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ARBUSCULAR MYCORRHIZAL FUNGI INDUCED DIFFERENT PROLINE ACCUMULATIONS IN TWO SORGHUM ACCESSIONS IN A RESPONSE TO DROUGHT STRESS

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Sorghum has good adaptability to drought stress conditions, but its early vegetative phase and the generative phase are susceptible to stress. Understanding the physiological response of plants under drought and mechanisms regulating drought tolerance in a plant, mediated by arbuscular mycorrhizal fungi (AMF) will be useful in developing a strategy to deal with drought. Here, a pot experiment was used to explore the growth performance, biomass production and physiological responses of two sorghum accessions (4183A and JP-1) inoculated by the AMF under drought stress, as well as the effect of AMF on soil enzyme and microbial stability. Based on growth observations, the AMF inoculation treatment had not significant effect on increasing the drought resistance of the two sorghum accessions. Drought stress decreased the rate of height increment for 4183A, and JP-1 accessions by 37% and 55%, respectively, compared to normal conditions. Shoot dry weight and root dry weight losses were up to 59% and 66%, respectively, compared to well-watered conditions. However, the interaction of AMF and plants to deal with drought can be captured through physiological response, particularly proline accumulation. AMF inoculation in JP-1 accession reduced proline accumulation (99.91 mM/leaf fresh weight) compared to non-AMF inoculated plants (149.86 mM/leaf fresh weight). It can be implied that mycorrhiza can reduce plant stress. In contrast to accession 4183A, there was an increase in the accumulation of proline in plants inoculated with mycorrhiza under drought conditions. Additionally, AMF inoculation improved acid phosphatase activity in the soil and proved crucial for maintaining the stability of the rhizosphere microorganisms under drought-stressed conditions.

Key words: abiotic stress, arbuscular mycorrhiza, drought resistances, proline, soil enzyme, sorghum

The global warming issue has captured the attention of many parties in the past few decades. Drought was one of the effects of climate change brought on by global warming (Dai 2011; Chiang *et al.* 2021). By the end of the twenty-first century, Earth's temperature had been rising steadily, ranging from 3 to 9°C (Trenberth *et al.* 2014; Khaleghi *et al.* 2019). Scientists anticipate that the risk of drought will rise with time. The agricultural sector will be impacted by this circumstance, particularly in semi-arid and dry regions that receive minimal rainfall. An increase in temperature will reduce pre-

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cipitation and decrease agricultural productivity in areas that depend on rainfall (Li *et al.* 2009). As a consequence, many types of research have been conducted to address the problem of drought, one of which explored new candidate plants that are resistant to drought and investigated how these plants respond to drought stress (Devnarain *et al.* 2016; Khaleghi *et al.* 2019; Martignago *et al.* 2020).

Sorghum [*Sorghum bicolor* (L.) Moench] is one of the food crops known to have resistance to drought stress. This native African plant has now spread worldwide (Devnarain *et al.* 2016). In Indonesia, sorghum is widely cultivated in the eastern regions because it has a suitable climate. All components of the sorghum plant can be utilised for a wide range of purposes, making it a versatile plant. The seeds are used as food (Ratnavathi & Patil 2013; Xiong *et al.* 2019), while the stove can be used as animal feed (Selle *et al.* 2018) or for industrial purposes, such as biocomposite materials (Yulianto *et al.* 2020) and fuel pellets (Wiloso *et al.* 2020). In addition, for sweet sorghum cultivars, the juice from the stems can be used as a fermentable sugar for bioethanol production (Ray *et al.* 2019) and citric acid (Kanti *et al.* 2018). As an essential cereal crop in the universe, sorghum has an average total production of around 61.23 tonnes per year in the 2012–2019 period (Food and Agriculture Organization (FAO), 2020). The United States is the largest sorghum producer. However, the information on sorghum production in Indonesia in the last ten years is restricted.

Sorghum has good adaptability to drought stress conditions, but in two phases in its life cycle is susceptible to stress. These are in the early vegetative stage and the generative stage (pre- and post-flowering) (Devnarain *et al.* 2016; Gano *et al.* 2021). In the early vegetative stage, sorghum seeds are in the process of adapting to environmental conditions. Several studies have found that drought stress during the seedling phase can negatively impact sorghum plant growth during the next stage (Tari *et al.* 2013; Gano *et al.* 2021). The sorghum plants require more water during the generative phase (pre- and post-flowering), so stress during this phase could reduce sorghum plant productivity (De Souza *et al.* 2020). The ability of sorghum cultivars to be planted in dry land conditions could be determined by their resistance in these two phases.

Many of the studies on the drought resistance of sorghum have relied heavily on the observation of agronomic characteristics. Research related to how the defence mechanism of sorghum deals with drought stress has not been widely disclosed. This causes limited information that can be used for the development of new cultivars to increase their resistance toward drought stress, for example through a molecular genetic approach. Plants naturally have a defence system in the face of environmental stresses with complex mechanisms, including drought stress (Goche *et al.* 2020). Agronomically, plants respond by rolling their leaves, producing a waxy coating on the leaf surface, or dropping leaves to reduce evaporation, thereby reducing water loss in plant cells (Bray 1997; Buchanan *et al.* 2005; Goche *et al.* 2020). In plant cells, it will activate several metabolic pathways to produce certain compounds that can reduce the adverse effects of cell damage by drought, such as the activation of antioxidant enzymes to reduce reactive oxygen species (ROS), which are produced when plants are stressed, and the production of osmolyte molecules that can prevent cell damage due to reduced water in cells, such as proline, sucrose, and mannitol (Devnarain *et al.* 2016; Khaleghi *et al.* 2019; Goche *et al.* 2020).

The resistance of plants to drought stress can also be induced by their symbionts, one of which is the arbuscular mycorrhizal fungus (AMF). Many studies have stated that AMF can optimise water absorption by the roots through hyphal nets in certain plants. AMF can increase the resistance of host plants through the induction of compounds that can reduce the adverse effects of drought stress, such as proline (Bahadur *et al.* 2019). However, because AMF interaction was host-specific, different plant species will respond differently to AMF inoculation. Sukri *et al.* (2019) stated that mycorrhizal compatibility with host plants varies greatly depending on the mycorrhizal species, type of host plant, and environmental conditions.

The interaction of AMF with plants is the focus of most studies on the use of AMF in increasing plant resistance. However, research on the effects of AMF application on soil microbial communities is still scarce. AMF has been shown to not only improve the adaptability and resistance of its host plants but also to maintain the stability of the soil microbi-

al community (Hestrin *et al.* 2022). Hestrin *et al.* (2022) demonstrated that applying AMF simultaneously can provide a protective effect on soil microbial communities exposed to drought stress. Soil inoculated with AMF had a higher microbial growth efficiency than soil not inoculated with AMF. The soil microbial community plays an important role in plant growth, including nutrient cycling (Jacoby *et al.* 2017). Consequently, it is critical to understand the effect of AMF inoculation on soil microbes and its relationship to supporting plant growth during drought. The impact of AMF application on soil microbial stability was observed in this study by measuring total microbial activity in the soil. Furthermore, the activity of the soil acid phosphatase enzyme, which is one of the important enzymes supplying the availability of phosphorus (P) that can be absorbed directly by plants, was measured. AMF is one of the enzyme producers of acid phosphatase (Liang *et al.* 2022).

This study used two sorghum accessions, JP-1, and 4183A. As reported by previous research (Wahyuni *et al.* 2019), JP-1 accession has high biomass productivity with low lignin content. Accession 4183A also has high productivity, but with a higher lignin content than JP-1 accession. In another study, it was reported that 4183A accession has the potential to be used as a raw material in the production of second-generation bioethanol due to its high productivity and high sugar content (Santoso *et al.* 2013). However, there have been no reports regarding the response of these two sorghum accessions to drought stress and AMF treatment. Therefore, this study aimed to determine the plant growth and biomass production, and physiological response of sorghum JP-1 and 4183A accessions under drought stress with AMF inoculation. In addition, this study was also conducted to determine the impact of AMF treatment on acid phosphatase and total microbial activity in soil under drought-stress conditions.

MATERIAL AND METHODS

Plant material and experimental design

The experiment was carried out in the greenhouse of the National Research and Innovation Agency at the Cibinong Science Center during the dry season (July-September). The sorghum seeds used were obtained from the Indonesian Center for Cereal Research (Balitsereal), Indonesian Ministry of Agriculture. It consists of two accessions, namely, 4183A and JP-1. The mycorrhizal inoculum used in this study contained spores, mycelium, infected maize root fragments, and zeolite, which were purchased from the Indonesian Center for Agricultural Land Resources Research and Development (ICALRD). A consortium of *Acaulospora* sp. and *Glomus* sp. with approximately 40 spores per 100 g of zeolite was used as the mycorrhizal inoculant.

This study used a factorial randomised block design consisting of three treatment factors. The first factor was accession (A), which consists of accession 4183A and accession JP-1. The second factor was the inoculation of arbuscular mycorrhizal fungi (B), which were inoculated with AMF and without the inoculation of AMF. The third factor was drought stress treatment (C), namely without stress and dry stress. The number of combinations of these three factors was eight combinations, and they were repeated three times, so there were 24 experimental units. Each experimental unit contained three plants grown in pots in a greenhouse. During the experiment's performance, the temperatures ranged from 25 to 33°C, the relative humidity ranged from 65.5% to 89.0%, and the light intensity ranged from 415 to 1439 lx.

Preparation of planting media, seedlings, fertilising, and watering

The planting medium used was 3 kg of unsterilized media per pot, consisting of Ultisol soil and basic fertiliser (manure made up 6% of the total media) that had been air-dried for one week. The physical and chemical properties of Ultisol soil used in the experiment were summarized in Table 1. The planting medium was put into a plastic pot with a diameter of 18 cm and a height of 13.5 cm. Mycorrhizal inoculum in the form of zeolite propagules was added along with seed sowing by placing 100 g/pot in the seed hole. All treatments were watered normally twice a week for the first 6 weeks (60% water holding capacity). Chemical fertilisers were applied in weeks 2 and 4 with the following total doses: N = 100 kg/ha, P = 60 kg/ha, and K = 30 kg/ha. Urea fertiliser was used as a source of N, SP-36 was

T a b l e 1

Ultisol soil physical and chemical properties used in the experiment.

Soil texture	Sand [%]	Silt [%]	Clay [%]	Organic C [%]	Total N [%]	Total P [mg/Kg]	Available P [mg/Kg]	Total K [mg/Kg]	CEC [cmol(+)/Kg]	pH (H_2O)
Clay	4	12	84	0.83	0.09	585.8	49.65	73.9	16.10	6.23

CEC – Cation exchange capacity

used as a source of P, and KCl was used as a source of K. Treatment for drought stress was started at 7, 8, and 9 weeks. The drought stress treatment group was not watered for 6 days, and on the 7th day, it was watered until it was saturated. After that, the drought stress was repeated for the same duration as before, so the plants received three cycles of drought treatment.

Measurement of soil water content

Soil water content measurement was carried out using the gravimetric method (Haney & Haney 2010). Soil samples in each treatment pot were taken at intervals of 0, 1, 2, 3, 4, 5, and 6 days after watering. Soil samples were dried at 105°C for 24 hours, and the soil moisture content was calculated using the following equation:

$$Soil\ water\ content\ (\%) = \frac{BB - BK}{BB}\ x\ 100\%$$

BB was the weight of fresh soil, and BK was the weight of the soil after drying.

Determination of plant growth parameters and biomass

The agronomic characteristics, such as plant height and the number of leaves were observed every week until nine weeks after sowing. Height measurements were carried out from the base of the stem to the highest tip using a rope and then converted using tape as a measure. Measurements of the number of leaves counted were taken on fully expanded leaves. Shoot and root were harvested in the ninth week and weighed to determine the fresh weight. The sample was subsequently dried in an oven at 70°C for three days, and the dry weight was determined

Measurement of AM fungal colonisation

The collected roots were washed with tap water to remove the soil. The roots were cut by 1 cm and put into a bottle containing 10% KOH, which was then heated for 20 minutes. The roots were rinsed to remove KOH and soaked in 10% H_2O_2 for 15 minutes. The cleared roots were rinsed with water and soaked in 3% HCl for 30 minutes, then stained with 0.05% lactoglycerol trypan blue (in 5:1:1 lactic acid: glycerol: distilled water) overnight (Birhane *et al.* 2017). Under a microscope, root segments were examined to determine whether they were colonised by AM fungal features such as hyphae, arbuscules, or vesicles were present (Sun *et al.* 2017). The percentage of root colonisation was calculated using the following formula (Bhale 2018):

$$Root\ colonisation\ (\%) = \frac{Number\ of\ colonised\ segments}{Total\ number\ of\ colonised\ segments\ examined} x\ 100$$

Measurement of plant physiological traits

Leaf relative water content (RWC)

The relative water content (RWC) was measured using the Oswal method (Rahimi & Madah Hosseini 2010), where the leaf sample was cut into 1 cm^2 size, and then weighed to determine the fresh weight of the leaf. The leaf sample was floated on the surface of the water for 4 hours to determine the maximum turgidity and then reweighed. The leaf sample was oven-dried for 12 hours at a temperature of 65°C, and then weighed to determine the dry weight of the leaf. The rate of water absorption was calculated using the following formula:

$$RWC = \frac{BB - BK}{BT - BK}\ x\ 100\%$$

RWC – relative water content,
BB – fresh weight of leaf,
BT – fresh weight of leaf after gaining maximum turgidity,
BK – dry weight of leaf.

Proline content

The determination of proline content was carried out using the method used by Khaleghi *et al.* (2019). The fresh leaves of the plant are crushed using a pestle and mortar. A total of 25 mg of crushed leaves was transferred into a 2 mL microtube and 500 µL of ethanol: water (70:30) was added. After that, the microtube was transferred to a block heater and incubated at 85°C for 20 minutes. The microtubes were then cooled to room temperature and centrifuged for 5 min at 14,000 g. If the extract was not analysed immediately, it was stored at –20°C. A total of 100 µL of the extract was transferred into a 2 mL microtube and mixed with 200 µL of reaction mix (ninhydrin 1% (w/v) in 60% acetic acid (w/v), 20% ethanol (v/v)). The microtube was then heated for 20 minutes in a block heater at 95°C. The microtube was then cooled to room temperature and spun down for 1 minute at 2,500 rpm. The supernatant was then aliquoted into a 96-well microplate, and its absorbance was measured at a wavelength of 520 nm. The sample concentration was determined using the standard L-proline curve with a concentration variation of 1 mM, 0.4 mM, 0.2 mM, 0.1 mM, and 0.04 mM.

Leaf chlorophyll content

Leaf chlorophyll content was determined using a Chlorophyll Meter SPAD-502 (Konica Minolta Sensing Inc., Japan). The chlorophyll content was the average value of three leaves (the third, fourth, and fifth leaf from the top) of each plant. The results of SPAD readings were transformed to chlorophyll content following the equation (Cerovic *et al.* 2012):

$$Chlorophyll\ (\mu g/cm^2) = (99 \times SPAD)/(144 - SPAD)$$

Malondialdehyde

The determination of malondialdehyde (MDA) in leaves followed the Cakmak and Host method (1991). Fresh leaves (1 g) were ground in 20 mL of 0.1% (v/v) trichloroacetic acid (TCA) solution and then centrifuged at 12,000 g. One mL of the supernatant was reacted with 4 mL of 20% TCA containing 0.6% thiobarbituric acid. The mixture was then incubated at 95°C for 30 minutes before being cooled. Each mixture was placed in a spectrophotometer, and the absorption at 532 and 600 nm wavelengths recorded were used to calculate the MDA concentration of that sample. The MDA concentration was determined by a molar coefficient of 155 mM/cm using the following equation:

$$MDA\ (nmol) = \Delta\ (A532nm - 600nm)/1.56 \times 10^5$$

Soil total microbial activity

Total microbial activity was determined using the fluorescein diacetate (FDA) hydrolysis method (Green *et al.* 2006; Liu *et al.* 2019). Soil samples were taken in the rhizosphere area at the end of drought treatment. A total of 2 g of fresh soil from each treatment was mixed with 15 mL of 60 mM potassium phosphate buffer pH 7.6 in a sterile Falcone tube. The reaction was started by adding 200 µL of FDA 1,000 g/mL solution into the tube as substrate and followed by incubation at 150 rpm, 30°C, for 1 hour. To stop the reaction, 950 µL of the soil suspension was aliquoted into a new 2 mL microtube containing the same volume of 2:1 (v/v) chloroform/methanol. After that, the obtained soil suspension was centrifuged for 6 minutes at 6,000 rpm and 300 µL of the supernatant was transferred to a 96-well microplate. The absorbance was measured at a wavelength of 490 nm using a spectrophotometer (Vario Scan, Thermo Scientific). The concentration of fluorescein produced was determined using a standard solution of Na-fluorescein with a concentration range of 1.0 – 7.0 g/mL.

Soil acid phosphatase

Acid phosphatase was measured based on the reduction of p-nitrophenyl phosphate (p-NPP) (Tabatabai 1994). Soil samples were taken in the rhizosphere area at the end of drought treatment. A total of 0.2 g of fresh soil from each treatment was put into a 100 mL Erlenmeyer and added to 4 mL of modified universal buffer (MUB pH 6.5), 0.25 mL of toluene, and 1 mL of 0.2 M p-NPP. The soil suspension was homogenised by swirling the Erlenmeyer and then covered with a cotton plug. After that, it was incubated in a water bath for 1 hour at 37°C. The reaction was stopped by adding 1 mL of 0.5 M $CaCl_2$ and followed by 4 mL of 0.5 M NaOH. Following that, the suspension was filtered through filter paper, and the filtrate was measured for absorbance at 420 nm using a spectrophotometer. For control, 1 mL of 0.2 M p-NPP substrate was added after the addition of $CaCl_2$ and NaOH just before extraction. Phosphatase activity was defined as g p-NP liberated by one g of soil per hour (µg p-NP/g dry soil h).

Statistical analysis

The obtained data were first checked for homogeneity. The data distribution was normal, thereby no transformation was required. The analysis of variance (ANOVA) and Duncan's new Multiple Range Test (DMRT) were used to compare the differences among treatments. The significant differences among factors were calculated at $p<0.05$. The data processing and visualising were carried out using an R software version 4.3.1. All data were reported as the mean of at least three replicates including standard error (SE).

RESULTS AND DISCUSSION

AM fungal colonisation

Our results showed that there was the colonisation of sorghum roots without AMF inoculation because unsterilised media were used. According to Diannastiti *et al.* (2022), there is no elimination of AMF in unsterilised soil, so indigenous AMF can still colonise the roots. Thus, the plants that had not been inoculated with AMF were colonised by indigenous AMF in the present study (Figure 1).

However, the percentage of colonisation was still high in the introduced AMF-inoculated sorghum roots, which were up to 77%, compared to colonisation by indigenous AMF, which only ranged from 37% to 48% (Figure 2). Yao *et al.* (2008) discovered differential colonisation in the roots of *Cyperus difformis* by native AMF, which is low, but appears to be associated with numerous vesicles. In contrast Diannastiti *et al.* (2022) that stated indigenous AMF was able to colonise corn roots by more than 89%. In the present study, the inoculated species of AMF had higher effect in infecting roots than the indigenous species. The drought treatment did not significantly affect the level of root colonisation by AMF (Figure 2).

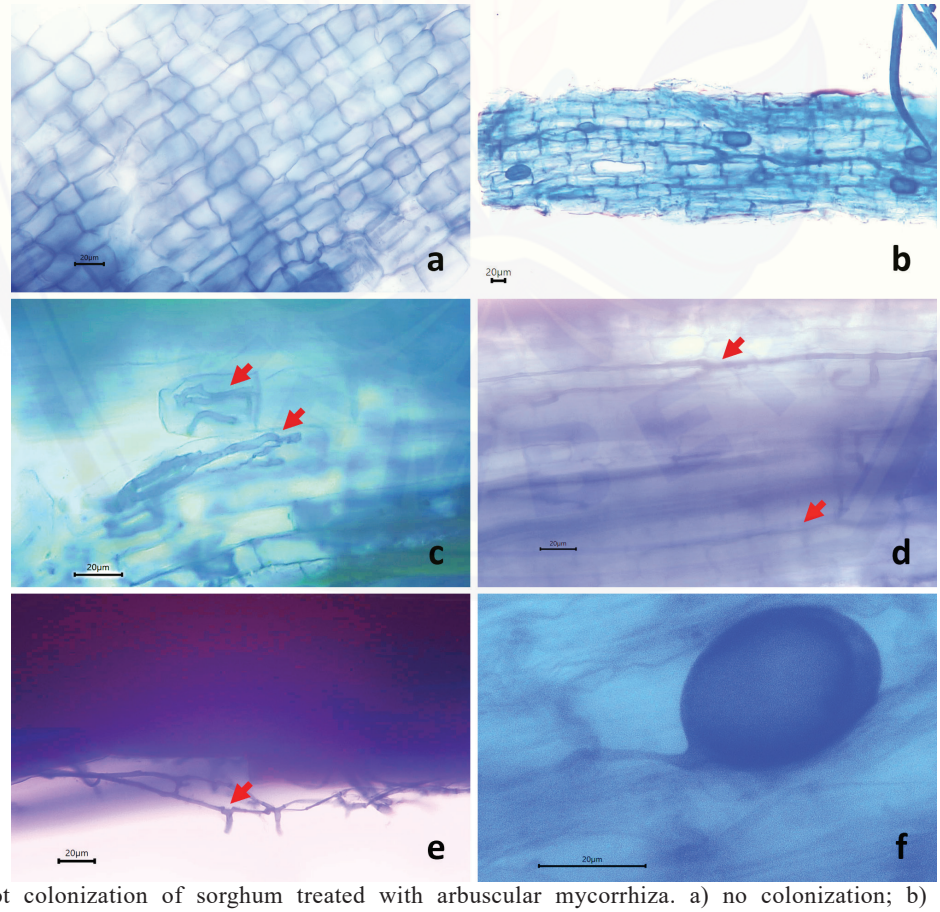

Figure 1. Root colonization of sorghum treated with arbuscular mycorrhiza. a) no colonization; b) colonised root; c) intercellular hyphae; d) intracellular hyphae; e) extraradical mycelium; f) vesicle. Bar scale = 20 µm.

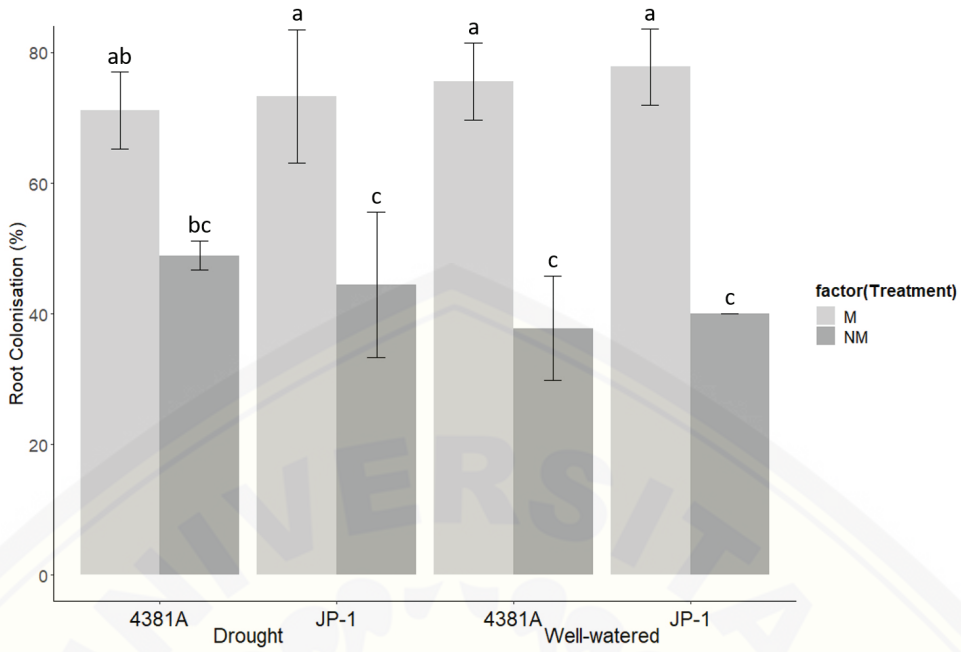

Figure 2. Root colonization of sorghum 4183A and JP-1 accession treated with arbuscular mycorrhiza and drought stress. M (with mycorrhiza), and NM (without mycorrhiza). The different letter over the bars indicate significant difference ($p < 0.05$) among treatment with the Duncan's new Multiple Rate Test. Vertical bars show the SE.

T a b l e 2

Height increment, leaf number increment, shoot fresh and shoot dry weight, and root fresh and dry weight of sorghum 4183A and JP-1 accession under arbuscular mycorrhiza and drought stress treatment. WW-NM (well-watered without mycorrhiza), WW-M (well-watered with mycorrhiza), D-NM (drought without mycorrhiza), and D-M (drought with mycorrhiza)

Accession	Treatment	Height increment [cm/week]	Leaf number increment [sheet/week]	Shoot fresh weight [g]	Shoot dry weight [g]	Root fresh weight [g]	Root dry weight [g]
4183A	WW-NM	11.93 ± 0.83^{ab}	1.00 ± 0.19^{ab}	130.17 ± 9.15^{a}	34.05 ± 1.42^{a}	13.63 ± 1.70^{c}	4.70 ± 0.48^{c}
	WW-M	14.86 ± 2.72^{a}	1.00 ± 0.11^{ab}	146.34 ± 9.83^{a}	36.74 ± 0.48^{a}	10.89 ± 0.66^{cd}	5.68 ± 0.49^{c}
	D-NM	7.53 ± 1.66^{cd}	0.78 ± 0.11^{b}	63.33 ± 3.00^{b}	13.99 ± 0.18^{b}	2.57 ± 0.06^{f}	1.58 ± 0.13^{e}
	D-M	10.21 ± 1.16^{bc}	0.78 ± 0.11^{b}	63.45 ± 3.32^{b}	14.52 ± 1.52^{b}	3.04 ± 0.17^{ef}	1.83 ± 0.11^{e}
JP-1	WW-NM	10.00 ± 0.82^{bc}	1.22 ± 0.11^{a}	139.77 ± 5.31^{a}	34.96 ± 1.77^{a}	24.82 ± 2.05^{b}	8.72 ± 0.28^{b}
	WW-M	10.72 ± 1.07^{abc}	1.22 ± 0.11^{a}	134.96 ± 0.62^{a}	35.69 ± 1.43^{a}	31.15 ± 1.73^{a}	10.47 ± 0.84^{a}
	D-NM	4.51 ± 0.50^{d}	1.11 ± 0.11^{ab}	67.85 ± 2.58^{b}	17.14 ± 0.58^{b}	7.16 ± 1.04^{de}	3.38 ± 0.22^{d}
	D-M	5.69 ± 0.43^{d}	0.89 ± 0.11^{ab}	65.68 ± 3.47^{b}	16.33 ± 0.54^{b}	9.33 ± 1.24^{cd}	3.26 ± 0.47^{d}
Significance	Accession (A)	**	*	ns	ns	***	***
	AMF (B)	ns	ns	ns	ns	ns	*
	Irrigation (C)	***	*	***	***	***	***
	A × B	ns	ns	ns	ns	*	ns
	A × C	ns	ns	ns	ns	***	***
	B × C	ns	ns	ns	ns	ns	*

Value within each column followed different superscript indicate significant difference ($p < 0.05$) among treatment with the Duncan's new Multiple Rate Test. 'ns'– not significant, '*' $p < 0.05$, '**' $p < 0.01$, '***' $p < 0.001$. Value are means ± SE (n=3).

Growth performance

The growth performance of the two types of accessions was significantly different, especially in the height increment and root biomass. Furthermore, this study showed that accession 4183A had a higher rate of height increment than accession JP-1. On the other hand, JP-1 accession had higher root biomass than 4183A accession (Table 2). However, both sorghum accessions have not significant difference in leaf number and shoot biomass. The differences in growth parameters of the two sorghum accessions were shown in Table 2.

In normal water conditions, the inoculation of AMF showed a significant effect only on the root weight increment of JP-1 accession (Table 2). Inoculation of AMF improves root fresh and dry weight in JP-1 accession by 25.5% and 20.1%, respectively, compared to plants without AMF inoculation. In contrast, inoculating accession 4183A with AMF did not result in a significant increase in root biomass. The 4183A accession showed an increase in height increment compared to non-inoculated AMF, but the difference was not significant ($p>0.05$). Based on root weights, it was seen that only JP-1 accession gave a positive response when inoculated with AMF. The increment root weight was one of the symbiotic effects of AMF on plants. AMF that has succeeded in infecting the roots will form hyphae that function to absorb nutrients from the soil, so that root weight increases (Begum *et al.* 2019). The increase in root weight with the AMF treatment was also reported by Hazzoumi *et al*. (2015), with an increase of 13% compared to the treatment without AMF. AMF treatment can increase root biomass higher than stem biomass, namely 24.2% in stems and 29.6% in roots. Wu *et al.* (2022) reported that the increase in biomass by AMF treatment depended on the organ type and functional group of the host plant (e.g., grain and non-grain, N-fixing, and non-N-fixing). The increase in plant stem biomass by AMF treatment was greater in non-grain plants, which was 54.9%, while in grain plants there was only a 17.4% increase. These are probably because AMF inoculation affects on yield of the plant, depending on the product produced by the plant. Regarding N-fixing plant, AMF treatment promotes the accumulation of rhizobia in the rhizosphere of host plants, which leads to increased yield and biomass (Wang *et al.* 2021)

Furthermore, the symbiosis of AMF with its host plant is specific, so the response given will not be the same for different plant species. Even on the same species of plant, different cultivars can give different responses. García de León (2020), who studied the response of six wheat cultivars to AMF inoculation, found that not all wheat cultivars tested increased growth in the presence of AMF inoculation. This report was in accordance with our findings in the response to 4183A accession to AMF inoculation. This makes the present study confirmatory.

The lack of response of plants to AMF inoculation is attributed to the lack of compatibility between the symbionts (Chagnon *et al.* 2013). AMF is widely known to non-host-specific, as a single fungus may associate with multiple plant species. However, preferential associations have been found in some studies (Torrecillas *et al.* 2012; Campos *et al.* 2018; Muneer *et al.* 2022). AMF preferential associations were influenced by several factors, such as soil type and nutrient availability, host plant species/cultivars, and AMF strains and origin (Chagnon *et al.* 2013, Werner & Kiers 2015; Symanczik 2018; Guo *et al.* 2021).

Drought treatment had a significant impact on the growth and biomass of sorghum. As shown in Table 2, the drought treatment significantly affected all growth parameters (height, number of leaves, shoot fresh and dry weight, root fresh and root dry weight). Drought stress decreased the rate of height increment for 4183A, and JP-1 accessions by 36.9% and 54.9%, respectively, compared to normal conditions. 4183A and JP-1 accessions also experienced a decrease in the biomass of both shoots and roots due to drought stress. For example, in accession 4183A, shoot dry weight and root dry weight losses were up to 58.9% and 66.4%, respectively, compared to well-watered conditions. However, in JP-1 accession, shoot dry weight, and root dry weight losses were up to 50.9% and 61.2%, respectively, compared to well-watered conditions. Drought stress causes various damages to plant tissues, including the diversion of cell metabolic pathways that are more focused on self-defence than growth and the inactivation of several enzymes. These con-

ditions will lead to decreasing plant growth and biomass (Zhang *et al.* 2020).

The growth performance of both sorghum accessions inoculated with AMF was relatively the same as with non-inoculated AMF plants in drought treatment (Table 2). Although there was an increase in growth parameters with AMF inoculation under drought, the values were not significantly different compared to non-inoculated AMF for both sorghum accession. 4183A and JP-1 accessions inoculated with AMF experienced a decrease in the rate of height increment under drought stress of about 14.4% and 43.1%, respectively. Moreover, 4183A and JP-1 accessions inoculated with AMF under drought treatment had reduced root dry weight by 61.1% and 62.6%, respectively. Nevertheless, these reduction to value were lower than the treatment without AMF inoculation under drought stress.

The role of AMF in increasing plant resistance to drought stress was not optimal in this study. This might be due to the drought stress applied at a severe level. The soil water content measurement results showed that the soil water content in the pot was in the range of 18–20% after six days of water-holding treatment. AMF cannot increase the drought tolerance of the plant host under severe conditions. In extreme drought stress conditions, AMF can be a competitor for plants, so instead of supporting plants, AMF prefers to defend itself (Li *et al.* 2019). Furthermore, severe stress causes serious damage to both the host plant and AMF, so AMF inoculation was ineffective (Li *et al.* 2020). Similar effects were previously reported by Chen *et al.* (2020) in a timber plant (*Catalpa bungei* C. A. Mey.). Application of AMF showed no effect on *Catalpa bungei* subjected to severe drought (30% of maximum field capacity) (Chen *et al.* 2020). Apart from the severe drought, it could be due to the incompatibility of the type of AMF used with the host plant or the AMF used are not resistant to drought. Each AMF has diverse characteristics as well as stress resistance in supporting its symbionts, for example, in conditions of drought stress, nutrient deficiency, saline stress, and others (Millar & Bennett 2016).

Plant physiological traits

The existence of environmental stress will encourage plants to immediately adapt through various mechanisms, and each accession has a different response and strategy. Likewise, with AMF treatment, the symbiosis that occurs can be responded to by plants in a different way, including through physiological responses. The physiological response of plants to abiotic stress, especially drought stress, can be observed from the relative water content (RWC), malondialdehyde (MDA), proline, and chlorophyll content.

Our results show that AMF treatment did not significantly affect the relative water content of sorghum accessions under drought stress (Table 3). Although AMF-inoculated plants had greater RWC values in drought conditions, their values were not significantly different statistically. Drought stress reduced the RWC value by up to half compared to well-watered. Regarding MDA content, drought stress increases the MDA content in leaves, which indicates damage to the cell membrane. The MDA was produced by membrane lipids in response to reactive oxygen species (ROS) that caused plasma membrane damage under environmental stress conditions (Zhang *et al.* 2021). The MDA content increased almost 2-fold in drought stress conditions. The sorghum 4183A and JP-1 accessions showed not significant difference in MDA content in both normal and drought conditions (Table 3). On the other hand, drought and AMF treatment had no impact on leaf chlorophyll. The chlorophyll content of sorghum 4183A and JP-1 accession remained stable in all treatments, with approximately 32 µg/cm^2 (Table 3). Regarding physiological responses, our study showed that the two sorghum accessions differed significantly only in proline content, particularly under AMF and drought treatment (Table 3). Inoculation of AMF increased the proline content under well-watered conditions, but the difference was not significant ($p > 0.05$). Interestingly, the two accessions had different responses to drought stress, both with and without AMF treatment. Accession JP-1 without AMF inoculation produced the highest proline content (149.86 mM/g leaf fresh weight), which was much higher than accession 4183A without AMF inoculation, which only had a proline content of 29.54 mM/g leaf fresh weight. The low accumulation of proline when under stress conditions was an indicator of a plant's resistance capability against these stresses (Chun *et al.* 2018). The high content

Table 3

Relative water content (RWC), chlorophyll, malondialdehyde (MDA), and proline content of sorghum 4183A and JP-1 accessions treated with arbuscular mycorrhiza and drought stress. WW-NM (well-watered without mycorrhiza), WW-M (well-watered with mycorrhiza), D-NM (drought without mycorrhiza), and D-M (drought with mycorrhiza)

Accession	Treatment	RWC [%]	Chlorophyll [μg/cm^2]	MDA [nmol/g leaf fresh weight]	Proline [mM/g leaf fresh weight]
4183A	WW-NM	72.88^a	31.81^a	0.254^b	18.05^c
	WW-M	75.17^a	33.56^a	0.241^b	22.16^c
	D-NM	36.49^b	30.13^a	0.645^a	29.54^c
	D-M	41.42^b	31.26^a	0.619^a	80.92^b
JP-1	WW-NM	76.44^a	32.42^a	0.275^b	15.07^c
	WW-M	79.94^a	33.14^a	0.241^b	17.52^c
	D-NM	35.33^b	31.58^a	0.714^a	149.86^a
	D-M	40.10^b	32.06^a	0.628^a	99.91^b
Significance	Accession (A)	ns	ns	ns	***
	AMF (B)	ns	ns	ns	ns
	Irrigation (C)	***	ns	***	***
	A × B	ns	ns	ns	**
	A × C	ns	ns	ns	***
	B × C	ns	ns	ns	ns

Value within each column followed the different superscript indicate significant difference ($p < 0.05$) among treatment with the Duncan's new Multiple Rate Test. 'ns'– not significant; '*' $p < 0.05$; '**' $p < 0.01$; '***' $p < 0.001$. Value are means ± SE (n=3).

of proline can be used as an indicator that the plant was under stress. When drought occurs, the water content in the cells will decrease, so it will trigger cell damage. Plants will try to minimise the impact of damage by several mechanisms, one of which is the accumulation of proline. Proline plays as an osmolyte in the cell that can maintain the osmotic balance in the cell under stress conditions, particularly drought (Havrlentová *et al.* 2021).

Furthermore, the proline content of accession JP-1 then decreased when inoculated with AMF. This indicated that AMF was able to reduce stress levels in JP-1 accession so that plants reduced proline accumulation in their cells. On the other hand, accession 4183A saw a drastic increase in proline content when inoculated with AMF during drought stress. Increased proline accumulation in AMF-inoculated plants during drought stress has also been widely reported, such as in *Poncirus trifoliata* (Fan & Liu 2011) and *Macadamia tetraphylla* L. (Yooyongwech *et al.* 2013). The accumulation of proline in plants inoculated with AMF during drought stress conditions was the mechanism by which AMF improved the host plant's resistance under stress conditions. A study reported by Alotaibi *et al.* (2021) showed that the increase in proline accumulation induced by AMF treatment was due to the upregulation of the glutamate synthesis pathway and downregulation of proline biodegradation. Proline accumulation by plants was highly dependent on the plant accession and the type of stress because it has different mechanisms in response to stress (Yang *et al.* 2013; Alotaibi *et al.* 2021). In addition, the accumulation of proline in plants also brings advantages for AMF because it can be used as their nitrogen and carbon source (Tang *et al.* 2022; Wang *et al.* 2022).

Soil microbial activity

The drought treatment showed a significant impact on reducing the total soil microbial activity (Figure 3a). The FDA hydrolysis activity per gram

of dry soil weight of the soil reflected the total soil microbial activity. Fluorescein diacetate (FDA) is a prefluorophore that can be hydrolyzed by a variety of extracellular enzymes and membrane-bound enzymes such as proteases, lipases, and esterases. FDA hydrolysis enzymatic activity correlates with several parameters, such as biomass, and optical density (Dzionek *et al.* 2018). Because soil microbes play an important role in the nutrient cycle required by plants, their stability during drought stress conditions might affect plant nutrient supply.

According to our findings, water limitation in soil reduced total soil microbial activity in 4183a and JP-1 accessions without AMF inoculation by 30% and 46.8%, respectively. In contrast, the total soil microbial activity with AMF inoculation showed not significant difference compared to the well-watered condition. Although there was a decrease in total microbial activity, the decrease was not as severe as in the treatment without AMF. These findings suggest that AMF treatment has a positive effect on maintaining soil conditions under drought stress, particularly by reducing the detrimental effects of drought on the microbial community in the rhizosphere. These results show that AMF has an important role in reducing drought stress in soil ecosystems. AMF can maintain soil moisture stability under drought stress conditions, ensuring that the microorganism community surrounding the root area is not adversely affected (Bahadur *et al.* 2019; Hestrin *et al.* 2022). However, under normal conditions, the AMF inoculation did not show a significant effect on the increase in total soil microbial activity.

Soil acid phosphatase

AMF has been widely known for its ability to optimise the absorption of nutrients for the plant, especially phosphorus (P). The optimization can be done by several mechanisms, such as producing acid phosphatase. Acid phosphatase decomposes the phosphate complex to provide available phosphate that can be absorbed by the roots (Liang *et al.* 2022). In addition, the protective effect of AMF inoculation suggested also protects soil microorganisms including acid phosphatase-microorganism, which subsequently influence the supply of available phosphate for the plant. This study showed that there was a significant increase in acid phosphatase activity in the soil treated with AMF both well-watered and drought treatment (Figure 3b). Both sorghum accessions had similar effects, and their acid phosphatase activity was not significantly different. What was found in this study confirmed the role of AMF in op-

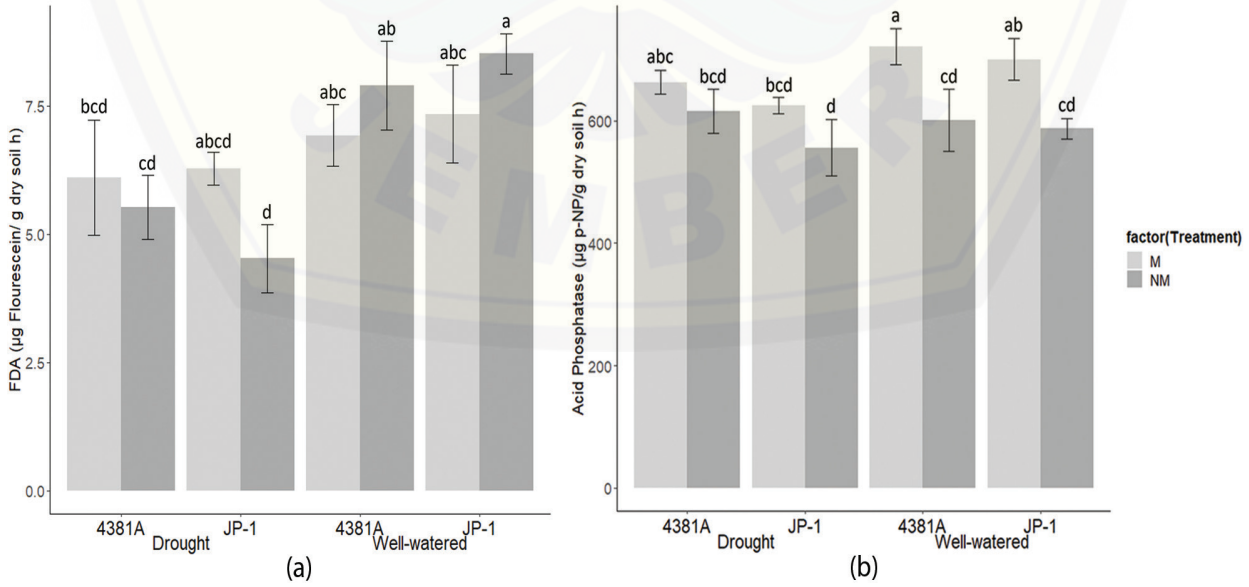

Figure 3. Total microbial activity (a) and acid phosphatase activity (b) of soil planted with sorghum 4381A and JP-1 accession under drought and mycorrhiza treatment. M (with mycorrhiza), and NM (without mycorrhiza). The different letter over the bars indicate significant difference ($p < 0.05$) among treatment with the Duncan's new Multiple Rate Test. Vertical bars show the SE.

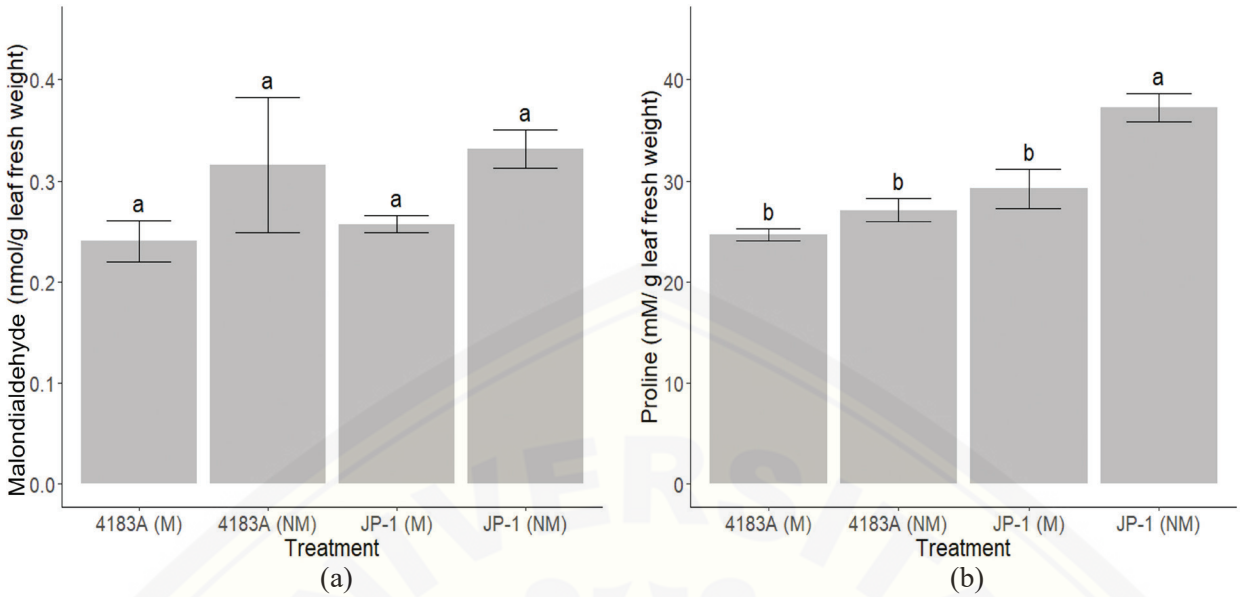

Figure 4. Malondialdehyde (a) and proline content (b) of sorghum 4183A and JP-1 accession treated with arbuscular mycorrhiza and drought stress after re-watering. M (with mycorrhiza), and NM (without mycorrhiza). The different letter over the bars indicate significant difference ($p < 0.05$) among treatment with the Duncan's new Multiple Rate Test. Vertical bars show the SE.

timising nutrients acquisition for plants, especially P through the production of acid phosphatase. Then the analysis of variance on the drought stress factor showed that acid phosphatase activity was not significantly different between drought stress and normal conditions. Even though there was a decrease in acid phosphatase under drought stress, the difference was not significant.

Recover after re-watering

Sorghum plants that had previously experienced drought stress were then re-watered. Observations after re-watering were aimed at studying the ability of the sorghum 4183A and JP-1 accessions to recover after drought stress. In addition, this was also done to see the role of mycorrhizae in accelerating plant recovery after being released from stress. The existence of post-stress recovery was observed through two parameters, namely lipid peroxidase, and proline. The two accessions had different speeds in the recovery process when viewed from the proline content, but none in the MDA content (Figure 4a and 4b). It can be implied that proline was more sensitive bioindicator than MDA to indicate plant stress. The 4183A and JP-1 accession differed significantly in their proline content after being re-watered. Accession JP-1 had a higher proline content (37.275 mM/g leaf fresh weight) than accession 4183A (27.127 mM/g leaf fresh weight), which was observed 24 hours after watering. It means that accession 4183A recover faster than JP-1 accession.

AMF inoculation has a significant impact on the ability of plants to recover after drought stress. Plants inoculated with AMF were able to recover faster than plants without AMF. Plants without AMF inoculation had higher proline content than plants inoculated with AMF on the JP-1 accession, in contrast to 4381A accession which had the same levels of proline between inoculated and without inoculation. The same similar results were reported by Boutasknit *et al.* (2020) in *Ceratonia siliqua* L., which experienced faster recovery after drought stress compared to treatment without AMF. These results further strengthen the critical role of AMF in helping plants reduce the adverse effects of stress, especially drought stress. Through its external hyphae, AMF helps provide water and nutrients so that plants can repair damaged cells more quickly and normalise the situation (Havrlentová *et al.* 2021).

CONCLUSIONS

Due to this study only evaluated a one-year experiment, only partial conclusions can be drawn from the findings. Our results demonstrate that drought treatment harmed the growth of sorghum 1483A and JP-1 accessions. Based on growth observations, the AMF inoculation treatment had not significant effect on increasing the resistance of the two sorghum accessions. Although this study was unable to demonstrate the beneficial effect of AMF on the growth performance of sorghum under drought stress, the effect of AMF treatment could be captured on the physiological response of sorghum plants, especially the proline accumulation.

Under drought conditions, AMF inoculation caused a significant difference in response to proline accumulation in both accessions. AMF inoculation in JP-1 accession reduced proline accumulation compared to non-AMF inoculated plants. In contrast to 4183A accession, there was an increase in the accumulation of proline in plants inoculated by mycorrhiza under drought conditions. The increase in proline accumulation improved plant resistance to drought stress because proline functions as an osmoprotectant. In terms of drought recovery ability based on proline content, 4183A accession recovery was faster than JP-1 accession. Furthermore, AMF inoculation was important in maintaining the stability of rhizosphere microorganisms under drought stress conditions and optimising available phosphate through acid phosphatase production, thereby increasing the possibility of phosphorus uptake by plants.

This experiment was carried out under conditions that were not fully controlled, so many factors still affect the result obtained. The symbiosis between the plants and AMF was influenced by several factors such as soil type, plant species or cultivars, and AMF species. The mechanism of proline accumulation in two sorghum accessions under mycorrhizal symbiosis needs to be investigated. The further experiment should be undertaken in metabolomic study to elucidate these mechanisms. Furthermore, studies on the effects of AMF on the stability of soil microbial communities should be expanded using metagenomic analysis. The compatibility of AMF types with symbiont plant types must be considered to increase the success of the experiment.

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