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

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Abstract

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

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

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

1. Introduction

Trace metals and metalloids (TMM) naturally occur in the environment due to their presence in the Earth’s crust. However, the past century has seen them become a major environmental issue. Concentrations of TMM above background levels have been observed in soils subject to agricultural and industrial activities, and are consequently transferred to the trophic web, notably via plants (He et al., 2005; Nawab et al., 2015; Sarwar et al., 2017). Phytoremediation is considered an innovative, cost-effective and ecologically beneficial biotechnology (Sawar et al., 2017). However, to select appropriate plant species for phytoremediation purposes, an understanding of plant stress tolerance mechanisms to TMM is required (Clemens et al., 2002; Antoniadis et al., 2017).

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

At the molecular and cell level, increases in the biosynthesis of some phytometabolites can counterbalance the generation (Michalak, 2006) and/or activity (Nimse et al, 2015) of reactive oxygen species (ROS). They are known to reduce photosynthetic activity through photosystem activity alteration and/or chlorophyll biosynthesis decrease (e.g. Clijsters and Assche, 1985; Assche and Clijsters, 1990; Maleva et al., 2012). Some metabolites have potential antioxidant activities, like phenolic compounds (e.g. flavonol, anthocyanidin, isoflavone), and may reduce ROS production/reactivity (Michalak, 2006; Nimse et al., 2015). Others, like phytochelatins and glutathione, which are thiol compounds, have complex properties (Yadav, 2010) and can bind TMM and transfer them into the vacuole where they are inactivated (Hall, 2002). However, the biosynthesis of these defence metabolites represents an associated cost for the plant, which needs to find a trade-off between growth (primary metabolism) and stress resistance (secondary metabolism) (Hems and Mattson, 1992). A higher allocation to one function will result in the decrease in the allocation to the other function (Caretto et al., 2015).

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

This study focuses on the chemical responses of *R. officinalis* in a field setting in the south-east of France, along a wide gradient of varying TMM soil pollutions resulting from past industrial activities. We hypothesized that TMM stress responses in *R. officinalis* in the field mobilizes identical mechanisms than those reported under controlled conditions and that the contamination gradient in the field is sufficient to generate contrasting results. Consequently, we aimed to i) determine patterns of TMM uptake and translocation to *R. officinalis* shoots across a broad range of TMM contamination levels, ii) highlight how contamination level affects secondary metabolites composition of *R. officinalis*, iii) determine the effect of heavy metal stress on the primary/secondary metabolism balance of *R. officinalis* due to change in resource allocation. Levels of several secondary metabolites, phenolic compounds including anthocyanins, thiol compounds and phytochelatin precursors, primary metabolites and chlorophylls were analysed in the shoots and correlated to contamination levels, notably lead (Pb), arsenic (As), antinomy (Sb) and zinc (Zn), in the soil and the plant.

2. Materials and methods

2.1. Study area

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

The former Pb smelter factory located in the Escalette Calanque, processed argentiferous galena ores from 1851 to 1925 by pyrometallurgical processes (Daumalin and Raveux, 2016). This activity generated massive Pb and Zn-rich slags (Gelly et al., 2019; Testiati et al., 2013) and atmospheric emissions of highly metal-concentrated particles, specifically with Pb, As and Sb (Laffont-Schwob et al., 2016; Testiati et al., 2013). Previous studies highlighted the metal and metalloid contaminations of the smelter surroundings, in particular by Pb, As, Sb and Zn (Heckenroth et al., 2016; Affholder et al., 2013; Testiati et al., 2013; Gelly et al., 2019).

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

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

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

2.2. Plant and soil sampling

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

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

2.3. Pseudo total TMM in soils

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

2.4. Mobile TMM in soils

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

2.5. Plant TMM analysis

Shoots samples were thoroughly washed using Milli-Q water to eliminate any soil particles. Samples were dried at 40 °C over 1 week, after which leaves and stems were separated and then ground at 0.2 mm (RETSCH zm 1000 blender with tungsten blades and titanium sieve). About 0.5 g dry matter of each sample was digested in a microwave mineralizer system (Milestone Start D) with a HNO_3–HCl mixture (volume proportion ratio 2/1). After filtration (0.45 µm), acid digests were analysed for Pb and Zn contents by ICP-AES, and for As and Sb contents by graphite furnace AAS (Rabier et al., 2007). Standard plant reference materials (DC 73349) from the China National Analysis Centre for Iron and Steel (NCS) was analysed as a part of the quality control protocol (accuracies within 100 ± 10 %). Translocation factors (TFs), i.e. ratios of shoot concentrations vs. roots concentrations (data of roots concentrations from Affholder et al., 2014) were calculated.

2.6. Plant phytometabolite analysis

2.6.1. Total free thiols analysis

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

Despite filtration, *R. officinalis* extracts contained aromatic molecules (phenols, flavones, etc) that absorbed in UV (Almela et al., 2006). To avoid background noise during UV spectrum measurement, samples were purified using cartridges filled with 50 mg of XAD-4 (styrene-divinyl benzene) resin previously conditioned by percolating 3 mL of acetonitrile and then rinsing with 3 mL of deionized water. A derivatisation was performed and consisted in the addition of ethylpropiolate. The reaction between ethylpropiolate and free thiols produces thioacrylate, a molecule presenting a maximal absorbance at 285 nm (Coulomb et al, 2017). Immediately before UV spectrum measurement, 10 µL of ethylpropiolate was added to 0.2 mL of purified sample and 0.2 M phosphate buffer pH 9 qs 10 mL. Calibration was undertaken with a solution of cysteine in a concentration ranging between 0 and 140 µM. Results are expressed in µmol g^{-1} of dry matter.

2.6.2. Cysteine and Glutathione analysis

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

2.6.3. Chlorophyll and phenol indices

Plant physiological indices were estimated optically using a Multiplex® 3 non-destructive measurement equipment (FORCE-A, Orsay, France; Agati et al., 2011). This portable fluorometric device uses fluorescence technology with multiple excitations to measure constitutive and induced epidermal phenols, flavonols, anthocyanins, chlorophylls and a chlorophyll-to-flavonoid ratio referred to as the nitrogen balance index (NBI) (Rabier et al., 2014). Different combinations of the blue-green, red and far-red fluorescence signals at the various excitation bands could be used as indices of the different compounds (Cerovic et al., 2008; Agati et al., 2011). In spring, each individual from 6 of the 8 sites (G1 to G6, 5 replicates per site) was flashed 25 times per individual. Data from sites G0 and S3 were analysed in autumn and are not included in this study as the phenological stage of plants differed. The indices obtained by Multiplex® cannot be directly converted into concentrations since calibration in the laboratory is not satisfactory. Indeed, Mutiplex® equipment is measuring metabolites from the surface tissues of the leaves while spectrometric analysis, needing a leaf extraction, make it difficult to distinguish the concentrations in upper and inner tissues. Therefore, the phenolics and chlorophyll indices obtained were not converted into concentrations. However, the measurement is free from the sampling geometry, allowing a field comparison between populations from the different areas as demonstrated in many experiments (Ben Ghozlen et al., 2010; Bürling et al., 2013; Louis et al., 2009).

2.7. Statistical analyses

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

The non-linear regressions observed between the shoots and soils pseudo-total concentrations were modelled by the Mitscherlich equation, using the functions available on the package *nls2* (Gothendieck, 2013) and *nlme* (Pinheiro et al., 2018). The equation used for the model was:

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

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

3. Results and discussion

3.1. TMM contamination in soils

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

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

3.2.1. Accumulation in shoots

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

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

For the 4 elements, results highlighted that below a certain level of soil contamination concentrations in shoots were linearly correlated with the soil concentrations, and, above, the shoot concentrations reached a plateau. This kind of behaviour has been previously observed, particularly for Cd and Zn (Dudka and Adriano, 1997; Hamon et al., 1999; Green, 2003). The observed plateau-type response of *R. officinalis* was modelled with the Mitscherlich plateau equation (significant correlation, $p \leq 0.001$) proposed by Logan and Chaney (1987), and already applied by Azizian et al. (2011, 2013) to model Cd uptake by lettuce and corn.

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

3.2.2 Translocation

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

Average TFs obtained per site are presented Table 1. Translocation factor values greater than 1 were highlighted on the less contaminated sites, mainly on site G6, where they reached 2.15, 3.37, 3.40 and 1.65 for As, Pb, Sb and Zn, respectively. Significant linear negative correlations (Pearson test, $p \leq 0.05$) with correlation coefficients of -0.55 for As, -0.71 for Pb, -0.73 for Sb and -0.81 for Zn were identified between TFs and pseudo-total soil concentrations (Log transformed). This shows a strong and linear decrease of TMM translocation when concentrations in soil is increased. In highly contaminated sites the average TF values were much lower than 1. In G0 for instance, values of 0.02, 0.03, 0.06 and 0.36 were obtained for As, Pb, Sb and Zn, respectively. In this case the highest TF values were associated with Zn, which is congruent with the results of De la Fuente et al. (2014). This may relate to the fact that Zn is an essential element for plants.

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

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

The occurrence of TMM in a plant’s shoots can lead to a decrease of chlorophyll biosynthesis and a subsequent reduction in photosynthesis (Assche and Clijsters, 1990). However, some species develop tolerance mechanisms to limit the stress induced by high TMM concentrations in their shoots (Antoniadis et al., 2017; Yadav et al., 2018). This mechanism involves the biosynthesis of phytometabolites mitigating the cause, by sequestering the TMM in the vacuole, or mitigating the consequences, by limiting the oxidative stress induced by the TMM (Singh et al., 2016; Sytar et al., 2013).

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

3.3.1. Stress tolerance phytometabolites

Complexing compounds: Thiols

Thiols include molecules like cysteine, glutathione and phytochelatins which are known to sequester and for their role against the oxidative stress associated to TMM occurrence in plants (Hall, 2002; Kawashima et al., 2004). Concentrations of total free thiol were determined in the shoots of *R. officinalis* from 6 sites (G1 to G6) and average concentrations are presented in Table 2. Results showed that the average concentrations of total free thiols in *R. officinalis*' shoots ranged from 8.7 ±2.3 to 30.4 ±7.5 µmol of $-SH.g^{-1}$ DW. Lower concentrations were found in *R. officinalis* individuals from sites G2 and G6 where, respectively, the highest and lowest concentrations of mobile TMM in the soil were observed for the 6 sites. As the concentrations in the shoots are not linearly correlated to the level of soil contamination, the relation free thiol/TMM in shoots was investigated per *R. officinalis* individuals and not per site. The results are presented Figure 2 and indicate a significant positive correlation between the concentrations of free thiols and Sb and Zn in *R. officinalis*' shoots (Spearman's test, $p \leq 0.05$, $\rho = 0.41$; 0.63 for Sb and Zn, respectively). Total free thiol biosynthesis seemed elicited when the concentration of Sb and Zn increased in *R. officinalis* shoots. It appears that total free thiols play an important role in *R. officinalis* ability to tolerate stresses induced by the occurrence of TMM in the shoots, either because of their antioxidant properties, or their ability to detoxify the TMM by sequestration.

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

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

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

Antioxidant compounds: phenolics

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

The average of the indices measured for *R. officinalis* from sites G1 to G6 are presented in Table 2. The lowest and highest indices were measured on *R. officinalis* individuals from G2 and G6, respectively. However, the analysis of the data per individuals has provided a better understanding of the impact of TMM concentration on the occurrence of phenolics. Positive correlations between As, Pb and Sb concentrations in *R. officinalis* shoots and the phenolic indices (Spearman's test, $p \leq 0.05$, table 3) were shown. Increase of phenolics in plants following a Pb contamination gradient has already been observed in *Phaseolus vulgaris* (Hamid et al., 2010). Phenolics appears to be involved in the tolerance of *R. officinalis* to TMM. They can act as TMM chelatants, or antioxidant compounds, limiting in both cases the oxidative stress caused by TMM presence (Michalak, 2006).

Health biomarker: Chlorophylls

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

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

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

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

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

5. Conclusions

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

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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: Average concentration per site of As, Pb, Sb and Zn: mobile concentrations in soil in mg.kg^{-1} DW and the mobile fraction (percent of pseudo-total concentration in soil), concentrations in Rosemary's shoots in mg.kg^{-1} DW and translocation factors (shoots vs roots 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

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

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

Table 3
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Table 3: Spearman correlation coefficients (ρ) between the concentrations of As, Pb, Sb and Zn, total free thiols, non-protein cysteine and glutathione in Rosemary's shoots and the chlorophyll and phenolic indices. Values in brackets correspond to the correlation 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
		(0.80)	***(0.69)***	*(0.09)*	*(-0.33)*	***(0.46)***	*(0.31)*	*(0.10)*	***(0.60)***	***(0.45)***
(2) Pb_{shoots}			**0.79**	**0.34**	-0.31	**0.60**	0.34	0.2	**0.37**	0.31
			(0.79)	*(0.30)*	***(-0.41)***	***(0.49)***	***(0.51)***	*(0.12)*	***(0.61)***	***(0.56)***
(3) Sb_{shoots}				**0.49**	-0.31	**0.43**	**0.41**	**0.45**	0.18	0.09
				(0.49)	***(-0.50)***	*(0.33)*	***(0.64)***	*(0.33)*	***(0.63)***	***(0.50)***
(4) Zn_{shoots}					-0.07	-0.17	**0.63**	**0.47**	0.24	0.08
					(-0.14)	*(-0.23)*	***(0.73)***	***(0.42)***	***(0.38)***	*(0.29)*
(5) Chlorophyll						**-0.47**	-0.33	-0.22	-0.35	-0.01
						(-0.53)	*(-0.38)*	*(-0.27)*	*(-0.38)*	*(0.00)*
(6) Phenolics							-0.05	0	-0.07	-0.3
							(-0.01)	*(-0.07)*	*(0.13)*	*(0.11)*
(7) Free thiols								**0.69**	**0.66**	0.18
								(0.72)	***(0.75)***	*(0.32)*
(8) Cysteine									0.22	**-0.61**
									(0.45)	***(-0.45)***
(9) Glutathione										**0.84**
										(0.82)
(10) GSH/Cys										

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Figure 1
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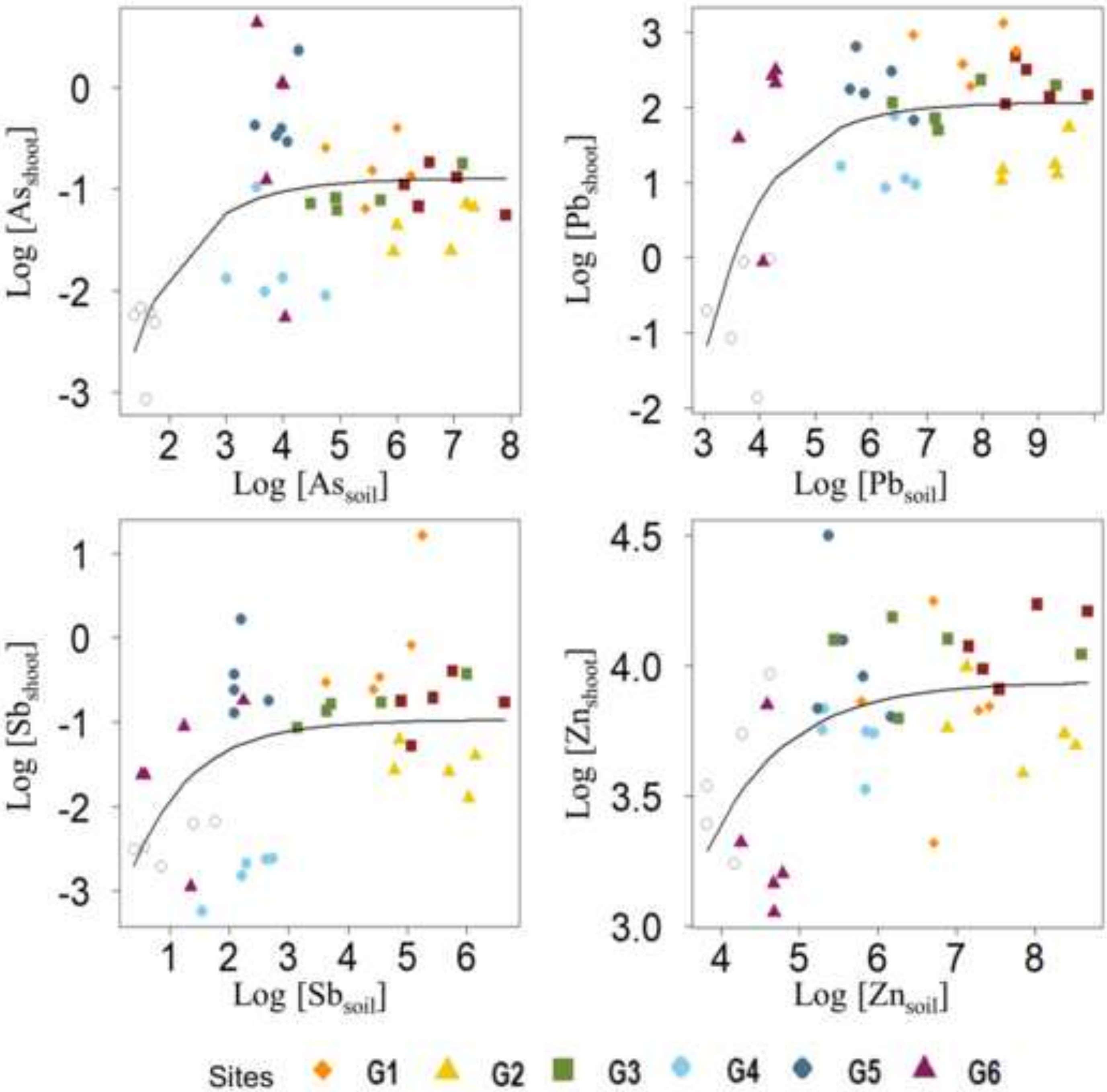

Figure 2

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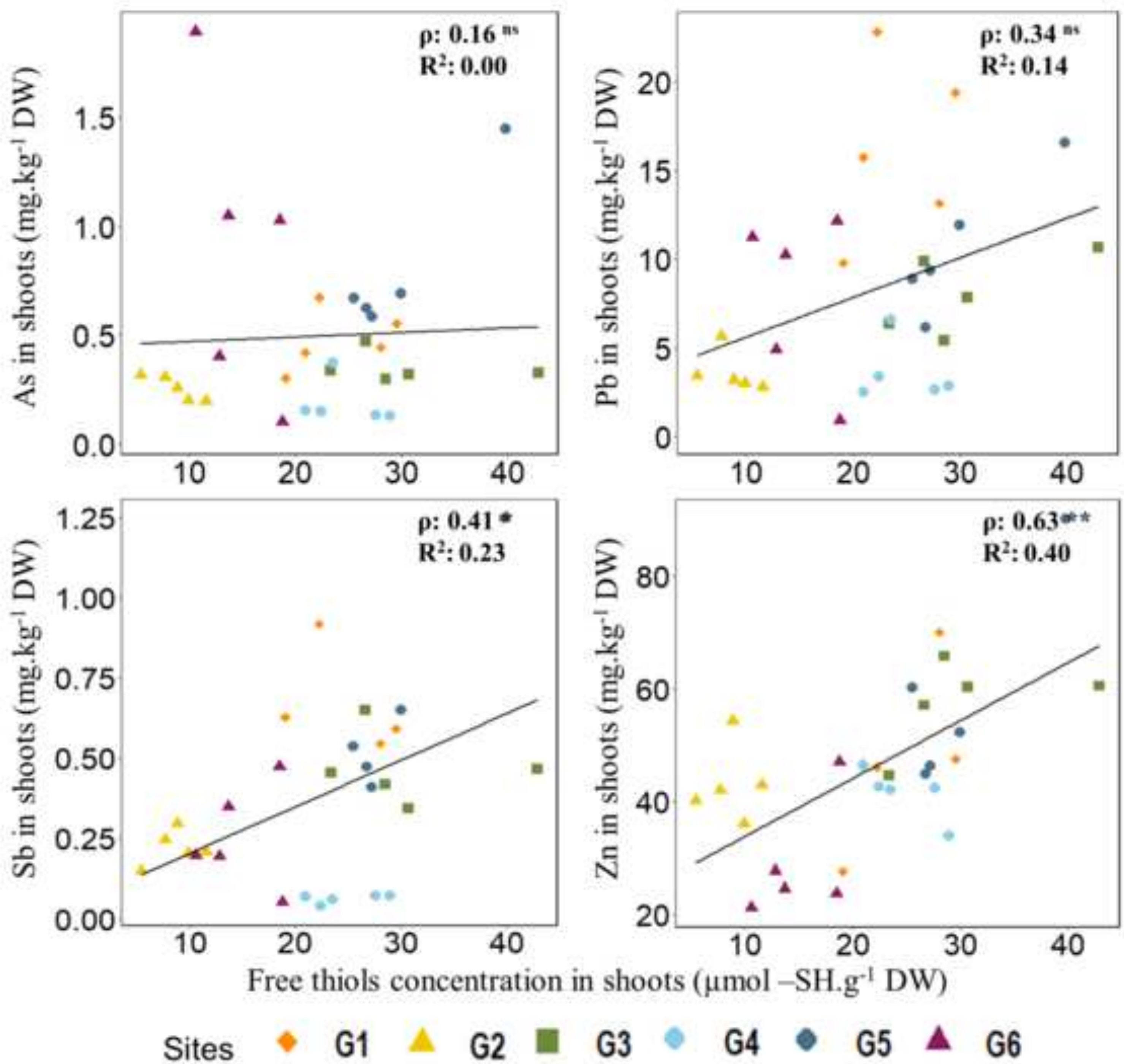

Figure 1: TMM concentrations in shoots depending on pseudo-total TMM concentration in the mycorrhizospheric soils for each Rosemary/soil couple (log transformed data) for As, Pb, Sb and Zn. The black lines represent the fitting of Mitscherlich model.

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

Supplementary Material

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