

Seed coating of native *Rhizophagus irregularis* and *Funneliformis* sp for improved production and moisture stress mitigation in finger millet, *Eleusine coracana*

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Abstract

Background

Commercial application of Bio-stimulants like AM fungi is limited due to higher quantity of inoculum required per unit area and per crop. AM fungal spore seed coating has the advantage of lowering dosage while simultaneously improving growth and yield metrics. In the present study, seed coating of two native AM fungi were evaluated for the first time to improve the production of finger millet under water deficit condition.

Methods

Two potential native AM fungi were identified using ITS primers. Seed coating method was standardized and combined and single inoculation of AM fungi was evaluated under water deficit conditions in finger millet. The Bio chemical, physiological, antioxidant properties and yield parameters were recorded and their root metabolites were analysed.

Result

Two potential native AM fungi were identified as *Rhizophagus irregularis* (OQ428879.1) and *Funneliformis* sp (OQ379911.1). Seed coating with combined inoculation of native AM fungi in finger millet significantly improved plant growth, yield parameters and root colonization potential under 50% deficit irrigated conditions. Similarly, this dual inoculation improved soil glomalin content, phosphatase activity, Chlorophyll stability index and antioxidant properties. Metabolomic study showed the presence of new compounds with fatty acid biosynthesis as predominant pathway, whereas glycolysis metabolism was predominant in AM fungi as soil inoculum.

Conclusion

Seed coating of *Rhizophagus irregularis* and *Funneliformis* sp. not only reduced the dosage to a tune of 150 to 200 g for one hectare seeds but also increased yield of 14.52% and improved soil biochemical, antioxidant properties of finger millet under water deficit conditions.

Introduction

AM fungi play a significant role in agricultural ecosystem function by different ways, it helps in crop uptake of phosphorous, Zn, water from distant places. It also confers resistance to biotic and abiotic stress of crop plants. Further, the colonization of AM fungi can improve the nutrient quality of crops (Khan et al. 2022). Owing to their ubiquitous symbiosis with angiosperms, factors such as host plant relation, soil condition, pH, nutrient availability and organic matter content determines the diversity of AM fungi (Mythili and Ramalakshmi 2022).

Although multiple benefits are attributed to AM fungi, its application in agricultural systems varies according to the location, For instance in India the recommended practice of AM fungi is 50 Kg per hectare of crop. Such high quantity adds more cost of production per crop and per unit area cultivation and very little literature addressed the issue of reducing the cost of AM fungi application. Hence, the application of AM fungi is static limited in agricultural crops especially for field crops (Malusà et al. 2016). Seed coating of AM fungal propagules is one of the efficient method in reducing the cost of AM fungal inoculum and precise method of delivery to crops (O'Callaghan 2016). Such coating enables early colonization of AM fungi on germinating seed and aids in better establishment of symbiosis at the initial stage of the crop. Several studies have been performed with seed coating of AM fungi, for instance seed coating of single and combined inoculation AM spores improved growth parameters of chick pea (Rocha et al., 2019). Similarly, Oliveira and Coworkers (2016) conducted study on seed coating of *R. irregularis* on wheat resulted in improved shoot length and increased nutrient levels in wheat shoots. Several biodegradable binder were tried to coat AM fungal spores to improve spore viability and enhance growth parameters in maize, it was found that 50% PVA and 50% tapioca starch as binder enhanced the spore germination (Marwanto et al., 2020). AM fungi spore coating in rice enhances insect resistance to crops. *Rhizophagus irregularis*, *Glomus aggregatum*, *Funneliformis mosseae*, and *Claroideoglomus etunicatum* when inoculated as seed treatment improved biomass and nutritional content besides conferring tolerance to rice water weevil (Bernaola et al., 2021). Although limited studies have been conducted delivering the AM fungi as seed coating, effect on moisture deficit conditions were not tried by the aforementioned study.

In this study, different polymers for effective seed coating of native AM fungi were assessed. The polymers coating prevent fungal infection and improves seed germinability besides safely carrying the bio inoculants. Further, experiments on performance of AM fungi coated seeds under 50% deficit irrigation were evaluated in finger millet, since AM fungal spores used in the present study were collected from minor millet growing regions of Tamil Nadu. Because, recent days drought stress is a serious issues across the country due to adverse climatic changes and they have negatively affects the field crops (He et al. 2020). One of the adaptation strategies developed by plants to help them deal with water stress is the AM fungi and plant symbiosis, which modifies plant water relations and response to drought (Augé 2001) (Ruiz-Lozano et al. 2006)

Materials and methods

AM fungal Spores and their molecular characterization

Rhizophagus intraradices and *Funneliformis mosseae* identified based on morphological characterization, previously by the authors from millet ecosystem. They showed superior root colonization potential and secreted efficient root metabolites when inoculated in maize (Mythili and Ramalakshmi 2022). These two spores were selected for the present study. The commercial AM fungal inoculum (*Rhizophagus intraradices*) and pink pigmented facultative methyilotrophic bacteria (PPFM)

were obtained from the Department of Agricultural Microbiology, Tamil Nadu Agricultural University, and Coimbatore for comparison studies.

The spores were also identified by molecular methods by extracting spore DNA and PCR amplification of ITS region carried out as per the method described by Manian et al., 2001. Briefly, the AM fungal spores were thoroughly washed in sterile distilled water and centrifuged at 11,000 g for one minute. Then the samples were frozen in liquid nitrogen and crushed using micro pestle. The DNA was extracted using lysis buffer and eluted in 50 µl of 1mMTrisHCL as per the procedure given by. Primer pairs viz., ITS1 and ITS4 were used for identification of AM fungi. Polymerase Chain Reaction (PCR) was carried out in Bio-Rad Thermal cycler T100. The 50 µl PCR mixture contained 20 ng of DNA, 1µM of each dNTP, 1 µM each primer, 1X buffer and 1 U of Taq polymerase. PCR conditions were as follows; initial denaturation for 2 min at 95°C; 35 cycles of 1 min at 94°C, 2 min at 55°C and 2 min at 72°C and final extension of 5 min at 72°C. The PCR product was purified using purification kit as per the method described by the manufacturers. The purified PCR product was sent for automated sequencing (Bio serve, Hyderabad). Sequencing was carried out using ITS1 primer and then the homology analysis of generated sequence was performed by BLAST (Basic Local Alignment Search Tool) of NCBI (<http://www.ncbi.nlm.nih.gov/Blast.cgi>). The sequencing and homology analysis was repeated three times.

Mass multiplication of AM fungi

The AM fungal inoculum was mass multiplied with maize as host and vermiculite as substrate for 60 days. The inoculum along with root bits was sieved through different sieves viz., 75, 180, 250 and 500micron. The presence of spores in sieved inoculum was estimated along with control (without sieved inoculum) by wet sieving and decantation method as outlined by (Gerdemann and Nicolson 1963).

Standardization of polymers for AM fungi seed coating

An experiment was carried out to evaluate the efficiency of AM fungal spores as seed inoculants using Roll towel method (Phaneendranath 1980). The purpose of this study is to reduce the inoculum load and improve the infection potential of AM fungi. In order to do this several polymers were tested as coating agents. Finger millet seeds (variety: Paiyur2) were surface sterilized with 70% ethanol for 1 minute followed by 5% sodium hypochlorite for 5 minutes and washed several times with sterile water. The surface sterilized seeds were coated with AM fungal inoculum at the rate of 10g per kg of seeds using different polymers at the rate of 0.1 per cent (Carboxy Methyl Cellulose (CMC), Polyvinylpyrrolidone (PVP), Biosticker, rice gruel). The CMC and PVP were purchased from Sigma-Aldrich® and the biosticker was obtained from Tropical Agro Systems Pvt (Ltd.), Coimbatore. The following parameters were recorded after 15 days of the experiment viz., germination percentage, vigour index, shoot length and root length as per method described by Hoagland and Arnon, 1950. Vigour index was expressed in number and was calculated using the formula, Vigour index = Germination percentage x (Root length + Shoot

length). AM colonization percentage in roots was assessed according to the method of Phillips and Hayman, 1970.

SEM analysis

The root samples were carefully removed from the treatment and control conditions after 10 days for arbuscules and after 30 days for vesicles and then were cut into small pieces of about 0.5 cm and rinsed with phosphate buffer saline. To check AM colonization, several hand-cut transverse sections were taken. Then the root samples were placed in a tiny plastic tube containing 2.5% glutaraldehyde (v/v) for fixation at room temperature for 1 hour. The root samples were again fixed in 1% osmium tetroxide at 4°C for 40 minutes and then dehydrated using 10% ethanol for 5 minutes. The samples were sputter coated with gold nanoparticles to improve the visibility of AM fungal colonization under SEM. The samples were then metal coated to improve resolution and to prevent the structural damage. The SC7620 Mini Sputter Coater/Glow Discharge System (Quorum Technologies Ltd, United Kingdom) was used to sputter coat the samples. The sputter coated root samples were examined under SEM (Quanta 250, FEI company, United States) and the AM colonization in finger millet seedlings were observed and documented.

Experimental design and description

A pot experiment was performed during two Rabi season (2021 and 2022) at green house, Department of Agricultural Microbiology, to evaluate the efficiency of new AM fungi viz., *Rhizophagus irregularis* and *Funneliformis sp* in finger millet crop grown under 50% deficit irrigation. Finger millet variety “ATL 1” was used for the experiment. Finger millet seeds were raised in trays containing mixture of soil and sand (1:1). The soil was collected from the Department of Millets, TNAU, Coimbatore. The physico-chemical chemical properties of soil were pH – 8.3 and EC – 0.35 dS m⁻¹, OC – 0.72%, N – 228.96 kg ha⁻¹, P – 39.12 kg ha⁻¹ and K – 289.93kg ha⁻¹. The 5 kg soil was filled in a pot measuring inner diameter, length and volume of 15 cm, 30 cm and 5.3 liter. The water holding capacity of soil was determined. 3 healthy seedlings were transplanted to each pot. Plants were grown under drought stressed condition. All the plants were watered regularly with tap water till it reach 50% of its water holding capacity (WHC). The 50% WHC was maintained based on gravimetric method on alternate days. The experiment was completely randomized with ten treatments and three replications per treatment.

Treatment details are T1 – Uninoculated control; T2 – *Rhizophagus irregularis* (soil application); T3 – *Rhizophagus irregularis* (seed inoculation); T4 – *Funneliformis sp* (soil application); T5 – *Funneliformis sp* (seed inoculation); T6 – *Rhizophagus irregularis*+ *Funneliformis sp* (soil application); T7 – *Rhizophagus irregularis*+ *Funneliformis sp* (seed inoculation); T8 – Commercial AM inoculum (soil application); T9 – Commercial AM inoculum (seed inoculation); T10– 1% PPFM spray

Seed coating of finger millet seeds were done using 500 micron sieved AM fungal inoculum (10g/kg of seed) coated with 0.8% carboxymethyl cellulose and 10g per plant was inoculated for soil application.

PPFM was used as a positive control for drought mitigation. The growth, yield, soil biochemical and antioxidant parameters were taken at 30 and 45 days after sowing under 50% deficit irrigation.

Plant growth and yield parameters

Three randomly selected seedlings from each treatment were used to measure the shoot length, root length at 30 and 45 days and expressed in centimeters. The yield parameters were recorded at the time of harvest and expressed as per cent increase over control.

Soil bio-chemical parameters

Root colonization potential of AM fungi in roots was assessed (Phillips and Hayman, 1970). The glomalin content in soil samples was measured using the method of Wright and Upadhyaya, 1998. The blue colour developed was read in a microplate reader (Spectramax i3X) using bovine serum albumin as standard at 595 nm and expressed as mg g^{-1} of soil.

The acid and alkaline phosphatase activity (pH – 6.5 for acid and pH – 11 for alkaline) was determined using the method of Tabatabai and Bremner, (1969). The intensity of yellow colour developed was measured at 420 nm in spectrophotometer (Systronics ® Spectrophotometer 166). The phosphatase activity was expressed in μg of p-nitrophenol released per g of soil per hour.

Antioxidant properties

The proline content in the finger millet leaf samples were determined by the method of Bates et al., (1973). The toluene layer was removed and absorbance was measured in spectrophotometer (Systronics ® Spectrophotometer 166) at 520 nm.

The catalase activity was estimated using the method (Chaparro-Giraldo et al. 2000) and absorbance was recorded at 240 nm using spectrophotometer (Systronics ® Spectrophotometer 166). The enzyme activity was expressed in $\text{g}^{-1} \text{min}^{-1}$ tissue.

The protein concentration in finger millet leaf samples was determined by the method (Lowry et al. 1951). The colour intensity was measured in spectrophotometer (Systronics ® Spectrophotometer 166) at 660 nm using Bovine serum albumin as standard.

Metabolic profiling of finger millet roots

The 40 days old finger millet seedlings were used for profiling of metabolomes as described by Meena et al., 2017. One seedling from each treatment was removed and the roots were thoroughly washed with

water. Then the roots were placed in a beaker containing 50 ml milli Q water. The solution was extracted with equal quantity of ethyl acetate and the ethyl acetate extract was dried and then processed for GC-MS analysis (PerkinElmer®, USA).

Data analysis

The Phylogenetic analysis of *Rhizophagus irregularis* and *Funneliformis* sp was performed using distance tree results of NCBI blast (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Pooled analysis was performed for two season experiments. The results of all experiments were statistically analyzed as per the method of Gomez and Gomez (1984). The mean values with standard errors were performed in triplicates. The results of all Enrichment analysis, Pathway analysis and Hierarchical Clustering heatmaps of AM fungi mediated root metabolites was performed using Metaboanalyst 5.0 (Pang et al. 2021).

Results

Molecular identification of AM fungi

Molecular identification results of *Rhizophagus intraradices* and *Funneliformis mossae* spores showed that their nucleotide sequence matched with *Rhizophagus irregularis* and *Funneliformis* sp respectively. Both nucleotide sequences were submitted to NCBI and their accession numbers are OQ428879.1 and OQ379911.1, respectively (Fig. 1).

AM fungi seed coating and SEM analysis

The mass multiplied AM fungal inoculum sieved through 500 micron mesh showed maximum spore count of 22.8 viable spores per g of finished product than un-sieved inoculum (8.2 viable spores per g of finished product). The 75, 180 and 250 micron sieved inoculum recorded 6.4, 8.6 and 12.8 viable spores per g of finished product, respectively. The results of polymers and AM fungal spore effect on seed germination and seedling vigour is presented in Fig. 2. The uninoculated control (without any treatments) and polymer alone coated treatments were on par with each other and registered lowest germination, seedling vigour and root colonization than sieved inoculum. Among the polymers, Carboxy methyl cellulose (CMC) coated seeds with 500 µm sieve of AM inoculum recorded highest germination ($93.33 \pm 3.36\%$) followed by Bio sticker coated seeds with 500 µm sieve AM inoculum ($86.66 \pm 3.24\%$) and least was recorded in Polyvinylpyrrolidone (PVP) coated seeds ($56.67 \pm 3.23\%$). Higher value of vigour index (2961.66 ± 70.84) was observed in seed coated with 500 µm sieve AM inoculum using CMC polymer (Fig. 2). The per cent root colonization of AM coated maize seeds ranged from 56.66 ± 3.43 to $96.66 \pm 3.33\%$. The maximum ($96.66 \pm 3.33\%$) was recorded in CMC coated seeds with 500 µm sieve than other polymers. The root colonization in AM coated roots was also confirmed by SEM analysis by visualizing vesicles and arbuscles (Fig. 3).

Plant growth and yield parameters

Shoot length and root length of finger millet was increased significantly by the inoculation of AM fungi both at 30 and 45 DAS respectively when compared to uninoculated control (Fig. 4). However, there were no significant differences between soil and seed inoculation of AM fungi at 30 and 45 DAS. Similarly, no significant differences in shoot length and root length were observed among two AM fungi tested. Combined inoculation of AM fungi improved shoot length (50.3 cm) and root length (20.31 cm) when applied as seed coated inoculum than soil application at 45 DAS. When compared to uninoculated, 54.76% and 53.74% improvement in shoot length and root length, respectively was observed at 45 DAS. The result on the yield attributes significantly revealed that the maximum per cent increase of thousand grain weight (74.19%) and grain yield (14.52%) were recorded in the combined inoculation of AM fungi as seed coating followed by soil application (67.74% and 14.27%, respectively) over control (Fig. 5). Whereas single inoculation as seed or soil application revealed 35.48 to 41.93 per cent increased in thousand grain weight and 9.14 to 9.36 per cent increased in grain yield. The drought mitigating PPFM spray also recorded the yield attributes significantly on par with the single inoculation (Fig. 5).

Soil Bio-chemical parameters

In the present study, per cent total glomalin content at 30 DAS had significantly improvement for soil (66%) and seed inoculation (63%) with AM fungi. Although the content of total glomalin was higher the percent improvement of total glomalin was observed to be reduced at 45 DAS. However the trend of glomalin content seems to be similar with 30 DAS where in 44% improved glomalin content was noticed in soil inoculation and seed inoculation (Fig. 4). In both the cases combined inoculation of AM fungi on seed resulted higher glomalin content 73% and 46% over control was observed at 30 and 45 DAS, respectively. AM fungal inoculated plants showed significant increase in phosphatase activity than control (Fig. 4). The acid and alkaline phosphatase activity ranged from 1.19 ± 0.01 to 2.83 ± 0.03 $\mu\text{g PNP g}^{-1} \text{ hr}^{-1}$ and 2.52 ± 0.04 to 4.27 ± 0.1 $\mu\text{g PNP g}^{-1} \text{ hr}^{-1}$, respectively. Maximum acid and alkaline phosphatase activity was recorded in combined seed inoculation followed by soil application. The AM fungal colonization percentage was significantly differed among the treatments (data not shown). The colonization percentage varied from 34 ± 1.01 to $92 \pm 1.02\%$. Maximum colonization ($92 \pm 1.02\%$) was observed in seed coating of *R. irregularis* and *Funneliformis sp* followed by $79 \pm 1.34\%$ in soil application. Minimum colonization of $34 \pm 1.01\%$ was observed in control.

Physiological and antioxidant parameters

The result of physiological and antioxidant parameters showed a significant variation among ten treatments. The chlorophyll stability index and antioxidant content were maximum in seed coating of *R. irregularis* along with *Funneliformis sp* followed by their soil application. AM plants had increased antioxidant enzyme activity thereby protecting the plants from oxidative damage and increased tolerance to drought. In the present study, the significant variation in the chlorophyll stability index was observed in

AM inoculated treatments than control (Table 1). The chlorophyll stability index ranged from 21.83 ± 0.24 to $27.83 \pm 0.75\%$. Seed coating of *R. irregularis* and *Funneliformis sp* showed highest chlorophyll stability index of ($27.83 \pm 0.75\%$) followed by soil application ($27.10 \pm 0.93\%$) and least ($21.83 \pm 0.24\%$) was observed in seed coating with *Funneliformis sp*.

Table 1
Effect of AM fungal inoculation on antioxidant properties in finger millet

Treatment details	Chlorophyll stability index (%)	Protein content (µg /g)	Proline content (µg /g)	Catalase activity (mg/min)
T1- Uninoculated control	19.30 ± 0.30 ^e	0.20 ± 0.005 ^g	7.30 ± 0.11 ^f	0.1 ± 0.01 ^f
T2 - <i>R. intraradices</i> (Soil)	22.43 ± 0.58 ^{cd}	0.26 ± 0.001 ^c (30)	7.99 ± 0.08 ^{de} (9.45)	0.19 ± 0.01 ^c (90)
T3 - <i>R. intraradices</i> (Seed)	26.57 ± 0.69 ^{ab}	0.27 ± 0.003 ^b (35)	8.38 ± 0.67 ^{abc} (14.8)	0.21 ± 0.003 ^c (110)
T4 - <i>F. mosseae</i> (Soil)	23.83 ± 0.73 ^c	0.22 ± 0.005 ^f (10)	8.19 ± 0.13 ^{cde} (12.19)	0.14 ± 0.01 ^e (40)
T5 - <i>F. mosseae</i> (Seed)	21.83 ± 0.24 ^d	0.24 ± 0.006 ^e (20)	8.00 ± 0.10 ^{de} (9.59)	0.16 ± 0.002 ^d (60)
T6 - <i>R. intraradices</i> + <i>F. mosseae</i> (Soil)	27.10 ± 0.93 ^{ab}	0.31 ± 0.004 ^a (55)	8.53 ± 0.12 ^{ab} (16.89)	0.21 ± 0.005 ^c (110)
T7 - <i>R. intraradices</i> + <i>F. mosseae</i> (Seed)	27.83 ± 0.75 ^a	0.26 ± 0.002 ^c (30)	8.59 ± 0.19 ^a (17.71)	0.27 ± 0.001 ^a (170)
T8 - Commercial AM inoculum (Soil)	25.63 ± 0.43 ^b	0.23 ± 0.002 ^f (15)	8.34 ± 0.07 ^{abc} (14.33)	0.24 ± 0.002 ^b (140)

(*R. intraradices* – *Rhizophagus intraradices* and *F. mosseae* – *Funneliformis mosseae*)

Values represent mean of three replications ± SE.

Values in parenthesis indicate percent increase over control.

Column values followed by different letters are significantly different from each other at 5% LSD.

Treatment details	Chlorophyll stability index (%)	Protein content (µg /g)	Proline content (µg /g)	Catalase activity (mg/min)
T9 - Commercial AM inoculum (Seed)	22.80 ± 0.17 ^{cd}	0.25 ± 0.002 ^d (25)	7.88 ± 0.08 ^e (7.95)	0.15 ± 0.002 ^{de} (50)
T10–1% PPFM spray	23.40 ± 0.30 ^{cd}	0.28 ± 0.003 ^b	8.26 ± 0.07 ^{bcd} (13.15)	0.21 ± 0.005 ^c (110)
SE (d)		0.005	0.15	0.007
CD	1.68	0.01	0.32	0.02
<i>(R. intraradices – Rhizophagus intraradices and F. mosseae – Funneliformis mosseae)</i>				
Values represent mean of three replications ± SE.				
Values in parenthesis indicate percent increase over control.				
Column values followed by different letters are significantly different from each other at 5% LSD.				

The inoculation of AM spores showed significant variation in leaf protein content when compared to uninoculated plant. Under stressed condition, leaf protein content was enhanced by 10 to 55% over uninoculated control. Seed coating of *R. irregularis* and *Funneliformis sp* showed 55% increase in leaf protein content over uninoculated control. Also, the native AM inoculum showed good response on leaf protein content over commercial AM application (Table 1). The Proline content ranged from 7.30 ± 0.11 to $8.59 \pm 0.19 \mu\text{g g}^{-1}$ and enhanced by 7.95 to 17.71% (Table 1). There was no significant difference in proline content among the combined inoculated AM fungi in both seed (17.71%) and soil (16.89%). The catalase activity in the finger millet leaves were significantly influenced by AM fungal inoculation than uninoculated control (Table 1). The catalase activity in the treatments ranged from 0.1 ± 0.01 to $0.27 \pm 0.001 \text{ mg min}^{-1}$ and showed 40 to 170% increase over control. There were no significant difference in catalase activity was noticed among soil and seed coated AM fungi. The seed coating of *R. irregularis* and *Funneliformis sp* showed maximum protein content of $0.26 \pm 0.004 \mu\text{g g}^{-1}$ with 55% increase over control. The Principle component analysis of soil and antioxidant parameters were analysed and presented in Fig. 6 and results revealed that the soil parameters viz., glomalin and phosphatase were positively correlated with antioxidant properties viz., proline, catalase and protein content etc in co inoculation of AM fungi as both soil and seed.

Metabolomics of root exudates by GC-MS

GC-MS analysis of root metabolites revealed that a total of 129 compounds were detected in the root samples grown under 50% deficit irrigation (FS 1). The roots treated with *R. irregularis* as soil inoculum had 12 compounds, the number of compounds got increased to 21 when the same *R. irregularis* was applied as seed treatment. Similar results were obtained when *Funneliformis sp* was inoculated in soil 12 compounds were detected. However, the diversity of compounds was enhanced when *Funneliformis sp* was applied as seed inoculum. When commercial inoculum was applied the soil inoculum has more number of compounds (20) than seed treated (14). The control sample had only 2 compounds present on it. Roots samples of Pink pigmented facultative methylotroph treated (a positive control) crop when applied as foliar spray under water deficit condition produced 14 compounds.

While analysing the compounds in comparison with seed and soil inoculation of *R. irregularis* number of compounds have increased in seed treatment than soil application and their enrichment analysis is presented in Fig. 7. In the present study, pathway analysis of AM fungi mediated root metabolites of Finger millet under moisture deficit condition revealed that fatty acid biosynthesis was predominant in seed coated treatment than soil application, whereas glycolysis metabolism was predominant in soil application than coated seeds (Fig. 8).

In the *R. irregularis* when applied in soil, two compounds Tridecane and Eicosane were present which was not observed when applied as seed treatment. Further, two new compounds namely Tetradecanoic acid and Octadecatrienoic acid were noticed in addition to existing compounds under fatty acid group. However, many new compounds under fatty acyl category were observed when *R. irregularis* was applied as seed treatment. For instance Octadecadienoic acid, Heptadecanoic acid and 9-Hexadecenoic acid are new compounds found in seed treatment of *R. irregularis*. γ -Thionodecalactone an hydrocarbon only found in seed treatment of *R. irregularis* and not observed in soil application. Phthalic acid, Hexa-t-butyl selenatrisiletane were newly observed in seed treatment alone. Nonanal, Decanal, 2-Nonadecanone, n-Butyl ricinoleate were new organo oxygen compounds noticed in seed treatment alone. 1,2-Benzenedicarboxylic acid, an salicylic acid usually present during the drought stress was observed in seed treatment alone (Fig. 9). Steroid compound, Androst-4-en-11-ol-3,17-dione, 8,14-Seco-3,19-epoxyandrostane were the other compounds noticed during seed treatment. Tri-O-methylphorbol, Oxiraneundecanoic acid, Dasycarpidan-1-methanol are the three compounds newly detected due to seed treatment with *R. irregularis*. In case of *Funneliformis sp* seed treatment yielded a new alkane compound Eicosane, in contrast to Tri-O-methylphorbol, Oxiraneundecanoic acid, Dasycarpidan-1-methanol are the three compounds newly detected due to seed treatment with *R. irregularis* where no such alkane compound was detected. Octadecadienoic acid, found in seed treatment with *R. irregularis* was also noticed with *Funneliformis sp*. A fatty acyl compound N-(2-Phenylethyl)undeca-(2Z,4E)-diene-8,10-dynamide) was noticed only in seed treatment. Two benzoid compounds Bergenin and 1-[1-(3-Chlorophenyl)-1,2,3-triazol-4-yl]cyclohexan-1-amine was observed in seed treatment of *Funneliformis sp* only and not in soil application. Seed treatment with *Funneliformis sp* also resulted in lactone compound 4-O-Methyl-12b,13,20-triacetoxy-2,9-dihydroxy-3a.

Further, it is interesting to note that when combination of spores *R. irregularis* and *Funneliformis* sp. were inoculated as soil inoculation a total of 33 different compounds under 11 different groups were noticed when compared to seed treatment, where in slight reduction number of compounds (30) were noticed. Presence of compounds in new groups namely fluorens, prenol group, organic acids and fatty aldehyde were also detected in seed treatment (Fig. 9).

Discussion

Molecular identification

Usually, the spore morphological characteristics are commonly used for identification of AM fungi. More than 200 different AM fungi in the order of Glomales were identified using these characteristics. In some cases morphological characters are difficult to determine as there are limited number only available. Application of molecular techniques particularly amplification of ITS region of rDNA is used for identification of AM fungi and to find out phylogenetic relationships among them (Redecker 2000). In most of the studies, morphological results matched with molecular results. For instance Lee et al. (2008) reported that molecular analysis 15 species of AM fungal spores had consistent results with morphological identification. However, in the present study among the two spores, molecular identification of *Funneliformis* sp was matched with morphological characterization, with respect to *Rhizophagus intraradices* the genus exhibits inconsistent results when sequenced by molecular means and it was matched with *Rhizophagus irregularis*. The reason for this is morphological descriptions of *Rhizophagus* genus often overlap with other species considerably. For example field level isolated spores often times will be similar to *R. venetianum* (Turrini et al. 2018), *R. aggregatum* and *R. intraradices* (Schenck and Smith 1982) and *R. irregularis*, *R. custos*, *R. Prolifer* (Declerck et al. 2000). It has been reported that limited molecular data are available for *Rhizophagus* is a major drawback in identification at species level with confidence (Walker et al. 2021).

Seed coating of AM fungi

AM fungi are mass multiplied in tanks using maize roots as source of inoculum and vermiculite as a carrier material and supplied as root cuttings mixed vermiculite to farmers. Although, P mobilization and other benefits such as nutrient uptake, biotic and a biotic stress tolerance to crops due to AM inoculation has been proven, their large scale applicability to field is hampered owing to high inoculum requirement per crop or per cultivation area and consequent cost associated with it. AM fungi are currently applied at a dosage of 50 Kg.ha⁻¹ as soil application where as others bio fertilizers namely *Azospirillum*, *Azotobacter*, *Rhizobium* and Phosphobacteria are applied at a dosage of 2 kg per ha. In the present study, AM fungal inoculum at the rate of 10g per kg of seeds (22 spores per g of product) was used. Hence, 150–200 g of inoculum is sufficient for one hectare seeds. It is concluded that seed coating of AM fungal spores reduces the dosage and cost which aid in effective delivery of beneficial microorganism. When seeds are coated with synthetic polymer, the pores in the seed coat are covered, preventing the entry of

water and fungus mycelia as well as physical damage (West et al. 1985). In the present study, CMC was found to be suitable for seed coating and this result is in accordance with the findings of (Meena et al. 2017), CMC when coated on wheat seeds was found to be enhancing growth and seed germination (Ren et al. 2019).

Evaluation of AM fungi in finger millet

In the present study, finger millet seeds treated with co-inoculation of native AM fungi *viz.*, *Rhizophagus irregularis* and *Funneliformis sp* increased shoot length, root length and grain yield at 54.76%, 53.74% and 14.52%, respectively, when compared with control under 50% deficit irrigation. The plant tolerance to drought may be primarily attributed to roots that have access to a lot of soil and fungi with extra-radical hyphae (Zhang et al. 2017). The same result was obtained by Daei et al. (2009) and they reported that *Glomus etunicatum* symbioses of wheat was responsible for the highest (38.3%) grain yield increases. Douds Jr et al. (2007) reported that 10 to 20% yield increase in potato was observed due to inoculation of AM fungi in high P content soil. Nicholas et al., (2021) reported that coinoculation *Rhizobium* spp with mycorrhiza improved drought stress and increased soybean yield size and fat content. This was in collaboration with Symanczik and co-workers (2018) who reported that co-inoculation of AM fungi under stress condition would help in improving plant growth and yield in sorghum. Lenin et al. (2010) stated that plants inoculated with AM fungi showed increased performance in germination percentage, shoot and root length than uninoculated plants. Songachan and Kayang (2014) reported that AM fungi can colonize a broad range of plant species in diverse environmental conditions. Accordingly, Sigüenza et al., (2006) stated that seedlings inoculated with indigenous fungi have higher colonization and biomass than seedlings inoculated with commercial AM inoculum owing to better nutrient uptake by AM fungi.

The glomalin content and phosphatase activity in soil was increased when AM fungi was applied in the soil compared to seed coating. The same result was observed by Gao et al. (2017) and they reported that glomalin related soil protein was increased due to inoculation of AM fungi in *Medicago sativa*. This glomalin produced by AM fungus increases soil fertility, upgrades nutrient cycling and enhances the microbial activity (Wu et al. 2008). It was described that co-inoculation of AM fungi significantly improved phosphorous uptake and enhanced the growth of citrus plant (Ortas and Ustuner 2014). The higher amount of phosphatase activity in the soil samples may be due to increased amount of available phosphorous. The phosphatase activity is also influenced by season, vegetation and mycorrhizal interaction (Nannipieri et al. 2011).

Chiang et al., (2006) reported that AM fungi has the potential to increase antioxidants activity when exposed to drought condition in addition to that C3 plants are capable of enduring drought stress when compared to C4 plants. Abdel-Salam et al., (2018) reported that rose leaves showed reduced photosynthetic pigments under stress condition but the AM fungal inoculation can increase photosynthetic pigments. Also, AM plants had increased antioxidant enzyme activity thereby protecting the plants from oxidative damage and increased tolerance to drought (Chiang et al. 2006). Fiorilli et al.,

(2018) reported increased leaf protein content was observed in mycorrhizal plants. The antioxidant activity and protein content was increased in our study due to inoculation of AM fungi. Under stressed condition, proline can accumulate in the leaves of tomato plant (Farouk 2011). The higher proline content in seed inoculated and soil inoculated AM fungi in our study corroborates with Rodríguez-Morelos et al., (2014), who showed that inoculation of three native AM fungal species showed higher proline content compared to uninoculated plants. Additionally, Talaat and Shawky, (2014) reported proline and amino acid concentration were higher in AM inoculated plants. Carole et al., (2018) reported that AM inoculation significantly increased the catalase enzyme activity in American taro plant. Kim and coworkers (2010) reported that plant response to AM fungal colonization depends on composition of inoculum and he stated that combination of AM fungi was more effective than monospecific inoculum.

Metabolomics of finger millet

Metabolomics study revealed that seed inoculation enhances the metabolite number in roots especially under deficit irrigated conditions. Nevertheless, there were no significant differences in number of compounds detected in the roots treated with combined inoculation both soil as well as seed treatment. However, there have been differences among the presence of compounds in both seed treated and soil inoculated. For example, the concentration and quantity of perol lipids, steroids, benzoid and organo oxygen compounds, fatty acyls and fatty acid compounds have been increased in seed treatment with *R. irregularis*. similar results were obtained when wheat was inoculated with *R. irregularis* the lipid compounds, benzoid compounds found to be upregulated and the fold change was also effective (Lucini et al. 2019). In addition, presence of new compounds like carboxylic acid and benzoid and hydrocarbon were noticed in seed treatment sample than soil inoculated one. A metabolic profiling study under drought in trifoliate orange revealed sugars, alcholols, fatty acid were the predominant root metabolite present (Liang et al. 2021). Bahadur et al., (2019) reported that AM fungi have variety of mechanisms including activation of metabolic pathways and increased drought responsive genes. Similar results were obtained by Saia and coworkers (2015), when wheat was inoculated with AM fungi and grown under drought conditions and the following compounds were upregulated or altered namely, amino acid and derivatives, organic acids, sugar, sugar acids, sugar alcohol, fatty alcohol, fatty acids, organic compounds, vitamins, and amine. A study conducted by Kang et al., (2019) showed that under drought root metabolites secreted sugars, carboxylic acid and phenols. Liu et al. (2023) reported that AM fungi changed the many metabolic pathways in peanut roots especially organic acids and amino acid metabolisms in both normal and stressed condition. The above study showed the potential of native AM fungal species *viz*, *Rhizophagus irregularis* and *Funneliformis sp* by soil application or seed coating in mitigation of drought stress in finger millet. The presence of multifunctional metabolites *viz*., antimicrobial, antioxidant, plant growth promoting compounds in AM inoculated plants will pave a pathway to develop a new, efficient and potent AM fungal consortium for sustainable crop production under biotic and abiotic stresses.

Conclusion

It can be concluded that, seed coating not only improves plant growth properties but also confers moisture stress mitigation potential of finger millet when coated as dual inoculation using *Rhizophagus irregularis* and *Funneliformis* sp. When compared to soil application, seed coating greatly reduces the inoculum load, precisely deliver the inoculum on seeds with enabled spore protection using polymers. Therefore seed coating of AM fungal could reduce the cost of inoculum considerably improved performance of the seed, enhances establishment of crop, and offers resistance biotic and a biotic stress for sustainable agricultural systems.

Declarations

Contributions

AR developed Conceptualization, Methodology, Validation, manuscript writing. AR and MM performed experiments and Investigation RK Experimental design and Agronomic traits AS: Physiological and antioxidant propertie, US Formal analysis, Supervision, Resources MB analyzed the data and guidance on statistical analysis

Data Availability

All data are presented in manuscript itself. Further details may be obtained through e-mail : ramalakshmi.a@tnau.ac.in.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Conflict of interest: The authors declared that there are no conflicts of interests.

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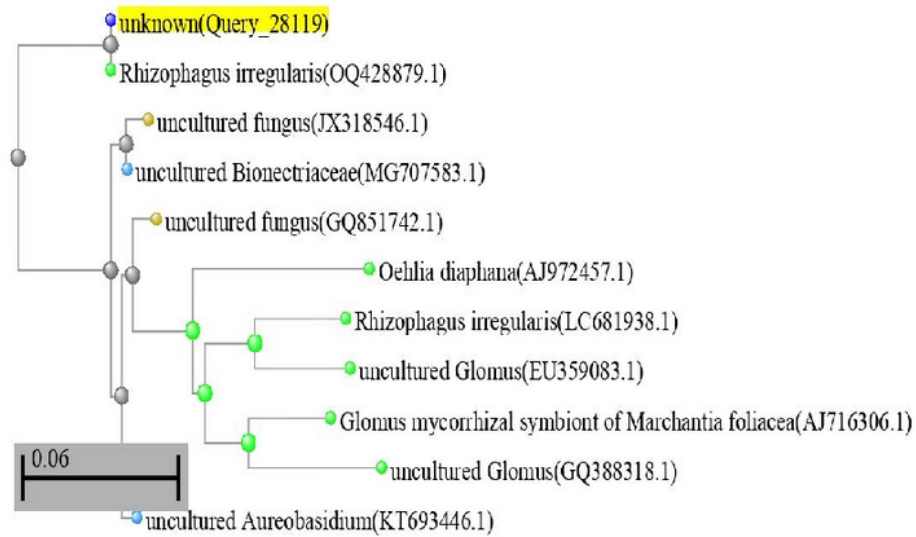
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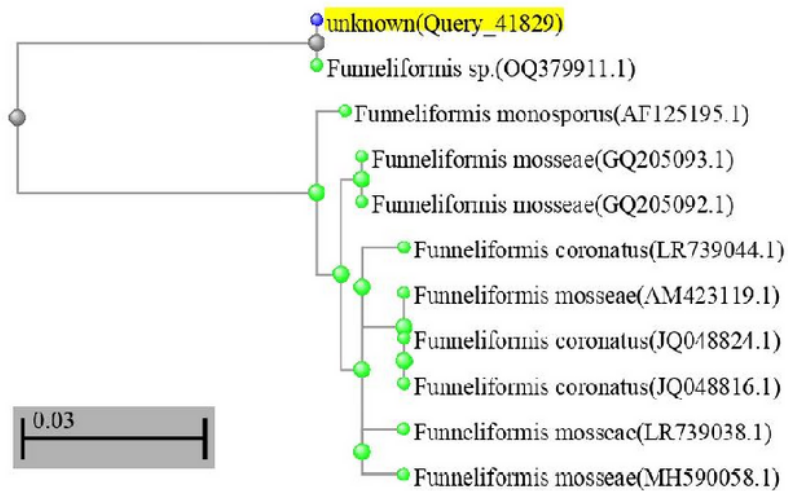
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Figures



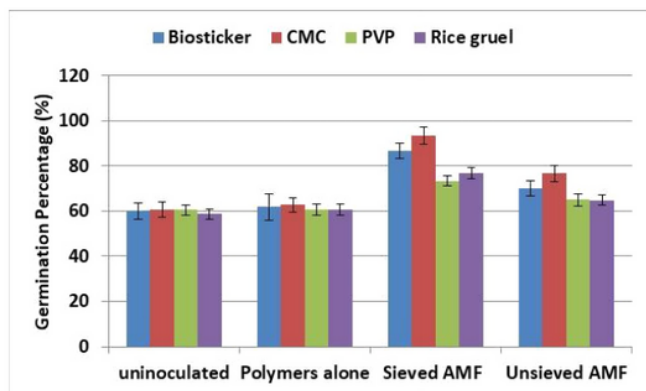
a. Rhizophagus intraradices



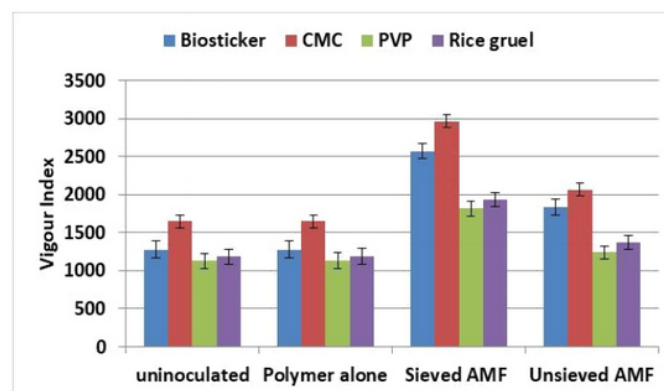
b. Funneliformis sp

Figure 1

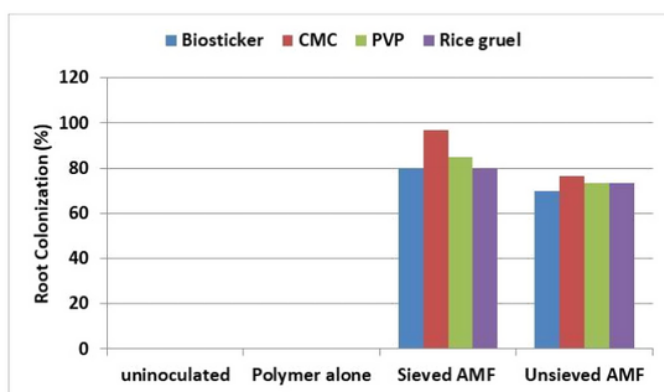
Phylogenetic analysis of *Rhizophagus intraradices* and *Funneliformis sp* isolated from millet soils



a. Germination percentage



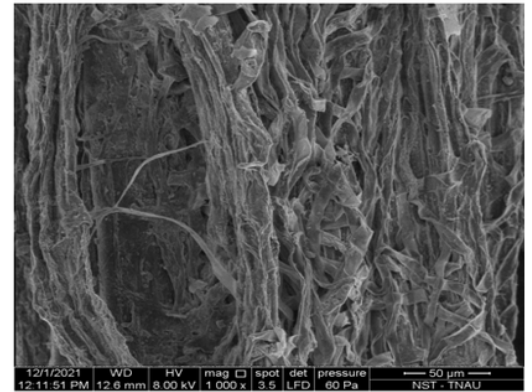
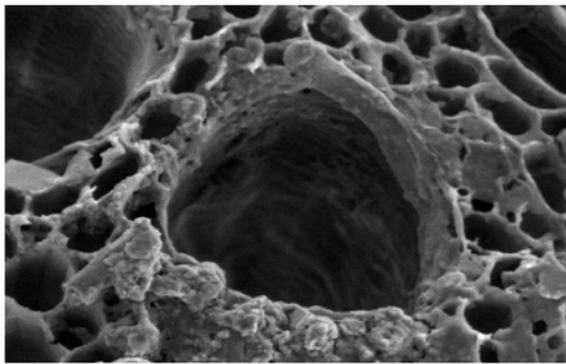
b. Vigour Index



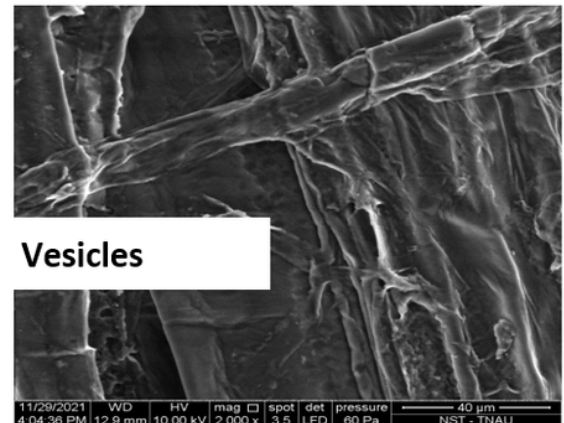
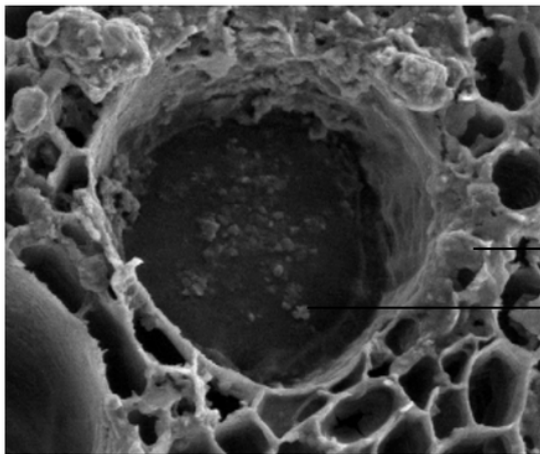
c. Root colonization

Figure 2

Standardization of polymers for AM fungi seed coating in finger millet



a. uninoculated root section



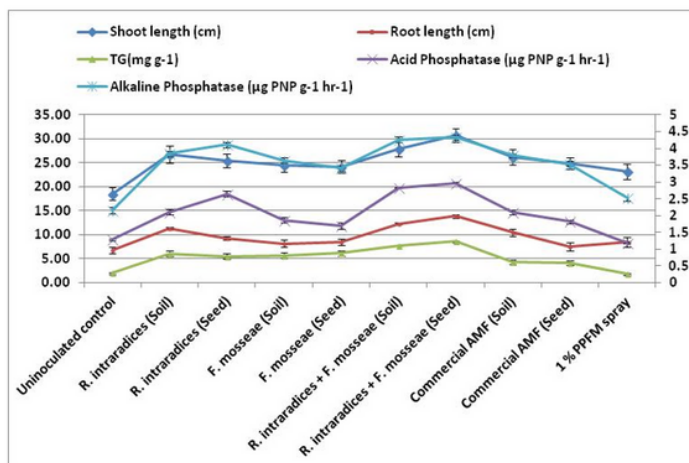
Arbuscules

Vesicles

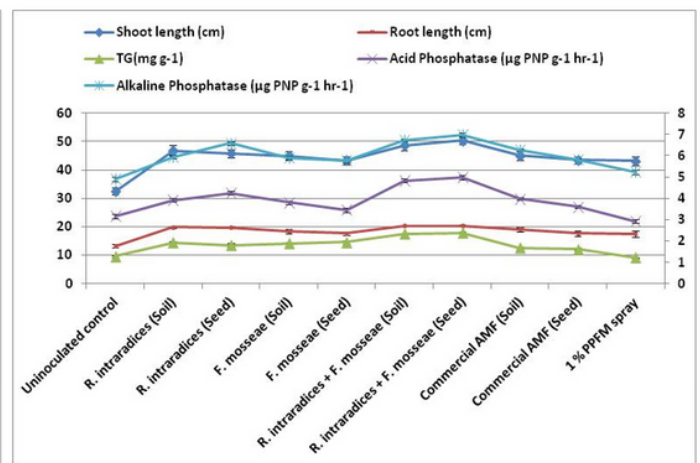
b. AM inoculated root section

Figure 3

SEM micrographs depicting colonization of AM fungi in the roots of finger millet



a.30 DAS



b.45 DAS

DAS: days after sowing

Figure 4

Effect of AM fungal inoculation on plant growth and soil parameters in finger millet

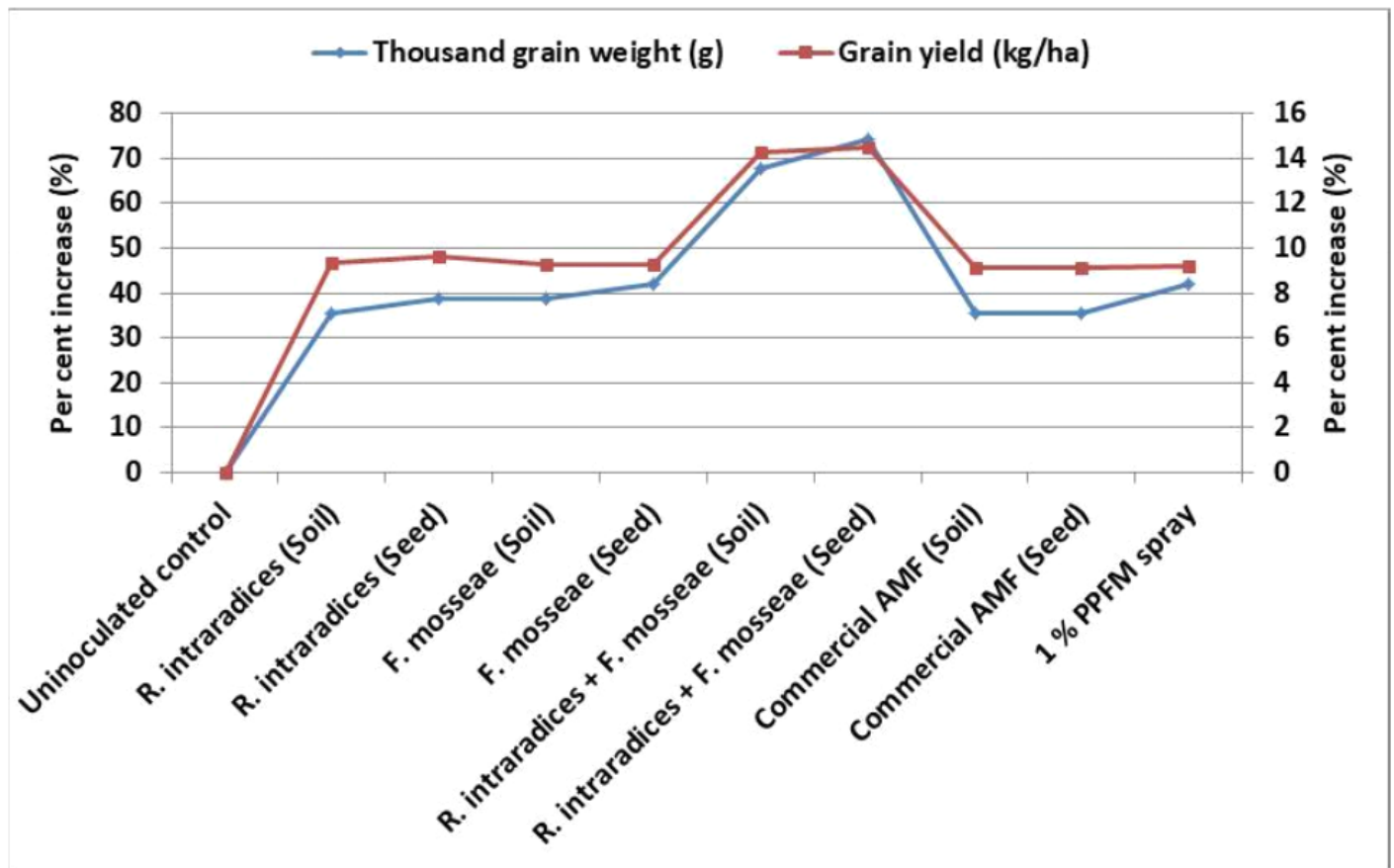
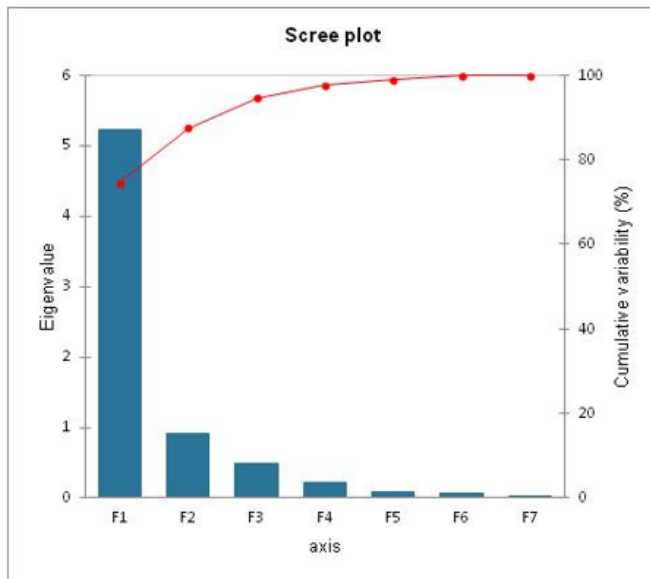
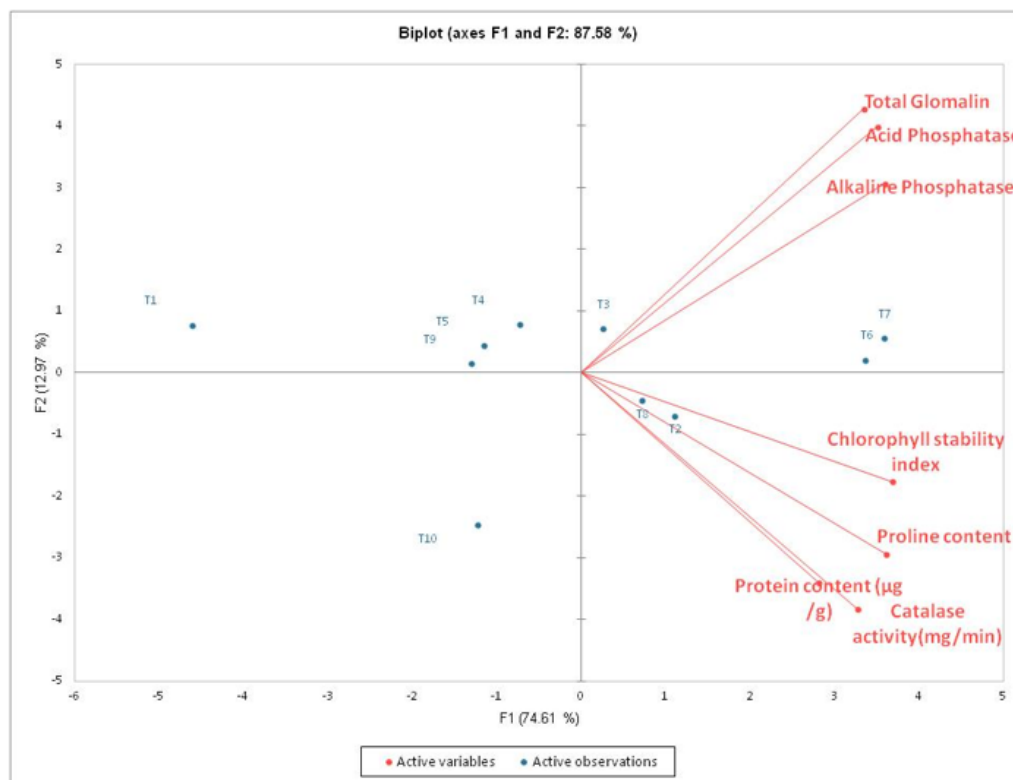


Figure 5

Effect of AM fungal inoculation on yield parameters in finger millet



a.Scree plot



b.Biplot

Figure 6

Principle component analysis of effect of AM fungi on soil and anti-oxidant properties

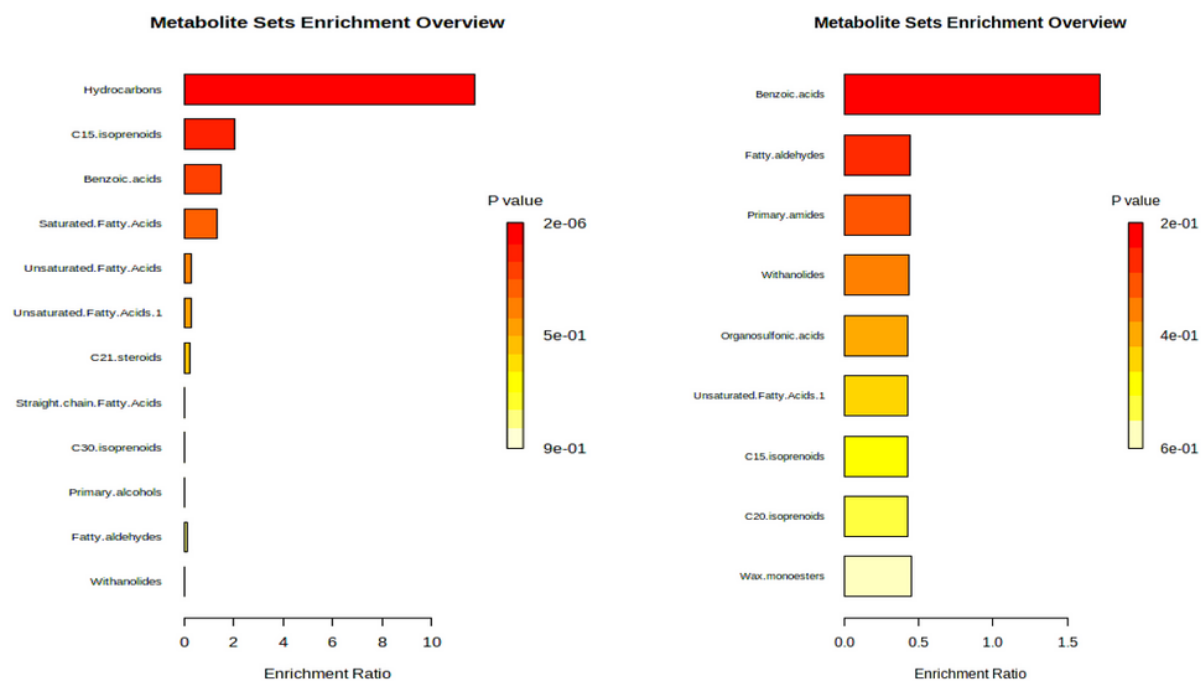


Figure 7

Enrichment analysis of AM fungi mediated root metabolites of Finger millet under moisture deficit condition

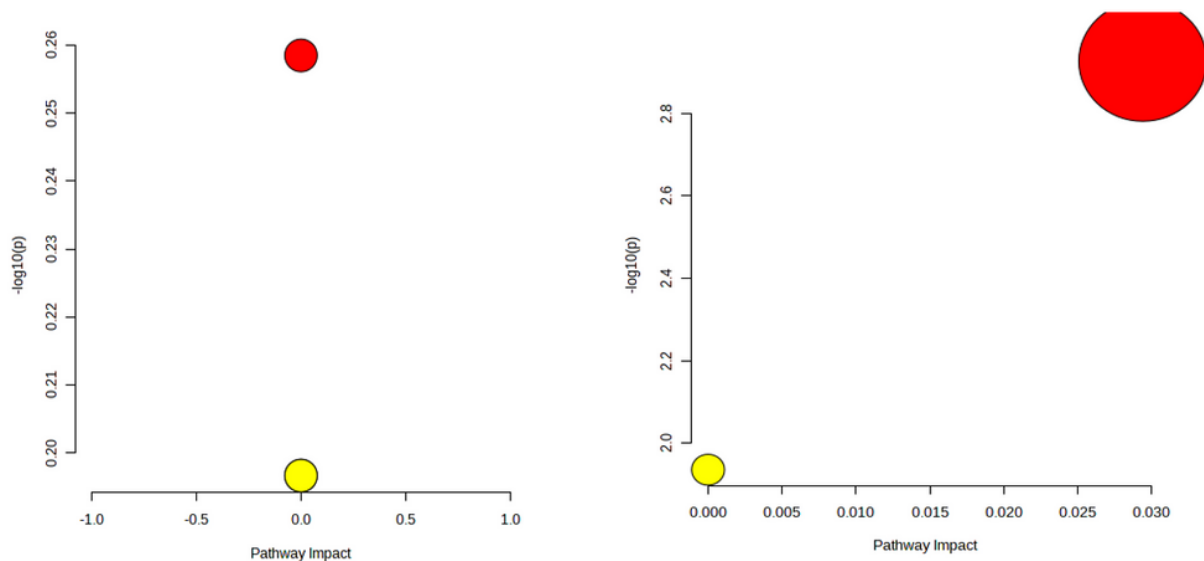


Figure 8

Pathway analysis of AM fungi mediated root metabolites of Finger millet under moisture deficit condition

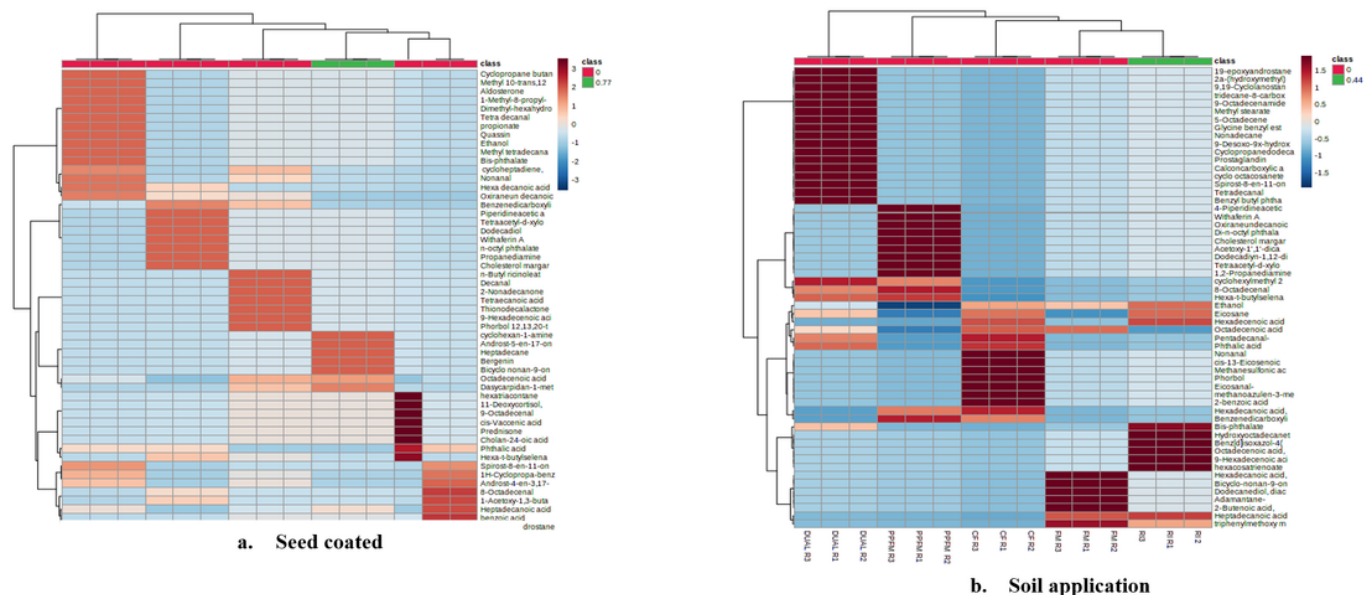


Figure 9

Hierarchical Clustering Heatmaps of AM fungi mediated root metabolites of Finger millet under moisture deficit condition

Dual: *Rhizophagus irregularis* and *Funnelformis sp*; PPFM: Pink pigmented facultative methylotrophs; CF: Commercial AM fungal inoculum; FM: *Funnelformis sp*; RI: *Rhizophagus irregularis*

Supplementary Files

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