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Identification and characterization of arbuscular mycorrhizal fungi associated with the roots of *Diospyros melanoxylon* Roxb.

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Isolation, identification and characterization of arbuscular mycorrhizal (AM) fungi associated with the roots of *Diospyros melanoxylon* Roxb. were studied by taking two random samples from plants grown in area from forest of Sathupally, Telangana state with physiological properties of samples. Properties of soil samples tested were- pH, moisture content, temperature and water holding capacity. Two samples containing roots were taken from two different randomly selected areas and were analyzed in laboratory which showed the presence of11 species belonging to 2 genera. Hence, an attempt has been made to screen the availability of AM fungi associated with forest angiosperm species *Diospyros melanoxylon* in Sathupally forest area of Telangana State. The rhizosphere samples along with roots of *Diospyros melanoxylon* were collected, AM fungi isolated and identified.The fungal spore numbers in soil were determined along with the percentage of root colonization.

Key words: *Diospyros melanoxylon,*rootcolonization, AM fungi

INTRODUCTION

Arbuscular Mycorrhizal (AM) fungi are obligate symbionts distributed abundantly in various soils and helping in nutrients uptake for the sustenance of vegetation.The roots of most plants form AM associations witha group of soil fungi belonging to Endogonaceae of Zygomycota. Recently the family has been designated as Glomaceae of Glomales. They produce specialized structures known as vesicles and arbuscules inside the cortical cells of the roots. AM fungi colonize the fine absorbing roots of the plants, invading only the primary cortex; while vascular tissue and secondary cortex and the thick fleshy roots that develop into main structural roots of perennial plants are not infected.

Essentially, therefore, AM infections involve only temporary structures with a limited functional life.In thisrespect they differ from more pathogenic infections by other fungi that cause lesions, distortion and discoloration of the invaded tissue and often spread into permanent tissues of the stele and beyond.These fungi produce their hyphae and reproductive structures outside the root system. An important part of the AM system is the extension of mycelium outside the root. It constitutes a strategically placed network of an additional absorbing surface that enables the plant to tap soil phosphate beyond depleting zone, which is otherwise not accessible to the unaided root.

Much work has been done on the physiology of the symbiotic association and it has been established beyond doubt that these AM roots act as efficient absorbing roots and help in the active uptakeof phosphorous and other micronutrie-nts (Hisamuddin *et al.,* 2015). AM fungi are distributed more abundantly in soils deficient in moisture and phosphorus. Semi-arid tropical soils supporting dry deciduous vegetation are nutritionally poor and harbora 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 (Marcel *et al.* 2015).Thepercentage of root colonization by thesefungi indicates mycorrhizal dependency and efficiency. AM fungi associated withforest soils are known tohelp in the establishment of forest seedlings, besides protecting them from forest pests

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(Naidoo *et al.* 2019).

Asmelash *et al*. (2016) have emphasized the significance of AM fungi in tropical soils. However, there is no such work on AM fungal flora from Indian forest soils in general and from southern dry deciduous forests of Telangana in particular.Hence, an attempt has been made to screen the availability of AM fungi from Sathupally forest area of Telangana state by selecting forest angiosperm species *Diospyros melanoxylon.* The rhizosphere soils of species were collected, isolate and identify AM fungi from the soil. The fungal spore numbers in soil determined along with the percentage of root colonization.

MATERIALS AND METHODS

Collection of Samples

Samples were collected randomly from the *Diospyros melanoxylon* plant grown area of Sathupally forest in the month of May, 2019 and collections were made by taking composite samples up to a depth of 10 cm, after scrapping off 3 cm of surface soil with a sterile trowel. A pit was dug with the trowel, which was sterilize with 1% $HgCl_2$ followed by three sub sequent washes with sterilized water and the root zone soil was collected infreshpolyethene bag along with root bits. The soil temperature was measured with the help of soil thermometer. The samples were immediately brought to the laboratory and the compositesoil samples of each plantspecies were thoroughly mixed and kept in onebig polyethene bag in refrigerator at 5^0 C until further study.

pH of Samples

To 2g of 2 mm sieved samples, 10 ml of glass distilled water was added. The contents were thoroughly shaken and kept undisturbed. The supernatant was carefully decanted in a wide mouth tube and the same is fed to the calibrated pH meter and the reading was recorded. Thus, the pH of the rhizosphere sample supporting the *Diospyros melanoxylon* plant under study was individually recorded.

Moisture Content

10 g of 2 mm sieved samples were taken in a known weight of cardboard paper boat.The same were kept in hot air oven adjusted at 105^0 C and left for 11 hours.Next day, the weight of the boat 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 beforedry ($W_2 - W_1$)	=	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 moisturecontent	=	$\frac{W6}{W4} \times 100$

Water holding Capacity

Water holding capacity of thetwosamples weredeterminedby themethod recommended by Keen and Raczkowski (Piper, 1944).2 mm sieved samples were taken ina knownweight of bottom pored brassbox having Whatman No.1filter 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 105^0C for11hours, another Whatman no.1filterpaper of same size was taken and its weight beforeand 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 samesized f.p	=	W_6 g
Weight of samesized f.p.after soaking	=	W_7 g
Weightof 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	=	$\frac{W13}{W9} \times 100$

Thus, the water holdingcapacityofthetwo samples was determined individually.Sampleswereretaken fromthe root zone soils supporting to *Diospyros melanoxylon*for determining number of AM fungal propagules after removing stonesand vegetable debrispresent. Root bits of each were fixed separately in 1:3 acetic alcohol.

Isolation and quantitative estimation of AM fungi

Foe isolation and quantitative estimation of AM fungal propagules, modified method of wet sieving and decanting technique (Gerdemannand Nicolson1963) was employed (McKenny and Lindsey1987).100 g of 2.0 mm sieved samples were taken and made into 4 equal parts and each part was put in 500 ml beaker. A pinch of sodium hexametaphosphate was added to prevent the aggregation of soil particles. 420mm, 250 mm, 105 mmand 45 mm sieves were arranged in the descending orderwith 45 mm sieve setting at the bottom.The contents of the beaker were thoroughly shaken mechanically for10 minand were allowed to settle for 15 min. The upper contents were decanted through the sieves. The debris retained on the sieves was carefully washed into 250 ml beakers separately for each sieve. The debris of 420 mm sieve was first filtered through single synthetic fibred white cloth. The cloth with debris was kept in a glass Petri dish with somewater and observed and isolated the AM fungal propagules with the help of a binocular dissecting microscope and injection needle.The process was repeated for debrisof othersieves.Permanent preparations of these propagules were made on slides with polyvinyl lacticacid as mounting medium.The sporocarps and spore aggregations were carefully isolated with micro needles and mounted in the same medium.

Polyvinyl lactic acid preparation

1.66 gram of Polyvinyl alcohol is dissolved in 10 ml of water at 80^0 C on water bath. Later on,10 ml of lactic acid is added and stirred with glass rod. Then add 1 ml of glycerin and stirred thoroughly till the glycerin is dissolved.

Identification

Six genera are in Endogonaceae of Mucorales in Zygomycolta (Glomaceae and Glomales) *viz*. *Glomus,Acaulospora entrophosphora, Gigaspora,Scutellospora* and *Sclerocystis* consisting of about 150 species (Schenck and Perez,1990).Thesespeciesform vesicles and arbuscules of same nature in host plant root tissue and cannot be identified on the basis these two structures or mycelium in or outside the host. The vesicles are formed in the host tissue intercellularfor probably the storehouses of reserve food material. Butit was found that some species do not form the vesicles. The arbuscules are formed in the host tissue intracellularlyand act ashaustoria.These AM fungi form azygosporesor chlamydosporesoutside the host in the rhizosphere soil. These are also generally called as propagules. Based on the nature ofthese propagules, the species were identified. The characters of these propagules to be studied arecolor,size, shape, wall characters *i.e*., thickness, color, number of layers andtheir thickness, inside and outside ornamentations of the wall,presence or absence of subtending hyphae and its nature, nature of poreand the contents are the features specific for each species.Various keys were takeninto account in the identification of AM fungi, such as Hall and Fish (1979), Schenck and Perez (1990) and Trappe (1982).

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 angiospermhost 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 at15lbsfor 15 minin 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 thatof having no such structures, as well as the number of cells with arbuscules with that of non-invaded cells.

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

Statistical Analysis

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

RESULTS

Temperature

As the soil samples were collected in the month of April, 2018 *i.e*., in the summer when the temperature of thesoilis slightly higherthanthe normal.The temperatureof rhizosphere soil supporting soil

sample 1was 31.4^{0}C, whereas the soil sample 2was 32.4^{0}C. (Table1).

Moisture content

The moisture content of the soil sample 1 was 2.58%,whereas that of soil sample 2 was 3.57%(Table1).

pHof the sample

The p^{H}of the soil was nearly neutral or slightlybasic. The p^{H}of soil sample 1 is 7.06, whereas that of soil sample 2 is 7.32. (Table1).

Water holding capacity

The water holding capacity of a soil provides an idea of biota living in that area.The water holding capacity of soil sample 1 is 38.4 %, whereas that of soil sample 2 is39.4 % (Table

Quantitative estimation AM fungi

Percentage colonization and number of propagules

Thepercentage colonization of AM fungi in soil samplewas found to be 73, where as that of soilsample 2 was75(Table2). The number of propagules of AM fungi in soil samples1and 2 were28/50gsoil and 20/ 50gsoilrespectively (Table2).

Qualitative estimation of AM fungi

Sample 1

Out of the25propagules in the soil1 supporting the angiospermplant, *Anogeissus latifolia*,14 belonging to *Acaulospora,*2 belongs to *Gigaspora* and 9 belongs to *Glomus* (Table3)

Sample 2

A total number of 16 propagules were obtained. Out of these12belongtothegenus *Acaulospora* and 4 belonging to thegenus *Glomus* (Table3)

Microsporic observation of root squashes

The conization of AM fungi within roots were also studied using a light microscope. The arbuscules and vesicles were evident, as shown in Fig.1 (a & b)

Characterization and description of species *Acaulospora appendicula* Spain, Sieverd.&N.C. Schenck

Azygosporesformed singly in Azygosporesglobose, 120 - 200 mmdiameter,whiteopaque becoming dull yellow cream to orange whenmature.Wall thickness 4 - 6 mm, yellow to brown with age with an irregular reticulate patternof finecracksthat serve as fracturelines when an azygospore is crushed, sub tending hyphae notpresent(Fig.2a).

***Acaulospora bireticulata* Rothwell & Trappe**

Sporocarps unknown. Azygospores are formed singly,sessile,spores globose 170 - 176 mmin diameter, sub - hyaline when youngbecoming orange andlightbrownatmaturity. Spore surfaceornamented witha polygonal reticulum, the ridges2 x1.5 - 2 mm with dark grayish green sides and apaler depressed central stratum, ridgesoccasionally branched towards the center of polygonsorforming irregular, isolated projectionsat polygon centers, polygons 6 - 18 mmlong,the enclosed spore surfacebeset with roundtipped, 4-6 sided processes ± 1 x1 mm to give the appearance of an inverted reticulum. Sporewall thickness 3.0 - 7. 5 mm, contents globular (Fig.2b).

***Acaulospora denticulate* Sieverd. & Toro**

Sporocarps unknown. Azygosporesformedsingly, Creamy yellowtopale brownish yellow, Globose, 200 - 213.75 mm in diameter. Spore wall yellow in colour, 4 - 6 mm in thickness.Irregular ridges arepresent appearing polygonal insurfaceview, at the periphery appearing like molarteeth (Fig.2c).

***Acaulospora foveata* Trappe & Janos**

Sporocarps unknown. Azygospores formed singly, spores globose tosub - globose, yellowish brown to dark reddish brown,161 - 225 mm in diameter.Spore surfaceuniformly pitted withroundto oblong or occasionally irregular depressions. Sporecomposite wall thickness is 10 - 12 mm,dark reddishbrown,laminated. Spore contents of small hyaline guttules (Fig.2d).

***Acaulospora lacunosa* Morton**

Spores formedsingly, spores are reddish yellowto

Table 1: The different physiological properties of soil

Sample	pH	Moisture	Temperature (°C)	Water holding capacity %
Soil Sample 1	7.06 ± 0.02	2.58 ± 0.02	31.4 ± 0.11	38.4 ± 0.14
Soil Sample 2	7.32 ± 0.04	3.57 ± 0.01	32.4 ± 0.25	39.3 ± 0.17
C.D (P = 0.05)	0.034	0.075	0.789	0.644
S.E (d)	0.048	0.026	0.277	0.226
S.E (m)	0.034	0.019	0.196	0.160

± standard error mean

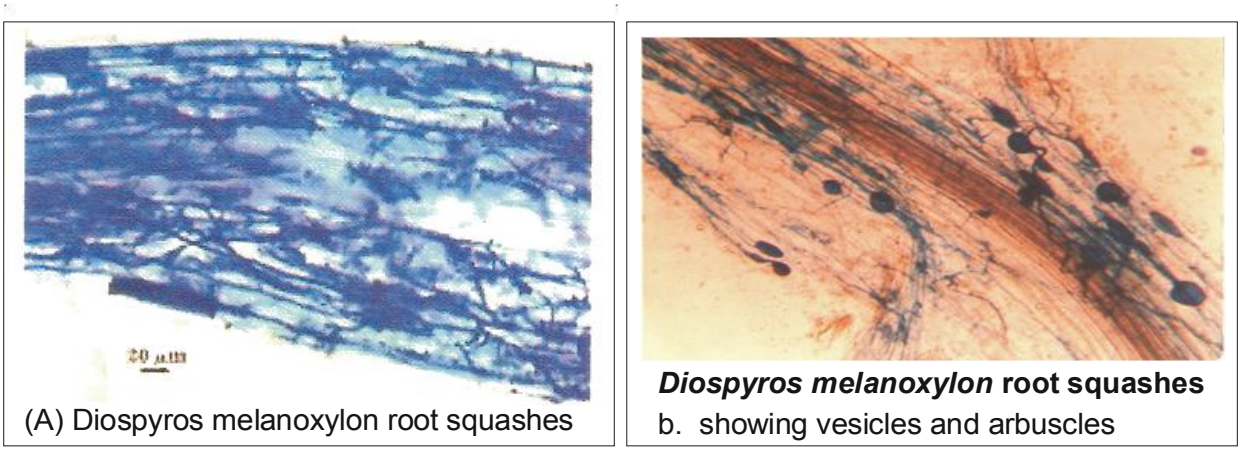

Fig. 1 : (a &b).AM fungal species within roots with vesicles and arbuscules

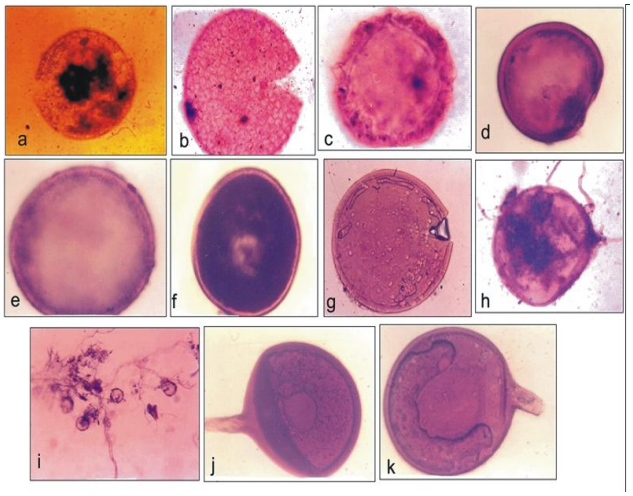

Fig. 2 : (a-k).(a).*Aculospora appendicula, (b) Aculasporabireticulata*, *(c) Aculospora denticulate (d) Aculosporafaveata, (e) Aculospora lacunose (f) Aculosporalaveis (g) Aculosporascrobiculata (h) Gigaspora gigantea (i) Glomus arborense (j) Glomus deserticola (k) Glomus geosporum*

dull brownish yellow, globose 175.25 – 180mm in diameter or sub globose145 - 150 x185 -190 mm, composite wall thickness is 5. 25 - 14 mm. The surface is ornamented with sausage shaped pitsand irregularly arranged ridges but minute pits are not present (Fig.2e).

Table 2: Values of % colonization and the number of propagules

Sample	Colonization (%)	Number of fungal spores
Sample 1	76.6	28 ± 1.73 /50 g soil
Sample 2	75.0	20 ± 2.02 /50 g soil
C.D (P = 0.05)		7.602
S.E (d)		2.667
S.E (m)		1.886

± standard error mean

Table 3: Qualitative estimation of AM fungi

Sample 1	Sample 2
1. *Acaulospora appendicula*	1. *Acaulospora appendicula.*
2. *Acaulosporabireticulata.*	2. *Acaulospora denticulate.*
3. *Acaulosporafoveata.*	3. *Acaulosporafoveata.*
4. *Acaulospora lacunose.*	4. *Acaulosporalaevis.*
5. *Acaulosporalaevis.*	5. *Acaulosporascrobiculata.*
6. *Gigaspora gigantean.*	6. *Glomus arborence.*
7. *Glomus arborense.*	7. *Glomus deserticola.*
8. *Glomus deserticola.*	
9. *G. geosporum*	

Acaulospora laevis Gerd. & Trappe

Sporocarps unknown.Spores formed singly,spores-aresessile. Vesicle lost in sieving. Spores smooth, size 116 - 236 mm in diameter or 391 -425 mm.

Shape globose tosub globose, colour dull yellowtogolden brown to reddish yellow. Sporewall consisting of 3layers, a rigid yellow brown to red brown outer wall and2 hyaline inner membranes. Composite wall thick, 4 - 18 mm, contents globose, spore surface smoothbut in olderones minutely perforated(Fig.2f).

***Acaulospora scrobiculata* Trappe**

Sporocarps unknown. Azygosporesformed singly, spores are globose175 - 190 mm in diameter. Spore surface evenly pitted with depressionswhich arecircular toelliptical.Thecomposite sporewallthickness is7 - 8.25 mm.Thecontents are consisting of small oil guttules(Fig.2 g).

***Gigaspora gigantea* Gerd. & Trappe**

Azygosporesformed singly, spores are globose 282 – 290 mm in diameter or slightlyoval 440 - 442 x 455 - 490 mm in size, pale greenish yellow to golden yellow. Thethickness of composite wall is 7.5 - 15 mm. It has an outer wall layer thin, evanescent oran inner, thick, brown and laminate. The diameter of suspensorat attachment is upto37. 5 mm andupto 56.25 mm at its maximum diameter tapering to10 mm. The suspensor hasone or two hyphal branches.Manyprotuberances probably germ tubesare present just near suspensor.Rarely, they contain onespore of *Acaulospora* sp. (Fig.2h).

***Glomus arborense Mc* Gee**

Hypogenous spore aggregations upto1mm-size.Chlamydospores formed terminally or subterminally.The spores and hyphae are hyaline; globoseto sub-globose, rarely irregular;30 – 30 .75 X 52. 5mm. The composite wall is very thin, less than3.75 mm.The surface is smooth to dull roughened, contents hyaline with globular oil drops and are cutoff infrequently by a septum. Hypha 2.5 - 10.5 mm in diameter at attachment and slightly flared (Fig.2i).

***Glomus deserticola* Trappe, Bloss & Menge**

Spores borne singly or in loose fascicles, spores are globose to slightly sub -globose, 120 - 146 mm in diameter, shiny smooth, reddish brown, with a single, sometimes laminated wall, 2.5 - 10.5 mm in thickness. The attached hypha is11.25 - 26.25 mm diameter at attachment, cylindric to occasionally somewhatfunnel - shaped, the walls thickened and reddish brown, especially thick adjacent to the sporebut not occluding thehypha.Interiorof the spore wall at the hyphalattachment thickenedatmaturitytoform aninner moundedcollar, whichappears tobe closed by a membranousseptum (Fig.2j).

***Glomus geosporum* (Nicol. & Gerd.) Walker**

Sporocarps unknown. Chlamydospores formed singly, spores are globose to sub-globose, 103 - 326 mm in diameter or105 x 120 mm, smooth and shinyor with a dull appearance or roughened from adherentdebris, light yellow - brown to dark red - brown at maturity. Spore walls 3.5 - 15 mm thick, 3 layeredwith a thin, hyaline,tightly adherent outer wall, most easily observedin youngspores and sometimes absent from mature specimen yellow to brown inner wall which forms a septum separating the spore contents from the lumen of the subtending hypha. Walls often becoming perforated with age, probably due to attack of soil microbes.Spores with one straight to recurved, simple to slightly funnel shaped subtending hypha (rarely with two adjacent attachments), 11 - 22 mm in diameter, with yellow to dark yellow - brown wall thickening. Occasionally spores lacking a subtending hypha due to breakage close to the spore base. Spore contents of uniform oil droplets when young, becomingincreasingly granular in appearance with age, cut off bya septum that protrudes slightly into hypha (Fig.2k).

DISCUSSION

The soil is acomplex medium. It is very difficult to interpret the part playedby 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 sucha way as to be calleda limiting factor, it is not safeto 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 soilswere collected during thedry days *i.e.*, April 2018. Hence, a study of quantitative and qualitative occurrenceof propagules and% colonization of AM fungi is madeto different

physiological properties of soil. However,the availability ofother micro and macro elements in the soil, also playa vital rolein the distribution of these AM fungi they were not considered here. Mishra *et al.,* (2012) described the seasonal distribution of AM and recorded high propagule countinrainy season soil and low count in summer season. In the present investigation also, the same thing hasbeen noticed that the number of spore propagules are less in summer with a high % of colonization. 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 rainy season. There are contradictory reports regarding the effect of soil moisture on the number of AM fungal propagules. Badr *et al*.(2020) revealed that the mycorrhizal plants are less sensitive than non mycorrhizal plants to water stress. Shukla *et al.* (2012) observed that mycorrhizal activity in soil was influenced by the moisture level. Increase in moisturecontent hasa negative effect on spore count and percentage infection. In the present investigation, the AM fungal propagules are less in number and morein % colonization. These results are in agreement with the conclusions of Penn (2019) who revealed that the availability of phosphorous is dependent on the soil reaction (p^H) and is particularly low in humid are as where deeply weathered and leached acid soils bind P in Fe and Al phosphates of low solubility. Khanam *et al*. (2006) cameto the same conclusion and stated that there is a direct correlation between soil pH andspore numbers. Mostof the AM spores are known to germinate between 4-7 pH at hightemperature. In the present work, where the pHis near 7 (neutral) and as tohigh temperature of summer, the AM fungal propagules are lessbut the % colonization is high. It has also been found that plants infected with mycorrhizal were dependent on soil temperature for growth. The best vesicleand spore formation in *Glycine max* took placeat 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 propagules is less when the temperature is at 31 or 32^0 C.

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