herbicides were used during 30 years, the pattern of the molecular composition and activity of humus- $\beta$ -glucosidase complexes were similar in covered and nude soils, although higher activity was retrieved in the former. This fact is probably related with the higher vegetal biomass incorporated and also with the different mode of action of the herbicides applied during the whole experiment, with different effects on both microbial activity and CO<sub>2</sub> fixation (Fedtke, 1982; Riah *et al.*, 2014; Kane, 2015). Tillage management increased carbon mineralization. As a consequence, the level of humic substances and the activity of  $\beta$ -glucosidase humic-bound were quite lower than in the rest of treatments. Given the ecological role of extracellular soil carbon cycling enzymes, the characterization of humus  $\beta$ -glucosidase complexes could be an adequate indicator of sustainability of agricultural management systems.

## References

- Benítez E, Nogales R, Masciandaro G, Ceccanti B, 2000. Isolation by isoelectric focusing of humic urease complexes from earthworm (*Eisenia fetida*)-processed sewage sludges. *Biol Fertil Soils* 31: 489-493. <http://dx.doi.org/10.1007/s003740000197>.
- Ceccanti B, Nannipieri P, Cervelli S, Sequi P, 1978. Fractionation of humus-urease complexes. *Soil Biol Biochem* 10: 39-45. [http://dx.doi.org/10.1016/0038-0717\(78\)90008-1](http://dx.doi.org/10.1016/0038-0717(78)90008-1).
- Ceccanti B, Alcaniz-Baldellou JM, Gispert-Negrell M, Gassiot-Matas M, 1986. Characterization of organic matter from two different soils by pyrolysis-gas chromatography and isoelectric focusing. *Soil Sci* 142: 83-90. <http://dx.doi.org/10.1097/00010694-198608000-00004>.
- Ceccanti B, Bonmati-Pont M, Nannipieri P, 1989. Microdetermination of protease activity in humic bands of different sizes after analytical isoelectric focusing. *Biol Fert Soils* 7: 202-206. <http://dx.doi.org/10.1007/BF00709649>.
- Ceccanti B, Masciandaro G, 2003. Stable humus-enzyme nucleus: the last barrier against soil desertification. In: Preserving soil quality and soil biodiversity-the role of surrogate indicators; Lobo MC, Ibanez JJ (eds), pp: 77-82. CSIC-IMIA, Madrid.
- Ceccanti B, Doni S, Macci C, Cercignani G, Masciandaro G, 2008. Characterization of stable humic-enzyme complexes of different soil ecosystems through analytical isoelectric focusing technique (IEF). *Soil Biol Biochem* 40: 2174-2177. <http://dx.doi.org/10.1016/j.soilbio.2008.02.004>.
- Doni S, Macci C, Chen H, Masciandaro G, Ceccanti B, 2012. Isoelectric focusing of  $\beta$ -glucosidase humic-bound activity in semi-arid Mediterranean soils under management practices. *Biol Fertil Soils* 48: 183-190. <http://dx.doi.org/10.1007/s00374-011-0615-8>.
- FAO, 1998. World Reference Base for Soil Resources. World Soil Resources Reports 84. FAO-ISRIC-ISSS, Roma. <http://www.fao.org/docrep/W8594E/w8594e00.htm>.
- Fedtke C, 1982. Biochemistry and physiology of herbicide action. Springer, Berlin. <http://dx.doi.org/10.1007/978-3-642-68375-6>.
- Garcia C, Hernandez T, Costa F, Ceccanti B, Masciandaro G, Calcinai M, 1993. Evaluation of the organic matter of raw and composted municipal wastes. *Soil Sci Plant Nutr* 39: 99-108. <http://dx.doi.org/10.1080/00380768.1993.10416979>.
- Govi M, Ciavatta C, Gessa C, 1994. Evaluation of the stability of the organic matter in slurries, sludges and composts using humification parameters and isoelectric focusing. In: Humic substances in the global environment and implications on human health; Senesi S, Miano TM (eds). pp: 1311-1316. Elsevier Sci., Amsterdam.
- Hayano K, Tubaki K, 1985. Origin and properties of  $\beta$ -glucosidase activity of a tomato-field. *Soil Biol Biochem* 17: 553-557. [http://dx.doi.org/10.1016/0038-0717\(85\)90024-0](http://dx.doi.org/10.1016/0038-0717(85)90024-0).
- Kane D, 2015. Carbon sequestration potential on agricultural lands: A review of current science and available practices. In association with National Sustainable Agriculture Coalition, Breakthrough Strategies and Solutions, LLC. <http://sustainableagriculture.net/publications/>.
- Moreno B, Garcia-Rodriguez S, Cañizares R, Castro J, Benitez E. 2009. Rainfed olive farming in south-eastern Spain: Long-term effect of soil management on biological indicators of soil quality. *Agric Ecosyst Environ* 131: 333-339. <http://dx.doi.org/10.1016/j.agee.2009.02.011>.
- Nannipieri P, Sequi P, Fusi P, 1996. Humus and enzyme activity. In: Humic substances in terrestrial ecosystems; Piccolo A (ed), pp: 293-328. Elsevier, Amsterdam. <http://dx.doi.org/10.1016/B978-044481516-3/50008-6>.
- Nannipieri P, Giagnoni L, Renella G, Puglisi E, Ceccanti B, Masciandaro G, Fornasier F, Moscatelli MC, Marinari S, 2012. Soil enzymology: classical and molecular approaches. *Biol Fertil Soils* 48: 743-762. <http://dx.doi.org/10.1007/s00374-012-0723-0>.
- Riah W, Laval K, Laroche-Ajzenberg E, Mougin C, Latour X, Trinsoutrot-Gattin I, 2014. Effects of pesticides on soil enzymes: A review. *Environ Chem Lett* 12: 257-273. <http://dx.doi.org/10.1007/s10311-014-0458-2>.
- Tabatabai MA, Bremner JM, 1969. Use of *p*-nitrophenol phosphate in assay of soil phosphatase activity. *Soil Biol Biochem* 1: 301-307. [http://dx.doi.org/10.1016/0038-0717\(69\)90012-1](http://dx.doi.org/10.1016/0038-0717(69)90012-1).
- Tiemann LK, Grandy AS, Atkinson EE, Marin-Spiotta E, McDaniel MD, 2015. Crop rotational diversity enhances belowground communities and functions in an agroecosystem. *Ecol Lett* 18: 1461-0248. <http://dx.doi.org/10.1111/ele.12453>.
- Yeomans JC, Bremner JM, 1988. A rapid and precise method for routine determination of organic carbon in soil. *Soil Sci Plant Anal* 19: 1467-1476. <http://dx.doi.org/10.1080/00103628809368027>.