

Phosphogypsum's impact on growth and nutrient acquisition in carob trees (*Ceratonia siliqua* L.) inoculated with native microbiota

Hamza Khassali (✉ hamza.khassali@ird.fr)

LSTM, Univ Montpellier, CIRAD, INRA, IRD, Montpellier SupAgro

Issam Jadrane

Cadi Ayyad, University

Alexandre Geoffroy

Fertil'innov Environnement, 34790 Grabels

Robin Duponnois

LSTM, Univ Montpellier, CIRAD, INRA, IRD, Montpellier SupAgro

Lahcen Ouahmane

Cadi Ayyad, University

Article

Keywords:

Posted Date: October 3rd, 2023

DOI: <https://doi.org/10.21203/rs.3.rs-3348818/v1>

License:   This work is licensed under a Creative Commons Attribution 4.0 International License. [Read Full License](#)

Additional Declarations: No competing interests reported.

Abstract

Phosphogypsum (PG) management is one of the major challenges of the phosphate industries in Morocco and worldwide. Its efficient management and valorization at a low-cost and without additional treatment are highly desirable. The purpose of this study is to evaluate the effects of Moroccan PG on the growth and development of carob tree (*Ceratonia siliqua*) in the presence and absence of its native microbiota. The analysis of the native microbiota revealed that the bacterial community was primarily dominated by Actinobacteria and Proteobacteria, while the fungal community was mainly composed of Ascomycota and Basidiomycota. In the absence of the consortium microbiota, the heights, root dry weights, shoot dry weights and leaflet numbers increased significantly ($p < 0.05$) in carob plants grown on soil substrates amended with PG at a ratio of 2 % compared to those grown without PG (0 %). Micronutrient (Zn, Fe, Cu, Mn, Ni and Co) contents also increased with PG doses in both the roots and leaves although toxic elements (As, Cd and Pb) were also detected, especially in carob plants grown at 10 and 20 % PG. Inoculation with consortium microbiota in combination with PG had similar beneficial effects on the different physiological parameters measured in the carob plants. The most significant growth was recorded in carob seedlings grown under PG doses of 1 and 2 % and these plants also displayed a better performance against toxic elements. In fact, toxic element concentrations in inoculated carob seedlings grown in PG amended soil were lower compared to non-inoculated plants and far below ranges considered phytotoxic for vascular plants for both inoculum treatments. Our results open up exciting perspectives for the valorization and more widespread use of phosphogypsum in combination with native microorganisms in crop cultivation particularly in regions with difficult soil conditions.

INTRODUCTION

The carob tree (*Ceratonia siliqua* L., Leguminosae) is one of the most important species native to the Mediterranean area and is widely cultivated for economic purposes¹ as well as environmental applications². However, this species has recently experienced declining populations³ due to various anthropogenic and natural challenges. Nutrient scarcity poses a significant problem for plant species in Mediterranean regions⁴, where they encounter harsh soil conditions characterized by aridity and nutrient deficiency. To improve plant nutrition under these challenging conditions, several alternatives have been implemented and investigated, with the use of soil microorganisms showing great promise⁵.

Arbuscular mycorrhizal fungi (AMF) and rhizobacteria are the microorganisms most widely used for these types of applications^{6,7} and several preselected strains of AMF and rhizobacteria have shown beneficial effects on the nutrient uptake of various plant species. However, this approach has several limitations such as the low symbiotic efficiency of some strains introduced under natural conditions and their environmental impact on the native microbiota^{8,9}. Indeed, under natural conditions, the rhizosphere associated with the carob is the site of important interactions and exchanges between all microorganisms including bacteria and fungi¹⁰, and each of these microorganisms intervene in different processes. Nevertheless, in some Mediterranean regions and despite microorganism's intervention in nutrient uptake, this process remains unsatisfactory due to land poverty in nutrients necessary for crop growth and plant development. To overcome this problem, the use of phosphogypsum waste as a source of nutrients can be considered as a possible solution.

Morocco is considered the world's leading phosphate reserve and the third-largest producer¹¹. The phosphate industry holds a crucial position in the Moroccan economy, generating approximately 30 million tons of acid phosphogypsum (PG) residues annually¹². Currently, a significant portion of this waste is disposed of in the sea without prior treatment, posing environmental concerns. However, this waste also represents a potentially valuable resource that remains untapped. The primary challenge lies in developing low-cost applications for the recovery of PG waste.

Recent studies have highlighted the beneficial use of PG in enhancing soil structure, plant nutrition, and agricultural production¹³. It offers an environmentally friendly solution to promote the soil's protective properties. Additionally, microbial processing has gained attention from the scientific community and the industrial sector as a promising, energy-efficient, and cost-effective alternative for the transformation and valorization of PG. Numerous studies have demonstrated that the combination of PG with microorganisms results in improved growth and development across different plant species^{14–17}.

Most of these studies have evaluated the combined effects of preselected microorganisms, including fungi and/or bacteria, with PG wastes on plant growth and development. However, to our knowledge, no comprehensive investigation has been conducted to assess the impact of PG wastes on plant growth using the plant's native microbiota. Therefore, our study aims to evaluate the effects of Moroccan PG wastes on the growth and nutrient uptake of *C. siliqua*, both in the presence and absence of its native microbiota.

RESULTS

Global composition of carob microbiota

The analysis of 16S rRNA gene sequences revealed that the bacterial community at the taxonomic level, was strongly dominated by *Actinobacteria* (including members of the genera *Agromyces*, *Gaiella*, *Mycobacterium*, *Nocardioides*, *Pseudonocardia*, *Rubrobacter*, *Solirubrobacter* and *Streptomyces*), and *Proteobacteria* (*Acidibacter*, *Dongia*, *Microvirga*, *Reyranella*, *Rhodoplanes*, *Skermanella* and *Sphingomonas*) (Figure 2). In terms of the fungal community, the phyla *Ascomycota* (including members of the genera *Cephalotrichum*, *Colletotrichum*, *Endoconidioma*, *Geopora*, *Gliocladium*, *Humicola* and *Preussia*), and *Basidiomycota* (*Inocybe* and *Lindtneria*) were the most abundant (Figure 3).

Parameters correlation

A strong positive correlation (ranging from +0.9 to 1.0) was observed between various growth parameters (height, shoot dry weights (SDW), root dry weights (RDW) and leaflet numbers)) in both inoculum treatments (Figure 4A, B). Notably, a particularly high correlation was recorded between SDW and height specifically within the inoculum treatment (Figure 4B). Furthermore, trace elements (As, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb and Zn) also had a positive correlation in shoot and root parts under both inoculum treatments. Interestingly, a significant positive correlations $r = +0.95$ to 1.0 was observed in uninoculated treatments (Figure 4A) between (Zn-l ; Pb-l), (Zn-l ; Cu-r) and (Cr-l ; Mn-r), and between (Mn-l ; Zn-l) under inoculated treatments. A moderate negative correlation was also found between Cu-l and the other trace elements under inoculated treatment. Conversely, correlations between growth parameters and trace elements were generally not detected or negative in both inoculum treatments.

Mycorrhizal colonization

Mycorrhizal intensity varied greatly between the different PG doses compared to the control (0 %). The GLM analysis revealed that the carob plants grown under 10 and 20 % of PG doses had a significant negative effect compared to the control (Figure 5). On the other hand, plants grown at 2 % PG were the most colonized by AMF compared to the other PG treatments. Mycorrhizal intensity did not differ between carob seedlings grown under 1 and 5 % of PG compared to the control.

Growth and biomass production under uninoculated treatment

The growth parameters of the carob plants responded differently to the different PG doses. The heights of carob seedlings grown under 2 % PG were positively affected by the treatment compared to the control whereas the heights of the carob plants were negatively impacted at 10 and 20 % of PG doses (Figure 6A). Shoot dry weights (SDW) and leaflet numbers responded similarly to the different PG doses, the plants grown under 1 and 2 % (5 % for leaflet number) showed a positive effect while all other doses showed no significant differences compared to the control (Figure 6B, D). The root dry weights (RDW) values of all the plants grown under the different PG doses were significantly higher than the control except for the plants grown with 20 % PG (Figure 6C).

Growth and biomass production under inoculated treatment

The response of the inoculated carob plants also varied greatly according to the different PG doses. The heights of plants grown in 1, 2 and 5 % PG were positively impacted whereas the plants grown under 20 % PG were negatively affected by the treatment (Figure 6A). Shoot dry weights (SDW) of plants grown under 1 and 2 % PG benefited the most from the PG treatment compared to the other plants while carob plants grown under 20 % PG benefited the least from the treatment compared to the other PG doses and the control (Figure 6B). The root dry weights (RDW) of the carob plants also responded differently to the different PG doses: a positive effect was recorded under 1, 2, 5 and 10 % PG compared to the control (Figure 6C). Leaflet numbers revealed a positive effect on carob plants grown under 1, 2 and 5 % whereas those grown under 20 % PG showed a negative effect compared to the control (Figure 6D).

Growth differential

The analysis of the growth differentials in response to inoculation revealed different effects on the measured parameters. Root dry weights (RDW) in inoculated plants increased the most (29 %) compared to uninoculated plants. This increase was significant for all phosphogypsum (PG) doses except for 20 %. Similarly, plant heights and shoot dry weights (SDW) also increased by 23 % and 22 % respectively in inoculated plants compared to the uninoculated plants. These enhancements were significant for all PG doses except 0 % for heights and 2 % and 20% for SDW. Leaflet numbers exhibited the smallest improvement, with a 15 % increase between inoculated and uninoculated plants. This increase was significant for all PG doses except 5 % and 20 %. (Table S1, Figure S1).

Trace elements in roots

The concentrations of the 10 trace elements in the roots varied with the different inoculum treatments and PG doses (Table 2; figure 7). Element concentrations in the roots increased with PG doses: all element concentrations increased significantly under 5, 10 and 20 % in uninoculated treatment, except for Cr and Fe under 10 and 5 % PG, respectively. In the inoculated plants, trace element concentrations increased significantly for many PG doses. For example, Ni and Pb concentrations increased for all PG doses while Fe and Zn concentrations increased only under 20 and 1 % of PG doses, respectively.

Trace elements in aerial parts

Trace element concentrations in the aerial part of the carob plants increased as a result of PG treatment in both inoculated and uninoculated plants (Table 3; figure 7). In the uninoculated plants, trace element contents increased for the different PG doses except for Cu concentration which showed no significant change under 2 % PG. In the inoculated plants, trace element concentrations also increased significantly in many PG doses but not as much as in the uninoculated plants; Co, Cr, Mn and Zn concentrations increased significantly in all PG doses. No significant change was recorded in Cu contents under the different PG doses.

Trace element differences between plant parts

Trace element concentrations differed greatly between the roots and aerial parts of the carob plants. Trace element concentrations in the roots were generally higher than the aerial part in both inoculated and uninoculated plants, except for Cr, Cu, Mn and Pb in inoculated plants and Cu, Mn and Zn in uninoculated plants in which no significant differences were detected. However, Zn concentrations in the inoculated plants were significantly higher in the aerial parts compared to the roots (Figure 7, table S1).

DISCUSSION

Phosphogypsum waste has emerged as a highly promising and cost-effective biofertilizer for agricultural applications³⁴. In our study, we aimed to assess the effects of different phosphogypsum doses (0, 1, 2, 5, 10 and 20 %) on carob plants, both in the presence and absence of their naturally associated root microbiota. Our findings indicate that phosphogypsum treatment positively impacts the yield of carob plants, particularly at doses of 1 and 2 %, and to a lesser extent at 5 %. These results align with similar observations in other plant species^{35–38}, suggesting a consistent positive influence of phosphogypsum at lower doses on plant growth and development. Conversely, higher doses of phosphogypsum (10 and 20 %) were associated with a significant accumulation of toxic elements such as As, Cd, and Pb, which imposed limitations on plant growth and development³⁹. Moreover, it appears that higher phosphogypsum doses may induce stress conditions in plants.

In this study, the use of PG doses at 1 % and 2 % significantly improved the growth of carob plants, especially when combined with the microbiota inoculum. This improvement was observed across all growth parameters. Several studies have shown the positive effect of the combined treatment between microorganisms (AMF and/or bacteria) and PG on plant growth and nutrition. However, it is important to note that these previous studies utilized preselected strains of AMF^{14–16} or bacteria^{40,41}. In a 2020 study¹⁷, a co-inoculation of AMF and phosphate-solubilizing bacteria, along with the application of PG, was employed to assess their combined impact on plant growth, a similar approach was used in our study where we recorded a significant positive effect of carob consortium microbiota in combination with different doses of PG on the growth of carob plants.

The global composition of the native microbiota in this study was consistent with global surveys at the phylum level^{42,43}, with *Actinobacteria*, *Proteobacteria*, *Ascomycota* and *Basidiomycota* as the most dominant taxa. Within these phyla, certain genera have been identified for their beneficial effects on plant growth and resistance. *Geopora*, a mycorrhizal fungus, enhances nutrient uptake and helps plants tolerate drought stress⁴⁴. *Gliocladium* shows potential as a disease suppressor⁴⁵. Additionally, *Rhodoplanes*, a bacterial genus, promotes plant growth through nitrogen fixation and phosphate solubilization⁴⁶.

The influence of soil bacteria on nutrient availability and uptake by plants has been well-documented⁴⁷ whereas mycorrhizal fungi are highly involved in water uptake by activating their aquaporins⁴⁸ and those of their plant symbiont⁴⁹. Mycorrhizal fungi have also been shown to enhance tolerance to metallic trace elements by reducing their direct toxic effect on plants⁵⁰. In our study, we observed a similar beneficial effect, as the inoculated carob plants demonstrated good tolerance to metallic trace elements under a 10 % PG treatment compared to the uninoculated plants. Additionally, mycorrhizal intensity broadly followed the growth results with high intensity for carob plants grown under 2 % and low under 10 and 20 % PG. The lower mycorrhizal intensity of carob seedlings grown on soil substrates amended with PG at a ratio of 10 and 20 % can be explained by the chemical properties (including PH and phosphorus) of PG which could decrease mycorrhizal spore production and root colonization⁵¹.

In addition to their role in water uptake and their positive effect against toxic elements, soil microorganisms also play a crucial role in influencing the bioavailability of macro (P, N, Ca, K, Mg) and micronutrients (Zn, Fe, Cu, Mn, Ni) ⁵². Previous studies in the Mediterranean regions focused mainly on the effect of macronutrients on plant growth, while the limiting effect of micronutrients on crop production has not been fully investigated ⁵³. Therefore, our study aimed to investigate the status of micronutrients, specifically Zn, Fe, Cu, Mn, Ni, and Co (which are the main components of Moroccan phosphogypsum), in different parts of carob plants in the presence or absence of inoculation, as these micronutrients play a critical role in plant growth and metabolic processes ⁵⁴. Our results indicated that PG amendment, especially at 5, 10 and 20 %, induced a significant increase in micronutrient concentrations in the roots and shoots of carob plants, regardless of the inoculation treatment. This increase can be attributed to the positive impact of PG on soil fertility and nutrient availability. Our findings are consistent with those of numerous surveys that reported improved nutrient uptake in the presence of PG ⁵⁵⁻⁵⁷.

In terms of distribution, the concentrations of micronutrients were found to be higher in the roots compared to the shoots of the carob plants. This observation is consistent with previous studies ^{56,58} that have reported greater micronutrient contents in belowground structures compared to aboveground parts. This difference can be attributed to the direct contact between roots and soil, providing easier access to micronutrients. Additionally, certain plant species tend to store a significant amount of micronutrients, including toxic elements, in belowground organs as a protective mechanism for aboveground tissues ^{59,60}. Unlike the other micronutrients, Zn was preferentially accumulated in the shoots of the plants compared to the roots under inoculum treatment, which confirms the hypothesis that it is easily transported to leaves from the roots ⁵⁸. This result can also be explained by the increased translocation of zinc from roots to shoots by mycorrhiza as reported in other studies ^{61,62}. Interestingly, the inoculum treatment did not significantly affect nutrient uptake in plants grown with 5, 10, and 20 % phosphogypsum (PG). This finding can be attributed to the improved nutrient uptake facilitated by PG itself, as it enhances the availability and accessibility of micronutrients in the roots, regardless of inoculation. The action of PG contributes to the overall nutrient uptake in both inoculated and uninoculated plants.

Higher doses of phosphogypsum (PG) were found to increase the concentrations of toxic elements such as arsenic (As), cadmium (Cd), and lead (Pb) in carob plants. These elements have no known biological function and have a negative impact on plant growth, as evidenced by the reduction in plant height observed in both inoculum treatments of carob plants grown with 20 % PG. However, when the carob plants were inoculated with a consortium of microbiota, the content of toxic elements in the aboveground parts of the plant was decreased. This reduction can be attributed to the ability of mycorrhizal fungi and bacteria to stabilize and restrict the translocation of certain toxic elements within the plant ^{63,64}. The findings of this study did not provide clear conclusions regarding the translocation of lead (Pb) between the roots and shoots of carob plants. Salazar et al. ⁶⁵ discovered that some mycorrhizal species can accumulate Pb in their spores, which limits its movement from belowground to aboveground plant organs. The role of microorganisms in Pb uptake, however, remains poorly documented. Despite the elevated concentrations of toxic elements (As, Cd, and Pb) in Moroccan PG, the inoculation with consortium microbiota helped restrict the transfer of these elements to the aboveground parts of carob plants. Moreover, the absolute concentration of toxic elements remained relatively low and well below the phytotoxic level reported by Chaney ⁶⁶ for vascular plants.

CONCLUSION

The findings of this study demonstrate the potential of Moroccan phosphogypsum (PG) as a soil amendment for promoting the growth and micronutrient uptake of carob trees. Application of PG directly to the soil resulted in enhanced growth and improved nutrient acquisition in carob plants, particularly when combined with their native microbiota. Furthermore, the inoculated plants exhibited a remarkable tolerance to toxic elements while maintaining their growth potential, unlike the uninoculated plants. These results present a promising perspective for the sustainable utilization of PG in conjunction with consortium microbiota inoculation as an effective agricultural strategy to enhance the growth and nutrition of diverse plant species.

MATERIALS AND METHODS

Phosphogypsum (PG) source and characterization

The PG samples used in this study were obtained from OCP group (Safi, Morocco), the largest exporter of raw phosphate, phosphoric acid and phosphate fertilizers in the world. The samples were taken to the laboratory to be mixed, ground and filtered through a 2-mm mesh and their physical and chemical properties were evaluated (Table 1). The major elements contained in the Moroccan PG sample were determined using X-ray fluorescence sequential spectrometer (XRF, Panalytical, AXIOS model) equipped with a Rh X-ray tube.

Biological material and experimental design

Mature seeds of *C. siliqua* were collected in July 2018 from the Ourika valley (High Atlas, Morocco) (31° 17' 45" N, 7° 42' 36" W). The seeds were scarified using concentrated sulfuric acid (96 %) for 30 min as a dormancy-breaking treatment, then transferred and soaked in distilled water for 48h. Germinated seeds were then transplanted into plastic pots containing sterilized *C. siliqua* soil, also sampled from the Ourika valley, to which PG waste was added at 0, 1, 2, 5, 10 and 20 %. Half of the carob seedlings were inoculated with 5g of consortium microbiota previously trapped and enriched in barley roots (*Hordeum vulgare* L.) using the trap culture technique¹⁸. The belowground microbiota of *C. siliqua* were propagated for 3 months using barley as a host plant (Figure 1), the non-inoculated plants also received 5g of sterilized barley roots. The plants were arranged in a randomized complete block design. Plantlets were watered regularly at 75 % field capacity (FC) for 6 months (Figure 1).

The experiment was carried out in a shaded-area in Safi, Morocco, under 32/18 °C day/night temperatures and a photoperiod of 16h. The experimental setup consisted of two treatment sets: The first was based on PG doses (0, 1, 2, 5, 10 and 20 %) and the second was based on inoculation status. All treatments were randomly placed in the shaded-area and repeated 3 times.

DNA extraction, gene amplification and sequencing

Total DNA was extracted from 500 mg of rhizospheric carob soil using the FastDNA SPIN kit for soil (MP Biomedicals Europe, Illkirch, France) following the manufacturer's instructions. In order to improve DNA purity, 40 mg of Polyvinylpyrrolidone (PVPP) was added during the initial step of DNA extraction. Furthermore, a washing step with 5.5 M guanidine thiocyanate was performed prior to the utilization of the washing buffer SEWS-M. Duplicate DNA extractions were conducted and the samples were stored at -20 °C for subsequent analysis. Amplification of a sub-region of the 16S rRNA gene and the internal transcribed spacer ITS2 was conducted for the analysis of bacterial and fungal communities, respectively. The amplified products were subjected to paired-end Illumina MiSeq sequencing (2 × 300 bp) performed by Get-PlaGe (Genotoul, Castanet-Tolosan, France). For the amplification of the 16S rRNA gene, polymerase chain reaction (PCR) was carried out using the V3F (5'-TACGGRAGGCAGCAG-3') and V4R (5'-GGACTACCAGGTATCTAAT-3') primers¹⁹. For ITS2, the primers ITS86F (5'-GTGAATCATCGAATCTTTGAA-3')²⁰ and ITS4 (5'-TCCTCCGCTTATTGATATGC-3')²¹ were employed. Further information on the amplification and sequencing procedures can be found detailed in Khassali et al.¹⁰.

Data processing

Illumina sequencing, base calling, and demultiplexing were conducted using RTA v1.18.54, MCS 2.6, and bcl2fastq2.17. Paired reads were assembled using vsearch v2.11.0²², and primer clipping was performed with cutadapt v1.9²³. Clustering was carried out using swarm v2.2.2²⁴, followed by chimera detection and taxonomic assignment as described in Maghnia et al.²⁵. For bacteria and fungi, the ribosomal database SILVA v132²⁶ and a custom version of the ITS database UNITE v7²⁷ were utilized, respectively. Details of data processing steps are provided in Khassali et al.¹⁰.

Plant growth parameters

After 6 months of growth, the plants were harvested and their heights and leaf numbers were determined. The dry weights of shoot and root parts were also determined after plants were oven dried at 80 °C for 48 h.

Mycorrhizal colonization

Fresh fine roots were washed thoroughly with tap water and clarified in 10 % KOH at 90°C for 2 hours then treated with 7.5 % H₂O₂ for 5 minutes and placed in 1 % HCl for 5 minutes. The roots were then soaked in 0.05 % trypan blue/lactoglycerol (v/v) for 20 minutes at 90 °C (Phillips and Hyman, 1970). Twenty fragments were randomly selected for microscopic observation to determine the different mycorrhizal parameters for each condition according to the method of Trouvelot et al.²⁸. The mycorrhizal colonization intensity (M %) was calculated using MYCOCALC software (<https://www2.dijon.inrae.fr/mychintec/Mycocalc-prg/download.html>).

Preparation and digestion of shoot and root samples

The shoots and roots of the plants were separated, oven-dried at 70 °C for 48 h, then ground and calcinated at 550 °C for 5 hours, 2g of the resulting fine powder were oxidized with 5 ml of H₂O₂, then heated on sand until dry. 5 ml of *Aqua-regia* (mixture of nitric acid HNO₃ concentrated with hydrochloric acid HCl (1:3)) are added to each sample previously treated with H₂O₂, all samples are then allowed to

mineralize by placing the Teflon beaker on a sanding plate and gradually heating it to about 200 °C for 2 hours. The digested samples were filtered using a membrane (0.45 mm) and diluted to 10 ml with deionized water.

Trace elements measurements

Measurements of 10 trace elements [arsenic (As), cadmium (Cd), cobalt (Co), chromium (Cr), copper (Cu), iron (Fe), manganese (Mn), nickel (Ni), lead (Pb) and zinc (Zn)] in the filtrate of the different samples were carried out using a coupled plasma emission spectrophotometer (ICPE-9000, Shimadzu, Japan).

Statistical analysis

The statistical analysis was performed using RStudio (version 4.1.3). A generalized linear model (GLM) was used to determine significant changes between the different PG doses under uninoculated or inoculated treatment with the carob control (0 %). The plots were generated using the R ggplot2 package (version 3.3.5) 29. Microbiota sequencing data were subjected to global rarefactions, based on the samples with the smallest sizes with R vegan package version 2.5-2 30 using the `rarefy()` function. Diversity (Shannon, inverse Simpson [1/D]), and richness (number of OTUs) were estimated using the R package vegan. Pearson correlations between plant growth, mycorrhizal intensity and trace elements were estimated using the `cor()` function from the R package stats 31, and visualized as heatmaps using `corrplot()` function from the R corrplot package (version 0.92) 32. Summary and interaction plots between uninoculated and inoculated conditions were performed using the `summ()` and `cat_plot()` functions from R jtools package (version 2.0.2) 33.

Declarations

ACKNOWLEDGEMENTS

The authors thank OCP group employees who provided us the phosphogypsum samples and for the various information along this survey. We express our gratitude to Hervé Sanguin and Frédéric Mahé for their valuable contribution in the analysis of the microbiota data.

Authors contribution statement

HK: Conceptualization, Methodology, Data curation, Formal analysis, Investigation, Writing - original draft, Writing - review & editing. **IJ:** Writing - original draft, Writing - review & editing. **AG:** Writing - original draft, Writing- review & Formal analysis. **RD:** Supervision, Conceptualization, Writing - review & editing. **LO:** Supervision, Conceptualization, Methodology, Investigation, Writing - review & editing.

All authors contributed to the article and approved the submitted version

Funding

This research received no external funding

Competing interests

The authors have no relevant financial or non-financial interests to disclose.

Ethical Approval

Not applicable.

Consent for Publication

The manuscript has not been published partly or fully elsewhere and is not currently being considered for publication in any journal. All the authors have seen the final version of the manuscript and agreed to submit it to this journal

References

1. Baumel, A. *et al.* Assessment of plant species diversity associated with the carob tree (*Ceratonia siliqua*, Fabaceae) at the Mediterranean scale. *Plant Ecol. Evol.* **151**, 185–193 (2018).
2. Ouahmane, L., Ibrahima, N., Abdessadek, M. & Abderrahim, F. Inoculation of *Ceratonia siliqua* L. with native arbuscular mycorrhizal fungi mixture improves seedling establishment under greenhouse conditions. *African J. Biotechnol.* **11**, 16422-16426–16426 (2012).

3. Viruel, J. *et al.* A strong east–west Mediterranean divergence supports a new phylogeographic history of the carob tree (*Ceratonia siliqua*, Leguminosae) and multiple domestications from native populations. *J. Biogeogr.* **47**, 460–471 (2020).
4. Correia, P. J. & Martins-Loução, M. A. The use of macronutrients and water in marginal Mediterranean areas: The case of carob-tree. *F. Crop. Res.* **91**, 1–6 (2005).
5. Meena, V. S. *et al.* Plant beneficial rhizospheric microorganism (PBRM) strategies to improve nutrients use efficiency: a review. *Ecol. Eng.* **107**, 8–32 (2017).
6. Islam, S., Akanda, A. M., Prova, A., Islam, M. T. & Hossain, M. M. Isolation and identification of plant growth promoting rhizobacteria from cucumber rhizosphere and their effect on plant growth promotion and disease suppression. *Front. Microbiol.* **6**, 1360 (2016).
7. Jadrane, I., Dounas, H., Kouisni, L., Aziz, F. & Ouahmane, L. Inoculation with selected indigenous mycorrhizal complex improves *Ceratonia siliqua*'s growth and response to drought stress. *Saudi J. Biol. Sci.* (2020).
8. Rodriguez, A. & Sanders, I. R. The role of community and population ecology in applying mycorrhizal fungi for improved food security. *ISME J.* **9**, 1053–1061 (2015).
9. Hart, M. M., Antunes, P. M., Chaudhary, V. B. & Abbott, L. K. Fungal inoculants in the field: Is the reward greater than the risk? *Funct. Ecol.* **32**, 126–135 (2018).
10. Khassali, H. *et al.* The belowground bacterial and fungal communities differed in their significance as microbial indicator of Moroccan carob habitats. *Ecol. Indic.* **114**, 106341 (2020).
11. Mehahad, M. S. & Bounar, A. Phosphate mining, corporate social responsibility and community development in the Gantour Basin, Morocco. *Extr. Ind. Soc.* (2019) doi:10.1016/j.exis.2019.11.016.
12. Meskini, S., Samdi, A., Ejjaouani, H. & Remmal, T. Valorization of phosphogypsum as a road material: Stabilizing effect of fly ash and lime additives on strength and durability. *J. Clean. Prod.* **323**, 129161 (2021).
13. Enamorado, S. *et al.* Transfer of Cd, Pb, Ra and U from phosphogypsum amended soils to tomato plants. *Water. Air. Soil Pollut.* **203**, 65–77 (2009).
14. Al-Karaki, G. N. & Al-Omouh, M. Wheat response to phosphogypsum and mycorrhizal fungi in alkaline soil. *J. Plant Nutr.* **25**, 873–883 (2002).
15. Ibrahim, M. Arbuscular mycorrhizal isolate and phosphogypsum effects on growth and nutrients acquisition of cotton (*Gossypium hirsutum* L.). *Advances in Horticultural Science* vol. 30 121–128 at <https://doi.org/10.2307/26525344> (2016).
16. Zielonka, D., Sas-Paszt, L., Derkowska, E., Lisek, A. & Russel, S. Occurrence of Arbuscular Mycorrhizal Fungi in Hemp (*Cannabis sativa*) Plants and Soil Fertilized with Sewage Sludge and Phosphogypsum. *J. Nat. Fibers* (2019) doi:10.1080/15440478.2019.1618779.
17. El Maaloum, S. *et al.* Effect of Arbuscular Mycorrhizal Fungi and Phosphate-Solubilizing Bacteria Consortia Associated with Phospho-Compost on Phosphorus Solubilization and Growth of Tomato Seedlings (*Solanum lycopersicum* L.). *Commun. Soil Sci. Plant Anal.* 1–13 (2020).
18. Ouahmane, L. *et al.* Improvement of *Cupressus atlantica* Gaussen growth by inoculation with native arbuscular mycorrhizal fungi. *J. Appl. Microbiol.* **103**, 683–690 (2007).
19. Alm, E. W., Oerther, D. B., Larsen, N., Stahl, D. A. & Raskin, L. The oligonucleotide probe database. *Appl. Environ. Microbiol.* **62**, 3557–3559 (1996).
20. Turenne, C. Y., Sanche, S. E., Hoban, D. J., Karlowsky, J. A. & Kabani, A. M. Rapid identification of fungi by using the ITS2 genetic region and an automated fluorescent capillary electrophoresis system. *J. Clin. Microbiol.* **37**, 1846–1851 (1999).
21. White, T. J., Bruns, T., Lee, S. & Taylor, J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR Protoc. Elsevier*, pp. 315–322 (1990).
22. Rognes, T., Flouri, T., Nichols, B., Quince, C. & Mahé, F. VSEARCH: a versatile open source tool for metagenomics. *PeerJ* **4**, e2584. at (2016).
23. Martin, M. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet. J.* **17** (1), 10–12 (2011).
24. Mahé, F., Rognes, T., Quince, C., de Vargas, C. & Dunthorn, M. Swarm v2: Highly-scalable and high-resolution amplicon clustering. *PeerJ* **3**, e1420 (2015) doi:10.7717/peerj.1420.
25. Maghnia, F. Z. *et al.* Habitat- and soil-related drivers of the root-associated fungal community of *Quercus suber* in the Northern Moroccan forest. *PLoS One* **12**, (2017).
26. Quast, C. *et al.* The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* **41**, D590–D596 (2012).
27. Kõljalg, U. *et al.* Towards a unified paradigm for sequence-based identification of fungi. *Mol. Ecol.* **22**, 5271–5277 (2013).

28. Trouvelot, A., Kough, J. L. & Gianinazzi-Pearson, V. Estimation of VA mycorrhizal infection levels. Research for methods having a functional significance. in *Physiological and Genetical Aspects of Mycorrhizae. Aspects physiologiques et genetiques des mycorrhizes, Dijon (France), 1-5 Jul 1985* (/1INRA, 1986).
29. Wickham, H. ggplot2: elegant graphics for data analysis. *R ggplot2 package version 3.0.0*. (Springer, 2016).
30. Oksanen, J. *et al.* Vegan: community ecology package, version 2.4-4. at (2019).
31. R Core Team. R: A language and environment for statistical computing. (2018).
32. Taiyun Wei, M. *et al.* Package 'corrplot' Title Visualization of a Correlation Matrix. (2017).
33. Long, J. A. jtools: Analysis and presentation of social scientific data. (R package version 2.0.1). (2019).
34. Abril, J. M. *et al.* Occupational dosimetric assessment (inhalation pathway) from the application of phosphogypsum in agriculture in South West Spain. *J. Environ. Radioact.* **100**, 29–34 (2009).
35. Takasu, E. *et al.* Soil Science and Plant Nutrition Effect of phosphogypsum application on the chemical properties of Andosols, and the growth and Ca uptake of melon seedlings Effect of phosphogypsum application on the chemical properties of Andosols, and the growth and Ca uptake of melon seedlings. *Soil Sci. Plant Nutr.* **52**, 760–768 (2006).
36. Vyshpolsky, F. *et al.* Optimizing the rate and timing of phosphogypsum application to magnesium-affected soils for crop yield and water productivity enhancement. *Agric. Water Manag.* (2010).
37. Mahmoud, E. & El-Kader, A. Heavy metal immobilization in contaminated soils using phosphogypsum and rice straw compost. *L. Degrad. Dev.* (2014) doi:10.1002/ldr.2288.
38. Smaoui-Jardak, M. *et al.* Effect of the phosphogypsum amendment of saline and agricultural soils on growth, productivity and antioxidant enzyme activities of tomato (*Solanum lycopersicum* L.). *Ecotoxicology* **26**, 1089–1104 (2017).
39. Muszyńska, E. & Labudda, M. Dual role of metallic trace elements in stress biology—from negative to beneficial impact on plants. *Int. J. Mol. Sci.* **20**, 3117 (2019).
40. Al-Enazy, A. A. R., Al-Oud, S. S., Al-Barakah, F. N. & Usman, A. R. A. Role of microbial inoculation and industrial by-product phosphogypsum in growth and nutrient uptake of maize (*Zea mays* L.) grown in calcareous soil. *J. Sci. Food Agric.* **97**, 3665–3674 (2017).
41. El-Shamy, M. A. *et al.* Quinoa response to application of phosphogypsum and plant growth-promoting rhizobacteria under water stress associated with salt-affected soil. *Plants* **11**, 872 (2022).
42. Delgado-Baquerizo, M. *et al.* A global atlas of the dominant bacteria found in soil. *Science (80-)*. **359**, 320–325 (2018).
43. Tedersoo, L. *et al.* Global diversity and geography of soil fungi. *Science (80-)*. **346**, (2014).
44. Gordon, G. J. & Gehring, C. A. Molecular characterization of pezizalean ectomycorrhizas associated with pinyon pine during drought. *Mycorrhiza* **21**, 431–441 (2011).
45. Vinale, F. *et al.* Trichoderma–plant–pathogen interactions. *Soil Biol. Biochem.* **40**, 1–10 (2008).
46. Zhang, H. *et al.* Subsoiling and conversion to conservation tillage enriched nitrogen cycling bacterial communities in sandy soils under long-term maize monoculture. *Soil Tillage Res.* **215**, 105197 (2022).
47. Abbasi, M. K., Sharif, S., Kazmi, M., Sultan, T. & Aslam, M. Isolation of plant growth promoting rhizobacteria from wheat rhizosphere and their effect on improving growth, yield and nutrient uptake of plants. *Plant Biosyst.* **145**, 159–168 (2011).
48. Xu, H., Cooke, J. E. K. & Zwiazek, J. J. Phylogenetic analysis of fungal aquaporins provides insight into their possible role in water transport of mycorrhizal associations. *Botany* **91**, 495–504 (2013).
49. Yooyongwech, S., Samphumphuang, T., Tisarum, R., Theerawitaya, C. & Cha-um, S. Arbuscular mycorrhizal fungi (AMF) improved water deficit tolerance in two different sweet potato genotypes involves osmotic adjustments via soluble sugar and free proline. *Sci. Hortic. (Amsterdam)*. **198**, 107–117 (2016).
50. Tumau, K. Heavy metal content and localization in mycorrhizal *Euphorbia cyparissias* zinc wastes in southern Poland. *Acta Soc. Bot. Pol.* **67**, 105–113 (2014).
51. Boldt-Burisch, K. & M. Anne, N. Phosphogypsum significantly alters root growth and fungal colonization of smooth brome and sheep fescue on a reclamation site. *Rhizosphere* **9**, 106–109 (2019).
52. Etesami, H. & Alikhani, H. A. Halotolerant plant growth-promoting fungi and bacteria as an alternative strategy for improving nutrient availability to salinity-stressed crop plants. in *Saline Soil-based Agriculture by Halotolerant Microorganisms* 103–146 (Springer Singapore, 2019). doi:10.1007/978-981-13-8335-9_5.
53. Kihara, J. *et al.* Application of secondary nutrients and micronutrients increases crop yields in sub-Saharan Africa. *Agron. Sustain. Dev.* **37**, 25 (2017).

54. Tripathi, D. K. *et al.* Micronutrients and their diverse role in agricultural crops: advances and future prospective. *Acta Physiol. Plant.* **37**, 1–14 (2015).
55. Nayak, A. K. *et al.* Efficiency of Phosphogypsum and Mined Gypsum in Reclamation and Productivity of Rice–Wheat Cropping System in Sodic Soil. <http://dx.doi.org/10.1080/00103624.2012.747601> **44**, 909–921 (2013).
56. Elloumi, N. *et al.* Effect of phosphogypsum on growth, physiology, and the antioxidative defense system in sunflower seedlings. *Environ. Sci. Pollut. Res.* **22**, 14829–14840 (2015).
57. Al-Enazy, A.-A., Al-Barakah, F., Al-Oud, S. & Usman, A. Effect of phosphogypsum application and bacteria co-inoculation on biochemical properties and nutrient availability to maize plants in a saline soil. *Arch. Agron. Soil Sci.* **64**, 1394–1406 (2018).
58. Bati, C. B., Santilli, E. & Lombardo, L. Effect of arbuscular mycorrhizal fungi on growth and on micronutrient and macronutrient uptake and allocation in olive plantlets growing under high total Mn levels. *Mycorrhiza* **25**, 97–108 (2015).
59. Díaz, G., Azcón-Aguilar, C. & Honrubia, M. Influence of arbuscular mycorrhizae on heavy metal (Zn and Pb) uptake and growth of *Lygeum spartum* and *Anthyllis cytisoides*. *Plant Soil* **180**, 241–249 (1996).
60. Yang, Y. *et al.* The roles of arbuscular mycorrhizal fungi (AMF) in phytoremediation and tree-herb interactions in Pb contaminated soil. *Sci. Rep.* **6**, 1–14 (2016).
61. Christie, P., Li, X. & Chen, B. Arbuscular mycorrhiza can depress translocation of zinc to shoots of host plants in soils moderately polluted with zinc. *Plant Soil* **261**, 209–217 (2004).
62. Usman, A. R. A. & Mohamed, H. M. Effect of microbial inoculation and EDTA on the uptake and translocation of heavy metal by corn and sunflower. *Chemosphere* **76**, 893–899 (2009).
63. Chen, B. *et al.* Uptake and intraradical immobilization of cadmium by arbuscular mycorrhizal fungi as revealed by a stable isotope tracer and synchrotron radiation μ X-Ray fluorescence analysis. *Microbes Env.* **00**, 0–000 (2018).
64. Podar, D. & Maathuis, F. J. M. The role of roots and rhizosphere in providing tolerance to toxic metals and metalloids. *Plant. Cell Environ.* **45**, 719–736 (2022).
65. Salazar, M. J. *et al.* Pb accumulation in spores of arbuscular mycorrhizal fungi. *Sci. Total Environ.* **643**, 238–246 (2018).
66. Chaney, R. L. Toxic element accumulation in soils and crops: protecting soil fertility and agricultural food-chains. 140–158 (1989) doi:10.1007/978-3-642-74451-8_10.

Tables

Table 1. Physico-chemical properties of phosphogypsum used in the experiments

Parameter	Values (Average $\pm$ SD)
pH	2.22 $\pm$ 0.3
H ₂ O (%)	27.88 $\pm$ 2.23
P ₂ O ₅ (%)	1 $\pm$ 0.15
CaO (%)	31.84 $\pm$ 0.17
MgO (%)	0.01 $\pm$ 0.01
SO ₃ (%)	46.88 $\pm$ 9.1
Fe ₂ O ₃ (%)	0.02 $\pm$ 0.04
Al ₂ O ₃ (%)	0.17 $\pm$ 0.03
Na ₂ O (%)	0.15 $\pm$ 0.06
SiO ₂ (%)	3.7 $\pm$ 0.5
Cd (mg/kg)	2 $\pm$ 0.7
As (mg/kg)	1.2 $\pm$ 1
Cr (mg/kg)	4.2 $\pm$ 1.9
Cu (mg/kg)	5.6 $\pm$ 1.1
Mn (mg/kg)	0.6 $\pm$ 0.2
Ni (mg/kg)	4 $\pm$ 3.3
Zn (mg/kg)	11.3 $\pm$ 6.5
Co (mg/kg)	0.3 $\pm$ 0.1
Pb (mg/kg)	12.3 $\pm$ 3.2

Values are means of three replicates (n = 3) per treatment $\pm$ Standard error

Table 2. Arsenic (As), cadmium (Cd), cobalt (Co), chromium (Cr), copper (Cu), iron (Fe), manganese (Mn), nickel (Ni), lead (Pb) and zinc (Zn) content (mg/kg) measured in carob roots grown under different phosphogypsum doses (PG doses) in uninoculated and inoculated plants

Inoculum status	PG doses (%)	As	Cd	Co	Cr	Cu	Fe	Mn	Ni	Pb	Zn
Uninoculated	0	0.413	0.028	0.3	1.95	0.83	772	11.83	1.22	0.46	3.01
	1	0.416	0.032	0.3	2.32***	1.59***	710	12.5	1.71***	0.54*	2.77
	2	0.446	0.032	0.35	2.11	1.76***	724	14.02***	1.31	0.62***	3.47
	5	0.48*	0.046***	0.4**	2.16*	1.91***	732	13.3**	1.41*	0.76***	5.13***
	10	0.63***	0.057***	0.423***	2.02	2.02***	917**	17.6***	1.78***	0.71***	5.24***
	20	0.72***	0.078***	0.42**	2.84***	2.17***	996***	20.24***	1.74***	0.73***	5.11***
Inoculated	0	0.33	0.027	0.27	1.85	1.8	764	12.58	0.96	0.42	3.4
	1	0.37	0.041***	0.31	2.04	1.24***	795	13.3	1.23***	0.55***	3.89*
	2	0.55***	0.034	0.39**	2.06	1.24***	687	12.84	1.49***	0.69***	3.6
	5	0.55***	0.036*	0.31	2.24**	1.31***	745	14.41***	1.67***	0.7***	3.84
	10	0.65***	0.041***	0.51***	2.29**	1.51***	724	15.79***	1.39***	0.73***	3.68
	20	0.66***	0.087***	0.62***	2.52***	2.44***	870*	16.59***	1.71***	0.82***	3.61

The stars indicate the significant values representing significant differences at the 5 % level by comparison to carob plants grown without PG doses (0 %) in uninoculated and inoculated conditions. Significant differences are reported with the following P-values: ***: P<0.001; **: P<0,01; *: P< 0,05; ns, not significant. Values are means of three replicates (n = 3) per treatment

Table 3. Arsenic (As), cadmium (Cd), cobalt (Co), chromium (Cr), copper (Cu), iron (Fe), manganese (Mn), nickel (Ni), lead (Pb) and zinc (Zn) content (mg/kg) measured in carob leaves grown under different phosphogypsum doses (PG doses) in uninoculated and inoculated plants

Inoculum status	PG doses (%)	As	Cd	Co	Cr	Cu	Fe	Mn	Ni	Pb	Zn
Uninoculated	0	0.106	0.014	0.088	0.96	1.2	186	5.12	0.45	0.33	2.18
	1	0.426***	0.029***	0.178***	1.35**	2.07***	399**	13.51***	0.8***	0.49***	3.9***
	2	0.4***	0.033***	0.264***	1.57***	1.46	548***	16.7***	1.02***	0.55***	4.69***
	5	0.203**	0.022*	0.191***	1.38**	1.81**	524***	12.33***	0.73***	0.58***	4.66***
	10	0.43***	0.028**	0.31***	2.36***	2.12***	618***	15.83***	1.1***	0.62***	5.13***
	20	0.566***	0.038***	0.25***	2.56***	2.17***	583***	15.38***	1.27***	0.68***	5.61***
Inoculated	0	0.36	0.03	0.19	1.35	1.7	480	8.61	0.95	0.62	2.47
	1	0.41*	0.03	0.23*	2.19***	1.83	538	11.61*	0.88	0.58	3.9***
	2	0.31	0.035*	0.26***	2.03**	1.88	694**	12.49**	1.15*	0.66	3.99***
	5	0.36	0.03	0.25**	2.49***	1.72	566	14.72***	0.87	0.68	4.47***
	10	0.32	0.035	0.273***	2.44***	1.58	677**	19.13***	1.17*	0.78**	5.58***
	20	0.45**	0.037*	0.29***	2.36***	1.69	651*	30.96***	1.35***	0.9***	7.5***

The stars indicate the significant values representing significant differences at the 5 % level by comparison to carob plants grown without PG doses (0 %) in uninoculated and inoculated conditions. Significant differences are reported with the following P-values: ***: P<0.001; **: P<0,01; *: P< 0,05; ns, not significant. Values are means of three replicates (n = 3) per treatment

Figures

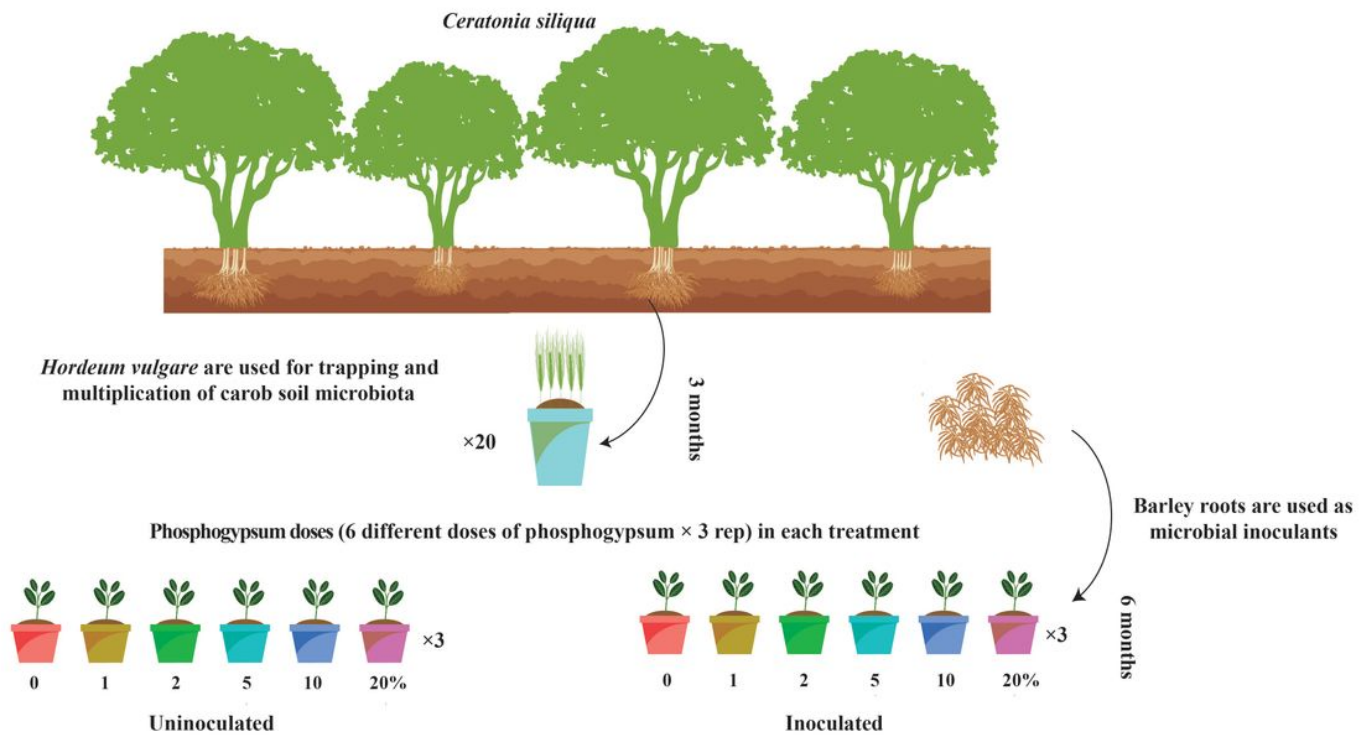


Figure 1

Flowchart of experimental procedures for the sampling of native soils from carob habitats and carob growth experimental setup under uninoculated or inoculated conditions

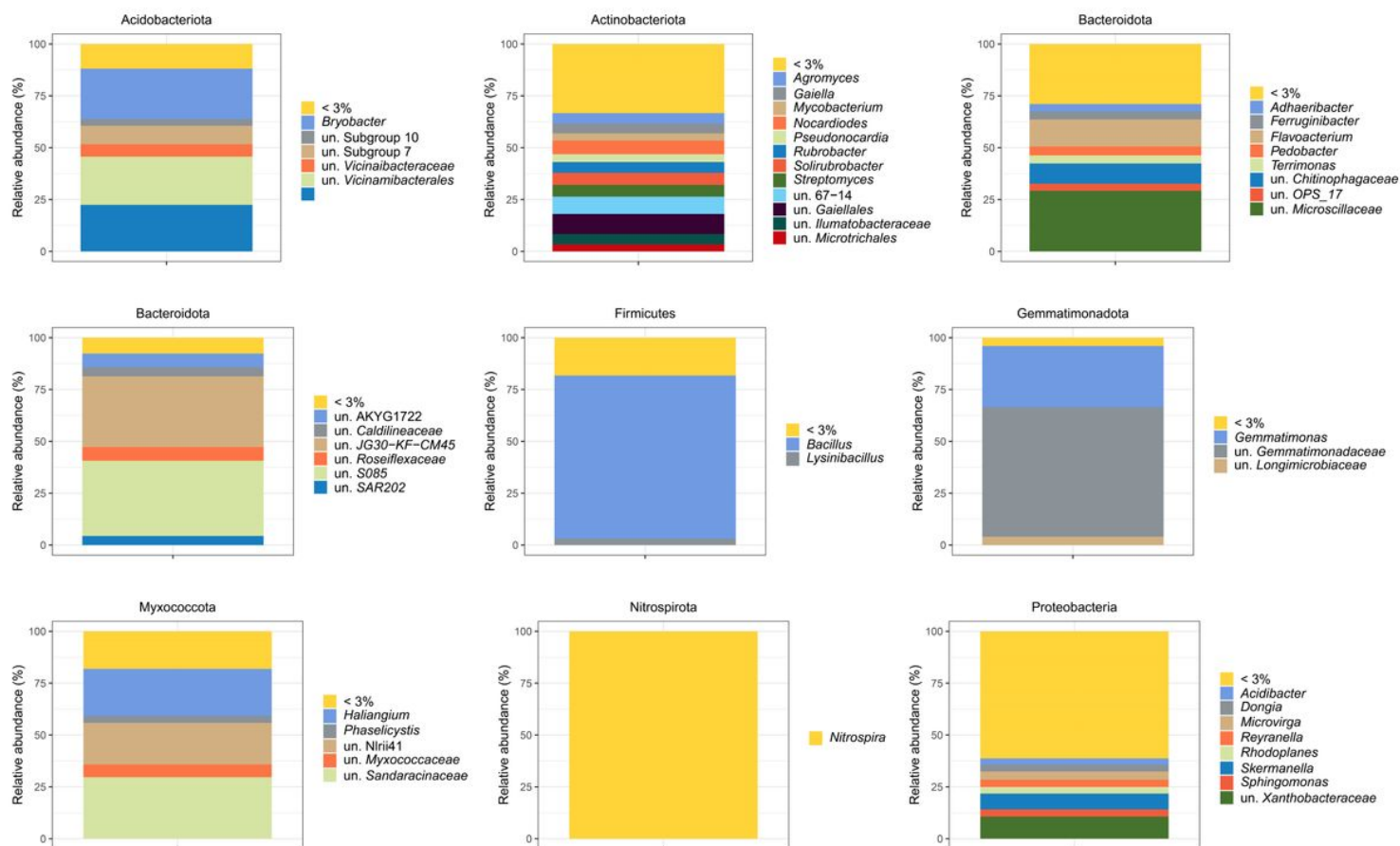


Figure 2

Bar chart of the relative abundance (%) of bacterial community compositions at the genus level in the carob rhizosphere

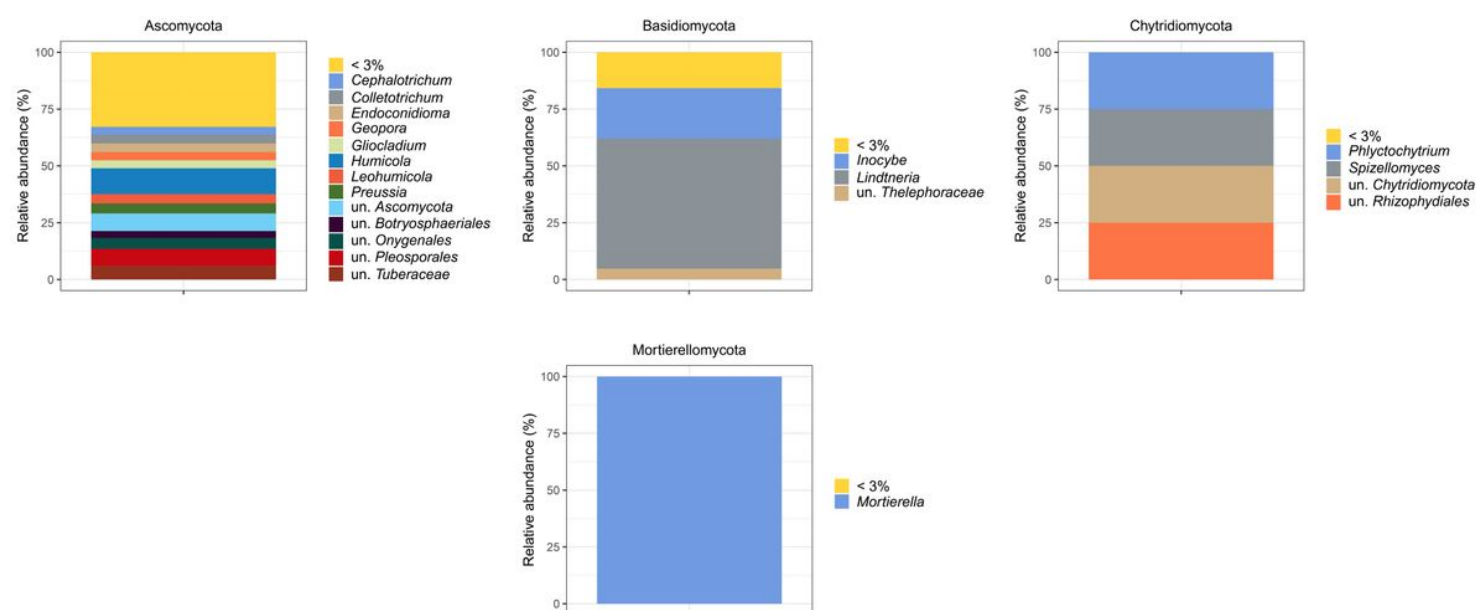


Figure 3

Bar chart of the relative abundance (%) of fungal community compositions at the genus level in the carob rhizosphere

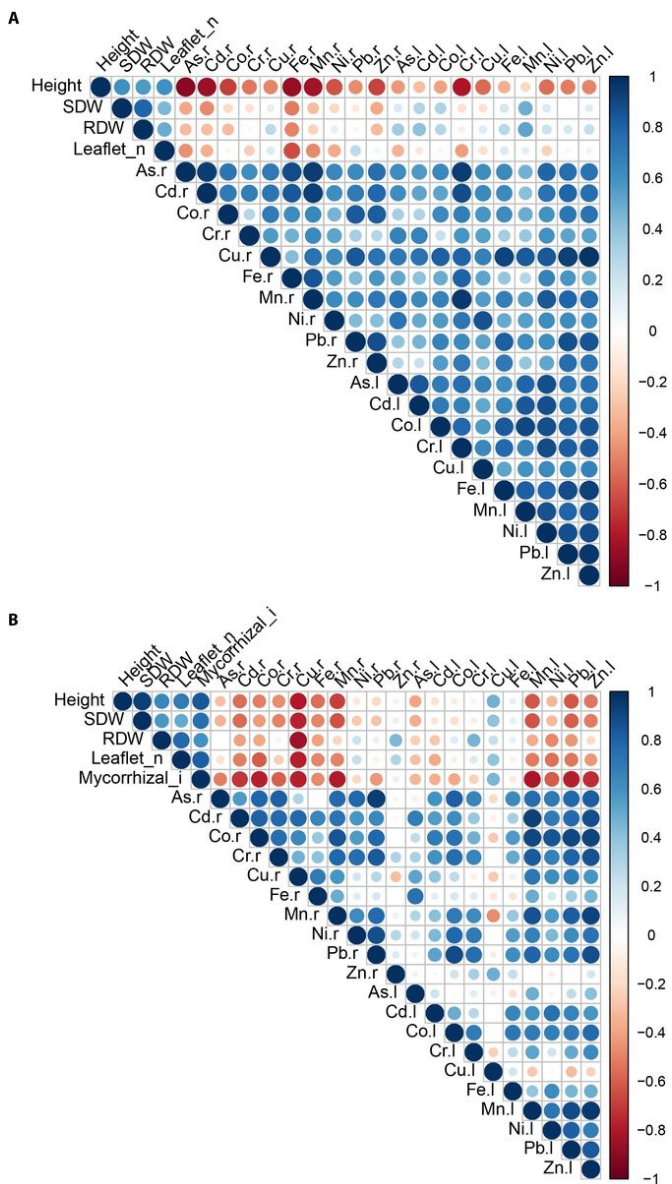


Figure 4

The correlation heatmap analysis of the relationships among the different parameters measured (growth and trace elements parameters) under uninoculated (A) or inoculated conditions (B). Pearson correlation values can vary between - 1 to + 1, showing a strong negative and positive correlation, respectively. Where the blue color indicates a positive correlation, while the orange color indicates a negative correlation, the white color indicates no correlation, in both colors (blue and orange), greater intensity indicates a greater degree of correlation, strong correlations are indicated by large circles, whereas small circles indicate weak correlations. "l" represents leaves, and "r" represents roots, for example, "As.l" represents arsenic concentrations in leaves, while "As.r" represents arsenic concentrations in roots

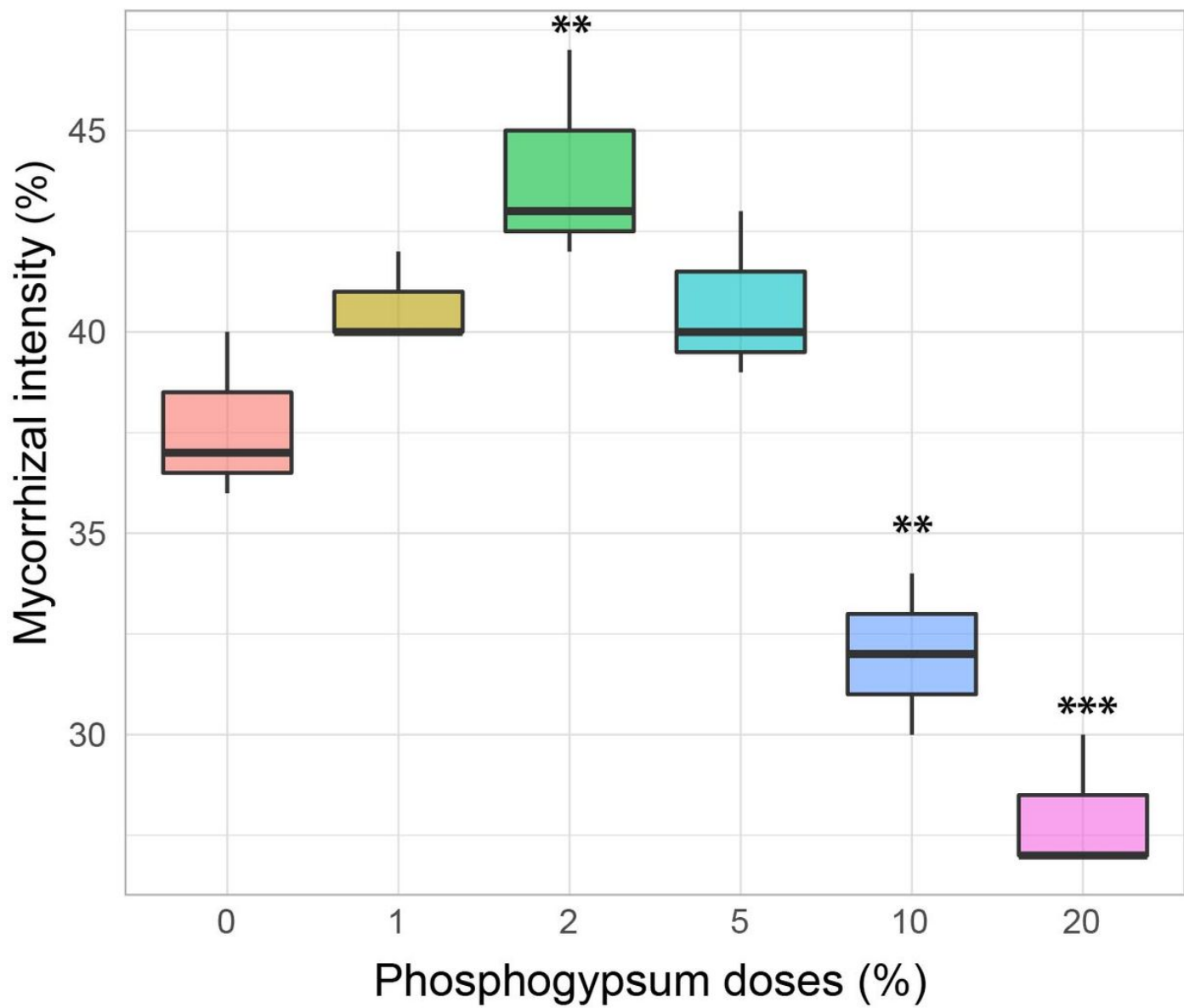


Figure 5

Boxplot for mycorrhizal intensity under different phosphogypsum (PG) doses. Significant differences are reported with the following P-values: ***: $P < 0.001$; **: $P < 0.01$; *: $P < 0.05$. Data presented of three replicates ($n=3$) per treatment.

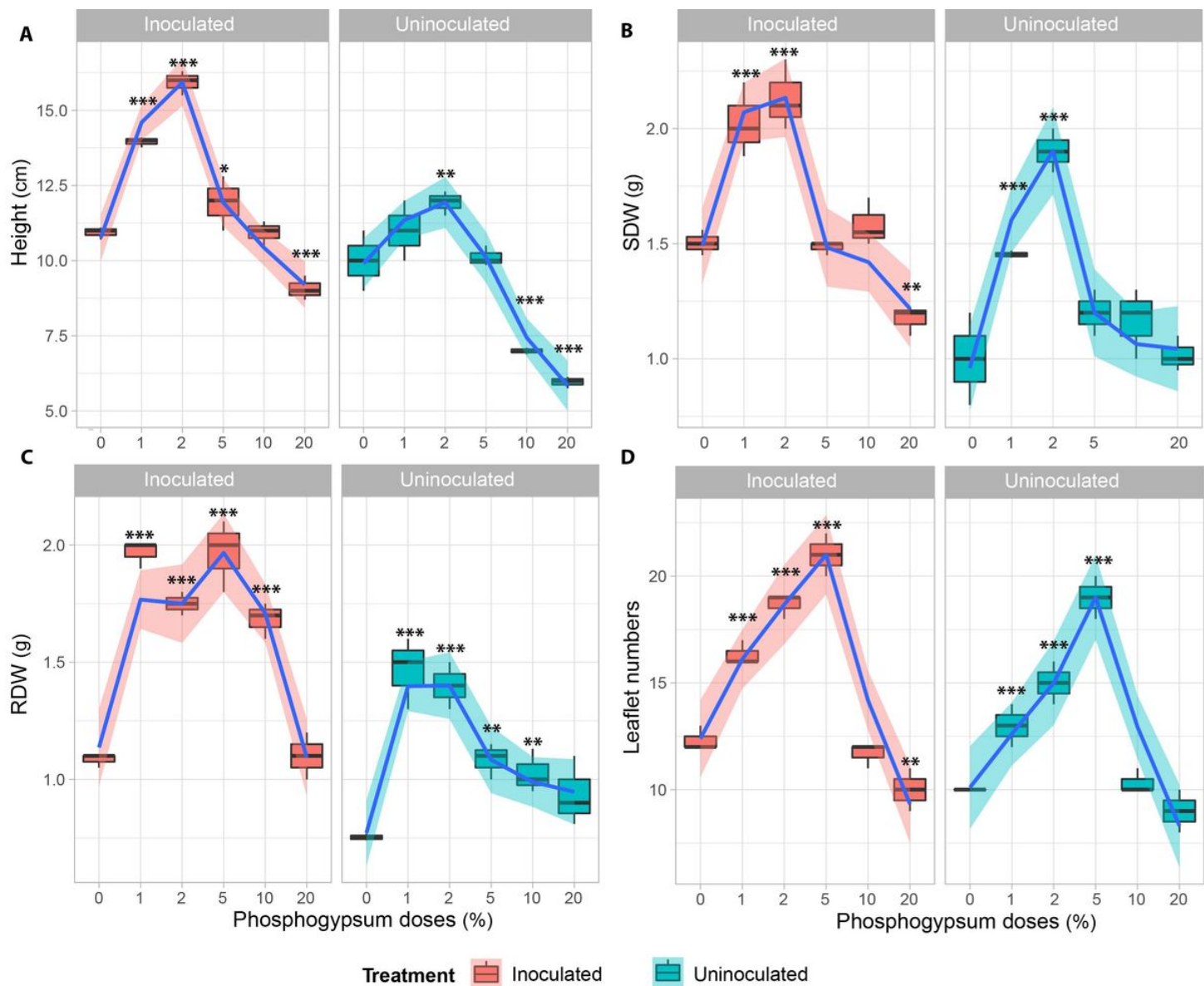


Figure 6

Boxplots for four growth parameters, height (A), shoot dry weight (B), root dry weight (C) and leaflet numbers (D) measured on six-months old carob plantlets under different phosphogypsum doses (PG doses) in uninoculated or inoculated treatment, significant differences are reported with the following P-values: ***: $P < 0.001$; **: $P < 0.01$; *: $P < 0.05$. Data presented of three replicates ($n=3$) per treatment.

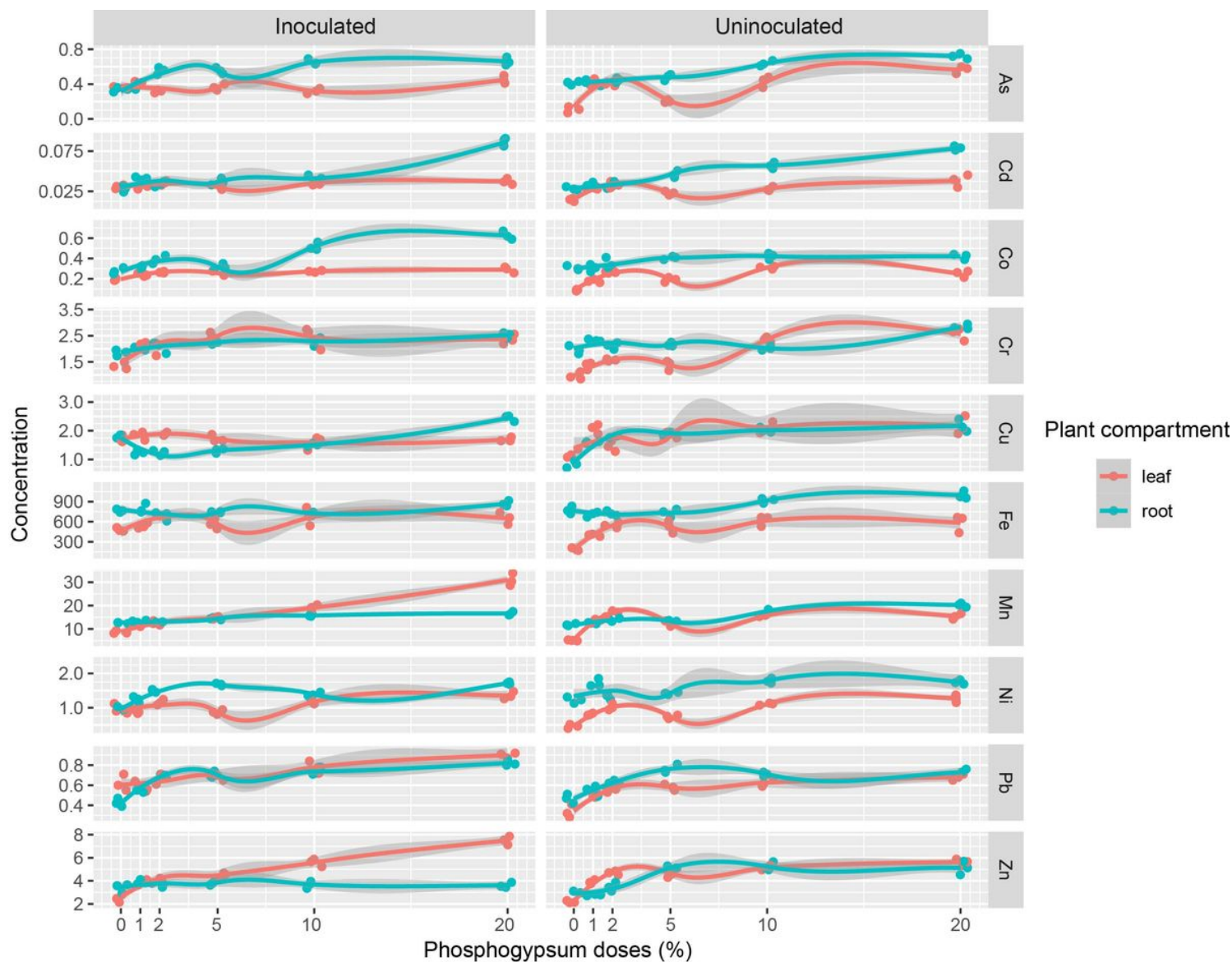


Figure 7

Plot interaction effect between uninoculated and inoculated plants for trace elements [arsenic (As), cadmium (Cd), cobalt (Co), chromium (Cr), copper (Cu), iron (Fe), manganese (Mn), nickel (Ni), lead (Pb) and zinc (Zn)] content in roots and leaves. Data presented of three replicates (n=3) per treatment.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Supplementaryfigure1.jpg](#)
- [Supplementarytable1.xlsx](#)