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Soil microorganisms and root exudation mediate rhizosphere acidification of the gypsum specialist Ononis tridentata Devesa & G. López

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Abstract

Background and Aims

Plants living on gypsum are adapted to uptake nutrients in extremely poor alkaline soils. Under such extreme conditions, processes affecting the chemical conditions of the rhizosphere may be crucial for plant survival and growth. Rhizosphere acidification in plants living on gypsum soils has never been reported before and the effect of root exudation and microbial fungi on the rhizosphere pH remains undescribed.

Methods

In this study we cultivated seeds of the gypsum specialist *Ononis tridentata* in rhizoboxes with natural gypsum soil and with fungi-sterile gypsum soil, and monitored changes in the rhizosphere pH with planar optodes coupled to a calibrated image recording system. Soil microbial life and root exudation were characterised.

Results

The acidification was steep in both treatments, more intense in the root tip. The higher presence of fungi led to lower pH values in the natural gypsum soil treatment. In the fungi-sterile treatment, however, rhizosphere acidification was more extensive across the root surface. Several organic acids and alcohols were exuded by plant roots, with a significantly higher concentration of some compounds in fungi-sterile roots, potentially due to the reduced fungal activity. However, the exudation of lactic acid, a compound related to rhizosphere bacteria, was higher in plants grown in the natural treatment.

Conclusion

Root exudation seemed to be a fundamental process to acidify the rhizosphere in gypsum soil, and fungal microbiota participated in the process without showing a dependency on plant growth. The direct visualization of pH changes at the rhizosphere helped to describe an important mechanism of plant life on gypsum.

Introduction

Soils with high contents of gypsum are present in arid and semiarid areas worldwide (Herrero & Porta, 2000). Its life-limiting properties such as water scarcity and nutrient imbalance have important consequences for plant nutrition and survival (Escudero et al, 2015; FAO, 1990). Gypsum chemical composition ($CaSO_4$. $2H_2O$) results in an alkaline soil solution with exceptionally high ionic concentrations of calcium and sulphate, oversaturating the cation-exchange complex and leading to remarkably low nutrient availability (Meyer et al, 1992). Thus, plants growing in gypsum soils have to cope with extremely high contents of Ca, S and Mg ions and extremely low concentrations of available P, N and K (Moore et al, 2014). However, some plant species have adapted to the harsh condition of

gypsum soils, which host a diverse flora rich in edaphic endemics, frequently named gypsophiles (Mota et al, 2003).

It is known that the gypsophile *Ononis tridentata* tends to accumulate S and Ca in the leaves as a strategy to uptake nutrients from gypsum soil while avoiding the toxicity derived from the high contents of these elements in the soil (Cera et al, 2021a). Root nutrient uptake is coupled with the uptake or release of protons, and therefore is commonly associated with root-induced changes in rhizosphere pH (Neumann& Römheld, 2012). The spatial extent of these pH changes into the rhizosphere strongly depends on the buffering capacity of the soil. Rhizosphere pH changes affect nutrient solubilisation in soils and nutrient uptake, so that, generally, cation uptake decreases with declining pH whereas anion uptake is inhibited when pH increases (Neumann & Romheld, 2012). Soil nutrients are not equally available for plants across the pH spectrum, and the optimal availability for most of them has been reported to be in a slightly acidic pH (Lauchli & Grattan, 2012). Therefore, rhizosphere acidification is necessary for nutrient solubilisation and uptake from this alkaline gypsum soil, however, it has never been observed *in situ* in gypsophiles.

The formation of mycorrhizal symbiosis is considered to be one of the most successful and widespread strategies to maximize the access of plant roots to available P, playing an important role in nutrient uptake efficiency (Smith and Read, 2008). Several studies have shown that plants living on gypsum soils rely on mycorrhizal fungi to acquire the scarce P available in the soil (Palacio et al, 2012; Cera et al, 2021b). Mycorrhiza is also known for alleviating different plant stresses (Smith et al, 2010) and so, this fungi-root symbiosis has been suggested as an important factor for plant edaphic adaptation in stressful habitats (Schechter and Bruns, 2008). Rhizosphere pH has been speculated to be decreased by the activity of mycorrhiza, as an indirect effect of mychorrizal facilitation of the P availability in the root (Rigou et al, 1995) or by external measures of the pH in agar with different ectomycorrhizal cultivation (Arvieu et al, 2003), but there is no direct evidence of a quantitative change in pH in the rhizosphere due to mycorrhizal infection. Several studies have assessed the diversity of AM fungi in gypsum plant communities (Alguacil et al, 2009; Menendez-Serra et al, 2018). Other works evaluated the differences in AM colonization between gypsum-tolerant and gypsum-exclusive plant species (Palacio et al, 2012), as well as the variability of colonization among seasons (Cera et al, 2021b). However, comparative studies aimed to elucidate the role of fungi in the interaction between plants and gypsum soils are lacking.

Besides AM fungi, previous studies indicate that gypsophiles may use alternative mechanisms (such as root exudation) to achieve an efficient nutrient uptake, whereas gypsum-tolerant plant species are more dependent on mycorrhizal fungi (Palacio et al, 2011). The production of root exudates to mobilize unavailable nutrients is a well-known mechanism to enhance nutrient acquisition in nutrient-limited soils (Marschner, 1995; Lambers et al, 2008). However, there are no studies analysing root exudation in gypsophiles. Major fractions of Low-Molecular-Weight (LMW) compounds are permanently lost from root cells by diffusion. They include sugars, organic acid anions, amino acids and various phenolics that may contribute to nutrient cycling as C and N sources for rhizosphere microorganisms (Jones et al., 2005). In addition, LMW compounds such as organic acids have also been proposed to be released as a

controlled excretion by roots in response to different stresses, such as a shortage of oxygen (Xia and Roberts, 1994), Al-toxicity in acid soils (Kochian et al., 2004) or P deficiency, to mobilize P in the soil (Neumann and Römheld, 2000). Thus, in a soil where fungi are scarce, root exudation should be the key agent in charge of nutrients uptake and mobilization by soil acidification (Yan et al., 2002). However, research on pH changes and root exudation under different scenarios of microorganism presence are needed to better understand the role of root activity in the adaptation of plants to nutrient-poor soils like gypsum. Such studies cannot take place directly in the field. However, rhizoboxes coupled with optode technology to track dynamic and spatial pH changes offer a unique opportunity to gain new knowledge on the effect of fungi on rhizosphere soil pH.

The aim of this study was to analyse the plant-soil interaction of a gypsophile plant cultivated in natural and a fungi-sterile gypsum soil with a special focus on processes at the rhizosphere level. We intended to determine the importance of soil fungi and the role of root exudation in the acidification of the rhizosphere of *Ononis tridentata* seedlings growing in alkaline gypsum soils with contrasting fungal presence. We hypothesized (1) that the presence of fungi in the soil will promote rhizosphere acidification, reaching lower pH in natural than in fungi-sterile soils. We also hypothesized (2) that root exudation will be promoted in the fungi-sterile soil treatment, as a response to a higher nutrient stress.

Materials & Methods

Experimental conditions

Ononis tridentata is a perennial gypsophile of up to 1.5 metres in height (Sánchez et al., 2012) that is widespread in gypsum soils across Spain and reaching Northern Africa (Mota et al, 2011). Seeds from *O. tridentata* were collected from mixed adult individuals from a natural population growing in Villamayor (41°41'48.3"N 0°44'34.7"W, Zaragoza province, Aragón, Spain). The soil for the experiment was collected in the same area from the top 0.5-1 m of the soil, removing the top litter and biological crust layer, and then, homogenized and cultivated in the Pyrenean Institute of Ecology (IPE-CSIC), Jaca (Huesca province, Aragón, Spain).

To understand the influence of fungi on the rhizosphere pH and the root exudation profile of *O. tridentata*, plants were grown in either natural soil or in fungi-sterile soil. Sterile soil was obtained after gamma irradiation (25 kGy) with a ^{60}Co source (Aragogamma, Granollers, Spain), and afterwards the sterilized soil was inoculated with a bacterial filtrate from the natural soil in order to add the bacterial community present in it. This bacterial inoculum was obtained by stirring natural soil in sterile distilled water in a proportion of 1/10 (weight/volume) for 5 minutes, and then passing the suspension sequentially through filter paper and a 9 µm ø filter to eliminate soil particles and fungal spores and propagules, but not bacteria. Each fungi-sterile pot was inoculated with 50 mL of this bacterial inoculum (Doubková et. al, 2011).

We cultivated *O. tridentata* plants in rhizoboxes (i.e. plastic containers of 220 x 170 x 15 mm with a transparent plexiglass window that enabled root observation, see Fig. 1A) and filled them with 561 ml of either natural soil or fungi-free soil. Five rhizoboxes per soil treatment were used.

Before cultivation, seeds were carefully scarified using a sandpaper, rehydrated with distilled water for 24 hours, and germinated in petri dishes humidified with distilled water. Afterwards, three germinated seeds of *O. tridentata* were planted in each rhizobox. Altogether there were 15 plants sown per treatment (natural and sterilized soil), although only one seedling per rhizobox was finally included in the soil pH measurements (the most vigorous one), amounting five replicates per treatment. During cultivation, the transparent windows of the rhizoboxes were covered with dark acetate sheets to prevent light from reaching the roots. Rhizoboxes were kept at a ~ 45º angle to ensure root growth towards the window (see Fig. 1B) and had several holes in its bottom to drain excess water. Plants were watered with distilled water every 12 hour for 1 minute, maintaining soil humidity at field capacity. However, due the low water retention of gypsum soils (Herrero and Porta, 2000), and to guarantee the constant humidity of the VisiSens sensor foils (see pH visualization), rhizoboxes were placed in a plastic tray that retained the excess irrigation water, which could be absorbed to keep the field capacity in the soil. Plants were grown in a room under natural non-direct sunlight and supplementary 12h artificial illumination (full spectrum LED light, 380nm-800nm). They were harvested after 22 days of cultivation.

pH visualization

Changes in rhizosphere pH were monitored by obtaining pictures of optodes placed at the root tip of selected seedlings and the resulting images were edited with the VisiSens TD® software (PreSens GmbH). VisiSens is a 2D pH mapping device with PC data acquisition and evaluation. Six days after the plants had been growing in the rhizobox, VisiSens sensor foils of 2 cm^2 were installed between the transparent plexiglas of the rhizobox and the soil, covering the root tip in the healthier seedling of each rhizobox (see Fig. 1C). After that, images were taken daily at 1 p.m. during a period of 14 days, to ensure the roots have grown and passed behind the sensor foil. The pictures were taken in a small dark chamber where the only light source came from the VisiSens TD camera. The camera was re-fixed for each image taken, placed in contact with the plexiglass, as near as possible of the sensor foil and focusing it whole (see graphic scheme in Fig. 1D and Fig. 1E). Images were edited by IDL evaluation pH software (VisiSens™ Analytical 2 Software) using previous calibration data of the sensor foils. Maximum, minimum and average pH were noted for each rhizobox, during the monitoring period (Fig. 1F).

Plant harvest and analysis of root exudates

After 14 days of pH measures, the transparent methacrylate (Plexiglas) of the rhizobox was carefully removed to access the roots. The aboveground plant part was separated from the root part. The roots were cleaned with a brush to remove rhizosphere soil. All the root tips of the rhizoboxes were weighed for fresh-weight on a precision scale (42 g / 0.00001 g, MS105DU, Mettler Toledo, Columbus-OH, USA) and, as quickly as possible, placed in a small vial with 1 mL of a 0.01% formic acid solution (obtained by adding 10 µL of 98% purity Formic acid to 100 mL Mili-Q water, the resulting solution had a pH of 3.06).

Fine roots were then shaken for 10 minutes at medium speed in an automated agitator (Rotabit, JPselecta). Exudates were then filtered through a 0.22 µm of 13 mm ø cellulose acetate filter (CA Syringe Filter 1mL, Filter-Lab. REF: JS1) and kept at -80 ºC. The rest of the root was weighed (fresh) and dried in an oven at 50 ºC for a minimum of three days, then weighted in a precision scale (42 g / 0.00001 g, MS105DU, Mettler Toledo, Columbus-OH, USA) to obtain the dry weight of roots.

Exudates were analysed at the metabolomics service/lab (CEBAS-CSIC) with a ultra-high-performance liquid chromatography system with a triple quadrupole mass spectrometer (UPLC-QToF-MS). Organic acids are the most common exudates, so organic standards were used and results are provided as µg/mL (ppm). Contrastingly, alcohols were analysed without standards, as the study focused on organic acids. Consequently, results for alcohol measurements are reported as a ratio of concentration, comparing each data with the highest value detected and for comparative purposes among treatments only.

PLFAs analyses

To characterize soil microbial communities and to detect shifts in the main functional groups of microbes, we used phospholipid fatty acid (PLFA) profiling. Aliquots of 2 g of lyophilized bulk soils were used for lipid extraction. Lipids were extracted with a one-phase chloroform–methanol-phosphate buffer solvent. Phospholipids were separated from non-polar lipids and converted to fatty acid methyl esters (FAMEs) before analysis, following the methodology described by Buyer and Sasser (2012). The resulting FAMEs were separated by gas chromatography using an Agilent 7890A GC System (Agilent Technologies, Wilmington, DE, USA) equipped with a 25-m Ultra 2 (5%-phenyl)-methylpolysiloxane column (J&W Scientific, Folsom, CA, USA) and a flame-ionization detector. The identification and quantification of FAMES was carried out using the PLFAD1 method of Sherlock software version 6.3 from MIDI, Inc (Newark, DE, USA). The internal standard 19:0 phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL, USA) was used for the quantification of the FAMEs. Microbial biomass was estimated by summing the contents of all individual PLFAs and reported as nanomoles of PLFAs per gram of soil.

Specific PLFAs were used as biomarkers to quantify the biomass of broad taxonomic microbial groups, according to their characteristic fatty acids: eukaryote, Gram negative and Gram positive bacteria (hereafter G- and G +, respectively), saprophytic fungi and arbuscular mycorrhizal fungi (AMF) (Frostegard and Baath, 1996). The ratio between cyclopropyl fatty acids and their monoenoic precursors [(cy17:0 + cy19:0) / (16:1ω7 + 18:1ω7); hereafter GN cy/pre] were calculated as proxies for physiological or nutritional stress in the bacterial communities (Frostegård et al. 2011; Willers et al. 2015).

Statistical analyses

We plotted in a boxplot the values for the minimum and mean pH observed in all the replicates filtered to obtain a clear observation of the root with PreSens VisiSens software. We also plotted the exudates detected with bars indicating the concentration or relative abundance of the compound according to the case in the different treatments. These graphs were designed with ggplot2 package 3.3.1 (Wickham, 2016). Statistical differences of pH among treatments were calculated with Kruskal Wallis test

(Hollander & Wolfe, 1973) as data were not parametric, as Shapiro Test indicated they were not normally distributed. The same procedure was used to analyse differences in the quantified exudates detected, creating an individual data set for each compound.

Relative abundance percentages of individual fatty acids were arcsine-transformed for normality before statistical analyses. A PCA was performed to represent and analyse the differences in soil microbial community composition (PLFAs) between experimental treatments. Then, we selected the parameters of the relative abundance of Saprophytic and Arbuscular Fungi, Gram Positive, Gram Negative and Actinobacteria; and the ratios Gram+/Gram- and G- cy/pre to project on the PCA plot to a facilitate visualization of the differences on the microbial composition on the soil of the different treatments. This plot was run with CANOCO software (Braak & Smilauer, 2002). The rest of the analyses were carried out using *R* version 4.0.0 (R Core Team, 2020).

The comparison between fungi-sterile soils and natural soils for the selected parameters related to fungi and bacterial composition or to microbiota stress were individually analysed by a one-way ANOVA test (Welch, 1951) or a Kruskal Wallis test (Hollander & Wolfe, 1973) when data were not parametric. Normality of the data was double checked by Shapiro Test (Royston, 1995) and also treating data as normally distributed and then checking the residuals of the *lm* model (Chambers, 1992) with DHARMa package (Hartig, 2020).

Results

Effect of experimental conditions on soil microbial communities

We observed a clear segregation of the treatments according to the presence and relative abundance of PLFAs in soil (Fig. 2), which indicates the existence of distinct microbial communities. Although we detected fungal presence in both treatments, the relative abundance of saprophytic fungi and AM fungi was, however, significantly lower in the “fungi-sterile” treatment ($p = 0.016$ and $p = 0.012$, respectively; Table 1). Therefore, our methodology worked in terms of reducing the presence of fungi, so for simplicity we will keep referring to natural and fungi-sterile treatments although we acknowledge that there was some fungal presence in the fungi-sterile treatment. Moreover, there was a higher relative abundance of Gram-negative bacteria ($p < 0.001$) and, consequently, a lower ratio Gram+/Gram- in the fungi-sterile soils ($p < 0.001$). On the other hand, the microbiota living in the fungi-sterile soil showed higher ratio of GN cy/pre ($p = 0.047$), indicating that it was suffering higher nutritional stress than that in the natural soil (White et al. 1996; McKinley et al. 2005)

Table 1
Significance of the differences ($p < 0.05$) between fungi-sterile and natural soils in different phospholipid fatty acids (PLFA) related to fungal and bacterial composition or to microbiota stress, based on one-way ANOVA or Kruskal Wallis test when data were not normally distributed. Significant *p*-values are in bold.

	F	Kruskal-Wallis chi-squared	*p*-value
% Total Fungi	8.60		**0.033**
% Saprophyte Fungi		5.77	**0.016**
% AM Fungi	12.14		**0.012**
Fungi/Bacteria	9.26		**0.026**
Total Bacteria	5.38		**0.049**
Gram+/Gram-	60.17		**< 0.001**
GN cy/pre	6.46		**0.047**
Gram -	46.64		**< 0.001**
Gram +	20.68		**0.003**
Actinobacteria	100.39		**< 0.001**

Rhizosphere pH acidification

Ononis tridentata seedlings developed a single apical radicle, whose position and morphology coincided with the images revealed by the VisiSens software. We observed an acidification in the rhizosphere for both treatments, with a pH decline of 2.4 units on average in natural replicates and 2.1 units in fungi-sterile replicates (Supplementary Table 1), calculated from the values of the mean of the pH maximum within the replicates. Figure 3 shows a darker blue area corresponding with the root tip, and how the intensity of this coloured signal descended as the root passed by the sensor over the days. The lowest minimum pH was reached in the rhizosphere of the seedlings growing in the natural soil, significantly lower than the minimum pH reached in the rhizosphere of seedlings growing in the fungi-sterile soil ($p = 0.014$; Fig. 4). However, the mean pH in the rhizosphere was lower in the fungi-sterile soil ($p < 0.001$, Fig. 4), indicating a greater acidification surface across the roots of plants grown with decreased fungal presence. The picture of the acidified rhizosphere was more intense and defined in the roots of the natural treatment, contrasting with the less intense but more widespread acidification in the fungi sterile rhizosphere.

Differences in exudation patterns

The UPLC analysis detected several organic acids (citric, malic, succinic, lactic, malonic and fumaric) as root exudates in both treatments, accounting for significantly higher quantities of malonic acid in the fungi-sterile treatment ($p = 0.036$). The isocitric acid was detected only in the fungi-sterile treatment, contrary to the natural treatment, whose seedlings did not exudate this compound. Conversely, lactic acid was significantly higher in the exudation profile of the seedlings growing in the natural treatment ($p = 0.016$). Some polyols were also detected in both treatments (myo-Inositol, galactinol, xylitol and sorbitol-mannitol), but only one of them presented significant differences in its abundance proportion: sorbitol-mannitol was significantly higher in the fungi-sterile treatment ($p = 0.021$; Table 2, Fig. 5). In general, as we expected, we found a greater proportion of root exudation in the fungi-sterile soil replicates, except for lactic acid, an organic acid related to microbial activity (Juturu & Wu, 2015).

Table 2
Significance of the differences ($p < 0.05$) between fungi-sterile and natural soils in root exudates, based on one-way ANOVA or Kruskal Wallis test when data were not normally distributed. Significant *p*-values are in bold.

	F	**Kruskal-Wallis chi square**	***p*-value**
Citric		0.5478	0.459
Isocitric		**5.5385**	**0.019**
Malic		0.0982	0.754
Succinic	0.2289		0.645
Lactic		**5.7709**	**0.016**
Malonic	**7.4533**		**0.036**
fumaric		1.3280	0.249
Myo_Inositol		0.2727	0.602
Galactinol	4.1786		0.093
Xilitol	4.2648		0.074
Sorbitol-Manitol	8.2484		**0.021**
Choline	3.9143		0.093

Discussion

Effect of experimental conditions on soil microbial communities

The lower G+/G- ratio found in the gamma irradiated soil might be a consequence that some G- bacteria are r-strategists that, after the inoculation of the sterilized soil, may have surpassed in growth rate Gram + bacteria under the elevated labile organic C conditions induced by radiation (McNamara et al., 2003; Zhang et al., 2016). Moreover, gamma-irradiation of soil is known to increase the pool of available inorganic nitrogen (Lensi et al., 1991; McNamara et al., 2003), which can stimulate the processes of nitrification and denitrification, both of them mostly conducted by Gram - bacteria (Hayatsu et al., 2008). Although increased relative abundance of Gram- bacteria after soil gamma-irradiation has been also reported in other researches (Yim et al., 2015; Zhang et al., 2016), it is far from being a general fact (Ogwu et al., 2019).

Plants from natural gypsum soil reached lower rhizosphere pH than those from fungi-sterile soil but the root acidification area was smaller

The key findings in our research were that (1) natural gypsum soils reached lower pH in the rhizosphere, showing a steeper rhizosphere gradient of pH but (2) fungi-sterile soils acidified a greater area in the soil, showing a larger extent of the rhizosphere. There is no previous literature about pH changes in such alkaline soils, especially in wild plant species. To the best of our knowledge, this is the first time an acidification of more than 2 pH units is shown *in situ* in a rhizosphere of a species not used for cropping purposes. The maximum pH observed in the soil (pH ~ 9.4, see Fig. 2 and Supplementary Figure S1could be due to the natural intrusions of calcium carbonate in the gypsum soil, which increases significantly the soil pH and reduces the Ca^{2+} activity (FAO, 1990). Given this high soil pH, plants found it necessary to acidify the soil to favour nutrient solubilisation and uptake. *O. tridentata* seedlings growing in natural soil seemed to be helped by its microbiota, which promoted a greater soil acidification by the release of H^+ protons to the rhizosphere (Zhu et al, 2023). In this way, a steeper rhizosphere acidification could be indicating a greater P and N availability in the soil (Ma et al, 2021; Cakmak & Marschner, 1990). Rhizosphere acidification may be also indicating the uptake of calcium and magnesium cations, which is balanced by a net release of protons (Neumann & Röhmheld, 2012). These cations can be found in excess in the gypsum soil (Cashby-Horton et al, 2015), and are accumulated in *O. tridentata* leaves (Cera et al., 2021a).

The fact that in plants grown in fungi-sterile soil the rhizosphere was more extended could be due to the different pH buffering capacity of the soil. Altered microbial composition could reduce the presence of secondary minerals that contribute to the pH buffering capacity (Bilyera et al, 2022). In addition, rhizosphere acidification under P-limiting conditions has often been associated with the release of organic acids, which were detected in both treatments. However, the rapid microbial decomposition of root exudates (Jones et al., 2005) could be affecting the natural replicates, but not the fungi-sterile rhizosphere, where exudates could be increasing the space and time residence in the soil (Boudot, 1992). Another possibility to explain this extended acidification could be due to an increased growth of the root of nutrient-deprived plants cultivated in the fungi sterile treatment to promote soil foraging (de la Fuente et al., 2020) or the extension of its bacterial microbiota due to the lack of fungi that also contributes to the acidification of the soil.

The release of root exudates differed between soil treatments

Until now, most studies investigating root exudation have focused on agricultural plants (Henry et al., 2008) but see Williams et al. (2020 and 2022). However, the exudation profiles in other ecosystems may significantly differ and play important roles in shaping the microbial community (Baudoin et al, 2003; Ma et al, 2021). It is difficult to find direct evidence of the influence of a certain root exudate on soil microbial communities, because of the complexity of the rhizosphere environment and the difficulty in studying belowground processes (Biedrzycki & Bais, 2009). Our work revealed the differences in the release of exudates at the rhizosphere of *O. tridentata* in plants interacting with contrasting microbial communities. These results have important implications for the understanding of the plant-soil interactions mediating plant nutrition and survival in alkaline nutrient poor soils like gypsum.

The main contribution of this work about root exudation was that plants growing with a reduced fungal population in the soil, exuded, in general, greater quantities of compounds, thus seeming to compensate for the lack of the fungal interaction on making nutrient available. Fungi-sterile replicates exuded higher quantities of different organic acids and a polyol, likely as a consequence of their nutrient deficiency due to a reduced fungal and bacterial activity in the soil. In low nutrient environments, root exudation could be employed as symbiotic signals to soil microbes involved in nutrients procurement (Dakora & Phillips, 2002). Moreover, our study showed that this species of plant adapted to such high pH soils is able to reduce the pH very significantly and, thus, facilitate nutrition processes through the exudation of different organic acids. Isocitric acid and malonic acid were found in greater quantities in the fungi-sterile rhizosphere. These compounds are in the class of aliphatic acids, whose function as root exudates includes growth regulation (Oliveros-Bastidas et al, 2009) and have been reported as major factors influencing the dissolution of minerals (Sokolova, 2019), thus contributing to *O. tridentata* nutrition.

We detected additional compounds in fungi-sterile replicates: the sugar alcohols sorbitol-mannitol. These compounds are also involved in the improvement of soil nutrient environment and soil nutrient cycling and, as such, they are beginning to be used as fertilisers in agriculture (Yu et al, 2014; Ichimura et al, 2016). Additionally, they have an important role in shaping and increasing different rhizosphere microbial communities (Yu et al, 2023).

Conversely, the exudation of lactic acid was significantly higher in *O. tridentata* replicates growing on natural soils, with an unaltered microbial composition. This compound has been reported to be a product of several bacteria associated to roots and is known by its beneficial effects on crops (Minervini et al, 2015). Significant increases in the abundance and diversity of soil bacteria communities have been reported after the artificial addition of lactic acid (Hengjing et al, 2011). In addition, this compound has also been reported to be exuded by plants in the rhizosphere to detoxify its high concentration in cell, as result of oxygen deprivation (Ryan et al, 2001; Badri & Vivanco, 2009). Other studies showed a biocontrol

function of lactic acid in tomato plants (Wang et al, 2019), or a detoxification function in response to copper or cadmium toxicity (Lyubenova et al, 2013; Chiang et al, 2006 respectively).

Besides our interesting results combining planar optodes with root exudation and a treatment of partial soil sterilization in a gypsum soil help with the understanding of *Ononis tridentata* rhizosphere behaviour, we should address that, the high soil humidity requirements of the pH sensor restricted our observations of root activity to well-watered conditions. This situation in gypsum ecosystems would take place in the most favourable moment of the year, when gypsum plants tend to grow and maximize their demand for N and P (Cera et al., 2021b).

It is also important to mention a potential consequence of the root exudation shown by plants living on gypsum, related to the use of gypsum crystallization water. Gypsum ($CaSO_4.2H_2O$) contains two water molecules in its crystalline structure, and several authors (Palacio et al, 2014; de la Puente et al, 2021; Huang et al, 2020) have reported the use of this water as the main water source by plants or other microorganisms growing on gypsum during drought. The mechanism to obtain this water proposed by Huang et al (2020) for cyanobacteria consists on gypsum dissolution by the acidification caused by the exudation of organic acids. However, the mechanisms displayed by plants to acquire gypsum crystallization water remain unknown. Our results show the ability to exude different organic acids by plants living on gypsum, which may affect the release of gypsum crystallization water. Similarly, the release of low molecular weight alcohols may also alter the thermodynamic conditions of calcium sulphate phases, modifying its hydration (Van Driessche et al, 2017; Tritschler et al, 2015). Although adult *O. tridentata* plants have been reported to rely mainly on deep water and not gypsum crystalline water (Palacio et al, 2014; de la Puente et al, 2021), the changes in rhizosphere pH observed in this study correspond to seedlings, whose access to gypsum crystalline water may be crucial for their survival at early stages. Preliminary results on root acidification similar to those observed in *O. tridentata* on other gypsum endemics plants with shallow root systems previously reported to rely on gypsum crystalline water, like *Helianthemum squamatum* or *Helianthemum syriacum* (Palacio et al, 2014; de la Puente et al, 2021) (see preliminary data in Supplementary Material Fig.S2), further support potential role of root exudation in gypsum plants as a mechanism to obtain gypsum crystalline water.

Conclusions

For the first time, we visualized *in situ* rhizosphere acidification in wild plants growing in alkaline gypsum soils. According to our results, rhizosphere acidification seems to be a necessary process to grow on gypsum. We conclude that soil microbial life participates in the rhizosphere acidification, as we observed a steeper pH decline in natural-soil replicates. However, the roots of *O. tridentata* also contributed directly to this acidification, through the exudation of different organic aliphatic acids. and sugar-alcohols. This indicates that *O. tridentata* is not fully dependent on microbiota for nutrient acquisition, although it is facilitated by their presence, and has ways to compensate for the symbionts being missing. We further conclude that root exudation is a determinant factor for plant adaptation to the atypical gypsum soil that could mediate plant nutrition and access to gypsum crystalline water.

Declarations

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Competing Interests

The authors declare that there is no conflict of interest for this study. The study was conducted in the absence of any commercial or financial relationship that could be constructed as a potential conflict of interest.

Author contributions

L.P. and L.E wrote the manuscript and prepared figures and tables, J.M.I interpreted PLFAs analysis and prepared the Figure associated; S.P & J.P.F gave the idea about manuscript and revised it critically and contributed for intellectual contents.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request

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Figures

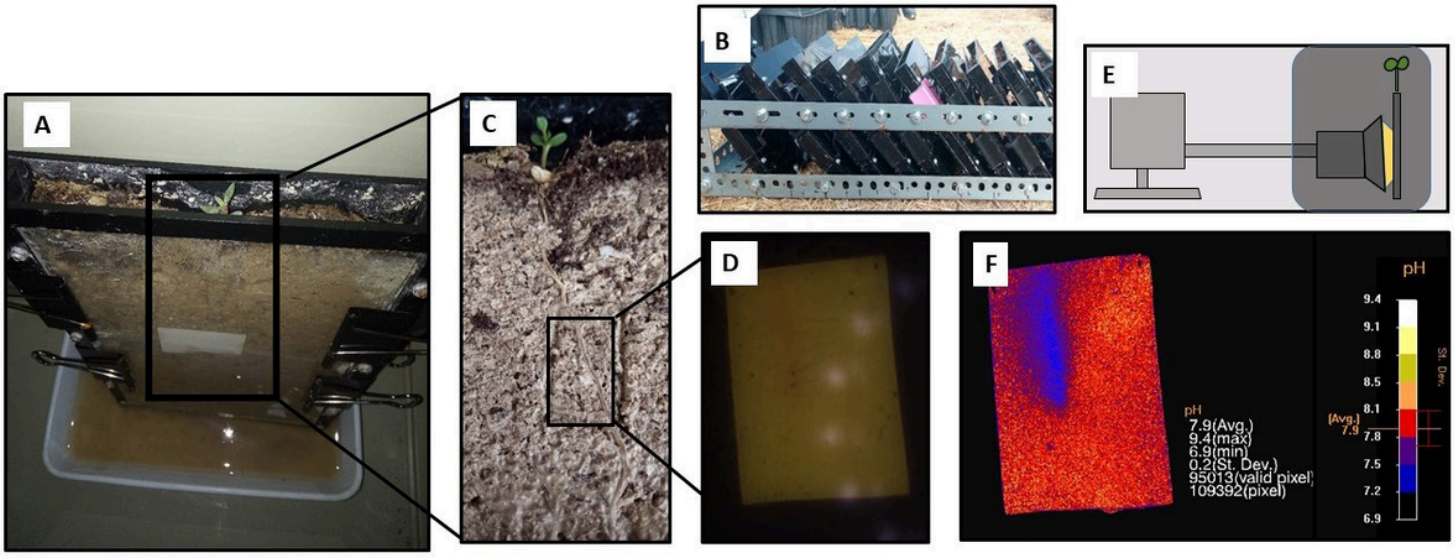


Figure 1

Methods. **A**. Picture of one of the rhizoboxes where seedlings were grown. **B**. rhizoboxes positioned *ca*. 45º to force the roots to grow along the plexiglass due to gravity and thus make as many of them visible as possible. **C**. Picture of an *Ononis tridentata* seedling 3 cm tall (mean size of the seelings analysed) and the area where the sensor foil was installed, taken after the pH measures. **D**. Image acquired with the VisiSens PreSens camera before its editing? with the calibrated pH. **E**. Graphic scheme of the procedure to take pictures. The software in the computer was connected to the camera, which was placed as close as possible to the sensor in contact with the transparent window. Pictures were taken with the camera light source. **F**. Example of the final image given by the software, indicating the maximum, minimum and mean pH of all the pixels corresponding to the sensor.

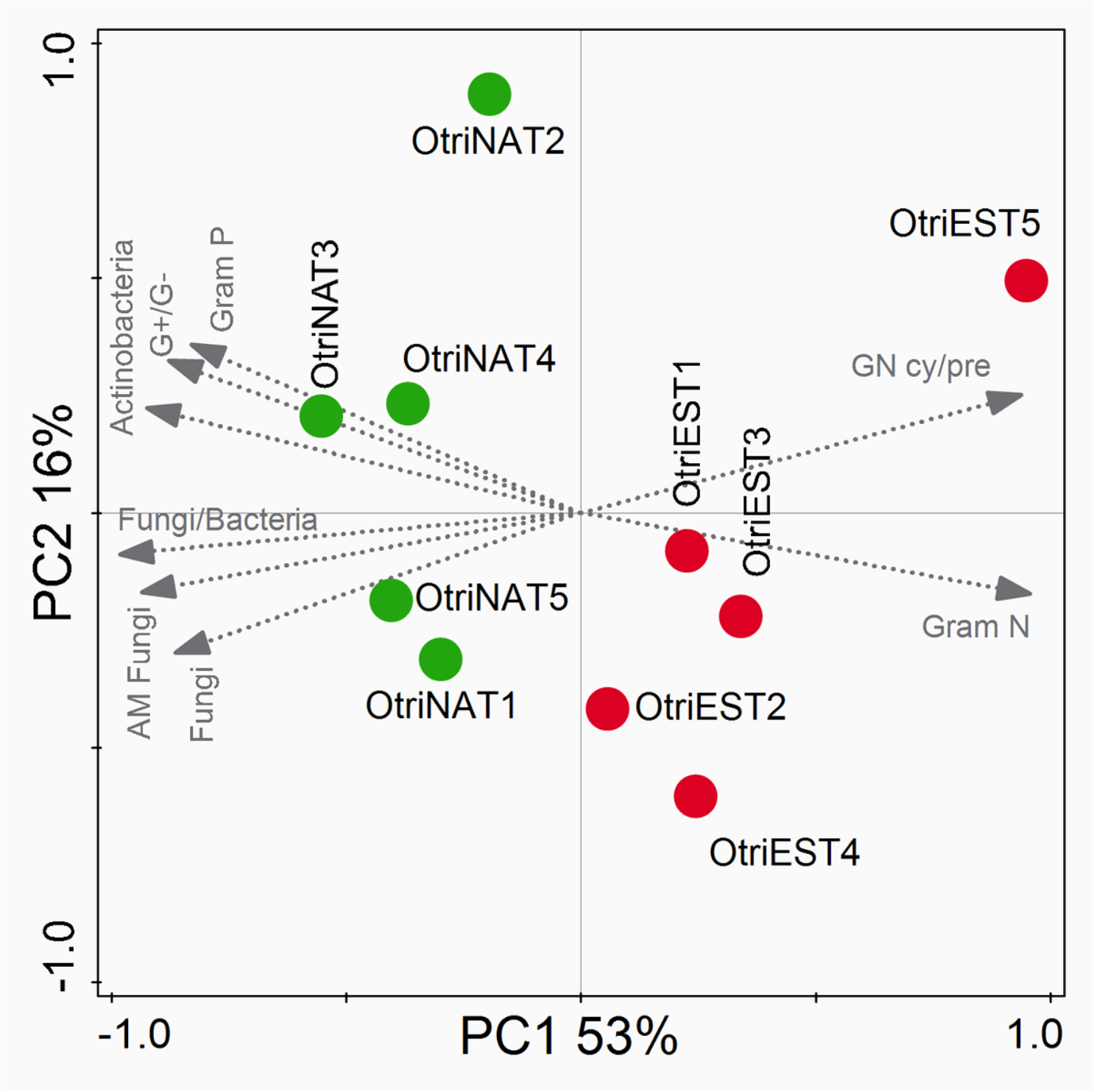


Figure 2

Biplot of the results of a PCA performed on the matrix of relative abundance of all PLFAs detected in soil samples. Arrows represent variables passively projected into the PCA diagram but not included in the calculation (accessory variables): relative abundance of Gram negative bacteria (Gram N), Gram positive bacteria (Gram P), Actinobacteria (Actinobacteria), saprophytic fungi (Fungi), mycorrizal fungi (AM Fungi), cyclopropyl fatty acids to monoenoic precursors ratio (GN cy/pre) and the ratio of the relative abundance of Gram Positive and Gram Negative Bacteria. Values on the axes indicate percentages of

total variation explained by each axis. Green points stand for the scores of the five replicates of natural soil treatments and red points for the five replicates of fungi-sterile treatment.

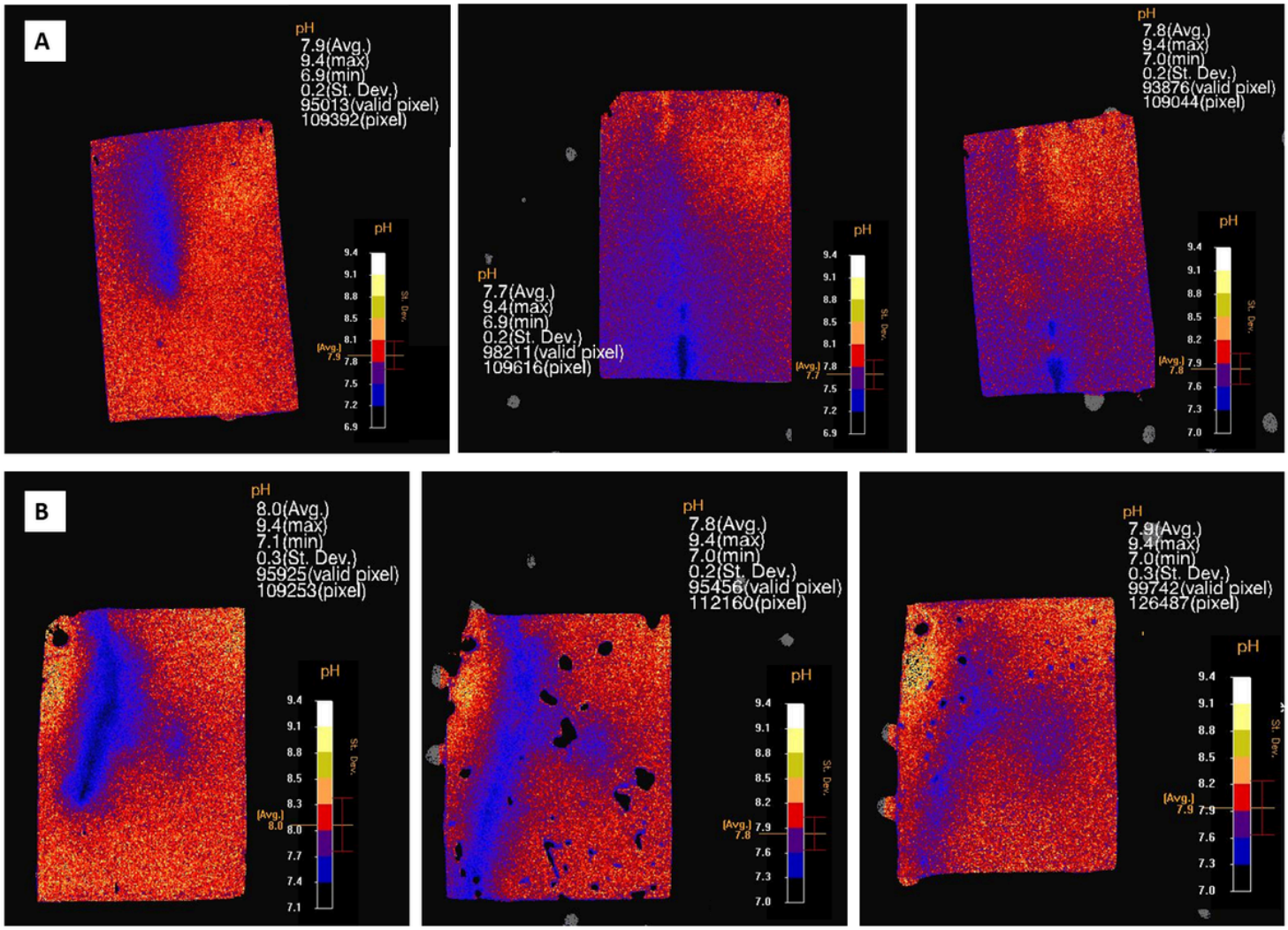


Figure 3

IDL evaluation images of growing roots taken with VisiSens PreSens. Sequential images show the movement of the acidification front due to the root tip passing through the sensor. **A**. Sequential images of one replicate of *Ononis Tridentata* (“rep1 natural”) seedling root growing in natural gypsum soil on different days (day 1, 4 and 9). **B.** Sequential images of one replicate of *Ononis Tridentata* (“rep 2 sterile”) seedling root growing in the fungi-sterile gypsum soil in different days (day 1, 3 and 9).

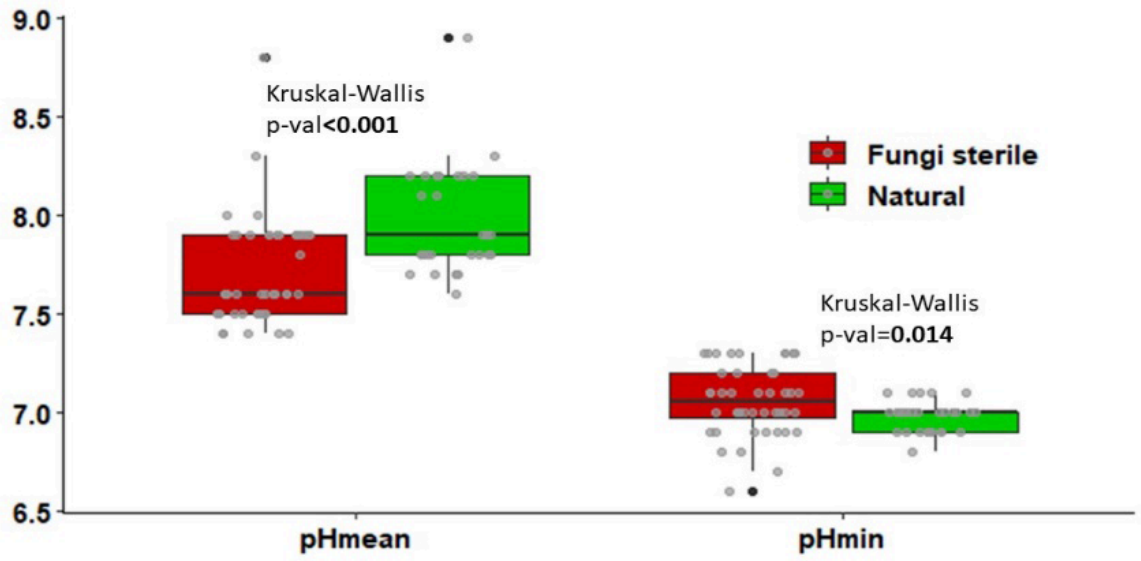


Figure 4

Boxplot of minimum and mean pH observed in the sensor foils placed in the roots of the growing seedlings of *Ononis tridentata*, compared per soil treatment: green boxes for the natural treatment, red boxed for the fungi-sterile treatment. *P*-values of Kruskal Wallis tests show the significance of the differences between treatments.

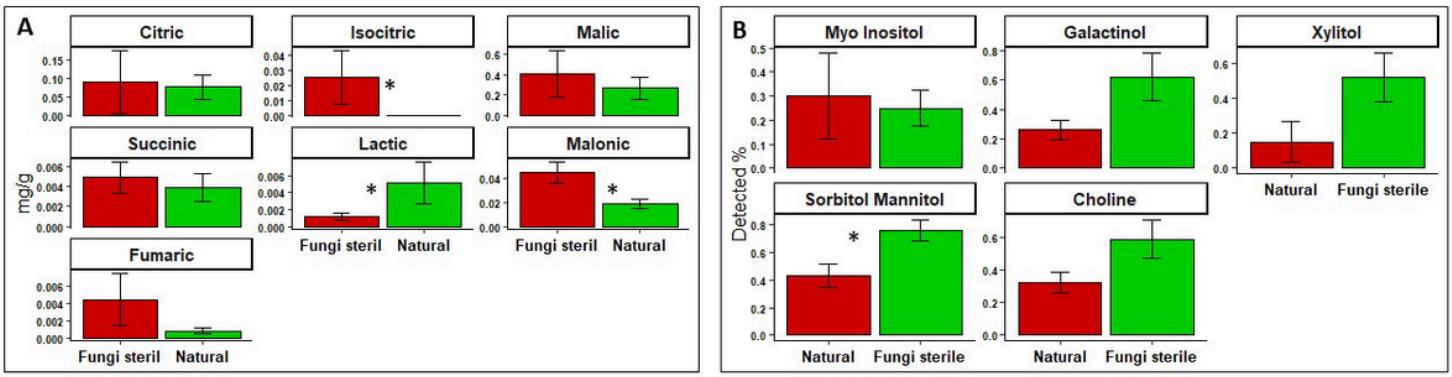


Figure 5

Bar plots showing the mean and SD of the exudates analysed in *Ononis tridentata* roots growing in natural gypsum soil (green bars) and in fungi-sterile gypsum soil (red bars). Organic acids were calculated as mg of the compound exuded by g of root (**A**), whereas the other exudates that belong to the polyols family or amine (such as choline) were calculated as ratio of detection, but not as exact concentration (**B**). The asterisk (*) indicate significant differences in each exudate between treatments (see also Table 2)

Supplementary Files

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