MORPHOLOGY AND QUANTIFICATION OF VAM on FIVE EXPERIMENTAL PLANTS
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Introduction

The importance of VA-mycorrhizal fungi enhancing host growth, both under natural and artificial conditions is well documented. This is due to the ability of fungal mycelium to spread in the soil and uptake of nutrients and then translocated to the host plants. A number of methods for estimating vesicular arbuscular colonization and related root physical properties are currently used. Amongst, the more popular are percentage of root segment, colonized percentage of root length, total root length, and specific root length. Although both intra and extra radical fungal mycelium involved in this process, but most experimentally work on VA-mycorrhiza is extensively studied with the development of the intraradical phase. It has been quantitatively assessed by different methods, but little quantitative data is available on morphology of VA-mycorrhiza in the hydrocarbon yielding plants.

The present work has been undertaken to study reveals the mycorrhizal variables, quantification of VAM colonization to determine the degree of root colonization.
Review of literature

The concept of fungal root symbiosis has since been a subject of extensive research. Vittadiny (1842) proposed that tree rootlets are nourished by certain fungal mycelia which mantle them. Later, this hypothesis was established to a theory of mutualistic symbiosis by Frank (1885), who named the fungus root organ called ‘Mycorrhizae’. Endomycorrhizal fungi grows inter and intracellularly and forms within the cortical cells specific fungal structure (Harley and Smith, 1983). Vesicualr arbuscular mycorrhizal fungi are known to colonize a number of tropical plants incuding many hydrocarbon yielding plants. Descriptive account of vesicular-arbuscular mycorrhizae has been given by the earlier workers in number of plants (Schlight 1889; Janse 1896; Gallud 1905; Peyronel 1924; Butler 1939; Strzemska 1949 and 1953; Harley 1950 and 1952; Mosse 1957 and 1959). Recently many excellent reviews and books have been published on structural, physiological, nutritional and ecological aspects of VA-mycorrhizae. (Harley 1969; Hacskaylo 1971; Mosse 1973; Smith 1974; Sanders et al., 1978; Powell and Bagyaraj 1984; Read et al., 1992; Varma and Hock 1998; Kapulnik and Douds Jr 2000). Vesicular-arbuscular mycorrhizae is of particular interest because of the association with roots of agricultural crops, tree species, horticultural crops and many
hydrocarbon yielding plants. The present study reveals the quantification of VAM colonization in five petroplants. Mycorrhizal variable amongst the plants quantification is needed to determine the degree of intensity of root colonization.

**Materials and Methods**

Roots and rhizospheric samples were collected from the different localities where plants were grown. Collected roots were washed thoroughly under tap water and observe directly under stereomicroscope to determine the extrametrical hyphae structure, colour and infected spots on roots. For quantifying vesicular-arbuscular mycorrhizal colonization followed the method of Phillips and Hayman (1970). Fine feeder roots (< 2 mm diam) of each collected samples kept in separate polythene bags with proper labeling. Some selected roots were cut into 1cm length and stored in alcohol-formaldehyde-acetic acid solution (200ml alcohol + 13ml formaline + 95ml acetic acid). The roots were cleared with 10% KOH, and neutralized with 1%HCl for 3-4 minutes, stained with Tryphan blue mounted in lactophenol. Gently tapped the cover slip to spread root tissue. And observed under the compound microscope.

The fungal mycelium took bright blue colour staine but the host tissue remains unstained. This gives clear contrast between mycorrhiza
and host tissues. The slides are thoroughly scanned for various structural components of VA-Mycorrhizae (i.e. vesicles, arbuscules, extra metrical chlamydospores etc.)

**Assessment of Colonization**

Assessment of VAM colonization of experimental plants were made as described by Giovanetti and Mosse (1980). The primary objective of this study is to determine the percentage of root colonization as well as intensity of colonization within the root.

The whole root sample is uniformly distributed in a petridish with the help of dissecting needles to eliminate clumping and to enhance light transmission. The number of susceptible root colonization in a sample determines the percentage of VAM infection. The roots are cut into 0.5cm bits spread on clean glass slides mount it observed under the microscope for the presence or absence of mycelium, vesicles and arbuscules. The percentage of root colonization is calculated after examining number of roots for the presence or absence of colonization. Colonized root samples were grouped as per the classification of (Institute for mycorrhizal research and development USDA forest service Athens, Georgia (1981). Class I: 0-5%, Class II 6-26%, Class II 26-50%, Class IV 51-75%, Class V 75-100% (Table-2), and their components had been examined.
Gridline Intersect Method

This method was used to estimate both the proportion of infected roots and their total length. The root sample was spread out evenly in a square petridish 10.2 x 10.2 cm. A grid of lines was marked on the bottom of the dish to form 0.5 in squares. The dimension of the grid squares is for measuring the total length of roots. Vertical and horizontal gridlines were scanned and the presence and absence of infection was recorded at each point where the roots intersected a line. Totally two grid line intersects were observed. By this technique the root length colonized by VAM was estimated. The data obtained given in (Table-2).

Estimation of Colonization by magnified intersections methods

Selected root samples were mounted in glycerine on microscopic slides covered 45 mm coverslips. Between 2-4 slides were used for each subsamples but all slides for a subsamples, were treated as a single unit. Roots were aligned parallel to the long axis of the slides and observed at magnification 100X. The field of view of the microscope was moved using the stage graticule to make four, six or eight complete passes across each slide perpendicular to its long axis. The number and distance between passes is constant for a subsample except where the cortex was missing all intersections between roots and the vertical eye piece.
crosshair were considered. The position on the root surface at which the center of the eye piece crosshairs entered through the slide of the root was taken as the point of intersection. Rotation of the vertical crosshair ensured each intersection was at right angles to the long axis of the root (Fig 2). Where the center of the crosshairs entered a root through an end rather than a side. The point of exit from the root through its side was taken as the point of intersection. Roots too wide to fit into the field of view at 100X magnification were examined in two or more width portions.

To examine each intersection the plane of focus was moved completely through the root and to know whether the vertical crosshair actually cut any arbuscules, vesicles and hyphae. The arbuscular colonization (AC) and vesicular colonization (VC) were calculated by dividing the count for the ‘arbuscules’ and ‘vesicules’ categories respectively by the total number of intersections examined. Hyphal colonization (HC) was calculated as the proportion of non-negative intersections. Mycorrhizal dependency of hydrocarbon yielding plants was estimated.
**Slide Method**

Root pieces, each approximately 1cm long, were selected at random from a stained sample and mounted on microscope slide in groups of 10. Length of cortex infected was assessed in millimeters for each root piece, averaged for the 10 pieces and expressed as a percentage (Root lengths in slide method). Alternatively presence or absence of infection was recorded in each of the 10 pieces and the result again expressed as a percentage (slide method ±). Each replicate comprised an additional sample of 10 root pieces.

**External mycelial phase**

VA-mycorrhizal roots are surrounded by extensive hyphal network which extends into the soil as much as 1-1.5cm. Hyphae also seen adhering to the outer root surface formed extensive wefts surrounding the roots of epidermis. But they never formed fungal mantle. The external hyphae were dimorphic and composed of course thick walled irregular non-septate rarely non septate hyphae. Most remarkable morphological feature was the variation in the diameter range from (3-25 μm) among the hyphal filaments have been noted in *Jatropha curcas*. The thick walled hyphae were almost file with densely cytoplasmic matrix, vacuoles, oil globules were commonly seen with multinucleated condition. Some cases hyphae were devoid of nuclei.
Most of the thick walled hyphae were smooth, with a few irregularities in outline and measuring (2-5 μm) in diameter thin walled hyphae arises laterally from the main hyphae. It was noted that the lateral thin walled hyphae grow directly out from the hyphae of the protoplasmic connection. Later, content of the hyphae disappeared and produced appressoria.

**Results and Observation**

All the experimental plant samples for microscopic observation for VAM colonization. The roots were colonized with the VA-mycorrhizae. Maceration and anatomical studies followed by tryphan blue staining revealed distinctly, stages and different components. Microscopic measurements provided an assessment of the relative abundance of mycelium in roots expressed as either the total mycorrhizal root length, the density of hyphae within roots, the length per entry point and the number of entry points, wall thickness, and pattern of outer epidermal cells. Numerous root hairs were seen in the infected roots of *Jatropa curcas* and *Jatropa gossifolia*. Course aseptate hyphal coils were oftenly seen from initial penetration points.
Colonization

VAM colonization examined in all the experimental plants, High percent of colonization with profusely branched mycelium were observed in *Jatropha curcas* and *Ricinus communis* (64 % and 50.7%). 26 – 50% of mycorrhizal colonization was noticed in *Jatrops gossifolia* and *Madhuca indica*. Colonization was started from the second set of lateral roots from the top, where as young terminal roots had more colonization. But colonization was not been observed in meristematic zone of the roots.

The experimental plants grown with rhizospheric soil inoculum in glass house condition and roots collected from natural habitat of these plants exhibited the same extent of colonization by VAM. The fresh preparation of colonization roots showed the signs of active growth in the cortical region. The plants that showed less percentage of colonization were due to the compactness of tissue system in *Jatropa gossifolia* and *Madhuca indica*.

The magnified intersection method gave additional accuracy to measure the fungal components in macerated roots. Magnified intersections were counted in the following categories; ‘Negative’ (no fungal material in root Table-3) ‘arbuscules’ ‘vesicles’ and hyphae only if the vertical crosshair cut one or more arbuscules or vesicles, The appropriate category was incremented by one, and similarly
for intersections where hyphae only were crossed. When both vesicles and arbuscles were scored at an intersection, the total number of intersections was only increased by one.

**Penetration Phase**

Appresorial hyphal structures were seen on the root surface prior to penetration. VA-mycorrhizal infection did not alter the morphology of roots. The size and colour of the extrametrical hyphae varies. In *Ricinus communis* (Var. Rosa) mycelial colour was yellow. It was brown colour in *Jatropha curcas*. Where as light yellow colour was observation in *Madhuca indica*. Root tips containing meristematic tissue wee free from hyphae. The different way of root penetration is linked to the anatomy of root, wall thickness, and pattern of outer epidermal cells. Root hairs were scanty in the infected roots of *Jatropha gossofolia* and *Jatropha curcas*. Course aseptate hyphal coils were oftenly seen from initial penetration points. It was difficult to obtain root hair infections. Number of penetrating hyphae found in a macerated root samples, However, the epidermal cell penetrations it was much easier to preserve the connection between internal and external mycelium in *Madhuka indica* (Plate-2, Fig.2).

All the experimental plant root samples subjected for microscopic observation for VAM colonization. Most of the roots were colonized with
VA-mycorrhizae. Maceration and anatomical studies followed by tryphan blue staining revealed distinctly, stages and different components of VA-mycorrhizae.

**External Mycelial Phase**

VA-mycorrhizal roots are surrounde by extensive hyphal net work which extended in to the soil as much as 1-1.5 cm. Hyphae was seen together with the outer root surface formed extensive wefts surrounding the roots of epidermis. But they never formed fungal mantle. The external hyphae were dimorphic and composed of course thick walled irregular non-septate rarely septat hyphae. Most remarkable morphological feature was the variation in the diameter range from (3-25 mm) among the hyphal filaments have been noted in *Jatropha curcas* (Plate-3, Fig. 2 &3 ). The thick walled hyphae were almost filled with dense cytoplasmic matrix; oil globules were commonly seen with multinucleated condition, in some cases hyphae were devoid of nuclei. Most of the thick walled hyphae were smooth, with a few irregularities in outline and measuring (2-5 m) in diameter, thin walled hyphae arises laterally from the main hyphae. It was noted that the lateral thin walled hyphae grown directly out from the hyphae of the protoplasmic connection. Later, content of the hyphae disappeared and produced appressoria (Plate –3, Fig.1).
**Internal mycelial phase**

The internal morphology of VA-mycorrhizae could be easily be observed by examining the whole mounts of stained preparation under relatively high power compound microscope. The hypha penetrates the epidermal cells of young roots behind the meristematic region. Penetration of root hairs were observed in host species namely; *Madhuca indica*, (Plate-3 Fig. 2). In the experimental plants roots, the mecelium in the host tissue develops from the hyphae penetrating through epidermal cells or root hairs. The hyphal growth initially intercellular in *Jatropa curcas, Madhuca indica* and *Ricinus communis* species, later it was observed that the hyphae were intracellular. Hyphae were irregular in shape and anastomous, complex coils and closed loops occurred between the cells observed in *Jatropa gossosfolia* (Plate –2, Fig. 3). The hyphae in the intercellular spaces were non-septae. In the older hyphae when growth ceases septa formation was noticed in *Jatropa curcas* (Plate – 4, Fig.1).

Colonization by the endophyte was observed from second, third and tertiary roots from the top of the root system, as seen in all the experimental plants. It was observed that the fungus grows throughout the cortex, but it does not invade the endodermis, stele or root meristem. The intracellular hyphal coils in the firs cell to be infected with similar coils being subsequently formed in neighboring cells.
Intracellular hyphae with coiled or linear orientation display signs of deterioration and degeneration of the fungal cytoplasm and subsequently hyphal wall collapsed. The fungus penetrated from one cell to another forming a new coil. Intercellular hyphae were usually found in the intermediate layers of the cortical parenchyma. Their diameter was 3-6 mm in *Ricinus communis* (var. Mysore local). The hyphae run parallel in between the parenchyma for considerable distance (up to several millimeter). In some areas the hyphae exhibited intermittent projections and were at times swollen. Longitudinal hyphal branches in the form of H and Y shape observed in *Jatropha curcas* (Plate - 4, Fig. 3). The septation of hypha were rare in activity growing mycelium.

**Arbuscules**

After infection hyphae spread in cortical cells and formed arbuscules look like haustoria or little tree with finger like projections. These arbuscules as seen in *Jatropha gossifolia* (Plate-2, Fig.3). Arbuscules are seen in various stages of the growth and some were in the state of disintegration.(Plate-2 & 3, Fig. 3 & 1). The host nucleus of infected cells were found slightly enlarged compared to the non-infected ones as observed in *Jatropha curcas*. Morphologically arbuscular branches were short and found that deteriorating and collapsing. In the older portion of the mycorrhizal roots senescent arbuscules compared to the
young mycorrhizal roots observed in *Ricinus communis* Var (Mysore local) (Plate – 4, Fig. 2). Arbuscules live only for 4-16 days in *Ricinus communis*. They degenerates and digested by the host cells.

**Vesicles**

Vesicles were globose, oval bodies formed by an intercalary or terminal swelling of hyphae. The different stages of fungal hyphae in the formation of vesicles observed in *Madhuca indica*, *Jatropha curcas* and *Ricinus communis* species (Plate –2, Fig. 2, Plate –3, Fig. 2. Plate–3, Fig. 1).

Vesicles were fond within the roots and formed intercellurally of intracellularly. Their size and shape diffused depending on the anatomy of the root. The vesicles size (42-48 mm) observed in *Madhuca indica* and *Ricinus communis* (Var. Mysore local.), where as vesicles were subglobose and large (89-106mm) in diameter was seen in *Jatropha curcas*. In *Jatropha gossifolia* vesicles were moderately less in number (Plate –2, Fig. 1). In certain areas the mycorrhizal roots possessed chains of vesicles observed in *Jatropha curcas* (Plate – 4, Fig. 1). The intercellular vesicles and host walls were distinct contact, where as the intracellular vesicles usually enclosed in a layer of cytoplasm. The dense granular cytoplasm with full of fat globules observed in matured vesicles. The outer walls of the vesicles appeared smooth without ornamentation.
### Table 2. VA-Mycorrhizal colonization in five hydrocarbon plants.

<table>
<thead>
<tr>
<th>Sl.</th>
<th>Plants</th>
<th>No of root samples examined</th>
<th>Mean root colonization rating</th>
<th>Intensity of colonization</th>
<th>No. of Localities presented</th>
<th>No. of samples infected</th>
<th>% of root segment mean+SD</th>
<th>% of root length infected per sample mean+SD (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Jatropha curcas</em> L.</td>
<td>21</td>
<td>7</td>
<td>5.3 ±0.00</td>
<td>16</td>
<td>8</td>
<td>62.1±4.2</td>
<td>64.0±0.00</td>
</tr>
<tr>
<td>2</td>
<td><em>J. gossifolia</em> L.</td>
<td>14</td>
<td>4</td>
<td>2.3±1.00</td>
<td>11</td>
<td>6</td>
<td>53.2±2.2</td>
<td>49.7±6.3 (0-72)</td>
</tr>
<tr>
<td>3</td>
<td><em>Madhuca indica</em> Gmel</td>
<td>19</td>
<td>3</td>
<td>1.7±4.2</td>
<td>5</td>
<td>4</td>
<td>44.4±7.1</td>
<td>41.0±3.3</td>
</tr>
<tr>
<td>4</td>
<td><em>Ricinus comminis</em> (var Mysorelocal)</td>
<td>31</td>
<td>9</td>
<td>7.5±2.0</td>
<td>14</td>
<td>9</td>
<td>59.5±3.5</td>
<td>67.7±5.6</td>
</tr>
<tr>
<td>5</td>
<td><em>Ricinus comminis</em> (Var. Rosa)</td>
<td>27</td>
<td>8</td>
<td>6.2±2.3</td>
<td>12</td>
<td>7</td>
<td>56.4±4.4</td>
<td>57.2±7.7 (0-69)</td>
</tr>
</tbody>
</table>

* Mean colonization rating 1) 0-5%  2) 6-25%  3) 26-50%  4) 51-75%  5) 76-100%

* Intensity of Colonization 1) Small widely scattered  2) large colonization  3) colonization in cortical cells.
Table 3. Selected examples data obtained using the magnified intersections method.

<table>
<thead>
<tr>
<th>Root Subsample</th>
<th>Microscope</th>
<th>Number of intersections</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Negative</td>
</tr>
<tr>
<td>1</td>
<td>A</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>Sum</td>
<td>181</td>
</tr>
<tr>
<td>2</td>
<td>A</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>Sum</td>
<td>79</td>
</tr>
</tbody>
</table>

Arbuscular colonization (AC), Vesicular colonization (VC), Hyphal colonization (HC).
Fig 2. Diagram shows how magnified intersection have taken to the long axis of the root at an angle to vertical cross hair from the center.
PLATE-2

Fig. 1 Less number of vesicles in *Jatropa gossifolia*

Fig. 2 Epidermal penetration in *Madhuca indica*

Fig. 3 Irregular hyphae and complex coils in *Jatropa ossifolia*
PLATE - 3

Fig. 1  Appressoria formation in Madhuca indica

Fig. 2 and 3. Hyphal filaments in Jatropha curcas
PLATE-4

Fig. 1 Septa formation and chains of vesicles in *Jatropha curcas* L.

Fig. 2 Senescent arbuscules in *Ricinus communis* L. Var. (Mysore local)

Fig. 3 Hyphal branches *H* and *Y* shape in *Jatropha curcas* L.
DISCUSSION

The relation between fungal and higher plants has been discussed with reference to vesicular arbuscular mycorrhizal fungi, which formed vesicles and arbuscules. In hydrocarbon bearing plants it appears to be caused by single or different types of endophytes in natural condition with typical non-septate hyphae. There was a marked dimorphism between internal and external mycelium, and frequent development of regular septation in certain external hyphae in outer cortical cells. These features noted in similar endophytes of many other plants (Mosse 1959 : Miller et al., 1989).

Distinguish among fungi in the field where, more than one fungi is present is to identify and quantify them. Spores are distinguishable (Daft and Hogarth 1983). Hyphae in the roots and the extra radical mycelium are crucial to the mycorrhizal processes and it is very important to identify them. In pot experiments where fungi have different morphological characteristics, they can be identified (Abbott 1982). To differentiate among populations of closely related species immunology is needed. (Monoclonal antibodies of chemical methods)

Morphological visual estimation has been used frequently to assess the VAM colonization followed the method of Mosse (1973), Abbott and Robson(1985). In the present study the magnified intersections method was adapted (McGonigle et al., 1990). This technique gave an important
advantage for the assessment of formation of arbuscules, vesicles and hyphae. Root samples clearing and staining technique adapted by Phillips and Hayman (1970) was ideal for identification of the symbiotic fungi. Though it was time consuming but the use of tryphan blue stain in this study was fungal specific that saved to observe morphological and structural details of the fungus. Large number of roots could be scanned for percentage colonization by this procedure (Grid line intersect method, Mosse and Giovanetti, 1980).

Plants benefited from mycorrhizal associations only in some of the least fertile soils in which they naturally occurred. Where as plants with roots that consistently resist colonization by mycorrhizal fungi, at least when they were young and healthy. Feeder roots were relatively short lived and the profuse development of mycelium suggested that their fungi may be able to exist saprophytic nature. The external and internal mycelium of hydrocarbon plants corresponds closely to many previous description of similar findings on other plants (Mosse, 1959; Mcgee, 1988; Jasper et al., 1989).

Our knowledge of this symbiotic association is not sufficient to conclude under which threshold or ideal percentage of root colonization will affect growth under different nutritional regimes. In growth response studies of the mycorrhizal plants the quantification of fungus in the roots
have to be examined to arrived at correlation values when growth is plotted against root colonization percentage.

VA-mycorrhizae in hydrocarbon yielding plants provide a physical channel for food transference and absorption. However this could equally well function to transfer material from mycelium into the root to the external mycelium. Therefore, physiological studies would be required to indicate in which direction any such transfer might takes place.
LOCATION OF THE PLACES SELECTED FOR THE STUDY