INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
21–0921/2021/26–6–750–760
DOI: 10.17957/IJAB/15.1891
http://www.fspublishers.org

Full Length Article

Enhanced Growth and Drought Resistance in Seedlings of *Acacia tortilis* due to Inoculation of Arbuscular Mycorrhiza Fungi and *Bacillus subtilis*

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Received 15 October 2021; Accepted 23 November 2021; Published 15 December 2021

Abstract

A shade house experiment was conducted in Saudi Arabia to evaluate the impact of a mixture of three arbuscular mycorrhiza fungi (AMF) namely *Funneliformis mosseae, Rhizophagus intraradices* and *Claroideoglomus etunicatum,* a bacterium *Bacillus subtilis*, and their combinations on the growth and drought resistance potential of *Acacia torilis* seedlings under moderate and water deficit-stress. Thus, inoculants treatments (AMF, *Bacillus subtilis*, AMF+*Bacillus*, and control) and several watering intervals (1, 2, 3 and 4 weeks) were applied. Inoculation of AMF and *Bacillus* to *A. tortilis* seedlings found effective in terms of improved seedling growth. AMF and combined inoculation resulted in a larger shoot (shoot fresh and dry weights, seedling height, leaf number, leaf area) and root development (root fresh and dry weights, root length, root surface area, and root volume) as compared to the non-inoculated seedlings. Single inoculants of *B. subtilis*, showed better improvement in 1- and 2-week watering intervals compared to the control. Inoculated seedlings showed lower proline accumulation than non-inoculated seedlings, and thus improved seedling resistance to water deficit-stress. Mycorrhizal and mixed inoculation enhanced the amount of chlorophyll in the seedling's leaves. Furthermore, seedlings with AMF and co-inoculants showed better drought tolerance even at 3- and 4-week watering intervals.

Keywords: *Acacia tortilis*; AMF; *Bacillus subtilis*; Co-inoculation; Deficit-stress

Introduction

Drought and climate change are great challenges that is being faced by forest ecosystems today (Bhuyan *et al.* 2017) and prediction of climate change models suggest that drought risk will rise in tropical forests during the next years. Results of different experiments state that drought can cause a reduction in trees development and amplified trees mortality (Richard 2016). Drought can cause significant environmental effects and is likely to increase in many places in the world with climate change (Amanda *et al.* 2016), particularly in the arid regions, where water is a limiting factor that controls plant growth and survival (Kondoh *et al.* 2006).

A large number of plants make symbiotic relationship with microorganisms in the soils to overcome the negative impacts of the drought (Wang and Qui 2006; Nadeem *et al.* 2014). Rhizosphere microorganisms have a decisive role on the growth of plants established under limiting soil environments (Hashem *et al.* 2019). Microorganism association provides essential resources to the plant, and that in turn will improve the performance of plant to cope with drought (Liddycoat *et al.* 2009). Different eco-physiological studies have stated that AMF symbiosis is a key factor that assists plants to cope with water stress and increase drought tolerance (Javaid 2009; Rapparini and Penuelas 2014). Yooyongwech *et al.* (2013) showed that AMF symbiosis enhanced chlorophyll content in woody trees under water deficit conditions. The inoculation of plant growth promoting bacteria (PGPB) singly or combined with other microorganisms (such as AMF) are widespread and their application is rising in global farming practices (Díaz-Zorita and Fernández-Canigia 2009; Sharf *et al.* 2021). The combination of AMF fungi and *Bacillus subtilis* increased the fresh and dry biomass production of aromatic plants (Alam *et al.* 2011). Hashem *et al.* (2015) concluded that there is a positive effect of *B. subtilis* on the growth of inoculated plants. Also, they stated that *B. subtilis* strain caused significant increase in chlorophyll *a* and *b* content in leaves of *Bassia indica*.

Acacia tortilis is one of the widespread tree across the dry-lands of African continent (especially Sudan) and Middle East and has a great role for several groups of pastoral communities (Andersen 2012). The tree is considered an important species of the arid region in many African and Asian countries, where provides building wood, shade, forage, shelter for people and animals, richness the biodiversity, and keep soil fertile, so that it is considered a keystone species (Maarten *et al.* 2015; Verma 2016).

The effect of water deficit-stress on plant life is affected by the plant growth period, length and strength of the water

To cite this paper: Abdelmalik AM, TS Alshahrani, AA Al-Qarawi (2021). Enhanced growth and drought resistance in seedlings of *Acacia tortilis* due to inoculation of arbuscular mycorrhiza fungi and *Bacillus subtilis*. *Intl J Agric Biol* 26:750–760

deficit-stress (Sharma *et al.* 2020). However, tree seedling stage is the most sensitive phase to the water deficit-stress for many plant species (Arrieta and Suárez 2006), because of their limited root networks that mostly found at the topsoil layer which make them experience further severe water deficiency than large trees and eventually, drought can lead to its destruction completely (Mueller *et al.* 2005). Seedling's stage of *A. tortilis* life cycle is the most critical stage; therefore, in this study we investigated the impact of co-inoculation of AMF and *B. subtilis* on growth and tolerance of *A. tortilis* seedlings to water deficit-stress.

Materials and Methods

Mycorrhizal fungi

The mycorrhizal fungi in our study consisted of a combination of *Funneliformis mosseae* (Syn. *Glomus mosseae*), *Rhizophagus intraradices* (Syn. *Glomus intraradices*) and *Claroideoglomus etunicatum* (Syn. *Glomus etunicatum*). These AMF fungi were extracted and isolated from the hair roots of *Conocarpus erectus* trees. AMF species were identified following the protocol defined by Redecker *et al.* (2013), where spores were separated and observed under computerized compound microscope. The identification process depended on the morphological characteristics of the spores.

Propagation of AMF

Inoculums of the mycorrhizal fungi were developed for 4 weeks in pots containing Sudan grass (*Sorghum sudanense*). The source inoculums were taken from the fine roots of *Conocarpus erectus* trees at the faculty of Agriculture and Food Sciences, King Saud University (KSU) and then placed in autoclaved sandy soils. After that, seeds of the host plant (Sudan grass) were spread in the pots. Pots were irrigated as needed until the host plant (Sudan grass) grown and established and become ready to be applied as inoculums.

Bacterium inoculants preparation

B. subtilis, was isolated previously from the roots of *Acacia seyal* Benth trees (Alqarawi *et al.* 2014; Hashem *et al.* 2015). The inoculants of *B. subtilis* were prepared in small flasks (250 mL), each flask has 100 mL of nutrient medium and then flasks were incubated on a shaker for three days at 25°C. Afterwards, the bacillus suspensions were adjusted to 3.6 × 109 cfu mL^{-1}.

Plant culture and growth conditions

Seeds of *A. tortilis* were provided by Forestry Research Centre, Khartoum, Soba, The Republic of the Sudan. The experiment was carried out in the shade house, Faculty of Food and Agriculture Sciences, KSU, from March to June. Seeds were sown in a plastic pot (50 cm height and 16 cm diameter). Pots were filled by a sandy loamy soil (3:1 v/v), with following characteristics: 0.42% of organic carbon with 0.075–0.10 mm particle size. The procedures described by Sommers (1982) and Miller (1987) was used for particle size and organic carbon analysis.

In each pot two seedlings were established in sterilized sandy loam soil. Pots were inoculated with AMF, *Bacillus*, and co-inoculants (AMF + *Bacillus*). Inoculation of AMF was done to the soil before seeding process. For bacillus treatment, seeds were dipped in the *B. subtilis* suspensions for 10 minutes and then talc powder was added as an adhesive material. After that, seeds were removed from suspension and dried at room temperature and then planted in the soil. Further suspension was added to the soil of *Bacillus* and combination treatments to increase cell number of *Bacillus* in the seedling's rhizosphere. Another group of seedlings was established under the same environment but without inoculants (control). Seedlings were irrigated frequently until the second true leaf was shown, after that, the seedlings were exposed to four irrigation intervals where pots irrigated by 250 mL of water every 1, 2, 3 and 4- weeks watering interval (water-deficit treatments).

Design of the experiment and layout

A split-plot arrangement in randomized complete block design was used to set up the experiment. Treatment consisted of four drought intervals (1, 2, 3 and 4 weeks) and four groups of microorganism's treatments; control (no microorganisms), AMF, *B. subtilis* and co-inoculants (AMF + *Bacillus*) with four replications (pot) per treatment.

Root colonization by mycorrhizal fungi

AMF were extracted from root hairs samples of AMF and co-inoculants treatments following the method defined by Daniels and Skipper (1982) and modified by Utobo *et al.* (2011). The roots were well washed with distilled water to remove the soil particles adherent to it, then washed with KOH (10%) and afterward stained with trypan blue in lactophenol, as followed by Phillips and Hayman (1970). The stained root hairs were cut to small segments, and then checked by a bright microscope at 400 × 23 magnification. Mycorrhizal fungi infection (mycelium, vesicles and arbuscules) in root hairs was measured using the following formula:

$$\% \text{ Colonization} = \frac{\text{Total number of AMF positive segments}}{\text{Total number of segments studied}} \times 100$$

Spore extraction

AMF Spores were separated using wet sieving and decanting method (Gerdemann and Nicolson 1963). A 100 g of soil samples were air dried and 800 mL of water was added to generate soil suspension. The suspension was filtered using gradual sieves. Then, suspension was filtered through gridded Whatman filter paper No. 1. The filter paper was

tested under microscope at 2.5 × 10 magnification and then spores number was recorded.

Measurements of areal and root part traits

Roots fresh weight, stems, and leaves were separated from each other and were weighed using a digital balance scale. Then, the roots, vegetative part (stem and leaves) were individually dried at 75°C for 48 h to achieve dry weights.

The following traits were measured during experiment: height of plant (cm) from the cotyledon scars to the seedling apex using a ruler, seedling stem diameter (mm) at the cotyledon scar using a digital caliper (± 0.04 mm), leaves number, leaf area by using portable leaf area meter (Model CI-202, CID, Bio-Science, Camas, USA) and branches number.

Seedlings were smoothly taken out from the soil, and then roots were separated from the shoot. Seedling roots were washed well from the adhesive soil and then spread gently over a scanner device connected with a computer and then and scanned at 600 dots per inch. The root images were saved in TIFF format to be evaluated and measured by a computer software. The root traits (total root length (cm), root surface area, root volume and root dimeter were measured using WinRhizo Pro software (Regent Instruments Inc and Christian 1996).

Estimation of chlorophyll a and b

Fresh leaf samples were collected from each treatment. Sample of fresh leaves with 0.5 g per treatment was weighted using digital balance scale and then placed at glass tube. Each tube filled with 5 mL of diemethyl formamide and left for 24 h at room temperature. After 24 h, leaf extracts were filtered and placed in spectrophotometer cuvette and read absorption at 664 nm, for chlorophyll a and at 620 nm for Chlorophyll b (Porra *et al.* 1989).

Estimation of Proline accumulation

Sadasivam and Manickam (1996) protocol was followed to measure seedlings proline content. Where, small sample from seedlings leaves (0.5 g) were clipped in the early morning and grounded in mortar and pestle by adding 10 mL of 3% sulphosalicyclic acid and the resulted homogenate was centrifuged at 18000 g for 1 h and purified. Then, 2 mL of filtered solution were added in test containers to glacial acetic acid (2 mL) and acid ninhydrin (2 mL) and test containers were watery bathed for 1 h at 100°C, followed by ice bath. The reaction blend was vortexed with toluene (4 mL). Layer of toluene was separated, and the absorbance was measured using spectrophotometer at 520 nm (Genesis 10-S, Thermo Fisher Scientific, Madison, USA). A standard curve of proline was used to identify proline accumulation.

Statistical analysis of the data

Analysis of variance (ANOVA) was used to analyze the data and means were separated using Fisher's least significance difference test (LSD) at $P < 0.05$. Statistical analyses were done using the SPSS software package version 22.0.

Results

Infection of *A. tortilis* roots by AMF

AMF colonization rate: AMF obviously colonized the roots of *A. tortilis* at mycorrhizal and co-inoculants treatments (Fig. 1). The highest colonization rate was recorded at co-inoculant treatments at all irrigation interval. The greatest colonization percentage (93.3% for mycelium, 77.2% for vesicle, and 68.1% for arbuscular) which was recorded at co-inoculant treatment at 1-week irrigation intervals. The lowest colonization rate (50% for mycelium, 14.7% for vesicle, and 17.9% for arbuscular) at 4, 2, 3-weeks irrigation interval respectively (Table 1).

Spores density: The spores' total densities varied between irrigation intervals and between AMF and co-inoculant treatment. The highest number of spores was (104 spores 10 g^{-1}) which recorded at co-inoculant treatment, and at 1-week watering interval, however, the lowest spore's number was (30 spores 10 g^{-1}) which found at the AMF treatment at 4-weeks irrigation interval (Table 1).

Effect of inoculants on shoot fresh and dry weight of *A. tortilis* seedlings

Results of statistical analysis showed significant impact for inoculants treatments (co-inoculant, AMF, *Bacillus*) on shoot fresh and dry weight in all irrigation intervals compared to control (Table 2). The seedlings treated with co-inoculants showed the highest averages for shoot fresh and dry weights in all irrigation intervals, followed by AMF-treated seedlings, and then *B. subtilis* treated seedlings. On other hand, control seedlings showed the lowest shoot fresh and dry weights (Table 2). In comparison to the control seedlings, co-inoculant increased shoot fresh weight by (207.07, 495.44, 916.77 and 792.65%) and shoot dry weight by (177.43, 1482.97, 891.17 and 651.24%) at 1, 2, 3 and 4weeks irrigation intervals respectively. However, AMF treated seedlings increased by 140.40, 1181.20, 659.02 and 626.95%, (for shoot fresh weight) and 148.44, 980.69, 668.38 and 636.93%, (for shoot dry weight), at 1, 2, 3 and 4-weeks irrigation interval. For *B. subtilis* treated seedlings, the percentage of increments were 26.63, 736, 5.62 and 5.32% (for shoot fresh weight) and 1.49, 511.02, 2.83 and 5.68% (for shoot dry weight), at 1, 2, 3 and 4-weeks irrigation interval.

Effect of inoculants on vegetative growth of *A. tortilis* seedlings

Statistical analysis indicated that co-inoculants treatment significantly affected seedlings height, leaf number and leaf area of the seedlings, compared to the control (Table 3). In the different irrigation interval, seedlings treated with co-

Table 1: Percentages of AMF colonization and spore's number in the roots and rhizosphere of *A. tortilis* seedlings

Irrigation interval (weeks)	Inoculant treatment	AMF Colonization rate (%)			Spores' numbers
		Mycelium	Vesicle	Arbuscular	
1	AMF	76.9	27.0	47.8	76
	Co-inoculant	93.3	77.2	68.1	104
2	AMF	66.6	20.0	26.6	52
	Co-inoculant	74.1	14.7	55.2	60
3	AMF	63.3	20.0	20.0	48
	Co-inoculant	85.5	25.8	17.9	46
4	AMF	50.0	23.3	30.0	30
	Co-inoculant	67.3	14.8	27.6	36

Table 2: Effect of co-inoculant on shoot fresh and dry weights of *A. tortilis* seedlings under different irrigation intervals

Irrigation interval (weeks)	Inoculant treatment	Shoot fresh weight (g/plant) (Means ±SE)	Shoot dry weight (g/plant) (Means ±SE)
1	Control	1.7900 ± 0.1127d	0.8867 ± 0.1186d
	AMF	4.3033 ± 0.2603b	2.2033 ± 0.1090ab
	Bacillus	2.2667 ± 0.2624c	0.9000 ± 0.0473d
	Co-inoculant	5.4967 ± 0.1849ab	2.4600 ± 0.1350a
2	Control	0.2500 ± 0.0416e	0.1533 ± 0.0145e
	AMF	3.2033 ± 0.3569bc	1.6567 ± 0.1617bc
	Bacillus	2.0900 ± 0.2312c	0.9367 ± 0.1004d
	Co-inoculant	5.6567 ± 0.0982a	2.4267 ± 0.1405a
3	Control	0.2367 ± 0.0133e	0.1167 ± 0.0120e
	AMF	1.7967 ± 0.2196d	0.8967 ± 0.1213d
	Bacillus	0.2500 ± 0.0470e	0.1200 ± 0.0208e
	Co-inoculant	2.4067 ± 0.3421c	1.1567 ± 0.1172cd
4	Control	0.2233 ± 0.0353e	0.1167 ± 0.0203e
	AMF	1.6233 ± 0.1849d	0.8600 ± 0.0924d
	Bacillus	0.2633 ± 0.0524e	0.1233 ± 0.0120e
	Co-inoculant	1.9933 ± 0.3023d	0.8767 ± 0.1091d
LSD $_{0.05}$		**1.2692**	**0.5691**
Sig		***	***

Mean ± standard errors. Values with same letters differ non-significantly at ($P > 0.05$)

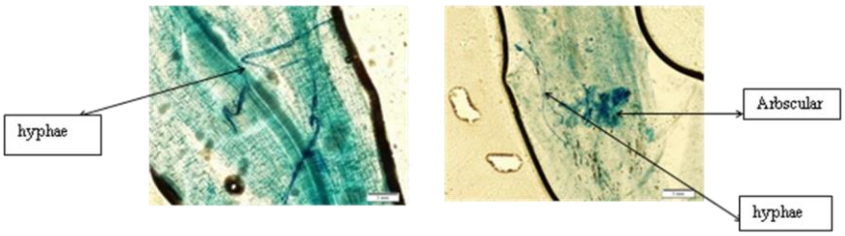

Fig. 1: Infection and colonization of *A. tortilis* roots by AMF

inoculants showed the greatest average of height which was 45.33 cm, 49.66 cm, 60.00 cm, and 50.66 cm at 1, 2, 3 and 4-weeks irrigation interval, respectively. The average of leaf number/plant was also the greatest in the all-different irrigation intervals with average of 34.333 (at 1-week irrigation intervals), 36.33 (at 2-weeks irrigation interval), 41.66 (at 3-weeks irrigation interval), and 17.33 (at 4-weeks irrigation interval). Leaf area also has the highest average in co-inoculants treatment, which was 92.667 cm^2, 131.85 cm^2, 37.663 cm^2 and 37.043 cm^2, at 1, 2, 3 and 4-week irrigation intervals, respectively (Table 3).

Effect of inoculants on root growth of *A. tortilis* seedlings

Co-inoculants, AMF and *Bacillus* treatments, significantly improved root fresh and dry weights in *A. tortilis* seedlings under the different drought conditions. Both co-inoculants and AMF treated seedlings showed the greatest average of shoot fresh and dry weights (Table 4). At 3 and 4-week irrigation intervals, drought decreased root fresh and dry weight of control and bacillus treated seedlings, however co-inoculated and AMF-seedlings were not affected by drought in terms of root fresh and dry weight (Table 4). Similarly, inoculum treatments had positive effect on the root parameters (root length, root surface area, root volume, and root diameter); where AMF and co-inoculants treatments showed the greatest root system at all irrigation intervals (Table 5 and Fig. 2–5).

Effect of inoculants on chlorophyll a and b

Inoculant's treatments significantly improved the content of chlorophyll-a in the leave of *A. tortilis* seedlings. No significant different was observed in chlorophyll b, however, seedlings inoculated with co-inoculants, AMF, and *B.*

Table 3: Effect of co-inoculant on height, leaf number and leaf area of *A. tortils* seedlings under different irrigation intervals

Irrigation Intervals (weeks)	Inoculant treatment	Height (cm) (Means ± SE)	Leaf number (Means ± SE)	Leaf area (cm^2) (Means ± SE)	Leaf temperature °C (Means ± SE)
1	Control	18.667 ± 4.6667fg	13.667 ± 0.3333fg	34.577 ± 2.1219ef	29.933 ± 0.6936a
	AMF	54.000 ± 1.7321a	46.000 ± 1.5275a	124.16 ± 3.0751a	26.367 ± 1.6707abcd
	Bacillus	29.667 ± 3.3830def	21.667 ± 1.2019de	50.253 ± 2.1040d	25.067 ± 0.4910bcde
	Co-inoc	45.333 ± 2.1858abc	34.333 ± 1.2019c	92.667 ± 3.1714b	24.033 ± 1.2811cde
2	Control	21.667 ± 1.4530efg	10.000 ± 0.5774gh	25.827 ± 1.1526fg	29.567 ± 0.3844ab
	AMF	39.333 ± 2.8480bcd	27.000 ± 0.5774d	43.887 ± 1.2714de	28.100 ± 0.8505abcd
	Bacillus	32.333 ± 1.4530cde	19.333 ± 0.6667ef	38.133 ± 0.1764def	23.833 ± 1.0525de
	Co-inoc	49.667 ± 0.3333ab	36.333 ± 0.3333bc	131.85 ± 3.8031a	21.633 ± 0.5840e
3	Control	12.000 ± 0.5774g	5.0000 ± 0.5774h	15.140 ± 0.1701gh	30.667 ± 0.4256a
	AMF	43.333 ± 0.3333ab	36.667 ± 2.0276bc	32.703 ± 2.7629ef	29.267 ± 0.2963ab
	Bacillus	14.667 ± 0.8819g	6.0000 ± 0.5774h	9.1400 ± 0.3100h	27.700 ± 0.9539abcd
	Co-inoc	54.667 ± 4.6667a	41.667 ± 2.7285ab	37.663 ± 1.6709def	26.967 ± 0.2186abcd
4	Control	13.000 ± 0.5774g	7.6667 ± 0.3333gh	11.533 ± 0.7860h	28.533 ± 1.3836abcd
	AMF	39.000 ± 3.0551bcd	18.333 ± 0.8819ef	68.973 ± 4.6321c	28.733 ± 0.2728abc
	Bacillus	14.333 ± 0.6667 g	10.000 ± 0.5774gh	6.4400 ± 0.4903h	28.733 ± 0.5696abc
	Co-inoc	50.667 ± 1.8559 ab	17.333 ± 0.8819ef	37.043 ± 3.1542ef	26.267 ± 0.2963abcd
$LSD_{0.05}$		**13.262**	**6.5313**	**12.986**	**4.8349**
Sig		*******	*******	*******	*****

Mean ± standard errors. Values with same letters differ non-significantly at ($P > 0.05$)

Table 4: Effect of co-inoculant on root length, root surface area, and root tips number of *A. tortils* seedlings under different irrigation intervals

Irrigation interval (weeks)	Inoculant treatment	Root fresh weight (g) (Means ± SE)	Root dry weight (g) (Means ± SE)
1	Control	2.7167 ± 0.2554bc	1.6933 ± 0.0736bcd
	AMF	4.3667 ± 0.4914ab	2.2500 ± 0.2616ab
	Bacillus	3.7567 ± 0.3779abc	1.4633 ± 0.1027bcd
	Co-inoculation	5.1167 ± 0.3805a	2.2767 ± 0.1172ab
2	Control	0.1767 ± 0.0536d	0.1133 ± 0.0120f
	AMF	3.6100 ± 0.3444abc	1.7633 ± 0.1387bcd
	Bacillus	2.3167 ± 0.2293c	1.0867 ± 0.0176cde
	Co-inoculation	4.1300 ± 0.3208abc	1.4833 ± 0.2350bcd
3	Control	0.1533 ± 0.0120d	0.0800 ± 0.0115f
	AMF	5.3167 ± 0.4667a	2.7833 ± 0.2530a
	Bacillus	0.2800 ± 0.0001d	0.1700 ± 0.0115ef
	Co-inoculation	3.1533 ± 0.5128bc	1.7767 ± 0.2826bcd
4	Control	0.2267 ± 0.0338d	0.1200 ± 0.0321f
	AMF	2.3733 ± 0.3457c	1.0033 ± 0.1586def
	Bacillus	0.3233 ± 0.0273d	0.2067 ± 0.0145ef
	Co-inoculation	3.9267 ± 0.6274abc	1.9867 ± 0.2765abc
$LSD_{0.05}$		1.8283	0.9614
Sig		*******	*******

Mean ± standard errors. Values with same letters differ non-significantly at ($P > 0.05$)

subtilis showed better content of chlorophyll-b than control seedlings (Table 6).

Effect of inoculants on proline accumulation

The proline accumulation in the leaves of all seedlings improved by increasing the irrigation intervals (Table 7). However, seedlings inoculated with co-inoculants, AMF, and bacillus showed lower content of proline than in control seedlings regardless of the irrigation intervals. This variation was more obvious under varied irrigation intervals.

Discussion

Responses of the plant to the soil's microbes are the consequence of interaction relationship between plants and microbes found in the soil. It obviously appears from our results that *A. tortilis* seedlings much improved and resisted drought when AMF and their combination with *B. subtilis* were applied.

Inoculation with AMF, *Bacillus* and co-inoculants improved plant height, leaf number, leaf area, and shoot fresh and dry weights. This improvement could be due to the increased presence of carbohydrates in the shoot part, enhancement of nutrients uptake (Verma *et al.* 2018) and increase of root system in treated seedlings. Our results in the same line with many studies that found, AMF improves plant growth by increasing nutrients amount in the soil and its absorption to the plant (Naheeda *et al.* 2020; Ya-Dong *et al.* 2021).

In general, seedlings inoculated with AMF, bacillus, and co-inoculants showed higher vegetative growth in comparison to the control seedlings. This finding is similar

Table 5: Effect of co-inoculant on root length, root surface area, root volume and root diameter of *A. tortils* seedlings under different irrigation intervals

Irrigation interval (Weeks)	Inoculant treatment	Root length (cm) (Means ± SE)	Root surface area(cm^2) (Means ± SE)	Root volume (Means ± SE)	Root diameter (Means ± SE)
1	Control	414.47 ± 24.056b	90.870 ± 6.9272bc	1.8367 ± 0.1135ce	0.7233 ± 0.0318bc
	AMF	697.14 ± 4.9770a	232.31 ± 34.631a	3.5067 ± 0.2373ab	1.1600 ± 0.0600a
	Bacillus	554.28 ± 66.016ab	168.39 ± 13.964ab	3.1333 ± 0.1648ab	0.7867 ± 0.0273bc
	Co-inoculation	618.13 ± 76.139ab	160.26 ± 8.8719ab	3.6233 ± 0.5732ab	0.7833 ± 0.0521bc
2	Control	152.39 ± 8.5729c	34.137 ± 4.4081bc	0.6933 ± 0.0751e	0.7400 ± 0.0404bc
	AMF	424.22 ± 31.882b	197.06 ± 60.199ab	3.2267 ± 0.3548ab	0.7000 ± 0.0404bc
	Bacillus	523.64 ± 83.834ab	132.62 ± 18.121abc	1.7533 ± 0.0120ce	0.7500 ± 0.0643bc
	Co-inoculation	348.25 ± 15.681bc	140.03 ± 9.3029ab	4.5767 ± 0.4421a	1.1233 ± 0.0426a
3	Control	96.890 ± 6.5094c	21.010 ± 1.1252c	0.4167 ± 0.041e	0.6400 ± 0.0764 bc
	AMF	373.33 ± 25.584bc	135.50 ± 3.0394abc	4.3667 ± 0.1913a	1.1633 ± 0.0940a
	Bacillus	135.13 ± 12.871c	39.097 ± 3.3340bc	0.9033 ± 0.0736ce	0.9233 ± 0.0233ab
	Co-inoculation	418.36 ± 33.081b	160.22 ± 18.291ab	3.4700 ± 0.7410ab	1.1433 ± 0.0581a
4	Control	152.38 ± 6.5865c	55.617 ± 9.9760bc	1.0233 ± 0.0433ce	0.5867 ± 0.0176c
	AMF	597.11 ± 43.652ab	146.42 ± 14.684ab	2.4000 ± 0.0651bc	0.6267 ± 0.0463c
	Bacillus	152.94 ± 22.496c	31.597 ± 3.8153bc	0.5200 ± 0.0557e	0.6567 ± 0.0296bc
	Co-inoculation	567.35 ± 57.092ab	174.36 ± 7.8967ab	4.0100 ± 0.2201a	0.7700 ± 0.0100bc
$LSD_{0.05}$		**245.47**	**124.84**	**1.5887**	**0.2734**
Sig		***	**	***	***

Mean ± standard errors. Values with same letters differ non-significantly at ($P > 0.05$)

Table 6: Effect of co-inoculant on chlorophyll-a, chlorophyll-b, and proline accumulation of *Acacia tortilis* seedlings under different irrigation intervals

Irrigation interval (Weeks)	Inoculant treatment	Chlorophyll-a (Means ± SE)	Chlorophyll-b (Means ± SE)
1	Control	1.9857 ± 0.0110 bc	1.3440 ± 0.0832ab
	AMF	2.9003 ± 0.0454a	1.7140 ± 0.0188ab
	Bacillus	2.9523 ± 0.0288 a	1.6060 ± 0.2441ab
	Co-inoculation	2.9477 ± 0.0268 a	1.7217 ± 0.2319ab
2	Control	2.2950 ± 0.0277b	1.2723 ± 0.0598b
	AMF	2.3320 ± 0.0519 b	1.4120 ± 0.1868ab
	Bacillus	2.7450 ± 0.0279 ab	1.1680 ± 0.2480b
	Co-inoculation	2.6863 ± 0.2295 ab	1.5350 ± 0.0594ab
3	Control	1.5680 ± 0.0150c	1.2320 ± 0.0576b
	AMF	1.9073 ± 0.0135bc	1.6567 ± 0.0640ab
	Bacillus	1.8723 ± 0.0306 bc	1.1723 ± 0.0351b
	Co-inoculation	2.4057 ± 0.0872ab	1.5570 ± 0.0188ab
4	Control	1.4153 ± 0.2040 c	1.2600 ± 0.1123b
	AMF	1.5793 ± 0.0946 c	1.4537 ± 0.2041ab
	Bacillus	1.5987 ± 0.0345 c	1.2657 ± 0.0198b
	Co-inoculation	2.4500 ± 0.2306 ab	2.0600 ± 0.0259a
$LSD_{0.05}$		**0.5655**	**0.7779**
Sig		***	**NS**

Mean ± standard errors. Values with same letters differ non-significantly at ($P > 0.05$): NS: not significant

Table 7: Effect of co-inoculant on proline accumulation of *Acacia tortilis* seedlings under different irrigation intervals

Irrigation interval (Weeks)	Inoculant treatment	Proline content (Means ± SE)
1	Control	1.1013 ± 0.1366abc
	AMF	0.7383 ± 0.0184bc
	Bacillus	0.5027 ± 0.0256c
	Co-inoculation	0.5490 ± 0.0142c
2	Control	1.0380 ± 0.1217abc
	AMF	0.8843 ± 0.0616bc
	Bacillus	0.7523 ± 0.1289bc
	Co-inoculation	0.4223 ± 0.0225c
3	Control	1.3727 ± 0.0353ab
	AMF	0.8570 ± 0.0749bc
	Bacillus	1.2637 ± 0.3669abc
	Co-inoculation	0.9873 ± 0.0552abc
4	Control	1.5873 ± 0.2492a
	AMF	0.8850 ± 0.0111bc
	Bacillus	0.8840 ± 0.0341bc
	Co-inoculation	0.7677 ± 0.0528bc
$LSD_{0.05}$		**0.7255**
Sig		*

Mean ± standard errors. Values with same letters differ non-significantly at ($P > 0.05$): NS: not significant

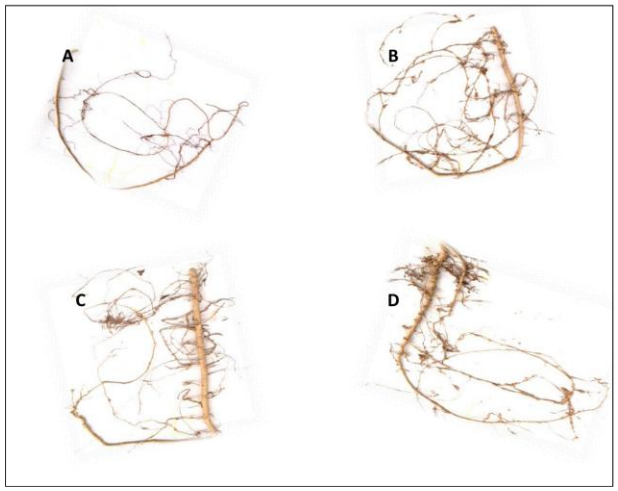

Fig. 2: Effect of inoculants on root architecture of *Acacia tortilis* seedlings at 1-week irrigation intervals
A: Control treatment; B: *Bacillus subtilis* treatment; C: AMF treatment; D: co-inoculation treatment

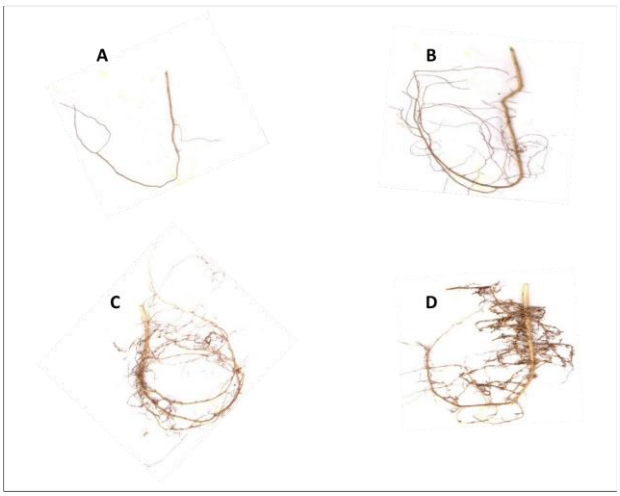

Fig. 4: Effect of inoculants on root architecture of *Acacia tortilis* seedlings at 3-weeks irrigation intervals
A: Control treatment; B: *Bacillus subtilis* treatment; C: AMF treatment; D: co-inoculation treatment

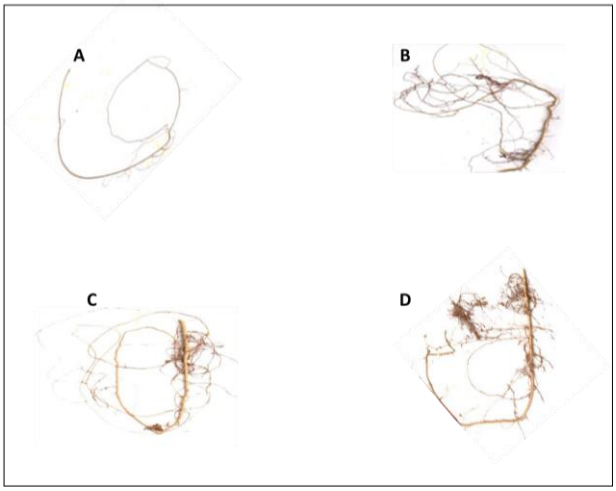

Fig. 3: Effect of inoculants on root architecture of *Acacia tortilis* seedlings at 2-weeks irrigation intervals
A: Control treatment; B: *Bacillus subtilis* treatment; C: AMF treatment; D: co-inoculation treatment

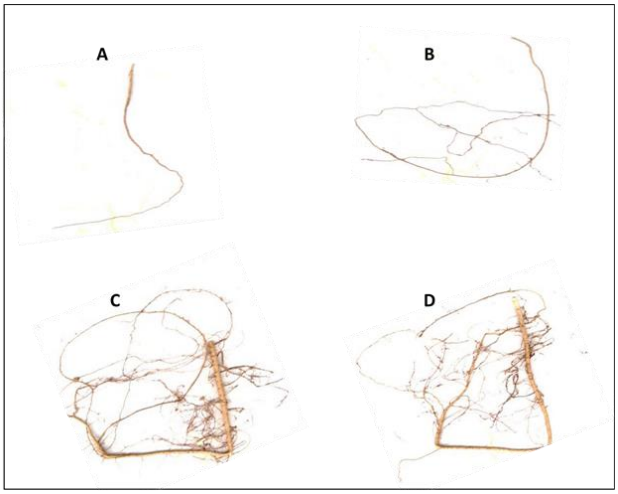

Fig. 5: Effect of inoculants on root architecture of *Acacia tortilis* seedlings at 4-weeks irrigation intervals
A: Control treatment; B: *Bacillus subtilis* treatment; C: AMF treatment; D: co-inoculation treatment

with our previous results in other acacia species (Abdelmalik *et al.* 2020). Addition of *B. subtilis* strain (pf4) (Anand *et al.* 2010), to plant resulted in a significant vigor index, shoot height and root length (Gowtham *et al.* 2020). Bacillus species can form endospores that are extremely resilient to harsh environmental conditions and can also produce metabolites that increase growth and vigor of plant. Also, many exopolysaccharides can be produced by bacillus which help water uptake by plant roots (Hashem *et al.* 2019). Zaidi *et al.* (2009) stated that *B. subtilis* acts directly involved in the dissolution of phosphorous and plays a synergistic role with the AMF. AMF alone increased the growth rate by 49.4%; however, when combined with *B. subtilis*, growth rate increased by 59.5% (Alam *et al.* 2011). The combined application of AMF and *B. subtilis* has a synergistic role and leads to promotion of plant growth (Hashem *et al.* 2019).

Results indicated to varied positive effect for AMF, co-inoculant and *B. subtilis*, in the different irrigation intervals and even at severe water-deficit conditions the inoculants showed positive effect on seedlings growth. Microorganisms have tremendous capabilities to reduce environmental stress and their interactions with plants, so that they offer both a local and systemic defense under various environmental stresses (Chialva and Bonfante 2018; Khoshru *et al.* 2020). Mycorrhizal fungi regulate and improve plant growth when exposed to harsh environmental conditions, where they

significantly enhanced the growth (Yadav *et al.* 2018; Xiao *et al.* 2019; Abdelmalik *et al.* 2020; Yasser *et al.* 2021) and biomass of tobacco plants under normal conditions and mitigated the decline caused by water deficit-stress (Begum *et al.* 2020). In this regard, that inoculation of *Onobrychisvicii folia*s seedlings with AMF reduces the damage resulted from water deficit-stress and improved the water deficit-stress resistance up to forty days (Kong *et al.* 2014). Interactions between AMF and plant growth promoting rhizobacteria (PGPR) in the plant rhizosphere has a synergistic role which improve growth and quality of the plant (Khalid *et al.* 2017). PGPR can greatly enhance plant growth and show beneficial interaction between plant and microbes. *B. subtilis* enhances stress tolerance in plants by stimulating the expression of stress response genes, hormones and metabolites related with drought stress (Lee *et al.* 2014; Hashem *et al.* 2019).

AMF, co-inoculants, and *B. subtilis* treated seedlings showed greater root length, root surface area, root diameter, and root volume than non-treated seedlings at all irrigation intervals, which would enable inoculated plants to explore great volume of rhizosphere and hence more nutrients availability to the seedlings. It clear that, the effects of AMF, bacillus, and co-inoculants on root morphology might be an important reason for enhanced nutrients uptake for the treated seedlings. Our results agree with a number of studies which reported that mycorrhizal fungi alters root morphology and increases plant tolerance to the severe environmental conditions (Khanna *et al.* 2019). Largest root morphology result in better nutrient uptake (Ya-Dong *et al.* 2021) and enhanced water relationships in the plants (Pallavi and Sharma 2021). The mixed inoculants (*B. subtilis* + AMF) improved root biomass and plant survival rate in comparison to those caused by sole inoculations and non-inoculated plants (Ibrahim *et al*. 2019). The combined use of PGPR may have a synergic effect on decreasing contrasting stress factors. The application of PGPR with useful fungi in farming is a suitable use in some stressful conditions (Deepmala *et al.* 2019; Hassan and Bernard 2020). Occurrence of PGPR is highly linked with plant rhizosphere and positive direct and indirect impacts on plant development; like a decline in environmental stress is reported. *Bacillus* species can make endospores that are tremendously resilient to severe environmental conditions and also can produce metabolites that motivate plant development and fitness (Hashem *et al.* 2019).

Inoculum's treatments were found to have significant contribution in the improvement of chlorophyll-a and b in *A. tortilis* seedlings under different irrigation intervals. The improvement of chlorophyll content in AMF and co-inoculated seedlings can be justified by the availability of nutrients and metabolism in the plant. However, AMF improve nutritional status of plants by absorbing and translocating mineral nutrients beyond rhizospheric zone (Rouphael *et al.* 2015). Microorganisms were found to improve the content of plant chlorophyll under normal and water deficit-stress conditions. Co-inoculated plants have higher chlorophyll content compared to single inoculants of AMF or bacteria (Mehdi *et al.* 2018). Also, the findings reported by Kim *et al.* (2010) and Berta *et al.* (2014) proved that co-inoculation (AMF and bacteria) increased the levels of chlorophyll content in plants leaves. The positive effect of AMF was extensively reported by scientists. Various research results explained that the association of microorganisms to the plant can change its physiological growth under many stress conditions (Xiaoying *et al.* 2014). Mycorrhizal inoculation highly improved the content of chlorophyll-a, b, and total chlorophyll (Naheeda *et al.* 2020) in the *Erythrina variegata* leaves. Yooyongwech *et al.* (2013) and Fang *et al.* (2018), showed that AMF symbiosis under water deficit conditions enhances chlorophyll fluorescence in woody tree nut species. Sonal *et al.* (2018) found that total chlorophyll content was more in AMF maize seedlings when compared to non-treated plants where AMF- maize seedlings had double of chlorophyll content as compared to control maize seedlings. AMF colonization could promote the synthesis of chlorophyll and carotenoid thereby enhancing the photosynthesis and biomass accumulation in plants through increasing the root absorption area and root activity, support the absorption and transport of water and other nutrients or mineral elements such as P, K, Mg, and Mn (Baslam *et al.* 2013). Also, in mycorrhizal plants the increase of chlorophyll contents can be associated with increased P and Mg uptake (Zhu *et al.* 2014).

Proline concentration increased greatly in the leaves of water deficit-stressed and non-inoculated seedlings compared to the well-watered and inoculated seedlings. The lower proline content in AMF and co-inoculation seedlings is an indicator of good drought tolerance of plant (Ruiz-Lozano 2003), therefore, the low content of proline in the inoculated seedlings in this study was linked with good seedlings drought tolerance that is induced by AMF and co-inoculant treatments. This finding is in agreement with Yooyongwech *et al.* (2013), where they stated that, AMF and co-inoculation in different plant species reduces proline content when water level is limited. The work done by Doubkova *et al.* (2013), explained that, when the concentration of proline increases in response to drought stress, a lower proline accumulation has been observed in AMF- plants. In the same way, Wu and Xia (2006) reported that, the content of proline was reduced significantly in orange seedlings inoculated by AMF under water stress conditions. The study conducted by Hazzoumi *et al.* (2015) reported that leaf proline accumulation was greater in non-AMF plants than AMF-plants under water deficit-stress. The synergistic effects between the bacillus and AMF were reported to increase nutritional status of inoculated plants and thus stimulate the plants resistance to the water deficit-stress (Ibrahim *et al.* 2019). The changes made by PGPR on root elasticity are one of the essential steps to enhanced tolerance to water shortage (Dimkpa *et al.* 2009). PGPR enhances the plant cell membranes by stimulating the

antioxidant system and increasing drought tolerance of many plant species (Gusain *et al.* 2015). Furthermore, AMF plants mostly had better leaf water status and high root volume, thus, plants suffer less water deficit and consequently had lower proline accumulation. The lower proline accumulation in the AMF plants may derive from the integration of the inhibition of glutamate synthetic pathway of proline with an enhancement of proline degradation (Zou *et al.* 2013).

Conclusion

Several soil microorganisms positively affect the growth and drought tolerance of *A. tortilis* seedlings, especially in the early stages of their growth, even when exposed to severe water deficit-stresses. The addition of inoculants in general and co-inoculants and AMF in particular resulted in increases in vegetative growth rates (fresh and dry weight, height, number of leaves, leaf area) and root traits (fresh and dry weight, root length, root surface area, root volume). In addition, the inoculants led to an improvement in some physiological characteristics such as chlorophyll-a and b. Also, inoculums reduced proline concentration levels in water deficit-exposed seedlings, and therefore improved seedlings drought tolerance and reduced damage resulting from water deficit due to more water content in inoculated plants. It can be said that, changes in proline levels are a response to tolerance or avoidance for water deficit. Based on the attained results, the inoculants can be used for *A. tortilis* seedlings, especially during the establishment stage. This will assist in the success of afforestation programs in the dry areas.

Acknowledgments

The authors thank the Deanship of Scientific Research, King Saud University, Saudi Arabia for supporting this work.

Author Contributions

AM, T S, and AA planed the experiment. AM conducted the experiment, data measurements and analysis, and wrote the first draft. TS analyzed root data and supervised all work. AA supervised the work.

Conflicts of Interest

All authors declare no conflicts of interest

Data Availability

Data presented in this study will be available on a fair request to the corresponding author

Ethics Approval

Not applicable in this paper

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