

INTERACTIVE EFFECTS OF COPPER STRESS AND ARBUSCULAR MYCORRHIZAL FUNGI ON PHOTOSYNTHETIC CHARACTERISTICS AND CHLOROPHYLL FLUORESCENCE PARAMETERS OF *ELSHOLTZIA SPLENDENS*

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Abstract

To determine interactive effects of added copper (Cu) and arbuscular mycorrhizal fungi (AMF) inoculation on the photosynthesis of *Elsholtzia splendens*, a greenhouse pot experiment was conducted. Four treatments were used, including -Cu-AMF (no Cu addition and no AMF inoculation), +Cu-AMF (Cu addition but no AMF inoculation), -Cu+AMF (no Cu addition and AMF inoculation), and +Cu+AMF (Cu addition and AMF inoculation). Cu addition did not change diurnal variation curves of the net photosynthetic rate (P_N), the intercellular CO₂ concentration (C_i), the stomatal conductance (g_s), or the transpiration rate (E); however, it significantly decreased the daily mean P_N , g_s , E , light-use efficiency (LUE), and carboxylation efficiency (CE). Furthermore, AMF inoculation significantly increased the daily mean P_N , g_s , LUE, and CE of *E. splendens*. In response to light, Cu addition significantly decreased the light-saturated net photosynthetic rate (P_{Nmax}), the light saturation point (LSP), the light compensation point (LCP), and the apparent quantum yield (AQY), while AMF inoculation significantly increased P_{Nmax} and AQY. In response to the CO₂ concentration, Cu addition significantly decreased P_{Nmax} and the CO₂ saturation point (CSP), while AMF inoculation significantly increased P_{Nmax} . Both Cu addition and AMF inoculation significantly decreased the relative chlorophyll content. Compared to the negative control treatment (-Cu-AMF), Cu addition significantly increased the minimal fluorescence, but significantly decreased maximal fluorescence, variable fluorescence, and maximum photochemical efficiency of PSII. These results suggest that AMF inoculations alleviate the inhibitory effect of copper stress on *E. splendens* plants by weakening its toxic effects on the photosynthetic apparatus and pigments.

Key words: AMF, Cu stress, Gas exchange, Light response, CO₂ response, Chlorophyll fluorescence

Introduction

Copper (Cu) is a redox-active transition metal, essential for many metabolic pathways, such as respiratory and photosynthetic electron transport, antioxidant activity, and protein and cell wall metabolism (Kamali *et al.*, 2012); however, an excess of Cu can potentially cause complete disruption of plant growth and development (Cook *et al.*, 1997, Wang *et al.*, 2012, Arunakumara *et al.*, 2013). Cu toxicity is very severe in agriculture due to the use of agrochemicals containing Cu as an active component (Chen *et al.*, 2013), in the greenhouse industry due to the application of electrolytically generated Cu to restrain algae and diseases (Zheng *et al.*, 2004), and in mine-waste tailings of Cu ores due to the residue of Cu particles in mine slurry (Kabata-Pendias & Pendias, 2001). Remediation techniques are urgently required to reduce the concentrations of this metal in the soil and to avoid its absorption by crop plants (Arunakumara *et al.*, 2013).

Arbuscular mycorrhizal fungi (AMF) are common root symbionts of terrestrial plants. AMF can significantly enhance the heavy metal tolerance of plants, including Cu stress (Carvalho *et al.*, 2006, Malekzadeh *et al.*, 2007, Hildebrandt *et al.*, 2007, Ferrol *et al.*, 2009; Meier *et al.*, 2011; 2012; 2015). The alleviative effect of AMF inoculation on Cu stress might be due to increasing water and nutrient absorption, particularly phosphate (Andrade *et al.*, 2009; Helgason & Fitter 2009; Smith & Smith, 2013), thus reducing the transfer of toxic metals into the shoots (Andrade *et al.*, 2009; Amir *et al.*, 2013), while increasing heavy-metal accumulation in plant tissues

(Orlowska *et al.*, 2012). However, few of these studies have investigated the physiological changes within plants under Cu stress induced by AMF colonization.

Photosynthesis is one of the central physiological processes in plants contributing to their growth (Cheng *et al.*, 2000). It is well documented that Cu exerts direct toxicity on photosynthesis (Lidon, 1999; Maksymiec, 1997) by disturbing lipid peroxidation of thylakoid membranes as well as the interaction between lipids and proteins in the chloroplast membrane (Szalontai *et al.*, 1999), thus severely affecting the photosynthetic electron transport chain (Myśliwa-Kurczel & Strazalka, 2002). Cu causes indirect toxicity by interfering with Calvin-Benson-cycle enzyme activities or with net CO₂ assimilation (Prasad & Strzalka, 1999), ultimately inhibiting plant growth (Vinit-Dunand *et al.*, 2002; Qian *et al.*, 2005). Mycorrhizal symbiosis can alter the photosynthesis of the host plant to aid against adverse environmental conditions (Zhu *et al.*, 2010). Several reports revealed that AMF inoculation could also affect photosynthesis in strawberries (Borkowska, 2002) and citrus fruits (Wu & Xia, 2006) under drought stress. Aloui *et al.* (2011) demonstrated that inoculation with the AMF *Glomus irregulare* resulted in positive effects on photosynthesis in the presence of Cd, while increasing photosynthesis-related proteins. However, the effects on photosynthesis of AMF symbiosis are related to the species of fungus, soil nutrient condition, and to the particular plant involved. Bittman *et al.* (2006) detected poor photosynthetic response to AMF inoculation under high nutrient condition. Syvertsen & Graham (1990) reported no influence of AMF infection on the net gas exchange characteristics of citrus leaves on plants. To our knowledge, no report exists that focuses on the

effect of AMF inoculation on the photosynthesis of plants under Cu stress.

Elsholtzia splendens is an annual herb from the Lamiaceae family and is a Cu-tolerant plant used as a metal hyperaccumulator (Jiang *et al.*, 2008). *E. Splendens* is widely distributed on Cu-polluted soils and Cu-mining wastes (Tang *et al.*, 1999, Lou *et al.*, 2004) and is reported to be an obligate symbiont with AMF (Yang *et al.*, 2010). AMF in turn plays a central role in plant uptake and accumulation of heavy metals (Wang *et al.*, 2006). In high concentrations, Cu significantly inhibits photosynthetic parameters (Ke *et al.*, 2007); however, inoculation with soil microbes can significantly increase the photosynthetic ability of *E. splendens* (Li *et al.*, 2015). We conducted a pot experiment and found significant interactions between mycorrhizal inoculation and Cu addition on the total seed number, vegetative biomass, and inflorescence number of *E. splendens* (Jin *et al.*, 2015). Here, we were using the same experimental system to explore interactions between AMF and Cu, affecting the photosynthetic capability of *E. splendens* to ascertain the following: 1) How are Cu and AMF interactively affecting the daily photosynthetic process of *E. splendens*? 2) How are Cu and AMF interactively affecting the photosynthetic capability of *E. splendens*? These results provide a basic reference for the application of hyperaccumulators in the phytoremediation and ecological restoration of Cu polluted soils.

Materials and Methods

Soil preparation: The culture medium that was used for the pot experiment consisted of vermiculite, sand, and peat soil (1:3:6, v/v/v). The soil medium was autoclaved under pressure (0.11 MPa) at 121°C for 2 h to neutralize all native microbial populations (Andrade *et al.*, 2009). Subsequent to autoclaving, each kilogram of soil had the following properties: 20.16±0.26 g organic matter, 14.61±0.53 mg total N, 17.86±0.49 mg available P, and 56.67±0.16 mg available K. The pH (in water) was 5.73±0.04.

Seed germination: On the 20th of December 2012, seeds of *E. splendens* were obtained from clean soil in the Tainan village, Hong'an County, Hubei Province, China (31°30.632'N, 114°32.620'E; altitude of 118 m) after which, they were transferred to an incubator at room temperature. On the 5th of May 2013, seeds were surface disinfected in a 0.5% solution of hypochlorite and thoroughly rinsed with sterile distilled water. Then, they were sowed into the autoclaved soil mixture within 4×8 trays for germination in a greenhouse at the Taizhou University in Zhejiang Province of China (121°17'E, 28°87'N).

Treatments: On the 1st of May 2013, plastic pots (15 cm deep, round, and with an inner diameter of 19 cm) were filled with 1.7 kg of autoclaved soil mixture, after sterilization via 75% ethanol. All pots were randomized and placed into the greenhouse under a relative humidity of 70% ± 10.5% and a temperature of 30.0 ± 5 °C during the days and 18.0 ± 2°C during nights. Plants were illuminated with natural light. The experiment consisted

of four treatments, including (1) -Cu+AMF (no Cu addition and AMF inoculation), (2) +Cu+AMF (both Cu addition and AMF inoculation), (3) -Cu-AMF (no Cu addition and no AMF inoculation), and (4) +Cu-AMF (Cu addition but no AMF inoculation). A total of 60 pots were used with 15 repetitions per treatment. On the 5th of May 2013, 50 mL aliquot of CuSO₄·5H₂O solution (34 mg mL⁻¹) were added to each pot of the treatment groups +Cu+AMF and +Cu-AMF. The available Cu content at the start of experiment in the soil of all four treatments was 18.90 ± 2.05 mg kg⁻¹.

On the 21st of December 2012, bulk sandy clay soil was collected from the top layer (0-20 cm) at a Cu mine tailing, which was located within the Chimashan Mountains, Yangxin County, Hubei Province, China (29°59.776'N, 115°05.856'E; altitude 138 m). The accompanying plants were *Xanthium sibiricum*, *Cynodon dactylon*, *Commelina communis*, *Artemisia capillaries*, and *Silene fortunei*. The soil was sieved with a 2-mm sieve to remove all litter and vegetation, subsequently stored at -20°C until further use as a resource of soil microbes. On the 6th of May 2013, soil obtained from a Cu mine tailing was taken out of the refrigerator and incubated at room temperature for 48 h. The AMF were inoculated, following a previously published procedure (Jin *et al.*, 2015). In the no AMF inoculation treatments, 50 mL filtrate was applied to each of the pots to compensate for the microbe treatment of the other groups. On the 5th of June 2013, one 12-cm-tall seedling was transplanted into each pot. All pots were well watered and the soil moisture content was monitored via weight.

Gas exchange measurement: On the 15th of August 2013, *In situ* photosynthetic traits measurements were made on a clear day on well-expanded, healthy leaves at similar positions on the main stems utilizing the portable photosynthesis system Li-6400XT (LICOR Inc., Lincoln, NE, USA). Three leaves per plant and pot were chosen, and three randomly selected *E. splendens* plants were measured from 06:00 to 18:00 h (Beijing time) at two-hour intervals. The parameters obtained included: net photosynthetic rate (P_N), intercellular CO₂ concentration (C_i), transpiration rate (E), stomatal conductance (g_s), air temperature (T_{AIR}), vapour pressure deficit (VPD), photosynthetically active radiation (PAR), leaf temperature (T_{Leaf}), relative humidity (RH), and ambient CO₂ concentration (C_a). Light-use efficiency (LUE) was defined as the ratio of P_N per PAR (Long *et al.*, 1993), water-use efficiency (WUE) was defined as the ratio of P_N per E (Hamid *et al.*, 1990), and carboxylation efficiency (CE) was defined as the ratio of P_N per C_i (Flexas *et al.*, 2001).

Light response curves: The light response curves were measured between 09:30 and 11:00 h (Beijing time) on fully expanded leaves of each plant under constant conditions (CO₂ concentration of 400 μmol mol⁻¹; relative humidity of 70 ± 5%; leaf temperature of 25°C). The light source was an internal LED red/blue (LI6400-02B). Prior to the measurements, the leaves were equilibrated under a PAR of 2,000 μmol m⁻²s⁻¹ for at least 15 min to reach steady-state photosynthesis. Once stable, the photosynthetic capacity of the leaves was measured at PARs of 2,000, 1,500, 1,200, 1,000, 800, 600, 400, 200,

100, 50, 20, and 0 $\mu\text{mol m}^{-2}\text{s}^{-1}$. The resulting light response curves were analyzed via the revised exponential equation (Ye, 2007);

$$P(I) = \alpha \frac{1 - \beta I}{1 + \gamma I} (I - I_c),$$

where α , β , and γ are coefficients, $P(I)$ is the net photosynthetic rate, I is the incident PAR, and I_c is the light saturation point. The maximum leaf light-saturated net photosynthetic rate ($P_{N_{\max}}$), light saturation point (LSP), light compensation point (LCP), and the apparent quantum yield (AQY) were calculated via the above equation (Ye, 2007).

CO₂ response curves: The CO₂ response curves were measured between 09:30 and 11:00 h (Beijing time) on fully expanded leaves from each plant with a leaf temperature of 25°C, a light saturating intensity of 1,500 $\mu\text{mol m}^{-2}\text{s}^{-1}$ (LI6400-02B; LED red/blue light source), and a relative humidity of 70 ± 5%. CO₂ was supplied from a small portable cylinder, filled to a specified CO₂ pressure. Prior to the measurements, the leaves were equilibrated at the light saturating intensity for at least 15 min to reach steady-state photosynthesis. Once stable, the photosynthetic capacity of the leaves was measured at a series of CO₂ concentrations of 1,500, 1,200, 1,000, 800, 600, 400, 200, 150, 120, 100, 80, and 50 $\mu\text{mol mol}^{-1}$. The interval between each CO₂ concentration was 300 s and the entire CO₂-response curves were analyzed via the rectangular hyperbolic equation (Ye & Yu, 2009).

$$P(C_a) = a \frac{1 - C_a}{1 + C_a} C_a - R_p,$$

where a is a coefficient, C_a is the concentration of atmospheric CO₂, $P(C_a)$ is the net photosynthetic rate, and R_p is the light respiration rate. The maximum leaf light-saturated photosynthetic rate ($P_{N_{\max}}$), the CO₂-saturation point (CSP), the CO₂-compensation point (CCP), and the apparent carboxyl efficiency (CE) were calculated via the above equation (Ye & Yu, 2009).

Chlorophyll content determination: The leaf chlorophyll values were obtained, using a CCM-200 plus chlorophyll content meter (Opti-Science Inc., Hudson, NH, USA). The third adult leaf counted from the apex of a plant was tested.

Chlorophyll fluorescence parameter determination: Chlorophyll fluorescence was measured between 08:00 and 11:00 h (Beijing time) using a OS30P portable fluorometer (Opti-Science Inc., Hudson, NH, USA) (Li *et al.*, 2012). The third healthy and mature leaf from the apex of a plant was tested after dark-adaptation for 30 min with dark leaf chips. The variable fluorescence (F_v), the minimal fluorescence yield (F_0), and the maximal fluorescence yield (F_m) were measured of dark-adapted leaf tissues. The maximum photochemical efficiency of PSII was defined as F_v/F_m to express the maximum PSII photochemical efficiency.

Rate determination of AMF colonization: Subsequent to the measurements, the fine roots of the plants were collected, and AMF infection was verified via mycorrhizal colonization rate. Analyses of AMF colonization of host plants were performed according to previously described staining methods (Kormanik *et al.*, 1980; Jin *et al.*, 2015), and observed via light microscopy. Vesicles, arbuscules, and intercellular hyphae were observed in root segments that were considered to be mycorrhizal. The rate of AMF colonization was calculated, using the following formula: Colonization (%) = (length of root infected / total length of root observed) × 100% (Graham & Syvertsen, 1985). In both the +Cu+AMF and -Cu+AMF treatments, the AMF colonization rates were 42.50% and 52.78%, respectively; however, they were zero in both non-inoculated plant treatments. These results suggest that AMF treatments were successfully colonized.

Statistical analysis: The differences of AMF inoculation, Cu addition, and their interactive effects on plant photosynthetic characteristic parameters were determined via two-way (ANOVA). Means among four treatments were compared using Fisher's LSD test at $p < 0.05$. Data are expressed as means with standard deviations (SD). SigmaPlot (version 13.0) was utilized to create all figures, and the SPSS software package (version 17.0) was used for all statistical analyses.

Results

Interactive effects of the daily photosynthetic process of *E. splendens*: The P_N , g_s , and E diurnal variation curves of *E. splendens* in all four different treatments showed similar single peaks without a midday depression, while the diurnal C_i variation curves showed a V-type curve (Fig. 1). Cu addition significantly decreased the daily mean P_N , g_s , LUE, and CE, while AMF inoculation significantly increased these parameters (Table 1). Cu addition significantly decreased the daily mean E , however, significantly increased C_i , while AMF inoculation had no significant effect (Table 1). Cu addition had no significant effect on WUE, while AMF inoculation increased it significantly. The interactive effect of AMF inoculation and Cu addition significantly affected the daily mean C_i but had no significant effect on the remaining three parameters (Table 1).

Interactive effect of light and CO₂ response curves in *E. splendens*: The light and CO₂ photosynthetic rate response curves of all four different treatments are shown in Figure 2. AMF-inoculated plants exhibited a significantly higher photosynthetic rate, while plants that were subjected to Cu-addition exhibited a significantly lower rate. In response to light, Cu addition significantly decreased $P_{N_{\max}}$, LSP, LCP, and AQY; however, AMF inoculation significantly increased $P_{N_{\max}}$ and AQY in *E. splendens*. The interactive effect of AMF inoculation and Cu addition significantly affected $P_{N_{\max}}$ and AQY in *E. splendens* (Fig. 3). In response to CO₂ concentration, Cu addition significantly decreased $P_{N_{\max}}$ and CSP in *E. splendens*; however, AMF inoculation significantly increased $P_{N_{\max}}$ only. Two-way ANOVA revealed that Cu had a significant effect on $P_{N_{\max}}$ and CSP, while AMF inoculation had a significant effect on $P_{N_{\max}}$, CSP, and CCP. The interactive effect of AMF inoculation and Cu addition on $P_{N_{\max}}$ was significant for *E. splendens* (see Fig. 4).

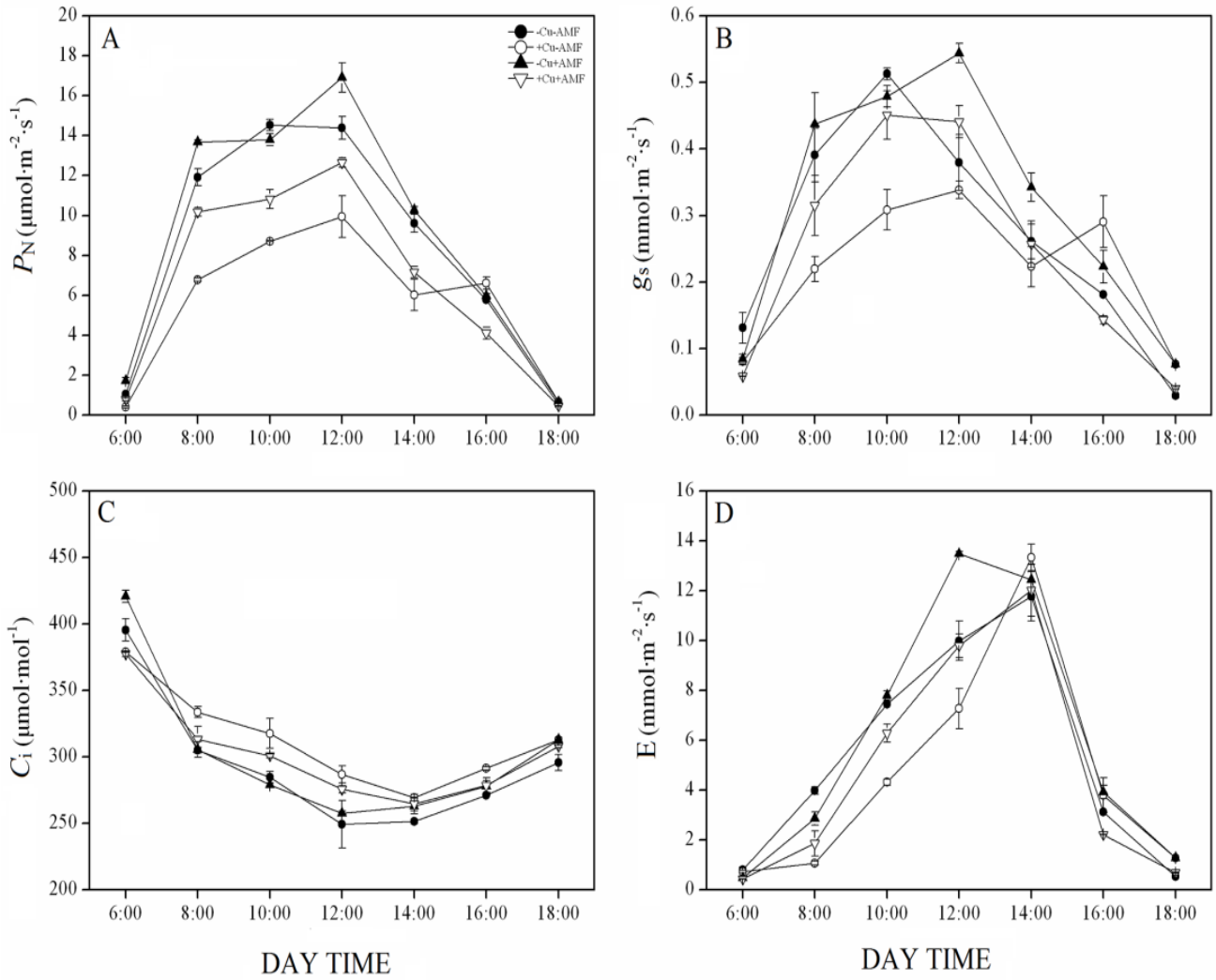


Fig. 1. Diurnal variation curves in *E. splendens* four different treatments. Data points represent average results of three plants per treatment \pm standard deviation. -Cu-AMF, +Cu-AMF, -Cu+AMF, and +Cu+AMF indicate no Cu addition and no AMF inoculation, Cu addition but no AMF inoculation, AMF inoculation but no Cu addition, Cu addition and AMF inoculation, respectively.

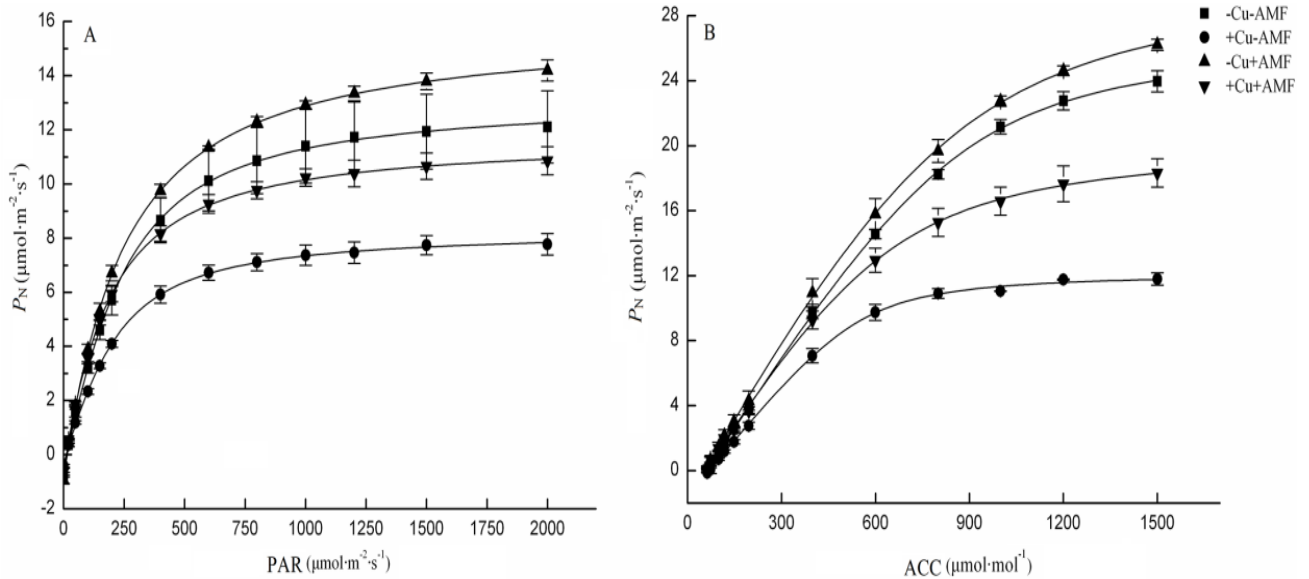


Fig. 2. Response of the net photosynthetic rate (P_N) on photosynthetically active radiation (A) and atmospheric CO_2 concentration (B) in *E. splendens*. Data points represent average results of three plants per treatment \pm standard deviation. -Cu-AMF, +Cu-AMF, -Cu+AMF, and +Cu+AMF indicate no Cu addition and no AMF inoculation, Cu addition but no AMF inoculation, AMF inoculation but no Cu addition, Cu addition and AMF inoculation, respectively.

Table 1. Interactive effects of Cu addition and AMF inoculation on P_N , g_s , C_i , E , LUE, WUE and CE in *E. splendens* in four different treatments, and the two-way ANOVA results.

Treatments	P_N / ($\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$)	g_s / ($\text{mmol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$)	C_i / ($\mu\text{mol}\cdot\text{mol}^{-1}$)	E / ($\text{mmol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$)	LUE/ ($\mu\text{mol}\cdot\text{mmol}^{-1}$)	WUE/ ($\mu\text{mol}\cdot\text{mmol}^{-1}$)	CE/ ($\mu\text{mol}\cdot\text{mmol}^{-1}$)
-Cu-AMF	8.255±0.231b	0.264±0.006b	293.282±3.518c	5.381±0.367ab	0.043±0.006a	1.628±0.056c	0.029±0.004b
+Cu-AMF	5.589±0.298d	0.239±0.002d	312.963±1.341a	4.543±0.387bc	0.029±0.002b	1.866±0.093b	0.020±0.002d
-Cu+AMF	8.993±0.220a	0.309±0.004a	299.175±0.596b	5.928±0.089a	0.044±0.003a	2.141±0.137a	0.032±0.001a
+Cu+AMF	6.599±0.216c	0.248±0.007c	302.397±3.252b	4.757±0.192c	0.031±0.004b	2.106±0.08a	0.023±0.002c
Two way ANOVA, F -value							
Cu	215.752**	29.455**	41.783**	24.477**	158.471**	2.173	1714.654**
AMF	25.753**	8.467**	1.739	3.513	9.448*	29.646**	184.552**
Cu×AMF	0.628	0.357	21.578**	0.675	1.915	3.920	2.828

Note: Different small letters indicated significant difference among different treatments at $p<0.05$. *, $p<0.05$; **, $p<0.01$

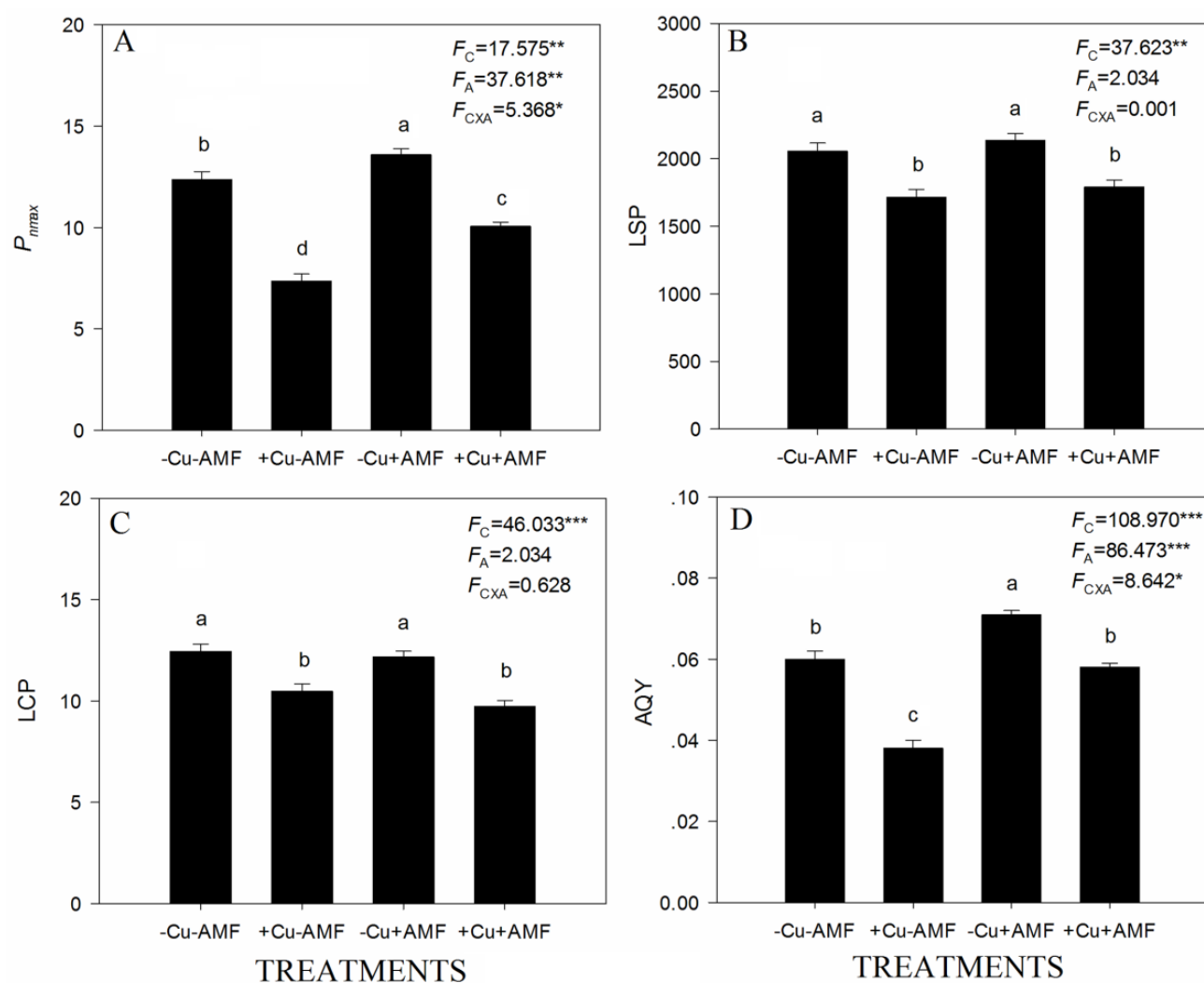


Fig. 3. Interactive effects of Cu addition and AMF on the maximum net photosynthetic rate (P_{Nmax} , A), light saturation point (LSP, B), light compensation point (LCP, C), and apparent quantum yield (AQY, D). -Cu-AMF, +Cu-AMF, -Cu+AMF, and +Cu+AMF indicate no Cu addition and no AMF inoculation, Cu addition but no AMF inoculation, AMF inoculation but no Cu addition, Cu addition and AMF inoculation, respectively. Different small letters indicate significant differences among different treatments at $p<0.05$. F_C indicates the effect of Cu addition. F -value and significance levels: *, **, and *** indicate significant differences at $p<0.05$, $p<0.01$, and $p<0.001$, respectively.

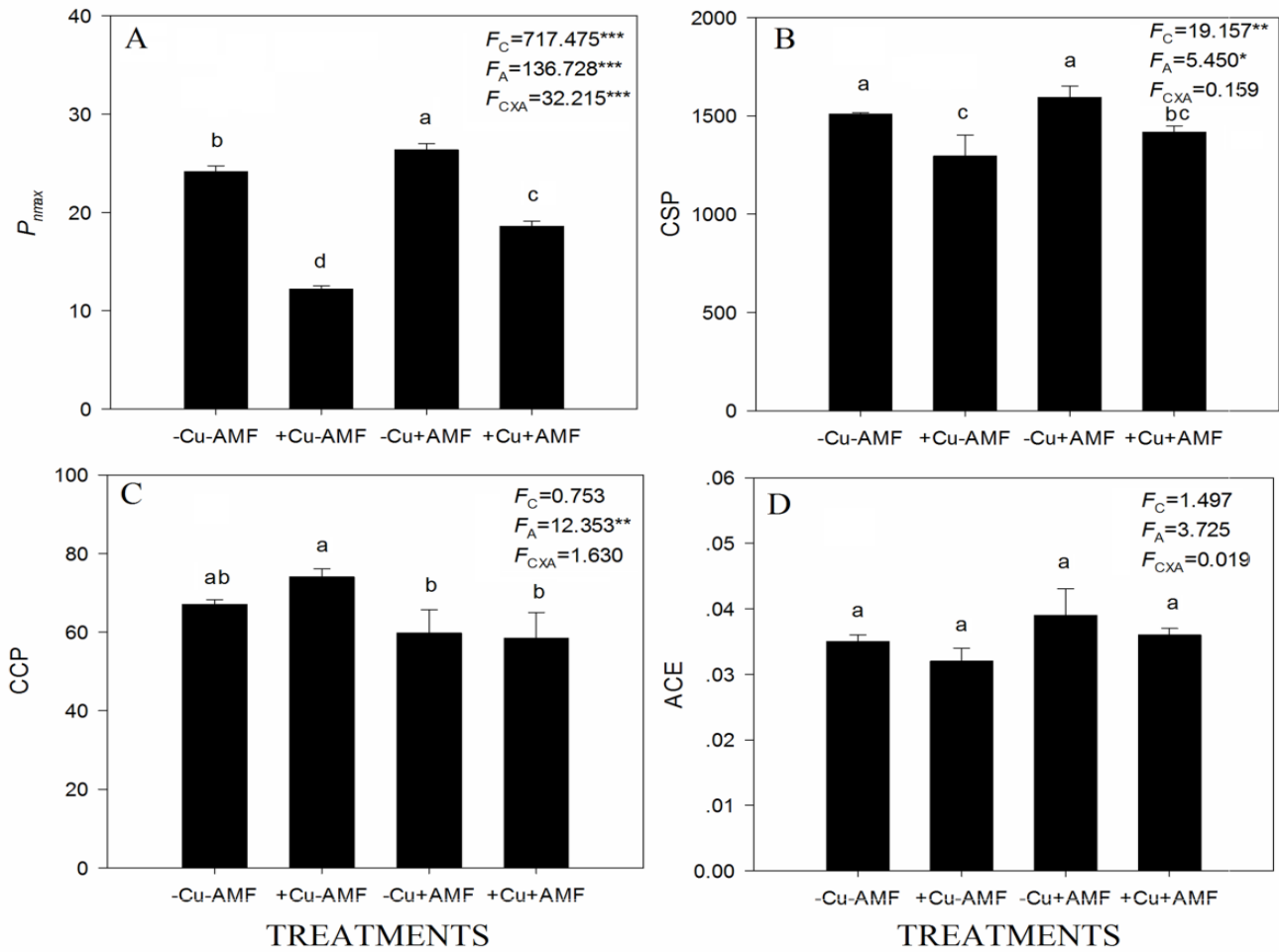


Fig. 4. Interactive effects of Cu addition and AMF on the maximum net photosynthetic rate (P_{Nmax} , A), CO₂ saturation point (CSP, B), CO₂ compensation point (CCP, C), and apparent carboxylation efficiency (ACE, D). -Cu-AMF, +Cu-AMF, -Cu+AMF, and +Cu+AMF indicate no Cu addition and no AMF inoculation, Cu addition but no AMF inoculation, AMF inoculation but no Cu addition, Cu addition and AMF inoculation, respectively. Different small letters indicate significant differences among the different treatments at $p < 0.05$. F_c indicates the effect of Cu addition. F -value and significance levels: *, **, and *** indicate significant differences at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

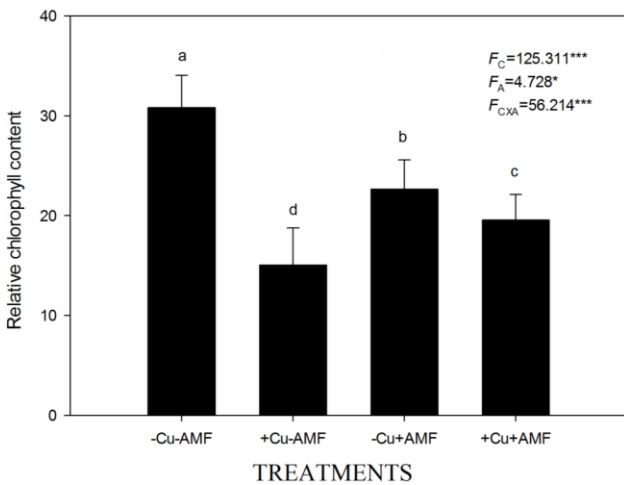


Fig. 5. Interactive effects of Cu addition and AMF on relative chlorophyll content. -Cu-AMF, +Cu-AMF, -Cu+AMF, and +Cu+AMF indicate no Cu addition and no AMF inoculation, Cu addition but no AMF inoculation, AMF inoculation but no Cu addition, Cu addition and AMF inoculation, respectively. Different small letters indicate significant differences among different treatments at $p < 0.05$. F_c indicates the effect of Cu addition. F -value and significance levels: *, **, and *** indicate significant differences at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

Interactive effects on relative chlorophyll contents: Both AMF inoculation and Cu addition significantly decreased the relative chlorophyll content of *E. splendens*, while their interaction significantly affected it (Fig. 5).

Interactive effect of the chlorophyll fluorescence parameters: Compared to the -Cu-AMF treatment, Cu addition significantly increased F_0 but significantly decreased F_m , F_v , and F_v/F_m . Compared to the -Cu-AMF treatment, AMF inoculation did not significantly affect any parameters (Fig. 6). Two-way ANOVA revealed that Cu addition had a significant effect on F_0 , F_m , F_v , and F_v/F_m , while AMF inoculation significantly affected F_0 , F_v , and F_v/F_m . The interaction between AMF inoculation and Cu addition significantly affected F_0 and F_v/F_m (Fig. 6).

Discussion

As we had hypothesized, photosynthesis in *E. splendens* was sensitive to both Cu addition and AMF inoculation. Our results revealed that Cu addition significantly changed the P_N , C_i , g_s , and E diurnal variation curves, and decreased the daily mean P_N , g_s , E, LUE, and CE values; however, AMF inoculation

significantly increased the daily mean P_N , g_s , LUE, and CE in *E. splendens*. These results indicate that Cu addition inhibits photosynthesis in *E. splendens* via an alteration of gas exchange capability and a weakening of the light utilization and carboxylation efficiency, while AMF inoculation could alleviate these inhibitory effects. A similar inhibitory effect of Cu stress has been reported for *E. splendens* (Ke *et al.*, 2007) and *Limoniastrum monopetalum* (Cambrollé *et al.*, 2013), and a similar enhancement effect of AMF inoculation has been reported for *Zea mays* (Zhu *et al.*, 2011). No interactions between AMF and Cu were observed in the above photosynthetic parameters, indicating that both factors might separately influence photosynthesis in *E. splendens*. Further study is required to verify these differences.

The observed decline in P_N might be ascribed to stomatal and/or non-stomatal limitations (Flexas & Medrano, 2002; Akhka *et al.*, 2017). Cambrollé *et al.* (2013) reported that excessive Cu reduced P_N and g_s but had no effect on C_i and the authors thus suggested that the observed reduction of photosynthetic activity might be a non-stomatal limitation. The significant C_i increase that accompanied the increase of P_N and g_s in *E. splendens* under Cu stress also indicated that the inhibition of

photosynthesis in this species via excessive Cu might be a non-stomatal limitation; however, it might possibly be related to the inactivation of Rubisco and the limitation of its regeneration via photosynthetic electron transport (Cornejo *et al.*, 2008, Zhu *et al.*, 2011). This explanation is in agreement with previous studies on *Cucumis sativus* seedlings (Vinit-Dunand *et al.*, 2002) as well as rice (Lidon *et al.*, 1999).

In this study, light and CO₂ response curves were used to further evaluate the photosynthetic capability of *E. splendens*, treated with the addition of Cu and inoculation of AMF. In response to light and CO₂, Cu stress significantly decreased P_{Nmax} , while AMF inoculation significantly increased it. This indicates that Cu stress weakens the photosynthetic efficiency due to toxicity for the photosynthetic apparatus (Danilov & Ekelund, 2001), while AMF could recover this efficiency. Furthermore, the interactive effect was significant. Cu stress significantly decreased LSP, LCP, AQY, and CSP, while AMF inoculation had no significant effect on these parameters, indicating that *E. splendens* requires greater light intensity to reach the saturation and compensation points (Ögren & Evans, 1993) as well as greater CO₂ concentration to reach the saturation point.

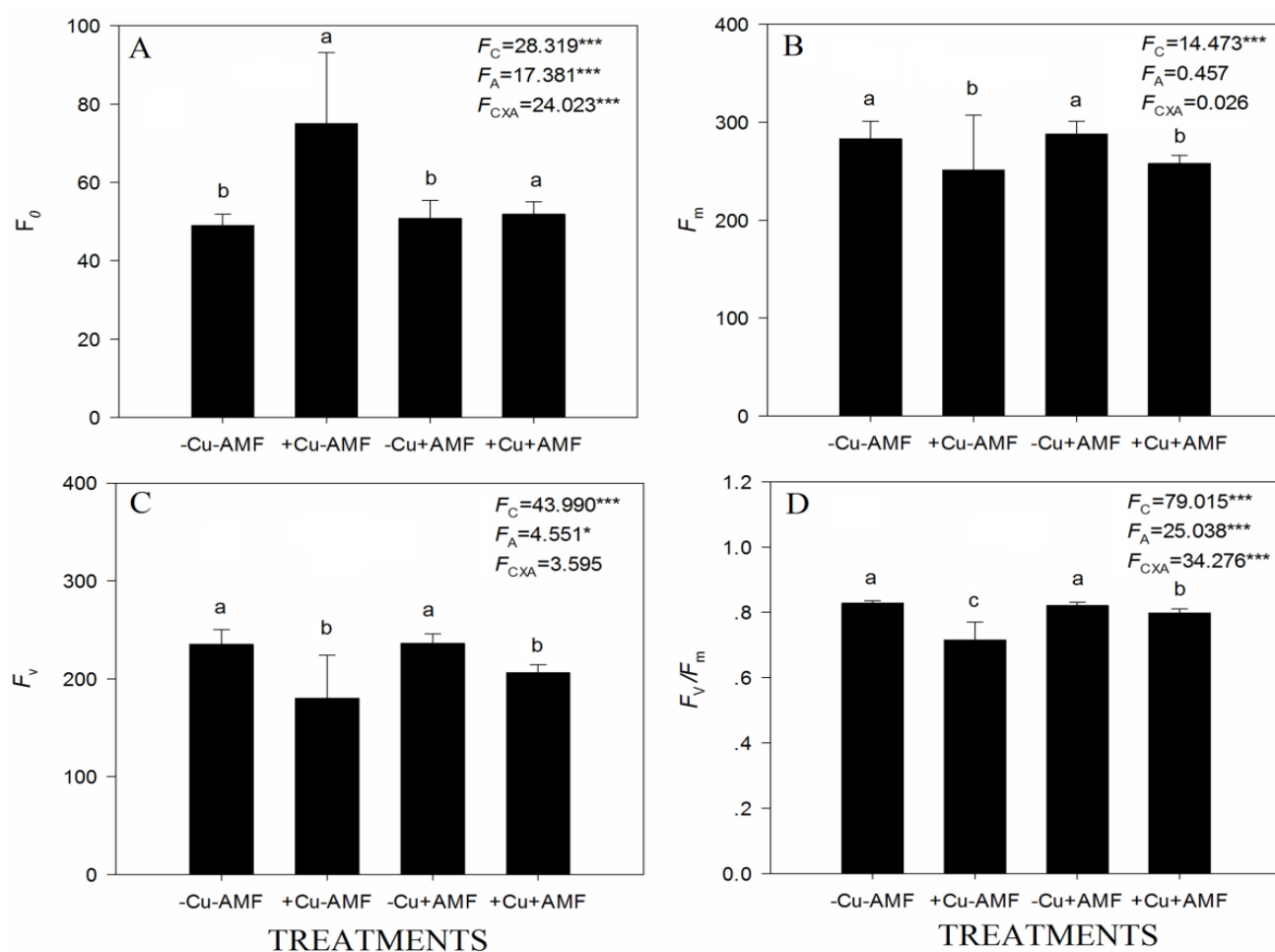


Fig. 6. Interactive effects of Cu addition and AMF on F_0 (A), F_m (B), F_v (C), and F_v/F_m (D). -Cu-AMF, +Cu-AMF, -Cu+AMF, and +Cu+AMF indicate no Cu addition and no AMF inoculation, Cu addition but no AMF inoculation, AMF inoculation but no Cu addition, Cu addition and AMF inoculation, respectively. Different small letters indicate significant differences among different treatments at $p < 0.05$. F_c indicates the effect of Cu addition. F-value and significance levels: *, **, and *** indicate significant differences at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

Three target sites of heavy metal interaction exist in photosynthesis, including photosynthetic pigments, photosynthetic enzymes, and photosystems (Aggarwal *et al.*, 2012). We focused on the photosynthetic pigments and photosystems in this study. Chlorophyll is a necessary pigment, allowing plants to capture light (Katz *et al.*, 1978). The chlorophyll content was previously found to be reduced due to excessive concentrations of heavy metals (Lagriffoul *et al.*, 1998), including Cu (Aggarwal *et al.*, 2012, Cambrollé *et al.*, 2012; 2013). Reduction of the chlorophyll content may be attributed to the reduced synthesis of chlorophyll due to enhanced activity of chlorophyllase induced by Cu (Abd_Allah *et al.*, 2015). We found that Cu addition significantly reduced the relative chlorophyll content, indicating that the reduction of chlorophyll might be the main cause for the observed photosynthetic inhibition. In our study, AMF treatment enhanced the chlorophyll content of *E. splendens* in the presence of Cu. This was likely due to AMF increasing the uptake of micronutrients (such as P and Mg) from the soil which play an important role as energy carriers during photosynthesis (Smith & Read, 2008). In particular, AMF require photosynthetically fixed carbon from the host to maintain a steady growth. The carbon sink strength due to mycorrhiza stimulates the host plants, thus increasing the photosynthetic rate which may be a key interaction for the generation of a carbon reserve to be mobilized in case of foliar stress (Kaschuk *et al.*, 2009).

The photosystem II (PSII) is a multi-subunit protein complex, which is distributed in the thylakoid membrane of the chloroplast that catalyzes the light-driven producer of molecular oxygen (Aggarwal *et al.*, 2012). Cu stress inhibits the photosynthetic apparatus by affecting its electron transport (Myśliwa-Kurczel, 2002). Chlorophyll fluorescence is one of the powerful tools with which to explore the function of the PSII (Zhu *et al.*, 2010; Hussein *et al.*, 2017). We observed that Cu addition significantly decreased chlorophyll fluorescence yields, including F_v , F_m , and F_v/F_m , but increased F_0 , which might reduce the energy transfer from the antennae to the reaction centers of the PSII (Aggarwal *et al.*, 2012). Cambrollé *et al.* (2013) previously reported similar results about the toxicity of Cu on *L. monoptalum*. The ratio of F_v/F_m can be considered a useful metric of the primary photochemical capacity of the PSII and is a sensitive indicator for damage when plants suffer from environmental stresses (Krause & Weis, 1991; Zhu *et al.*, 2010). Values of F_v/F_m between 0.80 and 0.86 are almost a constant for most higher plants under natural conditions (Li *et al.*, 2012). In this study, the F_v/F_m ratio of *E. splendens* in the +Cu-AMF treatment (0.715 ± 0.056) was lower than the ratio observed in the -Cu-AMF treatment (0.828 ± 0.008); those in the -Cu+AMF (0.822 ± 0.010) and +Cu+AMF (0.798 ± 0.013) treatments were higher than that in the +Cu-AMF treatment, but similar to that in the -Cu-AMF treatment. These results indicate that Cu addition imposed a minor inhibitory effect on photosynthesis in *E. splendens* and that AMF inoculation could alleviate this inhibition. It is possible that AMF ameliorated the non-stomatal limitation, caused by excessive Cu, and thus improved the electron transport or enzyme activities of the host plants. The significant interactive effect between AMF inoculation and Cu addition indicated an antagonistic effect.

In conclusion, Cu stress significantly inhibits the photosynthetic ability of *E. splendens* due to a toxic effect on photosynthetic apparatus and pigments. However, AMF inoculation significantly alleviates the inhibition produced by Cu stress on these plants, by weakening its toxicity on the photosynthetic apparatus and pigments. The alleviation of AMF on Cu stress suggests that utilization of AMF might be a potential method for the phytoremediation of Cu-contaminated soil. Further studies should therefore focus on the mechanisms underlying the alleviating effects of AMF inoculation.

Acknowledgments

We are grateful to W.S. Ke and M. Guan for their help in sampling soil and seeds. We also thank the editors and reviewers for their valuable comments. This study was financially supported by Zhejiang Provincial Nature Science Foundation (LY12C03002).

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(Received for publication 10 June 2016)