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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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- 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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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 investigated different traits which characterize the root-soil interaction of Parietaria 24 25 judaica, a wild plant species commonly known as "Pellitory-of-the- wall". P. judaica adopts the acidification-reduction strategy (Strategy I) for iron (Fe) acquisition from soil, 26 27 and it can complete its life cycle in highly calcareous environments without any symptoms of chlorosis. In a field-to-lab approach, the microbiome associated with P. judaica roots 28 29 was analyzed in spontaneous plants harvested from an urban environment consisting in an extremely calcareous habitat. Also, the phenolics and carboxylates content and root 30 plasticity and exudation were analyzed in P. judaica plants grown under three different 31 controlled conditions mimicking the effect of calcareous environments on Fe availability: 32 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.

37

38 Introduction

Variations in soil pH are among the abiotic factors affecting most plant growth, since they 39 40 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 41 micronutrients (Lindsay and Schwab, 1982; Schenkeveld and Kraemer, 2018). Calcareous 42 soils, representing 30% of the Earth's land surface, are characterized by high pH values 43 and may contain high HCO - ions in the soil solution. Iron deficiency-induced chlorosis, 44 otherwise known as lime chlorosis, is a major nutritional disorder observed in crops 45 growing in calcareous soils; on one hand, alkaline pHs dramatically reduce Fe solubility in 46 soil and on the other hand, the presence of HCO_3^- may interfere with the physiological 47 48 processes of Fe uptake (Chen and Barak, 1982; Romheld, 1987; Kim and Guerinot, 2007; 49 Díaz et al., 2012). Plant roots grown under calcareous conditions may exhibit a higher Fe 50 content than those grown under non-calcareous conditions, indicating that low Fe 51 availability in soil is not the unique factor causing lime-induced chlorosis; indeed, the 52 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 53 54 respiration and photosynthesis and other major metabolic processes in plants (Kobayashi and Nishizawa, 2012; Vigani and Murgia, 2018); the elucidation of plant adaptive growth 55 strategies in calcareous habitats represents a valuable approach for the identification of 56 57 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 58 59 been made (Ter'es et al., 2019).

The understanding of plant resilience to different biotic and abiotic stresses and to various 60 natural habitats and climate changes is an emerging and urgent goal, as outlined in the Plant 61 Science Decadal Vision 2020–2030 (Henkhaus et al., 2020). In particular, Decadal 62 63 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 64 environments, as well as the exploration and characterization of as-yet-undiscovered plant-65 associated biota. In the context of Fe nutrition, the issues raised by Plant Science Decadal 66 Vision suggest that the adapting processes of non-crop plants to calcareous habitats can 67

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

In most cases, crop domestication led to modifications of the composition of root 72 microbiota, with adverse effects on the association with beneficial plant microbes (Perez-73 74 Jaramillo et al., 2016). Root exudation of metabolic compounds plays a crucial role in influencing the recruitment of functional microbiota. In general, the variety of biotic and abiotic 75 stresses, such as Fe deficiency, encountered by plants during in vitro and in vivo growth 76 conditions impacts the biosynthesis of secondary metabolites (Cheynier et al., 2013; Isah, 77 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 enhance Fe availability in the rhizosphere. Indeed, plant metabolites released to 83 rhizosphere can have diverse effects on soil microbial com- munities by changing soil 84 85 properties or nutrient availability in the root vicinity, directly attracting microbes or being toxic for others (Vive- s-Peris et al., 2020). 86

In some cases, specific plant root exudates initiate a molecular dialogue mediated by both 87 88 partners' exometabolite production, resulting in the establishment of beneficial plantmicrobe associations (Sasse et al., 2018). Badri and co-workers found a strong positive 89 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 Felimiting soils: besides their established role in Fe mobilization in the rhizosphere (Rajniak 93 94 et al., 2018), coumarins are also involved in shaping root-associated micro- biomes (Voges et al., 2019). Furthermore, Harbort et al. (2020), by using synthetic microbiota and A. 95 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 be- tween 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 103 104 deficiency. P. judaica (L. 1753) is a wild perennial dicotyledonous plant capable to grow 105 in acidic and alkaline soils (Tato et al., 2020); it represents the most widespread plant species 106 found in highly calcareous and hostile environments such as wall cracks exposed to the sun, 107 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 108 109 approach to first investigate the microbiome associated with P. judaica roots in plants 110 growing spontaneously in an urban environment and harvested from an extremely calcareous habitat. We then analyzed phenolics content and root plasticity and exudation 111 112 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 113 114 discriminate the effects of low Fe availability from those caused by the presence of 115 carbonate and alkaline conditions (Tato et al., 2020).

116

117 Material and methods

118 P. judaica growth conditions

119 *P. judaica* plants were sampled in an urban area of Milan $(45^{\circ}28'36.8''N, 9^{\circ}13'39.2''E;$ 120 $45^{\circ}28'37.0''N, 9^{\circ}14'00.1''E; 45^{\circ}28'35.2''N, 9^{\circ}14'03.7''E)$: to sample the whole root 121 apparatus, the walls and the substrates where plants were growing were broken if 122 necessary.

Cuttings of P. judaica were allowed to radicate in aerated half-strength nutrient solution 123 for 10 days (Tato et al., 2020). Rooted plants were then transferred into 10 L plastic 124 tanks (40 plants/tank) under four different conditions: +Fe (control, complete nutrient 125 solution adjusted to pH 6.2 with NaOH), -Fe (complete nutrient solution without Fe, 126 adjusted to pH 6.2), Bic (complete nutrient solution supplemented with 5 mM CaCO₃ and 127 15 mM NaHCO₃, pH 8.3) and Tric (complete nutrient solution, buffered with Tricine at pH 128 8.3); the pH was adjusted with NaOH if required. The nutrient solution was changed every 129 130 two days. Plant size at the harvesting time has been previously reported in Tato et al. 131 (2013).

132 Treatments were carried out for 7 days in a growth chamber under 16/8 h light/d ark 133 regime with cool-white light 200 μ mol photons m⁻² s⁻¹, 27/21 °C temperature range, 134 65–75% relative humidity.

- 135
- 136 Sampling of P. judaica in urban sites and DNA extraction of their root-associated microbes

From each of the three urban sampling sites considered, three replicates of bulk soil (i.e. 137 the soil portion not affected by the root presence, according to Bulgarelli et al., 2012) and 138 139 root samples from *P. judaica* were collected. Large soil aggregates were removed from the roots by shaking as described in Bulgarelli et al. (2012), in order to leave only the 140 (rhizospheric) soil attached to the roots. Bulk soil and root samples with attached soil were 141 142 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 root endophytes (the rhizosphere + the endorhiza, called rhizosphere for the sake of 145 brevity hereinafter). 146

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

Extracted DNA was quantified using NanoDrop spectrophotometry (NanoDrop,
Wilmington, DE, USA) and normalized prior to library construction for the highthroughput sequencing.

156

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

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

- SILVA database (version 132, release date December 13, 2017; Quast et al., 2012; Yilmaz
 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
 implemented in QIIME2 (Pedregosa et al., 2011).
- All statistical analyses were conducted in R v3.6.1 (R Development Core Team, 2016). Rarefaction species richness curves were generated using the R package *vegan* (Oksanen et al., 2013). Alpha diversity in- dexes were calculated for Prokaryotic and Fungal OTU tables using the *vegan* package (Oksanen et al., 2013). The differences between soil and root samples were tested by using Tukey's Honest Significant Differences test, with the R package *TukeyC* (Faria et al., 2016).
- To correct for difference in sequencing depth, a subsampling at even sequencing depth from 181 182 each sample (9935 for Prokaryotic and 2632 for Fungal samples) was performed before the downstream analysis using the R package *phyloseq* (McMurdie and Holmes, 2013), 183 generating also the taxonomical composition of the whole microbial community. The 184 185 significance of Bray-Curtis dissimilarity between the soil and root samples were tested by permutational multivariate analysis of variance (PerMANOVA) using the adonis function 186 in the R package *vegan* with 9999 permutations. The multivariate homogeneity of group 187 dispersions was first assessed by means of the betadisper and permutest (with 9999 188 permutations) functions in the R package vegan. The differences in the composition of 189 190 Prokaryotic and Fungal communities in P. judaica soil and root samples were visualized by means of a Non-metric Multidimensional Scaling (NMDS) ordination carried out using 191 192 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 com- munities 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 topological network properties, as well as network modules (substructures of nodes with a 198 199 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

Roots of *P. judaica* plants sampled in urban sites (described above, n = 8) were carefully 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

For qualitative and quantitative determination of phenolic com- pounds, plant tissues (1 g 210 211 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, the combined solutions were first concentrated under vacuum, depigmented with petroleum 213 ether (bp 40-70 °C), filtered through 0.45 µm Millipore Millex-HN, and then used for the 214 215 determination of total phenolic content and HPLC-DAD determination of phenolic compounds (Lattanzio et al., 2001). Identification of phenolics was made by using retention 216 217 times (tR) and spectral data of different peaks compared with standard com- pounds (Extrasynthese, Genay, France). In addition, HPLC-MS/MS analyses of main peaks 218 219 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 um Millipore Millex-HN; the filtered 224 solution was analyzed for total phenolic content and HPLC determination of phenolic compounds. HPLC analyses were performed with Hewlett Packard Series 1100 liquid 225 G1312A. a G1315A with a binary gradient pump 226 chromatograph equipped spectrophotometric photodiode array detector was set at 325 nm, and G1316A Column with 227 228 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) Luna C18 (5) column (4.6 \times 250 mm) was used throughout this work. The solvent system 230 231 consisted of (A) MetOH 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.

HPLC-MS/MS analyses were performed on a QTrap MS/MS system, (Applied Biosystems,
Foster City, CA, USA), equipped with an ESI interface and a 1100 series micro-LC system
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.

243

244 Root morphology and biomass allocation

After 7 d treatments (+Fe, -Fe, Bic, Tric), three independent bio- logical samples from 245 each treatment were collected randomly, and their shoots and roots were harvested. Shoots 246 were dried at 70 °C for 48 h, and their dry weight (WS, g) was measured. The root system 247 was stained with 0.1% (w/v) toluidine blue O for 5 min and then divided into two root 248 orders: 'shoot-borne' or adventitious roots (AR), and their 1st- order lateral roots (LR) as 249 defined by Atkinson et al. (2014). Each root was scanned at 300 dpi resolution (WinRhizo 250 251 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 252 253 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 254 255 area (ST) and volume (VT) of the whole root system were calculated as the sum of the two 256 root types. Then, dry weights of the adventitious roots (WA, g) and total dry weight of 1storder lateral roots (WI, g) were measured after drying in an oven at 70 °C for 48 h. Total 257 root dry weight (WT, g) was the sum of the WA and WI. Plant dry weight (WP, g) was 258 259 obtained as the sum of WT and WS. Based on the measurements above, the following parameters were calculated for the whole root system: 260

- 261 root length ratio RLR = LT/WP (cm g⁻¹)
- 262 root mass ratio RMR = WT/WP (g g^{-1})
- 263 fineness F = LT/VT (cm cm⁻³)
- 264 tissue density $TD = WT/VT (g \text{ cm}^{-3})$

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: RLR = RMR x 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 laterals [aLI = LI/NI] (cm) were also calculated (Table S1). The 'branching zone' length 271 (BZ) that extends rootwards from the shoot base to the youngest emerged LR and the 272 273 '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).

Two-way ANOVA tested the effects of the different treatments on the root parameters. Tukey's 277 post hoc test comparison was applied to test the effects of each treatment at P < 0.05. To 278 correct for allometric effects (Coleman et al., 1994), the ln-transformed plant dry weight (ln 279 280 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 using SPSS software. To unveil the impact of the root morphology pattern on plant growth, 283 Pearson's test was used to test the correlation between the PC factor scores and plant DW. 284 Statistical analysis was conducted using the Systat v. 8.0 software package (SPSS Inc., 285 Evanston, IL, USA). 286

287

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

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

296

297 Iron reduction by phenolics compounds

The phenolics concentration in root exudates and the caffeic and citric acids ability to reduce Fe(III)-EDTA was measured spectrophotometrically by using BPDS (Chaney et al., 1972). Root exudates (100 μ g), prepared as described above, were incubated for 120 min in 1 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 to soil at rates 50 μ mol acid g⁻¹ soil according to Hu et al. (2005). The soil was watered to 308 field capacity, and then incubated at 20 °C for 30 min. Soluble soil fractions were collected 309 310 ac- cording to Mimmo et al. (2008). Extracts were filtered through 0.2 µm 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 fused silica capillary with extended light path BF3 (i.d. = 50 μ m, I = 72 cm, L = 80.5 cm). 313 314 Sample injection was at 50 mbar for 4 s with -30 kV voltage and detection at 350/80 nm wavelength. Compounds were identified using pure standards and anion contents were 315 expressed as $\mu g g^{-1}$ FW. 316

317

318 Miscellaneous

Iron and P content were determined by ICP-MS on oven-dried tissue samples (n = 3)319 320 mineralized in HNO₃. 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 CaSO₄ 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(NO₃)₂, 1.5 mM 2,2' bipyridyl (pH 5.5) at 25 °C. Tubes were covered with a cotton plug and N₂ was bubbled 325 through the solution. After 5 min, 1 mL of 250 mM Na₂S₂O₄ was added. The A₅₂₀ of the 326 solution (Fe[bipyridyl]3 $\varepsilon = 8.650 \text{ M}^{-1} \text{ cm}^{-1}$) was determined on 2 mL aliquots. The 327 aliquots employed for the determinations were returned to the tube and left for 1 h in the 328 dark. The determination was carried out every 1 h 30 min until a constant value was 329 obtained. Lignin visuali- zation was performed according to Donnini et al. (2011). After 330 being fixed at 4 °C overnight in 100 mM Na-phosphate buffer (pH 7.00) containing 4% 331 paraformaldehyde (w/v), root segments were dehydrated through an ethanol-tertiary 332

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

339

340 **Results**

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

P. judaica plants were harvested from soil of an urban site displaying alkaline and 342 calcareous conditions, with a pH value of 7.9 \pm 1.3 and an average 20% CaCO₃ content. 343 344 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 rarefaction curves indicated that a satisfactory sequencing depth was obtained for each 346 considered sample, for both fungal and bacterial amplicons (Fig. S1). The clustering of high-347 quality reads allowed us to obtain 349 fungal and 2327 prokaryotic operational taxonomic 348 349 units (OTUs), that were further assigned taxonomically (Supplemental files 1a, 1b). The standardized datasets were used to generate Venn diagrams of the community composition 350 351 and unique or shared OTUs among bulk soil and rhizosphere samples (Fig. 1). The results show a reasonable degree of microbiota diversity, especially for prokaryotes (more 352 than 2000 OTUs overall). Also, a lower number of OTUs unique to the rhizosphere 353 354 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 microbes recruited by roots under the occurring environmental conditions. 356

357 The analysis of Alpha diversity indexes outlines the structure of the considered microbial communities (Fig. S2). All indexes point to a higher microbial richness in the bulk soil 358 359 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 microbial composition of bulk soil and rhizosphere; however, the two compartments appear 362 363 more diverse when phylogenetic distances weighted by relative abundances were 364 considered in relation to fungal microbiota (weighted GUnifrac, Fig. S3). Notably, the prokaryotic taxa typically present and dominant in soils (Jansenn, 2006) were all detected 365 in the current experiment (Fig. 2), with Proteobacteria as the most represented in both 366 367 compartments. However, Acidobacteria were underrepresented; this result is consistent

368 with the high pH of the soil under investigation (around pH 7.9), as many species in this 369 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 383 improvement of mineral nutrition, at a cost for the plant host, since they take up 384 photosynthates from the roots. Hence, the presence of AM fungi of such urban soil was 385 also investigated; a few OTUs referring to AM fungi were retrieved in both the bulk soil 386 387 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 388 389 Glomeromycotina. Despite AM OTUs not being abundant in our dataset, arbuscule formation was observed in the same *P. judaica* roots sampled for the microbiome 390 391 sequencing, suggesting that AM fungi are an active microbial component of such an urban 392 niche.

393 Co-occurrences in the Prokaryotic and Fungal communities were assessed by performing 394 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 395 correlations between Prokaryotic and Fungal OTUs in the soil and root communities (Fig. 396 397 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-398 399 occur with other taxa under the experimental conditions considered, and are thought to be 400 ecologically important and to play potentially a key role in the structuring of the microbiota 401 (Hartman et al., 2018, Supplemental file 2).

Also, OTUs that might act as indicator species in such networks were sought; a species is 402 403 described as an "indicator" when it is characteristic of a group of samples or experimental 404 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 405 406 (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 407 408 to Actinobacteria (99.75% identity). Members of Actinobacteria are widespread in soils and 409 display tolerance to diverse extreme conditions (Ranjani et al., 2016).

Indicator species of the bulk soil comprise genera of fungi and bac- teria 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*.

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420 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 421 422 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 423 424 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 425 426 nutrients was determined. A reduction of Fe concentration was observed in leaves of plants 427 grown in the -Fe, Bic and Tric media, and in -Fe roots (Fig. 5). Phosphorus was slightly 428 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

431 and C). Indeed, the TD of the whole root system was lower in the -Fe and +Fe than in Bic- and

432 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 plants (Fig. 6D): Bic-treated plants decreased (-49%) the aLA in comparison to the +Fe 437 treatment and the Tric treatment reduced the LI and aLI by -83% and -71%, respectively, 438 compared to the +Fe plants (Fig. 6D). The Fe-deficient plants exhibited similar morphology in 439 both root types when compared with the +Fe plants (Fig. 6D). As expected in plants adapted 440 441 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).

The PCA was applied using only root parameters significantly changed by treatments as 444 445 observed in the univariate ANOVA. The PC1 (explaining 49% of the variance) consisted of high positive loads for the aLA and the BZ and negative loads for the TD, while the PC2 446 447 (explaining 38% of the variance) showed high positive loads for the LI and aLI (Table 1). Two-dimensional PCA score plots and subsequent hierarchical cluster analysis revealed a 448 449 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 associated with high TD, aLI and LI, thus exhibiting root architectures different to the +Fe 453 plants. Conversely, the root architectures of the Fe deficient- and Tric-treated plants 454 455 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 and the PC1 and PC2 scores. The PC1 was significantly and positively correlated with the 458 plant growth (r = 0.4890, p = 0.0139) differently to the PC2 (r = 0.0356, p = 0.55) 459 (Fig. 7B and C), suggesting that the high aLA, BZ and low TD but not the lateral roots (PC2) 460 explained most of the P. judaica growth. 461

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

The total phenolics and carboxylic acids released by *P. judaica* roots were recently monitored in the four conditions, i.e. control, -Fe, Bic, Tric (Tato et al., 2020). The profile of carboxylic acids and the total phenolics of *P. judaica*, both in roots and in their exudates are shown in Figs. S6 and S7, respectively. Malic, ketoglutaric and citric acids were detected in all tested root samples, with a higher accumulation of malic and citric acid in -Fe, Bic, and Tric treatments, whereas ketoglutaric acid con- centration increased only in -Fe and Tric-treated plant roots. Both malic and citric acids were present in all root exudates, with a higher accumulation in -Fe, Bic and Tric treatments. Notably, cis-aconitic acid was exuded only by the Tric-treated roots.

474 Total root phenolics were measured spectrophotometrically using Arnow's reagent, which 475 selectively determines the concentration of ortho-dihydroxy phenolic compounds (Fig. S7A). Only the Bic treatment induced a significant increase (87%) of total phenolic concentration 476 477 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 chromato- graphic 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 various positional isomers of mono- and di-caffeoylquinic acid esters. 5-O-caffeoylquinic acid 482 483 (chlorogenic acid) and 3,5-O-dicaffeoylquinic acid were the main constituents of the phenolics fraction in root extracts (Fig. 8A, left panel). Other caffeoylquinic derivatives identified in 484 roots were: 3-O- caffeoylquinic acid, 4,5-O- and 3,4-O-dicaffeoylquinic acids, and two p-485 coumaric acid glycosides. Accordingly, the phenolics content of plants harvested in the 486 487 urban site revealed that the main constituents of root extracts of P. judaica were represented by chlorogenic acid and 3,5-O- dicaffeoylquinic acid, which constitute together about 88% 488 489 of the total phenolic compounds present in root extracts (Fig. 8B).

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

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

- increase in total phenolics root exudates from -Fe, Bic and Tric treated plants (Fig. S7B).
 Again, the highest increase in phenolics was observed in Bic exudates.
- In Bic and Tric exudates, caffeic acid accounted for 67 and 44% of the total phenolics, respectively. Caffeic acid and chlorogenic acid together accounted for 76–84% of total phenolic compounds in both control and all the stressed exudates (Fig. 8A, right panel).
- 507

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

510 The ability of root exudates to favor Fe mobilization was tested by measuring the in vivo Fe reductase activities of exudates secreted from roots of P. judaica plants grown in -Fe, 511 Bic and Tric media. Such Fe reductase activity was increased by all three stress conditions, 512 513 and in particular, it was the highest in the Bic treatment (Fig. 9A). Since Bic- grown P. judaica roots exuded citric and caffeic acids, the effect of commercially available caffeic 514 515 and citric acids on the Fe(III) reduction was assayed. Caffeic acid displayed a higher Fe reduction capacity compared with citric acid (Fig. 9A). Besides Fe availability, calcareous 516 517 conditions also affect phosphorus (P) availability; the urban calcareous soil where plants were collected was incubated with caffeic and citric acid to study their potential effect on 518 PO_4^{3-} solubility. Soil incubation with both caffeic and citric acid enhanced the 519 concentration of PO ³⁻ in the soil soluble fraction with respect to the control, but the effect 520 521 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 intercellular spaces (Fig. 9B). Other than Fe mobilization, phenolics might be involved in 524 525 other re- actions, such as lignification process (Donnini et al., 2011). Therefore, the lignification rate of root tissues was investigated by a staining procedure and the root cross-526 527 sections were visualized microscopically. Roots of plants grown under Bic and Tric showed high lignification signals at rhizodermis, endodermis and cortex layers (Fig. 9C). 528 529 Such findings suggest that both alkaline growth conditions (Bic and Tric) induced the synthesis of lignin in the root cell walls. 530

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 all prokaryotic taxa typically present and dominant in soils. The comparison of the root-541 542 associated versus bulk soil microbiota highlighted some shifts in the microbial composition, with a higher relative abundance in the rhizo- sphere of some genera, 543 544 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 the second OTU points to an unidentified actinobacterium. Besides the Rhizobia's ability to 546 form N-fixing nodules on legume roots, they can also thrive in the rhizosphere of non-547 leguminous plants acting as Plant Growth Promoting Rhizobacteria (PGPR, Mehboob et 548 549 al., 2012), 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 of a wide range of hosts beneficial for the plants' health, and the interest in their use as 551 552 PGPR bacteria has raised in recent years (Sathya et al., 2017).

Taken together, our analyses of the root-associated microbiome of spontaneous urban *P*. *judaica* plants indicate that these plants retain the competence to actively recruit beneficial soil microbes such as PGPR, phosphate solubilizers and AM fungi, possibly excluding from their rhizosphere other components of the soil microbiota. These results are remarkable, given the limited microbial reservoir to which plant roots had access in the 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 rhizospheric microbiota, and the outcome and magnitude of such an influence can change, 560 depending on both fixed (e.g. plant genotype) and variable (biotic/abiotic stresses) factors. 561 In this scenario, root exudation can act as a crucial driver of microbiota recruitment (Sasse 562 et al., 2018). Among the different fractions of A. thaliana root exudates, phenolics are 563 very effective in shaping the soil microbiome, as they significantly correlate with 31 564 565 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, coumarins limit the growth of a Pseudomonas strain through a mechanism that involves the 567 568 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 rizhoplane. This suggests that

571 the negative effect demonstrated for coumarins on *Pseudomonas* growth might be extended 572 to other phenolic compounds, thus providing indications towards the engineering of 573 beneficial plant root microbiota.

574 Root morphological plasticity and exudation, which are two relevant processes driving 575 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 576 577 responses to the low availability of Fe due to a high pH and that caused by bicarbonate 578 (Tato et al., 2020). Among the morphological parameters of the whole root system, the TD 579 of P. judaica was the only affected trait, mainly by the calcareous condition (Bic) (Fig. 580 1C). The TD is an adaptive trait positively correlated with the lignification degree and cell 581 wall thickness (Ciamporova et al., 1998; Wahl and Ryser, 2000; Hummel et al., 2007) and, 582 in turn, is inversely related to the Arabidopsis adaptation to Fe deficiency (Barberon et al., 583 2016). As well, the root lignification degree has been interpreted as a Fe deficiency 584 sensitivity trait in a quince rootstock (Donnini et al., 2011).

585 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 586 approach could provide early information on the contributions of the different root types of 587 P. judaica to the adaptation in alkaline, calcareous and Fe deficient conditions. Indeed, root 588 types were found to respond differently to the environmental cues such as water (Romano 589 590 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), 591 allelochemicals (Abenavoli et al., 2004, 2008; Lupini et al., 2016), as well as rot (Roman-592 Aviles et al., 2004) and fungal colonization (Zad- worny and Eissenstat, 2011). In the 593 lateral roots of *P. judaica* were more modified by treatments than 594 present work. adventitious ones. This kind of root ideotype, which is characterized by an even extended 595 spread of roots throughout the soil, is useful for the acquisition of nutrients with restricted 596 597 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 598

599 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*.

judaica to low Fe availability caused by high pH (Tric-treatment) and calcareous environment 606 607 (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 their root axes. In particular, the low TD of the root axes observed in Fe-deficient and Tric 609 610 treatments was negatively correlated with root exudation which, in turn, is a fundamental physiological trait of the Fe deficiency syndrome (Ladygina and Hedlund, 2010; Hell and 611 612 Stephan, 2003; de Vries et al., 2019). This root trait, in association with a high aLA and RBZ, explained the higher fitness of *P. judaica* plants as shown by Pearson correlations. 613

614 Conversely to the -Fe and Tric-treated plants, the root architecture of the Bic-treated ones was characterized by higher LI but less TD. The Bic- induced calcareous environment affected the 615 616 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 plants. In such conditions, the soil exploration by roots might be a strategy to survive in 618 calcareous soils (White et al., 2013; Campestre et al., 2016; Ding et al., 2019). Indeed, P. 619 *judaica* exposed to the calcareous condition displayed higher variability of root plasticity 620 with respect to plants exposed to the other treatments, by reducing the branching zone, 621 622 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, caffeic acid, probably arising from hydrolysis of caffeoylquinic esters, is one of the components 625 626 of the root exudates. The accumulation of phenolics in plant tissues is a hallmark of plant stress: phenolic compounds may be synthesized *de novo* in plants as a response to various 627 628 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 low-temperature (Lattanzio and Van Sumere, 1987; Lattanzio et al., 2001; Lattanzio et al., 631 1994), wounding (Cantos et al., 2001), high UV-B irradiation and insect attack (Izaguirre 632 633 et al., 2007).

Caffeoylquinic acid (CQA) derivatives are caffeic acid (3,4-dihy- droxycinnamic acid) depsides, positional isomers of caffeic acid esters of quinic acid, which are broadly distributed in plants. The chelating activity of CQAs is attributed to their catechol ring (Kono et al., 1998). Low temperature stress induces, in artichoke tissues, an accumulation of constitutive phenolic compounds, mono- and di-caffeoylquinic acids, that protect chilled tissues from damage by free radical-induced oxidative stress (Lattanzio et al., 1994). Due to the presence of a catechol ring in its structure, chlorogenic acid can promote the reductive release of

ferritin Fe as mobile Fe²⁺ that, in turn, forms colourless complexes with the excess of 641 chlorogenic acid (Boyer et al., 1988). Hence, chlorogenic acid can act as a reductant of 642 Fe^{3+} as well as a ligand of Fe^{2+} . In addition, this paper shows that the exudation process 643 produces, likely by hydrolytic processes, caffeic acid, which has a high Fe reduction ability. 644 645 Overall, the results in the present work support the current view that secretion of phenolic compounds is a relevant component of the reduction strategy of Fe acquisition in non-646 647 graminaceous plants. In the past decades, several studies suggested that the secreted 648 phenolics could enhance Fe availability in the rhizosphere soil, as an alternative/reinforcement of the membrane-bound reductase, through chelation 649 and reduction of insoluble Fe. Initially, phenolics were thought to help with the solubilization 650 and reutilization of apoplastic Fe in red clover. This feature was not considered part of the 651 Fe uptake mechanism until coumarin derived phenolics were observed in Arabidopsis 652 under high pH conditions (Fourcroy et al., 2014). Other plant species such as peanut (Arachis 653 hypogaea L.) and rice (Oryza sativa) plants secrete other phenylpropanoids instead of 654 coumarins, which also facilitate the reduction of ferric Fe (Romheld and Marschner, 1983; 655 656 Ishimaru et al., 2011).

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

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

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

Furthermore, the exudation of phenolics into the rhizosphere in- fluences selectively some 693 694 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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- 707
- 708 Authorship

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716

717 **Declaration of competing interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

720

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723

724 **References**

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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.)



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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 1152 microbiome at the genus level. The bars show the relative abundance on the overall 1153 1154 microbial composition for the fungal and prokaryotic genera considered (n = 8). 1155 Asterisks show significant differences in the bulk soil vs rhizosphere composition for 1156 each genus displayed (p-value < 0.05). Note: the Rhizobium group comprises 1157 Allorhizobium, Neorhizobium, Pararhizobium and Rhizobium, as they are considered as a 1158 single genera according to the Silva taxonomy. Eight in- dependent biological replicates were considered (n = 8). 1159



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. 6. Morphological analysis of the whole root system of *P. judaica* grown in +Fe, -Fe, 1255 Bic, and Tric media. A) Images captured of root; B) Total root length and total root 1256 1257 surface; C) Root length ratio (RLR) and its components, i.e. root mass ratio RMR, 1258 fineness F and tissue density ratio TD; D) Morpho- logical analyses intra-root of lateral roots (LI, length; aLI, average length, NI, number) and adventitious roots (LA, length; 1259 1260 aLA, average length, NA, number) (abbreviation are also reported in Table S1); E) Root 1261 branching analysis of P. judaica (root branching zone's length (BZ), lateral root (LRFZ)). Different letters correspond to statistically significant 1262 formation zone differences among mean values (P < 0.05; Tukey test), n = 3. 1263



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). Cor- relation 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.



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).

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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 1

1369 Principal components of root traits of rooted cuttings of Parietaria judaica

1370 exposed to different treatments (+Fe, -Fe, Bic and Tric).

	Attribute loadings	
	PC1	PC2
Statistics		
Eigenvalue and variability		
Eigenvalue	2.474	1.919
Proportion of variability (%)	49	38
Variable		
Eigenvectors		
Tissue density (TD)	867	.071
Total length lateral roots (LI)	.192	.959
Average length adventitious roots (aLA)	.926	.073
Average length lateral roots (aLI)	074	.981
Root branching zone (BZ)	.907	.165

parameters	Adventitious root	1 st -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	

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



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.

Prokaryotic Alpha diversity





Fungal Alpha diversity

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).

co-occurence networks of Parietaria Bacterial OTUs



co-occurence networks of Parietaria Fungal OTUs



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 HLPC-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.