Exploring the synergistic effects of biochar and arbuscular mycorrhizal fungi on phosphorus acquisition in tomato plants by using gene expression analyses

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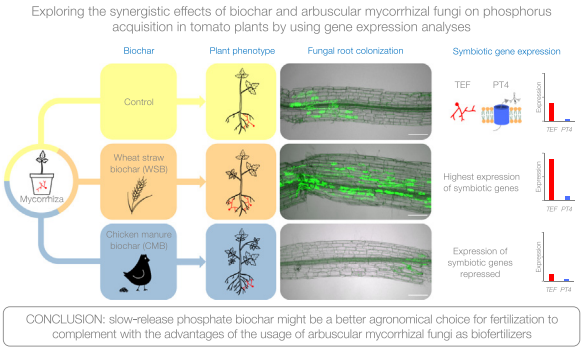
GRAPHICAL ABSTRACT



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ABSTRACT

Arbuscular mycorrhizal (AM) fungi are symbiotic organisms that contribute significantly to plant mineral nutrition, mainly phosphate. However, their benefits are constricted by the availability of phosphate in the soil, and thus they are recalcitrant as amendment in highly fertilized soils. Biochars are by-products of the pyrolysis of biomass in the absence of oxygen. They can improve soil properties and act as a source of nutrients for plants. However, depending on their origin, the final composition of biochars is extremely variable and thus, their efficiency unpredictable. In order to gain mechanistic insights into how the combined application of biochars and AM fungi contribute to plant phosphate nutrition and growth, we used gene expression analyses of key symbiotic marker genes. We compared for this analysis two biochars originated from very different feedstocks (chicken manure and wheat straw) on tomato plants with or without the AM fungus *Rhizophagus irregularis*. Our results show that the synergy between AM fungi and biochars as P biofertilizers is greatly governed by the origin of the biochar that determines the speed at which phosphate is released to the soil and absorbed by the plant. Thus, chicken manure biochar quickly impacted on plant growth by readily releasing P, but it turned out detrimental for symbiosis formation, decreasing colonization levels and expression of key symbiotic plant marker genes such as *SlPT4* or *SlFatM*. In contrast, wheat straw biochar was inferior at improving plant growth but stimulated the establishment of the symbiosis, producing plants with the same concentration of phosphate as those with the chicken manure. Taken together, slow P releasing biochars from plant residues appears to be a more promising amendment for long terms experiments in which biofertilizers such as AM fungi are considered. Furthermore, our results indicate that implementing plant transcriptomic analyses might help to mechanistically dissect and better understand the effects of biochars on plant growth in different scenarios.

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1. Introduction

Plant productivity is closely linked to the supply of phosphorus, mainly achieved in modern agriculture by addition of rock phosphate. Rock phosphate is a non-renewable resource and reserves of rock phosphate are rapidly declining leading to price increases and lack of food security in many areas of the world (Alewell et al., 2020; Bouwman et al., 2013; Gilbert, 2009). Despite its scarcity, phosphorus is mined and applied in intensive agriculture in excess. This is because orthophosphate (Pi), the main available form of phosphorus, is strongly adsorbed in soil by cations such as iron, aluminium, magnesium or calcium, depending on the soil pH, and therefore Pi concentration in the soil solution is as low as 10 μM or less (Schachtman et al., 1998). Thus, despite a high amount of phosphorus in the soil, plants quickly develop phosphate starvation zones around the roots (Smith and Smith, 2011) and have evolved highly conserved genetic mechanisms to withstand Pi starvation (Calderon-Vazquez et al., 2011). In addition, in some circumstances, excessive Pi from the soil solution can end into waterbodies by surface runoff and leaching, leading to their contamination and eutrophication (Chen et al., 2008; Schoumans et al., 2014). Therefore, it seems imperative to investigate more sustainable approaches of phosphate fertilization that help to diminish the amount of mineral fertilizers required and to close the phosphorus cycle.

One of the management strategies that is receiving increasing attention to improve soil fertility and structure is the use of biochar as a soil amendment (Hou et al., 2020). Biochars are co-products of the thermochemical treatment of biomasses at temperatures between 400 and 700 °C and in oxygen-limited conditions. This biomass pyrolysis produces energy carriers in gaseous form as well as solid and liquid products. Biochars are the solid residue of this pyrolysis process and their main purpose is, by definition, their subsequent application to soil to improve its properties (Lehmann and Joseph, 2009). Biochars are highly enriched in organic carbon, but depending on the feedstock origin and the pyrolysis parameters, the rest of their physicochemical properties can be significantly different (He et al., 2021). In some cases, biochar content in phosphorous and nitrogen might represent an interesting alternative to conventional fertilizers (Glaser et al., 2001; Kloss et al., 2012). Biochars are not only a source of mineral nutrients for plants but also a mean for long-term sequestration of carbon in the soil, reducing N_2O emissions and increasing soil water and mineral nutrient retention (Lehmann, 2007; Zimmerman, 2010). However, it is to be taken into consideration that biochars might also act as nutrient sequesters for instance by surface adsorption of nutrients or microbial immobilization processes (El-Naggar et al., 2019; Ohsowski et al., 2018). They can also be used to buffer the pH of acidic soils, for instance in the tropics where application of biochar or charcoal to the soil was shown to improve soil fertility (Chan et al., 2007; Glaser et al., 2002), or to improve soil structure (Lehmann et al., 2011; Weber and Quicker, 2018).

All these changes in the physiochemical properties of the soil have a major impact on its microbial composition and abundance (Gujre et al., 2021; He et al., 2021; Lehmann et al., 2011), including the rhizosphere (Kolton et al., 2017; Kolton et al., 2011). Changes in the soil microbial composition mediated by the amendment of biochar can impact on plant growth in different ways, ranging from direct effects of the microbes on the plant to effects mediated by the microbial alteration of the soil structure or of the nutrient cycles (He et al., 2021; Lehmann et al., 2011). Moreover, many of the positive effects of biochar application in plant growth associated to changes in the microbial diversity are related to changes in the microbial functioning (Jaiswal et al., 2017; Kolton et al., 2017). For instance, biochar amendment has been shown to suppress bacterial wilt of tobacco and tomato produced by *Ralstonia solanacearum* by altering the soil bacteria population (Gao et al., 2019; Zhang et al., 2017). Thus, biochar from rice or wheat straw favoured the expansion of Bacteroidetes over other groups of bacteria what ultimately protected tobacco and tomato plants from *R. solanacearum* (Gao et al., 2019; Zhang et al., 2017). But also, plants growing in soils added by biochars have been reported to be more resistant not only to diseases caused by soil borne pathogens but also towards foliar pathogens (Elad et al., 2010), indicating that systemic defense mechanisms might be invoked upon biochar application (Jaiswal et al., 2020; Jaiswal et al., 2017; Mehari et al., 2015; Meller Harel et al., 2012) either directly or by modifications of the soil microbiome diversity and amount (Kolton et al., 2017).

Given that soil microorganisms are key to close the soil nutrient cycles and in particular the plant microbiome plays an essential role in plant health and nutrition, it is of great importance to understand the role of biochar application in the composition and performance of microbes associated with plant roots. As a matter of fact, the impact of biochar application to the soil biology has been intensively of studied in the last decade (Gujre et al., 2021). One of the key soil microorganisms impacting on plant health are symbiotic arbuscular mycorrhizal (AM) fungi. AM fungi live in the roots of ca. 80 % of all land plants (Smith and Read, 2008). This symbiotic association has persisted for >400 million years helping plants to obtain mineral nutrients from poor nutrients soil in exchange for photosynthetically fixed carbon (Smith and Read, 2008). Because of the low mobility of Pi in soil, AM fungi are particularly effective at improving plant phosphate nutrition thanks to their ability to grow in the soil fine pores, that are unreachable by fine roots (Pearson and Jakobsen, 1993). But also because of their associated hyphal microbiome that contributes to release immobilized Pi from soil chelates or from organic forms (Finlay, 2008; Taktek et al., 2017; Taktek et al., 2015; Zhang et al., 2018), which can be then efficiently taken up by fungal phosphate transporters (Benedetto et al., 2005; Harrison and van Buuren, 1995; Maldonado-Mendoza et al., 2001).

Therefore, it is conceivable that a combined strategy of using biochar as soil amendment and AM fungi will help to maintain plant productivity and improve the soil structure and decrease the amount of mineral fertilizer required, thus contributing to the sustainability of agricultural systems. There are several reports about the combined use of biochar and AM fungi, including some where biochar was used as a matrix to study hyphal accessibility to phosphate in their micropores (Hammer et al., 2014), or as a substrate for AMF inoculum production and the capacity of this inoculum to improve growth of lettuce plants under drought conditions (Videgain-Marco et al., 2021). Most of the studies have concentrated on the ability of biochar to stimulate growth of plants, with different outcomes ranging from positive, not significant to even negative effects on plant growth (Amendola et al., 2017; Blackwell et al., 2010; Castañeda et al., 2020; Cobb et al., 2018; LeCroy et al., 2013; Solaiman et al., 2019; Vanek and Lehmann, 2015; Warnock et al., 2010; Zwetsloot et al., 2016). Negative effects have been often associated to a reduction in colonization due to changes in the soil phosphorus availability (Amendola et al., 2017; Cobb et al., 2018; LeCroy et al., 2013; Solaiman et al., 2019; Warnock et al., 2010). More recently, combined applications of biochar and AM fungi have been shown to contribute to a richer and connected root microbiome contributing to a better use of phosphorus utilization (Li et al., 2022) and improvement of soil quality (Gonzalez-Chavez et al., 2017; Ohsowski et al., 2018). Combined application of AM fungi and biochar has been also employed into phytoremediation processes, again with contrasting results (Liu et al., 2018; Ohsowski et al., 2018; Qiao et al., 2015; Vejvodová et al., 2020; Zhang et al., 2019).

However, as reviewed by Gujre et al. (2021), the functionality and dynamics of the combined used of biochar and AM fungi is only starting to be disclosed. This is because the combined amendment of biochar and mycorrhiza can influence the outcome in the plant through many different mechanisms which are often difficult to disentangle. In this study, we have explored the use of transcriptomic changes in the plant to investigate the functionality of the combined application of biochar and AM fungi regarding Pi nutrition. We have analyzed two biochars from substantially different feedstocks, a chicken manure biochar and a wheat straw biochar, in their ability to improve plant growth and their compatibility with a combined mycorrhizal inoculation. Since these types of biochars are known to have very different characteristics in terms of P content (Solaiman et al., 2019), and P is a main driver of the mycorrhizal symbiosis, we aimed here to investigate how the combined used of these amendments will affect the formation of the mycorrhiza symbiosis and impact on plant gene

expression. Our results indicate that indeed, effects in plant growth and phosphate nutrition were mirrored by changes in the symbiotic phosphate-related gene expression. We propose, therefore, that studies analyzing the plant transcriptomic response to amendments should be included to shade light on the molecular mechanisms governing the plant response and to better predict the agronomic outcome in complex environments such as biochars and mycorrhiza.

2. Material and methods

2.1. Biological materials and growth conditions

The arbuscular mycorrhizal fungus *Rhizophagus irregularis* DAOM 197198 (Schenck and Smith, 1982) was grown in vitro on monoxenic culture with *Agrobacterium rhizogenes*-transformed *Daucus carota* roots as previously described (Kuhn et al., 2010). Colonized carrot roots were chopped in 2 cm fragments and mixed with the corresponding substrate to serve as inoculum before potting the plants.

The plant species employed in the experiments described in this work are *Zea mays* cv. Golden Bantam, *Hordeum vulgare* cv. Golden promise, *Medicago truncatula* cv. Jemalong A17, *Solanum tuberosum* cv. Desirée and *Solanum lycopersicum* cv. Moneymaker. Plants were grown on 450 ml pots filled with a mixture of soil and biochar or vermiculite as control. The tested biochar:soil and vermiculite:soil ratios were 1:10, 1:5 and 1:3. Three seeds of maize, barley and barrel medic were germinated on each pot and plants were grown in a growth chamber (CLF Plant Climatics, Germany) at 25 °C, 64 % relative humidity. The light intensity was set to 357 μmol.m-^{2}.s-1 and the photoperiod to 16 h:8 h of light:darkness. All other experiments were performed using the 1:10 biochar:soil mixture.

M. truncatula seeds were sterilized for 20 min in 96 % sulfuric acid, washed 3 times with distilled water and placed on water-agar (9 g l^{-1}) plates. After one day of stratification at 4 °C, plates were left four days at 28 °C for seed germination, after which they were potted on the biochar-soil mixture. Maize and barley seeds were directly planted in the substrate without previous sterilization. Four days old potato cuttings were used to plant into the biochar soil mixture. Tomato seeds were sterilized by incubation in 70 % ethanol for 1 min, washed 3 times with distilled water, incubated for 10 min in a 3 % sodium hypochlorite solution while shaking, washed again 3 times with distilled water and placed on water-agar (9 g l^{-1}) plates. Seeds were germinated on plates for 3 days at 25 °C in dark conditions. Half of the pots were inoculated with *R. irregularis* inoculum. Plants grew in a growth chamber (conditions: 22 °C, 77 % relative humidity, 120 μML and 16 h:8 h light:darkness photoperiod) for 45 days after which they were harvested.

Plants were watered with distilled water and fertilized with half strength Long Ashton (without phosphate) for *M. truncatula* and potato, and with half strength Hoagland solution (20 μM phosphate) for tomato. Long Ashton: 750 μM $MgSO_4{\cdot}7H_2O$; 4 mM $NaNO_3$; 2 mM K_2SO_4; 2 mM $CaCl_2{\cdot}2H_2O$; 25 μM Na-FeEDTA; 5 μM $MnSO_4{\cdot}H_2O$; 500 nM $CuSO_4{\cdot}5H_2O$; 500 nM $ZnSO_4{\cdot}H_2O$; 50 μM NaCl; 25 μM H_3BO_3; 250 nM $Na_2MoO_4{\cdot}2H_2O$. Hoagland: 5 mM KNO_3; 5 mM $Ca(NO_3)_2{\cdot}4H_2O$; 1 mM $MgSO_4{\cdot}7H_2O$; 20 μM KH_2PO_4; 50 μM NaFe-EDTA; 1 μM $ZnSO_4{\cdot}7H_2O$; 0.5 μM $CuSO_4{\cdot}5H_2O$; 10 μM H_3BO_3; 0.2 μM $Na_2MoO_4{\cdot}2H_2O$; 2 μM $MnCl_2{\cdot}4\ H_2O$; 0.2 μM $CoCl_2{\cdot}6\ H_2O$; 25 μM HCl; 0.5 mM MES buffer. pH adjusted to 6.1 with NaOH.

2.2. Soil and biochars characteristics

Two different Biochars were used for this experimentation produced from chicken manure pellets (Chicken Manure Biochar, CMB) and bark-free wood (Wheat Straw Biochar, WSB), both originating from Germany. The biochar generation took place at a pilot scale experimentation at the STYX pilot-scale screw pyrolysis reactor at the Karlsruhe Institute of Technology (KIT) in Germany. The details of the testing facility as well as experimental set up are described in detail in Bergfeldt et al. (2018), Tomasi Morgano (2019) and Tomasi Morgano et al. (2015). Biochars were produced at 450 °C with a solids residence time of 10 min. Both the feedstock and the biochars were chemically characterized and an approximate analysis was performed to determine ash and volatile matter (VM) contents as described in Bergfeldt et al. (2018) and Tomasi Morgano (2019). The solubility degree in water (WS) (EN 15958) and/or neutral NH_4-citrate (NACS) (EN15957) and citric acid (2 %) (CAS) (EN 15920) of the main plant nutrients (NPK) contained in the biochar was analyzed. P and K in eluates were measured using ICP-OES while N was determined as TNb (Total Nitrogen bound) in EN 15958 eluate using the EN 12260 procedure. The mineralogical phase composition of the CMB had been previously analyzed using an X-Ray powder diffractometry (Bergfeldt et al., 2018). For the WSB, the analysis was performed in the frame of this work as follows: the XRD analysis was carried out in a D8 Discover (Bruker) using Cu K_α radiation (40 kV, 40 mA) in the 2 theta range from 2 to 82° (step size 0.02°, 0,4 s). Powdered samples were mixed with a defined amount of dry zinc oxide (ZnO) as additive to estimate the amorphous phase contribution. The composition of all crystalline phases was matched to the reference patterns given in the PDF-2 database and identified phases were refined using the Rietveld-method to extract the concentration of all crystalline components. Using the Reference-Index-Ratio (RIR) as given in the PDF-2, the concentrations were determined.

The soil used in all experiment was the substrate Floraton 3 soil (Floragard, Germany, https://www.floragard.de/de-de) with the following composition: pH ($CaCl_2$) 5.8, salt content 1 g l^{-1}, nitrogen ($CaCl_2$) 100 mg l^{-1}, phosphate (P_2O_5) (CAL) 100 mg l^{-1}, potassium oxide (K_2O) (CAL) 110 mg l^{-1}. The pH of the runoff of the biochar:soil mixtures after watering the pots at the end of the experiment with distilled water was determined using pH meter inoLab pH 7110 (Xylem Analytics Germany GmbH, Mainz, Germany) was used.

2.3. Analysis of phosphate in planta

The *Malachite Green Phosphate Assay* kit (Merck KGaA, Darmstadt, Germany) was used to determine the phosphate concentration in the shoots and roots of the tomato plants. A Retsch MM 400 (Retsch GmbH, Haan, Germany) mixer mill was used to homogenize the frozen tissue in 2 ml tubes containing metal beads. Three rounds of homogenization at 25 oscillations per second were carried out. Afterwards, 50 to 100 mg of frozen homogenized tissue was collected in a new tube and the specific weight per sample was annotated to express phosphate concentration per amount of fresh weight. Phosphate was isolated through a 1-minute incubation in 200 μl 250 mM NaOH (sodium hydroxide) at 95 °C and a 2 min incubation after adding 200 μl in 250 mM HCl (hydrochloric acid, for pH neutralization) at 95 °C. Cell debris was precipitated by centrifugation (13,000 rpm, 5 min) and the supernatant collected in a new tube. Phosphate determination was carried out according to manufacturer instructions. Two technical replicates per sample were prepared and their absorbance was measured at 620 nm using a Tecan Infinite M Nano (Tecan Group Ltd., Männedorf, Switzerland). As specified by the manufacturer, a calibration curve was prepared for the phosphate determination of the samples, which were then diluted using distilled water to match the range of the calibration curve.

2.4. Quantification and visualization of mycorrhizal

Mycorrhizal roots were incubated for 40 min in 10 % KOH at 95 °C and then washed three times with 1 x PBS (phosphate-buffered saline) containing 0.02 % Tween (PBST). pH was neutralized through a 5 min incubation in 1 % HCl at room temperature. Roots were then washed three times with distilled H_2O and once with PBST. Roots were incubated overnight at 4 °C with a 5 μg ml^{-1} WGA-FITC (wheat germ agglutinin-fluorescein isothiocyanate) in PBST buffer to label fungal structures. Once stained, mycorrhizal roots were washed three times with 1 × PBST and stored in PBS at 4 °C until observation.

The WGA-FITC-stained mycorrhizal roots were photographed using a Leica TCS SP5 (DM5000; Leica Biosystems GmbH, Nussloch, Germany)

confocal microscope with conventional photomultiplier tubes (PMT) detectors and a Leica DFC295 color camera. The LASAF v2.6 software was used to take the pictures. The FITC was excited with an argon laser at 488 nm. The emission of the fluorescein was detected in the spectrum from 505 to 525 nm. Pictures were then later processed with the open source software Fiji v1.0.

Fungal colonization levels were quantified by using the magnified intersections method described by (McGonigle et al., 1990). For each biological replicate 150 intersections of roots crossing the ocular lens gridline were scored for the presence of hyphae, arbuscules and/or vesicles at a ×200 magnification. The results were expressed in percentage.

2.5. Gene expression analyses

Total RNA was extracted using the *innuPREP Plant RNA Kit* (Analytik Jena GmbH, Thuringia, Germany) as specified by the manufacturer. The integrity of the RNA was visualized by gel electrophoresis and quantification determined spectrophotometrically using a nanodrop device DeNovix DS-11 + spectrophotometer (DeNovix Inc., United States).

500 ng of RNA were digested for 20 min with DNaseI (Amplification grade) and control PCRs were carried out using the *SlEF* gene (Solyc06g005060) to ensure the absence of genomic DNA. cDNA synthesis was carried out in 10 μl using Superscript II Reverse Transcriptase for 1 h at 42 °C following manufacturer instructions (Invitrogen, United States).

Transcript levels were determined using MESA Green qPCR MasterMix Plus for SYBR Assay with Fluorescein (Kaneka Eurogentec S.A., Seraing, Belgium) and the CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories Inc., California, United States). One μl of 1:5-diluted cDNA was used per reaction as template. The PCR protocol consisted of a 5 min incubation at 95 °C, followed by 40 cycles of 15 s at 95 °C, 15 s at 56 °C and 30 s at 72 °C. Gene expression of 4 to 5 biological replicates was analyzed per treatment in technical duplicates. Expression levels of plant genes and of *RiTEF* were shown as relative to *SlEF*, and fungal gene expression relative to *RiTEF* (DQ282611) using the delta-delta Ct method. Sequences of the primers used for real-time PCR and gene numbers can be found in Supplementary Table 1.

2.6. Statistics

Numerical data in Figs. 2, 3, 4 and 5a are represented as boxplots in which the horizontal lines of the boxes indicate the 3 quartiles (Q1, median and Q3), the mean is labelled as "x" and the colored dots represent the individual values of all biological replicates. Whiskers cover the range of values from minimum to maximum except when there are outliers, which are shown as black dots. The number of biological replicates per treatment was $n \geq 4$ and it is indicated in each figure legend. For statistical analysis and comparison of all the treatments against each other (Figs. 4c and 5a), a one-way ANOVA test and a Tukey HSD post-hoc test were carried out when normality and homoscedasticity could be assumed, these assumptions were checked through the Shapiro-Wilk and Levene's tests, respectively. If at least one of the groups was not normally distributed, treatments were compared with the Kruskal-Wallis test and the Mann-Whitney U post-hoc test. Differences were considered statistically significant if the p-value was <0.05 and are indicated on the plots with different letters. For the rest of the boxplots (Figs. 2, 3 and 4b), each treatment was individually compared with the corresponding control using a t-test if normality of the data could be assumed or a Mann-Whitney U test for comparisons in which at least one group wasn't normally distributed. In this case, significance was shown with * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) or ns ($p > 0.05$). All mentioned statistical tests for the boxplots were carried out with the online tools available at Statistics Kingdom (http://www.statskingdom.com).

In Fig. 5b scatter plots were used to compare two variables for which the linear correlation was assessed through Pearson's correlation coefficient "r". This value ranges from −1 to +1 and the correlation was considered statistically significant when the p-value was <0.05. A regression line was also included in the plots. This statistical analysis was done with the RStudio software (RStudio Team, 2021). All boxplots and scatter plots were generated using the RStudio software with the help of the ggplot2 and ggpubr packages, respectively.

3. Results

3.1. Biochar analyses

In both biochar samples no crystalline phases of phosphates could be recognized (Table 1). Crystalline phases identified for the chicken manure derived biochar were described in Bergfeldt et al. (2018). For the wheat straw derived biochar, the reduced degree of crystallinity in the analyzed samples turned out to be problematic for the determination of low concentrated side phases. The carbonization of organic material led to the formation of a dark phase (possibly charcoal), that increased the background and reduced the sample quality. While typical phosphate containing mineral phases can usually be determined using the method as described in Section 2.2, no such phases could be identified in the wheat straw derived biochar samples analyzed here that could be referred to known phases described in the literature. Therefore, the phosphates were either only side phases of low concentration or they mainly formed with an amorphous composition. In both cases, this shows the limitations of the analytical method used to determine possible amorphous phosphates. The chicken manure biochar contained ca. 28 g kg^{-1} of total P, >9 times the amount present in the wheat straw biochar (Table 1). And although, from this total amount <4 % was water soluble, and thus, ready available for plants and microbes, the wheat straw biochar contained a much lower proportion (<0.05 %).

3.2. Biochar concentration has to be adjusted for plant growth

To determine the optimal proportion of biochar for plant growth and the effects on soil pH, we tested several proportions of the biochars and soil. Soil pH is an important factor for plant growth and microbial activity, with a soil pH in the range of 6.5 and 7.5 considered optimum (reviewed in He et al., 2021). In control treatments, the same proportion of biochar was substituted with vermiculite. In this preliminary test, germination and growth of a mixture of plants (corn, barley and barrel medic) were assessed at 10 and 25 days after planting. It could be observed that the biochar from chicken manure (CMB) delayed the germination and early growth of plants

Table 1
The elemental composition as well as the solubility of the chicken manure biochar (CMB) and the wheat straw biochar (WSB) employed in this work.

		CMB	WSB
C_{total}	%	39.4**	63.7**
N_{total}		4.49**	1.28
VM		28.8**	19**
Ash		49.3**	22.8**
C/N		8.78	49.76
P_{total}	g/kg	27.99*	2.99**
P_{WS}	%	<4*	<0,05
P_{NACS}		84.1*	4.15
P_{CAS}		90*	12,5
Ca	g/kg	121.14	16.78
Mg		13.68	2.75
K		45.4	26.5
Fe	mg/kg	2910	
Al		3915	845
Zn			
Cu		140	
Mn		960	74
Ni		17	8
Pb		4	<2

Abbreviations: P, phosphorus; WS, water solubility; NACS, neutral NH_4-citrate solubility; CAS, citric acid solubility.
References: *Bergfeldt et al., 2018; **Tomasi Morgano, 2019.

when used at high concentration, but it did best at promoting plant growth, both at 10 and at 25 days, when diluted 1 to 10 with soil (Fig. 1). The negative effect of both biochars and in particular the CMB on plant growth at a high concentration might have different explanations. On one hand, some authors have observed that under high nitrogen conditions, absorption by biochars of plant signaling molecules required for the establishment of the symbiosis might negatively impact plant growth (LeCroy et al., 2013). In addition, when applied at a high concentration, some biochars might have high levels of heavy metals that might hinder plant growth (He et al., 2021; Jin et al., 2021). On the other hand, it is known that most biochars have alkalizing properties due to their ash content causing release of base cations, what has been related to their ability to suppress N_2O and NO production (Obia et al., 2015). In order to investigate whether there was a correlation between these results and changes in the pH due to the biochar amendment, the runoff pH was analyzed at the end of the experiment in all treatments. The results as shown in Table 2 indicate that both biochars, but most significantly the chicken manure biochar alkalinized the medium when used at high concentration. This effect was buffered by the soil for both biochars when they were diluted 10 times. It is possible, that dilution of biochars might have also mitigated some of the other possible above mentioned constrains to plant growth.

Table 2
pH measurements of the soil-biochar (or soil-vermiculite) treatments at three different concentrations. CMB, chicken manure biochar, WBS wheat straw biochar, V (vermiculite), S (soil).

pH	1/10	1/5	1/3
CMB: S	5	8	9
V: S	5	5	5
WSB: S	5	7 8	8

3.3. Biochar addition improves growth of several crop plants

The effect on plant growth of the chicken manure and the wheat straw biochars was analyzed in potato and barrel medic. Plants were grown on pots in these substrates diluted with soil in a 1/10 proportion and compared to the growth of control plants grown in soil with vermiculite. Amendment with the chicken manure produced the highest increase in plant growth, although the wheat straw biochar had also a significant but only moderated benefit on potato plants, but not on barrel medic (Fig. 2a, b, a). The chicken manure biochar was especially superior with regards to potato tuber production (Fig. 2b, c).

We then tested the effect of these biochars in tomato plants inoculated or not with the arbuscular mycorrhizal fungus *Rhizophagus irregularis* (Fig. 3a). In the absence of mycorrhizal inoculation, both biochars increased plant shoot growth (Fig. 3b), but only chicken manure was able to augment root growth (Fig. 3c). Interestingly, under mycorrhizal conditions, shoot growth of tomato plants grown on wheat straw biochar was no-longer better than shoot growth of control plants, which, due to mycorrhizal colonization, were as large as non-mycorrhizal plants amended with wheat straw (Fig. 3b). Shoot growth of tomato plants fertilized with chicken manure biochar and inoculated with mycorrhiza was even larger than with biochar alone. Mycorrhizal inoculation did not significantly change root growth in any of the treatments respect to the non-inoculated controls (Fig. 3c).

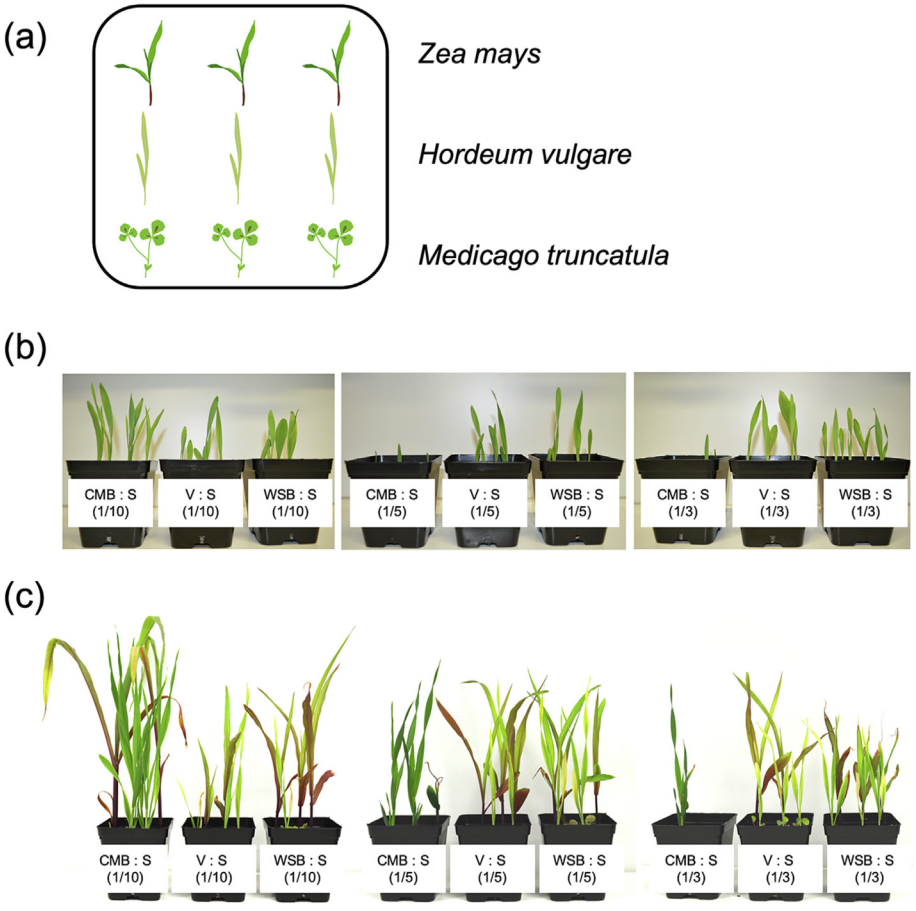


Fig. 1. Biochar concentration effect on plant germination and growth. (a) Three seeds of three different plants (*Zea mays*, *Hordeum vulgare* and *Medicago truncatula*) were set on each pot as in the cartoon. (b–c) Effects on plant germination and growth of two different biochars were evaluated at 10 (b) and 25 days (c) after planting. Chicken manure biochar (CMB) or wheat straw biochar (WSB) were mixed with soil (S) in three different proportions (1/10, 1/5, or 1/3) and their effect compared against soil mixed in the same proportions with vermiculite (V).

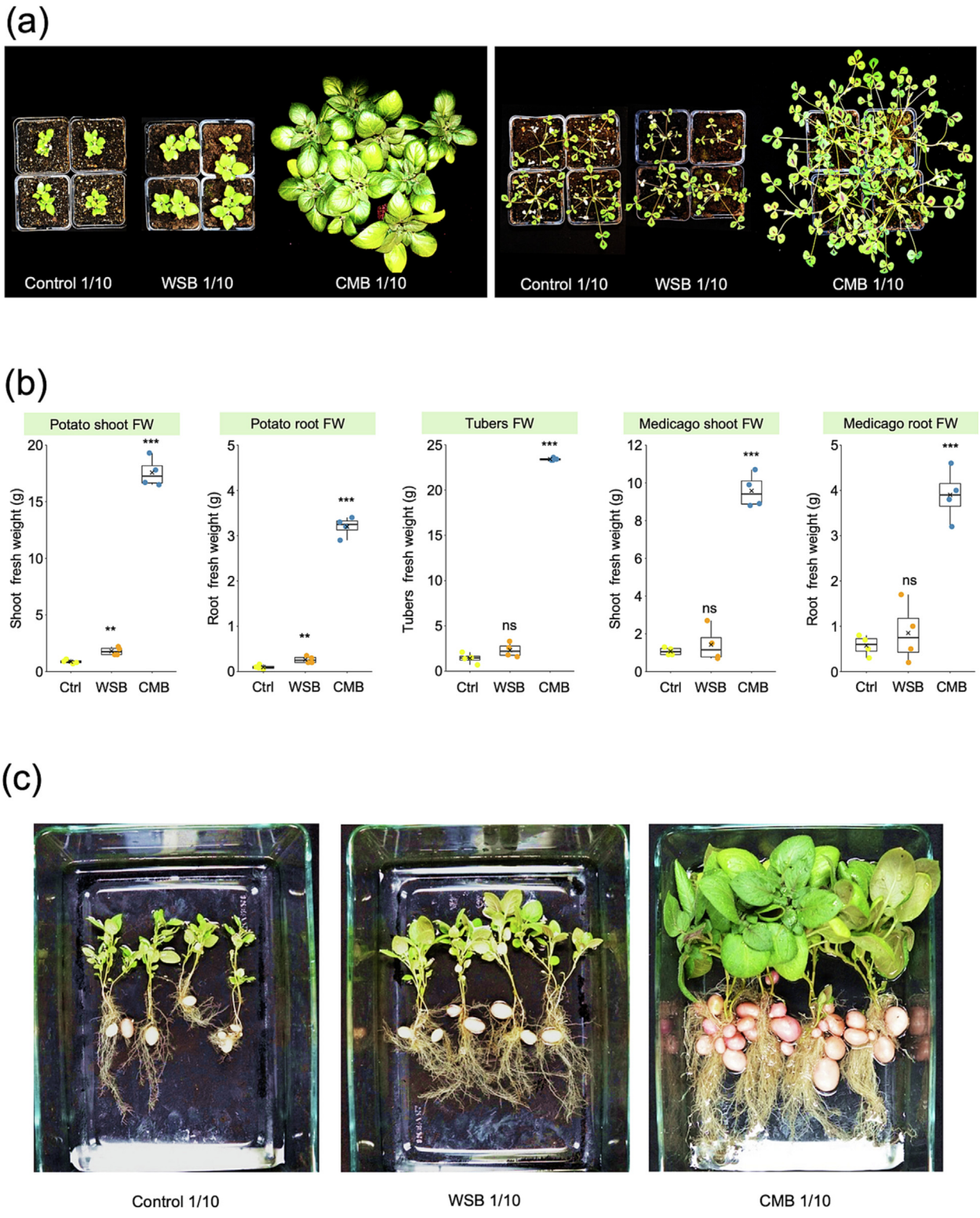


Fig. 2. Biochar effect on potato and lucerne growth. (a–b) *Solanum tuberosum* cuttings or *Medicago truncatula* seedlings were planted either on chicken manure biochar (CMB) or on wheat straw biochar (WSB) mixed with soil in a 1/10 dilution and grown for 46 (potato) and 30 (lucerne) days. Their growth was compared to that of control plants grown on a soil mixed with vermiculite in the same proportion (c) Biochars effect on potato production. Statistical significance was calculated using a *t*-test (data are normally distributed) comparing each treatment against the control as explained in the section "Material and methods". Significance is shown as * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) or ns ($p > 0.05$).

To investigate how all these treatments alone or in combination affected the phosphate nutrition of the tomato plant, phosphate concentration and content was measured in shoots and roots in all treatments (Fig. 3b, c). In non-mycorrhizal plants, only amendment with chicken manure biochar was efficient at increasing both, the concentration and the content of Pi, in shoots and roots. Interestingly, under mycorrhizal conditions, wheat straw biochar increased Pi concentration in shoots and roots over the control mycorrhizal treatments and was as efficient as the chicken manure

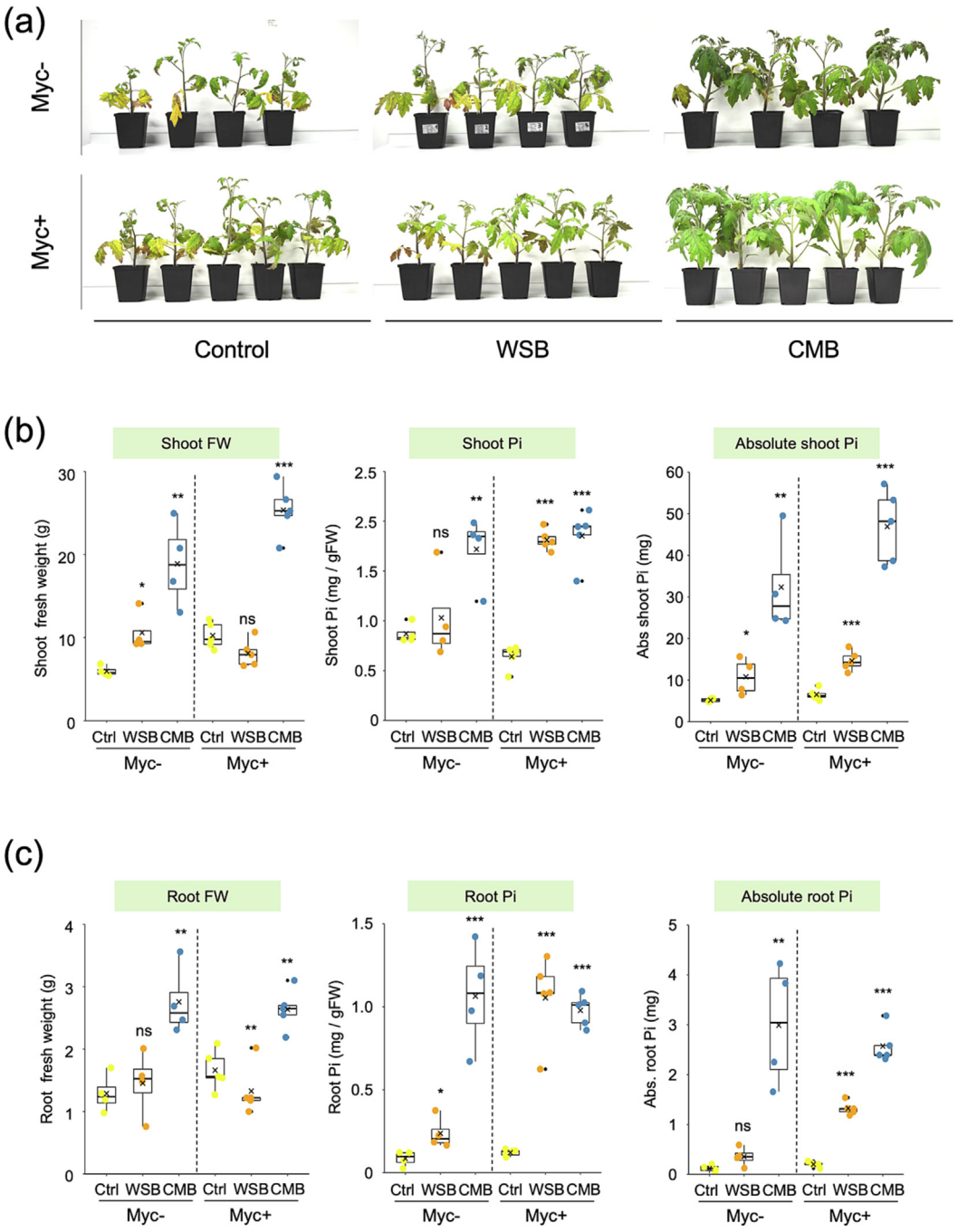


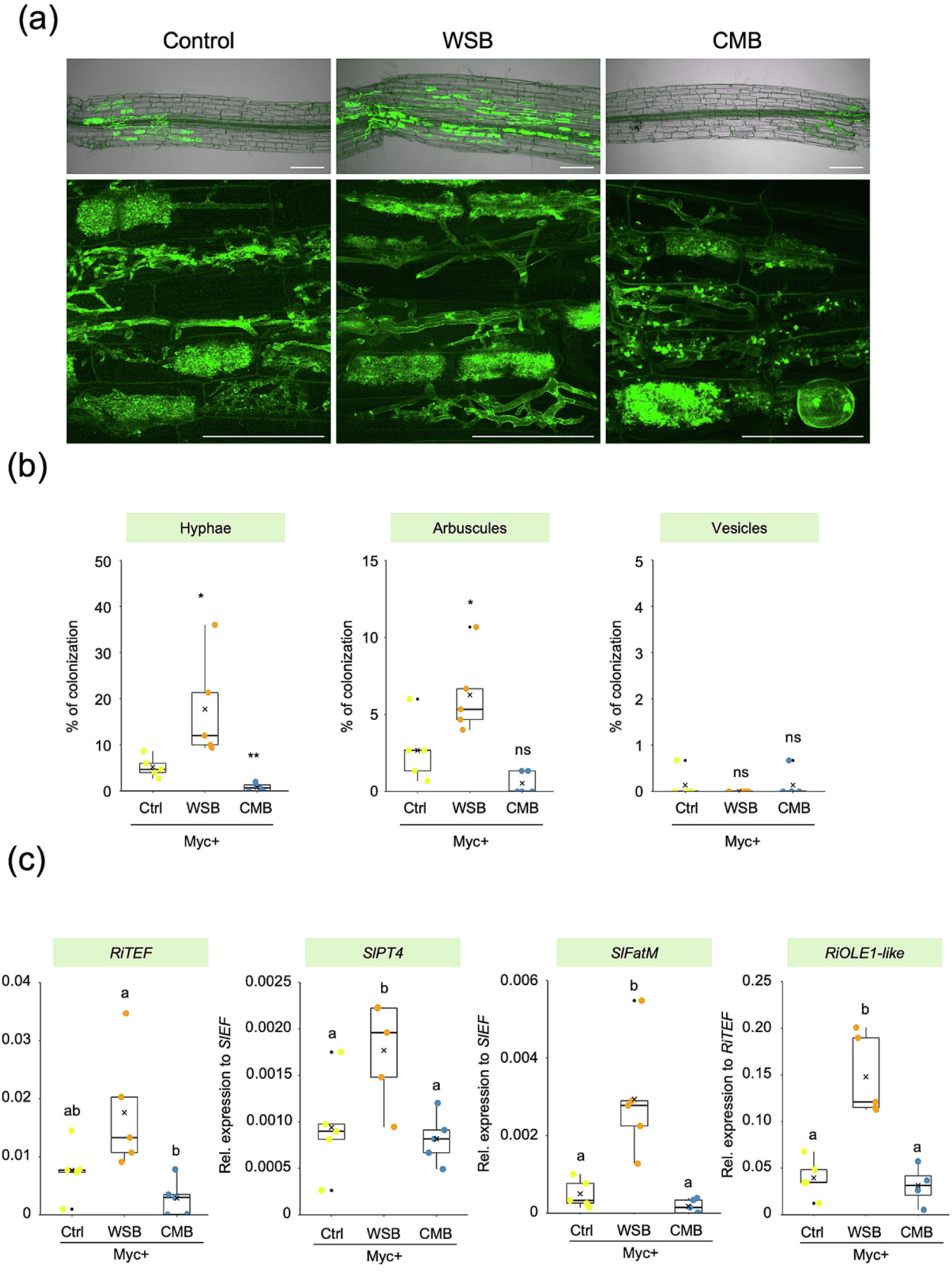
Fig. 3. Biochar and mycorrhizal effects on tomato growth. (a) *Solanum lycopersicum* seedlings were planted on either chicken manure biochar (CMB) or on wheat straw biochar (WSB) mixed with soil in a 1/10 dilution. Half of the pots were inoculated with the AM fungus *R. irregularis* (Myc+), while the other half served as uninoculated control (Myc−). Control plants were grown on soil mixed with vermiculite in the same proportion (b–c) Biomass (expressed as fresh weight) as well as Pi concentration and absolute content of shoots and roots of tomato plants was evaluated after 45 days. For the statistical analysis, each group was compared to the corresponding control using either a *t*-test or a Mann-Whitney *U* test depending on the normality of the data as specified on the section "Material and methods". Significance is shown as * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) or ns ($p > 0.05$). Population size: non-mycorrhizal $n = 4$ biological replicates, mycorrhizal $n = 5$.

biochar. Pi content under mycorrhizal conditions was highest in plants fertilized with chicken manure biochar.

3.4. Wheat straw biochar improved mycorrhizal colonization in tomato

Biochar addition had also an impact on the mycorrhizal colonization of tomato plants (Fig. 4a). Thus, while chicken manure biochar had a tendency towards suppressing the mycorrhizal colonization, albeit not significant, biochar from wheat straw augmented mycorrhizal colonization significantly, including the number of hyphae and arbuscules, but not of vesicles (Fig. 4b). In all treatments the differences observed were related only to the intensity of colonization but not to the morphology of the different fungal structures observed within the root (Fig. 4a). Changes in the colonization levels might be related to the differences observed in the amount of P present in both biochars, as observed in other experiments where biochars and mycorrhiza were combined (Solaiman et al., 2019; Vanek and Lehmann, 2015; Warnock et al., 2007).

The microscopic colonization results were mirrored by the quantification of fungal material in roots using qRT-PCR for the housekeeping gene *RiTEF* (Fig. 4c). But most interestingly, also the expression of other


(a)
Control
WSB
CMB
(b)
Hyphae
Arbuscules
Vesicles
% of colonization
Ctrl
WSB
CMB
Myc+
ns
(c)
RiTEF
SlPT4
SlFatM
RiOLE1-like
Rel. expression to SlEF
Rel. expression to RiTEF
ab
a
b

symbiotic markers, such as the plant symbiotic phosphate transporter *SlPT4*, the plant acyl-ACP thioesterase gene *SlFatM* and the fungal acyl-CoA desaturase gene *RiOLE1-like*, followed the same trend and their expression was enhanced in plants amended with wheat straw biochar (Fig. 4c).

3.5. Biochar amendment in combination with mycorrhizal colonization modifies the root phosphate uptake pathway

In order to further investigate the phosphate uptake of tomato plants grown on the different biochars with or without mycorrhiza inoculation, we also analyzed the expression in roots of two other tomato phosphate transporters *SlPT1*, *SlPT2*, which are primarily expressed in the rhizodermis (Daram et al., 1998; Liu et al., 1998), and of the phosphate starvation response marker *SlTPSI1* (Fig. 5a). *SlPT1* expression was not altered by the amendment of Biochar, with the exception of wheat straw biochar in mycorrhizal plants. These results were in line with the lack of correlation between the *SlPT1* expression and the levels of Pi in shoots or roots (Fig. 5b).

In contrast, *SlPT2* expression was inversely correlated with the levels of Pi in roots (Fig. 5b) although not with those of shoots. *SlPT2* expression was only elevated in non-mycorrhizal plants without biochar amended. Mycorrhizal colonization with or without addition of biochar suppressed *SlPT2* expression, even in mycorrhizal plants without biochar which did not show significant differences in their Pi levels with respect to non-mycorrhizal plants. This would possibly indicate a signaling mechanism of the mycorrhizal colonization leading to the downregulation of *SlPT2* even in the absence of a Pi benefit.

The expression of *SlTPSI1*, a phosphate starvation inducible gene (Liu et al., 1997), did not show large variations among the treatments, despite the differences observed in Pi concentrations and content (Fig. 5a). In contrast, correlation analyses showed that, as expected, its expression was inversely correlated with Pi levels in shoots and roots when considering all treatments together (Fig. 5b). Congruently, *SlTIPS1* expression was also inversely correlated with the expression of the mycorrhizal markers *SlPT4* and *RiTEF*, indicating that higher colonization levels and higher number of arbuscules, correlated with a lower phosphate starvation response in roots (Fig. 5b).

4. Discussion

This study aimed to investigate the effects of chicken manure and wheat straw biochar (CMB and WSB) on the growth and phosphate accumulation of tomato plants in the presence or absence of arbuscular mycorrhizal colonization. In the absence of mycorrhizal colonization, CMB, richer in soluble Pi than WSB, improved plant productivity, not only in tomato but also in potato or barrel medic plants. WSB also improved plant growth, but without increasing the Pi content of the plants. WSB had a positive effect on symbiosis establishment and functioning whereas CMB had a negative effect. Our results will be discussed with regards to the effect of biochar on (i) plant nutrition and plant growth, (ii) on symbiosis establishment and (iii) with regards to phosphate uptake mechanisms in the root.

Although both biochars increased plant growth, a detailed analysis in tomato revealed that under non-mycorrhizal conditions, plant growth promotion effects in response to the wheat straw biochar were not related to a significant increase in the Pi concentration nor the Pi content of those plants, correlating with the low Pi availability of this substrate. Thus, factors or nutrients others than phosphate in the WSB might have accounted for the positive effect that this substrate exerted on shoot growth. In contrast, the superior ability of the chicken manure biochar to improve growth can be associated with an increase both in the concentration and content of Pi in both shoots and roots. Tomato plants showed a positive correlation between the amount of Pi accumulated in shoots and roots and their growth responses. This was the case in both in the absence or presence of a mycorrhizal colonization for almost all treatments. However, this trend was surprisingly not observed in mycorrhizal plants amended with WSB. They contained higher Pi than their control counterparts, and as much as the CMB treated plants, but their shoot growth was not improved, and root growth was even repressed. This apparently contradictory effect has been attributed to a disequilibrium between the higher photosynthate demand of mycorrhizal roots to fulfil the fungal needs and the nutritional benefits provided by the fungus, particularly at early stages of the interaction. Such a disequilibrium might, in consequence, provoke a slowing down of plant growth reviewed in Jin et al. (2017). This effect has been also observed in other experiments with combined application of biochar and mycorrhiza (Akhter et al., 2015; LeCroy et al., 2013) and also attributed to a carbon drainage towards the fungus. This might have been the case in the tomato experiment here because plants grown on wheat straw biochar exhibited the highest colonization levels and Pi concentration, but they did not promote plant growth.

In support of this hypothesis, the expression of the fungal marker *RiOLE1-like* in plants amended with WSB was higher than in control plants. RiOLE1-like is an acyl-CoA desaturase that synthesizes palmitvaccenic acid (16:1111cis), an unusual fatty acid typical from AM fungi (Brands et al., 2020; Cheeld et al., 2020; Graham, 2000; Olsson et al., 1995). Its elevated expression suggests that the fungal lipid metabolism is boosted in those plants, possibly as a result of a higher lipid transfer from the plant. This is further supported by the increased expression of the plant gene *SlFatM*, an orthologue of the plant acyl-acyl carrier protein thioesterases *LjFatM* and *MtFatM* known to be required for lipid accumulation in arbucules and specifically recruited during the AM symbiosis (Brands et al., 2018; Bravo et al., 2017). Altogether these results indicate that WSB boosted fungal colonization in tomato plants thereby improved symbiotic phosphate acquisition. Regarding the mechanisms of how WSB increased the colonization by AM fungi versus control plants, some authors have suggested that biochars might counteract allellopathic substances that prevent or diminish the AM symbiosis (Elmer and Pignatello, 2011) and/or modify the root microbiome to facilitate AM fungal colonization through signaling mechanisms (Gujre et al., 2021; He et al., 2021). It could be observed that both biochars were toxic for plant growth when employed at higher concentrations but exerted positive effects at low doses. This effect known as hormesis, is often associated to the presence of phytotoxic organic compounds in biochars (Graber et al., 2010).

In contrast, fungal colonization in plants amended with CMB was similar or even lower to that of control plants, and thus, the benefits on plant Pi accumulation in those plants can be mainly attributed to the phosphate fertilizing ability of this substrate. However, in contrast to the hypothesis that under conditions of sufficient available Pi, the combination of biochar and AM fungi would not have positive effects on plant growth (Vanek and Lehmann, 2015), we observed a synergistic effect in the combined

Fig. 4. Mycorrhizal colonization of tomato plants grown on soil amended with wheat straw or chicken manure biochars. (a) Overview of mycorrhizal colonization of tomato roots grown on soil amended with wheat straw biochar or with chicken manure biochar as compared to control plants. Cleared roots were stained with WGA-FITC to visualize fungal structures. Size of the scalebars: 200 μm for the overview pictures; 100 μm for the close-up pictures. (b) Percentage of total colonization, arbuscules and vesicles was estimated using the magnified intersections method in all mycorrhizal samples. For statistical significance each treatment was compared against the control using the *t*-test or the Mann-Whitney *U* test depending on the normal distribution of the data as specified on the section "Material and methods". (c) The amount of living *R. irregularis* in the roots was estimated by analyzing the expression of the fungal gene *RiTEF* (translation elongation factor of *R. irregularis*) relative to the expression of the *SlEF* (translation elongation factor of *S. lycopersicum*) using qRT-PCR. The expression of other mycorrhizal marker genes was also measured: *SlPT4*, *SlFatM* (symbiotic Pi transporter and enzyme for lipid biosynthesis, respectively; both expression levels relative to *SlEF*) and *RiOLE1-like* (fungal Acyl-CoA desaturase specific of AMS; expression relative to *RiTEF*). Statistical significance was calculated by comparing the groups through a one-way ANOVA or Kruskal-Wallis test depending on the normal distribution and homoscedasticity of the data. Then the appropriate post-hoc test was applied, as explained on "Material and methods". Different letters indicate significant differences (p-value < 0.05).

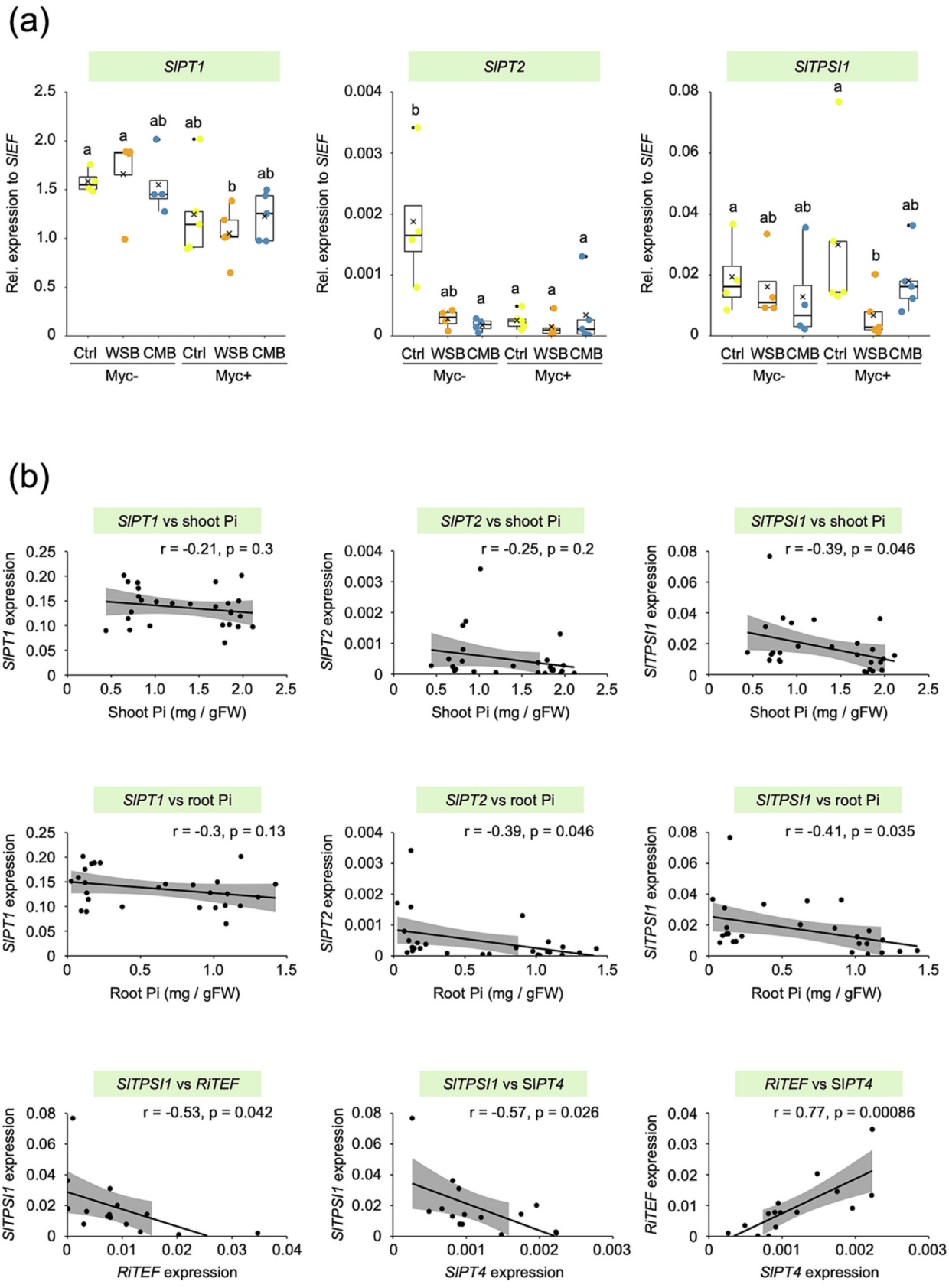


Fig. 5. Expression analysis of genes involved in Pi homeostasis and correlation of *in planta* Pi levels with the expression of several mycorrhizal marker and Pi-related genes. (a) Expression levels of Pi transporters *SlPT1* and *SlPT2* as well as Pi starvation responsive gene *SlTPSI1* relative to *SlEF* were measured through qRT-PCR. The different treatments were compared through the Kruskal-Wallis test plus the Mann-Whitney *U* as post hoc test as explained in "Material and methods". Different letters indicate significant difference (p-value < 0.05). (b) Correlation between pairs of variables expressed through the Pearson's correlation coefficient ($r > 0$ for positive correlations; $r < 0$ for negative correlations). The exact p-value (p) of each test is shown on the plots.

treatment of CMB and mycorrhiza, that produced the largest plants with the highest P content. Therefore, other mechanisms of how *R. irregularis* contributed to improve plant growth on CMB besides P nutrition might have played a role. It is possible that the known, but far less investigated, role of AM fungi to contribute to the mineral nutrition of many other elements besides P (Wang et al., 2017) might have played a role here, and should be further investigated in the future.

Although the colonization levels were very different among the three treatments, the structures within the roots of all tomato plants were very similar. They comprised coils, hyphae, arbuscules, arbusculate coils and vesicles, therefore resembling a Paris-type colonization pattern (Cavagnaro et al., 2001; Dickson, 2004). Because there were no obvious arbuscule size differences among the treatments, the higher expression of *StPT4* observed in mycorrhizal plants amended with WSB suggested that the number of functional arbuscules was higher in this treatment than in the others. *StPT4* is a plant phosphate transporter induced during arbuscular mycorrhizal symbiosis (Nagy et al., 2005; Poulsen et al., 2005). It is assumed to play an identical role to its orthologues in other mycorrhizal plants, which are located at the periarbuscular membrane and transport phosphate from the periarbuscular space into the cytoplasm of the arbuscule-containing cell (Harrison et al., 2002; Javot et al., 2007). Accordingly, the expression of the symbiotic phosphate transporters, including *SlPT4*, correlates with the Pi uptake in mycorrhizal tomato plants (Poulsen et al., 2005). It can be therefore considered a good indicator of the symbiosis performance in terms of Pi, and thus, it reflects that the Pi increase in shoot and root in mycorrhizal plants grown on the wheat straw biochar is mainly obtained through the symbiotic route.

The study of the expression of the root epidermal phosphate transporters *SlPT1* and *SlPT2*, responsible for the non-symbiotic Pi uptake pathway, further revealed insights into the phosphate homeostasis of the tomato plants in response to both the biochar amendments and the mycorrhiza inoculation. In agreement with previous studies (Nagy et al., 2005; Poulsen et al., 2005), *SlPT1* showed a rather constitutive expression in roots, independent of the Pi status, while *SlPT2* was repressed in response to high Pi. However, in general *SlPT1* expression was lower in mycorrhizal plants and even significantly downregulated in plants amended with wheat straw biochar. This mycorrhiza-effect was more evident in the negative regulation of *SlPT2*, which expression decreased in response to mycorrhizal colonization, even in the absence of a mycorrhizal Pi benefit. This supports the hypothesis that part of the transcriptional regulation of the epidermal transporter during symbiosis with AM fungi is regulated through other signaling mechanisms initiated with the fungal colonization of the root that are not necessarily correlated to the actual symbiotic Pi benefits (Burleigh and Harrison, 1997; Poulsen et al., 2005).

5. Conclusions

In conclusion, our study shows that the origin of biomass which is processed into biochars significantly determines their final properties as plant Pi fertilizers. Moreover, the compatibility of these biochars with other Pi biofertilizers such as in this case symbiotic AM fungi is also greatly governed by the origin of the biochar and thus its final properties. We showed that plant growth and phosphate nutrition were mirrored by changes in symbiotic phosphate-related gene expression. Thus, the negative effects of the chicken manure biochar on the root colonization by *R. irregularis* might have been mainly determined by its ability to instantly provide Pi to the plant, in accordance with the results of a study using 34 different biochars in which the P concentration of biochar negatively correlated with the mycorrhizal colonization (Solaiman et al., 2019). This hypothesis is also supported by the gene expression analysis, which clearly showed that the chicken manure biochar suppressed the expression of the symbiotic phosphate transporter *SlPT4*. High availability of phosphate in the soil systemically inhibits the development of the symbiosis and shuts down the expression of the symbiotic phosphate transporter (Breuillin et al., 2010). In turn, silencing or knocking out of this transporter are known to impair fungal development within the root (Javot et al., 2007) and the expression of metabolic genes required for fungal feeding such as *FatM* (Floss et al., 2017). We cannot rule out, however, that in addition, the chicken manure biochar might have exerted direct detrimental effects on fungal growth, given that the external mycelium of AM fungi has been shown to grow on biochars pores (Hammer et al., 2014), or through other mechanisms such as for instance altering the soil structural properties or its microbial composition (Lehmann, 2007). These results will help to design biochars more compatible with mycorrhizal fertilizers by taking into account the biomass origin and modelling the pyrolysis process towards obtaining a low Pi release biochar with an optimized surface for symbiotic fungal growth. We show here that transcriptomic analyses are useful to better understand the response of plants to the application of amendments in an specific environment. Thus, we propose to include this type of studies to predict the agronomic outcome of plants in complex environments such as biochars and mycorrhiza. We are aware, however, that the costs of these methods and the difficulty of the results interpretation in complex environment can be a limiting factor. Overall, our study provides insights into the potential benefits and drawbacks of using biochar in combination with arbuscular mycorrhizal inoculation as a soil amendment for plant growth and highlights the need for further research, including gene expression analyses, to better understand the mechanisms underlying their effects in planta.

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CRediT authorship contribution statement

Natalia Requena: Conceptualization, writing - original draft preparation, reviewing and editing. David Figueira-Galán: Investigation, methodology, data curation, manuscript reviewing. Stephanie Heupel: Methodology. Glykeria Duelli: Methodology, manuscript reviewing. Marco Tomasi Morgano: Methodology for biochar production. Dietmar Stapf: Manuscript reviewing.

Data availability

No data was used for the research described in the article.

Declaration of competing interest

The authors declare no competing, non-financial/financial interests or personal relationships with other people or organizations that could influence the work reported in this paper.

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