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Isolation and Screening of Arbuscular Mycorrhizal Fungi for Drought Tolerance in Green Gram (*Vigna radiata* L.)

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Abstract

The climate change over the years has led to the occurrence of abiotic stresses in crops. Drought stress has become the main limiting factor for crop growth, development and production. Arbuscular mycorrhizal fungi (AMF) establish symbiotic interaction with 80 per cent of known land plants. It has good impact on plant growth, water absorption and protection from abiotic stresses. In this view, a total of forty AM fungal isolates were isolated from the drought prone areas of Karnataka. Isolates were mass produced by using maize as host plant. MPN method was carried out to determine the number of infective propagules in each isolate. The efficient isolates were screened for drought tolerance in green gram at different levels of field capacity (FC) (25, 50, 75 and 100% FC). Plants were grown in polythene bags for 30 days under greenhouse condition and based on performance of mycorrhizal parameters and plant parameters, the efficient drought tolerant isolates were selected.

Keywords : Arbuscular mycorrhizal fungi, Drought tolerance, Green gram, Field capacity

The climate change over the years has led to abiotic stresses around the world. Drought stress is one of the major constraints for pulse production which negatively affecting its growth and production. It interferes with photosynthesis, plant water status, enzyme structure, biomass distribution, reduces nutrient uptake and causes nutritional imbalance in the plant. Water deficit condition also affects osmotic balance that can lead to changes in cell wall permeability and increased leakage of solutes across membranes (Kumar and Verma, 2018). In addition, drought stress triggers the production of reactive oxygen species (ROS). ROS disrupts normal metabolism through oxidative damage to carbohydrates, protein synthesis, peroxidation of membrane lipids and cell death in plant tissues (Xie *et al*., 2018). These factors ultimately affect plant growth and reduce the yield.

Recently there has been a great interest in miti gating drought stress by the use of beneficial micro organisms. Plants depend on root-associated microrganisms to overcome various environmental stresses. A number of Plant Growth Promoting Bacteria (PGPB) can also mitigate the impact of abiotic stresses on plants through a process called induced systemic tolerance (IST), which includes bacterial production of cytokinins, production of antioxidants and degradation of ethylene precursor 1-aminocyclopropane 1-carboxylate (ACC) by bacterial ACC deaminase. (Glick *et al.,* 2007).

The association of plant roots with arbuscular mycorrhizal fungi (AMF) is the most abundant symbiosis for 80 per cent of land plants. The symbiosis between AMF and plants enhances the plant growth, nutrient uptake and stress resistance. (Bi *et al,* 2019; Gupta 2020). Arbuscular mycorrhizal symbiosis improves plant performance under drought stress through various mechanisms such as, water/nutrient uptake through extraradical hyphae, increased photosynthesis and stomatal conductance, production of glomalin for soil aggregate stability, protect the plant from oxidative damage by producing antioxidant

enzymes and regulate the metabolic activity through osmotic adjustment (He *et al*., 2019; Wu *et al*., 2019; Zou *et al*., 2019). Therefore, use of AM fungi for drought tolerance in plants would be a better option.

Green gram is one of the important leguminous crop in India with high nutritional value. It contains 23 per cent of protein and very low levels of oligosaccharides (Ihsan *et al*., 2013). India is the largest producer and consumer of green gram in the world, with an area of 4.58 M ha, production of 2.50 Mt and productivity of 548 kg / ha (Gopakumar *et al*., 2022). This crop is a source of food, animal feed and income in arid and semi-arid regions, the amount of water available to the crop is the major limiting factor for crop growth and yield. In India, about 68 per cent of net sown area (140 million hectares) is reported to be vulnerable to drought conditions. In legume crops, mycorrhizal fungi were found to increase the vegetative growth and seed yield under drought stress conditions (Hashem *et al.,* 2019, Musyoka *et al.,* 2020). In this view, the present study was aimed to isolate and screen AM fungi for drought tolerance in green gram.

Material and Methods

The present investigation on isolation and screening of arbuscular mycorrhizal fungi for drought tolerance in green gram (*Vigna radiata* L.) was conducted in the Department of Agricultural Microbiology, University of Agricultural Sciences, Gandhi Krishi Vignana Kendra (GKVK), Bengaluru.

Soil Sample Collection and Extraction of AM Spores from the Soil

The soil samples were collected from rhizosphere region of the plants in the drought prone areas of Karnataka (Table 1). From soil samples, AM spores were extracted by wet sieving and decanting method given by Gerdemann and Nicholson (1963). The sievings were collected from each sieve separately in beakers. The collected sievings for recovering AM spores were subjected to the sucrose density gradient centrifugation method (Ohms, 1957).

Funnel Technique

Morphologically similar spores were picked and a single spore as representation of each morphotype (as distinguished by colour or size) and those spores were brought in to funnel technique (Fig. 1a). Sand and soil were mixed in 1:1 proportion and the isolated spores were placed inside the mixture, ragi (*Finger millet*) seeds were sown and maintained for 45 days. Hoagland's solution was applied at weekly intervals (Nicolson, 1967).

Table 1

Geographical details of the soil sample collection

Place (District)	Location	Latitude (N)	Longitude (E)	Number of isolates
Chitradurga	JN Kote	14° 17' 8420''	76° 54' 0783''	5
	Vaddikere	14° 11' 6572''	76° 59' 7156''	4
	Ramajogihalli	14° 17' 5931''	76° 62' 7469''	6
	Sanikere	14° 18' 0814''	77° 64' 3092''	6
	Challakere	14° 30' 1398''	76° 61' 7712''	4
Tumkur	Sira	13° 73' 5175''	76° 89'1865''	2
	Changavara	13° 93' 9765''	76° 85' 3383''	4
Bellary	Marammanhalli	14° 29' 5285''	77° 12' 8695''	3
	Karur	14° 56' 5754''	75° 73' 6439''	2
Raichur	Chilkaragi	16° 05' 3083''	76° 76' 5729''	4
			Total	40

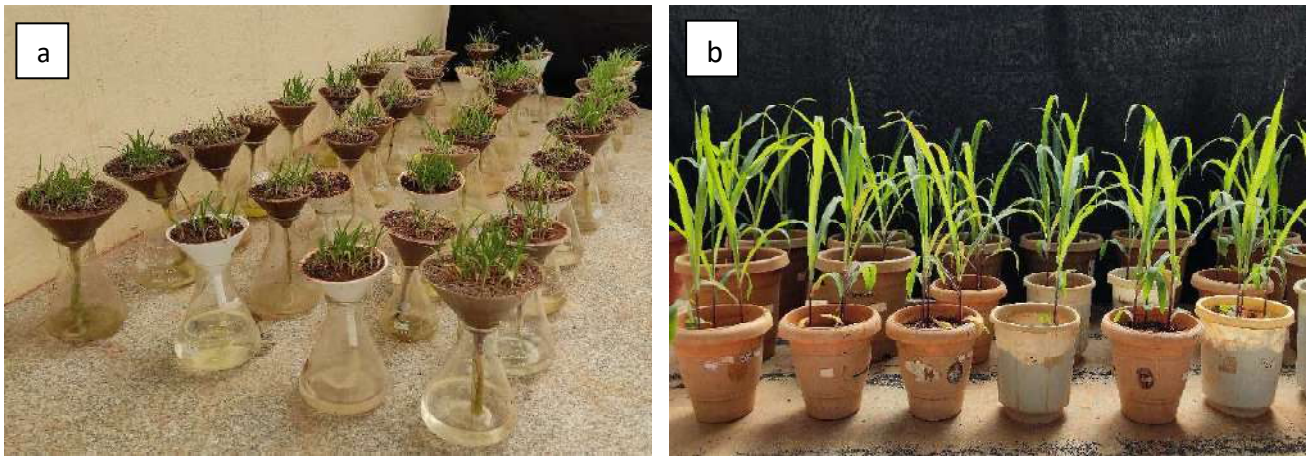

Fig.1 : (a) Funnel technique for isolation of fungal isolates; (b) Mass multiplication of AM fungal isolates

Mass Multiplication

Once the roots of plants started emerging from the stem-tips of the funnels, the contents were transferred to small plastic pots containing sterilized sand: soil substrate (1:1) after confirming the roots for AM colonization. The spores were multiplied up to 45 days using maize as host plant (Fig. 1b). After 45 days of growth, substrate containing spores, hyphae and root bits (cut into about 1 cm pieces) were used as inoculum.

Estimation of Infective Propagules of AM Fungi by MPN Method

The infective propagule (IP) numbers of the AM fungal isolates were estimated by the most probable number (MPN) method as described by Porter (1979). For this, 30 g of substrate based individual inoculum was mixed with 270g of sterile diluents *i.e.,* soilrite, perlite and vermiculite (each 90 g) in cups (4×2''). The mixture was mixed thoroughly to get 10^{-1} dilution. Then 30 g of this 10^{-1} dilution mixture was weighed and placed into another cup containing 270 g of sterile diluents to get 10^{-2} dilution. Likewise, up to 10^{-5} dilutions were made. Each dilution was distri buted into five plastic cups with five replications. Ragi (*Finger millet*) seeds were sown as host into each cup and watered regularly. After 45 days, the roots collected from each cup were stained with acid fuchsin and the presence or absence of mycorrhizal colonization for each replicate was determined under microscope. Counts of positive cups (those containing AM fungal structures) in different dilutions were used to calculate MPN values using the table.

Screening of AM Fungi for Drought Tolerance in Green Gram

Drought stress significantly reduces plant growth and development by inducing oxidative stress, disturbing membrane integrity, plant water relations, nutrient uptake and photosynthetic activity. AM fungal isolates were evaluated based on the ability of mycorrhizal fungi to colonize the plants and growth under drought stress in green house condition. To evaluate for drought stress tolerance, polythene bags were filled with soil and were inoculated with AM fungi prior to sowing of seeds. Seeds were sown and different field capacity levels (25, 50, 75 and 100% FC) were maintained during plant growth. Ability of AM fungal isolates to perform under drought stress conditions were recorded after 30 days of sowing.

Determination of AM Fungal Root Colonization

Staining of root segments was carried out as per the procedure proposed by Philips and Hayman (1970). Fresh root samples were collected after harvesting, roots were washed in tap water to remove the soil debris. Then the roots were cut into pieces of 1cm and the root segments were transferred to glass test tubes containing 10 per cent KOH solution and autoclaved at 121 °C for 15 minutes to soften the root pieces. The root pieces were then rinsed in water

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and 1 per cent HCl was added and kept aside to neutralize them for five minutes. The roots in the test tube were immersed in 0.05 per cent acid fuchsin in lacto glycerol staining solution for 24h. Then excess stain was removed by immersing the roots in lacto glycerol solution. The stained roots were arranged on grid-line plates and observed using microscope. Number of roots with AM fungal colonization was recorded and the per cent of mycorrhizal colonization was calculated by using formula.

$$\% \text{ root colonization} = \frac{\text{Number of root fragments +ve for AM fungal colonization}}{\text{Total number of root fragments observed}} \times 100$$

Total Biomass

The plant materials after harvest were dried in a hot air oven at 60 ^{o}C for 48 hours to a constant weight. Later the weight of shoot and root of each replication was weighed and expressed in grams (g) plant^{-1}.

Statstical Analysis

The data were subjected to two way Analysis of Variance by factorial complete block design and means were separated by the Duncan's Multiple Range Test (DMRT).

Results and Discussion

Isolation of AM Fungi from Soil

The soil samples were collected from rhizosphere region of the plants in the drought prone areas of Karnataka (Table 1). A total of forty AM fungal isolates were obtained from ten soil samples. This shows that rhizosphere soils of drought prone areas harbor a vast diversity of AM fungi. Earlier studies have revealed that in drought condition, highest external AM fungal development, fungal mycelium length, root mycorrhization rates and fungal diversity were observed (Benabdellah *et al.,* 2011; Calvo-Polanco *et al*., 2016). This diversity is due to AM fungi that evolved characteristics that are advantageous in dry environments. Some of the AMF can tolerate drought stress were extensively distributed and adapted to the soils of extreme environments. A number of investigations reported that *Glomus* species are dominant in arid and semi-arid regions and are able to grow under water deficit conditions (Opik *et al*., 2006 and Verma *et al*., 2008).

Estimation of Infective Propagules of AM Fungi by MPN Method

This method reveals all living infective propagules capable of colonizing with plant roots (Porter 1979). According to the fertilizer control order (FCO) specifications in India, AM fungal biofertilizers should contain 10 viable spores and 1200 infective propagules (IP) per gram of inoculum according to fertilizer fifth amendment order, July 2021(Agnihotri *et al*., 2022). The MPN method was carried out for forty AM fungal isolates to determine the number of infective propagules. Results revealed that the infective propagule number ranges from 120-1800 IP/g (Fig. 2). Among forty AM fungal isolates, twenty eight isolates showed more than 1000 IP/g and the isolates which showed less than 1000 IP/g were rejected. Total of twenty eight AM fungal isolates were selected for further analysis. Abinaya *et al*. (2018) reported that the MPN assay is considered to be the best for determining the quality of AM fungal inoculum.

Screening of AM Fungi for Drought Tolerance

Determination of per cent root colonization : Ability of AM fungal isolates to colonize with plant roots at different levels of field capacity (FC) were recorded by determining the per cent root colonization. The results revealed that the extent of the mycorrhizal colonization was significantly decreased in green gram roots as the level of the drought stress raised. Root mycorrhizal colonization was significantly higher under 100 per cent FC (without stress) than under 25, 50 and 75 per cent FC (with stress) conditions. The highest percentage of mycorrhizal colonization was recorded at 100 per cent FC with an average of 87.95 per cent and the lowest percentage of mycorrhizal colonization was recorded at 25 per

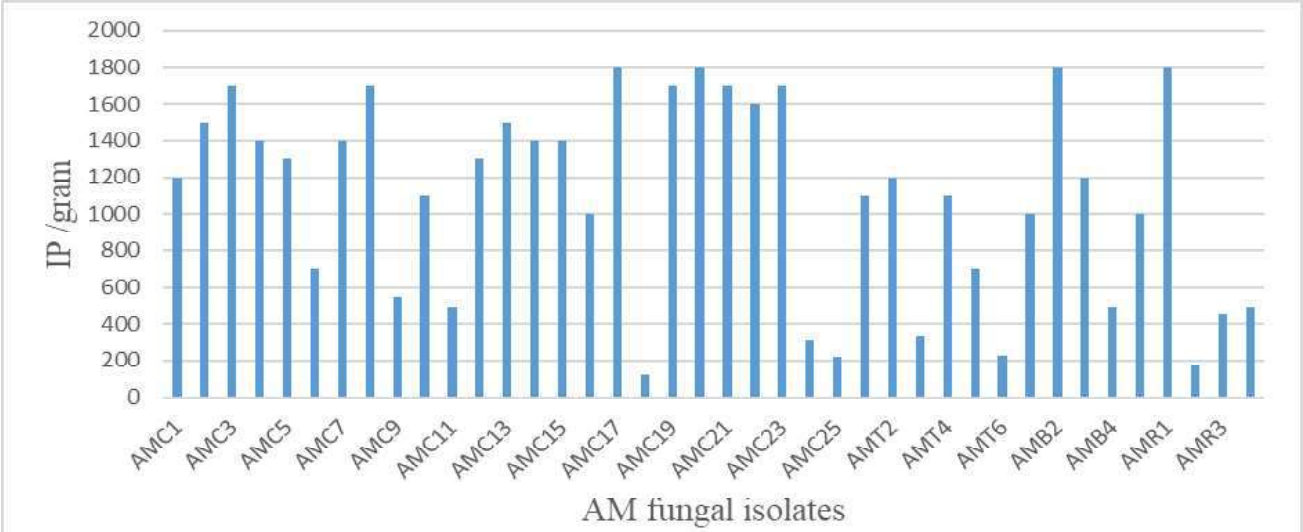

Fig. 2 : Infective propagule (IP) numbers in AM fungal isolates

cent FC with an average of 32.47, 50 and 75 per cent FC levels were recorded the root colonization with an average of 67.86 and 80.11 per cent respectively (Table 2). 50 per cent FC level significantly increased the per cent root colonization by 35.39 per cent as compared to 25 per cent FC. Likewise, From 50 to 75 per cent FC and 75 to 100 per cent FC levels increased the per cent root colonization by 12.25 and 7.84 per cent respectively.

These results depict that AM fungi are able to colonize the plant roots under water deficit conditions to protect the plants from stress but the extent of colonization differs with stress levels. The results were due to the influence of water availability on mycorrhizal colonization to plant roots. In water deficit condition, water shortage interferes with AMF development cycle, which affects the AMF spore germination, colonization capacity, sporulation and extra-radical hyphal elongation (Zhang *et al*., 2018).

Present results were similar with the result obtained by Abdel-Salam *et al.* (2018), who studied the levels of drought stress (100, 75, 50 and 25% FC) on mycorrhizal damask rose (*Rosa damascena* Mill.) plants and found a decrease in root colonization of damask plants by AMF under water deficit conditions from 83.67 to 54.17 per cent. The results were also consistent with the results of Musyoka *et al.* (2020), who performed the studies in green gram (*Vigna radiata* L.) and obtained the highest percentage of mycorrhizal colonization in watering regime R3 (irrigation after interval of 4 days) 36.93 per cent and the lowest percentage of mycorrhizal colonization of 31.73 per cent was recorded in watering regime R1 (irrigation after interval of 12 days). Treatment M3 (*Rhizophagus irregularis*) was recorded highest percentage colonization of 77.01 per cent.

In the present study, among twenty eight AM fungal isolates, AMC3 isolate was recorded the highest percentage mycorrhizal colonization at 75 and 100 per cent FC levels with 89.05 and 97.25 per cent respectively. AMB2 isolate was recorded the highest percentage mycorrhizal colonization at 25 and 50 per cent FC levels with 45.25 and 79.66 per cent respectively. At all the FC levels, AMB2 and AMC3 isolates were recorded highest percentage of root colonization with an average of 77.33 and 76.85 per

Fig.3 : Spores of AM fungal isolates (a) AMB2 (b) AMC3

TABLE 2

Mycorrhizal colonization of green gram plants grown under different field capacity levels

Isolates	Mycorhizal colonization (%)				
	25% FC	50% FC	75% FC	100% FC	Mean
AMC1	26.37 ^{l}	61.74 ^{k}	74.33 ^{ij}	82.50 ^{k}	61.23 ^{i}
AMC2	31.37 ^{h}	71.00 ^{gh}	82.1 ^{de}	89.08 ^{f}	68.39 ^{e}
AMC3	44.11 ^{b}	77.00 ^{b}	89.05 ^{a}	97.25 ^{a}	76.85 ^{a}
AMC4	29.39 ^{ij}	66.96 ^{i}	77.09 ^{h}	87.28 ^{g}	65.18 ^{f}
AMC5	36.29 ^{f}	72.59 ^{ef}	82.08 ^{def}	92.34 ^{bcde}	70.82 ^{d}
AMC7	25.86 ^{l}	59.65 ^{l}	73.73 ^{j}	77.26 ^{n}	59.12 ^{jk}
AMC8	39.28 ^{d}	75.05 ^{m}	84.13 ^{c}	91.40 ^{e}	72.46 ^{c}
AMC10	28.82 ^{jk}	64.4 ^{lj}	76.66 ^{h}	84.94 ^{i}	63.71 ^{g}
AMC12	30.23 ^{i}	66.03 ^{i}	76.90 ^{h}	87.98 ^{g}	65.28 ^{f}
AMC13	32.15 ^{h}	70.83 ^{gh}	80.99 ^{g}	92.11 ^{cde}	69.02 ^{e}
AMC14	31.90 ^{h}	70.53 ^{h}	80.53 ^{g}	91.32 ^{e}	68.57 ^{e}
AMC15	34.15 ^{g}	72.60 ^{ef}	82.33 ^{d}	93.40 ^{b}	70.62 ^{d}
AMC16	21.95 ^{o}	58.53 ^{mnop}	71.43 ^{kl}	78.82 ^{m}	57.68 ^{l}
AMC17	40.93 ^{c}	73.95 ^{cd}	84.72 ^{c}	93.10 ^{bc}	73.18 ^{bc}
AMC19	41.86 ^{c}	73.48 ^{de}	85.22 ^{bc}	92.32 ^{bcde}	73.22 ^{bc}
AMC20	37.84 ^{e}	73.33 ^{de}	85.11 ^{c}	92.18 ^{cde}	72.12 ^{c}
AMC21	37.06 ^{ef}	74.97 ^{c}	89.00 ^{a}	91.6 ^{de}	73.17 ^{bc}
AMC22	36.06 ^{f}	71.78 ^{fg}	80.77 ^{g}	89.20 ^{f}	69.45 ^{e}
AMC23	41.22 ^{c}	74.60 ^{c}	87.92 ^{a}	92.73 ^{bcd}	74.11 ^{b}
AMT1	23.17 ^{no}	58.41 ^{nopq}	74.15 ^{ij}	80.64 ^{l}	59.09 ^{jk}
AMT2	23.37 ^{n}	59.02 ^{mn}	73.86 ^{j}	83.36 ^{jk}	59.90 ^{j}
AMT4	27.98 ^{k}	64.16 ^{j}	75.09 ^{i}	84.02 ^{ij}	62.81 ^{gh}
AMB1	28.46 ^{jk}	60.66 ^{l}	74.33 ^{ij}	86.02 ^{h}	62.37 ^{h}
AMB2	45.25 ^{a}	79.66 ^{a}	88.08 ^{a}	96.33 ^{a}	77.33 ^{a}
AMB3	24.27 ^{mn}	57.41 ^{oq}	74.04 ^{ij}	80.49 ^{l}	59.05 ^{jk}
AMB5	26.04 ^{l}	55.84 ^{r}	71.03 ^{l}	82.35 ^{k}	58.81 ^{jkl}
AMR1	41.11 ^{c}	76.38 ^{b}	86.25 ^{b}	93.42 ^{b}	74.29 ^{b}
AMR2	24.76 ^{m}	58.56 ^{mno}	72.42 ^{k}	79.03 ^{m}	58.69 ^{kl}
Mean	32.47 ^{d}	67.86 ^{c}	80.11 ^{b}	87.95 ^{a}	

Note : Means with same superscrit, in a column do not differ significantly at P=<0.05 as per Duncan Multiple Range Test (DMRT); FC-field capacity

cent respectively. This is due the ability of plant species to be colonized by specific group of AM fungi (Grman, 2012).

Total Plant Biomass

AM fungal isolates were evaluated based on the plant performance of drought tolerance at different levels of field capacity. The results revealed that total dry biomass of plant had significantly increased with the level of reduction in drought stress. As the level of drought stress reduced from 25 FC to 100 per cent FC, the average values of total dry biomass of plant ranged from 0.62 to 1.21 g/plant respectively. From 25 to 100 per cent FC levels, two fold increase in total biomass of plant was observed. 50 per cent FC was recorded the total biomass of plant with an average of 0.86 g/plant and 75 per cent FC was recorded the total biomass of plant with an average of 1.09 g/plant (Fig. 4).

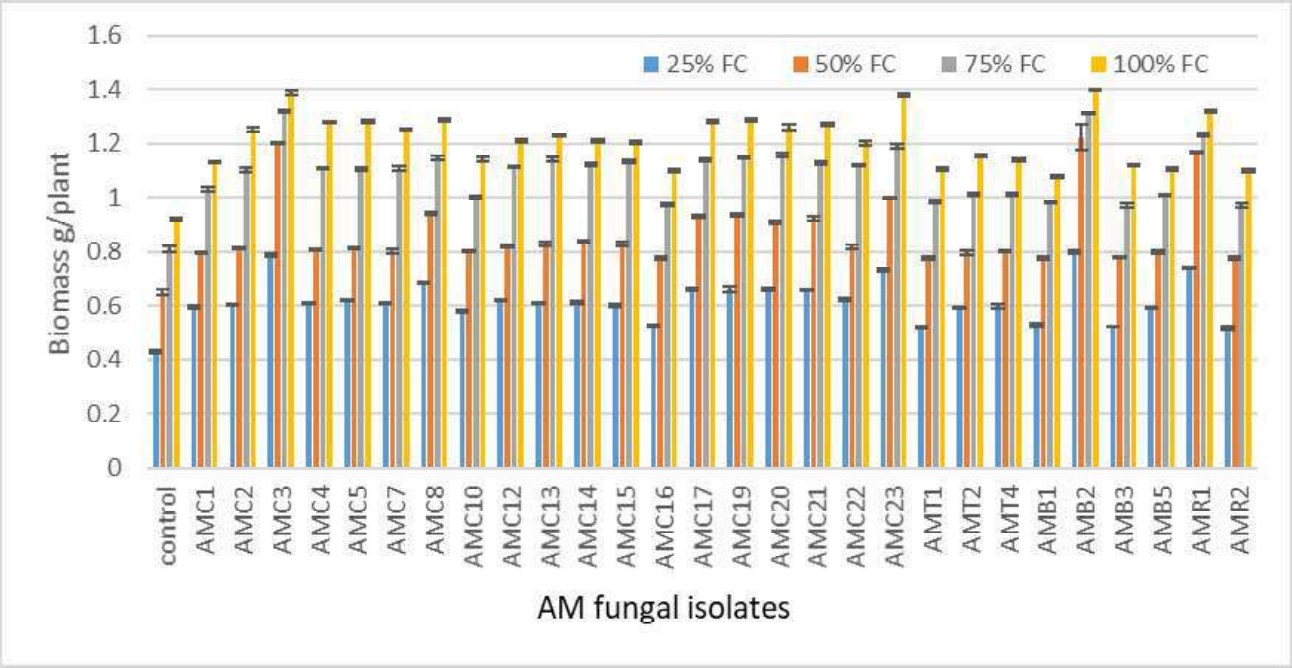

Fig. 4 : Total biomass of green gram plants as influenced by different AM fungi grown under different field capacity levels

These results were due to higher percentage of mycorrhizal colonization under well watered condition campared to water stress conditions and the higher AMF colonization has positive effect on the plant growth traits. Under well watered condition, the biomass of plant was 3.29 g/plant while under drought stress condition biomass of plant was 2.75 g/plant as reported by Zhang *et al*. (2020) in trifoliate orange (*Poncirus trifoliate*) which support the results of present study.

AMF inoculated plants showed maximum biomass of plant compared to uninoculated control (0.70g/plant) under different field capacity (25, 50, 75 and 100% FC) levels. Among twenty eight AM fungal isolates, AMB2 and AMC3 isolates recorded highest biomass of plant with an average of 1.165 and 1.16 g/plant. These results were due to the AM fungal colonization to plant roots under water deficit conditions, can able to protect the plant and increase the growth of plant through various mechanisms such as, water/nutrient uptake through extraradical hyphae, increased photosynthesis, stomatal conductance, root hydraulic conductivity and root architecture, protect the plant from oxidative damage by producing antioxidant enzymes and regulate the metabolic activity through osmotic adjustment, root hydraulic conductivity and root architecture (He *et al*., 2019 and Wu *et al*., 2019). The effect of AM fungal colonization in increasing the plant performance has been well documented by many researchers in many plant species (Nagarathna *et al*., 2013; Musyoka *et al*., 2020; Azizi *et al*., 2021 and Jabborova *et al*., 2021) augment these results.

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