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Comparative Fastness of AM Development in Roots of Different Rice Cultivars Due to Their Varying Responsiveness to AMF

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Abstract: ***Mycorrhiza varies in their responsiveness among the different plant and also within the species of a plant. This was established in case of various rice varieties in our previous study. Our hypothesis was that as AM fungi behaves like pathogen with the independent or negatively dependent variety group, a defence mechanism may triggered which restricted the fungi to grow in those plant root where as dependent variety group should allow the fungi to grow and there may be some differences in the penetration phase to colonization phase of the AM fungi between the two variety groups. Based on the above, 8 rice varieties were selected with varying responsiveness for the study with challenged inoculation. Root colonization process for AM development was examined at 10 to 15 days of seed germination. The colonization processes in low or non responsive variety groups are slower than that of the high responsive variety groups. Although, there is no difference in pre and post colonization structures but differ in intensity, the high responding varieties allowing faster and earlier establishment of mycorrhiza than the low or non responsive varieties.***

Keywords: Arbuscular mycorrhizal, Rice, Responsiveness, Root colonization

1.Introduction

Arbuscular mycorrhizal infection is initiated from spores, hypha or previously colonized root pieces – external or internal hyphae, vesicles, etc. AM fungal spores germinate normally on water but the germ tubes fail to grow and branch, unless in the vicinity of plant roots [1], [2]. The germ hypha of AM fungal spores undergo morphological differentiation – growth and branching, bending, swelling and production of fan like structures [3] or tufts [4] in close vicinity of the host roots before attachment and penetration.

Structural development of arbuscular mycorhiza by the 5 recognized AMF genera (*Glomus, Gigaspora, Acaulospora, Scutellospora and Entrophospora*) in all hosts follow a common pattern at the pre-penetration phase. Germinated spores form one or a few germ tubes which in the vicinity of roots soon develop as branched hyphae and move towards roots to establish contact. Old hyphal pieces, infected root pieces etc. that are randomly distributed in soil may get attached with a passing lateral root and may renew or give rise to new hyphal growth. These hyphae (*surface colonizing hypha*), originating either from spores or hyphae or pre-infected root pieces branch, proliferate, curl or form tufts on root surface or in close proximity of the roots. These hyphae, either directly or by producing lateral finer branches (*infective hypha*) establish contact with roots at multiple points. At such points of contact swollen knob or hyphopodia resembling appresoria are formed by these hyphae. The appresoria in turn produce penetration hyphae to penetrate the intact epidermis which first proliferate inside the host epidermis, hypodermis and then the cortex.

The penetration hyphae more commonly enter the host through the space in between two epidermal cells or sometimes by penetrating the wall of an epidermal cell. In both the cases, especially former, one or a few cells of the epidermis or the hypodermis or even the outer cortex may be colonized by intracellular hyphae as source point of further proliferation. From there on two morphological types of intraradical development take place. In the first and more common type, the hypahe grow rapidly and extensively through intercellular spaces of the upper cortex, often running length wise, parallel to the cortical cells, to ultimately reach and ramify in the inner cortex. The intercellular hyphae penetrate a series of cortical cells, mostly in the inner cortex with lateral branch hyphae which form much branched terminal arbuscules in the penetrated cells. Cortical colonization of this type is predominantly intercellular. In the other type, intraradical proliferation is rather slow and intracellular with cell to cell penetration by branch hyphae in the epidermis, hypodermis and cortex. In the cortical cells there is extensive formation of intracellular hyphal coils with or without a few less developed intercalary arbuscules. Intraradical colonization of this type is wholly intracellular.

AM colonization in upland rice were elaborated by Rajeshkannan *et. al.* in 2009 [5]. Among the two varietal groups selected to examine the AM development, the Black Gora & ARC 12737 are showing negative response to AM inoculation at early stage presumes that AM behaves like pathogen for these two cultivars should restrict the AM fungal growth particularly at the initial age of the plant growth but that do not seem to be reach at levels that would prevent colonization [6]. Whereas, MTU 7029 and TN1 showing positive response to AM inoculation at early stage of plant growth behaved a symbiotic association should allow the fungus to grow. Although there is no difference in the process of penetration or colonization and structure development among the two varietal groups but there is a significant difference in respect of comparative fastness of the plant to allow the fungus to grow or develop structures in the roots of the rice cultivar.

2. Materials and Methods

For histological examination of AMF colonization structures and intensity at the pre-penetration and post-penetration stages 2 varieties each of the low responsive (Black gora and ARC 12737) and high responsive (MTU 7029 and TN1) varieties were examined and the seedlings were grown in low volume sterilized soil in 250 ml thermocole cups keeping 3 seedlings per cup for 15 days. Sterilized soil was inoculated with pure root based AMF inoclum at the rate of 5 g inoculum per kg soil to raise the Infective inoculum density to approx. 3.5 X 10^5 propagules per g soil. Seedlings were raised from surface sterilized seeds in inoculated soil and whole plant harvests were made between 10-15 days of seedling growth. Roots taken from the harvested plants were preserved, processed, stained and examined for the AMF structures for required number of replicates.
For assessment of surface colonization intensity and primary root infection intensity data were recorded at 10 days after seed germination and for assessing the later parts of the colonization process plants were allowed to grow for 15 days of seed germination.

Results are presented in Tables 1 - 4 and Plate No. 1 have been appended to show the structural features of AM development in rice roots at different stages.

The slide micrometric method of root infection intensity assessment [7] was followed to asses root infection intensity of experimental plants. Stained root segments of about 1 cm length, mounted in lactophenol were observed under microscope. Root and hyphal length was measured by ocular micrometer; number of vesicles and arbuscules were counted.

For assessment of pre-and post-infection colonization structures, mainly the Chlorazol Black stained root material was used. Whole plant root samples were spread in squared paper and the lateral branch roots from 3 random 1cm^2 segments were separated, bulked per plant and cut into 1.5 cm pieces. Such cut root pieces were stained separately for each plant sample and 100- stained root pieces per plant sample were mounted suitably in slides for examination and data recording. Minimum 3 plants were used as replicates for a variety so that ultimate data were based on 3 x 100 – 1.5 cm or 450 cm root length examination for each plant variety. All pre-penetration structures were counted and measured by micrometry under low magnification and data were expressed as number or length of AMF structures per unit root length or per unit root surface area or per unit surface length of surface colonizing hyphae. For count and estimates of internal colonization structures, a 4-point scoring system was devised from repeated observation of stained root pieces as accurate estimation of spread of the AMF in root cortex, especially with large number of samples was difficult. Considering that only two dimensional views were possible from micrometry of a tubular root piece, only surface view of fungal spread in 1.5 cm root pieces of nearly equal width (0.30mm) were taken to represent the colonization. The extent of surface spread of intracellular and intercellular AMF hyphae were divided into 5 categories attaching scores in simple arithmetic series.

No internal spread of AMF structures (-)	0
10 % of root surface area covered by either Intracellular or intercellular hyphae or arbuscules in the cortical cells (+)	1
11-25 % of root surface area covered by either Intracellular or intercellular hyphae or arbuscules in the cortical cells (++)	2
26-40 % of root surface area covered by either Intracellular or intercellular hyphae or arbuscules in the cortical cells (+++)	3
41 % to 60% % of root surface area covered by either Intracellular or intercellular hyphae or arbuscules in the cortical cells (++++)	4

During the relatively short experimental time period allowed for root colonization, highest root surface area covered by internal spread rarely exceeded 50 %, so that keeping the limit of spread up to 60 % was realistic for the situation. Root pieces individually showing the colonization grades as above were counted and the total number of root pieces belonging to each grade was multiplied by score value. The summation of scores of all the root pieces examined was divided by the total number of samples to arrive at the relative score of internal colonization by either intracellular or intercellular hyphae or arbuscules. For the purpose a minimum of 3 x 100 – 1.5 cm root pieces were examined.

Structural features of AM development related to AMF colonization responses of the differently responding rice varieties was assessed from root samples taken from inoculated soil pots For the purpose of assessment, the colonization process was divided into the following 5 distinct stages.

(i) Surface colonization through production of colonizing hyphae from spores, hyphal pieces and pre-infected root pieces
(ii) Surface contact and development of penetration points through infective hyphae, appresoria
(iii) Penetration of epidermis and entry into epidermal region
(iv) Intercellular and intracellular hyphal development in the cortex
(v) Development of arbuscules (and vesicles) in the cortical cells

3. Results

Data presented in Table 1 showed that at 10 days pre-penetration and post-penetration colonization intensities of the two variety groups varied significantly in terms of root pieces showing the designated pre-penetration and post-penetration structures of colonization. There was evidence that intensity of both pre- and post-penetration colonization was higher in the high responding varieties than the low or non-responsive varieties. This showed that at the given period of time when initial colonization was being attempted the differently AM responding varieties might differ in the rate at which both surface and internal colonization were being established. It would seem that the process of colonization in the non- or low responsive varieties was slower than that of the high responsive varieties. However, so far as the structural features of the colonization were concerned there were no differences between the varieties.

Table 1: AMF colonization intensity – pre- and post infection responses of the rice varieties between 10 days after seed germination in inoculated soil

Features	*4 low/ non-responsive varieties*	*4 high responsive varieties*	*Est-t*
Percent root pieces showing pre-penetration structures – surface colonization hyphae, infective hyphae, penetration hyphae, and post penetration intraradical hyphae, appresoria etc	54.4 ± 8.6	79.1± 11.2	4.321**
Percent root pieces with both pre-penetration surface and post-penetration internal structures – inter- and intracellular hyphae, hyphal coils and arbuscules and vesicles	34.3 ± 4.7	63.7 ± 9.3	7.274**
Percent root pieces with surface colonization & penetration structures but without any post-penetration internal structures	20.1 ± 2.8	15.4 ± 3.6	NS

Based on 3 x 100 – 1.5 cm root pieces of the 8 varieties sampled from 3 random quadrants of whole root mass; Table t at 3 df - 0.05 *p* 3.182, 0.01*p* 5.841

Table 2: Pre-penetration AM structures on root surface of differently dependent rice varieties between 10 days after seed germination in inoculated soil

Variety	*Number of surface colonization sites*		*Average length of surface colonizing hypha*		*No. of penetration points from surface colonization*			
	per mm root length	Per mm ^{2} Root surface	Per colonization site (mm)	Per mm root Length (mm)	Per colonization site	Per mm hyphal length	Per mm root length	Per mm^2 root surface
Mean of two negatively responding varieties	0.1± 0.03	0.076± .007	7.0± 0.95	0.67± 0.05	5.6± 1.20	0.76±0.03	0.51± 0.03	0.38± 0.02
Mean of two positively responding varieties	0.2± 0.05	0.180± .047	35.2± 0.043	6.1± 0.082	9.4± 1.84	0.78±0.04	1.86± 0.61	1.02± 0.32
Est. t $_{0.05}$	5.59 **	8.61 **	91.38 **	18.40**	5.59 **	8.13**	9.43**	8.42**
Table t at 5 df	0.05 *p* 2.571				0.01 *p* 4.032			

Based on 3 x 100 - 1.5 cm root pieces sampled from 3 random quadrants of the whole root mass

Upon assessment of the intensity of pre- and post penetration colonization of the varieties, as above, differences in the structural details of pre-penetration colonization between the two variety groups, if any, were assessed from two varieties each of the two groups. Results are presented in Table 2. Data showed that based on the three distinct features of pre-penetration development – number of surface colonization sites per unit root surface area (representing an individual spore or a hyphal or a pre-infected root piece associated with the sampled root), length of such hyphae per unit root length and colonization site, and the number of penetration attempts (through penetration hyphae) per unit surface colonization site and surface area of the root, the high and low AM responding varieties had significant differences. At the given period of time the intensity of pre-penetration surface colonization through root associated hyphal development prior to appresorium formation was significantly higher in the high responding varieties than the low (or early non-) responding varieties. These details confirmed that the progress of surface colonization for establishing mycorrhiza in the non- or low responding varieties was comparatively slow than that in the high responding varieties.

These results were then followed by assessment of the possible differences in actual penetration intensity of the varieties by counting the number of appresoria formed by surface colonizing and penetration hyphae per unit surface colonized root surface area and per unit length of surface colonizing hypha. The estimates (Table 3) showed that at the given point of time appresorium formation intensity on the two high responding varieties was significantly higher than that on the low responding varieties. Slower progress of appresorium formation on the root surface of the low AM responding varieties was again indicated by the results.

Table 3: AMF penetration response of differentially AM responsive varieties at 10 days after seed germination in inoculated soil

Features	*2 low responsive varieties*	*2 high responsive varieties*	*Est. t*
Percent root pieces showing appresorium development on surface from penetration hypha	32.2 ± 3.2	44.5 ± 4.5	7.14**
Mean number of appresoria per mm ^{2} root surface showing surface hyphal colonization	11.4 ± 1.5	21.8 ± 1.9	13.68**
Mean number of appresoria per mm length of surface attached hypha	0. 8 ± 0.04	1.3 ± 0.08	18.63**

Based on 3 x 100 – 1.5 cm root pieces sampled from 3 random quadrants of whole roots Table t at 5 df - 0.05 *p* 2.571, 0.01 *p* 4.032

Variations, if there were any between the two variety groups in internal colonization response – intra- and intercellular hypha development and arbuscule formation were then assessed. These estimates (Table 4) also showed that internal colonization intensity as judged by score values for intercellular, intracellular and arbuscule development in the cortex was significantly higher in high responding varieties than the low responding varieties.

All these estimates of pre- and post penetration colonization of the host varieties by the AMF fungi used in the study pointed out differences in the intensities in host-fungus interactions between the two differently responding variety groups at the early seedling stage, not for any qualitative aspects of pre- and post-penetration colonization structures but for the intensity of both pre- and post- penetration colonization. There were no apparent differences in host response between the variety groups also. The observed difference was interpreted as difference in the rapidity with which colonization was established in the two variety groups, the high responding varieties allowing faster and earlier establishment of mycorrhiza than the low or non-responsive varieties.

Table 4: Post-penetration AMF development response of differentially AM responsive varieties at 15 days of seed germination in inoculated soil

Features (by 0-4 point scale)	*Average of 2 low responsive varieties*	*Average of 2 high responsive varieties*	*Est. t*
Relative score of intercellular hypha development in upper cortex of the colonized root pieces	2.2±0.06	3.4±0.08	38.33**
Relative score of intracellular hypha including coils development of the colonized root pieces	1.4± 0.03	2.7±0.07	58.14**
Relative score of arbuscule and / or vesicles development in inner cortex of colonized root pieces	1.2± 0.04	2.3±0.06	49.19**

Based on 3 x 100 - 1.5 cm root pieces sampled from 3 random quadrants of whole root; Table t at 5 df - 0.05 *p* 2.571, 0.01 *p* 4.032

4. Discussion

Results of the previous studies on inter-variety differences in AMF response reviewed by many researchers [8] - [11] and read with the early data on mutational origin of resistance to AMF colonization [12] have shown the possible existence of two sets of independently acting genes behind the functional compatibility of the AMF with their hosts and identification of several novel nutrient transporters has revealed some of the cellular processes that underlie symbiosis [13]. There may be one set of genes that control whether or not colonization occurs [14] and another set that controls the degree of benefit derived from the symbiosis [10]. In the present case, the negatively or low AM responding varieties were all colonized by the AM, though at a slower rate than the high responding varieties. It seems that AMF colonization in these varieties was not blocked either at pre – or post-penetration stages, so that AM colonization responsive genes were likely to be present in these varieties also. It is possible that the genes that control AM responsiveness were not equally present or expressed in these varieties as in the high responding varieties. Hetrick et. al. 1993 [10] observed that wheat cultivars which do not benefit from AM symbiosis may suffer from growth depressions. Hetrick *et al*. (1995) [15] from their data on formal genetic analyses of differently AM responding wheat cultivars suggested for the existence of 'mycorrhiza responsive' genes in the cultivars which respond to AM colonization. Whether the present results point out such a phenomenon being universal becomes a pertinent point to consider and may be important for considerations of exploitation of AMF in low input systems of cultivation through selection and breeding 'high AM symbiosis responding' varieties.

These interpretations take us to consider the reasons for the delay or slower AMF colonization rates of the negative or low responding varieties. Results of the histological study on colonization features of the differently AM responding varieties showed that the structural pattern of colonization either at the pre- or post-penetration stage were not different between the two variety groups. The difference that was there was in the relative rapidity with which colonization was established in the two variety groups. The results were inadequate to understand the reasons for the slower rate of colonization in the negative or low AM responsive varieties. The only possible speculative explanation that can be offered is the expression of some degree of incompatibility between the host varieties and the AMF, especially at the early seedling stage. We are not aware of any previous example of such delayed or slower rate of histological colonization by the AMF of varieties of a plant species, except the cases of low or reduced AMF colonization of the *rmc* mutant of tomato [16] and the recently described low AMF penetration, and low response mutant phenotype of pea which has low nodulation phenotype also [17]. The latter has two blocks to AMF colonization, one at penetration of epidermis and the other at cortex invasion stage. Both the *rmc* and the low *Pen*, low *coi* phenotypes are however,

arbuscule positive. Although these phenotypes are not exactly comparable to the presently observed slow colonization phenotype of rice varieties, existence of varietal or genotypic differences in compatibility to AMF colonization rates cannot be overruled among the plant species.

5. Future Scope of Study

Results of the investigation have helped in identification of physiological correlates of variable of AM responsiveness or dependency of rice, chosen as the model plant, in a low nutrient soil and have provided a base for further genetic studies relating to selection and breeding of high mycorhiza responsive varieties for low input cultivation with arbuscular mycorrhiza. Results of the studies on inter-variety differences in AMF response have provided basis for further study on the presence of two sets of independently acting genes behind the functional compatibility of the symbionts and the role of elicitor – receptor in the host – parasite interactions.

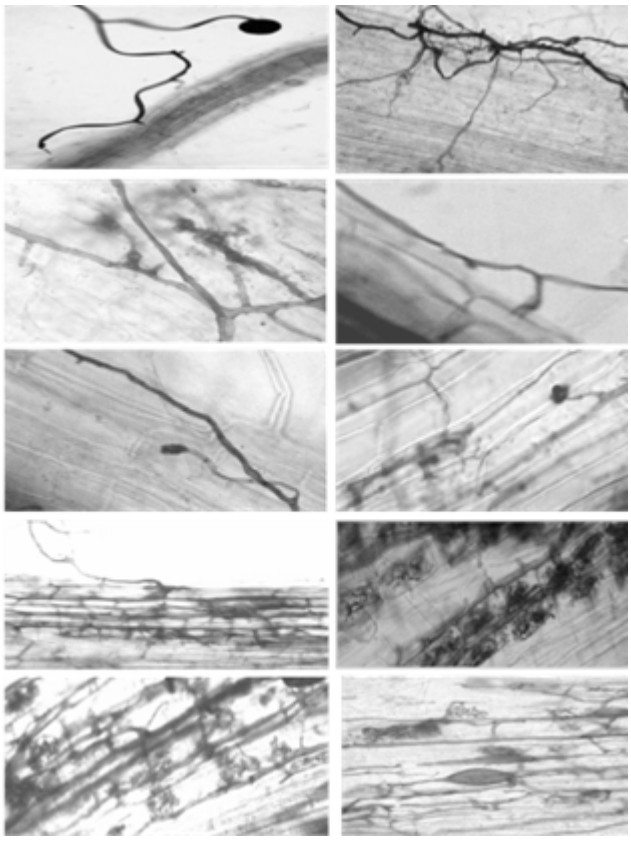

Plate 1: Microscopic observation of surface colonization, appresoria formation, penetration, post-penetration colonization, arbuscule and vescicle formation in rice root by AMF

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Dr. Bidisha Mondal was graduated from University of Calcutta with Botany Honours and was a gold medallist in Genetics & Plant Breeding (Ag.) from University of Calcutta. She was selected for training in Plant Biotechnology: Molecular Marker Technology by DST, Govt. of India among the 10 researcher from all over India. She has worked as guest research worker in Bose Institute after completing her Master's degree. She obtained doctorate degree from Bidhan Chandra Krishi Viswavidyalaya in 2005 with funding by a ICAR-NATP-CGP project of Govt. of India. She has 17 publication in indexed journal out of which 4 are internationally highly reputed one. She has 6 full papers in national &international level proceedings. Adding to this she has written 5 chapters of Subsidiary Botany Book of Netaji Subhas Open University and edited two books of Genetics and Evolution of the same University. Other than that she has written 5 popular articles in Bengali magazines. She has a research career of more than 13 years and a teaching career of 5 years. She served Netaji Subhas Open University as Assistant Professor in Botany from 2009 to 2012 and also taken classes of Post Graduate Diploma in Medicinal & Aromatic Plants of the same University. As Guest Lecturer she took classes of Molecular Biology, Biotechnology & Microbiology. At present she is working as Scientist in Bidhan Chandra Krishi Viswavidyalaya. She has earned the prestigious Bio-CARe Women Scientist award of Department of Biotechnology, Govt. of India in 2012. She has delivered several radio lectures as anchor and distance teacher on several socio-scientific issues. Her research interest involves biotechnology, molecular biology, genetics, plant physiology, plant nutrition and environment.