



Fungal Endophyte *Phomopsis liquidambari* Improves Iron and Molybdenum Nutrition Uptake of Peanut in Consecutive Monoculture Soil

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Received: 29 May 2018 / Accepted: 3 January 2019 / Published online: 5 February 2019
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Abstract

Iron (Fe) and molybdenum (Mo) are essential trace elements for plants, especially for peanut. However, peanut planted in consecutive monoculture soil usually suffers from malnutrition. Thus, the objective of this study was to investigate the effects of the fungal endophyte *Phomopsis liquidambari* on Fe and Mo nutrition uptake of peanut in consecutive monoculture soil. Plant growth parameters, physiological parameters, the accumulation of Fe and Mo elements, gene expression related to absorption and transformation of Fe and Mo *in planta*, and the low-molecular-weight organic acids (LMWOAs) concentration in rhizosphere soil were analyzed by greenhouse pot experiments. The results showed that the application of *P. liquidambari* improved growth parameters, chlorophyll content, nitrate reductase (NR) activity, and Fe and Mo nutrition of peanut. Furthermore, LMWOAs in peanut rhizosphere soil and the expression levels of *AhFROI*, *AhIRT1*, and *AhMOT1* were significantly changed by the application of fungal endophyte. These findings indicated that the improvement of Fe and Mo nutrition in peanut was mostly derived from the promoting growth function of *P. liquidambari* and the regulation of peanut-*P. liquidambari* symbiosis with rhizosphere LMWOAs and Fe- and Mo-related gene expression in continuous monoculture soil.

Keywords Fungal endophyte · Peanut · Iron · Molybdenum · Nutrition uptake · Consecutive monoculture soil

1 Introduction

Peanut (*Arachis hypogaea* L.), the main economic and oil crop in China, accounts for approximately 60–85% of the dry farming area in recent years (Tahir et al. 2016). Peanut is commonly monocropped continuously for over 10 years to gain the maximum profit (Dai et al. 2013). Unfortunately, long-term consecutive monoculture has led to a series of soil problems, such as barren soil, imbalance of nutrient elements, declines in crop yield, and increased numbers of soil-borne pathogens. These problems can be generally described as “soil sickness” or “replanting disease” (Li et al. 2016). Li et al.

(2012) found that a decline in the activity of urease and sucrose resulted in a reduction of the peanut yield in consecutive monoculture soil. In addition, soil mineral nutrient shortages or barrenness can also be a main constraint on plant morphogenesis. In a continuous monoculture system, the degradation of the soil structure and the imbalance of soil nutrient components may be the main causes of plants dwarfing and chlorosis (Li et al. 2016).

The peanut growth process requires the absorption of a variety of elements, especially Fe and Mo. As legumes, peanuts have the capacity to fix dinitrogen (N₂) by establishing mutualistic symbiosis with compatible rhizobia (mainly from the genus *Bradyrhizobium*) (Zhang et al. 2017). Molybdenum-iron protein is the active site of nitrogenase (Keable et al. 2017). Previous studies have demonstrated that Fe deficiency causes serious chlorosis, inhibiting the growth of peanut seedlings and decreasing the concentration of soluble Fe and chlorophyll in peanuts at the same time (Kong et al. 2015). Peanut has developed a reduction-based strategy in response to Fe deficiency, but physical and chemical pretreatment methods can mod-

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ulate and enhance the catalytic capabilities of iron oxides in soils (Pizarro et al. 2018). The acidification of rhizosphere soil was shown to be the first step in dissolving soil trace elements (Wang et al. 2016); then, Fe(III)-chelate is reduced to Fe(II) by ferric reductase oxidase (FRO), and the Fe(II) is absorbed by the iron-regulated transporter (IRT) (Robinson et al. 1999). In addition, *NRAMP* genes code for a number of proteins and some of the *NRAMP* transport proteins are capable of transporting metal ions, including iron. In *Arabidopsis thaliana*, the iron transport process involves the *IRT1* and *FRO2* genes (Curie et al. 2000). Mo is taken up by plants through membrane transporters in the dissolved form as molybdate (MoO_4^{2-}), and transport proteins coded by *MOT* genes play multiple functions in Mo uptake (Ide et al. 2011). Tang et al. (2017) designed experiments with citric acid, nitric acid, and ethylene diamine tetraacetic acid to recover rare earth elements containing mainly Fe, Mn, Al, Si, and Pb. Pires et al. (2007) reported that there was a positive relationship between rhizosphere organic acids and utilizable trace element. Similarly, a deficiency of Mo has threatened plant growth and development. Thus, Fe and Mo concentrations in soil play key roles in plant growth and metabolism.

To alleviate the contradiction between soil element deficiency in continuously cropped soil and peanut normal growth, various methods have been used. Recently, using biological methods to enhance plant growth and soil element uptake in barren soil has aroused the attention of researchers. *Phomopsis liquidambari*, which is a plant growth-promoting rhizobacterium (PGPR), mainly colonizes the roots of plants, such as rice and peanut (Zhang et al. 2016). Further research found that the endophyte *P. liquidambari* has the ability to establish a mutualistic relationship with rice and improve nitrogen uptake and nitrogen metabolism in rice plants (Yang et al. 2014). Subsequently, Zhang et al. (2017) demonstrated that the fungal endophyte *P. liquidambari* improves the efficiency of symbiosis between peanuts and *Bradyrhizobium* and promotes peanut nodulation and growth under continuous monoculture conditions.

Therefore, we wanted to explore whether *P. liquidambari* is related to the absorption of Fe and Mo by peanut in consecutive monoculture soil. In the present study, soil from peanut consecutive monoculture for 10 years was used, and we carried out pot experiments to obtain peanut with or without endophyte *P. liquidambari* colonization, an artificial climate incubator. This study evaluated the growth parameters of peanut and the factors associated with the absorption of Fe and Mo in consecutive monoculture soil. The objective was to investigate the effects of fungal endophyte *P. liquidambari* application on peanut growth and the potential mechanism of Fe and Mo uptake under consecutive monoculture conditions for agricultural purposes.

2 Materials and Methods

2.1 Fungal Strain

The fungal endophyte was isolated from the inner bark of *Bischofia polycarpa*, and identified as *Phomopsis liquidambari* (Shi et al. 2004). It was stored at 4 °C on potato dextrose agar (PDA, containing 200 g potato extract, 20 g glucose, and 20 g agar, in 1 L water, pH 7.0). *Phomopsis liquidambari* was first activated in 100 mL potato dextrose broth (PDB, containing 200 g potato extract and 20 g glucose, in 1 L water, pH 7.0) in an Erlenmeyer flask (250 mL) for 48 h at 180 rpm in an orbital shaker at 28 °C. To evaluate the dry mycelia weight, 10 mL of culture broth was removed; the mycelia were washed twice with sterile distilled water, and then dried in an oven at 60 °C to a constant dry weight. To prepare the fungal suspension for inoculating germinating seeds, a total of 0.21 g dry weight fungal mycelia was collected and washed twice with sterilized distilled water, and then diluted with sterilized water to a final volume of 50 mL.

2.2 Soil in the Experiments

Peanut continuous cropping soils were collected from the Ecological Experimental Station of Red Soil, Chinese Academy of Sciences, Yujiang, Jiangxi province (28°13' N, 116°55' E). The region's 50-year mean annual precipitation is 1750 mm, with rainfall generally concentrated from April to late June. The basic chemical properties of the soils were as follows: pH (1:5 H₂O) 5.6, organic matter 13.35 g kg⁻¹, total N 0.75 g kg⁻¹, total P 0.75 g kg⁻¹, total K 8.85 g kg⁻¹, alkali-hydrolyzable N 17.50 mg kg⁻¹, rapidly available P 62.67 mg kg⁻¹, and rapidly available K 260.64 mg kg⁻¹. The soils for the experiment were air-dried and mixed to homogeneity, sieved (2-mm mesh) to remove plant tissues. All peanut seedlings were cultivated in sterilized soil to avoid the interference of soil indigenous microbes. And the sterilized soil was weighed 1.5 kg split charging to per plastic pot (15 cm diameter, 13.5 cm high).

2.3 Plant Growth Conditions and Inoculation

The peanut used in pot the experiments is a common cultivar grown in Jiangxi province, southern China, named Guanhua-5. The peanut seeds were placed in 75% ethanol for 5 min, rinsed twice with sterile water, sterilized for 5 min in 10% NaClO, and then rinsed six times in sterile distilled water. The sterilized seeds and the last washing water were placed on PDA plates and cultivated for 5 days at 28 °C as a sterility check (Feng et al. 2006). The thoroughly sterilized seeds were randomly divided into two groups. For the endophyte-inoculated group (E+ treatment), 100 seeds were soaked in a 200-mL fungal suspension. The endophyte-non-inoculated

group (E⁻ treatment, control) was treated with 200 mL sterilized deionized water. For the pot experiments, seeds were germinated and grown in a growth chamber at 28 °C for 16-h days/25 °C 8-h nights, with light at 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and 70% relative humidity. After germination, the peanuts were transplanted into the plastic pot with the sterilized continuous cropping soil. To achieve maximum benefits, after 5 days, 30 mL *P. liquidambari* inoculums (equivalent to 0.12 g dry weight fungal mycelia) was added to the E⁺ plant rhizosphere soil, and for the control, we added 30 mL sterilized water instead of the inocula (the E⁻ treatment). Each treatment was replicated 30 times.

2.4 Sample Collection and Preparation

Plant samples and rhizosphere soil were collected from each treatment and used for analysis of the physiological and biochemical indexes at 5, 10, 20, 30, and 45 days after *P. liquidambari* inoculation. Each period samples included six peanut plants and plant rhizosphere soil (0–3 mm from the root surface into the soil), and three fresh plants were immediately frozen separately in liquid nitrogen and stored at –80 °C, mainly used to test chlorophyll contents, nitrate reductase activity, root vitality, and relative expression levels of genes involved in Fe and Mo uptake. After measuring plant growth parameters, the other three fresh plants were placed in an oven at 105 °C for 30 min to inactivate enzymes and then dried at 70 °C to a constant weight. The dry weights were recorded; the dried samples were milled to pass through a sieve (1-mm mesh), mainly used to test the concentration of Fe and Mo in roots, stems, and leaves of peanuts. Rhizosphere soil stored at 4 °C used to test low-molecular-weight organic acids.

2.5 Analysis of Peanut Root Vitality

Root activity was analyzed by the α -naphthylamine oxidation method (Tatsumi and Kono 1983). In brief, 500 mg fresh root was immersed in a 20-mL solution of 50 $\mu\text{g mL}^{-1}$ α -naphthylamine and 0.1 mol L⁻¹ pH 7.0 phosphate buffer, and the same solution without roots was arranged as the control. In the dark at 25 °C after 60 min, 2 mL solution was added to the reaction mixed solutions containing 10 mL water, and 1 mL 1% sulfanilic acid and 1 mL 0.01% sodium nitrite were added to the reactions. The absorbance of the mixed solution at 510 nm was determined using a spectrophotometer. Root activity was expressed as α -naphthylamine oxidation intensity. Root vitality = amount of α -naphthylamine oxidation (μg) fresh root weight (g)⁻¹·time (h)⁻¹.

2.6 Determination of Total Iron and Molybdenum in Peanut Plants

After homogenization of the peanut samples mentioned above, 400 mg dried peanut leaves, stems, and roots from E⁻ and E⁺ plants were transferred to a PTFE vessel for digestion. The digestion process followed the method described by Alam et al. (2003). After digestion, the digested samples were transferred into a volumetric flask and diluted to 10 mL with deionized water. Thereafter, all samples were determined using inductively coupled plasma atomic emission spectroscopy (Prodigy, Leeman, USA).

2.7 Determination of Chlorophyll Content in Peanut Leaves

Fresh leaves (200 mg) were ground to a fine powder in liquid nitrogen before adding 10 mL 95% alcohol (v/v). The homogenates were incubated at –20 °C for 24 h and then centrifuged at 10,000g and 4 °C for 10 min. The supernatant was used to determine the chlorophyll content. The absorbance of the solution at 665 and 649 nm was determined using a spectrophotometer, and chlorophyll a and b levels were calculated using the extinction coefficients and the equations given by Porra et al. (1989).

2.8 Nitrate Reductase (NR) Activity in Peanut Leaves

To assay nitrate reductase activity, 500 mg fresh peanut leaves were washed with running water. Following the instructions of the Nitrate Reductase Assay Kit (Jiancheng, Nanjing, China), fresh leaves were induced in an inducer for 2 h and then ground in a prechilled mortar and pestle on ice in 4.5 mL of extraction buffer. The mixture was centrifuged at 3,500 rpm at 4 °C for 10 min, and then 0.2 mL supernatant was added to the reaction mixture. The mixture was incubated at 37 °C for 30 min in the dark. The 1-mL color-developing agent was added to the mixture, gently mixed, and then centrifuged at 3500 rpm for 10 min. One unit of NR activity was calculated as $\text{min}^{-1} \text{g}^{-1}$ protein catalyze μg^{-1} substrate in A540 nm.

2.9 Quantitative Real-Time PCR Analysis

Total RNA from peanut roots was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The RNA was treated with DNaseI (Invitrogen) to remove genomic DNA, reverse-transcribed using the PrimeScript™ II 1st strand cDNA Synthesis Kit (TaKaRa, Tokyo, Japan), and then subjected to real-time PCR analysis using gene-specific primers. PCR amplification was conducted with an initial step at 95 °C for 30 s followed by 35 cycles of denaturation at 95 °C for 5 s, annealing at 60 °C for 40 s, and extension at 72 °C for 30 s. Real-time PCR was carried out in a StepOnePlus™ Real-Time

PCR System (ABI, Foster City, CA, USA). The relative expression of the target genes was calculated by the $\Delta\Delta C_t$ method (Livak and Schmittgen 2012). The *ubiquitin* gene was used as an internal reference for calculating relative transcript abundance (Luo et al. 2005). The RT-PCR reaction system was optimized so that the amplification efficiencies of each target gene and reference gene were approximately equal. Each reaction was repeated at least three times to assess the reproducibility.

2.10 Determination of LMWOAs in Peanut Rhizosphere Soil

The extraction of LMWOAs in rhizosphere soil and HPLC analysis were performed using the method described by Ding et al. (2006). Potassium fluoride (1.0 M, pH 4.0) was applied as an extractant and mixed with 0.1 M sodium hydroxide to sufficiently extract the LMWOAs. The sample was separated through a C_{18} (4.6 mm \times 250 mm \times 5 μ m, Agilent Technologies, USA) chromatographic column at 25 °C and detected at a 220-nm wave length with a mobile phase of 95% 10 mM KH_2PO_4 (pH 2.8)/5% methanol (v/v). The flow rate was 0.8 mL min^{-1} , and the injection volume was 20 μ L. The HPLC system used in this study was the 1100 series RP-HPLC system of Agilent Technologies, USA. All of the samples and solutions of the mobile phase were filtered through a 0.45- μ m filtration membrane and vacuum degassed before entering the chromatographic column. The detection limit was defined as a ratio of 3 for signal-to-noise (S/N), and all values reported for LOD are based on peak area.

2.11 Statistical Analyses

Data were subjected to analysis of variance (ANOVA). All analyses were conducted using the program SPSS 13.0 (SPSS, Chicago, IL, USA). All data shown are average values of three biological replicates and standard error (SE). Data were considered to be significantly different at $P < 0.05$ by using one-way ANOVA and Tukey's test.

3 Results

3.1 Seedlings Growth Parameters

In this study, peanuts were planted in pots with sterilized continuous cropping soil to evaluate the effects of endophyte *P. liquidambari* on peanut growth parameters. The results showed that aboveground height, root length, root vitality, and seedling dry weight were increased to varying degrees by *P. liquidambari* application. Compared with the E⁻ treatment, the aboveground height of the E⁺ treatment was significantly increased by 25.66%, 15.71%, 13.05%, and 28.47% at

5, 20, 30, and 45 DAI, respectively, when compared with the E⁻ treatment (Table 1). However, no significant difference was noticed between the two treatments at 10 DAI. The length of the roots is related to absorbing mineral nutrients and water from the soil. Root length in the two treatments was not significantly different until 20 DAI, and it was markedly increased by 43.96%, 32.67%, and 34.62% at 20, 30, and 45 DAI, respectively (Table 1). Similar to the variation in root lengths, root vitality was not pronounced between the two treatments until 20 DAI. Then, the root vitality of the E⁺ treatment was 17.65–29.22% higher than that of the E⁻ treatment. With respect to seedling dry weight, that of the E⁺ treatment was significantly heavier than that of the E⁻ treatment throughout the experiment, especially at 45 DAI, and the dry weight in the E⁺ treatment increased to 50.72% (Table 1).

3.2 Chlorophyll Content and NR Activity in Peanut Leaves

To investigate whether *P. liquidambari* application changed the levels of photosynthesis and nitrogen assimilation, we measured the chlorophyll contents and NR activities in peanut leaves at different growth points (Figs. 1 and 2). In the E⁻ and E⁺ treatments, the content of chlorophyll increased but did not significantly differ until 30 DAI. The chlorophyll content in the E⁺ treatment remarkably increased by 8.98% and 25.37% at 30 and 45 DAI, respectively (Fig. 1). Nitrate reductase activity in the plant leaves increased gradually as the number of days after inoculation increased and then decreased in both treatments. Moreover, NR activity peaked at 30 DAI. NR activity significantly increased by 38.46%, 24.58%, 20.58%, and 32.73% at 10, 20, 30, and 45 DAI, respectively, compared with the E⁻ treatment (Fig. 2).

3.3 Assessment of the Concentration of Fe and Mo in Peanut Organs

In most cases, the concentrations of Fe and Mo increased in peanut roots, stems, and leaves by *P. liquidambari* application. In the E⁺ treatment, the Fe concentration in the roots was significantly enhanced by 18.45%, 37.39%, 9.62%, and 22.55% at 10, 20, 30, and 45 DAI, respectively, compared with the E⁻ treatment (Table 2). In addition, the Fe concentration in the E⁺ treatment peanut stems and leaves peaked at 36.00% and 28.30% at 45 DAI, respectively, in contrast to the E⁻ treatment (Table 2). The Mo concentration displayed approximately consistent changes in Fe content. The Mo concentration in plant root tissue increased gradually as the days after inoculation increased in the E⁺ treatment; however, in the E⁻ treatment, the Mo concentration in plant root tissue increased from 5 DAI to 20 DAI and then decreased to a stable level (Table 2). The Mo concentration in the E⁺ treatment peanut roots markedly increased by 34.53%, 193.53%, and

Table 1 Peanut plant aboveground height, root length, root vitality, and dry weight of peanut at different periods

Time DAI	Endophyte status	Aboveground height (cm)	Root length (cm)	Root vitality ($\mu\text{g NA}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$)	Dry weight ($\text{g}\cdot\text{plant}^{-1}$)
5 days	E-	22.00 ± 2.00 b	9.33 ± 0.58 a	45.53 ± 2.83 a	0.46 ± 0.01 b
	E+	27.67 ± 1.53 a	10.67 ± 1.15 a	43.73 ± 2.49 a	0.68 ± 0.02 a
10 days	E-	28.50 ± 1.00 a	12.82 ± 0.58 a	73.46 ± 3.85 a	0.70 ± 0.10 b
	E+	28.67 ± 1.76 a	15.00 ± 1.32 a	82.23 ± 7.18 a	1.01 ± 0.02 a
20 days	E-	29.67 ± 0.58 b	15.17 ± 1.26 b	124.33 ± 6.62 b	0.93 ± 0.03 b
	E+	34.33 ± 2.08 a	21.83 ± 0.76 a	146.27 ± 9.06 a	1.34 ± 0.12 a
30 days	E-	33.17 ± 0.76 b	16.83 ± 1.04 b	116.75 ± 6.00 b	1.22 ± 0.05 b
	E+	37.50 ± 0.87 a	22.33 ± 1.04 a	140.49 ± 4.49 a	1.61 ± 0.07 a
45 days	E-	39.83 ± 1.89 b	17.33 ± 0.58 b	108.57 ± 7.48 b	2.07 ± 0.26 b
	E+	51.17 ± 1.26 a	23.33 ± 1.53 a	140.29 ± 8.86 a	3.12 ± 0.19 a

Values are an average of three biological replicates ± standard error (SE). Letters correspond between E- and E+ status to one-way ANOVA, Tukey’s test ($P < 0.05$)

DAI days after inoculation, E- endophyte uninoculated, E+ endophyte inoculated

235.12% at 20, 30, and 45 DAI, respectively, compared with the E- treatment (Table 2). The difference in the concentration of Mo in the roots, stems, and leaves in the two treatments was not statistically significant at 5 and 10 DAI. In plant stems and leaves, the Mo concentration was much lower than that in the roots by almost one order of magnitude. The concentration of Mo in the stems and leaves increased the most in the E+ group, by 335.71% and 150.00%, respectively, at 30 DAI and 45 DAI, compared with the E- treatment (Table 2).

3.4 Expression Levels of Genes Involved in Fe and Mo Uptake in Peanut Roots

To investigate whether the application of *P. liquidambari* reorganized genes concerning Fe and Mo uptake in peanuts, we quantified transcript levels of genes *AhFRO1*, *AhIRT1*,

AhNRAMP1, *AhMOT1*, and *AhMOT2* by real-time PCR. The expression of *AhFRO1* and *AhIRT1* in the E+ treatment peanut roots was significantly higher than that in the E- treatment roots at a later growth stage (Fig. 3a and b). Compared with the E- treatment, *AhFRO1* and *AhIRT1* expression was upregulated in the E+ treatment by 25.45% and 12.44% at 20 DAI, 55.52% and 42.38% at 30 DAI, and 71.89% and 38.64% at 45 DAI, respectively. Unlike *AhFRO1* and *AhIRT1*, the expression of *AhNRAMP1* in the E+ treatment was always higher than in the E- treatment peanut roots throughout the experimental period, but there was no significant difference between the two treatments (Fig. 3c). The expression of the *AhMOT1* gene gradually increased after the inoculation time. The expression of *AhMOT1* in the E+ treatment was upregulated 119.74%, 159.70%, and 201.59% at 20, 30, and 45 DAI,

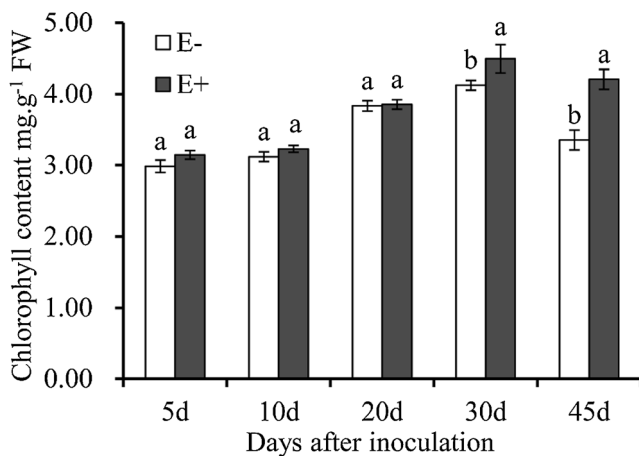


Fig. 1 Chlorophyll contents in peanut leaves at different periods. Values are an average of three biological replicates ± standard error (SE). Letters correspond between E- and E+ status to one-way ANOVA, Tukey’s test ($P < 0.05$). DAI, days after inoculation; E-, endophyte uninoculated; E+, endophyte inoculated; FW, fresh weight

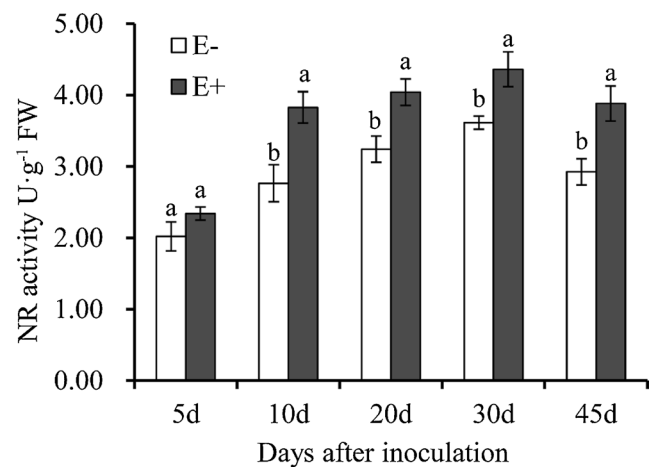


Fig. 2 Nitrate reductase (NR) activity in peanut leaves at different periods. Values are an average of three biological replicates ± standard error (SE). Letters correspond between E- and E+ status to one-way ANOVA, Tukey’s test ($P < 0.05$). DAI, days after inoculation; E-, endophyte uninoculated; E+, endophyte inoculated; FW, fresh weight

Table 2 The concentration of Fe and Mo in peanut roots, stems, and leaves at different periods

Time DAI	Endophyte status	Fe content (mg·kg ⁻¹ DW)			Mo content (g·kg ⁻¹ DW)		
		Roots	Stems	Leaves	Roots	Stems	Leaves
5 days	E-	1.39 ± 0.01 a	0.53 ± 0.01 a	0.56 ± 0.02 a	1.61 ± 0.02 a	0.27 ± 0.03 a	0.061 ± 0.002 a
	E+	1.40 ± 0.02 a	0.52 ± 0.01 a	0.55 ± 0.01 a	1.60 ± 0.02 a	0.25 ± 0.02 a	0.068 ± 0.002 a
10 days	E-	4.01 ± 0.16 b	0.64 ± 0.02 b	0.72 ± 0.01 a	1.88 ± 0.03 a	0.29 ± 0.01 a	0.039 ± 0.002 a
	E+	4.75 ± 0.07 a	0.83 ± 0.01 a	0.77 ± 0.01 a	1.88 ± 0.01 a	0.27 ± 0.01 a	0.039 ± 0.004 a
20 days	E-	3.37 ± 0.05 b	0.31 ± 0.01 b	0.43 ± 0.01 b	2.23 ± 0.03 b	0.27 ± 0.01 b	0.114 ± 0.012 b
	E+	4.63 ± 0.07 a	0.42 ± 0.01 a	0.51 ± 0.01 a	3.00 ± 0.05 a	0.43 ± 0.01 a	0.156 ± 0.009 a
30 days	E-	3.12 ± 0.09 b	0.31 ± 0.01 b	0.44 ± 0.01 b	2.01 ± 0.11 b	0.28 ± 0.02 b	0.122 ± 0.012 b
	E+	3.42 ± 0.05 a	0.36 ± 0.01 a	0.53 ± 0.01 a	5.90 ± 0.21 a	1.22 ± 0.13 a	0.305 ± 0.014 a
45 d	E-	3.77 ± 0.05 b	0.50 ± 0.01 b	0.53 ± 0.01 b	2.05 ± 0.11 b	0.45 ± 0.03 b	0.116 ± 0.005 b
	E+	4.65 ± 0.04 a	0.68 ± 0.01 a	0.68 ± 0.02 a	6.87 ± 0.35 a	1.10 ± 0.04 a	0.290 ± 0.007 a

Values are an average of three biological replicates ± standard error (SE). Letters correspond between E- and E+ status to one-way ANOVA, Tukey's test ($P < 0.05$)

DAI days after inoculation, E- endophytic uninoculated, E+ endophyte inoculated, DW dry weight

respectively, compared with the E- treatment plant roots (Fig. 4a). Unexpectedly, the transcript level of *AhMOT2* in the E+ treatment was lower than that in the E- treatment, especially at 20, 30, and 45 DAI (Fig. 4b). The expression level of *AhMOT2* in the E+ treatment was downregulated by 26.67%, 53.49%, and 53.33% at 20, 30, and 45 DAI, respectively, compared to the E- treatment. In particular, the expression of *AhMOT2* in the E+ treatment remained relatively stable during the growth stage.

3.5 The Concentration of LMWOAs in Peanut Rhizosphere Soil

The concentration of citric acid in E+ treatment peanut rhizosphere soil was significantly increased throughout the entire sampling time (Fig. 5a). Unlike the dynamic changes in citric acid concentration, the concentration of oxalic acid significantly increased after 30 DAI (Fig. 5b), and that of formic acid significantly increased after 20 DAI (Fig. 5c). Interestingly, the concentrations of oxalic acid and formic acid in the E+ treatment

were lower than those in the E- treatment during the early growth stage at 5, 10, and 20 DAI (Fig. 5b and c). Specifically, the concentration of citric acid in the E+ treatment increased 27.51%, 38.69%, and 32.74% relative to the E- treatment at 5, 20, and 45 DAI, respectively (Fig. 5a). Compared with the E- treatment control, the concentration of oxalic acid in the E+ treatment was significantly increased by 57.86% and 110.73%, respectively, at 30 and 45 DAI (Fig. 5b). In comparison with the E- treatment, the concentration of formic acid in the E+ treatment significantly increased by 156.05% and 101.16%, respectively, at 30 and 45 DAI (Fig. 5c).

4 Discussion

In consecutively monocultured soil, plants usually suffer from replanting diseases, such as malnutrition affecting plant growth or declines in biomass and yield (Li et al. 2014). In this study, we demonstrated that *P. liquidambari* inoculation of peanut can promote peanut growth parameters under consecutive

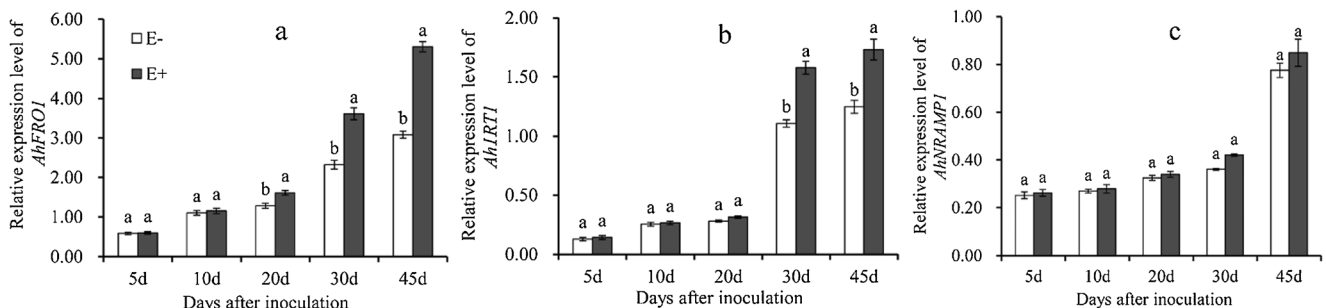


Fig. 3 Relative expression levels of genes involved in Fe uptake at different periods. **a** *AhFRO1*, **b** *AhIRT1*, **c** *AhNRAMP1*. Values are an average of three biological replicates ± standard error (SE). Letters

correspond between E- and E+ status to one-way ANOVA, Tukey's test ($P < 0.05$). DAI, days after inoculation; E-, endophyte uninoculated; E+, endophyte inoculated

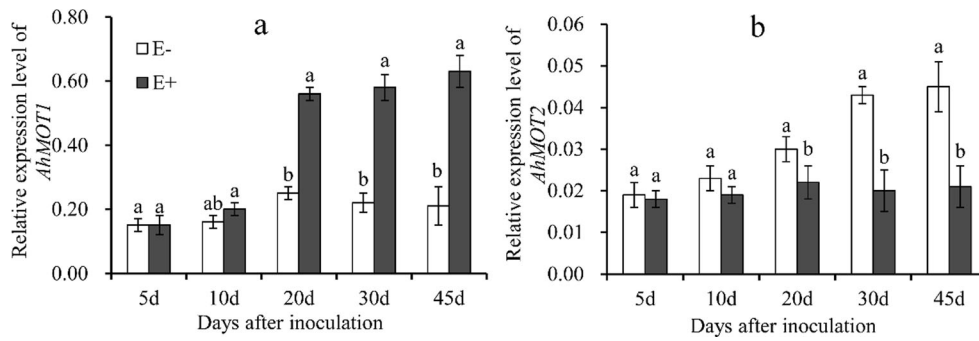


Fig. 4 Relative expression levels of genes involved in Mo uptake at different periods. **a** *AhMOT1*, **b** *AhMOT2*. Values are an average of three biological replicates ± standard error (SE). Letters correspond

between E– and E+ status to one-way ANOVA, Tukey’s test ($P < 0.05$). DAI, days after inoculation; E–, endophyte uninoculated; E+, endophyte inoculated

monoculture (Table 1). During the peanut growth periods, the aboveground height and root length of the E+ treatment plants were higher than those of the E– treatment plants. The increased plant height led to a higher biomass (Table 1). Similar results were reported by Santamaria et al. (2017), who described that fungal endophyte *Byssosclamyces spectabilis* inoculation increased *Ornithopus compressus* yield by approximately 42%. In addition, the root vitality under continuous cropping conditions was determined. The results of root vitality were in agreement with the plant growth parameters. The root system is the main organ by which plants absorb water and nutrients from soil, contributing to plant growth (Hong 2012). Fe accumulation in plants is significantly positively correlated with root length and root activity (Long et al. 2017). These results indicated that *P. liquidambari* might promote element uptake by enhancing root length and root activity.

Chlorophyll is the primary pigment in photosynthesis, and a deficiency of Mo and/or Fe causes a decrease in chlorophyll content (Gao and Shi 2007). Our results indicated that the chlorophyll content was enhanced by *P. liquidambari* inoculation (Fig. 1). Zhou et al. (2017) reported that most genes concerning carbohydrate metabolism, secondary metabolism, and amino acid metabolism were upregulated in rice infected with *P. liquidambari*. This is consistent with the results of Shi et al. (2009), who found that seeds soaked in endophytes can improve the content of chlorophyll in *Beta vulgaris* while

increasing the accumulation of sugar and improving the quality of *Beta vulgaris*. In addition, chlorophyll and plant dry weight responded to Fe application to tobacco roots under Fe-deficient conditions (Bastani et al. 2018). It is possible that *P. liquidambari* activated plant carbohydrate metabolism under nutritional restriction conditions, considering the promoted plant biomass.

Previous studies have shown that Mo deficiency decreased the activity of nitrate reductase (Nie et al. 2016). Our results showed that the activity of nitrate reductase was significantly increased except at 5 DAI in the E+ treatment (Fig. 2), which hinted that the peanut-*P. liquidambari* symbiosis had accumulated sufficient Mo. In addition, *P. liquidambari* can enhance the nitrogen metabolism of plants. Chen et al. (2013) found that ammonia-oxidizing bacteria increased in litter soil after the application of *P. liquidambari*, inferring that the endophyte *P. liquidambari* promoted nitrification and NH_4^+ -N release. Furthermore, the expression of the gene encoding nitrate reductase was stimulated by fungal endophyte *Piriformospora indica* colonization in tobacco and *Arabidopsis* roots. Therefore, we surmised that endophyte-infection changed or affected peanut nitrate reductase activity by inducing related gene expression in the host, and these changes made the plant grow faster and be more adapted to consecutively monocultured soil.

In addition, the accumulation of Fe and Mo was increased in the E+ treatment peanut under consecutive monoculture

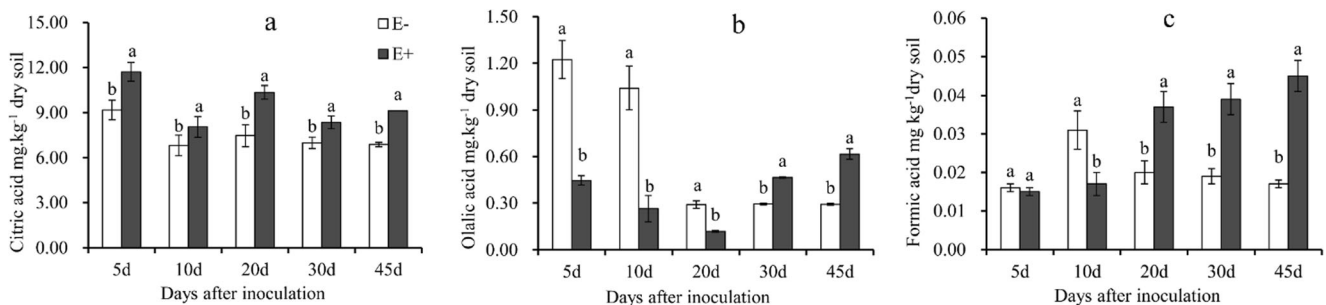


Fig. 5 Low-molecular-weight organic acid content in peanut rhizosphere soil at different periods. **a** citric acid, **b** oxalic acid, **c** formic acid. Values are an average of three biological replicates ± standard error (SE). Letters

correspond between E– and E+ status to one-way ANOVA, Tukey’s test ($P < 0.05$). DAI, days after inoculation; E–, endophyte uninoculated, E+: endophyte inoculated

(Table 2). Interestingly, the energy cost to accumulate Mo and Fe accumulation did not compromise peanut dry weight (Table 1). Preliminary experiments showed that the application of *P. liquidambari* and *Atractylodes Lancea* powder could activate trace elements (Mo, B, Fe, Zn, and Mn) in rhizosphere soil continuously cropped with peanut (Su et al. 2016). Santamaria et al. (2017) found that the fungal endophyte *Stemphylium sp.* improved the nutritive value of *Ornithopus compressus* by increasing the accumulation of essential minerals (B, Mo, P, and S). The accumulation of Fe and Mo was in accordance with the increase in chlorophyll content and nitrate reductase activity of peanut, respectively, indicating that the functional role of Fe and Mo in chlorophyll biosynthesis and nitrogen metabolism could be indispensable.

Moreover, our results indicated that the enhanced Mo-uptake effect could last longer in peanut with *P. liquidambari* inoculation (Table 2). The altered concentration of Mo and the time distribution in organs showed that the Mo absorbed from the soil was transported from the roots to the stems and leaves. To summarize, the increase in chlorophyll content and NR activity was followed by the uptake and accumulation of Fe and Mo elements, subsequently enhancing peanut growth in consecutively monocultured soil. The observed accumulation of Fe and Mo in the roots, stems, and leaves of peanut colonized by fungal endophytes suggested that *P. liquidambari* might be an important participant in plant element uptake in consecutively monocultured soil.

In this study, fungal endophytes significantly upregulated the transcription level of *AhFRO1* and *AhIRT1* (Fig. 3a and b). However, we did not observe a significant difference between E⁻ and E⁺ treatment peanut at the transcription level of *AhNRAMP1* (Fig. 3c). Phosphate transporter (*PiPT*)-related genes from the endophyte *Piriformospora indica* could improve the nutritional status of *P. indica*-colonized host *Zea mays* (Yadav et al. 2010). We hypothesized that symbiont *P. liquidambari*-peanut failed to stimulate the expression of *AhNRAMP1* but could upregulate the gene expression of *AhFRO1* and *AhIRT1*.

Concerning the genes encoding transporters of Mo, our results showed that *P. liquidambari* inoculation significantly upregulated the transcription level of *AhMOT1* in later experiments (Fig. 4a). In contrast, the transcription level of *AhMOT2* in E⁺ treatment peanut was always lower than in E⁻ treatment peanut (Fig. 4b). Recently, a report revealed that the *Medicago truncatula* genome has five MOT1 members, but only MtMOT1.3 is uniquely expressed in nodules (Tejada-Jiménez et al. 2017). *AhMOT1* could be the major transporters in peanut roots, while *AhMOT2* plays roles in other tissues. This could explain why the function of *AhMOT2* was affected by the interaction of the two transporters and/or the fungal endophyte. The mechanism by which the endophyte *P. liquidambari* affects the expression of the transporter genes *AhMOT1* and *AhMOT2* requires further study.

Considering the insoluble or microscale traceability of Fe and Mo in consecutively monocultured soil, we explored the potential mechanism involved in peanut and soil mediated by *P. liquidambari*. Here, our results revealed that the concentrations of citric acid and formic acid significantly increased in *P. liquidambari*-infected peanut rhizosphere soil (Fig. 5a and c). Yang et al. (2015) discovered that the total concentration of organic acids in rice root exudates was 64.3% higher after fungal endophyte inoculation than the control. Fe in the soil always exists in the form of ferric chelates, and root epidermal cells secrete proton ATPase AHA2 to acidify the rhizosphere soil (Jeong et al. 2017). The increase in the concentration of LMWOAs and the transcription level of Fe- and Mo-related transport genes are conducive to element translocation from soil to roots and from roots to shoots. The oxalic acid content in the E⁺ treatment peanuts was lower than that in the E⁻ treatments at the preliminary stage and then increased and became higher than that in the E⁻ treatment peanuts (Fig. 5b). Previous work has shown that phenolic acids accumulate more in continuous cropping soil, while *P. liquidambari* inoculation can effectively reduce the phenolic acid concentration in continuous cropping soil (Xie et al. 2016). We conjectured that LMWOAs, a type of metal chelation, could release Fe and Mo in consecutively monocultured soil, while plants colonized by *P. liquidambari* might be involved in the production of LMWOAs.

5 Conclusion

In conclusion, this study revealed that the application of *P. liquidambari* improved the plant growth and nutrient uptake, and the concentration of Fe and Mo in all tested tissues including roots, stems, and leaves showed a significant increase at 20, 30, and 45 DAI. Moreover, peanut-*P. liquidambari* symbiosis effected the rhizosphere environment by LMWOAs, and regulated gene expression related to Fe and Mo. Thus, the application of *P. liquidambari* to consecutive monoculture soil could be beneficial for nutrient uptake and could reduce the burden of applying chemical fertilizers, implying that *P. liquidambari* has some functions for alleviating continuous cropping obstacles. Even though these results have an importance for the prospect of application of *P. liquidambari* under continuous cropping conditions, further studies on plant-endophyte interactions and the mechanisms of Fe and Mo uptake in peanut are needed.

Funding Information This work was supported by the National Natural Science Foundation of China (NSFC) under Grant number 31870478 and the Priority Academic Program Development (PAPD) of Jiangsu Higher Education Institutions of China.

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References

- Alam MG, Snow ET, Tanaka A (2003) Arsenic and heavy metal contamination of vegetables grown in Samta village, Bangladesh. *Sci Total Environ* 308:83–96
- Bastani S, Hajiboland R, Khatamian M, Saket-Oskoui M (2018) Nano iron (Fe) complex is an effective source of Fe for tobacco plants grown under low Fe supply. *J Soil Sci Plant Nutr*. <https://doi.org/10.4067/S0718-95162018005001602>
- Chen Y, Ren CG, Yang B, Peng Y, Dai CC (2013) Priming effects of the endophytic fungus *Phomopsis liquidambari* on soil mineral N transformations. *Microb Ecol* 65:161–170
- Curie C, Alonso JM, Le MLJ, Ecker JR, Briat JF (2000) Involvement of NRAMP1 from *Arabidopsis thaliana* in iron transport. *Biochem J* 347:749–755
- Dai CC, Chen Y, Wang XX, Li PD (2013) Effects of intercropping of peanut with the medicinal plant *Atractylodes lancea* on soil microecology and peanut yield in subtropical China. *Agrofor Syst* 87:417–426
- Ding JH, Wang XX, Zhang TL, Li QM, Luo MB (2006) Optimization of RP-HPLC analysis of low molecular weight organic acids in soil. *J Liq Chromatogr Relat Technol* 29:99–111
- Feng Y, Shen D, Song W (2006) Rice endophyte *Pantoea agglomerans* YS19 promotes host plant growth and affects allocations of host photosynthates. *J Appl Microbiol* 100:938–945
- Gao L, Shi YX (2007) Genetic differences in resistance to iron deficiency chlorosis in peanut. *J Plant Nutr* 30:37–52
- Hong Y (2012) The relationship between root traits and aboveground traits in peanut (*Arachis hypogaea* L.). *Afr J Agric Res* 7:6234–6238
- Ide Y, Kusano M, Oikawa A, Fukushima A, Tomatsu H, Saito K, Hirai MY, Fujiwara T (2011) Effects of molybdenum deficiency and defects in molybdate transporter MOT1 on transcript accumulation and nitrogen/sulphur metabolism in *Arabidopsis thaliana*. *J Exp Bot* 62:1483–1497
- Jeong J, Merkovich A, Clyne M, Connolly EL (2017) Directing iron transport in dicots: regulation of iron acquisition and translocation. *Curr Opin Plant Biol* 39:106–113
- Keable SM, Vertemara J, Zadovnyy OA, Eilers BJ, Danyal K, Rasmussen AJ, De Gioia L, Zampella G, Seefeldt LC, Peters JW (2017) Structural characterization of the nitrogenase molybdenum-iron protein with the substrate acetylene trapped near the active site. *J Inorg Biochem* 180:129–134
- Kong J, Dong YJ, Zhang XW, Wang QH, Xu LL, Liu S, Hou J, Fan ZY (2015) Effects of exogenous salicylic acid on physiological characteristics of peanut seedlings under iron-deficiency stress. *J Plant Nutr* 38:127–144
- Li PD, Dai CC, Wang XX, Zhang TL, Chen Y (2012) Variation of soil enzyme activities and microbial community structure in peanut monocropping system in subtropical China. *Afr J Agric Res* 7: 219–228
- Li XG, Ding CF, Zhang TL, Wang XX (2014) Fungal pathogen accumulation at the expense of plant-beneficial fungi as a consequence of consecutive peanut monoculturing. *Soil Biol Biochem* 72:11–18
- Li ZG, Zu C, Wang C, Yang JF, Yu H, Wu HS (2016) Different responses of rhizosphere and non-rhizosphere soil microbial communities to consecutive *Piper nigrum* L. monoculture. *Sci Rep* 6:35825
- Livak KJ, Schmittgen TD (2012) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. *Methods* 25:402–408
- Long WJ, Gu T, Wan NX, Peng BY, Kong FL, Yuan JC (2017) Effect of low iron stress on root growth and iron uptake and utilization of different maize cultivars at seedling stage. *Chin J Eco-Agric* 25: 1163–1172
- Luo M, Dang P, Bausher MG, Holbrook CC, Lee RD, Lynch RE, Guo BZ (2005) Identification of transcripts involved in resistance responses to leaf spot disease caused by cercosporidium personatum in peanut (*Arachis hypogaea*). *Phytopathology* 95:381–387
- Nie ZJ, Hu CX, Tan QL, Sun XC (2016) Gene expression related to molybdenum enzyme biosynthesis in response to molybdenum deficiency in winter wheat. *J Soil Sci Plant Nutr* 16:979–990
- Pires AMM, Marchi G, Mattiazzo ME, Guilherme LRG (2007) Organic acids in the rhizosphere and phytoavailability of sewage sludge-borne trace elements. *Pesq Agrop Brasileira* 42:917–924
- Pizarro C, Escudey M, Gacitua MA, Fabris JD (2018) Iron-bearing minerals from soils developing on volcanic materials from Southern Chile: application in heterogeneous catalysis. *J Soil Sci Plant Nutr*. <https://doi.org/10.4067/S0718-95162018005002001>
- Porra RJ, Thompson WA, Kriedemann PE (1989) Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. *Biochim Biophys Acta* 975:384–394
- Robinson NJ, Procter CM, Connolly EL, Guerinot ML (1999) A ferric-chelate reductase for iron uptake from soils. *Nature* 397:694–697
- Santamaria O, Lledó S, Rodrigo S, Poblaciones MJ (2017) Effect of fungal endophytes on biomass yield, nutritive value and accumulation of minerals in *Ornithopus compressus*. *Microb Ecol* 74:841–852
- Shi Y, Dai CC, Wu YC, Yuan ZL (2004) Study on the degradation of wheat straw by endophytic fungi. *Acta Sci Circumst* 24:144–149
- Shi YW, Lou K, Li C (2009) Effects of endophytic fungus on sugar content and key enzymes activity in nitrogen and sugar metabolism of sugar beet (*Beta vulgaris* L.). *Acta Agron Sin* 35:946–951
- Su CL, Wang HW, Xie XG, Zhang W, Li XG, Wang XX, Dai CC (2016) Effects of endophytic fungi and *Atractylodes lancea* powder on rhizosphere microflora and trace elements during continuous peanut cropping. *Acta Ecol Sin* 36:2052–2065
- Tahir M, Lv YJ, Gao L, Hallett PD, Peng XH (2016) Soil water dynamics and availability for citrus and peanut along a hillslope at the Sunjia Red Soil Critical Zone Observatory (CZO). *Soil Tillage Res* 163: 110–118
- Tang H, Shuai W, Wang X, Liu Y (2017) Extraction of rare earth elements from a contaminated cropland soil using nitric acid, citric acid, and EDTA. *Environ Technol* 38:1980–1986
- Tatsumi J, Kono Y (1983) Effect of shading on α -naphthylamine oxidation by the root system of rice plants. *Jpn J Crop Sci* 52:104–105
- Tejada-Jiménez M, Gil-Diez P, León-Mediavilla J, Wen J, Mysore KS, Imperial J, González-Guerrero M (2017) *Medicago truncatula* Molybdate transporter type 1 (MtMOT1.3) is a plasma membrane molybdenum transporter required for nitrogenase activity in root nodules under molybdenum deficiency. *New Phytol* 216:1223–1235
- Wang X, Tang C, Severi J, Butterly CR, Baldock JA (2016) Rhizosphere priming effect on soil organic carbon decomposition under plant species differing in soil acidification and root exudation. *New Phytol* 211:864–873
- Xie XG, Dai CC, Li XG, Wu JR, Wu QQ, Wang XX (2016) Reduction of soil-borne pathogen *Fusarium solani* reproduction in soil enriched with phenolic acids by inoculation of endophytic fungus *Phomopsis liquidambari*. *BioControl* 62:111–123
- Yadav V, Kumar M, Deep DK, Kumar H, Sharma R, Tripathi T, Tuteja N, Saxena AK, Johri AK (2010) A phosphate transporter from the root endophytic fungus *Piriformospora indica* plays a role in phosphate transport to the host plant. *J Biol Chem* 285:26532–26544
- Yang B, Ma HY, Wang XM, Jia Y, Hu J, Li X, Dai CC (2014) Improvement of nitrogen accumulation and metabolism in rice (*Oryza sativa* L.) by the endophyte *Phomopsis liquidambari*. *Plant Physiol Biochem* 82:172–182
- Yang B, Wang XM, Ma HY, Yang T, Jia Y, Zhou J, Dai CC (2015) Fungal endophyte *Phomopsis liquidambari* affects nitrogen transformation

- processes and related microorganisms in the rice rhizosphere. *Front Microbiol* 6:982
- Zhang W, Wang HW, Wang XX, Xie XG, Siddikee MA, Xu RS, Dai CC (2016) Enhanced nodulation of peanut when co-inoculated with fungal endophyte *Phomopsis liquidambari* and *bradyrhizobium*. *Plant Physiol Biochem* 98:1–11
- Zhang W, Wang XX, Yang Z, Siddikee MA, Kong MJ, Lu LY, Shen JX, Dai CC (2017) Physiological mechanisms behind endophytic fungus *Phomopsis liquidambari*-mediated symbiosis enhancement of peanut in a monocropping system. *Plant Soil* 416:325–342
- Zhou J, Li X, Chen Y, Dai CC (2017) De novotranscriptome assembly of *phomopsis liquidambari* provides insights into genes associated with different lifestyles in rice (*Oryza sativa* L.). *Front Plant Sci* 8:121