Mycorrhiza Fungus *Rhizophagus intraradices* Mediates Drought Tolerance in *Eleusine coracana* Seedlings

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Abstract:

Under abiotic stress conditions, arbuscular mycorrhizal (AM) fungi help plants by improving nutrient and water uptake. Finger millet is an arid crop having soils with poor water holding capacity. Therefore, it is difficult for the plants to obtain water and mineral nutrients from the soil to sustain life. To understand the role of mycorrhizal symbiosis in water and mineral up-take from the soil, we studied the role of *Rhizophagus intraradices* colonization and its beneficial role for drought stress tolerance in finger millet seedling. Under severe drought stress condition, AM inoculation led to the significant increase in plant growth (7%), phosphorus, and chlorophyll content (29%). Also, the level of osmolytes including proline and soluble sugars were found in higher quantities in AM inoculated seedlings under drought stress. Under water stress, the lipid peroxidation in leaves of mycorrhized seedlings was reduced by 29%. The flavonoid content of roots in AM colonized seedlings was found 16% higher compared to the control, whereas the leaves were accumulated more phenol. Compared to the control, ascorbate level was found to be 25% higher in leaf tissue of AM inoculated seedlings. Moreover, glutathione (GSH) level was increased in mycorrhiza inoculated seedlings with a maximum increment of 182% under severe stress. The results demonstrated that AM provided drought tolerance to the finger millet seedlings through a stronger root system, greater photosynthetic efficiency, a more efficient antioxidant system and improved osmoregulation.

Key words: Finger millet, Mycorrhiza, Drought, ROS, Antioxidant

1. INTRODUCTION

Finger millet (*Eleusine coracana* L.) is grown worldwide in more than 4 m ha, and is the staple food for millions of people in less developed countries of Africa and Asia (1). It is rich in calcium, phosphorus, iron, and amino acids like -cysteine, tyrosine, tryptophan, and methionine

56   (2), which are crucial for human health. This plant is grown in semiarid and tropical regions

57   where the soils are suffering from the deficiency of nutrients, low precipitation, high

58   evapotranspiration rates and other restrictive environmental factors. Drought is one of the most

59   challenging threat that may cause serious losses in crop yield, and by 2025 up to 30% of the

60   global crop yield losses are expected due to drought (3). Drought conditions threat crop

61   productivity with finger millet no exception to it, so there is an urgent need to find solutions

62   which can provide an optimum yield under the drought stress.

63   Many studies have been focused to understand the molecular and physio-chemical

64   mechanism of drought tolerance in crop plants. Under water stress, various biochemical

65   reactions occur in plants like reduction in chlorophyll content and increase in the production of

66   reactive oxygen species (ROS) (4). Like other environmental stresses, the homeostasis between

67   production and detoxification of ROS in plants affect the development and growth under water

68   stress (5). These irregularities cause several cellular damages such as oxidative damage of

69   proteins, nucleic acids and lipids (6-7). Water limitation influence many physiological processes

70   by altering the production and accumulation of secondary metabolites like phenols and

71   flavonoids (8). These are efficient chain-breaking antioxidants that can inhibit lipid peroxidation

72   and reduce oxidative damage during water stress and helps in scavenging of ROS (9).

73   Recently the use of arbuscular mycorrhiza (AM) has received increased attention in crop

74   physiology, because mycorrhized plants are generally more tolerant to abiotic stresses than non-

75   mycorrhized plants. AM symbiosis protects the host plants against the harmful effects through

76   different physiological mechanisms of drought avoidance (10-11). Promotion of plant growth

77   under stress is due to establishment of the extensive hyphal networks, secretion of biomolecules

78   like glomalin for improving soil structure, and increasing water and nutrient uptake (12).

79   Moreover, due to the presence of extra radical mycelium (ERM) the plant can effectively absorb

80   water from the tightly held soil water around the roots, thus increase the soil-root hydraulic

81   conductance (13). Previous reports have suggested that the AM symbiosis can help plant to

82   achieve drought tolerance due to physical, nutritional, physiological and cellular processes (14).

83   The effect of AM symbiosis for nutrient absorption and other growth parameters attributes in

84   finger millet has been studied in recent past (15-16). But the studies to evaluate the AM

85   symbiosis to mitigate abiotic stress in this millet crop are very less. In the previous study, plant

86   growth promoting rhizobacteria (PGPR) and AM symbiosis was evaluated for reducing the

87   effects of water stress (17). They found that the symbiosis of PGPR along with AMF has positive

88   role on plant growth parameters during watered and water deficient conditions. But the

89   underlying biochemical mechanism behind this association was not revealed, as only proline and

90   superoxide dismutase (SOD) content were estimated. In the present study, we have evaluated the

91   physiological and biochemical impacts of symbiotic association under drought in depth and

92   reported here.

93   **2. EXPERIMENTAL PROCEDURES**

94   **2.1 Plant material, soil and drought stress treatment**

95   Finger millet seeds (cv. Ragi Korchara) were surface sterilized with 2% of sodium hypochlorite

96   for 2 min followed by washing with sterilized distilled water for three times. The sterilized seeds

97   were germinated in Petri plates containing sterilized wet filter paper with distilled water at 27±2

98   °C. Three days old germinated seeds with uniform length of radical were transferred to pots (1.5

99   l size, 2 seedlings /pot) with the mixture of double autoclaved sand and soil in 1:1 proportion.

100   The potting mixture was analysed for various soil parameters at Soil Testing Laboratory, IARI,

101   New Delhi with standard established methods. The results of analysis showed that, it contained

102   0.10 % organic carbon (OC), 4.11 g kg^{-1} of P and 18.57 g kg^{-1} K, pH 8.33, electrical

103   conductivity (EC) 0.34 ds/m, and field capacity (FC) of 33 %.

104   The seedlings with and without inoculation of AM fungus- *Rhizophagus intraradices* were

105   exposed to drought stress conditions. For this, the starter culture of AM fungi was maintained

106   and multiplied with maize seedlings in pots with autoclaved soil and sand in 1:1 ratio. The

107   numbers of spores present in the inoculum were counted and 2 g of inoculum (50 spores g^{-1}) was

108   used by making holes at the immediate vicinity of the germinated seeds. For control treatment,

109   microbial wash from same quantity of inoculum was added, which was prepared by filtering AM

110   inoculum through Whatman filter paper. Seedlings were grown in glass house under controlled

111   conditions with 28 ºC temperature, 16 h photoperiod (2500 lx) and 60–70 % relative humidity.

112   The experiment was carried out in a completely randomized design with three replications of

113   each treatment.

114   Initially, the seedlings were irrigated with tap water to 100 % field capacity (FC) for one month.

115   Later, water stress treatments were given by maintaining the soil water status to 100 % (well-

116   watered), 60 % (mild stress) and 40 % (severe stress) of FC (18-19). To achieve the soil water

117   status at 60 % and 40 % FC, pots were allowed to dry to reach the required level. During stress

118   period of 10 days, the pots were weighed daily, and the amount of water lost by

119   evapotranspiration replenished by re-watering. After 10 days of drought stress, seedlings were

120   harvested by firmly shaking the pots to loosen the soil and then tilting the pots at < 45° of angle

121   for smoothly pulling out the intact soil ball from the pots, without damaging the roots. For

122   agronomical and biochemical estimation, treated and control samples were stored separately in

123   plastic bags. The short duration storage was done at 4 °C for physiological observation, and for

124   biochemical analysis the samples were stored at -80 °C.

125   **2.2 Morphological parameters of finger millet seedlings**

126   After drought treatment, randomly selected seedlings from each treatment were used to measure

127   the plant height, number of leaves in plant and root length. Shoot and root dry weights of plant

128   was estimated after drying at 75ºC for 48 h in oven until a constant weight was obtained.

129   Phosphorus content in seedlings of all the treatments was measured in oven dried samples (20).

130   **2.3 Estimation of root colonization**

131   Mycorrhizal colonization percentage in roots was measured according to the established method.

132   Briefly, after washing with distilled water, roots were cleared in 5 % KOH solution at 95 °C for

133   1 h, and then treated with 5 % HCl for 10 min. The cleared roots were stained with 0.05 %

134   Trypan blue-lactic acid solution (v/v). The colonization frequency was estimated by grid-line

135   intersect method (21), and three replicates per treatment were used for the measurements.

136   **2.4 Chlorophyll content in leaves**

137   Chlorophyll content in the leaves was estimated by adding 0.1 g of finely chopped leaf samples

138   in 7 ml of dimethyl sulfoxide (DMSO) followed by incubation in water-bath at 65°C for 30 min

139   until green tissues turned colourless (22). The cooled samples were filtered, and volume was

140   made up to 10 ml by adding more DMSO. After vortexing for few seconds, UV light absorption

141   was measured using spectrophotometer (UV/Vis-1800, Shimadzu, Japan) at 645 and 663 nm.

142   DMSO without any plant sample was used as a control. The amount of total chlorophyll present

143   in DMSO extract was measured as mg chlorophyll g^{-1} tissue according to the following formula

144   (23).

145   Total Chl (g l^{-1}) = 0.0202× A_{645} + 0.00802 × A_{663}

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147

2.5 Determination of proline content

The proline content in finger millet tissues was determined by previously described method (24-25). Briefly, for this 0.1 g of fresh plant tissue was homogenized in 1.5 ml of 3 % sulfosalicylic acid and centrifuged for 5 min at 13,000 rpm. The supernatant of around 300 µl was transferred into a new tube followed by the addition of 2 ml each of acid ninhydrin [1.25 g of ninhydrin in 20 ml of phosphoric acid (6M) and 30 ml of glacial acetic acid] and glacial acetic acid. The mixture was kept in water bath (100°C) for 1h, and immediately cooled on ice. Toluene (1 ml) was added to the reaction and vigorously mixed for a few seconds. Toluene containing chromophore layer was removed from the aqueous phase and kept at room temperature. Absorbance of each sample was measured in spectrophotometer at 520 nm against Toluene blank. The standard curve was used to calculate the concentration of proline, with three independent replicates.

2.6 Estimation of total soluble sugar (TSS)

Total soluble sugars from the finger millet tissues were extracted and analysed according to the method reported earlier (26). In short, 0.1 g of tissue was homogenised in 2 ml of 80 % (v/v) ethanol, and vortexed for few seconds. The homogenates were allowed to stand at room temperature for 30 min and centrifuged at 10,000 rpm for 20 min. The resulted supernatants were stored at 4 $^\circ$C until further analysis. Later, 5 ml of supernatant was mixed with 3 ml of freshly prepared anthrone reagent (200 mg anthrone, 100 ml of 72 % sulphuric acid), and followed by the incubation in the water bath at 100°C for 10 min, after which the absorbance was measured at 620 nm. The TSS was determined using glucose as a standard and expressed as mg g^{-1} fresh weight (FW) of plant tissue.

171 **2.7 Measurement of lipid peroxidation**

172 Measurement of lipid peroxidation was evaluated in terms of malondialdehyde (MDA) content

173 as reported by Li et al., (27). The analysis contained 1.0 g of fresh grinded tissue mixed with 5

174 ml solution of 0.6 % TBA in 10 % Trichloroacetic acid (TCA). The mixture was subsequently

175 centrifuged at 12,000 rpm for 20 min, and 2 ml of the resultant supernatant was supplemented

176 with 2 ml of 0.6 % thiobarbituric acid (TBA) in 10 % TCA. The reaction was incubated in

177 boiling water for 15 min, and then quickly cooled on ice. Afterward it was centrifuged at

178 12,000rpm for 10 min again, and the absorbance of the supernatant was measured at 450, 532

179 and 600 nm.

180 The MDA content was calculated on a fresh weight bases using the below mentioned formula

181 μM MDA g^{-1} of FW $= 6.45 \times (OD_{532} - OD_{600}) - 0.56 \times OD_{450}$.

182 **2.8 Estimation of hydrogen peroxide content**

183 Method suggested earlier was used for the estimation of hydrogen peroxide (H_2O_2) content (28).

184 Briefly, 0.5 g of plant tissue was homogenized in 0.1% TCA, and homogenized mixture was

185 centrifuged at 12000 rpm. Later, the reaction mixture containing 0.5 ml of 10 mM potassium

186 phosphate buffer (pH 7.0) and 1 ml of potassium iodide solution were thoroughly mixed to 0.5

187 ml of the supernatant and absorbance was measured at 390 nm. A standard curve plotted using a

188 known concentration of H_2O_2, was used to calculate the content of H_2O_2 .

189 **2.9 Estimation of antioxidant compounds - Glutathione, Ascorbate, Phenols, and**

190 **Flavonoids**

191 Glutathione (GSH) content was determined by following the method suggested in the previous

192 report (29). Briefly, plant tissue (0.1 g) was homogenized in 1 ml of 5 % TCA under cold

193 condition, and centrifuged at 10000 rpm for 10 min. 100 μl of the supernatant was made up to

194   1.0 ml with 100 mM potassium phosphate buffer (pH 7.0) and 2 ml of 5,5-dithio-bis-(2-

195   nitrobenzoic acid) (DTNB) solution. The resultant mixture was vortexed thoroughly for few

196   seconds and incubated for 10 min for colour development. The intensity of the yellow colour

197   developed was measured at 412 nm with spectrophotometer. The values were expressed as nM

198   GSH g^{-1} plant sample. GSH standards were prepared for concentrations ranging between 0 and

199   50 ng ml^{-1}.

200   The amount of ascorbic acid (AsA) was determined from 0.25 g of fresh tissues that were

201   crushed in 10 ml of 6 % TCA (30). The homogenised mixture was centrifuged for 10 min at 4º C

202   at 1000 rpm followed by the addition of 0.5 ml of 2 % dinitrophenyl hydrazine solution. A drop

203   of thiourea solution (10 % thiourea in 70 % ethanol) was S to the mixture and boiled for 20 min.

204   The resultant mixture was placed on ice to decrease the temperature to 25 ºC followed by the

205   addition of 5 ml of 80 % sulphuric acid (v/v) under cold conditions.   The absorbance was

206   measured at 530 nm, and AsA content was estimated by comparing it with the standard curve

207   prepared by a known standard of ascorbic acid.

208   For the estimation of phenols and flavonoid content in finger millet seedlings, fresh plant tissues

209   were collected and dried at 25°C in the dark. The dried tissues were grounded into fine powder.

210   Out of this powdered tissue, 0.1 g was extracted in 10 ml methanol by shaking it overnight at

211   room temperature, followed by sonication for 30 min. The resultant mixture was filtered, and

212   filtrate was used for phenol and flavonoid estimation. For flavonoid estimation 500 µl of

213   methanol extract was added to 0.5 ml of an 2% aluminium chloride solution in methanol (31).

214   Incubation was done at room temperature for 60 min and absorbance was measured at 240 nm.

215   The resulting yellow colour intensity indicated the presence of flavonoids. Standard curve

216   plotted for solution of quercetin at varying concentrations (10, 20, 40, 80, and 160 µg ml^{-1}) was

217   used for quantification, and total flavonoid content was expressed as quercetin (mg g^{-1} dry

218   weight).

219   Total phenolic content was determined by Folin-Ciocalteu method (32). A 1.16 ml of distilled

220   water and 100 µl of Folin-Ciocalteu reagent were added to 20 µl aliquot of methanol extract,

221   followed by addition of 300 µl of 20% Na_2CO_3 solution. The mixture was kept in a shaking

222   incubator at 40 ºC for 30 min and its absorbance was measured at 760 nm. Gallic acid was used

223   as a standard for the preparation calibration curve. Total phenolic contents were expressed as

224   gallic acid (mg g^{-1} dry weight).

225   **2.10 Statistical analysis**

226   All experiments were performed in triplicate (n=3) and were expressed as average ± standard

227   deviation. The data was analysed using two-way analysis of variance (ANOVA), with

228   inoculation treatment (with and without AM) and water level as source of variation. Duncan's

229   multiple range test (DMRT) was performed by SPSS software (33) for comparative analysis

230   under all water levels in all treatments.

231   **3. RESULTS**

232   **3.1 Effect of AM inoculation on morphological parameters and phosphorous uptake**

233   To evaluate the response of finger millet seedlings to drought, the seedlings were subjected to

234   well-watered (100 % FC), mild stress (60 % FC) and severe stress (40 % FC) condition for 10

235   days. It was found that length, fresh weight, and dry weight of shoot and root were reduced under

236   soil moisture depletion under mycorrhized and non-mycorrhized conditions (Fig. 1). However,

237   compared to the non-mycorrhized seedlings, the reduction rate of morphological characters was

238   negligible in mycorrhiza inoculated seedlings. Seedlings height was also more in the case of AM

239   inoculated plants i.e. 44, 40, and 38 cm (18, 11 and 7 % more than non-inoculated seedlings)

240   (Table 1). Mycorrhiza treated seedlings showed maximum increase in the fresh weight of shoot

241   and root at 60 % FC (mild stress) *i.e.* 54.46 and 62 %, respectively compared to the control.

242   Number of leaves was more in the cases of treated plants, and highest percent increase was also

243   observed under mild stress i.e. 33 % more than control. Mycorrhizal colonization had significant

244   effect on the root length under severe drought stress, that showed 15 % more root length

245   compared to the non-mycorrhizal seedlings. Highest increase in shoot dry mass was observed

246   under severe stress (40 % FC), and it was found to be 70 % more compared to the control

247   seedlings (Table 1). The results indicated that mycorrhiza inoculum has improved the biomass,

248   especially the root biomass under water stress. To analyse the representative nutrient status of the

249   seedlings, the phosphorous content was estimated; and found that it was significantly higher in

250   AM seedlings. The highest percent increase (44 %) was observed at 40 % FC (2.27 mg g^{-1} tissue)

251   as compared to the control (1.57 mg g^{-1} tissue) (Table 1).

3.2 Mycorrhiza colonization of roots under water stress

253   In the present study, no colonization by mycorrhiza was observed in non-inoculated seedlings. In

254   the inoculated seedlings, it was decreased with the aggravation of drought stress, and was 54, 48

255   and 25 % under well-watered, mild stress and severe stress, respectively (Table 1).

3.3 Chlorophyll content in seedlings under stress

257   AM inoculation was significantly increased the total chlorophyll content, even in drought stress.

258   In AM-inoculated seedlings it was significantly more than un-inoculated seedlings, and highest

259   increase observed at 100 % FC was 43 % (0.56 mg g^{-1} FW). During the severe stress at 40 % FC,

260   the total chlorophyll in AM-inoculated seedlings was 29 % (0.31 mg g^{-1} FW) more than control

261   (Fig. 2).

262

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3.4 Effect of drought on biochemical parameters of finger millet

3.4.1 Proline content

Because of the drought treatment, proline content was found to be more in leaves of AM treated finger millet compared to the non-inoculated seedlings (Fig. 3A). No significant differences were observed between AM-inoculated and control seedlings under well-watered condition. But under severe stress condition, proline content was 27 μg g^{-1} of fresh weight in AM inoculated seedlings, *i.e.* 13.71 % higher than control (24.14 μg g^{-1} FW). We also found that root accumulated higher proline content compared to the leaves. In AM treated seedlings, proline content was significantly up-regulated under moderate and severe drought stresses, and highest concentration was found at 40 % FC i.e. 63.57 μg g^{-1} of FW of root, whereas 33.55 μg g^{-1} of FW in control (89.4 % more than control) (Fig. 3B).

3.4.2 Total soluble sugars (TSS) content

Our results indicated an increase in TSS content with the reduction in soil moisture in both the treatments (Fig. 3C and 3D). Soluble sugar level in leaves was more in AM-inoculated seedlings under both stresses, and higher content of TSS osmolyte was found in AM seedlings (173.73 mg g^{-1} FW). The TSS osmolyte content in non-AM plants was found to be158.93 mg g^{-1} FW under severe stress (40 % FC). Soluble sugar concentration in the roots of AM seedlings was increased significantly under 60 and 40 % FC, which was 53 % (90.82 mg g^{-1} of FW) and 34 % (93.37 mg g^{-1} of FW) higher compared to the control (59.19 mg g^{-1} of FW and 90.82 mg g^{-1} of FW) (Fig 3D).

3.4.3 Malondialdehyde (MDA) content

To analyse lipid peroxidation, the MDA content was measured. In leaves, the inoculation of AM fungus was more persistent in improving plant membrane stability by decreasing the level of

286   MDA in finger millet under drought stress. Under severe stress condition, MDA content in

287   leaves of AM treated seedlings was found to be less (44.66 nM g^{-1} FW) than non-AM (54 nM g^{-1}

288   of FW), that indicated the presence of less oxidative damage due to the AM treatment. It showed

289   a 22 % decrease of MDA in non-AM seedings (Fig. 3E).   In the cases of roots, MDA

290   accumulation was higher compared to the leaf tissues. Higher MDA content was observed under

291   60 % and 40 % FC, and that was 18 % and 28 % more in non-AM plants compared to the

292   mycorrhizal roots (Fig. 3F). These findings indicated that higher degree of oxidative stress was

293   observed in control plants, and mycorrhiza helps in mitigating oxidative damage.

294   **3.4.4 Hydrogen peroxide (H_2O_2) content**

295   The H_2O_2 content in finger millet tissues was increased in all the stress treatments, but we found

296   that, in all the AM inoculated seedlings H_2O_2 level was decreased significantly compared to the

297   control. Highest H_2O_2 content (0.59 µM g^{-1} of FW) was observed in non-AM plants under severe

298   stress (40 % FC), whereas in AM treated plants it was found to be 0.50 µM g^{-1} of FW (Fig. 4A).

299   In the ecase of roots, the level of H_2O_2 followed the same trend of leaves, where AM inoculation

300   showed its significant effect under both stress levels. Higher variation among the control and

301   treated roots was seen at 40% FC, where AM- seedlings showed 16 % less H_2O_2 accumulation

302   than control (Fig.4B).

303   **3.4.5 Ascorbate (AsA) and Glutathione (GSH) content**

304   In the present study, ascorbate content (AsA) was found to be decreased with the increase in

305   level of water stress, and AM inoculated plants had showed higher ascorbate content than control

306   under all stress levels. In well-watered conditions, the differences in both non-mycorrhized and

307   mycorrhized seedlings were non-significant, indicating nearly same ascorbate redox status (Fig.

308   4C).   With the depletion of water content, AM fungus was more able to improve the ascorbate

309   showed a significant increase of 10 % to 25 % under 60% and 40 % FC, respectively. Roots

310   accumulated less ascorbate content compared to the leaf tissue and no significant changes were

311   observed under mild and severe stress (Fig. 4D).

312   We also found that, GSH was highly affected by AM fungi; specially in the case of leaf tissue. It

313   was increased in all drought stress treatments, but higher variation was found at 40 % FC with a

314   significant increase of 182 % in AM inoculation (2.4 μM g^{-1} FW) compared to control seedlings

315   (0.85 μM g^{-1} FW) (Fig.4D). Root accumulates significantly less amount of glutathione compared

316   to the leaves, while the results were significant and in favour with the AM seedlings (Fig. 4E).

3.4.6 Phenol and flavonoid content

318   Total phenolic and flavonoid content were also assayed to understand the influence of water

319   stress on secondary metabolites in finger millet seedlings. With the induced water stress, phenol

320   level was increased in all the treatments (Fig.5A). No significant change was observed at 100 %

321   FC, but AM showed its significant influence on phenol accumulation in roots under mild stress

322   (139 mg gallic acid g^{-1}of DW), which was 13 % more as compared to the non-AM seedlings. In

323   the case of leaves under severe stress condition, mycorrhiza treatment was not effective in

324   respect of increase in phenol content. Roots accumulated less phenol than leaf tissues; significant

325   and high variation could be seen between roots of treated and control seedlings. High phenol

326   content was observed in AM-inoculated roots under mild and severe stress (39.64 mg g^{-1} of DW

327   and 48.66 mg g^{-1} of DW, respectively). The increase was 35 % and 46 % more compared to the

328   control (Fig. 5B).

329   Total flavanoid content in the leaves and roots was also increased in both treatments with the

330   reduction in soil moisture level. No significant difference was observed under well-watered

331   condition, but in the leaves of mycorrhizal treated seedlings, there was a 30 % (0.025 mg g^{-1} of

332   DW) and 50 % (0.030 mg g^{-1} of DW) increase in flavonoid content compared to non-AM plant

333   under mild (0.015 mg g^{-1} of DW) and severe stress (0.022 mg g^{-1} of DW) (Fig.5C). Results also

334   showed that finger millet roots accumulated more flavonoid than leaves (Fig. 5D). At 60 % FC,

335   flavonoid content in the AM-inoculated roots was nearly same as compared to the control, but

336   under severe stress condition mycorrhiza showed a significant effect with 16.48 % increase

337   compared to the control.

338   **4. DISCUSSION**

339   Seedling stage is more sensitive to the drought stress than the subsequent ones. It severely limits

340   the crop stand and subsequent yield of various crop plants. The aim of the present study was to

341   evaluate the effect of mycorrhizal symbiosis during water stress on growth of finger millet

342   seedlings. Avoidance and tolerance are the two main strategies through which mycorrhized

343   plants cope up with abiotic stress (11). Extensive hyphal network makes it perfect drought

344   avoider by maintaining an adequate hydration status inside the plant cell and promote the plant

345   growth through enhanced absorption of nutrition's from the soil (10). Along with this, the effect

346   of AM on the stress tolerance has often been measured in terms of osmolyte, secondary

347   metabolite accumulation and biomass production (34).

348   In the present study, AM symbiosis enhanced the growth and biomass of finger millet seedlings

349   under water stress. Drought stress has reduced the shoot and root biomass of finger millet

350   seedlings (both AM and non-AM seedlings). However, the length, fresh and dry weights of the

351   roots and shoots in AM seedlings were found to be in treated plants under mild and severe

352   drought stress. Similar results were previously reported in *Pistacia vera* L (35) and *Zea mays*

15

353   (36) where AM fungi had enhanced the tolerance potential to the abiotic stresses by improving

354   the physiological parameters. Drought affects plant chlorophyll content, indicating a lower

355   photosynthetic capacity. But chlorophyll content in AM treated finger millet seedlings was more

356   by 23.68 % and 29.16 % under mild and severe stress condition, respectively. This indicated that

357   lessor damage to photosynthetic ability of finger millet was might be due to greater availability

358   of nutrient and water content from the soil through AM symbiosis. Similar results were also

359   reported in other crops under severe drought stress (37-38). We found enhanced accumulation of

360   phosphorous in mycorrhizal seedlings under well-watered and severe stress conditions, and

361   similar finding was previously observed in many other plant species (39).

362   Drought stress in finger millet seedlings significantly affected the mycorrhizal colonization.

363   Similar results have been reported in *Helianthemum almeriense* and *Terfezia claveryi* orchards

364   (40). The results have confirmed the hypothesis that after establishment of initial symbiosis of

365   AM with plant, water stress reduces the AM growth in the soil by inhibiting the spore

366   germination and spread of extra radical mycelium (ERM) through branching (41). The lesser the

367   photosynthetic efficiency in the host plant induced by drought stress, lesser the quantity of root

368   carbohydrates, and hence the rate of AM colonization (42-43).

369   Proline has been broadly considered as a drought-inducible metabolite with an osmoprotective

370   role. It has been reported that, accumulation and interaction of proline and soluble sugars

371   preserved a high antioxidant protection in leaves of *Arabidopsis thaliana* under drought stress

372   (44). As expected, we also found high proline content under drought stress. The higher proline

373   accumulation in AM treated finger millet root and leaf was more than that of non-AM plant

374   under severe stress and was in agreement with the previous findings (17). It was also found that

375   leaves have lower proline content as compare to roots, which might be probably because, its

376   synthesis occur in the shoots and then transported to the roots to maintain the growth at low

377   water level (45).

378   Along with proline, soluble sugars also play an important role in protecting membrane integrity

379   through osmotic adjustment (5). In our study, drought was found to increase the accumulation of

380   soluble sugars more in the presence of AM compared to the control. Leaves in AM treated

381   seedlings were accumulated more carbohydrate than roots and may be an outcome of the

382   enhanced photosynthetic efficiency and the sink effect of fungal demand for sugars from leaves

383   to roots (45-46).

384   We also found that AM inoculation resulted in reduced the lipid peroxidation and hydrogen

385   peroxide concentration in seedlings under the drought stress. In roots, higher lipid peroxidation

386   was observed under severe stress, and less MDA was accumulated in AM treated roots. Most of

387   the studies have demonstrated that lipid peroxidation is a biomechanism of cellular damage in

388   living organisms and can be used as an indicator of oxidative stress. The increase of MDA

389   content in the leaves indicates that the bulk oxidative lipid synthesis was induced by drought,

390   suggesting a close relationship between drought and oxidative stress (47-48).

391   Interestingly ascorbate protects the plant cell against oxidative damage by its ability to function

392   as an electron donor in a broad range of enzymatic and non-enzymatic reactions (50). Both AsA

393   and GSH participate in the AsA-GSH cycle and helps to neutralise H_2O_2 into water and oxygen

394   (51). The H_2O_2 content was increased with drought in control seedlings, but in treated plants it

395   was significantly reduced. The H_2O_2 content was highly affected at mild stress that shows 44 %

396   less H_2O_2 content in AM seedlings than control. It was reported that AM symbiosis improve the

397   response of plants to drought largely through the accumulation of the antioxidant compound like

398   glutathione. The glutathione was found to be associated with a reduction in oxidative damage to

399   membrane lipids and cellular H_2O_2 (11,52). On the other hand, ascorbate levels were less in the

400   mycorrhizal plants compared to the non-mycorrhizal counterparts. Similar findings were found

401   in the present study as well, where the leaves of AM inoculated finger millet had increased

402   glutathione level in severe drought stress, and ascorbate levels were decreased in the mycorrhizal

403   plants. This is in accordance with the previous reports in rice where antioxidant compound

404   glutathione was higher in AM plants compared to the non-AM (53).

405   In our study, phenols and flavonoids content in finger millet leaves were increased during

406   drought stress. High content of phenol and flavonoid was also observed in AM treated seedlings.

407   Recent studies have shown that accumulation of phenols, flavonoids can significantly increase in

408   the plants under mild drought stress (54). As ROS is highly responsible for oxidative burst in

409   plant cell, prevention of ROS production is achieved by compounds such as phenolic, flavonoids,

410   and antioxidants. The drought stress lead to enhancement of these metabolites in the seedlings.

411   **5. CONCLUSION**

412   From the results, we conclude that AM fungi symbiosis with the finger millet seedlings has

413   improved it's growth performance under drought stress. The beneficial effect of the AM

414   symbiosis was linked to the effective osmotic adjustment mechanism by accumulation of proline

415   and soluble sugars; along with soluble phenols and AsA–GSH cycle. The drought stress

416   decreased the shoot and root yield, but enhanced the accumulation of phosphorus and water,

417   which could help seedlings to cope up with the water stress conditions. Thus, the arbuscular

418   mycorrhizal fungi *Rhizophagus intraradices* can be an efficient plant growth promoting fungi to

419   enhance drought tolerance in finger millet seedlings. Further research on the molecular aspects of

18

420 this AM assisted drought tolerance can reveal more to understand the molecular basis of drought

421 tolerance.

AUTHOR CONTRIBUTIONS

423 JT performed the experiments, NS performed data analysis and paper drafting; AKS, AV, and

424 RNP designed the experiments, supervised the work and finalized the manuscript. All the authors

425 have read the manuscript and provided comments.

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References

431 1.   Upadhyaya, H. D.; Ramesh, S.; Sharma, S.; Singh, S. K.; Varshney, S. K.; Sarma, N. D.;

432   Ravishankar, C. R.; Narasimhudu, Y.; Reddy, V. G.; Sahrawat, K. L.; Dhanalakshmi, T. N.

433   Genetic diversity for grain nutrients contents in a core collection of finger millet (*Eleusine*

434   *coracana* (L.) Gaertn.) germplasm. *Field Crops Research,* 2011, *121*, 42-52.

435 2.   Kumar, A.; Gaur, V. S.; Goel, A.; Gupta, A. K. De novo assembly and characterization of

436   developing spikes transcriptome of finger millet (*Eleusine coracana*): A minor crop having

437   nutraceutical properties. *Plant Molecular Biology Reporter,* 2015, *33*, 905-922.

438 3.   Rosegrant, M. W.; Cai, X.; Cline, S. A. World water and food to 2025: Dealing with scarcity. *Intl*

439   *Food Policy Res Inst*, 2002

440 4.   Kurepin, L. V.; Ivanov, A. G.; Zaman, M.; Pharis, R. P.; Hurry, V.; Hüner, N. P. Interaction of

441   glycine betaine and plant hormones: Protection of the photosynthetic apparatus during abiotic

442   stress. In *Photosynthesis: Structures, mechanisms, and applications*. Springer, Cham, 185-

443   2022017

444 5.   Choudhury, F. K.; Rivero, R. M.; Blumwald, E.; Mittler, R. Reactive oxygen species, abiotic

445   stress and stress combination. The Plant Journal, 2017, 90(5), 856-867.

446 6.   Noctor, G.; Mhamdi, A.; Foyer, C. H. The roles of reactive oxygen metabolism in drought: not

447   so cut and dried. *Plant Physiology,* 2014, *164*, 1636-1648.

7. Petrov, V.; Hille, J.; Mueller-Roeber, B.; Gechev, T. S. ROS-mediated abiotic stress-induced programmed cell death in plants. *Frontiers in plant science,* 2015, *6,* 69.

8. Ma, D.; Sun, D.; Wang, C.; Li, Y.; Guo, T. Expression of flavonoid biosynthesis genes and accumulation of flavonoid in wheat leaves in response to drought stress. *Plant Physiology and Biochemistry,* 2014, *80*, 60-66.

9. Das, K.; Roychoudhury, A. Reactive oxygen species (ROS) and response of antioxidants as ROS-scavengers during environmental stress in plants. *Frontiers in Environmental Science,* 2014, *2*, 53.

10. Augé, R. M.; Toler, H. D.; Saxton, A. M. Arbuscular mycorrhizal symbiosis alters stomatal conductance of host plants more under drought than under amply watered conditions: a meta-analysis. *Mycorrhiza,* 2015, *25*, 13-24.

11. Dastogeer, K. M. G.; Stephen J. W. Plant–Fungi Association: Role of fungal endophytes in improving plant tolerance to water stress. In *Plant-Microbe Interactions in Agro-Ecological Perspectives*, Springer, Singapore, 2017, 143-159.

12. Miransari, M. Contribution of arbuscular mycorrhizal symbiosis to plant growth under different types of soil stress. *Plant Biology,* 2010, *12*, 563-569.

13. Gonzalez-Dugo, V.; Durand, J. L.; Gastal, F. Water deficit and nitrogen nutrition of crops -A review. *Agronomy for sustainable development,* 2010, *30,* 529-544.

14. Zou, Y. N.; Wang, P.; Liu, C. Y.; Ni, Q. D.; Zhang, D. J.; Wu, Q. S. Mycorrhizal trifoliate orange has greater root adaptation of morphology and phytohormones in response to drought stress. *Scientific reports,* 2017, *7*, 41134.

15. Karasawa, T., and Takebe, M., Temporal or spatial arrangements of cover crops to promote arbuscular mycorrhizal colonization and P uptake of upland crops grown after nonmycorrhizal crops. *Plant and soil,* 2012, *353*, 355-366.

16. Ramakrishnan, K.; Bhuvaneswari, G. Effect of inoculation of am fungi and beneficial microorganisms on growth and nutrient uptake of *Eleusine coracana* (L.) Gaertn.(Finger millet). *International Letters of Natural Sciences,* 2014, *8,* 2.

17. Kamal, R.; Gusain, Y. S.; Sharma, I. P.; Sharma, S.; Sharma, A. Impact of arbuscular mycorrhizal fungus, *Glomus intraradices*, *Streptomyces* and *Pseudomonas* spp. strain on finger millet (*Eleusine coracana* L.) cv Korchara under water deficit condition. *African Journal of Biotechnology,* 2015, *14,* 3219-3227.

18. Colman, E. A laboratory procdure for determining the field capacity of soils. *Soil science,* 1947, *63,* 277-284.

481 19. Veihmeyer, F.; Hendrickson, A., Methods of measuring field capacity and permanent wilting
482   percentage of soils. *Soil science,* 1949, *68*, 75-94.

483 20. Nouri, E.; Breuillin-Sessoms, F.; Feller, U.; Reinhardt, D. Phosphorus and nitrogen regulate
484   arbuscular mycorrhizal symbiosis in *Petunia hybrida*. *PLoS One,* 2014, *9(3),* e90841.

485 21. Giovannetti, M.; Mosse, B. An evaluation of techniques for measuring vesicular arbuscular
486   mycorrhizal infection in roots. *New phytologist,* 1980, *84,* 489-500.

487 22. Hiscox, J.T.; Israelstam, G. A method for the extraction of chlorophyll from leaf tissue without
488   maceration. *Canadian Journal of Botany,* 1979, *57*, 1332-1334.

489 23. Arnon, D. I. Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris. Plant*
490   *Physiology,* 1949, *24*, 1.

491 24. Bates, L.; Waldren, R.; Teare, I. Rapid determination of free proline for water-stress studies.
492   *Plant and soil,* 1973, *39,* 205-207.

493 25. Tyagi, J.; Pudake, R. N. Spectrophotometric assays to evaluate the rhizospheric microbes
494   mediated drought tolerance in plants. In: Modern tools and techniques to understand microbes.
495   Springer, 2017, 413-429.

496 26. Wang, S.; Zhu, Y.; Jiang, H.; Cao, W. Positional differences in nitrogen and sugar concentrations
497   of upper leaves relate to plant N status in rice under different N rates. *Field Crops Research,*
498   2006, *96,* 224-234.

499 27. Li, Y.; Zhao, H.; Duan, B.; Korpelainen, H.; Li, C. Effect of drought and ABA on growth,
500   photosynthesis and antioxidant system of *Cotinus coggygria* seedlings under two different light
501   conditions. *Environmental and Experimental Botany,* 2011*, 71*, 107-113.

502 28. Junglee, S.; Urban, L.; Sallanon, H.; Lopez-Lauri, F. Optimized assay for hydrogen peroxide
503   determination in plant tissue using potassium iodide. *American Journal of Analytical Chemistry,*
504   2014, *5*, 730.

505 29. Moron, M.S.; Depierre, J.W.; Mannervik, B. Levels of glutathione, glutathione reductase and
506   glutathione S-transferase activities in rat lung and liver. *Biochimica et Biophysica Acta (BBA)-*
507   *General Subjects,* 1979, *582*, 67-78.

508 30. Mukherjee, S.; Choudhuri, M. Implications of water stress-induced changes in the levels of
509   endogenous ascorbic acid and hydrogen peroxide in Vigna seedlings. *Physiologia Plantarum,*
510   1983, *58*, 166-170.

511 31. Bettaieb, I.; Hamrouni-Sellami, I.; Bourgou, S.; Limam, F.; Marzouk, B. Drought effects on
512   polyphenol composition and antioxidant activities in aerial parts of *Salvia officinalis* L. Acta
513   *Physiologiae Plantarum,* 2011, *33,* 1103-1111.

514   32.   Slinkard, K.; Singleton, V. L. Total phenol analysis: automation and comparison with manual
515   methods. *American Journal of Enology and Viticulture,* 1977, *28*, 49-55.

516   33.   Verma, J. Data analysis in management with SPSS software. Springer Science & Business Media,
517   2012.

518   34.   Quiroga, G.; Erice, G.; Aroca, R.; Chaumont, F.; Ruiz-Lozano, J. M. Enhanced drought stress
519   tolerance by the arbuscular mycorrhizal symbiosis in a drought-sensitive maize cultivar is related
520   to a broader and differential regulation of host plant aquaporins than in a drought-tolerant
521   cultivar. *Frontiers in plant science,* 2017, *8,* 1056.

522   35.   Abbaspour, H.; Saeidi-Sar, S.; Afshari, H.; Abdel-Wahhab, M. Tolerance of mycorrhiza infected
523   pistachio (*Pistacia vera* L.) seedling to drought stress under glasshouse conditions. *Journal of
524   plant physiology,* 2012, *169,* 704-709.

525   36.   Bárzana, G.; Aroca, R.; Bienert, G. P.; Chaumont, F.; Ruiz-Lozano, J. M. New insights into the
526   regulation of aquaporins by the arbuscular mycorrhizal symbiosis in maize plants under drought
527   stress and possible implications for plant performance. *Molecular Plant-Microbe Interactions*,
528   2014, 27, 349-363.

529   37.   Ashraf, M.;   Harris, P. Photosynthesis under stressful environments: an overview
530   *Photosynthetica,* 2013, *51,* 163-190.

531   38.   Yooyongwech, S.; Samphumphuang, T.; Tisarum, R.; Theerawitaya, C.; Chaum, S. Arbuscular
532   mycorrhizal fungi (AMF) improved water deficit tolerance in two different sweet potato
533   genotypes involves osmotic adjustments via soluble sugar and free proline. *Scientia
534   Horticulturae,* 2016, *198,* 107-117.

535   39.   Burghelea, C.; Zaharescu, D. G.; Dontsova, K.; Maier, R.; Huxman, T.; Chorover, J. Mineral
536   nutrient mobilization by plants from rock: influence of rock type and arbuscular mycorrhiza.
537   *Biogeochemistry,* 2015, 124, 187-203.

538   40.   Morte, A.; Navarro-Ródenas, A.; Nicolás, E. Physiological parameters of desert truffle
539   mycorrhizal *Helianthemun almeriense* plants cultivated in orchards under water deficit
540   conditions. *Symbiosis,* 2010, *52,* 133-139.

541   41.   Augé, R. M. Water relations, drought and vesicular–arbuscular mycorrhizal symbiosis.
542   *Mycorrhiza,* 2001, *11,* 3–42.

543   42.   Amiri, R.; Nikbakht, A.; Etemadi, N. Alleviation of drought stress on rose geranium
544   [*Pelargonium graveolens* (L.) Herit.] in terms of antioxidant activity and secondary metabolites
545   by mycorrhizal inoculation. *Scientia Horticulturae,* 2015, *197,* 373-380.

43. Wu, Q. S.; Zou, Y. N.; Huang, Y. M. The arbuscular mycorrhizal fungus *Diversispora spurca* ameliorates effects of waterlogging on growth, root system architecture and antioxidant enzyme activities of citrus seedlings. *Fungal ecology,* 2013, *6,* 37-43.

44. Sperdouli, I.; Moustakas, M. Interaction of proline, sugars, and anthocyanins during photosynthetic acclimation of Arabidopsis thaliana to drought stress. *Journal of plant physiology,* 2012, *169,* 577-585.

45. Sharma, S.; Villamor, J.G.; Verslues, P. E. Essential role of tissue-specific proline synthesis and catabolism in growth and redox balance at low water potential. *Plant Physiology,* 2011, *157,* 292-304.

46. Aroca, R.; Porcel, R.; Ruiz-Lozano, J. M. Regulation of root water uptake under abiotic stress conditions. *Journal of experimental botany,* 2012, *63,* 43-57.

47. Zhang, Z.; Zhang, J.; Huang, Y. Effects of arbuscular mycorrhizal fungi on the drought tolerance of Cyclobalanopsis glauca seedlings under greenhouse conditions. *New forests,* 2014, *45,* 545-556.

48. Bacon, C. W.; White, J. F. Functions, mechanisms and regulation of endophytic and epiphytic microbial communities of plants. *Symbiosis* 2016, *68,* 87-98.

49. Huang, Y. M.; Zou, Y. N.; Wu, Q. S. Alleviation of drought stress by mycorrhizas is related to increased root $H(2)O(2)$ efflu.x in trifoliate orange. *Scientific reports,* 2017, *7,* 42335.

50. Akram, N. A.; Shafiq, F.; Ashraf, M. Ascorbic acid-a potential oxidant scavenger and its role in plant development and abiotic stress tolerance. *Frontiers in plant science,* 2017, *8,* 613.

51. Sharma, P.; Jha, A. B.; Dubey, R. S.; Pessarakli, M. Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. *Journal of Botany,* 2012, *2012,* 1-26.

52. Li, J.; Wang, Y.; Pritchard, H.W.; Wang, X. The fluxes of H2O2 and O2 can be used to evaluate seed germination and vigor of *Caragana korshinskii*. *Planta,* 2014, *239,* 1363-1373.

53. Ruiz-Sánchez, M.; Aroca, R.; Muñoz, Y.; Polón, R.; Ruiz-Lozano, J. M. The arbuscular mycorrhizal symbiosis enhances the photosynthetic efficiency and the antioxidative response of rice plants subjected to drought stress. *Journal of plant physiology,* 2010, *167,* 862-869.

54. Gharibi, S.; Tabatabaei, B. E. S.; Saeidi, G.; Goli, S. A. H. Effect of drought stress on total phenolic, lipid peroxidation, and antioxidant activity of *Achillea* species. *Applied Biochemistry and Biotechnology,* 2016, *178,* 796-809.

578   **Figure legends**:

579   **Fig.1** Effect of mycorrhiza on plant growth at 100% field capacity (A), 60% field capacity (B),

580   and 40% field capacity (C); effect of different field capacity on root length of control (NM) plant

581   (D) and root length of mycorrhizal (M) plants (E); microscopic images of stomata from leaves of

582   finger millet control leaves in which stomata are open (F); closed stomata in leaves exposed to

583   drought stress (G); trypan blue stained finger millet plant roots (a) spores; (b) arbuscules; and (c)

584   intraradical hyphae (H).

585   **Fig.2** Effect of different soil water levels (100%, 60%, and 40% FC) and AM colonization on

586   chlorophyll content in finger millet leaves. Values are the means of three replications ± SD.

587   Mean with same letter are not significantly different (P < 0.05). Control (non-mycorrhizal NM)

588   and treated (mycorrhizal M), FC: Field capacity, WW: well-watered, MS: mild-stress, and SS:

589   severe-stress

590   **Fig. 3** Effect of water stress and AM on proline content in leaves (A) and roots (B), Total soluble

591   sugars (TSS) leaves (C) and roots (D), Malondialdehyde (MDA) content in leaves (E), and root

592   (F) in Finger millet seedlings. Values are the means of three replications ± SD. Mean with same

593   letter are not significantly different (P < 0.05). Control (non-mycorrhizal NM) and treated

594   (mycorrhizal M), FC: Field capacity, WW: well-watered, MS: mild-stress, and SS: severe-stress

595   **Fig.4** Effect of drought stress on hydrogen peroxide (H_2O_2) content leaves (A) and roots (B),

596   ascorbate-glutathione status in leaves (C) (E) and roots (D) (F) at different moisture levels

597   (100%, 60%, and 40% field capacity (FC). Values are the means of three replications ± SD.

598   Mean with same letter are not significantly different (P < 0.05). Control (non-mycorrhizal NM)

599   and treated (mycorrhizal M), FC: Field capacity, WW: well-watered, MS: mild-stress, and SS:

600   severe-stress

601   **Fig.5** Effect of drought stress on antioxidant metabolites in finger millet total phenol in leaves

602   (A) and roots (B), Total flavonoid in leaves (C) and roots (D) with and without AM inoculation.

603   Values are the means of three replications ± SD. Mean with same letter are not significantly

604   different (P < 0.05). FC: Field capacity, WW: well-watered, MS: mild-stress, and SS: severe-

605   stress

606

Control (F)　　　　　Drought (G)
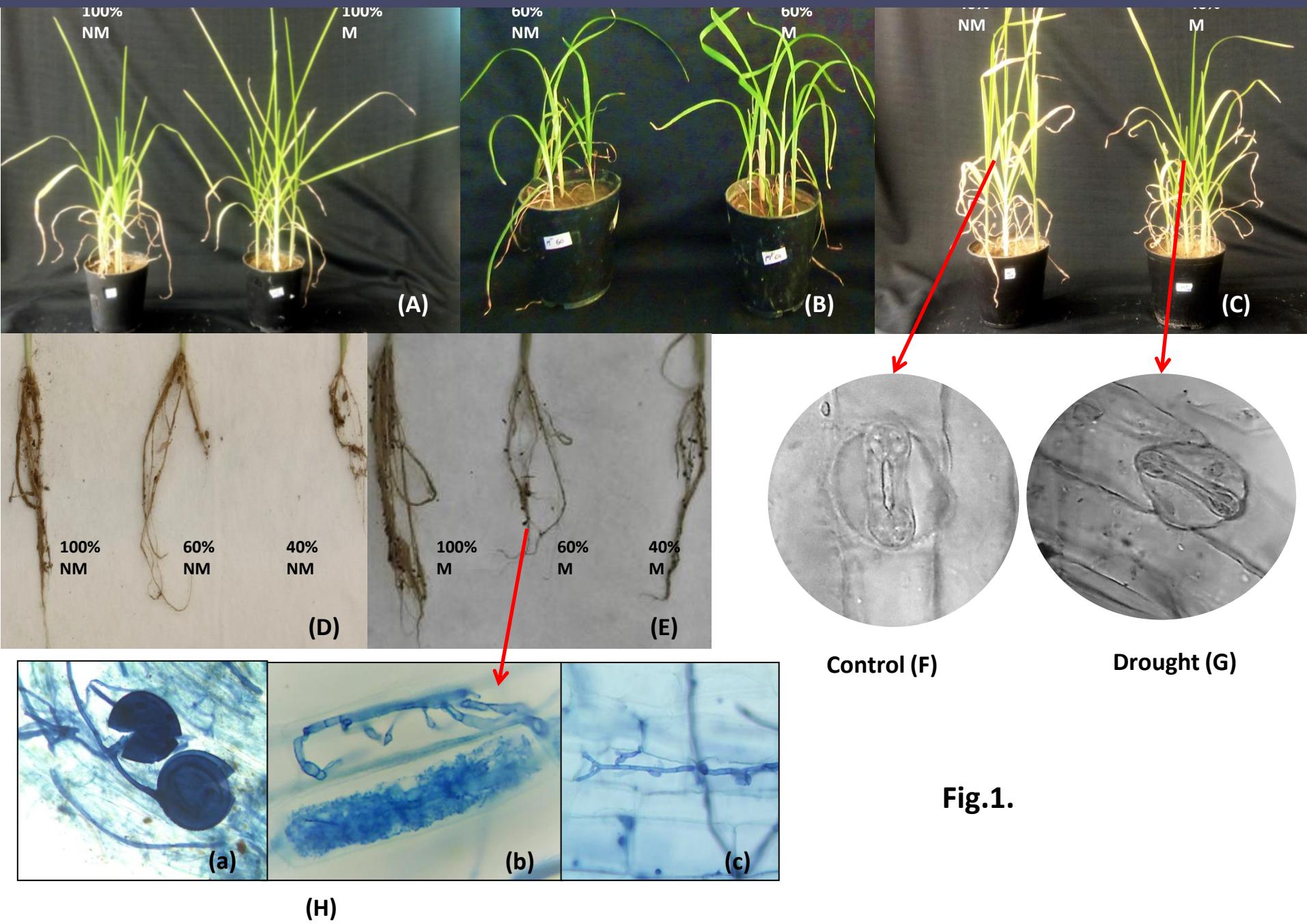
Fig.1.

Fig.2

Fig.3

(A)

(B)

(C)

(D)

(E)

(F)
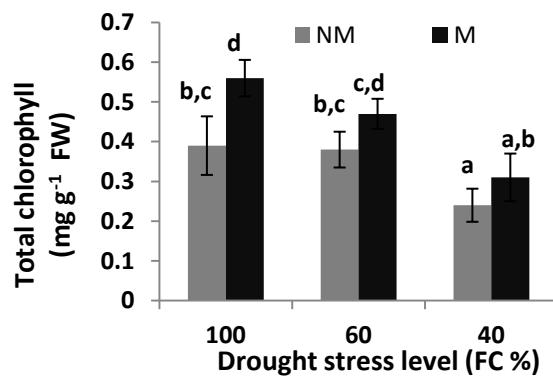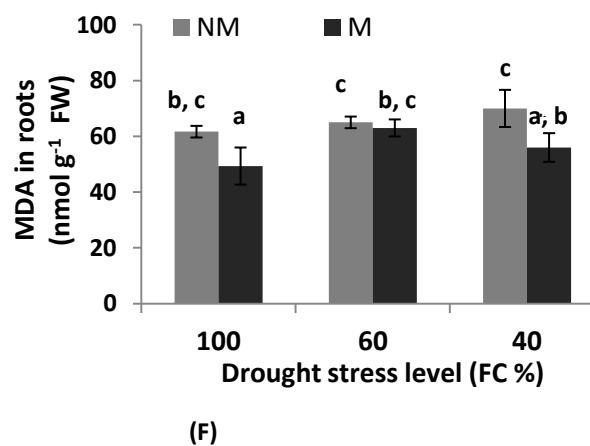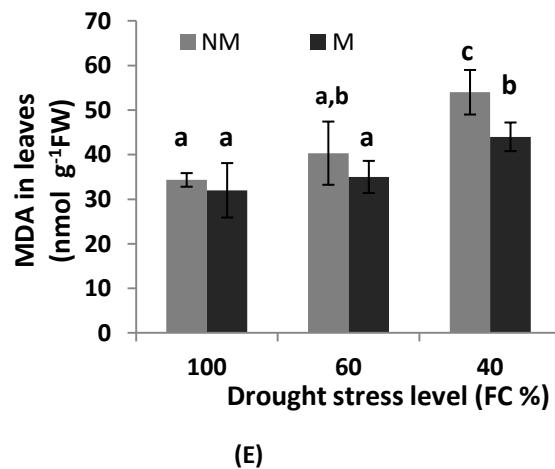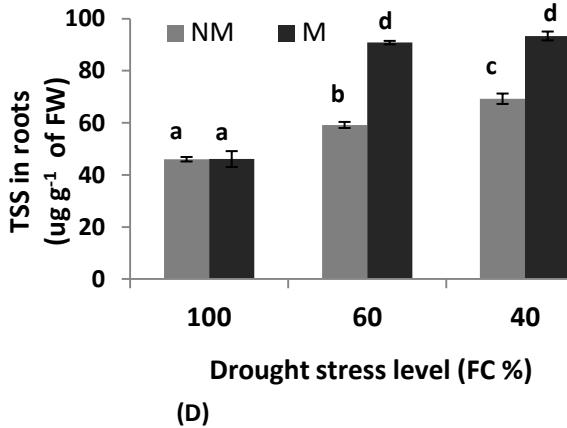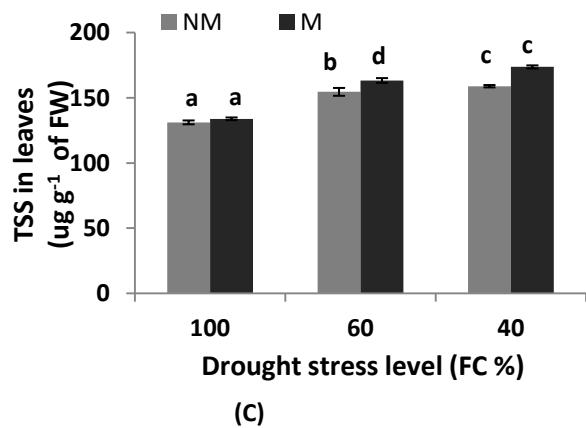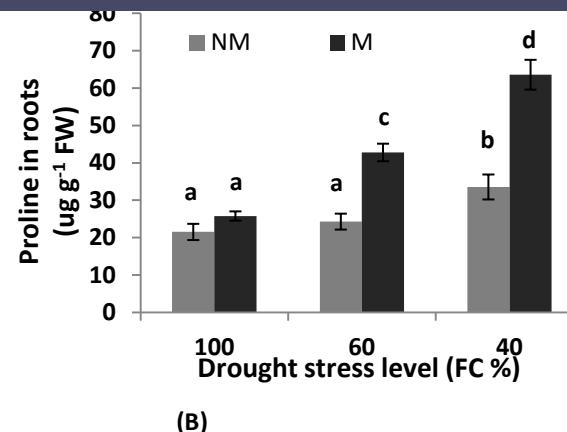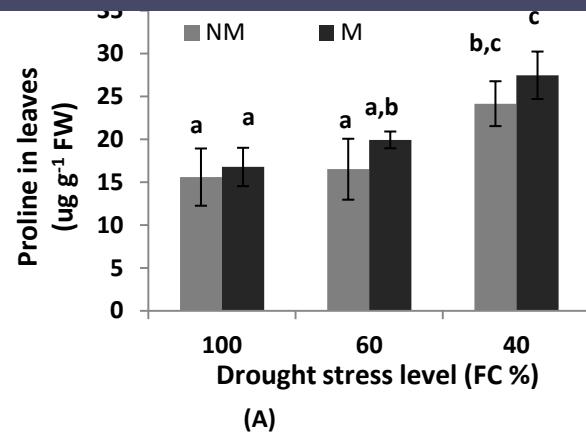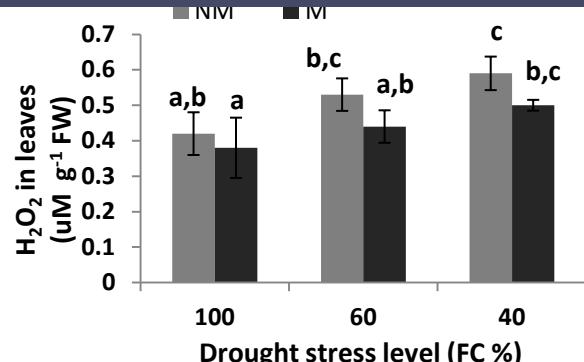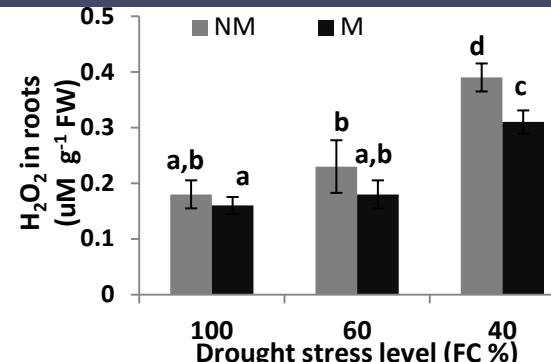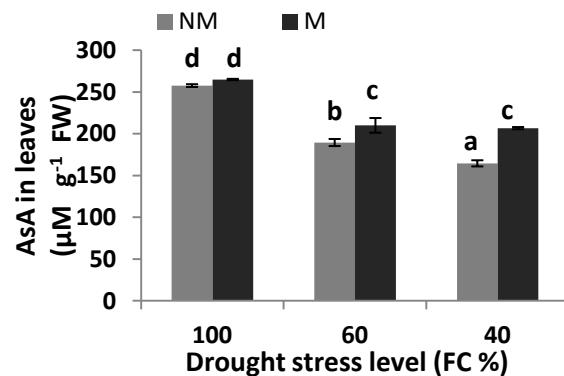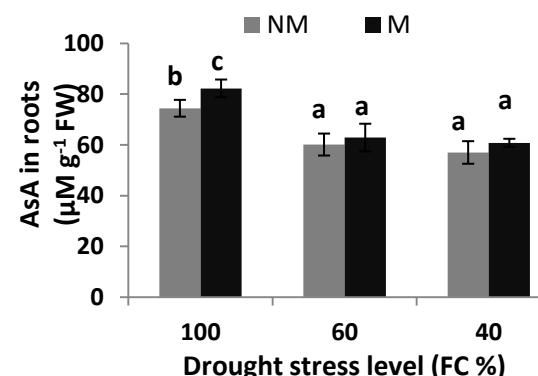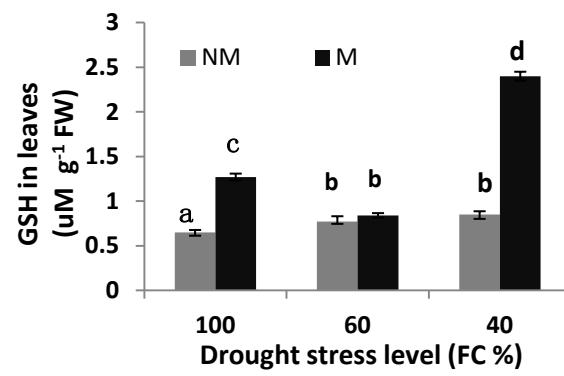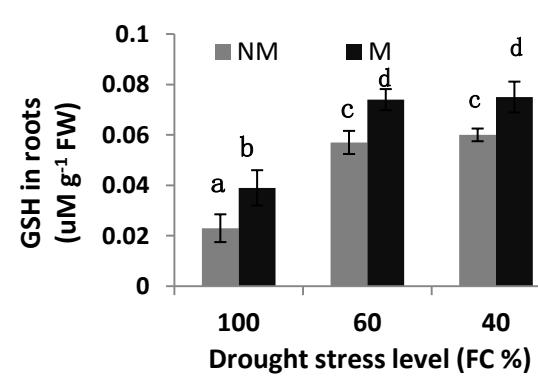
Fig. 4

Fig. 5
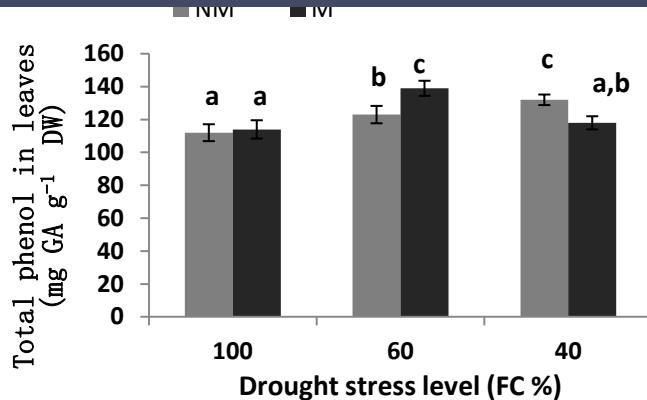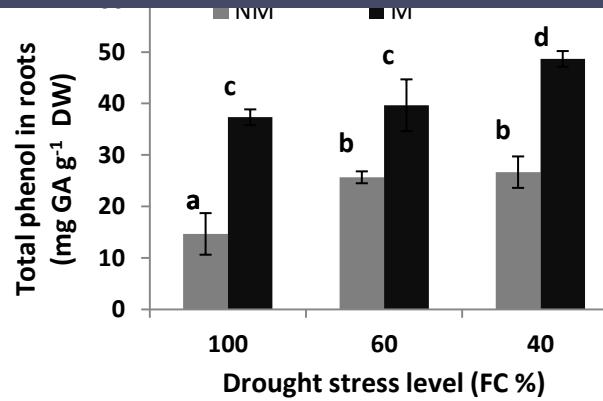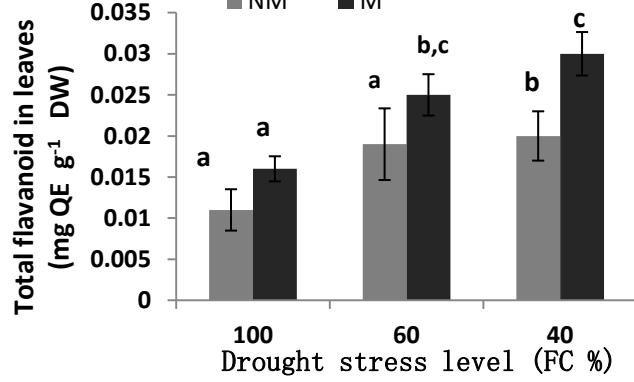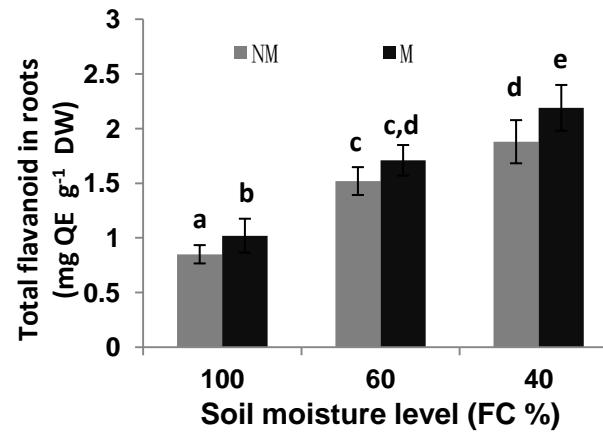
Table.1 : Effect of mycorrhiza on finger millet plant characteristics – shoot and root length, shoot and root fresh weight, shoot and root dry weight, number of leaves per plant , root colonization percentage (%) and phosphorus content, **under different moisture regimes**.

Values are the means of three replications ± SD, and same letters indicate that means are not significantly different (P < 0.05). **NM**: non-inoculated control and **M**: inoculated with mycorrhiza, **FC**: Field capacity, **WW**: well-watered, **MS**: mild-stress, and **SS**: severe-stress

Drought Treatment	Fungal Inoculants	Shoot length (cm)	Root length (cm)	Shoot fresh weight (gm)	Shoot dry weight (gm)	Root fresh weight (gm)	Root dry weight (gm)	No. of Leaves/ Plant	Phosphorus content (mg/g tissue)	Mycorrhizal Colonization (%)
100 % FC (WW)	NM	37.6 ± 0.36a,b	17.9 ± 0.56c	1.98 ± 0.11b	0.20 ± 0.03b	0.18 ± 0.02b	0.036 ± 0.02b,c	7.3 ± 0.57b,c	3.66 ± 0.14 c	0.00 ± 0.0a
	M	44.7 ± 2.08d	20.6 ± 1.21d	2.76 ± 0.18c	0.29 ± 0.01c	0.28 ± 0.02c	0.049 ± 0.003c	9.3 ± 0.57d	4.29 ± 0.44d	54.0 ± 5.29c
60 % FC (MS)	NM	36.4 ± 0.81 a,b	14.8 ± 0.47b	1.12 ± 0.13a	0.17 ± 0.03b	0.16 ± 0.01b	0.028 ± 0.004a,b	6.3 ± 0.57a,b	3.43 ± 0.20c	0.00 ± 0.0a
	M	40.5 ± 1.46c	15.6 ± 1.28b	1.73 ± 0.28b	0.21 ± 0.03b	0.26 ± 0.01c	0.03 ± 0.001a,b,c	8.0 ± 1.0c	3.83 ± 0.08c,d	48.0 ± 8.0c
40 % FC (SS)	NM	36.0 ± 1.25 a	12.1 ± 0.66a	0.90 ± 0.24a	0.10 ± 0.02a	0.13 ± 0.02a	0.015 ± 0.002a	5.3 ± 0.57a	1.57 ± 0.29a	0.00 ± 0.0a
	M	38.7 ± 0.87 b,c	14.0 ± 1.70a,b	1.15 ± 0.13a	0.17 ± 0.03b	0.17 ± 0.02b	0.018 ± 0.003a,b	6.6 ± 0.57b	2.27 ± 0.24b	25.3 ± 4.93b