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

Beatriz Moreno, Emilio Benitez

Background . Laccases, mostly laccase-like multicopper oxidases (LMCO), are probably the most common ligninolytic enzymes in soil. Although, in recent studies, laccase-encoding genes have been successfully used as molecular markers in order to elucidate the role of bacteria in soil organic C cycling , further research in this field is necessary . In this study, using rainfed olive farming as an experimental model, we determined the stability and accumulation levels of humic substances and appliedthese 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) of DNA/RNA 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, showed the highest humic acid levels as well as the largest bacterial population, rich in laccase genes and transcripts after more than 30 years of experiments. 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 DGGEanalysis, 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 different laccase substrates derived from vegetation cover decay when herbicides are used.

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.

1   IMPACT OF AGRICULTURAL MANAGEMENT ON BACTERIAL LACCASE-ENCODING GENES, WITH

2   POSSIBLE IMPLICATIONS FOR SOIL CARBON STORAGE IN SEMI-ARID MEDITERRANEAN OLIVE

3   FARMING

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10   **ABSTRACT**

11

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13   most common ligninolytic enzymes in soil. Although, in recent studies, laccase-encoding genes

14   have been successfully used as molecular markers in order to elucidate the role of bacteria in soil

15   organic C cycling, further research in this field is necessary. In this study, using rainfed olive

16   farming as an experimental model, we determined the stability and accumulation levels of humic

17   substances and applied these data to bacterial laccase-encoding gene expression and diversity in

18   soils under four different agricultural management systems (bare soils under tillage/no tillage and

19   vegetation cover under chemical/mechanical management).

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23   like multicopper oxidase (LMCO)-encoding genes and transcripts were also carried out.

24   **Results.** Soils under spontaneous vegetation, eliminated in springtime using mechanical

25   methods, showed the highest humic acid levels as well as the largest bacterial population, rich in

26   laccase genes and transcripts after more than 30 years of experiments. The structure of the

27   bacterial community based on LMCO genes also pointed to phylogenetic differences between

28   these soils due to the impact of different management systems. Soils where herbicides were used

29   to eliminate spontaneous vegetation once a year and those where pre-emergence herbicides

30   resulted in bare soils clustered together for DNA-based DGGE analysis, which indicated a

31   certain amount of microbial selection due to the application of herbicides. When LMCO-

32   encoding gene expression was studied, soils where cover vegetation was managed either with

33   herbicides or with mechanical methods showed less than 10% similarity, suggesting that the

34   different laccase substrates derived from vegetation cover decay when herbicides are used.

35   **Conclusions.** We suggest that the low humic acid content retrieved in the herbicide-treated soils

36   was mainly related to the type (due to vegetal cover specialization) and smaller quantity (due to

37   lower vegetal biomass levels) of phenolic substrates for laccase enzymes involved in

38   humification processes. We also found that spontaneous vegetal cover managed using

39   mechanical methods could be the best option for achieving C stabilization in rainfed

40   Mediterranean agroecosystems.

41

42   **INTRODUCTION**

43

44   Due to their importance for human welfare, ecosystem services have, in recent years, been

45   attracting increasing attention in the scientific literature. Although, traditionally, agroecosystems

46   have been primarily regarded as service providers, their contribution to other types of ecosystem

47   services have recently been seen as equally crucial (MEA, 2005). Apart from providing food,

48   forage and bioenergy, agricultural ecosystem services have other benefits such as water and

49   climate system regulation, soil fertility and C sequestration (Chen et al., 2016). The latter

50   involves the removal of C from the atmosphere and its storage in soils through physical and

51   biological processes. As land-based biological C-mitigation strategies are regarded as important

52   and viable pathways to climate stabilization (Canadell & Schulzel, 2014), it is crucial to evaluate

53   the effects of land-use changes on ecosystem functions. However, in semi-arid climates,

54   agricultural soils are particularly vulnerable due to adverse environmental conditions and

55   centuries-old farming systems (Metzger et al., 2006).

56   One of the principal agroecosystems in semi-arid Mediterranean areas is that used for perennial

57   olive-orchard cropping. Olive is the most extensively cultivated fruit crop in the world, with

58   more than 10 million hectares under cultivation in 2013 (FAOSTAT, 2015). Its economic and

59   social importance is enormous, as reflected in the increasing per capita consumption of olive oil

60   around the world. However, olive growing also has a considerable ecological impact in the

61   Mediterranean region, where olive orchards constitute a fundamental part of the landscape.

62   Nevertheless, these agroecosystems are particularly exposed to degradation because of low soil

63   organic C (SOC) content.

64   The possibility of increasing the C-storage capacity of soils, with no significant reduction in

65   yields, through the use of an appropriate soil management system, has been widely discussed

66   (Scholes, Palm & Hickman, 2014). A recent meta-analysis concluded that the most appropriate

67   strategy for maximizing SOC levels in the Mediterranean region is to combine organic inputs

68   with reduced tillage management (Aguilera et al., 2013). However, in rainfed olive farming,

69   SOC is mainly dependent on plant-derived C-breakdown (Castro et al., 2008; Moreno et al.,

70   2009). SOC decomposition is generally thought to be regulated by biological and environmental

71   conditions (Schmidt et al., 2011). Litter provides the carbon that supports microbial activity,

72   returns part of the C to the atmosphere and stores the rest in the form of humic substances, a

73   highly stable organic fraction considered to be the true agent responsible for soil C-sequestration

74   mechanisms (Miralles et al., 2014). There is overwhelming evidence which shows that C storage

75   in soils is mainly limited by the incorporation of new C rather than by the decomposition of

76   existing C and also shows the critical role played by the soil microbial community in these

77   processes (Lange et al., 2015). Thus, the microbial degradation of plant-waste lignin has been

78   recognized as a C turnover bottleneck in soil (Theuerl & Buscot, 2010; Torres et al., 2014). The

79   microbial breakdown of lignin has been well characterized, as have the enzymes involved,

80   including laccase, lignin peroxidase and manganese peroxidase. However, the capacity to

81   degrade lignin varies. By comparing the three classes of enzymes, Chen et al. (2011) have shown

82   that laccases exhibit the most abundant aromatic-aromatic molecular interactions as well as the

83   highest binding affinity. Laccases, generally laccase-like multicopper oxidases (LMCOs), whose

84   kinetics have been widely studied, are probably the most common class of ligninolytic enzymes

85   in soil. Although they have traditionally been regarded as fungal enzymes, there is mounting

86   evidence which shows that bacterial LMCOs make a major contribution to lignin degradation

87   and SOC turnover (Alexandre & Zhulin, 2000; Sinsabaug, 2010). Laccase-encoding genes have

88   been successfully used in recent studies as molecular markers for SOC cycling under many

89   different environmental conditions and demonstrate the known potential of bacteria to degrade

90   lignin (Baldrian & López-Mondéjar, 2014). However, more studies are needed to elucidate the

91   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.

114 Cover crop + herbicides (CH): Covered soils where weeds were left to grow each year to be

115 eliminated in spring with herbicide glyphosate.

116 Cover crops + mower (CM): Covered soils where weeds were left to grow each year to be

117 eliminated in spring with various passes of a chain mower.

118 Two samples were collected from the centre of each plot, using a modified soil-sample ring kit

119 (Eijkelkamp), which consists of a 20 cm-deep sampling cylinder specifically manufactured for

120 this purpose, and then bulked. To isolate the nucleic acids, subsamples of fresh soil were

121 immediately frozen in liquid nitrogen. The samples were stored at -80ºC until molecular analyses

122 were carried out.

123

124 **Humic substances and isoelectric focusing**

125

126 In this study, humic C was measured in sodium pyrophosphate extract (pH 7.1), a good

127 extractant of humic compounds (Nannipieri et al., 1974; Piccolo et al., 1998). Humic substances

128 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

129 soil:extract ratio (Ceccanti et al., 1978). The suspension was then centrifuged at 12,400 g and

130 filtered through a 0.22 μm Millipore membrane. The $>10^4$ Da humic-derived fraction was

131 obtained through ultrafiltration on the AMICON PM10 membrane of the extract. The C content

132 of humic C ($>10^4$ Da) was determined by acid digestion with 0.17 M $K_2Cr_2O_7$ and concentrated

133 (97%) H_2SO_4 at 140 °C for 2 h. A spectrophotometric method was used to quantify the Cr^{3+}

134 produced by the reduction of Cr^{6+} (λ=590 nm; Yeomans & Bremner, 1988).

135 Isoelectric focusing (IEF) of humic C ($>10^4$ Da) was carried out in cylindrical gel rods (0.5×8

136 cm) containing polyacrylamide gel (5% w/v) and carrier ampholines in the pH 4–6 range (Bio-

137   Rad Laboratories, Richmond, CA, USA) at a final concentration of 2% (Ceccantiet al., 1986;

138   1989). N,N,N′,N′-Tetramethyl-1,2-diaminomethane and ammoniumperoxy-disulfate were also

139   added in a gel solution at 0.03%. At the top of the gel rod (cathode), 100 µl of humic C ($>10^4$

140   Da) at 4.4% of glycerine were applied. A small amount (5 µl) of glycerine at 2.2% was added to

141   the top of the sample to avoid mixing with the cathodic solution (NaOH 0.02N); 0.01 M H_3PO_4

142   was used for the anodic cell. A pre-run of 1 h at the same current intensity and voltage used for

143   the samples run was carried out for each gel tube (1.5 mA for each tube, 100–800 V); the

144   samples run was then carried out for 2 h or longer until stable IEF banding was reached. The

145   electrophoretic bands were scanned using a Bio-Rad GS 80 densitometer, giving a typical IEF

146   profile for each soil investigated. The IEF peak area was determined for each soil IEF profile,

147   assuming as 100% the area under the entire IEF profiles (representative of the total loaded C).

148   Gel pH was measured at 0.5 cm intervals with an Orion microprocessor (model 901, Orion

149   research) connected to a microelectrode gel-pHiler (Bio-Rad Laboratories, Richmond, CA,

150   USA).

151

152   **Aromatic Hydrocarbons**

153

154   Aromatic hydrocarbons were extracted ultrasonically from 5 g of freeze-dried soils using 10 ml

155   of hexane/acetone (1:1 v/v). 50 µl of C3-naphthalene in dichloromethane was added as a GC

156   internal standard. After centrifugation and concentration, the solvent was dried by passing it over

157   anhydrous sodium sulfate. The aromatic hydrocarbons were analyzed using a large volume

158   injection-gas chromatography mass spectrometry (LVI–GC–MS) method. GC analyses were

159   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 $0.9\,\mathrm{ml\,min^{-1}}$. 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**

174

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 ml 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 × $\log_{10}$ (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

PCR-DGGE analyses

The community structure of bacterial LMCO-encoding genes and transcripts was subjected to DGGE analysis, for which Cu1AF-DGGE and Cu2R primers were used. Primer Cu1AF-DGGE contained the same sequence as Cu1AF but with an additional 40-nucleotide GC-rich sequence (GC clamp) at the 5′ end of the primer.

DGGE analyses were conducted using 550 ng of the final PCR product loaded onto a 45-60 % urea-formamide–polyacrylamide gel. An INGENYphorU System (Ingeny International BV, The Netherlands) was run at 200 V for 10 min followed by 75 V for 18 h at 58 ºC in order to separate the fragments. Gels were silver stained with the Bio-Rad Silver Stain according to the standard DNA-staining protocol and photographed under UV light (λ = 254 nm) using a UVItec Gel Documentation system (UVitec Limited, Cambridge, UK).

Data analyses

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. P-values of under 0.05 were considered to be evidence of statistical significance.

Band patterns in different DGGE lanes were compared with the aid of UVImap Analysis software (UVItec Limited, Cambridge, UK). DGGE banding data were used to estimate the Raup and Crick probability-based index of similarity (SRC) (1979). Raup and Crick's probability method was used to determine whether similarities within and between samples were stronger or

252 weaker than the randomized prediction. For this purpose, band-matching data were stored as a

253 binary matrix and analyzed using the aforementioned probability-based index. The SRC index

254 measures the probability of the randomized similarity being greater than or equal to the similarity

255 actually observed; SRC values of over 0.95 or under 0.05 indicate similarity or dissimilarity,

256 respectively, which are not random assortments of the same species (bands or OTUs) (Rowan et

257 al., 2003). SRC and cluster analyses were carried out using the PAST (Paleontological Statistics)

258 software program version 1.82b (Hammer, Harper & Ryan, 2001).

259

260 **RESULTS**

261

262 **Organic Carbon Fractions**

263

264 We found that the high-molecular-weight (C $>10^4$ Da) humic acid (HA) fractions behaved in a

265 significantly different way from total SOC (Table 1). HA levels were much higher in CM,

266 comparable in CH and NC and considerably lower in the T treatment. Figure 1 shows the IEF

267 patterns of HAs. All the treatments showed the highest optical-density values (IEF peaks) in the

268 pH range 4.5–4.2 (band 1 and band 2).

269

270 **Aromatic hydrocarbons**

271

272 The results of the analysis of major aromatic hydrocarbons are shown in Table 2. Three main

273 polycyclic aromatic hydrocarbons were detected in soil samples. 2,6-diisopropylnaphthalene

274 (m/z=239+199) was found to be present in all soils, although values were close to the detection

275   limit in the CM treatment. A similar trend was observed for pp-DDA, a polar metabolite of DDT,

276   with significantly higher levels being found in the NC treatment. The third compound, present

277   only in the NC and CH soils, was a mixture of four aromatic hydrocarbons (m/z=265+266+331),

278   whose mass spectra did not match any compounds in the library. To gain a better understanding

279   of this mixture, its mass spectra are shown as supplementary material (Supplemental Files: mass

280   spectra). The data appear to suggest that the C and D compounds, containing an odd number of

281   N atoms, are dimers of A and B, respectively.

282

283   **Real-time PCR assays and LMCO gene expression**

284

285   Table 3 shows LMCO-encoding gene and transcript-copy numbers in the four treatments, both of

286   which were much higher in CM soil and comparable in the other treatments.

287

288   **LMCO-encoding gene and transcript diversity**

289

290   Figure 2 shows the DNA- and RNA-based DGGE dendrograms generated by Raup and Crick

291   cluster analyses (see Supplemental Files: DGGE bands). The SRC values, obtained by

292   comparing the four treatments, are also summarized in Figure 2. With respect to LMCO-

293   encoding genes (Fig. 2a), T and NC soils differed significantly (SRC < 0.05). For the other pairs

294   of DNA-DGGE profiles compared, the similarity was no greater than that the random prediction

295   (0.95 > SRC > 0.05). However, SRC values comparing CM and the other treatments were very

296   close to the dissimilarity boundary (SRC < 0.05), as evidenced by the solitary cluster where these

297   soils were located.

298   When the LMCO transcripts were evaluated (Fig. 2b), T and NC soils clustered together,

299   showing a high degree of similarity (SRC ≈ 1). After comparing CM with the other soils, the

300   similarity was found to be no greater than that randomly predicted (0.95 > SRC > 0.05).

301   Both DNA- and RNA-based DGGE cluster analyses found significant similarities within replicas

302   (SRC > 0.95; data not shown).

303

304   **DISCUSSION**

305

306   In recent years, scientists have been discussing the relationship between agricultural management

307   practices and soil-C sequestration. The long-term impact of several types of agricultural

308   management on SOC dynamics has been extensively evaluated, some of which have been

309   proposed as a means of offsetting anthropogenic CO_2 emissions (Söderströmet al., 2014). In

310   particular, the advantage of including cover crops in cropping systems, as opposed to other

311   management practices that increase SOC, has been highlighted (Poeplau & Don, 2015).

312   Data on SOC dynamics obtained in our previous studies suggest that no absolute advantages in

313   terms of C sequestration appear to be associated with any of the four types of agricultural

314   management tested. A previous paper reported comparable amounts of aboveground vegetal

315   biomass per year in T, CH and CM treatments, as well as highly variable levels of annual

316   biomass production which is typically found in semi-arid Mediterranean habitats, while leaves

317   and root exudates from olive trees were the only C biomass input in the NC soil (see C vegetal

318   biomass in Table 1 from Castro et al., 2008). SOC, measured after 30 years of experiments, was

319   also shown to be significantly lower in NC as compared to the T and CM treatments, though

320   similar (p < 0.05) to the levels found in CH soils. However, no significant differences were

321   observed between the former and latter treatments (SOC in Table 1 in Cañizares, Moreno &

322   Benitez, 2012). Nevertheless, the outcomes for HAs have differed significantly, given that C-

323   sequestration mechanisms are primarily based on the stabilization of $C>10^4$ Da (Miralles et al.,

324   2014). HA levels were much higher in CM soils, where spontaneous vegetation was allowed to

325   grow each year and then eliminated in spring using mechanical methods. The IEF profiles

326   confirm the stability levels of the humic substances isolated: larger quantities of organic C

327   compounds were found at higher pH values (4.5–4.2) in the IEF gel, thus providing

328   overwhelming evidence that the higher the isoelectric point, the more humified the organic

329   matter (Ceccanti & Nannipieri, 1979; Govi, Ciavatta & Gessa, 1994).

330   These results provide supporting evidence that reduced tillage and green manure incorporation

331   could represent the best option for C input and stabilization in the Mediterranean region (Alvaro-

332   Fuentes et al., 2009; Aguilera et al., 2013; Garcia-Ruiz & Gomez-Muñoz, 2014; Garcia-Franco

333   et al., 2015). This has usually been explained by the greater physical-chemical protection of the

334   organic matter by microaggregates that this type of management provides. Tillage disrupts a

335   larger proportion of aggregates, thus releasing higher levels of organic C as compared to no-

336   tillage treatments (Six, Elliott & Paustian, 1999), which could explain why the T treatment

337   exhibited the lowest HA levels. However, much of the current debate still revolves around SOC

338   stabilization mechanisms, with existing evidence pointing to the synthesis of polyphenols as an

339   essential step in HA formation. Polyphenols could be derived from lignin-degradation products

340   or from microbial resynthesis, reflecting the importance of the specific type of vegetation and the

341   critical role played by microbial ligninolytic enzymes in the soil, specifically phenoloxidase,

342   laccase and peroxidase activities (Stevenson, 1994). Laccases are probably the largest class of

343   ligninolytic enzymes in soil and perform different oxidative and polymerative functions. The

344   enzymes of the former group are mainly involved in lignin breakdown, while the latter are

345   chiefly involved in polymerizing soluble phenols, thereby contributing to humification

346   (Gianfreda, Xu & Bollag, 1999). The data gathered in the present study suggest that there is a

347   relationship between the number and expression of bacterial LMCO genes on the one hand and

348   the quantity and stability of HAs on the other. Soils under vegetal cover managed by mechanical

349   methods, where the highest HA levels were retrieved after 30 years of experiments, showed the

350   largest bacterial population rich in laccase genes. Also, the environmental conditions facilitated a

351   corresponding higher level of gene expression in these soils as compared to other treatments. The

352   structure of the bacterial community based on LMCO genes also points to a phylogenetic

353   difference in CM soils due to the management system used.CH and NC soils clustered together

354   in the DNA-based DGGE analysis, suggesting a certain amount of microbial selection due to the

355   application of herbicides. With respect to LMCO-encoding gene expression, CH and CM soils

356   showed low similarity (<10%), indicating how different laccase substrates derived from

357   vegetation cover decay when herbicides are used. Indeed, differences in weed community

358   composition and the weed seed bank have previously been highlighted following long-term

359   applications of preemergence herbicides as compared to non-chemical weed control management

360   (Sosnoskie, Herms & Cardina, 2006; Barroso et al., 2015; Fracchiolla et al., 2015).

361   However, the results above did not provide confirmatory evidence concerning the role played by

362   bacterial laccase in HA formation. Laccase substrates are quite diverse and extracellular bacterial

363   laccase can oxidize other compounds such as dyes, pesticides and polycyclic aromatic

364   hydrocarbons (Majeau, Brar & Tyagi, 2010). Nevertheless, in this study, almost all predominant

365   aromatic hydrocarbon and herbicide metabolites were retrieved when herbicides were part of the

366 agricultural management system; neither the type nor amount correlated with LMCO gene

367 copies, transcript numbers or diversity.

368 Bringing together the above results, we found that management systems involving the

369 application of herbicides could influence bacterial laccase activity and diversity either directly

370 through their effect on soil microorganisms or indirectly through vegetal cover specialization.

371 The literature provides considerable evidence to show that different types of lignin phenols are

372 found in specific types of vegetation (Radosevich, Holt & Ghersa, 1997; Caseley, Cussans &

373 Atkin, 2013), although there is no consensus on the effects of herbicides on microbial

374 community structure and activity in soil (Jacobsen & Hjelmsø, 2014). Many authors have

375 described the side-effects of glyphosate on microorganisms as of "little ecological significance"

376 (Wardle & Parkinson, 1990), while others have contended that it has a considerable ecological

377 impact (Nannipieriet al., 2003; Zobioleet al., 2011). In this long-term experiment, the claim that

378 herbicides have no significant effect on soil bacteria after 30 years of herbicide applications is

379 supported by previous data; soils under vegetation cover showed similar bacterial biomass and

380 activity levels and characteristics whether spontaneous weeds were eliminated by mechanical or

381 by chemical methods (Cañizares, Moreno & Benitez, 2012). However, apart from a different

382 plant community pattern, herbicides could produce a collateral effect on certain bacterial

383 activities related to the soil C cycle. Weed biomass and leaf/root exudates from olive trees were

384 the sole C biomass input (Moreno et al., 2013), while litter-derived C was incorporated into both

385 more and less chemically stable compounds. However, after 30 years of experiments, we

386 detected an unexpected lower humification rate in CH as compared to CM soils. After reviewing

387 the history of CH soils, we found that the herbicides diquat and paraquat had been applied over

388 the years until their prohibition (Moreno et al., 2009) and were later replaced by glyphosate. On

389 the basis of their mode of action, bipyridylium herbicides act by inhibiting photosynthesis

390 (Dodge, 1971), while glyphosate inhibits the formation of phenolic precursors necessary for the

391 production of lignin (Franz, Mao & Sikorski, 1997). We can thus assume that the lignin content

392 of weeds incorporated into the soil in both circumstances was lower or simply different from that

393 incorporated into the CM treatment, and it is also generally accepted that the synthesis of

394 polyphenols from lignin is a critical step in C stabilization. Moreover, CH soils, where

395 vegetation cover was eliminated once a year with herbicides, and NC soils, where weeds were

396 prevented from growing by the application of herbicides, presented comparable levels of HAs as

397 well as similar bacterial laccase gene-copy numbers, diversity and expression.

398

399 **CONCLUSIONS**

400

401 A long-term experiment comparing four types of agricultural management was conducted in a

402 semi-arid Mediterranean agroecosystem. After 30 years of experiments, we found comparable

403 stable levels of C in both non-tillage + no cover soils and soils under spontaneous vegetation

404 cover managed using chemical methods. We suggest that the lower humic acid content retrieved

405 in the herbicide-treated soils was mainly related to the type, due to vegetal cover specialization,

406 and smaller quantity, due to lower vegetal biomass levels, of phenolic substrates for laccase

407 enzymes involved in humification processes. We also conclude that spontaneous vegetation

408 cover managed with mechanical methods is the best option to achieve C stabilization.

409

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411

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416

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558

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
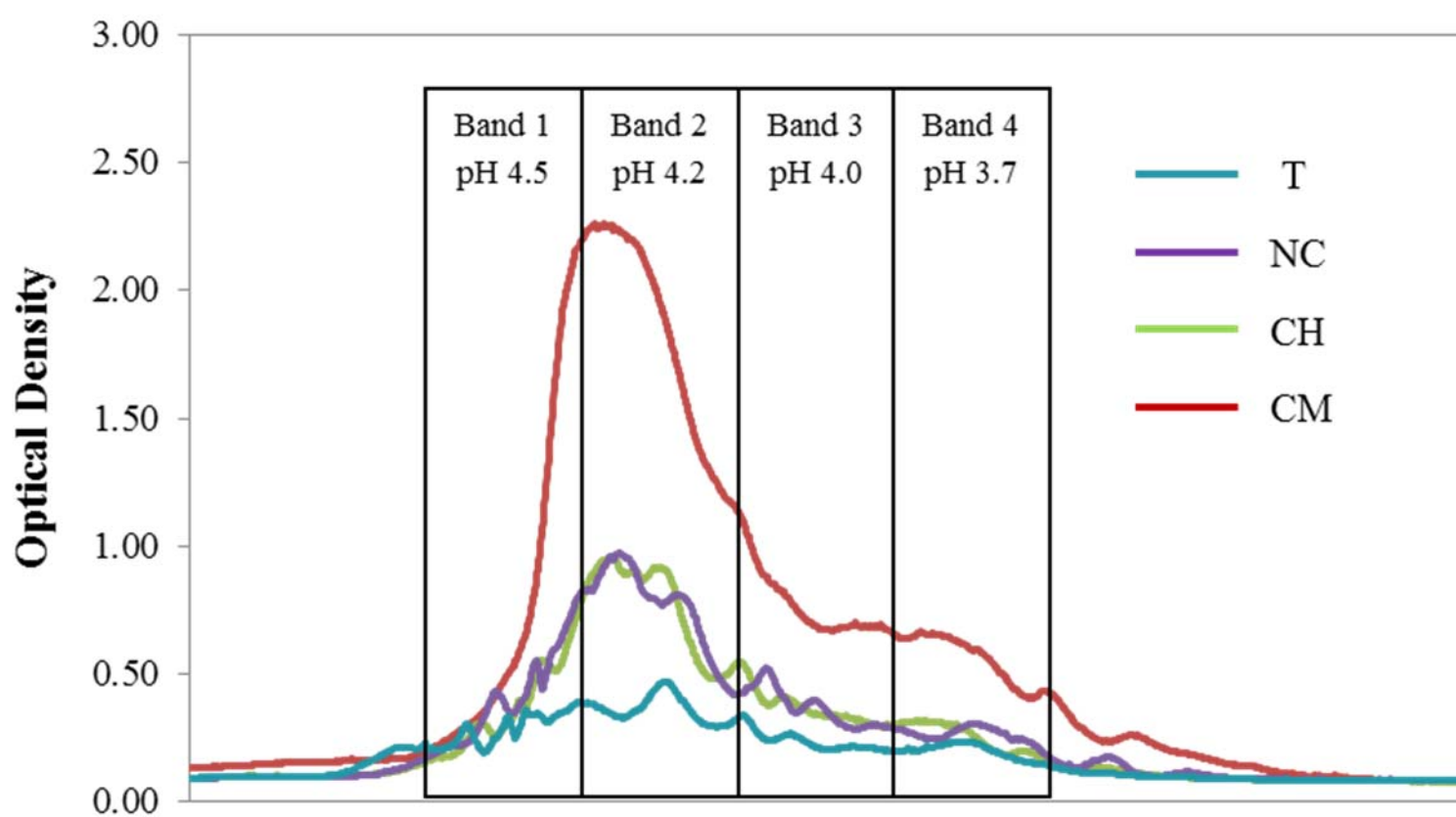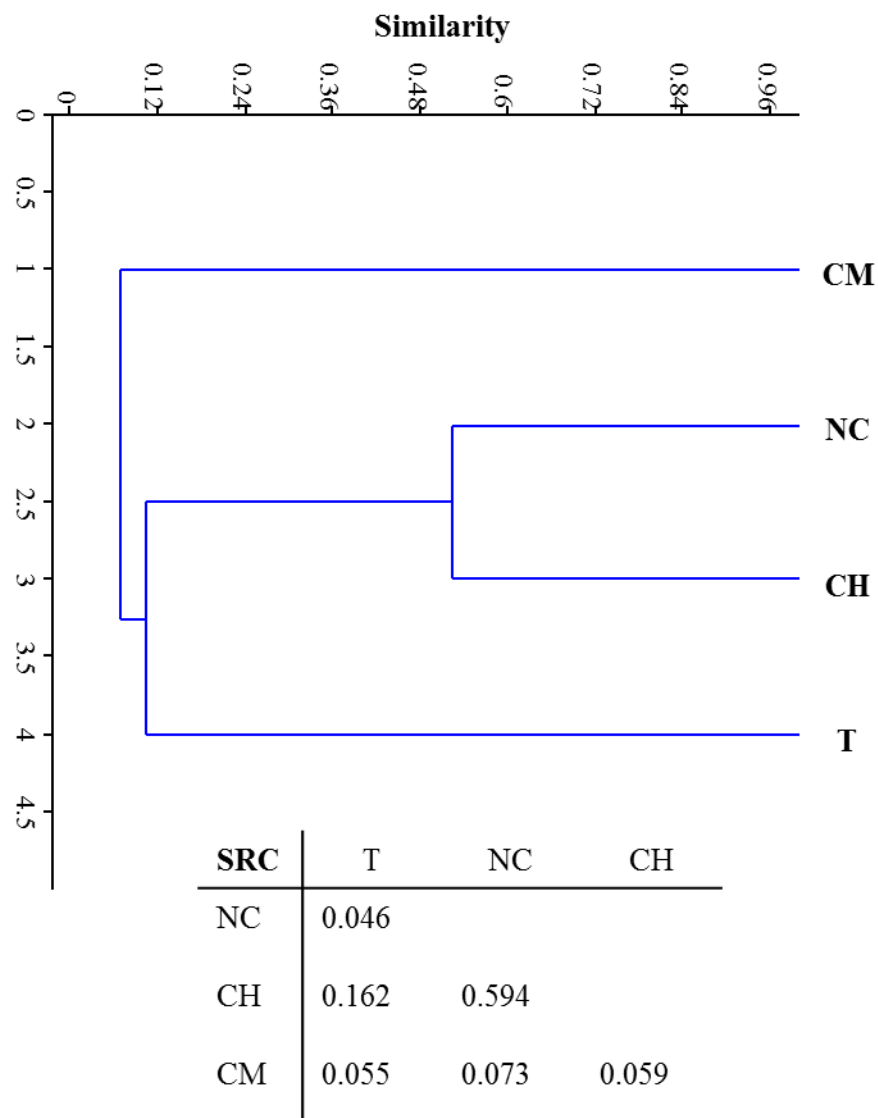
IEF profile

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