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Arbuscular mycorrhiza improved drought tolerance of maize seedlings by altering photosystem II efficiency and the levels of key metabolites

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Abstract

Background: Water shortage can limit plant growth, which can be ameliorated by arbuscular mycorrhizal (AM) symbiosis through physiological and metabolic regulations. Deciphering which physiological and metabolic processes are central for AM-mediated regulations is essential for applications of mycorrhizal biotechnology in dryland agriculture.

Methodology: In this study, the influence of AM symbiosis on growth performance, photosynthesis, and organ accumulation of key C and N metabolites were assessed by growing maize (Mo17, Lancaster Sure Crop) seedlings inoculated with or without AM fungus (*Rhizophagus irregularis* Schenck & Smith BGC AH01) under different water regimes in greenhouse.

Results: Drought stress reduced shoot growth, while AM symbiosis significantly improved growth performances, with significant changes of photochemical processes and organ concentration of the key metabolites. AM symbiosis increased root levels of the metabolites in ornithine cycle and unsaturation of fatty acids regardless of water conditions. Root putrescine (Put) concentration was higher in AM than non-inoculated (NM) plants under well-watered conditions; the conversion of Put via diamine oxidase to γ -aminobutyric acid (GABA) occurred in roots of AM plants under drought stress. Leaf concentration of Put, the tricarboxylic acids, and soluble sugars significantly increased in AM plants under drought stress, showing higher values compared to that of NM plants. Moreover, photosystem II efficiency and chlorophyll concentration were higher in AM than NM plants regardless of water status.

Conclusion: Fatty acid- and ornithine cycle-related metabolites along with soluble sugars, Put, and GABA were the key metabolites of AM-mediated regulations in response to drought stress.

Keywords: Arbuscular mycorrhiza, Drought tolerance, Fatty acids, Putrescine, γ -Aminobutyric acid, Urea cycle, *Zea mays*

Background

In drylands, water deficit can cause plant physiological and metabolic disorders and limit plant growth. Stomatal responses are sensitive to even minor changes of

water availability. A decline in stomatal conductance is in common when plants are subjected to drought stress. This stomatal response is beneficial to maintain plant water budget; whereas it inevitably restricts CO₂ availability, which may inhibit photosynthesis. Non-stomatal factors are also important determinants for plant photosynthetic capacity. Severe drought can result in chlorophyll degradation, chloroplast dysfunction, and membrane lipid peroxidation, showing an energy

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distribution imbalance in photosystems and accumulation of reactive oxygen species (ROS). Drought stress can induce C re-partitioning between metabolites; the starch–sugar interconversion in source and sink tissues is a typical plant adaptation to drought [1]. Soluble sugars (e.g., sucrose, glucose and fructose) have been demonstrated to increase in response to drought stress [2, 3]. Increased soluble sugars play important roles in stabilizing cell turgor pressure. In addition, these carbohydrates play roles in cellular respiration, cell homeostasis, and secondary metabolic processes [2]. Moreover, drought stress can induce changes of photoassimilate allocation between plant organs. In most plants, sucrose (Suc) is a main C form translocated from shoot sources to root sinks [4].

Drought-caused alterations in C metabolism can influence N metabolism, for instance, inducing changes in compositions of free amino acids [5, 6]. Some intermediate metabolites such as oxaloacetic acid (OAA) and aspartic acid (Asp), pyruvic acid (Pyr) and alanine (Ala), and α -ketoglutaric acid (α -KA) and glutamate (Glu) are the targets in drought-induced regulations on plant C and N metabolisms. The concentration of other amino acids linked to photorespiration, such as serine or glycine, has also been found to vary in response to water shortage [7]. Numerous metabolites are mutually transformational to meet growth requirement and drought adaptation. Nonetheless continuing drought leads to substantial energetic/metabolic imbalance in plants. How to offset the imbalances is a key issue in improving plant drought tolerance.

Considerable evidence supports beneficial effects of Arbuscular mycorrhizal (AM) symbiosis on plant drought tolerance. Firstly, AM symbiosis improves root water absorption and plant water relations [8]. Extraradical hyphae of AM fungi can access water resource unavailable for roots [9] and directly participate in water uptake and transport. AM inoculation can regulate root-hair growth possibly through the influence on auxin synthesis and transport [9]. Moreover, AM symbiosis can influence cell wall components of host plants through stimulating phenylpropane or lignin synthesis [10]. Structural modifications of roots by AM fungi increase root hydraulic conductance and cell-to-cell water transport [11, 12]. AM symbiosis can modulate plant water relations through influencing stomata functioning, photosynthesis, and metabolic responses to drought stress [13]. In early publications [14], AM-mediated improvement in plant water relations were related to improved P nutrition, as physiological and biochemical processes regulating plant water relations depend on P nutrition status [15].

AM symbiosis increases the sink strength to unload more carbohydrates from phloem [16]. It is estimated

that approximately 20% of shoot photoassimilates flow to root systems for mycorrhizal growth [17]. In early studies, sugars were considered major C sources transferred from host plants to AM fungi. Root concentration of soluble sugars has been demonstrated to increase significantly in AM plants [18]. A possible pathway is that AM symbiosis enhances source–sink translocation of the photoassimilates through inducing expression of several sucrose transporters (SUTs) [19]. In recent studies, fatty acids (FAs) were confirmed to be another C sources supplied by host plants to AM fungi [17, 20]. Some recent studies [21, 22] confirmed significant AM effects on fatty acid compositions, but showing inconsistent results (such as C15.0, C15.1 and C18.0). However, the mechanisms underlying the different AM effects are largely unknown. Nutrient (such as N and P) supply may be an important determinant in AM-induced changes of FA compositions [21]. An alternation of fatty acid saturation is physiologically important in AM-mediated improvements of plant drought tolerance [22].

Arginine (Arg) is proved to be mycelium-specific AA [23]; translocation and breakdown of fungi Arg are active in rapid plant growth or early drought. Changes of fungi Arg can influence N storage, mobilization, and nutrient balance of plant tissues [24]. Arg catabolism serves as defense mechanisms of plants against drought stress [25] because Arg-derived metabolites, including nitric oxide (NO), proline (Pro), and polyamines (PAs), are drought-sensitive and physiologically important. Putrescine (Put) is an important intermediate metabolite in the Arg-derived PA pathway, and is reported to enhance plant dehydration [26]. Putrescine catabolism is physiologically significant in plant drought tolerance [27], as Put degradation contributes to accumulations of Pro and γ -aminobutyric acid (GABA) in plant tissues [28]. Pro and GABA are multifunctional amino acids (AAs), which play important roles in cellular homeostasis, signaling transduction, C/N balance, and osmotic adjustment. Pro and GABA commonly accumulate in response to drought stress [29]; however, it is not fully clear whether this is a metabolic regulation or an adaptive response to mitigate environmental stress [30].

In this study, we hypothesized that unsaturation of fatty acids and ornithine cycle along with the metabolism of carbohydrates and polyamines were the primary targets in AM-mediated metabolic regulations in response to drought stress. Through greenhouse pot experiments, we examined accumulation patterns of the key C and N metabolites in leaves and roots of maize plants under drought stress and AM symbiosis. We also examined photosynthetic responses to AM

symbiosis and drought stress by analyzing stomatal and non-stomatal factors.

Materials and methods

Host plants and AM fungus

Maize seeds (Mo17, Lancaster Sure Crop) were surface-sterilized by using 0.5% K_2MnO_4 for 20 min. After soaking (for 6 h) and incubating (at 30 °C), well-germinated seeds were selected for pot experiments. A mixture of peat, sand and vermiculite (4:1:1 v/v/v) was used as growing substrate. Before sowing, the substrate was sterilized by autoclaving at 120 °C for 1 h, during 2 consecutive days. Seeds were planted in 3.25-L pots with one seed per pot. A total of 40 pots were divided into two groups: one consisted of 20 pots inoculated with AMF *Rhizophagus irregularis* Schenck & Smith BGC AH01 (AM), and the other corresponded to 20 pots as non-mycorrhizal (NM) plants. The fungal inoculum was provided as a mixture of sand, spores, mycelia, and colonized root fragments. For the inoculated treatment, 40 g inoculum (i.e., approximately 2000 spores) was applied per pot. For the NM treatment, an equivalent amount of sterilized inoculum was mixed with the soil together with a 5-mL inoculum filtrate to ensure similar microbial populations for all experimental treatments excluding AM fungus. Maize seedlings were grown in a greenhouse with a ventilation system and natural light, and daily watered (200 mL H_2O plant⁻¹ day⁻¹; corresponding to approximately 14% of soil water-holding capacity).

Drought stress

14 days after emergence, the non-inoculated and inoculated seedlings were subjected to the following treatments: (1) W: (10 pots) watered with 200 mL H_2O plant⁻¹ day⁻¹ for 14 days; (2) D: (10 pots) plants were not watered for 14 days. At the end of the experiment, plant samples were collected for morphological, metabolic and physiological measurements.

Sampling and treatments

After carrying out non-destructive measurements (e.g., plant height, gas exchange rates, and photochemical efficiency), shoots and roots were harvested separately. For clearing substrate particles attached to fine roots, the root system was thoroughly washed, first with tap water followed by distilled water; root surface moisture was removed with filter paper. The samples were separated into three parts for further analyses, namely: (1) fresh weight (FW) and dry weight (DW) determinations for calculation of water content. Dry tissues were subsequently ground to powder and used for mineral element

determinations; (2) for root morphology and AMF colonization observations; and (3) for determining metabolite concentrations and enzyme activities after liquid-nitrogen grinding to powder.

Biomass and water content

After record of fresh weight, dry weight was measured after oven-drying at 65 °C to a constant weight. Water content (WC) of shoots and roots was obtained.

Assessment of mycorrhizal status

On the 14th day of drought treatment, mycorrhizal colonization percentage was determined by using the KOH bleaching–acid fuchsin staining method [31]. Root samples were cut into 1-cm-long segments, then cleaned (10% KOH), acidified (2% HCl), and stained (0.01% acid fuchsin in 5% lactic acid), respectively. Thirty stained root segments per plant were used for microscope observation and calculation of AM colonization. AMF colonization was observed by using an Olympus Bx43 fluorescence microscope (Tokyo, Japan).

C, N, and P concentrations

One-hundred milligrams of dried samples were treated by H_2SO_4 – H_2O_2 Kjeldahl digestion method. Regarding sample digestion, 0.1 g of dried samples was placed into 1 mL of H_2SO_4 – H_2O_2 acid for 12 h at room temperature. Then, the samples were digested at 320 °C in the digestion instrument until clarification; subsequently, the liquid was diluted to 10 mL. Total C and N concentrations were determined using a Vario EL III analyzer (Elementar Analysensysteme, Hanau, Germany). Total P was measured by vanadate-molybdate-yellow method [32].

Gas exchange rates

On the 14th day of the treatments, gas exchange rates of the third fully expanded leaf (from the top) were measured using a photosynthesis system Li-6400 (LI-COR, Lincoln, Nebraska, USA). Net CO_2 assimilation rate (Pn) and stomatal conductance (g_s) were determined at 380 $\mu L \cdot L^{-1}$ CO_2 , 65% RH, and 1000 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PPFD (photosynthetic photon flux density). Measurements were performed in the morning between 09:00 a.m. and 11:00 a.m.

Chlorophyll fluorescence variables

On the 14th day of the treatments, photochemical and nonphotochemical quenching variables of photosystem II were measured using a portable fluorometer (Handy PEA, Hansatech Instruments Ltd., Norfolk, UK).

Leaves were first dark-adapted for 30 min, then exposed to a saturating red light pulse (650 nm, 3000 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) provided by an array of six light-emitting diodes. Chlorophyll fluorescence variables were calculated automatically in Handy PEA v 1.3 software [33].

Total free fatty acids, total sugars and soluble sugars

Fresh samples (leaves and roots) were ground in liquid N, then 100 mg samples were weighed and hydrolyzed in 1 mL deionized water and vortexed. After 3 h, the homogenates were centrifuged at $8000\times g$ at 4 °C, then 0.4 mL of the supernatant was used for the chromogenic reaction of free fatty acid (free FA) with copper acetate, and 200 μL was used to estimate the concentration of total free FAs by measuring absorbance at 715 nm. Additionally 100 mg samples were hydrolyzed, boiled, diluted, centrifuged, and used in the chromogenic reaction of reducing sugar with 3,5-dinitrosulfosalicylic acid, then absorbance was measured at 540 nm. For determination of soluble sugars, 100 mg fresh samples (leaves and roots) were ground in liquid N and then hydrolyzed in 1 mL deionized water. The homogenates were transferred to a 1.5-mL centrifuge tube and boiled at 95 °C for 15 min, then cooled with tap water. The tubes were then centrifuged at $8000\times g$ 25 °C for 10 min. The metabolite concentration was estimated using soluble sugar assay Kit (Comin Biotechnology Co. Ltd, Suzhou, China) and the manufacturer's instructions.

The concentration of free FAs, total sugars, and soluble sugars was determined separately using linear regression equations of the standard curves.

Quantitative analysis of fatty acids composition

One-hundred milligrams of fresh samples were ground in liquid N and transferred to a 15-mL centrifuge tube. 2 mL of 5% methanol-hydrochloric acid, 3 mL chloroform methanol (1:1, v:v), and 100 μL of methyl nonadecylate as the internal standards were added in sequence into the tube. After boiling at 85 °C for 60 min, and then cooling to 25 °C, 1 mL hexyl hydride was added into the tube, oscillation extraction for 2 min and stood for 1 h. The supernatant (100 μL) was consequently collected, filtrated by 0.45- μm filter membrane, and analyzed by GS-MS/MS (ThermoFisher Trace 1310 ISQ). Chromatographic separation was conducted on a TG-5MS (30 m \times 0.25 mm \times 0.25 μm) (a flow rate of 1.2 mL $\cdot\text{min}^{-1}$; injection port temperature of 290 °C).

Determination of free amino acids

Five hundred milligrams of fresh leaves and roots were ground to fine powder in liquid N and then hydrolyzed in 5 mL deionized water. Samples were diluted and

derivatized as done by [34]. The derivatized supernatant was quantified by HPLC-MS/MS (UltiMate 3000 [Thermo Fisher Scientific Inc., Waltham, MA, USA]-API 3200 QTRAP [AB Sciex, Boston MA, USA]) using MSLAB HP-C18 column (150 mm long, 4.6 mm diameter, 5 μm particle size; Beijing Amino Acid Medical Research Co.), with a flow rate of 1 mL $\cdot\text{min}^{-1}$, column temperature of 50 °C; solvent A (water with 0.1% methanoic acid) and solvent B (acetonitrile with 0.1% methanoic acid). Mass spectrometry (MS) conditions were set as IS: +5500 V; TEM: 500 °C; EP: +10; and CXP: +2.0.

Free polyamine analysis

Two-hundred milligrams of fresh tissue samples were ground in liquid N and extracted in 1.5 mL 5% (v/v) perchloric acid. Samples were centrifuged at 15,000 g and 4 °C for 30 min, and the supernatant was kept for further analysis. Following dansylation [35], free polyamines (Put, Spd, and Spm) in the supernatant were determined by HPLC-MS/MS (Ultimate3000-API 3200 Q TRAP) using a HP-C18 column (150 \times 4.6 mm, 5 μm). Two solvents, solvent A (water) and solvent B (acetonitrile), were delivered to the column at a flow rate of 1 mL $\cdot\text{min}^{-1}$. Elution was operated as described by [34].

Determination of the tricarboxylic acid cycle

Two-hundred milligrams of fresh tissue samples were ground in liquid N and hydrolysed in 1.5 mL deionized water. Then 50 μL of the solution was added to 200 μL of methanol (containing the internal standards). After left standing for 1 min, samples were centrifuged at 13,000 $\times g$ and 4 °C for 4 min. The supernatant was consequently collected and analyzed by HPLC-MS/MS (Ultimate3000-API 3200 Q TRAP) with ESI in negative ion mode. Chromatographic separation was conducted on a MSLab HP-C18 column (150 \times 4.6 mm, 5 μm ; a flow rate of 1 mL $\cdot\text{min}^{-1}$; column temperature of 50 °C). Two solvents, solvent A (water with 2 mmol $\cdot\text{L}^{-1}$ ammonium formate) and solvent B (acetonitrile with 2 mmol $\cdot\text{L}^{-1}$ ammonium formate), were used as mobile phases. Elution was operated as described by [34].

Determination of the key enzyme activities

The activities of key N metabolic enzymes were assayed separately using glutamine synthetase (GS), and glutamate synthetase (GOGAT) assay kits (Comin Biotechnology Co. Ltd, Suzhou, China). The activities of polyamine (i.e., ADC-arginine decarboxylase, and DAO-diamine oxidase,) metabolic enzymes were assayed separately using enzyme-linked immunosorbent assay (ELISA) Kits (MLBIO Biotechnology Co. Ltd, Shanghai, China). Assays were performed in 96-well

culture plates. Two-hundred milligrams of fresh tissue samples were ground in liquid N and homogenized in 500 µL extraction buffer (PBS, pH 7.4). Thereafter, they were centrifuged at 2500g and 4 °C for 20 min. The supernatants were collected, diluted and heat incubated at 37 °C for 60 min according to [36]; the processes followed the manufacturer’s instructions. Enzyme activity of the samples was calculated based on the absorbance values and the standard curve.

Statistical analysis

Before analysis of variance and testing for statistical significance, the normality of data distribution and homogeneity of variance were checked using the Shapiro–Wilk test and Levene’s test, respectively. For the data that did not meet the assumptions of normality and homogeneity of variance, logarithmic or the other transformations were applied. Then the data were assessed by two-way analysis of variance (ANOVA), followed by Duncan’s test to examine the differences between experimental treatments. Differences were considered significant at $P < 0.05$. If the data after transformations still did not meet normality distribution or homogeneity of variance, non-parametric estimations (Mann–Whitney U test or Kruskal–Wallis test) were applied. Correlation of metabolite concentration and plant water parameters was analyzed by using Pearson correlation coefficient (r) and linear regression analysis ($P < 0.05$). The analyses were performed using Excel software (Microsoft Office Standard 2013, Microsoft, Redmond, WA, USA), and IBM SPSS Statistics 20.0 (IBM Corp., Armonk, NY, USA).

Results

AM colonization and plant growth

Root colonization by *R. irregularis* was extensive in inoculated plants, with mycorrhizal colonization rate of about 60% under well-watered conditions, whereas root colonization was not detected in non-inoculated plants (Table 1). Drought stress had no significant impact on mycorrhizal colonization.

Drought stress decreased shoot biomass (DW) and height (SH), water content (WC), and N concentration of NM plants, without significant impact on C and P concentrations. AM symbiosis significantly increased shoot biomass, SH, WC, and P concentrations regardless of water conditions. Shoot N concentration was significantly higher in AM than NM plants under drought stress. Root-to-shoot biomass ratio (R:S) both of NM and AM plants significantly increased under drought stress.

Leaf photosynthesis and the levels of some metabolites

Drought stress decreased leaf photosynthesis (P_n , g_s and PI_{abs} values) of NM plants, with significant increase of DIO/RC and without a change of Chl concentration. Chl and PI_{abs} values were higher, but DIO/RC values were lower in AM than NM plants regardless of water conditions (Table 2). P_n and g_s values of AM plants were higher under well-watered conditions, but lower under drought stress as compared to NM plants (Table 2).

Leaf concentration of SS increased, but that of OAs decreased in NM plants under drought stress, without concentration changes of TS and free FAs. Leaf concentrations of SS and free FAs were significantly higher in AM than NM plants regardless of water conditions.

Table 1 Growth performance of maize shoots under drought stress and arbuscular mycorrhizal fungal inoculation (mean ± SD, n = 5)

Treatment	MC (%)	DW (g)	R:S	SH (cm)	WC (g)	C (mg·g ⁻¹)	N (mg·g ⁻¹)	P (mg·g ⁻¹)
W								
NM		1.24 ± 0.30b	0.39 ± 0.08b	54.19 ± 3.04b	11.21 ± 2.91 ^c	399 ± 4.81b	34.53 ± 1.08a	2.12 ± 0.35b
AM	65.20 ± 14.94a	2.17 ± 0.18a	0.20 ± 0.02d	62.80 ± 2.61a	21.15 ± 0.69a	413 ± 3.34a	35.25 ± 3.51a	3.46 ± 0.44a
D								
NM		0.94 ± 0.13c	0.55 ± 0.11a	50.57 ± 1.73c	6.47 ± 0.64d	401 ± 1.54b	30.32 ± 3.29b	2.38 ± 0.90b
AM	57.20 ± 10.25a	2.10 ± 0.18a	0.28 ± 0.06c	60.82 ± 3.25a	16.32 ± 3.97b	410 ± 5.96a	35.76 ± 1.15a	3.39 ± 0.51a
Two-way ANOVA								
D	ns ¹	ns	**	*	*	ns	**	ns
I	–	**	**	**	*	**	**	**
D × I	–	ns	ns	ns	–	ns	*	ns

Different lowercase letters in the same column indicate statistically significant differences between treatments at $P < 0.05$ by Duncan’s test

D drought stress, I inoculation treatment, W well-watered, AM arbuscular mycorrhizal fungal inoculation, NM non-mycorrhizal fungal inoculation, C carbon, DW dry weight, MC mycorrhizal colonization percentage, N nitrogen, P phosphorus, R:S root to shoot biomass ratio, SH shoot height, WC water content, ns not significant

* $P < 0.05$, ** $P < 0.01$

¹ ANOVA outputs by nonparametric statistical test

Table 2 Levels of leaf photosynthesis, sugars, tricarboxylic acids, and fatty acids of maize seedlings under drought and arbuscular mycorrhizal fungal inoculation (mean \pm SD, $n = 5$)

Treatment	Chl (mg·g ⁻¹)	Pl _{abs}	Dlo/RC	Pn (μmol·m ⁻² ·s ⁻¹)	G _s (mol·m ⁻² ·s ⁻¹)	TS (mg·g ⁻¹)	SS (mg·g ⁻¹)	OAs (μg·g ⁻¹)	FA (nmol·g ⁻¹)
W-									
NM	0.85 \pm 0.06c	0.74 \pm 0.15b	0.83 \pm 0.07b	22.19 \pm 1.68b	0.15 \pm 0.02b	26.26 \pm 1.80b	10.23 \pm 0.75c	1489 \pm 160b	13.90 \pm 1.59c
AM	1.11 \pm 0.11b	1.43 \pm 0.39a	0.61 \pm 0.09c	28.10 \pm 0.96a	0.22 \pm 0.03a	27.32 \pm 2.20b	12.63 \pm 1.56b	822 \pm 111c	29.08 \pm 5.15a
D									
NM	0.89 \pm 0.02c	0.63 \pm 0.06b	0.95 \pm 0.07a	16.19 \pm 1.01c	0.09 \pm 0.00c	27.92 \pm 3.24b	13.50 \pm 1.10b	908 \pm 94c	14.94 \pm 1.53c
AM	1.25 \pm 0.12a	1.50 \pm 0.28a	0.56 \pm 0.05c	10.95 \pm 1.30d	0.06 \pm 0.01d	32.17 \pm 2.58a	23.94 \pm 2.07a	2134 \pm 281a	20.66 \pm 1.93b
Two-way ANOVA									
D	**	ns	ns	**	**	*	**	**	ns
I	**	**	**	**	*	*	**	**	**
D \times I	ns	ns	*	**	**	ns	**	**	**

Different lowercase letters in the same column indicate statistically significant differences between treatments at $P < 0.05$ by Duncan's test

D drought stress, I inoculation treatment, W well-watered, AM arbuscular mycorrhizal fungal inoculation, NM non-mycorrhizal fungal inoculation, Chl chlorophyll, Dlo/RC the amount of energy dissipated per active reaction center, FA free fatty acids, g_s stomatal conductance, OAs organic acids in TCA cycle, Pl_{abs} photosystem II performance index on absorption basis, Pn net photosynthetic rate, SS soluble sugars, TS total sugars, ns not significant

* $P < 0.05$, ** $P < 0.01$

Table 3 Metabolic levels of free amino acids and polyamines in leaves of maize plants under drought and arbuscular mycorrhizal fungal inoculation (mean \pm SD, $n = 5$)

Treatment	AAs (μmol·g ⁻¹)	Arg (ng·mg ⁻¹)	Orn (ng·mg ⁻¹)	Put (ng·mg ⁻¹)	GABA (ng·mg ⁻¹)	ADC (U·g ⁻¹)	ODC (U·g ⁻¹)	DAO (U·g ⁻¹)	GAD (U·g ⁻¹)
W									
NM	71.89 \pm 8.50b	1.34 \pm 0.08a	0.77 \pm 0.14c	1.06 \pm 0.16c	4.13 \pm 0.49c	1.49 \pm 0.32a	0.69 \pm 0.09b	0.15 \pm 0.02b	4.16 \pm 0.76a
AM	56.20 \pm 6.06c	1.25 \pm 0.08a	1.01 \pm 0.10b	3.00 \pm 0.57b	2.99 \pm 0.50d	1.45 \pm 0.09a	1.01 \pm 0.04a	0.18 \pm 0.01a	3.23 \pm 0.07a
D									
NM	85.50 \pm 8.13a	1.17 \pm 0.39a	0.82 \pm 0.03c	4.73 \pm 0.63a	13.49 \pm 0.36a	1.63 \pm 0.22a	0.97 \pm 0.08a	0.12 \pm 0.02c	3.85 \pm 1.45a
AM	79.65 \pm 3.85ab	1.51 \pm 0.35a	1.46 \pm 0.18a	5.21 \pm 0.63a	7.79 \pm 0.38b	1.42 \pm 0.31a	0.79 \pm 0.12b	0.15 \pm 0.02b	3.96 \pm 1.02a
Two-way ANOVA									
D	**	ns	**	**	**	ns	ns	* ¹	ns ¹
I	**	ns	**	**	**	ns	ns	* ¹	ns ¹
D \times I	ns	ns	**	**	**	ns	**	–	–

Different lowercase letters in the same column indicate statistically significant differences between treatments at $P < 0.05$ by Duncan's test

D drought stress, I inoculation treatment, W well-watered, AM arbuscular mycorrhizal fungal inoculation, NM non-mycorrhizal fungal inoculation, AAs free amino acids, ADC arginine decarboxylase, Arg arginine, DAO diamine oxidase, GABA γ -aminobutyric acid, GAD glutamic acid decarboxylase, ODC ornithine decarboxylase, Orn ornithine, Put putrescine, ns not significant

* $P < 0.05$, ** $P < 0.01$

¹ ANOVA outputs by nonparametric statistical test

Arbuscular mycorrhiza induced the accumulation of leaf OAs and TS under drought stress.

Drought stress increased leaf concentration of free AAs (excluding Arg and Orn) of NM plants. AM symbiosis induced the accumulation of free AAs (including Orn) under drought stress compared to that under well-watered condition. Moreover, drought stress increased Put and GABA concentration of NM plants, being accompanied with changes of ODC (increased) and DAO (decreased) activity and without changes of ADC and GAD activity. Leaf concentration of Put was higher

and of GABA lower in AM than NM plants regardless of water conditions (Table 3). Leaf ODC activity was lower and DAO was higher in AM than NM plants under drought stress.

Correlations of GABA concentration with stomatal conductance and malic acid concentration

Leaf GABA concentration had a significantly negative correlation with leaf g_s for both NM and AM plants subjected to drought stress (Fig. 1). Moreover, significant correlations between GABA and malic acid

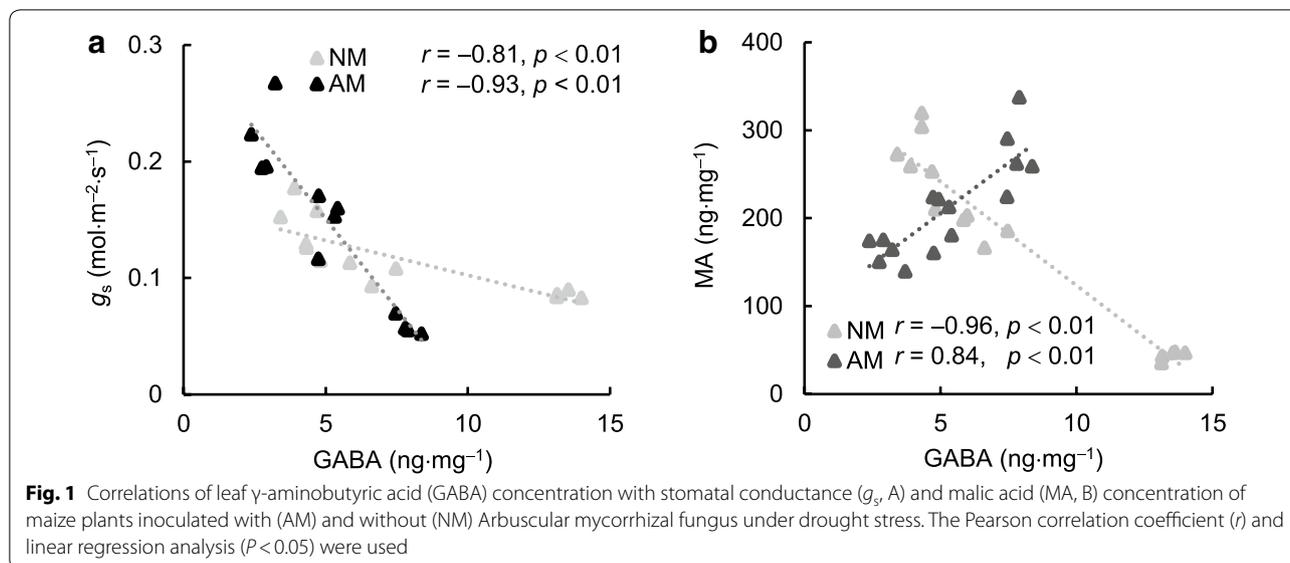


Table 4 Root levels of P and C, sugars, and tricarboxylic acids of maize seedlings under drought and arbuscular mycorrhizal fungal inoculation (mean \pm SD, $n = 5$)

Treatment	P (mg·g ⁻¹)	C (%)	R:S C ratio	SS:TS	Gly (mg·g ⁻¹)	OAs (μ g·g ⁻¹)
W						
NM	2.42 \pm 0.53b	41.22 \pm 0.78a	0.40 \pm 0.07b	0.33 \pm 0.02b	4.95 \pm 0.70b	311 \pm 63a
AM	3.80 \pm 0.28a	41.34 \pm 0.81a	0.17 \pm 0.01d	0.40 \pm 0.02b	3.62 \pm 0.21c	281 \pm 44a
D						
NM	2.49 \pm 0.13b	40.72 \pm 0.93a	0.56 \pm 0.10a	0.41 \pm 0.06b	8.11 \pm 0.89a	254 \pm 50a
AM	3.26 \pm 0.45a	40.18 \pm 1.43a	0.30 \pm 0.03c	0.53 \pm 0.11a	5.72 \pm 0.48b	289 \pm 31a
Two-way ANOVA						
D	ns	ns	**	**	**	ns
I	**	ns	**	*	**	ns
D \times I	ns	ns	ns	ns	ns	ns

Different letters above the columns indicate statistically significant differences between means at $P < 0.05$ by Duncan's test

D drought stress, I inoculation treatment, W well-watered, AM arbuscular mycorrhizal fungal inoculation, NM non-mycorrhizal fungal inoculation, Gly glycogen, OAs organic acids in the tricarboxylic acid cycle, SS soluble sugars, TS total sugars

concentrations were found in leaves of NM (negatively) and AM (positively) plants during drought stress.

Root levels of ornithine cycle, polyamines, and fatty acids

Root C and P concentrations were not affected by drought stress, showing higher root P levels in AM than NM plants regardless of water conditions (Table 4). Drought stress induced increases of R:S C ratio, SS:TS ratio, and Gly concentration in roots both of NM and AM plants. SS:TS ratio was higher and both R:S C ratio and Gly concentration were lower in AM than NM plants regardless of water conditions. Drought and AM

symbiosis did not impact root OAs level both of NM and AM plants.

Drought stress had no impact on root concentration of the unsaturated FAs, but decreased its proportion in total FAs. Root levels of the unsaturated FAs were significantly higher in AM than NM plants regardless of water conditions (Table 5). C16.1 concentration was about 50- and 36-folds higher in AM than NM plants under watered and stressed condition, respectively. Concentrations of C18.1N9 and C18.2N6 were about twofold higher in AM than NM plants both under watered and stressed conditions. Concentrations of the major SFAs (except C16.0) increased under drought stress, without significant difference (except C24.0) in root concentration between

Table 5 Root levels of free fatty acids (FAs) of maize seedlings under drought and arbuscular mycorrhizal fungal inoculation (mean \pm SD, $n = 5$)

Treatment	UFAs ⁱ (ng·mg ⁻¹ FW)	SFAs ⁱⁱ (ng·mg ⁻¹ FW)												
		(%, in the total FAs)	C16.1	C18.1N9	C18.2N6	C18.3N6	C16.0	C18.0	C20.0	C22.0	C24.0			
W														
NM	193.66 \pm 8.64b	30.35 \pm 1.01b	0.93 \pm 0.26c	57.41 \pm 16.13b	109.27 \pm 13.76b	14.97 \pm 5.52b	329.63 \pm 18.14b	47.53 \pm 3.98b	9.48 \pm 0.66b	7.08 \pm 0.84b	36.32 \pm 3.14c			
AM	462.44 \pm 101.61a	39.45 \pm 2.24a	46.69 \pm 13.51b	119.22 \pm 38.44a	253.18 \pm 54.89a	26.26 \pm 5.04a	585.28 \pm 113.65a	44.56 \pm 10.19b	7.64 \pm 1.14b	7.05 \pm 0.40b	43.87 \pm 4.60b			
D														
NM	197.28 \pm 11.94b	28.67 \pm 1.12c	1.85 \pm 0.75c	65.86 \pm 6.11b	92.78 \pm 9.81b	23.22 \pm 7.28ab	344.99 \pm 25.28b	57.41 \pm 1.78a	11.04 \pm 1.78a	10.54 \pm 2.01a	49.55 \pm 5.03b			
AM	447.58 \pm 69.30a	37.26 \pm 0.60a	67.66 \pm 6.23a	124.36 \pm 20.17a	224.32 \pm 56.72a	20.63 \pm 3.45ab	563.12 \pm 61.86a	56.01 \pm 1.10a	11.87 \pm 1.10a	11.58 \pm 0.85a	58.38 \pm 2.21a			
Two-way ANOVA														
D	ns	*	ns ¹	ns	ns	ns	ns ¹	**	**	**	**	**	**	**
I	**	**	** ¹	**	**	ns	** ¹	ns	ns	ns	ns	ns	ns	**
D \times I	ns	ns	-	ns	ns	*	-	ns	ns	ns	ns	ns	ns	ns

Different lowercase letters above the same column indicate statistically significant differences between means at $P < 0.05$ by Duncan's test

D drought stress, I/inoculation treatment, W/well-watered, AM arbuscular mycorrhizal fungal inoculation, NM non-mycorrhizal fungal inoculation, UFAs unsaturation fatty acids, SFAs saturation fatty acids, ns not significant

* $P < 0.05$, ** $P < 0.01$

ⁱ Presents all the unsaturation fatty acids that were detected by GS-MS/MS

ⁱⁱ Presents the major compositions (with a concentration > 10 ng·mg⁻¹ FW) of free FAs

¹ ANOVA outputs by nonparametric statistical test

Table 6 Metabolic levels of free amino acids and polyamines in roots of maize plants under drought and arbuscular mycorrhizal fungal inoculation (mean ± SD, n = 5)

Treatment	AAs (μmol·g ⁻¹)	Urea-N (mg·g ⁻¹)			Ornithine cycle (ng·mg ⁻¹)			Polyamines (ng·mg ⁻¹)			GABA (ng·mg ⁻¹)	DAO (U·g ⁻¹)	GAD (U·g ⁻¹)
		Arg	Orn	Cit	Asp	Put	Spd	Spm					
W													
NM	18.70 ± 2.80c	0.08 ± 0.01b	8.69 ± 1.81c	0.90 ± 0.15c	1.06 ± 0.08c	18.57 ± 5.57c	2.74 ± 0.88b	0.0036 ± 0.002a	0.016 ± 0.005a	12.31 ± 1.40b	0.12 ± 0.02b	3.84 ± 0.77a	
AM	51.84 ± 4.73b	0.07 ± 0.01b	16.41 ± 2.93a	2.91 ± 0.72a	2.47 ± 0.20a	28.52 ± 4.45b	6.00 ± 1.02a	0.0043 ± 0.001a	0.016 ± 0.005a	9.28 ± 2.20c	0.13 ± 0.02ab	3.19 ± 0.18ab	
D													
NM	12.86 ± 1.76d	0.11 ± 0.02a	8.49 ± 1.90c	1.24 ± 0.13c	1.02 ± 0.11c	26.85 ± 13.28b	1.47 ± 0.54c	0.0037 ± 0.002a	0.014 ± 0.002a	20.96 ± 1.94a	0.11 ± 0.02b	4.08 ± 0.83a	
AM	69.53 ± 7.11a	0.09 ± 0.01b	11.44 ± 1.77b	2.11 ± 0.23b	2.02 ± 0.09b	53.10 ± 19.1a	1.41 ± 0.38c	0.0031 ± 0.001a	0.020 ± 0.010a	19.35 ± 3.21a	0.15 ± 0.03a	3.91 ± 1.25a	
Two-way ANOVA													
D	ns ¹	**	ns	ns	*	*	**	ns	ns	** ¹	ns	ns	
I	*	*	**	**	**	*	*	ns	ns	ns ¹	**	ns	
D x I	-	ns	*	**	ns	-	**	ns	ns	-	*	ns	

Different lowercase letters in the same column indicate statistically significant differences between treatments at P < 0.05 by Duncan's test

D drought stress, I inoculation treatment, W well-watered, AM arbuscular mycorrhizal fungal inoculation, NM non-mycorrhizal fungal inoculation, AAs free amino acids, Arg arginine, Asp aspartic acid, Cit citrulline, DAO diamine oxidase, GABA γ-aminobutyric acid, GAD glutamic acid decarboxylase, Orn ornithine, Put putrescine, Spd spermidine, Spm spermine, ns not significant

*P < 0.05, **P < 0.01

¹ ANOVA outputs by nonparametric statistical test

NM and AM plants. Root C16.0 concentration was significantly higher in AM than NM plants regardless of water conditions.

Drought stress decreased free AAs concentration and led to urea accumulation in roots of NM plants. Compared to NM plants, free AAs concentration significantly increased in AM plants regardless of water conditions; urea-N concentration remained a lower level. Drought stress did not impact root ornithine cycle (except for Asp); in contrast, AM symbiosis stimulated ornithine cycle, showing significant increases in root concentrations of Arg, Orn, Cit, and Asp (Table 6). Correspondingly, root Put concentration was significantly higher in AM than NM plants under well-watered conditions. Root Put concentration decreased and GABA concentration significantly increased both in NM and AM plants with drought stress. AM symbiosis alone and in combination with drought can increase DAO activity, but did not impact GAD activity.

Discussion

In recent studies [37], the importance of mycorrhizal symbiosis in improving plant drought tolerance was emphasized. Considerable evidence supports the beneficial AM effects on plant water relations and nutrition status. AM-induced plant adaptations to drought involve complex changes of numerous metabolites and metabolic pathways [13], which are accompanied with concurrent changes of plant growth and nutrient availability [38]. Deciphering which metabolic processes are central for AM-mediated regulations is essential for the application of AM-based biostimulators in improving agricultural production in drylands. Our results support the hypothesis that AM symbiosis significantly improved plant drought tolerance through altering ornithine cycle and fatty acid compositions in roots, and accumulation patterns of polyamines and carbohydrates in plant organs.

AM-mediated improvements in growth performance and photosystem II efficiency

Maize as a C₄ plant has high water and nutrient requirements for rapid plant growth. Maize plants are sensitive to even minor changes of water availability [39]. So obviously decreased water and nutrient status occurred in maize cultivars (e.g., Doge and Luce [40]; Mo17 in this study) when subjected to drought stress. In this study, R:S biomass ratio of NM plants increased by 41% under drought stress compared to that under well-watered conditions. The increased R:S ratio is mainly due to a reduction of shoot biomass, because root biomass was not changed significantly (data not shown). Drought stress led to limitations of water and nutrient (such as N and P) availability, an adaptive strategy of plants is that root

growth-related metabolisms were enhanced for maintaining water and nutrient requirements, whereas shoot growth-related metabolisms were downregulated to reduce consumptions of water and nutrients [41]. Previous studies showed that AM fungi have a high capacity to colonize maize roots, with a colonization rate of approximately 40–80% after 30 days of inoculation [27, 42]. Our results were within such range of colonization (Table 1). Mycorrhizal colonization can substantially improve crop growth both under well-watered and drought-stressed conditions [43], which has also been verified by the present study (Table 1). Increasing P availability is a major contributor for AM plants to improve growth performance and drought tolerance [15]. Our previous studies [44] have demonstrated the beneficial effects of AM-mediated improvement in P availability on photosynthesis (stomatal conductance and net photosynthetic rate), water use efficiency, and growth performances.

In this study, AM symbiosis produced beneficial effects on allocations of the absorbed light in photosystems, which increased the energy proportion used for photochemical processes (e.g., PI_{abs}) but decreased the proportion for non-photochemical dissipation (e.g., DIO/RC). Several previous studies [45] reported consistent results, but they did not explain the underlying mechanisms. The increased accumulation of PAs in leaves of AM plants may be a cue for the beneficial effects (Table 3). PAs are mainly present in chloroplasts and photosynthetic sub-complexes (such as thylakoids, LHCII complex, and PSII membranes) [46]; so they can impact photochemical processes through regulations of photosystem II activity, photophosphorylation, and light energy dissipation [47]. The accumulation of intracellular PAs can also stimulate light-independent chlorophyll biosynthesis from protochlorophyllide [48]. The above cues may provide a reasonable explain why Put level, Chl concentration, and PI_{abs} values were conformably higher in leaves of AM plants (compared to NM plants) both under well-watered and drought-stressed conditions.

AM effects on the accumulation of soluble sugars and saturation of fatty acids

Previous studies have demonstrated the flow of C into mycorrhizal roots for the mutualistic interaction [17]. The amount of C allocated to mycelium or storage lipids in AM fungi is a potential determinant of root C allocation [49]. In this study, there is no significant difference in root C concentration and root biomass between NM and AM plants. It seemly indicates that AM symbiosis did not impact root C allocation. The C concentration-based assessment may underestimate the real amount of C transferred from shoot to mycorrhiza, because a proportion of C flows to different components of soil C pools for

nutrient (such as N and P) exchanges between the interfaces of soil and plant root system [50]. Another evidence is that fatty acid concentration in roots was significantly higher in AM than NM plants. Root C16:1 concentration showed an AM-specific response, whereby C16:1 concentration was 50-fold higher in AM than NM plants (Table 2). In some studies [51], 16-carbon fatty acids were considered AM fungi-specific fatty acids. Moreover, the unsaturated compositions (C18.1N9, C18.2N6, and C18.3N6) also significantly increased in roots of AM plants (compared to NM plants) regardless of water status. In contrast, the saturated compositions of FAs (such as C18.0, C20.0, C22.0, and C24.0) were not affected by mycorrhizal colonization, but the root concentration increased with drought stress. Therefore, our results confirm that AM symbiosis substantially increased unsaturation of root fatty acids. AM-induced changes in FA unsaturation have the physiological significance to plant drought adaptation. Because an increase in FA unsaturation can alleviate the oxidative damage to plants under drought stress conditions [22].

Soluble sugars (such as sucrose) are very sensitive to drought stress. Organ concentration of SS and SS:TS ratio increased under drought stress compared to well-watered conditions. Soluble sugars are involved in osmotic and metabolic regulations. Molecular evidence showed the roles of SS in regulations on expressions of both growth- and stress-related genes [2]. AM symbiosis had significantly positive impacts on the accumulation of soluble sugars in leaves regardless of water status. An increased photosynthetic rate may be a contributor for AM plants to the accumulation of carbohydrates in leaves under well-watered conditions. However, under drought stress, showing a lower Pn value in leaves of AM than NM plants, leaf SS concentration was still significantly higher in AM than NM plants. This result indicates that AM may regulate soluble sugar metabolism through the other mechanisms. A possible pathway may be that AM regulates expression of sugar metabolism-related proteins (such as sucrose phosphate synthase, neutral invertase, and sucrose:fructan 6-fructosyltransferase) and/or the enzyme activities (such as acid invertase and sucrose synthase activity) [3]. An alternative pathway is that AM symbiosis can stimulate glycolysis process through upregulating expression of the glycolysis-related genes [10]. The alternative pathway mentioned above may be linked to our result that leaf OAs concentration was significantly higher in AM plants (compared to NM plants) under drought stress. Citrate is a potential target of AM actions on TCA cycle; a recent study by Zhang et al. [52] demonstrated the roles of AM symbiosis in regulating the gene expression of key metabolic enzymes (such as citrate synthase and citrate lyase). Moreover,

citrate is an alternative C source for fatty acid synthesis [53]. Therefore, citrate may be very important in organic C partitioning between tricarboxylic acids and fatty acids in AM plants. In this study, opposite changing patterns of OAs and free FA concentrations occurred in leaves of AM plants under well-watered and drought stress conditions (Table 2). However, our result cannot ensure whether citrate is really involved in AM-induced changes of OA and FA concentrations or not.

AM symbiosis altered ornithine cycle and polyamine metabolism

Arginine is considered a major fungal AA after N uptake and assimilation by the external hyphae [23]. Mycelium Arg translocation is proven under the N supplies [54]. Fungal Arg translocation may increase Arg level in AM roots and further cause qualitative or quantitative alterations of free AAs. Our results support this view by the evidence that root levels of free AAs and the ornithine cycle (showing concentration increases of Arg, Orn, Cit, and Asp) were significantly higher in AM than NM plants regardless of water status. Urea is a metabolic product in the conversion of Arg and Orn. So root urea level was predicted to increase in a precondition of increasing ornithine cycle, whereas in this study, root urea concentration was significantly lower in AM than NM plants under drought stress. This result can occur if AM symbiosis increased the conversion efficiency from urea to NH_4^+ in roots, further stimulating root-to-shoot N translocation. In this study, drought stress led to a decrease of shoot N concentration of NM plants, being accompanied with a significant accumulation of root urea, in contrast, the N concentration of AM plants was not changed significantly in shoots under well-watered and drought stress conditions.

Putrescine is a coupling metabolite in the ornithine cycle; so the result that root Put level was higher in AM than NM plants was expected under well-watered conditions. However, the changes of root Put concentration that decreased both in NM and AM plants with drought stress did not depend on concentration changes of Arg or Orn, and may be a responsive strategy for maize plants. Decreased Put concentration in plant organs was also reported in drought-responsive experiments, which was attributed to metabolic conversions into PAs (spermidine and spermine) or catabolites [55]. A conversion of Put via diamine oxidase (DAO) to GABA may be a reason for the decline of Put concentration in roots of AM plants [27]. Under drought stress, root GABA concentration increased by about 110% in AM plants, in contrast, only by 70% in NM plants. Moreover, there was a positive correlation between root GABA and malic acid concentration in AM plants (data not shown). A recent study by

[30] proposed that an increase in GABA concentration can restrict efflux of malate²⁻ from root cytosol through down-regulations of ALMT gene expression, which may improve osmoregulation capacity of root cells.

A high sensitivity of GABA to drought has been demonstrated by the genetic mutation. GABA concentration in plant tissues is estimated to alter even within seconds in response to stresses [56]. Leaf GABA accumulation contributes to preservation of plant water under drought stress [57]. In this study, a negative correlation between leaf GABA concentration and stomatal conductance existed during drought stress. Several previous studies have demonstrated the roles of GABA in regulating stomatal opening. A possible mechanism is assumed to be GABA-induced repression of the gene expression of 14-3-3 proteins and subsequent inactivation of the KAT1, K channel, finally leading to a repression of inward K⁺ flow (I_{K_{in}}) [58]. Another possible mechanism is through GABA-induced repression of the expression of aluminum-activated malate transporter (ALMT) genes in plasma membrane and subsequent stimulation on anions (malate²⁻ and Cl⁻) efflux from cytosol, but restrict influx of the anions from apoplast [30]. Some of these hypothetical mechanisms will ultimately trigger stomatal closure and restrict water loss through leaf transpiration.

Conclusions

Arbuscular mycorrhizal symbiosis significantly improved maize growth and drought tolerance. The physiological (such as photochemical processes) and metabolic (such as soluble sugars, fatty acids, and ornithine cycle) improvements by AM symbiosis are central for maize seedlings in growth regulation and drought response. Our results confirm significant positive effects of AM symbiosis on organ Put level under well-watered conditions, which may be due to AM-induced increases in Orn concentration and ODC activity, whereas the mechanisms causing the differences in organ Put response to drought (decreased in roots and increased in leaves) were unknown. Future studies are proposed to focus on the signal pathways and molecular mechanisms of AM-induced regulations on the central processes.

Abbreviations

AAs: Free amino acids; ADC: Arginine decarboxylase; AM: Arbuscular mycorrhizal fungal inoculation; Arg: Arginine; Asp: Aspartic acid; Chl: Chlorophyll; Cit: Citrulline; DAO: Diamine oxidase; Dlo/RC: The amount of energy dissipated per active reaction center; DW: Dry weight; FA: Free fatty acids; GABA: γ -Aminobutyric acid; GAD: Glutamic acid decarboxylase; Gly: Glycogen; g_s: Stomatal conductance; MC: Mycorrhizal colonization percentage; NM: Non-mycorrhizal fungal inoculation; OAs: Organic acids in TCA cycle; ODC: Ornithine decarboxylase; Orn: Ornithine; P_{abs}: Photosystem II performance index on absorption basis; Pn: Net photosynthetic rate; Put: Putrescine; SFAs: Saturation fatty acids; Spd: Spermidine; Spm: Spermine; SS: Soluble sugars; TS: Total sugars; UFAs: Unsaturation fatty acids; WC: Water content.

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Authors' contributions

YBH and BDC designed the experiments and wrote the paper. YBH performed the experiments. YBH and W X analyzed the data. All authors read and approved the final manuscript.

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Availability of data and materials

The data sets generated and/or analyzed during the current study are not publicly available (because all of the data were gathered by the research team), but are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

I approved the ethics guideline of the journal.

Consent for publication

All of authors have informed regarding submitting the manuscript to the journal of *Chemical and Biological Technologies in Agriculture*.

Competing interests

The authors declare that they have no competing interests.

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