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RESEARCH ARTICLE

Standardization of spore load for AMF inoculation and its effect on rice root colonization and root system architecture *in vitro*

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ABSTRACT

Arbuscular Mycorrhizal Fungi (AMF) can form symbiotic relationship with rice, preferably in the modern rice production systems such as System of Rice Intensification (SRI), aerobic and upland conditions. Their association with rice roots under *in vitro* conditions was investigated in this study. A suspension containing one lakh (1,00,000) spores of *Rhizophagus irregularis* was employed to standardize optimum AMF spore load/seedling and evaluate its effect on Root System Architecture (RSA). The results indicated that different spore loads of AMF inoculated with rice seedlings, under *in vitro* conditions, modified most of the traits of RSA of rice at early stages significantly better than the uninoculated Control. The *in vitro* study clearly showed that the seedlings of rice with AMF inoculated with a dose of 14 µl/ seedling (31 spores/ seedling) recorded maximum root colonization of 100 per cent by AMF in rice.

Keywords: *AM Fungi, Spore load, Rice (Oryza sativa), RSA, in vitro, Principal Component Analysis (PCA)*

INTRODUCTION

Rice (*Oryza sativa* L.) is one of the predominant cereal and staple crop cultivated in India. Though India has got largest area under rice cultivation, its average productivity is too low. For increasing its production, Arbuscular Mycorrhizal Fungi (AMF) can be employed for improving its sustainability as it imparts multifunctional benefits to the crop plants. AMF placed under the phylum Glomeromycota is one of the oldest groups of living organisms found in symbiosis with terrestrial plants (Redecker, Kodner and Graham, 2000).

AMF act as beneficial microbial symbionts in most of the plant species (Saritha *et al.,* 2014). They render direct and indirect impact on plant nutrition (Smith and Smith, 2012), improve plant water use in natural and agricultural ecosystems (Smith and Read, 2008), soil structure (Rillig and Mummey, 2006), carbon sequestration in soil aggregates (Jastrow *et al.*,1998) and develops induced systemic resistance (Azcon-Aguilar and Barea, 1997) in crop plants. AM fungal colonization depends on the structure and function of plant root system (Smith and Read, 2008; Maiti *et al.*, 1995; Sawers *et al.*, 2008; Campos-Soriano *et al.*, 2010). Cosme *et al.* (2011) reported that AMF (indigenous or native species) colonize cereal and rice crops. From this, it is well known that Arbuscular Mycorrhizal Fungi (AMF) can form symbiotic association with rice roots.

Root system architecture is an important morphological feature and plays a key role in acquiring nutrients from soil volume (Lynch, 1995). In response to environmental stresses (nutrient limitation in particular), root system architecture can be modified to promote the nutrient-acquiring capacity (Sorgona *et al*., 2007). On colonization with rice roots, AMF provide essential nutrients in their available form. It is important to understand the dynamics of AMF interaction with rice to enhance its yield. In this context, rice is used as a model plant to study the components like AMF spore load/ rice seed and its effect on root system architecture with the objective of achieving sustainability in rice farming.

MATERIAL AND METHODS

Experimental location and conditions

The experiment was conducted at the Department of Agricultural Microbiology, Tamil Nadu Agricultural University (TNAU), Coimbatore located between latitude 11°07'47"N and longitude 76°55'59"E with an altitude of 2,56,000mm above mean sea level. The rice seedlings were grown under *in vitro* conditions in the Plant Growth Chamber maintained at 24°C with 12 h day/night light cycle.

Materials used

Seed material

Short duration rice variety CO51 was used in this

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study. The seeds were collected from Central Farm of Tamil Nadu Agricultural University, Coimbatore.

AMF inoculum and seed coating polymer

Liquid spore suspension of AMF was provided by Biofarm, Coimbatore, manufacturer of bioinputs for agriculture. This suspension consists of surface sterilized spores of *Rhizophagus irregularis* at a concentration of 1 lakh spores/50ml sterile distilled water. The seed coating polymer for coating the rice seeds with AMF spores was also obtained from the above firm.

Growth media and preparation of seedling agar tubes for in vitro study

Rice seedlings were grown in Murashige and Skoog (MS) medium (Murashige *et al*., 1962) @ half strength (½ MS) using 0.6% Cleri Gel TMT (Hi-media) as solidifying agent to standardize the AMF spore inoculum load for rice seedlings. MS medium @ half strength was dispensed to seedling agar tubes (30 cm height and 3 cm dia) to one third of its volume, plugged with Non absorbent cotton and sterilized.

Surface sterilization and germination of rice seeds

The seeds were surface sterilized with sodium hypochlorite (5% available chlorine) for 10min. Then the chemical sterilant was drained and washed with sterile distilled water thrice and allowed to germinate for a period of 72 hrs on the moistened germination paper in the sterile Petri plate. Three days after incubation, germinated seeds with good emergence of radicle and plumule were aseptically transferred using a sterile long forceps to the seedling agar tubes @ 1 seed/tube.

Inoculation of rice seedlings with AMF spore suspension

Different doses of spore suspension of AMF were inoculated at the hypocotyl region of the germinated rice seeds on 4th day under axenic condition. To achieve different spore load, 11 different treatments with doses of 2, 4, 6, 8, 10, 12, 14, 16, 18, 20µl of the AMF spore suspension/seedling were inoculated with a suitable un-inoculated control. Seven days after inoculation, growth of hyphal network was observed on all the inoculated tubes and % AM colonization was calculated.

Per cent AM Colonization

Per cent Arbuscular Mycorrhizal (% AM) colonization was determined using Tryphan blue staining of the processed root bits collected from the seedling agar tubes (Phillips and Hayman, 1970 and Koske and Gemma, 1989). The stained rice root segments (one cm in length, 50 segments used as replicate for each sample) were observed under Stereo-zoom microscope at 5 X magnification. The presence of mycelia and spores in the root samples was recorded to assess the % AM colonization based on the formula given below (Giovannetti and Mosse, 1980).

$$\% \text{ AM Colonization} = \frac{\text{No. of root segments infected}}{\text{Total No. of root segments studied}} \times 100$$

Determination of RSA variables by PCA method

The rice seedlings, grown as single plant in half strength MS medium in seedling agar tubes, were observed for root system architecture (RSA) variables using 'GiA root' imaging system. The rice RSA variables after 32 days of rice-AMF co-inoculation were compared among 11 treatments with varying dosage of AMF spores (0, 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 µl/seedling). A set of different RSA related variables such as average root width (AVRW), maximum number of roots (MXNR), median number of roots (MDNR), root network connecting components (NOCC), network business (NWBS), network solidity (NWSD), network width (NWWI), perimeter (NWPM), total network area (NWAR), network volume (NWVL), root network width to depth ratio (NWWD), specific root length (SPRL), network length (NWLN), length distribution (NWLD), depth (NWDP), surface area (NWSA) major (MAEA) and minor ellipse axes (MIEA) and ellipse axes ratio (ELAX) were compared and the results are presented along with spore count (SPCN) and infection percentage (INFP) as follows.

Principal Component Analysis (PCA) was performed to identify the RSA variables in AMF inoculated rice plants (Wold *et al.*, 1987). The inoculated and uninoculated root objects were given with their scores and loadings, which were plotted against each other in a graph. Similar objects in a score plot will be placed close to each other. Positively correlated variables will be placed close to each other whereas negatively correlated objects will be placed opposite to each other in terms of loading plot.

RESULTS AND DISCUSSION

Assessment of different spore loads of AM Fungi on root colonization of rice under in vitro conditions

The mycorrhizal colonization in *Prunus* roots was investigated by (Dumas *et al*., 1989; Wyss *et al.,* 1990; Schellenbaum *et al*., 1992). Per cent AM colonization was assessed on 25 days old *in-vitro* grown rice seedlings. In the present investigation, the spore load of the different doses of the AMF spore suspension used for inoculating the rice hypocotyl region was assessed. The spore load was found to be 5.0, 9.0, 13.0, 17.0, 22.0, 28.0, 31.0, 35.0, 42.0 and 48.0 for the different doses *viz*., 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20µl/seedling respectively. The % AM colonization with the different

doses of AMF spore suspension inoculated rice seedlings was assessed. When 20 µl of AMF spore suspension (T_{10}) with a spore load of 48.0/seedling was used, maximum % AM colonization (100%) on rice roots was noticed (Table 1) followed by T_9 (42.0 spores/seedling), T_8 (35.0 spores/seedling) and T_7 (31.0 spores/seedling) which also recorded 100% AM colonization. The other treatments *viz*., T_6 (28.0 spores/seedling), T_5 (22.0 spores/seedling), T_4 (17.0 spores/seedling), T_3 (13.0 spores/seedling), T_2 (9.0 spores/seedling) and T_1 (5.0 spores/seedling) recorded the % AM colonization of 85.0, 60.0, 50.0, 42.8, 37.5 and 20.0 respectively. No AM colonization was found in the un-inoculated control. However, a minimum of 5 spores is required for AM colonization in rice roots.

Table 1. Effect of different doses of AMF spore suspension on the spore load/seedling and %AM colonization in rice roots under *in vitro* condition

Treatment	No. of spores	%AM Colonization
T1: 2 l AMF/seedling	5.00	20.0
T2: 4 l AMF/ seedling	9.00	37.5
T3: 6 l AMF/ seedling	13.0	42.8
T4: 8 l AMF/ seedling	17.0	50.0
T5: 10 l AMF/ seedling	22.0	60.0
T6: 12 l AMF/ seedling	28.0	85.0
T7: 14 l AMF/ seedling	31.0	100
T8: 16 l AMF/seedling	35.0	100
T9: 18 l AMF/ seedling	42.0	100
T10: 20 l AMF/ seedling	48.0	100
T11: Un-inoculated-Control	-	-
SEd	0.92	3.05
CD (0.05)	1.87	6.19

* Values are means of three replicates

The results clearly indicated that % AM colonization increased with increase in dosage of AMF spores applied (Table 1 and Plate 1 A, B and C). It might be due to the mycorrhizal fungi which differ in their ability to infect and colonize roots. *Glomus* species has ability to infect and colonize plant roots faster than *Gigaspora* species, making it highly competitive (Kurle and Pfleger, 1994). The higher mycorrhizal colonization in maize could be due to strigolactones exuded by host plant roots and taken up by AMF since strigolactones stimulated fungal metabolism and branching Parniske, (2008). The role of strigolactones as a key signaling compound in the interaction between plants and soil-borne symbiotic AMF has been suggested by Soto *et al.,* (2010), which is in conformity with the present findings.

RSA variables and Principle component analysis

Principle component analysis (PCA) was performed for all the 11 treatments to score the best treatment based on its spore count, infection percentage and rice RSA parameters. The PCA loading plot showing the variables and scoring plot due to the inoculation of different doses of AMF spore suspension are presented as Figure 1. The principal component (PC1) adds 55.27 % variability, while PC2 adds additional 26.50% to the total cumulative variability of 81.77%. All the observed variables significantly influenced by the different doses of AMF and contributed equally to the total variability of PC (Figure 1). Corresponding to the scoring plot, the best treatments (10, 12,14,16,18 and 20 µl doses of AMF/seedling) showing positive correlation to the selected variables were orthogonally positioned in the positive plot (+ for both PC1 and PC2; top right hand quadrant). The treatments ranging from uninoculated control to AMF spore suspension inoculated seedlings in gradient up to 6 µl/seedling recorded poor results and positioned in negative quadrant plot. The results clearly indicated that, with increase in the doses of AMF spore suspension, the % AM infection, spore count and rice root architecture variables also increased. When considering the variables (loading) plot, those variables positively responded to AMF-inoculation such as maximum number of roots (MXNR), median number of roots (MDNR), root width (AVRW), volume (NWVL), surface area (NWCA), spore count (SPCN) and infection percentage (INFP) orthogonally positioned in the treatments with higher AMF loads. The correlation matrix relating the assessed RSA variables is presented in Table 2. Among the assessed RSA variables, the network parameters such as network area (NWAR), perimeter (NWPM), network length (NWLN), network width (NWWI) and network convex area (NWCA) had maximum significant correlation with other assessed variables. While the specific root length and network width to depth ratio (NWWD) did not show much significant correlation with any of the other assessed variables. All the positively responded variables had significantly higher loading scores (>50%) and their contribution to the principal component is nearly uniform (ranged from 1 to 11%) (Table 3). In case of PC1, all the variables except network solidity (NWSD) and specific root length (SPRL) had higher contribution to the significant component. Whereas, in case of PC2, spore count (SPCN), infection percentage (INFP), maximum number of roots (MXNR), median number of roots (MDNR), average root width (AVRW), ellipse axis ratio (ELAX), network solidity (NWSD), network depth (NWDP) and network volume (NWVL) alone recorded higher contribution to the significant component (Table 3).

Root system architecture reflects the shape, three-dimensional distribution, branching pattern and age of the primary and post-embryonically generated roots Ambreetha *et al*., (2018).

Table 2.Correlation matrix [Pearson (n^{-1})] of assessed rice root system architecture variables as influenced by AMF inoculation

Variables	SPCN	INFP	NWBS	NOCC	NWLD	MXNR	MDNR	AVRW	MAEA	MIEA	ELAX	NWSD	SPRL	NWWD	NWWI	NWDP	NWPM	NWLN	NWAR	NWCA	NWSA	NWVL
SPCN	1.00																					
INFP	0.96	1.00																				
NWBS	-0.28	-0.29	1.00																			
NOCC	0.17	0.12	0.61	1.00																		
NWLD	0.42	0.38	0.50	0.87	1.00																	
MXNR	0.58	0.60	0.19	0.56	0.72	1.00																
MDNR	0.85	0.89	-0.36	0.08	0.37	0.70	1.00															
AVRW	0.87	0.88	-0.41	0.12	0.24	0.35	0.80	1.00														
MAEA	0.08	0.01	0.22	0.73	0.69	0.52	0.12	-0.04	1.00													
MIEA	0.50	0.43	0.39	0.86	0.91	0.86	0.48	0.34	0.73	1.00												
ELAX	0.59	0.59	0.40	0.49	0.57	0.66	0.51	0.50	-0.04	0.65	1.00											
NWSD	0.35	0.43	-0.68	-0.67	-0.64	-0.25	0.34	0.52	-0.66	-0.52	-0.05	1.00										
SPRL	-0.88	-0.88	0.37	-0.18	-0.28	-0.40	-0.79	-0.99	-0.01	-0.39	-0.53	-0.49	1.00									
NWWD	-0.23	-0.33	0.10	0.48	0.34	0.22	-0.23	-0.36	0.88	0.41	-0.38	-0.63	0.31	1.00								
NWWI	0.45	0.42	0.49	0.87	0.91	0.86	0.43	0.26	0.73	0.98	0.63	-0.56	-0.32	0.42	1.00							
NWDP	0.59	0.64	0.47	0.58	0.71	0.71	0.55	0.50	0.09	0.70	0.95	-0.15	-0.53	-0.31	0.73	1.00						
NWPM	0.20	0.17	0.25	0.69	0.72	0.77	0.30	0.00	0.92	0.82	0.17	-0.60	-0.05	0.76	0.84	0.29	1.00					
NWLN	0.22	0.18	0.23	0.67	0.70	0.77	0.32	0.01	0.92	0.82	0.18	-0.58	-0.06	0.76	0.84	0.28	1.00	1.00				
NWAR	0.56	0.51	0.03	0.66	0.72	0.84	0.61	0.43	0.81	0.89	0.37	-0.31	-0.47	0.54	0.86	0.45	0.90	0.90	1.00			
NWCA	0.27	0.21	0.38	0.85	0.84	0.74	0.28	0.10	0.92	0.92	0.32	-0.66	-0.16	0.70	0.93	0.43	0.95	0.95	0.90	1.00		
NWSA	0.57	0.51	0.03	0.65	0.71	0.85	0.62	0.42	0.80	0.89	0.38	-0.30	-0.47	0.54	0.86	0.45	0.89	0.90	1.00	0.90	1.00	
NWVL	0.76	0.69	-0.14	0.53	0.62	0.78	0.77	0.69	0.61	0.82	0.47	-0.04	-0.72	0.30	0.75	0.50	0.69	0.70	0.94	0.73	0.94	1.00

Values in bold are different from 0 with a significance level p=0.01. The abbreviation of all the variables have been explained in Results

Root phenotyping remains a major bottleneck to the discovery of genes that control important RSA traits and constraints are experienced on at least two levels (de Dorlodot *et al.*, 2007; Zhu *et al.*, 2011). The first constraint concerns the observation of root systems in their native soil environment.

Table 3. Loading values and per cent contribution of assessed variables on the axis identified by the principal component analysis

Variables	PC1		PC2	
	Loading	% contribution	Loading	% contribution
SPCN	0.59	2.90	0.74	9.40
INFP	0.55	2.50	0.79	10.75
NWBS	0.25	0.51	-0.52	4.60
NOCC	0.79	5.12	-0.35	2.13
NWLD	0.88	6.31	-0.16	0.44
MXNR	0.89	6.55	0.12	0.25
MDNR	0.60	2.95	0.69	8.24
AVRW	0.43	1.52	0.84	12.05
MAEA	0.75	4.65	-0.54	4.91
MIEA	0.98	7.83	-0.09	0.14
ELAX	0.58	2.75	0.42	2.98
NWSD	-0.41	1.36	0.81	11.36
SPRL	-0.48	1.90	-0.81	11.23
NWWD	0.42	1.45	-0.74	9.31
NWWI	0.96	7.61	-0.15	0.38
NWDP	0.67	3.67	0.36	2.19
NWPM	0.86	6.02	-0.42	2.96
NWLN	0.86	6.03	-0.40	2.75
NWAR	0.95	7.49	-0.01	0.00
NWCA	0.92	6.93	-0.38	2.46
NWSA	0.95	7.43	-0.01	0.00
NWVL	0.89	6.52	0.29	1.48

Values in bold explained >50% contribution to the significant component. The abbreviation of all the variables explained in Results.

In the field, even the most advanced procedures offer only a partial glimpse of RSA (Zhu *et al.*, 2011). To overcome this limitation, several laboratory and greenhouse growth methods have been developed that provide relatively easy access to the roots of a variety of plant species (Gregory *et al.*, 2009). While each of these methods offers clear advantages to the study of root development, most of them suffer from certain drawbacks such as failing to preserve

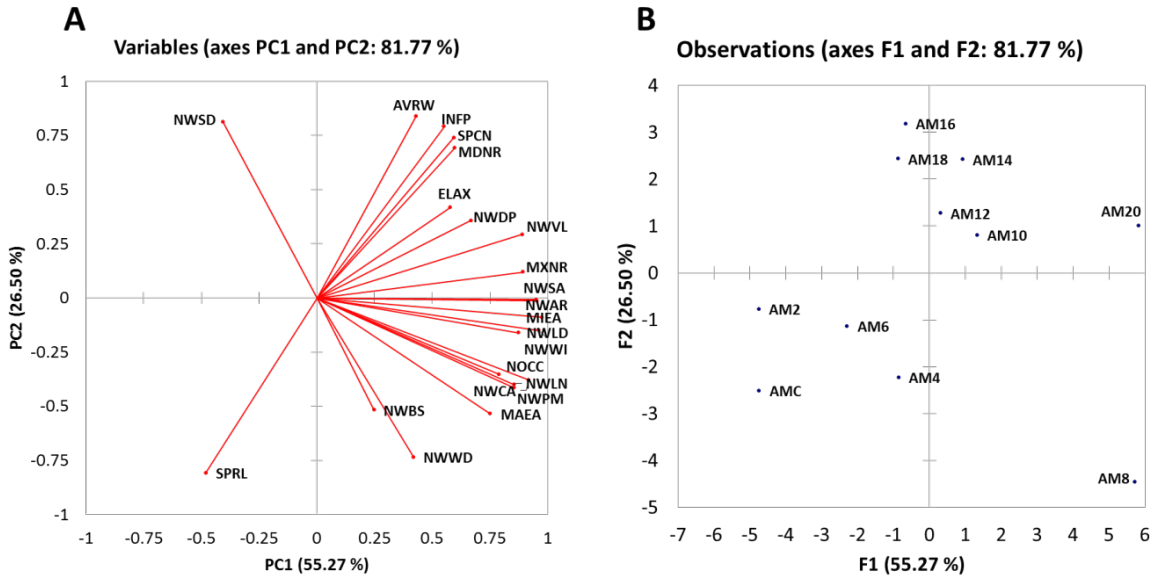
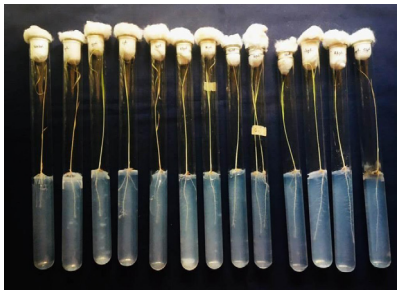


Figure 1. Scoring plot of samples (A) and loading plot of variables (B) in Principal component analysis of RSA variables of rice as influenced by AMF inoculation. AMC – Uninoculated control; AM2-AM20 - AMF inoculated rice seedling (2-20µl/seedling); Mean of three replicates were plotted. The % variance explained by each component (PC1and PC2) are given in parentheses in axes. Description of each variables is given in Results.

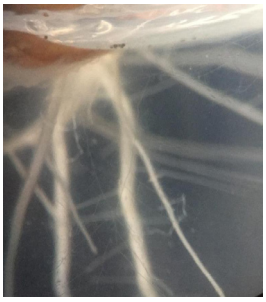
three-dimensional RSA, lacking resolution and throughput, being cost-prohibitive or preventing repeated measurements of the same root system over time. The second constraint involves quantifying RSA traits from field or laboratory grown plants. Information about RSA is typically collected by digital imaging or scanning, and then quantified by methods with various levels of automation. High-throughput, semi-automated methods offer the greatest ability to quantify complex RSA on a large number of genotypes, replicates and conditions as required in large genetic experiments (Ingram *et al.*, 2012).

The objective of the present study was to assess the variation of RSA in rice as influenced by AMF inoculation, when the rice seedling was growing under nutritionally and environmentally controlled conditions. To capture information about complex three-dimensional RSA, we used imaging and analysis platform (GIA Roots software) Galkovskyi *et al.* (2012) that quantifies 20 different root traits on multiple two-dimensional images of plants growing in half strength MS medium. Using this system, we found robust and reproducible differences in RSA between AMF-inoculated and uninoculated control plant. The twenty RSA traits assessed were maximum number of roots (MXNR), median number of roots (MDNR), average root width (AVRW), number of connecting components (NOCC), specific root length (SPRL), ellipse aspect ratio (ELAX), major ellipse axes (MAEA), minor ellipse axes (MIEA), network bushiness (NWBS), network solidity (NWSD), network depth (NWDP), network length (NWLN), network length distribution (NWLD), network width (NWWI), network perimeter (NWPM), network area (NWAR), network convex area (NWCA), network surface area (NWSA), network volume (NWVL) and network width depth ratio (NWWD). The description of each RSA related variable as explained by Iyer-Pascuzzi *et al.* (2010). Ingram *et al.* (2012) used this software to assess the root architecture of *Brachypodium* as influenced by nutrient deficiency. They could identify the RSA traits that were affected due to nitrogen and phosphorus deficiencies. Similarly, the hormonal balancing of rice on RSA was assessed by Gia Roots software Singh *et al.* (2015) and could able to identify that exogenous application of IAA and benzyl amino purine (BAP) changed several of the above RSA traits in young rice seedlings. Topp *et al.* (2013) used this software and identified the genes that regulate the root architecture of rice to improve the root traits and agronomical quality of rice. In the

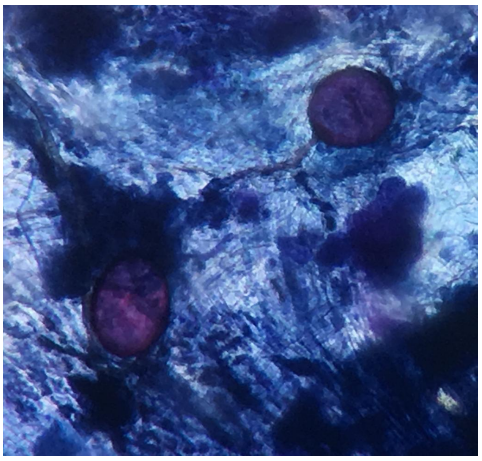
present work, we used this root imaging and phenotyping software to assess the impact of AMF inoculation on the changes in the RSA traits of rice seedlings grown under *in vitro* condition. The principal component analysis used in the present investigation relating the assessed RSA variables to the AMF inoculation further confirmed that the above variables are the most responsive RSA traits influenced by AMF inoculation. Hence it is concluded that AMF inoculated rice seedlings under controlled condition, improved the traits of RSA of rice in the earlier stages of growth of rice seedlings. However, it is important that the RSA-modification by AMF has to be evaluated under natural conditions in soil medium for further confirmation and to relate it for the growth of rice plant.



A. Seedling agar tube experiment for standardizing AMF spore load for rice



B. Microscopic view of mycorrhizal colonization in rice root



C. Mycorrhizal colonization with spore formation in rice roots

Plate 1. Assessment of different spore loads of AM Fungi on root colonization of rice under *in vitro* condition

CONCLUSION

The different spore loads of AMF, inoculated under *in vitro* conditions, modified the traits of RSA of rice at early stages significantly better than the uninoculated control. The AMF inoculation enhanced the RSA traits gradually as the dosage was increased and maximum positive effect was documented in 20 µl/seedling treatment. A substantial increase in most of the RSA variables was documented in all AMF treated seedling than those of uninoculated control. This revealed that AMF inoculation had a signal transduction role in root morphogenesis of rice.

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