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Implication of phytometabolites on metal tolerance of the pseudo-metallophyte *-Rosmarinus officinalis-* in a Mediterranean brownfield

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1 **Implication of phytometabolites on metal tolerance of the pseudo-**
2 **metallophyte *Rosmarinus officinalis* in a Mediterranean brownfield**

3

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16

17 Abstract

18 This study highlights the trace metal and metalloid (TMM) accumulation in *Rosmarinus*
19 *officinalis* L. and its chemical responses when exposed to high levels of contamination. *R.*
20 *officinalis* individuals growing along a gradient of mixed TMM soil pollutions, resulting from
21 past industrial activities, were analysed. Several plant secondary metabolites, known to be
22 involved in plant tolerance to TMM or as a plant health indicator, were investigated. The
23 levels of thiol compounds and phytochelatin precursors (cysteine and glutathione) in the
24 shoots were measured in the laboratory, while a portable non-destructive instrument was used

25 to determine the level of phenolic compounds and chlorophylls directly on site. The level of
26 Pb, As, Sb and Zn contaminations within the soil and plants was also determined.
27 The results highlighted a decrease of TMM translocation with increases of soil
28 contamination. The concentration of TMM in the shoots followed the Mitscherlich equation
29 and reached a plateau at 0.41, 7.9, 0.37, 51.3 mg.kg⁻¹ for As, Pb, Sb and Zn, respectively. In
30 the shoots, the levels of thiols and phenols were correlated to concentrations of TMM.
31 Glutathione seems to be the main thiol compounds involved in the tolerance to As, Pb and
32 Sb. Phenols indices, using non-destructive measurements, may be considered as an easy way
33 to establish a proxy to estimate the TMM contamination level of the *R. officinalis* shoots. The
34 study highlights metabolic processes that contribute to the high potential of *R. officinalis* for
35 phytostabilisation of TMM in contaminated areas in the Mediterranean.

36

37 Keywords

38 Field experiment, metalloids, metal translocation, rosemary, stress responses, trace metals.

39

40 1. Introduction

41

42 Trace metals and metalloids (TMM) naturally occur in the environment due to their presence
43 in the Earth's crust. However, the past century has seen them become a major environmental
44 issue. Concentrations of TMM above background levels have been observed in soils subject
45 to agricultural and industrial activities, and are consequently transferred to the trophic web,
46 notably via plants (He et al., 2005; Nawab et al., 2015; Sarwar et al., 2017). Phytoremediation
47 is considered an innovative, cost-effective and ecologically beneficial biotechnology (Sawar
48 et al., 2017). However, to select appropriate plant species for phytoremediation purposes, an

49 understanding of plant stress tolerance mechanisms to TMM is required (Clemens et al.,
50 2002; Antoniadis et al., 2017).

51

52 To counteract TMM toxicity, plants have complex mechanisms of detoxification, ranging
53 from the whole plant to molecular level (see review by Singh et al., 2016). At the whole plant
54 level, the reduction of the translocation is argued as an important mechanism for plant
55 tolerance to high TMM concentrations. Previous studies have highlighted a non-linear
56 relation between TMM concentration in soil and in shoots (De Oliveira and Tibbett, 2018;
57 Green et al., 2006). Physiological mechanism arguably attenuates TMM uptake and transfer
58 to the aerial parts when the soil concentration goes beyond a certain level, leading to a
59 plateau pattern (Hamon et al., 1999). Several authors have applied the Mitcherlich equation to
60 fit their data to help describe the observed plateau (Hamon et al., 1999; Chaney et al., 1997;
61 Logan and Chaney, 1987).

62 At the molecular and cell level, increases in the biosynthesis of some phytometabolites can
63 counterbalance the generation (Michalak, 2006) and/or activity (Nimse et al, 2015) of
64 reactive oxygen species (ROS). They are known to reduce photosynthetic activity through
65 photosystem activity alteration and/or chlorophyll biosynthesis decrease (e.g. Clijsters and
66 Assche, 1985; Assche and Clijsters, 1990; Maleva et al., 2012). Some metabolites have
67 potential antioxidant activities, like phenolic compounds (e.g. flavonol, anthocyanidin,
68 isoflavone), and may reduce ROS production/reactivity (Michalak, 2006; Nimse et al., 2015).
69 Others, like phytochelatins and glutathione, which are thiol compounds, have complex
70 properties (Yadav, 2010) and can bind TMM and transfer them into the vacuole where they
71 are inactivated (Hall, 2002). However, the biosynthesis of these defence metabolites
72 represents an associated cost for the plant, which needs to find a trade-off between growth
73 (primary metabolism) and stress resistance (secondary metabolism) (Hems and Mattson,

74 1992). A higher allocation to one function will result in the decrease in the allocation to the
75 other function (Caretto et al., 2015).

76 To elucidate those mechanisms and get a better understanding of the phytometabolites
77 involved, many papers consider model plant species, metallophytes, under controlled
78 conditions (see reviews by Memon and Schröder, 2009; Hossain et al., 2012; Singh et al.,
79 2016). However, it is less common to consider wild species in field conditions. Metallophytes
80 are plant species that have evolved adaptive mechanisms, such as metal tolerance, enabling
81 them to grow efficiently on TMM-rich soils (Frérot et al., 2006). Pseudo-metallophytes are
82 plants found both in TMM polluted and non-polluted areas, but the phenotypic adaptations to
83 TMM are far less understood (Salducci et al., 2019). *Rosmarinus officinalis* L., a perennial
84 plant species native to the Mediterranean is able to grow in soils with high levels of TMM
85 contamination (Testiati et al., 2013; Gelly et al., 2019; Madejon et al., 2009). Chemical
86 investigations are therefore necessary to improve understanding of the TMM tolerance
87 mechanisms of this wild plant species. Tolerance mechanisms refers to all mechanisms from
88 the absorption to the compartmentalization of TMM in plant parts to avoid TMM toxicity.
89 Organic acids, amino acids and thiols are ligands that enable chelation of TMM (Singh et al.,
90 2016). Phenolic compounds and notably anthocyanins are also part of plant TMM stress
91 defence response (Cheynier et al., 2013).

92 This study focuses on the chemical responses of *R. officinalis* in a field setting in the south-
93 east of France, along a wide gradient of varying TMM soil pollutions resulting from past
94 industrial activities. We hypothesized that TMM stress responses in *R. officinalis* in the field
95 mobilizes identical mechanisms than those reported under controlled conditions and that the
96 contamination gradient in the field is sufficient to generate contrasting results. Consequently,
97 we aimed to i) determine patterns of TMM uptake and translocation to *R. officinalis* shoots
98 across a broad range of TMM contamination levels, ii) highlight how contamination level

99 affects secondary metabolites composition of *R. officinalis*, iii) determine the effect of heavy
100 metal stress on the primary/secondary metabolism balance of *R. officinalis* due to change in
101 resource allocation. Levels of several secondary metabolites, phenolic compounds including
102 anthocyanins, thiol compounds and phytochelatin precursors, primary metabolites and
103 chlorophylls were analysed in the shoots and correlated to contamination levels, notably lead
104 (Pb), arsenic (As), antimony (Sb) and zinc (Zn), in the soil and the plant.

105

106 2. Materials and methods

107

108 2.1. Study area

109 The study area, located in the Calanques hills, is a peri-urban area of Marseille in South-East
110 France. It is characterized by a Mediterranean climate and matorral vegetation dominated by
111 *Rosmarinus officinalis*, *Cistus albidus*, *Quercus coccifera* and *Pistacia lentiscus*.

112 The former Pb smelter factory located in the Escalette Calanque, processed argentiferous
113 galena ores from 1851 to 1925 by pyrometallurgical processes (Daumalin and Raveux, 2016).

114 This activity generated massive Pb and Zn-rich slags (Gelly et al., 2019; Testiati et al., 2013)
115 and atmospheric emissions of highly metal-concentrated particles, specifically with Pb, As
116 and Sb (Laffont-Schwob et al., 2016; Testiati et al., 2013). Previous studies highlighted the
117 metal and metalloid contaminations of the smelter surroundings, in particular by Pb, As, Sb
118 and Zn (Heckenroth et al., 2016; Affholder et al., 2013; Testiati et al., 2013; Gelly et al.,
119 2019).

120 Eight sites were selected having similar soil characteristics, vegetation and climatic
121 conditions and included in the National Park of Calanques. Sampling areas were chosen
122 along a transect going away from the former smelter factory through the Garenne valley
123 following the direction of the prevailing wind, and then returning to the sea through the

124 Mounine valley (see map in supplementary data 1). Seven sites were selected along a
125 suspected contamination gradient: G0 on the site of the factory (close to the horizontal
126 chimney exit), G1, G2, G3, in the Garenne valley, G4 and G5 in the Mounine valley, and G6
127 in Sormiou cove which is away from the Escalette and the urban area and is considered
128 scarcely contaminated from the mapping of soil element concentrations conducted in an
129 extended area around the factory site (Laffont-Schwob et al., 2016). The last site, S3 in the
130 never industrialized part of the Calanques hills, was considered as a reference site as
131 described by Affholder et al. (2014).

132 The soils were stony, and their thicknesses varied from place to place but were generally less
133 than 50 cm. Soils were alkaline with an average pH (ISO 10390) of between 7.8 and 8.1,
134 belonging to the typical pH range of soils from calcareous areas. Soil fertility was low with
135 total organic carbon contents varying from 3.6 to 14.2 %, total Kjeldahl nitrogen (ISO 11261)
136 contents from 0.28 to 0.72 %, assimilable phosphorous (ISO 11263) from 0.010 to 0.057 g P.
137 kg⁻¹ and Cation Exchange Capacity (CEC, ISO 22036) from 15 to 42 cmol⁺.kg⁻¹.

138

139 2.2. Plant and soil sampling

140 Sampling was undertaken, as reported by Affholder et al. (2013), on 5 individuals of *R.*
141 *officinalis* on each site. To obtain representative samples of each site, an area of 100 m² (10 ×
142 10 m) was delimited on the 8 sites. The plant cover was over 60 % on the selected areas. The
143 5 individuals of *R. officinalis* were selected according to a cross pattern inside the delimited
144 area, spaced by around 2 m, with similar sizes, i.e. heights and collar diameters, and same
145 phenological stage. A total of 5 plant/soil couples were taken on each site. Shoots were
146 collected for TMM analysis and phytometabolite (phenolics and thiols) analysis. Soil samples
147 were collected from the top 15 cm (after removal of the litter) in the mycorrhizospheric area

148 of the plants. Fresh plant and soil samples were stored in clean plastic bags for transport to
149 the laboratory.

150 Soil samples were sieved to 2 mm on site, air-dried at room temperature in the laboratory and
151 then ground (RETSCH zm 1000 with tungsten blades) to pass through a 0.2 mm titanium
152 sieve.

153

154 2.3. Pseudo total TMM in soils

155 Soils were mineralized in a microwave mineralizer (Milestone Start D) using aqua regia (1/3
156 HNO_3 + 2/3 HCl). The mineralization products were filtered with a 0.45 μm mesh and the
157 TMM concentrations were determined by ICP-AES (inductively coupled plasma atomic
158 emission spectroscopy, Jobin Yvon Horiba, Spectra 2000) for Zn and Pb (Lotmani et al.,
159 2011), and by GF-AAS (graphite furnace atomic absorption spectroscopy, Thermo Scientific
160 ICE 3000) for As and Sb. Quality controls and accuracy were checked using standard soil
161 reference materials (CRM049–050, from RTC-USA) with accuracies within $100 \pm 10 \%$.

162

163 2.4. Mobile TMM in soils

164 A 0.05 M EDTA ($\text{pH} = 7.00 \pm 0.05$) solution was used as extractant and was prepared
165 following the protocol of the CBR (Community Bureau of Reference) (Quevauviller, 1998).
166 A volume of EDTA solution corresponding to a ratio of 1/10 w/v was added to dry and
167 ground soil sample and placed into a PTFE (Teflon) tube (triplicates per soil sample). The
168 mixture was stirred at room temperature on an orbital shaker (Fisher Scientific Bioblock
169 SM30B) at 125 rpm for 1 h. The tubes were then centrifuged for 10 min at 8000 rpm (JP
170 SELECTA, Médifriger BL-S), and the supernatants were collected and filtered to 0.45 μm .
171 The resulting solutions were stored at 4 °C until analysed by ICP-AES or GF-AAS.

172

173 2.5. Plant TMM analysis

174 Shoots samples were thoroughly washed using Milli-Q water to eliminate any soil particles.
175 Samples were dried at 40 °C over 1 week, after which leaves and stems were separated and
176 then ground at 0.2 mm (RETSCH zm 1000 blender with tungsten blades and titanium sieve).
177 About 0.5 g dry matter of each sample was digested in a microwave mineralizer system
178 (Milestone Start D) with a HNO₃–HCl mixture (volume proportion ratio 2/1). After filtration
179 (0.45 µm), acid digests were analysed for Pb and Zn contents by ICP-AES, and for As and Sb
180 contents by graphite furnace AAS (Rabier et al., 2007). Standard plant reference materials
181 (DC 73349) from the China National Analysis Centre for Iron and Steel (NCS) was analysed
182 as a part of the quality control protocol (accuracies within 100 ± 10 %). Translocation factors
183 (TFs), i.e. ratios of shoot concentrations vs. roots concentrations (data of roots concentrations
184 from Affholder et al., 2014) were calculated.

185

186 2.6. Plant phytometabolite analysis

187 2.6.1. Total free thiols analysis

188 Total free thiols in shoots were analysed by UV spectrophotometer (JASCO V-670) after
189 lyophilization, grinding and derivatization. Thiols were extracted accordingly to the modified
190 protocol of Potesil et al. (2005). A 0.5 g of ground sample of *R. officinalis* and 5 mL of 0.2 M
191 phosphate buffer at pH 7.2 were introduced into a centrifugation tube. The mixture was
192 stirred for 30 min at 30 rpm (Fisher Scientific Bioblock SM30B) and at 10 °C. The samples
193 were then centrifuged at 14000 g for 30 min at 4 °C (JP Selecta, Médifriger BL-S). The
194 supernatant was recovered and filtered on 0.45 µm PES (polyethersulfone) filters and stored
195 at -80 °C until analysis and purification.

196 Despite filtration, *R. officinalis* extracts contained aromatic molecules (phenols, flavones, etc)
197 that absorbed in UV (Almela et al., 2006). To avoid background noise during UV spectrum

198 measurement, samples were purified using cartridges filled with 50 mg of XAD-4 (styrene-
199 divinyl benzene) resin previously conditioned by percolating 3 mL of acetonitrile and then
200 rinsing with 3 mL of deionized water. A derivatisation was performed and consisted in the
201 addition of ethylpropiolate. The reaction between ethylpropiolate and free thiols produces
202 thioacrylate, a molecule presenting a maximal absorbance at 285 nm (Coulomb et al, 2017).
203 Immediately before UV spectrum measurement, 10 μ L of ethylpropiolate was added to
204 0.2 mL of purified sample and 0.2 M phosphate buffer pH 9 qs 10 mL. Calibration was
205 undertaken with a solution of cysteine in a concentration ranging between 0 and 140 μ M.
206 Results are expressed in μ mol g^{-1} of dry matter.

207

208 2.6.2. Cysteine and Glutathione analysis

209 Fresh plant samples were ground in liquid nitrogen. Amino acids and glutathione were
210 extracted accordingly to the modified protocol of Bates et al. (1973). A mixture of sample
211 and sulfosalicylic acid at 3 % were sonicated and centrifuged for 10 min at 8000 rpm (JP
212 selectam medifriger-BL-S). Supernatants were filtrated at 0.45 μ m and stored at -20 $^{\circ}$ C until
213 analysis. Analysis was performed using a high-pressure ion chromatography (ICS 3000,
214 Dionex) equipped with an AminoPacTM PA 10, constituted by a guard column and an
215 analytical column (2 x 250 mm), and a pulsed amperometric detector (ED40- Dionex).

216

217 2.6.3. Chlorophyll and phenol indices

218 Plant physiological indices were estimated optically using a Multiplex[®] 3 non-destructive
219 measurement equipment (FORCE-A, Orsay, France; Agati et al., 2011). This portable
220 fluorometric device uses fluorescence technology with multiple excitations to measure
221 constitutive and induced epidermal phenols, flavonols, anthocyanins, chlorophylls and a
222 chlorophyll-to-flavonoid ratio referred to as the nitrogen balance index (NBI) (Rabier et al.,

223 2014). Different combinations of the blue-green, red and far-red fluorescence signals at the
224 various excitation bands could be used as indices of the different compounds (Cerovic et al.,
225 2008; Agati et al., 2011). In spring, each individual from 6 of the 8 sites (G1 to G6, 5
226 replicates per site) was flashed 25 times per individual. Data from sites G0 and S3 were
227 analysed in autumn and are not included in this study as the phenological stage of plants
228 differed. The indices obtained by Multiplex® cannot be directly converted into
229 concentrations since calibration in the laboratory is not satisfactory. Indeed, Mutiplex®
230 equipment is measuring metabolites from the surface tissues of the leaves while spectrometric
231 analysis, needing a leaf extraction, make it difficult to distinguish the concentrations in upper
232 and inner tissues. Therefore, the phenolics and chlorophyll indices obtained were not
233 converted into concentrations. However, the measurement is free from the sampling
234 geometry, allowing a field comparison between populations from the different areas as
235 demonstrated in many experiments (Ben Ghozlen et al., 2010; Bürling et al., 2013; Louis et
236 al., 2009).

237

238 2.7. Statistical analyses

239 All statistical analyses and graphical presentation was performed using R software (version
240 3.5.0, R Core team, 2018). Spearman's correlation test was used for the correlations
241 involving the phytometabolites as the data did not follow a normal distribution even after log
242 transformation. For the correlation involving only the TMM concentrations in the soils and
243 TFs, Pearson's correlation test was used after logarithm transformation of the data. Both
244 correlation tests were performed using the function Rcorr() from the *Hmisc* package (Harrell
245 et al., 2018).

246 The non-linear regressions observed between the shoots and soils pseudo-total concentrations
247 were modelled by the Mitscherlich equation, using the functions available on the package

248 *nls2* (Gothendieck, 2013) and *nlme* (Pinheiro et al., 2018). The equation used for the model
249 was:

$$250 \quad y=a+b(1-e^{-u}),$$

251 where y is Log (TMM in shoots), a is the intersection of the model with the y axis i.e. the
252 TMM background concentration in shoot tissues, b is the asymptote (plateau) of TMM in
253 shoots, u is the slope of the line in the area between the intersection with the y axis and the
254 asymptote, with $u= (x-a)/b$ for Pb and $u=(x-a)$ for Sb and Zn where x is Log (total TMM in
255 soil).

256

257 3. Results and discussion

258 3.1. TMM contamination in soils

259 The average mobile concentration of TMM in soils is presented in Table 1, having
260 maximums values of 155, 3522, 17 and 560 mg.kg⁻¹ of dry weight (DW) for As, Pb, Sb and
261 Zn, respectively. Maximum average concentrations were measured in G0 for As and Sb and
262 in G2 for Pb and Zn, both sites being near to the former smelter. The average mobile
263 fractions (percentage of pseudo-total concentrations) are presented Table 1. Results indicate
264 maximum mobile fractions of 10.9, 57, 4.2 and 33 % for As, Pb, Sb and Zn, respectively. On
265 average, Pb and Zn presented the highest mobile fractions, explaining the measured high
266 mobile concentrations. For Pb, Sb and Zn, the average mobile fractions were in the same
267 range of value for all the sites, highlighting that mobility is not related to the contamination
268 level. For As, the mobile fractions ranged between 10.9 and 0.2 %, that increased with
269 contamination levels, excepting S3. We understand that this is the first report on the mobile
270 fraction of these elements in the Calanques and will provide improved insights around the
271 potential risk of transfer to the biota.

272

273 3.2. TMM accumulation and translocation in shoots of *R. officinalis*

274

275 3.2.1. Accumulation in shoots

276 TMM accumulation in shoots of *R. officinalis* is presented in Table 1. Results showed
277 maximum average concentrations in shoots of 0.89, 16.2, 1.20 and 59.9 mg.kg⁻¹ DW for As,
278 Pb, Sb and Zn, respectively. Therefore, *R. officinalis* may not be identified as an
279 hyperaccumulator species according to Baker and Brooks (1989) since, regardless of the soil
280 contamination level, none of the *R. officinalis* individuals presented elemental concentrations
281 in shoots greater than 1000 mg.kg⁻¹ for Pb and 10000 mg.kg⁻¹ for Zn. Maximal TMM
282 concentrations were not measured in *R. officinalis* individuals growing in the most
283 contaminated soils. For instance, for Pb and Sb, the highest shoot concentrations were
284 measured in an individual from the site G1. This was growing in a soil presenting mobile
285 concentrations about 5 times lower for Sb and 1.5 times lower for Pb, compared to the
286 highest mobile concentration found in the soils.

287

288 However, regarding the high discrepancy of soil contamination, the results were also
289 processed by plant-soil couples. Figure 1 shows, for each plant-soil couple, the shoots
290 concentrations in function of the pseudo-total soil concentrations (logarithm transformed
291 values).

292 For the 4 elements, results highlighted that below a certain level of soil contamination
293 concentrations in shoots were linearly correlated with the soil concentrations, and, above, the
294 shoot concentrations reached a plateau. This kind of behaviour has been previously observed,
295 particularly for Cd and Zn (Dudka and Adriano, 1997; Hamon et al., 1999; Green, 2003). The
296 observed plateau-type response of *R. officinalis* was modelled with the Mitscherlich plateau

297 equation (significant correlation, $p \leq 0.001$) proposed by Logan and Chaney (1987), and
298 already applied by Azizian et al. (2011, 2013) to model Cd uptake by lettuce and corn.

299

300 Asymptote values for As, Pb, Sb and Zn concentrations in *R. officinalis* shoots were
301 calculated from the value of b obtained from the Mitschelich model. These values are 0.41,
302 7.9, 0.37 and 51.3 mg.kg^{-1} for As, Pb, Sb and Zn, respectively. The plateau could be
303 explained either by mechanisms occurring in the soil, limiting the presence of TMM in the
304 soil solution, or by plant physiological mechanisms mitigating TMM uptake and/or
305 translocation (Hamon et al., 1999). In this study, a decrease of the concentration of TMM in
306 the soil solution from heavily contaminated sites could be excluded (Antoniadis et al., 2017).
307 Indeed, mobile fractions are important and by consequent mobile concentrations in soils of
308 the heavily contaminated sites are high. Physiological mechanisms limiting the translocation
309 seem therefore more likely.

310

311 3.2.2 Translocation

312 Translocation factors highlight the plant's ability to translocate TMM into the shoots. For a
313 phytostabilization process, and in the case of *R. officinalis* which is an edible plant, TFs lower
314 than 1 are expected (Mendez and Maier, 2008).

315 Average TFs obtained per site are presented Table 1. Translocation factor values greater than
316 1 were highlighted on the less contaminated sites, mainly on site G6, where they reached
317 2.15, 3.37, 3.40 and 1.65 for As, Pb, Sb and Zn, respectively. Significant linear negative
318 correlations (Pearson test, $p \leq 0.05$) with correlation coefficients of -0.55 for As, -0.71 for Pb,
319 -0.73 for Sb and -0.81 for Zn were identified between TFs and pseudo-total soil
320 concentrations (Log transformed). This shows a strong and linear decrease of TMM
321 translocation when concentrations in soil is increased. In highly contaminated sites the

322 average TF values were much lower than 1. In G0 for instance, values of 0.02, 0.03, 0.06 and
323 0.36 were obtained for As, Pb, Sb and Zn, respectively. In this case the highest TF values
324 were associated with Zn, which is congruent with the results of De la Fuente et al. (2014).
325 This may relate to the fact that Zn is an essential element for plants.

326 For the 4 elements studied, when the concentration increased in roots, the translocation was
327 not enhanced. Previous studies showed similar results, namely that *R. officinalis* accumulated
328 more TMM in the roots than in the shoots indicating a low and controlled transport of the
329 contaminants (De la Fuente et al., 2014; Parra et al., 2014). Affholder et al. (2014) has
330 suggested the involvement of a root filter phenomena involving arbuscular mycorrhizal fungi
331 (AMF) and dark septate endophyte (DSE) colonisation, promoting TMM root containment.
332 The limitation of the translocation of the TMM from the roots to the shoots is an important
333 mechanism for the reduction of stress due to TMM occurrence in the plant. However, part of
334 the TMM are still translocated into the shoots. Root to shoot TMM transfer occurs via the
335 xylem (Clemens et al., 2002). In the xylem TMM are presents as hydrated ions or as
336 complexes with chelates, mainly organic acids, amino acids or peptides and phytochelatin
337 (Briat and Lebrun, 1999; Clemens et al., 2002). Occurrence of TMM in the shoots may
338 activate tolerance mechanisms to limit the oxidative stress induced by the contaminants.

339

340 3.3. Phytometabolites involved in *R. officinalis* tolerance to TMM and identification of stress
341 biomarkers

342 The occurrence of TMM in a plant's shoots can lead to a decrease of chlorophyll biosynthesis
343 and a subsequent reduction in photosynthesis (Assche and Clijsters, 1990). However, some
344 species develop tolerance mechanisms to limit the stress induced by high TMM
345 concentrations in their shoots (Antoniadis et al., 2017; Yadav et al., 2018). This mechanism
346 involves the biosynthesis of phytometabolites mitigating the cause, by sequestering the TMM

347 in the vacuole, or mitigating the consequences, by limiting the oxidative stress induced by the
348 TMM (Singh et al., 2016; Sytar et al., 2013).

349 This study considered several tolerance phytometabolites, an amino acid (cysteine) and a
350 peptide (glutathione) precursors of the phytochelatins, the total free thiols and the phenolics
351 to reveal the tolerance mechanisms (Singh et al., 2016; Yadav et al., 2018) involved in the
352 shoots of *R. officinalis* individuals growing in a gradient of contaminated soils. Chlorophyll, a
353 biomarker of health status of the plant, was monitored as a proxy of the effect of TMM on the
354 primary metabolism and indirectly of the photosynthetic activity of *R. officinalis* (Shakya et
355 al., 2008; Maleva et al., 2012; Chandra and Kang, 2015) .

356

357 3.3.1. Stress tolerance phytometabolites

358 Complexing compounds: Thiols

359 Thiols include molecules like cysteine, glutathione and phytochelatins which are known to
360 sequester and for their role against the oxidative stress associated to TMM occurrence in
361 plants (Hall, 2002; Kawashima et al., 2004). Concentrations of total free thiol were
362 determined in the shoots of *R. officinalis* from 6 sites (G1 to G6) and average concentrations
363 are presented in Table 2. Results showed that the average concentrations of total free thiols in
364 *R. officinalis*' shoots ranged from 8.7 ± 2.3 to 30.4 ± 7.5 $\mu\text{mol of } -\text{SH.g}^{-1}$ DW. Lower
365 concentrations were found in *R. officinalis* individuals from sites G2 and G6 where,
366 respectively, the highest and lowest concentrations of mobile TMM in the soil were observed
367 for the 6 sites. As the concentrations in the shoots are not linearly correlated to the level of
368 soil contamination, the relation free thiol/TMM in shoots was investigated per *R. officinalis*
369 individuals and not per site. The results are presented Figure 2 and indicate a significant
370 positive correlation between the concentrations of free thiols and Sb and Zn in *R. officinalis*'
371 shoots (Spearman's test, $p \leq 0.05$, $\rho = 0.41$; 0.63 for Sb and Zn, respectively). Total free thiol

372 biosynthesis seemed elicited when the concentration of Sb and Zn increased in *R. officinalis*
373 shoots. It appears that total free thiols play an important role in *R. officinalis* ability to tolerate
374 stresses induced by the occurrence of TMM in the shoots, either because of their antioxidant
375 properties, or their ability to detoxify the TMM by sequestration.

376 The thiol compounds known to be involved in TMM detoxification are the phytochelatins,
377 alongside glutathione (GSH) and non-protein cysteine, which are both involved in the
378 phytochelatin metabolic pathway (Cobbett, 2000). Concentrations of cysteine and GSH were
379 measured in *R. officinalis*' shoots (Table 2). The average concentrations measured in shoots
380 ranged between [0.28 and 19.7] and [15.7 and 265] nmol.g⁻¹ FW for cysteine and glutathione,
381 respectively.

382

383 Positive significant correlations were highlighted between the concentration of free thiol and
384 cysteine ($\rho=0.69$, $p<0.05$), and glutathione ($\rho=0.66$, $p<0.05$) (Table 3). The free thiols
385 measured in the shoots of *R. officinalis* were at least partly constituted by non-protein
386 cysteine and GSH. Surprisingly, cysteine and glutathione concentrations showed no
387 correlation, although cysteine level is known as one of the factors controlling glutathione
388 synthesis (Noctor et al., 1998). This could indicate that in this study cysteine level is not a
389 limiting factor for the biosynthesis of glutathione. Indeed, except for the individuals growing
390 in site G4, the results did not show a significant decrease of cysteine when glutathione
391 concentration was increasing. *R. officinalis* was able to efficiently maintain the level of
392 cysteine in the shoots despite an increase of glutathione biosynthesis. The low level of
393 cysteine in G4 is the reason for extremely high average GSH/Cys ratio at this site. One
394 possible explanation would be a sulfur (S) deficiency in the plants from this site. Indeed,
395 cysteine synthesis is dependent on a sufficient sulfate supply from the roots (Wirtz and
396 Droux, 2005).

397

398 The results also highlighted some significant correlations between the concentrations of free
399 thiols, non-protein cysteine and glutathione and the concentrations of TMM in the shoots
400 (Table 3) but not with the concentrations of TMM in the soil or in the roots (results not
401 shown). This indicates that the production of thiols, including cysteine and glutathione, is
402 triggered by the occurrence of contaminants in the shoots. The correlations, in the data
403 excluding G4 where the cysteine synthesis seemed disturbed, suggest that glutathione is the
404 main thiol compound involved in the stress alleviation of As, Pb and Sb, which is in
405 agreement with previous studies (Li et al., 2009; Pourrut et al., 2011; Ortega et al., 2017).
406 Concerning Zn, the results indicate the involvement of cysteine. This is congruent with the
407 results of Zeng et al. (2011), that showed involvement of cysteine in the Zn homeostasy
408 instead of phytochelatin or glutathione in *Arabidopsis paniculata*.

409

410 Antioxidant compounds: phenolics

411 The toxicity of the TMM in plants can be caused by the formation of ROS, creating an
412 oxidative stress (Gamalero et al., 2009; Muszynska and Labudda, 2019). Oxidative damages
413 in biological system are varied and can affect DNA, amino acids and proteins, as well as
414 lipids from the cell membrane, modifying their properties (Briat and Lebrun, 1999; Farid et
415 al., 2020). Phenolics can inhibit the lipid peroxidation phenomena by trapping the lipid
416 alkoxyl (Michalak, 2006).

417 The average of the indices measured for *R. officinalis* from sites G1 to G6 are presented in
418 Table 2. The lowest and highest indices were measured on *R. officinalis* individuals from G2
419 and G6, respectively. However, the analysis of the data per individuals has provided a better
420 understanding of the impact of TMM concentration on the occurrence of phenolics. Positive
421 correlations between As, Pb and Sb concentrations in *R. officinalis* shoots and the phenolic

422 indices (Spearman's test, $p \leq 0.05$, table 3) were shown. Increase of phenolics in plants
423 following a Pb contamination gradient has already been observed in *Phaseolus vulgaris*
424 (Hamid et al., 2010). Phenolics appears to be involved in the tolerance of *R. officinalis* to
425 TMM. They can act as TMM chelatants, or antioxidant compounds, limiting in both cases the
426 oxidative stress caused by TMM presence (Michalak, 2006).

427

428 Health biomarker: Chlorophylls

429 Among the primary metabolites in plants, chlorophylls are particularly interesting to study as
430 they provide information about a plants photosynthetic ability (Blankenship, 2010). The
431 results did not highlight any significant correlation between chlorophyll index and the
432 concentration of TMM in the shoots of *R. officinalis*. This means that the contamination level
433 in the shoots is either not important enough to generate a destruction of the photosynthetic
434 system, or that the TMM detoxification mechanisms are efficient enough to avoid a
435 deterioration of the chlorophyll pool (Yadav et al., 2018; Maleva et al., 2012).

436 Chlorophylls index is negatively correlated with phenolics index. This may be related to a
437 trade-off made by *R. officinalis* in order to deal with the contamination. Indeed, providing an
438 adequate adaptation mechanism against environmental stresses has a cost for the plant. Plants
439 exposed to high metal have to make a trade off to synthesize protection metabolites instead of
440 primary metabolites like chlorophylls, and allocate more carbon towards secondary than
441 primary metabolism (Hems and Mattson, 1992; Caretto et al., 2015).

442

443 4. *Rosmarinus officinalis* as a good model to study pseudo-metallophyte adaptations to TMM
444 pollution in field

445

446 This field study corroborated numerous results obtained with agronomic plant species under
447 controlled conditions. Even with the potential genetic diversity of a wild plant species in the
448 field and the heterogeneity of field conditions, TMM stress response mechanisms were
449 clearly observed in wild *R. officinalis*. Chlorophylls were not significantly altered, which
450 appears to be linked to efficient mitigating mechanisms associated with chelating compounds
451 and antioxidant molecules. This study's results contribute to improving understanding of the
452 underlying parameters of the biochemical plasticity of this plant species enabling its growth
453 on highly TMM contaminated soils. These results also confirm that this perennial is a good
454 candidate for phytostabilisation of metallurgical brownfields in the Mediterranean (Pandey et
455 al., 2019; Bozdoğan Sert et al., 2019). Recent studies on another native plant growing nearby
456 the same brownfield, *Astragalus tragacantha*, showed the capacity of this other
457 Mediterranean pseudo-metallophyte to cope with TMM soil pollution (Salducci et al., 2019).
458 However, no significant implication of the studied phytometabolites had been revealed in the
459 TMM tolerance of this plant species.

460 Our results also highlighted that even after 95 years since the former factory's closure,
461 diffuse soil pollution is still significant, and TMM mobility is not negligible. The soil diffuse
462 pollution in this area is widespread (Laffont-Schwob et al., 2016; Gelly et al., 2019) and since
463 the area is now within a protected area (namely, the Calanques National Park),
464 phytostabilisation with native plant species would likely be favoured (Heckenroth et al.,
465 2016). This could include colonization of wild *R. officinalis* in areas of non-vegetated TMM
466 contaminated soils for this study area. Considering that *R. officinalis* is common in the
467 matorrals of the site, the non-destructive in-situ monitoring of the phenol index of plant
468 leaves, using the Multiplex® device, at the geographical scale of the Massif des Calanques
469 could be a relatively easy way to establish proxy of contamination areas.

470

471 5. Conclusions

472 Our study has shown that *R. officinalis* provides a good model to study pseudo-metallophyte
473 adaptations to TMM pollution in a field environment. TMM tolerance mechanisms appear to
474 be driven by phenolic and cysteine-rich compounds preventing TMM translocation in the
475 shoots. Consequently, low TMM content in shoots and its capacity to grow spontaneously in
476 highly TMM contaminated-soils, shows that *R. officinalis* is likely to be a good candidate for
477 TMM phytostabilisation. Determination of the roles of phytochelatins and metallothioneins in
478 *R. officinalis*, as well as the intracellular localization of the TMM-chelates formed, using
479 imagery techniques, would be the next step to gain a deeper understanding of the
480 translocation and detoxification mechanisms involved in this species.

481

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490

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Credit author statement

Conceptualization: MCA, ILS, PP; **Methodology:** MCA, BC, JLB, JR; **Validation:** MCA, ILS, JR, BC, JLB, PP; **Formal analysis:** MCA, AB; **Investigation:** MCA, CD; **Resources:** MCA, ILS, JR, BC, JLB, CD, PP; **Writing – Original draft:** MCA, ILS, PP; **Writing- Review and Editing:** MCA, ILS, JR, BC, JLB, AB,PP; **Visualisation:** MCA; **Supervision:** ILS, PP; **Funding acquisition:** ILS

Table 1[Click here to download Table: table_1.docx](#)

1 Table 1: Average concentration per site of As, Pb, Sb and Zn: mobile concentrations in soil in
 2 mg.kg⁻¹ DW and the mobile fraction (percent of pseudo-total concentration in soil),
 3 concentrations in Rosemary's shoots in mg.kg⁻¹ DW and translocation factors (shoots vs roots
 4 concentrations),. Mean ±SD, n=5.

Site	As	Pb	Sb	Zn	As	Pb	Sb	Zn
Mobile concentration				Shoot concentration				
G0	155 ±213	2631 ±2270	17.1 ±23.4	429 ±350	0.37 ±0.08	10.3 ±2.90	0.48 ±0.14	59.9 ±8.31
G1	27.6 ±22.6	1847 ±691	3.3 ±1.9	165 ±71	0.48 ±0.14	16.2 ±5.11	1.20 ±1.20	47.6 ±15.1
G2	72.8 ±57.4	3522 ±2551	8.9 ±7.1	560 ±421	0.26 ±0.06	3.62 ±1.15	0.22 ±0.06	43.2 ±6.82
G3	26.2 ±45.7	1337 ±1565	3.1 ±5.4	309 ±411	0.35 ±0.07	8.04 ±2.22	0.47 ±0.11	57.7 ±7.95
G4	0.28 ±0.23	180 ±91	0.13 ±5.4	56.0 ±38.5	0.19 ±0.10	3.61 ±1.71	0.06 ±0.01	41.5 ±4.54
G5	0.37 ±0.32	182 ±179	0.10 ±0.08	53.0 ±63.7	0.80 ±0.36	10.6 ±3.92	0.66 ±0.34	58.8 ±18.6
G6	0.24 ±0.21	13.9 ±5.6	0.07 ±0.05	11.1 ±9.1	0.89 ±0.69	7.90 ±4.79	0.25 ±0.16	28.8 ±10.5
S3	0.56 ±0.30	18.5 ±8.0	0.07 ±0.02	14.3 ±7.3	0.09 ±0.03	0.58 ±0.37	0.09 ±0.02	37.0 ±10.9
Mobile fraction				Translocation factors				
G0	10.9 ±5.6	30 .6 ±5.1	4.2 ±2.1	17.2 ±3.3	0.022 ±0.009	0.034 ±0.035	0.061 ±0.019	0.36 ±0.18
G1	7.3 ±3.2	54.7 ±9.9	2.8 ±1.1	13.9 ±3.7	0.032 ±0.021	0.046 ±0.37	0.13 ±0.19	0.44 ±0.36
G2	7.4 ±1.5	43.7 ±8.7	3.1 ±0.8	21.2 ±5.7	0.007 ±0.004	0.006 ±0.05	0.016 ±0.012	0.17 ±0.14
G3	3.9 ±3.0	41.4 ±3.5	1.6 ±1.1	16.5 ±2.7	0.094 ±0.087	0.051 ±0.037	0.087 ±0.074	0.66 ±0.42
G4	0.9 ±0.5	36.0 ±7.4	1.3 ±0.9	33.4 ±20.9	0.15 ±0.15	0.14 ±0.09	0.81 ±1.43	1.32 ±0.84
G5	0.8 ±0.8	57.0 ±25.9	1.3 ±0.6	21.8 ±19.9	0.49 ±0.20	0.46 ±0.10	0.54 ±0.26	2.68 ±3.16
G6	0.2 ±0.3	21.1 ±16.0	1.9 ±1.9	11.4 ±8.3	2.15 ±0.17	3.37 ±2.06	3.40 ±2.35	1.65 ±0.68
S3	9.8 ±7.9	45.4 ±46.7	2.6 ±1.5	19.2 ±19.6	0.086 ±0.030	0.065 ±0.035	0.29 ±0.14	2.92 ±1.63

5

6

1 Table 1: Concentrations of non-protein cysteine (nmol.g^{-1} FW), glutathione (nmol.g^{-1} FW)
 2 and total free thiols ($\mu\text{mol.g}^{-1}$ DW), ratio of glutathione over cysteine concentration and the
 3 chlorophyll and phenolic indices (no unit) in the shoots of Rosemary. Mean per site \pm SD
 4 (n=5). n.d: not detected, n.a: not analysed.

Site	Cysteine	Glutathione	Ratio GSH/Cys	Free thiol	Chlorophyll	Phenolics
G0	19.7 \pm 3.7	63.9 \pm 24.3	3.24 \pm 0.93	n.a	n.a	n.a
G1	5.2 \pm 1.0	98.5 \pm 72.8	19.2 \pm 14.3	24.0 \pm 4.6	1.9 \pm 0.2	59.8 \pm 9.3
G2	n.d	n.d	-	8.7 \pm 2.3	2.3 \pm 0.2	39.5 \pm 9.7
G3	9.1 \pm 5.8	152.7 \pm 36.9	24.0 \pm 17.9	30.4 \pm 7.5	2.1 \pm 0.3	48.2 \pm 10.3
G4	0.28 \pm 0.38	264.7 \pm 75.4	806.7 \pm 657.5	24.7 \pm 3.4	1.9 \pm 0.1	41.3 \pm 12.0
G5	19.5 \pm 4.3	206.2 \pm 93.8	10.9 \pm 5.8	29.8 \pm 5.8	1.9 \pm 0.2	43.0 \pm 8.0
G6	n.d	58.1 \pm 36.9	-	14.9 \pm 3.6	2.0 \pm 0.1	65.1 \pm 21.0
S3	13.0 \pm 6.8	15.7 \pm 3.7	1.56 \pm 1.00	n.a	n.a	n.a

5

Table 3

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- 1 Table 3: Spearman correlation coefficients (ρ) between the concentrations of As, Pb, Sb and
 2 Zn, total free thiols, non-protein cysteine and glutathione in Rosemary's shoots and the
 3 chlorophyll and phenolic indices. Values in brackets correspond to the correlation
 4 coefficients when the site G4 was excluded. Values in bold: $p < 0.05$.

	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
(1) As_{shoots}	0.85	0.72	0.17	-0.22	0.57	0.16	0.15	0.34	0.22	
	<i>(0.80)</i>	<i>(0.69)</i>	<i>(0.09)</i>	<i>(-0.33)</i>	<i>(0.46)</i>	<i>(0.31)</i>	<i>(0.10)</i>	<i>(0.60)</i>	<i>(0.45)</i>	
(2) Pb_{shoots}		0.79	0.34	-0.31	0.60	0.34	0.2	0.37	0.31	
		<i>(0.79)</i>	<i>(0.30)</i>	<i>(-0.41)</i>	<i>(0.49)</i>	<i>(0.51)</i>	<i>(0.12)</i>	<i>(0.61)</i>	<i>(0.56)</i>	
(3) Sb_{shoots}			0.49	-0.31	0.43	0.41	0.45	0.18	0.09	
			<i>(0.49)</i>	<i>(-0.50)</i>	<i>(0.33)</i>	<i>(0.64)</i>	<i>(0.33)</i>	<i>(0.63)</i>	<i>(0.50)</i>	
(4) Zn_{shoots}				-0.07	-0.17	0.63	0.47	0.24	0.08	
				<i>(-0.14)</i>	<i>(-0.23)</i>	<i>(0.73)</i>	<i>(0.42)</i>	<i>(0.38)</i>	<i>(0.29)</i>	
(5) Chlorophyll						-0.47	-0.33	-0.22	-0.35	-0.01
						<i>(-0.53)</i>	<i>(-0.38)</i>	<i>(-0.27)</i>	<i>(-0.38)</i>	<i>(0.00)</i>
(6) Phenolics							-0.05	0	-0.07	-0.3
							<i>(-0.01)</i>	<i>(-0.07)</i>	<i>(0.13)</i>	<i>(0.11)</i>
(7) Free thiols								0.69	0.66	0.18
								<i>(0.72)</i>	<i>(0.75)</i>	<i>(0.32)</i>
(8) Cysteine									0.22	-0.61
									<i>(0.45)</i>	<i>(-0.45)</i>
(9) Glutathione										0.84
										<i>(0.82)</i>
(10) GSH/Cys										

Figure 1
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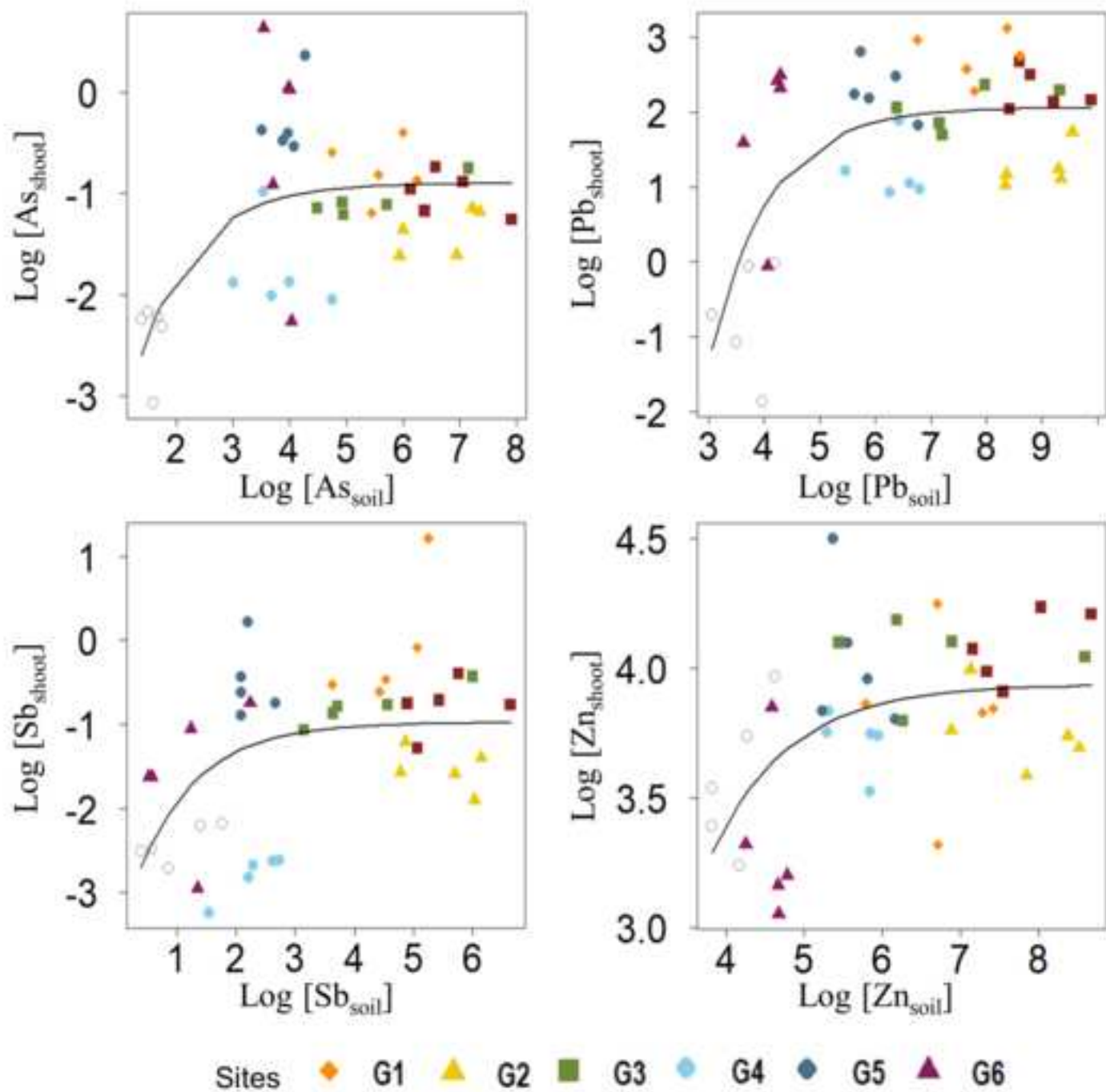
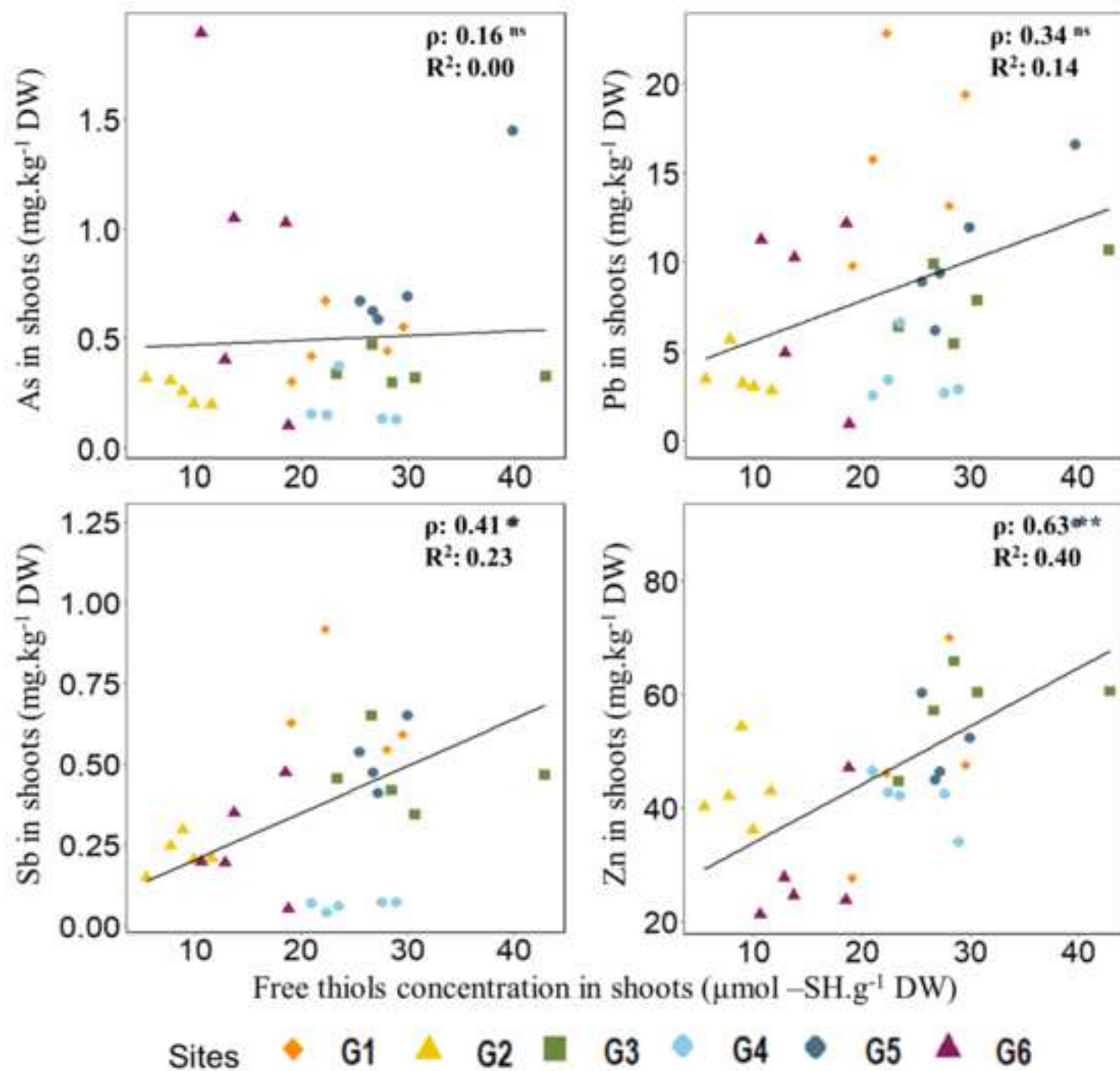


Figure 2

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1 Figure 1: TMM concentrations in shoots depending on pseudo-total TMM concentration in
2 the mycorrhizospheric soils for each Rosemary/soil couple (log transformed data) for As, Pb,
3 Sb and Zn. The black lines represent the fitting of Mitscherlich model.

4

5 Figure 2 : Concentrations of As, Pb, Sb and Zn in the shoots of Rosemary individuals (in
6 mg.kg^{-1} DW) depending on the concentration in total free thiols ($\mu\text{mol -SH.g}^{-1}$ DW) for sites
7 G1 to G6. ρ : Spearman's correlation coefficients, R^2 : coefficient of determination of the
8 linear regression. Significance of the correlation: ns; *, **, ***= not significant; significant at
9 $P < 0.05$; 0.01 or 0.001 respectively.

Supplementary Material

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