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# A Contribution to the Vesicular Arbuscular Mycorrhizal Fungal status on twenty selected medicinal plants of Pandam forest in Darjeeling Himalaya, West Bengal, India

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***Abstract:*** *Twenty medicinal plants were investigated for Vesicular-arbuscular mycorrhizal (VAM) association in a survey of the Pandam area of Darjeeling Himalaya, West Bengal. Vesicular-arbuscular mycorrhiza was found to be of universal occurrence in almost all plants located at different regions of the globe except a few one under varied eco-habitats. Great variations were found in the VAM infection percentage compared to those at the other sites including seasons also. Herbaceous plants showed more infection in comparison with the shrubby plants found in the same locality. The association showed the first time information about the extent of the VA-mycorrhizal study along with the study of the soil characteristics at Pandam. Spore density of rhizospheric soils was also determined which showed the range in between 12 to 650 number per 100 gram of soil sample that may be a guide line to study more in the same site of Pandam for ready inoculums preparation at the later stage to develop mycorrhizal bio-fertilizer in restoration of ecosystem in hill.*

***Keywords:*** *VA-Mycorrhizae, Root Colonization, Seasonal variations, medicinal plants-Pandam-Darjeeling.*

## I. Introduction

Mycorrhiza is a symbiotic association between a soil borne fungus and the roots of a plant (Kirk et al., 2001). Arbuscular mycorrhizas are symbiotic relationships between the plant roots and soil fungi belonging to phylum Glomeromycota (Schussler et al. 2001). The ubiquity of arbuscular mycorrhizal fungi (AMF) at the interface between soil and plant roots makes them a key functional group of soil biota. AMF are known to benefit plant establishment by increasing resistance to environmental stresses, enhancing plant nutrient acquisition, water relations, disease resistance and improving soil quality (Smith and Read, 1996).

Mycorrhizal fungi have existed since the first plants appeared on dry land more than 450 million years ago. They form a close symbiotic relationship with plant roots. They are called mycorrhizae from the Greek "mukés", meaning fungus, and "rhiza," meaning roots. Mycorrhizae form a network of filaments that associate with plant roots and draw nutrients from the soil that the root system would not be able to access otherwise. This fungus-plant alliance stimulates plant growth and accelerates root development.. However, in soil that has been disturbed by human activity, the quantity of mycorrhizae decreases drastically so that there are not enough of them to produce a significant benefit on plant growth and health, hence the importance to compensate for this mutuality effect. Soil structure refers to the soil particle aggregates as well as pore spaces. Maintainance of soil structure is of critical importance to the preservation of soil functions and fertility. Mycorrhizal fungi play a major role in soil aggregation through hyphae networking and glomalin (biological glue) production. Therefore, their presence in the soil is essential to maintain physical soil properties. This includes greater water infiltration and water holding capacity, more permeability to its better root development, high degree of microbial activity and nutrient cycling, better resistance to surface sealing, better resistance to erosion and better resistance to compaction.

Vesicular-arbuscular mycorrhizal (VA) fungi colonize plant roots and ramify into the surrounding bulk soil extending the root depletion zone around the root system. They transport water and mineral nutrients from the soil to the plant while the fungus is benefiting from the carbon compounds provided by the host plant. Therefore VA-fungi have a pervasive effect upon plant form and function (Bethlenfalvay et al., 1994). Little is known about the natural ecology of these fungal-plant associations and the effects of certain soil amendments with natural waste products.VA-fungi are associated with improved growth of many plant species due to increased nutrients uptake, production of growth promoting substances, tolerance to drought, salinity and transplant shock and synergistic interaction with other beneficial soil microorganisms such as N-fixers and P-solubilizer (Sreenivasa and Bagyaraj, 1989 ).

The VAM fungal association is the most common and widely occurring in the mycorrhizal symbiosis. The mycorrhizal fungi associated with more than 80 percent of the plant species in the 90 percent of the plant families (Wang and Qui, 2006). Mycorrhizal fungal colonization present present in angiosperms, gymnosperms, pteridophytes and some bryophytes (Smith and Read, 1997). In the mycorrhizal association commonly divided

in to ecto-mycorrhizas and endo-mycorrhizas both are important component of soil life and soil fertility including soil chemistry. AM fungi are most important factor for rhizosphere microflora in natural ecosystem and play a main role in restoration of nutrient cycling in local ecosystem (Peterson et al., 1985). The major benefit of mycorrhiza is its greater soil exploration and increasing uptake of P, N, K, Zn, cu, S, Fe, Mg, Ca, and Mn (Sundar and Parthipan 2010). The induce disease resistance of plant by AM fungi has become a hot spot in chemo-ecological study and biocontrol of plant diseases (Huang and Zenq, 2003). The AM fungal colonization not only improved the growth but also enhanced the active principle content of the medicinal plants (Zubek and Blaszkowski, 2009).

## II. Objectives Of Study

**Study includes-**

1. To study the important medicinal plants available in the locality.
2. To study of Physico-chemical properties of mycorrhizospheric soils of Pandam forest area.
3. To study the status of VAM fungi in roots of selected medicinal plants.
4. To study the colonization status of roots of selected medicinal plants,
5. Suggestion forwarding scientific management to use the importance of VAM to restore the local ecosystem.

**Area under Study**

The study was conducted at Pandam area of Darjeeling, West Bengal, located at Eastern Himalayan region in India. The annual mean maximum temperature is 14.9° C and annual mean minimum temperature is 8.9° C and average annual rainfall is 3092 mm. (Sharma, 2013). The altitudinal range of this hilly region varies from 150 to 3636 meter resulting in a huge contrast and diversity in climate and vegetation (Saha et al, 2011). The district is surrounded by Bhutan in the east, Nepal in the west and Sikkim of India in the north. Due to similar environmental and cultural conditions, the major inhabitants of Darjeeling hills and its surrounding areas are bonded together by Nepali language, the medium of communication among the different ethnic groups, viz. Lepchas, Bhutias, Rai, Sherpa, Tamang, Mangar, Gurung and Kagatay of the Nepali communities (Rai and Bhujel, 1999). Traditionally, chief occupation of the people of Darjeeling had been agriculture, agro forestry, horticulture and animal husbandry. A wide range of microclimatic sites under a wide array of climatic zones are available here, that allow growing more luxuriant biota in a proper naturally managed environment. The climates favour the luxuriant growth of diversified floral and faunal elements and make the vegetation as a climatic climax. Therefore, the gradient of this region is also diverse with gentle range of natural flora. So, the area boosts luxuriant growth of ground vegetation of angiosperms along with different mosses, liverworts, lichens, fungi, algae and cyano-bacteria. The shrubby vegetation of different members aggregate the small patches along the dominant tree species there in a two or three layered canopy system, which have high coverage of litter fall (Das, 2014).

## III. Materials And Methods

**Selection for study plants in the field**

Twenty medicinal forest plants were taken to study. The selected medicinal plants were taken during monsoon and post monsoon seasons only. All these twenty plants were collected from Pandam forest areas of Darjeeling Himalaya for the study of preliminary kind to develop local inocula.

**Collection of Mycorrhizospheric soil sample and root sample**

Soil samples and roots were collected from the rhizosphere soil of twenty selected medicinal plants in two different seasons consecutively monsoon and post monsoon from the study area. The samples consisting of feeder roots + soil were collected with the help of a soil digger (0-15cm depth) so as to represent the complex root zone. Root systems of common plant species were excavated taking care to ensure that fine roots predominate in the sample and to exclude entangled roots of other species. Sufficient samples were taken to determine any variation and consistency in the degree of mycorrhizal root colonization between or within the sampling sites. All soil samples were dried and sealed in polyethylene bags and stored in laboratory for future analyses. These include study of Physico-chemical properties of soil and mycorrhizal sustenance.

**Isolation of VAM spores from soil**

Numerous techniques were available to recover VAM spores from soil. The most basis of this is wet sieving and decanting (Gerdemann and Nicolson, 1963), which remove the clay and sand particles, organic matter etc. and retaining spores and other similar sized soil particles on sieves of various meshes (53, 175, 250, 300 and 450μm). For the isolation of spores, 100g of soil was weighed and is added to 1000 ml of water taken in a conical flask. Then the flask was shaken well in a vortex mixture and allowed to settle soil particles and was immediately transferred to a series of sieves. This sieving was collected in respected jars by washing with water

after filtration by Whatman filter paper no 110. Then transferred the sieving on a gridded petriplate and observed it under the stereomicroscope. The number of spores were counted and expressed as the number of spores/100g of rhizosphere soil sample. These isolated spores were picked up using wooden dowel and were mounted in Poly Vinyl Lacto Glycerol (PVLG) to make permanent slides.

**Preparation of diagnostic slides for Examination**

The preparation of good semi permanent diagnostic slide is critical in making as for the determination for specimen of VAM fungi. Semi permanent mounts, such as PVL (Polyvinyl lacto-phenol) or PVLG (Polyvinyl lacto-glycerol) allow slides to remain usable for years (Koske and Tessier, 1983). Therefore, a drop of mountant was placed on one of the corner of a clean dry microscopic slide allowing one end of the slide for labelling. Selected spores were picked with a wooden dowel and placed on slide. In order to disperse the spores, the mountant along with the spores were mixed gently and is allowed to set for 3-5 minutes to become more viscous. Then a clean dry cover slip was moved at $45^0$ angle towards the drop of mountant containing spores until it contacts the mountant. After the contact, the mountant was allowed for few seconds to spread along the cover slip. Then gentle pressure was applied over the cover slip in order to break the spore walls. This mountant with spores were allowed to dry for several hours in a flat position. After that, the spores were observed under stereomicroscope. Finally, the edges of the cover slip were sealed and dried.

**Clearing and staining of root specimens for Examination**

The roots were collected first by hand sieving and were placed in plastic cassettes and were then washed vigorously with water. Then specimens were placed the same in a beaker containing 10% KOH solution and keep cassettes in water bath at 60 $^0$C for 15 minutes (Phillips and Heyman, 1970). Then KOH was poured off and rinse the cassettes using at least three complete changes of tap water until no brown colour appears in rinsing water. Then the cassettes with coloured roots were covered with alkaline H2O2 at room temperature for 30 minutes until the roots were bleached. The cassettes were then thoroughly rinsed to remove H2O2 completely. Then covered the cassettes with 1 % HCl and soaked for 3-4 minutes. After this step, the roots are not washed with water because the specimen must be acidified for proper staining. The cassettes were then treated with common chelpark ink rather than with 0.01% acid fuschin 0.05% trypan blue and kept in the period 30 minutes for staining (Utobo et al., Ghosh, 2014). Ink is used because dyes are corrosive and health hazardous. Then the roots were removed from cassettes and were placed in a gridded petriplate with a grid of 0.5 x 0.5 squares affixed to the base. The stained roots were observed under the compound microscope following standard slide method to examine the number of vesicles, arbuscules, coiled hyphae, other structures; inter radicular vesicles (IRVs) and infection threads. The degree of mycorrhizal colonization were also measured by counting the total number of colonized root segments /total number of root segments examined x 100

## IV. Results And Discussion

Arbuscular Mycorrhizal Fungi have been described as keystone which mutualise in ecosystem due to their unique place at the root-soil interface (Kumar et al., 2010). The present investigation is the first time information for occurrence of VAM fungal diversity in some selected medicinsl plants from great variations in Pandam, Darjeeling. The physico-chemicals parameters were noted in table 1 while plants along and with their VAM fungal characterizations are noted in table 2 given below. The density of spores in each community under specific plant is also given in table 3.

**TABLE 1**
**Physico-chemical properties of mycorrhizospheric soils of Pandam**

| Sl. No. | Name of Plants | Range of $p^H$ of Soil | Moisture content of soil | Soil Organic carbon (OC) | Soil Organic matter (SOM) |
|---|---|---|---|---|---|
| 1. | **Artemisia vulagris** | 6.30 ± 0.4 | 40.8±0.4 | 0.61±0.034 | 0.990±0.4 |
| 2. | **Calamintha umbrosa** | 6.44 ± 0.4 | 38.8±0.4 | 0.67±0.034 | 1.155±0.4 |
| 3. | **Curculigo recurvata** | 6.45 ± 0.4 | 40.2±0.4 | 0.70±0.034 | 1.051±0.4 |
| 4. | **Drymeria cordata** | 6.26 ± 0.4 | 35.20 ± 0.4 | 0.58±0.034 | 1.206±0.4 |
| 5. | **Drymeria villosa** | 6.26 ± 0.4 | 35.20 ± 0.4 | 0.58±0.034 | 1.206±0.4 |
| 6. | **Erigeron belliloides** | 6.36 ± 0.4 | 34.20 ± 0.4 | 0.59±0.034 | 1.017±0.4 |
| 7. | **Eupatorium canabinum** | 6.26 ± 0.4 | 35.20 ± 0.4 | 0.58±0.034 | 1.206±0.4 |
| 8. | **Fagopyrum dibotrys** | 6.37 ± 0.4 | 33.20 ± 0.4 | 0.59±0.034 | 1.017±0.4 |
| 9. | **Galium aperine** | 6.45 ± 0.4 | 40.2±0.4 | 0.70±0.034 | 1.051±0.4 |
| 10. | **Hydrocotyle himalaica** | 6.45 ± 0.4 | 40.2±0.4 | 0.70±0.034 | 1.051±0.4 |
| 11. | **Impatiens arguta** | 6.45 ± 0.4 | 40.2±0.4 | 0.70±0.034 | 1.051±0.4 |
| 12. | **Impatiens stenantha** | 6.25 ± 0.4 | 34.20 ± 0.4 | 0.55±0.034 | 0.948±0.4 |
| 13. | **Persicaria nepalensis** | 6.30 ± 0.4 | 40.8±0.4 | 0.61±0.034 | 0.990±0.4 |
| 14. | **Plantago sp.** | 6.26 ± 0.4 | 35.20 ± 0.4 | 0.58±0.034 | 1.206±0.4 |
| 15. | **Pouzolzia indica** | 6.40 ± 0.4 | 39.80±0.4 | 0.67±0.034 | 1.155±0.4 |
| 16. | **Selenium tenuifolium** | 6.30 ± 0.4 | 40.8±0.4 | 0.61±0.034 | 0.990±0.4 |
| 17. | **Stellaria media** | 6.680 ± 0.4 | 36.22±0.4 | 0.67±0.034 | 1.155±0.4 |
| 18. | **Swertia bimaculata** | 6.30 ± 0.4 | 40.8±0.4 | 0.61±0.034 | 0.990±0.4 |
| 19. | **Swertia chiryata** | 6.26 ± 0.4 | 35.20 ± 0.4 | 0.70±0.034 | 1.206±0.4 |
| 20. | **Urtica dioica** | 6.95 ± 0.4 | 36.2±0.4 | 0.80±0.034 | 1.379±0.4 |

**Note: Value: Mean ± SD**

Rhizospheric soil pH could have major impact on diversity pattern of AM fungal species in natural ecosystem and agricultural ecosystem (Porter and Abbott, 1987). As a result the rhizospheric soil confirmed the properties of soil were almost normal and pH in between 6.26 ± 0.4 to 6.95 ± 0.4. Soil organic carbon content of the said area ranges between 0.58±0.03 to 0.80±0.03. Similarly Soil Organic Matter (SOM) of the same area ranging between 0.90±0.4 to 1.31±0.4.

In the present study twenty plant species were studied under the influence of mycorrhizal association that have great role. All the selected plants have been taken from 2 different seasons at Pandam in Darjeeling District of West Bengal State. Vesicular Arbuscular Mycorrhizal (VAM) fungal colonization and diversity of spores in soils were noted from Pandam area. Therefore, these may be documentation for first time which is the insight of the VAM research or AMF research in local area of hill. The inoculums produced from reported mycorrhiza increases the growth and yield of experimental plants. The present result shows that all medicinal and ornamentals have great variations in spore density and diversity. However, the VAM fungal status was surveyed in 20 selected medicinal plant species belonging to 11 different families under 18 different genera of Darjeeling District of Eastern Himalaya during monsoon and post monsoon seasons only. Most of the plants were colonized by VAM fungi. The VAM colonization percentage differs among different plant species. In the study the maximum root infection was observed in case of Calamintha umbrosa (95 %) belongs to the family Lamiaceae and lowest root infection was noticed in case of Galium aperine (9 %) belongs to the family Rubiaceae which was less dependent in Pandam forest area. Within 20 plant species, 3 of them are non-mycorrhizal viz. Fagopyron dibotrys, Pouzolzia indica and Urtica dioica. This may be due to the fungal toxins present in root tissue that reduces the mycorrhizal association (Tester and Smith, 1987).

The highest spore population was recorded in the plant species like Calamintha umbrosa (450±0.2/100gmsoil) belongs to the family Lamiaceae. The lowest spore population was observed in the species like Fagopyum dibotrys followed by Impatiens stenantha (12±0.2/100gm of soil) belongs to the Polygonaceae and Balsaminaceae respectively. The spore density was observed in between 12 to 450 per 100 gm of soil.

Totally four AMF were noted in which dominant species was Glomus (Sarwade and Bhale, 2011). This is similar to our observation which showed Glomus as dominant AMF species. The study shows mycorrhizal diversity less in monsoon, moderate to high degree in post monsoon season. Glomus species recorded as dominat mycorrhzal genus followed by other three species like Gigaspora, Acaulospora, and Scutellospora. These species of AMF type have a great role and major impact on the host plants in those areas even under any environmental conditions as are presented climatic and chemical factors in our Eastern Himalaya.

**TABLE 2**
**Arbuscular Mycorrhizal Fungal (AMF) colonization in roots of twenty plant species**

| Sl. No. | Plant Species | Arbuscule | Vesicle | Colonization % | Other Structures |
|---|---|---|---|---|---|
| 1. | **Artemisia vulagris** | 60±0.4 | 60±0.4 | 60 | CH |
| 2. | **Calamintha umbrosa** | 95±0.4 | 95±0.4 | 95 | IRV |
| 3. | **Curculigo recurvata** | 22±0.4 | 53±0.4 | 38 | CH |
| 4. | **Drymeria cordata** | 88±0.4 | 06±0.4 | 47 | - |
| 5. | **Drymeria villosa** | 72±0.4 | 66±0.4 | 69 | - |
| 6. | **Erigeron belliloides** | 80±0.4 | 40±0.4 | 60 | - |
| 7. | **Eupatorium canabinum** | 84±0.4 | 48±0.4 | 66 | - |
| 8. | **Fagopyron dibotrys** | Nil | Nil | Nil | -- |
| 9. | **Galium aperine** | 12±0.4 | 06±0.4 | 09 | - |
| 10. | **Hydrocotyle himalaica** | 54±0.4 | 66±0.4 | 75 | - |
| 11. | **Impatiens arguta** | 16±0.4 | 10±0.4 | 26 | CH |
| 12. | **Impatiens stenantha** | 60±0.4 | 06±0.4 | 33 | - |
| 13. | **Persicaria nepalensis** | Nil | Nil | Nil | - |
| 14. | **Plantago sp.** | 20±0.4 | 02±0.4 | 15 | - |
| 15. | **Pouzolzia indica** | Nil | Nil | Nil | - |
| 16. | **Selenium tenuifolium** | 89±0.4 | 23±0.4 | 56 | IRV |
| 17. | **Stellaria media** | 38±0.4 | 04±0.4 | 21 | - |
| 18. | **Swertia bimaculata** | 60±0.4 | 10±0.4 | 35 | - |
| 19. | **Swertia chiryata** | 64±0.4 | 50±0.4 | 57 | - |
| 20. | **Urtica dioica** | Nil | Nil | Nil | - |

**N.B:** CH-Coiled hyphae, IRV-Interradicular vesicles

**TABLE 3**
**Spore density of Rhizosphere soil in Study area**

| Sl. No. | Selected Plant Species | Spore number/100gm soil |
|---|---|---|
| 1. | Artemisia vulagris | 240±0.4 |
| 2. | Calamintha umbrosa | 450±0.4 |
| 3. | Curculigo recurvata | 122±0.4 |
| 4. | Drymeria cordata | 189±0.4 |
| 5. | Drymeria villosa | 220±0.4 |
| 6. | Erigeron belliloides | 72±0.4 |
| 7. | Eupatorium canabinum | 185±0.4 |
| 8. | Fagopyrum dibotrys | 12±0.4 |
| 9. | Galium aperine | 179±0.4 |
| 10. | Hydrocotyle himalaica | 354±0.4 |
| 11. | Impatiens arguta | 16±0.4 |
| 12. | Impatiens stenantha | 12±0.4 |
| 13. | Persicaria nepalensis | 09±0.4 |
| 14. | Plantago sp. | 420±0.4 |
| 15. | Pouzolzia indica | 79±0.4 |
| 16. | Selenium tenuifolium | 156±0.4 |
| 17. | Stellaria media | 145±0.4 |
| 18. | Swertia bimaculata | 240±0.4 |
| 19. | Swertia chiryata | 164±0.4 |
| 20. | Urtica dioica | 104±0.4 |

**Photographs**

**Figure 1. Land scape of Pandam Forest Area in Darjeeling Himalaya; Figure 2. A team of Collectors and workers from Microbiology Laboratory, Post Graduate Department of Botant, Darjeeling Govt. College (DGC), Darjeeling; Figure 3. 4th Semester Students, Botany, 2015 of DGC; Figure 4. Leader person Sri Bishnu Prasad Sharma of Pandam Village (Standing) and Dr. Debabrata Das, author (Sitting) after specimen collection from village forest.**

**Fig. 5 Magnified view of landscape diversity of Pandam forest in Eastern Himalaya**

**Figure 6. Small Village in the lap of Hill slope at Darjeeling Himalaya**

**Figure 7. Left one is the house wife of Pandam village, right three are the students of 4th semester Botany, during field visit, 2015 and carrying plant specimens including soil**

**Figure 8. Scenic view of Hill range from Pandam, Darjeeling**

**Figure 8. Top of the peak is Tiger Hill, nearer part is Senchal Sanctuary, a deep vegetation in Darjeeling**

**Figure 9. In the valley some villages situated nearer to 'Tin mile forest range' right side is Pandam forest.**
**PHOTOPLATE**

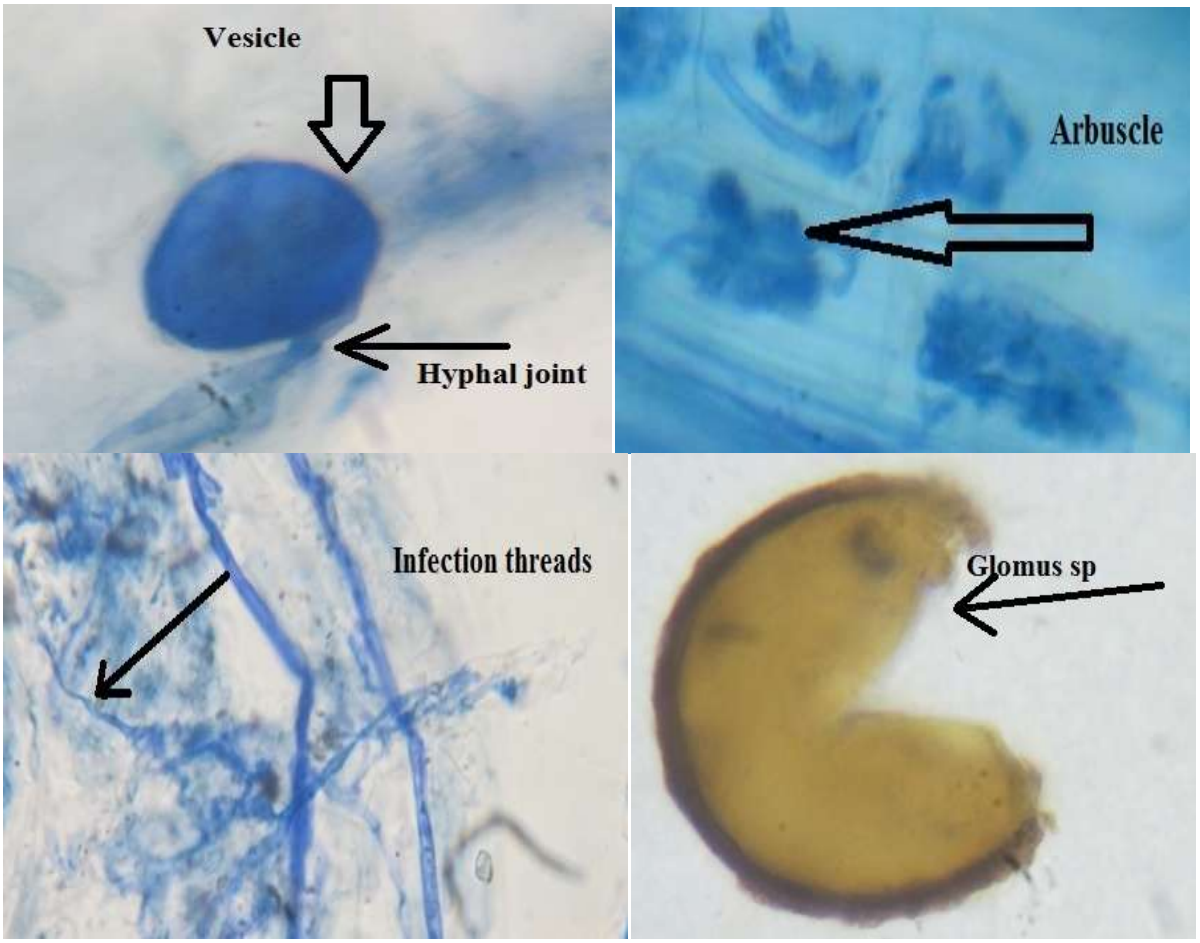


**Figure 10.** Vesicle of Mycorrhizal fungus, Figure 11. Arbuscule; Figure 12. Infection threads; Figure 13. Spore of VAM fungus after isolation through wet sieving and decanting technique.

## V. Conclusion

Vesicular Arbuscular mycorrhizal (VAM) fungal type of symbiosis is found in vast taxonomic range of both herbaceous and woody plants that are found in diverse habitats ranging from arctic to the tropics, arid to aquatic environments and stable plant communities to highly disturbed ecosystems. However, the AM fungal community and AM colonization of plant roots may vary greatly in different soil types (Sambandan, 2014). However, the AM fungal community and AM colonization of plant roots may vary following different types of habitats and seasons. The AM fungi differ widely in the level of root infection of plants and in their impact on nutrient uptake particularly phosphate. Vesicular Arbuscular Mycorrhizal (VAM) fungi are ubiquitous in soils and are associated with roots of majority of plants (Brown, 1985). However, families of Cruciferae, Chenopodiaceae, Compositae, Cyperaceae, Fumariaceae, Polygonaceae and Urticaceae recorded to be non mycorrhizal (Gerdemann, 1968). Later on mycorrhizal association was reported in certain members of Chenopodiaceae, Cyperaceae and Cruciferae (Mejstrik, 1972; Ross and Harper, 1975). Our result revealed the same as per the previous author's suggestion. Report also revealed that herbaceous plants are more infective than the shrubs or woody perennials in the study by VAM fungi which is similar to the study of Gorsi (2002).

Change of Soil pH is a factor that can govern the adversely affect the growth and development of plants especially in different stress prone zones. Therefore, mycorrhizal plants are of great interest in bioremediation since the management of mycorrhizal systems which is necessary for the success of establishing the onset of problematic plants. In this study, 20 plants were selected as a study type in Darjeeling area to generate the potential biofertlizer from VAM fungi. Study revealed climatic conditions which are strongly influence the percentage of colonization and AM spore population in the present study. Physio-chemical analyses of mycorrhizosphere soils showed that all the study sites had high percentage of Carbon, and high moisture content. The potential adaptation of Glomus sp. as dominant indigenous AM fungi and their ability to colonize in hill soils may be effective if artificially inoculated to economic plants. To generate the local biofertilizer and to develop artificially induced plant communities in the said area it would be helpful to restore the ecosystem pristine. Hope that in near future we will develop the local techniques to make home inocula from indigenous strains.

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