# Effect of arbuscular mycorrhizal (AM) fungi on growth and physiological responses of water stressed *Azadirachta indica* A. Juss seedlings

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ABSTRACT

A neem nursery experiment was conducted to investigate the effect of AM on germination, growth, physiological traits and to evaluate the effect of AM fungi in tolerating water stress. The experiment comprised of two treatments; AM-inoculated and non-inoculated three months old neem seedlings, of which the treatments were subjected to water stress condition. A slightly higher germination rate of 7.77 % was observed in the *Glomus mosseae* inoculated treatment relative to the non-inoculated treatment. Moreover, water stress exhibited significant reductions in various morphological parameters and relative water content, with more effects pronounced in non-inoculated treatment. The AM–inoculated treatment showed prompt recovery during water resumption period which was reflected in the reduction of relative stress injury. Proline and soluble carbohydrate contents were significantly more in leaves of non-inoculated treatment as compared to AM-inoculated treatment during water stress. Although, water stress caused a reduction in mycorrhizal abundance, growth, and soil moisture content, AM considerably maintained plant growth performance hence retained better soil moisture content.



## 1 Introduction

In the changing scenario of the climate change regime, the availability of water resource has been decreasing over the years in many regions of the world (Anjum *et al.*, 2011). Water limitation may prove to be critical constraints to primary productivity under future scenarios of more arid climate due to global environmental change (Fischer *et al.*, 2001). Amongst several environment stress factors, drought constitutes one of the most important factors limiting plant growth, development, and quality forest seedling production (Sani and Boureima, 2014). With increasing climate change, the neem tree is considered suitable for its evergreen, drought resistance with no specific site quality, though in severe drought situations may shed most or nearly all its leaves (Nandal and Bahadur, 1997). The tree has been centered global pivot for its multiple uses more particularly the biologically active ingredient in its seed kernels (Puri and Swamy, 2001), its significance and suitability in rehabilitation, restoration of wastelands, desertification control, reforestation, and integration in various agroforestry systems programs in arid and semi-arid lands (Pagano and Cabello, 2011; Banerjee *et al.*, 2013).

Growth performance of tree species responds sensitively to drought stress in various ways (Fischer and Polle, 2010). Many plants physiological processes are disturbed and toxic elements such as reactive oxygen species

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(ROS) produced during drought stress period create oxidative damage to the cellular organization (Anjum *et al.*, 2011; Jangid and Dwivedi, 2016). The seeds of *Azadirachta indica* are known for their erratic viability and long storage period results in poor seed germination due to seed ageing as well as seed deterioration (Pandey and Pati, 2017). Therefore, seed selection and knowledge about their germination behaviour are also of utmost importance for quality seedling production.

Studies on biological properties of soils are now of ultimate interest. Mycorrhizal fungi inoculation is amongst the adaptive strategies that have proven to mitigate climate change and may improve seed germination and nursery seedling growth under water stress conditions thus resulting in improving reforestation programs (Allen, 2007; Pandey and Pati, 2017). Mycorrhizae are symbiotic association established between soil fungi and most vascular plants, where both partners exchange nutrients and energy (Brundnett, 2002; Choi *et al.*, 2018).

Therefore, mycorrhizal interaction for reforestation and use of mycorrhizal technology had been found fundamental in optimizing plant fitness, plant nutrition, environment quality and improvement of forest products (Azcon-Aguilar *et al.*, 2009). Rapid production of high-quality forest seedlings in a nursery is mandatory to meet wood and forest product demand (Banerjee *et al.*, 2013). Amendment of soils with endo or ectomycorrhizal fungus has been widely studied having a positive impact on germination, mineral uptake, growth and survival in neem and other forest species (Beniwal *et al.*, 2010, 2011; Bhattacharya *et al.*, 2012; Banerjee *et al.*, 2013; Gutowski, 2015; Rashmi and Dixit, 2015; Pandey and Pati, 2017; Ouledali *et al.*, 2018; Bahadur *et al.*, 2019; Campo *et al.*, 2020).

Therefore this study aimed to investigate the ameliorative effects of arbuscular mycorrhizal (AM) fungi under drought tolerance on *Azadirachta indica* nursery seedlings to find the solution to the afforestation problem in drought-prone conditions keeping in view the following objectives (i) To study seed germination behaviour in relation to AM fungi inoculation, (ii) Evaluate drought-induced changes in growth and morpho-physiological traits in neem seedlings, and (iii) Observe the effects of AM fungi in overcoming water stress.

# 2 Materials and methods

### Study site and climatic condition

In the present study, neem seedlings were maintained in the green-net chamber of Department of Forestry, CCS Haryana Agricultural University, Hisar, India. The study was purposely conducted in Hisar as it has characteristics of semi-arid climate with hot, dry summer and extremely cold winter, naturally available in the habitat of this specie. Hisar average minimum and maximum temperature for July–December, was about $18.8°C$ and $32.1°C$, respectively with an average rainfall of about 162.6 mm occurring mostly during the month of July–August.

### Mycorrhizal inoculation

Mycorrhizal inoculum *Glomus mosseae* was used for AM fungi multiplication through the germination of *Pennisetum glaucum* (bajra) plants. Sandy soil obtained from Balsamand Research Farm was autoclaved at $121°C$ for 2 h and filled in 6 plastic pots. Glomus mosseae inoculum was mixed 1 cm upper the pot soil and kept for 40 days under nursery condition. Soil and infected rootlets obtained from the root horizon of *Pennisetum glaucum* were used as inocula for *Azadirachta indica* seeds.

### Growth medium

The experiment was performed using a proportion of 2:1 sand and farmyard manure (FYM) mixture for pot filling. Sandy soil used in this study was collected from Balsamand Research Farm, CCS HAU, Hisar while FYM was obtained from the nursery area of the Department of Forestry. Soil mixture was autoclaved at $120°C$ for

30 min after sieving. In non –AM treatments, *Azadirachta indica* seeds were sown in pots containing 8 kg of untreated soil; whilst in AM –inoculated treatments, seeds were sown in pots containing 7.9 kg of untreated soil with a portion of 100 g of G*lomus mosseae* inoculums added in top 7-10 cm pot soil.

### Experimental design and growth condition

Seeds of neem were collected from a plus tree growing in the campus area of CCS HAU, Hisar. Seeds of uniform size were selected, hand processed (de-pulped) and dried for 2 days. 5 seeds were sown and thinned to 3 seedlings per pot after germination. Each pot received normal watering about 106 days of growth (water withdrawal took place at 106 days). The experiment was performed using two treatments: AM fungi (*Glomus mosseae*) inoculation and non-inoculation, each made up of three soil water regimes (normal watering, drought and re-watering). For each water regime in AM –inoculated and non-inoculation treatments, 12 pots each for normal watering (NW), drought (D), and re-watering (RW) were maintained. Hence, a total of 72 pots were used in this study, consisting of 36 pots, each in AM –inoculated and control treatments. During water withholding phase, normal watering (NW) plants were supplied with tap water on alternate days in the morning and rest of the seedlings were maintained without watering to observe the effects of water stress on neem seedlings. Seedlings were subjected to drought for 63 days by withholding water till leaves drooping which was indicated by symptoms such as leaf rolling, defoliation, loss of leaf turgor and wilting of lower leaves. At 63 days of drought stage (drought harvest) relative water content was taken to determine the amount of water retained by the plant, similarly after 7 days of re-watering (RW) relative water content was observed. During drought harvest, there was a decline in relative water content, therefore, neem seedlings re-watering (RW) treatments were again exposed to normal watering for 7 days, and the plants [drought (D)] which were maintained to determine the aftereffect of drought were harvested. Morpho-physiological parameters were recorded after each stage of water stress. Experiment pots were arranged in a complete randomized design in a greenhouse.

### Analysis of endo-mycorrhizal colonization

A fraction of neem roots per treatment were carefully washed, cut into small segments ($0.5-1.0\,cm$) and boiled for 50 min in a water bath at $95^{\circ}C$ in 10% KOH (w/v) solution until soft and tanning material has been removed (Phillips and Hayman, 1970). Afterwards, roots were washed thrice with distilled water, dipped in an alkaline solution of 10% $H_2O_2$ at room temperature for 5-15 min until roots were bleached and then rinsed thoroughly in distilled water and acidified with 5% HCL for about 1-3 min. Finally, roots were stained in 0.05% trypan blue in lactophenol. Quantification of AM infection percentage under a microscope was counted by the following formula: Root infection (%) = (No. of root bits showing colonisation/ Total number of root bits observed) $\times$ 100.

## Morphological Parameters

### Germination parameters

First cotyledon leaf emergence (days after sowing, DAS) was recorded by counting the number of days from sowing to emerging of first seed leave(s) under the two treatments (AM –inoculated and non-inoculated). A total number of seeds germinated under each variable was recorded and expressed in term of per cent. Germinative energy (%) and Energy period (days) were determined based on the standard method prescribed by Seward (1980). Germination value was also estimated by the method prescribed by Czabator (1962) as; Germination value = (Final daily germination speed %) $\times$ (Peak value daily germination speed %).

### Growth parameters

At each harvest [normal watering (NW), drought (D) and at harvest after seven days of water resumption (RW)] plants were harvested and the following parameters were measured: collar diameter, shoot length, root length,

leaf area, fresh and dry weight (FW, DW). Collar diameter was recorded at the collar region (or alternatively at seedlings basal area) using digital vernier calliper and fully developed fresh leaves were used to measure leaf thickness using a digital vernier calliper. Whole plant fresh and dry weight was determined at each harvest and plant materials were oven-dried at $60°C$ for 1 week for the determination of dry mass. To determine leaf area, five leaves per seedling of five samples per treatment were scanned using area meter to determine per unit mass leaf area. Soil moisture content was also determined at D harvesting stage by the formula; soil moisture content (%) = (original weight – oven dry weight)/ (original weight) $\times$ 100.

## Physiological Parameters

### Total soluble carbohydrate (TSC) and proline estimation

Total soluble sugar was estimated using anthrone with the method of Yemm and Willis (1954) using a graded concentration of glucose as the standard curve. The extraction was done according to Barnett and Naylor (1966). Fresh leaf samples (200 mg) were homogenized separately in 80% ethanol, refluxed for 15 min on a steam bath and centrifuged. The residue was further refluxed with 40% ethanol and the extraction was repeated thrice. The supernatant from different extraction was pooled and volume made to 5 ml with 80% ethanol. The extract obtained was used for estimation of TSC with absorbance recorded at 620 $\eta$m using Spectrophotometer-117. For proline determination, 300 mg fresh leaf samples were separately homogenized in 5 ml of 3% sulphosalicylic acid and then centrifuged at 1000 rpm for 15 minutes and the supernatant was taken (Bates *et al.*, 1973). 2 ml of extract was added to with 2 ml reagent acid ninhydrin. This mixture was then kept in a boiling water bath for 1 h at $100°C$ and cooled. 4 ml of toluene was added and given vigorous shaking. The upper organic phase (3 ml) formed was taken after attainment at room temperature and the absorbance was recorded at 520 $\eta$m.

### Relative water content (RWC, %) and Relative stress injury (RSI, %)

The relative leaf water content was determined using the third top fully expanded trifoliate leaf, taken between 9-10:00. RWC (%) = (Fresh weight – dry weight)/ (Turgid weight – dry weight) $\times$ 100 (Weatherly, 1950). Turgid weight was taken after 5 h at room temperature till the tissues were fully saturated in a perfectly humid environment and constant dry weight was attained after 72 h in an oven at $65°C$. Relative stress injury was measured as per cent proportion of ion leakage into the external aqueous medium at room temperature to the total ion concentration of the stressed tissue as measured by the electrical conductivity (EC) of the external medium (Sullivan and Ross, 1979) and calculated as $RSI(\%) = (EC_1/EC_2) \times 100$.

### Chlorophyll and Carotenoid content

Leaf total chlorophyll and carotenoid contents were assayed in leaves of 5 randomized plants according to Hiscox and Israelstam (1979) using dimethyl sulphoxide (DMSO). Pigments estimation was done using 30 mg of freshly harvested leaf samples and placed in an oven at $70°C$ for 3 h to facilitate the extraction of the pigments. After incubation, tubes were cooled to the room temperature and absorbance of the supernatant was recorded at 645, 665 and 454 $\eta$m by spectrophotometer (GBC UV- spectrophotometer).

## Statistical Analysis

Morpho-physiological parameters were assessed with Software Package SPSS Stat. Differences between means were considered significant when the $P \leq 0.05$.

# 3 Results

## 3.1 Effect of mycorrhizal inoculation on different growth parameters of Azadirachta indica grown under water stress and re-watering condition

### Chlorophyll and Carotenoid content

The response of neem to inoculation with AM fungi showed varying features. The germination per cent differed slightly among AM and non-inoculated treatments (Table 1), however, soil amendment with AM –fungi resulted in 7.77% higher cumulative germination per cent. First cotyledon leaf emergence and energy period coincided 5th and 8th day of sowing, respectively, both in mycorrhizal and non-mycorrhizal treatments, with a slight improvement in germination energy (6.67%) in AM –inoculated against non-inoculated pots. Germination value was recorded 13.36% more in AM treated neem seeds compared to non-inoculated seeds.

**Table 1:** Summarizing the effects of AM –inoculation presence or absence on germination behaviour of *Azadirachta indica* seeds under normal watering (seed per pot $n = 5$)

| Treatment | Emergence of first cotyledon leaf (DAS) | Germination percent (%) | Germination energy (%) | Energy period (DAS) | Germination value |
|---|---|---|---|---|---|
| AM-inoculated | 5 | 94.44 | 70.56 | 8 | 59.54 |
| Non-inoculated | 5 | 86.67 | 63.89 | 8 | 46.18 |

### Seedling growth

In the presented study, about 3 months old neem seedlings were subjected to water stress for 63 days by withholding water till the dropping of the leaves. However, drought symptoms appeared extremely slow among all the treatments due to the maintenance of seedlings under the green-net chamber. Findings of this study found water stress caused depression and a decrease in growth parameters. Water stress significantly declined collar diameter, shoot length, primary and secondary root length, with the higher decline observed in non-inoculated neem seedlings (Fig 1).

### Collar diameter, plant height and root growth

A significant increment in diameter growth was exhibited among the G. mosseae inoculated plants compared to un-inoculated plants (Fig.1). Results indicated that the collar diameter of AM fungal inoculated plants were 13.65, 20.28 and 20.99% higher against the respective un-inoculated seedlings at three stages of the watering condition. Similar significant effects of *G. mosseae* in inoculated plants were also realized in shoot length, primary and secondary root elongation of neem plants (Fig 1). Overall, *G. mosseae* inoculation enhanced better secondary root growth in comparison to primary root growth and when compared to non-inoculated plants. Nevertheless, water stress declined shoot length by 79.26%, primary root 57.18% and secondary root 75.14% in non-inoculated plants against inoculated plants.

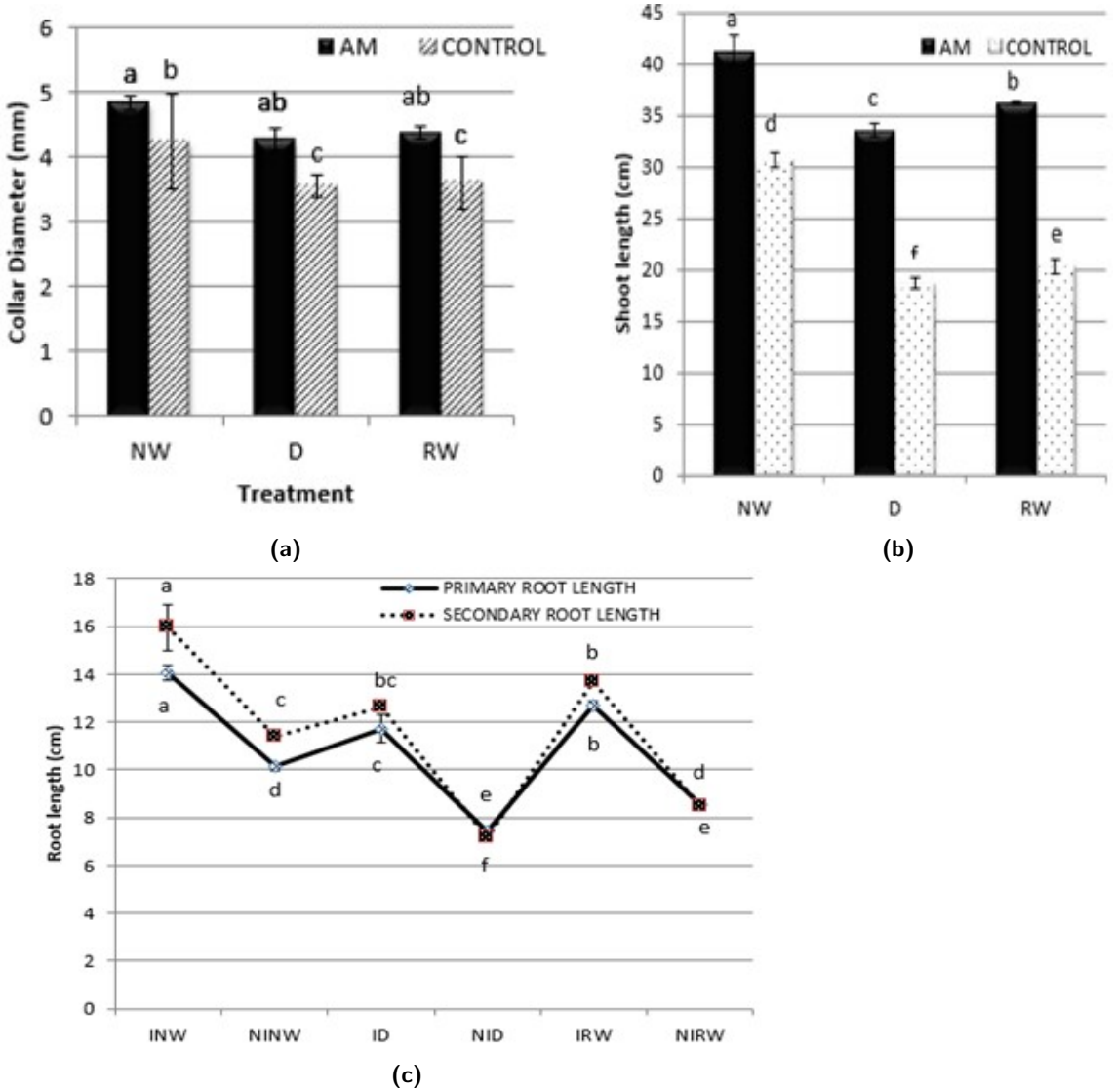


**Figure 1:** Effect of water stress on collar diameter (a), shoot (b) and root length (c) of AM –inoculated *Azadirachta indica* seedlings maintained at three watering regimes. (NW normal watering, D drought, RW re-watering, INW inoculated normal watering, NINW non-inoculated normal watering, ID inoculated drought, NID non-inoculated drought, IRW inoculated re-watering, NIRW non-inoculated re-watering seedlings. Data show means ($\pm$SE, $n = 24$). Different letters indicate significant differences at $P \leq 0.05$ (SPSS, Duncan test)

## Biomass and leaf area

AM –inoculated plants exhibited 40.78% more fresh biomass during the well-watering regime and 95.18%, 74.96% more correspondingly in water-stressed and re-watered regime, with drastic reductions observed in both fresh and dry biomass accumulation during water stress, though reductions were subsequently higher in non-inoculated plants (Fig. 2). Biomass production inhibition was substantially high in non-inoculated neem plants due to low soil water content observed which decreased the intake of both carbon dioxide and photosynthetic activities. However, biomass production slightly increased after water resumption, though non-inoculated seedlings yielded less compared to mycorrhizal plants. Greater leaf defoliation was observed in non-inoculated water-stressed

seedlings, consequently, the number of leaves/specific leaf area (not shown) and total leaf area per plant were negatively affected more in water-stressed non-inoculated seedlings. Water deficit reduced leaf area by 51.30% (Fig. 2). During recovery from water stress for 7 days, all the stressed re-watered seedlings recovered their hydration level; however, the non-inoculated plants could not recover their drooping leaves and remained wilted till the harvest of the seedlings.

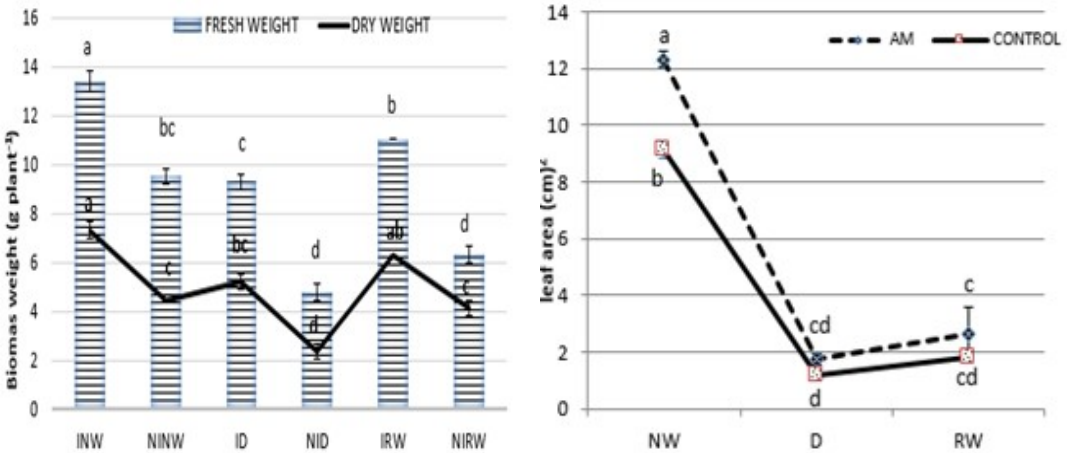


**Figure 2:** Effect of water stress on biomass (left) and leaf area (right) of AM –inoculated *Azadirachta indica* seedlings maintained at three watering regimes. (INW inoculated normal watering, NINW non-inoculated normal watering, ID inoculated drought, NID non-inoculated drought, IRW inoculated re-watering, NIRW non-inoculated re-watering, NW normal watering, D drought, RW re-watering seedlings. Data show means ($\pm$SE, $n = 24$). Different letters indicate significant differences at $P \leq 0.05$ (SPSS, Duncan test)

## 3.2 Effect of mycorrhizal inoculation on physiological parameters of *Azadirachta indica* seedlings grown under water stress and re-watering condition

**Table 2:** Effect of water stress on physiological parameters of AM –inoculated *Azadirachta indica* seedlings maintained at three water regimes

| Parameters | Treatment | | | | | |
|---|---|---|---|---|---|---|
| | AM-inoculated | | | Non-inoculated | | |
| | Water regimes | | | Water regimes | | |
| | NW | D | RW | NW | D | RW |
| Total chlorophyll content (mg/g FW) | $5.07 \pm 0.31a$ | $3.52 \pm 0.11c$ | $4.31 \pm 0.07b$ | $4.88 \pm 0.026a$ | $2.40 \pm 0.21e$ | $2.78 \pm 0.11d$ |
| Carotenoid content (mg/g FW) | $9.89 \pm 0.47a$ | $9.43 \pm 0.22a$ | $9.82 \pm 0.27a$ | $7.82 \pm 0.42b$ | $5.85 \pm 0.24d$ | $7.20 \pm 0.29c$ |
| Proline content ($\mu$moles/g DW) | $12.00 \pm 0.53c$ | $20.45 \pm 0.71b$ | $10.78 \pm 0.12c$ | $13.48 \pm 0.49c$ | $29.49 \pm 0.63a$ | $18.59 \pm 0.24b$ |
| Total sugar content (mg/g DW) | $85.07 \pm 0.40de$ | $86.83 \pm 0.91bc$ | $82.62 \pm 0.63cd$ | $97.30 \pm 0.39e$ | $106.39 \pm 079a$ | $74.45 \pm 0.56b$ |
| Relative water content (%) | $88.89 \pm 0.46a$ | $66.22 \pm 1.18c$ | $78.48 \pm 1.11b$ | $88.36 \pm 0.04a$ | $60.24 \pm 0.11e$ | $63.18 \pm 0.78d$ |
| Relative stress injury (%) | $12.04 \pm 0.43e$ | $22.38 \pm 0.17c$ | $19.5 \pm 0.12d$ | $12.16 \pm 0.33e$ | $27.08 \pm 0.06a$ | $23.6 \pm 0.26b$ |

Note: AM stands for arbuscular mycorrhizae, NW normal watering, D drought, RW re-watering seedlings. Data show means ($\pm$ SE, $n = 12$). Different letters indicate significant differences at $P \leq 0.05$ (SPSS, Duncan test)

### Chlorophyll and carotenoid pigments

Water stress induced significant reductions in chlorophyll and carotenoid pigments in leaves which lead to massive oxidative stress. Normal water seedlings displayed significantly higher chlorophyll and carotenoid contents in leaves. The water-stressed AM –plants displayed significantly higher total chlorophyll contents in their leaves compared to similar conditioned non-inoculated plants. This study, however, observed that AM –plants recovered faster the depleted amount of leaves chlorophyll during 7 days of re-watering (Table 2).

### Proline and Total sugar content

Proline and soluble carbohydrates displayed higher accumulation in leaves during water stress which helped to lower osmotic potential by maintaining cell turgor and higher osmotic adjustment. Proline accumulation in leaves of neem increased significantly in non-inoculated control water-stressed leaves (22.32%) compared to AM –plants while re-watering after 7 days led to a decrease in the proline accumulation, though, the reduction in proline was recorded more in AM –plants (49.34%) than non-inoculated plants (Table 2). It was also observed that proline accumulation showed no significant effect among AM and non –AM plants during the normal watering stage, however, its build-up differences were observed significant only in stressed leaves. Similarly, there were more soluble carbohydrates in the leaves of all the water-stressed plants in comparison to their respective controls, which lead to an increase in total sugar concentrations in them at the time of drought harvest before re-watering.

### Leaf relative water content (RWC) and leaf relative stress injury (RSI)

The reduction of leaf relative water content (RWC) after 63 days of water stress caused significant dehydration in the water status of the *Azadirachta indica* seedlings in all variables and tremendously resulted in a significantly higher relative stress injury in non-inoculated plants (17.36%) compared to their normal watered plants and AM –plants. The reduction of relative water content in non-inoculated water-stressed (NID) neem seedlings was 9.93% more compared to AM –inoculated water-stressed (ID) leaves. At the phase of recovering, re-watering resulted in the improvement of the hydration level of the seedlings, but it was more noticeable in AM –inoculated seedlings (Table 2).

**Table 3:** Effect of water stress on mycorrhizal colonization of AM –inoculated *Azadirachta indica* seedlings maintained at three water regimes

| Treatment | Mycorrhizal colonization (%) | | |
|---|---|---|---|
| | **NW** | **D** | **RW** |
| AM-inoculated | 92.70 | 76.80 | 78.20 |
| Non-inoculated | 4.87 | 2.45 | 3.74 |

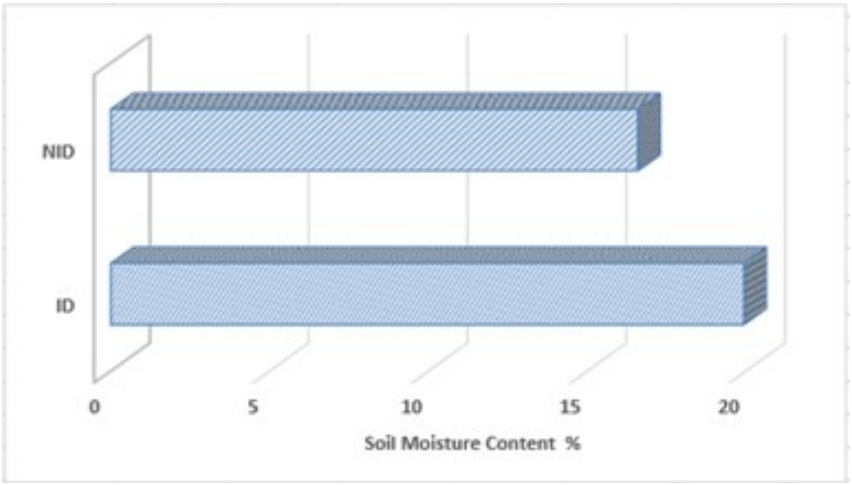


**Figure 3:** Effect of water stress on the reduction of soil moisture in absence or presence of soil amendment with AM fungi at drought harvest.

### 3.3 Mycorrhizal quantification and soil moisture content (SMC)

On the microscopic evaluation of root segments, it was found that Glomus mosseae penetrated the cortical cells of feeder roots intracellular and made large vesicles and arbuscles in root cells. Results displayed that well-watered neem seedlings inoculated with AM –fungi exhibited high infection (92.70%) while it was noted marginally (4.87%) in non-inoculated plants (Table 3). Water deficit inhibited mycorrhizal multiplication and sporulation thus, caused a major reduction in the AM abundance (76.80%) in AM –inoculated plants roots (Table 3). However, re-watering of neem seedlings enhanced AM colonization (78.20%) in the roots in comparison to water stress harvest. The soil moisture content at drought harvest in AM –inoculated pots were better maintained for plant growth compared to the pots without any soil amendment and was 3.34% higher in soil amended with mycorrhiza (Fig. 3).

## 4 Discussion

### 4.1 Effects of water stress and mycorrhizal inoculation on growth parameters of Azadirachta indica seedlings

The mycorrhizal fungus –Glomus mosseae had been associated with enhancement of neem growth rate, root colonisation and water stress tolerance particularly in areas with less water potential (Banerjee *et al.*, 2013; Jangid and Dwivedi, 2016). The present study examined morphological aspects of *Azadirachta indica* related to water relation and drought tolerance in AM –inoculated and non-inoculated seedlings exposed to water stress. The study observed the effectiveness of AM –*Glomus mosseae* symbiosis effect on germination and growth enhancement of neem at the seedling stage to improve plant tolerance to drought stress.

Notably, this reflected positive results of the presence of mycorrhizal inoculum for its huge impact in accelerating germination and growth (Vijayakumari, 2004; Kumar and Mishra, 2009; Sidibe *et al.*, 2012; Gutowski, 2015; Pandey and Pati, 2017; Al-Hmoud and Al-Momany, 2017; Li *et al.*, 2019). Our increase in growth and biomass of inoculated seedlings in the present study may prove AM seedlings to have a better water desiccation tolerance in comparison to non –AM plants. A significantly enhanced growth of AM –plants could be due to higher root colonization (Table 3), which ought to facilitate maximally root absorption surface area by their extra radical fungal (Venkateswarlu *et al.*, 2008; Banerjee *et al.*, 2013; Li *et al.*, 2019).

Findings were also in harmony with significant evidence of AM –inoculation on various tree seedlings as of *Tectona grandis* (Rajan *et al.*, 2000), *Azadirachta indica* (Banerjee *et al.*, 2013) and *Gmelia arborea* (Rashmi and Dixit, 2015) as well Zhu *et al.* (2012) who had reported that AM fungus significantly improved plant growth and described AM –symbiosis significance through enhancement of gas exchange capacity by decreasing stomatal resistance and increasing transpiration influxes. Higher root colonization observed in AM –seedlings (Table 3) allowed more host-fungus contact which helped in growth acceleration of AM –plants through a well-established root system which is vital for the exploitation of limiting root zone water, thus with the help of extra metrical hyphae that increase the surface area, it enhances a better penetration of root growth (especially secondary roots) in search of water and nutrients (Beniwal *et al.*, 1992; Banerjee *et al.*, 2013).

In spite our finding to had proven the previous hypothesis that AM –inoculated seedlings enhanced better growth, root colonization and soil water retention, growth and biomass reduction substantially observed, inhibited high in non-inoculated plants against its counterparts during water deficit period. This was proven that water stress leads to substantial water scarcity to plants by decreasing the soil water content (Fig. 2) inducing a severe reduction in biomass which ascribed to stomata closure. Closing of stomata results in suppression of leaf and shoot expansion through a decline in cell enlargement, leaf senescence (Marron *et al.*, 2003; Beniwal *et al.*, 2010), limiting water loss by transpiration and rick of xylem metabolism (Braatne *et al.*, 1992; Marron *et al.*, 2003), reduction in vessel diameter (Beniwal *et al.*, 2010) as well as fewer photosynthates production (Beniwal *et al.*, 2010; Anjum *et al.*, 2011).

## 4.2 Effects of water stress and mycorrhizal inoculation on physiological parameters of Azadirachta indica seedlings

Biochemical analysis such of chlorophyll status is a key major for assessing photosynthesis efficiency in plants. In the present study, well-watered AM –plants displayed higher chlorophyll pigments (3.89%) compared to non –AM counterparts (Table 2). Similar results on increased chlorophyll pigments were observed earlier by Wu and Xia (2006) in Citrus tangerine seedlings. Although water stress declined chlorophyll pigments in neem, the neem plants grown with AM –inoculation showed less reduction in chlorophyll pigments during water stress and could maintain significantly higher chlorophyll pigments (46.67%) over non –AM plants, which Wu and Xia (2006); Makbul *et al.* (2011) and Amit (2014) also evidently reported same. This suggests that water deficiency/stress interfered less with chlorophyll synthesis in mycorrhizal treated plants in comparison to non-mycorrhizal plants (Zhu *et al.*, 2012). Therefore, chlorophyll degradation has been well documented in its sensitivity and prone to soil dehydration (Anjum *et al.*, 2011; Amit, 2014) causing a massive sign of oxidative stress thus marginal inactivation of photosynthesis activities which reflected in the poor growth of neem plants.

Our results further confirmed that re-watering to AM –plants managed to maintain chlorophyll pigments towards hydrated levels and showed significant recovery (55.04%) compared to non –AM plants. Additionally, *Glomus mosseae* showed a massive effect on carotenoid pigments, and our study revealed that carotenoid pigments were 26.47% higher in AM well-watered seedlings against similar conditioned non –AM seedlings. This study also observed that water stress caused significant reductions in carotenoid contents, whereas, re-watering triggered a better performance, and increased carotenoid pigment by 36.39% in AM –inoculated seedlings over non –AM neem seedlings (Table 2). Correia *et al.* (2014) finding also agreed with our study, that photosynthetic rate in Eucalyptus globulus genotype declined in response to water stress, however, 7 days water resumption helped in faster recovery of plants photosynthetic pigments.

During water stress, *Azadirachta indica* accumulated some organic solutes in the cytosol such as proline and sugar to lower osmotic potential and helping in maintaining cell turgor and higher osmotic adjustment. During the water deficit stage, our study indicated that proline accumulation was lesser with 22.32% in AM plant leaves compared to non –AM plants. These results suggested that AM colonisation in inoculated plants enhanced plants with an ability to withstand drought. This was reported by Ruiz-Lozano (2003) and Ouledali *et al.* (2018) who

observed less proline content in AM -plants due to its better inoculant tolerance to drought. Therefore, high proline accumulation in non –AM seedlings played a greater role in drought tolerance enabling neem seedlings surviving water stress. The accumulation of proline in water-stressed neem seedlings also assumed to maintain a proper balance between extracellular and intracellular osmolarity.

Similar significant responses of proline solutes to water stress condition were realized by Wu and Xia (2006) in *Citrus tangerine*, Zheng *et al.* (2010) in *Azadirachta indica* and Amit (2014) in *Dalbergia sissoo* seedlings. Wu and Xia (2006) in citrus and Amit (2014) in Shisham reported less proline content in AM –inoculated stressed seedlings compared to non –AM stressed counterparts. Our results were also in harmony with Adivappar *et al.* (2003) that detected lower proline content in G. fasciculatum inoculated papaya plant leaves after drought imposition (for 10 and 20 days) compared to non-mycorrhizal plants. Other authors such as Sanchez *et al.* (1998) and Ruiz-Lozano (2003) have also reported similar results. Proline is well documented in other literature as an influence of protein solvation and maintaining membrane integrity under dehydration stress and reduces oxidation of lipid membranes (Demiral and Turkan, 2004).

During the experiment, our outcomes observed an enhanced accumulation of soluble sugars in drought conditioned leaves of neem plants. Non-inoculated water-stressed neem seedlings accumulated significantly higher total soluble carbohydrates (8.61%) in leaves than corresponding AM –inoculated neem leaves (Table 2). Drought stress increased total soluble carbohydrates more in non –AM plant leaves compared to AM –plants leave leading to similar results obtained in neem (Vijayakumari, 2004), poplar (Beniwal *et al.*, 2010), shisham (Amit, 2014) and *Olea europea* (Ouledali *et al.*, 2018). Proline accumulation may have resulted in greater osmotic adjustment in non –AM neem seedlings, allowing non –AM seedlings to accumulate more carbohydrate.

Relative water content (RWC) is the most significant index for dehydration tolerance that measures plant water status thus reflecting the metabolic activity in tissues (Anjum *et al.*, 2011). In the present investigation, leaf RWC of well-watered AM –inoculated neem plants did not significantly differ from that of non –AM plants (Table 2). Similar hydration levels have been broadly consistent as well in well-watered shisham (Amit, 2014), *Santalum album* (Binu *et al.*, 2015) and *Olea europea* (Ouledali *et al.*, 2018) seedlings. Our findings stipulated AM –inoculated seedlings displaying high values of relative water content during drought as compared to non –AM seedlings. Despite significant dehydration at 63 days of water-stress, our results indicated that AM –fungi may have explored deeper and farther soil horizons and maintained soil moisture in the root zone, consequently resulting in AM –seedlings during water stress having better plant water status.

Reduction in relative water content may be due to high transpiration influx which leads to stomatal closure, thus in return limiting carbon uptake by the leaves and reduce leaf water status (Chaves *et al.*, 2002). Protective effects of mycorrhizal inoculation on plants exposed to water stress have also been elaborated other studies (Beniwal *et al.*, 1992, 2010, 2011; Luo *et al.*, 2009; Yang and Miao, 2010; Zheng *et al.*, 2010; Ouledali *et al.*, 2018). Water stress contributes to solute accumulation leading to damaging membrane integrity associated with an increase in cell membrane leakiness, and in our experiment relative stress injury (RSI) to the membrane was significantly more in non –AM water-stressed seedlings (17.36%) compared to AM –inoculated seedlings (Table 2). Overall, our results confirmed that soil amendment with arbuscular mycorrhizal fungi contributed to the enhancement of plant water status, and this may have resulted in a better performance of AM –plants that outgrow non –AM plants under limited water supply.

# 5 Conclusion

This experiment confirmed the use of Glomus mosseae to have significantly enhanced growth, water retention and physiological status of AM –plants compared to those of non –AM plants. Though there were significant enhancements in proline and sugar content accumulation in drought-stressed plants as compared to normal watering plants; however, drought-stressed AM –plants had fewer enhancements compared to non-AM stressed

plants. This indicates that the utilization of AM –fungi can be signified for healthy and vigorous neem seedlings production purposely for rehabilitation as well improvement of agroforestry modalities.

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