

Synergistic effect of colonization with arbuscular mycorrhizal fungi improves growth and drought tolerance of *Plukenetia volubilis* seedlings

Yao-hua Tian · Yan-bao Lei · Yu-long Zheng · Zhi-quan Cai

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Abstract The objective of this study was to investigate the effects of arbuscular mycorrhizal fungus (AMF) inoculation on plant growth and drought tolerance in seedlings of a promising oilseed crop, Sacha Inchi (*Plukenetia volubilis* L.), under well-watered or drought conditions. AMF inoculation was applied in four treatments: without AMF inoculation, *Glomus versiforme*, *Paraglomus occultum*, or combination of both microorganism inoculations. The results showed that AMF colonization significantly enhanced the growth of Sacha Inchi seedlings regardless of soil water conditions, and the greatest development was reached in plants dually inoculated under well-watered conditions. *G. versiforme* was more efficient than *P. occultum*. Plants inoculated with both symbionts had significantly greater specific leaf area, leaf area ratio and root volume when compared with the uninoculated control, *G. versiforme*, and *P. occultum* treatments alone, indicating a synergistic effect in the two AMF inoculation. Photosynthetic rate and water-use efficiency were stimulated by AMF, but not stomatal conductance. Inoculation with AM fungus increased antioxidant enzymes activities including guaiacol peroxidase and catalase, thus lowering hydrogen peroxide accumulation

and oxidative damage, especially under drought stress conditions. However, proline content showed little change during drought stress and AMF colonization conditions, which suggested that proline accumulation might not serve as the main compound for osmotic adjustment of the studied species. These results indicate that AMF inoculation stimulated growth and enhanced drought tolerance of Sacha Inchi seedlings, through alterations in morphological, physiological and biochemical traits. This microbial symbiosis might be an effective cultivation practice in improving the performance and development for Sacha Inchi plants.

Keywords Arbuscular mycorrhizal symbiosis · Drought · Growth · Oxidative stress · *Plukenetia volubilis*

Abbreviations

AMF	Arbuscular mycorrhizal fungi
CAT	Catalase ($\mu\text{mol H}_2\text{O}_2 \text{ min}^{-1} \text{ mg}^{-1}$)
Chl	Leaf chlorophyll content (g m^{-2})
GPX	Guaiacol peroxidase ($\mu\text{mol min}^{-1} \text{ mg}^{-1}$)
G_s	Stomatal conductance ($\text{mol m}^{-2} \text{ s}^{-1}$)
H_2O_2	Hydrogen peroxide ($\mu\text{mol g}^{-1}$)
MDA	Malondialdehyde (nmol g^{-1})
P_{max}	Light-saturated photosynthetic rate ($\mu\text{mol m}^{-2} \text{ s}^{-1}$)
RGR	Relative growth rate ($\text{mg g}^{-1} \text{ day}^{-1}$)
ROS	Reactive oxygen species
SLA	Specific leaf area ($\text{cm}^2 \text{ g}^{-1}$)
LAR	Leaf area ratio ($\text{cm}^2 \text{ g}^{-1}$)
WUE	Water-use efficiency ($\mu\text{mol mol}^{-1}$)

Introduction

Drought stress is considered to be one of the most important abiotic factors that can limit plant growth and crop

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Y. Tian
Yunnan Institute of Tropical Crops, Jinghong 666100, China

Y. Lei · Y. Zheng
Key Laboratory of Tropical Forest Ecology, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Mengla 666303, China

Z. Cai (✉)
Laboratory of Tropical Plant Resource Science, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Mengla 666303, China
e-mail: zhiquan.cai@126.com

yield. Plants can avoid the damages induced by drought through maximizing water uptake (e.g., tapping ground water by deep roots) or minimizing water loss (e.g., stomatal closure, small leaves, etc.) (Chaves et al. 2003). Apart from these morphological changes, plants have evolved to employ a variety of physiological and biochemical processes ranging from photosynthesis to antioxidant defenses and solute accumulation as components of drought tolerance (Lei et al. 2006; Ruíz-Sánchez et al. 2011). Production of excessive reactive oxygen species (ROS) and generation of oxidative damage are typical stress-derived physiological responses. ROS accumulation, such as superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^{\cdot}OH$), can seriously impair the normal function of plants by lipid peroxidation, protein degradation, DNA nicking and may even cause cell death (Beligni and Lamattina 1999). To keep ROS under a favorable level, plants possess antioxidative systems which are composed of metabolites such as ascorbate, glutathione, tocopherol, etc., and enzymatic scavengers such as superoxide dismutase (SOD), peroxidases (GPX) and catalases (CAT) (Asada 1999). Moreover, the other potentially important mechanism of drought tolerance is osmotic adjustment as a result of the accumulation of compatible solutes in protoplasm, which allows cell enlargement, stomata open and CO_2 assimilation during drought conditions (Hare et al. 1998; Augé 2001).

Plants can benefit from external methods, i.e., the application of various beneficial microorganisms including arbuscular mycorrhizal fungus (AMF) as a low-cost, eco-friendly and sustainable alternative technique to enhance their drought resistance and growth performance (Ruíz-Sánchez et al. 2011). The majority (probably 70–80 %) of terrestrial plant species are capable of interacting with AMF in nature as a result of coevolution over at least 450 million years (Smith et al. 2008). AMF brings about an array of favorable influences on the host plants, such as absorption of more water and access to poorly available nutrients due to the fine exploration of the rhizosphere by the hyphae (Navarro et al. 2009). In this sense, AMF inoculation has been considered as an effective approach to promote plant growth and favor survival under either biotic or abiotic stress conditions (Smith et al. 2008). So far, AMF-induced tolerance has been shown to be involved in enhancement of drought, high salinity, chemical pollution and oxidative stress, in numerous plants (Bressano et al. 2010; Abdel Latef and He 2011). It has also been found that colonization by different AMF might result in different responses in the same plant species (Abdel Latef and He 2011). However, whether there is synergistic, adding or antagonistic effect in the interacting of different AMF, symbiosis remains not well elucidated.

Plukenetia volubilis (commonly known as Sacha Inchi), a promising oilseed crop indigenous to the rain forests of the Andean region of South America, has been successfully introduced to Xishuangbanna, southwest Yunnan, China. It is well known that Sacha Inchi seeds contain high concentration of omega fatty acids, which is beneficial to human health (Hamaker et al. 1992; Cai 2011; Cai et al. 2011). Although the composition and properties of *P. volubilis* seeds are relatively well known, to date there is a lack of detailed information about its cultivation potential. Intensive research on this species can contribute to future implementation of *P. volubilis* into the agricultural systems of South China as an alternative crop which can reduce local farmers' dependence on cultivation of rubber trees (Cai et al. 2012). We hypothesized that (1) when exposed to drought stress, seedlings of *P. volubilis* will adjust some morphological, physiological and biochemical changes to acclimate the damage; (2) AMF inoculation can alleviate the deleterious effect of water deficit and enhance its growth; (3) different AMF symbiosis might display contrasting effect under separate and combined colonization.

Materials and methods

Plant materials and experimental design

Seeds of *P. volubilis* were sown in Xishuangbanna Tropical Botanical Garden (21°56'N, 101°15'E, 560 m asl), Chinese Academy of Sciences in February, 2011. When the seedlings were approximately 15 cm tall, uniform seedlings were transplanted into 20 × 30 cm pots containing 3 kg autoclaved topsoil from the nearby forest. Four to six seedlings were harvested 2 weeks after transplantation for measurements of initial biomass. The remaining 120 pots were grown inside a naturally lit greenhouse between March and September 2011.

The treatments consisted of two levels of irrigation (well-watered vs. drought), and four AMF conditions (without AM inoculation, *Glomus versiforme*, *Paraglomus occultum*, or combination of both *G. versiforme* and *P. occultum* inoculation) in a randomized block design. Each treatment included five replications, and three seedlings per replication were used. For the AMF treatment, AM inoculum (containing approx. 500 spores; i.e., 10 g per pot, dry weight) of *G. versiforme*, *P. occultum*, and every 5 g per pot from *G. versiforme* and *P. occultum* (both AM treatment) was placed at 5 cm depth of soil mixture. These mycorrhizal inocula were provided by the Institute of Plant Nutrition and Resources, Beijing Academy of Agriculture and Forestry Sciences, China. Water treatment began after 1 month of acclimation in greenhouse conditions. During

the experiment, well-watered and drought stress pots were controlled at 80 and 40 % of relative soil water content. The water status in the substrate was daily determined and the amount of water loss was supplied to each pot to keep the designed soil water content according to our previous method (Lei et al. 2006). At the end of the experiment, leaf and whole-plant traits were measured.

Leaf water potential, chlorophyll and proline content measurements

The pre-dawn leaf water potential was measured just before the sunrise using a pressure chamber (PMS-1000, PMS Instrument Co., Corvallis, Oregon). Total chlorophyll was extracted with 80 % (v/v) acetone in the dark and assayed with a spectrophotometer (UV-B 2501; Shimadzu, Japan) based on the method of Cai et al. (2009). Free proline was extracted and determined with acid ninhydrin as described by Bates et al. (1973). The absorbance at 520 nm was determined using L-proline as standard.

Photosynthesis measurement

Gas exchange measurements were made between 0900 and 1100 hours with a portable infrared gas analyzer in open system mode (LI-6400XT, Li-Cor). Five plants per treatment were selected for photosynthetic measurements; one leaf per plant was measured. Photosynthetic parameters were measured on fully expanded, recently matured sun canopy leaves. We measured net photosynthetic rate (P_{\max}) and gas conductance (G_s) under a light saturating irradiance (photon flux density = $1,500 \mu\text{mol m}^{-2} \text{s}^{-1}$, provided by an internal red/blue LED light source; LI6400-02B) under ambient CO_2 concentration (380 ppm). Leaf temperature and vapor pressure deficit (VPD) in the cuvette were kept at 25–26 °C and less than 1 kPa, respectively. Intrinsic water-use efficiency (WUE) was calculated as the ratio of P_{\max} to G_s .

Oxidative stress measurement

The levels of H_2O_2 were measured by monitoring the absorbance of the titanium-peroxide complex at 415 nm as described by Lin and Wang (2002). Absorbance values were calibrated to a standard curve generated using known concentrations of H_2O_2 . Leaf oxidative damage to lipids was expressed as equivalents of malondialdehyde (MDA) contents. After extraction and reaction with thiobarbituric acid, the absorbance at 450, 532 and 600 nm was determined. The MDA content was calculated according to Hodges et al. (1999).

For enzyme extracts and assays, 500 mg fresh leaves were frozen in liquid nitrogen and then ground in 4 ml

solution containing 50 mM phosphate buffer (pH 7.0), 1 % (w/v) polyvinylpyrrolidone, and 0.2 mM ascorbic acid. The homogenate was centrifuged at 15,000g for 30 min, and the supernatant was collected for enzyme assays. Guaiacol peroxidase (GPX) (EC 1.11.1.7) activity was based on the determination of guaiacol oxidation (extinction coefficient $26.6 \text{ mM}^{-1} \text{ cm}^{-1}$) at 470 nm by H_2O_2 (Lin and Wang 2002). The activity of catalase (CAT, EC 1.11.1.6) was determined as a decrease in absorbance at 240 nm for 1 min following the decomposition of H_2O_2 (coefficient of $39.4 \text{ mM}^{-1} \text{ cm}^{-1}$). The reaction mixture contained 50 mM phosphate buffer (pH 7.0) and 15 mM H_2O_2 (Beligni and Lamattina 1999).

Whole-plant traits and observation of AM development

At the end of the experiment, ten seedlings per treatment were harvested and separated into leaves (including petioles), stems, and roots. Leaf areas were determined with a leaf area meter (LI-3100A; Li-Cor, Lincoln, NE). The whole-plant root systems were carefully washed out from the pots and cleaned before being scanned and the digital images were quantified with DT-SCAN image analysis software (Delta-T Devices, Cambridge, UK) for measuring root morphological parameters, such as root length, root surface area, and root volume (Cai et al. 2009). All tissues were dried to a constant weight at 70 °C for 48 h. Leaf mass ratio (LMR, g leaf/g plant), stem mass ratio (SMR, g stem/g plant), root mass ratio (RMR, g root/g plant), specific leaf area (SLA, $\text{cm}^2 \text{ leaf/g leaf}$) and leaf area ratio (LAR, $\text{cm}^2 \text{ leaf/g plant}$) were calculated. Relative biomass growth rate (RGR, $\text{mg g}^{-1} \text{ day}^{-1}$) was calculated as:

$$\text{RGR} = [\ln(\text{final plant mass}) - \ln(\text{initial plant mass})]/\text{time}.$$

A segment taken from the middle part of the ten roots for each treatment was carefully washed, cut into 1-cm-long sections and cleared with 10 % (w/v) KOH. Then the root segments were stained with 0.05 % (w/v) trypan blue prepared in lactophenol for 10 min. Examination of AM development was done by a microscope (Olympus BH2, Japan) equipped with a Nikon DXM1200 digital camera. The pictures were collected as JPEG digital files. The AM colonization was quantified according to the following formula (Wu and Xia 2006):

$$\text{AM colonization rate (\%)} = \frac{\text{root length infected}}{\text{root length observed}} \times 100 \%$$

Statistical analysis

A two-way ANOVA analysis was performed for each variable, with water and AM fungus inoculation as main fixed factors, using SPSS 16.0. Prior to analysis, data were

checked for normality and homogeneity of variance and, if necessary, were transformed by taking \log_{10} - or square roots to satisfy the assumptions of ANOVA. Means were separated by the LSD test ($P < 0.05$).

Results

Symbiotic development and leaf water potential

AM colonization was not observed in non-inoculated plants. There were no significant water \times AMF interactions for mycorrhizal root colonization and pre-dawn leaf water potential, implying that the responsiveness to drought did not differ among AMF treatments (Fig. 1). Drought significantly decreased the percentage of mycorrhizal root colonization (Fig. 1b). Inoculation with *G. versiforme* alone had a greater effect than *P. occultum* alone on the root mycorrhizal colonization, while the highest percentage of mycorrhizal root colonization was observed when both symbionts were inoculated together. Pre-dawn leaf water potential increased in response to increased water availabilities across all AMF treatments. AM colonization affected water potential under drought conditions, whereas not under well-water conditions (Fig. 1a).

Growth and morphological traits

There was no significant water \times AMF interactions for RGR of Sacha Inchi seedlings (Table 1), indicating that AMF colonization led to a similar, modest, increase in RGR with an increase in water availability. Drought stress significantly decreased, whereas either *G. versiforme* or *P. occultum* inoculation enhanced the relative growth rate (RGR) in seedlings of *P. volubilis* under well-watered or drought stress conditions. Relative to *P. occultum*, *G. versiforme* exhibited stronger growth stimulation. Furthermore, when both symbionts were inoculated together, the increase in the biomass accumulation was greater than their separate symbiosis, indicating the synergistic effect of AMF association. For example, the RGR under *G. versiforme*, *P. occultum* and both AMF inoculations were 1.19, 1.03 and 1.31 times that of the non-AMF colonized plants under well-watered conditions, respectively; while under drought stress the ratio was 1.32, 1.15 and 1.55, respectively. These results also indicated the more profound enhancement by AMF under drought stress than under well-watered condition. Drought exhibited little effect on biomass allocation, whereas AMF colonization decreased SMR, leaving more biomass allocated to roots and leaves, especially when associated with both symbionts. Drought decreased LAR, whereas AMF colonization increased SLA

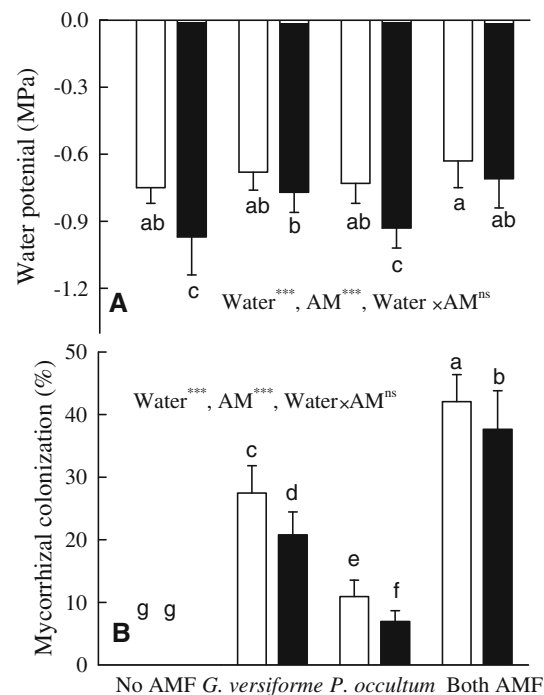


Fig. 1 Water potential (a) and AM colonization rate (b) of *P. volubilis* seedlings in response to watering and AM inoculations. The values (mean \pm SE, $n = 10$) followed by a different letter indicate significant difference at $P < 0.05$ level. Open and closed bars represent well-watered and drought treatment, respectively. ns not significant, $P > 0.05$; ** $P < 0.01$; *** $P < 0.001$

and LAR. Compared to the treatment with no inoculation, inoculation with AM fungi significantly enhanced root growth as indicated by more total root length, larger root surface area, and more root volume under well-watered or drought stress conditions (Table 1).

Photosynthetic traits

There were no significant water \times AMF interactions for all leaf gas exchange parameters, implying that the responsiveness to drought did not differ among AMF treatments (Fig. 2). Drought significantly decreased P_{\max} and G_s , whereas increased WUE. AMF colonization affected P_{\max} and WUE, with the highest values found when both symbionts were inoculated together; whereas it had no effect on G_s . To further analyze intraspecific variation in growth rate under different treatments, RGR was related to some of its underlying components. RGR was significantly positively correlated with P_{\max} , SLA or LAR, but not with LMR (Fig. 3).

Leaf Chl and proline content and oxidative stress

Chl content decreased significantly by drought stress and had no significant differences among all AMF treatments under drought conditions (Fig. 4a). AMF colonization did

Table 1 The whole-plant traits of *P. volubilis* seedlings in responses to drought and AMF inoculations

Treatment	RGR (mg g ⁻¹ day ⁻¹)	LMR (%)	SMR (%)	RMR (%)	SLA (cm ² g ⁻¹)	LAR (cm ² g ⁻¹)	Root length (cm)	Root surface (cm ²)	Root volume (cm ³)
No AM									
Well-watered	23.2 ± 1.11 c	33.2 ± 3.74 ab	42.7 ± 2.25 a	24.1 ± 3.85 b	230.4 ± 20.1bc	263.3 ± 45.8b	644.5 ± 113.5bc	274.9 ± 65.4c	5.51 ± 0.50d
Drought	16.8 ± 2.58e	33.7 ± 4.36a	37.5 ± 3.26bc	28.9 ± 4.03a	209.8 ± 15.7c	210.3 ± 39.1c	507.7 ± 66.7d	206.3 ± 35.4e	4.47 ± 0.24e
G. versiforme									
Well-watered	27.7 ± 1.47b	31.8 ± 2.71ab	40.9 ± 3.69ab	27.2 ± 3.28ab	260.4 ± 31.0ab	279.3 ± 59.5ab	725.3 ± 94.0ab	380.1 ± 32.8b	7.31 ± 0.42bc
Drought	22.2 ± 2.03c	32.5 ± 3.06ab	37.7 ± 2.57bc	29.9 ± 3.12a	249.1 ± 18.3ab	251.9 ± 40.7b	584.0 ± 102.8cd	264.7 ± 36.7cd	6.77 ± 0.28c
P. occultaum									
Well-watered	23.9 ± 1.64c	29.5 ± 3.24b	41.4 ± 4.41ab	29.1 ± 6.14a	250.0 ± 15.9ab	240.1 ± 23.8b	641.4 ± 79.5bc	270.6 ± 30.4cd	5.35 ± 0.25d
Drought	19.5 ± 2.20d	30.7 ± 2.33ab	41.1 ± 5.12ab	28.1 ± 5.19ab	235.1 ± 20.8b	223.2 ± 42.4bc	519.9 ± 84.4d	221.8 ± 30.0de	5.03 ± 0.24e
Both AM									
Well-watered	30.4 ± 1.71a	33.9 ± 4.36a	34.4 ± 2.29d	31.7 ± 4.60a	291.5 ± 17.6a	352.2 ± 67.4a	810.1 ± 73.6a	446.9 ± 83.8a	8.23 ± 0.16a
Drought	25.8 ± 1.81b	31.6 ± 2.25ab	36.5 ± 2.70d	31.9 ± 3.01a	287.3 ± 22.6a	288.1 ± 43.2ab	612.1 ± 73.3c	337.1 ± 31.5b	7.89 ± 0.17b
Two-way ANOVA (significant level)									
Water	***	ns	*	ns	ns	**	***	***	***
AMF	***	*	***	**	***	*	***	***	***
Water × AM	ns	ns	*	ns	ns	ns	ns	*	*

The values (mean ± SE, $n = 10$) followed by a different letter in each column indicate significant difference at $P < 0.05$ level. $P > 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. ns not significant, RGR relative growth rate, LMR, SMR and RMR leaf, stem and root mass ratio, respectively, SLA specific leaf area, LAR leaf area ratio

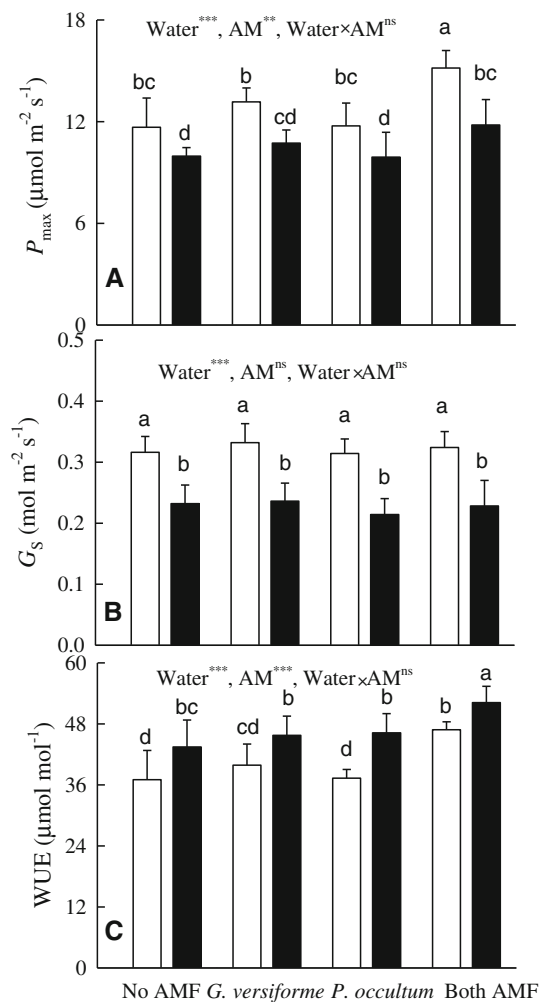


Fig. 2 Leaf photosynthetic traits of *P. volubilis* seedlings in response to watering and AM inoculations. The values (mean \pm SE, $n = 5$) followed by a different letter indicate significant difference at $P < 0.05$ level. Open and closed bars represent well-watered and drought treatment, respectively. ns not significant, $P > 0.05$; ** $P < 0.01$; *** $P < 0.001$

not affect leaf proline content, and drought also induced little change of proline content within each AMF treatment (Fig. 4b). Symbiosis of AMF significantly alleviated oxidative damage, which was indicated by the lower contents of H_2O_2 (Fig. 4c) and MDA (Fig. 4d) compared with non-mycorrhizal plants, especially under drought conditions. On the other hand, AMF colonization enhanced the antioxidant enzyme activities, including GPX (Fig. 4e) and CAT (Fig. 4f), especially for GPX under both well-watered and drought conditions.

Discussion

Drought stress incurred a large set of parallel changes in the morphological, physiological and biochemical responses,

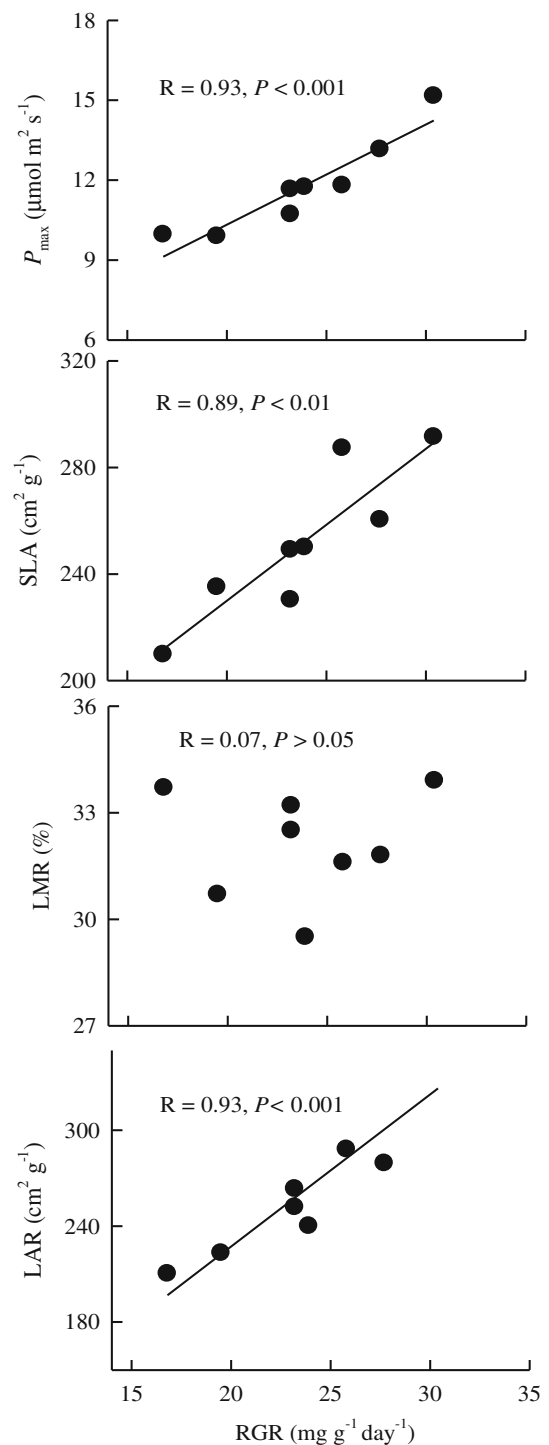


Fig. 3 Relationship between relative growth rate (RGR) and the maximum photosynthetic rate (P_{\max}), specific leaf area (SLA), leaf area ratio (LAR) and leaf mass ratio (LMR) across all treatments of *P. volubilis* seedlings

whereas AMF symbiosis can protect host plants against the detrimental effects (Lei et al. 2006; Bressano et al. 2010; Huang et al. 2011; Abdel Latef and He 2011). In our study, AMF inoculation enhanced the water potential in leaves of

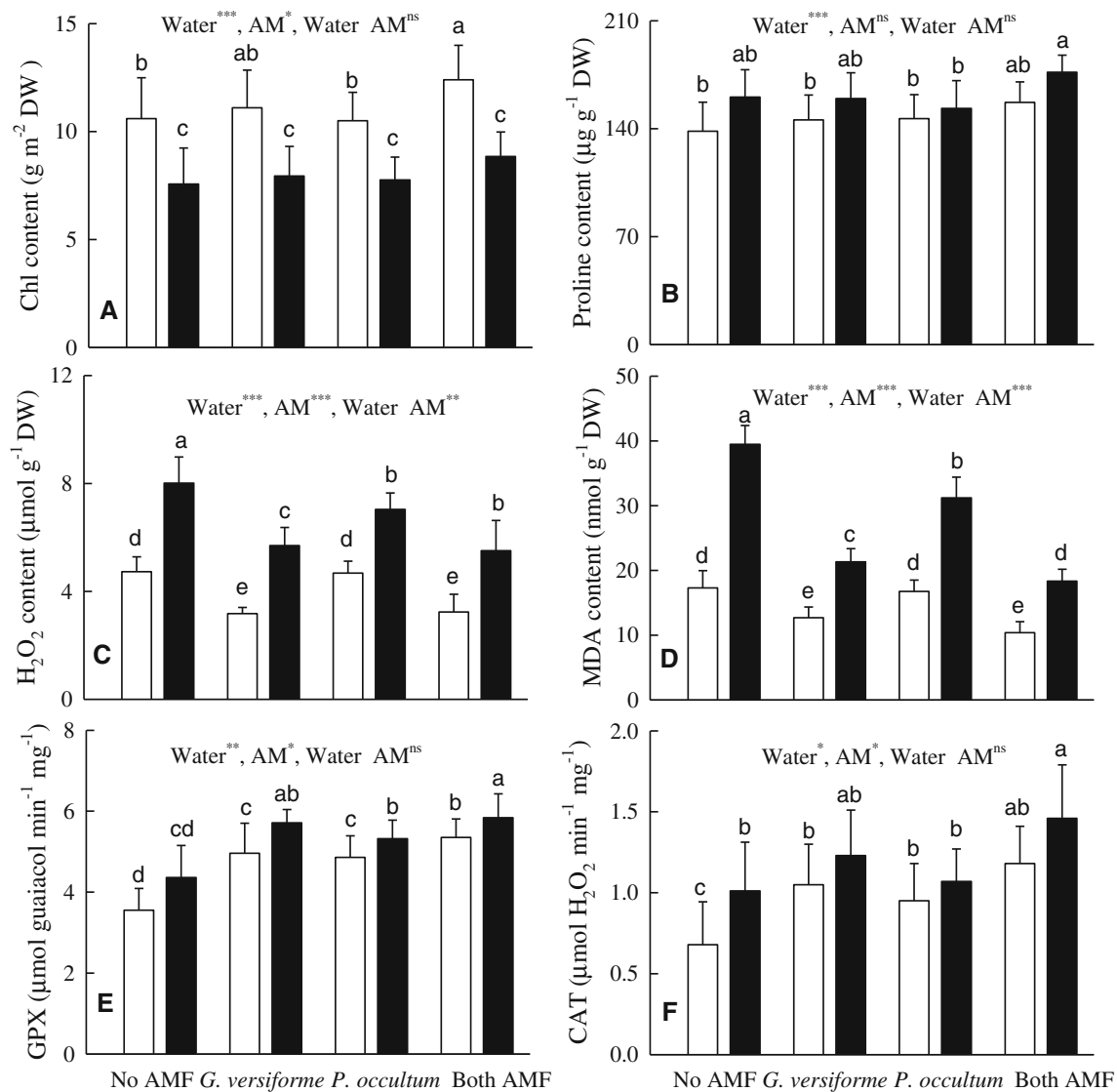


Fig. 4 Leaf chlorophyll content (a), proline (b), H₂O₂ (c), MDA (d) contents, and GPX (e) and CAT (f) activities of *P. volubilis* seedlings in response to watering and AM inoculations. The values (mean ± SE, *n* = 5) followed by a different letter indicate significant

difference at *P* < 0.05 level. Open and closed bars represent well-watered and drought treatment, respectively. *ns* not significant, *P* > 0.05; **P* < 0.05; ***P* < 0.01; ****P* < 0.001

P. volubilis seedlings (Fig. 1), which was consistent with other studies (Ruíz-Sánchez et al. 2011). Colonization by AMF improved the growth and development of *P. volubilis* seedlings, indicated by the increase in P_{max} (Fig. 2a), SLA, LAR and RGR (Table 1). SLA is an indicator of leaf density and thickness, which is positively associated with the fraction of leaf mass in structural tissue (Poorter et al. 2009). SLA is also associated with N allocation to photosynthesis (Lei et al. 2011); and plants with higher SLA allocate a higher fraction of leaf N to photosynthesis and have a higher P_{max} , thus higher RGR. Therefore, high SLA might contribute to the ability to opportunistically capture available resources for better growth and reproduction

(Cai et al. 2009; Abdel Latef and He 2011). AMF symbiosis did not affect G_s , whereas significantly increased WUE. Thus, AM-inoculated *P. volubilis* seedlings needed less water to growth than non-AM plants since they can use water more efficiently. RGR was positively correlated with P_{max} , SLA or LAR (Fig. 3). The high P_{max} and LAR are likely to have a higher light-capturing ability and total photosynthetic carbon gain, and thus lead to the higher RGR. This is in accordance with studies comparing fast-growing and slow-growing woody species (Poorter 1999; Cai et al. 2009). P_{max} was found to correlate positively with G_s across all treatments ($r = 0.72$; $P < 0.001$), which seems to be a common feature of drought-adapted species

(Chaves et al. 2003). Therefore, the variation observed in P_{\max} in the present study may be largely due to variations in G_s , caused by changes in stomatal aperture.

AM associations have been shown to induce modifications in root architecture and morphogenesis in herbaceous plants as well as in trees, but with inconsistent results for different plants and/or fungal species. Some studies found that AMF colonization enhanced root branching as well as root length, such as in *Vitis vinifera* (Schellenbaum et al. 1991) and carob (Cruz et al. 2004). In contrast, that AM fungi inhibited root growth as indicated by less total root length, smaller root surface area, and less root volume in trifoliolate orange and soybean were also found (Bressano et al. 2010; Wang et al. 2011). These indicated that the effects of AM association on root architecture may be plant and/or fungal species dependent. We found that AMF colonization significantly enhanced the total root length, the root surface area, and the root volume in *P. volubilis* seedlings, regardless of soil water conditions (Table 1). Thus, that can improve water and nutrient uptake ability to enhance growth and drought tolerance.

Water deficit also incurred serious oxidative damage as the significant increases in H_2O_2 and MDA contents (Fig. 4). Under drought stress, high level of reactive oxygen species maybe due to the partial stomata closure indicated by the decrease in G_s , which may result in not only the limitation of CO_2 fixation and lower photosynthetic capacity, but also the inefficient dissipation of the excessive excitation energy (Asada 1999). The H_2O_2 accumulation inflicted significant oxidative damage, manifested by the increase in lipid peroxidation product of MDA (Asada 1999) (Fig. 4d). AMF are obligate symbionts and acquire carbon as energy source from their host plants to complete their life cycle (Bago et al. 2000). In return, the fungi provide multiple benefits for their hosts, including enhanced mineral nutrition and tolerance to abiotic and biotic stresses (Smith et al. 2011). Several studies on the topic have demonstrated that the contribution of the AMF symbiosis to plant drought tolerance results from a combination of physical, nutritional, physiological, and cellular effects. An improved drought tolerance may result not only from direct water supply by extra-radical mycorrhizal fungal hyphae (Augé 2001), but also from indirect mycorrhizal effects, such as an improved nutrient status (Smith et al. 2011; Wang et al. 2011), hormonal regulation of stomata (Smith et al. 2008), a better osmotic adjustment (Ruíz-Sánchez et al. 2011), and increased antioxidant levels in AMF plants (Huang et al. 2011). AMF alleviated the drought-induced oxidative damage of *P. volubilis* seedlings, which maybe through enhancement of enzyme activities, including GPX and CAT (Fig. 4). The more efficient antioxidant system caused lower accumulation of H_2O_2 and lipid peroxidation product MDA. In addition, the

stimulation of photosynthetic ability and water-use efficiency might also contribute to the drought tolerance of *P. volubilis* seedlings (Asada 1999). On the other hand, there was no significant increase in proline content under drought stress or AMF colonization, suggesting that proline maybe not as the main compatible solute participating in osmoregulation and osmoprotection in *P. volubilis* seedlings. Actually, the precise role of proline remains a controversial subject. For example, proline accumulation seems to be a symptom of injury rather than an indicator of salinity resistance in rice. Indeed, salt-resistant in rice cultivars accumulate lower amounts of free proline than salt-sensitive ones (Lutts et al. 1999) and exogenously applied proline even exacerbates the salt-induced injuries (Garcia et al. 1997). As a stress-related signal, the synthesis of proline could be one of the earliest metabolic responses triggered in the signal transduction pathway that links the perception of many environmental stresses to the elicitation of physiological response at the cellular level (Hare et al. 1998). Therefore, to clarify whether a change in proline content is actually a signaling component and how it regulates gene expression and adjusts metabolic process will undoubtedly be a challenge question and needs more exploration.

Due to the large physiological and morphological variability among AMF, we can expect changes in their behavior during the establishment of symbiosis, causing responses in host plants ranging from a very efficient symbiosis to the lack of effects or the occurrence of deleterious effects on growth. Zhang et al. (2010) found three AM fungi enhanced drought tolerance of *Casuarina equisetifolia* seedlings more pronouncedly than the other three fungi belong to *Glomus*. Reis et al. (2011) also found that *Pinus pinaster* benefited more from the association with ectomycorrhizal fungi *Pisolithus arhizus* than with *Paxillus involutus* in the antioxidant potential. Our result showed that different AMF might display different effects. For example, *G. versiforme* exhibited stronger stimulation than *P. occultum*, in well accordance with the pervious literature in which *Glomus* has been considered as the best adapted genus for habitats subjected to drought (Pagano et al. 2009). In addition, there was a synergistic effect when the two symbionts were inoculated together, resulting in a greater benefit (increased water status, growth rate and increased antioxidant levels, and decreased drought-induced oxidative damage, etc.) for the *P. volubilis* seedlings with co-inoculation than inoculation with AM fungi alone. Babu and Reddy (2011) found that the combination of AM fungi and phosphate solubilizing fungus *Aspergillus tubingensis* elicited a synergistic effect by increasing plant growth and uptake of nutrients with reducing metal translocation in fly ash ponds. The *P* solubilizing microorganisms increase the mycorrhizal root colonization by

producing specific metabolites such as vitamins, amino acids and hormones apart from *P* solubilization (Barea et al. 2005). Rhizobia AMF have developed similar recognition systems for plant flavonoids and then present common properties in their interactions with plants. Thus, a highly effective inoculation occurs if there is established a synergistic interaction among effective symbionts of rhizobia and/or mycorrhizal fungi. These results can be considered as a hypothetical signal of a symbiotic differential compatibility of mycorrhizal fungus for a host. There is considerable functional diversity among plant–AM fungal symbioses in terms of benefits and costs (C supply to the fungus). Individual plants in the field will be colonized by many fungal taxa, and the sum total benefits and costs contribute to the success, in terms of growth and reproduction of host plants (Abdel Latef and He 2011; Wang et al. 2011). The outcomes of the symbioses are determined by interactions between plant and AMF genomes as well as environmental conditions. This indicates a high probability of being able to more generally identify, isolate, and culture fast colonizing AMF for use as inoculants in agriculture and horticulture strategies, including *P. volubilis* plants. Moreover, long-term field experiments are needed for evaluating how the AMF affected ecophysiological traits, seed yield and seed chemicals of *P. volubilis* plants.

In conclusion, our results show that AMF colonization improved the growth of Sacha Inchi seedlings under both drought and well-watered conditions, which mainly contributed to the higher SLA, LAR and P_{max} . At the molecular level, higher antioxidant enzyme activities were detected in the AM plants, consistent with the enhanced drought tolerance. The enzymes responsible for elimination of ROS and alleviation of oxidative stress were induced to a larger degree in the AMF plants. However, proline may be not as the main compatible solute participating in osmoregulation in Sacha Inchi plants. All of these results demonstrate that AMF inoculation results in growth promotion and a well-established defense mechanism against the drought, in which mitigation of oxidative stress might be a crucial part. Thus, the encouragement of this symbiosis symbiotic association between AMFs and Sacha Inchi plants is therefore of great interest in its commercial production, especially in many arid and semi-arid regions.

Author contribution Y.H.T. and Z.Q.C. conceived and designed the experiments; Y.H.T., Y.B.L. and Y.L.Z. collected, analyzed the experimental data and prepared the first draft. All authors contributed substantially to revising the manuscript.

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