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ORIGINAL ARTICLE

Isolation and Morphological Identification of AM fungi and Physicochemical properties of Rhizosphere samples associated with Beedi tree (*Diospyros melanoxylon* Roxb.)

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ABSTRACT
Isolation, identification and characterization of AM fungi associated with the roots of Diospyros melanoxylon Roxb. were studied by taking three random Rhizosphere soil samples in the plant-grown area from the tree grown areas of Khammam and Bhadradri kothagudem districts, Telangana State and comparing the physicochemical properties of the three samples. Measured all the properties of the samples, i.e., p^H, temperature, electrical conductivity, moisture content, temperature, and water holding capacity, available organic carbon, available nitrogen and phosphorus. Three Rhizosphere samples, which contain the roots taken from three different randomly selected areas and analysed in a laboratory. The samples, which contain 11 species of AM fungi, belongs to the three genera. Hence, an attempt has been made to screen the availability of AM fungi associated with the plant species Diospyros melanoxylon in the Khammam and Bhadradri kothagudem districts areas of Telangana State. The fungal spore numbers in the Rhizosphere soil were determined, along with the percentage of root colonization.
KEY WORDS:*AM fungi; Colonization; Rhizosphere; Diospyros melanoxylon; Physicochemical properties.*

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INTRODUCTION

In India, tendu (*Diospyrosmela noxylon* Roxb.) a member of the Ebenaceae family, is regarded as a minor forest product (MFP). The tendu plant is widely spread over Telangana in order to commercialize the leaves used to make beedi as a traditional cigarette.

AM fungi are obligate symbionts distributed abundantly in various soils and help in the uptake of nutrients for the sustenance of vegetation. The roots of most plants form AM associations with a group of soil fungi belonging to the Endogonaceae of Zygomycota [2]. Recently, the family has been designated Glomaceae of Glomales [1].An important publication "Manual for the Identification of VA Mycorrhizal Fungi" has been published by Schenck and Pérez [18] which compiled all summary species descriptions. Now AM fungi are upgraded into the division Glomeromycota Redecker *et al.,* [16].

AM fungi They produce specialized structures known as vesicles and arbuscules inside the cortical cells of the roots [19]. In addition to other chemicals, the mycorrhizal fungi create organic acids that aid in the metabolism of organic compounds and the solubilization of mineral nutrients [19]. The maintenance of host plant growth under biotic and abiotic stress is another advantage of mycorrhizal symbiosis [5].

AM roots act as efficient absorbing roots and help in the active uptake of phosphorous and other micro nutrients [2]. AM fungi are distributed more abundantly in soils deficient in moisture and phosphorus; as such semi-arid tropical soils supporting dry deciduous vegetation are nutritionally poor and harbor a greater number of AM fungal propagules. The AM fungal propagules vary in soil and their number may be

from a few to 800/g dry soil. It is thought that 95 % of the Angiosperms are mycorrhizal dependent and some Bryophytes and Pteridophytes do possess AM associations [11]. The percentage of root colonization by thesefungi indicates mycorrhizal dependency and efficiency.AM fungi associated with forest soils are known to help in the establishment of forest seedlings, besides protecting them from forest pests [17].

MATERIAL AND METHODS

Study area

The study was conducted at forest plantgrown area of Khammam and Bhadradri kothagudem districts forest of Telangana from spring and autumn 2019.,The study sites were in the part of Telangana zone with hot semi-arid climatic conditions. The three rhizosphere soil samples were collected randomly from above the said area.

Collection of Soil Samples

The upper soil layer of the *Diospyros melanoxylon* rhizosphere was cleared of litter, bushes and grass before soil samples were taken at 2 points diagonally at a depth of 15- 30 cm. The500gof rhizosphere samples were obtained from each site [14] which were properly packed in a zip lock bag and labelled, which were kept in the laboratory at 5^{o}C until the next study.

Soil analysis

Soil samples collected during the survey were analysed for P^{H}, EC, temperature, moisture content, water holding capacity, organic carbon, available nitrogen, and available phosphorus.

Soil P^{H}, temperature and electrical conductivity (EC)

Soil P^{H} was determined in 1:2.5 soil-water suspensions by the method of potentiometry using a digital pH meter with a glass electrode. The soil-water suspension used for pH determination was allowed to stand for 24 hours. The soil temperature was measured with the help of a soil thermometer. The electrical conductivity of the supernatant liquid was determined by the method of conductometry using a digital conductivity meter and was expressed in dSm-1 [15].

Moisture Content

10 g of 2 mm sieved samples were taken in a known weight of card board paper. The same is kept in hot air oven at 105^{0} C for 11 hours. Next day, the weight of the board including dry soil was taken, when it was cool. The moisture content in % was determined as follows:

Weight of empty paper	= W_1 g
Weight of paper + sample	= W_2 g
Weight of paper + sample (after dry)	= W_3 g
Weight of sample before dry ($W_2 - W_1$)	=W_4 g
Weight of sample after dry ($W_3 - W_1$)	=W_5 g
Moisture content ($W_4 - W_5$)	= W_6 g

Percentage of moisture content = $\frac{W6}{W4} \times 100$

Water holding Capacity

Water holding capacity of the three samples were determined by the method recommended by Keen and Raczkowski [15]. 50 grams of 2 mm sieved samples were taken in a bottom pored brass box having whatman No.1 filter paper distilled water was added till the samples is saturated. The thin film of water on the reverse of bottom was cleaned with the help of a filter paper and weight was taken. The same was kept in hot air oven at 105˚C for11 hours, another whatmanno.1 filter paper of same size was taken and its weight before and after soaking in distilled water was measured. Next day, when the contents became cool the weight of brass box with filter paper and sample was determined. The water holding capacity in percentage was calculated as follows:

Weight of brass box	= W_1 g
Weight of box + filter paper (f.p)	= W_2 g
Weight of box + f.p + sample	=W_3 g
Weight of box + f.p + sample (wet)	=W_4 g
Weight of box + f.p + sample (dry)	=W_5 g
Weight of same sized f.p	=W_6 g
Weight of same sized f.p. after soaking	=W_7 g
Weight of water absorbed by f. p. (W_7- W_6)	=W_8 g
Weight of collected sample($W_3 - W_2$)	=W_9 g
Weight of wet sample + $W_4 - W_2$	=W_{10} g

Water absorbed by f.p.

Weight of dry sample $W_5 - W_2$	=W_{11} g

Weight of water absorbed $W_{10} - W_{11}$ $=W_{12}$ g

Water absorbed by soil + f. p.

Weight of water absorbed by sample $W_{12} - W_8 = W_{13}$ g

Water holding capacity in percentage $= \frac{W13}{W9} \times 100$

Organic carbon

Organic carbon in the soil sample was analysed by wet chromic acid digestion, as outlined by Walkley and Black [24]. 10 g of 2 mm sieved soil in a 500 ml conical flask kept on an asbestos sheet, 10 ml of 1 N potassium dichromate and 20 ml of concentrated sulfuric acid were added and mixed gently for a minimum of 30 minutes. After 30 minutes, 200 ml of distilled water, 10 ml of orthophosphoric acid and 1 ml of diphenylamine indicator were added. Then the solution was back titrated against 0.5 N ferrous ammonium sulphate until it appeared brilliant green. A blank was run without soil simultaneously. Organic carbon was calculated and expressed as a percentage.

Soil available nitrogen

Available nitrogen content in soil was estimated by the alkaline permanganate method as described by Subbaiah and Asija (1956). 10 g of 2 mm sieved soil sample, 25 ml each of 0.32 percent alkaline potassium permanganate and 2.5 percent sodium hydroxide were added, and the contents were distilled. Liberated ammonia was absorbed in 25 ml of a 2 percent boric acid solution containing mixed indicators (bromocresol green and methyl red). Soil nitrogen was estimated by titrating the above sample mixture against 0.01 N sulfuric acid until the bluish-green colour changed to the original pink colour. Simultaneously, a blank without a soil sample was run. Available nitrogen was calculated and expressed in kg ha-1 or mg pot-1 [9].

Soil available phosphorous

Available phosphorous was extracted from soil by Olsen‘s extractant and estimated by colorimetry using the reduced molybdate ascorbic acid blue colour method outlined by Watanabe and Olsen (1965). 10 g of 2 mm sieved soil sample, 40 ml of Olsen‘s 50 extractant (0.5 N sodium bicarbonate; pH 8.5) and a pinch of charcoal were added and kept in a shaker for 30 minutes. After shaking, the contents were thoroughly filtered through Whatman No. 40 filter paper. For the assay, 5 ml of the extract was taken in a 25-ml volumetric flask acidified with 2.5 M sulfuric acid. To this, 4 ml of reagent B (1.056 g ascorbic acid in 200 ml of water) reagent A (12 g ammonium molybdate in 200 ml of distilled water and 0.2908 g of antimony potassium tartrate in 100 ml of distilled water added to 1000 ml of 2.5 M sulfuric acid and made up to 2 litres) was added, and volume was made up to 25 ml with distilled water. Standards prepared using potassium dihydrogen orthophosphate were processed simultaneously to obtain a standard curve. After 10 minutes, the intensity of the colour developed was measured in a spectrophotometer at 420 nm. Available phosphorous was calculated and expressed in kg ha-1 or mg pot-1 [9, 15].

Isolation and quantitative estimation of AM fungi

For isolation and quantitative estimation of AM fungal propagules, a modified method of wet sieving and decanting technique (Gerdemann J. W and Nicolson T. H., 1963) was employed (McKenney and Lindsey, 1987). 100 g of 2 mm sieved samples were taken and made into 4 equal partsand each part was put in a 500 ml beaker. A pinch of sodium hexametaphosphate was added to prevent the aggregation of soil particles. 420mm, 250 mm, 105 mm, and 45 mm sieves were arranged in descending order with the 45 mm sieve at the bottom. The contents of the beaker were thoroughly shaken mechanically for 10 minutes and allowed to settle for 15 minutes. The upper contents were decanted through the sieves. The debris retained on the sieves was carefully washed into 250ml beakers separately for each sieve. The debris of the 420mm sieve was first filtered through a single synthetic fibre white cloth. The cloth with debris was kept in a glass Petri dish with some water, and we observed and isolated the AM fungal propagules with the help of a binocular dissecting microscope and injection needle. The process was repeated for debris from other sieves. Permanent preparations of these propagules were made on slides with polyvinyl lactic acid as the mounting medium. The sporocarps and spore aggregations were carefully isolated with microneedles and mounted in the same medium.

Colonization of AM fungi

The magnitude of infection of AM fungi to the host was determined in terms of percentage (Toth and Toth, 1982). The collected and fixed root bits of the angiosperm host plant were used to determine the percentage infection following the method of Phillips and Hayman (1970).The fixed root bits were cut into 1 mm pieces. They were autoclaved at 15 lbs for 15 min in 10% KOH solution and rinsed in tap water. Later, they were acidified in dilute HCl for 3 - 4 min and stained. The percentage of colonization was determined taking into account the number of root bits having vesicles and arbuscules with that of having no such structures as well as the number of cells with arbuscules with that of non-invaded cells.

$$\% \text{ colonization} = \frac{Number\ of\ bits\ (cells)\ having\ vesicle}{Total\ number\ of\ bits\ (cells)} \times 100$$

Morphological Identification of AMF Spores

Arbuscular mycorrhizal fungi were identified based on similarities in the morphological properties of their spores, such as colour, spore shape, size and decoration [20].(Gerdemann and Trappe, 1982;Walker, 1984;Schenck and Perez, 198;Redecker, 2013and INVAM, 2014).

The following characters were taken for morphological identification of AMF.

1. Spore colour can be determined by utilizing a commonly accepted colour wheel. Mycorrhizal spores come in a variety of hues, including hyaline yellow, greenish yellow, brown, and reddish brown and blackish brown.
2. Spore shape - In general, spores have a globe, sub globose, or oval shape.
3. Spore size and spore wall layers
4. Has spore contents and ornaments like bulbous spines, delicate threads like hair, and others.

Statistical Analysis

All the data presented were subjected to the one-way Analysis of Variance (ANOVA) at level of P≤0.05 by using OPSTAT software. All the values were considered as significant

RESULTS

Physicochemical properties of collected soil samples

The soil pH, electrical conductivity, available nitrogen, available phosphorous, available potassium, and organic carbon of collected soil samples were analyzed and results are presented in Table.1.

Three soil samples were red sandy loam and sandy loam type with soil P^H ranged between 7.06 (S1) to 7.32 (S3).The electrical conductivity of soil ranged between 0.363dS m-1 (S1) to 0.457dS m-1(S3). Soil temperature was ranged between 31.4ºC (S1) to 29.36ºC (S3). Moisture content was ranged between 2.58 (S1) to 3.24 (S3)and water holding capacity was ranged between 38.4 (S3) to 36.8 (S3).

The available organic carbon ranged between 2.25 per cent (S1) to 1.85 per cent(S3), available nitrogen in collected soil samples ranged between 184.38kg ha-1(S1) to 116.92 kg ha-1 (S3) and available phosphorous ranged between 38.52 kg ha-1 (S1)to 41.33 kg ha-1 (S3).

Table.1. The different physicochemical properties of collected samples of rhizosphere (*Diospyros melanoxylon* Roxb.)

Sample	P^H	Electrical conductivity (dS m^{-1})	Temperature (°C)	Moisture content	Water holding capacity (%)	Available Organic carbon (%)	Available Nitrogen (kg ha-1)	Available phosphorus (kg ha^{-1})
Sample 1	7.06 ± 0.02	0.363 ± 0.005	31.4 ± 0.11	2.58 ± 0.02	38.4 ± 0.14	2.25 ± 0.01	184.38 ± 0.91	35.82 ± 2.57
Sample 2	7.50 ± 0.04	0.384 ± 0.002	32.4 ± 0.25	3.57 ± 0.01	39.3 ± 0.17	3.42 ± 0.01	175.54 ± 1.93	28.51 ± 0.82
Sample 3	7.32 ± 0.08	0.457 ± 0.003	29.36 ± 0.39	3.24 ± 0.04	36.8 ± 0.25	1.85 ± 0.01	116.92 ± 0.74	41.33 ± 0.75
C.D (P≤0.05)	**0.034**	**0.624**	**0.789**	**0.075**	**0.644**	**0.135**	**8.614**	**3.827**
S.E (d)	**0.048**	**0.241**	**0.277**	**0.026**	**0.226**	**0.082**	**6.526**	**2.164**
S.E (m)	**0.034**	**0.164**	**0.196**	**0.019**	**0.16**	**0.063**	**5.269**	**2.031**

Values were expressed in mean of three replications ± standard deviation.

Quantitative estimation AM fungi

Percent colonization

The percentage colonization of AM fungi in soil sample 1 is 76.6, where as that of soil sample 2 is 75.0 and Sample 3 was 73.25. Results were presented in Table-2.

Number of fungal spores

The number of fungal spores in sample 1 is 19/50gof soil, where as that of sample 2is17/ 50gofsoil and sample 3 was 15/50 g of soil. Results were presented in Table-2

Qualitative estimation of AM fungi

Sample 1

Total of 19 spores were isolated in sample 1 out of these, 9 belonging to *Acaulospora,*3 belongs to *Gigaspora* and 7 belongs to *Glomus* (Table-3 and 4)

Sample 2

A total number of 17spores were obtained in sample 2. Out of these8 belongs to the genus *Acaulospora* and 9 belonging to the genus *Glomus* (Table-3 and 4)

Sample 3

A total number of 15spores were obtained. Out of these6 belongs to the genus *Acaulospora*, 4 belonging to the genus *Glomus* and 5 belongs to *Gigaspora*.(Table-3 and 4).

Characterization and Description of species

The spore morphological characters of genus *Acaulospora*, *Glomus* and *Gigaspora* shown in Table-5.

Table.2.Percentage root colonization and the number of fungal spores with vesicles and arbuscules of roots of *D. melanoxylon* by AMF.

Sample	Colonization (%)	Number of fungal spores	Vesicles	Arbuscules
Sample 1	76	19 ± 1.0 /100 g soil	+	+
Sample 2	75	17 ± 2.0 /100 g soil	+	+
Sample 3	73	15 ± 1.0/100 g soil	+	+
C.D (P≤0.05)	-	**7.602**	-	-
S.E (d)	-	**2.667**	-	-
S.E (m)	-	**1.886**	-	-

Values were expressed in mean of three replications ± standard deviation.

Table 3.Diversity and abundance of AMF spores associated with *Diospyros melanoxylon* Roxb.in different samples.

S. No.	Genus	No. of species isolated		
		Sample 1	Sample 2	Sample 3
1	*Acaulospora*	**5**	**5**	**2**
2	*Gigaspora*	**1**	**-**	**1**
3	*Glomus*	**3**	**2**	**2**

Table.4. Qualitative estimation of AM fungi species from different samples.

Sample 1	Sample 2	Sample 3
1.*Acaulosporaappendicula* 2. *Acaulosporabireticulata* 3. *Acaulosporafoveata* 4. *Acaulosporalacunosa* 5. *Acaulosporalaevis* 6. *Gigasporagigantea* 7. *Glomus aggregatum* 8. *Glomusdeserticola* 9. *Glomusgeosporum*	1. *Acaulosporaappendicula* 2. *Acaulosporadenticulata* 3. *Acaulosporafoveata* 4. *Acaulosporalaevis* 5. *Acaulosporascrobiculata* 6. *Glomus aggregatum* 7. *Glomusdeserticola*	1.*Acaulosporaappendicula* 2. *Acaulosporabireticulata* 3. *Gigasporagigantea* 4. *Glomus aggregatum* 5. *Glomusgeosporum*

DISCUSSION

The soil is a complex medium. It is very difficult to interpret the part played by different factors, as some of them tend to exert a positive influence, while others have reverse effect or no effect. Unless a factor behaves in such a way as to be called a limiting factor, it is not safe to ascribe the observations to a single factor. There are many factors playing important role at micro-environmental level which are very difficult to analyze, assign or to define. In spite of having a constellation of physiological and biological factors, still the soil maintains a dynamic equilibrium of microorganisms. However, there some information available to study the quantitative and qualitative distribution of AM fungi. In the present study the rhizosphere soils were collected during the spring and autumn 2019. Hence, a study of quantitative and qualitative occurrence of propagules and % colonization of AM fungi is made to different physiological properties of soil. However, the availability of other micro and macro elements in the soil also plays a vital role in the distribution of these AM fungi they were not considered here. Mahindra K Mishra *et al.,* [10] described the seasonal distribution of AM and recorded high propagule count in rainy season soil and low count in summer season. Even though the water holding capacity of the soil may be more, such situation occurs when soils were satisfied with full water capacity in the collected samples. There are contradictory reports regarding the effect of soil moisture on the number of AM fungal propagules. Ashok Shukla *et al.,* [2] observed that mycorrhizal activity in soil was influenced by the moisture level. Increase in moisture content has a negative effect on spore count and percentage infection. In the present investigation; the AM fungal propagules are less in number and more in % colonization. These results are in agreement with the conclusions of Chad J Penn [3] revealed that the

availability of phosphorous is dependent on the soil reaction (p^H) and is particularly low in humid areas where deeply weathered and leached acid soils bind P in Carbon and N phosphates of low solubility. Delowara Khanam *et al*., [4] came to the same conclusion and stated that there is a direct correlation between soil pH and spore numbers. Most of the AM spores are known to germinate between 4 - 7 pH at high temperature. In the present work, where the pH is near 7 (neutral) and as to high temperature of summer, the AM fungal propagules are less but the % colonization is high. The best vesicle and spore formation in *Glycine max* took place at 35^0 C, the optimum temperature for arbuscule formation was 30^0 C and the mycelium development best between 28 and 30^0 C. In the present work, the number of AM spores is less when the temperature is at 31 or 32^0 C.

Table No-5 Identification and Characterization of AM fungi of different samples of *Diospyrosmelanoxylon*Roxb in the Khammam and Bhadradrikothagudem Districts Forest of Telangana

Morphological charaters of spores	**Shape**	**Size**	**Colour**	**Spore wall**	**Hyphal attachment**	**Auxiliary cell**
Glomus geosporum	Globose to subglobose	103 - 326 µm diam	Light yellow - brown to dark red - brown	Three layers (L1,L2,L3)	Septate hyphae	none
Glomus deserticola	Globose to subglobose	120 - 146 µ diam	pale yellow to orange	Two layers (L1,L2)	Septate hyphae	none
Glomus aggregatum	Subglobose	85 - 157 µ diam	Dark brown to black	Two layers (L1,L2)	Septate hyphae	none
Gigasporagigantea	Globose to subglobose	282 – 290 µ diam	Pale greenish yellow to golden yellow	Two layers (L1,L2)	Septate hyphae	None
Acaulospora scrobiculata	Globose, subglobose	175 - 190 µ diam	Yellowish white to pale yellow	Three layers (L1,L2,L3)	Septate hyphae	none
Acaulospora laevis	Globose, subglobose	116 - 236 µ diam	Dull yellow to golden brown to reddish yellow	Three layers (L1,L2,L3)	Septate hyphae	none
Acaulospora lacunosa	Globose, subglobose	175.25 – 180 µm diam	Reddish yellow to dull brownish yellow	Two layers (L1,L2)	Septate hyphae	none
Acaulospora foveata	Globose, subglobose	240-360 µm diam	Red-orange to dark red-brow	Three layers (L1,L2,L3)	Septate hyphae	none
Acaulosporaden ticulata	Globose	200 - 213.75 µm diam	Creamy yellow to pale brownish yellow	Three layers (L1,L2,L3)	Septate hyphae	none
Acaulospora bireticulata	Globose, subglobose	170 - 176 µm diam	young becoming orange and light brown at maturity	Three layers (L1,L2,L3)	Septate hyphae	None
Acaulosporaapp endicula	Globose to ovoid	120 - 200 µm diam	white opaque becoming dull yellow cream to orange when mature	Three layers (L1,L2,L3)	Septate hyphae	none

Fig. No	Surface ornamenta- tion	Sporocarp
i	No, but Outer layers of the spore wall often slough as the spore ages	presence
Ii	The ornamentation of the upper surface of the spore wall layer 2 consisting of a polygonal reticulum overded spines.	presence
iii	Irregular ridges are present appearing polygonal in surface view, at the periphery appearing like molar teeth.	presence
iv	Spore contents of small hyaline guttules.	presence
V	The surface is ornamented with sausage shaped pits and irregularly arranged ridges but minute pits are not present).	presence
vi	No, spore surface smooth but in older ones minutely perforated	presence
vii	No, but Outer layers of the spore wall often slough as the spore ages.	presence
Viii	No	Presence
ix	No, The surface is smooth	presence
x	No, The surface is smooth	presence
xi	No, but Outer layers of the spore wall of- ten slough as the spore ages	Presence

Classification of AM fungi (Redecker*et al.,*2013)

Kingdom	Fungi	Fungi	Fungi
Division	Glomeromycota	Glomeromycota	Glomeromycota
Class	Glomeromycetes	Glomeromycetes	Glomeromycetes
Order	Diversisporales	Gigasporales	Glomerales
Family	Acaulosporaceae	Giagasporaceae	Glomeraceae
Genus	*Acaulospora*	*Gigaspora*	*Glomus*

Fig 1: Diospyros melanoxylon root squashes showing vesicles and

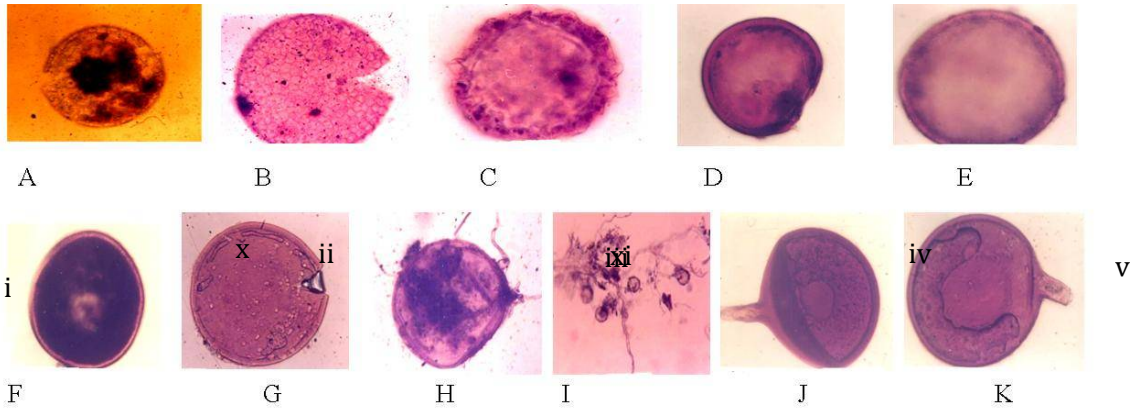

Figure 2: Showing the AM fungi species with vesicles and arbuscules sample of rhizosphere of *Diospyros melanoxylon* Roxb.

A. Acaulospora appendicula, B. Acaulosporabireticulata, C. Acaulosporadenticulata D. Acaulospora foveata, E. Acaulospora lacunosa F. Acaulospora laevis G. Acaulosporascrobiculata H. Gigaspora gigantea I. Glomus aggregatum J. Glomus deserticola K. Glomus geosporum

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