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Isolation, Identification and Characterization of Arbuscular Mycorrhizal Fungi in Apple (*Malus domestica Borkh*) Growing Area of Kashmir Himalaya

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A B S T R A C T

Samples from rhizospheric soil and root pieces collected from 10 villages of district Pulwama of Kashmir Himalaya were processed for isolation of arbuscular mycorrhizal spores and for their identification with the help of INVAM. The isolated genera were identified as *Acaulospora*, *Scutellospora*, *Gigaspora*, and *Glomus*. *Glomus* spores were more predominant while as *Scutellospora* spores were least predominant in the district. Spores of *Gigaspora* were larger in diameter than others. *Glomus sp.* showed higher root colonization from sunsomil, *Acaulospora sp.* from Pinglin, *Gigaspora sp*. from Shiekhar and *Scutellospora sp*. from Drubgam. Highest spore population and phosphatase activity was recorded in Pinglin. The highest biological activity was due to adequate application of organic manures in the soil and also due to application of fertilizers.

Introduction

Apple (*Malus domestica* Borkh.) is a premier table fruit, rich in nutrients especially fats, carbohydrates, calcium, phosphorus and iron. It is cultivated in temperate regions of the world and, to some extent, in cool highlands of some sub-tropical regions such as East Africa, Northern parts of India etc. India in terms of apple fruit production ranks fourth in the world. In India, apple cultivation alone accounts for 55 per cent of the total area and 75 per cent of the total production under temperate fruits in the country (Chadha, 1993). Commercial cultivation of apple fruit in India is confined to the States of Jammu and Kashmir and Himachal Pradesh, and some selected areas of Uttrakhand, Arunachal Pradesh, Manipur and Sikkim. In Jammu and Kashmir apple is grown on an area of 1.07 lakh ha with annual production of 12.08 lakh metric tonnes, which constitutes about 84 per cent of total fruit production in the State (Malik *et al.*, 2016). In Horticulture sector the largest area of 43.53% is occupied by apple out of total area under fruit and 65.46% out of fresh fruit area (Anonymous, 2016) thereby making it the largest contributor to the state GDP among the horticulture produce.

District Pulwama is an important part of Kashmir valley with respect to the

agricultural perspective and is surrounded by Srinagar in the north, Budgam and Poonch in the west and Anantnag and Shopian in the east and south side. The district is situated between 33°46´ to 33°58´ N Latitude and 74°45´ to 75°13´ E longitude with a mean elevation of about 1630 m amsl. It contributes a total geographical area of 0.109 m ha out of which 0.02365 m ha is under agriculture and 0.0412 m ha under forest cover, rest being used for other purposes. Pulwama soils are shallow to deep, mostly loam to silty loam and silty clay to clay. The wide variations in soil characteristics are mostly associated with slope aspect. The soils are mostly subjected to moderate to severe erosion and have moderate surface stoniness at some places.

The natural vegetation of the district consists of trees like *Salix Alba, Populus Alba.* The high hill ranges are covered with forests and dominant species are *Pinus sylvestris, Pinus walichiana, Robinia pseudoacacia, Cedrus deodara, Abies pindrow and Picea smithiana*. Several shrubs and herbs of medicinal value are also found in the forest. Agriculture is the main occupation of the people in the districts. Paddy, oil-seeds, fodder, saffron are the main agricultural crops of istrict Pulwama and fruits especially apple is the main horticultural crop of District. Pulwama is also known for both quantity and best quality apple in the valley.

Arbuscular mycorrhizal fungi play an important role in sustainable agriculture as well as agricultural ecosystem management. The important genera of endomycorrhizal fungi reported so far are *Acaulospora, Entrophosphora, Gigaspora, Glomus, Sclerocystis* and *Scutellospora* (Morton, 1988). All these fungi are obligatory associated with plant roots and develop symbiotic relationship with their hosts. Different species can be differentiated on the basis of sporocarp size, spore dimension, presence/absence of hyphal mantles, spore ornamentation (warts, wrinkles, pits, reticulum, spines etc.), spore walls, spore content, hyphal attachment, manner of spore germination, histochemical reaction etc. (Schenck *et al.,*1982). Keeping in view the importance of arbuscular mycorrhizal fungi in phosphorus solubization and very little work having been done on arbuscular mycorrhizae with respect to apple, the present investigation was taken up with the objective of isolation and purification of arbuscular mycorrhizal spores from the rhizospheric soil of apple, morphological characterization of the spores upto generic level and their root colonization studies.

Microbial enzymes constitute an important part of soil matrix as the extracellular enzymes, influencing soil microbial activity, exert control over soil enzymes production, control nutrient availability and soil fertility. Soil enzymes play key biochemical function in overall processes of organic matter decomposition in soil. Soil enzymes are significant in catalyzing several important reactions necessary for life process of an organism in soil, stabilizing soil structure, decomposition of organic wastes, organic matter formation and nutrient cycling. The enzyme activity has been reported to be important in determination of soil quality (Makoi and Nolakidema, 2008). These soil enzymes have also been suggested as potential indicator of soil health because of their essential role in soil biology (Riffaldi *et al.,* 2002)

Phosphatases have been extensively studied in soil because they catalyze the hydrolysis of ester-phosphate bonds, leading to the release of phosphate, which can be taken up by plants or microorganisms. It has been shown that the activities of phosphatases (like those of many hydrolases) depend on several factors such as soil properties, soil organism interactions,

plant cover, leachate inputs and the presence of inhibitors and activators. Phosphatases are enzymes catalyzing the hydrolysis of both esters and anhydrides of phosphoric acid.

Materials and Methods

Study area

Pulwama with geographic coordinates of 33.87162° N, 74.8946° E is one of the major apple growing district of Jammu and Kashmir with apple production 1, 39, 2, 88 MT in the year 2016-17 and the land under apple cultivation is 14290 H.

Ten villages (Rajpora, Shadimarg, Nikas, Drubgam, shiekhar, Tikkin, Sunsomil, Pinglin, Gungoo, Puhoo) were selected from district Pulwama. From each village three orchards were randomly chosen and from each orchard five rhizosphere soil samples were drawn which were composited into one representative sample. Most of the orchardists followed the pesticide schedule as per Department of Horticulture, Government of Jammu and Kashmir.

Collection of root and soil samples

Extensive field survey was carried out in order to collect the root and rhizospheric soils samples of three years old apple trees (var. Red delicious) from ten Villages of district Pulwama in June 2014-15.

Samples were collected randomly from the feeder roots on all sides of the canopy of the tree (fruiting stage). Rhizospheric soils at a depth of 0-30 cm from 5 different locations in each study site were collected in sterile polyethylene bags using soil auger.

A portion of the soil samples was analyzed for soil chemical parameters like soil organic carbon, available phosphorus, available potassium and available sulphur as per standard methods (Walkley and Black, 1934; Olsen, 1954; Stanford and English, 1949; Chesnin and Yien, 1951). Soil used during the present study contained 1.74% organic carbon, 357.43 kg/ha available nitrogen, 17.05 kg/ha phosphorus, 12.02 kg/ha available sulphur and 185.38 kg/ha available potassium and all these were in medium range.

Remaining soil samples of 200g was used to isolate AM fungal spores. The root samples were washed thoroughly with running tap water to remove the adhered soil particles. Then roots were cut into small pieces of about 1cm and fixed in Std. FAA solution until it is used for the assessment of % colonization of AMF.

Isolation and identification of arbuscular mycorrhizal fungal spores

Isolation of AM fungal spores from the rhizospheric soil samples was done by following Wetsieving and decanting method (Gerdemann and Nicolson, 1963). The spores were counted under microscope Olympus CH20i with magnification of 10×40.).

Spore population was then expressed in terms of number of spores per 100 gm of dry soil. Clean and intact spores were isolated using a specially designed needle, spores were mounted with PVLG (poly vinyl alcohol+ lactic acid+ glycerol) + Melzer's Reagent and observed under microscope and photographed.

Identification of spores up to generic level was based on spore size, spore colour, wall layers and hyphal attachments using the species descriptions provided by INVAM (http://invam.caf.wvu.edu) (INVAM, 2005) and other suitable references(Schenck *et al.*,1990; Morton and Benny,1990; Almeida

and Schenck, 1990; Bentivenga and Morton,1995; Walker and Vestberg, 1998).

Assessment of AM fungal colonization of isolated spores

The isolated spores were further purified and mass multiplied on maize. Surface sterilized healthy maize seeds, pre-germinated in Petri plates under aseptic conditions, were sown in polythene bags containing sterilized soil + sand mixture (1:2 w/w). These bags were aseptically inoculated with identical AM spores at 5 cm depth (Jackson, 1973). The bags were kept in a greenhouse at 25±3°C and irrigated with sterile water. The plants were uprooted after 45 days.

The roots were collected, washed with sterile water to remove adhering soil debris and observed for mycorrhizal infection. The infectivity was proved by noticing the presence of hartig net, vesicles, asbuscules or hyphae of endophytes on roots.

For estimating mycorrhizal root colonization, the root samples were collected and washed carefully to remove the adhering debris. The tertiary roots were cut into small pieces of approximately 1cm length and subjected to differential staining (Phillips and Hayman, 1970).

The estimation of mycorrhizal infection in roots was made by visual observation (Giovannetti and Mosse, 1980). A randomly selected aliquot of stained root segments, suspended in water, was spread in a Petridish viewed under a dissecting microscope at a magnification of 10 and 40×. In case of AM colonization, root segments containing vesicles and arbuscules of endophyte and number of mycorrhizal short roots were considered infected (Beckjord, 1984).

Per cent mycorrhizal infection = Number of infected root segments / Total number of segments examined × 100

The data recorded during the investigation was statistically analyzed with the help of Pearson correlation (Gomez and Gomez, 1984).

Results and Discussion

Morphological characterization of arbuscular mycorrhizal spores

Spore morphology and wall characteristics were considered for the identification of arbuscular mycorrhizal fungi. Four types of genera viz., *Glomus*, *Acaulospora*, *Scutellospora* and *Gigaspora, were* recovered and identified. 3 to 6 unidentified spores per gram from all studied locations were tagged as unidentified spores (Table 1).

The spore colour of the species of *Glomus* was of wide range. It varied from red-brown to almost black or straw to dark orange but most was yellow brown in colour. Spores possessed globose to sub-globose shape, about 40 to 120 µm in size.

Spore wall consisted of three layers (L1, L2 and L3). Our findings corroborate with those of many other workers (Koske, 1984; Koske and Gemma, 1990). *Acaulospora* spores were present singly in the soil and develop laterally on the neck of asporiferous saccule. Spores were light orange to yellowish brown (Table 2 and Figures 1, 2, 3 and 4) globose to sub-globose in shape and 150 to 210 µm in diameter.

These spores were triple layered with L1 which forms the spore surface light yellow to apricot yellow in colour and 0.7 to 2.0 µm in thickness. L2 was laminate and light orange to yellowish brown, 6.8 to 7.4 µm in thickness. L3 was laminate, hyaline, 0.8 to 1.6 µm in thickness and usually tightly adherent to L2. Similar observations have been reported by others also (Walker, 2007;

Sharma, 2009). *Scutellospora* spores were with or without ornamentations. Spores consisted of a bilayered spore wall and two bilayered flexible inner walls.

Thin-walled auxillary cells with smooth to knobby surfaces were produced on hyphae in the soil near the root surface and were also reported by Morton (2002).

Gigaspora spore wall consisted of a permanent outer layer enclosing a laminate layer, each with different properties that distinguish species (e.g. color, thickness, etc). Our observations corroborate with those of Koske (1987) and Bentivenga *et al.*, (1995).

There was no evidence of any ectomycorrhizal association with apple roots, and this corroborates with the findings of [29]. *Glomus* species was common and made up for more than 75% of total isolates followed by *Acaulospora, Gigaspora* and *Scutellospora.*

Dominancy of *Glomus* in the present study is in agreement with the findings of many other workers (Mridha and Dhar, 2007; Burni, 2009; Sharma, 2009). The predominance of *Glomus* spp. under varying soil conditions might be due to the fact that they are widely adaptable to the varied soil conditions and survive in acidic as well as in alkaline soils (Pande and Tarafdar,2004).

Root colonization studies of arbuscular mycorrhizal fungi

In the current study, the AM colonization in the apple roots from Pulwama district varied between 65.87 and 79.78% (Table 3, Figures 5, 6, 7, and 8). The results are in conformity with the Kandula *et al.*, (2006) who also observed higher colonization in the apple roots and confirmed the ubiquitous nature of AMF spores. The highest root colonization was recorded in response to the inoculation with *Glomus* spp. (79.78%) followed by *Acaulospora* species (79.56%), *Gigaspora* species (73.56%) and *Scutellospora* species (71.23%). Similar results were reported by some workers (Gosal *et al.*, 2003; Smith and Read, 2008).Results of the present study indicate that the nutrient contents of the soils played a significant role in occurrence of different species of arbuscular mycorrhizal fungi and it is evident from the Perusal of the data presented in table 2 which revealed that AM spore population of district Pulwama was positively and significantly correlated with organic carbon (r=0.887**). The results are in conformity with those of (Lipinski *et al.*, 2003) who also reported a significant positive correlation between soil organic carbon and AM spore population. There was a significant correlation between AM spore population and root colonization (r=0.512*) in district Pulwama.

Table.1 Isolation of arbuscular mycorrhizal spores from rhizospheric soil of apple from Different locations of District Pulwama

Locations	Spore count per gram of soil (Identified Genera)				
	Acaulospora	***Scutellospora***	***Gigaspora***	***Glomus***	**Unidentified Genera**
Rajpora	-	-	1	2	4
Shadimarg	2	-	3	3	4
Nikas	1	2	-	-	4
Drubgam	-	1	1	2	3
Shiekar	2	-	1	-	5
Tikkin	3	-	-	3	4
Sunsomil	-	-	2	2	4
Pinglin	3	2	1	-	6
Gungoo	-	1	-	4	3
Puhoo	3	-	-	-	5

Table.2 Morphological features of isolated genera of AM fungi

Genera	Spore size (µm diameter)	Spore shape	Spore Colour	Spore wall	Hyphal colour
Acaulospora	115-170	Globose to sub globose	Yellow brown to dark brown	Three layered (L1.L2 and L3)	Grey white
Gigaspora	200-300	Globose to sub globose	White to cream usually a rose pink tint.	Bilayered layered (L1 and L2)	Orange brown
Scutellospora	100-170	Sub globose to ellipsoid to oblong	Cream to yellow or pale orange brown to dark orange brown	Bilayered spore wall (L1 and L2)	Hyaline to orange white.
Glomus	40 – 120	Globose to ellipsoid	red brown to almost black most are yellow brown	three layered (L1,L2 and L3)	Hyaline to yellowish.

Table.3 *In vitro* root colonization by AM fungal spores isolated from District Pulwama

Locations	Genera	Root Colonization (%)
Rajpora	*Gigaspora* sp. *Glomus* sp.	68.23 72.05
Shadimarg	*Acaulospora* sp. *Gigaspora* sp. *Glomus* sp.	65.87 70.03 72.13
Nikas	*Scutellospora* sp. *Acaulospora* sp.	69.09 70.05
Drubgam	*Scutellospora* sp. *Glomus* sp. *Gigaspora* sp.	73.23 76.67 69.08
Shiekar	*Gigaspora sp.* *Acaulospora* sp.	73.56 68.67
Tikkin	*Acaulospora* sp. *Glomus* sp.	72.13 68.78
Sunsomil	*Gigaspora* sp. *Glomus* sp.	67.67 79.55
Pinglin	*Acaulospora* sp. *Gigaspora* sp. *Scutellospora* sp.	79.56 67.80 65.87
Gungoo	*Scutellospora* sp. *Glomus* sp.	69.77 79.78
Puhoo	*Acaulospora* sp.	76.86

Table.4 Mean soil phosphatase activity under field conditions of District Pulwama

Villages	Phosphatase (µgPNP/g/24hr)
Rajpura	29.76
Shadimarg	30.12
Nikas	33.62
Drubgam	31.78
Shiekar	26.67
Tikkin	33.08
Sunsomil	29.78
Pinglin	34.56
Gungoo	32.78
Puhoo	30.34
Mean	**31.24**
CD(P≤0.05)	**1.709**
CV	**3.133**

Table 5 Correlation between spore population and other studied parameters of District Pulwama

Parameters	Spore population
Spore population	1
Organic carbon	0.887**
Available Nitrogen	0.815*
Available Phosphorus	0.797
Available Sulphur	0.910
Available Potassium	0.614*
Soil Phosphatase	0.458
Root colonization	0.512*

* Correlation is significant at the 0.05 level; ** Correlation is significant at the 0.01 level

Fig.1 Spores of the genus *Acaulospora*

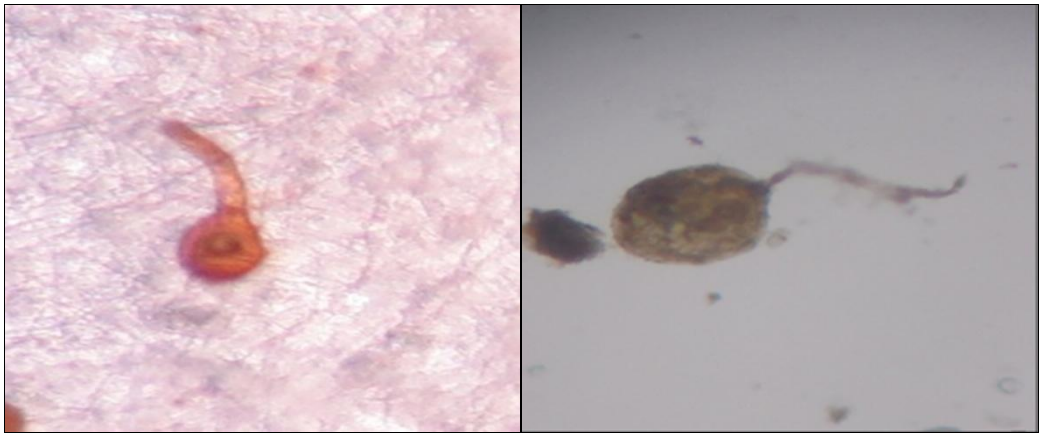


Fig.2 Spores of the genus *Glomus*

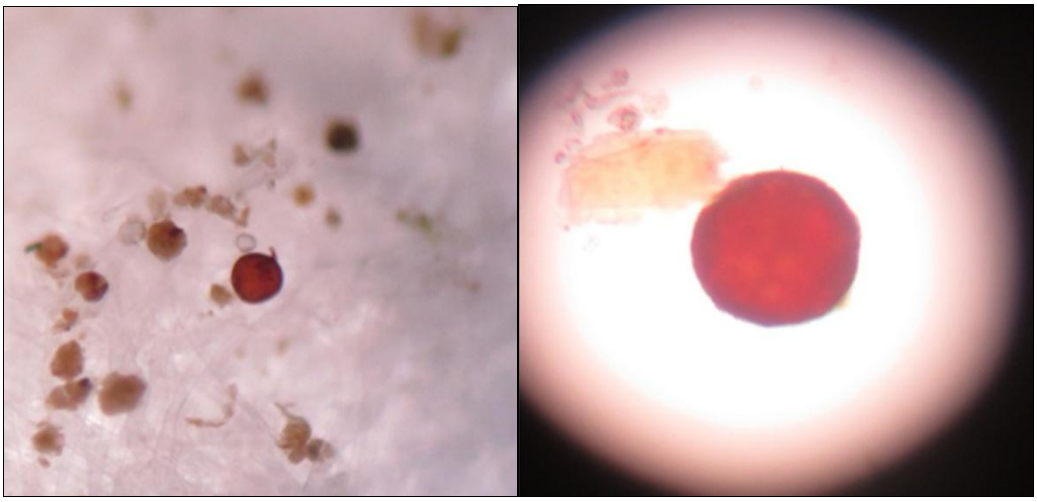


Fig.3 Spores of the genus *Gigaspora*

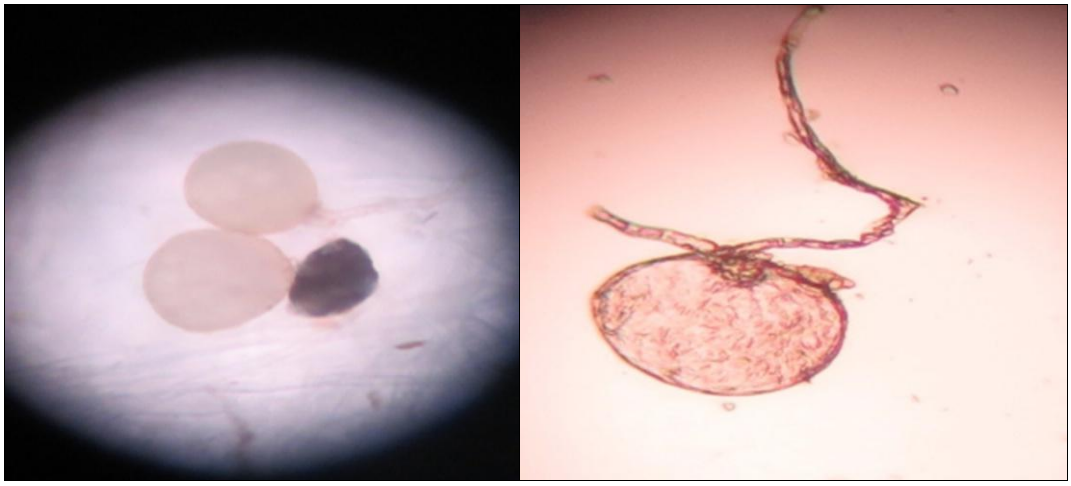


Fig.4 Spores of the genus *Scutellospora*

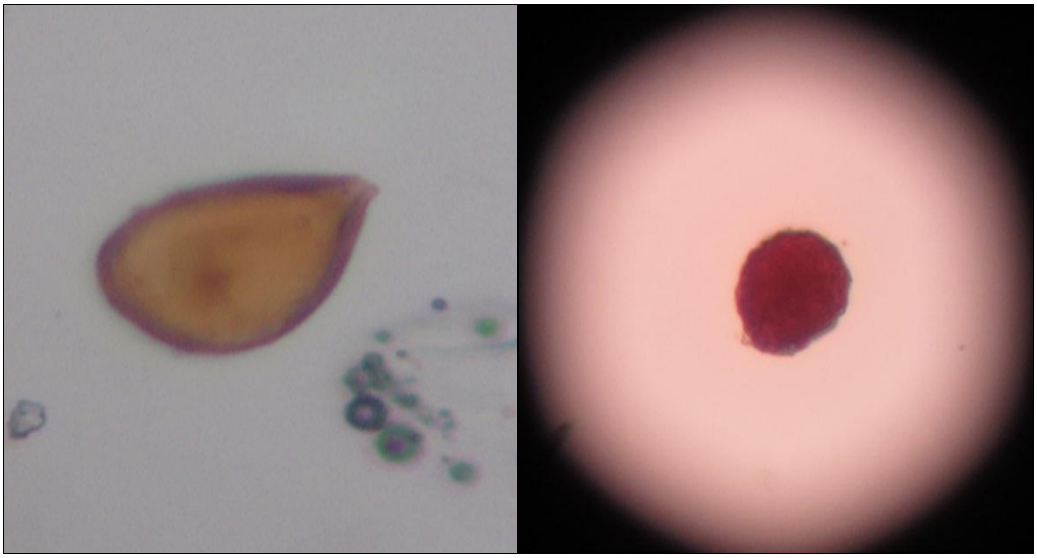


Fig.5 Root colonisation of the genus *Acaulospora*

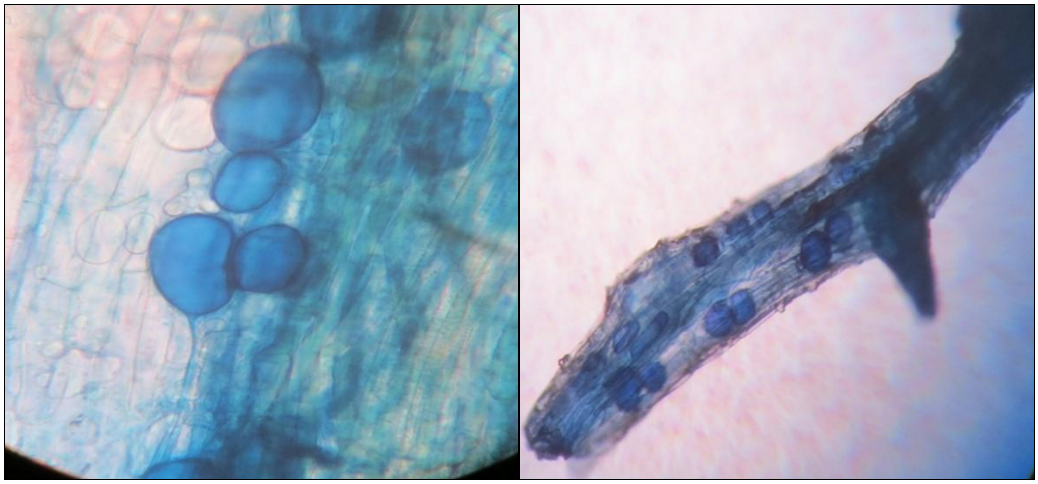


Fig.6 Root colonisation of the genus *Scutellospora*

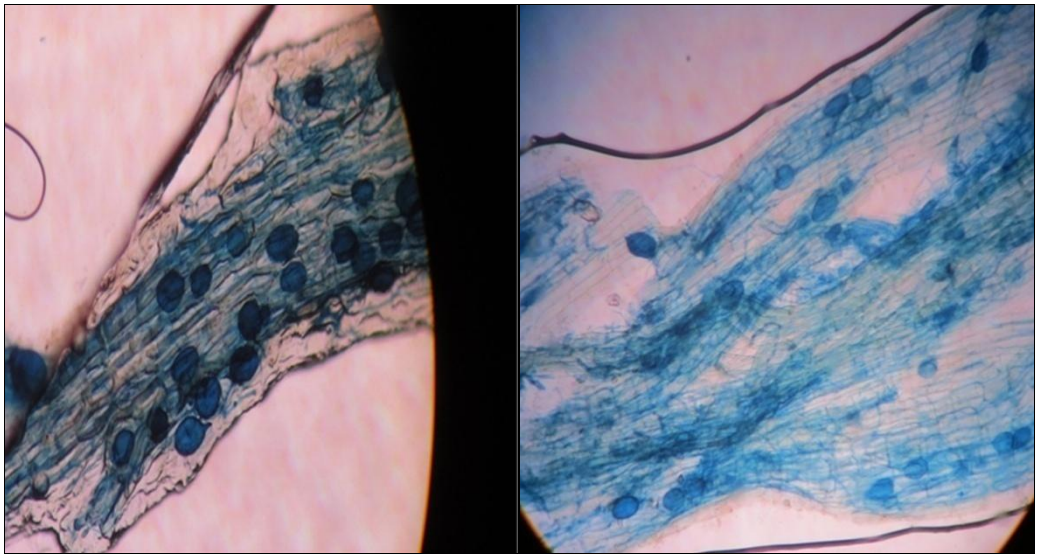


Fig.7 Root colonisation of the genus *Glomus*

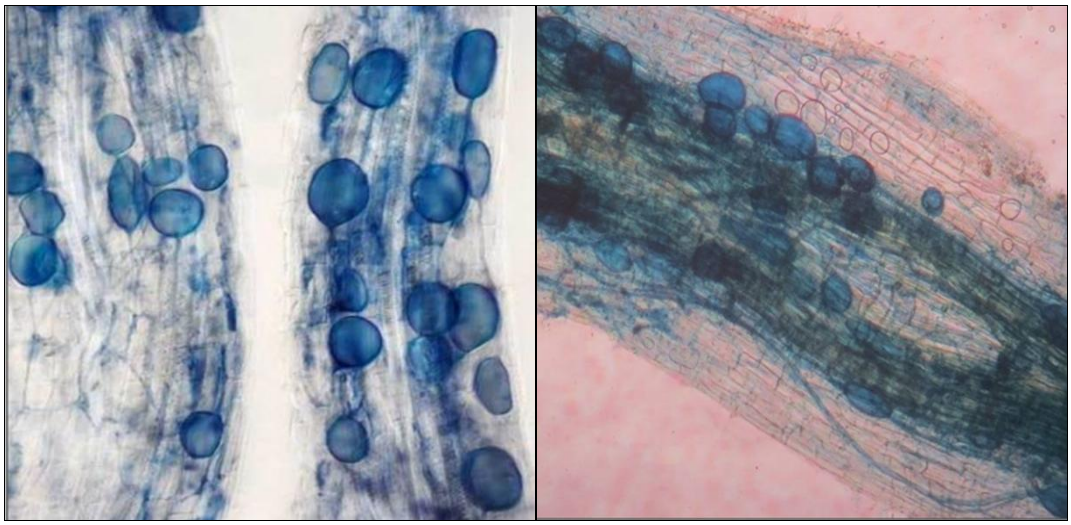
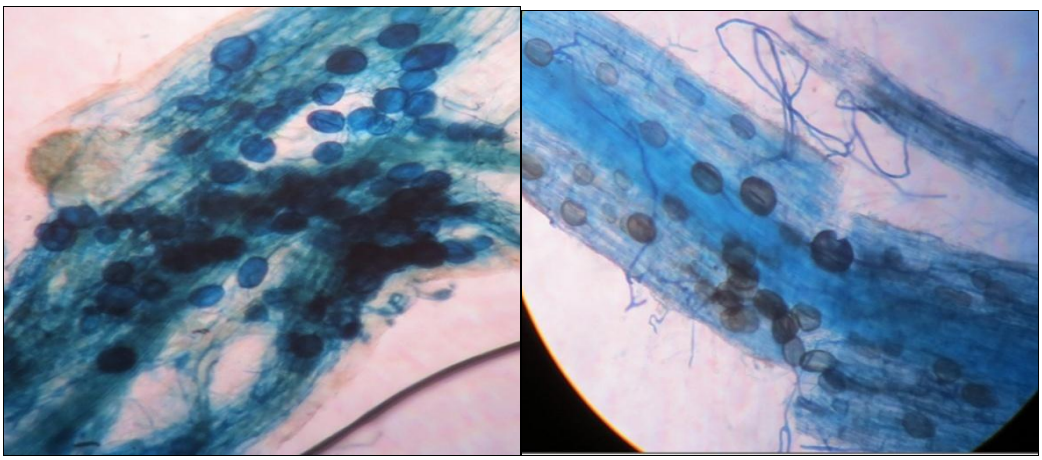


Fig.8 Root colonisation of the genus *Gigaspora*



Pulwama with geographic coordinates of 33.87162° N, 74.8946° E

Kumar also found a significant positive correlation between mycorrhizal spores and colonization (Kumar *et al.,* 2013).Yang *et al.,* (2010) found a positive correlation between and mycorrhizal colonization and spores. These results are also supported by Li *et al.,* (2009). The positive and significant correlation between AM spores and available nitrogen (r=0.815*) was found which might be due to the fact that nitrogen and organic carbon are required by micro-organisms for their special requirements and as a result high nitrogen and organic carbon in the soil increased infection and population of AM fungi. Similar results were reported (Venkatrao *et al.,* 1972). Since the climatic conditions of the study area fall under temperate zone which are conducive to the mycorrhizal development, it is possible that concentration of such propagules may be higher (Akhter, 2005). Moreover, influence of apple roots through their exudates cannot be ruled out which needs further studies.

Soil phosphatase activity

Phosphatase activity of soils of District Pulwama was found to be 26.67 to 34.56 µgPNP/g/24hr with an average value of 31.24 µgPNP/g/24hr (Table 5). Maximum phosphatase activity (34.56 µgPNP/g/24hr) was recorded in Pinglin which was statistically at par with Nikas and Tikkin, however, significantly superior to other villages. The results are in conformity with the results of Eichler *et al.,* (2004) who observed the phosphatase activity between 15.8 to 35.2 µgPNP/g/24hr in the soil. Ohm *et al.,* also observed the phosphatase activity of 19 to 38 µgPNP/g/24hr (Ohm *et al.*, 2013).

Soil acid phosphomonoesterase activity was higher at low available P content of soil than at high content which was supported by other findings (Speir and Cowling, 1991; Santruckova, 2004) as they found that higher enzymatic hydrolysis of organic P depended on the higher microbial P immobilisation but not on the higher mineralisation of organic P compound.

The reason could be that when available phosphorus is deficient in soil, soil biota increases the production of extracellular phosphatase to enhance the supply of inorganic P in soil. Relationships between soil P supply and phosphatase activities are regulated by negative feedback mechanis (Olander and Vitousek, 2000).

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