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# 1 Development of a root exudate collection protocol for metabolomics analysis using 2 Nuclear Magnetic Resonance

3 Mélanie Fortier<sup>a,b</sup>, Julie Lemyre<sup>a</sup>, Edouard Ancelin<sup>b</sup>, Hassan Oulyadi<sup>a</sup>, Azeddine Driouich<sup>b</sup>, Maïté Vicré<sup>b</sup>,  
4 Marie-Laure Follet-Gueye<sup>b\*</sup> and Laure Guilhaudis<sup>a\*</sup>

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6 <sup>a</sup> Laboratoire COBRA (UMR 6014 & FR 3038), Normandie Université, UNIROUEN, INSA de Rouen, CNRS, Rouen,  
7 France

8 <sup>b</sup> Université de Rouen Normandie, Laboratoire Glyco-MEV UR 4358, SFR Normandie Végétal FED 4277, Innovation  
9 Chimie Carnot, F-76000 Rouen

10 \*Equal contribution

## 11 Abstract

12 Large amounts of root exudates are released by plant roots into the soil. Due to their importance in  
13 regulating the rhizosphere properties, it is necessary to unravel the precise composition and function of  
14 exudates at the root-soil interface. However, obtaining root exudates without inducing artefacts is a  
15 difficult task. To analyse the low molecular weight molecules secreted by pea roots, a protocol of root  
16 exudate collection was developed to perform a metabolomics analysis using Nuclear Magnetic Resonance  
17 (NMR). To date a few NMR studies are dedicated to root exudates. Plant culture, exudates collection and  
18 sample preparation methods had thus to be adapted to the NMR approach. Here, pea seedlings were  
19 hydroponically grown. The obtained NMR fingerprints show that osmotic stress increases the quantity of  
20 the exudates but not their diversity. We therefore selected a protocol reducing the harvest time and using  
21 an ionic solvent and applied it to the analysis of faba bean exudates. NMR analysis of the metabolic  
22 profiles allowed to discriminate between pea and faba bean according to their exudate composition. This  
23 protocol is therefore very promising for studying the composition of root exudates from different plant  
24 species as well as their evolution in response to different environmental conditions or pathophysiological  
25 events.

26  
27 **Keywords:** Metabolomics, Nuclear Magnetic Resonance (NMR) spectroscopy, *Pisum sativum*, root  
28 exudates, *Vicia faba*

## 30 1. Introduction

31 Plants actively and passively release large amounts of exudates containing a wide diversity of molecules  
32 into the soil (Bais et al., 2006). These are primary and secondary metabolites, of a low molecular weight,  
33 such as amino acids, sugars, organic acids, fatty acids and vitamins, or of a high molecular weight, such as  
34 proteins, polypeptides and glyco-polymers of mucilage (Oburger and Jones, 2018; Vives-Peris et al., 2019).  
35 It is believed that up to 40% of the carbon photosynthesized by the plant is released as root exudates  
36 (Lynch and Whipps, 1990). It is known that the species, stages of plant development and biotic and abiotic  
37 stresses strongly influence both quantity and quality of root exudates (Badri and Vivanco, 2009; De-la-  
38 Peña et al., 2008; Sasse et al., 2018). Due to the position of roots at the plant-soil interface, exudates are  
39 involved in a number of interactions between the plant and its rhizospheric environment (Haichar et al.,  
40 2014). They participate in biological interactions within the rhizosphere, as they are an essential carbon  
41 source for soil microorganisms, either beneficial, neutral, or pathogenic (Lynch and Whipps, 1990; Knee et  
42 al., 2001; Sasse et al., 2018). They are able to recruit beneficial microorganisms such as Plant Growth  
43 Promoting Rhizobacteria (PGPR) and are involved in the establishment of mycorrhizae. Root exudates also  
44 interfere with belowground pathogenic microorganisms and are involved in root defense (Badri and

45 Vivanco, 2009; Baetz and Martinoia, 2014; Lanoue et al., 2010). Moreover, abiotic factors such as water  
46 status influence the quality and quantity of root exudation (e.g., water excess or deficit impact the release  
47 of organic carbon into the rhizosphere). Mutual connections between plant root exudates and  
48 microbiomes upon drought stress impact plant resistance to water deficit by modifying the perception of  
49 stress (Zolla et al., 2013). Root exudates provide plant protection against drought, by modulating the  
50 composition and functioning of the microorganism communities in the rhizosphere (Chen et al., 2022;  
51 Hartman and Tringe, 2019; Williams and de Vries, 2020). Root exudates are also at the heart of the  
52 physico-chemical processes in soils such as changing the soil pH or stabilising soil particles (Badri and  
53 Vivanco, 2009; Galloway et al., 2020; Lynch and Whipps, 1990; Oburger and Jones, 2018). Therefore, the  
54 production and release of the exudate compounds represent a considerable metabolic cost for the plant.  
55 The released compounds are of a central importance as they contribute actively to interactions of roots  
56 with the rhizosphere.

57 A number of studies focus on root exudates characterization and function (Lanoue et al., 2010; Sasse et  
58 al., 2018; Zhao et al., 2001). With such a diversity of compounds released including amino acids,  
59 hormones, organic acids, flavonoids, a complete inventory is difficult with the use of only one type of  
60 analysis method (Oburger and Jones, 2018). The analysis is then often targeted on a single type of  
61 chemicals or chemical classes. Most studies are based on chromatography techniques: Ion  
62 chromatography (IC), High Pressure Liquid Chromatography (HPLC), Gas or Liquid Chromatography  
63 coupled with Mass Spectrometry (GC-MS, LC-MS) (Dundek et al., 2011; Oburger and Jones, 2018). In our  
64 study, a metabolomics characterization is envisaged in order to provide a non-targeted analysis of the  
65 metabolites (van Dam and Bouwmeester, 2016). Such an approach appears very promising for studying  
66 the composition of root exudates (Gregory et al., 2022) and their evolution under different conditions. In  
67 this study, Nuclear Magnetic Resonance (NMR) analysis was chosen to focus on the composition of low  
68 molecular weight compounds of our exudate samples. This analytical technique allows the detection of  
69 both primary and secondary metabolites from various chemical classes (Kim et al., 2010). Unlike other  
70 analytical techniques, NMR requires a very simple sample preparation (i.e. dissolution in a solvent), which  
71 preserves exudates from degradation and loss of compounds during the purification steps. In addition, for  
72 each sample, a single NMR analysis is sufficient; technical replicates are not necessary (Keun et al., 2002;  
73 Kim et al., 2010). Non-selectivity, reproducibility and non-destructiveness are thus significant advantages  
74 of this method (Dundek et al., 2011; Emwas et al., 2019; Letertre et al., 2021; Oburger and Jones, 2018;  
75 Rivas-Ubach et al., 2013). As the signal intensity is proportional to the concentration, it also allows an easy  
76 quantification of the identified compounds with a unique internal or external calibrant reference  
77 (Emwas et al., 2019; Kim et al., 2010). By comparing one 1D  $^1\text{H}$  spectrum by sample, it is easy to highlight  
78 the appearance or the disappearance of compounds between different samples. Thus, NMR appears as an  
79 analytical technique of choice for studying root exudates and investigating plant responses to biotic and

80 abiotic stresses. The Principal Component Analysis (PCA) is used in metabolomics as an unsupervised  
81 method to compare different samples and separate them into specific groups (Farhadi et al., 2019;  
82 Ramadan et al., 2006). Thus, NMR coupled to statistical analyses appears suitable for monitoring root  
83 exudation under different plant growth conditions. Although NMR is a robust technique, its low sensitivity  
84 represents a significant drawback (Emwas et al., 2019; van Dam and Bouwmeester, 2016). The detection  
85 limit of a compound is approximately 100nM (Nagana Gowda and Raftery, 2019).

86 In addition, we paid particular attention to improving the exudate collection protocols, as it determines  
87 the biological significance of the obtained results. In the literature, there are many strategies for  
88 harvesting exudates which depend on the growing plant medium and the type of analyses to be done  
89 (Escolà Casas and Matamoros, 2021; Vranova et al., 2013). In the present study, growing plants in  
90 hydroponics was chosen to perform the metabolomics characterization of root exudates. While root  
91 exudates are mainly harvested in hydroponics, the methods differ according to the type of analysis and  
92 the targeted metabolites (Escolà Casas and Matamoros, 2021; Vives-Peris et al., 2019). To remove growth  
93 medium nutrients, a preliminary rinsing of the roots can be carried out, mainly with distilled water, but  
94 the volumes and durations are rarely specified in the literature. Roots are immersed in a harvest solvent:  
95 distilled water (Cannesan et al., 2011, 2012; Escudero et al., 2014; Fan et al., 1997), ionic solutions (Fan et  
96 al., 2001; Zancarini et al., 2012; Zhao et al., 2001, 2016), or a nutrient medium similar to the culture  
97 medium (Lanoue et al., 2010; Song et al., 2021). The sampling strategy is based on the solubility of the  
98 compounds in the solvent. The choice of solvent is made as a compromise between the osmotic stress  
99 that can be caused in plants, and the concentration of ions that can interfere with the analysis. The  
100 immersion times are very variable in the literature: from a few minutes (Cannesan et al., 2012; Escudero  
101 et al., 2014; Fan et al., 2001) to several hours (Fan et al., 1997; Zhao et al., 2001, 2016) even a few days  
102 (Song et al., 2021). As the metabolite concentrations obtained are very low (Oburger and Jones, 2018),  
103 the analysis requires compound concentration, by freeze-drying or vacuum evaporation (Dundek et al.,  
104 2011). Furthermore, adaptation of the culture method, exudate collection and sample preparation for  
105 NMR analysis are essential, especially since NMR is not frequently used in the literature to characterize  
106 root exudates (Escudero et al., 2014; Fan et al., 1997, 2001; Zhao et al., 2016). Indeed, NMR analysis  
107 mainly targets human biofluids (Nagana Gowda and Raftery, 2019). Protocols are available on plant  
108 samples (Deborde et al., 2017, 2019; Pereira et al., 2006; Rivas-Ubach et al., 2013), but they are mostly  
109 used for whole tissue and not for root exudate analyses (Selegato et al., 2019). It was also necessary to  
110 assess that NMR sensitivity was sufficient to detect and identify compounds in root exudates under basal  
111 condition with minimum stress. In such control condition, exudation is expected to be reduced.

112 We are interested in pea (*Pisum sativum*), a legume of agricultural importance used for its high protein  
113 content in animal and human food. This species has been extensively used as a model to examine root  
114 interactions with microorganisms in the rhizosphere. Several studies have investigated the composition of

115 pea root mucilage with regards to high molecular weight compounds (Knee et al., 2001; Wen et al., 2007).  
116 In contrast, there is a lack of knowledge regarding the characterization of low molecular weight  
117 compounds of pea root exudates. These molecules are of importance as they are believed to play a major  
118 role in the response of root to microbial pathogens (Badri and Vivanco, 2009; Laloum et al., 2021).  
119 In this study, using pea as a plant model, we have developed a specific protocol for growing seedlings, in  
120 hydroponics and harvesting root exudates, with the aim of performing a non-targeted metabolomics  
121 analysis of low molecular weight compounds using NMR. The effects of both exudate collection medium  
122 (distilled water *versus* ionic solutions) and exudate harvest time were investigated to achieve an  
123 experimental procedure with limited artefacts. Indeed, it is essential to obtain reproducible data in basal  
124 conditions for subsequent detection of responses to biotic and abiotic stresses. This protocol was then  
125 applied to faba bean (*Vicia faba*) another plant species that belongs to the same family as pea (i.e.,  
126 Leguminosae family). Our protocol has proven to be effective in providing NMR exudation profiles which  
127 are species-specific, and to accurately identify compounds present in root exudates. The advantages and  
128 applications of this new and promising protocol for exudates characterization are discussed.

129

## 130 **2. Material and methods**

### 131 *2.1 Growing seedlings in hydroponics*

132 Pea (*Pisum sativum*, Astronaute variety, RAGT) and faba bean (*Vicia faba*, Fanfare variety, RAGT) seeds  
133 were surface sterilized successively in 70% ethanol for 10 minutes and in 1% (v/v) sodium hypochlorite for  
134 10 minutes and 6 times with sterile distilled water. They were left in distilled water overnight for  
135 imbibition and sown on filter paper imbibed with 20 mL MS ½ (Murashige and Skoog diluted 1:2, Duchefa  
136 Biochemie) liquid medium (Murashige and Skoog, 1962) in Petri dishes (**Fig. 1**). Four days after sowing, 30  
137 seedlings (with 3-4cm-root length) were transferred to a mesh support in microboxes with filter (0170555  
138 Dutscher), containing 250 mL MS ½ medium and are cultivated 3 more days at 21 °C, 16-hours-day/8-  
139 hours-night.

140

### 141 *2.2 Collecting root exudates*

142 The root exudates were collected in sterile conditions from 7-day-old seedlings. First, the mesh support  
143 with the seedlings was transferred for a few seconds to a new microbox containing distilled water to rinse  
144 the roots. Then, it was left in a harvest box with 250 mL distilled water or 0.1 mM calcium chloride (CaCl<sub>2</sub>)  
145 for 15 or 120 minutes. The entire exudates collection medium was centrifuged 10 minutes at 10,000 x g,  
146 and the supernatant was freeze-dried (**Fig. 1**).

147

### 148 *2.3 Exudate sample preparation and NMR spectra acquisition*

149 The exudates powder (from 2 to 4 mg) was dissolved in 700  $\mu$ L phosphate buffered deuterated water (50  
150 mM pH 7.4) containing 500  $\mu$ M DSS (Sodium trimethylsilylpropanesulfonate) as an intensity and chemical  
151 shift reference compound. The aqueous dissolution solvent and the fixed pH correspond to the ones of  
152 the reference spectra of the databases. The solution was homogenized, sonicated for 5 minutes and  
153 centrifuged at 10,000 xg for 10 minutes. 600  $\mu$ L of the supernatant were transferred to a 5 mm NMR tube  
154 (Garcia-Perez et al., 2020). All NMR experiments were performed at 298 K on a Bruker Avance III 600 MHz  
155 spectrometer equipped with a cryogenically cooled probe CPTXI. For metabolite fingerprinting, one-  
156 dimensional  $^1\text{H}$  Nuclear Overhauser Effect spectroscopy NOESY 1D (NOESY1dgppr sequence) spectra were  
157 acquired with a relaxation delay of 5 s, a mixing time of 10 ms and 2048 scans. 64k time domain points  
158 were used, and the spectral width was set to 9615.38 Hz. Water signal was suppressed using a weak  
159 presaturation pulse during the relaxation delay. Prior to Fourier transform, spectra were apodized by  
160 multiplication with an 0.3 Hz exponential decay and zero-filled to 128k points. Spectra were manually  
161 phased, baseline corrected and referenced to DSS at 0.0 ppm (Harris et al., 2008).

162 To allow metabolite identification, additional two-dimensional experiments were carried out:  $^1\text{H}$  J-RES (J-  
163 resolved Spectroscopy),  $^1\text{H}$ - $^1\text{H}$  COSY (COrelated Spectroscopy),  $^1\text{H}$ - $^1\text{H}$  TOCSY (TOtal Correlated  
164 Spectroscopy),  $^1\text{H}$ - $^{13}\text{C}$  HSQC (Heteronuclear Single Quantum Correlation) and  $^1\text{H}$ - $^{13}\text{C}$  HMBC (Heteronuclear  
165 Multiple Bond Coherence) (Garcia-Perez et al., 2020). 2D experiments were recorded with 2k data points  
166 and 512 increments, except for J-RES for which 128 increments were used.  $^1\text{H}$  spectral width was set to  
167 9615.38 Hz. HSQC and HMQC spectra were acquired with  $^{13}\text{C}$  spectral widths of 30183.83 Hz and  
168 33202.71 Hz respectively. The number of scans was comprised between 32 and 80 for  $^1\text{H}$ - $^1\text{H}$  experiments  
169 and between 48 and 104 for  $^1\text{H}$ - $^{13}\text{C}$  spectra.  $^1\text{H}$  J-RES were carried out with 80 transients. Topspin  
170 software 3.2.0 and 3.6.2 (Bruker Biospin, Wissembourg France) were used for NMR data acquisition,  
171 processing, and analyses (**Fig. 1**).

172 1D and 2D spectra of commercial compounds, not found in databases, were also recorded under the  
173 same conditions (1 mg in 600  $\mu$ L of phosphate buffered deuterated water 50 mM; pH 7.4 containing 500  
174  $\mu$ M DSS; 298 K), using the experimental parameters given above. These compounds correspond to  
175 molecules identified previously by our team in pea and/or faba bean exudates: p-coumaric acid, trans-  
176 ferulic acid, and formononetin (Laloum et al., 2021). Formononetin was found to be insoluble in aqueous  
177 solution.

178

#### 179 *2.4 Metabolomic fingerprint study*

180 The comparison of the metabolomics fingerprint of the different root exudate samples was carried out  
181 with the NMRProcFlow 1.4 software (Jacob et al., 2017). The first step consisted in removing the residual  
182 signal of the water from 4.704 to 4.876 ppm, then the spectra were aligned. Spectral intensities were  
183 reduced to integrated regions, referred to as buckets. These buckets were calculated automatically with

184 intelligent bucketing method and factor resolution of 0.5, normalized with constant sum normalization  
185 method, and analysed with the R 3.6.1 software. Four principal component analyses (PCA) were carried  
186 out to compare the pea exudation according to the exudate harvesting conditions: two solvents were  
187 used, distilled water and 0.1 mM calcium chloride solution, as well as two exudation times of 15 or 120  
188 minutes, to establish the optimal conditions for the study. For each harvest condition, 4 biological  
189 replicates were performed. Then, a fifth PCA was made to compare metabolite profiles of pea and faba  
190 bean and to discriminate the two species. For each species, 4 exudate samples were analysed to evaluate  
191 the biological variation of plants. Given the good reproducibility of the analytical technique, only one  
192 measurement per sample was sufficient (Keun et al., 2002; Kim et al., 2010).

193

### 194 *2.5 Metabolite identification or metabolite profiling*

195 Bayesil (Ravanbakhsh et al., 2015) and Workflow4Metabolomics (Giacomoni et al., 2015) software  
196 allowed the comparison of the one-dimensional experimental spectrum with those of the Human  
197 Metabolome Database (HMDB) compounds (Wishart et al., 2018). The signal assignment was then  
198 checked manually with two-dimensional experimental spectra (Dona et al., 2016). For each signal,  
199 chemical shift, multiplicity, coupling constants and two-dimensional correlations were verified. It is  
200 affirmed that an identified compound is indeed present in the sample if all the experimental spectra  
201 match the data in the HMDB, Madison Metabolomics Consortium Database (Cui et al., 2008),  
202 BioMagResBank (Ulrich et al., 2008) or Birmingham Metabolite Library (Ludwig et al., 2012) databases.  
203 For an easier identification, spectra corresponding to all harvest conditions were analysed. Identified  
204 compounds are given in supplementary material (**Supplementary Table S1**). Compounds which did not  
205 meet with all the above criteria (missing  $^1\text{H}$ - $^{13}\text{C}$  or  $^1\text{H}$ - $^1\text{H}$  correlations on 2D spectra) were labelled as  
206 putatively present compounds (Sumner et al., 2007) (**Supplementary Table S2**).

207

## 208 **3. Results**

### 209 *3.1 Impact of exudate collection on the pea exudate composition*

210 The experimental set-up for the *in vitro* growth of pea and faba bean provided between 2 and 4 mg of  
211 root exudates from 30 seedlings. This amount was sufficient to perform NMR analyses and to obtain  
212 metabolomics fingerprints. The chosen analytical method is therefore sufficiently sensitive to detect  
213 compounds in root exudates, despite the low sensitivity mentioned as a drawback in the literature.  
214 Our objective is to monitor exudation under controlled conditions, while limiting the stress during both  
215 the pea seedling growth and the following root exudate harvesting steps. Two exudate collection  
216 parameters were compared, the collection medium and the exudation time. Four conditions were thus  
217 used to assess their impact on the composition of NMR pea root exudates. These conditions are:  
218 exudation for 120 minutes in an aqueous solution of calcium chloride, 120 minutes in water, 15 minutes

219 in an aqueous solution of calcium chloride and 15 minutes in water. The comparison of one-dimensional  
220 spectra, on an example spectrum for each condition, shows compositional similarities between the  
221 samples (**Fig. 2**). The NMR analysis allowed to obtain a characteristic metabolic fingerprint of pea root  
222 exudates. However, variations occur in signal intensity, revealing different concentrations of metabolites  
223 according to the protocol used for exudate collection. The same compounds are therefore detected, but  
224 in different quantities. The exudates collected in water have higher concentrations of metabolites than  
225 those collected in the calcium chloride solution. Similarly, higher quantities of metabolites are present in  
226 samples obtained after a longer harvest time.

227 Four different biological replicates were carried out to confirm these observations and to refine the  
228 protocol for harvesting exudates for NMR analyses. Biological variations were observed between the  
229 replicates: there are slight variations in the concentrations of the compounds (**Supplementary Fig. S1**). It  
230 was then necessary to statistically compare the metabolomic fingerprints of pea according to the  
231 harvesting conditions of root exudates. To this end, we have chosen to perform Principal Component  
232 Analyses (PCA), which are multivariate and unsupervised methods. Four different analyses were carried  
233 out: exudation during 15 minutes in calcium chloride solution or in water (**Fig. 3A**); exudation in calcium  
234 chloride solution for 15 or 120 minutes (**Fig. 3C**); exudation during 120 minutes in calcium chloride  
235 solution or in water (**Fig. 3B**); exudation in water for 15 or 120 minutes (**Fig. 3D**). The first principal  
236 component (Dim 1) of the different plots explained ~60% of the total variability and the second (Dim 2)  
237 nearly 20%. In each case, the individuals, corresponding to the different samples of root exudates, are  
238 therefore well represented on the PCA two-dimensional plan. The variables correspond to the values of  
239 the buckets obtained with the <sup>1</sup>H NMR spectra on NMRProcFlow software.

240 The first principal component analysis aims to compare the exudation profiles according to the harvest  
241 solvent with an exudation time of 15 minutes (**Fig. 3A**). The confidence ellipse around the mean appears  
242 much wider for the samples of exudates collected in water than in the calcium chloride aqueous solution.  
243 This indicates greater variability in exudation profiles. For exudation times of 120 minutes, the variability  
244 of the samples seems similar regardless of the harvest medium (**Fig. 3B**). The third PCA consists in  
245 comparing the profiles of the metabolites according to the harvesting time of the exudates, meaning the  
246 duration of root immersion in the harvesting solution. For the analysis of the exudation time in the  
247 calcium chloride solution, the samples are separated into two groups according to the exudation time  
248 (**Fig. 3C**). This separation is much less clear in the case of exudation in water, where the confidence  
249 ellipses around the mean overlap (**Fig. 3D**).

250 These four analyses (**Fig. 3**) show that the choice of medium and exudation time is of considerable  
251 importance since it is responsible for variability in exudation profiles not in terms of metabolite diversity  
252 but in terms of metabolite quantities. Moreover, the exudation profiles in the calcium chloride solution,  
253 with short times exposure appear the least variable. In the following, an exudation of the seedlings in a

254 solution of calcium chloride for 15 minutes was therefore selected to compare the exudation profiles  
255 between species. Indeed, these harvesting conditions can be considered as corresponding to a basal level  
256 of exudation.

257  
258 *3.2 Application of the exudate collection experimental procedure to compare pea and faba bean exudation*  
259 *fingerprints by NMR*

260 PCA was also performed to compare samples of pea and faba bean exudates (**Fig. 4**). The first two  
261 dimensions explain nearly 90% of the variability. The two species are clearly separated on these  
262 components. Within one species, the exudate samples are similar but different from the other species  
263 showing that biological variability does not modify the exudation profile of a species (**Supplementary Fig.**  
264 **S1 and S2**), but mainly affects the concentrations of metabolites. Our protocol has therefore the  
265 advantage of being able to discriminate the exudation fingerprint of a species, and to obtain a profile  
266 characteristic of each species, corresponding to a metabolomic fingerprint.

267  
268 *3.3 First assessment of pea and faba bean exudate composition according to the Human Metabolome*  
269 *Database*

270 Among the four biological replicates carried out for each species, a representative <sup>1</sup>H NMR spectrum was  
271 chosen for pea (**Fig. 5**) and another for faba bean (**Fig. 6**), to illustrate the compound identifications. One  
272 and two-dimensional experimental spectra were compared with those of the Human Metabolome  
273 Database (HMDB) compounds (**Supplementary Tables S1 and S2**). In total, 30 compounds have been  
274 identified (21 without ambiguity) in pea and 36 (22 without ambiguity) in faba bean exudates. For each  
275 species, three main regions stand out on the one-dimensional spectrum, characteristic of classes of  
276 chemical compounds. Most often, high chemical shifts indicate the presence of aromatic protons. We  
277 have been able to identify trigonelline and uracil in pea exudates and L-tyrosine in those of faba bean.  
278 These high chemical shifts also correspond to protons bound to carboxyl groups of carboxylic acids, such  
279 as formic acid and fumaric acid present in both species. There are many signals in the second zone  
280 corresponding to chemical shifts between 3 and 6 ppm. The exudates are thus very rich in sugars, like  $\alpha$ -  
281 D-glucose and  $\beta$ -D-glucose, alcohols, such as glycerol and myo-inositol, and carboxylic acids like L-lactic  
282 acid or glycolic acid. Many organic and amino acids are also found in the samples, with signals present in  
283 the third zone, under 3 ppm shifts. In both species, we find, for example, succinic acid, L-glutamine, acetic  
284 acid, L-alanine, lactic acid, and L-valine. Some compounds are specific to pea, such as L-asparagine, L-  
285 methionine, acetone, and L-threonine whereas others are present only in faba bean exudates, including L-  
286 aspartic acid, L-proline, L-glutamic acid, and L-lysine. Most of the identified compounds without ambiguity  
287 are common to both species, 8 are specific to pea and 9 are specific to faba bean. Therefore, the  
288 exudation profiles of pea and faba bean differ in their composition. Pea exudates appear to be enriched in  
289 amino acids, whereas those of faba bean contain more sugars and organic acids. The one-dimensional

290 experimental spectra were also compared with those of p-coumaric acid, trans-ferulic acid and  
291 formononetin. These compounds were not identified in root exudates of both species. In addition, some  
292 species-specific metabolites could not be identified (**Supplementary Table S3**). There are therefore  
293 specificities for each species in the exuded metabolites.

294

#### 295 **4. Discussion**

296 The experimental protocol developed in this study is based on the production of root exudates from pea  
297 seedlings grown in hydroponics. Our aim is to offer a reproducible and reliable protocol to collect and  
298 analyse root exudate in order to characterize the environmental impact on plant exudation. The major  
299 requirements of such protocol are the limitation of stress applied to plant, the reduction as much as  
300 possible of the evolution of sample composition and the use of a reproducible and quantitative analytical  
301 method.

302 In the literature, several studies raise the importance of the exudate collection method (Escudero et al.,  
303 2014; Fan et al., 2001, 1997; Knee et al., 2001; Schapire et al., 2009; Valentinuzzi et al., 2015; Vranova et  
304 al., 2013; Zancarini et al., 2012; Zhao et al., 2001), but a wide variety of crop media are used to obtain  
305 root exudates (Oburger and Jones, 2018; Vranova et al., 2013). From the choice of a method derives the  
306 ecological representativeness and the exhaustive inventory of exudates. Soil or other solid substrate  
307 cultures are the most ecologically representative since the plants are present in their natural habitat and  
308 conditions (Oburger and Jones, 2018; Vives-Peris et al., 2019). However, the integrity of the root system is  
309 often altered during harvesting and the composition of the exudates difficult to determine accurately.  
310 A part of the exudates can be eliminated by microorganisms or confused with the degradation of organic  
311 matter products in the soil (Oburger and Jones, 2018; Paterson et al., 2009). The hydroponic culture has  
312 the advantage of preserving the roots and allowing easy and complete metabolite harvesting even if it is  
313 not fully ecologically representative (Escolà Casas and Matamoros, 2021; Oburger and Jones, 2018; Vives-  
314 Peris et al., 2019). Recently Ulrich et al. (2022), in a study dedicated to follow root exudate composition of  
315 *Bouteloua gracilis* plant under drought, proposed a mix protocol consisting of soil culture and hydroponic  
316 exudate collection. Before exudate collection, an antiseptic treatment was applied to remove  
317 microorganisms. The authors reported that such procedure could disturb plants. Thus, an impact on  
318 exudate composition due to this treatment cannot be excluded. Therefore, in this study, we made the  
319 choice of hydroponics to respect the standardization constraints of the experiment, carried out in a sterile  
320 medium, by excluding environmental variation factors which greatly influence exudation (Oburger and  
321 Jones, 2018).

322 Root exudates were collected from the entire root immersion medium. For NMR spectroscopy, no sample  
323 purification was performed and exudates were simply dissolved in an aqueous solution. The signals  
324 observed during the analysis thus correspond to all the soluble compounds present in the collected

325 exudates. Different collecting conditions were tested. Two harvest media: distilled water and 0.1 mM  
326 calcium chloride aqueous solution used in the literature and compatible with NMR analyses (Escudero et  
327 al., 2014; Zhao et al., 2001, 2016), and two exudation times (15 and 120 minutes) were studied. With the  
328 aim of conducting NMR analyses, a low sensitivity analytical method, under basal exudation conditions, it  
329 was first necessary to check that the collection protocol allowed to obtain a sufficient quantity of  
330 exudates to acquire a metabolomics fingerprint. It turned out that the quantities of exudates obtained  
331 were satisfactory for obtaining NMR profiles, for following changes in concentrations, and for identifying  
332 compounds present in the samples. The samples collected with the condition of root immersion in water  
333 appeared the most variable in compound concentrations. One hypothesis is that the increase in  
334 metabolites released is linked to increased stress of the seedlings when the roots are immersed in water,  
335 which could lead to increased exudation, even more important as the duration of immersion is longer.  
336 This could be explained by an osmotic stress of the seedlings, increased during the transfer from the  
337 nutrient medium to water and reduced with immersion in 0.1 mM calcium chloride solution instead.  
338 Changes in concentrations were detected, however no change in composition was found. We thus chose  
339 to favour a short root immersion time. This choice also allowed to reduce the biological variability.  
340 Indeed, the longer is the harvesting time, the greater is the biological variability between samples. The  
341 conditions of root immersion during 15 minutes in a calcium chloride solution, allow to limit the seedling  
342 stress, and therefore can correspond to a basal level of exudation, which nevertheless remains sufficient  
343 to detect compounds by an NMR approach.

344 Unlike other analytical methods, no special sample treatment or separation is required prior to the NMR  
345 analysis (Nagana Gowda and Raftery, 2019). In view of detecting and quantifying the differences between  
346 root exudates obtained in different growth conditions, it is essential to limit purification processes that  
347 might alter the sample composition and bias sample comparisons. Although, direct sample introduction  
348 strategies coupled to Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS), an ultra-  
349 high-resolution mass spectrometer, have been increasingly used in MS-based metabolomics studies  
350 (Ghaste et al., 2016), optimization is still required. Recently, a study investigating the ability of direct  
351 introduction FTICR-MS coupled to different ionization sources to detect metabolites in pea root exudates  
352 in a non-targeted way was published (Calabrese et al., 2023). Metabolites were identified by UHPLC-MS  
353 and they showed that MALDI-FTICR-MS, by reducing matrix effects due to the presence of salt, allowed to  
354 detect and identify more metabolites than the usually used ESI source. Despite these interesting results,  
355 the authors indicated that future studies were required to address questions regarding sample  
356 homogeneity and reproducibility for root exudate.

357 In our study, the step of rinsing the roots proves to be crucial to eliminate the culture medium. Since the  
358 freeze-dried exudates are in the form of powder, they are first dissolved in the analysis solvent. A wide  
359 range of solvents is available and this choice is essential to ensure the good solubilisation of the

360 compounds (Bothwell and Griffin, 2011; Holmes et al., 2006). Deuterated water is the reference in most  
361 databases used for NMR metabolomics studies (Bothwell and Griffin, 2011). Protons are replaced by  
362 deuterium atoms to get rid of their signal in the analysis. As the chemical shifts are variable depending on  
363 the pH, it is recommended to use a buffered solution (Beckonert et al., 2007). In order to quantify the  
364 concentration of the compounds, and to compare the spectra, a calibration is necessary with a chemical  
365 shift reference (Martineau et al., 2019). Here, the exudate powder obtained after centrifugation and  
366 freeze-drying of the harvest medium was resuspended in a phosphate buffer with deuterated water and  
367 DSS as a reference compound. This easy preparation coupled to NMR allowed to discriminate under  
368 controlled conditions two closely related species belonging to the Fabaceae family. The metabolomics  
369 data obtained here are complementary of other approaches previously used to study high molecular  
370 weight compounds of pea (Cannesan et al., 2012; Knee et al., 2001; Laloum et al., 2021; Wen et al., 2007)  
371 and faba bean (Laloum et al., 2021).

372 In this study, we highlighted small compounds known to be present in root exudates (Escudero et al.,  
373 2014; Fan et al., 2001; Vives-Peris et al., 2019). Among the metabolites identified herein, many are also  
374 found in other plant species, such as tomato for example, where the presence of amino acids (leucine,  
375 isoleucine, alanine), acetate, malate, succinate, glycerol, sugars, and formic acid was demonstrated using  
376 NMR analyses (Escudero et al., 2014). Some compounds could not be identified with certainty because  
377 not all the expected signals were visible on the experimental spectra. NMR being of low sensitivity (van  
378 Dam and Bouwmeester, 2016), this could be explained by a too low concentrations of these molecules.  
379 Moreover, the protons of molecules of the same chemical classes have close chemical shifts, so that the  
380 signals can overlap (Gómez et al., 2014). It is then difficult to differentiate each signal, which can make the  
381 identification quite challenging. However, as several signals can correspond to the same molecule, the  
382 observation of a specific signal can make it possible to identify a compound. NMR can then be coupled  
383 with chromatography and mass spectrometry techniques to complete the analysis (Holmes et al., 2006)  
384 and confirm the assignment of certain compounds. The analysis of root exudates with the FTICR-MS  
385 method made it possible to find compounds of seven molecular classes in pea exudates: nucleosides,  
386 amino acids, fatty acids, fatty acid amides, alkaloids, oligopeptides, pwhosphocholines, triterpenes,  
387 polyphenols, glucosyl-phenols and indoles (Calabrese et al., 2023). With HPLC analyses, phenolic  
388 compounds, such as quercetin, p-coumaric acid, trans-ferulic acid, pisatin and formononetin have been  
389 detected in pea exudates as well as tryptophan in pea and faba bean exudates (Laloum et al., 2021).

390 These molecules are not found in NMR databases; spectra of commercial p-coumaric acid, trans-ferulic  
391 acid and formononetin were recorded under the same experimental conditions, but these compounds  
392 were not found in the experimental spectra of the exudates. For p-coumaric and trans-ferulic acids, this  
393 can be explained by their low concentrations in the samples, these compounds being stress markers,  
394 produced in very small quantities under controlled conditions. For formononetin, it is probably due to low

395 water solubility unlike the HPLC analysis solvent. The exudation profiles obtained correspond to profiles  
396 of water-soluble metabolites and are therefore not exhaustive. A pseudo-extraction could be considered  
397 to dissolve more compounds. This type of extraction requires the use of other solvents for NMR analysis,  
398 such as chloroform and methanol (Bothwell and Griffin, 2011). But this raises the difficulty of finding or  
399 using databases with these types of solvent for which acquisition conditions are not fixed. Indeed, open  
400 access software for metabolite identification by NMR, such as Bayesil and WorkFlow4Metabolomics, use  
401 reference spectra acquired in deuterated water, at a pH around 7 (7 or 7.4) and a temperature of 298K.  
402 One of the most popular databases, which includes NMR spectra of more than a thousand metabolites  
403 recorded in these fixed conditions is the HMDB (Wishart et al., 2018). It is used by Bayesil for automatic  
404 identification and was used manually here to confirm the identification of metabolites of pea and faba  
405 bean root exudates from NMR spectra. The use of the HMDB database is a limiting factor in the analysis,  
406 since it contains compounds found in human biofluids, and not plant-specific compounds (Wishart et al.,  
407 2022). Although many low molecular weight compounds are universal, the lack of plant-specific  
408 compounds does not allow exhaustive analysis. This raises the need to develop *in-house* database, to  
409 enrich the databases, or to combine several databases to analyse the results. For example, the Madison  
410 Metabolomics Consortium Database (Cui et al., 2008), the BioMagResBank (Ulrich et al., 2008) or the  
411 Birmingham Metabolite Library (Ludwig et al., 2012) databases are well-provided but not used by open  
412 access software. Alternatively, as NMR is a non-destructive method (Nagana Gowda and Raftery, 2019),  
413 other analytical techniques, such as liquid chromatography or liquid chromatography coupled to mass  
414 spectrometry can be implemented on the same samples after NMR analysis to complement the  
415 identification of molecules.

416 In this work, we have chosen to consider the exudation of seedlings, therefore on young growing roots.  
417 To complete the analysis, it would be interesting to evaluate the impact of seedling age on exudation and  
418 to examine the dynamics of compound release. We noticed that root exudate samples collected at close  
419 dates in time had little biological variability between them. There are likely variations due to the batches  
420 of seeds and their conservation, which explain the variability within the species.

421 The metabolic profile of exudates is widely described as a witness to the interactions of the plant with its  
422 environment, and thus constitutes a good indicator of stress conditions. Here we developed a simple,  
423 repeatable and reproducible protocol. This protocol allowed discriminating compounds from closely  
424 related plant species despite biological variability. The possible applications are numerous: effects of  
425 microbial pathogens or elicitors, effects of abiotic stresses including drought, cold or heavy-metal  
426 pollutants. Quantitative and kinetics studies of exudation can also be implemented. This opens very  
427 interesting prospects in unravelling composition and function of root exudates.

428

## 429 **5. Conclusion**

430 The protocol developed in this study to analyse the low molecular weight molecules secreted by roots by  
431 NMR using a target-free approach allowed to discriminate pea and faba bean seedlings according to their  
432 exudate composition. This protocol therefore appears very promising for studying the composition of root  
433 exudates from different plant species and their evolution in response to biotic and abiotic stresses.

434

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664

Fig. 1. Schematic description of the experimental procedure used for growing pea seedlings *in vitro* and collecting and preparing root exudates for NMR analyses.

Note that the first steps (1-4) are performed under sterile conditions. Four collecting conditions of root exudates were tested: exudation for 120 minutes in an aqueous solution of calcium chloride ( $\text{CaCl}_2$ ), 120 minutes in water, 15 minutes in an aqueous solution of calcium chloride and 15 minutes in water. The exudate harvesting conditions written in red (15 minutes in an aqueous solution of calcium chloride) were selected to compare the exudation profiles of pea and faba bean and to identify the metabolites. DSS: Sodium trimethylsilylpropanesulfonate; NOESY: Nuclear Overhauser Effect spectroscopy; J-RES: J-resolved Spectroscopy; COSY: COrelated Spectroscopy; TOCSY: TOtal COrelated Spectroscopy; HSQC: Heteronuclear Single Quantum Correlation; HMBC: Heteronuclear Multiple Bond Coherence.

Fig. 2. Comparison of  $^1\text{H}$  1D NOESY spectra (600 MHz, 298 K) of pea exudates according to exudation time and collection medium.

Fig. 3. Principal component analyses of pea metabolomic fingerprints according to the root exudate harvest conditions.

Individuals are coloured according to the harvesting conditions. A: exudation during 15 minutes in calcium chloride solution (black dot) or in water (blue triangle); B: exudation during 120 minutes in calcium chloride solution (black dot) or in water (blue triangle); C: exudation in calcium chloride solution for 15 minutes (orange dot) or 120 minutes (red triangle); D: exudation in water for 15 minutes (orange dot) or 120 minutes (red triangle). The plotted ellipses represent a 95% confidence interval around the centroid corresponding to the largest point.

Fig. 4. Principal component analyses of pea and faba bean metabolomics fingerprints.

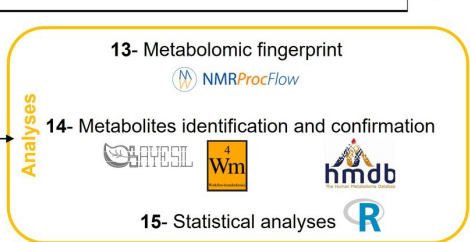
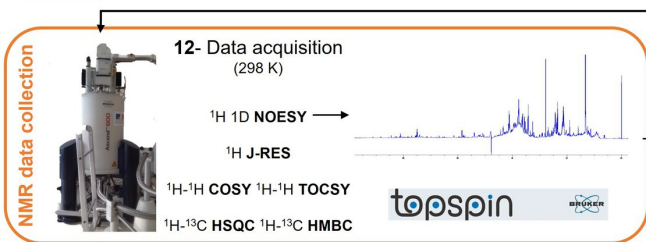
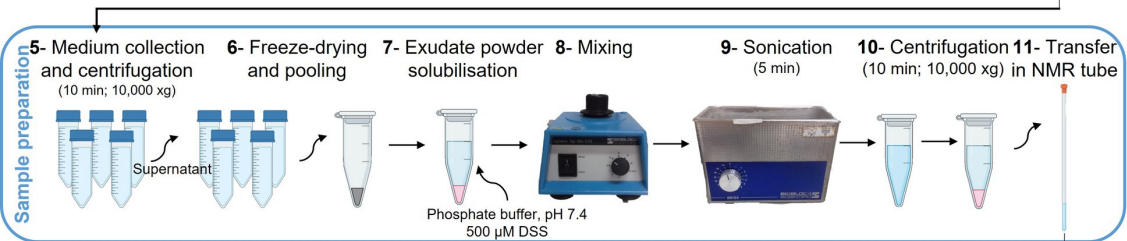
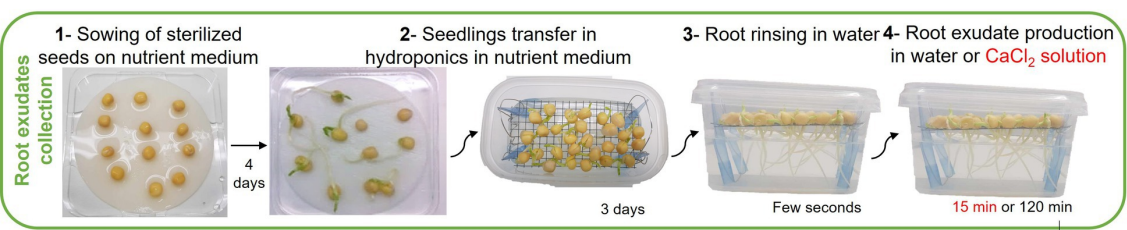
The exudation is compared in the same conditions: the exudates were harvested in calcium chloride aqueous solution for 15 minutes. Individuals are coloured by species: faba bean with purple dot and pea with green triangle. The plotted ellipses represent a 95% confidence interval around the centroid corresponding to the largest point.

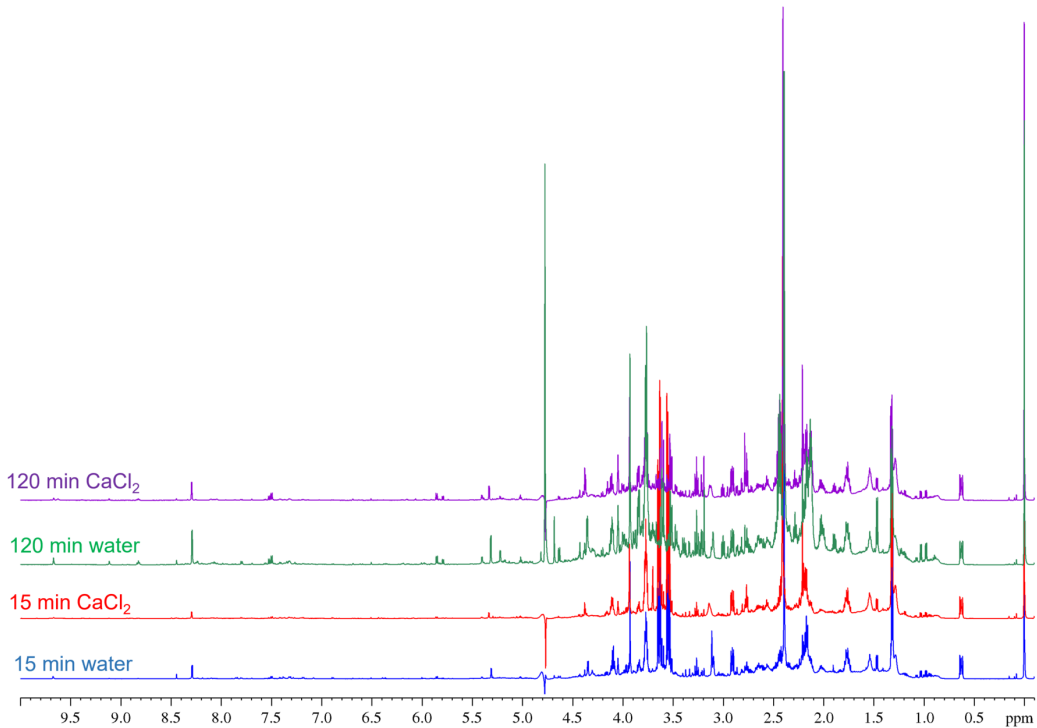
Fig. 5. Identification of pea metabolites on  $^1\text{H}$  1D NOESY spectrum (600 MHz, 298 K).

Three regions corresponding to aromatic compounds (top), sugars and alcohols (middle) and amino and organic acids (down) are displayed. Identified compounds are indicated by arrows; compounds specific of species are coloured in orange. Peaks corresponding to unknown compounds specific of each species are indicated by an orange asterisk.

Fig. 6. Identification of faba bean metabolites on  $^1\text{H}$  1D NOESY spectrum (600 MHz, 298 K).

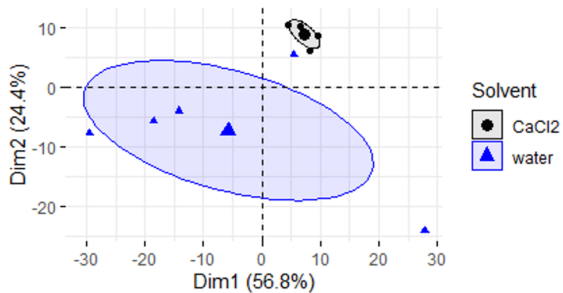
Three regions corresponding to aromatic compounds (top), sugars and alcohols (middle) and amino and organic acids (down) are displayed. Identified compounds are indicated by arrows; compounds specific of species are coloured in orange. Peaks corresponding to unknown compounds specific of each species are indicated by an orange asterisk.



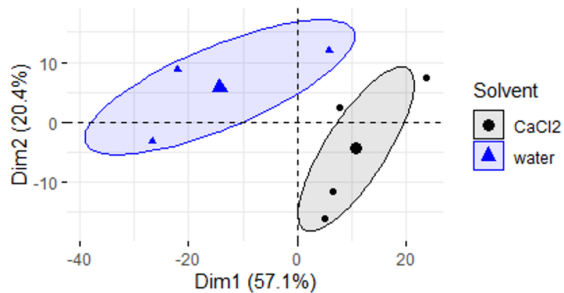
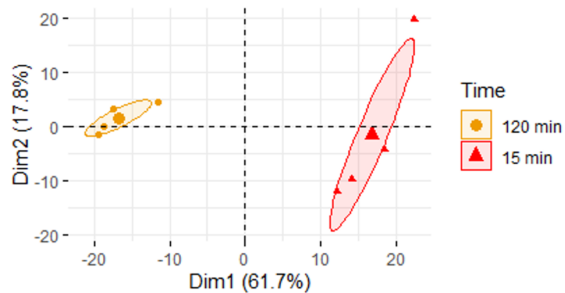


**A**

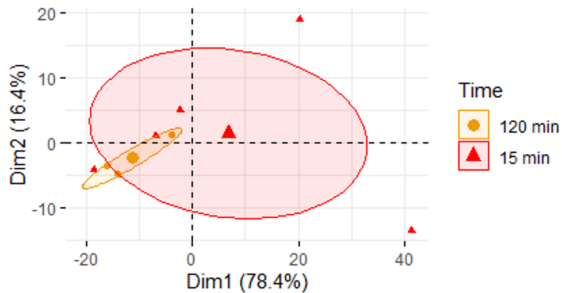
15 minutes exudation

**B**

120 minutes exudation

**C**CaCl<sub>2</sub> exudation**D**

Water exudation



# 15min, CaCl<sub>2</sub> exudation

