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Analysis and Quantification of Spread of Arbuscular Mycorrhizal Infection in Root of Vetiveria Zizanioides

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ABSTRACT

Arbuscular mycorrhizal fungi (AMF) are important component of rhizosphere microbial communities in natural ecosystems, forming symbiotic associations with the majority of land plant roots. Colonization is restricted to root cortex and does not enter the vascular cylinder. Arbuscular mycorrhizal fungi invade cortical cells inter- and intra-cellularly and form clusters of finely divided hyphae known as arbuscules in the cortex. They also form membrane-bound organelles of varying shapes known as arbuscules inside and outside the cortical cells. The fungal hyphae spread into the soil from host plant roots and improve the efficiency of nutrient uptake, such as immobile phosphate ions. The aim of this work was to determine the various morphological and anatomical parameters associated with extent of AM colonization in roots of Vetiveria zizanioides in control and three test plots (P1, P2 and P3) for three growth years i.e. from June 2011 to June 2014.

KEYWORDS: arbuscular mycorrhizal fungi, arbuscules, am colonization, vetiver

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INTRODUCTION

The term “mycorrhiza” was coined by A. B. Frank[1]. It means “fungus-root,” and stands for the mutualistic association existing between a group of soil fungi and higher plants. Arbuscular mycorrhizal fungi (AMF) are important component of rhizosphere microbial communities in natural ecosystems, forming symbiotic associations with the majority of land plant roots[2]. It has been proved that arbuscular mycorrhizae (AM) can be found in almost all sorts of soils in different tropical, mild and cold habitats[3]. The fungal hyphae spread into the soil from host plant roots and improve the efficiency of nutrient uptake, such as immobile phosphate ions[4, 5]. Today, mycorrhizal symbiosis are found associated with more than 90% of terrestrial plants, distributed in all climates and ecosystems regardless of soil type, vegetation and environmental conditions[6]. Colonization is restricted to root cortex and does not enter the vascular cylinder. In natural communities, approximately 80% of higher plants are obligatorily dependent on fungal associates and 18% typically non mycorrhizal[7.] This is in contrast to the antagonistic interactions of plants and pathogenic fungi, with defense mechanism of Arbuscular mycorrhizal fungal relationship with plants which can increase the growth of plants by enhancing phosphate uptake mainly and perhaps the other minerals such as K, Fe, Cu, Ca and Zn[8]. Arbuscular mycorrhizal fungi invade cortical cells inter- and intra-cellularly and form clusters of finely divided hyphae known as arbuscules in the cortex. They also form membrane-bound organelles of varying shapes known as vesicles inside and outside the cortical cells. Outside the root in the soil extensive, branched, external mycelium grows from the infection units[9]. A single root axis might contain two or more infection units per centimeter on average[10], each producing an external mycelium that can extend many centimeters beyond the rhizosphere and potentially colonize different parts of the same root system and other plants. Arbuscular mycorrhizal (AM) fungi, which coevolved with plant roots, form symbiotic associations with around 82 % of angiosperms[11]. These fungi are well known to improve plant growth on nutrient-poor soils and enhance the uptake of P, Cu, Ni, Pb and Zn[12].

MATERIALS AND METHODS

Plants of *Vetiveria zizanioides* (Linn.) were grown under natural conditions in experimental plots for three years from June 2011 to June 2014 and screened for the presence of arbuscular mycorrhiza in fine root segments at regular intervals.

Collection of plant and soil samples

Soil was collected from different localities in and around Delhi. These sites are (a) Shastri park area- dumping ground for burnt and half burnt electronic waste and disposal ground for nickel-cadmium batteries, (b) Dump yard of Okhla sewage treatment plant where treated sewage is converted into manure, (c) Sanganer soil from Rajasthan where effluents from dying industries are dumped into the soil, (d) Yamuna soil from the three different regions i. e. near okhla barrage, Wazirabad barrage and ITO barrage. These parts of Yamuna are its flood plains and receive water from Yamuna during rainy season. The water of Yamuna is highly polluted as it receives waste domestic sewage as well industrial effluents of Delhi yet there is large scale cultivation of vegetables and horticulture plants at its bank, (e) Uncontaminated garden soil from Sri Aurobindo college**.** The soils collected from above sites contain moderate level of Ni so Nickel was supplied in the form of soluble nickel salt dissolved in double distilled water (DDW), was mixed thoroughly in the soil and the soil was homozinised and kept in polythene bags and labeled to avoid a mix-up of the different soil samples and later analyzed for their Ni contamination. Plant samples were collected carefully using hand trowel to dig the soil around the plant and the plants were pulled out carefully, ensuring that no part of the root was lost. The different plant samples were kept in different polythene bags and properly labeled. Soil samples were collected from the same point where the plant samples were uprooted. Clums of *Vetiveria zizanioides*(Linn.) procured from Central Soil Salinity And Research Institute, Karnal, Haryana and planted in prepared plots (4x4inch) of 15 kg soil capacity containing soil collected from the different locations. Four plots were used for cultivating the plants. The prepared plots were placed in field conditions to expose the growing plants to natural environment. Ten plants per plot were maintained. Plants and soil was analyzed at 15 days interval for investigating the uptake potential, spore density and % root colonization. Every year plants of Vetiver grass uptake Ni from the soil and again the healthy clums of Vetiver grass were planted in the same soil. The culms of *Vetiveria zizanioides* (Linn.) grass with root (10 cm) and shoot (20 cm) were selected for the study. This experiment was conducted for three consecutive growth period with three treatments to *Vetiveria zizanioides*.

Assessment of roots for AM colonization

Measurements and analysis: Ten randomly chosen plants were harvested after an interval of 15 days after planting the tillers. Shoot dry weight was determined after drying the shoot tissue in an oven at 800C for 48 h. Oven-dried shoot tissue was ground and sieved through a 0.5 mm sieve. Assessment of

roots for AM colonization was made at the end of the experiment by random sampling of roots. The roots were clarified and stained according to the method of Phillips &Hayman[13]. All AM fungal structures (hyphae, arbuscules and vesicle) found in the roots were counted under the microscope and the extent of the colonization was estimated in terms of percentage of mycorrhizal root.

Estimation of percentage mycorrhizal colonization: Several methods have been described to quantify arbuscular rmycorrhizal fungi in roots of several plants[14,1 5, 16, 17, 18, 19].

For the present study the mycorrhizal status of plants were analyzed by:

For rough calculation of percentage of infection, the technique of Nicolson[17] was employed. The number of root segments infected by AM fungi and uninfected was recorded in this technique and the percentage root infection was then determined as follows:

$$\% \text{ root infection} = \frac{\text{Number of infected segments}}{\text{Total No. of segments infected}} \times 100$$

The analysis of infected root segments was then done in detail in the manner as described below:

I. External spread of VAM fungi:

1. Hyphae running parallel to the root surface,
2. Hyphae running radially across, the root surface,
3. Hyphae running in longitudinal, parallel and spiral manner on the root surface,
4. Presence or absence of extrametrical vesicles.

II. Entry of VAM fungi into the root tissue:

1. Direct Entry

(a) Number of entry points per mm. of root tissue.

(b) Thickness of penetrating hyphae.

2. Indirect Entry

(a)Number of appressoria per mm. of root tissue and shape of appressorium

(b) Branching of infection hyphae.

Intramatrical hyphal network:

1. Spread of infection hyphae internally

(a) Hyphae running parallel to the root tissue
(b) Hyphae running readily
(c) Hyphae running in all directions
(d) Diameter of hyphae in the cortex

2. Infection hyphae forming secondary appressorium or appressoria; that develop secondary infection hyphae respectively, which then reach either upto the inner cortex only upto the stellar region also.

3. Presence or absence of

(a) Coiled hyphae (intercellular or intracellular)
(b) Looped hyphae (intercellular or intracellular)
(c) Projections on internal hyphae.

III. Formation of arbuscules:

1. Presence or absence

2. Diameter of arbuscular trunk

3. Shape and size of arbuscules

4. Arbuscular abundance (no./mm infected root)

5. Proportion of cell occupied by the arbuscule, expressed as % arbuscular area i.e.

% arbuscular area = Area of the arbuscule/ Area of the cell *100

IV. Formation of intramatrical spores of VAM fungi

Preparation of root segments for anatomical studies:

Roots were rinsed with distilled water, cleared by 10% KOH, 30-45 min at 90°C and acidified in 1% HCl for 5-10 min. Then they were stained using Trypan Blue (0.05% in lacto-glycerol) for 10 min. They were left in lacto-glycerol at 90°C for 45 min for elimination of undesired dye particles. For quantification of AMF colonization, 70 one cm sections were selected randomly and left them on slides

under microscope (80×) and percentage root colonization (PRC) was calculated according to Phillips and Hayman[13] procedure.

Experimental setup

The experiment was conducted in 3 micro-plots of 10 m2 soil (up to 30 cm depth) was fumigated twice with 0.1% formaldehyde at an interval of 15 days. Then the soil was allowed to dry and the fumigant was dissipated. *Vetiver zizanoides* was grown in three types of experimental plots containing sterilized soil with a known quantity of Ni salts as present in polluted soils. Control without any kind of AM spores + Normal non polluted garden soil

(i) AM inoculum produced from AM spores collected from contaminated soils + test plants + Ni contaminated soil.
(ii) AM inoculums produced from AM spores collected from normal garden soil + test plants + Nickel contaminated soil.
(iii) No Am spores + Test plants + Nickel contaminated soil.

Clums rate was kept uniform for all treatments and when clums were 15 days old, thinning was done to maintain spacing of 10 cm between the plants and 20 cm within the rows. The plants were allowed to grow and no fertilizer or pesticide was added to the soil during the course of the experiment. Weeding was done mechanically at regular intervals and plots were irrigated with tap water.

P1 (Plot 1) – Nickel contaminated soil+ AM fungi spores inoculum produced from spores obtained from Ni contaminated soil + Vetiver test plant

P2 (Plot2) – Nickel contaminated soil + AM fungi spores inoculum produced from spores obtained from normal garden soil + Vetiver plant

P3 (plot 3) - Nickel contaminated soil + without AM fungal Control – normal non contaminated garden soil without AM spores + vetiver plants

OBSERVATION AND RESULTS

Comparison and Quantification of spread of colonization / infection internally and externally in roots of Plants of different treatments.

1. External spread of Am fungi (Table 1 and Plate 4)

a) Hyphae running parallel to the root surface-This was observed after the AM spores germinate on the surface of root and develops external hyphae first. Control and P3 showed almost negligible (Only 2nd and 3rd year of growth in control otherwise nil) parallel external hyphae. Plots P1 and P2 showed tremendous external hyphae in all three years but maximum external hyphae were observed in 3rd year of growth i.e. 37 in P1 and 29 in P2.

b) Hyphae running radially across the root surface-There was extensive network of radially running hyphae in P1 and P2 treatments and showed maximum number only in 3rd growth reaching up to 36 and 29 at one point of time in P1 and P respectively. Control and P3 showed negligible radial hyphae.

c) Hyphae running longitudinal, parallel and spiral manner on root – This also follows the same trend as the above two parameters. P1 and P2 showed up to 12 and 10 such external hyphae respectively.P3 and control showed negligible hyphae.

d) Presence and absence of extrametrical hyphae – Extrametrical hyphae were only observed in Plot P1 and P2. In P3 and control extrametrical hyphae were absent.

2. Entry of am fungi into root tissue (Table 1 And Plate 3)

a) Direct entry:-Direct entry of AM hyphae was observed in all treatments during all three years of study but in plot 3 the direct entry was only observed in 3rd year of study and not in 1st and 2nd year of study.

b) Number of entry points:-Maximum number of entry points were observed in plot 1 and plot 2. The entry points in these plots vary between 4 to 10 but maximum entry points in these plots at any point of time were observed only in 3rd year of growth. The entry points were maximum up to 2 in control and absent in 1stand 2nd year of growth and only 1 in 3rdyear of growth.

c) Thickness of penetrating hyphae:-Thickness of the penetrating hyphae was more than double (4.7micrometer) as compared to control (2.1micrometer) and the thickness of the hyphae in plot 2 was also double then the control but only in 3rd year of growth. Thickness could not be taken in plot 3.

d) Indirect entry:–Indirect entry pattern followed the same trend as was observed indirect entry i.e. indirect entry of AM hyphae was observed in all treatments during all three years of study but in plot 3 the direct entry was only observed in 3rd year of study and not in 1st and 2nd year.

i) Shape and number of appresoria per mm of root tissue-Number of appresoria varied from three to five in plot 1 with shapes ranging from swollen, flat, elliptical and compressed. In plot 2 the number of appresoria varied from 2, 1 and 5 per mm of root length with shape ranged from swollen to flat and compressed. In control on an average only one appresoria present at any time of study and the shape appeared only swollen. In plot 3 none were observed in 3rd and 2nd year of growth and only 1 flat appresoria in 1styear was observed per mm of root length.

ii) Branching of infection hyphae-Branching of infection hyphae was not observed in control and plot 3. In plot 1 in extensive branching was observed in 2ndyear of study and nominal branching was observed in 1st year in all three years of study in plot 2. (Table 4.6)

3) Intrametrical Hyphal Network (Table 1 And Plate 1)

Spread of internal hyphae internally

i) Hyphae running parallel to the root tissue-Parallel hyphae was seen in all the treatments including control except 1stand 2ndyear plants taken from plot P3.

ii) Hyphae running radially-Radial hyphae was observed only in plants of plot P1 and P2 and in the 3rdyear of growth of plants in control.

iii) Secondary appresoria- Secondary appresoria was observed only in 2nd and 3rd year plants taken from Plot P1 and 3rd year plants taken from plot P3.

4) Presence And Absence Of (Table 1 And Plate 3)

i) Coiled Hyphae –Coiled hyphae was found to be present incontrol in all years in plot P1 and P2 and none in P3.

ii) Looped Hyphae- Looped hyphae was observed only in plot P1 in all years and plot in 2nd and 3rdyear.

iii) Projection in Internal Hyphae-It followed the same trend as was looped hyphae.

5) Formation Of Arbuscules (Table 1 And Plate 2)

a) Present or absent –arbuscules were seen clearly and well formed in 3rd year plants of control and all years in Plot 1 and 2.

b) Size of arbuscules- Maximum size was observed in PlotP1 i.e. up to 18 micrometer and minimum in control upto 6 – 7 micrometer.

c) Arbuscular Abundance-Maximum number of arbuscules per mm of infected root was 25 in plot 1 (3rd year) and 17 in plot 2. They are completely absent in plot 3.

d) % arbuscular area-Maximum percent arbuscular area was observed in plot 1 i.e.42% followed by 36% in plot 2.

TABLE AND PLATES

Table 1- Analysis and Quantification of spread of infection in root of test plants of *Vetiveria zizanioides* in three years of study

S.No.	Features of Colonization	Control			Plot P1			Plot P2			Plot P3		
		Ist Yr.	IInd Yr.	IIIrd Yr.	Ist Yr.	IInd Yr.	IIIrd Yr.	Ist Yr.	IInd Yr.	IIIrd Yr.	Ist Yr.	IInd Yr.	IIIrd Yr.
1	External spread of AM fungi												
a)	Hypae running parallel to root surface (Maximum at one time)	nil	nil	2	14	28	37	11	20	29	nil	nil	nil
b)	Hypae running radially across the root surface (Max. at any time)	nil	nil	1	12	36	41	14	22	29	nil	nil	nil
c)	Hypae running in longitudinal, Parallel and Spiral Manner on the root surface	1	1	3	10	12	12	7	11	10	nil	nil	1
d)	Presence or absence of Extramatical Hyphae	absent	absent	absent	Present	Present	Present	absent	Present	Present	absent	absent	absent
2	Entry of AM Fungi into Root Tissue												
a)	Direct Entry	√	√	√	√	√	√	√	√	√	x	x	√
(i)	No. of Entry Points	1	1	2	4	7	10	3	8	9	0	0	1

(ii)	**Thickness of penetrating Hyphae**	**2 µm**	**2.1 µm**	**2.1 µm**	**4 µm**	**4.5 µm**	**4.7 µm**	**3.1 µm**	**3.7 µm**	**4.2 µm**	**NA**	**NA**	**NA**
b)	**Indirect Entry**	√	√	√	√	√	√	√	√	√	**x**	**x**	√
(i)	**No. of appresoria per mm of Root tissue and shape**	**1**	**1**	**1**	**3**	**3**	**5**	**2**	**1**	**5**	**0**	**0**	**1**
(ii)	**Branching of Infection Hyphae**	**Not observed**	**Not observed**	**Not observed**	**Present**	**Extensive**	**Extensive**	**Present**	**Present**	**Present**	**Not observed**	**Not observed**	**Not observed**
3	**Intramatical Hyphae Network**												
a)	**Spread of Infection Hyphae internally**												
(i)	**Hyphae Running Parallel to the root tissue**	√	√	√	√	√	√	√	√	√	**x**	**x**	√
(ii)	**Hyphae Running Radially**	**–**	**–**	√	√	√	√	√	√	√	**x**	**x**	**x**
(iii)	**Secondary Apppresoria**	**–**	**–**	**–**	**–**	√	√	**–**	**–**	√	**–**	**–**	**–**
4	**Presence or absence of**												
a)	**Coiled Hyphae**	**–**	**–**	**Present**	**Present**	**Present**	**Present**	**Present**	**Present**	**Presnt**	**–**	**–**	**–**
b)	**Looped Hyphae**	**–**	**–**	**–**	**P**	**P**	**P**	**–**	**P**	**P**	**–**	**–**	**–**
c)	**Projection in internal Hyphae**	**–**	**–**	**–**	**P**	**P**	**P**	**–**	**P**	**P**	**–**	**–**	**–**
5	**Formation of arbuscules**										**–**	**–**	
a)	**Present or absent**	**–**	**–**	**P**	**P**	**P**	**P**	**P**	**P**	**P**	**–**	**–**	
b)	**Shape & size of arbuscule**	**–**	**–**	**6-7 µm**	**10 µm**	**12 µm**	**18 µm**	**7 µm**	**10 µm**	**15 µm**	**–**	**–**	
c)	**Arbuscular abundance (No./mm infected root)**	**–**	**–**	**6 to 7**	**15**	**18**	**25**	**11**	**11**	**17**	**–**	**–**	
d)	**% arbuscular area**	**–**	**–**	**2%**	**12%**	**30%**	**42%**	**7%**	**19%**	**36%**	**–**	**–**	

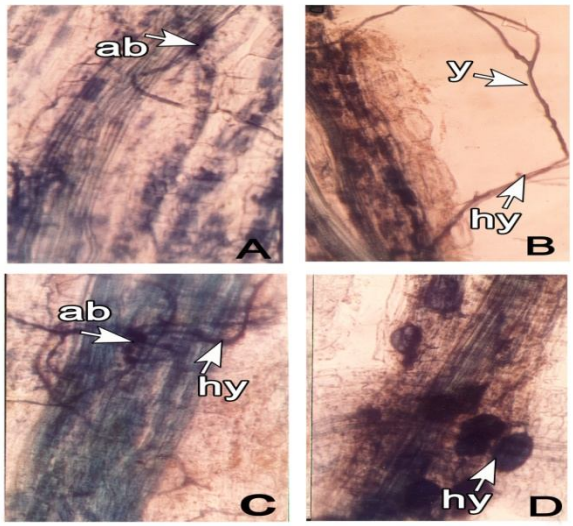

Plate-I

A. Arbuscules on the side branch of internal hyphae.

B. External hyphae showing appresorium.

C. Superficial mycelium colonizing stele as well as cortex.

D. Oval arbuscules present in cortex as well as in stele.

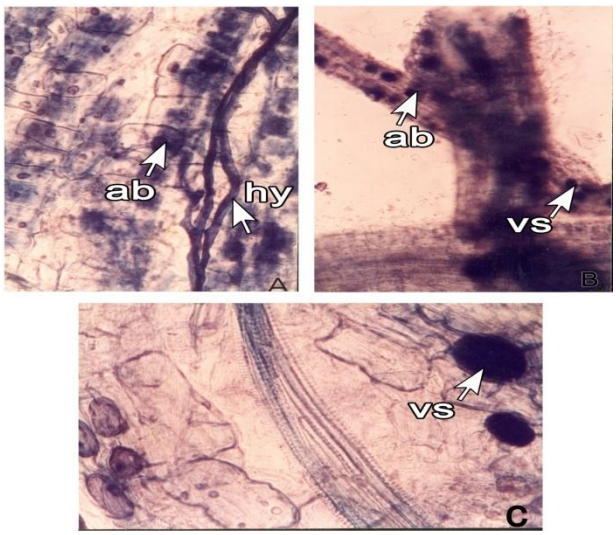

Plate-II

A. Superficial mycelium showing appressoria & arbuscules

B. Fine root segment showing heavy colonization.

C. Round to oval arbuscules present in the external cortex

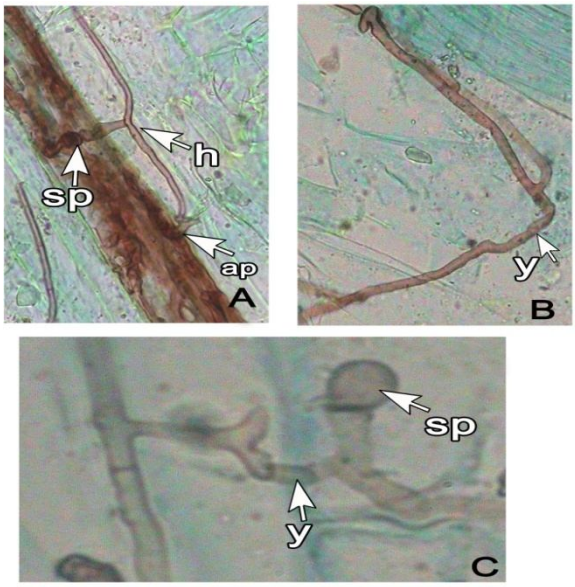

Plate-III

A. Penetration of root by hyphae through appressioum

B. Hyphal coil

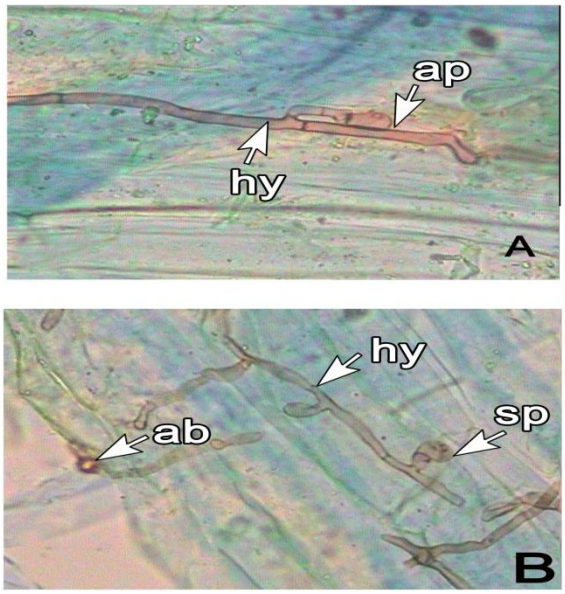

Plate-IV

A. Root showing appressoria.

B. Hyphae running in longitudinal and Parallel manner on Root surface

PLATES: I-IV Showing arbuscular mycorrhizal fungal components in macerated root sections of test plant.

DISCUSSION

Arbuscular rmycorrhizae were of general occurrence in all families except *Utricaceae, Casuarinace:ae, Nyctaginaceae, Portulaceac, Caryophyllaceae, Amaranthaceae, Chenopodiaceae, Oleaceae, Zygophyllaceae, Tamaricaceae and Euphorbiaceae*[20.] Nicolson[21], in his review emphasized that this type of mycorrhiza, although designated as endotrophic, was composed of a two phase mycelia system: an internal mycelium within the cortex of a mycorrhizal sheet and an external mycelium in the silwhich varies considerably in extent but which may be very extensive in some cases, even obscuring the root. However well-defined pseudoparenchymatous sheath around the roots is never formed in case of arbuscular rmycorrhizae. Structure produced by VA fungi within host roots include a hyphal system extending into the soil, short lived intracellular arbuscules generally thought to function in nutrient transfer between the symbionts; and enlarged intercalary or terminal vesicles that appear to function as endophytic storage organs. There are many reports on the anatomy of the host and endophyte association[22, 23, 24, 5, 6, 25].

Development of Infection:

Details of the infection process have been studied chiefly using spores or infected segments of root as inoculums, either in axenic culture in agar[26] on slides buried in soil[27], production of entry points has also been studied in pot experiments[28, 29, 30, 31] using sequential harvests, which do not, however, permit continual observation of the same infection. When spores are used as inoculum, germination is followed by considerable growth of one or several germ tubes, so that a simple mycelium in which total length of hyphae of a few centimeters is produced. Growth is sometimes increased if susceptible roots are present, so that it was at first thought that exudates from the roots might provide substrates for hyphal growth after the reserves in the spores had been used up. The role of root exudates in the development of infection is still receiving considerable attention. In spite of the increased mycelia growth in the presence of roots, hyphae may not appear to make directional growth towards roots until they are very close to them, i.e. within a few millimeters[26, 27]. Formation of an appressorium on the root epidermis is normally followed rapidly by penetration of the epidermal and cortical cells by hyphae and development of typical mycorrhizal structures within the root. Infection peg push into the cell wall. The later buldges round the hyphae and, in cortical cells, becomes much thinner[8]. This buldging implies the exertion of pressure by the growing hyphae and a degree of extensibility, existent or induced, in cell wall. Whether enzyme production is also involved is not known, but it seems unlikely that the hyphae

can generate much hydrolytic activity in view of the poor saprophytic ability of the fungi concerned. Nevertheless, changes in the middle lamella, as seen by electron microscopy[7], when the intercellular spaces are colonized by hyphae, might be thought to give some credence to the suggestions that fungal enzymes may be important.

NOTE: The result of this investigation clearly show that all the experimental plant species examined is mycorrhizal, their type being arbuscular and it does not show any other type of mycorrhiza. This result is in accordance with the detailed survey of Johnston[2].

REFERENCES

1. Frank, A. B."Über die auf Würzelsymbiose beruhende Ehrnährung gewisser Bäum durch unterirdische Pilze". Berichte der Deutschen Botanischen Gesellschaft.1885;3: 128–145.
2. Johnston, A. Vesicular-arbuscular mycorrhiza in sea Island cotton and other tropical plants. Trop. Agric. Trin. 1949; 26: 118-121
3. Lakshman, H. C. Selection of Suitable AM Fugus to *Atrocarpus heterophyllus* Lam. A Fruit/Timber for an Ecofriendly Nursery, M.D.Publisher, New Delhi, pp.50- 61. 2009
4. Graham, J.H. Effects of Citrus root exudates on germination of Chlamydospores on the Vesicular-arbuscular mycorrhizal fungus. Glomus epigaeum. Mycologia.1982; 74: 831-835.
5. Graham, J.H., R.T. Leonard, and J.A. Menge. Interaction of light intensity and soil temperature with phosphorus inhibition of vesicular-arbuscular mycorrhiza formation. New Phytologist. 1982; 91:683–690.
6. Fontana, A., Bonfanto-Fasolo, P. and Schubert, A. Morphological characteristics of Vesicular-arbuscular mycorrhizae associated with vine. Soil and Fertilizer.1978; 44: 4738.
7. Kinden, D.A. and Brown, M.F. Electron microscopy ofVesicular-arbuscular mycorrhizae of yellow poplar. II. Intracellular hyphae and vesicles. Can. J. Microbiol. 1975b; 21: 1768-1780.
8. Cox, G. and Sanders, F.E. Ultrastructure of the host-fungus interface in a vesicular- arbuscular mycorrhiza. New Phytol. 1974; 73: 901-912.
9. Smith, S.E., Read, D.J. Mycorrhizal symbiosis. New York: Academic Press. 800p. 2008
10. Harley, J.L, Smith S.E. Mycorrhizal symbiosis. London: Academic Press. 483 p. Harrison MJ. 2005. Signaling in the arbuscular mycorrhizal symbiosis. Ann Rev Microbiol.1983; 59:19–42.
11. Brundrett, M.C. Coevolution of roots and mycorrhizas of land plants. *New Phytol*; 2002 ; 154(2):275–304

12. Khan A.G, Kuek C., Chaudhry T.M, Khoo C.S., and Hayes WJ. Role of plants, mycorrhizae and phytochelators in heavy metal contaminated land remediation.*Chemosphere*. 2000; 41(1-2):197–207.
13. Phillips, J. M., & Hayman, D. S. Improved procedure for clearing roots and staining of mycorrhizal fungi for rapid assessment of infection. *Transactions of the British Mycological Society,* 1970; 55:158–161.
14. Ambler, J. R. and Young, J.L. Techniques for determining root length infected by Vesicular-arbuscular mycorrhizae. Soil Sci. Soc. Am. J. 1977; 41: 551-556.
15. Ames, R.N. and Lindermann, R.G. Acaulospora trappei sp. Nov. Mycotaxon.1977; 3: 565.
16. Davies, F.T., Puryear J.D., Newton R.J., *et al*. Mycorrhizal fungi increase chromium uptake by sunflower plants: Influence on tissue mineral concentration, growth, and gas exchange. Jour Pl Nutri. 2002; 25: 2389-2407.
17. Nicolson, J.H. The mycotrophic habit in grasses. Thesis, Notingham University. 1985
18. Read,D.J., Koucheki. H.K. and Hodgson, J. Vesicular arbuscular mycorrhizae in natural vegetation systems. I. The occurrence of infection. New phytol. 1976; 77: 641-653.
19. Sutton, J.C. Development of vesicular-arbuscular mycorrhiza in crop plants. Can. J. Bot. 1973; 51: 2487-2493.
20. Khan, A.G. The effect of Vesicular-arbuscular mycorrhizal associations on growth of cereals. II. Effects on Wheat growth. J. Appl. Biol. 1973.
21. Nicolson, T.H. Vesicular-arbuscular mycorrhiza- a universal plant symbiosis. Sci. Prog., Oxford 1967; 55: 561-581.
22. Abbott, L.K. Comparitive anatomy of Vesicular arbuscular mycorrhizas formed on subterranean clover. Aust. J. Bot. 1982; 30: 485-499.
23. Abbott, L.K. and Robson, A.D. A quantitative study of the spores and anatomy of mycorrhizas formed by a species of Glomus, with reference to its taxonomy. Aust. J. Bot. 1979; 27: 363-375.
24. Carling, D.E. and Brown, M.F. Anatomy and Physiology of Vesicular-arbuscular and non mycorrhizal roots. Phytopath. 1982; 72: 1108-1114.
25. Sanders, F.E, Tinker, P.B. Mechanism of absorption of phosphate from soil by *Endogone* mycorrhizas. Nature. 1971; 233: 278-279.
26. Mosse, B. and Heppar, C.M. Vesicular-arbuscular mycorrhizal infections in root organ cultures. Physiol. Pl. Path. 1975; 5: 215-223.

27. Powell, C.L. Development of mycorrhizal infection from Endogone spores and infected root segments. Trans. Br. Mycol. Soc. 1976;66: 439-443.
28. Carling, D.E., Brown, M.F. and Brown, R.A. Colonization rates and growth responses of soyabean plants infected by Vesicular arbuscular mycorrhizal fungi. Can. J. Bot. 1979; 57: 1769-1771.
29. Smith, F.A., Smith, S.E. Mycorrhizal infection and growth of Trifolium subterraneum: comparison of natural and artificial inocula. New Phytol. 1981; 88: 311–325.
30. Smith, S. E. and Bowen, G. D. Soil temperature, mycorrhizal infection and nodulation of Medicago truncatula and Trifolium subterraneum. Soil Biol. Biochem. 1979; 11: 469-473.
31. Smith, S. E. and Walker, N.A. Aquantitative study of mycorrhizal infection in Trifolium: separate determination of the rates of infection and of mycelia growth. New Phytol. 1981 ; 89: 225-240.