3. MATERIALS AND METHODS

3.1 Study site

The study site was located in Karbi Anglong Hills (92°45' and 93°54' East Longitude and 25°45' and 26°35' North Latitude) of Assam, India. Study sites showing different degrees of disturbances were selected for the present study. Degree of disturbances at selected sites was assessed from plant coverage, vegetation types and amount of forest floor organic matter removal. Three forests representing undisturbed forest (UF), monoculture forest (MF) and slash-and-burn field (SBF) were consequently selected.

3.2 Sampling procedure

Sampling was carried out in between June 2009 and January 2011. Soil was sampled in June 2009/2010 (start of rainy season), September 2009/2010 (end of rainy season), November 2009/2010 (start of dry season) and January 2010/2011 (middle of dry season). Sixteen soil samples, each of approximately 200 g, were collected in each season from the rhizosphere region of plants at sampling points 20 m apart on the middle line transect of each site. Soil was sampled with a shovel to a depth of 15 cm, and stored in plastic bags, transported within 12 h to the Department of Botany, Gauhati University, Guwahati, Assam, air dried on an open bench for 72 h, and then stored in a refrigerator until analysis.

3.3 Trap culture establishment

Trap culture was established from fresh soil samples mixed with autoclaved sand in a ratio of 1:1. Onion (*Allium cepa*) was used as host or trap plant. Cultures were grown under natural ambient light and temperature. Soil samples were collected from trap culture pot and was air dried for spore isolation.

3.4 Single spore culture

For establishment of single spore culture, soil and sand in the ratio of 1:1 was autoclaved twice, first at 121 °C at 15 lb for 15 min and was left as such overnight. The second
autoclaving was done next day. Averages of 50 spores isolated from the field soil (identical in shape, size and colour and identified up to genus level) were inoculated into the pot culture with onion (*Allium cepa*) as the host plant. Cultures were grown under natural ambient light and temperature for a period of 24 weeks. Freshly produced spores were isolated by wet sieving and decanting method and permanent slides were prepared.

### 3.5 Determination of soil character

Soil pH was determined in 1:1, soil: water suspension after the soil samples were brought to the laboratory. Soil nitrogen was determined by following the Indophenol Blue Method as described by Allen (1974). The Ascorbic Acid procedure was followed for the determination of total soil phosphorus (Okaelebo 1993). The soil potassium content was determined by flame photometer as described by Okaelebo (1993).

### 3.6 Spore isolation and identification of AMF

Soil or substrate (100 g) from each field sample or trap culture was dispersed in 1 l water and decanted through a series of sieves (300, 250, 212, 150, 90, 75, 63, and 45 μm) arranged in decreasing order according to Gerdemann and Nicolson (1963). Residues were filtered through filter papers and all intact spores (non-collapsed spores with cytoplasmic contents), spore clusters and sporocarp were counted through a stereo-zoom microscope (Labomed CZM4). Thereafter, spores of similar size and colour were separated, counted and later identified with a compound microscope at up to 400x magnifications (Labomed made). Intact AM fungal spores were transferred using a wet needle to polyvinylalcohol-lactophenol (PVL) with or without Melzer’s reagent on a glass slide for microscopic identification. Spores mounted in PVL with Melzer’s reagent were crushed in order to observe reaction of different spore wall layers. Identifications were based on current online species descriptions, [http://www.amf-phylogeny.com](http://www.amf-phylogeny.com); International Culture collection of Arbuscular and Vesicular-Arbuscular Endomycorrhizal Fungi, INVAM: [http://invam.caf.wvu.edu](http://invam.caf.wvu.edu); University of Agriculture in Szczecin, Poland: [http://www.agro.ar.szczecin.pl/~jblaszkowski/](http://www.agro.ar.szczecin.pl/~jblaszkowski/). Permanent voucher slides are stored at the Microbial Ecology Laboratory, Department of Botany, Gauhati University, Assam, India.
3.7 Root colonization

Fine roots of Costus, Ixora and Mellastoma were sampled from the three study sites. Root samples were gently washed with tap water to remove adhering soil patches and particles. Washed root samples were cleared in 10 % (w/v) KOH for 30 minutes at 90 °C, acidified in 2 % (v/v) lactic acid for 10 min, and stained for 30 min at 90 °C with 0.05 % (w/v) Trypan blue (Phillips and Hayman 1970). Roots that remain dark after clearing were bleached in alkaline H₂O₂ prior to acidification with lactic acid. The stained roots were examined with compound microscope and the percentage root length colonization and frequency of colonization was estimated according to magnified intersection method (McGonigle et al. 1990). The colonization was calculated using the following formula:

\[
\text{% Colonization} = \frac{\text{Total length of root segments colonized}}{\text{Total length of root segments examined}} \times 100
\]

Frequency of colonization was estimated as \( \frac{\text{total number of root segments colonized}}{\text{total number of root segments examined}} \times 100. \)

3.8 Statistical analyses

AM fungal composition in field samples was evaluated based on spore frequency (F), density, relative abundance (RA), Shannon-Wiener index of diversity (H'), Simpson diversity index (D) and Sorenson's similarity coefficients (Cs). Frequency was calculated as the percentage of samples from which spores of a particular genus or species were isolated. Spore density was calculated from direct counts of spores or sporocarps per 100 g soil. Relative abundance was calculated as the number of given species spore divided by total number of spores. Since different study sites usually have different spore numbers, therefore, calculating different diversity indexes (e.g., Shannon-Wiener and Simpson index) can led us to compare Glomeromycotan diversity from communities with different numbers of spores. The Shannon-Wiener index of diversity was used to evaluate the AM fungal diversity, which is the product of the two main components of diversity, i.e. evenness and the number of species. H' value was calculated according to the formula

\[
H' = - \sum p_i \ln p_i
\]

where \( p_i \) is the relative spore abundance of the ith species compared to all species identified in a sample.
Simpson's diversity index (D), which measures the concentration of species, was calculated from the formula

$$D = 1 - \sum \frac{n_i (n_i - 1)}{N (N-1)}$$

where \(n_i = \text{number of individuals of species} \) and \(N = \text{Total number of species in community} \).

Sorenson's similarity coefficients (Cs) was employed to evaluate the degree of community similarity of AM fungi among the three sites according to the formula

$$Cs = \frac{2j}{(a + b)}$$

where \(j\) is the number of AM fungus species co-existing in both sites, \(a\) is the total number of AM fungal species in one site (e.g. UF) and \(b\) is the total number of AM fungal species in other site (e.g. SBF or MF).

Statistical software SPSS 16.0 (SPSS Inc. 2007) was used to conduct one-way ANOVA and post hoc comparisons. In order to investigate and visualize the influence of disturbance (management type) on the distribution of AMF, ordination analyses were conducted in CANOCO for windows version 5 (Šmilauer and Leps 2014) using relative abundance and spore density data for each AM fungal species.

### 3.9 Molecular analysis

#### 3.9.1 Root sampling

Fine roots of *Costus*, *Ixora* and *Mellastoma* were sampled from the three study sites. Roots were washed under running tap water to remove attached soil and debris. Samples were stored at 4 °C for subsequent assessment of root colonization and molecular analyses.

#### 3.9.2 DNA extraction from roots

The genomic DNA was extracted from roots by following the CTAB protocol (Li et al. 2009). Aliquots (1 g) of root samples (sampled from each study site) were homogenized in 1mL of CTAB extraction buffer (2 % CTAB, 10mM EDTA, 200mM Tris HCl, pH-8 and 2M NaCl). The mixture was incubated at 65 °C for 1 h, and centrifuged at 10000 rpm for 10 min. Then, the supernatant was collected in a fresh 1.5 ml micro-centrifuge tube and equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added. The mixture was centrifuged at 10000 rpm for 15 min. Thereafter, aqueous phase was
collected in a fresh tube and 2/3 volume of Isopropanol was added and centrifuged again at 12000 rpm for 5 min. DNA pellet was washed with 75 % ethanol. DNA pellet was air dried, and re-suspended in TE buffer, pH-7.5. The DNA samples were checked on a 1 % agarose gel, and diluted 100 times for subsequent nested PCR reaction.

### 3.9.3 Nested PCR amplification

Small subunit rDNA genes (SSU rDNA) were amplified via the polymerase chain reaction (PCR) using universal and glomeromycotan specific primers. A two-step procedure (nested PCR) was conducted. The first amplification of a partial sequence of the SSU rDNA gene was performed with forward and reverse universal eukaryotic primers NS1 (GTA GTC ATA TGC TTG TCT C) and NS4 (CTT CCG TCA ATT CCT TTA AG) developed by White et al. (1990). PCR was carried out using 10 mM dNTPs (2.5mM each), 20 pmol of each primer, 5 U of Taq DNA polymerase and the supplied reaction buffer in total volume of 20 μl as follows: initial denaturation at 94 °C for 3 min, followed by 30 cycles at 94 °C for 30 s, 1 min primer annealing at 40 °C, 1 min extension at 72 °C, followed by a final extension period of 10 min at 72 °C. The first PCR product was diluted 1/100 with 1 × Tris EDTA (TE) buffer. The dilutions was used as template DNA in a second PCR reaction to be performed using the forward and reverse fluorescently labelled primers AML1 (5'-ATC AAC TTT CGA TGG TAG GAT AGA-3') and AML2 (5'-GAA CCC AAA CAC TTT GGT TTC C-3') developed by Lee et al. (2008). Second PCR reactions was carried out as follows: 3 min initial denaturation at 94 °C, followed by 30 cycles of 1 min denaturation at 94 °C, 1 min primer annealing at 50 °C and 1 min extension at 72 °C, followed by a final extension period of 10 min at 72 °C.

### 3.9.4 Restriction analyses

Nested PCR products were digested with restriction enzymes Hinf I and Alu I. The reactions were performed using 10 μl of PCR product, 7.75 μl nuclease free water, 2 μl of restriction enzyme buffer, and 0.5 μl of each restriction enzyme (Hinf I and Alu I). The reaction mixture was incubated at 37 °C for 24 hours. Restriction fragments were separated in 2 % agarose gel. The gels were stained with ethidium bromide and photographed using an ultraviolet transilluminator camera.