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1 Exopolysaccharides in the rhizosphere: A comparative study of extraction methods.
2 Application to their quantification in Mediterranean soils

3
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10

11 **Keywords**

12 Extracellular polymeric substance (EPS); Exopolysaccharide (EPSac); EPS extraction method;
13 Cation Exchange Resin (CER); mid-infrared spectroscopy (MIR); Rhizosphere

14 **Definitions:**

15 EPS = Extracellular Polymeric Substances

16 EPSac = Exopolysaccharides

17

18 **Abstract**

19 Quantifying and characterizing Extracellular Polymeric Substances (EPS) and especially
20 exopolysaccharides (EPSac) is an issue for understanding the hydro-physical and biological
21 functioning of the rhizosphere. However, few comparative studies of extraction techniques
22 have been carried out on soils and none on calcareous Mediterranean soils. Three soil-
23 bound EPS extraction techniques, i.e. Cation Exchange Resin (CER), EDTA and
24 NaOH+Formaldehyde (NaOH+F) were compared on three contrasted Mediterranean soils.
25 CER presented the lowest extraction efficiency of EPSac, but also the lowest contamination
26 of EPS by extractants and extracellular compounds. Contamination with intracellular
27 compounds was low and similar with the three methods. Mid-Infrared (MIR) spectra enabled
28 the best discrimination of the EPS extracts when they were prepared with CER. CER is then
29 identified to be the suitable extraction technique of EPS (including EPSac) from soils,
30 including calcareous soils. This technique was applied on rhizospheric and bulk soils
31 harvested in an experimental field of tomato cultivation. A rhizospheric effect was

32 highlighted during the growth of plants of two cultivars with both the soil-bound EPSac
33 amounts (total sugar equivalent of extracted EPS) and the MIR spectra of extracted EPS.
34 Extraction of soil-bound EPS and their further analysis by spectral and chemometric
35 approaches is a promising way for relating EPS chemical characteristics to their biological
36 and hydric impacts within the plant rhizosphere in a context of agro-ecological transition and
37 climatic change.

38

39 **1. Introduction**

40 Rhizosphere is the thin soil zone around plant roots, in which roots, soil and associated
41 microorganisms highly interact. It is a hot spot for biological activity and metabolic processes
42 (Chakraborty et al., 2012). Regarding soil-plant hydric relations, the properties of the
43 rhizosphere are critical because all the water transpired by the plant has to go through this
44 thin soil zone (Bengough et al., 2012). One of the specificities of the rhizosphere is the
45 dynamics of organic substrates, driven by exudation by roots and consumption/excretion by
46 microorganisms. Among these organic compounds produced by roots, but also by the
47 microorganisms, Extracellular Polymeric Substances (EPS) and especially exopolysaccharides
48 (EPSac) are biopolymers that occur as hydrogels (McCully and Boyer, 1997). EPSac adsorb on
49 soil particles and bind them together (depending on the available cations and the type of
50 EPSac), participating in the soil aggregation (Crouzet et al., 2019), and modifying the local
51 soil porosity and the pore size distribution (Czarnes et al., 2000). Recent studies have shown
52 in a variety of edaphic environments (e.g. in soil biocrusts, Rossi et al., 2018; and in
53 rhizosphere, Ahmed et al., 2016; Czarnes et al., 2000) that EPSac can exhibit contrasted
54 hydric properties: They are both water-retentive and hydrophilic, when wet, but become
55 hydrophobic when they lose their water in a dry environment. EPSac are recognized for their
56 role in protecting microorganisms from water stress (Bérard et al., 2015) and in maintaining
57 the continuity of the liquid phase between soil aggregates, which probably favours water
58 and nutrient extraction by plants from drying soils (Benard et al., 2019).

59 The EPSac mostly involved in these soil properties are those tightly bound to the soil
60 particles (Chen et al., 2014). Moreover, compared to loosely-bound EPS, the soil-bound EPS
61 fraction seems more « preserved » from exo-enzymatic activity, more condensed (Rossi et
62 al. 2018) and less soluble and thus less dependent on the hydric conditions prevailing at the
63 site during or just before the sampling (Redmile-Gordon et al., 2014; 2020).

64 Quantifying and characterizing these complex polymer molecules are therefore an issue for
65 understanding the hydro-physical and biological functioning of the rhizosphere, especially
66 under soil water deficit conditions, which are increasingly encountered in Mediterranean
67 agricultural areas (Jia et al., 2019). This necessitates above all an efficient method for
68 extracting particle-bound EPS from soils, but very few comparison studies have been
69 devoted to soils (Redmile-Gordon et al., 2014; Wang et al., 2019) and none, to the best of
70 our knowledge, were tested on Mediterranean calcareous soils and on contrasted texture
71 soils.

72 Various EPS extraction techniques have been performed or compared on various media,
73 such as: activated sludge, sediments, photosynthetic biofilms and microbial crust. In
74 particular, two techniques are widely used for these environmental samples: a physico-
75 chemical Cation Exchange Resin (CER) technique (Frolund et al., 1996; Gerbersdorf et al.,
76 2005; Redmile-Gordon et al., 2014) and a chemical EDTA technique (Rossi et al., 2018;
77 Underwood et al., 1995). A third chemical extraction technique makes use of NaOH (Liu et
78 Fang, 2002). This last technique is more drastic and is known for its effectiveness in
79 extracting organic materials. These three techniques were simultaneously compared on
80 activated sludge only (Comte et al., 2006, Liu and Fang 2002). However, to our knowledge,
81 the only two comparison studies for soil-EPS extraction (Redmile-Gordon et al., 2014; Wang
82 et al., 2019) did not include both CER and EDTA. The first objective of our study was
83 therefore to compare these three EPS extraction methods by using three different
84 Mediterranean soils contrasting in their texture, organic matter and vegetation cover, as
85 texture and CEC, level of carbonates and organic matter may interfere in the efficiency of
86 the extraction. In particular we look at the method offering the best compromise in
87 extracting enough tightly-bound EPS (especially tightly-bound EPSac) for further chemical
88 analysis, while limiting contamination by the extractants and other products resulting from
89 microbial cell lysis, or co-extracted from soil like humic substances widely present in soils
90 (Comte et al., 2007; Redmile-Gordon et al., 2014). The second objective of this work was to
91 apply the chosen extraction method (after the first step of comparing techniques) to soil
92 samples originating from a field context (Mediterranean tomato cultivation), in order to
93 assess whether this technique allowed to highlight rhizospheric effects when comparing EPS
94 measurements of rhizospheric soils and adjacent bulk soils.

95

96 **2. Material and methods**

97 2.1. Comparison of extraction methods

98 2.1.1 Investigated soil samples

99 For the comparison of extraction methods, three Mediterranean soils were selected on the
100 basis of their contrasted texture, organic C, total N, CEC (Cation Exchange Capacity) and
101 WHC (Water Holding Capacity) (Table 1). These are a sandy clay loam from an irrigated
102 permanent grassland ("Crau", "C"), a silty clay loam soil from a conventional agricultural field
103 ("Lysi", "L") and a sandy soil from a pine forest ("Tavel", "T"). The bulk soils were
104 collected from the upper 20 cm (after removing the top litter), air dried and sieved (0–2
105 mm), and then stored at laboratory temperature before use. Water Holding Capacity (WHC,
106 gravimetric water content), Water Stable Aggregate percentage (WSA, laboratory wet-
107 sieving method) and Microbial Biomass (MB, Glucose-Induced Respiration) measurements
108 were performed following Seybold and Herrick (2001) and Ben Sassi et al. (2012). All other
109 soil analyses were performed by a laboratory dedicated to soil analysis (LAS-INRAe
110 <https://www6.hautsdefrance.inra.fr/las>) according to AFNOR norms. Six sample replications
111 were performed for each of the three soils and each of the three extraction methods (six
112 replicates per extraction technique and per soil, 54 total of soil samples).

113 2.1.2. EPS extraction methods

114 For each EPS extraction method, the same amount of 0.5 g of soil (Dry Weight DW) was
115 used. Before soil-bound EPS extraction, loosely bound EPS were first using 5 mL CaCl_2 10^{-2} M
116 (with salts of equal valence to that of the EPS binding sites, thus maintaining the stability of
117 the bound-EPS prior to the further extraction, Redmile-Gordon et al., 2014; 2020) under
118 rotary agitation (50 rpm, HEIDOLPH REAX2 agitator, Germany) for one hour at room
119 temperature. The soil/ CaCl_2 mixtures were then centrifugated (8000 x g for 15 min at 10°C),
120 and the supernatants were stored at -20°C for further cell lysis controls (section 2.2.4). The
121 remaining centrifuge pellets were then used to compare the three bound-EPS extraction
122 methods.

123 Cation Exchange Resin (CER) extraction

124 In this method, the mechanical action of beads onto soil particles combined with the resin
125 exchange of the divalent cation Ca^{2+} and Mg^{2+} of soil that bind the polymer chains of EPS to
126 soil particles result in the release of EPS into the solution.-The CER (named "CER technique")
127 extraction was performed according to Frolund et al. (1996), Redmile-Gordon et al. (2014)

128 and Gerbersdorf et al. (2005). The CER (Dowex Marathon C, Na⁺ form, Sigma Aldrich,
129 Steinheim, Germany) was preliminary washed with a phosphate buffer (consisting of 2 mM
130 Na₃PO₄ · 12H₂O, 4 mM NaH₂PO₄ · H₂O, 9 mM NaCl, 1 mM KCl, adjusted to pH 7 with 1M HCl
131 and stored at 4 ° C) until the pH on the solution was stabilized to 7. The CER was added to
132 the centrifuge tube containing the pellet sample in order to obtain a ratio of 70 g dry CER
133 per 1 g organic matter (Table 1). Five mL of phosphate buffer pH 7 were then added to the
134 mixture. After manual stirring, the sample was incubated for 16 hours, for a more effective
135 extraction (Frolund et al., 1996 and Gerbersdorf et al. 2005), under rotary agitation (50 rpm,
136 agitator (HEIDOLPH REAX2, Germany) at room temperature.

137 EDTA extraction

138 EDTA is a chelating agent that sequester the ions (Mg²⁺ and Ca²⁺) that link EPS to soil
139 particles and then releases EPS into the solution. The EDTA (named “EDTA technique”)
140 extraction was performed according to Underwood et al. (1995) and Rossi et al. (2018).
141 25mL of EDTA (0.1 M) were added to the centrifuge tube containing the soil pellet sample.
142 After manual stirring, the sample was incubated for 16 hours overnight, as for CER
143 extraction, under rotary agitation (50 rpm, agitator (HEIDOLPH REAX2, Germany) at room
144 temperature (Chen et al., 2014).

145 NaOH+Formaldehyde extraction

146 The sodium hydroxide (NaOH) sharply increases the soil solution pH, which ionizes the
147 functional groups (e.g. carboxylic group) and causes a strong repulsion between the EPS and
148 the soil particles. This extraction method uses a preliminary incubation of soil with
149 Formaldehyde to limit the microbial cell lysis caused by NaOH (More et al., 2014). The
150 NaOH+Formaldehyde (named “NaOH technique”) extraction was performed following
151 Comte et al. (2007), Felz et al. (2016) and Liu and Fang (2002). 20 mL of distilled water with
152 0.3 mL of formaldehyde (37%) were added to the centrifuge tube containing the soil pellet
153 sample, the tube was incubated at 4°C for 1 h. The NaOH was then added (8 mL 1M NaOH)
154 and the mixture was then incubated for a further 3 h at 4°C.

155 All samples from each extraction method were subsequently centrifuged at 8000 g for 15
156 min (10°C), and all supernatants were dialysed (12kD Dialysis tubing cellulose membrane -
157 Sigma Aldrich, Germany - against NaCl 0.1M for 24h to remove extractant residues and then
158 against ultrapure water for a further 48h to remove NaCl, Renard and Giniès, 2009) to
159 remove the extractant residues that could interfere with subsequent analysis (as EDTA or

160 NaOH+F) and to purify the EPSac before analysis (Chen et al., 2014; Rossi et al. 2018).
161 Dialysis was applied for all samples to avoid any variations between the extraction methods.
162 The dialysed samples were weighed and divided into (i) aliquots of 200 μL (microtubes
163 stored at -20°C) for analyses of total sugars, proteins, 'Humic acid equivalent' (HAE), and for
164 measurements of cell lysis and (ii) an aliquot was freeze-dried for subsequent measurements
165 using Mid-Infrared Spectroscopy (MIR).
166 Another series of 6 independent extractions by each of the three techniques was carried out
167 on one of the three soils ("Lysi" soil, "L"). The obtained extracts were not dialysed in order to
168 compare them with dialysed extracts.

169 2.1.3. Analytical methods

170 Total sugars

171 Soil-bound Exopolysaccharides (EPSac) were measured as equivalent total sugars of soil-
172 bound EPS using the phenol-sulphuric acid assay with 200 μL of 5% phenol and 1 mL sulfuric
173 acid added to 200 μL of the extract and vortexed. Measurement was read after 30 minutes
174 (water bath 30°C) using a spectrophotometer (Biotek EL800, USA) at 490 nm with glucose as
175 a standard for the calibration curve (Dubois et al., 1956; Gerbersdorf et al., 2005).

176 Protein and 'Humic Acid Equivalent' (HAE)

177 Protein and HAE content of soil-bound EPS were measured following Frolund et al. (1995)
178 and Redmile-Gordon et al. (2013), using the modified Lowry method, with bovine serum
179 albumin (Sigma A-6003) and humic acid (Sigma 1675-2) as the respective standards.

180 Mid-Infrared Spectroscopy (MIR) measurements.

181 MIR spectra of the soil-bound EPS were collected on freeze-dried aliquots of EPS extracts
182 with a Tensor 27 FTIR spectrometer (Bruker Optics, Wissembourg, France) equipped with a
183 single-reflectance horizontal ATR cell (Golden Gate equipped with a one internal reflection
184 diamond crystal, Bruker Optics). The freeze-dried homogenized samples of soil extracts were
185 placed at the surface of the diamond crystal and were pressed with a system press tip flap
186 (Bureau et al. 2012). The samples were scanned between 4000 to 600 cm^{-1} and each
187 spectrum was obtained by averaging 16 successive scans to have a good ratio of signal to
188 noise. For each sample, four replications on different aliquots were made to integrate the
189 sample heterogeneity. These analytical replicates were averaged. The spectra of the
190 extractant solutions used in the three techniques as well as non-dialysed samples from the
191 "Lysi" soil (L) were also measured.

192 2.1.4. Cell lysis estimation

193 Given the very different extraction solutions that were used (in particular a very alkaline
194 medium with NaOH+F), two complementary methods were applied to estimate cell lysis: the
195 amounts of DNA (Liu and Fang, 2002) and the activity of the intracellular enzyme G6PDH
196 (Glucose-6-Phosphate Dehydrogenase) of the samples which were compared before and
197 after application of each extraction technique (Caudan et al., 2012) (three to four
198 replications).

199 After adding RNase to the CaCl₂ pre-extracted samples and to the soil-bound EPS extracts,
200 the DNA was precipitated with 2.5 vol of -20°C absolute ethanol. The sample was then
201 centrifuged (10 000 g, 20 min, 4 °C). This step was repeated twice. The resulting pellet was
202 dried and solubilized in pure water. The amount of DNA was measured by reading the
203 absorbance at 260 nm using NanoQuant plate and microplate reader (Infinite 200 Pro;
204 Tecan).

205 The activity of the intracellular G6PDH was measured using a colorimetric method in CaCl₂
206 pre-extracted samples and in soil-bound EPS CER and EDTA extracts using the Sigma KIT
207 MAK015 (Sigma-Aldrich Chimie, Lyon, France) at 37°C. Absorbance values (450 nm) were
208 recorded every 5 min with a microplate reader (Infinite 200 Pro; Tecan). Only CER and EDTA
209 techniques have been tested with G6PDH, NaOH+F being known to be incompatible with
210 this method of cell lysis evaluation (denaturation of the G6PDH protein).

211

212 2.2. Application of the optimal extraction method to rhizospheric and bulk soils

213 The CER extraction method was applied to an agronomic Mediterranean context, for soil
214 from an experimental site of an industrial tomato field near Avignon (France). Main soil
215 properties (“Piolenc”) are presented in Table 1. The cultural practices applied in this
216 experimental field are those from to the current conventional practices (fertilization,
217 pesticides and drip irrigation to compensate 100% replacement of evapotranspiration;
218 Castro Vilas Boas et al., 2017). The influence of two industry-type cultivars of *Solanum*
219 *lycopersicum* (H1015 and Terradou) on the soil were compared by sampling their
220 corresponding rhizospheric and bulk soil (5 subplots per H1015 cultivar and 4 subplots per
221 Terradou cultivar). Two soil sampling campaigns were conducted, in June (beginning of
222 flowering stage) and August 2018 (beginning of fructification stage). For each subplot, the
223 proximal root system of three representative plants per cultivar was excavated with a spade.

224 The plants were then shaken vigorously by hand, but without breaking roots, until no more
225 soil aggregates detached from roots (less than 5 minutes) (Barillot et al., 2013; Göttlein,
226 2006; Luster et al., 2009). Remaining aggregates still adhering to roots were then collected
227 as rhizosphere soil and the soil from the three plants was pooled. The corresponding bulk
228 soil was sampled in each subplot in the inter-row near the plants (depth 0-20 cm). The
229 collected soils were then air-dried and sieved (0–2 mm) before use (Fig. S2. A total of 36 soil
230 samples was analyzed.

231 Soil-bound EPS were extracted from the samples with the CER extraction technique detailed
232 in 2.1.2 section. The soil-bound EPS were quantified by their total sugars content (Dubois
233 method, EPSac) and by acquiring their MIR spectra, as described in section 2.1.3. Soil
234 microbial biomass was assessed with the MicroRespTM technique (Ben Sassi et al. 2012)
235 using a 96-deep-well microplate filled with soil subsamples (soil moisture preliminary
236 adjusted to 40% of WHC). 25 µl of glucose (6.7 mg g⁻¹ dw soil) was added in each deep-well.
237 The plates were then tightly covered with a colorimetric CO₂-trap microplate and incubated
238 in the dark (23 °C±1) for six hours. Absorbance was measured at 570 nm. A calibration curve
239 of absorbance *versus* headspace equilibrium CO₂ concentration (measured by gas
240 chromatography) was fitted to a regression model, which was used to compute the amounts
241 of released CO₂. Glucose-induced respiration was used as a proxy of active microbial
242 biomass (Anderson and Domsch, 1978).

243

244 2.3. Statistical analysis

245 Quantitative data were analysed using XLSTAT statistical software. Statistical differences
246 were considered at a level of significance of $p < 0.05$. Homoscedasticity of variances (Bartlett
247 test) and normality (Shapiro test) were tested before data treatments. Comparisons
248 between each extraction technique, or between each soil (EPSac, HAE, DNA, G6PDH), or
249 between EPSac amounts extracted from "Lysi" soil before and after dialysis, were made with
250 non-parametric tests (Kruskal-Wallis tests followed by a Dunn test and Mann-Whitney tests).
251 Data from field experiment were analysed with a two-way ANOVA followed by a pairwise
252 comparisons with Tukey post hoc tests, to test the influence of rhizosphere and cultivar
253 factors. Comparisons between rhizospheric and corresponding bulk soils were performed

254 with t test paired samples. Pearson correlation was performed to investigate links between
255 amounts of EPSac and microbial biomass in rhizospheric and bulk soils.

256 MIR Spectra pre-processing and data treatment were performed with Matlab 7.5
257 (Mathworks Inc., Natick, MA) software using the SAISIR package (Bertrand and Cordella,
258 2008). Before any data treatment, different pre-processing methods were compared:
259 baseline correction, standard normal variate correction (SNV), first and second
260 derivatives. The spectral range between 2000 and 700 cm^{-1} was used insofar as it was the
261 most discriminant area considered as the fingerprint region with intense specific bands of
262 polysaccharides (Ludwig et al., 2008). A Principal Component Analysis (PCA) was performed
263 on spectral data (2000 and 700 cm^{-1}) to preliminary eliminate outliers and qualitatively
264 discriminate the soil-bound EPS extracts, according to the tested extraction techniques and
265 different samples from the tomato field such as sampling date, rhizospheric and bulk soils,
266 and cultivars. A factorial discriminant analysis (FDA) was performed to test the possibility of
267 MIR to classify samples according to the known qualitative groups (rhizospheric/bulk soils,
268 cultivars). It was carried out in two steps: 1) The Principal Component Analysis (PCA) was
269 done on the spectral data to visualize the samples distribution according to the most
270 discriminating spectral ranges identified with the eigenvector display and 2) The FDA was
271 applied on the gravity centers of each qualitative group assessed on the normalized principal
272 component scores (Bertrand et al., 1990).

273

274 **3. Results**

275 3.1. Comparison of extraction methods

276 3.1.1. EPS analysis

277 The total sugar amounts of the soil-bound EPS (EPSac) were not significantly different
278 between the three extraction techniques, with an exception for the EPSac of "Crau" soil,
279 which were extracted in higher amounts by the NaOH+F technique than by CER technique
280 (Kruskal-Wallis test) (Fig.1). Whatever the extraction technique used, the extracted EPSac
281 amount decreased according to "Crau"> "Lysi"> "Tavel". All the three techniques allowed
282 the differentiation between "Crau" and "Tavel" soils, the soil "Lysi" showing intermediate
283 EPSac amounts (Kruskal-Wallis test, Fig.1). Concerning the two soils with the highest EPSac
284 amounts ("Crau" and "Lysi"), the CER technique exhibited the lowest variability, between
285 soils and within each soil type for the six repeated extractions (Fig.1).

286 The analysis of EPS extracted with the CER technique before or after dialysis resulted in
287 comparable amounts of EPSac, while those extracted with the EDTA technique showed
288 significantly higher EPSac amounts before dialysis than after (Mann-Whitney tests, Table 2).
289 Concerning the NaOH+F technique, the undialysed samples could not be analysed due to
290 interference between NaOH + formaldehyde and the reagents of the Dubois method
291 (preliminary measurements showed aberrant pink colour of the mixture, which was
292 probably caused by the strong alkaline effect of the NaOH).

293 Freeze-dried EPS from extracted soils were further processed with MIR spectroscopy, but
294 the only the "Crau" and "Lysi" soils were analysed because the amount of extracted EPS
295 from "Tavel" soil was too low.

296 The MIR spectra revealed differences in the chemical structures of the bound-EPS extracted
297 by the three techniques (Fig.2a). Especially for the CER technique whose MIR spectra of EPS
298 were located on the left on the PC1 axis (principal component) (76.4% variance) of the PCA
299 and were characterised by a high absorption at 1004 cm^{-1} (Fig. 2a-2, eigenvector analysis).
300 The MIR spectra of EPS extracted by EDTA discriminated the soils "Crau" and "Lysi" (along
301 PC2 axis: 14.8% of variance, Fig.2b) whereas the NaOH+F technique did not allow this
302 discrimination (Fig.2c). The discrimination was the most efficient with the CER technique
303 along the PC1 axis (84.6% of variance, Fig.2d). Moreover, with this last technique the EPS-
304 spectra of "Crau" were located and clumped on the left of PC1 and were characterized by a
305 high absorption at 1035 cm^{-1} (eigenvector analysis, Fig.S1). A PCA were also performed on
306 spectra of the non-dialysed EPS samples extracted by each technique from "Lysi" soil and of
307 the extractant solutions (Fig.S3). Based on their spectral data, dialysed and non-dialysed
308 "Lysi" EPS extracted by the EDTA and NaOH+F techniques were clearly discriminated on PC1
309 axis (more than 60% of variance), whereas no separation (at least on the PC1 axis for EDTA)
310 was observed between the blanks' spectra (i.e. the extractant solutions) and those from the
311 non-dialysed extracted samples. Concerning the CER technique, the dialysed and non-
312 dialysed "Lysi" samples were only separated on the PC2 axis (16% of variance) whereas the
313 extractant solution and the non-dialysed samples were separated along PC1 axis (78.8%
314 variance) (Fig.S3c).

315 No protein was detected in dialysed EPS samples (data not shown). The quantities of HAE
316 gave varying mean values according to the soil and the extraction technique, in particular
317 with the NaOH+F technique. With EDTA and CER techniques, a HAE quantitative gradient

318 was observed in the following increasing order "Crau"> "Lysi"> "Tavel", as previously seen
319 for EPSac amounts. Both the CER and EDTA techniques allowed the differentiation between
320 "Crau" and "Tavel" soils, the soil "Lysi" presenting intermediate EPS-HAE amounts (Kruskal-
321 Wallis test, $p < 0.05$). The CER technique resulted in lower and less variation of the amounts
322 of HAE extracted than EDTA extraction (significant for the "Crau" soil, Mann-Whitney test,
323 Table 2).

324 3.1.4. Cell lysis estimation

325 The amounts of DNA measured in the solution of extracted EPS were (inexplicably) highly
326 variable. Although it seemed that the mean quantities of DNA measured in the EPS extracted
327 by the three techniques were higher than those measured before the extractions, this
328 increase was not significant for the three methods (except for the DNA measured in the EPS
329 extracted by the NaOH+F technique, which was in higher quantities than that measured
330 before extraction in the soil of Tavel) (Kruskal–Wallis tests, Table 2).

331 A higher G6PDH activity was measured after CER extraction for Lysi and Tavel soils, whereas
332 no significant difference was observed between CER and EDTA extraction techniques (Mann-
333 Whitney tests) and G6PDH activities did not appear to be greater to the results obtained
334 (one replication) before extraction (Table 2).

335

336 3.2. EPS extracted from soils of the experimental field site

337 The EPSac amounts extracted from the soils of the experimental field tomato site were
338 higher in August than in June (ANOVA) and correlated with the microbial biomasses ($r_{\text{all soils}} =$
339 0.478 , $p = 0.04$) and particularly in the rhizospheric zone ($r_{\text{rhizospheric soils}} = 0.496$, $p = 0.036$; r_{bulk}
340 $\text{soils} = 0.439$, $p = 0.078$; Fig.3). Moreover, only in June the amounts of EPSac were significantly
341 higher in the rhizosphere compared to bulk soil (Fig.4). No difference was observed in the
342 amounts of EPSac between the two cultivars in June, but in August the amounts of soil
343 bound-EPSac of the cultivar Terradou became significantly higher than that of the cultivar
344 H1015 (Fig.3). Differences between June and August soil samplings were also apparent from
345 the MIR spectra, with a temporal discrimination of soil-bound EPS along the PC1 axis (Fig 5).
346 This discrimination could be attributed to the main absorptions at 1016 cm^{-1} for August
347 samples (left side of PC1 axis) and at 899 cm^{-1} for June samples (right side of PC1 - Fig 5b). A
348 Factorial Discriminant Analysis (FDA) performed on the spectral data of EPS enabled the
349 ranking of samples according to the soil zone (rhizospheric or bulk soil) and cultivar

350 (Terradou or H1015). Results are expressed as a percentage in matrices of confusion. A good
351 classification of samples was generally observed (Tab.3, Tab.4). All bulk soils were well
352 classified in June (100%) against 89% in August whereas around the same percentage of
353 classification (80%) was obtained for rhizospheric soils whatever the sampling date (Tab.3).
354 Concerning cultivars, 100% of Terradou samples were well-classified in June and August
355 against around 80% for H1015 (Tab.4).

356

357 **4. Discussion**

358 4.1. Efficiency and discrimination of EPS extraction techniques from calcareous soils

359 The three extraction techniques seemed consistent since they showed the same trends
360 between the three tested soils. They extracted higher amounts of EPSac in the "Crau" soil
361 compared to "Lysi" and "Tavel" soils. The "Crau" soil is covered with a permanent grassland
362 (strongly influenced by the roots of this permanent vegetation cover), rich in organic matter
363 and nutrients (nitrogen), with high microbial active biomasses and higher water retention
364 (Water Holding Capacity) and structural stability (Water Stable Aggregate). The "Lysi" soil is
365 from an agricultural field with conventional practices for annual crops, showing intermediate
366 organic matter and microbial biomass contents, while the sandy soil of "Tavel" originates
367 from a slope far from neighbouring trees and shows the lowest organic matter, microbial
368 biomass levels and physical characteristics (Table 1). Interestingly, the very recent study by
369 Redmile-Gordon et al. (2020) (comparing unfertilised grassland, fertilised arable land and
370 fallow), suggests that the type of land use strongly influence the soil-bound EPS
371 concentrations which, in turn, influence soil physical characteristics such as aggregation.

372 The NaOH+F extraction technique was the most effective for extracting EPSac from soils as
373 already observed on sludges by Liu and Fang (2002). This is more evident for the "Crau" soil,
374 rich in organic matter, which could interfere on the linkage between EPS and soil aggregates,
375 limiting the effectiveness of EDTA and CER techniques. As already mentioned, very few
376 authors have applied and compared these EPS extraction methods on soils (Redmile-Gordon
377 et al., 2014; Wang et al., 2019) and, to our knowledge, were never applied on
378 Mediterranean calcareous soils. A specificity of these alkaline Mediterranean soils is their
379 high concentration of cations and in particular Ca^{2+} ions that saturate the Cation-Exchange
380 Capacity (CEC) of soil (Table 1). The chemical mode of action of CER and EDTA techniques is
381 the exchange or sequestration of the divalent cations (mainly Ca^{2+} and Mg^{2+}) present in the

382 soil that bind EPS chains to soil particles. A soil highly concentrated in divalent cations could
383 limit the efficiency of extraction of these two techniques, which could also explain the higher
384 extraction efficiency of the NaOH+F technique. The CER extraction was the lesser efficient
385 for the quantitative determination (EPSac), in agreement with Redmile-Gordon et al. (2014),
386 but was the most repeatable on the basis of six replicates (Fig.1). Moreover, even if the
387 three techniques gave a similar classification of soils according to the quantities of EPSac,
388 the CER technique was the most discriminating according to the MIR spectra whereas the
389 NaOH+F technique was the least. These spectra revealed some wavenumbers to be
390 important to discriminate soils: The absorption at 1004 cm^{-1} discriminated the EPS by the
391 CER compared to the other techniques (Fig. 2a-2) and the "Crau" soil (containing the highest
392 quantities of EPSac measured by colorimetry) was discriminated from the "Lysi" soil using
393 the CER technique by the wavenumber 1035 cm^{-1} (Fig.S1-c). According to the review of
394 Movasaghi et al. (2008) and as observed by Velmourougane et al. (2017) on soil
395 microorganisms, by Artz et al. (2008) and Ludwig et al. (2008) on soil and litter, and by
396 Ellerbrock et al. (2019) on root mucilages, absorbances around 1200 and 900 cm^{-1} are
397 explained by vibrations of C-OH and C-O-C of functional groups of polysaccharides and
398 carbohydrates. This suggests that despite a lower overall efficiency of EPSac extraction by
399 the CER (as measured by colorimetry with Dubois method), this extraction technique
400 seemed to be able to discriminate by MIR soils extracellular polymeric substances through
401 the content and/or chemical structure of their exopolysaccharide.

402 Finally, it should be noticed that the discrimination of soils was better observed qualitatively
403 according to their MIR spectral characteristics than quantitatively according to their EPSac
404 amounts determined by colorimetric measurements.

405 4.2. EPS contamination

406 The two measurements of DNA and G6PDH did not show significant differences between
407 techniques in terms of cellular contamination even if small differences were observed on
408 soils before extraction. Moreover, the amounts of DNA (0 to 19 mg g^{-1} soil organic matter)
409 and G6PDH activities were comparable or less than those obtained in the literature after EPS
410 extractions (Liu and Fang, 2002; Aguilera et al., 2008). These results suggest that the three
411 techniques did not induce significant intracellular contamination of EPS, despite the long
412 extraction time applied for CER and EDTA techniques (Frolund et al., 1996). We estimated
413 the extracellular contamination by comparing the HAE amounts extracted with each

414 technique (Table 2). It is difficult to evaluate the NaOH+F technique from this point of view
415 because the results were, inexplicably, very variable. Between EDTA and CER extraction
416 techniques, CER extracted the lowest amounts of HAE with the highest repeatability,
417 suggesting a lower extracellular contamination induced by the latter technique. No protein
418 was detected in the dialysed EPS samples, suggesting that the dialysis allowed to eliminate
419 the protein content (Chen et al., 2014), without losing any EPSac, at least for the EPS
420 samples extracted by the CER, as no significant decrease of the EPSac from the "Lysi" soil
421 before and after dialysis was observed.

422 As suggested by Comte et al. (2006), it is also important to consider EPS contamination
423 related to extractant residues, when comparing extraction techniques. According to Pan et
424 al. (2010), who used fluorescence measurements to characterize EPS extracted from aquatic
425 microbial biofilms, dialysis eliminated extractant residues (including EDTA and NaOH +
426 formaldehyde), allowing a better visualization of these extracted EPS through fluorescence
427 peaks that appeared after dialysis. In our experiment, the MIR spectra of the same samples
428 were different depending on whether the samples were dialysed or not, but close to those
429 of their respective chemical extractants (EDTA and NaOH + formaldehyde). The hypothesis
430 of Pan and al. (2010) on the attachment of extractant residues to EPS, preventing EPS
431 visualization by spectral technique, was confirmed here. Similarly, it was pointed out by
432 infrared analysis that the EPS contamination due to EDTA extractants occurred at around
433 1300 cm^{-1} and $1600\text{-}1550\text{ cm}^{-1}$ (Fig. S3) (Comte et al. 2006; Feng et al., 2019). It is possible
434 that these contaminations due to EDTA extractant may have interfered during the
435 measurement of EPSac using the colorimetric phenol sulfuric acid method. Moreover, using
436 the EDTA technique, the amounts of EPSac measured on the non-dialysed extracts were
437 significantly higher than the dialysed ones. The EDTA technique also extracted the highest
438 amount of HAE which could have induced an overestimation of total sugars by the phenol
439 sulfuric acid method according to Felz et al. (2019). Concerning the NaOH+F extraction
440 technique, colour artefacts were found here with the non-dialysed NaOH+F extracts
441 (possibly due to important pH variations), preventing this analysis.

442 From these results it seems necessary to dialysate the EPS samples extracted by EDTA and
443 NaOH+F techniques in order to be able to analyse their composition both, quantitatively
444 (colorimetric phenol sulfuric acid method of Dubois et al., 1956) or qualitatively (for example
445 MIR spectroscopy or other methods such as size fractions using the size exclusion

446 chromatography measurements, or monosaccharide composition by gaz chromatography;
447 Chen et al., 2014). Dialysis does require an additional time-consuming step in the processing
448 of the extracted EPS samples, which in addition, has probably induced a higher variability in
449 the results, possibly in relation with the variations in volume of liquid observed in the
450 dialysis bags.

451 4.3. Conclusion on the comparison of extraction techniques

452 To reach a global conclusion on the comparison of the EPS extraction techniques applied to
453 soil, the ergonomic, safety, economic and environmental aspects have also to be considered,
454 as for example, proposed by the new collective "Labos 1point5"
455 (<http://labos1point5.org/en/home/>). The most time-consuming extraction was the CER, due
456 to the sample preparation, the washing with a buffer and the necessity to weigh the CER
457 amount for each soil sample. In contrast, EDTA required the fewest sample manipulations.
458 However, CER extraction is the only technique that did not require a further dialysis, saving a
459 lot of handling time and processing errors. Recently, Feng et al (2019) proposed the use of
460 centrifugal filter devices that could advantageously replace dialysis to purify and
461 discriminate EPS in sludge. These systems, although quite expensive, would deserve further
462 testing for soil EPS purification. The CER technique is also the least dangerous for the
463 manipulator, whereas the NaOH+F technique with formaldehyde requires working under
464 fume cupboard (carcinogen), and NaOH is a strong base. From a cost point of view, the three
465 techniques are comparable (around 0.6 euros for extraction products per 0.5 g of soil
466 sample). CER is more expensive due to the resin price but larger volumes of extraction
467 products are required for EDTA and NaOH+F techniques inducing higher handling for
468 purification and freeze-drying. These lower volumes of extractant in the CER technique make
469 possible to increase the quantity of the analysed sample in order to take into account soil
470 heterogeneity and thus promote better results. Finally, from an environmental point of view,
471 the NaOH+F (Formaldehyde and pH rise) and EDTA (weakly biodegradable and high chelating
472 power) techniques present ecotoxic hazards. In addition, all three extraction techniques
473 require energy, particularly for centrifugation and agitation, and CER and EDTA techniques
474 involved a 16-hour agitation. One perspective would be to test shorter
475 agitations/incubations. However, the energy issue is probably the conservation of all the
476 extracted samples of EPS before analysis through freezing and/or freeze-drying and the low
477 volumes of extracts (e.g. with CER) are an advantage here.

478 We hereafter consider CER as the “optimal” method for extraction of bound-EPS from our
479 soil samples because this method provides a good compromise between efficiency,
480 repeatability, discrimination, cost and handling time and requires lower volumes of low toxic
481 extractants.

482

483 4.4. Field experiment

484 4.4.1. Seasonal and rhizospheric effects on soil-bound EPS

485 A difference in EPS extracted from soil with the CER technique was observed between the
486 two sampling campaigns: both quantitatively (EPSac measured with the phenol sulfuric acid
487 method of Dubois et al., 1956) and qualitatively (MIR). The PCA of MIR spectra discriminated
488 June and August soil samples and this discrimination was characterised by two main
489 opposite absorptions: one at 899 cm^{-1} for June (right side of PC1) and 1016 cm^{-1} for August
490 (left side of PC1) (Fig. 5b). The wavenumber 1016 cm^{-1} is specific of sugars whereas 899 cm^{-1}
491 is specific of aromatic molecules and of clay-mineral characteristics (Movasaghi et al., 2008;
492 Velmourougane et al., 2017; Artz et al., 2008; Ludwing et al., 2008). Soils sampled in August
493 were characterized by higher amount of sugars as measured with the reference Dubois
494 analysis (Fig. 4a). This suggests that between June and August the soil would have received
495 an input of fresh carbon (carbohydrates) through the mucilage of developed tomato plants
496 root (Artz et al., 2008). Moreover, our results showed that the rhizosphere of tomato plants
497 was richer in EPSac than bulk soil (Fig. 4a) and correlated with microbial biomasses (Fig.3).
498 MIR data allowed for a good classification of samples in both Rhizospheric and Bulk classes
499 (Tab.3) suggesting an effect of root tomato mucilage on rhizospheric soil carbon
500 composition. These results highlight the important role of soil EPS in plant-microorganism
501 interactions in our agronomic context (Oburger and Jones, 2018), beyond their constitutive
502 microbial aspect Marchus et al., 2018).

503 4.4.2. A cultivar effect on soil-bound EPS

504 The studied tomato cultivars influenced quantitatively and qualitatively the soil-bound EPS.
505 The total root exudates represent about 5-21% of the products of photosynthesis (Bakker et
506 al., 2013). The Terradou cultivar, which is more productive than the H1015 (de Castro Vilas
507 Boas et al., 2017), may have probably a higher mucilage production that is reflected by its
508 higher amounts of EPSac in the rhizosphere observed in August (Fig. 4-b). The MIR data

509 allowed to classify samples in the two Terradou and H1015 classes, and the percentage of
510 classification remained similar in June and in August (Tab.4).

511 To our knowledge, this is the first time that quantitative and qualitative differences in soil-
512 bound EPS have been identified in rhizospheric soils collected in the field. These first results
513 concerning the soil-bound EPS during growth of a tomato crop are promising insofar as most
514 of the experimental data on mucilage are collected on young plants and without soil, and
515 then little information is available on differences in mucilage composition according to soil,
516 age and plant species/variety (Oburger and Jones, 2018). However, these molecules exuded
517 into the soil induce a cascade of feedback loops between the roots, the associated
518 microbiome and the soil aggregates. Thus, through actions such as aggregation,
519 detoxification, stimulation of microorganisms and modification of water flow in soil, these
520 molecules can directly and indirectly promote plant growth and stress resistance (Oburger
521 and Jones, 2018; Czarnes et al., 2000; de Vries et al., 2019). Understanding this environment
522 of plant-soil feedbacks for water is a recent and open way for alleviating the impact of water
523 deficits on crop productivity (Ahmed et al., 2018). According to the study by de Castro Vilas
524 Boas et al. (2017) on the same tomato cultivars grown in comparable soil, the Terradou
525 cultivar had a higher water use efficiency, compared to H1015 cultivar, especially under
526 water stress conditions. It is possible that higher amounts and different characteristics of
527 bound-EPS in the soil surrounding the Terradou cultivar may contribute to its higher
528 efficiency? This hypothesis is to be confirmed with additional experiments and
529 measurements of hydro-physical parameters of rhizospheric soils.

530

531 **5. Conclusion**

532 This study applied on calcareous Mediterranean soils allowed to determine the most
533 suitable method, i.e. the CER, to extract and quantify soil-bound EPS, including EPSac. It
534 appears interesting too to use a standardized extraction technique such as the CER
535 extraction, applied to a wide range of environmental matrices (sludges, sediments, microbial
536 biofilms, algae and soils) to compare different environmental matrices and contexts as
537 suggested by Redmile-Gordon et al (2014). Beyond quantitative measurements, the interest
538 of MIR spectroscopy coupled to chemometric approaches (fast, inexpensive and
539 environmentally friendly technique) was confirmed to discriminate soil EPS. However, we
540 did not analyse loosely-bound EPS that may also have important ecological and agronomic

541 roles in the rhizosphere, for example as being an available of carbon source that is readily
542 degradable by microbial communities, which can influence nutrient supply by plants (Chen
543 et al., 2014). In addition, some soluble EPS molecules may be large and ignoring them may
544 underestimate their role in soil structure and water retention. For example, loosely-bound
545 EPS may be involved in hydrophobicity, as it was demonstrated for cyanobacteria and
546 cyanobacterial biocrusts (Mugnai et al., 2018). However, because of their solubility, mobility
547 and biodegradability, the interpretation of their measured quantities in soil remains a
548 challenge (Redmile Gordon et al. 2014, 2020). This loosely-bound EPS fraction needs further
549 study to be better understood.

550 We applied this extraction technique to soil samples taken from the field at an experimental
551 site for the comparison of industrial tomato varieties. Our initial results showed a
552 rhizospheric effect during plant growth, as well as differences in soil-bound EPS between the
553 two cultivars studied. This approach is therefore promising for improving our knowledge on
554 soil EPS characteristics and properties, with the aim of better understanding the hydro-
555 physical and biological functioning of the rhizosphere (Oburger and Jones, 2018; Lipiec et al.
556 2013).

557

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567

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733 **FIGURES captions**

734 **Figure 1** Boxplots of Bound-EPSac amounts extracted from the three soils (Crau, Lysi, Tavel)
735 with each technique (EDTA, CER, NaOH+F). The different letters indicate significant different
736 values between soils for each technique (n=6; Kruskal–Wallis test).

737

738 **Figure 2** Results of the Principal Component Analysis performed on Mid-Infrared spectra **a-**
739 representing the 3 techniques (E=EDTA, C=CER, N=NaOH+F) for dialysed EPS (soils Crau and
740 Lysi, mean of 4 spectra): **a1.** PCA eigen vectors for PC1 (**a2**) and PC2 (**a3**) respectively. **b-** PCA
741 of the EDTA technique for dialysed EPS (soils CD=Crau and LD=Lysi). **c-** PCA of the NaOH+F
742 technique for dialysed EPS (CD and LD). **d-** PCA of the CER technique for dialysed EPS (CD and
743 LD). Ellipses are calculated with a confidence of 0.05.

744

745 **Figure 3** Correlation between Microbial Biomass and Bound-EPSac of soils sampled in an
746 industry tomato field.

747

748 **Figure 4** Boxplots of bound-EPSac of soils sampled in an industry tomato field. **a-** Comparison
749 between rhizospheric and bulk soils. **b-** Comparison between cultivars. Stars indicate
750 significant difference between Bulk soils and rhizospheric soils in June and between tomato
751 cultivars (Terradouand “H1015”) in August (ANOVA).

752

753 **Figure 5** Results of the Principal Component Analysis performed on Mid-Infrared spectra
754 (mean of 4 spectra) of EPS extracted from rhizospheric and bulk soils sampled in an industry
755 tomato field. **a-** PCA representing the 2 dates of sampling (June and August). **b-** PCA
756 eigenvector of PC1.

757

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Fig.1

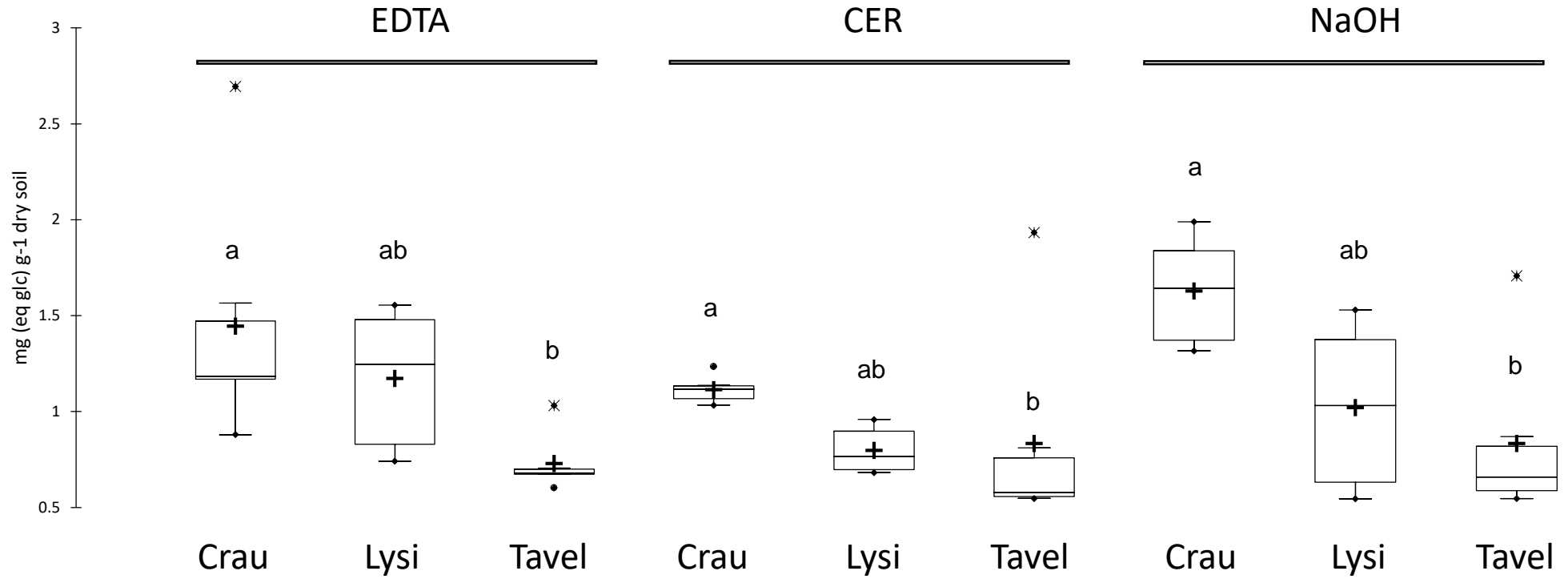


Fig.2

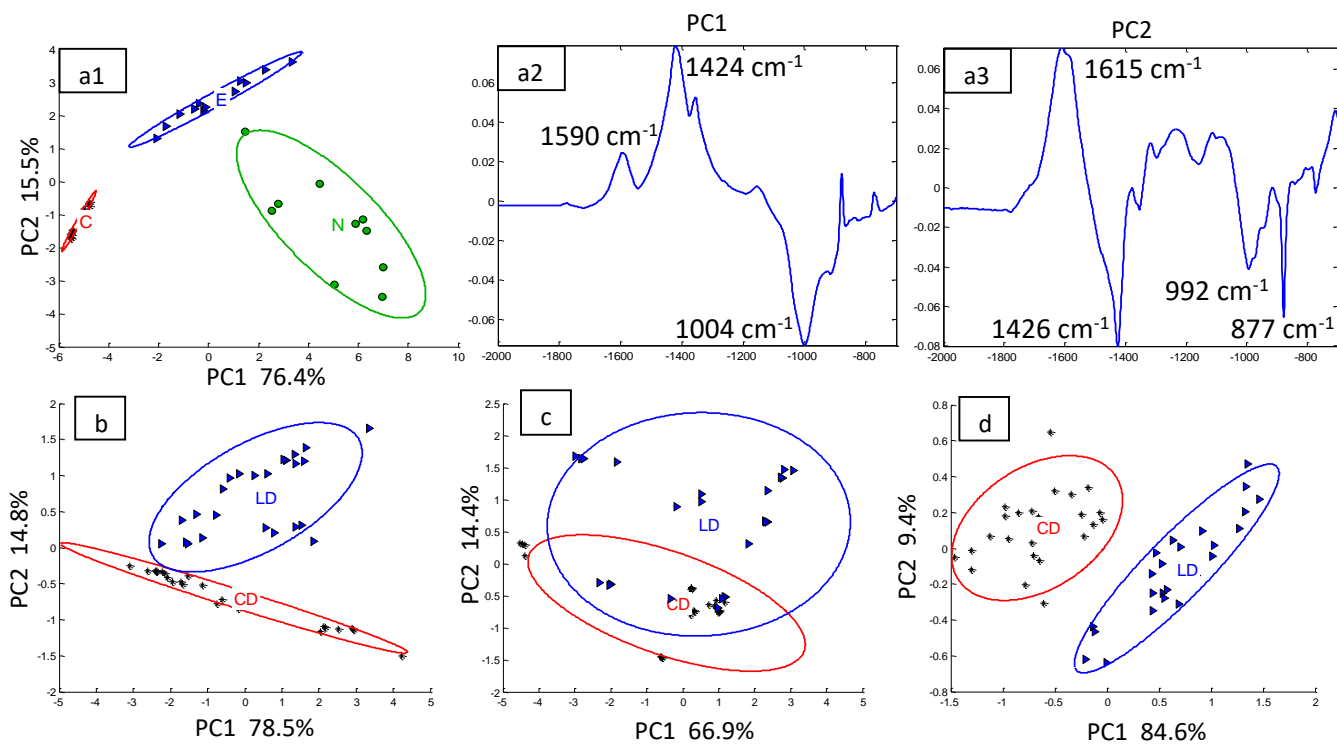


Fig.3

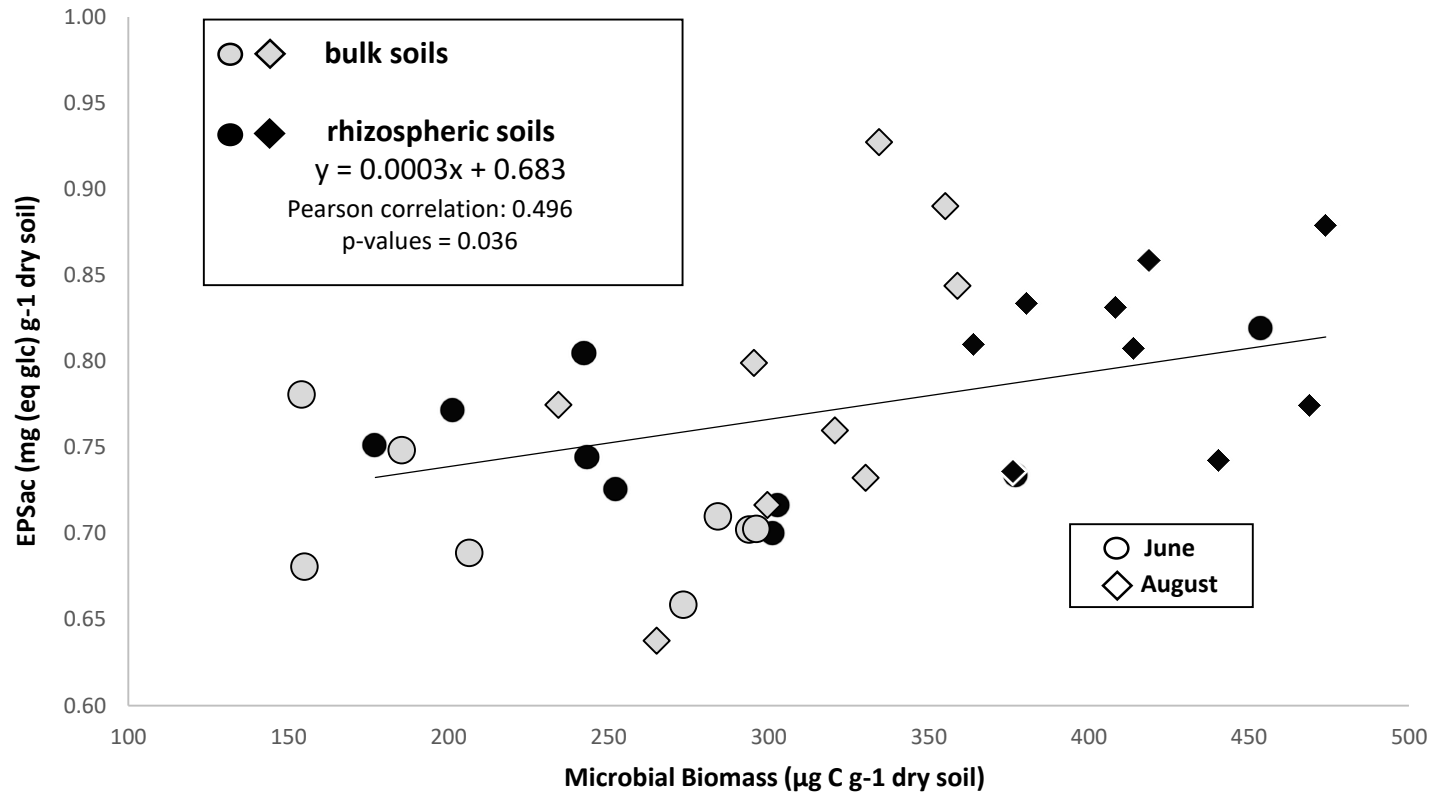


Fig.4

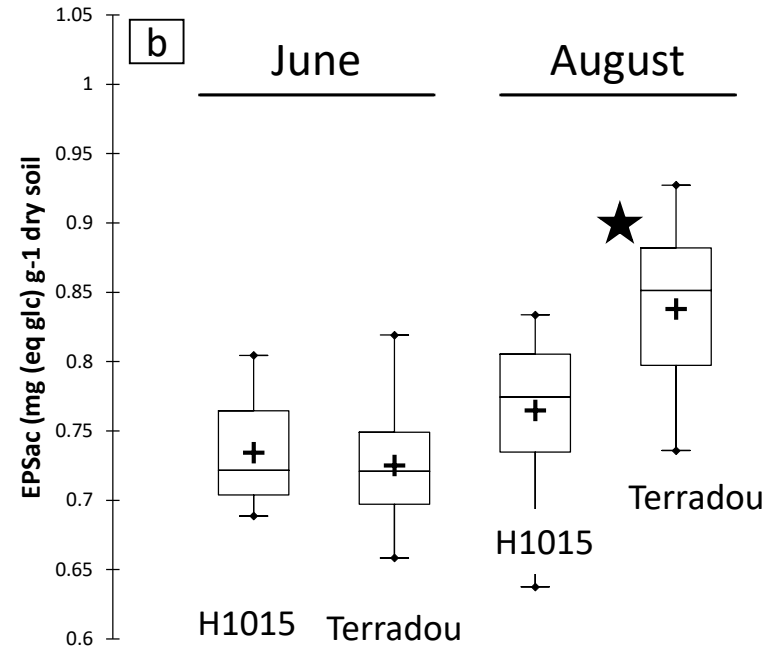
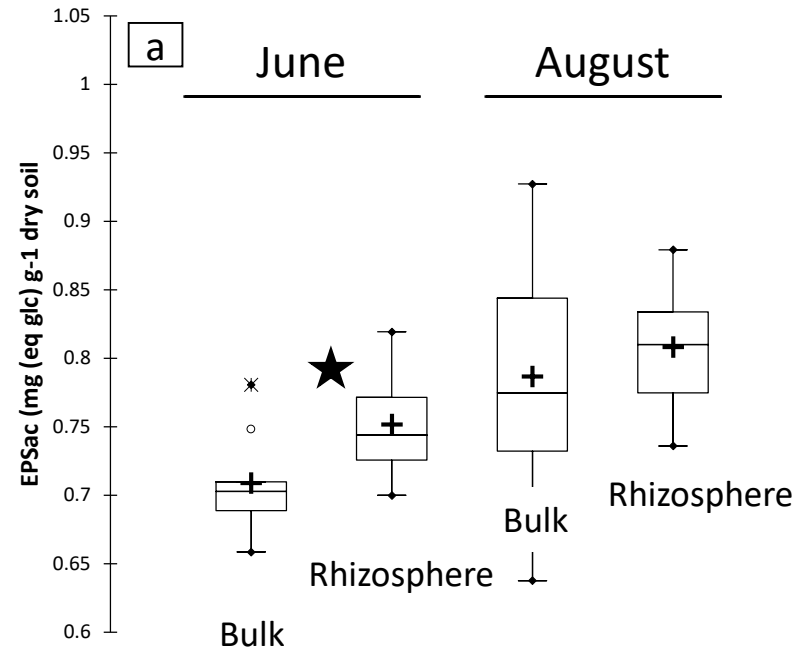
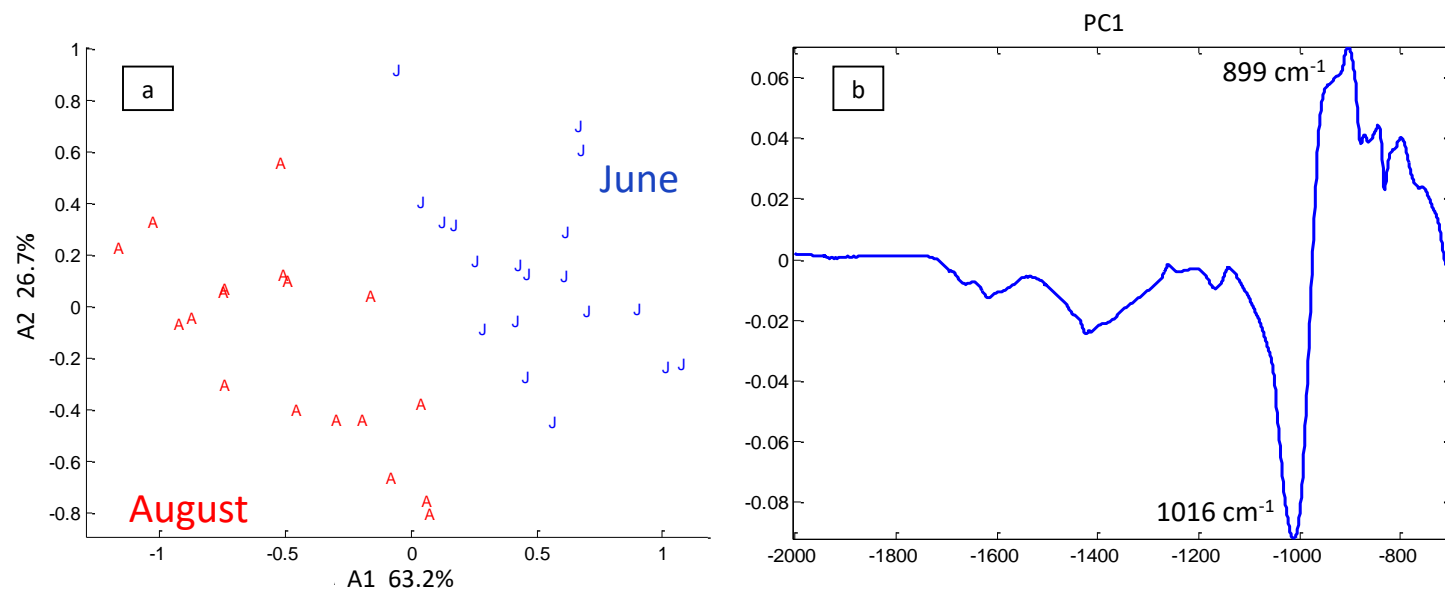


Fig.5



TABLES (4 tables)

Table 1. Main physical, chemical and microbial properties of the soils used for this study: Crau, Lysi and Tavel soils were used for the comparison of the three soil-bound EPS extraction methods, and the Piolenc soil was the soil from the tomato field experiment used as a test case for extraction with CER method of bound-EPS from rhizospheric and bulk soil samples.

Soil	Crau	Lysi	Tavel	Piolenc
Geographical context	Mediterranean 43°38'N/5°00'E	Mediterranean 43°54'N/4°52'E	Mediterranean 44°02'N/4°42'E	Mediterranean 44°11'N/4°48'E
Agronomical context	Grassland (irrigated)	Agricultural soil	Forest soil (slope)	Agricultural soil
Soil Type	Fersialsol	Fluvisol	Colluviosol	Redoxisol
Texture	sandy clay loam	silty clay loam	Sand	loam
Clay (g kg ⁻¹)	261	369	49	290
Loam (g kg ⁻¹)	246	256	128	331
Sand (g kg ⁻¹)	475	31	634	379
SOC (g kg ⁻¹)	31.8	15.5	3.09	12.5
TN (g kg ⁻¹)	3.6	1.6	0.1	1.1
C/N	8.9	9.5	28.1	11
CaCO ₃ (g kg ⁻¹)	18	339	188	484
Ca ²⁺ (cmol+ kg ⁻¹)	19.3	16.2	6.58	14.6
Mg ²⁺ (cmol+ kg ⁻¹)	2.0	1.42	0.098	0.595
CEC (cmol+ kg ⁻¹)	13.3	11.9	2.26	12.7
pH (water)	7.94	8.44	8.72	8.5
WHC (% g water g ⁻¹)	72	55	30	56
MB (mg C g ⁻¹)	372	213	60	231*
WSA (%)	75	43	20	36

SOC: Soil Organic Carbon, TN: Total Nitrogen, CEC: Cation Exchange Capacity, WHC: Water Holding Capacity, MB: Microbial Biomass, WSA: Water Stable Aggregates

* Bulk soil sampled in June 2018

Table 2. Characteristics of the EPS extracts. Mean of 6 replications

Extraction technique	soil	EPSac			EPSac nd			HAE			DNA			G6PDH		
		mean	min/max		mean	min/max		mean	min/max		mean	min/max		mean	min/max	
		mg eq glc g ⁻¹ dry soil						mg g ⁻¹ dry soil			µg g ⁻¹ dry soil			U/L		
CER	Crau	1.12	1.03	1.23				3.30	2.33	3.99	179	124	275	0.06	0.00	0.18
	Lysi	0.80	0.68	0.96	0.88	0.82	0.97	2.46	1.66	3.50	188	31	429	0.76	0.49	1.12
	Tavel	0.83	0.55	1.93				0.25	0.00	0.45	46	29	59	0.34	0.00	0.54
EDTA	Crau	1.45	0.88	2.69				7.63	4.69	15.2	202	80	290	0.18	0.11	0.24
	Lysi	1.17	0.74	1.55	4.74	4.30	5.06	6.15	1.59	15.2	119	56	218	0.19	0.14	0.24
	Tavel	0.73	0.60	1.03				0.66	0.00	3.44	90	10	154	0.06	0.00	0.15
NaOH+F	Crau	1.63	1.32	1.99				1.26	0.87	1.84	104	0	196			
	Lysi	1.02	0.54	1.53				9.29	3.40	20.3	61	3	105			
	Tavel	0.83	0.55	1.71				0.00	0.00	0.00	152	99	234			
before soil bound EPS extraction	Crau										18	16	20	0.38		
	Lysi										13	10	17	0.34		
	Tavel										2	1	3	0.20		

nd: EPS extracts not dialysed, CER: Cation Exchange Resin, NaOH+F: [NaOH + Formaldehyde], EPSac: exopolysaccharides, HAE: Humic-Acid Equivalent. G6PDH activities.

Table 3. Matrices of confusion (expressed in percentage) of the Factorial Discriminant Analysis (FDA) using PC scores of the PCA with soils as tested factors: rhizospheric and bulk soil sampled in June and in August. The total number of samples per sampling date was 18.

Soil		Bulk	Rhizospheric
June	Bulk	100	0
	Rhizospheric	20	80
August	Bulk	89	11
	Rhizospheric	22	78

Table 4. Matrices of confusion (expressed in percentage) of the Factorial Discriminant Analysis (FDA) using PC scores of the PCA with cultivar plot as tested factors: Terradou and H1015 plots sampled in June and in August. The total number of samples per sampling date was 18.

Cultivar plot		H1015	Terradou
June	H1015	78	22
	Terradou	0	100
August	H1015	80	20
	Terradou	0	100