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Response of growth and drought tolerance of *Acacia seyal* Del. seedlings to arbuscular mycorrhizal fungi

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Abstract: Considering the improvement of acacia species growth in arid and semi-arid environment, a pot experiment was conducted to evaluate the role of arbuscular mycorrhizal fungi (AMF); *Funneliformis mosseae* (syn. *Glomus mosseae*), *Rhizophagus intraradices* (syn. *Glomus intraradices*) and *Claroideoglomus etunicatum* (syn. *Glomus etunicatum*) on growth and drought tolerance of *Acacia seyal* Del. seedlings under drought cycles (7, 14, 21 and 28 days). AMF-inoculated seedlings showed a clear colonisation percentage (36–68%). AMF treatment significantly improved seedlings shoot and root growth under all drought cycles compared to non-AMF control seedlings. Moreover, AMF treatment enhanced seedlings drought resistance by increasing root surface area (root length increased by 483.76% and root tips number increased by 1 463.94% under 28 days of drought cycle), there was a strong linear relation between proline accumulation, AMF and drought stress (proline content decreased in treated seedlings by 31.3% and 14.3% and increased by 97.5% and 80.4% in untreated seedlings under drought cycles of 21 and 28 days, respectively). In conclusion, the AMF inoculation improved growth and enhanced drought tolerance of *A. seyal* seedlings and can be used as a natural biostimulator for acacias seedlings establishment in arid areas.

Keywords: abiotic stress; water limitation; forest ecosystem; reforestation; stress symbiosis; biomass

Trees are usually subjected to many abiotic stresses that negatively affect their growth and development. Drought is one of the major constraints of plant growth (Rowland et al. 2015) in arid and semi-arid regions where water is a limiting factor that controls plant growth and survival (Kondoh et al. 2006). Vulnerability to drought varies depending on tree species, sizes, age classes, growth rates, and sites (Bennett et al. 2015). The seedling stage is the most critical phase of the plant growth cycle in many plant species (Arrieta and Suárez 2006). Drought can constrain the development of *Acacia* seedlings, especially at an early age. Arbuscular mycorrhizal fungi (AMF) association provides essential resources to the plant, and that, in turn, improves the ability of acacias to cope with drought. Approximately 80% of the plants on earth are associated with mycorrhizal fungi (Wang and Qui 2006). These fungi can contribute to forest ecosystems by increasing dry matter accumulation and making a network between trees through which nutrients can be absorbed (Futai et al. 2008). Mycorrhiza improves the development of host plants at the seedling stage (Balota et al. 2011). Under nutrient and water limitations, mycorrhizal symbiosis constitutes an important mechanism through which plants improve the uptake of nutrients and water (Birhane et al. 2015). Different eco-physiological studies have stated that AMF symbiosis is a key factor that assists plants to cope with water stress, and that increases drought tolerance (Rapparini and Penuelas 2014). Acacia trees are long-lived, drought-resistant, and stay

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green for a long period; therefore, they constitute an important resource and are considered to be multipurpose trees (Andersen 2012). *Acacia seyal* Del. is used as forage, where its bark is extensively used to feed livestock during the dry period of the year (Mohammed 2011). During February and March, small branches are cut, and the bark and small leaves are browsed on by animals. The leaves, seedpods, and flowers are considered important sources of fodder produced in the early dry season for sheep and goats throughout Africa (Hall 1994). *A. seyal* provides firewood, which is used widely as cooking energy in rural areas of Africa. The smoke of its wood is pleasantly fragrant; in Sudan, it is thus used by women as perfume. *A. seyal* produces gum with a brown colour but inferior in quality compared with *A. senegal* gum (Mariod et al. 2014). Using *Acacia* species for afforestation and reforestation requires an enhancement of their ability, particularly in the seedling stage, to tolerate extremely unfavourable growth conditions particularly drought by producing longer roots, which is confirmed by El-Atta et al. (2016) who reported an increase of root length and root fresh and dry weights of *A. asak* seedlings with increasing of water stress. In addition, some species develop a mutual association with AMF, which is one of the mechanisms for drought stress tolerance. Water limitation is a detrimental factor for the growth of indigenous *Acacia* species, and early mortality of seedlings is the main threat in successful establishment of plantations in semi-arid and arid areas. Therefore, this study was undertaken to determine the role of AMF on seedling growth and tolerance to drought stress and the relation between proline accumulation, AMF, and drought stress.

MATERIAL AND METHODS

Mycorrhizal fungi. The AMF in the study contained a mixture of *Funneliformis mosseae* (syn. *Glomus mosseae*), *Rhizophagus intraradices* (syn. *Glomus intraradices*), and *Claroideoglomus etunicatum* (syn. *Glomus etunicatum*). These mycorrhizal fungi were isolated from the roots of conocarpus trees (*Conocarpus erectus*).

Inoculum propagation. Inoculums of AMF were grown in small pots with Sudan grass (*Sorghum sudanense*). Original inoculums were collected from the roots of conocarpus tree (*Conocarpus erectus*) at the College of Food and Agriculture Sciences, King Saud University (KSU), and then mixed with autoclaved sandy soils. Then, seeds of Sudan grass (host plant) were sowed. Pots were watered as needed, and then the host plants developed well and became ready to use as inoculums.

Plant materials, growth conditions, and drought stress treatments. Seeds of *A. seyal* were introduced by the National Tree Seeds Centre, Soba, Forestry, and Gum Arabic Research Centre, the Republic of Sudan. The experiment was conducted in the nursery, College of Food and Agriculture Sciences, KSU. Seeds were sown in a cylindrical plastic tube (50-cm height and 16-cm diameter). The growth medium consisting of sand + loam (3:1 *v*/*v*) was used, which was characterised by 0.42% organic carbon content and particle size of 0.075–0.10 mm. The soil medium was analysed for particle size and organic carbon content, following the procedures described by Miller and Miller (1987) and Nelson and Sommers (1982), respectively.

Each tube contained two seedlings established in autoclaved sandy loam soil inoculated with AMF. Inoculation with AMF was applied to the soil before sowing the seeds. Moreover, another group of seedlings was established under the same conditions but without AMF (control). Seedlings were watered regularly as needed during the first two weeks until the second true leaf was fully unfolded. After that, drought treatments were applied by adding 250 mm per tube in the interval of 7, 14, 21, and 28 days (developed drought cycles) throughout the experiment duration (seven months).

Experiment design. The experiment has two factors, AMF (seedlings treated with AMF and non-AMF (control) seedlings and drought treatments comprising 4 levels of drought cycles (7-, 14-, 21- and 28-day watering interval). The experiment was set as a split-plot arrangement in complete randomised block design.

Status of AMF colonisation in the root. Mycorrhizal fungi were extracted from root samples in each treatment using the method described by Daniels and Skipper (1982) and modified by Utobo et al. (2011). The roots were cleaned carefully in distilled water to remove the adhering soil, then cleaned with KOH (10%) and subsequently stained with trypan blue in lactophenol, as described by Phillips and Hayman (1970). The stained root segments were tested using a light microscope at 400 × 23 magnification. Infection by mycorrhizal fungi (mycelium, vesicles, and arbuscules) in roots were calculated as colonisation (%) = (total number of AMF × segments)/total number of segments studied.

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Data collection

Measurement of the fresh and dry weight of shoots and roots. Fresh weight of the roots, stems, and leaves was obtained by separation from one another and weighing the parts by using a digital balance scale. Thereafter, the roots, stems and leaves were separately dried at 75 °C for 48 h to obtain dry weights.

Shoot growth traits. During the experiment, the following traits were measured: plant height (cm) from the cotyledon scars to the stem apex using a ruler, stem diameter (mm) at the cotyledon scar using a digital caliper (± 0.04 mm), number of leaves, leaf area by using leaf area meter (CI-202 Portable Laser Leaf Area Meter, CID, Bio-Science, Camas, USA) and number of branches.

Root growth traits. Seedlings were gently removed from the soil. Seedling roots were well cleaned from soil particles and then spread gently over a computer scanner and scanned at 600 dots per inch. The images were saved in TIFF format to be analysed later by PC software (WinRhizo Pro 2012b, Regent Instruments Inc., Quebec, Canada). The total root length (cm), root surface area and number of root tips were measured using WinRhizo Pro software (Regent Instruments Inc. 1996).

Measurement of proline. The method of Sadasivam and Manickam (1996) was used to determine the proline content in seedling leaves. Based on this method, fresh leaves (0.5 g) from each species were collected and ground in a mortar and pestle with 10 mL of 3% sulphosalicylic acid and the homogenate was centrifuged at 18 000 *g* for 1 h. The homogenate was filtered and then 2 mL of filtrate was added into test tubes to 2 mL of glacial acetic acid and 2 mL of acid ninhydrin. The test tubes were kept for 1 h at 100 °C in a water bath, followed by ice bath. The reaction mixture was vortexed with 4 mL of toluene. The toluene layer was removed and the absorbance was read at 520 nm in a spectrophotometer (Genesis 10-S, Thermo Fisher Scientific, Madison, USA). A standard curve of proline was used to determine proline concentration.

Statistical analysis. The data were subjected to analysis of variance (ANOVA) and means were compared by the Fisher's least significance difference test (*LSD*) at $P < 0.05$, while the general linear models (GLM) was used to test the interaction between the tested factors: AMF, non-AMF (control) and 4 drought cycles. All statistical analyses were performed using the SAS software package 9.2 for Windows (SAS Institute Inc. 2010).

For the correlation between drought stress, AMF treatment and proline accumulation, simple linear regression analysis was used.

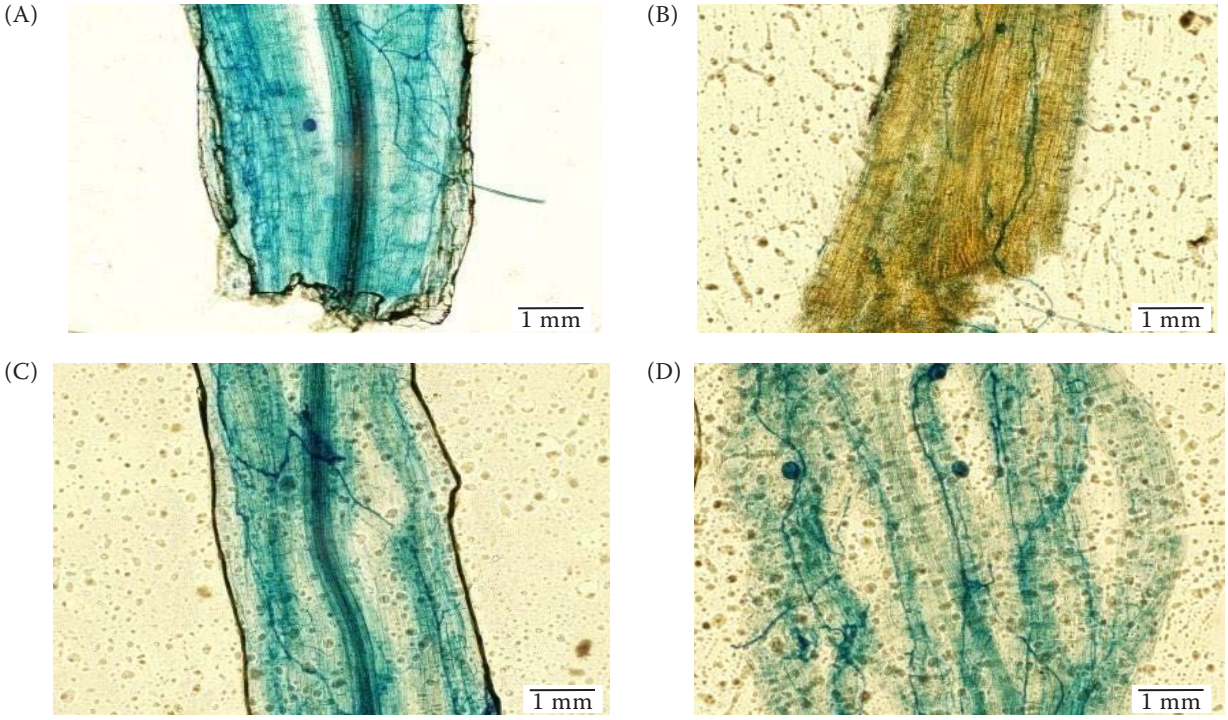

Figure 1. Colonisation of *Acacia seyal* seedlings roots by arbuscular mycorrhizal fungi under four drought cycles. (A) 7-days drought cycle; (B) 14-days drought cycle; (C) 21-days drought cycle, and (D) 28-days drought cycle

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Table 1. Arbuscular mycorrhizal fungi (AMF) colonisation rates and spore density in seedling roots of *Acacia seyal* under different drought cycles

Drought cycle (days)	AMF (%)	Spore density/10 g in the soil
7	68.2	162
14	45.10	86
21	36.06	62
28	55.33	45

RESULTS AND DISCUSSION

Colonisation of roots by AMF and spore density in the soil. Non-AMF-treated seedlings showed no root colonisation with AMF; however, AMF-inoculated seedlings showed clear colonisation by AMF (Figure 1). The AMF colonisation rates were 68.2, 45.10, 36.6 and 55.33% at 7, 14, 21 and 28 days of drought cycle, respectively (Table 1). The average of the total density of spores in seedlings soils varied depending on the drought cycles. The total numbers were 162 spores/10 g, 86 spores/10 g, 62 spores/10 g, and 45 spores/10 g at 7, 14, 21 and 28-day drought cycles, respectively (Table 1, Figure 1).

Seedlings growth performance. Statistical analysis indicated significant effects of AMF on seedling shoot growth, fresh weight ($P = 0.014$) and dry weight ($P = 0.022$) in all drought cycles, compared to control seedlings (Table 2). The seedlings with AMF showed a significant increase in their shoot fresh and dry weights in comparison to non-inoculated (control) seedlings. AMF treatment improved seedlings height by 17.8, 170.5, 50.0 and 91.3%, branch number increased by 134.5, 111.0, 144.0 and 164.25%, and leaf area by 147.0, 91.8, 104.2 and 464.6% under 7, 14, 21 and 28-day drought cycles respectively, compared to non-AMF seedlings growth. Although all seedlings treatments with AMF were superior to control seedlings in all drought cycles, maximum shoot fresh and dry weights (4.8 g and 3.4 g) were recorded in seedlings grown in 7-day drought cycle and treated by AMF (Table 2). Improvement of seedlings growth under AMF inculcation is inconsistent with Shao et al. (2018) who concluded that AMF inoculation had positive effects on plant growth performance, root morphology of *Camellia sinensis* (L.) O. Kuntze cv. Fuding Dabaicha. The results indicated a positive role of AMF inoculation to improve height, leaf number and leaf area of seedlings subjected to the four drought cycles. This improvement of plant growth can be explained by the ability of AMF to help host plants absorb more water and nutrients from the soil by developing extra radical hyphae (Cheng et al. 2012, Chen et al. 2016). In the same way, the study conducted by Li et al. (2019) revealed a decrease in biomass production of the studied plant because of the drought stress conditions, but the application of AMF enhanced the plant growth compared with non-AMF plants, regardless of drought stress. Inoculation of AMF had a highly significant effect on root growth in terms of total root length ($P = 0.0001$), total root tips ($P = 0.0001$), fresh weight

Table 2. Interaction effects of arbuscular mycorrhizal fungi (AMF) inoculation and drought cycles on shoot growth of *Acacia seyal* seedlings

Drought cycle (days) × inoculant treatment	Seedling height (cm)	Shoot biomass (g/plant)		Leaf number (leaf/plant)	Total leaf area (cm^2)
		fresh weight	dry weight		
7 × non-AMF (control)	58.0 ± 10.3^{b}	4.27 ± 0.45^{a}	1.90 ± 0.13^{b}	12.33 ± 0.88^{c}	38.16 ± 1.48^{d}
7 × AMF	68.3 ± 3.8^{a}	4.79 ± 1.04^{a}	3.43 ± 0.52^{a}	30.00 ± 1.00^{a}	94.26 ± 3.01^{a}
14 × non-AMF (control)	17.7 ± 0.3^{d}	1.36 ± 0.19^{bc}	0.25 ± 0.06^{c}	9.67 ± 0.89^{c}	28.68 ± 0.25^{d}
14 × AMF	46.0 ± 5.2^{bc}	1.40 ± 0.04^{bc}	0.69 ± 0.07^{b}	19.67 ± 0.33^{b}	55.00 ± 1.52^{c}
21 × non-AMF (control)	30.7 ± 4.1^{c}	0.28 ± 0.02^{c}	0.11 ± 0.04^{c}	9.33 ± 0.67^{c}	36.38 ± 0.25^{d}
21 × AMF	45.6 ± 2.2^{bc}	2.33 ± 0.17^{b}	1.15 ± 0.10^{b}	22.33 ± 2.19 ^{b}	74.29 ± 10.45^{b}
28 × non-AMF (control)	23.7 ± 3.2^{d}	0.28 ± 0.03^{c}	0.14 ± 0.02^{c}	4.67 ± 0.67^{d}	9.71 ± 1.29^{e}
28 × AMF	44.7 ± 2.6^{bc}	1.69 ± 0.15^{bc}	0.89 ± 0.10^{b}	12.34 ± 0.88^{c}	54.82 ± 4.66^{c}
LSD	17.86	1.42	0.81	4.01	15.36
Significant level	*	*	**	*	*

Different letters in a column indicate mean differences within treatments; *$P < 0.05$; **$P < 0.01$; *LSD* – least significant difference

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Table 3. Interaction effects of arbuscular mycorrhizal fungi (AMF) inoculation and drought cycles on root growth of *Acacia seyal* seedlings

Drought cycle (days) × inoculant treatment	Major roots length (cm)	Root biomass (g/plant)		Total root tips (root tip/plant)
		fresh weight	dry weight	
7 × non-AMF (control)	495.54 ± 59.48^{c}	9.21 ± 080^{b}	4.48 ± 0.48^{b}	1 920 ± 447.44^{d}
7 × AMF	1 331.80 ± 65.18^{a}	19.88 ± 1.86^{a}	10.97 ± 0.68^{a}	4 153.70 ± 587.59^{c}
14 × non-AMF (control)	313.97 ± 23.20^{d}	1.63 ± 0.17^{d}	0.81 ± 0.14^{d}	1 392.30 ± 159.39^{de}
14 × AMF	1 285.19 ± 39.19^{a}	7.71 ± 0.54^{b}	3.51 ± 0.13^{b}	1 805.00 ± 19.30^{d}
21 × non-AMF (control)	293.63 ± 39.93^{d}	0.69 ± 0.10^{d}	0.31 ± 0.04^{d}	709.67 ± 57.22^{e}
21 × AMF	1 136.70 ± 76.88^{b}	5.05 ± 0.57^{c}	2.52 ± 0.32^{c}	5 488.00 ± 197.00^{b}
28 × non-AMF (control)	246.16 ± 30.94^{d}	0.78 ± 0.04^{d}	0.33 ± 0.03^{d}	552.00 ± 24.43^{e}
28 × AMF	1 436.10 ± 30.14^{a}	5.40 ± 0.44^{c}	2.80 ± 0.26^{c}	8 633.00 ± 577.50^{a}
LSD	177.30	2.82	1.09	1077.40
Significant level	***	**	***	***

Different letters in a column indicate mean differences within treatments; $^{**}P < 0.01$; $^{***}P < 0.001$); *LSD* – least significant difference

($P > 0.001$) and dry weight ($P > 0.0063$). For all drought cycles, AMF-treated seedlings showed the highest average of total root length and total root tips (Table 3). The percentage increases of total root length for AMF seedlings compared with the control were 168.75, 339.59, 287.13 and 483.76% for 7, 14, 21 and 28-day drought cycles, respectively. The increase rate for total root tips was 116.30% (7-day drought cycle), 29.66% (14-day drought cycle), 674.04% (21-day drought cycle) and 1 463.94% (28-day drought cycle) in comparison with the control seedlings. AMF-treated seedlings showed the maximum average of root fresh and dry weight, whereas non-treated seedlings had lower root fresh and dry weight in all drought cycles (Table 3). The experiment resulted from the superior root system in the AMF seedlings (Figure 2). The results concluded that AMF-treated seedlings had maximum root length, biomass, and number of root tips (Figure 3) in all drought cycles, compared to non-treated seedlings (Figure 4). This finding may be attributed to improvement resulted from the increase of fungal hyphae located around the seedlings' roots, where these hyphae increase water and nutrient uptake, and this is directly reflected in an increase of shoot and root fresh and dry weights. AMF living hyphae can absorb nutrients available in the soil. Then, these nutrients arrive at the root hair mantle and move through the root towards the shoots of the plant (Plett et al. 2011). AMF-colonised seedlings showed high shoot and root dry weights. Untreated control seedlings subjected to severe drought showed the lowest shoot and root dry and fresh weights (Zarik et al. 2016). Similarly, Solís-Domínguez et al. (2011) found that

Figure 2. Photo of root system morphology under the effect of arbuscular mycorrhizal fungi

Figure 3. Photo of root system morphology under the effect of non-arbuscular mycorrhizal fungi control

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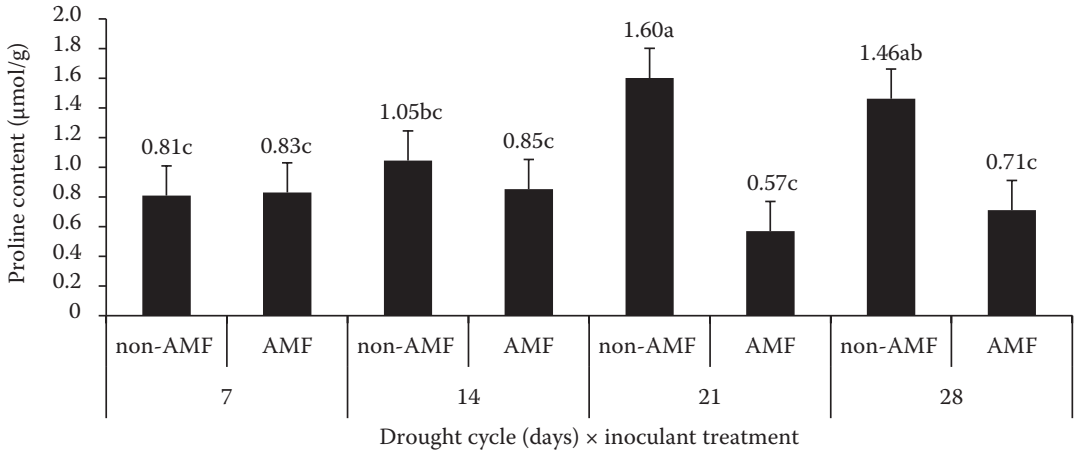

Figure 4. Interaction effects of arbuscular mycorrhizal fungi (AMF) inoculation and drought cycles on proline accumulation in seedlings leaves. Different letters indicate mean differences within treatments at $P < 0.05$

AMF-inoculated plants of *Prosopis juliflora* showed an increase in dry biomass and root length and had effective AMF colonisation compared with non-inoculated plants, where the AMF-inoculated plants had a 44% to 76% increase in dry biomass. The AMF inoculant increased the shoot and root fresh and dry weights of *A. seyal* seedlings for all drought cycles compared with non-mycorrhizal seedlings.

Variation of proline content. A significant increase ($P > 0.017$) of proline content was recorded in leaves of non-AMF treated seedlings. The results revealed that leaf proline concentration was higher in leaves of non-AMF treated seedlings. The increasing percentages of proline content of non-AMF seedlings in comparison with the AMF-treated seedlings were as follows: 29.1, 97.5 and 80.4% at drought cycles of 14, 21 and 28 days, respectively, while proline content decreased by 2.6, 31.3 and 14.3% with increasing drought cycles from 7 days to 14, 21 and 28 days (Figure 4). Linear regression ($R^2 = 0.785$) and correlation ($R = 0.886$) analysis stated a strong positive relation between proline accumulation in non-AMF plants with increasing drought days, while a negative relation was observed in AMF plants ($R = -0.636$) (Figure 5). This observation in line with Pavithra and Yapa (2018) reported a higher leaf proline concentration in soybean in non-AMF treated plants than AMF-treated plants at water-stressed conditions.

Enhancement of seedling tolerance to drought. These results agree with many studies that reported that the association of AMF with the plant reduced the severe impact of water stress and increased drought tolerance in plants (Ruiz-Lozano 2003). In this matter, Bárzana et al. (2012) concluded that the plants associated with AMF had high ability and flexibility to switch between water transport pathways according to the demand of plant shoots as a response of these plants to water shortage. In dry soils, water is retained in smaller pores where the plant roots cannot grow and uptake water, but fungal hyphae with their very small diameter (2–5 µm) eventually grow in tiny pores and uptake water; that in turn increases plant survival (Ruiz-Lozano 2003, Allen 2007, Lehto and Zwiazek 2011). The non-inoculated seedlings were more highly impacted by drought than the AMF-inoculated seedlings. In this regard, a recent study reported that drought stress affected the vegetative systems of plants, although AMF always enhanced

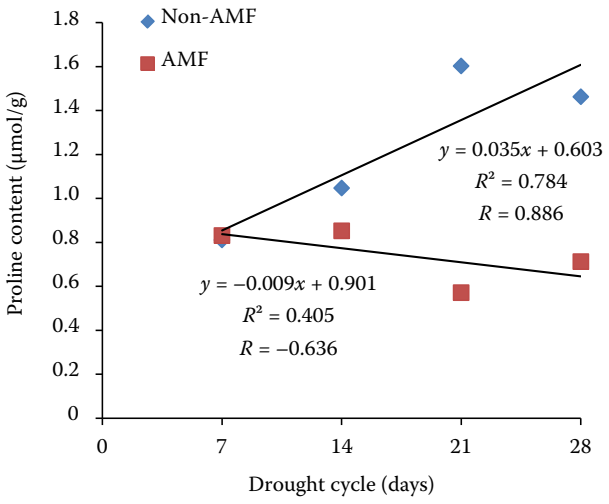

Figure 5. Linear relations between proline accumulation in arbuscular mycorrhizal fungi (AMF) inoculation, non-AMF plants, and drought cycles

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the values of plant shoots and root dry weights compared with non-mycorrhizal plants. However, many studies proved the positive influence of AMF on plant drought tolerance. For example, AMF increased tolerance to drought for the host plant, which was confirmed by greater biomass production of these plants under water deficit treatment, as reported by Begum et al. (2019). The experiment resulted from the superior root system in the AMF seedlings, which made it possible to utilise the great volume of soil, resulting in increasing water uptake and, therefore, increasing the drought tolerance of the seedlings. Kong et al. (2014) found that inoculated seedlings of *Onobrychis viciifolia* Scop. with AMF slowed down the damage from drought and improved the drought resistance, even though the dry period extended to 40 days. A review study done by Mohan et al. (2014) concluded that the main impact of AMF symbiosis is in a plant stress decrease under drought conditions. In the majority of studies, AMF had a positive role in plant productivity under drought stress conditions compared with non-AMF-treated plants. In conclusion, the AMF application enhanced the growth and drought tolerance of *A. seyal* seedlings for drought cycles of as much as 28 days. Therefore, AMF can be used as a natural biostimulator for the establishment of *A. seyal* seedlings, especially in areas subjected to severe drought. The results of our study concluded that arbuscular mycorrhizal fungi positively increased *A. seyal* seedling development and its tolerance to drought *via* increasing root system (root length, root tip, root surface area, root volume) and vegetative growth under several drought conditions. Arbuscular mycorrhizal fungi have confirmed their role in increasing seedlings drought tolerance in arid and semi-arid regions, especially during seedlings establishment stage.

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