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

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**Keywords**

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

**Definitions:**

EPS = Extracellular Polymeric Substances

EPSac = Exopolysaccharides

## Abstract

Quantifying and characterizing Extracellular Polymeric Substances (EPS) and especially exopolysaccharides (EPSac) is an issue for understanding the hydro-physical and biological functioning of the rhizosphere. However, few comparative studies of extraction techniques have been carried out on soils and none on calcareous Mediterranean soils. Three soil-bound EPS extraction techniques, i.e. Cation Exchange Resin (CER), EDTA and NaOH+Formaldhyde (NaOH+F) were compared on three contrasted Mediterranean soils. CER presented the lowest extraction efficiency of EPSac, but also the lowest contamination of EPS by extractants and extracellular compounds. Contamination with intracellular compounds was low and similar with the three methods. Mid-Infrared (MIR) spectra enabled the best discrimination of the EPS extracts when they were prepared with CER. CER is then identified to be the suitable extraction technique of EPS (including EPSac) from soils, including calcareous soils. This technique was applied on rhizospheric and bulk soils harvested in an experimental field of tomato cultivation. A rhizospheric effect was highlighted during the growth of plants of two cultivars with both the soil-bound EPSac amounts (total sugar equivalent of extracted EPS) and the MIR spectra of extracted EPS. Extraction of soil-bound EPS and their further analysis by spectral and chemometric approaches is a promising way for relating EPS chemical characteristics to their biological and hydric impacts within the plant rhizosphere in a context of agro-ecological transition and climatic change.

## 1. Introduction

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

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

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

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

## 2. Material and methods

### 2.1. Comparison of extraction methods

### 2.1.1 Investigated soil samples

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

### 2.1.2. EPS extraction methods

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

#### Cation Exchange Resin (CER) extraction

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

and Gerbersdorf et al. (2005). The CER (Dowex Marathon C, Na+ form, Sigma Aldrich, Steinheim, Germany) was preliminary washed with a phosphate buffer (consisting of 2 mM $Na_3PO_4 . 12H_2O$, 4 mM $NaH_2PO_4 . H_2O$, 9 mM NaCl, 1 mM KCl, adjusted to pH 7 with 1M HCl and stored at 4 ° C) until the pH on the solution was stabilized to 7. The CER was added to the centrifuge tube containing the pellet sample in order to obtain a ratio of 70 g dry CER per 1 g organic matter (Table 1). Five mL of phosphate buffer pH 7 were then added to the mixture. After manual stirring, the sample was incubated for 16 hours, for a more effective extraction (Frolund et al., 1996 and Gerbersdorf et al. 2005), under rotary agitation (50 rpm, agitator (HEIDOLPH REAX2, Germany) at room temperature.

<u>EDTA extraction</u>

EDTA is a chelating agent that sequester the ions ($Mg^{2+}$ and $Ca^{2+}$) that link EPS to soil particles and then releases EPS into the solution. The EDTA (named “EDTA technique”) extraction was performed according to Underwood et al. (1995) and Rossi et al. (2018). 25mL of EDTA (0.1 M) were added to the centrifuge tube containing the soil pellet sample. After manual stirring, the sample was incubated for 16 hours overnight, as for CER extraction, under rotary agitation (50 rpm, agitator (HEIDOLPH REAX2, Germany) at room temperature (Chen et al., 2014).

<u>NaOH+Formaldehyde extraction</u>

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

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

NaOH+F) and to purify the EPSac before analysis (Chen et al., 2014; Rossi et al. 2018). Dialysis was applied for all samples to avoid any variations between the extraction methods. The dialysed samples were weighed and divided into (i) aliquots of 200 µL (microtubes stored at -20 ° C) for analyses of total sugars, proteins, 'Humic acid equivalent' (HAE), and for measurements of cell lysis and (ii) an aliquot was freeze-dried for subsequent measurements using Mid-Infrared Spectroscopy (MIR).

Another series of 6 independent extractions by each of the three techniques was carried out on one of the three soils ("Lysi" soil, "L"). The obtained extracts were not dialysed in order to compare them with dialysed extracts.

### 2.1.3. Analytical methods

#### Total sugars

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

#### Protein and 'Humic Acid Equivalent' (HAE)

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

#### Mid-Infrared Spectroscopy (MIR) measurements.

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

### 2.1.4. Cell lysis estimation

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

After adding RNase to the $CaCl_2$ pre-extracted samples and to the soil-bound EPS extracts, the DNA was precipitated with 2.5 vol of -20°C absolute ethanol. The sample was then centrifuged (10 000 g, 20 min, 4 °C). This step was repeated twice. The resulting pellet was dried and solubilized in pure water. The amount of DNA was measured by reading the absorbance at 260 nm using NanoQuant plate and microplate reader (Infinite 200 Pro; Tecan).

The activity of the intracellular G6PDH was measured using a colorimetric method in $CaCl_2$ pre-extracted samples and in soil-bound EPS CER and EDTA extracts using the Sigma KIT MAK015 (Sigma-Aldrich Chimie, Lyon, France) at 37°C. Absorbance values (450 nm) were recorded every 5 min with a microplate reader (Infinite 200 Pro; Tecan). Only CER and EDTA techniques have been tested with G6PDH, NaOH+F being known to be incompatible with this method of cell lysis evaluation (denaturation of the G6PDH protein).

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

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

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

Soil-bound EPS were extracted from the samples with the CER extraction technique detailed in 2.1.2 section. The soil-bound EPS were quantified by their total sugars content (Dubois method, EPSac) and by acquiring their MIR spectra, as described in section 2.1.3. Soil microbial biomass was assessed with the MicroResp$^{TM}$ technique (Ben Sassi et al. 2012) using a 96-deep-well microplate filled with soil subsamples (soil moisture preliminary adjusted to 40% of WHC). 25 µl of glucose (6.7 mg $g^{-1}$ dw soil) was added in each deep-well. The plates were then tightly covered with a colorimetric $CO_2$-trap microplate and incubated in the dark (23 °C±1) for six hours. Absorbance was measured at 570 nm. A calibration curve of absorbance *versus* headspace equilibrium $CO_2$ concentration (measured by gas chromatography) was fitted to a regression model, which was used to compute the amounts of released $CO_2$. Glucose-induced respiration was used as a proxy of active microbial biomass (Anderson and Domsch, 1978).

### 2.3. Statistical analysis

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

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

## 3. Results

### 3.1. Comparison of extraction methods

#### 3.1.1. EPS analysis

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

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

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

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

No protein was detected in dialysed EPS samples (data not shown). The quantities of HAE gave varying mean values according to the soil and the extraction technique, in particular with the NaOH+F technique. With EDTA and CER techniques, a HAE quantitative gradient was observed in the following increasing order "Crau"> "Lysi"> "Tavel", as previously seen for EPSac amounts.  Both the CER and EDTA techniques allowed the differentiation between "Crau" and "Tavel" soils, the soil "Lysi" presenting intermediate EPS-HAE amounts (Kruskal-Wallis test, $p<0.05$). The CER technique resulted in lower and less variation of the amounts of HAE extracted than EDTA extraction (significant for the “Crau” soil, Mann-Whitney test, Table 2).

### 3.1.4. Cell lysis estimation

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

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

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

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

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

## 4. Discussion

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

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

The NaOH+F extraction technique was the most effective for extracting EPSac from soils as already observed on sludges by Liu and Fang (2002). This is more evident for the "Crau" soil, rich in organic matter, which could interfere on the linkage between EPS and soil aggregates, limiting the effectiveness of EDTA and CER techniques. As already mentioned, very few authors have applied and compared these EPS extraction methods on soils (Redmile-Gordon et al., 2014; Wang et al., 2019) and, to our knowledge, were never applied on Mediterranean calcareous soils. A specificity of these alkaline Mediterranean soils is their high concentration of cations and in particular $Ca^{2+}$ ions that saturate the Cation-Exchange Capacity (CEC) of soil (Table 1). The chemical mode of action of CER and EDTA techniques is the exchange or sequestration of the divalent cations (mainly $Ca^{2+}$ and $Mg^{2+}$) present in the soil that bind EPS chains to soil particles. A soil highly concentrated in divalent cations could limit the efficiency of extraction of these two techniques, which could also explain the higher extraction efficiency of the NaOH+F technique. The CER extraction was the lesser efficient for the quantitative det ermination (EPSac), in agreement with Redmile-Gordon et al. (2014), but was the most repeatable on the basis of six replicates (Fig.1). Moreover, even if the three techniques gave a similar classification of soils according to the quantities of EPSac, the CER technique was the most discriminating according to the MIR spectra whereas the NaOH+F technique was the least. These spectra revealed some wavenumbers to be important to discriminate soils: The absorption at 1004 cm$^{-1}$ discriminated the EPS by the CER compared to the other techniques (Fig. 2a-2) and the "Crau" soil (containing the highest quantities of EPSac measured by colorimetry) was discriminated from the "Lysi" soil using the CER technique by the wavenumber 1035 cm$^{-1}$ (Fig.S1-c). According to the review of Movasaghi et al. (2008) and as observed by Velmourougane et al. (2017) on soil microorganisms, by Artz et al. (2008) and Ludwig et al. (2008) on soil and litter, and by Ellerbrock et al. (2019) on root mucilages, absorbances around 1200 and 900 cm$^{-1}$ are explained by vibrations of C-OH and C-O-C of functional groups of polysaccharides and carbohydrates. This suggests that despite a lower overall efficiency of EPSac extraction by the CER (as measured by colorimetry with Dubois method), this extraction technique seemed to be able to discriminate by MIR soils extracellular polymeric substances through the content and/or chemical structure of their exopolysaccharide.

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

## 4.2. EPS contamination

The two measurements of DNA and G6PDH did not show significant differences between techniques in terms of cellular contamination even if small differences were observed on soils before extraction. Moreover, the amounts of DNA (0 to 19 mg g$^{-1}$ soil organic matter) and G6PDH activities were comparable or less than those obtained in the literature after EPS extractions (Liu and Fang, 2002; Aguilera et al., 2008). These results suggest that the three techniques did not induce significant intracellular contamination of EPS, despite the long extraction time applied for CER and EDTA techniques (Frolund et al., 1996). We estimated the extracellular contamination by comparing the HAE amounts extracted with each technique (Table 2). It is difficult to evaluate the NaOH+F technique from this point of view because the results were, inexplicably, very variable. Between EDTA and CER extraction techniques, CER extracted the lowest amounts of HAE with the highest repeatability, suggesting a lower extracellular contamination induced by the latter technique. No protein was detected in the dialysed EPS samples, suggesting that the dialysis allowed to eliminate the protein content (Chen et al., 2014), without losing any EPSac, at least for the EPS samples extracted by the CER, as no significant decrease of the EPSac from the "Lysi" soil before and after dialysis was observed.

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

From these results it seems necessary to dialysate the EPS samples extracted by EDTA and NaOH+F techniques in order to be able to analyse their composition both, quantitatively (colorimetric phenol sulfuric acid method of Dubois et al., 1956) or qualitatively (for example MIR spectroscopy or other methods such as size fractions using the size exclusion chromatography measurements, or monosaccharide composition by gaz chromatography; Chen et al., 2014). Dialysis does require an additional time-consuming step in the processing of the extracted EPS samples, which in addition, has probably induced a higher variability in the results, possibly in relation with the variations in volume of liquid observed in the dialysis bags.

### 4.3. Conclusion on the comparison of extraction techniques

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

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

## 4.4. Field experiment

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

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

### 4.4.2. A cultivar effect on soil-bound EPS

The studied tomato cultivars influenced quantitatively and qualitatively the soil-bound EPS. The total root exudates represent about 5-21% of the products of photosynthesis (Bakker et al., 2013). The Terradou cultivar, which is more productive than the H1015 (de Castro Vilas Boas et al., 2017), may have probably a higher mucilage production that is reflected by its higher amounts of EPSac in the rhizosphere observed in August (Fig. 4-b). The MIR data allowed to classify samples in the two Terradou and H1015 classes, and the percentage of classification remained similar in June and in August (Tab.4).

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

## 5. Conclusion

This study applied on calcareous Mediterranean soils allowed to determine the most suitable method, i.e. the CER, to extract and quantify soil-bound EPS, including EPSac. It appears interesting too to use a standardized extraction technique such as the CER extraction, applied to a wide range of environmental matrices (sludges, sediments, microbial biofilms, algae and soils) to compare different environmental matrices and contexts as suggested by Redmile-Gordon et al (2014). Beyond quantitative measurements, the interest of MIR spectroscopy coupled to chemometric approaches (fast, inexpensive and environmentally friendly technique) was confirmed to discriminate soil EPS. However, we did not analyse loosely-bound EPS that may also have important ecological and agronomic roles in the rhizosphere, for example as being an available of carbon source that is readily degradable by microbial communities, which can influence nutrient supply by plants (Chen et al., 2014). In addition, some soluble EPS molecules may be large and ignoring them may underestimate their role in soil structure and water retention. For example, loosely-bound EPS may be involved in hydrophobicity, as it was demonstrated for cyanobacteria and cyanobacterial biocrusts (Mugnai et al., 2018). However, because of their solubility, mobility and biodegradability, the interpretation of their measured quantities in soil remains a challenge (Redmile Gordon et al. 2014, 2020). This loosely-bound EPS fraction needs further study to be better understood.

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

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**FIGURES captions**

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

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

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

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

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

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Fig.1

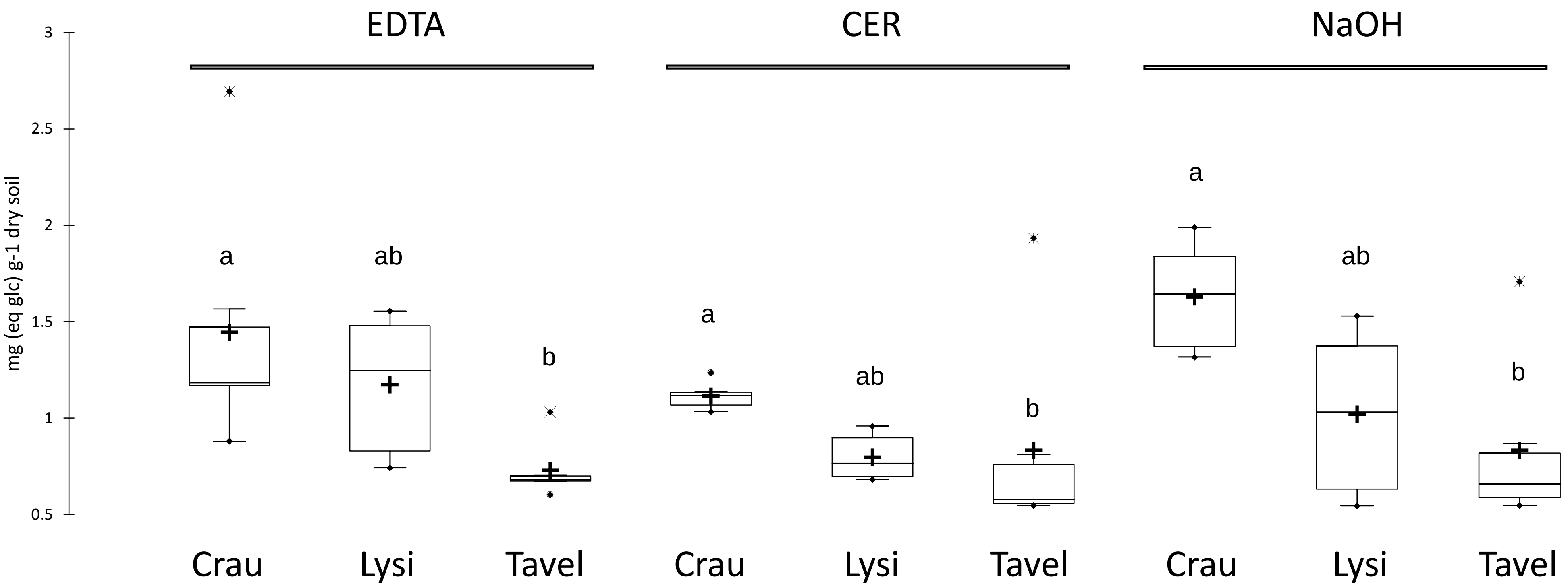

EDTA
CER
NaOH
mg (eq glc) g-1 dry soil
3
2.5
2
1.5
1
0.5
a
ab
b
a
ab
b
a
ab
b
Crau
Lysi
Tavel
Crau
Lysi
Tavel
Crau
Lysi
Tavel

Fig.2

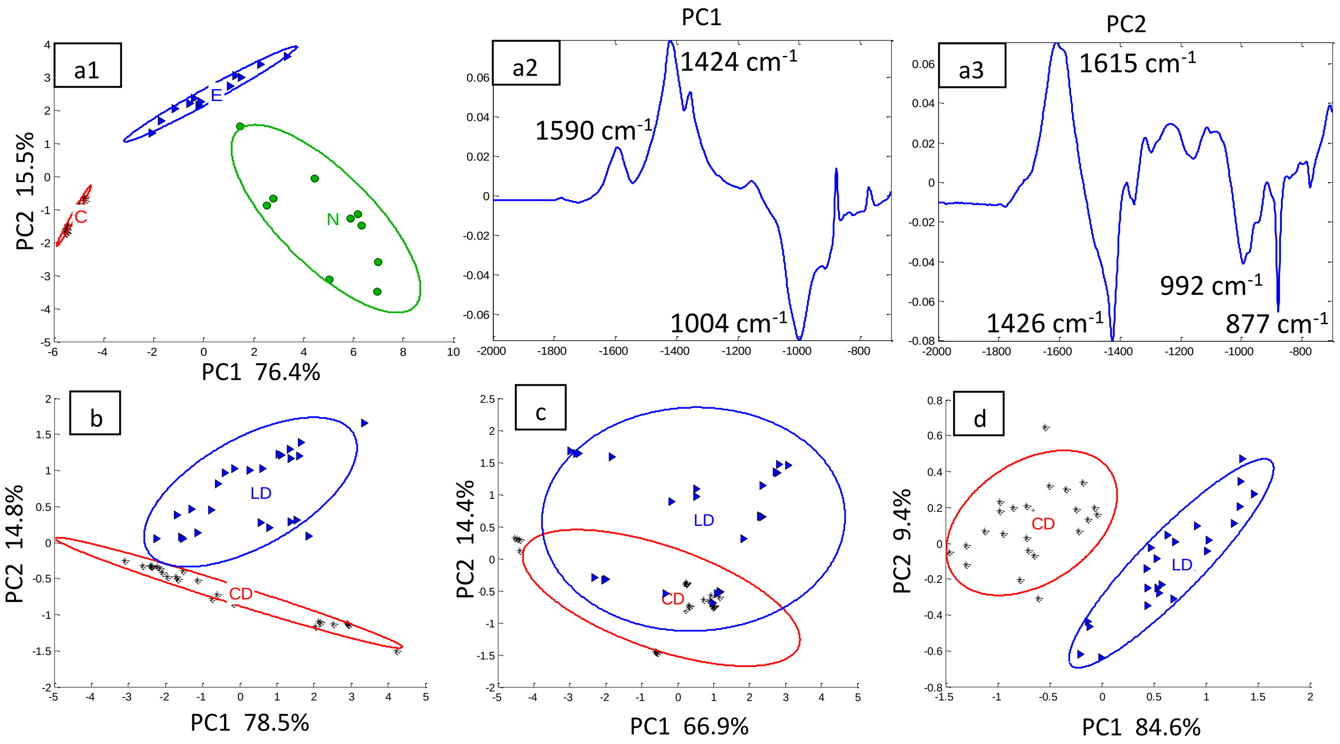
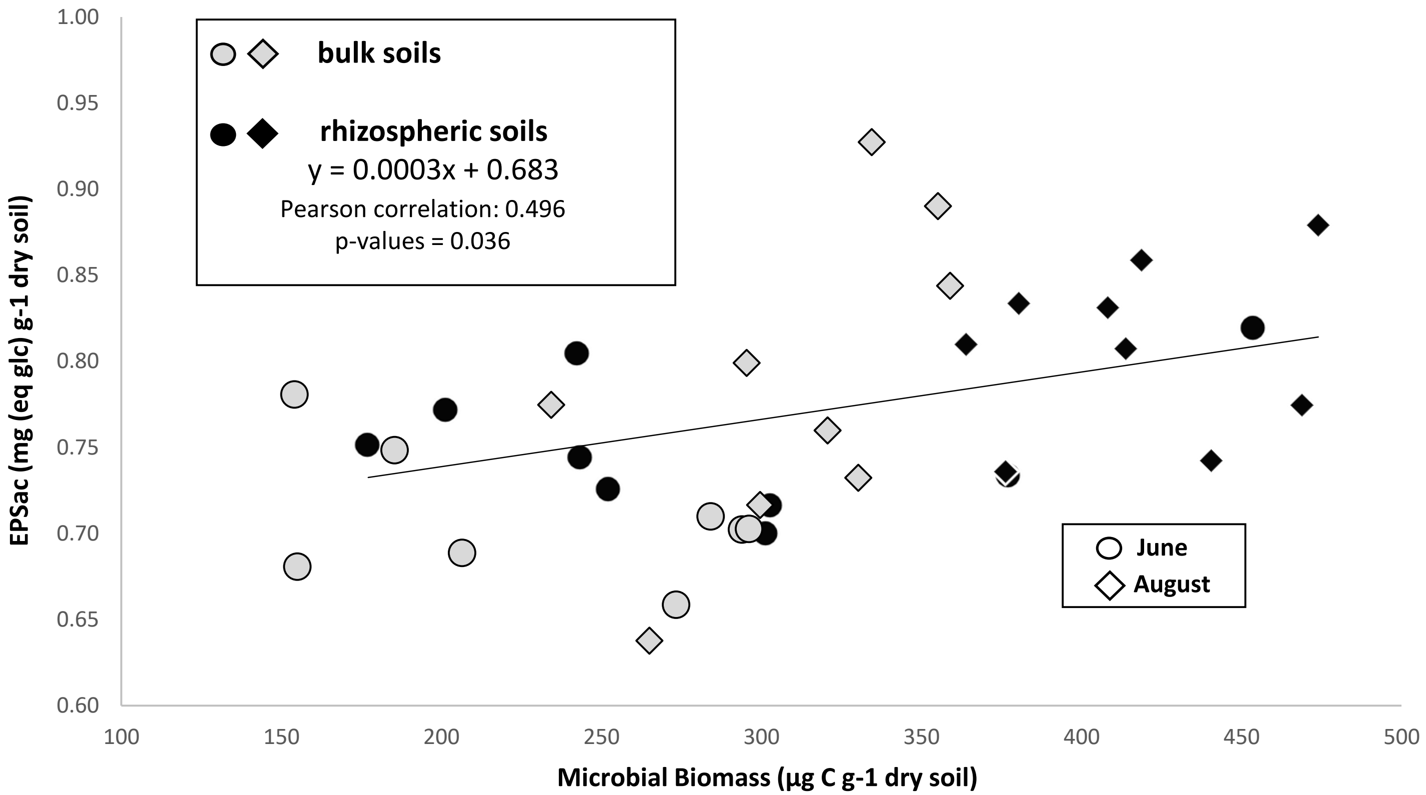

a1
E
C
N
PC2 15.5%
PC1 76.4%
PC1
a2
1424 cm$^{-1}$
1590 cm$^{-1}$
1004 cm$^{-1}$
PC2
a3
1615 cm$^{-1}$
1426 cm$^{-1}$
992 cm$^{-1}$
877 cm$^{-1}$
b
LD
CD
PC2 14.8%
PC1 78.5%
c
LD
CD
PC2 14.4%
PC1 66.9%
d
CD
LD
PC2 9.4%
PC1 84.6%

Fig.3



○ ◇ bulk soils
● ◆ rhizospheric soils
y = 0.0003x + 0.683
Pearson correlation: 0.496
p-values = 0.036
○ June
◇ August
EPSac (mg (eq glc) g-1 dry soil)
Microbial Biomass (µg C g-1 dry soil)
1.00
0.95
0.90
0.85
0.80
0.75
0.70
0.65
0.60
100
150
200
250
300
350
400
450
500

Fig.4

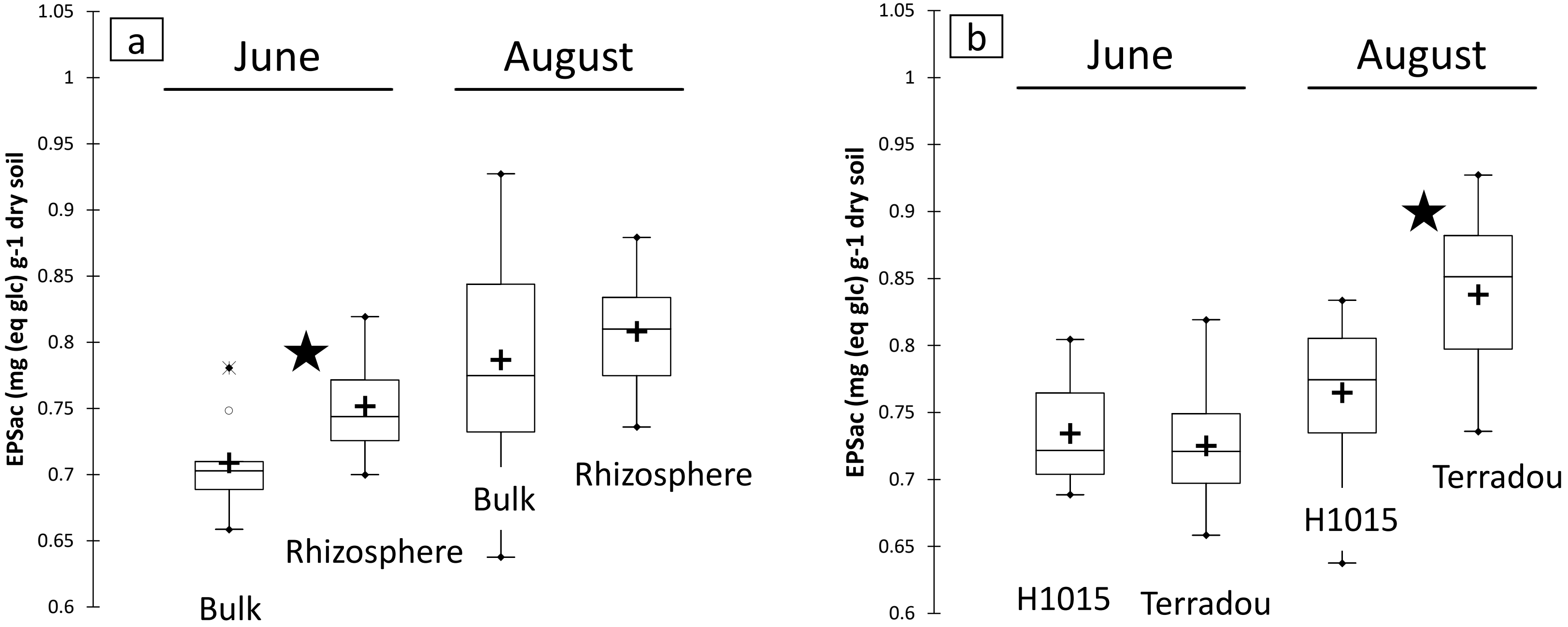
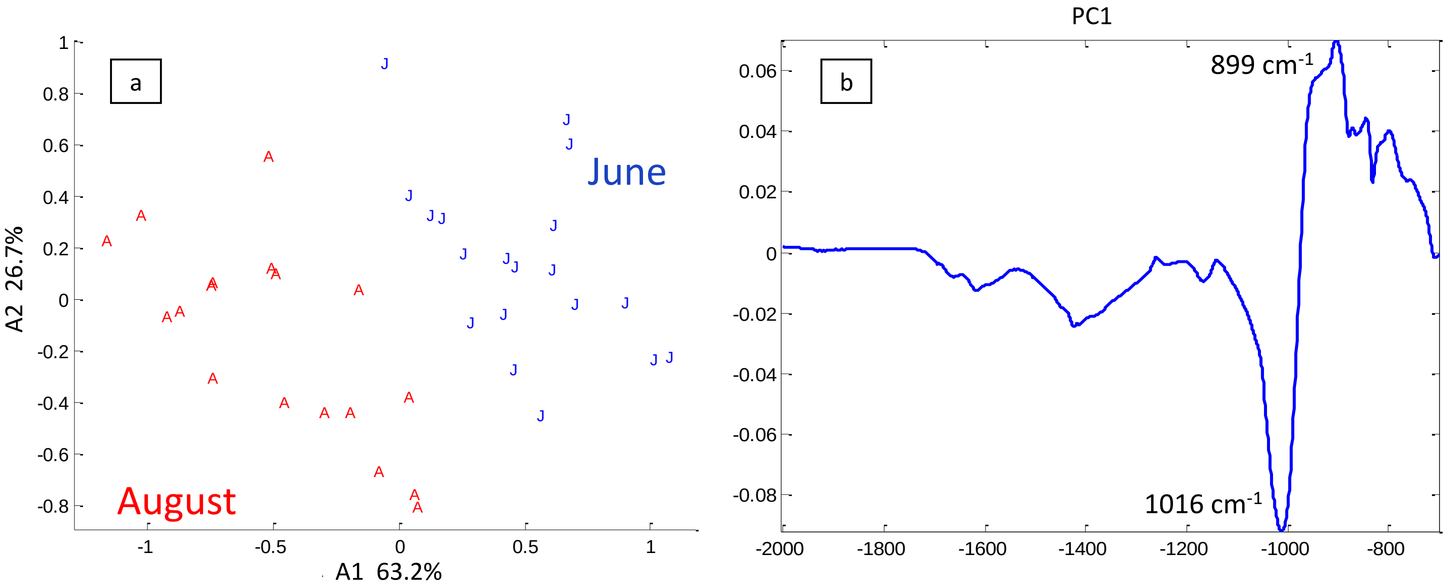

a
June
August
EPSac (mg (eq glc) g-1 dry soil
1.05
1
0.95
0.9
0.85
0.8
0.75
0.7
0.65
0.6
Bulk
Rhizosphere
Bulk
Rhizosphere
★
b
June
August
H1015
Terradou
H1015
Terradou
★

Fig.5



a
A2 26.7%
A1 63.2%
June
August
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
-0.5
0
0.5
1
b
PC1
899 cm-1
1016 cm-1
0.06
0.04
0.02
0
-0.02
-0.04
-0.06
-0.08
-2000
-1800
-1600
-1400
-1200
-1000
-800

## 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<br>43°38’N/5°00’E | Mediterranean<br>43°54’N/4°52’E | Mediterranean<br>44°02’N/4°42’E | Mediterranean<br>44°11’N/4°48’E |
| Agronomical context | Grassland (irrigated) | Agricultural soil | Forest soil (slope) | Agricultural soil |
| Soil Type | Fersialsol | Fluviosol | 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 |
| CaCO3 (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 + Formaldhyde], 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 |