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Plasticity, exudation and microbiome-association of the root system of Pellitory-of-the-wall plants grown in environments impaired in iron availability

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1 Plasticity, exudation and microbiome-association of the root system of Pellitory-of-the-
2 wall plants grown in environments impaired in iron availability

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17

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19 of-the-wall, Phenols, Rhizosphere, Root architecture, Urban habitat

20

21 **Abstract**

22 The investigation of the adaptive strategies of wild plant species to extreme environments
23 is a challenging issue, which favors the identification of new traits for plant resilience. We
24 investigated different traits which characterize the root-soil interaction of *Parietaria*
25 *judaica*, a wild plant species commonly known as “Pellitory-of-the- wall”. *P. judaica*
26 adopts the acidification-reduction strategy (Strategy I) for iron (Fe) acquisition from soil,
27 and it can complete its life cycle in highly calcareous environments without any symptoms
28 of chlorosis. In a field-to-lab approach, the microbiome associated with *P. judaica* roots
29 was analyzed in spontaneous plants harvested from an urban environment consisting in
30 an extremely calcareous habitat. Also, the phenolics and carboxylates content and root
31 plasticity and exudation were analyzed in *P. judaica* plants grown under three different
32 controlled conditions mimicking the effect of calcareous environments on Fe availability:
33 results show that *P. judaica* differentially modulates root plasticity under different Fe

availability-impaired conditions, and that it induces, to a high extent, the exudation of caffeoylquinic acid derivatives under calcareous conditions, positively impacting Fe solubility.

Introduction

Variations in soil pH are among the abiotic factors affecting most plant growth, since they influence the bioavailability of essential nutrients and toxic elements for plants. The presence of carbonates in soils causes a decrease in the solubility of iron (Fe) and other micronutrients (Lindsay and Schwab, 1982; Schenkeveld and Kraemer, 2018). Calcareous soils, representing 30% of the Earth's land surface, are characterized by high pH values and may contain high HCO_3^- ions in the soil solution. Iron deficiency-induced chlorosis, otherwise known as lime chlorosis, is a major nutritional disorder observed in crops growing in calcareous soils; on one hand, alkaline pHs dramatically reduce Fe solubility in soil and on the other hand, the presence of HCO_3^- may interfere with the physiological processes of Fe uptake (Chen and Barak, 1982; Romheld, 1987; Kim and Guerinot, 2007; Díaz et al., 2012). Plant roots grown under calcareous conditions may exhibit a higher Fe content than those grown under non-calcareous conditions, indicating that low Fe availability in soil is not the unique factor causing lime-induced chlorosis; indeed, the composition of the soil itself can also influence Fe uptake from the apoplast (Mengel, 1994). Iron is an essential micronutrient for the energy-yielding electron transfer reactions of respiration and photosynthesis and other major metabolic processes in plants (Kobayashi and Nishizawa, 2012; Vigani and Murgia, 2018); the elucidation of plant adaptive growth strategies in calcareous habitats represents a valuable approach for the identification of tolerance traits under soil constraints. In this context, a characterization of *Arabidopsis thaliana* demes locally adapted in their native habitat to soils with high carbonate has recently been made (Ter'es et al., 2019).

The understanding of plant resilience to different biotic and abiotic stresses and to various natural habitats and climate changes is an emerging and urgent goal, as outlined in the Plant Science Decadal Vision 2020–2030 (Henkhaus et al., 2020). In particular, Decadal Vision's proposed actions include, among others, the selection of ecologically diverse plant lineages for an in-depth analysis of their morphology, anatomy, ecology in their natural environments, as well as the exploration and characterization of as-yet-undiscovered plant-associated biota. In the context of Fe nutrition, the issues raised by Plant Science Decadal Vision suggest that the adapting processes of non-crop plants to calcareous habitats can

68 potentially uncover traits lost in domesticated crops. Moreover, they also support the
69 importance of studying the root microbiota associated with non-crop plants (or wild crop
70 relatives). Interestingly, urban calcareous habitats can also constitute a potential source of
71 information of the resilience of plants in inhospitable soils.

72 In most cases, crop domestication led to modifications of the composition of root
73 microbiota, with adverse effects on the association with beneficial plant microbes ([Perez-
74 Jaramillo et al., 2016](#)). Root exudation of metabolic compounds plays a crucial role in influencing
75 the recruitment of functional microbiota. In general, the variety of biotic and abiotic
76 stresses, such as Fe deficiency, encountered by plants during *in vitro* and *in vivo* growth
77 conditions impacts the biosynthesis of secondary metabolites ([Cheynier et al., 2013](#); [Isah,
78 2019](#)). Several works have reported an increased number of phenolic compounds in plants
79 and root exudates as a response to different environmental stresses ([Cesco et al., 2010](#);
80 [Caretto et al., 2015](#)), and as an adjustment of secondary metabolism occurring in response
81 to Fe mobilization ([Jin et al., 2007](#)). Some species, such as *P. judaica*, can secrete ortho-
82 dihydroxy phenolic compounds showing reducing and metal chelating properties that
83 enhance Fe availability in the rhizosphere. Indeed, plant metabolites released to
84 rhizosphere can have diverse effects on soil microbial communities by changing soil
85 properties or nutrient availability in the root vicinity, directly attracting microbes or being
86 toxic for others ([Vives-Peris et al., 2020](#)).

87 In some cases, specific plant root exudates initiate a molecular dialogue mediated by both
88 partners' exometabolite production, resulting in the establishment of beneficial plant-
89 microbe associations ([Sasse et al., 2018](#)). Badri and co-workers found a strong positive
90 correlation between phenolics and Plant Growth Promoting Bacteria (PGPB) species,
91 including *Rhizobium*, *Bacillus*, *Sphingomonas*, *Streptomyces* and *Frankia* ([Badri et al., 2013](#)). A
92 similar mechanism of recruitment of PGPB has been demonstrated for coumarins in Fe-
93 limiting soils: besides their established role in Fe mobilization in the rhizosphere ([Rajniak
94 et al., 2018](#)), coumarins are also involved in shaping root-associated micro- biomes ([Voges
95 et al., 2019](#)). Furthermore, [Harbort et al. \(2020\)](#), by using synthetic microbiota and *A.
96 thaliana* plants deficient in the exudation of secondary metabolites, demonstrated that
97 coumarins are important drivers for the assembly of the rhizospheric bacterial community
98 under Fe deprivation. All these recent works on the tripartite interaction between roots'
99 exudates, soils and microorganisms support the emerging view that metabolites exuded
100 under peculiar environmental conditions can recruit microbiota components able to
101 alleviate the specific stress experienced by the plant.

102 The present work aims to unravel root-related processes of *Parietaria judaica*'s response to Fe

deficiency, and particularly its root plasticity and metabolite exudation under Fe deficiency. *P. judaica* (L. 1753) is a wild perennial dicotyledonous plant capable to grow in acidic and alkaline soils (Tato et al., 2020); it represents the most widespread plant species found in highly calcareous and hostile environments such as wall cracks exposed to the sun, where it displays phenotypic changes, though without any chlorosis symptoms (Dell’Orto et al., 2003; Donnini et al., 2012; Tato et al., 2020). In this work, we adopted a field-to-lab approach to first investigate the microbiome associated with *P. judaica* roots in plants growing spontaneously in an urban environment and harvested from an extremely calcareous habitat. We then analyzed phenolics content and root plasticity and exudation in *P. judaica* plants grown under various controlled laboratory conditions mimicking the effects of calcareous environments; in particular, we applied conditions that allowed us to discriminate the effects of low Fe availability from those caused by the presence of carbonate and alkaline conditions (Tato et al., 2020).

Material and methods

P. judaica growth conditions

P. judaica plants were sampled in an urban area of Milan (45°28’36.8’’N, 9°13’39.2’’E; 45°28’37.0’’N, 9°14’00.1’’E; 45°28’35.2’’N, 9°14’03.7’’E): to sample the whole root apparatus, the walls and the substrates where plants were growing were broken if necessary.

Cuttings of *P. judaica* were allowed to radicate in aerated half- strength nutrient solution for 10 days (Tato et al., 2020). Rooted plants were then transferred into 10 L plastic tanks (40 plants/tank) under four different conditions: +Fe (control, complete nutrient solution adjusted to pH 6.2 with NaOH), -Fe (complete nutrient solution without Fe, adjusted to pH 6.2), Bic (complete nutrient solution supplemented with 5 mM CaCO₃ and 15 mM NaHCO₃, pH 8.3) and Tric (complete nutrient solution, buffered with Tricine at pH 8.3); the pH was adjusted with NaOH if required. The nutrient solution was changed every two days. Plant size at the harvesting time has been previously reported in Tato et al. (2013).

Treatments were carried out for 7 days in a growth chamber under 16/8 h light/dark regime with cool-white light 200 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$, 27/21 °C temperature range, 65–75% relative humidity.

Sampling of P. judaica in urban sites and DNA extraction of their root-associated microbes

137 From each of the three urban sampling sites considered, three replicates of bulk soil (i.e.
138 the soil portion not affected by the root presence, according to [Bulgarelli et al., 2012](#)) and
139 root samples from *P. judaica* were collected. Large soil aggregates were removed from the
140 roots by shaking as described in [Bulgarelli et al. \(2012\)](#), in order to leave only the
141 (rhizospheric) soil attached to the roots. Bulk soil and root samples with attached soil were
142 then stored at -20°C until DNA extraction. To obtain the samples that represent the root-
143 associated microbial diversity, the root-adhering soil plus the root itself were processed
144 together. This condition accounts both for the externally associated microbes and for the
145 root endophytes (the rhizosphere + the endorhiza, called rhizosphere for the sake of
146 brevity hereinafter).

147 The rhizosphere samples were first lyophilized for 24 h and then ground to a fine powder
148 with liquid nitrogen. DNA was obtained as described in [Edwards et al. \(2015\)](#) extracting
149 from a 300-mg bulk soil samples and 50 mg rhizospheric samples (dry weight), by using
150 the MoBio PowerSoil DNA Isolation Kit (Qiagen Inc., Hilden, Germany) and DNeasy Plant Mini
151 Kit (Qiagen Inc., Hilden, Germany), respectively, according to the manufacturer's
152 instructions.

153 Extracted DNA was quantified using NanoDrop spectrophotometry (NanoDrop,
154 Wilmington, DE, USA) and normalized prior to library construction for the high-
155 throughput sequencing.

156

157 *Molecular, bioinformatic and statistical analyses of the microbes associated with P. judaica roots*

158 The extracted genomic DNA was used to amplify the V3–V4 region of the prokaryotic 16S rRNA
159 gene, using the modified primer pair pro341f/ pro805r ([Takahashi et al., 2014](#)) with the
160 standard Illumina overhang. Fungal ITS2 rDNA cistron was amplified using the modified
161 primers fITS7 ([Ihrmark et al., 2012](#)) and ITS4ngs ([Tedersoo et al., 2014](#)), with the standard
162 Illumina overhang adapters. Purified PCR products were combined in equimolar amounts,
163 and the corresponding metabarcoding libraries were sequenced on the Illumina MiSeq
164 platform (Illumina, San Diego, CA, USA) with paired-end 2×300 bp sequencing mode
165 at the IGA Technology Services (Udine, Italy).

166 Raw data were processed and analyzed following the pipelines of QIIME2 version
167 2019.7.0 ([Bolyen et al., 2019](#); [Caporaso et al., 2010](#)). The high-quality reads were clustered
168 into operational taxonomic units (OTUs) at a 97% identity level and chimeric sequences
169 were filtered using UCHIME ([Edgar et al., 2011](#)), as implemented in the QIIME2 pipeline.
170 Taxonomy assignment of both prokaryotic and fungal OTUs was performed using the

171 SILVA database (version 132, release date December 13, 2017; Quast et al., 2012; Yilmaz
172 et al., 2013) and the ITS UNITE database (UNITE QIIME release for Fungi, Version November
173 18, 2018. <https://doi.org/10.15156/BIO/786334>) respectively using *sklearn* algorithm as
174 implemented in QIIME2 (Pedregosa et al., 2011).

175 All statistical analyses were conducted in R v3.6.1 (R Development Core Team, 2016).
176 Rarefaction species richness curves were generated using the R package *vegan* (Oksanen
177 et al., 2013). Alpha diversity indices were calculated for Prokaryotic and Fungal OTU
178 tables using the *vegan* package (Oksanen et al., 2013). The differences between soil and
179 root samples were tested by using Tukey's Honest Significant Differences test, with the R
180 package *TukeyC* (Faria et al., 2016).

181 To correct for difference in sequencing depth, a subsampling at even sequencing depth from
182 each sample (9935 for Prokaryotic and 2632 for Fungal samples) was performed before the
183 downstream analysis using the R package *phyloseq* (McMurdie and Holmes, 2013),
184 generating also the taxonomical composition of the whole microbial community. The
185 significance of Bray-Curtis dissimilarity between the soil and root samples were tested by
186 permutational multivariate analysis of variance (PerMANOVA) using the *adonis* function
187 in the R package *vegan* with 9999 permutations. The multivariate homogeneity of group
188 dispersions was first assessed by means of the *betadisper* and *permutest* (with 9999
189 permutations) functions in the R package *vegan*. The differences in the composition of
190 Prokaryotic and Fungal communities in *P. judaica* soil and root samples were visualized
191 by means of a Non-metric Multidimensional Scaling (NMDS) ordination carried out using
192 *metaMDS* function in the R package *vegan*. The R package *gunifrac* (Chen et al., 2012)
193 was used to test differences in the microbial composition of soil and root samples. Co-
194 occurrences in the Prokaryotic and Fungal communities were assessed by performing
195 network analysis using the Spearman rank correlations between OTUs ($\rho > 0.7$ and $p <$
196 0.001). All networks were visualized with the Fruchterman-Reingold layout with 9999
197 permutations in the R package *igraph* (Csardi and Nepusz, 2006). Descriptive and
198 topological network properties, as well as network modules (substructures of nodes with a
199 higher density of edges within the group than outside it) were calculated as described in
200 Hartman et al. (2018).

201

202 *Morphological analysis of arbuscular mycorrhizal (AM) root colonization*

203 Roots of *P. judaica* plants sampled in urban sites (described above, $n = 8$) were carefully
204 washed with tap water, stained overnight in a solution of methyl blue (0.1% w/v) in 80%

205 lactic acid (v/v) and clarified in lactic acid in order to remove the excess of staining
206 solution. 80 segments, 1 cm long each, were obtained from each root apparatus, placed on
207 glass slides and observed under a light microscope.

208

209 *HPLC analysis of phenolic compounds*

210 For qualitative and quantitative determination of phenolic compounds, plant tissues (1 g
211 FW) of *P. judaica* were first homogenized for 3 min with 30 mL hot MeOH–EtOH (1:1) and then
212 refluxed under nitrogen for 30 min ($2 \times$). After centrifugation and pooling of the extracts,
213 the combined solutions were first concentrated under vacuum, depigmented with petroleum
214 ether (bp 40–70 °C), filtered through 0.45 µm Millipore Millex-HN, and then used for the
215 determination of total phenolic content and HPLC-DAD determination of phenolic
216 compounds (Lattanzio et al., 2001). Identification of phenolics was made by using retention
217 times (tR) and spectral data of different peaks compared with standard compounds
218 (Extrasynthese, Genay, France). In addition, HPLC-MS/MS analyses of main peaks
219 identified in *P. judaica* and standard compounds were used for structure characterization.
220 Metabolites released by root (root exudates fraction) were collected according to Tato et
221 al. (2020) from plants grown hydroponically. The collected materials were acidified to pH
222 3.5–4.0 with HCl to maintain the structural stability of phenolic compounds, freeze-dried,
223 suspended in 3 mL methanol and filtered through 0.45 µm Millipore Millex-HN; the filtered
224 solution was analyzed for total phenolic content and HPLC determination of phenolic
225 compounds. HPLC analyses were performed with Hewlett Packard Series 1100 liquid
226 chromatograph equipped with a binary gradient pump G1312A, a G1315A
227 spectrophotometric photodiode array detector was set at 325 nm, and G1316A Column with
228 the thermostat set at 45 °C. The Hewlett Packard Chem Station (Rev. A. 06.03) software
229 was used for spectra and data processing. An analytical Phenomenex (Torrance, CA, USA)
230 Luna C18 (5) column (4.6 × 250 mm) was used throughout this work. The solvent system
231 consisted of (A) MeOH and (B) acetic acid-water (5/95, v/v). The elution profile was as
232 reported by Lattanzio and Van Sumere (1987). The flow rate was 1 mL min^{−1}. Samples of
233 (25 µl) were applied to the column using a 25 µl loop valve. UV absorption spectra were
234 acquired in the 235–450 nm range.

235 HPLC-MS/MS analyses were performed on a QTrap MS/MS system, (Applied Biosystems,
236 Foster City, CA, USA), equipped with an ESI interface and a 1100 series micro-LC system
237 comprising a binary pump and a microautosampler (Agilent Technologies, Waldbronn,

Germany). The ESI interface was used in positive ion mode, with the following settings: temperature (TEM) 350 °C; curtain gas, nitrogen, 30 psi; nebuliser gas air, 10 psi; heater gas, air, 30 psi; ion spray voltage + 4500 V. Full scan chromatograms were acquired in the mass range 100–800 amu, MS/MS chromatograms were acquired at collision energy of 20 V. LC conditions were as for the HPLC-DAD analysis.

Root morphology and biomass allocation

After 7 d treatments (+Fe, -Fe, Bic, Tric), three independent biological samples from each treatment were collected randomly, and their shoots and roots were harvested. Shoots were dried at 70 °C for 48 h, and their dry weight (WS, g) was measured. The root system was stained with 0.1% (w/v) toluidine blue O for 5 min and then divided into two root orders: ‘shoot-borne’ or adventitious roots (AR), and their 1st-order lateral roots (LR) as defined by [Atkinson et al. \(2014\)](#). Each root was scanned at 300 dpi resolution (WinRhizo STD 1600, Instruments Regent Inc., Canada) to determine length (LA, cm), volume (VA, cm³) and surface area (SA, cm²) of the adventitious roots and total length (LI, cm), total volume (VI, cm³) and total surface area (SI, cm²) of the 1st-order laterals using the WinRhizo Pro v. 4.0 software package (Instruments Regent Inc.). Length (LT), surface area (ST) and volume (VT) of the whole root system were calculated as the sum of the two root types. Then, dry weights of the adventitious roots (WA, g) and total dry weight of 1st-order lateral roots (WI, g) were measured after drying in an oven at 70 °C for 48 h. Total root dry weight (WT, g) was the sum of the WA and WI. Plant dry weight (WP, g) was obtained as the sum of WT and WS. Based on the measurements above, the following parameters were calculated for the whole root system:

$$\text{root length ratio RLR} = \text{LT/WP (cm g}^{-1}\text{)}$$

$$\text{root mass ratio RMR} = \text{WT/WP (g g}^{-1}\text{)}$$

$$\text{fineness F} = \text{LT/VT (cm cm}^{-3}\text{)}$$

$$\text{tissue density TD} = \text{WT/VT (g cm}^{-3}\text{)}$$

where RLR expresses the root order’s potential for the acquisition of below-ground resources, the RMR indicates the relative biomass allocated to the root and F and TD represent the structural root parameters. The relationship among these parameters is: $\text{RLR} = \text{RMR} \times \text{F/TD}$ ([Ryser and Lambers, 1995](#)).

269 The adventitious (NA) number and the 1st-order laterals (NI) were directly counted from
270 the images. The average length of the adventitious [$aLA = LA/NA$] and the 1st-order
271 laterals [$aLI = LI/NI$] (cm) were also calculated (Table S1). The 'branching zone' length
272 (BZ) that extends rootwards from the shoot base to the youngest emerged LR and the
273 'lateral formation zone' (LFZ) that spreads from below the youngest emerged LR up to the
274 2–6 mm from the root apex were also measured, as described in Dubrovsky and Forde
275 (2012). NI/BZ calculated the root branching density (BD, number of laterals in cm of
276 branching zone).

277 Two-way ANOVA tested the effects of the different treatments on the root parameters. Tukey's
278 post hoc test comparison was applied to test the effects of each treatment at $P < 0.05$. To
279 correct for allometric effects (Coleman et al., 1994), the ln-transformed plant dry weight (ln
280 WP) was used as a covariate to analyze the root morphology and biomass allocation, when
281 significant correlations between lnWP and these root traits were found. A multivariate
282 statistical PCA (principal components analysis) and a cluster analysis were performed
283 using SPSS software. To unveil the impact of the root morphology pattern on plant growth,
284 Pearson's test was used to test the correlation between the PC factor scores and plant DW.
285 Statistical analysis was conducted using the Systat v. 8.0 software package (SPSS Inc.,
286 Evanston, IL, USA).

287

288 *Estimation of total ortho-dihydroxy phenolic compounds (Arnow's reagent)*

289 One mL of extract sample was placed in a test tube and 1 mL 0.5 N HCl was added. The tube
290 was well mixed and then 1 mL Arnow's reagent (10 g Na nitrite and 10 g Na molybdate in a
291 final volume of 100 mL distilled water) was added (which resulted in a yellow color), mixed,
292 and 1 mL 1 N NaOH was added (solution turned into red color). The solution was then
293 brought to a final volume of 5 mL with distilled water and absorbance was measured at
294 500 nm. The concentration was calculated and expressed as mg g^{-1} FW. Chlorogenic acid was
295 used as a standard, in a range of 0–0.15 mg mL^{-1} .

296

297 *Iron reduction by phenolics compounds*

298 The phenolics concentration in root exudates and the caffeic and citric acids ability to
299 reduce Fe(III)-EDTA was measured spectrophotometrically by using BPDS (Chaney et al.,
300 1972). Root exudates (100 μg), prepared as described above, were incubated for 120 min in 1
301 mL 100 mM Fe(III)-EDTA, 100 mM BPDS solution, in the dark, at 26 °C and under

302 shaking. A solution containing caffeic (50 mM) and/or citric acid (50 mM), 100 mM Fe(III)-
303 EDTA and 100 μ M BPDS, in the dark at 26 °C and under shaking, was also prepared, according
304 to [Hu et al. \(2005\)](#). The absorbance at 535 nm was measured as in [Donnini et al. \(2009\)](#).

305

306 *Soil incubation with caffeic and citric acids*

307 Solutions of caffeic and citric acids were adjusted to pH 5.5 using diluted NaOH and added
308 to soil at rates 50 μ mol acid g^{-1} soil according to [Hu et al. \(2005\)](#). The soil was watered to
309 field capacity, and then incubated at 20 °C for 30 min. Soluble soil fractions were collected
310 according to [Mimmo et al. \(2008\)](#). Extracts were filtered through 0.2 μ m filters and then
311 analyzed by an Agilent 7100 Capillary Electrophoresis System (Agilent Technologies, Santa
312 Clara, CA, US). Phosphate anions were determined by capillary electrophoresis, using a bare
313 fused silica capillary with extended light path BF3 (i.d. = 50 μ m, I = 72 cm, L = 80.5 cm).
314 Sample injection was at 50 mbar for 4 s with -30 kV voltage and detection at 350/80 nm
315 wavelength. Compounds were identified using pure standards and anion contents were
316 expressed as μ g g^{-1} FW.

317

318 *Miscellaneous*

319 Iron and P content were determined by ICP-MS on oven-dried tissue samples ($n = 3$)
320 mineralized in HNO_3 . and carboxylic acids contents in roots were determined according to
321 [Tato et al. \(2020\)](#). Apoplastic Fe was determined according to [Tato et al. \(2020\)](#). Briefly,
322 roots from 5 plants per treatment were transferred to a beaker with 0.5 mM $CaSO_4$ under
323 vigorous aeration. After 10–15 min, roots were transferred to 40 mL tubes with 21 mL of
324 10 mM 2-(N-morpholino)-ethanesulfonic acid (MES), 0.5 mM $Ca(NO_3)_2$, 1.5 mM 2,2'-
325 bipyridyl (pH 5.5) at 25 °C. Tubes were covered with a cotton plug and N_2 was bubbled
326 through the solution. After 5 min, 1 mL of 250 mM $Na_2S_2O_4$ was added. The A_{520} of the
327 solution ($Fe[bipyridyl]_3 \epsilon = 8.650 M^{-1} cm^{-1}$) was determined on 2 mL aliquots. The
328 aliquots employed for the determinations were returned to the tube and left for 1 h in the
329 dark. The determination was carried out every 1 h 30 min until a constant value was
330 obtained. Lignin visualization was performed according to [Donnini et al. \(2011\)](#). After
331 being fixed at 4 °C overnight in 100 mM Na-phosphate buffer (pH 7.00) containing 4%
332 paraformaldehyde (w/v), root segments were dehydrated through an ethanol-tertiary

333 butanol series and embedded in paraffin (Paraplast Plus, Sigma). Serial sections (5 μ m)
334 were cut with a micro- tome, mounted on silanized slides, deparaffinized in xylene and
335 rehydrated through an ethanol series. Sections were then stained with the safranin/fast
336 green method (Johansen, 1940), mounted with cover slides, observed by optical
337 microscopy (Leica DMR) and images were acquired using a digital camera (Leica
338 DC300F).

339

340 **Results**

341 *The root-associated microbiome of P. judaica collected from urban sites*

342 *P. judaica* plants were harvested from soil of an urban site displaying alkaline and
343 calcareous conditions, with a pH value of 7.9 ± 1.3 and an average 20% CaCO₃ content.

344 The root-associated microbiota of the collected plants (rhizosphere samples) and that of the
345 soil not affected by the root presence (bulk soil samples) were then analyzed. The
346 rarefaction curves indicated that a satisfactory sequencing depth was obtained for each
347 considered sample, for both fungal and bacterial amplicons (Fig. S1). The clustering of high-
348 quality reads allowed us to obtain 349 fungal and 2327 prokaryotic operational taxonomic
349 units (OTUs), that were further assigned taxonomically (Supplemental files 1a, 1b). The
350 standardized datasets were used to generate Venn diagrams of the community composition
351 and unique or shared OTUs among bulk soil and rhizosphere samples (Fig. 1). The
352 results show a reasonable degree of microbiota diversity, especially for prokaryotes (more
353 than 2000 OTUs overall). Also, a lower number of OTUs unique to the rhizosphere
354 compartment was detected, while the highest OTU number was present in the bulk soil
355 only. The OTUs shared between rhizosphere and bulk soil likely represent the core of
356 microbes recruited by roots under the occurring environmental conditions.

357 The analysis of Alpha diversity indexes outlines the structure of the considered microbial
358 communities (Fig. S2). All indexes point to a higher microbial richness in the bulk soil
359 than the rhizosphere, in agreement with the higher OTU number detected in the former
360 compartment. A general analysis of Beta diversity on prokaryotic and fungal communities
361 was also conducted. The Bray-Curtis index did not find a significant dissimilarity in the
362 microbial composition of bulk soil and rhizosphere; however, the two compartments appear
363 more diverse when phylogenetic distances weighted by relative abundances were
364 considered in relation to fungal microbiota (weighted GUnifrac, Fig. S3). Notably, the
365 prokaryotic taxa typically present and dominant in soils (Jansenn, 2006) were all detected
366 in the current experiment (Fig. 2), with Proteobacteria as the most represented in both
367 compartments. However, Acidobacteria were underrepresented; this result is consistent

with the high pH of the soil under investigation (around pH 7.9), as many species in this phylum are indeed acidophilic.

The bulk soil and rhizosphere compartments do not show a dramatic difference in the overall community structure, although some shifts in microbial composition can be detected already at the phylum level (Fig. 2). The rhizosphere showed an increase in the relative abundance of Proteobacteria, Actinobacteria and Firmicutes phyla in comparison to the bulk soil. In contrast, other phyla such as Bacteroidetes, Chloroflexi, Planctomycetes and Verrucomicrobia were significantly more abundant in the bulk soil compartment than in the rhizosphere (Fig. 2, upper panel).

A more in-depth analysis of microbiome composition (at genus level) revealed a higher relative abundance of *Bacillus*, the *Rhizobium* group and *Streptomyces* in the rhizosphere, and for the last two genera the increase was statistically significant (Fig. 3, upper panel). However, the fungal genera *Mortierella* and *Wallemia* were more abundant in the bulk soil (Fig. 3, lower panel). This latter genus comprises a few known species for their xerotolerant and halophilic behaviour (Zajc and Gunde-Cimerman, 2018).

Arbuscular mycorrhizal (AM) fungi provide many services to plants, including the improvement of mineral nutrition, at a cost for the plant host, since they take up photosynthates from the roots. Hence, the presence of AM fungi of such urban soil was also investigated; a few OTUs referring to AM fungi were retrieved in both the bulk soil and the rhizosphere compartment (data not shown). They point to *Funneliformis mosseae*, a widespread species common in diverse soils, and to another AM fungus also belonging to Glomeromycotina. Despite AM OTUs not being abundant in our dataset, arbuscule formation was observed in the same *P. judaica* roots sampled for the microbiome sequencing, suggesting that AM fungi are an active microbial component of such an urban niche.

Co-occurrences in the Prokaryotic and Fungal communities were assessed by performing network analysis and visualising the positive, significant correlations among OTUs ($\rho > 0.7$ and $p < 0.001$, Fig. S4). Similarly, meta-networks were constructed to visualize correlations between Prokaryotic and Fungal OTUs in the soil and root communities (Fig. 4). Within this network, we identified keystone OTUs (Fig. 4), defined as the top 1% node with the highest degree of interactions. These OTUs are microbial taxa that frequently co-occur with other taxa under the experimental conditions considered, and are thought to be ecologically important and to play potentially a key role in the structuring of the microbiota (Hartman et al., 2018, Supplemental file 2).

Also, OTUs that might act as indicator species in such networks were sought; a species is described as an “indicator” when it is characteristic of a group of samples or experimental treatments and/or is highly sensitive to the changes entailed by the treatment. Only two bacterial OTUs could be identified as indicators for the rhizosphere compartment (Supplemental file 3): the first one refers to the *Rhizobium* genus (probably *Rhizobium grahamii*, 100% sequence identity), the second points to an uncultured isolate belonging to Actinobacteria (99.75% identity). Members of Actinobacteria are widespread in soils and display tolerance to diverse extreme conditions (Ranjani et al., 2016).

Indicator species of the bulk soil comprise genera of fungi and bacteria known to be widespread in soils, including saprotrophs such as *Mortierella* as well as microbes that tolerate extreme environments, such as the fungi *Coniosporium apollinis* and *Naganishia albida*, as well as the bacteria *Microvirga*, *Brevundimonas*, *Altererythrobacter* and *Rhodospirellula* (Supplemental File 3).

The microbiome associated with the roots displayed an increase in P solubilizing microbial genera (as defined by Kalayu, 2019), mainly belonging to Rhizobiaceae and *Streptomyces* (Fig. 3, upper panel). Conversely, some soil generalist microbes seem to be rather excluded from the rhizosphere of *P. judaica*.

Root morphology of P. judaica grown in calcareous, alkaline or Fe- deprived media

P. judaica plants sampled from the urban sites were allowed to radicate in half-strength complete nutrient solution and then transferred into one of four different media (i.e. +Fe, -Fe, Bic and Tric), to discriminate between the effects of low Fe availability due to a high pH and those of bicarbonate itself (Tato et al., 2020). Since alkaline and calcareous conditions mainly affect Fe and P availability, the leaf and root concentration of these nutrients was determined. A reduction of Fe concentration was observed in leaves of plants grown in the -Fe, Bic and Tric media, and in -Fe roots (Fig. 5). Phosphorus was slightly decreased with respect to the control only in the roots of plants grown in Bic (Fig. 5).

The morphology of the whole root systems of *P. judaica* was not significantly modified by all the treatments in comparison with the +Fe condition, with exception of the TD (Fig. 6A, B and C). Indeed, the TD of the whole root system was lower in the -Fe and +Fe than in Bic- and Tric- treated plants (Fig. 6C).

The root system of *P. judaica* consisted of the adventitious roots (AR), also named “shoot-borne” roots, and the lateral roots (LR), which emerged from AR, suggesting the “within-root” approach to analyze the root morphology. Differently from the whole root system, some

436 treatments significantly modified the LI, the aLI and the aLA in comparison to those of the +Fe
 437 plants (Fig. 6D): Bic-treated plants decreased (—49%) the aLA in comparison to the +Fe
 438 treatment and the Tric treatment reduced the LI and aLI by —83% and —71%, respectively,
 439 compared to the +Fe plants (Fig. 6D). The Fe-deficient plants exhibited similar morphology in
 440 both root types when compared with the +Fe plants (Fig. 6D). As expected in plants adapted
 441 to alkaline soils (White et al., 2013), Bic-treated *P. judaica* plants exhibited an increase in
 442 the BD (+61%) associated to a reduction of the BZ, but these parameters were unchanged
 443 in the Fe deficiency and Tric treatments (Fig. 6E).
 444 The PCA was applied using only root parameters significantly changed by treatments as
 445 observed in the univariate ANOVA. The PC1 (explaining 49% of the variance) consisted
 446 of high positive loads for the aLA and the BZ and negative loads for the TD, while the PC2
 447 (explaining 38% of the variance) showed high positive loads for the LI and aLI (Table 1).
 448 Two-dimensional PCA score plots and subsequent hierarchical cluster analysis revealed a
 449 sharp separation among the treatments (Fig. 6A; Fig. S1). In particular, a first cluster
 450 included the +Fe plants, a second cluster comprised the Bic-treated plants, and a third one
 451 incorporated both the Tric- and Fe deficient-treated plants (Fig. 7A; Fig. S5). As shown by
 452 Fig. 6A and Table 1, the Bic-treated plants were characterized by lower aLA and BZ
 453 associated with high TD, aLI and LI, thus exhibiting root architectures different to the +Fe
 454 plants. Conversely, the root architectures of the Fe deficient- and Tric-treated plants
 455 exhibited lower aLI and LI but intermediate values of aLA, RBZ and TD between the Bic
 456 treated and the +Fe plants (Fig. 6A). The importance of these different root architectures
 457 for the *P. judaica* fitness was tested by a Pearson correlation between the plant dry weight
 458 and the PC1 and PC2 scores. The PC1 was significantly and positively correlated with the
 459 plant growth ($r = 0.4890$, $p = 0.0139$) differently to the PC2 ($r = 0.0356$, $p = 0.55$)
 460 (Fig. 7B and C), suggesting that the high aLA, BZ and low TD but not the lateral roots (PC2)
 461 explained most of the *P. judaica* growth.

462

463 *Carboxylic acids and phenolic compounds in root tissues and in exudates of P. judaica grown in* 464 *calcareous, alkaline or Fe-deprived media*

465 The total phenolics and carboxylic acids released by *P. judaica* roots were recently monitored
 466 in the four conditions, i.e. control, -Fe, Bic, Tric (Tato et al., 2020). The profile of carboxylic
 467 acids and the total phenolics of *P. judaica*, both in roots and in their exudates are shown in

468 Figs. S6 and S7, respectively. Malic, ketoglutaric and citric acids were detected in all tested
 469 root samples, with a higher accumulation of malic and citric acid in -Fe, Bic, and Tric
 470 treatments, whereas ketoglutaric acid concentration increased only in -Fe and Tric-treated
 471 plant roots. Both malic and citric acids were present in all root exudates, with a higher
 472 accumulation in -Fe, Bic and Tric treatments. Notably, cis-aconitic acid was exuded only
 473 by the Tric-treated roots.

474 Total root phenolics were measured spectrophotometrically using Arnow's reagent, which
 475 selectively determines the concentration of ortho-dihydroxyphenolic compounds (Fig. S7A).
 476 Only the Bic treatment induced a significant increase (87%) of total phenolic concentration
 477 in roots. A non-significant increase was observed in the other two conditions, -Fe and Tric
 478 (+28% and +17% respectively). These data are consistent with the results obtained from
 479 chromatographic HPLC-MS analyses where an increase of total phenolics in plants grown under
 480 Bic condition (+73%) and in plants grown in Tric conditions (+18%) was found (Fig.
 481 S7B). In detail, the phenolic fraction of root tissues was characterized by the presence of
 482 various positional isomers of mono- and di-caffeoylquinic acid esters. 5-O-caffeoylquinic acid
 483 (chlorogenic acid) and 3,5-O-dicaffeoylquinic acid were the main constituents of the phenolics
 484 fraction in root extracts (Fig. 8A, left panel). Other caffeoylquinic derivatives identified in
 485 roots were: 3-O-caffeoylquinic acid, 4,5-O- and 3,4-O-dicaffeoylquinic acids, and two *p*-
 486 coumaric acid glycosides. Accordingly, the phenolics content of plants harvested in the
 487 urban site revealed that the main constituents of root extracts of *P. judaica* were represented
 488 by chlorogenic acid and 3,5-O-dicaffeoylquinic acid, which constitute together about 88%
 489 of the total phenolic compounds present in root extracts (Fig. 8B).

490 From a qualitative viewpoint, isomerization phenomena have been observed in the
 491 phenolic fraction of stressed plants in comparison with the control. Overall, such
 492 isomerization phenomena in all stressed plant extracts was associated to an increase of total
 493 mono-caffeoylquinic acids while the total content of di-caffeoylquinic acids decreased (Fig.
 494 8A, left panel).

495 The nutritional stress conditions -Fe, Bic, Tric also affected the composition of phenolic
 496 fractions of *P. judaica* root exudates (Fig. 8A, right panel) in which 3-O- and 5-O-
 497 caffeoylquinic acids, 3,5-O-dicaffeoylquinic acid and the caffeic acid aglycone were identified;
 498 the latter compound is absent in root extracts. This suggests that secretion of phenolics by *P.*
 499 *judaica* roots (both control and stressed roots) also leads to partial hydrolysis of caffeoylquinic
 500 esters. Although total phenolics concentration determined by Arnow's reagent method revealed
 501 an increase mainly in root exudates of -Fe plants (Fig. S7A), HPLC-MS results revealed an

502 increase in total phenolics root exudates from -Fe, Bic and Tric treated plants (Fig. S7B).
503 Again, the highest increase in phenolics was observed in Bic exudates.
504 In Bic and Tric exudates, caffeic acid accounted for 67 and 44% of the total phenolics,
505 respectively. Caffeic acid and chlorogenic acid together accounted for 76–84% of total
506 phenolic compounds in both control and all the stressed exudates (Fig. 8A, right panel).

507

508 *Fe mobilization properties of P. judaica's root exudates in calcareous, alkaline or Fe-deprived*
509 *media*

510 The ability of root exudates to favor Fe mobilization was tested by measuring the *in vivo*
511 Fe reductase activities of exudates secreted from roots of *P. judaica* plants grown in -Fe,
512 Bic and Tric media. Such Fe reductase activity was increased by all three stress conditions,
513 and in particular, it was the highest in the Bic treatment (Fig. 9A). Since Bic- grown *P.*
514 *judaica* roots exuded citric and caffeic acids, the effect of commercially available caffeic
515 and citric acids on the Fe(III) reduction was assayed. Caffeic acid displayed a higher Fe
516 reduction capacity compared with citric acid (Fig. 9A). Besides Fe availability, calcareous
517 conditions also affect phosphorus (P) availability; the urban calcareous soil where plants
518 were collected was incubated with caffeic and citric acid to study their potential effect on
519 PO_4^{3-} solubility. Soil incubation with both caffeic and citric acid enhanced the
520 concentration of PO_4^{3-} in the soil soluble fraction with respect to the control, but the effect
521 of citric acid was stronger than that of caffeic acid (Fig. S8).

522 Bic treatment led to a significant accumulation of Fe in the apoplast, suggesting that the
523 higher synthesis of phenolics compounds might be induced by the high Fe content in the
524 intercellular spaces (Fig. 9B). Other than Fe mobilization, phenolics might be involved in
525 other re- actions, such as lignification process (Donnini et al., 2011). Therefore, the
526 lignification rate of root tissues was investigated by a staining procedure and the root cross-
527 sections were visualized microscopically. Roots of plants grown under Bic and Tric
528 showed high lignification signals at rhizodermis, endodermis and cortex layers (Fig. 9C).
529 Such findings suggest that both alkaline growth conditions (Bic and Tric) induced the
530 synthesis of lignin in the root cell walls.

531

532 Discussion

533 The study of plants living in natural and extreme environments, in which different stressors
534 naturally coexist, allows to unravel the morpho-anatomical and physiological traits
535 enabling them to survive in these extreme environments (Bartoli et al., 2013; Bechtold,

536 [2018](#)). In this paper, we investigated different traits characterizing the root-soil interaction
 537 of *P. judaica*, a wild plant species commonly known as “Pellitory of the wall”, including
 538 morphological plasticity, exudation, and association with the microbiome.
 539 The analysis of the root microbial community associated to spontaneous *P. judaica* plants
 540 harvested from the urban environment revealed a good degree of microbiota diversity, detecting
 541 all prokaryotic taxa typically present and dominant in soils. The comparison of the root-
 542 associated versus bulk soil microbiota highlighted some shifts in the microbial
 543 composition, with a higher relative abundance in the rhizo- sphere of some genera,
 544 including beneficial species for plants. Among these beneficial bacterial species, two
 545 OTUs are noteworthy; the first OTU refers to a bacterium from the genus *Rhizobium*, whereas
 546 the second OTU points to an unidentified actinobacterium. Besides the Rhizobia’s ability to
 547 form N-fixing nodules on legume roots, they can also thrive in the rhizosphere of non-
 548 leguminous plants acting as Plant Growth Promoting Rhizobacteria (PGPR, [Mehboob et al., 2012](#)),
 549 thus providing benefits even in the absence of nodule formation. Also, besides
 550 the well-studied *Streptomyces* genus, many other Actinobacteria can associate with roots
 551 of a wide range of hosts beneficial for the plants’ health, and the interest in their use as
 552 PGPR bacteria has raised in recent years ([Sathya et al., 2017](#)).
 553 Taken together, our analyses of the root-associated microbiome of spontaneous urban *P.*
 554 *judaica* plants indicate that these plants retain the competence to actively recruit beneficial
 555 soil microbes such as PGPR, phosphate solubilizers and AM fungi, possibly excluding
 556 from their rhizosphere other components of the soil microbiota. These results are
 557 remarkable, given the limited microbial reservoir to which plant roots had access in the
 558 urban environmental niche where these plants were growing.
 559 It is now well acknowledged that plants play an active role in the assembly of the
 560 rhizospheric microbiota, and the outcome and magnitude of such an influence can change,
 561 depending on both fixed (e.g. plant genotype) and variable (biotic/abiotic stresses) factors.
 562 In this scenario, root exudation can act as a crucial driver of microbiota recruitment ([Sasse et al., 2018](#)).
 563 Among the different fractions of *A. thaliana* root exudates, phenolics are
 564 very effective in shaping the soil microbiome, as they significantly correlate with 31
 565 bacterial OTUs ([Badri et al., 2013](#)). Also, a role for root-secreted coumarins in shaping the
 566 *A. thaliana* rhizospheric microbiota has been found ([Voges et al., 2019](#)). In particular,
 567 coumarins limit the growth of a *Pseudomonas* strain through a mechanism that involves the
 568 production of reactive oxygen species. Interestingly, we could not highlight any significant
 569 enrichment for the *Pseudomonas* genus in the *P. judaica* rhizosphere, although some other
 570 genera of PGP bacteria seemed to be actively recruited in the rizhophane. This suggests that

the negative effect demonstrated for coumarins on *Pseudomonas* growth might be extended to other phenolic compounds, thus providing indications towards the engineering of beneficial plant root microbiota.

Root morphological plasticity and exudation, which are two relevant processes driving plant-soil interaction, were investigated in *P. judaica* grown in three different controlled conditions inducing Fe-deficiency, i.e. -Fe, Bic, Tric, to discriminate between root responses to the low availability of Fe due to a high pH and that caused by bicarbonate (Tato et al., 2020). Among the morphological parameters of the whole root system, the TD of *P. judaica* was the only affected trait, mainly by the calcareous condition (Bic) (Fig. 1C). The TD is an adaptive trait positively correlated with the lignification degree and cell wall thickness (Ciamporova et al., 1998; Wahl and Ryser, 2000; Hummel et al., 2007) and, in turn, is inversely related to the *Arabidopsis* adaptation to Fe deficiency (Barberon et al., 2016). As well, the root lignification degree has been interpreted as a Fe deficiency sensitivity trait in a quince rootstock (Donnini et al., 2011).

Besides studying the morphology of the whole root system, a ‘within- root analysis’ was applied, looking at the morphological changes of the different root types. Such a phenotyping approach could provide early information on the contributions of the different root types of *P. judaica* to the adaptation in alkaline, calcareous and Fe deficient conditions. Indeed, root types were found to respond differently to the environmental cues such as water (Romano et al., 2013; Tellah et al., 2014; Abenavoli et al., 2016), salt (Stevanato et al., 2013), and combined P/drought stress (Ho et al., 2005), N deficiency (Sorgona et al., 2007), allelochemicals (Abenavoli et al., 2004, 2008; Lupini et al., 2016), as well as rot (Roman-Aviles et al., 2004) and fungal colonization (Zadworny and Eissenstat, 2011). In the present work, lateral roots of *P. judaica* were more modified by treatments than adventitious ones. This kind of root ideotype, which is characterized by an even extended spread of roots throughout the soil, is useful for the acquisition of nutrients with restricted phytoavailability in alkaline soils (White et al., 2013). Indeed, *P. judaica* exhibited an increase of the BD (+61%), maintaining the LI, in response to the Bic treatment (Fig. 5D and E).

Recently, several works pointed out the importance of the synergism and/or antagonism among the different root traits for understanding the root architecture adaptation to diverse environments (York et al., 2013; Miguel et al., 2015; Rangarajan et al., 2018), suggesting using a multivariate rather than a univariate approach for analyzing *P. judaica* root architecture. The “root multi-trait” pattern, as determined in the present work, also in agreement with the results of within-root morphology, might reflect the adaptation of *P.*

606 *judaica* to low Fe availability caused by high pH (Tric-treatment) and calcareous environment
 607 (Bic). Indeed, the root architecture of the -Fe and Tric-treated plants was characterized by
 608 the development of adventitious roots and branching zone associated with lower TD of
 609 their root axes. In particular, the low TD of the root axes observed in Fe-deficient and Tric
 610 treatments was negatively correlated with root exudation which, in turn, is a fundamental
 611 physiological trait of the Fe deficiency syndrome (Ladygina and Hedlund, 2010; Hell and
 612 Stephan, 2003; de Vries et al., 2019). This root trait, in association with a high aLA and
 613 RBZ, explained the higher fitness of *P. judaica* plants as shown by Pearson correlations.
 614 Conversely to the -Fe and Tric-treated plants, the root architecture of the Bic-treated ones was
 615 characterized by higher LI but less TD. The Bic- induced calcareous environment affected the
 616 availability of different nutrients, including P, Mn, B, and Zn (Tyler, 2003). In this study, a low
 617 P content in roots and an Fe accumulation in the root apoplast was observed in Bic-treated
 618 plants. In such conditions, the soil exploration by roots might be a strategy to survive in
 619 calcareous soils (White et al., 2013; Campestre et al., 2016; Ding et al., 2019). Indeed, *P.*
 620 *judaica* exposed to the calcareous condition displayed higher variability of root plasticity
 621 with respect to plants exposed to the other treatments, by reducing the branching zone,
 622 increasing the branching density and lateral spread.
 623 Direct (-Fe) as well as induced Fe deficiency (Bic, Tric), all caused an increase in caffeoylquinic
 624 acid derivatives, especially in Bic-treated roots (both tissues and exudates). Interestingly,
 625 caffeic acid, probably arising from hydrolysis of caffeoylquinic esters, is one of the components
 626 of the root exudates. The accumulation of phenolics in plant tissues is a hallmark of plant
 627 stress: phenolic compounds may be synthesized *de novo* in plants as a response to various
 628 biotic and abiotic stresses, including nutrient deficiency (Osmond et al., 1987; Cheynier et
 629 al., 2013; Lattanzio, 2019). Several studies have reported the increase in chlorogenic acid
 630 and/or mono- and di-caffeoylquinic acid in response to different abiotic stresses such as
 631 low-temperature (Lattanzio and Van Sumere, 1987; Lattanzio et al., 2001; Lattanzio et al.,
 632 1994), wounding (Cantos et al., 2001), high UV-B irradiation and insect attack (Izaguirre
 633 et al., 2007).
 634 Caffeoylquinic acid (CQA) derivatives are caffeic acid (3,4-dihydroxycinnamic acid)
 635 depsides, positional isomers of caffeic acid esters of quinic acid, which are broadly distributed
 636 in plants. The chelating activity of CQAs is attributed to their catechol ring (Kono et al., 1998).
 637 Low temperature stress induces, in artichoke tissues, an accumulation of constitutive
 638 phenolic compounds, mono- and di-caffeoylquinic acids, that protect chilled tissues from
 639 damage by free radical-induced oxidative stress (Lattanzio et al., 1994). Due to the presence
 640 of a catechol ring in its structure, chlorogenic acid can promote the reductive release of

641 ferritin Fe as mobile Fe^{2+} that, in turn, forms colourless complexes with the excess of
 642 chlorogenic acid (Boyer et al., 1988). Hence, chlorogenic acid can act as a reductant of
 643 Fe^{3+} as well as a ligand of Fe^{2+} . In addition, this paper shows that the exudation process
 644 produces, likely by hydrolytic processes, caffeic acid, which has a high Fe reduction ability.
 645 Overall, the results in the present work support the current view that secretion of phenolic
 646 compounds is a relevant component of the reduction strategy of Fe acquisition in non-
 647 graminaceous plants. In the past decades, several studies suggested that the secreted
 648 phenolics could enhance Fe availability in the rhizosphere soil, as an
 649 alternative/reinforcement of the membrane-bound reductase, through chelation and
 650 reduction of insoluble Fe. Initially, phenolics were thought to help with the solubilization
 651 and reutilization of apoplastic Fe in red clover. This feature was not considered part of the
 652 Fe uptake mechanism until coumarin derived phenolics were observed in *Arabidopsis*
 653 under high pH conditions (Fourcroy et al., 2014). Other plant species such as peanut (*Arachis*
 654 *hypogaea* L.) and rice (*Oryza sativa*) plants secrete other phenylpropanoids instead of
 655 coumarins, which also facilitate the reduction of ferric Fe (Romheld and Marschner, 1983;
 656 Ishimaru et al., 2011).

657 Root exudates collection was performed using a hydroponic-only system which is useful
 658 for the characterization of specific compounds, avoiding alteration via sorption processes
 659 to the soil matrix and microbial decomposition (Oburger and Jones, 2018). However,
 660 soil-specific resource availability and microbiome activity are important factors affecting
 661 plant metabolism and root exudation, and therefore hydroponic-only systems are less
 662 suitable to provide useful information about the metabolites released by roots (Oburger and
 663 Jones, 2018). Nevertheless, by setting up different treatments mimicking an alkaline or
 664 calcareous conditions, our approach allowed us to identify differential phenolics exudation
 665 patterns in direct (-Fe) and induced Fe deficiency (Bic and Tric) conditions. However,
 666 further analyses are required to provide more details on the root exudation from *P. judaica*.
 667 In addition, it has been suggested that the root Fe deficiency response also includes the
 668 dynamic use of a large Fe reservoir bound to cell wall components in the root apoplast,
 669 secretion of phenolic compounds in the apoplast, and inhibition of suberization of
 670 endodermal cells in order to allow apoplastic and transcellular radial transport of Fe
 671 (Romheld and Marschner, 1983; Jin et al., 2007, 2008; Ishimaru et al., 2011; Connorton et
 672 al., 2017). Accordingly, the increased cell wall lignification, together with the high
 673 apoplastic Fe accumulation observed in Bic-treated roots, are in agreement with these
 674 findings. Recently, it has been suggested that kiwifruit plants activate two different

675 strategies to acquire and translocate Fe from the -Fe or + Bic nutrient solution (Wang et al.,
676 2020). Under -Fe conditions, a foraging-reusing strategy increased the mobilization of Fe
677 (by the release of hemicellulose Fe from the cell wall and the redistribution of water-soluble
678 Fe and apoplastic Fe in roots). However, under + Bic conditions, roots employed a
679 resisting-inactivating strategy due to the bicarbonate-mediated inhibition of Fe
680 translocation from root to shoot, resulting in an accumulation of water-soluble and
681 apoplastic Fe and slowing down the release of hemicellulose Fe in the cell wall (Wang et
682 al., 2020).

683 The approach employed highlighted several differences between direct (-Fe) and induced
684 Fe deficiency (Bic and Tric) treatments. Other than the presence of bicarbonate, such
685 differences are also attributable to the high pH. Recently it has been demonstrated that the environ-
686 mental pH is an important determinant of global gene expression which tunes Fe
687 acquisition to the prevailing edaphic conditions in Arabidopsis plants. Under high pH, Fe
688 deficiency responses are affected, and the production and secretion of Fe-mobilizing
689 coumarins is induced, prioritizing the most effective strategy to mobilize Fe from otherwise
690 inaccessible pools. Furthermore, at transcriptional level, Fe-deficient plants grown at high
691 pH displayed an increased expression of genes involved in the orchestration of defence
692 responses to pathogens (Tsai, 2020).

693 Furthermore, the exudation of phenolics into the rhizosphere influences selectively some
694 microbial soil species that produce either siderophores or auxins that support Fe
695 acquisition by the plant (Jin et al., 2008; Stringlis et al., 2018). The overall microbiome
696 associated with the roots of *P. judaica* differed from that of the bulk soil, indicating that
697 plants in urban soil carry out microbial recruitment. The coumarin mechanism for shaping
698 root-associated microbioma is mainly associated to the catechol moiety of such compounds,
699 which can mobilize Fe and produce ROS, playing a detrimental effect on the growth of
700 some microbial genera (Voges et al., 2019). A mechanism similar to that suggested for
701 coumarins in the rhizosphere might also occur for *P. judaica* phenolics, as they also display
702 both the catechol moiety in their chemical structures and Fe reducing activity.

703

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712 characterization and data elaboration; V.La, V.Li phenols characterization; M.A, A.S, root
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716

717 **Declaration of competing interest**

718 The authors declare that they have no known competing financial interests or personal
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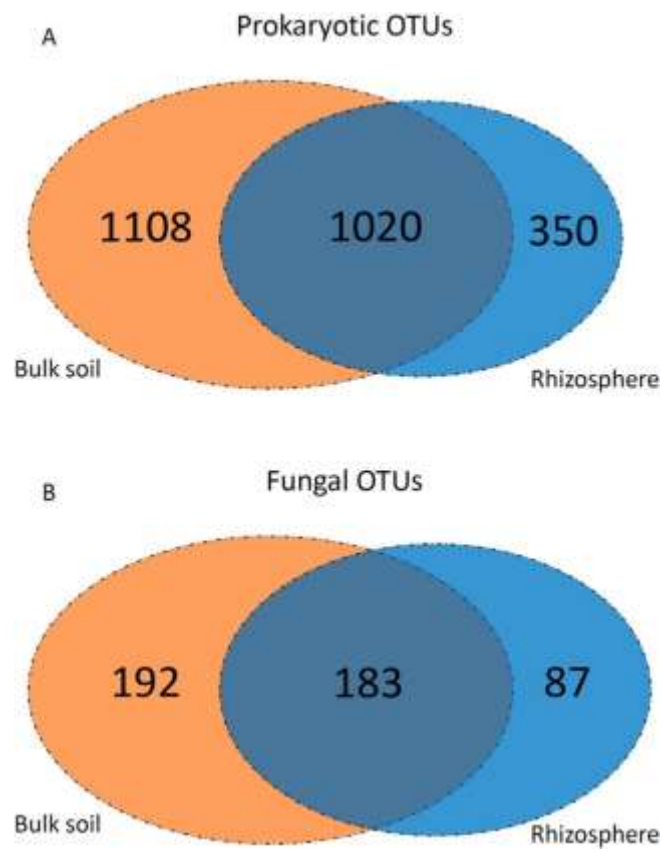


Fig. 1. Venn diagrams of the distribution of prokaryotic (A) and fungal (B) OTUs detected in bulk soil and rhizosphere samples. For each panel the number of identified OTUs in the bulk soil (in orange), and in the rhizosphere (in blue) are reported, as well as the number of overlapping OTUs. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

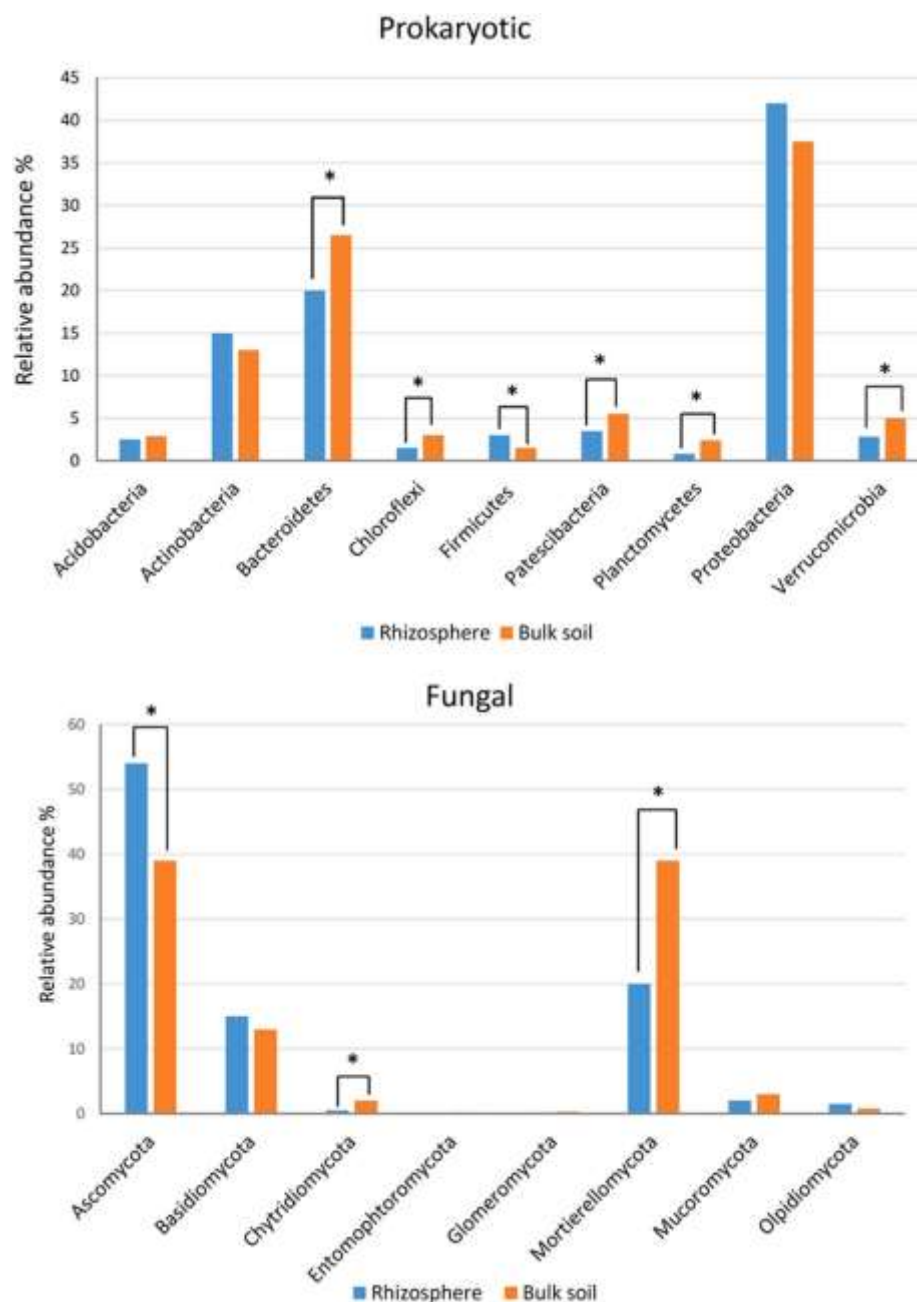


Fig. 2. Shifts in the microbial composition between the bulk soil and the rhizosphere microbiome at the phylum level. The bars show the relative abundance of each phylum on the overall microbial composition for the fungal (upper panel) and prokaryotic (lower panel) microbiome. Asterisks show significant differences in the bulk soil vs rhizosphere composition for each phylum displayed (p -value < 0.05). Eight independent biological replicates were considered ($n = 8$).

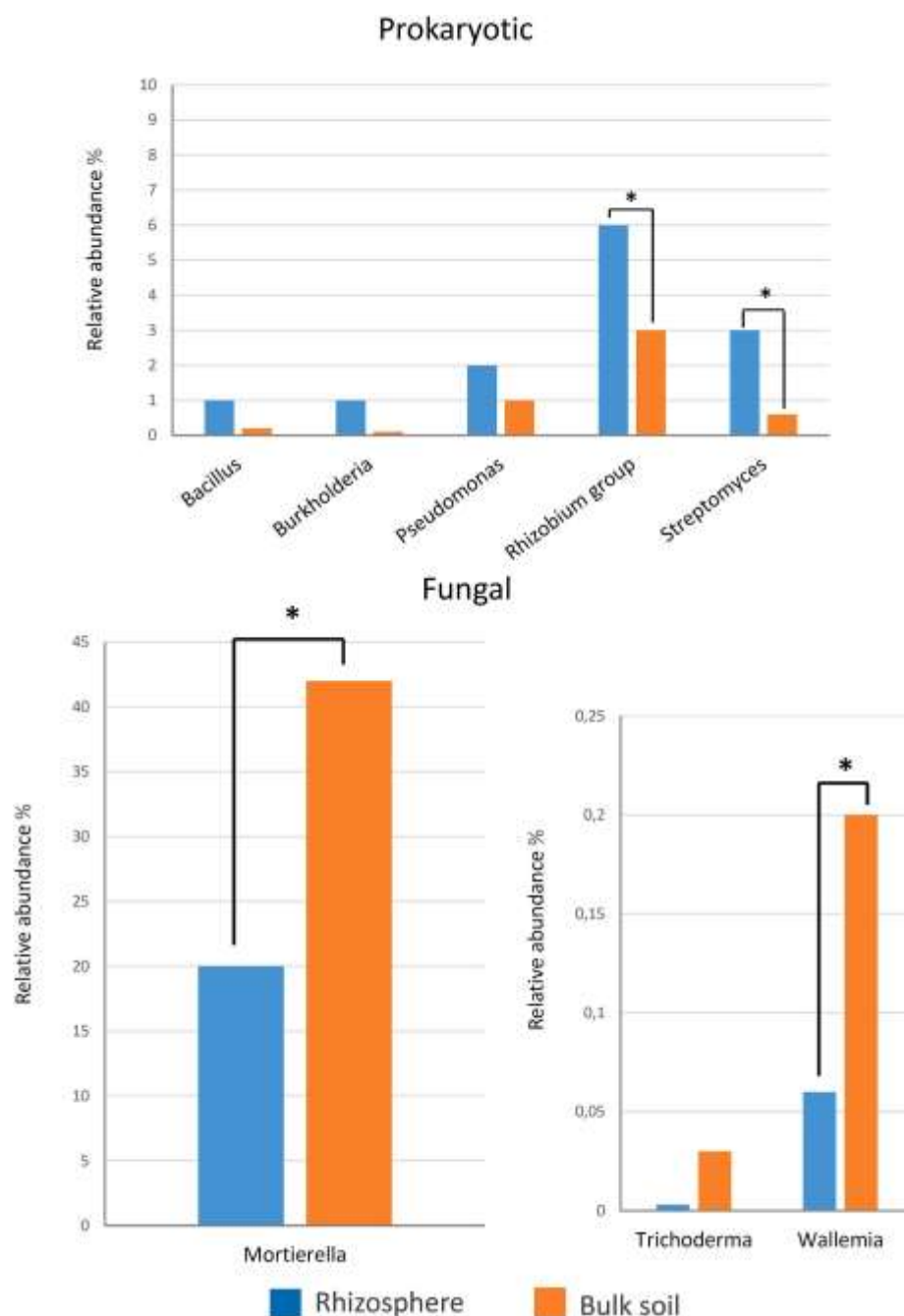


Fig. 3. Shifts in the microbial composition between the bulk soil and the rhizosphere microbiome at the genus level. The bars show the relative abundance on the overall microbial composition for the fungal and prokaryotic genera considered (n = 8). Asterisks show significant differences in the bulk soil vs rhizosphere composition for each genus displayed (p-value < 0.05). Note: the Rhizobium group comprises *Allorhizobium*, *Neorhizobium*, *Pararhizobium* and *Rhizobium*, as they are considered as a single genera according to the Silva taxonomy. Eight independent biological replicates were considered (n = 8).

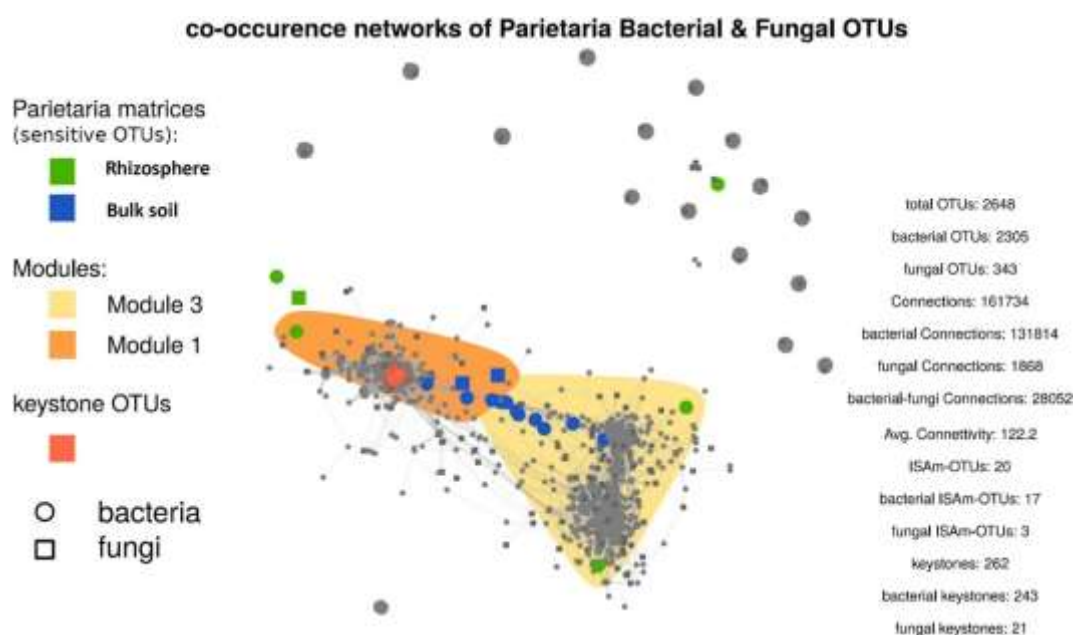


Fig. 4. Co-occurrence network (meta-network) visualising correlations between prokaryotic and fungal OTUs in the bulk soil and rhizosphere communities. The sensitive OTUs shown in green and blue represent the OTUs identified as indicator species for the rhizosphere and bulk soil condition, respectively (listed in supplemental file 3). Red triangles represent the Keystone OTUs (listed in supplemental file 2, red triangles) are also represented. Modules are defined as areas that show a high density of connections among OTUs. The Gray symbols represent “hot spots” of overlapping OTUs. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

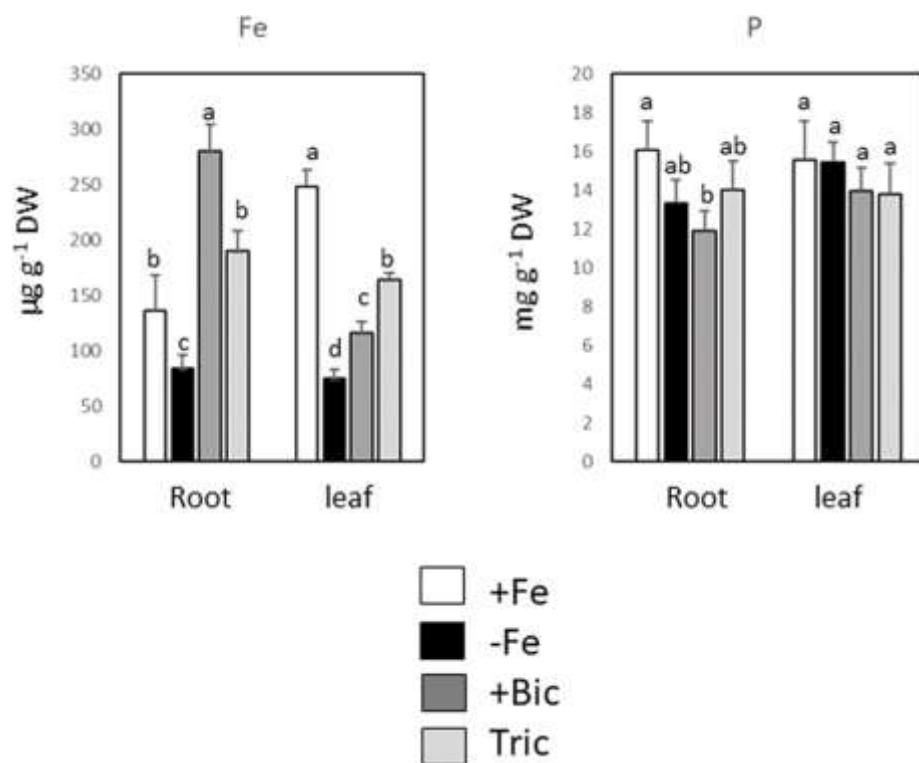


Fig. 5. Phosphorous (P) and iron (Fe) content in root and leaf tissues of *P. judaica* grown in +Fe, -Fe, Bic, and Tric treatments. Different letters correspond to significant differences among means ($P < 0.05$; Tukey test), $n = 3$.

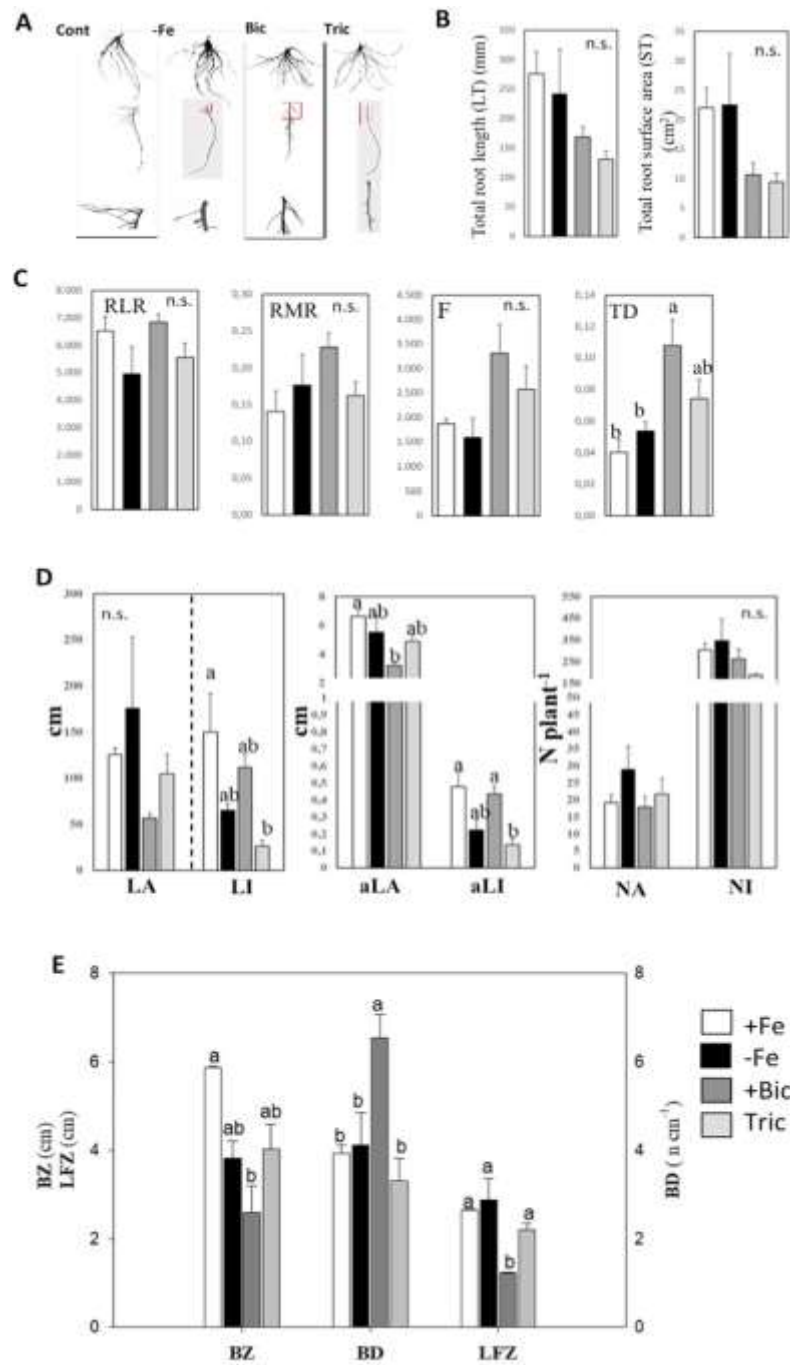


Fig. 6. Morphological analysis of the whole root system of *P. judaica* grown in +Fe, -Fe, Bic, and Tric media. A) Images captured of root; B) Total root length and total root surface; C) Root length ratio (RLR) and its components, i.e. root mass ratio RMR, fineness F and tissue density ratio TD; D) Morphological analyses intra-root of lateral roots (LI, length; aLI, average length, NI, number) and adventitious roots (LA, length; aLA, average length, NA, number) (abbreviation are also reported in Table S1); E) Root branching analysis of *P. judaica* (root branching zone's length (BZ), lateral root formation zone (LRFZ)). Different letters correspond to statistically significant differences among mean values ($P < 0.05$; Tukey test), $n = 3$.

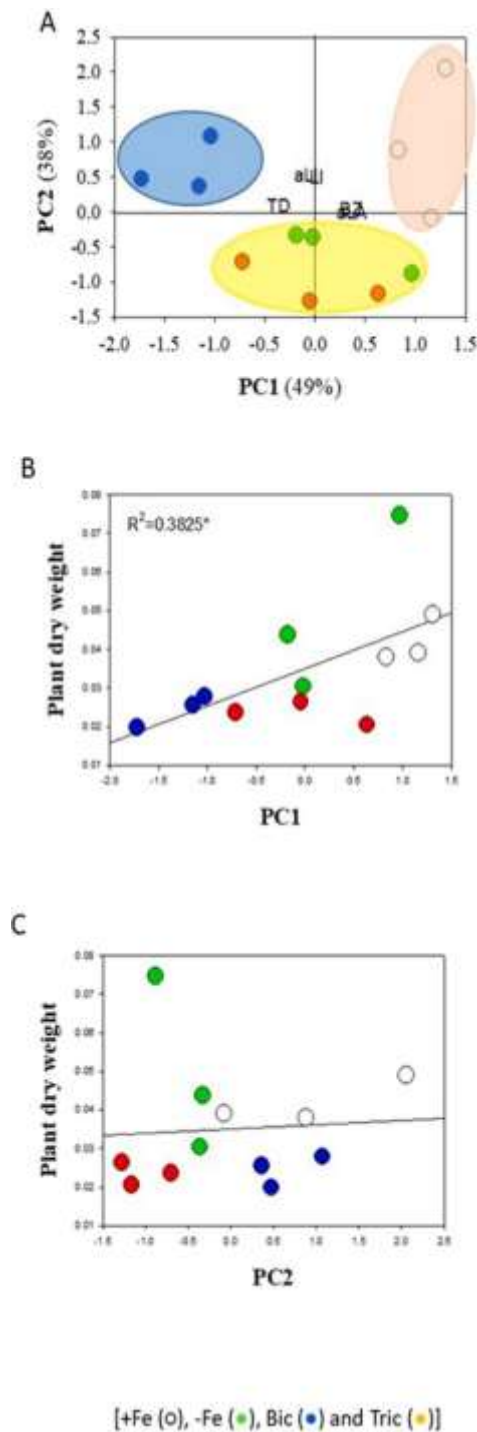


Fig. 7. A) Score and loading plots of principal component analysis of root traits from *P. judaica* plants exposed to + Fe, -Fe, Bic and Tric treatments. The proportion of variability explained by each PC is given within the bracket. The ellipses denote the grouping of the samples after Hierarchical Cluster Analysis (Ward's method with distance measure by squared Euclidean distance). Correlation between plant dry weight and PC1 (B) and PC2 (C) in *P. judaica* plants exposed to different treatments (+Fe, -Fe, Bic and Tric). The coefficient of determination and p-values are reported.

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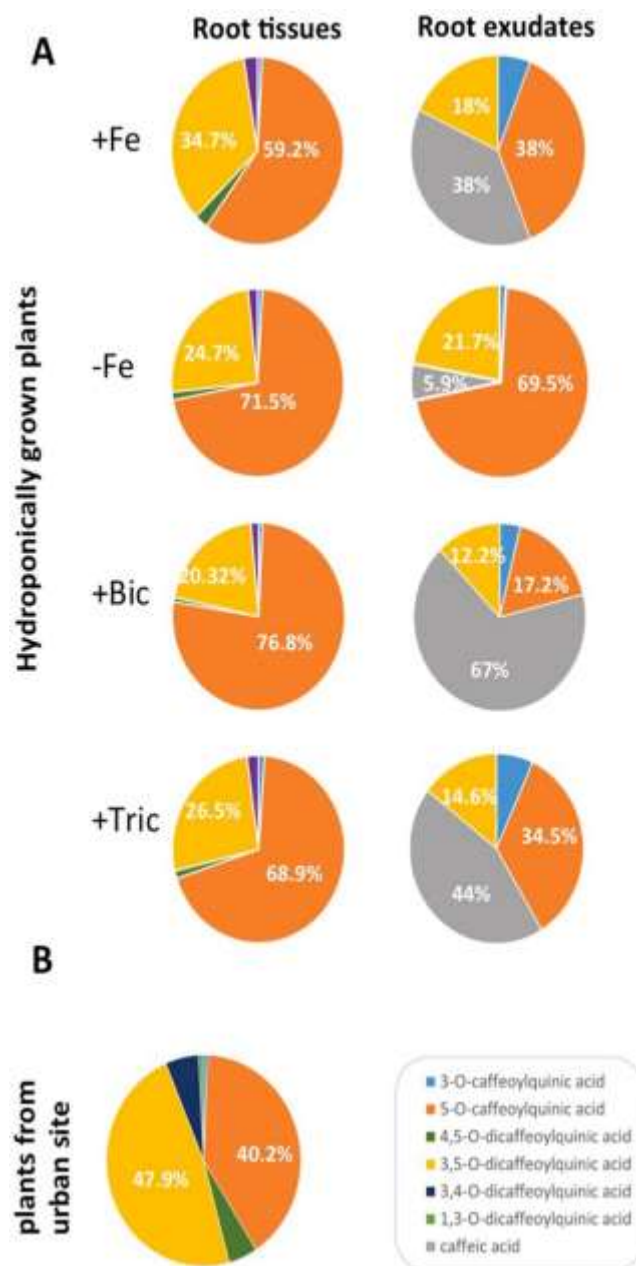


Fig. 8. Profile of caffeoylquinic acid derivatives identified in extracted fraction from tissues (left panel) and exudates fraction (right panel) of root of *P. judaica* grown hydroponically in +Fe, -Fe, Bic, and Tric treatments (A). Profile of caffeoylquinic acid derivatives identified in *P. judaica* harvested from the soil (urban soil) is reported in B. Percentage Pie chart is related to a representative experiment with three independent replicates (n = 3).

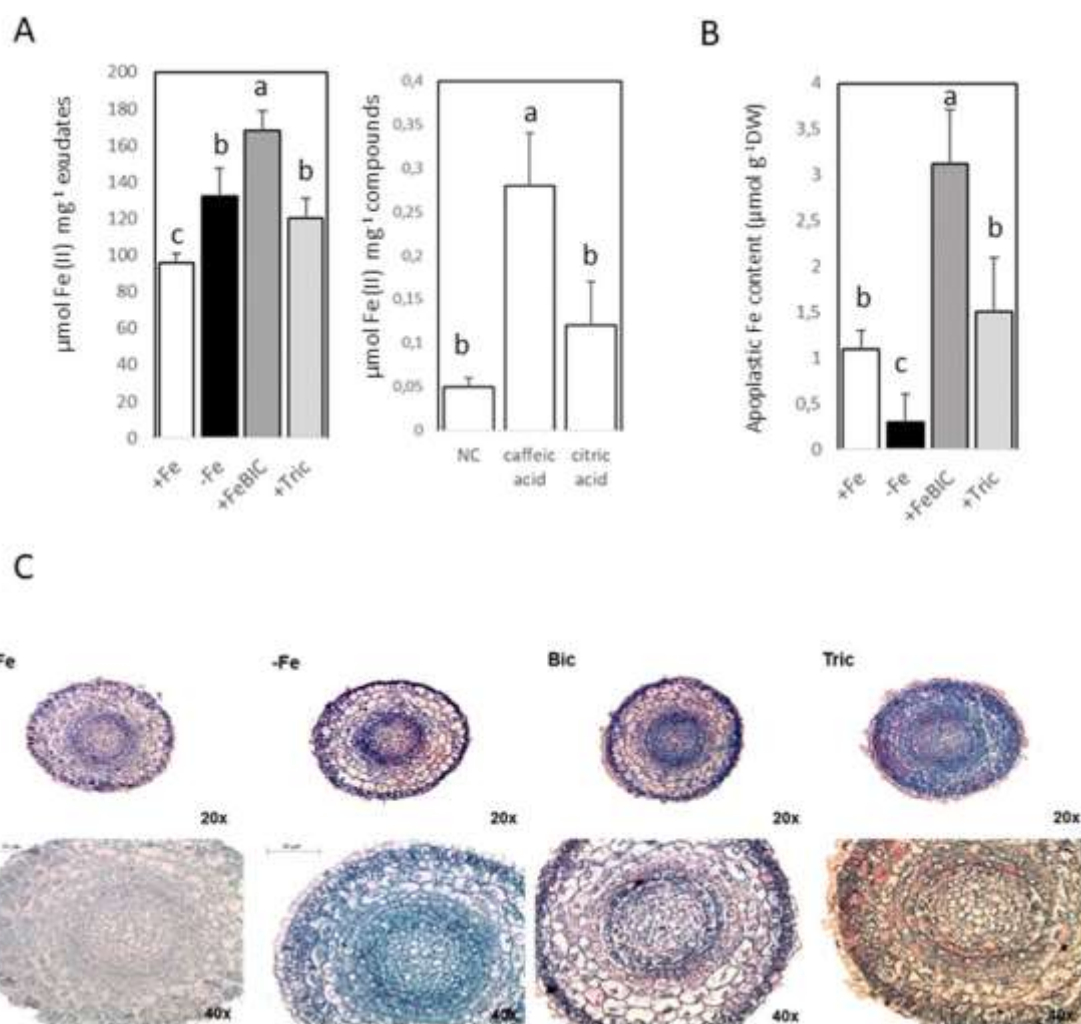


Fig. 9. A) Fe (III) reduction activity of root exudates, caffeic and citric acids; B) Fe content in the root apoplast fraction and C) lignin visualization (red color) in root cross sections of *P. judaica* grown in +Fe, -Fe, Bic, and Tric treatments (20x and 40x magnification). Different letters correspond to significant differences among means ($P < 0.05$; Tukey test), $n = 3$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table S1. Abbreviation of root parameters determined and mentioned in the text

parameters	Adventitious root	1st-order laterals roots	Whole roots
length	LA	LI	LT
number	NA	NI	-
average lenght	aLA	aLI	-
surface	SA	SI	ST
volume	VA	VI	VT
ary weight	WA	WI	WT

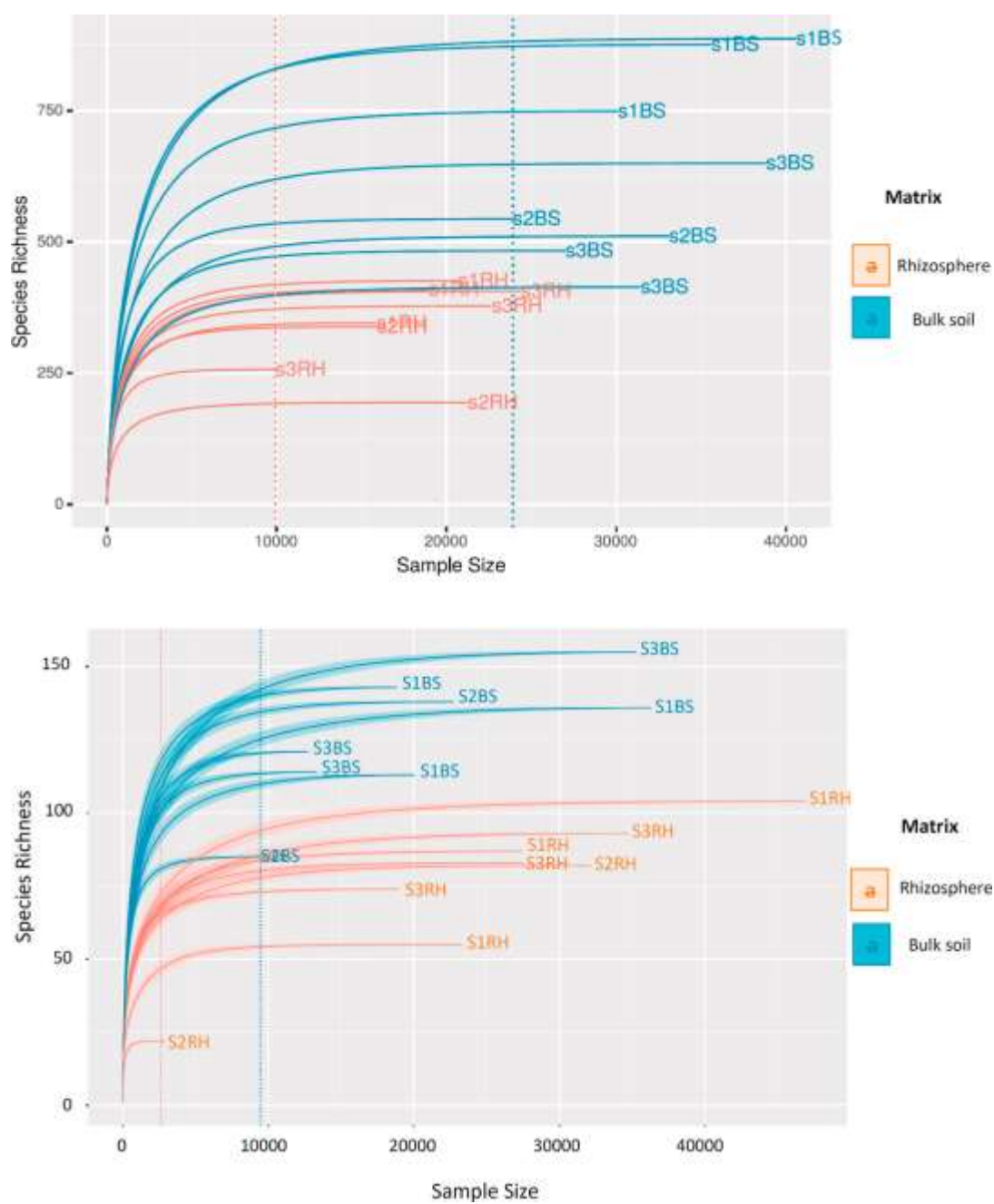


Fig. S1. Rarefaction curves showing the reaching of a satisfactory sequencing depth for each of the sequenced samples. Upper panel: prokaryotic libraries; lower panel: fungal libraries.

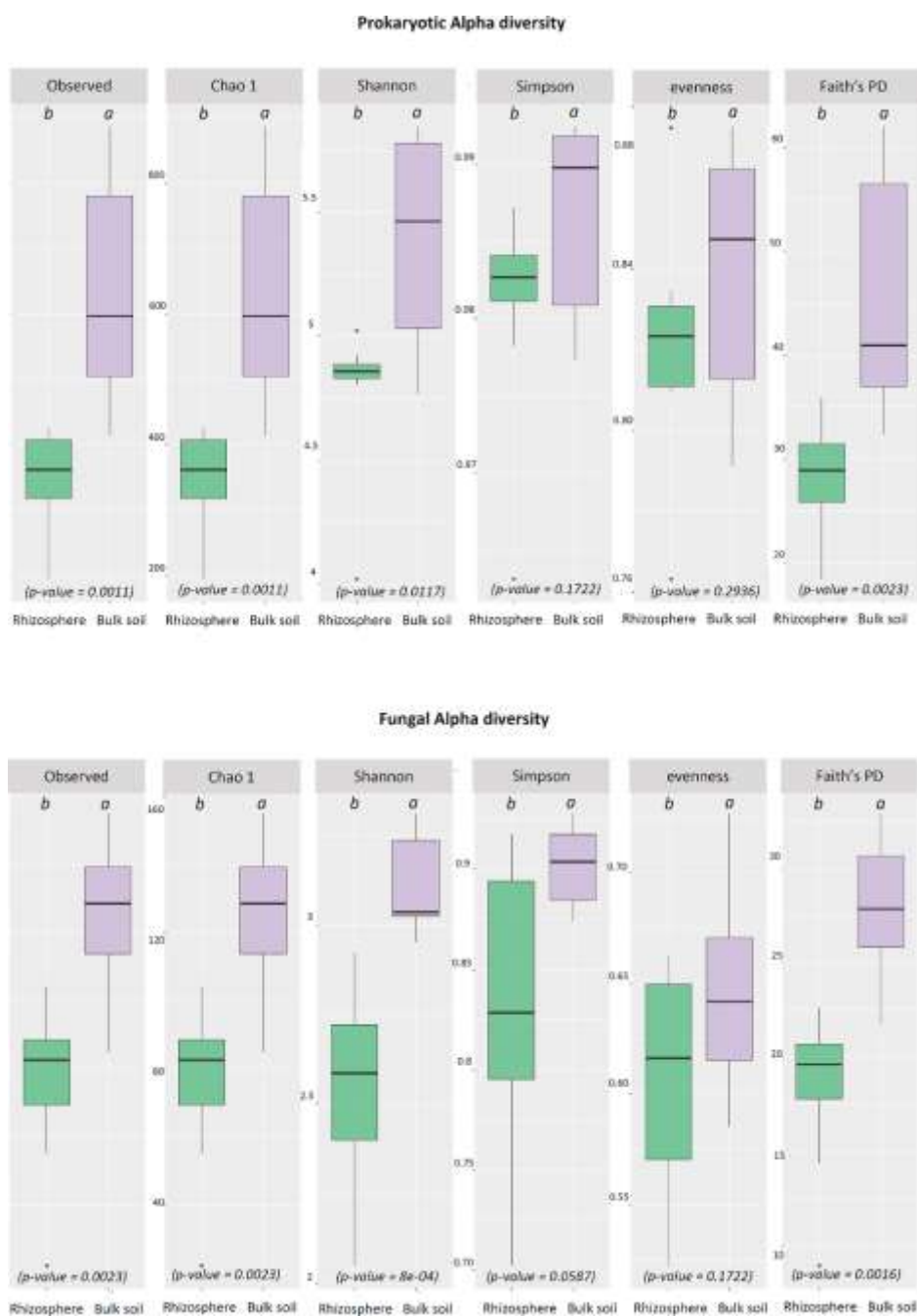


Fig. S2. Alpha indexes showing the OTUs diversities between rhizosphere and bulk soil.

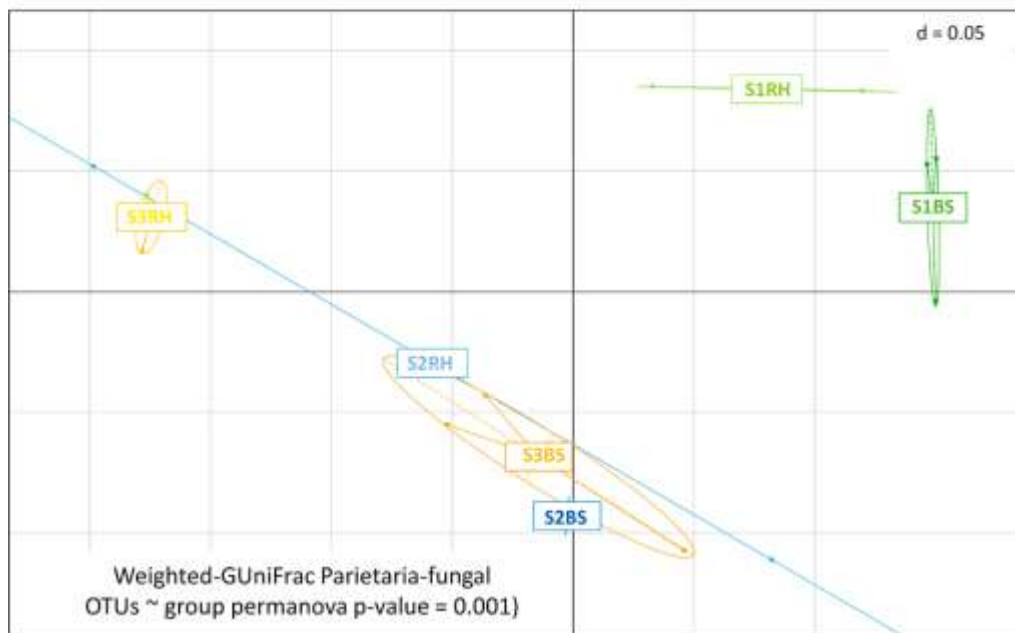
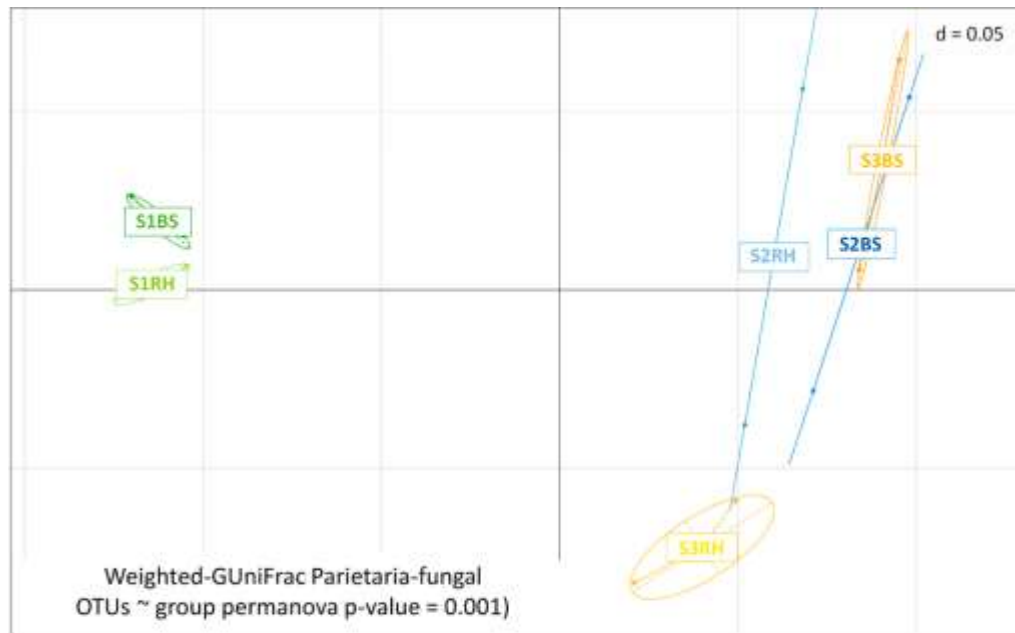


Fig. S3. Weighted Generalized Unifrac analysis showing the intra-groups diversity of the Prokaryotic (upper diagram) and Fungal (lower diagram) microbial communities between the two conditions considered (BS= Bulk soil; RH = Rhizosphere samples).

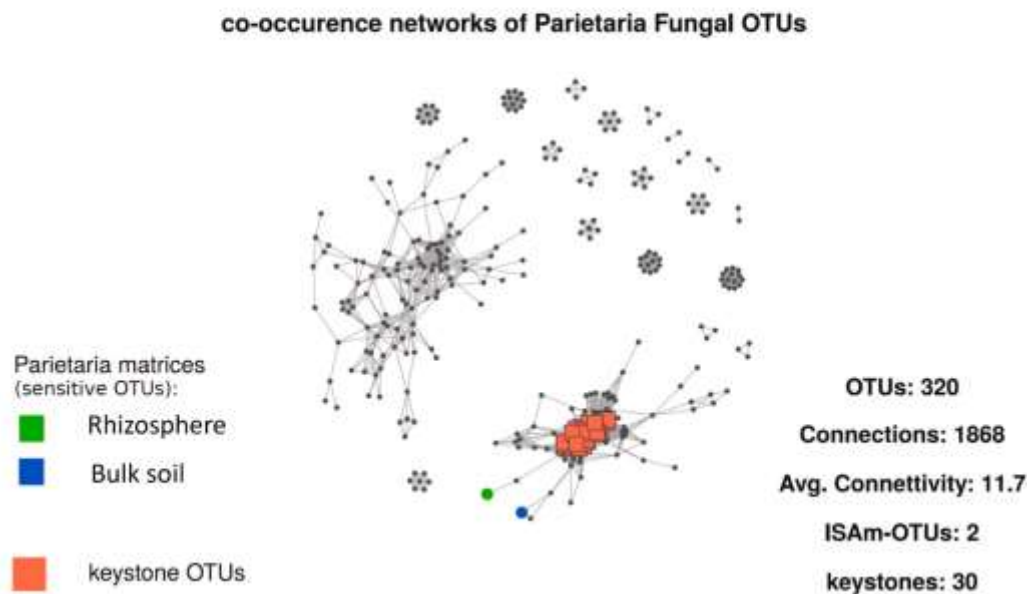
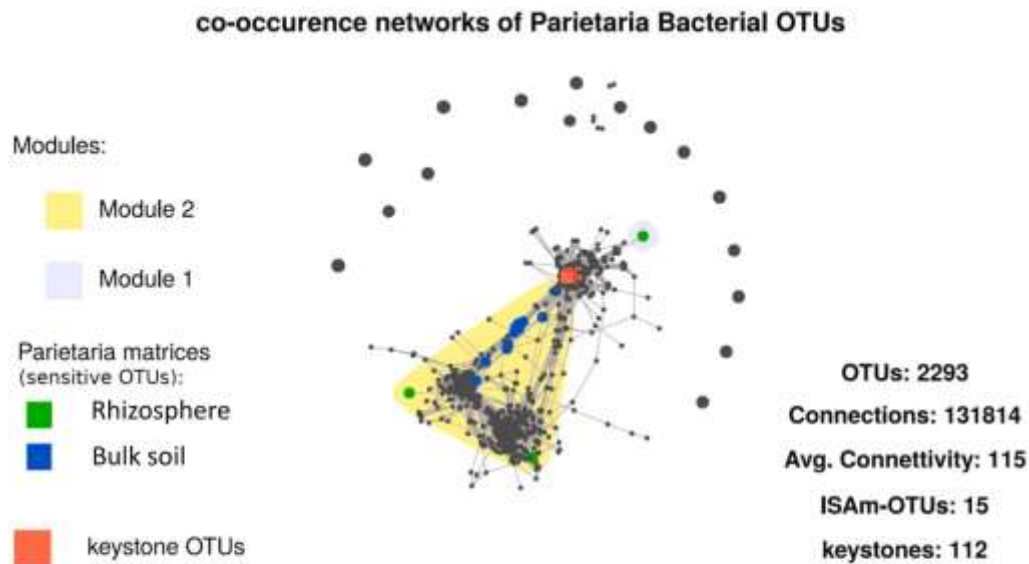


Fig. S4. Co-occurrence networks visualising the positive, significant correlations ($\rho > 0.7$ and $p < 0.001$) among prokaryotic (upper diagram) and fungal OTUs (lower diagram) from the Rhizosphere and the Bulk soil microbial communities. The sensitive OTUs shown in green and blue represent the OTUs identified as indicator species for the rhizosphere and bulk soil condition, respectively (listed in supplemental file 3). Red squares represent the keystone OTUs (listed in supplemental file 2). Modules are defined as areas that show a high density of connections among OTUs. The Gray symbols represent “hot spots” of overlapping OTUs.

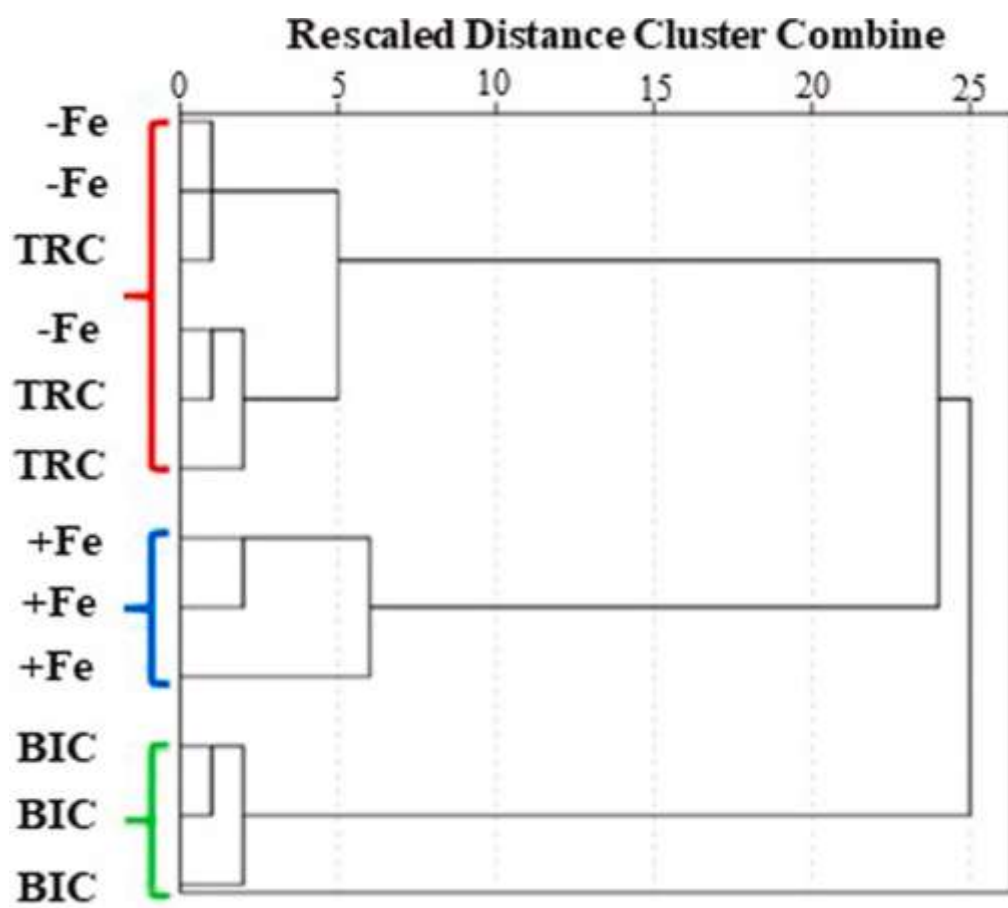


Fig. S5. Dendrogram of Hierarchical Cluster Analysis of the scores of the PCA using the Ward's method with distance measure by squared Euclidean distance.

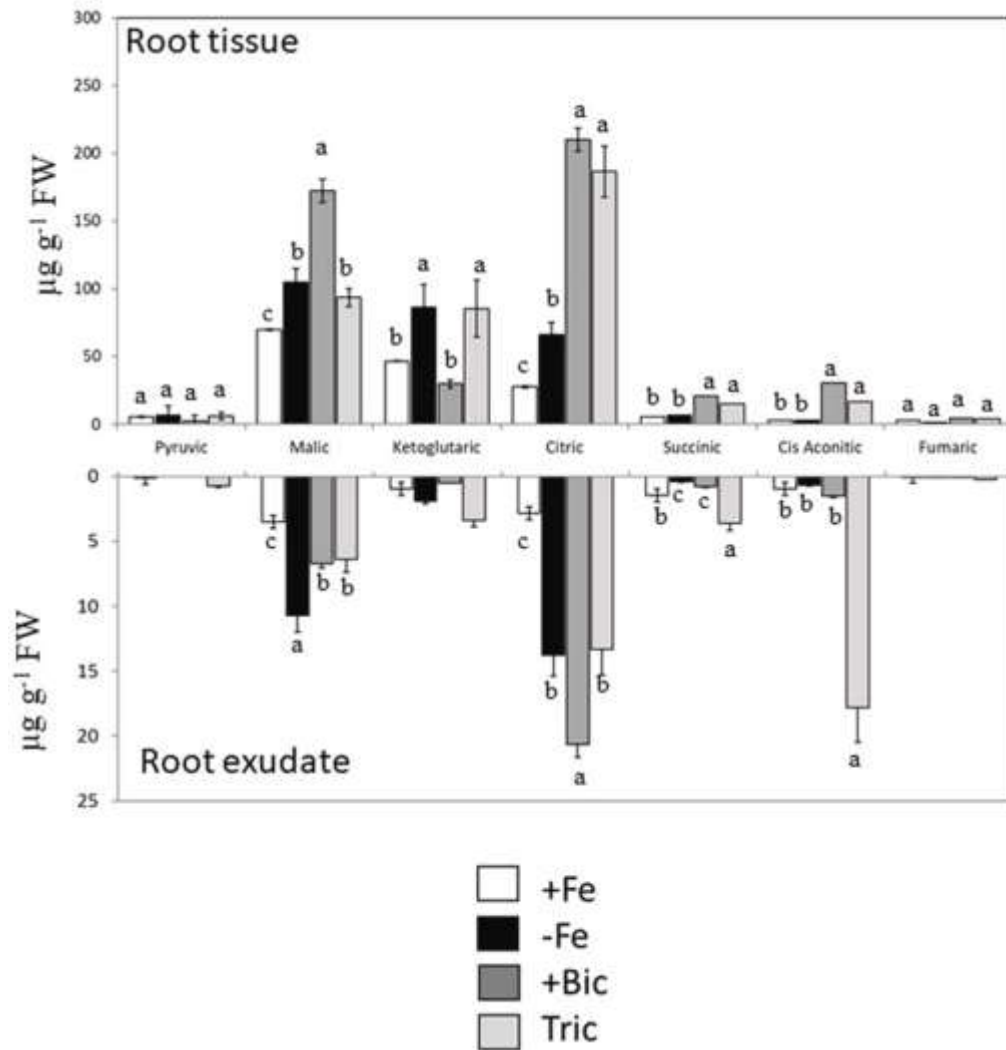


Fig. S6. Characterization of carboxylic acids concentrations in root tissues (upper panel) and root exudates (lower panel) of *P. judaica* grown in +Fe, -Fe, Bic, and Tric treatments. Different letters correspond to significant differences among mean values ($P < 0.05$; Tukey test), $n = 3$.

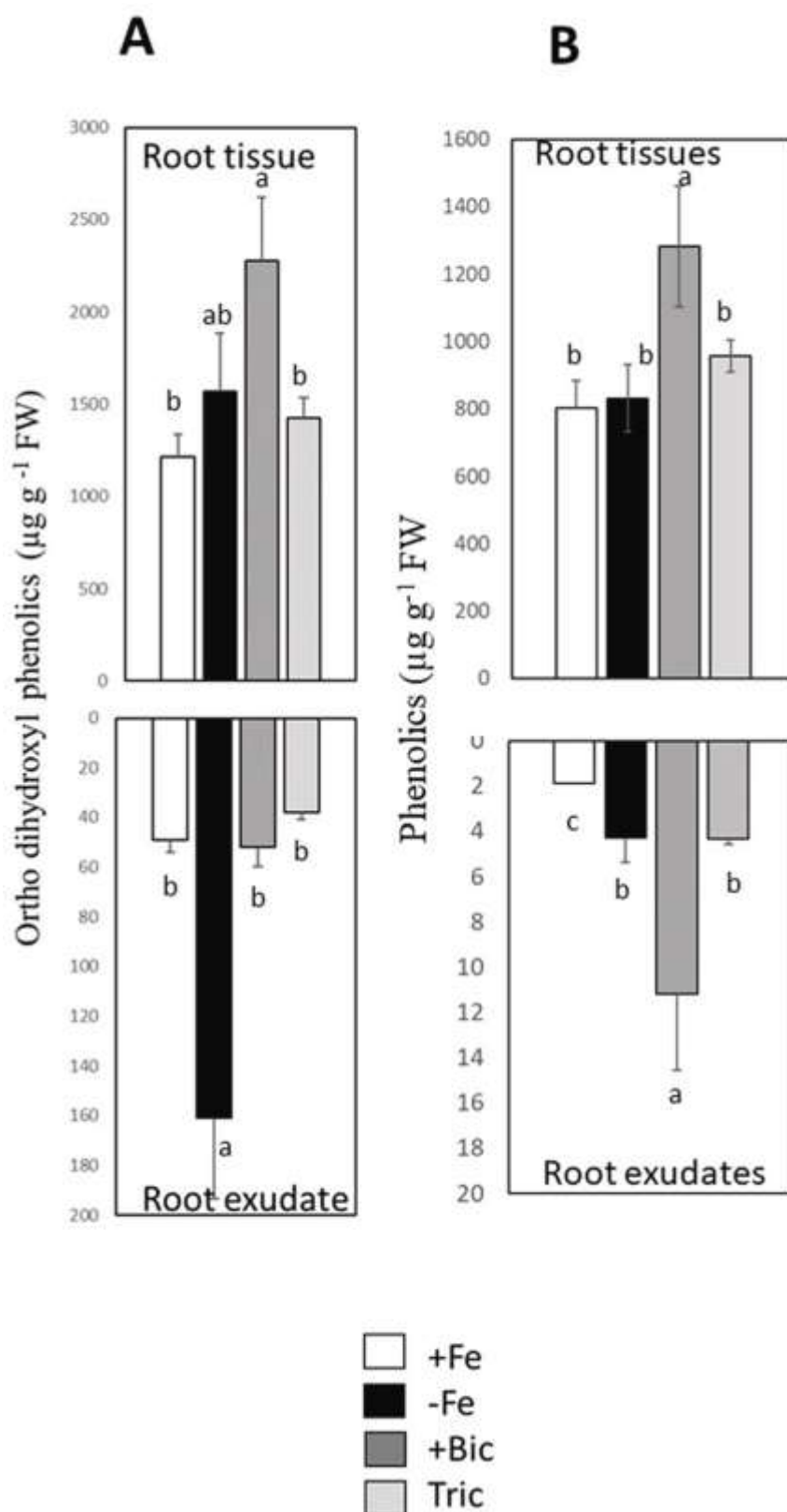


Fig. S7. Quantification of the phenolics compounds by Arnowns' reagent (A) and by HPLC-DAD (B) approaches. Different letters correspond to significant differences among means ($P < 0.05$; Tukey test), $n = 3$

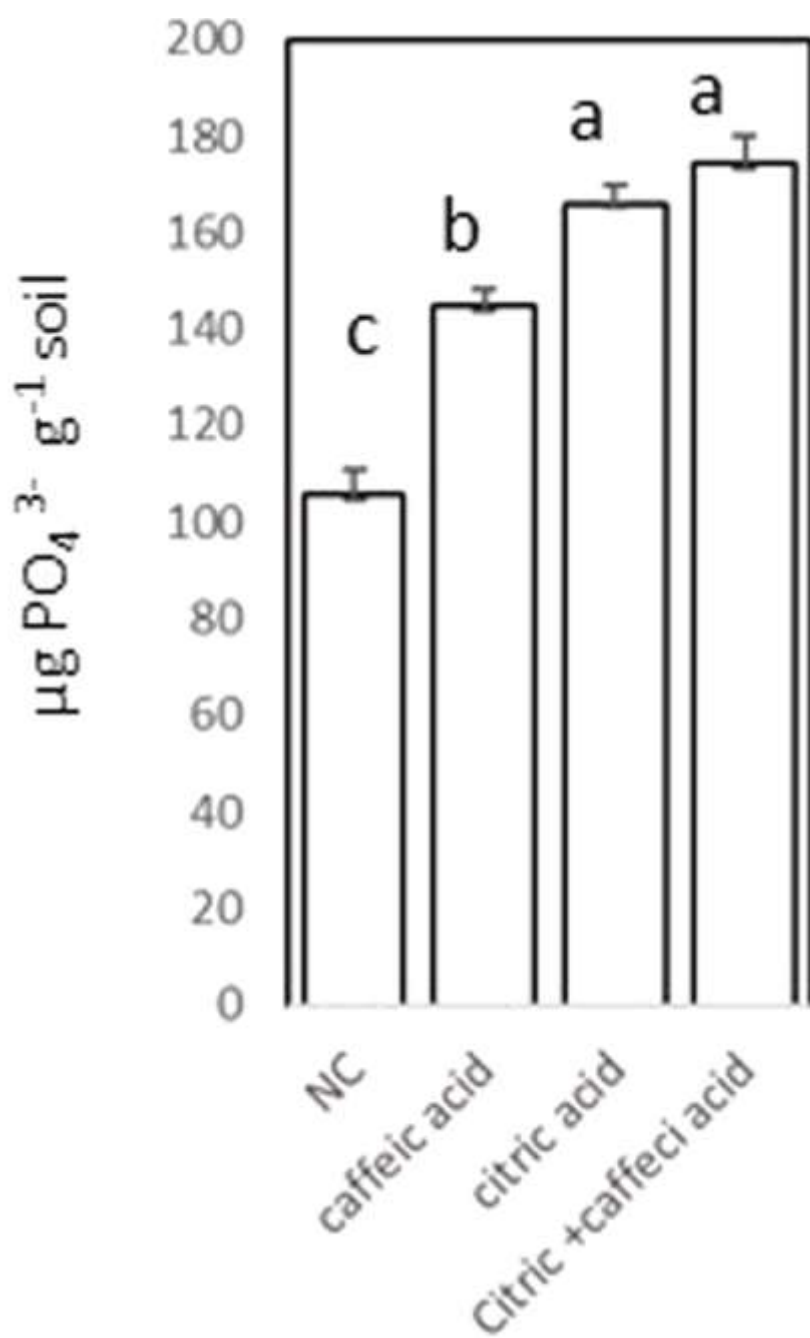


Fig. S8. Content of phosphate anions in soluble fraction of urban soils after incubation with caffeic and citric acids. Different letters correspond to significant differences among mean values ($P < 0.05$; Tukey test), $n = 3$.