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Mycorrhizal mass multiplication in trap culture method in *Cynodon* sp. and evaluation of growth parameters

Ray, A., Das, N. and Saha, A.*

Plant Pathology Laboratory, Department of Botany, University of North Bengal, Raja Rammohanpur, Darjeeling, West Bengal, India 734103.

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Abstract In the present study, one such method known as the trap culture method is utilized for the mass multiplication of mycorrhiza in *Cynodon dactylon,* and it is found to be successful for the mass production of the mycorrhizal spores. Mycorrhiza increased P and K in soil following application of mycorrhizal spore preparation. The mycorrhizal spores improved plant growth which was manifested by vigorous growth in treated plants. Biochemical analysis of twigs of *C. dactylon* revealed that protein content, reducing sugar, PAL content, and Chlorophyll content increased but total phenol and total sugar content reduced. Root colonization percentage, spore quantification, and soil health increased in the treated sets as compared to the controlled sets. Thus, mass multiplication of mycorrhiza in the trap culture method improved plant health as well as soil health in an eco-friendly manner.

Keywords: Mycorrhiza, *Cynodon* sp.*,* Mass multiplication, Trap culture

Introduction

Mycorrhiza is one type of soil-inhabiting fungus that forms a mutually beneficial symbiosis with plant roots. In today's world, about 80% of plants are associated with mycorrhiza (Smith and Read, 2010). It belongs to the phylum Glomeromycota (Gianinazzi, 1986). Mycorrhiza is not host-specific but shows host preference. According to their colonization process, there are two major types Ectomycorrhiza and Endomycorrhiza. Mycorrhiza accumulates essential nutrients like N, P, K, Zn, Mn, Co, Fe, etc. from soil and subsequently transfers them to the host plant. In exchange, the host provides food and shelter to the colonizing fungus. It is highly effective in increasing the root surface area which helps the plant to survive in adverse conditions and also increases the growth and yield of plants (Clark and Zeto, 2000; Goussous and Mohammad, 2009; Cozzolino *et al*., 2010).

Cynodon dactylon is widely cultivated in warm climates all over the world between about 30° S and 30° N latitude, and it gets between 625 and 1,750 mm (24.6 and 68.9 in) of rainfall a year (or less, if irrigation is available). In any ecological background, grasses play a vital role as they

* **Corresponding Author:** Saha, A.; **Email:** asahanbu@yahoo.co.in

are the pioneer of any fertile land as well as the main source of food and shelter for grazing animals and insects. It also maintains soil structure and prevents soil erosion. So, it has a huge role in maintaining the flow of the ecological pyramid. In the past few years, global temperatures have changed sharply due to climate change and have severely affected the production of crops as well as it also impacted the growth and activity of symbiotic arbuscular mycorrhizal (AM) fungi (Kytoviita and Ruotsalainen, 2007). The purpose was to evaluate the effect of a mixture of mycorrhizal spores (*Glomus, Gigaspora, Acaulospora, Scutellospora,* etc.) on growth, photosynthetic pigments, sugar content, phenol production, and protein content in the leaves of *Cynodon* under the field to further understanding the effect of mycorrhizae in ecological perspective.

Material and methods

Soil and root sample collection from rhizosphere

The upper 5-10 cm part of the rhizospheric soil was collected without hampering the plant root. A total of Three replicas of the rhizospheric soil were taken from the host plant randomly. Each soil sample was taken in individual plastic bags and labeled properly. For observing root colonization some fine roots were separately collected in plastic bags and tagged.

Wet sieving and decanting

Spores of arbuscular fungi from the rhizospheric soil of plants were isolated by using the wet sieving and decanting method described by Gerdemann and Nicolson (1963). In 1.5 litre of water 150ml of soil was suspended and kept for 10 minutes to settle down the heavy debris then the liquid was decanted through sieves of decreasing size (BS 60, BS 80, BS 100, BS 150, and BS 200). The spores were washed with water and passed through the sieving sets to get the different sizes of spores which will help in identification.

Spore quantification

A filter paper was folded in 4 equal parts and each part was marked as 1, 2, 3, and 4. The spores on the filter paper were spread using a brush and the number of spores present in each part was counted under a simple microscope finally total number of spores was measured by adding the number of spores present in each fold. This process was repeated five times in the case of collected rhizospheric soil samples.

Clearing and staining root specimens for the experiment

The staining solution was used 20% KOH: 20 g of KOH in 100 ml of distilled water. 5% HCL: 5 ml of conc. HCL in 95 ml of distilled water, and 0.05% Trypan Blue: 0.05g Trypan Blue in 100 ml of Aceto-glycerol.

Clearing and staining root specimens

Soil-free washed root samples were stained following the method of Phillips and Hayman (1970) with certain modifications. Sufficient KOH and staining solution were added so that roots were not tightly clumped together while processing. To ensure uniform staining the roots were chopped into smaller (1-1.5 cm) segments. More than 200 root segments were taken for the staining procedure. Root specimens were first washed thoroughly under running tap water and placed in a beaker containing 20% KOH solution for about 45 minutes. The concentration of KOH and time of incubation of roots varied depending upon the age and tenderness of the roots. The KOH solution was decanted off and the roots were rinsed well in a beaker using tap water until any brown color came out. Roots contain a high amount of phenols, so the root samples need to be white or transparent after KOH treatment. The roots were dipped in 5% HCL and soaked for 15-30 min. The solution was then decanted off and washed. The roots were then incubated with a staining solution (0.05% trypan blue in lactophenol) and kept overnight for staining.

Root colonization percentage

The stained roots were observed under the compound microscope following the standard method to examine the number of vesicles, arbuscules, coiled hyphae; inter-reticular vesicles (IRVs), and infection threads. Root colonization percentage was measured using the following formula of Kullu and Behera, 2016.

$$\%\ Colonization = \frac{Total\ number\ of\ colonized\ root\ segments}{Total\ number\ of\ root\ segments\ examined} \times 100$$

Field preparation

The experiment was set up in the field with sufficient irrigation. Two plots (6 ft x 6 ft dimension) were prepared, one for the experimental set and another for the control set in the garden of the Department of Botany, University of North Bengal. The fields were prepared by plucking out unwanted plants and weeds. Thereafter ten kg of soil from the top layer of each plot was cut, autoclaved, and mixed with dry cow dung powder (1:1 ratio). The mixture was poured into the plots from where it was cut. Before

pouring the cut area soil was left for 2-3 days for sun-drying. The cut area was also subjected to fumigation by formaldehyde. A bunch of sterilized young *Cynodon* twigs was then collected from the nursery and planted onto the prepared field. The *Cynodon* seedlings were grown in both fields and after 45 days the treated field was inoculated with the mycorrhizal spores and the control field was kept blank.

Soil quality assessment

The rhizospheric soil from the *Cynodon-infested* field was taken for quantification of soil pH, nitrogen, phosphorus, and potassium concentration before and after inoculation. The soil quality was assessed by the Department of Tea Science, University of North Bengal.

Spore inoculation

Spores (approximately 250 number) were mixed with 1 litre of water to make a spore suspension. The spore suspension was applied in the 45-day-old *Cynodon* plants while the 'Control' set remained uninoculated.

Plant growth assessment

Morphological parameters

The effects of AMF (Arbuscular Mycorrhizal Fungi) i.e *Glomus* sp., *Gigaspora* sp., *Acaulospora* sp., and *Scutellospora* sp. as a biofertilizer were measured in terms of different growth parameters like plant height, internodal length, number of branches, the total number of leaves, leaf length, leaf breadth, fresh weight of shoot, dry weight of shoot, the total number of root branches, fresh weight of root, dry weight of root, total biomass, maximum root length and girth of the plant. The growth parameters were measured after every 15 days post-inoculation up to 45 days.

Biochemical parameters

The effects of AMF (Arbuscular Mycorrhizal Fungi) i.e *Glomus* sp., *Gigaspora* sp., *Acaulospora* sp., and *Scutellospora* sp. as a biofertilizer were also measured in terms of different biochemical parameters like- total chlorophyll reducing sugar, total sugar, total phenol, total protein and phenylalanine ammonia-lyase content (Sadasivam and Manickam, 1992). The growth parameters were measured after 45 days post-inoculation.

Results

The AMF (Arbuscular Mycorrhizal Fungi) i.e *Glomus* sp., *Gigaspora* sp., *Acaulospora* sp., and *Scutellospora* sp. spores were mass

multiplied in the rhizosphere of *Cynodon* sp. Following the multiplication of mycorrhizae, its quantity, diversity of the spores; root colonization percentage, and their effect on plant growth was estimated.

Quantification of AMF spores in Cynodon rhizosphere

The number of spores of *Glomus* sp., *Gigaspora* sp., *Acaulospora* sp., and *Scutellospora* sp. per 150 g of rhizospheric soil of the AMF inoculated *Cynodon* plants were compared with the uninoculated ones up to 45 days post-inoculation. From the results, it was shown that the spore count in the rhizosphere had significantly increased in the AMF inoculated (treated) set compared to the uninoculated control set (Figure 1).

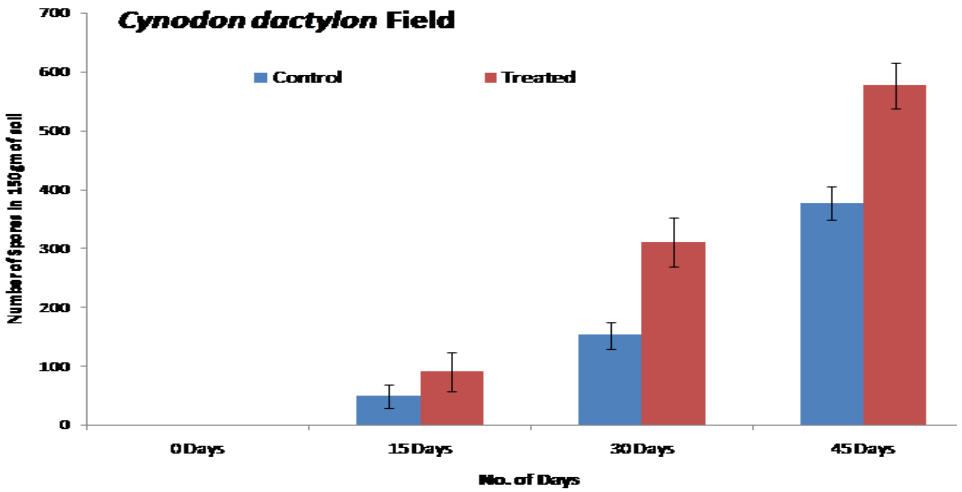

Figure 1. Number of spores per 150 g of rhizospheric soil in AMF inoculated (treated) and uninoculated (control) in field condition

Diversity of AMF spore after inoculation in Cynodon rhizosphere

All four major genera- *Glomus, Gigaspora, Acaulospora,* and *Scutellospora* as AM fungi were observed in the rhizosphere of *Cynodon* after 45 days of inoculation. Out of which *Glomus mosseae* was found to be the most frequently occurring AM fungal species, followed by *Gigaspora, Acaulospora,* and *Scutellospora* (Figure 2).

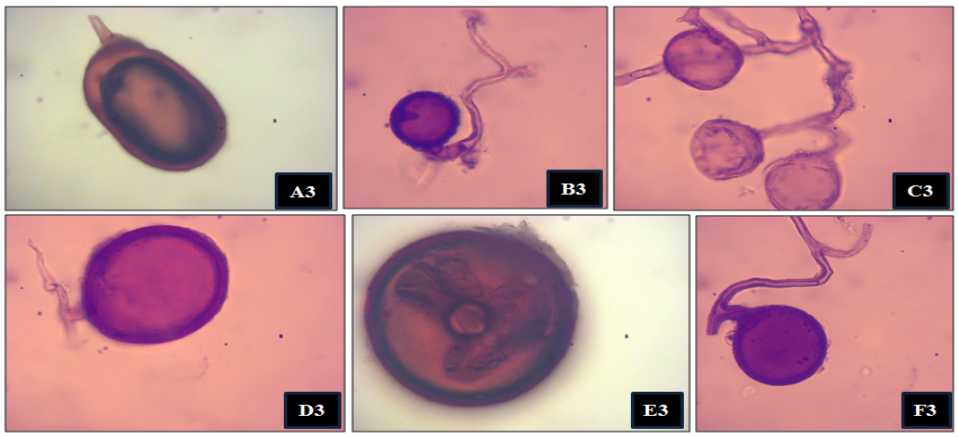

Figure 2. AMF spores from *Cynodon* plant rhizosphere under light microscope: A3, C3, D3, F3: *Glomus* sp.; B3: *Gigaspora* sp.; E3: *Scutellospora* sp. The size of the *Glomus* spores was measured at 90-140 µm, *Gigaspora* spores were measured at 260-350 µm, and *Scutellospora* spores were measured at 150-240 µm

Percentage of root colonization after mass multiplication of AMF spores in Cynodon rhizosphere

The percentage of root colonization indicated the successful establishment of symbiosis between mycorrhizae and the host plant. In the present study, it gradual increase in root colonization up to 45 days post-inoculation was observed in the *Cynodon* plants inoculated with AMF spores compared to the uninoculated control (Figure 3). Different kinds of symbiotic structures had also been observed in the inoculated ones (Figure 4).

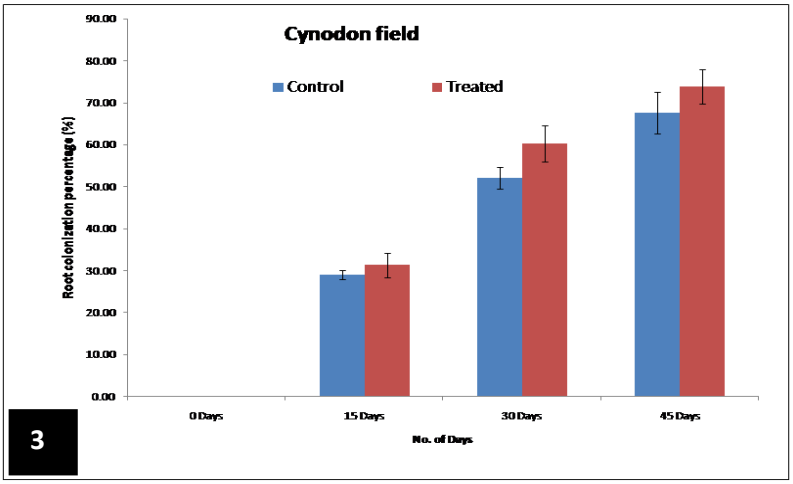

Figure 3. Root colonization percentage of AMF spores within *Cynodon* sp. in control and treated sets up to 45 days in the field

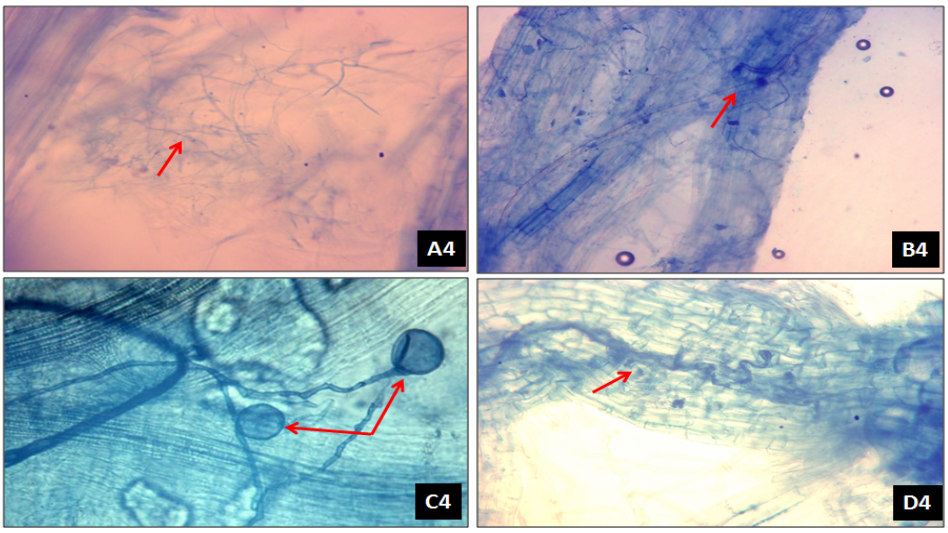

Figure 4. Different kinds of symbiotic structures of AMF after successful root colonization images in *Cynodon* A4: ectomycorrhizal hyphae; B4: endomycorrhizal hyphae; C4: vesicles; D4: hyphae penetrated the cortical cells

Assessment of plant growth after mass multiplication of AMF spores in Cynodon rhizosphere

Morphological parameters

Different morphological parameters (i.e., plant height, internodal length, the number of branches, the total number of leaves, leaf length, leaf breadth, fresh weight of shoot, dry weight of shoot, the total number of root branches, fresh weight of root, dry weight of root, total biomass, maximum root length, and girth) increased after mass multiplication of AMF spores in *Cynodon* rhizosphere in comparison to control (Figures 5 and 6). This indicated a significant role of AMF symbiosis in the improvement of plant health in the field (Figure 7).

It was found that in treated plants plant height increased proportionally with time in the treated set. But internodal length, branch number, and leaf number gradually increased up to 30 days and gradually decreased after that. Leaf length and leaf breadth increased up to 30 days and then gradually decreased. Root branch no. they were increased with time. Root dry biomass and root fresh biomass decreased after 30 days in both treated and control sets. Shoot dry biomass, shoot fresh biomass, total biomass and maximum root length increased with time. However, in treated plants, these parameters had higher values compared to the control plants. Plant girth remains constant in the case of control plants but in the case of treated plants, it increases with time.

Biochemical parameters

The result of biochemical tests showed that total protein content, reducing sugar, PAL, and chlorophyll increased. In contrast, total phenol content and total sugar decreased in treated plants compared to control plants (Figure 8).

Soil health assessment after mass multiplication of AMF spores in *Cynodon* rhizosphere

From different nutrient contents present in the soil of *Cynodon* rhizosphere it was evident that before the application of mycorrhiza (at the time of field preparation) in the field the soil pH, organic carbon, nitrogen, K_2O, and P_2O_5 content was lower. After 45 days of AMF inoculation the concentration of K_2O in the control and treated increased from an initial 9 ppm to 22 ppm in the control set and 40 ppm in the treated set. Similarly, after 45 days of AMF spore inoculation, the concentration of P_2O_5 in control and treated increased from an initial 7 ppm to 15 ppm and 25 ppm respectively (Table 1).

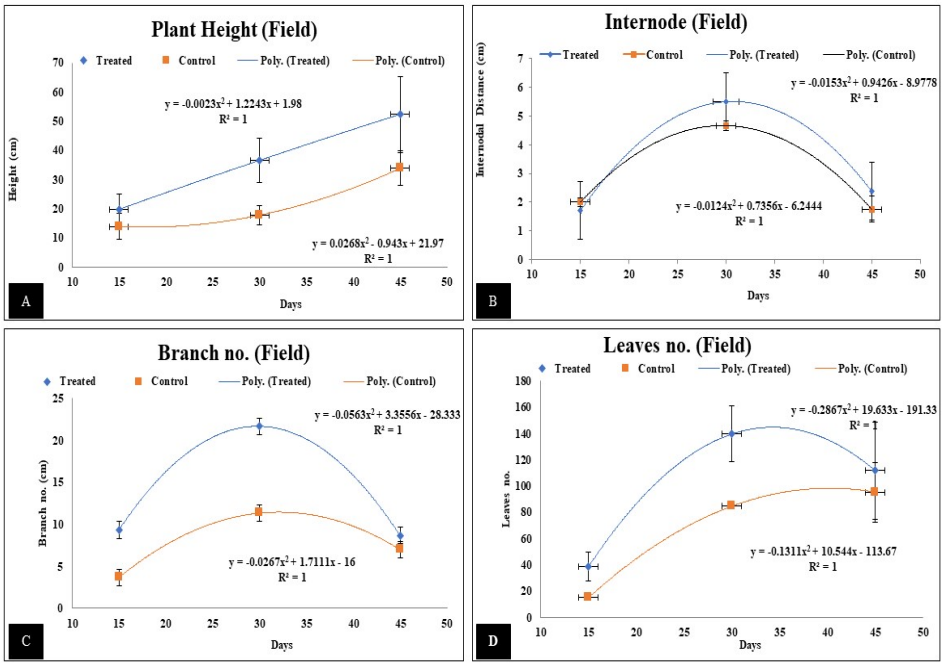

Figure 5. Effect of mass multiplication of AMF spores in *Cynodon* rhizosphere on **(A)** Height of plants **(B)** internodal length **(C)** branch number and **(D)** leaf number after inoculation in the field

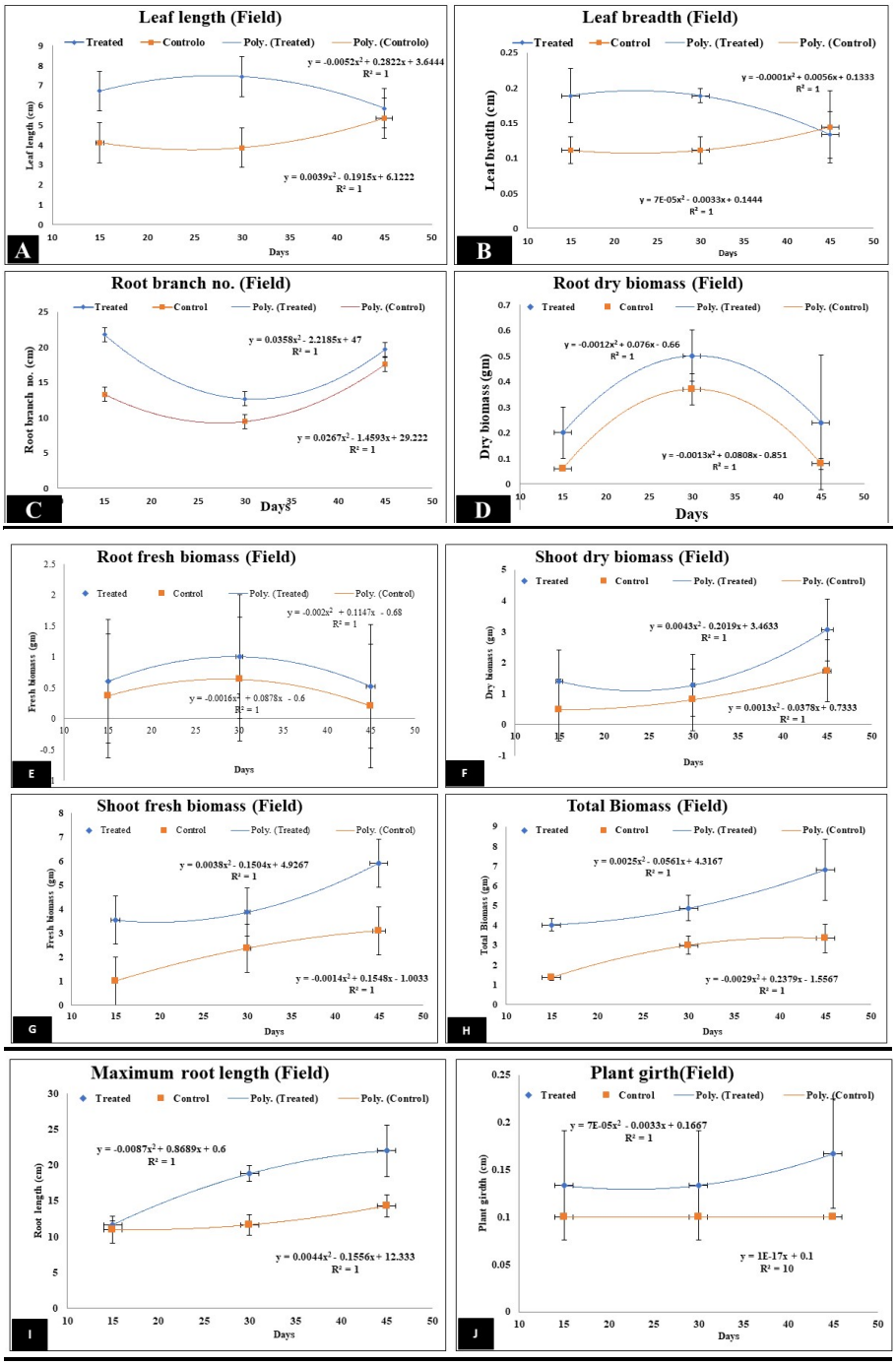

Figure 6. Effect of mass multiplication of AMF spores in *Cynodon* rhizosphere on (**A**) leaf length (**B**) leaf breadth (**C**) root branch no. (**D**) root dry biomass **(E**) root fresh biomass (**F**) shoot dry biomass **(G**) shoot fresh biomass **(H**) total biomass **(I**) maximum root length and **(J**) plant girth after inoculation in the field

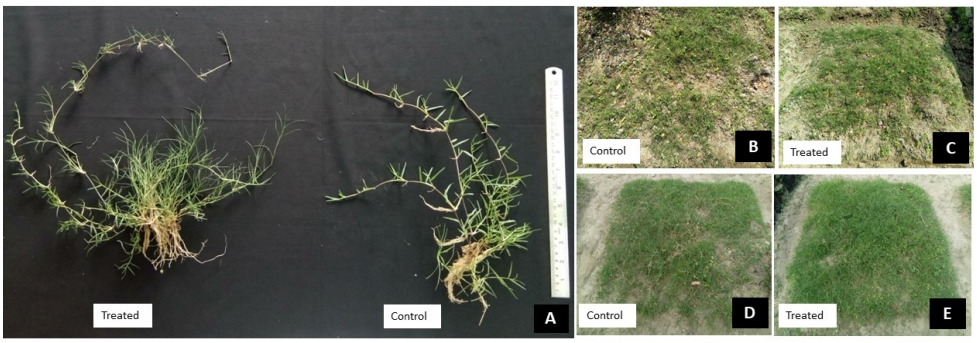

Figure 7. A- Comparison between treated and control plant in field condition; **B**- Field of control set (after 15 days); **C**- Field of treated set (after 15 days); **D**- Field of control set (after 45 days); **E**- Field of treated set (after 45 days)

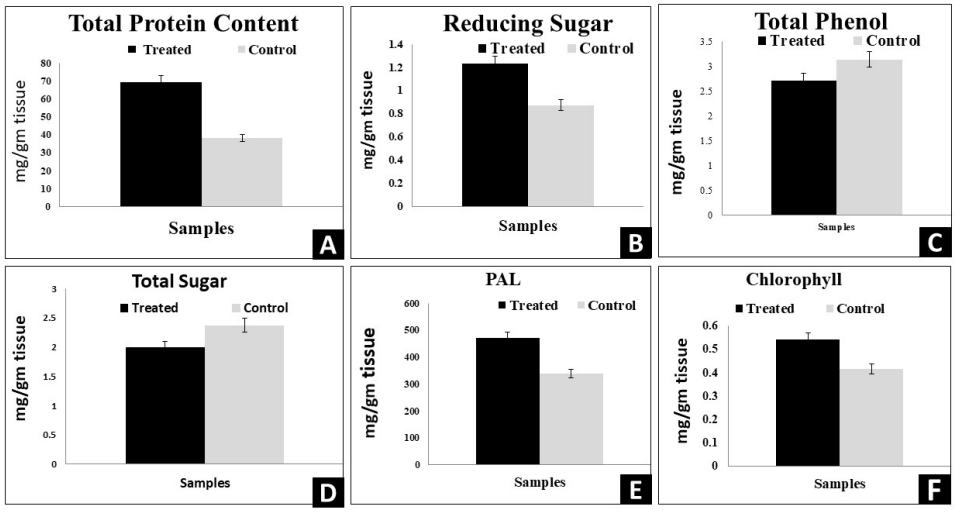

Figure 8. Effect of mass multiplication of AMF spores in *Cynodon* rhizosphere after 45 days on (**A**) protein content (**B**) reducing sugar (**C**) total phenol (**D**) total sugar (**E**) PAL content and (**F**) Chlorophyll content after inoculation in field

Table 1. Nutrient (organic carbon, % nitrogen, content, K_2O, and P_2O_5) content in *Cynodon* rhizospheric soil before and after application of AMF spores [L= Low, O= Optimum, H= High]

Sample	pH	Organic carbon	Nitrogen (%)	K_2O	P_2O_5
Field Preparation	5.26	1.58±0.06	0.14	9±0.08	7±0.039
Control (45 days)	4.15	1.38±0.02	0.13	22±0.09	15±0.05
Treated (45 days)	5.54	1.56±0.26	0.12	40±0.045	25±0.48

Discussion

According to Vestberg *et al.* (1995), colonization of mycorrhizal fungi with the host plant may be done by more than one species of fungus showing a broad range of specificity. In the present study, mixed spores of mycorrhiza (originally collected from the tea rhizosphere) have been used for inoculation and our results also indicated that all the species that were inoculated could colonize the root system regardless of specificity. Many researchers reported that AM fungi could affect plant growth, nutrition uptake and transport (Wang *et al.*, 2002; Hawkes *et al.*, 2008), and metabolite content (Charest *et al.*, 1993; Paradis *et al.*, 1995). In our study, the effect of the AMF could increase rooting mass as well as root morphology which enabled the host to absorb water and nutrients.

Mycorrhizal formation helps the agricultural plant to absorb N, P, K, Ca, Mg, Zn, and Mn through extra radical hyphae (Upreti *et al.*, 2016; Wu *et al.*, 2012; 2016). In our study, it was revealed that the number of AMF spores and root colonization percentage increased arithmetically in the root system and rhizospheric soil. AMF spores inoculation led to plant health improvement. It was measured through morphological parameters (plant height, intermodal length, number of branches, total number of leaves, leaf length, leaf breadth, fresh weight of shoot, dry weight of soot, total number of root branches, fresh weight of root, dry weight of root, total biomass, maximum root length and girth of the plant) and biochemical (total chlorophyll, reducing sugar, total sugar, total phenol, total protein, and phenylalanine ammonia-lyase content) parameters. Increased root hair density of the host plants was also observed by Wu *et al.* (2016) with the application of AMF in citrus. AM colonization could affect chlorophyll concentration in the leaves of maize and wheat plants (Paradis *et al.*, 1995). Our results also showed increased chlorophyll content, reducing sugar, total protein, and PAL activity as suggested by Charest *et al.* (1993).

AMF stimulates the synthesis of phenolic compounds in host plants, it increases when plants are subjected to abiotic or biotic stress (Chen *et al.,* 2017) but in our study, it was revealed that phenol and total sugar less in AMF-treated plants probably due to biotic and abiotic stress less condition. From the results, it was also evident that plant growth, as well as health, was increased significantly following the mass multiplication of AMF spores in the *Cynodon* rhizosphere. This indicated a significant role of AMF symbiosis in the improvement of plant health. AM fungi are obligate symbionts and acquire carbon from their host plants to complete their life cycle (Bago *et al.*, 2000). In return, the fungus provides multiple benefits to the plant, including enhanced mineral nutrition and tolerance to abiotic and biotic stresses (Sawers *et al.*, 2008). AM fungi not only stimulate the growth of plants but also contribute to enhancing plant tolerance to abiotic and biotic stresses, such as low temperature, drought, nutrient deficiency, etc.

(Charest *et al.,* 1993). The soil pH and nutrient contents were also studied before and after application of the AMF spores in the soil which indicated increased K_2O and P_2O_5 content after AMF inoculation. Along with increased soil fertility, mycorrhiza also plays a significant role in the regulation of soil biological activity because of their abundance throughout the uppermost soil layer. Mycorrhiza is directly accessible to the plant, fixes carbon, and makes it available to soil-living microorganisms and animals (Jakobsen and Rosendahl, 1990; Finlay and Soderstrom, 1992). The soil profiling of the control and treated sets revealed that the organic carbon content slightly increased in the treated one. As per Babu and Reddy (2011) the inoculation of AMF triggers the development of *Cynodon dactylon* in red mud compared to the controls. Thus, our results are in agreement with that of Babu and Reddy (2011), and our trap culture study mass multiplication of the desired mycorrhiza was accomplished in *Cynodon dactylon*.

The present study would help to improve the scientific knowledge of future workers, working on the relationship between mycorrhiza and plant health. Finally, it can be concluded that the application of mycorrhiza in the rhizosphere can improve plant health as well as soil health in an eco-friendly manner. Further work on molecular and physiological mechanisms, which are being played during plant-mycorrhiza interaction, may provide better insight into the use of hazard-free biofertilizers (AMF) in several economically important crop systems.

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