Impact of agricultural management on bacterial laccase-encoding genes with possible implications for soil carbon storage in semi-arid Mediterranean olive farming

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Background . In this work, we aimed to gain insights into the contribution of soil bacteria to carbon sequestration in Mediterranean habitats. In particular, we aimed to use bacterial laccase-encoding genes as molecular markers for soil organic C cycling. Using rainfed olive farming as an experimental model, we determined the stability and accumulation levels of humic substances and applied these data to bacterial laccase-encoding gene expression and diversity in soils under four different agricultural management systems (bare soils under tillage/no tillage and vegetation cover under chemical/mechanical management).

Materials and Methods. Humic C (>10^4 Da) was subjected to isoelectric focusing. The GC-MS method was used to analyze aromatic hydrocarbons. Real-Time PCR quantification and denaturing gradient gel electrophoresis (DGGE) for functional bacterial laccase-like multicopper oxidase (LMCO)-encoding genes and transcripts were also carried out.

Results. Soils under spontaneous vegetation, eliminated in springtime using mechanical methods for morethan 30 years, showed the highest humic acid levels as well as the largest bacterial population rich in laccase genes and transcripts. The structure of the bacterial community based on LMCO genes also pointed to phylogenetic differences between these soils due to the impact of different management systems. Soils where herbicides were used to eliminate spontaneous vegetation once a year and those where pre-emergence herbicides resulted in bare soils clustered together for DNA-based DGGE analysis, which indicated a certain amount of microbial selection due to the application of herbicides. When LMCO-encoding gene expression was studied, soils where cover vegetation was managed either with herbicides or with mechanical methods showed less than 10% similarity, suggesting that the type of weed management strategy used can impact weed community composition and consequently laccase substrates derived from vegetation decay.

Conclusions. We suggest that the low humic acid content retrieved in the herbicide-treated soils was mainly related to the type (due to vegetal cover specialization) and smaller quantity (due to lower vegetal biomass levels) of phenolic substrates for laccase enzymes involved in humification processes. We also found that spontaneous vegetal cover managed using mechanical methods could be the best option for achieving C stabilization in rainfed Mediterranean agroecosystems.

IMPACT OF AGRICULTURAL MANAGEMENT ON BACTERIAL LACCASE-ENCODING GENES, WITH POSSIBLE IMPLICATIONS FOR SOIL CARBON STORAGE IN SEMI-ARID MEDITERRANEAN OLIVE FARMING

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ABSTRACT

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INTRODUCTION

Due to their importance for human welfare, ecosystem services have, in recent years, been attracting increasing attention in the scientific literature. Although, traditionally, agroecosystems

have been primarily regarded as service providers, their contribution to other types of ecosystem services have recently been seen as equally crucial (MEA, 2005). Apart from providing food, forage and bioenergy, agricultural ecosystem services have other benefits such as water and climate system regulation, soil fertility and C sequestration (Chen et al., 2016). The latter involves the removal of C from the atmosphere and its storage in soils through physical and biological processes. As land-based biological C-mitigation strategies are regarded as important and viable pathways to climate stabilization (Canadell & Schulze, 2014), it is crucial to evaluate the effects of land-use changes on ecosystem functions. However, in semi-arid climates, agricultural soils are particularly vulnerable due to adverse environmental conditions and centuries-old farming systems (Metzger et al., 2006).

One of the principal agroecosystems in semi-arid Mediterranean areas is that used for perennial olive-orchard cropping. Olive is the most extensively cultivated fruit crop in the world, with more than 10 million hectares under cultivation in 2013 (FAOSTAT, 2015). Its economic and social importance is enormous, as reflected in the increasing per capita consumption of olive oil around the world. However, olive growing also has a considerable ecological impact in the Mediterranean region, where olive orchards constitute a fundamental part of the landscape. Nevertheless, these agroecosystems are particularly exposed to degradation because of low soil organic C (SOC) content.

The possibility of increasing the C-storage capacity of soils, with no significant reduction in yields, through the use of an appropriate soil management system, has been widely discussed (Scholes, Palm & Hickman, 2014). A recent meta-analysis concluded that the most appropriate strategy for maximizing SOC levels in the Mediterranean region is to combine organic inputs with reduced tillage management (Aguilera et al., 2013). However, in rainfed olive farming,

SOC is mainly dependent on plant-derived C-breakdown (Castro et al., 2008; Moreno et al., 2009). SOC decomposition is generally thought to be regulated by biological and environmental conditions (Schmidt et al., 2011). Litter provides the carbon that supports microbial activity, returns part of the C to the atmosphere and stores the rest in the form of humic substances, a highly stable organic fraction considered to be the true agent responsible for soil C-sequestration mechanisms (Miralles et al., 2014). There is overwhelming evidence which shows that C storage in soils is mainly limited by the incorporation of new C rather than by the decomposition of existing C and also shows the critical role played by the soil microbial community in these processes (Lange et al., 2015). Thus, the microbial degradation of plant-waste lignin has been recognized as a C turnover bottleneck in soil (Theuerl & Buscot, 2010; Torres et al., 2014). The microbial breakdown of lignin has been well characterized, as have the enzymes involved, including laccase, lignin peroxidase and manganese peroxidase. However, the capacity to degrade lignin varies. By comparing the three classes of enzymes, Chen et al. (2011) have shown that laccases exhibit the most abundant aromatic-aromatic molecular interactions as well as the highest binding affinity. Laccases, generally laccase-like multicopper oxidases (LMCOs), whose kinetics have been widely studied, are probably the most common class of ligninolytic enzymes in soil. Although they have traditionally been regarded as fungal enzymes, there is mounting evidence which shows that bacterial LMCOs make a major contribution to lignin degradation and SOC turnover (Alexandre & Zhulin, 2000; Sinsabaug, 2010). Laccase-encoding genes have been successfully used in recent studies as molecular markers for SOC cycling under many different environmental conditions and demonstrate the known potential of bacteria to degrade lignin (Baldrian & López-Mondéjar, 2014). However, more studies are needed to elucidate the controversial role played by bacteria in soil C cycling (Kellner et al., 2008).

92 In this study, we identify the management practices which best help to increase SOC stocks in
93 semi-arid agriculture in order to gain an insight into the contribution of soil bacteria to carbon
94 sequestration in Mediterranean habitats. Using rain-fed olive farming as an experimental model,
95 we thus determined the stability and accumulation levels of humic substances and then related
96 these data to bacteria laccase-encoding gene expression and diversity levels in soils under four
97 different agricultural management systems.
98
99 **MATERIALS AND METHODS**
100
101 **Experimental design**
102
103 The main characteristics of the experiment and soils are described in Castro et al. (2008) and
104 Moreno et al. (2009). The study was conducted in Jaen in south-east Spain (Supplemental Files:
105 sampling locations), with a random block experimental plot design consisting of four treatments
106 and four replicates. Each plot consisted of 16 olive trees, with the central four monitored trees
107 surrounded by guard rows. The experiment, involving bare vs. covered soils, was begun in 1976
108 and was carried out over a period of 30 years. Samples were collected in May 2011. The
109 treatments tested were:
110 Tillage (T): Bare soils where weeds were eliminated by 3–4 annual passes with a disk harrow (at
111 30 cm deep) and/or a cultivator in spring, followed by a tine harrow in summer.
112 Non-tillage and no-cover (NC): Bare soils where weeds were eliminated by applying the pre-
113 emergence herbicide oxyfluorfen in autumn. 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 herbicide glyphosate.

Cover crops + mower (CM): Covered soils where weeds were left to grow each year to be eliminated in spring with various passes of a chain mower.

Two samples were collected from the centre of each plot, using a modified soil-sample ring kit (Eijkelkamp), which consists of a 20 cm-deep sampling cylinder specifically manufactured for this purpose, and then bulked. To isolate the nucleic acids, subsamples of fresh soil were immediately frozen in liquid nitrogen. The samples were stored at -80ºC until molecular analyses were carried out.

Humic substances and isoelectric focusing

In this study, humic C was measured in sodium pyrophosphate extract (pH 7.1), a good extractant of humic compounds (Nannipieri et al., 1974; Piccolo et al., 1998). Humic substances were extracted at 37 °C for 24 h under shaking, using $Na_2P_4O_7$ (0.1 M, pH 7.1) in a 1:10 w/v dry soil:extract ratio (Ceccanti et al., 1978). The suspension was then centrifuged at 12,400 *g* and filtered through a 0.22 μm Millipore membrane. The $>10^4$ Da humic-derived fraction was obtained through ultrafiltration on the AMICON PM10 membrane of the extract. The C content of humic C ($>10^4$ Da) was determined by acid digestion with 0.17 M $K_2Cr_2O_7$ and concentrated (97%) H_2SO_4 at 140 °C for 2 h. A spectrophotometric method was used to quantify the Cr^{3+} produced by the reduction of Cr^{6+} (λ=590 nm; Yeomans & Bremner, 1988).

Isoelectric focusing (IEF) of humic C ($>10^4$ Da) was carried out in cylindrical gel rods (0.5×8 cm) containing polyacrylamide gel (5% w/v) and carrier ampholines 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 a gel solution at 0.03%. At the top of the gel rod (cathode), 100 µl of humic C (>10^4 Da) at 4.4% of glycerine were applied. A small amount (5 µl) of glycerine at 2.2% was added to the top of the sample to avoid mixing with the cathodic solution (NaOH 0.02 N); 0.01 M H_3PO_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 was carried out for each gel tube (1.5 mA for each tube, 100–800 V); the samples run was then carried out for 2 h or longer until stable IEF banding was reached. The electrophoretic bands were scanned using a Bio-Rad GS 80 densitometer, giving 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).

Aromatic Hydrocarbons

Aromatic hydrocarbons were extracted ultrasonically from 5 g of freeze-dried soils using 10 ml of hexane/acetone (1:1 v/v). 50 µl of C3-naphthalene in dichloromethane was added as a GC internal standard. After centrifugation and concentration, the solvent was dried by passing it over anhydrous sodium sulfate. The aromatic hydrocarbons were analyzed using a large volume injection-gas chromatography mass spectrometry (LVI–GC–MS) method. GC analyses were conducted on a Varian 450-GC fitted with a 1079 injector in split/splitless mode, a CTC

160 Analytics CombiPal refrigerated autosampler and a Varian 240 Ion Trap as a mass spectrometer
161 detector. The samples were injected in large volume mode using the 1079 PTV injector. 10 µl
162 were injected with the split open (split ratio of 1/100) for 0.5 min to purge the solvent, the split
163 was then closed for 3 min, with the injector temperature ramped up, and the split was finally
164 opened again (split ratio of 1/50). The injector temperature started at 60 °C for 0.5 min and then
165 ramped up by 100 °C/min to 280 °C for 8 min. A Thermo TG-5SILMS fused silica capillary
166 column (30 m × 0.25 mm × 0.25 µm) was used. Temperature ramp: 70 °C (3.5 min)>>25 °C/min
167 to 180 °C (10 min) >>4 °C/min to 300 °C (13 min). 10 µl of the extracts were injected according
168 to the LVI method. The carrier gas was He at 0.9 ml min^{-1}. Electron-impact ionization and
169 detection in full scan (m/z 50 to 500) mode were used. Structural and quantitative information
170 were obtained by comparing the mass spectra and retention times of the samples and standards
171 with the aid of MS Workstation 6.9.1 software.
172
173 **Nucleic acid isolation from soils and cDNA synthesis**
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175 For each soil-sample replicate, the total community-DNA was separately extracted from four 1 g
176 subsamples using the bead-beating method by following the manufacturer's instructions for the
177 MoBioUltraClean Soil DNA Isolation kit (MoBio laboratories, Solana Beach, CA, USA). The
178 extracts were pooled and further concentrated at 35 ºC to a final volume of 20 µl using a Savant
179 Speedvac® concentrator.
180 Total RNA was extracted from four 2 g subsamples of each replicate following the
181 manufacturer's instructions for the MoBio RNA PowerSoil Total RNA Isolation kit (MoBio
182 laboratories, Solana Beach, CA, USA). To remove residual DNA, DNase I enzyme was added

183 using the RNase-Free DNase Set (Roche Applied Science, Penzberg, Germany) following the
184 manufacturer's instructions. The extracts were pooled and further concentrated at 35 ºC to a final
185 volume of 80 µl using a Savant Speedvac® concentrator. The cDNA was synthesized from 1-2
186 µg of total DNase-treated RNA using the Transcriptor High Fidelity cDNA Synthesis Kit
187 according to the manufacturer's instructions (Roche Applied Science, Penzberg, Germany). The
188 synthesis reaction was carried out at 50 ºC for 30 min. The concentration and quality of the final
189 DNA/RNA/cDNA samples were checked with the aid of a Nanodrop® ND-100 spectrometer
190 (Nanodrop Technologies, Wilmington, DE, USA).
191
192 **Quantitative real-time PCR assays**
193
194 Real-time PCR quantification of DNA/cDNA for bacterial functional LMCO-encoding genes
195 and transcripts was based on amplification with primers Cu1AF (5'-ACM WCB GTY CAY
196 TGG CAY GG-3') and Cu2R (5'-G RCT GTG GTA CCA GAA NGT NCC-3'), designed by
197 Kellner et al. (2008). Agarose-gel electrophoresis was carried out to confirm the specific PCR
198 product, resulting in a single band of the expected size (approximately 144 bp), with primer
199 sequences excluded. To determine whether the primers were specific for amplification and
200 detection of bacterial LMCO genes and transcripts of the soils tested, an LMCO gene-clone
201 library was established after PCR of soil DNA samples. A total of 46 sequences were analysed:
202 38 showed homology to LMCO-genes, 6 showed homology to the cloning vector, 2 were badly
203 sequenced and 1 did not show homology to the LMCO-genes (Supplemental Files: homology).
204 Each 21-µl PCR reaction contained between 4 and 5.5 ng of DNA or between 0.2 and 0.3 µg of
205 cDNA, 10.5 µl 2x iQ SYBR Green Supermix (Bio-Rad, Munich, Germany) and 400 nM of each

206 primer. For each DNA/cDNA extracted, real-time PCR experiments were carried out three times,
207 with the threshold cycle (Ct) determined in triplicate. The real-time PCR program consisted of 2
208 min at 50 ºC for carryover prevention, 5 min at 94 ºC for enzyme activation, followed by 5
209 cycles consisting of 94 °C for 15 s, 57-54 °C for 15 s, while decreasing the temperature by 0.5
210 °C in every cycle, 72 °C for 30 s, and 30 cycles consisting of 94 °C for 15 s, 54 °C for 15 s and
211 72 °C for 30 s; the fluorescence signal was measured at the 72 ºC step for both DNA and cDNA.
212 The final step lasted 7 min at 72 °C. The PCR amplification procedure was checked using a heat
213 dissociation protocol (from 70 ºC to 100 ºC) following the final PCR cycle. The LMCO-
214 encoding gene copy number was quantified on an iQ5 thermocycler using iQ5-Cycler software
215 (Bio-Rad, Munich, Germany).
216 A standard curve was generated by using a recombinant plasmid containing one copy of the
217 LMCO gene from soil bacteria. The curves were drawn according to the method described by
218 Moreno et al. (2013). The relationship between the threshold cycle (Ct) and target-gene copy
219 number and the copy numbers of the real-time standard was calculated as described by Qian et
220 al. (2007) using the formula $Ct = -3.089 \times \log_{10}(LMCO) + 30.254$, $R^2 = 0.984$. Target molecules
221 were linear from 10^4 to 10^9 copies.
222 The potential presence of qPCR inhibitors was tested by mixing 1 µl (4-8 ng) of soils DNA
223 extracts or 1 µl (50-350 ng) of soils cDNA extracts with a known amount of recombinant
224 plasmid DNA (pCR®2.1, Invitrogen, Carlsbad, CA, USA) with the appropriate primers.
225 Controls, where DNA and cDNA templates were replaced by filter-sterilized milliQ water, were
226 carried out simultaneously. Ct values did not significantly differ between the DNA/cDNA
227 extracts and the controls.
228

229 **PCR-DGGE analyses**

230

231 The community structure of bacterial LMCO-encoding genes and transcripts was studied by
232 DGGE analysis, for which Cu1AF-DGGE and Cu2R primers were used. Primer Cu1AF-DGGE
233 contained the same sequence as Cu1AF but with an additional 40-nucleotide GC-rich sequence
234 (GC clamp) at the 5′ end of the primer.
235 DGGE analyses were conducted using 550 ng of the final PCR product loaded onto a 45-60 %
236 urea-formamide–polyacrylamide gel. An INGENYphorU System (Ingeny International BV, The
237 Netherlands) was run at 200 V for 10 min followed by 75 V for 18 h at 58 ºC in order to separate
238 the fragments. Gels were silver stained with the Bio-Rad Silver Stain according to the standard
239 DNA-staining protocol and photographed under UV light (λ = 254 nm) using a UVItec Gel
240 Documentation system (UVItec Limited, Cambridge, UK).

241

242 **Data analyses**

243

244 Results are the means of 12 replicates (three per plot) for chemical analyses. With regard to
245 nucleic acids, 4 extractions of each sample were carried out and then grouped together to obtain
246 a single sample per plot. The results were subjected to a factorial analysis of variance (ANOVA)
247 using the STATISTICA software program (StatSoft Inc., Tulsa, OK, USA). The *post hoc* Tukey
248 HSD test in a one-way ANOVA was used. P-values of under 0.05 were considered to be
249 evidence of statistical significance.
250 Band patterns in different DGGE lanes were compared with the aid of UVImap Analysis
251 software (UVItec Limited, Cambridge, UK). DGGE banding data were used to estimate the Raup

252 and Crick probability-based index of similarity (SRC) (1979). Raup and Crick's probability
253 method was used to determine whether similarities within and between samples were stronger or
254 weaker than the randomized prediction. For this purpose, band-matching data were stored as a
255 binary matrix and analyzed using the aforementioned probability-based index. The SRC index
256 measures the probability of the randomized similarity being greater than or equal to the similarity
257 actually observed; SRC values of over 0.95 or under 0.05 indicate similarity or dissimilarity,
258 respectively, which are not random assortments of the same species (bands or OTUs) (Rowan et
259 al., 2003). SRC and cluster analyses were carried out using the PAST (Paleontological Statistics)
260 software program version 1.82b (Hammer, Harper & Ryan, 2001).
261
262 **RESULTS**
263
264 **Organic Carbon Fractions**
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266 We found that the high-molecular-weight (C $>10^4$ Da) humic acid (HA) fractions behaved in a
267 significantly different way from total SOC (Table 1). HA levels were much higher in CM,
268 comparable in CH and NC and considerably lower in the T treatment. Figure 1 shows the IEF
269 patterns of HAs. All the treatments showed the highest optical-density values (IEF peaks) in the
270 pH range 4.5–4.2 (band 1 and band 2).
271
272 **Aromatic hydrocarbons**
273

The results of the analysis of major aromatic hydrocarbons are shown in Table 2. Three main polycyclic aromatic hydrocarbons were detected in soil samples. 2,6-diisopropylnaphthalene (*m/z*=239+199) was found to be present in all soils, although values were close to the detection limit in the CM treatment. The levels of pp-DDA, a polar metabolite of DDT also found in all soils, were significantly higher in the NC treatment. The third compound, present only in the NC and CH soils, was a mixture of four aromatic hydrocarbons (*m/z*=265+266+331), whose mass spectra did not match any compounds in the library. To gain a better understanding of this mixture, its mass spectra are shown as supplementary material (Supplemental Files: mass spectra). The data appear to suggest that the C and D compounds, containing an odd number of N atoms, are dimers of A and B, respectively.

Real-time PCR assays and LMCO gene expression

Table 3 shows LMCO-encoding gene and transcript-copy numbers in the four treatments, both of which were much higher in CM soil and comparable in the other treatments.

LMCO-encoding gene and transcript diversity

Figure 2 shows the DNA- and RNA-based DGGE dendrograms generated by Raup and Crick cluster analyses (see Supplemental Files: DGGE bands). The SRC values, obtained by comparing the four treatments, are also summarized in Figure 2. With respect to LMCO-encoding genes (Fig. 2a), T and NC soils differed significantly (SRC < 0.05). For the other pairs of DNA-DGGE profiles compared, the similarity was no greater than that the random prediction

297 (0.95 > SRC > 0.05). However, SRC values comparing CM and the other treatments were very
298 close to the dissimilarity boundary (SRC < 0.05), as evidenced by the solitary cluster where these
299 soils were located.
300 When the LMCO transcripts were evaluated (Fig. 2b), T and NC soils clustered together,
301 showing a high degree of similarity (SRC ≈ 1). CH and CM soils showed low similarity (<10%).
302 After comparing CM with the other soils, the similarity was found to be no greater than that
303 randomly predicted (0.95 > SRC > 0.05).
304
305 **DISCUSSION**
306
307 In recent years, scientists have been discussing the relationship between agricultural management
308 practices and soil-C sequestration. The long-term impact of several types of agricultural
309 management on SOC dynamics has been extensively evaluated, some of which have been
310 proposed as a means of offsetting anthropogenic CO_2 emissions (Söderström et al., 2014). In
311 particular, the advantage of including cover crops in cropping systems, as opposed to other
312 management practices that increase SOC, has been highlighted (Poeplau & Don, 2015).
313 Data on SOC dynamics obtained in our previous studies suggest that no absolute advantages in
314 terms of C sequestration appear to be associated with any of the four types of agricultural
315 management tested. A previous paper reported comparable amounts of aboveground vegetal
316 biomass per year in T, CH and CM treatments, as well as highly variable levels of annual
317 biomass production which is typically found in semi-arid Mediterranean habitats, while leaves
318 and root exudates from olive trees were the only C biomass input in the NC soil (see C vegetal
319 biomass in Table 1 from Castro et al., 2008). SOC, measured after 30 years of experiments, was

320 shown to be significantly lower in NC as compared to the T and CM treatments, though similar
321 ($p < 0.05$) to the levels found in CH soils. However, no significant differences were observed
322 between the former and latter treatments (SOC in Table 1 in Cañizares, Moreno & Benitez,
323 2012). Nevertheless, the outcomes for HAs have differed significantly, given that C-
324 sequestration mechanisms are primarily based on the stabilization of $C>10^4$ Da (Miralles et al.,
325 2014). HA levels were much higher in CM soils, where spontaneous vegetation was allowed to
326 grow each year and then eliminated in spring using mechanical methods. The IEF profiles
327 confirm the stability levels of the humic substances isolated: larger quantities of organic C
328 compounds were found at higher pH values (4.5–4.2) in the IEF gel, thus providing
329 overwhelming evidence that the higher the isoelectric point, the more humified the organic
330 matter (Ceccanti & Nannipieri, 1979; Govi, Ciavatta & Gessa, 1994).
331 These results provide supporting evidence that reduced tillage and green manure incorporation
332 could represent the best option for C input and stabilization in the Mediterranean region (Alvaro-
333 Fuentes et al., 2009; Aguilera et al., 2013; Garcia-Ruiz & Gomez-Muñoz, 2014; Garcia-Franco
334 et al., 2015). This has usually been explained by the greater physical-chemical protection of the
335 organic matter by microaggregates that this type of management provides. Tillage disrupts a
336 larger proportion of aggregates, thus releasing higher levels of organic C as compared to no-
337 tillage treatments (Six, Elliott & Paustian, 1999), which could explain why the T treatment
338 exhibited the lowest HA levels. However, much of the current debate still revolves around SOC
339 stabilization mechanisms, with existing evidence pointing to the synthesis of polyphenols as an
340 essential step in HA formation. Polyphenols could be derived from lignin-degradation products
341 or from microbial resynthesis, reflecting the importance of the specific type of vegetation and the
342 critical role played by microbial ligninolytic enzymes in the soil, specifically phenoloxidase,

laccase and peroxidase activities (Stevenson, 1994). Laccases are probably the largest class of ligninolytic enzymes in soil and perform different oxidative and polymerative functions. The enzymes of the former group are mainly involved in lignin breakdown, while the latter are chiefly involved in polymerizing soluble phenols, thereby contributing to humification (Gianfreda, Xu & Bollag, 1999). The data gathered in the present study suggest that there is a relationship between the number and expression of bacterial LMCO genes on the one hand and the quantity and stability of HAs on the other. Soils under vegetal cover managed by mechanical methods, where the highest HA levels were retrieved after 30 years of experiments, showed the largest bacterial population rich in laccase genes. Also, the environmental conditions facilitated a corresponding higher level of gene expression in these soils as compared to other treatments. The structure of the bacterial community based on LMCO genes also points to a phylogenetic difference in CM soils due to the management system used. CH and NC soils clustered together in the DNA-based DGGE analysis, suggesting a certain amount of microbial selection due to the application of herbicides. With respect to LMCO-encoding gene expression, CH and CM soils showed low similarity (<10%), indicating that the type of weed management strategy used can impact weed populations and consequently laccase substrates derived from vegetation decay. Indeed, differences in weed community composition and the weed seed bank have previously been highlighted following long-term applications of preemergence herbicides as compared to non-chemical weed control management (Sosnoskie, Herms & Cardina, 2006; Barroso et al., 2015; Fracchiolla et al., 2015).

However, the results above did not provide confirmatory evidence concerning the role played by bacterial laccase in HA formation. Laccase substrates are quite diverse and extracellular bacterial laccase can oxidize other compounds such as dyes, pesticides and polycyclic aromatic

366 hydrocarbons (Majeau, Brar & Tyagi, 2010). Nevertheless, in this study, almost all predominant
367 aromatic hydrocarbon and herbicide metabolites were retrieved when herbicides were part of the
368 agricultural management system; neither the type nor amount correlated with LMCO gene
369 copies, transcript numbers or diversity.
370 Bringing together the above results, we found that management systems involving the
371 application of herbicides could influence bacterial laccase activity and diversity either directly
372 through their effect on soil microorganisms or indirectly through vegetal cover specialization.
373 The literature provides considerable evidence to show that different types of lignin phenols are
374 found in specific types of vegetation (Radosevich, Holt & Ghersa, 1997; Caseley, Cussans &
375 Atkin, 2013), although there is no consensus on the effects of herbicides on microbial
376 community structure and activity in soil (Jacobsen & Hjelmsø, 2014). Many authors have
377 described the side-effects of glyphosate on microorganisms as of "little ecological significance"
378 (Wardle & Parkinson, 1990), while others have contended that it has a considerable ecological
379 impact (Nannipieri et al., 2003; Zobiole et al., 2011). In this long-term experiment, the claim that
380 herbicides have no significant effect on soil bacteria after 30 years of herbicide applications is
381 supported by previous data; soils under vegetation cover showed similar bacterial biomass and
382 activity levels and characteristics whether spontaneous weeds were eliminated by mechanical or
383 by chemical methods (Cañizares, Moreno & Benitez, 2012). However, apart from a different
384 plant community pattern, herbicides could produce a collateral effect on certain bacterial
385 activities related to the soil C cycle. Weed biomass and leaf/root exudates from olive trees were
386 the sole C biomass input (Moreno et al., 2013), while litter-derived C was incorporated into both
387 more and less chemically stable compounds. However, after 30 years of experiments, we
388 detected an unexpected lower humification rate in CH as compared to CM soils. After reviewing

the history of CH soils, we found that the herbicides diquat and paraquat had been applied over the years until their prohibition (Moreno et al., 2009) and were later replaced by glyphosate. On the basis of their mode of action, bipyridylium herbicides act by inhibiting photosynthesis (Dodge, 1971), while glyphosate inhibits the formation of phenolic precursors necessary for the production of lignin (Franz, Mao & Sikorski, 1997). We can thus assume that the lignin content of weeds incorporated into the soil in both circumstances was lower or simply different from that incorporated into the CM treatment, and it is also generally accepted that the synthesis of polyphenols from lignin is a critical step in C stabilization. Moreover, CH soils, where vegetation cover was eliminated once a year with herbicides, and NC soils, where weeds were prevented from growing by the application of herbicides, presented comparable levels of HAs as well as similar bacterial laccase gene-copy numbers, diversity and expression.

CONCLUSIONS

A long-term experiment comparing four types of agricultural management was conducted in a semi-arid Mediterranean agroecosystem. After 30 years of experiments, we found comparable stable levels of C in both non-tillage + no cover soils and soils under spontaneous vegetation cover managed using chemical methods. We suggest that the lower humic acid content retrieved in the herbicide-treated soils was mainly related to the type, due to vegetal cover specialization, and smaller quantity, due to lower vegetal biomass levels, of phenolic substrates for laccase enzymes involved in humification processes. We also conclude that spontaneous vegetation cover managed with mechanical methods is the best option to achieve C stabilization.

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REFERENCES

Aguilera, E., Lassaletta, L., Gattinger, A., Gimeno, B.S., 2013. Managing soil carbon for climate change mitigation and adaptation in Mediterranean cropping systems: a meta-analysis. Agriculture, Ecosystems and Environment 168, 25-36.

Alexandre, G., Zhulin, I.B., 2000. Laccases are widespread in bacteria. Trends in Biotechnology 18, 41–42.

Alvaro-Fuentes, J., López, M.V., Arrúe, J.V., Moret, D., Paustian, K., 2009. Tillage and cropping effects on soil organic carbon in Mediterranean semiarid agroecosystems: Testing the Century model. Agriculture, Ecosystems and Environment 134, 211-217.

Ausec, L., Zakrzewski, M., Goesmann, A., Schlüter, A., Mandic-Mulec, I., 2011. Bioinformatic Analysis Reveals High Diversity of Bacterial Genes for Laccase-Like Enzymes. PLoS ONE 6(10): e25724. doi:10.1371/journal.pone.0025724.

Baldrian, P., López-Mondéjar, R., 2014. Microbial genomics, transcriptomics and proteomics: new discoveries in decomposition research using complementary methods. Journal of Applied Microbiology and Biotechnology 98, 1531-1537.

435 Barroso, J., Miller, Z.J., Lehnhoff, E.A., Hatfield, P.G., Menalled, F.D., 2015. Impacts of
436 cropping system and management practices on the assembly of weed communities. Weed
437 Research 55, 1365-3180.
438 Canadell, J.G., Schulze, E.D., 2014. Global potential of biospheric carbon management for
439 climate mitigation. Nature Communications 5, Article number: 5282.
440 Cañizares, R., Moreno, B., Benitez, E., 2012. Bacterial β-glucosidase function and metabolic
441 activity depend on soil management in semiarid rainfed agriculture. Ecology and Evolution 2,
442 727–731.
443 Caseley, J.C., Cussans, G.W., Atkin, R.K., 2013. Herbicide resistance in weeds and crops.
444 Oxford/Boston: Butterworth-Heineman.
445 Castro, J., Fernandez-Ondoño, E., Rodrıguez, C., Lallena, A.M., Sierra, M., Aguilar, J., 2008.
446 Effects of different olive-grove management systems on the organic carbon and nitrogen
447 content of the soil in Jaen (Spain). Soil & Tillage Research 98, 56-67.
448 Ceccanti, B., Nannipieri, P., 1979. Concerning the reliability of the isoelectric focusing
449 technique to separate the soil humic substances. In: Frigerio, A., Renoz, L. (Eds.) Recent
450 Developments in Chromatography and Electrophoresis. Elsevier, Amsterdam, vol 10, pp 225–
451 232.
452 Ceccanti, B., Nannipieri, P., Cervelli, S., Sequi, P., 1978. Fractionation of humus–urease
453 complexes. Soil Biology & Biochemistry 10, 39–45.
454 Chen, M., Zeng, G., Tan, Z., Jiang, M., Li, H., Liu, L., Zhu, Y., Yu, Z., Wei, Z., Liu, Y., XieG.,
455 2011. Understanding lignin-degrading reactions of ligninolytic enzymes: binding affinity and
456 interactional profile. PloS one 6(9), e25647, doi:10.1371/journal.pone.0025647.

457 Chen M., Zeng, G., Zhang, J., Xu, P., Chen, A., Lu, L., 2015. Global Landscape of Total Organic
458 Carbon, Nitrogen and Phosphorus in Lake Water. Scientific Reports 5, Article number:
459 15043, doi:10.1038/srep15043.
460 Chen M., Qin, X., Zeng, G., Li, J., 2016. Impacts of human activity modes and climate on heavy
461 metal "spread" in groundwater are biased. Chemosphere 152, 439-445.
462 Dodge, A.D., 1971. The mode of action of the bipyridylium herbicides, paraquat and diquat.
463 Endeavour 30, 130-135.
464 FAOSTAT, 2015. Food and Agriculture Organization of the United Nations Statistics division,
465 available at: http://faostat3.fao.org (last access: 28 May 2015).
466 Fracchiolla, M., Terzi, M., Frabboni,L., Caramia, D., Lasorella, C., De Giorgio, D., Montemurro,
467 P., Cazzato, E., 2015. Influence of different soil management practices on ground-flora
468 vegetation in an almond orchard. Renewable Agriculture and Food Systems, 9 pp,
469 doi:10.1017/S1742170515000241.
470 Franz, J.E., Mao, M.K., Sikorski, J.A., 1997. Glyphosate: a unique global herbicide. American
471 Chemical Society: Washington, DC.
472 Garcia-Franco, N., Albaladejo, J., Almagro, M., Martínez-Mena. M. 2015. Beneficial effects of
473 reduced tillage and green manure on soil aggregation and stabilization of organic carbon in a
474 Mediterranean agroecosystem. Soil & Tillage Research 153, 66–75.
475 Garcia-Ruiz, R., Gomez-Muñoz, B., 2014. Potential contribution of natural plant of the
476 intercanoppy of fruity crops on carbon sequestration. In:
477 http://www.redremedia.org/events/images/stories/ponencias/poster/suelo-
478 planta/45_garcia_roberto.pdf.

479 Gianfreda, L., Xu, F., Bollag, J.M., 1999. Laccases: A Useful Group of Oxidoreductive
480 Enzymes. Bioremediation Journal 3, 1-26.
481 Govi, M., Ciavatta, C., Gessa, C., 1994. Evaluation of the stability of the organic matter in
482 slurries,sludges and composts using humification parameters and isoelectric focusing. In:
483 Senesi, N., Miano, T.M. (Eds.) Humic substances in the global environment and implications
484 on human health. Elsevier Science, Amsterdam, pp 1311–1316.
485 Hammer, Ø., Harper, D.A.T., Ryan, P.D., 2001. Past: Paleontological statistics software package
486 for education and data analysis. Palaeontologia Electronica 4, 9 pp.
487 Jacobsen, C.S., Hjelmsø, M.H., 2014. Agricultural soils, pesticides and microbial diversity.
488 Current Opinion in Biotechnology 27, 15–20.
489 Kellner, H., Luis, P., Zimdars, B., Kiesel, B., Busco, F., 2008. Diversity of bacterial laccase-like
490 multicopper oxidase genes in forest and grassland Cambisol soil samples. Soil Biology &
491 Biochemistry 40, 638-648.
492 Lange, M., Eisenhauer, N., Sierra, C.A., Bessler, H.,Engels, C., Griffiths, R.I., Mellado-Vázquez,
493 P.G., Malik, A.A., Roy, J., Scheu, S., Steinbeiss, S., Thomson, B.C., Trumbore, S.E.,
494 Gleixner, G., 2015. Plant diversity increases soil microbial activity and soil carbon storage.
495 Nature Communications 6:6707, doi: 10.1038/ncomms7707.
496 Majeau, J.A., Brar, S.K., Tyagi, R.D., 2010. Laccases for removal of recalcitrant and emerging
497 pollutants. Bioresource Technology 101, 2331-2350.
498 MEA Millennium Ecosystem Assessment, 2005. Ecosystems and Human Well-Being: Synthesis.
499 Island Press, Washington, DC.

500 Metzger, M.J., Rounsevell, M.D.A., Acosta-Michlik, L., Leemans, R., Schröter, D, 2006. The
501 vulnerability of ecosystem services to land use change. Agriculture, Ecosystems and
502 Environment 114, 69-85.
503 Miralles, I., Piedra-Buena, A., Almendros, G., González-Vila, F.J., González-Pérez, J.A., 2014.
504 Pyrolytic appraisal of the lignin signature in soil humic acids: Assessment of its usefulness as
505 carbon sequestration marker. Journal of Analytical and Applied Pyrolysis 113, 107–115.
506 Moreno, B., Cañizares, R., Nuñez, R., Benitez, E., 2013. Genetic diversity of bacterial β-
507 glucosidase-encoding genes as a function of soil management. Biology and Fertility of Soils
508 49, 735-745.
509 Moreno, B., Garcia-Rodriguez, S., Cañizares, R., Castro, J, Benitez, E., 2009. Rainfed olive
510 farming in south-eastern Spain: Long-term effect of soil management on biological indicators
511 of soil quality. Agriculture, Ecosystems and Environment 131, 333–339.
512 Nannipieri, P., Ascher, J., Ceccherini, M.T., Landi, L., Pietramellara, G., Renella, G., 2003.
513 Microbial diversity and soil functions. European Journal of Soil Science 54, 655-670.
514 Nannipieri, P., Ceccanti, B., Cervelli, S., Sequi, P., 1974. Use of 0.1 M pyrophosphate to extract
515 urease from a podzol. Soil Biology & Biochemistry 6, 359–362.
516 Piccolo, A., Conte, P., Scheunert, I., Paci, M., 1998. Atrazine interactions with soil humic
517 substances of different molecular structure. Journal of Environmental Quality 27, 1333–1724.
518 Poeplau, C., Don, A., 2015. Carbon sequestration in agricultural soils via cultivation of cover
519 crops-A meta-analysis. Agriculture, Ecosystems and Environment 200, 33–41.
520 Qian, H., Hu, B., Cao, D., Chen, W., Xu, X., Lu, Y., 2007. Bio-safety assessment of validamycin
521 formulation on bacterial and fungal biomass in soil monitored by real-time PCR. Bulletin of
522 Environmental Contamination and Toxicology 78, 239-244.

523 Radosevich, S.R., Holt, J.S., Ghersa, C., 1997. Weed ecology: implications for management.
524 John Wiley & Sons, New York.
525 Raup, D.M., Crick, R.E., 1979. Measurement of Faunal Similarity in Paleontology. Journal of
526 Paleontology 53, 1213-1227.
527 Rowan, A.K., Snape, J.R., Fearnside, D., Barer, M.R., Curtis, T.P., Head, I.M., 2003.
528 Composition and diversity of ammonia-oxidising bacterial communities in wastewater
529 treatment reactors of different design treating identical wastewater. FEMS Microbiology
530 Ecology 43, 195-206.
531 Schmidt, M.W.I., Torn, M.S., Abiven, S., Dittmar, T., Guggenberger, G., Janssens, I.A., Kleber,
532 M., Kögel-Knabner, I., Lehmann, J., Manning, D.A.C., Nannipieri, P. Rasse, D.P., Weiner, S.,
533 Trumbore, S.E., 2011. Persistence of soil organic matter as an ecosystem property. Nature
534 478, 49–56.
535 Scholes, R.J., Palm, C.A., Hickman, JE., 2014. Agriculture and climate change mitigation in the
536 developing world. CCAFS Working Paper No. 61. Copenhagen, Denmark: CGIAR Research
537 Program on Climate Change, Agriculture and Food Security (CCAFS).
538 Sinsabaug, R.L., 2010. Phenol oxidase, peroxidase and organic matter dynamics of soil. Soil
539 Biology & Biochemistry 42, 391-404.
540 Six, J., Elliott, E.T., Paustian, K., 1999. Aggregate and soil organic matter dynamics under
541 conventional and no-tillage systems. Soil Science Society of America Journal 63, 1350-1358.
542 Söderström, B., Hedlund, K., Jackson, L.E., Kätterer, T., Lugato, E., Thomsen, I.K., Jørgensen,
543 H.B., 2014. What are the effects of agricultural management on soil organic carbon (SOC)
544 stocks? Environmental Evidence 3(2), doi:10.1186/2047-2382-3-2.

545 Sosnoskie, L.M., Herms, C.P., Cardina, J., 2006. Weed seedbank community composition in a
546 35-yr-old tillage and rotation experiment. Weed Science 54, 263-273.
547 Stevenson, F.J., 1994. Humus chemistry. John Wiley & Sons, New York.
548 Theuerl, S., Buscot, F., 2010. Laccases: toward disentangling their diversity and functions in
549 relation to soil organic matter cycling. Biology and Fertility of Soils 46, 215-225.
550 Torres, I.F., Bastida, F., Hernández, T., Bombach, P., Richnow, H.H., García, C., 2014. The role
551 of lignin and cellulose in the carbon-cycling of degraded soils under semiarid climate and
552 their relation to microbial biomass. Soil Biology & Biochemistry 75,152-160.
553 Tveit, A., Schwacke, R., Svenning, M.M., Urich, T., 2013. Organic carbon transformations in
554 high-Arctic peat soils: key functions and microorganisms. The ISME Journal 7, 299–311.
555 Wardle, D.A., Parkinson, D., 1990. Effects of three herbicides on soil microbial biomass and
556 activity. Plant Science 122, 21-28.
557 Yeomans, J.C., Bremner, J.M., 1988. A rapid and precise method for routine determination of
558 organic carbon in soil. Communications in Soil Science Plant Analysis 19, 1467–1476.
559 Zobiole, L.H.S., Kremer, R.J., Oliveira, R.S., Constantin, J., 2011. Glyphosate affects micro-
560 organisms in rhizospheres of glyphosate-resistant soybeans. Journal of Applied Microbiology
561 110, 118–127.

Table 1(on next page)

Table 1

Table 1 - Organic carbon fractions for tillage (T), non-tillage and no-cover (NC), cover vegetation + herbicides (CH), and cover vegetation + mower (CM) treatments (means ± SE). For each parameter, significant differences are indicated by different letters ($P < 0.05$, ANOVA, Tukey post-hoc).

	C vegetal biomass (kg ha^{-1} year^{-1})*	SOC (g kg^{-1})**	HAs (C>10^4 Da) (mg kg^{-1})
T	3743 a	9.4 ± 3.1 a	306 ± 1.1 c
NC	----	4.6 ± 0.7 b	390 ± 2.9 b
CH	2617 a	6.8 ± 3.2 ab	443 ± 29 b
CM	4059 a	8.3 ± 2.0 a	914 ± 6.6 a

1 SOC: Soil Organic Carbon; HAs: Humic Acids; * from Castro et al., (2008); ** from Cañizares et al., (2012)

2

Table 2(on next page)

Table 2

Table 2 - Main aromatic hydrocarbons in tillage (T), non-tillage and no-cover (NC), cover vegetation + herbicides (CH), and cover vegetation + mower (CM) soils (means ± SE). For each parameter, significant differences are indicated by different letters ($P < 0.05$, ANOVA, Tukey post-hoc).

	CAS 24157-81-1 (239+199)	p,p-DDA	A+B+C+D (265+266+331)
T	0.22 ± 0.13 a	0.007 ± 0.005 b	nd
NC	0.19 ± 0.05 a	0.021 ± 0.001 a	0.36 ± 0.05 a
CH	0.25 ± 0.01 a	0.014 ± 0.004 b	0.22 ± 0.05 a
CM	0.04 ± 0.03 b	0.011 ± 0.001 b	nd

1 nd: not detected

2

Table 3(on next page)

Table 3

Table 3 - LMCO-encoding gene and transcript copy numbers for tillage (T), non-tillage and no-cover (NC), cover vegetation + herbicides (CH), and cover vegetation + mower (CM) soils (means ± SE). For each parameter, significant differences are indicated by different letters ($P < 0.05$, ANOVA, Tukey post-hoc).

	LMCO gene copies g^{-1}	LMCO transcript copies g^{-1}
T	$9.79 \times 10^4 \pm 5.74 \times 10^3$ b	$1.34 \times 10^3 \pm 3.16 \times 10^2$ b
NC	$1.02 \times 10^5 \pm 8.64 \times 10^3$ b	$3.02 \times 10^3 \pm 8.38 \times 10^2$ b
CH	$7.89 \times 10^4 \pm 1.10 \times 10^4$ b	$6.67 \times 10^3 \pm 3.21 \times 10^3$ b
CM	$5.71 \times 10^5 \pm 4.03 \times 10^4$ a	$4.12 \times 10^4 \pm 4.86 \times 10^3$ a

1

Figure 1(on next page)

Figure 1

Fig 1 - Isoelectric focusing profiles of HAs from tillage (T), non-tillage and no-cover (NC), cover vegetation + herbicides (CH), and cover vegetation + mower (CM) soils

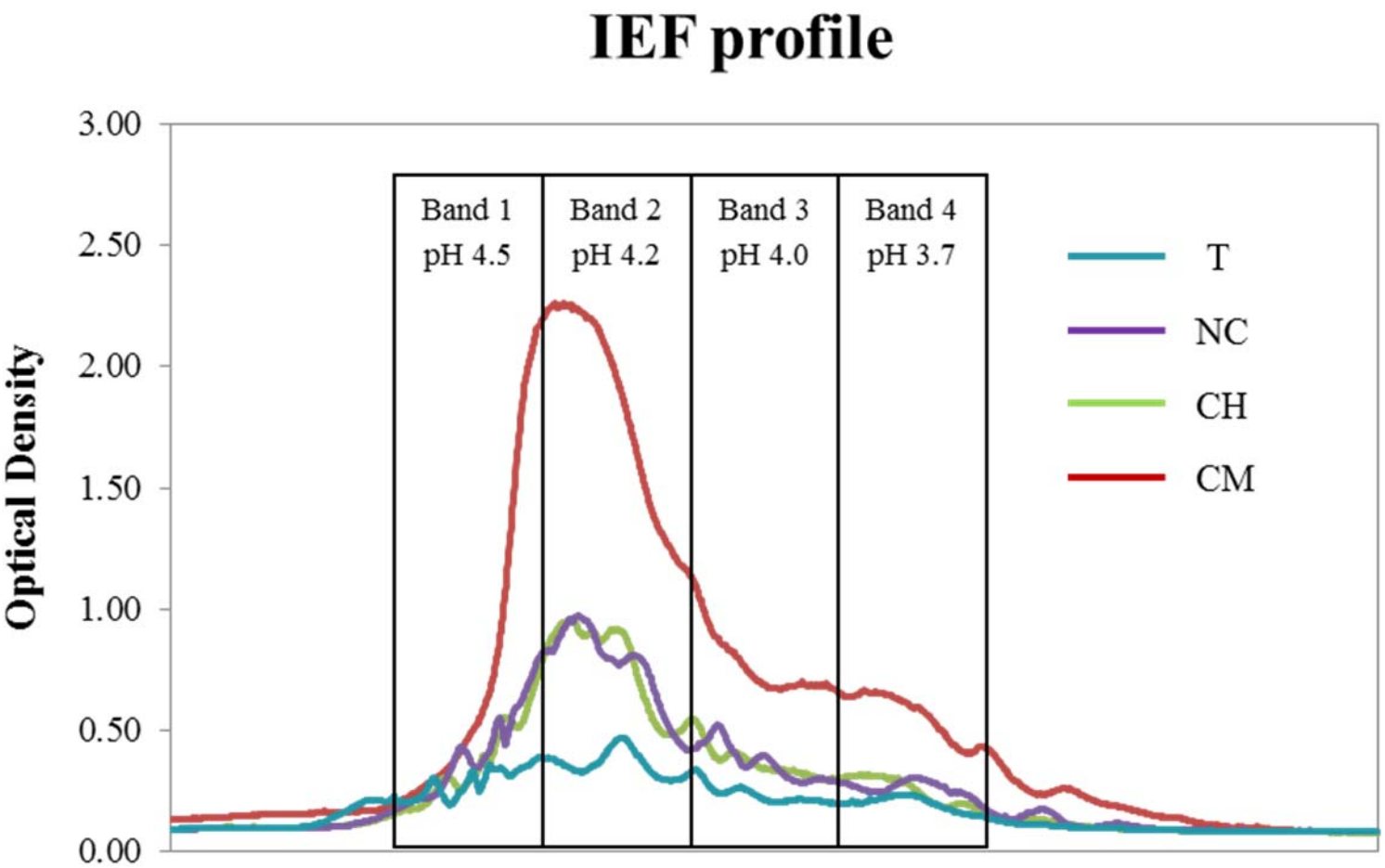
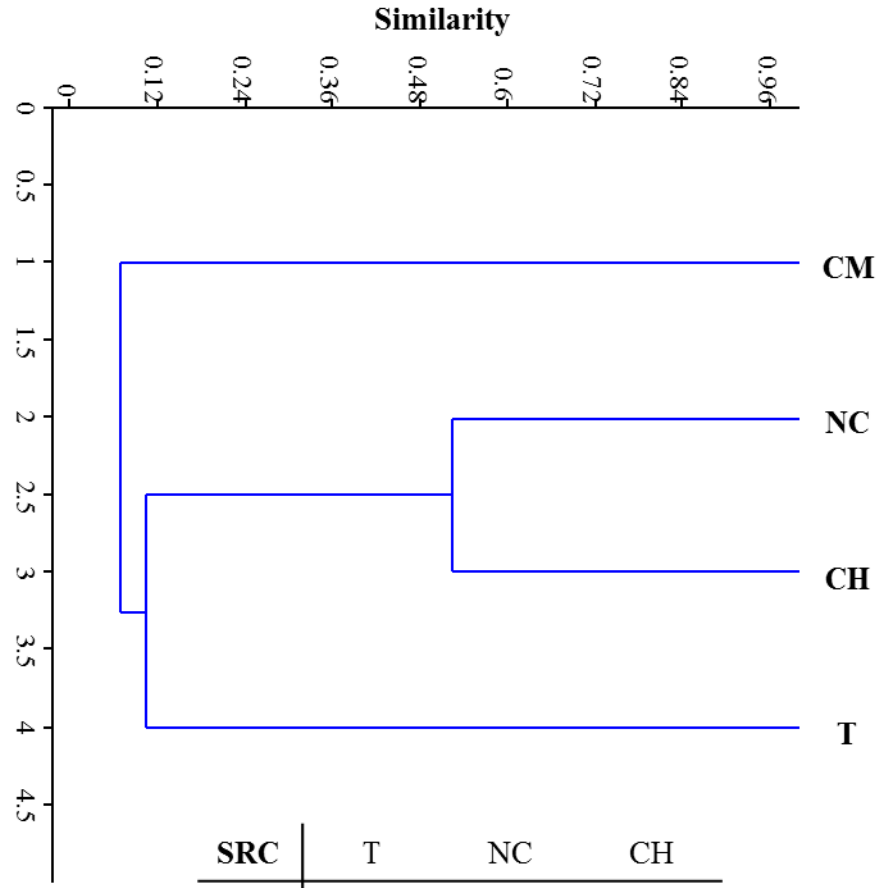

IEF profile
Optical Density
3.00
2.50
2.00
1.50
1.00
0.50
0.00
Band 1
pH 4.5
Band 2
pH 4.2
Band 3
pH 4.0
Band 4
pH 3.7
T
NC
CH
CM

Figure 2(on next page)

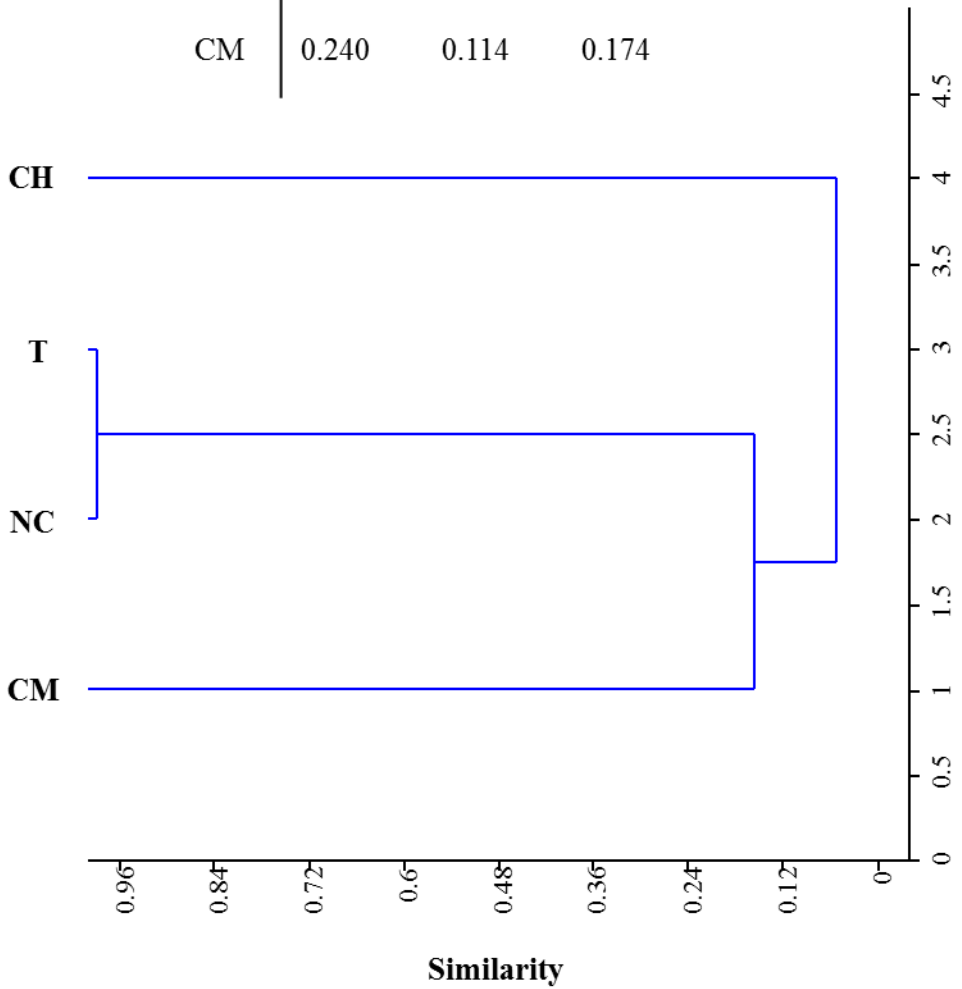
Figure 2

Fig 2 - Raup and Crick probability-based index of similarity cluster analyses and similarity values (S_{RC}) between samples for profiles of DNA- (a) and RNA- based DGGE analysis of LCMO-encoding genes (b) for tillage (T), non-tillage and no-cover (NC), cover vegetation + herbicides (CH), and cover vegetation + mower (CM) soils

a)



SRC	T	NC	CH
NC	0.046		
CH	0.162	0.594	
CM	0.055	0.073	0.059

b)

SRC	T	NC	CH
NC	0.986		
CH	0.011	0.003	
CM	0.240	0.114	0.174