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Altitudinal variation and colonization patterns of Arbuscular Mycorrhizal Fungi associated with organically grown Mandarin Oranges of Sikkim Himalayas

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Three distinct altitudes in Upper Dzongu, Lower Dzongu of North Sikkim and Lower Chuba of East Sikkim were selected for investigations on the altitudinal variation and patterns of Arbuscular Mycorrhizal Fungi (AMF) associated with organically grown Mandarin oranges in the diverse landscapes of Sikkim Himalayas. Field sampling was conducted at multiple locations across varying altitudes. Spore population in field samples of lower altitude were relatively higher than those of higher altitude region. Similarly AMF colonization patterns and types of AMF structure also significantly varied in all the root samples across the altitudinal gradient. The location exhibited a diverse mycorrhizal community, including *Rhizophagus*, *Funneliformis*, *Gigaspora*, and *Acaulospora*. *Acaulospora* isolated from the highest elevation was dominant in both the field and trap cultures, while, *Glomus*, *Rhizophagus* and *Funneliformis* were dominant in lower altitudinal region. These findings suggest a clear trend of increased mycorrhizal colonization and spore abundance at lower altitudes and highlight the influence of altitude and local environmental conditions on the composition and abundance of mycorrhizal fungi in Sikkim's diverse ecosystems.This current investigation also reports for the first time the AMF diversity in Mandarin oranges grown in Sikkim Himalayas. These findings contribute to our understanding of AMF dynamics in Sikkim Himalayas and their significance in organically grown Mandarin orange cultivation.

Keywords : Arbuscular Mycorrhizal Fungi (AMF), diversity, Mandarin oranges, Trap Culture

INTRODUCTION

Arbuscular mycorrhizal fungi (AMF) exist in the roots and soil and can form symbiotic relationships with 80% of terrestrial vascular plants (Shi *et al*. 2020) AMF are widely found in almost all types of terrestrial ecosystems and plays an important role in stabilizing ecosystem functions and soil qualities thereby influencing above ground vegetations.

AMF are also found to regulate inter-root microbial communities in the soil and its nutrient cycling like N, P and K (Rodrigues and Rodrigues, 2020; Qiang *et al* 2019; Wu *et al*. 2017; Chakraborty *et al*. 2011). AMF are also reported to enhance plant adaptation to environments (Hinni, 2024). However, AMF communities and their effect are widely influenced by a variety of environmental factors like altitude, plant communities, soil properties and climatic factors. (Ezeokoli *et al*. 2019). AMF colonization is effected by ecosystems showing variation in altitudes. Though the environmental factors associated to altitudes tend to change at a smaller scales but their effect on AMF structure and colonization pattern have been reported by earlier investigators (Deng *et al*. 2020, Zanzottera *et al*. 2020). Studies have also shown that AMF community significantly decreased with increase

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in elevation in temperate climates (Vieira *et al.* 2019) Moreover, slope orientation, soil physiochemical properties and vegetation types have shown to influence AMF community structure (Sun *et al*. 2019; Guo *et al.* 2022; Zhang *et al.* 2021). There are many studies on AMF distribution pattern and diversity, however a conclusive and distinct pattern of diversity and distribution of AMF along various altitudinal are the areas where it needs much attention and focus (Li *et al*. 2014). Morphological Characters based identification and characterization of AMF is one of the most widely used methods. Spore collected either directly from the field or traps are characterized to study their diversity by almost all the investigators (Gai *et al*. 2006). In certain cases, spores collected from field and natural environment lack basic informative taxonomic characters and hamper a more accurate identification. Moreover, in the field conditions, AMF spores may be less in number or some species may be absent at the time of sampling even though they may be present within roots. Assessment of species composition of a given location or environment is improved by establishment of trap cultures which produce clear and healthy spores, these spore can provide sufficient data for morphological characterization and identification. On the other hand, all AMF species present in the field cannot be enumerated through trap cultures, the selected host plant for trap establishment may also favour or hinder sporulation of few AMF species (Muthukumar and Udaiyan, 2002). Some cryptic AMF species shows enhanced sporulation in trap cultures which were not sporulating in the field conditions (Strumer, 2004).

The Sikkim Himalayas, nestled in the North Eastern region of India, represent a unique ecological hotspot characterized by diverse ecosystems and microclimates owing to its steep altitudinal gradient. This pristine region is not only renowned for its breathtaking landscapes but also for its rich agricultural diversity, with mandarin oranges (*Citrus reticulata*) being a prominent crop in the area. The cultivation of mandarin oranges in Sikkim is of particular significance due to the growing global demand for organic and sustainably produced agricultural products (Bhutia *et al.* 2019).

In this current investigation, we aim to investigate the altitudinal variation in AMF patterns associated with organically grown mandarin oranges in the Sikkim Himalayas. We will explore how the unique environmental conditions at different altitudes influence the composition and diversity of AMF communities in mandarin orange orchards. Additionally, we will assess the potential implications of these AMF-plant interactions for mandarin orange growth and quality in the context of organic agriculture. Our findings have the potential to inform sustainable farming practices in this ecologically sensitive region and contribute to the broader understanding of AMF ecology and their roles in agro-ecosystems worldwide.

MATERIALS AND METHODS

Study site and Sampling

Three distinct regions in North Sikkim were designated for sampling as indicated in Table 1, Fig 1. Samples were collected randomly from the feeder roots surrounding the host (Mature plants of *Citrus reticulata* of 5-8 years old) Rhizospheric soils along with fine roots were collected from a depth of 0-30 cm at 10 distinct locations within each study site, and they were carefully placed into sterile polyethylene bags. These samples were then transported to the laboratory for the isolation of AMF spores. pH and soil types were recorded.

Isolation of AMF Spores

Required amount of soil from soil samples collected from various location of study sites (stored in the proper condition) were taken for isolation of AM spores. Spores were isolated by wet-sieving and decanting method (Gerdemann and Nicolson, 1963) with modifications as suggested by Pacioni *et al.* (1992). 100g of sample soil taken were air-dried and suspended in 1000 ml of tap water, agitated manually for 5 minutes and the soil suspension was left for 10 minutes enabling heavier particles to settle. The remaining soil, roots, hyphal and spore suspension were slowly poured through a stack of sieves of different pore size ranging from 800-40 µm. Extracts were collected in a clean beaker of 100 ml. It was transferred to 50 ml centrifuge tube and centrifuged at 1750 rpm for 5 minutes. Spore suspended pellets obtained after removal of the supernatant was again centrifuged by the sucrose gradient process for 1 minute (Walker *et al.* 1983). Supernatant obtained from the centrifugation was filtered with nylon cloth (25 µm pore size) using filter funnel to remove water and clear sugar molecules from AM fungal spores. The filtrate was placed in clean, sterilized Petri dishes and spores were picked up using white bristle brush and observed under Nikon binocular Stereo microscope (20-120x magnification) and then AMF species were identified from the spores observed and photography were taken for each species and recorded. Live spores were stored in eppendorf tubes at 4^0C. For quantification of spore population, soil samples taken from the various location of study site were homogeneously mixed before extraction and the spores were isolated by wet-sieving and decanting method followed by sucrose density gradient centrifugation (Daniels and Skipper, 1982)

Establishing AMF Trap Cultures

In order to access the dominant AMF species in the native soil samples, Trap Cultures in *Sorghum bicolour* were established as described by Walker (2021) with modifications.

Trap cultures were set-up within a week of sample collection. All trap cultures were established in 1.5 l pots, previously rinsed with 70 % (v/v) ethanol. For preparation of the soil traps, seeds of *Sorghum bicolour* (Sorghum) were surface sterilised in 10 % (v/v) sodium hypochlorite and 0.5 % (v/v) Tween 20 for 10 min and thoroughly rinsed with autoclaved water. Sterilised seeds were then sown in an autoclaved mixture of 1:1 (v/v) Soil:Sand as well as 300g of unsterilized soil obtained from the field as inoculum source. Plants were not supplied with any fertiliser during the first month, after which they were watered with a quarter-strength Hoagland's solution when they showed signs of nutrient deficiency. Plants were checked for sporulation and mycorrhizal colonisation after 5–6 months as described by Walker (2005).

Characterization of AMF species and identification

Intracellular AMF colonization was studied as outlined by (Wilkes *et al*. 2019). Spores obtained from the above method observed in water and mounted on slides using PVA (polyvinyl alcohol) with and without Melzer's reagent. These slides were then examined using a compound microscope to document characteristics like shape, size, color, bulbous suspensor, sporocarp, surface ornamentation, vesicles, and more. Spores were categorized into different types based on their morphology, and detailed observations were conducted on each spore type. Selected morphotypes were further examined under a De-Winter (CROWN) digital compound microscope. Other taxonomic keys used by various Germplasm banks were also referred for identification (Schenck and Perez, 1990; Walker, 1992; Sunar *et al*. 2014, 2015, 2016).

Spore Density

Spores were extracted using wet sieving and decanting method proposed by Gerdemann and Nicolson (1963). 50 grams of soil were thoroughly mixed with a 500 mL and stirred with a glass rod to create a uniform suspension. After allowing the suspension to sit for five minutes, the mycorrhiza spores floated to the top. The suspension was then passed through various sieves of different mesh sizes (60, 100, 300 BSS). This process was repeated 8-9 times to ensure the capture of all AM fungal spores. The population of AM fungi was subsequently determined. Grids were created on a 90 mm petri dish and the number of spores per square centimeter was counted under a compound microscope, including both intact and damaged spores.

Colonization assessment

The root clearing and staining technique proposed by Phillip and Hayman (1970) were employed to investigate mycorrhizal root colonization. Roots were cleared by treating 10% KOH at room temperature for 24 hrs. Following this, the KOH solution was decanted, and the root pieces were washed with water until the brown color disappeared. Subsequently, the roots were acidified using a 1% HCl solution for 3-5 minutes, and the acid was then decanted. The root segments were stained with a 0.5% Trypan blue solution for 24 hours. After staining, the Trypan blue solution was removed. The colonization was assessed by frequency distribution method (Biermann and Lindermann, 1981).

AMF Spore population from both field and traps were tabulated and mean and standard errors were calculated. Simpson's index (D), Shannon-Wiener index (H'), Evenness (E) and species richness (SR) were calculated as described by Mashebo *et al*. (2023). The variation of AMF spore density, root colonization structures between the areas were tested using Pearson Correlation Coefficient (https://www.socscistatistics.com). Significance of difference was determined by one-way ANOVA.

RESULTS AND DISCUSSION

Isolation and Spore count

This current investigation was undertaken with an objective to access the abundance of AMF population and their diversity in the rhizosphere of Mandarin Oranges grown in higher altitude regions of Sikkim hills. Both rhizospheric soil and root samples were collected from specific elevations as mentioned in Table 1 & Fig 1. These three distinct altitudes were representative of those areas where Mandarin Oranges are generally cultivated in organically manured Orchards. Information provided in Table 1 also indicates that these sampling sited received more or less equal amount of annual rain fall and were exposed to equal temperature during the season of collection. From each location a minimum of fifteen samples were randomly collected covering a wide range of distance. Analysis of soil samples revealed that they were slightly acidic in nature and the pH of the soil of upper Dzongu (elevation 1055 mt asl) ranged from 5.5 to 6.5; lower Dzongu (elevation 843 mt asl) 5.5 to 6.5 and lower Chuba (elevation 750 mt asl) from 5.5 to 6.5. All these locations revealed significant difference in AMF population measured in terms of total number of spores present per gram soil. Upper Dzongu, North Sikkim area which is the highest elevated site for our investigation recorded an average of 170 to 270 spores per gram, whereas Lowed Dzongu, North Sikkim the site located at second level of elevation recorded an average of 190 to 300 spores per gram soil. On the other hand, the site at the lowest elevation, Lower Chuba located at East Sikkim recorded maximum of 280 to 430 spores per gram soil (Fig 2a). Feeder roots from mature Orange trees were collected and were aseptically transported to the laboratory for further analysis. Roots were stained and checked for presence of AMF structures. Root colonization assays of the root samples also revealed altitude specific colonization pattern. Roots obtained from higher elevation of Upper and Lower Dzongu area showed more hyphal proliferation and much lesser arbuscules and vesicles within the cortical cells. On the basis of presence and absence of AMF structures, root colonization percentages were calculated. Samples from high altitude area like Upper Dzongu (1055 mt. asl) showed an average colonization percentage ranging from 25 to 40 % while samples from mid altitude of 843 mt asl, lower Dzongu showed root colonization percentage ranging from 55-60 %, whereas samples collected from the lowest elevation of 750 mt asl, Lower Chuba showed root colonization ranging from 55 to 70%. (Fig 2b). However abundant arbuscules were found in the cortical cells of the roots obtained from the lower elevations (Fig 3). In order to test the correlation between these various set of data, Pearson correlation coefficient test was conducted and significance and correlation was expressed by the coefficient "R". This statistical analysis measures linear correlation between two sets of data and hence two variables: Root colonization percentage and Spore count per gram soil were tested for their interrelationships. Data were pooled and correlation among and between the parameters were tested using Pearson Correlation Coefficient calculation tool. The measured linear correlation represented by 'R" suggests that root colonization % and spore count are significantly influenced by each other in all

Table 1: Sampling area and variation in altitudes and other climatic conditions

Site code	Site S-1	Site S-2	Site S-3
Area	Kayem, Upper Dzongu, North Sikkim	Hee Gyathang, Lower Dzongu, North Sikkim	Lower Chuba Busty, East Sikkim
Geographical location	N27°32'154" E088°29'052"	N27°28'743" E088°31'062"	N27° 16.435', E088° 34.169'
Altitude	1055 meters asl	843 meters asl	750 meters asl
Average soil pH	5.20	5.15	6.45
Soil type			
Host	*Citrus reticulata*	*Citrus reticulata*	*Citrus reticulata*
Farming type	Orchard	Orchard	Orchard
Manure type	Farm Yard Manure	Farm Yard Manure	Farm Yard Manure
Age of Plantation	5-7 yrs	5-7 yrs	5-7 yrs
Number of Gird points per sampling area	10	10	10
Average temperature	-4-18°C	-4-18°C	5-23°C
Average rainfall Seasonal (August)	364 mm	364 mm	397 mm

Source: Annual report of Met Centre Gangtok, IMD, Ministry of Earth Science

the three locations. This correlation indicates that at a given altitude, the root colonization patterns and spore populations are significantly correlated with each other. At the same time difference in "R" values of among all the pairs also suggests that each site had a unique interaction pattern the data did not show any significant correlation between the individual sites (Fig 4). Occurrence of AMF spore and colonization patterns is distinct and different for all locations There are numerous reports where AMF association in *Citrus reticulata* grown in high altitude areas have been reported, in a study conducted by Allay *et al*. (2021), AMF were isolated from the rhizosphere of three *Citrus* spp, wherein *Gigaspora* spp, *Glomus* spp, . *Scutellospora* spp., *Acaulospora* spp. and *Entrophospora* spp. have been found to be present in abundance. Wu *et al.* (2020) have also successfully isolated different beneficial isolates AMF like *Funneliformis mosseae, Diversispora versiformis* and *Rhizoglomus intraradices* from *Citrus reticulata* and have demonstrated their potential use in *Citrus* cultivation. Panja and Chaudhuri (2004) have also reported that AMF in Mandarin Oranges are the main root colonizers when grown in pot cultures in green house conditions. Sharma *et al.* (2009) have also reported for the first time the diversity of AMF in *Citrus* and their uses in sustainable horticultural practices. Varghese and Ray (2025), in their study have shown that AMF structures like arbuscules and hyphae are the key components of AMF association which are also associated with nutrient uptake and growth responses.

AMF diversity in field samples

Individual spores were carefully segregated and grouped on the basis of their shape, size and ornamentation. These spores were than treated with PVLG and Melzers' reagent, microscopic observations were carried out after 12-24 hrs of incubations. A wide variety of AMF species were observed from each location. AMF spores were photographed and identified up-to genus level using taxonomic keys and reference specimen. Since the primary objective of this investigation was to study the populations of various groups of AMF in a given sample, molecular and species based identification was not carried out at this

Table 2: Spore density, species abundance and colonization pattern in trap cultures

Area/ GPS location	Trap No	Soil pH	Average AMF spore density (Number of spores/g soil)	Average root colonization percentage	Dominant Species
Kayem,	C/RH/UDZ/T-01	6.53	283.117±11.46	55.116±0.847	a,c,d
Upper Dzongu,	C/RH/UDZ/T-02	5.52	221.432±09.33	53.145±1.166	a,c,d
North Sikkim	C/RH/UDZ/T-03	5.46	156.133±08.66	58.562±1.221	a,,c
	C/RH/UDZ/ T-04	5.22	148.122±12.12	53.441±1.161	a,,c,d
N27^{O}32'154"	C/RH/UDZ/ T-05	5.18	218.122±12.68	51.263±1.166	a,,c,f
E088^{O}29'052"	C/RH/UDZ/ T-06	5.50	224.563±11.44	60.117±2.116	a,,c,f
Site-S-1	C/RH/UDZ/ T-07	5.50	250.126±11.48	68.116±0.932	a,c
	C/RH/UDZ/ T-08	6.18	268.343±13.21	68.212±0.886	a,c,d
	C/RH/UDZ/ T-09	6.13	220.220±09.46	63.412±1.414	a,b,d
	C/RH/UDZ/ T-10	5.15	286.225±09.11	60.188±1.226	a,b,d
Hee Gyathang,	C/RH/LDZ/ T-01	6.50	216.115±12.166	50.178±1.116	a,b,d
Lower Dzongu,	C/RH/LDZ/ T-02	6.22	233.188±12.113	52.336±1.383	a,b
North Sikkim	C/RH/LDZ/ T-03	5.18	146.343±11.144	71.432±2.177	a,b
N27^{O}28'743"	C/RH/LDZ/ T-04	6.33	218.168±09.312	61.224±2.133	a,b,d
E088^{O}31'062"	C/RH/LDZ/ T-05	6.46	313.213±07.155	66.781±2.161	a,b,d
Site-S-2	C/RH/LDZ/ T-06	6.18	316.221±11.416	65.436±1.413	a,b,d,f,g
	C/RH/LDZ/ T-07	6.23	337.336±11.883	62.188±1.133	a,b,f
	C/RH/LDZ/ T-08	6.26	218.121±10.443	61.446±1.133	a,b,c,e,g
	C/RH/LDZ/ T-09	6.44	215.316±11.446	58.190±2.166	a,b,c,e
	C/RH/LDZ/ T-10	6.21	286.336±12.188	64.176±2.136	a,b,c
Lower Chuba	C/RH/CHU/ T-01	6.55	316.117±16.133	72.432±2.338	a,b,e
Busty, East	C/RH/CHU/ T-02	6.18	338.142±16.133	73.166±2.336	a,b,e
Sikkim	C/RH/CHU/ T-03	6.30	416.223±17.186	74.221±2.166	a,b
	C/RH/CHU/ T-04	6.16	418.176±13.188	61.217±1.866	a,b
N27° 16.435',	C/RH/CHU/ T-05	5.54	316.224±13.167	59.144±1.813	a,b,d,e
E088° 34.169"	C/RH/CHU/ T-06	5.33	332.144±12.182	63.422±2.111	a,b,c
Site-S-3	C/RH/CHU/ T-07	5.17	350.176±12.133	79.166±2.111	a,b,c,e
	C/RH/CHU/ T-08	6.14	358.313±09.233	81.443±2.276	a,b,c,d,g
	C/RH/CHU/ T-09	6.18	338.122±09.146	76.117±2.144	a,b,c,g
	C/RH/CHU/ T-10	6.50	417.433±09.118	76.122±2.163	a,b,d,g

Values are average of 10 replicates; ±=SE; Dominant species refers to those groups of AMF whose spore counts were above 10/g soil; a -*Glomus* spp; b -*Rhizophagus* spp, c -*Funneliformis* spp, d - *Acaulospora*, e - *Entrophospora spp,* f- *Scutellospora spp; g - Gigaspora*

stage. Dominant species was designated on the basis of maximum spore count belonging to that Spore-type. On the basis of our microscopic examination and morphological characterization, the most dominant AMF species recorded for upper Dzongu region was *Acaulospora* spp followed by *Glomus* spp. These two AMF species were the most dominant and were present in all the soil samples collected from that region. For lower Dzongu region, the most dominant species of AMF to be recorded were *Acaulospora* spp. followed by *Scutellospora* group. Maximum diversity in terms of AMF species was recorded in the soil samples collected from lower elevation of East Sikkim. Soil samples of this area contained AMF spores of *Glomus* spp, *Funneliformis* spp, *Rhizophagus* spp and few spores of *Acaulospora* spp. *Rhizophagus* group of spores were most abundant. Microscopic images and their morphological details have been presented in Fig.5. In a similar study AMF species were identified based on occurrence, morphology by Gaonkar and Rodrigues (2020) and have reported thirty-two AM fungal species belonging to nine genera viz., *Acaulospora, Claroideoglomus, Entrophospora, Funneliformis, Gigaspora, Glomus, Rhizophagus, Sclerocystis,* and *Scutellospora*, wherein *Acaulospora* was found to be the dominant genus and *A. dilatata* was the dominant AM species in mangroves of Chorao island. In our current investigation, we report the genus *Acaulospora* to be the most dominant geneus in the higher altitude orchard of *Citrus reticulata*. Other genera of *Glomus*, *Rhizophagus* and *Funneliformis* were dominant in lower altitudes. Diversity of AMF communities

Table 3: Analysis of Different Diversity indexes based on spore population and spore types obtained both from field and trap cultures

Site/location	Spore type	Field per g soil	Field D	Field 1/D	Field H'	Field SR	Field E	Trap per g soil	Trap D	Trap 1/D	Trap H'	Trap SR	Trap E
Kayem,	ST 1	106						138					
Upper Dzongu,	ST 2	53						43					
N. Sikkim	ST 3	28	0.24	4.25	1.60	6.0	0.894	37	0.29	3.49	1.56	7.0	0.802
1055 meters	ST 4	43						18					
asl	ST 5	26						12					
	ST 6	16						23					
	ST 7	-						14					
Hee Gyathang,	ST 1	128						132					
Lower Dzongu,	ST 2	56						62					
N. Sikkim	ST 3	26	0.23	4.37	1.69	7.0	0.870	13	0.28	3.53	1.54	7.0	0.790
843 meters	ST 4	48						26					
asl	ST 5	17						16					
	ST 6	22						28					
	ST 7	24						08					
Lower Chuba	ST 1	183						233					
Busty,	ST 2	67	0.24	4.19	1.67	7.0	0.856	86	0.370	2.70	1.33	7.0	0.684
E.Sikkim	ST 3	58						25					
750 meters asl	ST 4	37						31					
	ST 5	44						13					
	ST 6	12						05					
	ST 7	37						22					

Spore type- ST; Simpson's index-D; Simpson's Reciprocal Index (1/D); Shannon diversity index (H'); Species Richness (SR); Species Evenness (E)

Host in Field :*Citrus reticulata* and Host in Trap culture: *Sorghum bicolour*

varies along different altitudes. Previous studies have shown that diversity of AMF communities are directly or indirectly influenced by increasing or decreasing altitude (Egan *et al.* 2017; Shi *et al.* 2019). In our study we have shown that AMF community decreases with increase in altitude which is in agreement with the previous studies conducted by Zhang *et al.* (2022) and Guo *et al.* *(*2022).

AMF diversity in trap cultures

All the spores from natural environment cannot be characterized due to loss of spore walls and attachment of soil particles to their exterior surfaces. To evaluate the total types of spores present in a natural soil sample, an indirect method of trapping these spores in a desired host is used to obtain those spores which cannot be recorded in original samples. Trap cultures with *Sorghum bicolour* as host were established with soils obtained from different locations; after an appropriate growth period, soils were sieved, their spore population were recorded and characterized. In our current investigation, we could obtain successful root colonizations on all the trap cultures which revealed that the AMF of higher altitude could also be cultivated in alternate hosts outside their native environment. Further root colonization assays revealed that these AMF could form profuse, interwoven extra radical hyphae, on certain points they could from profuse intercalary arbuscules. Vesicles were also observed in few cases; in original samples of *Citrus reticulata* vesicles were not observed (Fig 6-a-c). Trap cultures are very helpful for rejuvenating the AMF population in a given sample. In a study conducted by Makdon and Kayang (2019), trap cultures with *Oryza sativa* and *Zea mays* as host plants were successfully used to access AMF population and diversity of fifteen years old abandoned coal mine. As a result five genera of AMF were successfully recovered and identified which included AMF genera of *Acaulospora*, *Claroideoglomus*, *Funneliformis*, *Glomus* and *Rhizophagus*, where, *Acaulospora* and *Glomus* were found to be dominant.

Similarly spores were segregated on the basis of their morphological characters and grouped. Microscopic observations and morphological characterization of few of the dominant spores

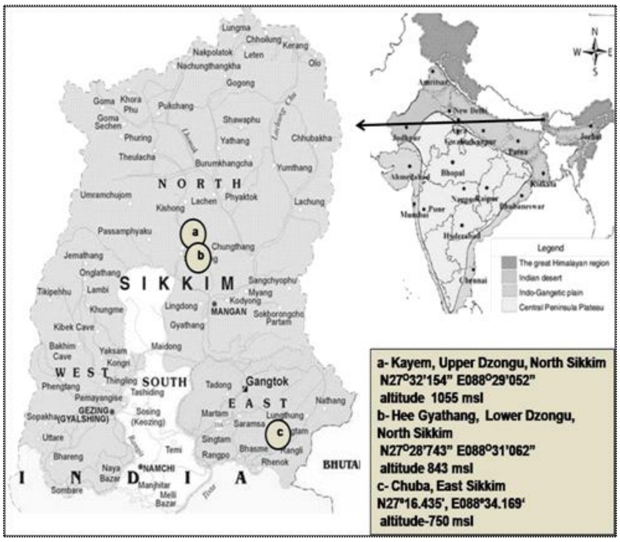

Fig. 1: Three Distinct Orchards of *Citrus reticulata* (Mandarin Orange) were selected. Each location represented a specific elevation/altitude where oranges are grown in Sikkim Himalayas. From each location, 15-20 samples were randomly collected. (Map source- Sikkim tourism)

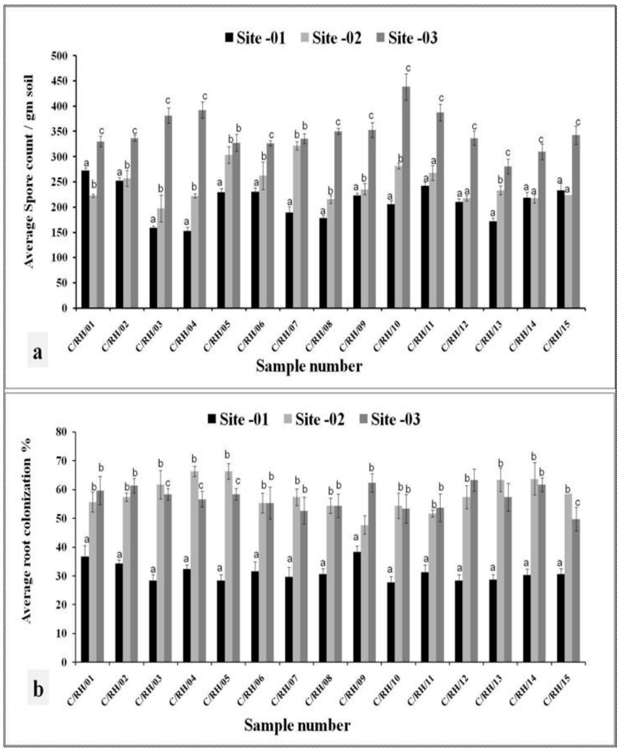

Fig. 2: Average Spore count (A) and Root colonization % (B) examined from the soil and root samples obtained from different sites of Sikkim.

Site 1- Lower Chuba Busty, East Sikkim; Site-2 Hee Gyathang, Lower Dzongu, North Sikkim; Site 3- Kayem, Upper Dzongu, North Sikkim

Values are average of fifteen replicate samples, bars represent SE. Bars with different letters indicate significant differences between the sampling sites (a, b- P =0.01; c- P=0.05). Similar letters indicate insignificant.

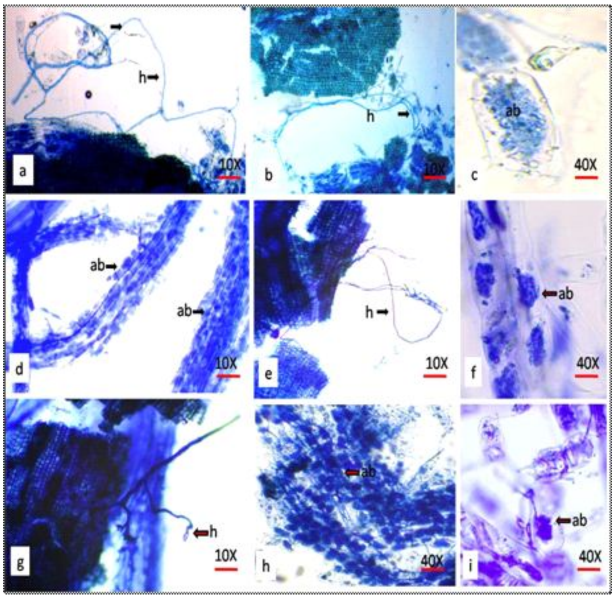

Fig. 3: Root colonization patterns of *Citrus reticulata* by indigenous AMF species obtained from different locations of Sikkim. (a-c)- Kayem, Upper Dzongu, North Sikkim, elevation 1055 mts asl; (d-f)- Hee Gyathang, Lower Dzongu, North Sikkim, elevation 843 mts asl; (g-i)-Roots obtained from Lower Chuba Busty, East Sikkim, elevation 750 mts asl. (Bar- 100µm for 10 X and 10 µm for 40 X)

were carried out. Unique sores of *Glomus* spp. were obtained from Chuba East Sikkim (Lowest elevation), these spores were round, small in size with unique thick outer wall layer. This outer thick wall layer was light brown in colour and was laminated (Fig 6-d,e). Similarly large spores approximately 300-400 µm in dia, probably belonging to *Gigaspora* spp. were also obtained from the traps of Lower Chuba soil samples. The spores of *Gigaspora* are large and stained dark purple in Melzers' reagent. This outer layer was rigid and smooth followed by inner laminated wall layers (Fig 6-f, g). Unique spores of *Acaulospora* spp were obtained from the traps raised from soils collected from lower Dzongu, North Sikkim. Spores belonging to this group were round to globose or even oblong with outer saccule attached to the most of the spores. The inner spore walls are golden brown in colour and are highly ornamented and contain pits. (Fig 6- h-k). Among these spores, one of the spores showed oblong spore shape with dark brown wall layers (Fig 6, k). Trap cultures obtained from the upper Dongu region contained some very unique AMF species which were not observed in the field samples. These isolates included *Rhizophagus spp, Scutellospora* spp. and *Funneliformis* spp. (Fig 6. l-o)

In our present investigation, six genera of AMF were isolated (*Acaulospora*, *Funneliformis*, *Glomus, Rhizophagus, Gigaspora* and *Scutellospora*) where, *Acaulospora* and *Glomus* were dominant in the higher altitudinal region, our findings are also consistent with the findings of Choudhury *et al.* (2010) and Singh and Jamaluddin (2011), where they found these two genera to be dominant on spoil of all age groups. *Glomus* and *Acaulospora* species have short sporulation time and high competitive interaction and adaptability as compared to that of *Gigaspora* and *Scutellospora* species in the same environment, allowing them to establish better than the others (Nandakwang *et al,* 2011, Singh *et al,* 2008). *Acaulospora* species are often associated with acidic soil (Teixeira-Rios *et al,* 2013, Mosbah *et al,* 2018) and a wide range of host species (Straker *et al.* 2010).

Diversity indexes based on spore population

In terms of diversity analysis, AMF spores were grouped based on their shape, size and morphology and were designated as spore types or morphotypes. Spore population in the field sample were comparatively less than those isolated from the trap cultures. Among the field samples lowest ppore population was recorded for the site -1, upper Dzongu which is located at the higher elevation of 1055 meters asl, followed by site-2, lower Dzongu located at an elevation of 843 meters asl and maximum spore population was found in samples collected from site-3, lower Chuba, elevation 750 meters asl. In all the cases only a few number of distinct morphotypes were isolated from the rhizosphere of *Citrus reticulata* (Table 3). Our findings also align with previous studies by Hinni et al, 2024, where they have reported low species richness of AMF in natural conditions. Factors like elevation, plant age and environmental conditions may affect their population in natural conditions (Schneider *et al,* 2017). Simpson's index (D) for AMF population from field samples was also comparative lower than that of trap cultures, similarly Shannon diversity index (H'), Species Richness (SR) and Species Evenness (E) was also less for field samples. Among the field samples both the locations Lower Dongu and Lower Chuba recorded same species richness of 7.0, whereas among the trap cultures, all the three locations had the same SR. Highest Simpson's index of 0.370 was recorded for AMF population obtained from traps established from Lower Chuba soil samples. It has been shown in earlier reports that the diversity index is influenced by AMF species composition and relative abundance (Yang *et al,* 2011). Moreover a specific trap plant used to cultivate AMF species may also play an important role in sporulation and abundance of AMF spores and it have been reported that host plants have an influence on the AMF development and colonization in trap cultures (Jansa *et al,* 2002, Leal *et al,* 2009, Makdoh and Kayang, 2019). In our current investigation we have also found that the root colonization percentage was also comparatively lower in samples collected from the field, lowest in upper Dzongu region and highest in lower Chuba. This finding is aligning with previous studies where it has been reported that availability of roots for colonization in natural conditions influences AMF colonization and sporulation (Lovelock *et al.* 2003; Ahulu *et al.* 2006; Songachan and Kayang, 2011).

CONCLUSION

The Arbuscular Mycorrhizal Fungal population associated with *Citrus reticulata* grown in the higher altitudinal regions of Sikkim gives a clear understanding about their association and relative abundance and distribution. Spore population and root colonization decreased with increasing altitude, suggesting the influence of altitude and climatic conditions on AMF colonization and survival. Distribution of AMF genera along the three altitudes were also distinct and unique. Orchards located at the highest elevation of 1055 mts asl showed less spore population but *Acaulospora* was the most dominant genus. In the lower altitudinal region of Lowe Chuba, East Sikkim *Glomus, Rhizophagus* and *Funneliformis* were the most dominant genera. Traps cultures were successfully established which facilitated to recover those genera that were not isolated from field samples. *Gigaspora* and *Scutellospora* were such genera that were successfully isolated from traps and characterized. Though our study is not extensive and does not cover the entire Mandarin cultivated areas of Sikkim hills, we report for the first time the occurrence of AMF species

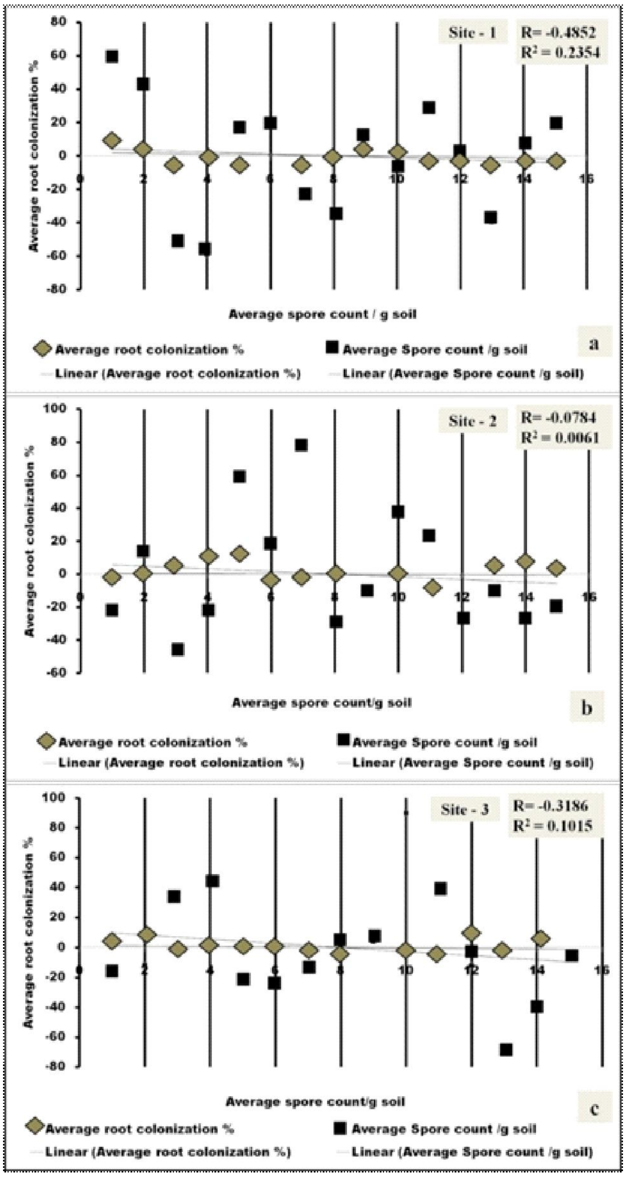

Fig. 4: Pearson Correlation Coefficient of average root colonization % and Average spore count/ g soil. Data were pooled and correlation among and between the parameters were tested using Pearson Correlation Coefficient calculation tool. The measured linear correlation represented by 'R" suggests that root colonization % and spore count are significantly influenced by each other in all the three locations. Occurrence of AMF spore and colonization patterns is distinct and different for all locations.

Site 1- Lower Chuba Busty, East Sikkim; Site-2 Hee Gyathang, Lower Dzongu, North Sikkim; Site 3- Kayem, Upper Dzongu, North Sikkim

in High altitudinal regions of Sikkim. We have also shown in a very preliminary study, the dominant AMF genera that can survive in high altitudinal climatic conditions of Sikkim Hills. Our study paves the way for more elaborative study of the region for better understanding of AMF in hill ecosystem.

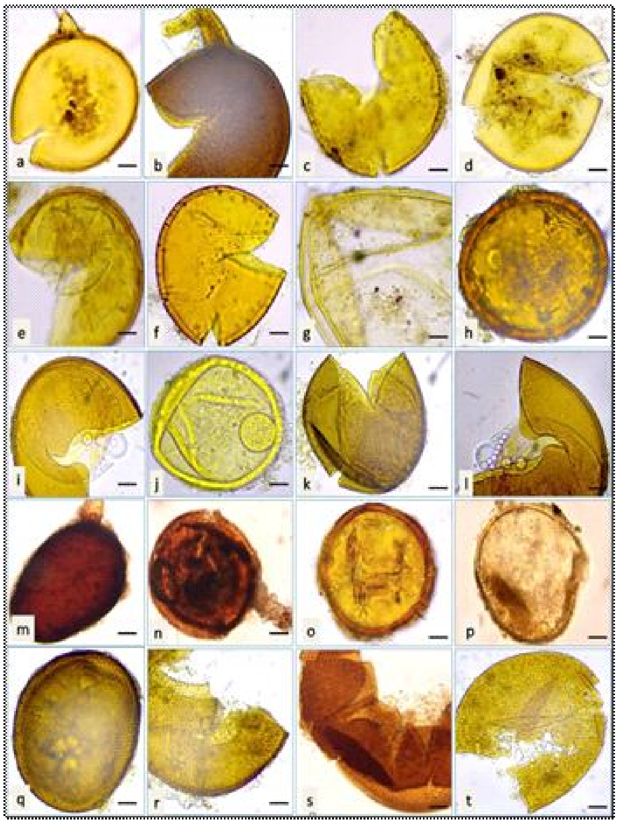

Fig. 5: Spores of Arbuscular Mycorrhizal Fungi belonging to different groups/species obtained from the field samples collected from different locations. (a-d)- *Funneliformis* group; (e-h)- *Glomus* group; (i-l)- *Scutellospora* group; (m-p)- *Rhizophagus* group; (q-t)- *Acaulospora* group. Bar- 100μm for 10 X

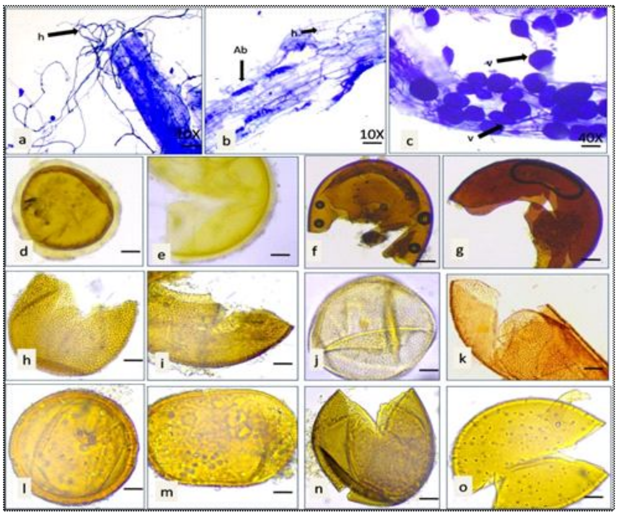

Fig. 6: Root colonization patterns and spores obtained from different trap cultures established using soil samples collected from different locations of Sikkim.

(a-c)- Root colonization in *Sorghum bicolour* (Trap host) showing hyphae (h), Arbuscules (Ab) and Vesicles (v). (d-e; f-g)- Dominant genera of AMF obtained from Lower Chuba Busty, East Sikkim; (h-k)- Hee Gyathang, Lower Dzongu, North Sikkim; (l-o)- Kayem, Upper Dzongu, North Sikkim (Bar- 100μm for 10 X and 10 μm for 40 X) d & e- *Glomus* spp., f&g- *Gigaspora* spp., h-k- *Acaulospora* spp., l&m-*Rhizophagus* spp. n- *Scutellospora* spp., o- *Funneliformis* spp.

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DECLARATION

Conflict of Interest: All the authors of collaborating institutions declare no conflict of interest.

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