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Seed priming effect of arbuscular mycorrhizal fungi against induced drought in rice

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Abstract
Rice (*Oryza sativa* L.) is most susceptible to water deficit (Lafitte and Bennet, 2003).Water requirement of paddy is approximately 1200 mm in flooded, puddled condition. In total, world rice production uses about 1,578 km^3 of water, which is 30% of the fresh water used worldwide (Trijatmiko, 2005). Due to changing climate, failure or delayed onset of monsoon etc., water scarcity is ever increasing worldwide and threatens the sustainability of rice ecosystem. Various adaptation and mitigation strategies were followed worldwide to resolve this abiotic stress. Microbial mitigation using PPFM, AMF and PGPR is one among the method used to alleviate drought. The present investigation was aimed to increase the drought sustaining capacity of rice through priming with Arbuscular Mycorrhizal Fungi (AMF). Here the drought was induced by PEG 6000 and the effect of AMF on increased drought resistance was assessed *in vitro.* PEG 6000 was given at different concentration *viz.*, 0%, 5%,10%,15%,20%, 25%, 30% and 35% and AMF was given as seed treatment. AMF treated plants withstand up to 35% PEG concentration for longer duration compared to control and sporulation of AMF also observed up to 30% PEG concentration. The study will be further up scaled to field level to assess the real-time impact of AMF against drought.

Keywords: AM fungi, induced drought, PEG, proline and endogenous ABA

Introduction
Rice and rice-based systems are predominant in Indian agriculture especially in south India, Tamil Nadu. Rice cultivated mostly under conventional method of irrigation consumes larger amount of fresh water. Approximately 50% of the fresh water used in Asian agriculture goes to rice production. There are two main water related problems which will affect the rice cultivation are moisture stress during critical crop stages of rice cultivation and untimely flooding during harvest time. Even in the previous year (2017) also Tamil Nadu state severely affected by moisture stress during paddy cultivation in as many as some 1,090 hectares.

Ever increasing water scarcity necessitates the development of alternate water saving methods such as system rice intensification, alternate wetting and drying, upland rice etc. Apart from the agronomic practices other techniques such as drought resistance breeding and microbial mitigation are followed to alleviate drought. Microbial mitigation of drought by PGPR organisms are an indirect measures by increasing growth and nutrient uptake and reduced evopotransipiration through various physical barriers and chemical signalling. Hence the present investigation was carried out to assess the seed priming effect of Arbuscular Mycorrhizal Fungi against induced drought by poly ethylene glycol (PEG 6000) *in vitro.*

Arbuscular mycorrhizal (AM) fungi are one of the most important microbial symbionts for the majority of plants. Under phosphate-limited conditions, AM fungi (AMF) can influence plant community development, nutrient uptake, water relations and aboveground productivity (Jeffries *et al.*, 2003) [8]. In the warm and dry Mediterranean environments, arbuscular mycorrhizal (AM) symbiosis could play an important role in alleviating the effects of drought on crop yield. Arbuscular mycorrhizal fungal (AMF) symbiosis could significantly improve plant tolerance to drought via increased dehydration-avoidance and increased tolerance (Ruiz-Lozano and Azcón, 2000). However, its efficacy should be linked to the plant AMF functional compatibility. The root colonization by the mycorrhiza increases active absorptive surface area and stimulates water uptake even in water stress conditions. The AMF symbiosis could increase the drought tolerance via the increased soil water movement to the plant roots (Ruiz-Lozano, 2003) [19], and the ability of the mycorrhizal plants to mediate the osmotic stress. These osmotic regulations are related to the sugar level adjustments in the plant shoots (Wu and Xia, 2006) [23]. AMF plants postpone declines in water potential during drought stress (Porcel and Ruiz-Lozano, 2004) [15]. The ability of the fungus to enhance the water uptake and transport it in the plant, could explain these effects.

The effects of AMF to the drought - plant relation also extend to the soil environment by promoting soil aggregation and thus improving the overall soil water conditions to the rhizosphere (Rillig *et al.*, 2002) [18].

Simulation of drought stress under *in vitro* conditions during the regeneration process constitutes a convenient way to study the effects of drought on the morphogenic responses. *In vitro* culture techniques minimize environmental variations due to defined nutrient media, controlled conditions and homogeneity of stress application. In addition, the simplicity of such manipulations enables studying large plant population and stress treatments in a limited space and short period of time. Polyethylene glycols (PEG) of high molecular weights have been long used to simulate drought stress in plants as non-penetrating osmotic agents lowering the water potential in a way similar to soil drying (Larher *et al.*, 1993) [10]. Many plant species naturally accumulate protein and proline as major organic osmolytes when subjected to different abiotic stresses. These compounds are thought to play adaptive role in mediating osmotic adjustment and protecting sub cellular structures in stressed plants. Thus, different approaches have been contemplated to increase the concentrations of these compounds in plants grown under stress conditions to increase their stress tolerance.

One of the mechanisms by which ABA enhances plant drought tolerance is via regulation of leaf transpiration and root hydraulic conductivity (Aroca *et al.*, 2007) [3]. Arkhipova *et al.* (2007) found an increase of ABA levels in lettuce plants inoculated with *Bacillus* sp., did not cause any difference in stomatal aperture between inoculated and noninoculated plants. This behavior could be caused by the counterbalance of higher levels of Cytokinins (CKs), since the ratio between ABA and CKs determines stomatal aperture (Arkhipova *et al.*, 2007). Regarding root hydraulic conductivity, Sarig *et al.* (1992) showed a positive effect of a PGPR (*Aazspirillum brasilense*) inoculation on root hydraulic conductivity under control and osmotic stress conditions. It is possible that this positive effect could be caused by an up-regulation of aquaporins.

Phytohormones help to regulate plant growth and development in response to stressful environments, such as salinity (Pozo *et al.*, 2015) [16]. AMF are well known to influence plant growth by affecting plant hormone production (Nadeem *et al.*, 2014), particularly in response to salt stress. For example, lettuce inoculated with AMF decreased abscisic acid (ABA) but increased strigolactone production to acclimate salt stress (Aroca *et al.*, 2013) [4]. Similarly, AMF inoculated *Sesbania sesban* maintained higher levels of indole-3-acetic acid (IAA) and gibberellic acid (GA) and allayed the negative effects of salinity (Allah *et al.*, 2015) [2]. Clearly, AMF influence hormones associated with salt stress (Pozo *et al.*, 2015) [16]. However, whether or not inoculation by AMF can ameliorate the growth of commercial crops through hormone regulation under salt stress is mostly unclear. The altered levels of cytokinin and auxin in mycorrhizal plants synergistically regulate root architecture (Fusconi, 2014) [7]. AM symbiosis can also regulate ABA to alter root hydraulic properties, enhancing water uptake in unfavorable conditions (Ruiz-Lozano *et al.*, 2012).

Materials and Methods

The present investigation was done during January, 2017 in Department of Agricultural Microbiology, Tamil Nadu Agricultural University with the objective to assess the impact of impact of AMF against drought *in vitro.* Paddy variety (CO 47) was taken for the study with two treatments *viz*., mycorrhizal treated and non-treated. Water Soluble Powder (WSP) formulation of Arbuscular Mycorrhizal spores were prepared as per the work of Radha *et al.* (2013).

AMF water soluble formulation (with 10,000 spores) was given at the rate of 4g/ acre of paddy seeds. Seeds were surface sterilized with 0.1 per cent mercuric chloride for 2-3 min and washed with sterile distilled water. Then the seeds were dried and coated with AMF formulation with an adhesive agent. The treated seeds were shade dried for 45 minutes and seeds were germinated on soft agar at RT for three days. Similarly non-treated plants were also surface sterilized and pre germinated in soft agar. The germinated paddy seeds were transferred to the germination tubes containing 70 ml of liquid MS media with different concentrations of PEG-6000 (0, 5, 10, 15, 20, 25, 30 and 35%). Plants were grown up to 20 days and their growth, mycorrhization behavior, root proline and endogenous ABA content were observed.

The Osmotic Pressure (OP) of PEG 6000 solutions was calculated using the following formula (Michel and Kaufmann, 1973). $OP= (-1.18 \times 10^{-2}) \times C - (1.18 \times 10^{-4}) \times C + (2.67 \times 10^{-4}) \times CxT + (8.39 \times l0^{-7}) \times C^2T$, here C=PEG concentration;, T=Tempearture. The osmotic pressure of the solute was given in table (1).

Table 1: Osmotic pressure of the solute with PEG 6000 at different concentrations

PEG 6000 Concentration (%)	PEG 6000 g/70 ml	PEG6000 g/kg	Bars of OP at 25^0C
0	-	-	-
5	3.5	35	-0.30
10	7	70	-0.83
15	10.5	105	-1.61
20	14	140	-2.62
25	17.5	175	-3.87
30	21	210	-5.36

Assessment of AM fungal colonization in roots

The AM colonization in paddy roots was estimated by adopting the procedure described by Philips and Hayman (1970). The roots were cut into 1 cm bits and immersed in FAA solution (Formaldehyde 5 ml: Glacial acetic acid 5 ml: Alcohol 90 ml) and were immersed in 10 per cent potassium hydroxide. After autoclaving at 5 lb pressure for 10 minutes, potassium hydroxide was poured out and the root bits were washed with water for 3-4 times. Then immersed in 30 per cent hydrogen per oxide solution for 10 - 15 minutes, then decanted and rinsed with water. These were then immersed in 2 per cent hydrochloric acid for 5 minutes and the excess acid was decanted. The root bits were stained with 0.05 per cent tryphan blue in lacto phenol (Lactic acid, Glycerol: water at 40 ml: 40 ml: 20 ml) and boiled for about 10 minutes. One hundred root bits were examined for each sample under stereo zoom binocular microscope for studying AM colonization and colonization per cent was calculated as below

$$\text{AM Colonization per cent} = \frac{\text{Total number of root bits infected}}{\text{Total number of root bits examined}} \text{ X } 100$$

Estimation of abscisic acid (ABA) from mycorrhizal roots

Abscisic acid (ABA) was extracted from the leaves of mycorrhizal treated and control paddy. For ABA analysis roots were immediately frozen at -80°C after harvest, and ABA concentration was quantified following the method of Siciliano *et al.* (2015) [22]. Briefly, 200 mg of fresh plant tissue, previously ground in a chilled mortar, was homogenized with 80% (v/v) methanol and filtered using Whatman No. 41 filter paper. The extract was concentrated at 40°C and pH was adjusted 8. The alkaline extract was separated with petroleum ether thrice and discards the petroleum ether fraction. The pH of the aqueous fraction was readjusted to 2.7 with 1N HCL. Then the acidic extract was separated twice with ethyl acetate evaporated to dryness at 40°C in a rotary evaporator. Finally the residue was dissolved in minimum volume of methanol and use for chromatographic separation of ABA and for subsequent bioassays. In order to quantify ABA concentrations in the samples, the external standard method was used. A standard curve with an original standard of ABA (Sigma-Aldrich; purity of 98.5%) was built, using concentrations ranging from 10 to 100 µg ml^{-1}.

Estimation of proline

Proline content was estimated for the leaves subjected to control and water-stressed plants using the method of Bates *et al.* (1973) [5]. Leaves (100 mg) from control and water-stressed plants were separately homogenized in 10 mL of 3% sulphosalicylic acid using mortor and pestle and centrifuged at 5000 rpm for 10 min and the supernatant was collected for the estimation of Proline. Ninhydrin (1.25 g) was dissolved in 30 mL of glacial acetic acid and then 20 mL of 6 M phosphoric acid was added and kept for 24 h at 40□C. To 2 mL of plant extract, 2 mL of acid Ninhydrin and 2 mL of glacial acetic acid were added and the mixture was boiled at 100□C for 1 h in a water bath. Then, the solution was cooled and the reaction was terminated. About 4 mL of toluene was added to the contents and mixed vigorously for few sec and OD values for the coloured component was measured at 520 nm using toluene as the blank. From the OD values, proline content (µmoles/g fresh wt.) was calculated separately.

Results and Discussion

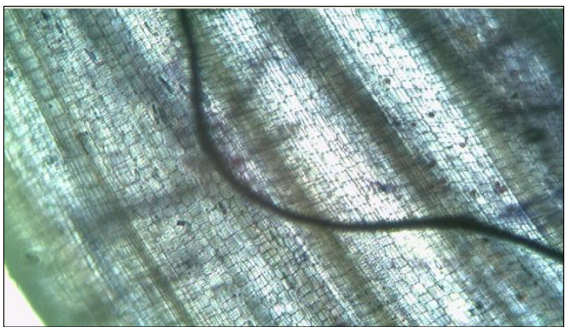
Laboratory experiment was conducted to study the influence of AMF on drought tolerance of crop plants. The drought was induced artificially using PEG 6000. The inoculation effect was studied using rice as model plant. Paddy Seeds (CO 47) were grown with different concentrations of PEG-6000 (0, 5, 10, 15, 20, 25 and 30%). Plants were grown up to 20 days and their growth and mycorrhization behavior was observed (plate 1). Though mycorrhization was observed up to 20 per cent PEG-6000 (12 %) the rate of colonization was very less and ranges from 12 to 44 per cent. Treatment without PEG recorded higher mycorrhizal colonization (44%).

While considering the root length and shoot length of rice under induced drought, little or negligible variation was observed between with AM and without AM. However survival efficacy of AM treated plants was higher compared to controls. AM treated plants remains green more than 20 days and no further progress in growth. At the same time, treatments having only PEG-6000, started drying and wilted. The result shows that AM treated plants withstand drought up to -5.36 bars of Osmotic Potential with in a limited period (Table 2). Our results had coherence with the study of Lum *et al.* (2014) [12] they concluded that the drought-tolerant variety, Pulot Wangi tolerated PEG at the highest drought level (-8 bar) and showed no significantly difference relation to control. However, drought-sensitive variety, Kusam was markedly affected even at the lowest drought level used. In the current study drought sensitive rice variety (CO 47) tolerated up to -5.36 bars of Osmotic Potential with the aid of AM treatment.

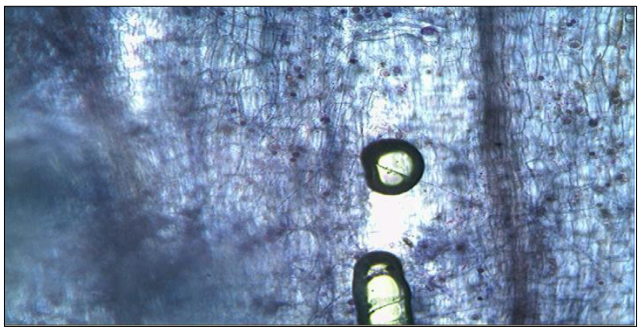
Similarly results of Abdelmoneim *et al.* (2014) [1] give evidence about the drought sustaining capacity of AM treated plants. They evaluated the effects of *Glomus mosseae* in three levels of soil infestation (300, 600 and 900 spores pot^{-1}) to improve tolerance of maize plants (*Zea mays* L.) for drought stress conditions. The result shown that the drought treatment causing decrease in values of almost PGP, except plant root dry weight, which was increased when comparing with well irrigation treatment. The plants treated by *G. mossea* were recorded a significant ($P<0.05$) increase in all PGP comparing with untreated plants in both normal irrigation and drought stress. The highest PGP values were recorded when plant inoculated by 900 spores pot^{-1}.

PEG 6000@15%

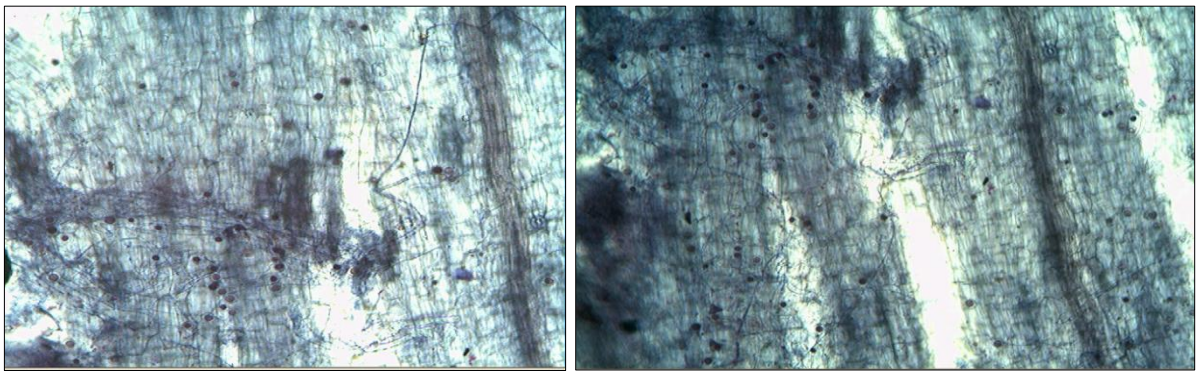
PEG 6000@20%



AM^+ PEG 6000@5%



AM^+ PEG 6000@10%



AM+ PEG 6000@15% AM+ PEG 6000@20%

Plate 1: Effect of AMF on stress induced by PEG-6000

Table 2: Effect of AMF against induced drought on biometrical and mycorrhizal parameters of rice

Treatments	Root length (cm)			Shoot length (cm)			Mycorrhization
	Without AM	With AM	% increase over control	Without AM	With AM	% increase over control	With AM
T_1 (PEG @ 0 %)	2.50	3.20	70	7.5	8	50	44
T_2 (PEG @ 5 %)	1.20	1.35	15	5	5.7	70	21
T_3 (PEG @10 %)	0.80	0.90	10	3.9	4.6	70	21
T_4 (PEG @ 15 %)	0.80	0.90	10	3.4	4.2	80	21
T_5 (PEG @ 20 %)	0.60	0.75	15	3	3.7	70	12
T_6 (PEG @ 2 5 %)	0.30	0.30	0	2.8	3	20	-
T_7 (PEG @ 30 %)	0.20	0.22	2	2.1	2.2	10	-
SEd.	0.02	0.03		0.13	0.16		
CD (0.05)	0.05	0.06		0.29	0.34		

Shoot proline and endogenous ABA content

To overcome the effect of stress, plants have evolved adaptive mechanisms which may be classified into four categories. Three of these adaptations are developmental traits (e.g. time of flowering), structural traits (e.g. leaf waxiness) and physiological mechanisms (e.g. ability to exclude salt while maintaining the absorption of water and the ability to compartmentalize ions with in vacuoles) involve complex interaction. The fourth one is the metabolic responses such as alteration in photosynthetic metabolism and accumulation of organic osmolytes, most commonly proline. One mechanisms utilized by the plants for overcome the water stress effects might be via accumulation of compatible osmolytes, such as proline and soluble sugars.

Production and accumulation of free amino acids, especially proline by plant tissue during drought, salt and water stress is an adaptive response. Proline has been proposed to act as a compatible solute that adjusts the osmotic potential in the cytoplasm. Thus, proline can be used as a metabolic marker in relation to stress. Moreover, under drought stress, the accumulation of total soluble sugars in different plant parts would be increased. However, the rate of additional production or accumulation of proline and soluble sugar is varying in different plant parts. In the present study highest proline accumulation (48.12 & 37.28 µg/g)) was registered in 25 per cent PEG with AM and without AM respectively. The mechanism by which AM symbiosis affects these physiological parameters is still unclear. The role played by ABA has been suggested as one of the nonnutritional mediated mechanisms by which AM symbiosis influence stomatal conductance and other physiological traits when plants are drought stressed (Ludwig-Müller, 2010) [11].

In contrast to proline, ABA accumulation was lower in AMF treated plants than the non-treated plants irrespective of the PEG dosages. Higher value was registered in 15 % PEG without AM (35.92 µg/g) and 46.89 µg/g with 20% PEG (with AMF). Lower value recorded in 30% PEG without AM (8.19 µg/g) and with AM (8.17 µg/g) (Table 3).

In support of our experimental results, recent studies have shown that ABA levels increased in response to water deficit and increased more in non mycorrhizal plants than in mycorrhizal plants, suggesting that AM plants experience less intense drought stress (Doubková *et al.*, 2013) [6]. Furthermore, these physiological processes may vary depending on host plant and especially on fungal species.

Table 3: Effect of AMF against induced drought on proline content and endogenous ABA content of rice

Treatments	Shoot proline (µg/g)		ABA(µg/g)	
	With AM	Without AM	With AM	Without AM
T_1 (PEG @ 0 %)	10.50	12.11	12.17	18.14
T_2 (PEG @ 5 %)	28.27	22.12	27.79	33.12
T_3 (PEG @10 %)	31.27	24.57	22.17	33.27
T_4 (PEG @ 15 %)	36.42	30.17	35.92	45.37
T_5 (PEG @ 20 %)	43.02	34.35	29.36	46.89
T_6 (PEG @ 2 5 %)	48.12	37.28	10.70	12.21
T_7 (PEG @ 30 %)	34.12	32.47	8.19	8.17
SEd.	1.73	1.57	1.0	1.27
CD (0.05)	3.61	3.27	2.08	2.65

Conclusion

Nowadays water scarcity problem is obvious and whole world is affected because of changing climate by anthropogenic and other factors. Our agricultural sector is mainly affected by the water deficit and crops do not meet out their crop water requirement during critical crop stages. Alternative sources like microbial mitigation are one among the prime strategies against the environmental stresses. Hence microbial inoculants such as PPFM and AM fungi were used to alleviate the stresses. Our present research confirms that AM fungi

exert higher tolerances against drought stress. Hence the potentiality of AM fungi must be utilized to overcome the ever growing moisture stress.

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