Arbuscular mycorrhizal fungi alter carbohydrate distribution and amino acid accumulation in *Medicago truncatula* under lead stress

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ABSTRACT

This study aims to understand the effect of arbuscular mycorrhizal (AM) inoculation on the regulation of carbon and nitrogen metabolites in *Medicago truncatula* plants under lead (Pb) stress. Root morphology, nutrient, photosynthesis, sucrose cleaving enzymes, the transcription levels of sucrose transporters, and metabolite profiling were evaluated via a two-factor (AM inoculation and Pb stress) pot experiment. AM inoculation alleviated the damage caused by Pb to photosynthesis and increased phosphorus uptake. AM inoculation decreased the shoot sucrose content but increased root sucrose content. The transcription of *MtSUT1-1* and *MtSUT4-1* in roots was upregulated by AM inoculation, which indicates that AM plant has a higher ability of sucrose transport. Both Pb stress and AM inoculation stimulated the activity of acid invertase and sucrose synthetase in roots. In shoots, minor amino acids such as cysteine were increased by AM inoculation. Sucrose content was negatively related to asparagine content. Therefore, AM inoculation may alleviate Pb toxicity by promoting the transport of sucrose from shoot to root, promoting the cleaving of sucrose in roots, and increasing minor amino acids accumulation.

1. Introduction

Recently, many human activities, such as mining, extracting fossil fuels, and using sewage sludge amendments, have been the primary sources of heavy metal pollution (Xiao et al., 2017). As a toxic compound, lead (Pb) mainly enters the soil through lead-zinc mining (Yabe et al., 2018). More than 800,000 tons of Pb have been released into the environment globally, and most Pb has accumulated in the soil environment and negatively affected plant growth and development (Yang et al., 2018). In addition, exposure to Pb pollution can endanger human health and interfere with behavior and learning abilities by damaging the nervous system (Mason et al., 2014; Song and Li, 2015). Phytoremediation as a cost-effective and noninvasive solution has been proposed to remediate soil that is polluted by heavy metals (Mahar et al., 2016). Methods of improving the phytoremediation effect by improving plant heavy metal resistance have drawn increasing attention.

Arbuscular mycorrhizal (AM) fungi are known to establish mutualistic symbioses with 90 % of terrestrial plants and to form tree-shaped subcellular structures within root cells (Parniske, 2008). Several studies demonstrated that AM fungi could increase plant biomass and help host plants survive abiotic stresses such as heavy metal stress (Dhawi et al., 2016; Zhan et al., 2018). Recently, mycorrhizal plants have been considered to be potential candidates for phytoremediation (Lenoir et al., 2016; Ma et al., 2019). The main benefit of AM fungi to plants is improved absorption of nutrients such as phosphorus and nitrogen; in return, host plants provide organic carbon to AM fungi (Zhang et al., 2016). Pb stress induces the loss of available nutrients in the soil, which increases barriers for roots and AM hyphae to absorb nutrients (Seshadri et al., 2017). Moreover, heavy metal stress could negatively affect photosynthesis and reduce the production of carbohydrates (Rehman et al., 2016; Sorrentino et al., 2018a,b). Therefore, Pb stress induces a challenge to AM plants in the exchange of organic carbon and nutrients.

Photosynthesis-mediated carbon assimilation is the main method of production of carbohydrates. The chlorophyll fluorescence parameter is usually used to characterize photosynthesis (Sheng et al., 2008). Sucrose can be transported from photosynthetic tissues into non-photosynthetic tissues such as roots (Kircher and Schopfer, 2012). Nitrogen is primarily absorbed as inorganic salts from the soil solution and is assimilated through the amino acid synthase pathway (Xu et al., 2012). Amino acids can be transported from roots into photosynthetic

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tissues for physiological metabolic processes, such as protein synthesis (Tegeder and Masclaux-Daubresse, 2018). The nitrogen assimilation efficiency affects photosynthesis and the partitioning of the assimilated carbon (Yadav et al., 2015). In return, carbohydrate partitioning affects amino acid composition and distribution (Busch et al., 2018). The carbon and nitrogen dynamic balance in plants is usually associated with stress resistance (Lu et al., 2015; Reyes et al., 2016). However, under abiotic stress, especially heavy metal stress, both nutrient absorption and carbon assimilation are disturbed. How AM plants maintain mutual symbiosis by regulating carbon metabolites and nitrogen metabolites under Pb stress is unclear.

We hypothesized that AM inoculation promotes heavy metal resistance by altering the synthesis and distribution of both carbohydrates and amino acids. Therefore, the aim of this study was to determine the effect of Pb stress and AM inoculation on carbohydrate and amino acid metabolism regulation by measuring chlorophyll fluorescence parameters, evaluating sugar-related gene expression, and measuring both carbohydrate and nitrogen metabolites. This study provides a new understanding of the balance of carbon and nitrogen metabolism in mycorrhizal plants under heavy metal stress.

2. Materials and methods

2.1. Experimental design

The experiment was performed via two AM fungi treatments (with or without AM fungi colonization) and two Pb concentrations (0 or 800 mg kg^{-1} substrate) as two factorial experiments. Each treatment contained four biological replicates. Due to the low biomass of *Medicago truncatula*, four randomly selected seedlings with four pots were combined and then considered as one biological replication. Plastic pots (10 cm *10 cm diameter * height, 785 cm^3 volume) were used to plant one *M. truncatula* seedling. Each pot contained 450 g sterilized substance (sand: soil = 1: 1). The sand was washed with tap water until the supernatant was clear. After drying, the sand was sterilized in the oven at 170 °C for 3 h. Soil was collected from the nursery garden of Northwest A&F University. The soil (loam, pH 8.6) contained 7.57 g kg^{-1} organic matter, 20.16 mg kg^{-1} Olsen phosphorus, 34.12 mg kg^{-1} available nitrogen, and 74.23 mg kg^{-1} rapidly available potassium. Soils were sterilized in the autoclave at 121 °C for 2 h. AM-treated seedlings were inoculated with 20 g inoculum at the beginning of planting. Nonmycorrhizal (NM)-treated seedlings were inoculated with 20 g of sterilized inoculum (Oven, 170 °C for 3 h). A 50 mL Pb solution (7.52 g L^{-1}) was added to the Pb-treated substances one time after the seedlings were cultivated for two weeks. The Pb stress lasted for 12 weeks. Twenty milliliters of modified Hoagland's nutrient solution (5 mM Ca (NO$_3$)$_2$, 5 mM KNO$_3$, 2 mM MgSO$_4$, 0.2 mM KH$_2$PHO$_4$, 46 μm H$_3$BO$_3$, 9 μm MnCl$_2$, 0.8 μm ZnSO$_4$, 0.3 μm CuSO$_4$, 0.1 μm H$_2$MoO$_4$, 18 μm FeNaEDTA) was added to each treatment once a week. *M. truncatula* seedlings were grown in the greenhouse at 28 °C/24 °C day/night temperature under 16 h daylight and 40–60 % humidity. Tap water was supplied every day throughout plant growth to maintain soil moisture.

2.2. Plant material and AM fungi inoculum

Seeds of *M. truncatula* (Jemalong A17) were kindly provided by Prof. Philipp Franken (Plant Physiology Department, Humboldt University of Berlin). Seeds of *M. truncatula* were sterilized by concentrated sulfuric acid for 10 min and washed by sterile water ten times. Sterilized seeds were placed in the wet filter paper at 4 °C for 1 d in the dark and then placed at 25 °C for 1 d in the dark. After 25 °C for 1 d in the light, the seeds were germinated, and uniformed seeds were selected for pot experiment. The AM inoculum of *Rhizophagus irregularis* (Bank of Glomales in China, No. BGC BJ09) was provided by Beijing Academy of Agriculture and Forestry Sciences (Beijing, China). The AM inoculum was propagated on maize for 8 months. The inoculum

consisted of the sandy substrate that contained spores (approximately 21 spores per gram), mycelium, and colonized root fragments.

2.3. Plant sampling and biomass measurement

Seedlings from each treatment were harvested 14 weeks after planting. Every 4 pots with 4 seedlings of each treatment were randomly merged into one sample as one biological replication. The shoots were cut, and the roots were separated. Fresh shoot and root biomass were weighed. Roots were scanned by a root scanner, and then parts of the roots were fixed with FAA solution (37 % formaldehyde: glacial acetic acid: 95 % ethanol, 9:0.5:0.5, v:v:v) for the measurement of AM colonization. The remaining samples were ground to powder using liquid nitrogen and stored at −80 °C for the following study.

2.4. AM colonization

Colonization was measured using the modified method of McGonigle et al. (1990). The decolorized root segments were placed parallel to the long axis of the slide and then covered with a transparent coverslip. Five slides were used for each sample. Another coverslip with a vertical line was placed over the transparent coverslip. All intersections between roots and the vertical line were counted. Total colonization = (hyphae + vesicles + arbuscules counted number)/total counted number. Arbuscular colonization = arbuscules counted number/total counted number.

### 2.5. Measurement of chlorophyll fluorescence parameters

Measurement of chlorophyll fluorescence was carried out on 14 week old seedlings. All the measurements were conducted at room temperature of 25 °C–27 °C. According to the Mini-Imaging-Pam manual, the saturation pulse method was used in the MINI-PAM (MINI-PAM, Waltz, Germany). Plants were darkened for 2 h prior to fluorescence measurements. The 3rd leaves from the top of seedlings without visible injury symptoms were selected for the measurements, and the area of the vein was avoided as the measuring. The maximum fluorescence (Fm) and initial fluorescence (Fo) yields were determined by the saturation pulse light (Zai et al., 2012). The actinic light was opened, and the saturation pulses light was opened every 30 s until 5 min. The maximal quantum efficiency of PSII (Fv/Fm), the maximum quantum yield in the light-adapted state (Fv'/Fm'), the actual quantum yield of PSII (ΦPSII), non-photochemical quenching (NPQ), photochemical quenching (qP) were calculated according to the following equations: Fv/Fm = (Fm-Fo)/Fm; ΦPSII = (Fm'-Fs)/Fm'; NPQ = (Fm-Fm')/Fm'; qP = (Fm'-Fs)/(Fm'-Fo').

2.6. Root system parameters

The fresh roots were scanned by a root scanner (Epson perfection, Japan). The scanned images of the roots were analyzed using WinRHIZO (Regent Instrument Inc.). The analytical protocol was to follow the software manual. The root surface (cm^2), root diameter (cm), root tips, root forks, total root length (cm), and root volume (cm^3) were measured and reported from the software (Pang et al., 2011).

2.7. Phosphorus and Pb contents

The dried samples (0.05 g) were digested by 10 mL HClO$_4$ + HCl (4:1) at 300 °C for 5 h. H$_2$O$_2$ was added after the brown smoke was produced. The digest solution was used to determine Pb content by atomic absorption spectrometry (PinAAcle500, America). Phosphorus content was determined by the method of Veneklaas et al. (2003).
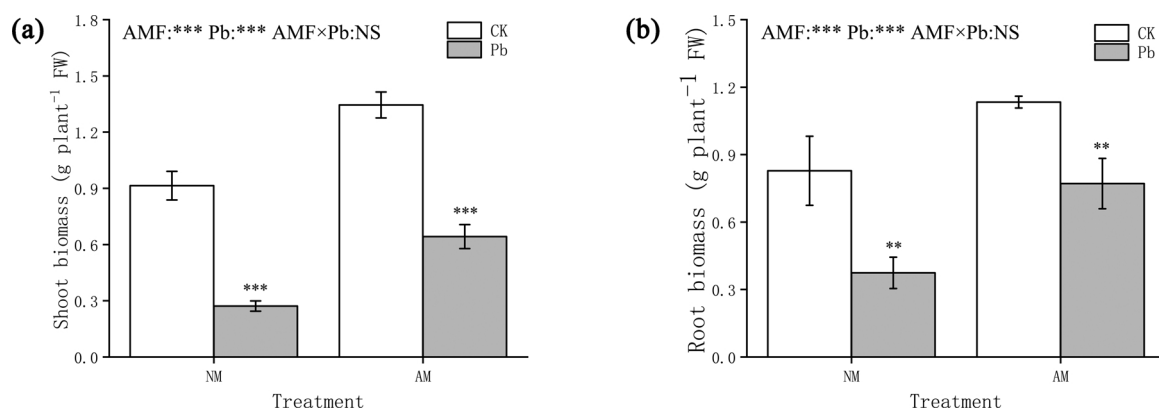
Fig. 1. (a–b) The fresh weight both in shoots and roots of *M. truncatula* plants inoculated with/without the AM fungi *R. irregularis* under Pb stress. The data are the means $\pm$ standard deviation (n = 4). Different symbols above the columns indicate a significant difference between Pb stress and non-Pb stress plant of fresh weight by Duncan's test (P < 0.05). NM = non-AM inoculation; AM = AM inoculation; CK = Non-Pb treatment; Pb = Pb treatment; AMF = AM fungi treatment; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, NS not significant.

2.8. RNA isolation and quantitative real-time PCR (qRT-PCR) analysis

The root samples (0.05 g) were ground in liquid nitrogen and used for total RNA extraction according to the manual of E.Z.N.ATM plant RNA kit (Omega Bio-Tek, Norcross, GA, USA). The RNA quality was evaluated by 1 % agarose gels stained with DuRed. RNA concentrations were determined by NanoDrop 2000 (Thermo Scientific, Pittsburgh, PA, USA). First-strand cDNA synthesis was obtained from 2 μg total RNA using the PrimerScript® First-strand cDNA Synthesis Kit (TaKaRa Bio, Dalian, China) following the manufacturer's instructions. Gene-specific primers for *MtSUT*s genes were designed as described in Supplemental Table 1S. The *M. truncatula* elongation factor 1-alpha gene was used as an internal control (Gomez et al., 2009). qRT-PCR was performed based on SYBR Green PCR. CF96X Real-time PCR system (Bio-Rad, Hercules, CA, USA) was used to perform the qRT-PCR experiments. The reaction volume was 20 μL containing 0.5 μL each gene-specific primer (10 μM), 2.0 μL of cDNA, 7 μL RNase-free H_2O, and 10 μL SYBR Green PCR master mix (Roche Diagnostics, Basel, Switzerland). qRT-PCR was performed under the following thermal cycles: 10 min at 95 °C and 40 cycles of denaturation at 95 °C for 15 s, annealing at 55 °C for 15 s, extension at 72 °C for 20 s, followed by heating from 60 to 95 °C at a rate of 0.5 °C per 10 s. The specificity of the primer pairs was indicated by the melting curve. The amplification efficiency of each primer pairs was measured by the method of the standard curve.

2.9. Extraction, derivatization, and analysis of M. truncatula leaf and root metabolites using GC-MS

The fresh samples were extracted in 1.4 mL of methanol, and 60 μL of the internal standard that contained 0.2 mg ribitol mL^{-1} water was added as a quantification standard (Roessner-Tunali et al., 2003). The homogenate was mixed using an ultrasonic cell crusher (Sonics, American) at 4 °C for 2 min with 40 % power, and then incubated at 70 °C for 20 min. Deionized water (1.4 mL) was added after incubation and then 0.75 mL chloroform was added to separate nonpolar metabolites. After centrifugation at 2200 g, the upper phase (methanol/water) was taken and dried in a vacuum dryer.

Residues were redissolved by 40 μL of 20 mg mL^{-1} methoxyamine hydrochloride in pyridine for 90 min at 37 °C. N-methyl-N-[tri-methylsilyl] trifluoroacetamide (60 μL) was added subsequently at 37 °C for 30 min. The GC–MS was performed on a TG-5MS column with 0.25-μm film thickness (Thermo, Massachusetts, USA) in TRACE 1310ISQLT (Thermo, Massachusetts, USA). The injection temperature was set at 230 °C, the interface at 250 °C, and the ion source temperature was 200 °C. Helium was used as the carrier gas. The measurement was performed under the following temperature program: 5 min of isothermal heating at 70 °C, followed by a 5 °C min^{-1} oven temperature ramp to 330 °C, and a final 5 min heating at 310 °C. Mass spectra were recorded at 2 scan s^{-1} with a mass-to-charge ratio of 50–600 scanning range. The concentrations of various metabolites were determined by the internal standard (ribitol).

2.10. Acid invertase, sucrose synthetase, and sucrose phosphate synthase activity

The powdered samples of roots were homogenized with an extraction buffer that contained 50 mM potassium phosphate buffer, 1 mM EDTA and 1 % polyvinylpyrrolidone (PVP). The homogenate was centrifuged at 14,000 g for 40 min at 4 °C. The supernatant was dialyzed and then used to determine the antioxidant enzyme activity. Total soluble protein was determined with G250 as the color agent, and bovine serum albumin was used as a standard, following the method of Sedmak and Grossberg (1977).

Acid invertase activity was determined by the production of reducing sugar content. The amounts of reducing sugar measurement were followed by the method of Miller (1959). Sucrose synthetase and sucrose phosphate synthetase activities were determined by the production of sucrose content. The amount of sucrose was followed by the method of Pavlinova et al. (2002).

2.11. Statistical analysis

Statistical analysis was performed using SPSS 19.0 statistical program (SPSS Inc., Chicago, lL, USA). The experimental data that used for ANOVA have complied with the normal distribution. Two-way ANOVA was conducted followed by Duncan's test when the ANOVA was significant. The cluster analysis and PLSD analysis were conducted by MetaboAnalyst 4.0 (Chong et al., 2018). Pearson's correlation analysis was made at the levels of 5 %.

3. Results

3.1. Biomass and AM colonization

AM inoculation greatly increased the shoot biomass and root biomass compared with the biomass of the NM plants (Fig. 1a, b). Pb stress significantly decreased the shoot biomass and decreased the root biomass compared with the biomass of the nonstressed plants.

Pb stress decreased AM colonization in AM roots. No mycorrhizal roots were observed in NM roots (Supplemental Table 2S).
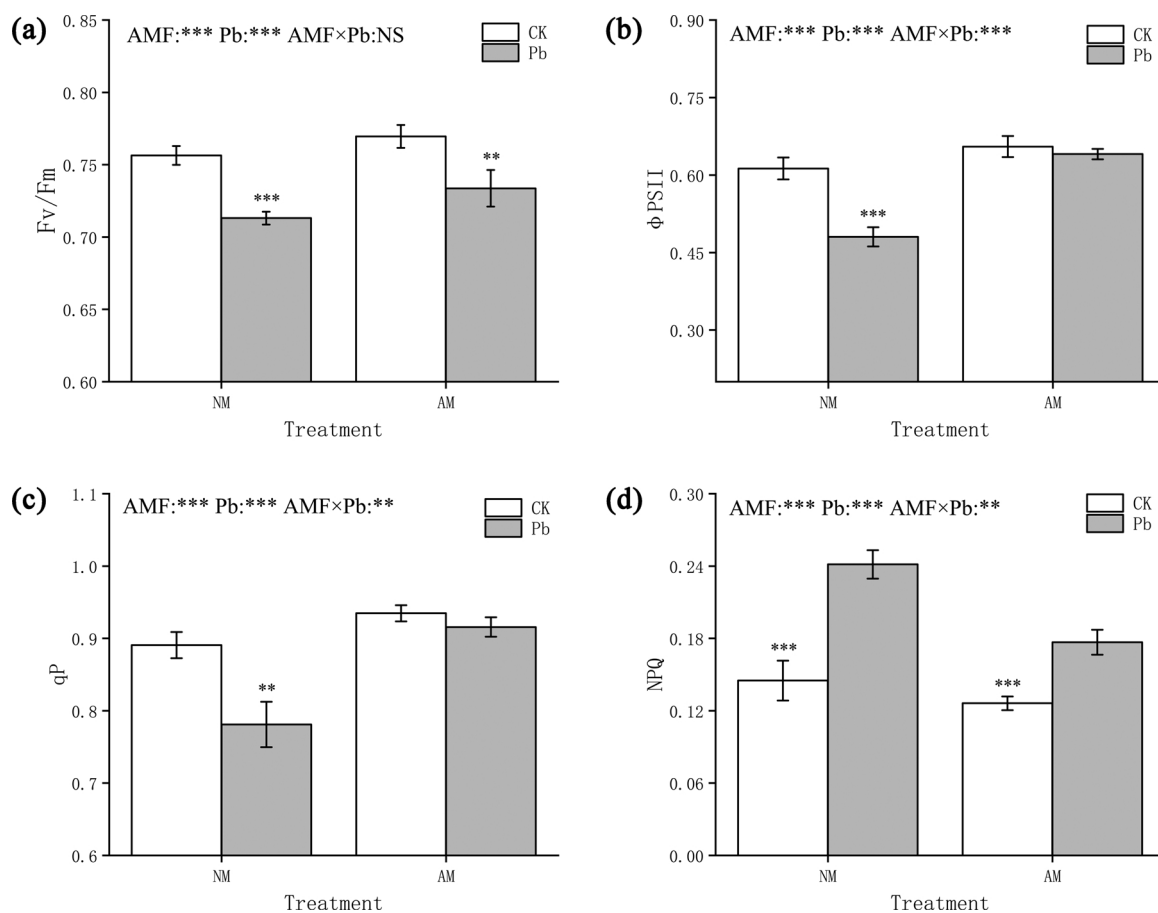
Fig. 2. (a–d) The chlorophyll fluorescence parameters of *M. truncatula* plants inoculated with/without the AM fungi *R. irregularis* under Pb stress. The data are the means ± standard deviation (n = 4). Different symbols above the columns indicate a significant difference between Pb stress and non-Pb stress plant of chlorophyll fluorescence parameters by Duncan's test (P < 0.05). The abbreviation is consistent with the above. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, NS not significant.

3.2. Chlorophyll fluorescence parameters

Pb stress significantly decreased the parameters of Fv/Fm and qP with or without AM inoculation (Fig. 2a, d). In NM plants, Pb stress decreased the ΦPSII and increased the NPQ compared to those of the nonstressed NM plants (Fig. 2b, c). In AM plants, Pb stress did not dramatically affect ΦPSII and qP. AM inoculation increased the parameters of Fv/Fm, ΦPSII, and qP with or without Pb stress. AM inoculation decreased NPQ compared to the NPQ of the NM plants.

3.3. Root morphology parameters

Pb stress significantly decreased the root surface, root forks, total root length, and root volume compared to those of the nonstressed plants (Fig. 3). Pb stress did not affect root diameter with or without AM inoculation. No significant influence of Pb stress on root length, root forks, or root tips was found in AM plants. Under Pb stress conditions, AM inoculation dramatically increased the root surface, total root length, root diameter, root volume, and root forks.

3.4. Phosphorus and Pb content

In NM plants, Pb stress decreased the phosphorus content in shoots (Fig. 4a). Pb stress did not affect the phosphorus content of shoots or roots in AM plants. AM inoculation greatly increased the phosphorus content both in shoots and roots compared to the phosphorous content in the NM plants.

Pb stress dramatically increased the Pb content both in shoots and roots (Fig. 4c, d). AM inoculation decreased the Pb content in roots. No

significant difference was found between the shoot Pb content of AM and NM plants.

3.5. Sucrose cleaving enzyme activity

Pb stress increased the acid invertase activity in roots (Fig. 5a). AM inoculation significantly increased the acid invertase activity, the sucrose synthetase activity, and the sucrose phosphate synthetase activity (Fig. 5a-c). Pb stress decreased the sucrose phosphate synthetase activity with or without AM inoculation.

3.6. Transcription levels of sucrose transporters in roots

Pb stress upregulated *MtSUT1-1* expression in AM roots but did not influence *MtSUT1-1* expression in NM roots (Fig. 6). Pb stress had no significant effect on *MtSUT2* transcription levels. Pb stress upregulated *MtSUT4-1* expression in roots. AM inoculation increased *MtSUT4-1* transcription with or without Pb stress.

3.7. Metabolites profiles of M. truncatula

From the GC–MS results, there are 122 kinds of polar metabolites that were identified (the complete data are presented in Supplemental Table 3S). The rotation time of the main metabolites is displayed in Supplemental Fig. 1S.

In shoots, Pb stress increased the content of organic acids (butanedioic acid, pyrogallol, threonic acid, 2-isopropylmalic acid, tartaric acid, dodecanedioic acid, shikimic acid, and galacturonic acid) and amino acids (valine, leucine, isoleucine, threonine, asparagine, aspartic
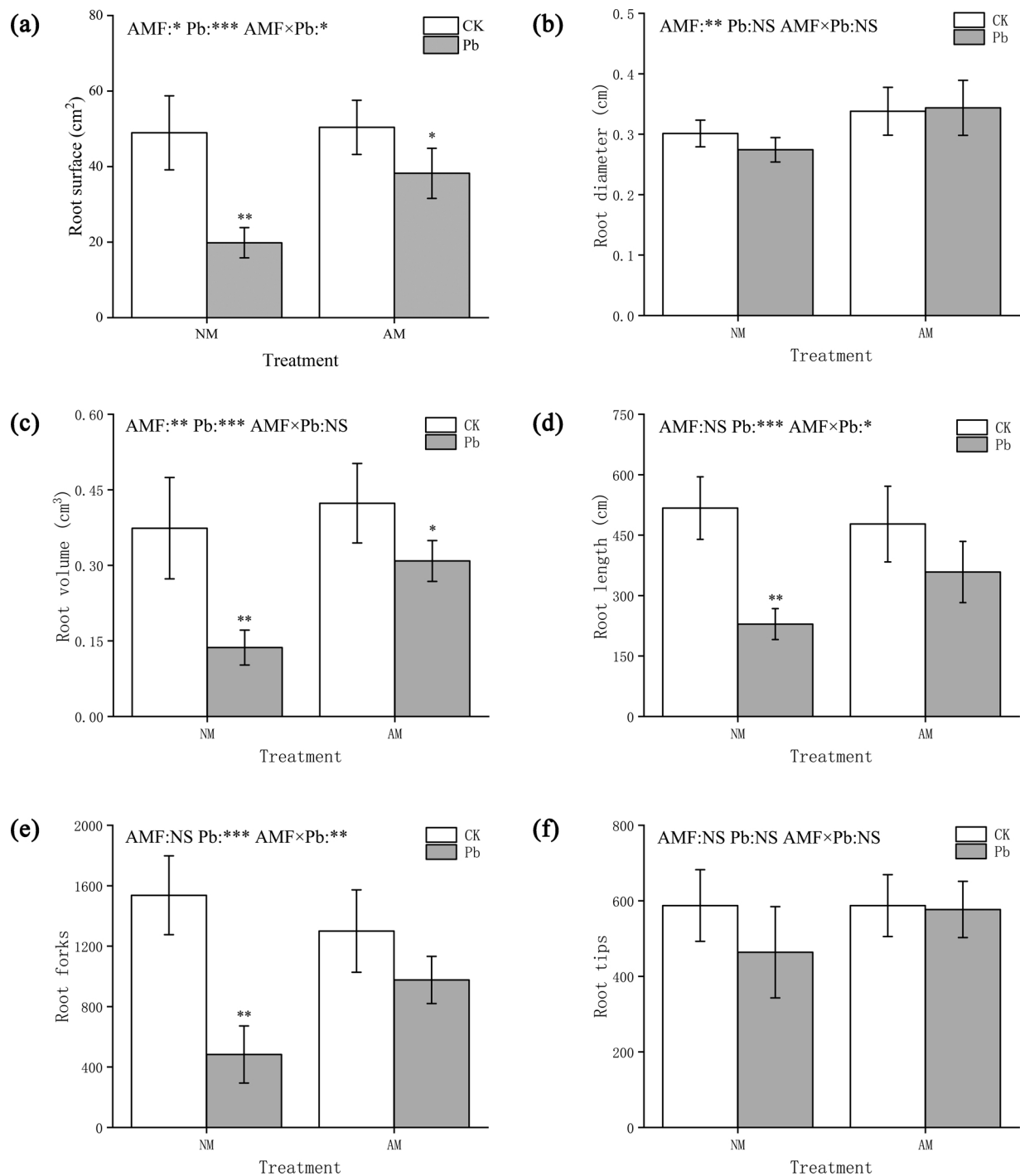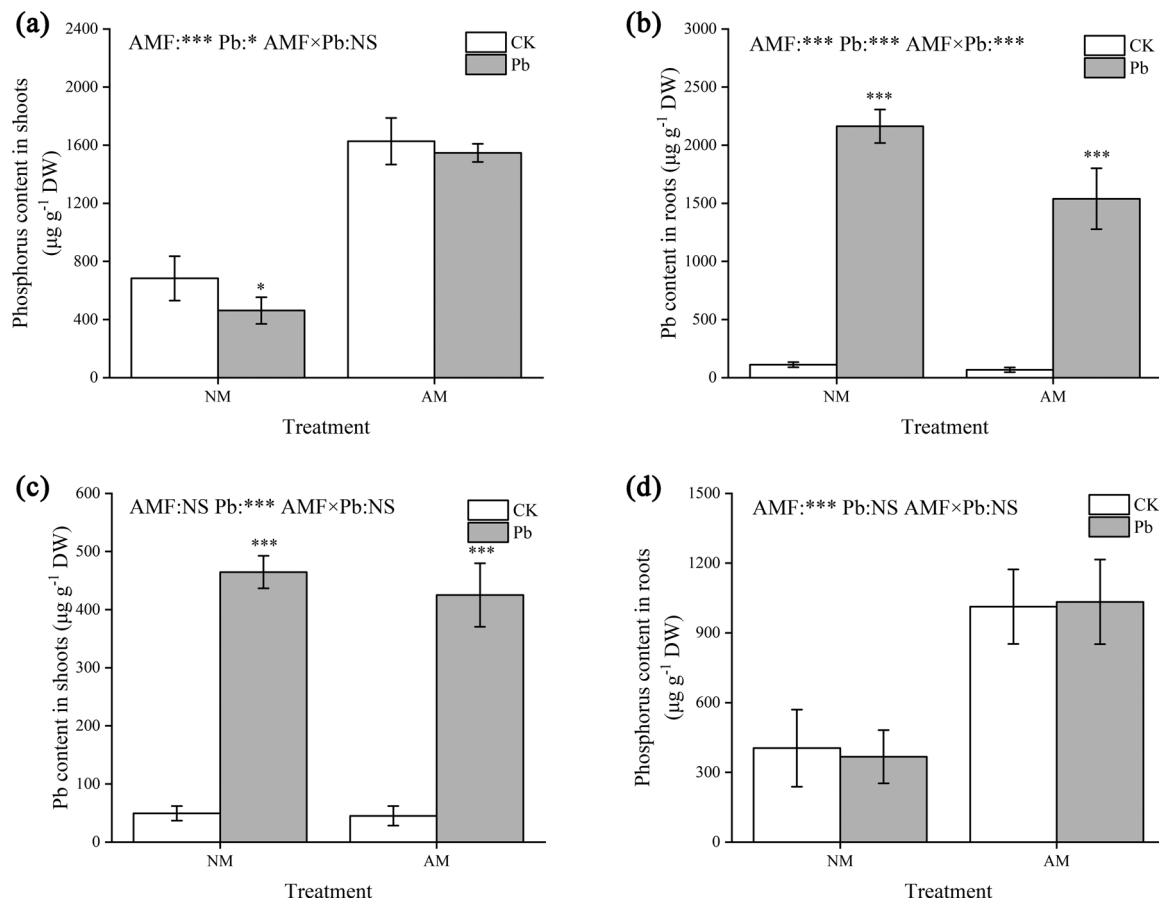
Fig. 3. (a–f) The root morphology parameters of *M. truncatula* plants inoculated with/without the AM fungi *R. irregularis* under Pb stress. The data are the means ± standard deviation (n = 4). Different symbols above the columns indicate a significant difference between Pb stress and non-Pb stress plant of root morphology parameters by Duncan's test (P < 0.05). The abbreviation is consistent with the above. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, NS not significant.

acid, glutamic acid, phenylalanine, cysteine, and lysine) (Fig. 7a). Pb decreased the sugars content (fructose, talose, sucrose, xylose, and glucose) in shoots (Table 1). AM inoculation increased the content of organic acids (4-hydroxybutanoic acid, salicylic acid, galactaric acid, 4-coumaric acid, isoferulic acid, shikimic acid, ascorbic acid, and 4-O-feruloylquinic acid) and amino acids (threonine, cysteine, norvaline) (Table 2) in shoots. Malic acid and sucrose content in leaves were decreased by AM inoculation and Pb stress.

In roots, AM inoculation increased the content of amino acids (aspartic acid and asparagine), sugars (glucose and sucrose), and organic acids (butanedioic acid, malic acid, salicylic acid, ribonic acid, hexonic

acid, gluconic acid, and 4-O-feruloylquinic acid). The content of 4-coumaric acid was decreased by AM inoculation and Pb stress in roots. Pb stress increased the content of amino acids (valine, leucine, iso-leucine, threonine, aspartic acid, asparagine, glutamic acid, phenyla-lanine, cysteine, norvaline) (Table 3), organic acids (threonic acid, 2-isopropylmalic acid, salicylic acid, 2-aminoadipic acid, and shikimic acid), and sugar (xylose) in roots. Pb stress decreased the sucrose content in roots (Table 1). The ratio of shoot/root of xylose and glucose was decreased by Pb stress. AM inoculation decreased the ratio of shoot/root of sucrose with or without Pb stress (Table 1).

Fig. 4. (a–d) The phosphorus and lead content of *M. truncatula* plants inoculated with/without the AM fungi *R. irregularis* under Pb stress. The data are the means ± standard deviation (n = 4). Different symbols above the columns indicate a significant difference between Pb stress and non-Pb stress plant of phosphorus or lead content by Duncan's test (P < 0.05). The abbreviation is consistent with the above. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, NS not significant.

3.8. Correlation analysis and PLSDA results

Correlation analysis showed that sucrose content was negatively related to asparagine content ($r = -0.602$, $P < 0.001$) (Supplemental Table 4S). Cysteine content was positively correlated with fructose content ($r = 0.575$, $P < 0.001$) and xylose content ($r = 0.417$, $P < 0.05$). Glucose content was positively correlated with valine content ($r = 0.530$, $P < 0.01$), leucine content ($r = 0.551$, $P < 0.01$), serine content ($r = 0.540$, $P < 0.01$), threonine content ($r = 0.678$, $P < 0.001$), and norvaline content ($r = 0.504$, $P < 0.01$).

In shoots, the *PLSDA* result showed that component 1 accounted for 15.4 % of the variance, and component 2 accounted for 36.3 % of the variance (Fig. 7). Samples of AM and NM treatments were separated by shoot metabolite contents. In roots, the PLSDA result showed that component 1 accounted for 23.1 % of the variance and component 2 accounted for 12.3 % of the variance. The treatments of AM inoculation without Pb stress and non-AM inoculation with Pb stress were separated by root metabolite contents.
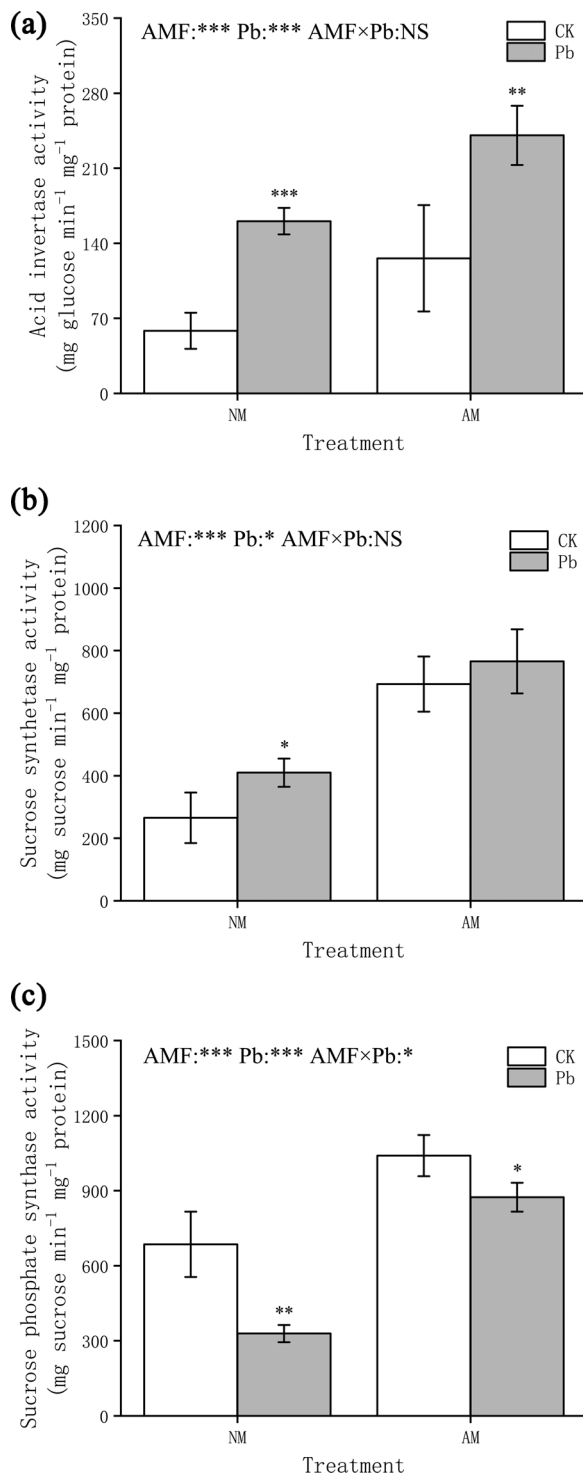
4. Discussion

4.1. AM fungi alleviated the Pb toxicity of M. truncatula

Due to the beneficial effects of AM fungi on host plants under abiotic stresses such as heavy metal stress (Zhang et al., 2019), drought (Xie et al., 2018), and salinity (Amanifar et al., 2019), combining plants with AM fungi to remediate soil heavy metal contamination has received increasing attention (Ma et al., 2019; Shi et al., 2019). In this study, Pb stress negatively affected AM and non-AM plant growth,

indicating that *M. truncatula* is sensitive to Pb toxicity. Some studies suggest that heavy metal pollution induces deficiencies in the absorption of nutrients (Cao et al., 2009; Khan et al., 2015). Moreover, heavy metal stress inhibits photosynthesis capacity, which negatively affects carbon fixation (Chandra and Kang, 2016; Sorrentino et al., 2018a,b). A similar result of Pb stress on phosphorus content and actual photosynthetic potential (ΦPSII) was also observed in this study. AM fungi improved host plant growth under Pb stress, which indicates that AM fungi could enhance heavy metal stress resistance. Under Pb stress, the AM plant maintained the transport of phosphorus to shoots and maintained photosynthetic activity. Similar improvements in phosphorus nutrient uptake and photosynthesis due to AM fungi have been observed under other abiotic stress conditions, such as drought (Hu et al., 2017) and salt-alkali stress (Lin et al., 2017). Heavy metal stress also results in oxidative damage. Many studies suggest that AM inoculation alleviates oxidative damage by increasing antioxidant enzyme activity and increasing the accumulation of antioxidant (Zou et al., 2015; Mollavali et al., 2016). In our previous study, AM inoculation stimulated the antioxidant activity in the roots of *M. truncatula* under two levels of Pb stress (Zhang et al., 2019), which means that AM inoculation may promote Pb resistance by stimulating antioxidant response. Therefore, AM fungi alleviate the Pb toxicity and increase the Pb resistance of *M. truncatula*.
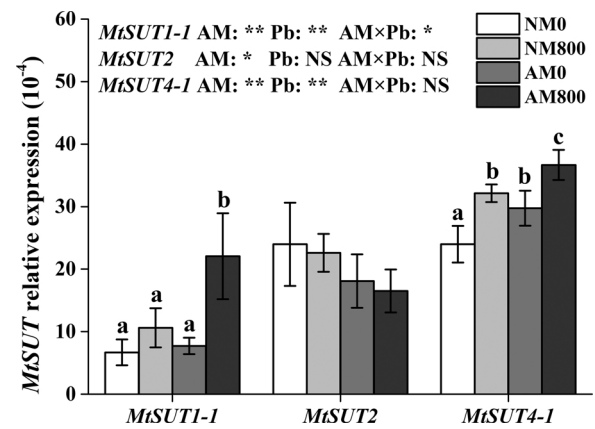
4.2. AM fungus promoted the transport of sucrose from shoot to root under Pb stress

The maintenance of mutualistic symbiosis between AM fungi and the host plant is primarily through the exchange of nutrient and carbon

(a) AMF:*** Pb:*** AMF×Pb:NS

(b) AMF:*** Pb:* AMF×Pb:NS

(c) AMF:*** Pb:*** AMF×Pb:*

Fig. 5. (a–c) The sucrose cleaving enzyme activity of *M. truncatula* plants inoculated with/without the AM fungi *R. irregularis* under Pb stress. The data are the means ± standard deviation (n = 4). Different symbols above the columns indicate a significant difference between Pb stress and non-Pb stress plant of sucrose cleaving enzyme activity by Duncan's test (P < 0.05). The abbreviation is consistent with the above. * P < 0.05, ** P < 0.01, *** P < 0.001, NS not significant.

MtSUT1-1 AM: ** Pb: ** AM×Pb: *
MtSUT2 AM: * Pb: NS AM×Pb: NS
MtSUT4-1 AM: ** Pb: ** AM×Pb: NS

Fig. 6. The relative expression of *MtSUT* in *M. truncatula* plants inoculated with/without the AM fungi *R. irregularis* under Pb stress. The data are the means ± standard deviation (n = 4). Different uppercase and lowercase above the columns indicate significant difference among the means of *MtSUT* relative expression by Duncan's test (P < 0.05). NM0 = non-AM inoculation in the absent of Pb treatment; NM800 = non-AM inoculation with 800 mg kg^{-1} Pb treatment; AM0 = AM inoculation in the absent of Pb treatment; AM800 = AM inoculation with 800 mg kg^{-1} Pb treatment. The abbreviation is consistent with the above. * P < 0.05, ** P < 0.01, *** P < 0.001, NS not significant.

plant may not be affected by Pb stress. In *Lycium barbarum*, AM-induced phosphate transporter 1 expression was not affected by drought stress (Hu et al., 2017). AM fungi significantly improved the phosphorus concentration in *Glycyrrhiza uralensis* under drought stress (Xie et al., 2018). AM roots had larger root surface area and higher levels of organic acids, which may benefit phosphorus absorption. Taken together, these results suggest that the host plant obtained sufficient phosphorus from the AM fungi under abiotic stress. According to the biological market theory, host plants should supply enough carbon metabolites to AM fungi. The process of carbon fixation in plants starts with photosynthesis. AM plants had higher photosynthesis potential under Pb stress than NM plants, indicating that AM alleviates the adverse impact of Pb stress. Certainly, the photosynthesis process in AM plants were disturbed by Pb stress, as seen by the performance of Fv/Fm and NPQ (Mishra et al., 2012). Therefore, Pb stress also had a negative effect on the carbon supply of AM plants. Under Pb stress, to maintain an equivalent carbon and nutrient trade, the host plants should enhance the transfer of carbon metabolites to mycorrhizal roots.

Glucose, xylose, fructose, talose, and sucrose are the primary carbohydrates in *M. truncatula*. In this study, an 84-day Pb stress period negatively regulated the content of the five major carbohydrates in shoots. Padmapriya et al. (2016) found that heavy metal-treated plant had a lower carbohydrate content than the control. Wu et al. (2017) observed that 71 days of drought stress decreased the leaf sucrose, glucose, and fructose content. However, in cucumber, salt stress stimulates the synthesis of carbohydrates in leaves (Zhu et al., 2016). The accumulation of carbohydrates in shoots may alleviate the imbalance of osmotic pressure caused by short-term salt stress. The carbohydrate content in the shoots showed a downward trend after 10 days of salt stress, indicating that long-term abiotic stress inhibits the synthesis of leaf carbohydrates. Interestingly, AM fungi decreased the sucrose content in shoots and decreased the shoot/root sucrose content under Pb stress. This result is inconsistent with the parameter of chlorophyll fluorescence. Sucrose is the main long-distance transport sugar in plants from source leaves to heterotrophic organs, such as the roots (Yadav et al., 2015). Boldt et al. (2011) suggested that AM plants preferentially distribute sucrose to mycorrhizal roots. Bao et al. (2019) observed that carbohydrates were allocated to AM roots in exchange for P under flooding conditions. We considered that AM *M. truncatula* may distribute more sucrose to mycorrhizal roots under Pb stress for nutrient exchange.
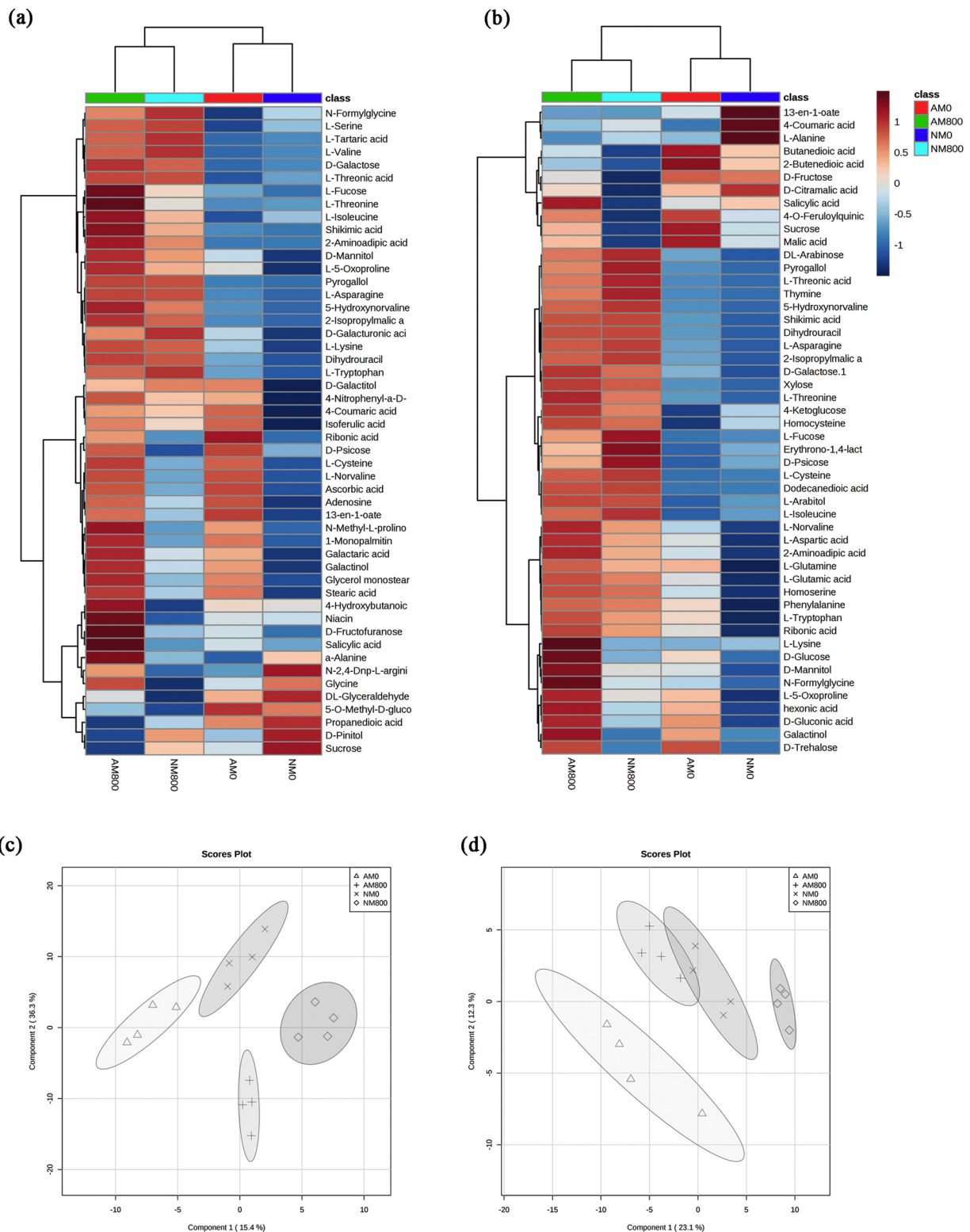
metabolites. According to biological market theory, the supply of carbon metabolites from the host plant and the nutrients from AM fungi are regulated through a feedback mechanism (Kiers et al., 2011). Pb stress had no effect on the phosphorus concentration in AM plants, indicating that the supply of phosphorus from AM fungi to the host

(a)

(b)

(c)

(d)

Fig. 7. (a), (b) The cluster analysis of metabolites both in shoots and roots of *M. truncatula* plants inoculated with/without the AM fungi *R. irregularis* under Pb stress. The analysis was conducted using data for significant different metabolites in shoots and roots. All the data were transformed $\log_{10}$ and samples were normalized by median. Blue rectangles indicate a decrease in metabolite content, and red rectangles represent an increase in metabolite content.The abbreviation is consistent with the above (c), (d) The principal component analysis of antioxidant-related parameters both in shoots and roots of *M. truncatula* plants inoculated with/without the AM fungi *R. irregularis* under Pb stress. The analysis was conducted using data for all metabolites in shoots and roots. Each icon represents a kind of treatment in this experiment. All the data were transformed $\log_{10}$. The abbreviation is consistent with the above (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Table 1

The carbohydrate components of *M. truncatula plants* inoculated with/without the AM fungi *R. irregularis* under Pb stress.

	Carbohydrate	NM0	NM800	AM0	AM800	P_{AMF}	P_{Pb}	$P_{AMF \times Pb}$
Shoot	Glucose (nmol g^{-1})	626.5 ± 193.2b	357.8 ± 39.7a	445.2 ± 104.1a	290.8 ± 127.8a	NS	**	NS
Shoot	Xylose (nmol g^{-1})	376.8 ± 65.2ab	343.5 ± 53.9ab	440.3 ± 87.0b	330.7 ± 22.9a	NS	*	NS
Shoot	Fructose (nmol g^{-1})	392.9 ± 262.9a	156.3 ± 90.5a	404.0 ± 116.0a	177.4 ± 88.5a	NS	*	NS
Shoot	Talose (nmol g^{-1})	253.9 ± 80.6bc	119.1 ± 48.1a	317.8 ± 104.5c	155.1 ± 53.8ab	NS	**	NS
Shoot	Sucrose (nmol g^{-1})	849.1 ± 171.9b	647.4 ± 147.1ab	594.4 ± 176.3a	425.7 ± 112.3a	**	*	NS
Root	Glucose (nmol g^{-1})	1.6 ± 1.1a	2.0 ± 0.4a	4.6 ± 3.4a	10.4 ± 4.3b	**	NS	NS
Root	Xylose (nmol g^{-1})	5.1 ± 2.7a	58.7 ± 32.6b	8.5 ± 6.8a	65.9 ± 12.8b	NS	***	NS
Root	Fructose (nmol g^{-1})	300.2 ± 108.7b	96.6 ± 14.3a	328.5 ± 142.1b	214.7 ± 84.0ab	NS	**	NS
Root	Talose (nmol g^{-1})	244.9 ± 94.3ab	133.5 ± 103.5a	305.4 ± 98.3b	197.6 ± 109.6ab	NS	NS	NS
Root	Sucrose (nmol g^{-1})	553.3 ± 115.3ab	402.4 ± 65.6a	810.3 ± 165.2b	635.1 ± 63.6c	**	*	NS
S/R	Glucose	497.4 ± 316.2b	183.0 ± 50.4ab	222.5 ± 288.5ab	34.6 ± 22.5a	NS	*	NS
S/R	Xylose	90.4 ± 48.0b	7.5 ± 4.3a	67.7 ± 27.7b	5.1 ± 0.7a	NS	***	NS
S/R	Fructose	1.53 ± 1.3a	1.6 ± 0.9a	1.3 ± 0.5a	0.9 ± 0.6a	NS	NS	NS
S/R	Talose	1.1 ± 0.4a	1.5 ± 1.0a	1.2 ± 1.1a	1.2 ± 1.1a	NS	NS	NS
S/R	Sucrose	1.5 ± 0.1b	1.6 ± 0.2b	0.7 ± 0.07a	0.7 ± 0.1a	***	NS	NS

The data are the means ± standard deviation (n = 4). Different letters indicate significant differences supported by Duncan's test (P < 0.05). NM0 = non-AM inoculation in the absent of Pb treatment; NM800 = non-AM inoculation with 800 mg kg^{-1} Pb treatment; AM0 = AM inoculation in the absent of Pb treatment; AM800 = AM inoculation with 800 mg kg^{-1} Pb treatment; S/R = The ratio of shoot/root of carbohydrate content; Pb = Pb treatment; AMF = AM fungi treatment; * P < 0.05, ** P < 0.01, *** P < 0.001, NS not significant.

Table 2

The amino acid content of *M. truncatula* shoots inoculated with/without the AM fungi *R. irregularis* under Pb stress.

Amino acid	NM0	NM800	AM0	AM800	P_{AMF}	P_{Pb}	$P_{AMF \times Pb}$
Alanine (nmol g^{-1})	34.7 ± 23.7a	22.8 ± 13.8a	28.5 ± 9.1a	11.2 ± 1.7a	NS	NS	NS
Valine (nmol g^{-1})	4.7 ± 1.8a	11.5 ± 3.4b	4.0 ± 1.2a	10.6 ± 5.3b	NS	**	NS
Leucine (nmol g^{-1})	1.0 ± 0.2a	1.6 ± 0.7ab	1.0 ± 0.2a	2.2 ± 0.8b	NS	*	NS
Proline (nmol g^{-1})	6.7 ± 5.9a	5.6 ± 5.1a	6.8 ± 7.1a	22.8 ± 26.7a	NS	NS	NS
Isoleucine (nmol g^{-1})	3.6 ± 1.1a	6.4 ± 1.8a	2.1 ± 1.0a	14.1 ± 6.6b	NS	**	*
Glycine (nmol g^{-1})	3.0 ± 1.8b	1.3 ± 0.3a	2.0 ± 0.6ab	2.9 ± 0.5b	NS	NS	*
Serine (nmol g^{-1})	13.1 ± 4.2ab	24.2 ± 8.4b	8.9 ± 2.4a	23.4 ± 10.7b	NS	**	NS
Threonine (nmol g^{-1})	5.4 ± 2.2a	6.6 ± 0.5a	5.1 ± 1.7a	11.5 ± 2.4b	*	**	*
Aspartic acid (nmol g^{-1})	5.7 ± 6.6a	17.6 ± 17.5ab	9.1 ± 5.5ab	43.3 ± 39.1b	NS	***	NS
Asparagine (nmol g^{-1})	174.2 ± 228.5a	989.0 ± 393.6b	108.5 ± 39.4a	989.3 ± 78.8b	NS	*	NS
Glutamic acid (nmol g^{-1})	14.3 ± 12.2a	44.6 ± 30.9ab	17.7 ± 15.2a	87.2 ± 66.5b	NS	**	NS
Phenylalanine (nmol g^{-1})	0.4 ± 0.1a	5.2 ± 4.0ab	1.4 ± 0.8a	15.3 ± 13.3b	NS	*	NS
Cysteine (nmol g^{-1})	1.7 ± 0.7a	2.3 ± 0.8a	4.5 ± 1.6b	5.2 ± 2.2b	**	NS	NS
Norvaline (nmol g^{-1})	6.6 ± 1.3a	9.1 ± 3.2a	20.1 ± 5.0b	20.5 ± 6.2b	***	NS	NS
Lysine (nmol g^{-1})	0.1 ± 0.1a	13.4 ± 7.4bc	1.2 ± 0.9ab	19.5 ± 14.6c	NS	**	NS

The data are the means ± standard deviation (n = 4). Different letters indicate significant differences supported by Duncan's test (P < 0.05). The abbreviation is consistent with the above. * P < 0.05, ** P < 0.01, *** P < 0.001, NS not significant.

When sucrose is transferred to roots, it can be released to the apoplast, and then it may be retrieved into adjacent root cells by sucrose transporters or may be hydrolyzed by invertase (Yadav et al., 2015). SUT1 is considered to unload sucrose in the sink tissue of the mature plant, and SUT4 contributes to sucrose transport in AM roots (Doidy et al., 2012). AM fungi upregulated the transcription levels of *MtSUT1-1* and *MtSUT4-1* under Pb stress, which supported the hypothesis that the decrease in sucrose in AM shoots may be caused by the transport and

Table 3

The amino acid content of *M. truncatula* roots inoculated with/without the AM fungi *R. irregularis* under Pb stress.

Amino acid	NM0	NM800	AM0	AM800	P_{AMF}	P_{Pb}	$P_{AMF \times Pb}$
Alanine (nmol g^{-1})	17.5 ± 3.5b	4.8 ± 1.7a	4.3 ± 2.2a	3.7 ± 2.9a	***	***	**
Valine (nmol g^{-1})	2.2 ± 0.4ab	2.5 ± 0.8b	1.4 ± 0.4a	2.9 ± 0.8b	NS	*	NS
Leucine (nmol g^{-1})	0.5 ± 0.3ab	0.7 ± 0.1ab	0.4 ± 0.1a	0.7 ± 0.2b	NS	*	NS
Proline (nmol g^{-1})	1.5 ± 0.5a	2.1 ± 0.8a	1.3 ± 1.5a	5.8 ± 5.8a	NS	NS	NS
Isoleucine (nmol g^{-1})	1.2 ± 0.3a	2.7 ± 0.6b	1.0 ± 0.3a	2.4 ± 0.9b	NS	***	NS
Glycine (nmol g^{-1})	1.0 ± 0b	0.5 ± 0.1a	0.6 ± 0.1a	0.6 ± 0.1a	NS	NS	*
Serine (nmol g^{-1})	2.4 ± 0.3a	2.2 ± 0.3a	2.5 ± 1.2a	3.1 ± 0.5a	NS	NS	NS
Threonine (nmol g^{-1})	1.7 ± 0.3a	2.9 ± 0.3b	1.9 ± 0.8a	3.3 ± 0.3b	NS	**	NS
Aspartic acid (nmol g^{-1})	4.6 ± 0.9a	24.8 ± 9.9b	15.3 ± 5.4ab	43.9 ± 18.7c	*	**	NS
Asparagine (nmol g^{-1})	116.5 ± 43.5a	939.2 ± 292.2b	214.9 ± 159.5a	794.3 ± 108.2b	NS	***	NS
Glutamic acid (nmol g^{-1})	11.9 ± 5.8a	59.9 ± 14.2ab	37.4 ± 23.3b	73.5 ± 10.2c	*	***	NS
Phenylalanine (nmol g^{-1})	0.2 ± 0.1a	10.8 ± 6.6b	4.0 ± 2.9ab	10.6 ± 8.6b	NS	*	NS
Cysteine (nmol g^{-1})	0.5 ± 0.1a	2.1 ± 1.4b	0.5 ± 0.1a	1.6 ± 0.5ab	NS	**	NS
Norvaline (nmol g^{-1})	4.7 ± 0.5a	13.2 ± 3.2bc	8.6 ± 2.6ab	19.4 ± 8.4c	NS	**	NS
Lysine (nmol g^{-1})	0.1 ± 0.1a	0.1 ± 0.1a	0.04 ± 0.03a	0.9 ± 0.9b	NS	NS	NS

The data are the means ± standard deviation (n = 4). Different letters indicate significant differences supported by Duncan's test (P < 0.05). The abbreviation is consistent with the above. * P < 0.05, ** P < 0.01, *** P < 0.001, NS not significant.

unloading of sucrose from shoots to mycorrhizal roots. Pb stress stimulated the decomposition of sucrose in roots by enhancing sucrose synthetase activity and acid invertase activity. Therefore, the sucrose content in the root was decreased by Pb stress. AM fungi improved the sucrose cleaving ability of the plant, which suggested the higher carbohydrate demand in AM roots under Pb stress. In citrus, Wu et al. (2007) found that mycorrhizal seedlings had lower shoot/root sucrose under drought stress. According to proteome data, AM *M. truncatula* resisted cadmium stress by expending sucrose in roots (Aloui et al., 2017). In wheat roots, AM fungi up-regulated sucrose synthesis to resist drought stress (Bernardo et al., 2017). These results indicate that AM plants may need more sucrose to be distributed in roots to survive Pb stress conditions.

4.3. AM fungus promoted the accumulation of minor amino acids in shoots under Pb stress

Although AM fungi have been considered to mainly benefit phosphorus absorption (Sawers et al., 2017), many studies have also suggested that AM fungi stimulate nitrogen fixation and assimilation (Hodge and Storer, 2015; Püschel et al., 2017). The inorganic nitrogen taken up from the soil is either assimilated into amino acids in the roots or transported to photosynthetic tissue (Yadav et al., 2015). In this study, Pb stress promoted the primary amino acids content in both roots and shoots. Exogenous application of silicon could improve alkaline stress by increasing total free amino acids (Abdel Latef and Tran, 2016). AM-inoculated wheat had higher levels of free amino acids than non-AM wheat when exposed to salinity (Talaat and Shawky, 2014). Taken together, these results suggest that the accumulation of amino acids is beneficial for Pb stress resistance.

However, AM fungi only promoted several minor amino acids (low concentration in plants), such as cysteine, in shoots. Phytochelatins and metallothioneins are cysteine-rich heavy-metal-binding protein molecules (Cobbett and Goldsbrough, 2002). Higher cysteine accumulation in AM roots led to greater heavy metal resistance by promoting the synthesis of phytochelatins and metallothioneins. In wheat, minor amino acids accumulated under salinity and high light stress, which indicated their key role in stress resistance (Woodrow et al., 2017). Fritz et al. (2006) suggested that the minor amino acids increase was accomplished by the increase in carbohydrates. Therefore, we considered that AM fungi may improve Pb stress resistance by minor amino acids accumulation.

Correlation analysis revealed that sucrose content was negatively correlated with primary amino acids such as asparagine and glutamic acid. This confirmed that sucrose transport to roots stimulates primary amino acids transport to shoots. Fellbaum et al. (2012) observed carbon and nitrogen exchange at the mycorrhizal interface, indicating that forms of carbon such as sucrose that were transferred to AM fungi stimulated nitrogen uptake. In both compartmental systems and soybean/maize intercropping systems, exogenous nitrogen application stimulated photosynthesis and carbon fixation in AM plants (Tomè et al., 2015; Wang et al., 2016). According to our results, AM plants transfer large amounts of sucrose to roots, which may benefit nitrogen absorption. Through shading experiments, AM fungi preferentially supply nutrients to nonshaded plants (Fellbaum et al., 2014). However, *R. irregularis* also supplied shaded plants with sufficient nitrogen. Zhang et al. (2015) found that plant carbon limitation had little effect on nitrogen transfer in AM *Plantago lanceolata*. These results indicate that the effect of photosynthetic enhancement of AM plants on root nitrogen assimilation is weaker than the effect of root nitrogen uptake on photosynthesis. The minor amino acids are positively correlated with the monosaccharide content, suggesting that sucrose cleaving promotes minor amino acids accumulation. Taken together, we considered that AM fungi increasing minor amino acids accumulation is an efficiency mechanism for Pb stress resistance.

4.4. Conclusion

To our knowledge, this is the first study analyzing the effect of AM fungi on the contents of carbon and nitrogen organic metabolites under Pb stress, which provided new evidence about the Pb stress-tolerance mechanism of AM plants. The photosynthesis potential was protected by AM inoculation. AM inoculation decreased the sucrose contents in shoots but increased the sucrose contents in roots. The accumulation of minor amino acids was enhanced by AM inoculation. AM inoculation stimulated phosphorus absorption. All in all, we considered that the exchange between carbohydrates and nutrients was not inhibited by Pb stress. The accumulation of minor amino acids in shoots and sucrose in AM roots might explain why AM plants perform better under Pb stress conditions.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

CRediT authorship contribution statement

Xiangyu Zhang: Conceptualization, Methodology, Writing - original draft, Writing - review & editing. **Huijuan Zhang:** Investigation. **Yongxin Zhang:** Software. **Yaqin Liu:** Visualization. **Haoqiang Zhang:** Resources. **Ming Tang:** Supervision, Funding acquisition, Project administration.

Declaration of Competing Interest

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

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.envexpbot.2019.103950.

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