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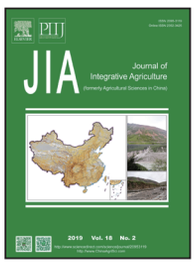

RESEARCH ARTICLE

Arbuscular mycorrhizal fungi combined with exogenous calcium improves the growth of peanut (*Arachis hypogaea* L.) seedlings under continuous cropping

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Abstract

The growth and yield of peanut are negatively affected by continuous cropping. Arbuscular mycorrhizal fungi (AMF) and calcium ions (Ca^{2+}) have been used to improve stress resistance in other plants, but little is known about their roles in peanut seedling growth under continuous cropping. This study investigated the possible roles of the AMF *Glomus mosseae* combined with exogenous Ca^{2+} in improving the physiological responses of peanut seedlings under continuous cropping. *G. mosseae* combined with exogenous Ca^{2+} can enhance plant biomass, Ca^{2+} level, and total chlorophyll content. Under exogenous Ca^{2+} application, the F_v/F_m in arbuscular mycorrhizal (AM) plant leaves was higher than that in the control plants when they were exposed to high irradiance levels. The peroxidase, superoxide dismutase, and catalase activities in AM plant leaves also reached their maximums, and accordingly, the malondialdehyde content was the lowest compared to other treatments. Additionally, root activity, and content of total phenolics and flavonoids were significantly increased in AM plant roots treated by Ca^{2+} compared to either *G. mosseae* inoculation or Ca^{2+} treatment alone. Transcription levels of *AhCaM*, *AhCDPK*, *AhRAM1*, and *AhRAM2* were significantly improved in AM plant roots under exogenous Ca^{2+} treatment. This implied that exogenous Ca^{2+} might be involved in the regulation of *G. mosseae* colonization of peanut plants, and in turn, AM symbiosis might activate the Ca^{2+} signal transduction pathway. The combination of AMF and Ca^{2+} benefitted plant growth and development under continuous cropping, suggesting that it is a promising method to cope with the stress caused by continuous cropping.

Keywords: *Arachis hypogaea* L., arbuscular mycorrhizal fungi, continuous cropping, exogenous calcium

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1. Introduction

Peanut (*Arachis hypogaea* L.) is an important oil and economic crop. The seeds contain essential proteins, vitamins, and minerals (Higgs 2003). Peanut plants can also be used for bio-energy (Li *et al*. 2011). Therefore, increasing peanut plant

yield is a primary goal. However, peanut has increasingly been continuously cropped on the same land without any crop rotation because of the pressure on farmland from the increasing human population and agro-industrialization (Li *et al*. 2014), especially in China. Continuous cropping can potentially disrupt the structural and functional diversity of soil microbial communities and enzyme synthesis (Shipton 2003; Xiong *et al*. 2015; She *et al*. 2017), which negatively affect the cycling of soil nutrients and soil functions. Furthermore, plant stress resistance is weakened, and disease pressure on peanut plants is increased by continuous cropping (Chen *et al*. 2012). In addition, autotoxic compounds from the plant accumulate in rhizosphere soil under continuous cropping, which destroys the rhizosphere environment (Huang *et al*. 2013). Continuous cropping will thus cause a series of stresses in the soil environment that affect plant growth and development. These effects include decreases in plant biomass, photosynthesis, and yield, along with deterioration in plant quality. Fortunately, plants have some strategies to deal with adverse soil conditions, including the formation of plant-microbe symbiosis and improving signaling transduction pathways (Maclean *et al*. 2017).

Arbuscular mycorrhizal fungi (AMF), part of the *Glomeromycotina* subphylum, can form symbioses with about 80% of extant land plants (Smith and Read 2008). These symbioses are characterized by the formation of hyphal structures in the cortical cells of host plants, called arbuscules, which can provide numerous soil nutrients to plants in exchange for carbohydrates (Köhl *et al*. 2015). The formation of these symbiotic associations is a complex molecular process, and it has been reported that some specific marker genes are involved in the formation of AM symbiosis. For example, *REDUCED ARBUSCULAR MYCORRHIZA 1* (*RAM1*) encodes a GRAS-type transcription factor and *RAM2* encodes a *glycerol-3-phosphate acyl transferase*, both of which are necessary for the colonization of the root by AMF and arbuscule formation. Plants that are defective in *RAM1* and *RAM2* cannot be colonized by AMF (Wang *et al*. 2012; Rich *et al*. 2015).

For many crops, AMF can improve plant nutrient uptake, which increases plant biomass (Govindarajulu *et al*. 2005; Javot *et al*. 2007; Garcia *et al*. 2017). It has also been reported that AMF can positively improve plant growth by enhancing total chlorophyll and PSII efficiency (Yang Y R *et al*. 2015), alleviating abiotic and biotic stresses by increasing scavenging capacity for reactive oxygen species (ROS) (Wu and He 2010; Chandrasekaran *et al*. 2014), and improving plant resistance against pathogens by enhancing defensive capacity (Kazan and Manners 2009; Cameron *et al*. 2013). It has been shown that AMF can improve peanut plant nutrient uptake and production (Carling *et al*. 1995). However, it is still unclear whether AMF can alleviate the stress caused by continuous cropping on peanut plants.

Calcium is an essential major element for plant growth and represents 0.1 to 5% of plant dry biomass (Jaffe *et al*. 1975). It is the second messenger in signal transduction and participates in plant physiological and biochemical processes. Plants are subject to exchangeable Ca^{2+} deficiency in many soils because Ca^{2+} is not very mobile. Therefore, supplementation with exogenous Ca^{2+} is often necessary. It has been shown that exogenous Ca^{2+} application may protect plants against environmental stresses, e.g., drought stress (Bowler and Fluhr 2000), cold injury (Zhang *et al*. 2014), and salt stress (Yin *et al*. 2015), by enhancing nutrient uptake, photosynthesis, membrane integrity, and antioxidant enzymes. Peanut is a calciphilous crop. In peanut, the application of exogenous Ca^{2+} can enhance plant resistance to stress by improving PSII efficiency, and increase the activities of antioxidant enzymes (superoxide dismutase (SOD) and catalase (CAT)), and decrease malondialdehyde (MDA) content under stress (Yang *et al*. 2013; Li *et al*. 2015). When exposed to a stressor, the transient elevation of free Ca^{2+} in the cytoplast will trigger a full range of signal transduction pathways *via* Ca^{2+}-binding proteins, such as calmodulins (CaM) and calcium-dependent protein kinases (CDPK). Previous studies have shown that exogenous Ca^{2+} can increase the transcription of *AhCaM* and *AhCDPK* under environmental stress (Yang *et al*. 2013; Li *et al*. 2015), which suggests that these genes play an important role in protecting plants against stress. However, it is still not clear what role Ca^{2+} plays in plant physiological and biochemical processes when peanut is being continuously cropped.

It has been reported that AM symbiosis can promote the Ca^{2+} uptake of plants (Bati *et al*. 2015; Cabral *et al*. 2016). Both AMF and exogenous Ca^{2+} have important roles in alleviating plant stress. However, the role of AMF, exogenous Ca^{2+}, and their combination during peanut growth in continuously cropped soils is not clear. In this study, we examined the impact of AMF, exogenous Ca^{2+}, and their combination on plant biomass and physiological indicators. Our observations suggested that AMF combined with exogenous Ca^{2+} could improve the growth of peanut seedlings under continuous cropping.

2. Materials and methods

2.1. Plant growth condition, AMF inoculation, and exogenous Ca^{2+} treatment

The AMF *G. mosseae* (BEG HEB02) was maintained as soil-sand-based inoculums. Soil that had been continuously cropped with peanut for 5 years (0–15 cm depth) was twice sterilized at 121°C for 30 min with an interval of 24 h between the two sterilizations. Huayu 22, a typical peanut cultivar

with large seeds and an upright phenotype, was used as the experimental material. Seeds were first surface-sterilized with 70% alcohol for 3 min and rinsed six times with sterile water. They were then germinated in the dark at 25°C for 3 days. The germinated seeds were transferred to pots filled with sterilized continuously cropped soil and inoculated with about 400 *G. mosseae* spores contained in 10 g mycorrhizal inoculums. The same sterilized mycorrhizal inoculums without *G. mosseae* spores were used as the control. The peanut seedlings were grown in a greenhouse at 24°C/18°C with a 16/8 h photoperiod, at 70% relative humidity, and at a photosynthetic photo flux density of 700 μmol m^{-2} s^{-1}. One seedling was planted in each pot (the pot diameter and height were 12 and 11 cm, respectively). One week after sowing, each seedling was watered regularly with 100 mL of modified (+Ca^{2+}, 20 mmol L^{-1} Ca^{2+}) Hoagland's solution (20 mmol L^{-1} $Ca(NO_3)_2 \cdot 4H_2O$, 5 mmol L^{-1} KNO_3, 2 mmol L^{-1} $MgSO_4 \cdot 7H_2O$, 1 mmol L^{-1} KH_2PO_4, 0.1 mmol L^{-1} EDTA-Na_2, 0.1 mmol L^{-1} $FeSO_4 \cdot 7H_2O$, 46 μmol L^{-1} H_3BO_4, 0.32 μmol L^{-1} $CuSO_4 \cdot 5H_2O$, 0.77 μmol L^{-1} $ZnSO_4 \cdot 7H_2O$, and 0.11 μmol L^{-1} H_2MoO_4) or solution without Ca^{2+} (20 mmol L^{-1} NH_4NO_3, 5 mmol L^{-1} KNO_3, 2 mmol L^{-1} $MgSO_4 \cdot 7H_2O$, 1 mmol L^{-1} KH_2PO_4, 0.1 mmol L^{-1} EDTA-Na_2, 0.1 mmol L^{-1} $FeSO_4 \cdot 7H_2O$, 46 μmol L^{-1} H_3BO_4, 0.32 μmol L^{-1} $CuSO_4 \cdot 5H_2O$, 0.77 μmol L^{-1} $ZnSO_4 \cdot 7H_2O$, and 0.11 μmol L^{-1} H_2MoO_4). In this study, 20 mmol L^{-1} NH_4NO_3 was used to balance nitrogen content from $Ca(NO_3)_2$. In total, there were four experimental treatments, including Ca_0+AM, Ca_0–AM, Ca_{20}+AM, and Ca_{20}–AM; 0 and 20 represent the Ca^{2+} concentrations (mmol L^{-1}). A toatal of 20 mmol L^{-1} of $Ca(NO_3)_2$ was optimal according to our previous experiments (unpublished data). Each treatment contained 12 peanut seedlings and was replicated three times. Six weeks later, the plants were harvested and analyzed.

2.2. Mycorrhizal quantification, and determination of dry weight and Ca^{2+} content

Ninety root sections per sample were examined by light microscopy (CX41; OLYMPUS, Japan) to estimate the extent to which the root had been colonized by hyphae and arbuscules (McGonigle *et al*. 1990). The fresh shoots and roots of plants were placed at 105°C for 30 min and then dried at 80°C until the weight was constant to determine the plant dry weight. The Ca^{2+} content in the leaves of AM and nonmycorrhizal (NM) plants from the different treatments were measured according to Yang *et al*. (2013).

2.3. Measurements of total chlorophyll and chlorophyll fluorescence

Total chlorophyll content was measured according to Arnon (1949). Chlorophyll fluorescence was measured under high irradiance stress with a portable fluorimeter (FMS2, Hansatech, UK) according to Van Kooten and Snel (1990). To induce high irradiance stress, the detached leaves were floated on the water with the adaxial side facing up. Then they were irradiated with 1 200 μmol m^{-2} s^{-1} PFD at room temperature (25°C). Initial fluorescence (F_o) was obtained using modulated light (about 10 μmol m^{-2} s^{-1}). The maximal fluorescence (F_m) was determined by 0.8 s of saturating light at 8 000 μmol m^{-2} s^{-1} on a leaf that had been dark adapted for 15 min. Variable fluorescence (F_v) was calculated from $F_v=F_m-F_o$. Maximal photochemical efficiency (F_v/F_m) of PSII was calculated using $F_v/F_m=(F_m-F_o)/F_m$

2.4. Determination of antioxidant enzyme activities and malondialdehyde content

The third fully expanded leaves from the apical tips were collected to assay antioxidant enzyme activities. Peroxidase (POD) activity was measured according to Sung and Jeng (1994). Fresh leaves were hand-homogenized separately at 4°C in a mortar and pestle with 5 mL of 5% (v/v) trichloracetic acid to precipitate proteins, and then centrifuged at 14 000×g for 20 min. The supernatant was used for POD determination. SOD activity in the fresh leaves was assayed according to Stewart and Bewley (1980). CAT activity and MDA content were determined according to Lin *et al*. (2006) and Yang S *et al*. (2015), respectively.

2.5. Determination of root activity, and total phenolics and flavonoids contents

Root activity was estimated by tetrazolium chloride (TTC) reduction according to the method reported by Comas *et al*. (2000). Total phenolics content in the roots was detected using the method described by Chun *et al*. (2003). Flavonoid content in the roots was measured according to Jia *et al*. (1999).

2.6. Quantitative real-time PCR

Total RNA was isolated from the roots, and cDNA was synthesized for qRT-PCR analyses using SYBR Premix *Ex Taq* Polymerase (TaKaRa, Japan) according to the manufacturer's protocol. The selected genes were analyzed using a Bio-Rad iQ1 Real-Time PCR machine (Bio-Rad, USA). The primers are shown in Table 1. The control reactions were conducted using primers Tua5-F and Tua5-R, which were reported by Chi *et al*. (2012). At least three replicates were tested per sample. Relative mRNA (fold) differences were assessed with the $2^{-\Delta\Delta Ct}$ formula (Livak and Schmittgen 2001).

2.7. Statistical analysis

Analysis of variance was performed using SSPS Software version 16.0 for Windows. One-way analysis of variance (ANOVA) was used, followed by Duncan's test. The values obtained are the mean±SE for the three replicates in each treatment. A *P*-value≤0.05 was considered to be significant.

3. Results

3.1. Effects of AM symbiosis combined with exogenous Ca^{2+} on root colonization

Six weeks after *G. mosseae* inoculation, 34.34 and 34.56% of the plant roots were colonized at 0 and 20 mmol L^{-1} of Ca^{2+}, respectively (Fig. 1-A). The application of Ca^{2+} seemed to have little effect on the degree of *G. mosseae* colonization.

3.2. AM association combined with exogenous Ca^{2+} improved plant biomass yield and Ca^{2+} content

Root dry weight per plant significantly increased in the Ca_0+AM treatment compared to the Ca_0–AM treatment, and it increased even more when Ca^{2+} was applied. The AM plants treated with 20 mmol L^{-1} Ca^{2+} had the highest root dry weight among all treatments (Fig. 1-B). The shoot dry weight per plant had the same trend as the roots (Fig. 1-C). Additionally, the Ca^{2+} content was significantly higher in AM plants than in NM plants under 0 and 20 mmol L^{-1} Ca^{2+} treatments (Fig. 1-D), which showed that AM symbiosis increases Ca^{2+} uptake. Furthermore, the application of exogenous Ca^{2+} significantly enhanced Ca^{2+} content of plants compared with Ca_0–AM and Ca_0+AM plants (Fig. 1-D). This indicated that supplemental exogenous Ca^{2+} is the main Ca^{2+} source for plants growing in continuously cropped soil, and AM association could further increase Ca^{2+} content.

3.3. AM association combined with exogenous Ca^{2+} increased total chlorophyll and chlorophyll fluorescence

Total chlorophyll content significantly increased in Ca_0+AM

Table 1 Primer sequences for quantitative real-time PCR amplification

Gene ID[1)]	Gene name	Primer sequence (5′→3′)
Au.N7IXY	*AhRAM1*	F: CACCTCCAATAATGCCAACC
		R: GTGAAGTGGGCGAATTTGAT
Au.GJ0WT	*AhRAM2*	F: GTCTGGTGGCAAGATGAGCA
		R: AGCAGCCCATGAGAATAGGGT
AY517930	*AhCaM*	F: GGTGCTCGACAAGGATCAA
		R: ACTCCTCGTAGTTGATCTGC
KC207812	*AhCDPK*	F: CCACACCAAAGGAAAACA
		R: GTCGATACAGAGGTACGTCA
GO264294	*AhTUA5*	F: CTGATGTCGCTGTGCTCTTGG
		R: CTGTTGAGGTTGGTGTAGGTAGG

[1)] Au.N7IXY and Au.GJ0WT represent *RAM1* and *RAM2* ID from *Arachis duranesis* which is thought to be one of the diploid ancestor, respectively.

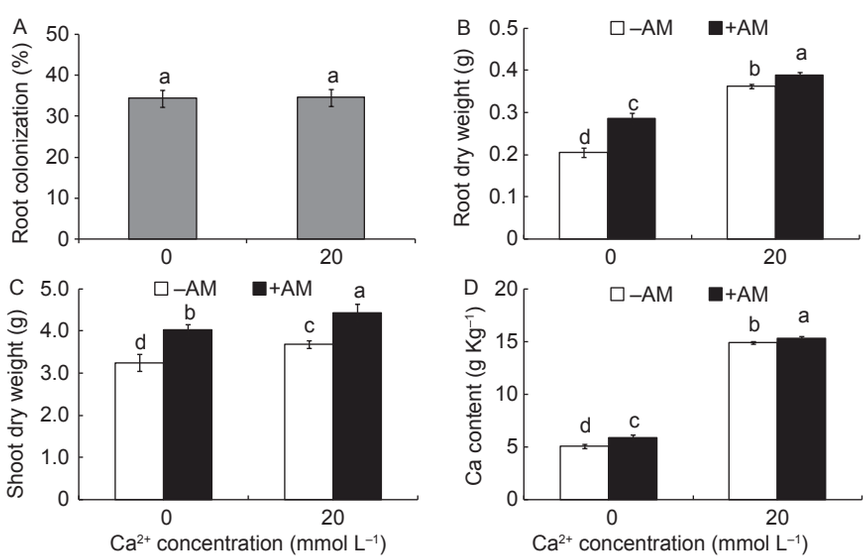

Fig. 1 Effects of arbuscular mycorrhizal (AM) symbiosis on plant growth following exogenous Ca^{2+} treatment. The fungal colonization rates were estimated in the roots of AM plants under different concentrations of $Ca(NO_3)_2$ (A). Root (B) and shoot (C) dry weight per plant for AM and non-mycorrhizal (NM) plants under 0 and 20 mmol L^{-1} Ca^{2+} treatments were measured. The Ca^{2+} content (D) was determined in AM and NM plant leaves treated with Ca^{2+} for 6 wk. Different letters show that the columns are significantly different (P≤0.05). n=6. Experiments were independently replicated three times. Bars mean SD.

plants compared with Ca_0–AM plants, and increased even more in Ca_{20}+AM treatment (Fig. 2-A). As an indicator of photoinhibition, the maximum photochemical efficiency of PSII (F_v/F_m) has been widely studied. When exposed to the high irradiance stress, F_v/F_m decreased in both AM and NM plant leaves. At the end of the stress period, F_v/F_m in the Ca_0–AM, Ca_0+AM, Ca_{20}–AM, and Ca_{20}+AM treatments decreased by 21.45, 14.82, 8.20, and 4.75% of their initial values, respectively (Fig. 2-B).

3.4. AM association combined with exogenous Ca^{2+} enhanced antioxidant enzyme activities

POD, SOD, and CAT are important antioxidant defense system enzymes. Their activities significantly increased in Ca_0+AM plant leaves, and exogenous Ca^{2+} application further significantly increased their activities (Fig. 3-A–C). The activities of these enzymes were the highest in the Ca_{20}+AM treatment. The MDA content, which reflects

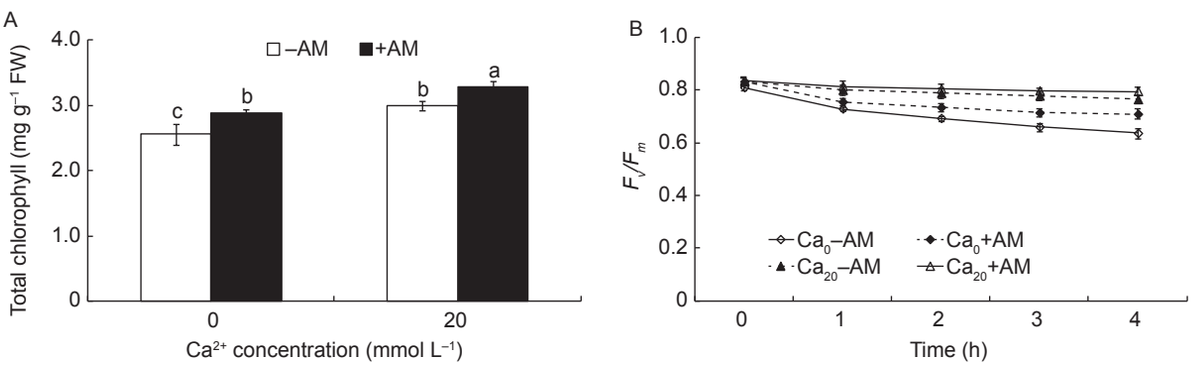

Fig. 2 Effects of arbuscular mycorrhizal (AM) symbiosis combined with exogenous Ca^{2+} on total chlorophyll (A) and on the maximum photochemical efficiency of PSII (F_v/F_m) (B) in peanut plants. Ca_0–AM, peanut plants without AM association and Ca^{2+} application; Ca_0+AM, peanut plants with AM symbiosis, but without Ca^{2+} application; Ca_{20}–AM, peanut plants were only treated by 20 mmol L^{-1} $Ca(NO_3)_2$; Ca_{20}+AM, peanut plants with AM symbiosis and 20 mmol L^{-1} $Ca(NO_3)_2$ was applied. Different letters show significant difference at $P \leq 0.05$. n=6. Experiments were independently replicated three times. Bars mean SD.

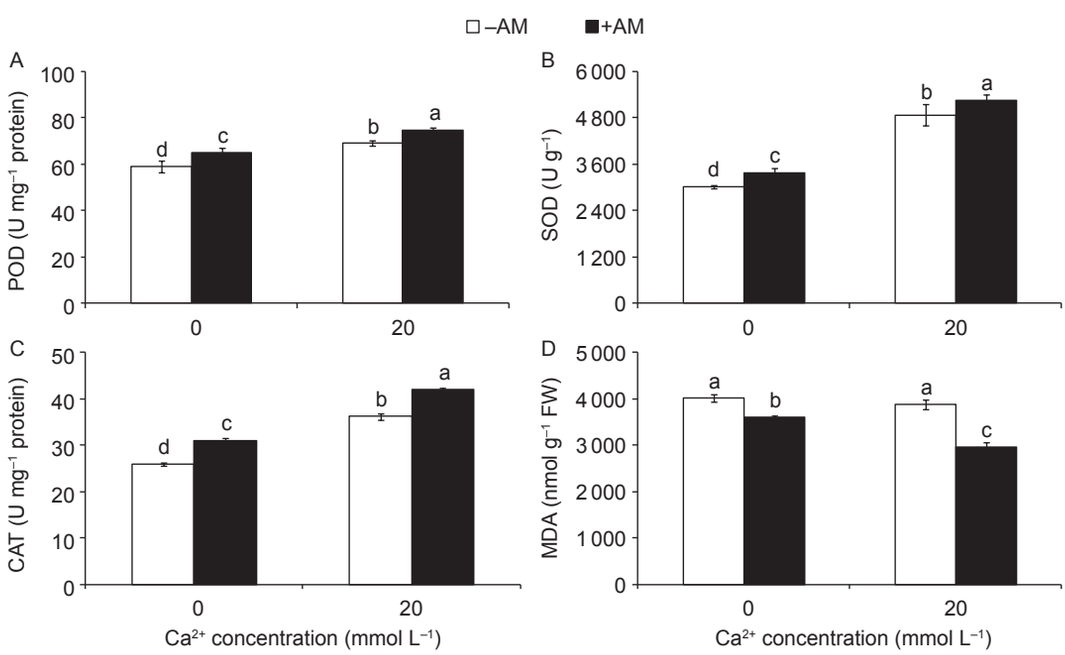

Fig. 3 Effects of arbuscular mycorrhizal (AM) symbiosis combined with exogenous Ca^{2+} on the peroxidase (POD, A), superoxide dismutase (SOD, B), and catalase (CAT, C) activities, and malondialdehyde (MDA) content (D) in the leaves of plants growing in continuously cropped soil. Different letters show that the columns are significantly different at $P \leq 0.05$. n=6. Experiments were independently replicated three times. The data presented are the mean value±SD of three individual experiments.

the degree of membrane lipid peroxidation, significantly decreased in Ca_0+AM plants (Fig. 3-D), and decreased even further in AM plants after Ca^{2+} treatment compared to those with no application of exogenous Ca^{2+}.

3.5. AM association combined with exogenous Ca^{2+} improved root activity and increased the content of total phenolics and flavonoids

AM symbiosis has no obvious effect on root activity under no exogenous Ca^{2+} application. However, Ca^{2+} significantly increased root activity, which was the highest in the Ca_{20}+AM treatment (Fig. 4-A). Meanwhile, the total phenolics content in the roots of AM plants was not significantly different from the NM plants under no supply of exogenous Ca^{2+}, but it was significantly accumulated in the Ca_{20}–AM treatment, and its content was the highest in the Ca_{20}+AM treatment (Fig. 4-B). A similar trend was observed for the total flavonoids content (Fig. 4-C).

3.6. AM symbiosis combined with exogenous Ca^{2+} changed the transcript level of genes involved in AM symbiosis and the Ca^{2+} signal transduction pathway

The transcript levels of two AM-specific marker genes and two Ca^{2+}-related genes were assayed to estimate the impact of AMF combined with Ca^{2+} on AM formation and the Ca^{2+} signal transduction pathway. *AhRAM1* and *AhRAM2* expression levels were significantly up-regulated in roots of AM plants under both 0 and 20 mmol L^{-1} Ca^{2+} treatments, but their expression levels were the highest in the Ca_{20}+AM treatment (Fig. 5-A and B). In addition, the *AhCaM* expression level was significantly up-regulated in AM plants after applying Ca^{2+}, and it was the highest in plant roots with Ca_{20}+AM treatment (Fig. 5-C). The *AhCDPK* transcript level was only up-regulated in AM plant roots and was not affected by exogenous Ca^{2+} (Fig. 5-D). These results indicated that AM symbiosis was not only regulated by exogenous Ca^{2+}, but also participated in the Ca^{2+} signal transduction pathway.

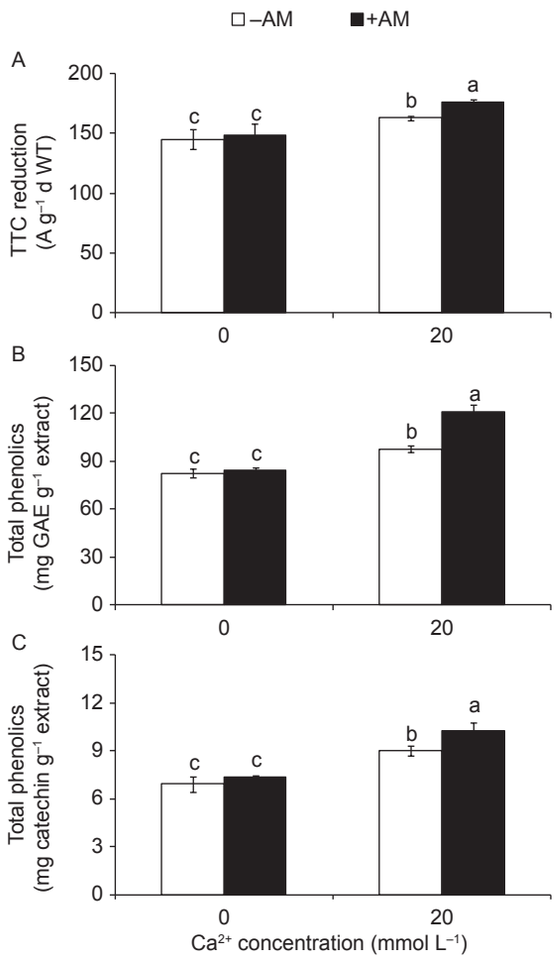

Fig. 4 Effects of arbuscular mycorrhizal (AM) symbiosis on plant roots supplied with exogenous Ca^{2+}. The root activity (A), total phenolics content (B), and flavonoids content (C) were determined in the roots of AM and NM plants under 0 and 20 mmol L^{-1} Ca^{2+} conditions. TTC, tetrazolium chloride; A, activity; GAE, gallic acid equivalent. Different letters show that the columns are significantly different at $P \leq 0.05$. $n=6$. Experiments were independently replicated three times. The data presented are the mean value±SD of three individual experiments.

4. Discussion

When subject to continuous cropping, peanut seedlings are seriously compromised by decreases in the activities of antioxidant enzyme and in leaf photosynthesis, leading to decreased plant biomass and yield (Liu *et al*. 2015). AM fungi not only enhance the ability of plants to absorb mineral nutrition, but also provide non-nutritional benefits to the host, including tolerance to abiotic stress and resistance against pathogens (Nadeem *et al*. 2014). Moreover, Ca^{2+} can also protect plants against environmental stresses (Yin *et al*. 2015).

4.1. AM symbiosis combined with exogenous Ca^{2+} improves plant growth and development

The combination of AM symbiosis and Ca^{2+} increases mineral nutrition uptake, since both Ca^{2+} content (Fig. 1-D) and potassium content in plants were increased with the supply of exogenous Ca^{2+} (Lei *et al*. 2014), thus increasing the root and shoot dry weights. Besides nutrient uptake, significant increases in the root and shoot dry weight of peanut might also be related to increases in photosynthesis (Fig. 2) and root activity (Fig. 4-A) due to AM symbiosis

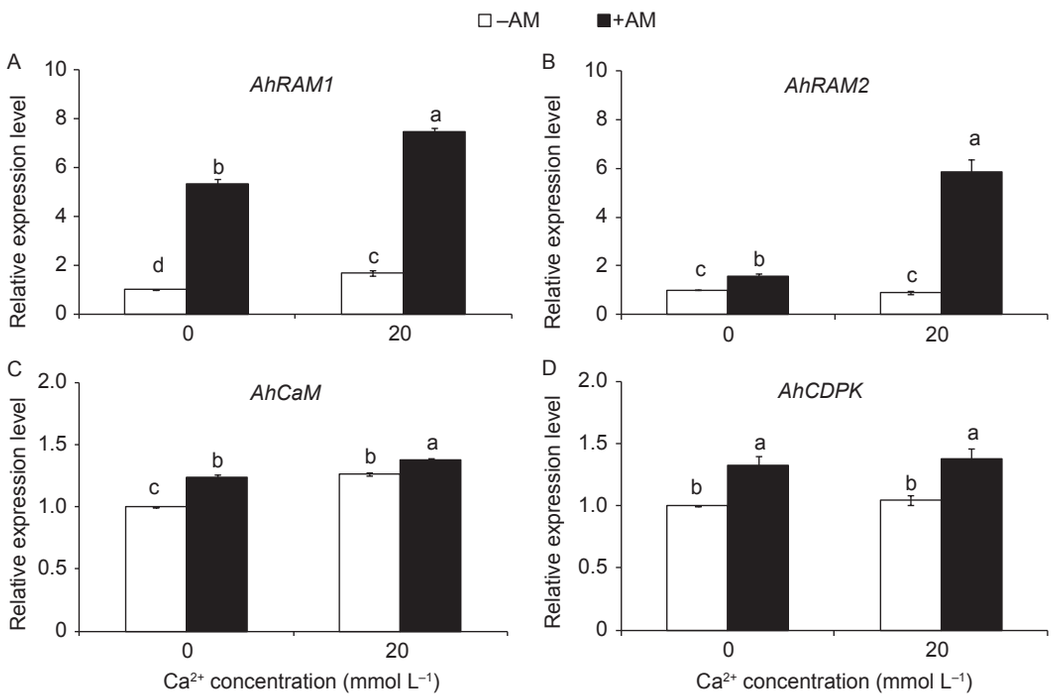

Fig. 5 Effects of arbuscular mycorrhizal (AM) symbiosis combined with exogenous Ca^{2+} on the expression levels of genes involved in AM function and the Ca^{2+} signal pathway. The transcript levels of the AM-specific marker genes *AhRAM1* (A) and *AhRAM2* (B), and Ca^{2+} signal pathway genes *AhCaM* (C) and *AhCDPK* (D) were determined in AM and non-mycorrhizal (NM) peanut roots grown under the 0 and 20 mmol L^{-1} Ca^{2+} treatment conditions for 6 wk. Different letters show that the columns are significantly different at $P \leq 0.05$. $n=6$. Experiments were independently replicated three times. Bars mean SD.

combined with exogenous Ca^{2+}. The results indicated that *G*. *mosseae* combined with exogenous Ca^{2+} improves plant dry biomass when peanut is continuously cropped.

4.2. AM symbiosis combined with exogenous Ca^{2+} enhances plant stress-resistance

AM symbiosis can enhance photosynthetic ability (Andrade *et al*. 2015). For peanut seedlings in this study, AM symbiosis increased photosynthetic ability by increasing both chlorophyll content and the maximum photochemical quantum efficiency of PSII, which was further increased by Ca^{2+} application (Fig. 2). These results suggest that *G*. *mosseae* combined with exogenous Ca^{2+} is involved in the mechanism that protects peanut plants under continuous cropping from more severe PSII photoinhibition induced by environmental stress (Yang *et al*. 2013; Yang S 2015). It has been proven that the increase in photosynthetic efficiency stems from improved ROS scavenging ability (Yang Y R *et al*. 2015; Garcia *et al*. 2017). In the present study, AM symbiosis combined with Ca^{2+} application further increased the activities of antioxidant enzymes in peanut leaves, including POD, SOD, and CAT (Fig. 3), which are responsible for scavenging the ROS induced by continuous cropping. These results indicated that *G*. *mosseae* combined with exogenous Ca^{2+} increases ROS scavenging and alleviates oxidative damage caused by continuous cropping.

Simultaneously, improved ROS scavenging ability was also an important factor for maintaining higher root activity (Fig. 4-A) induced by AM symbiosis combined with exogenous Ca^{2+} compared with Ca^{2+} application alone. Moreover, the increased root activity was closely related to the accumulation of phenolics and flavonoids, which improve root tolerance to abiotic stress, and to alterations in the soil environment (Norman *et al*. 1996; Leifheit *et al*. 2014). As secondary metabolites, phenolics and flavonoids could improve plant tolerance to stress by removing toxic radicals and maintaining membrane stability (Pietta 2000; Michalak 2006; Wahid and Ghazanfar 2006). It has been reported that AM symbiosis regulated the biosynthesis of phenylpropanoid derivatives, including increasing the flavonoids content (Adolfsson *et al*. 2017). In the present study, exogenous Ca^{2+} further increased the content of total phenolics and flavonoids in AM plants (Fig. 4-B and C), suggesting that *G*. *mosseae* combined with exogenous Ca^{2+} increased the synthesis of phenolics and flavonoids, which could enhance root development in continuously cropped soil.

4.3. Exogenous Ca^{2+} improves the formation of AM symbiosis

As AM-specific marker genes, *AhRAM1* and *AhRAM2* play an important role in formation of the AM symbiosis (Wang *et al*. 2012; Rich *et al*. 2015). Exogenous Ca^{2+} application significantly up-regulated the expression levels of these two genes, indicating that exogenous Ca^{2+} positively affected AM association. *CaM* and *CDPK* were important components in the Ca^{2+} transduction pathway (Liese and Romeis 2013; Ruge *et al*. 2016), and the expression levels of *AhCaM* and *AhCDPK* were up-regulated by the application of exogenous Ca^{2+} in peanut plants under stress (Yang *et al*. 2013; Li *et al*. 2015). However, the *AhCDPK* transcript was not affected by exogenous Ca^{2+} (Fig. 5-D), it was consistent with the NtCDPK expression that was induced by fungal elicitors (Yoon *et al*. 1999). Interestingly, *AhCaM* and *AhCDPK* were significantly up-regulated in AM plant roots, suggesting that AM symbiosis might be involved in the Ca^{2+} signal transduction pathway.

5. Conclusion

Arbuscular mycorrhiza symbiosis enhanced the Ca^{2+} content in peanut plants, and Ca^{2+} participated in AM symbiosis signaling *via* the Ca^{2+} signal transduction pathway, which plays an important role in protecting plants against stresses. Therefore, the interaction between AM symbiosis and exogenous Ca^{2+} can increase resistance to stress caused by continuous cropping and improved growth of peanut seedlings. Further studies will be needed to validate the molecular mechanism that operates when *G*. *mosseae* combined with exogenous Ca^{2+} improves plant growth under continuous cropping.

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