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The Relationship of Stable Isotope Accumulation in Ectomycorrhizas on *Pinus sylvestris*

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Abstract: ***The stable isotopes of nitrogen (^{15}N) and carbon (^{13}C) have been widely used to investigate nutrient transfer between ectomycorrhizal (ECM) plants and fungi. In this study, the $^{13}CO_2$ was used to label mycorrhizal Pinus sylvestris saplings collected from the Culbin forest and ^{15}N injected into the intact blocks of turf containing the saplings. The carbon allocation from the plant to ectomycorrhizas was hypothesised to be positively related to the acquisition and accumulation of nitrogen from the soil by the fungi. There were multiple colonisations of ECM fungi were found in individual sapling root systems.The analysis of stable isotopes showed that morphologically distinct groups of ECM fungi accumulated different amounts of ^{15}N and ^{13}C. Contrary to our hypothesis, the analysis also demonstrated a strong negative correlation between ^{15}N and ^{13}C in the ECM tips. This suggests the dynamics of carbon allocation and nitrogen acquisition may be offset, that some ECM fungi are using carbon from hosts to acquire other growth limiting nutrients (e.g. Phosphorus) that has not labelled, or that accumulation of carbon and nitrogen in ECM tips does not accurately reflect nutrient transfer processes.***

Keywords: Ectomycorrhiza, stable isotopes ^{15}N and ^{13}C, nutrient transfer, carbon allocation, *Pinus sylvestris*.

1. Introduction

The analysis of stable isotopes is widely used by ecologists, archaeologists and geologists,but now, they have been used to study fungal ecology[1]. For example, stable isotopes have been used to understand and quantify the uptake of nitrogen (N) by different plant species, which showed spatial variation in N utilisation among plant species in the field[2]. Ostle *et al.*[3] used pulse-labelling of $^{13}CO_2$ to monitor assimilation of CO_2*in situ* in grassland and demonstrated the potential of *in situ* isotope labelling techniques in the realm of ecological and agricultural research. Stable isotope analysis can be useful for studying macrofungi that are difficult to isolate on agar media or in laboratory microcosms[1]. Studies of ectomycorrhizal (ECM) and saprotrophic fungi using natural abundance isotope approaches have revealed that the fungi have different $\delta^{13}C/\delta^{15}N$ profiles. ECM fungi are usually enriched in ^{15}N and depleted in ^{13}C compared to saprotrophic fungi from the same habitat[4-6]. This finding undoubtedly reflects the particular modes of nutrition of the fungi, and may also reflect preferences for specific forms of nitrogen in soil.

A key feature of isotopic analyses is the ability to quantify the movement of carbon from host plants to mycorrhizal fungi, and nitrogen from soil to host plants via mycorrhizal fungi. Thus, the relationship between energy gained by the fungus from the plant, and the benefit in terms of nitrogen uptake to the plant can be determined. Such reciprocal exchange of resource likely underpins the mycorrhizal symbiosis. Most plants fix carbon from the atmosphere and allocate large amounts (typically 5-20%) to their mycorrhizal fungi[7]. The fungi are heavily dependent on this carbon, and use it for growth and the acquisition of primarily nitrogen and phosphorus from the soil, a large proportion of which is translocated to the plant. The development of economic models to represent 'biological trade'[8] has increased our understanding of the evolution of specialization in mutualistic mycorrhizal symbioses. For example, it would be expected that fungi that are particularly beneficial to plants in terms of nutrient transfer would be 'rewarded' with carbon. This idea has been proposed[9] and placed in an evolutionary theoretical framework[10] yet little empirical work has been published to support it. What experimental evidence there is supporting these predictions has thus far focused either on uni-directional resource transfer[11-12]or has been based on highly simplified experimental situations[13]. For example, by investigating the uni-directional movement of carbon from plants to fungi, it was found that onions allocated more carbon to the most beneficial of two arbuscular mycorrhizal fungi[11]. Similarly, when Scots pine (*Pinus sylvestris*)seedlings were supplied with elevated concentrations of CO_2, the specialist ECM fungus *Suillus bovinus* used recent plant assimilate more efficiently, enabling it to acquire more nitrogen and reward the plant compared to the generalist ECM fungus *Laccaria bicolor*[12]. Kiers *et al.*[13] was the first to demonstrate that plants could reward arbuscular mycorrhizal fungi with carbon, and these fungi then enforced the cooperation by rewarding the plant with more phosphorus. However, much of the evidence was derived from agar plate-based systems using transformed carrot roots as the host 'plant' that lacked any photosynthetic tissue that makes the findings difficult to extrapolate to the situation in nature.

In ECM fungi, carbon and nitrogen can be measured at the level of the mycorrhiza, and this can be used to establish the relationship between these nutrients. In a pioneering field-based $^{13}CO_2$ pulse labelling study of Scots pine shoots in combination with additions of $^{15}NH_4Cl$ to soils, it was found that ^{13}C and ^{15}N content of ECM roots were positively correlated[14]. This finding suggested that the roots that are the strongest sink for recent assimilate are also the strongest sink for nitrogen acquired from the soil. However, the relationship was quite variable, which may suggest a key role of ECM fungal diversity. For example, the 'source-sink' relationship between ECM fungi and plants may differ

depending on morphological and physiological traits of the fungi. It is well established that different species of ECM fungi have different abilities to acquire mineral nutrients from the soil. *Betula popuilifolia* colonised by *Piloderma bicolor* had significantly shoot nitrogen content compared to plants colonised by *Amanita flavorubescens* or *Cenoccocum geophilum* [15]. Pena *et al.*[16] found ECM fungi species showed a large interspecific variation of ^{15}N accumulation, e.g, among the short-distance exploration types they isolated from cored soil, *Cenococcum geophilum* showed the lowest ^{15}N accumulation, suggesting *Cenoccocum geophilum* has differences in nitrogen demand or metabolism in comparison with *Humaria* sp. and *Sebacina* sp. In this study, the reciprocal $^{13}CO_2$ and ^{15}N labelling was used to test the relationship between C and N acquisition in ECM roots of naturally regenerating Scots pine saplings. Further, the morphological analyses were used to test how this relationship holds among ECM morphotypes. This is because the ECM fungi were hypothesised to supply the most nitrogen to the host plant would receive more carbon from the host plant in return.

2. Materials and Method

The main objective of this study was to investigate the relationship between ^{15}N and ^{13}C accumulation in ECM root tips that were directly harvested from Scots pine saplings collected from Culbin forest. The sapling shoots were labelled with $^{13}CO_2$ and shortly after $(^{15}NH_4)_2SO_4$ was used to label the soil.

2.1 Sampling Method and Area

Scots pine saplings (approximately 10 cm height) were collected from Culbin pine forest in Morayshire, Scotland. Culbin forest is located on the southern shore of the Moray Firth in the northeast of Scotland (57°38'08" N, 03°42'07"W) and it is a 125 year Scots pine (*Pinus sylvestris* L.) plantation. The site has no obvious environmental gradient, has no other ground layer vegetation and covered with dominant bryophytes; *Rhytidiadelphus triquestrus* (Hedw.) Warnst. and *Hylocomium splendens* (Hedw.) B.S.G[17-18]. The soil profile of this site consists of an organic horizon ((bryophyte/litter (L, *c.* 0-2cm); fermentation (F, *c.* 2-4cm) and humic (H, *c.* 4-12cm)) above deep Aeolian sand deposits [19]. The saplings were collected by carefully cutting the soil around the saplings in a square with a spade. The blocks of soil with the saplings were transferred into pots with dimension 29 cm (w) x 19 cm (d) x 13 cm (h). One sapling was transferred into one pot. The saplings were then kept in the glass-house in the temperature was maintained at 10°C for seven months prior to labelling. Distilled water was used to water the saplings with 200 ml per pot twice for every 7 days.

2.2 Labelling the pine saplings with ^{15}N and ^{13}C

The ^{15}N labelled solution was prepared by diluting 0.5 g $(^{15}NH_4)_2SO_4$into 200 ml distilled water. Each pot received 200 ml of $(^{15}NH_4)_2SO_4$ by injecting the first 100 ml using a syringe into each pot, which were allowed to settle. After 1 hr, another 100 ml was injected into each pot and the pots were left for 30 min. This allowed the soil to absorb the $(^{15}NH_4)_2SO_4$. 100 ml of distilled water was then injected into each pot to ensure the $(^{15}NH_4)_2SO_4$ was uniformly distributed in the soil. The water holding capacity of representative sapling soils was determined as the maximum volume of water the soil can hold. This process was conducted before labelling the soil with ^{15}N to avoid leaching losses of $^{15}(NH_4)_2SO_4$ from the soil when the soil is labelled with $^{15}(NH_4)_2SO_4$. The water holding capacity of this soil was 50.9%.The pots were left for 24 hr before conducting the ^{13}C labelling. After 24 hr, the pots were transferred into a growth chamber and were placed inside a plastic container (4 pots per container). A light meter was also placed inside the box, which was illuminated (190 – 250 µmol m^{-2} s^{-1}). Compressed air, in which the ambient CO_2 was replaced with 370 ppm 99 atom % $^{13}CO_2$, was blown under cylinder pressure at a flow rate of 1.0 L per minute through the labelling container to maintain atmospheric CO_2 concentrations inside it. The saplings were continuously exposed to the $^{13}CO_2$ for 6 hr. Once the labelling was terminated, a pair of shoots was collected from each sapling for initial isotopic analysis to ensure that the saplings had taken up the $^{13}CO_2$. The pots were taken out of the plastic chamber and placed in the growth chambers for another 48 hr before harvesting. This time period was considered to be long enough to allow the carbon to be transported into the ECM root tips and nitrogen to be taken up by the saplings shoots.

2.3 Harvesting the saplings

The saplings were harvested 48 hr after the ^{13}C labelling had finished. The saplings were first soaked in tap water for 30 min prior to harvesting to expand and loosen the soil. The soil was then delicately removed from the sapling roots to avoid snapping the roots and losing ECM root tips. Clean saplings were then placed onto tissue paper, wrapped in plastic bags and were frozen prior to analysis. The saplings were frozen to stop any biological activity.

2.4 Analysing the saplings

The saplings were thawed overnight at 4°C prior to analysis. A dissecting microscope was used to select and group the ECM tips according to physical morphology, which were then photographed. The saplings shoots and the grouped ECM root tips were weighted and oven dried at 70°C for 48 hr. After 48 hr, the shoots and ECM root tips were finely ground for isotopic analysis. A maximum of 1 mg finely ground shoots and ECM root tips (minimum 10 tips) were weighted and placed into individual tin capsules (5 mm diameter x 8 mm height). The capsules were properly sealed and folded into a round ball. Flour with a known carbon and nitrogen percentage was used as a control. The capsules were then loaded into an isotope mass spectrometer analysis SerCon 'Callisto CF-IRMS' system for ^{13}C and ^{15}N determination.

2.5 Ammonium extraction

The amount of ammonium-N (NH_4-N) present in the soil was determined before labelling the soil with ^{15}N. The soil was thoroughly mixed before sieving through a 3 mm sieve. Ten grams of fresh sieved soil were transferred into a 250 ml

conical flask and 100 ml of 1M potassium chloride was added into each flask. Two blank flasks just containing the extractant were used as controls. The samples were shaken for one hour on a rotary shaker. After 1 hr, the samples were filtered using Whatman No 1 (lowest blanks) filter paper. The samples were then analysed for ammonium content colorimetrically using a flow injection auto-analyser(Tecator FIAstar 5000 Analyzer, Croydon, UK) as per method by Allen *et al.*[20]. From the analysis conducted, the ammonium-N content of the soil was 4 x 10^{-4}mg/g.

2.6 Statistical Analysis

All ECM tips collected from the saplings were grouped as described in section 2.4. Different saplings may vary in efficiency to allocate the ^{13}C to their ECM root tips, possibly due to different numbers of shoots between saplings and the saplings may also be of different age. To reduce these effects, for each sapling the data were expressed as the proportion of ^{13}C in saplings shoots to ^{13}C allocation in ECM root tips. This method therefore considers the enrichment of root tips by ^{13}C relative to the amount fixed by the trees. All statistical analysis was conducted using R[21]. All data were explored for the normality of residuals and homogeneity of variance and where necessary, data were log_{10}-transformed to fulfil the data normality. A one way ANOVA test was conducted to test the mean differences of ^{15}N and ^{13}C accumulation between ECM root tips groups. A correlation test was conducted to determine a relationship between the nitrogen acquisition and allocations of recent plant assimilate in below ground ECM root tips.

3. Results and Discussion

A collection of ectomycorrhizal root tips harvested from Pinus sylvestris saplings

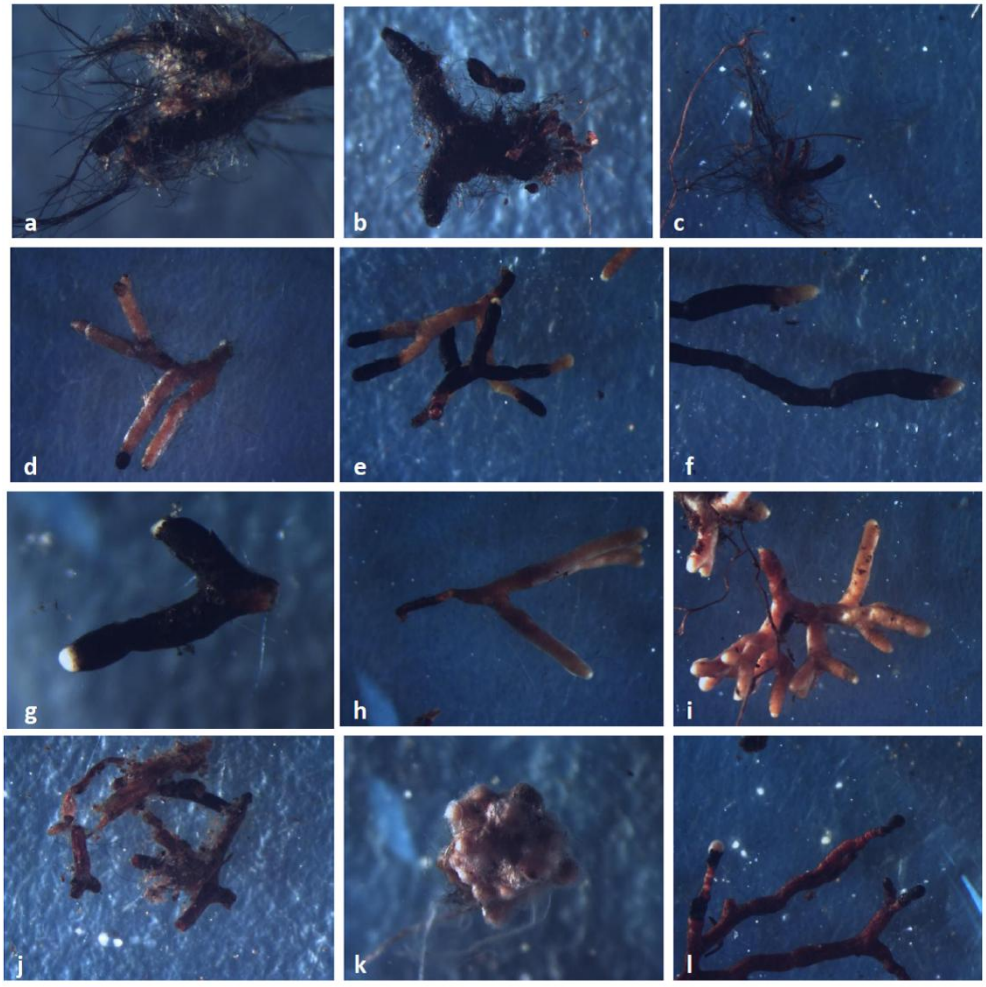

Figure 1: Ectomycorrhizal root tips harvested from four *Pinussylvestris*saplings collected from Culbin forest and were grouped into seven groups based on physical morphologies; a,b&c) group 1 , d&e) group 2 , f&g) group 3 , h&i) group 4, j) group 5 , k) group 6 and l) group 7.

Table 1: Physical morphologies of ectomycorrhizal root tips harvested from four *Pinussylvestris* saplings collected from Culbin forest.

Group	Description of physical morphology
1	Charcoal black tips. The tips texture was woolly: with copious thick emanating black hyphae and hyphal strands. This group is suspected to be *Cenococcum* sp.
2	The tips were branching like Y shape; dichotomous: the root tips meristem divides into two branches that grow to similar lengths and may divide and grow repeatedly. There were black hyphae emanating out from the branches. Some of the branches have a black end tips like a match. The branches were light brown to brown with black end tips and some were black with white end tips. The tips were straight: linear, smooth sided.

3	The tips were straight: linear, smooth sided. The tips were black with end tips were white.
4	As group 2 but the tips were light brown to brown, not shining, with white fungus sheath covered end tips. The tips were straight: linear, smooth sided The end tips were white.
5	The tips texture was woolly: with copious thick emanating hyphae and hyphal strands. The tips were brown.
6	The tips were clustered and white creamy. The tips were tuberculate and were covered with white fungal sheath. This group is suspected to be *Suillus* sp.
7	As group 2 but no hyphae emanating out from the branches. The tips were brown and end tips were black like matches head.

Figure-1 shows groups of ECM root tips harvested from four Pinussylvestris saplings collected from Culbin forest.

Meanwhile table-1 shows the physical morphologies of the ECM root tips.

Accumulation of ^{15}N and ^{13}C in ectomycorrhizal root tips

The concentration of ^{15}N in ECM root tips was not significantly different between the groups ($F_{6,10}$ = 0.86, P= 0.55). The concentration of ^{13}C in ECM root tips was significantly different between the groups ($F_{6.10}$=4.45,P=0.02). There was a significant relationship between ^{15}N and ^{13}C accumulation in ECM root tips (rs[1352]= -0.657,P=0.005). This shows that the ECM root tips that accumulated ^{15}N had the least relative enrichment of ^{13}C (Figure-2).There was no relationship between ^{13}C and ^{15}N within morphotype.

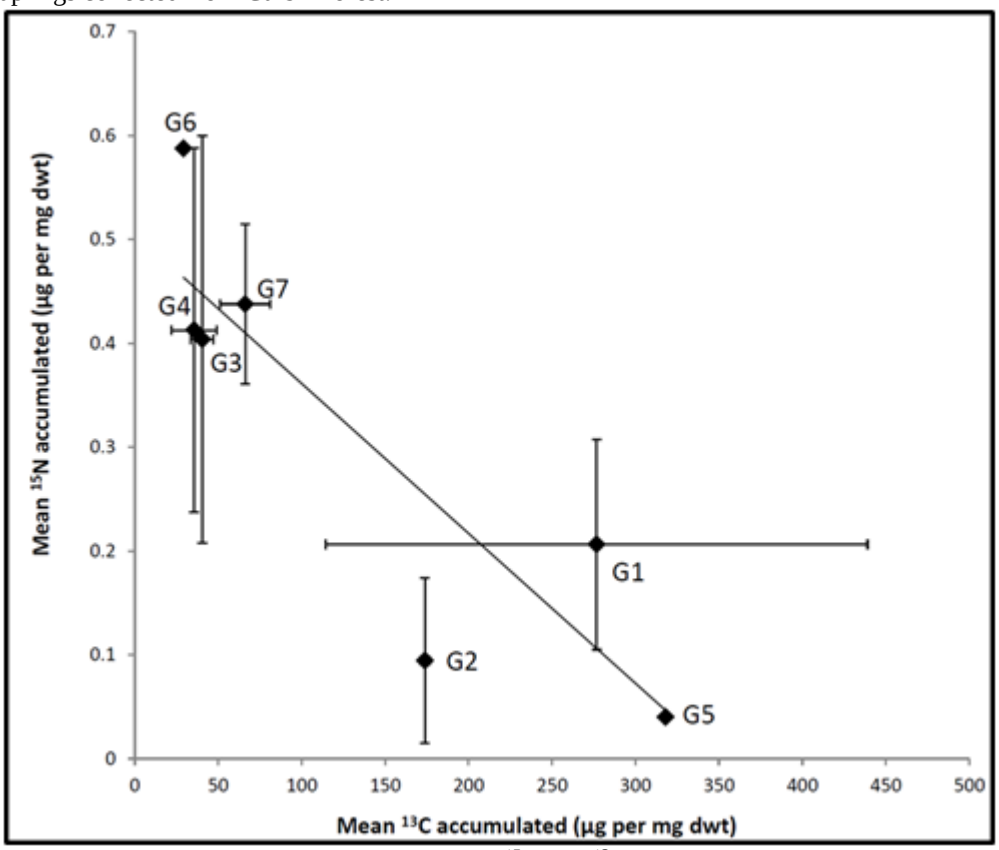

Figure 2: The relationship between the mean concentration of ^{15}N and ^{13}C in seven groups of ectomycorrhizal tips harvested from four *Pinussylvestris*saplings root systems. Abbreviations G1 = group 1, G2 = group 2, G3 = group 3, G4 = group 4, G5 = group 5, G6 = group 6 and G7 = group 7. The minimum number of root tips per group is approximately 10 tips. The ^{13}C data was $\log_{10}$-transformed before ANOVA analysis, but was back-transformed for presentation of this scatter-plot. Bars are standard error of the mean.

The ECM root tips that showed high accumulations of ^{15}N were hypothesised would also show high accumulation of ^{13}C. Högberg *et al.*[14] suggested that the ECM roots that have the largest sinks of carbon were the same roots that provided the host plant with the most nitrogen from the soil. In this study, a negative relationship was found between ^{15}N and ^{13}C accumulation within the ECM root tips groups, opposite of what have expected. The saplings were collected randomly from the Culbin forest site.

This negative relationship was probably due to several factors. The different amount of ^{15}N and ^{13}C accumulation observed between the ECM root tips groups (Figure-2) is probably due to each ECM root tips group having a different foraging strategy, and efficiency and speed of uptake of nutrients. Figure-2 shows group 1 which was suspected to be a *Cenococcum* sp. showed high accumulation of ^{13}C but low accumulation of ^{15}N. Meanwhile group 6, which was suspected to be a *Suillus* sp. showed high accumulation of ^{15}N but low accumulation of ^{13}C. *Cenococcum* sp. is known to have a wide range of host plants [22] meanwhile *Suillus* sp. is known to develop ectomycorrhizas only on the genus *Pinus* [23]. These also might influence the accumulation of ^{15}N and ^{13}C observed in both groups in Figure-2.

Pena *et al.* [16]investigated the ability of ECM fungi associated with beech roots in an old-growth forest to access nitrogen from ^{15}N-labelled beech litter bags. They found different

ECM fungi with different soil exploration types had different nitrogen accumulation over time. After 6 months of exposing to the labelled litter bags, they found ^{15}N accumulation was highly increased in all ECM root tips, except the root tips colonised by *Betula pruinatus* (long-distance soil exploration). After 14 months, the root tips colonised by *Cortinarius* sp. (medium-distance soil exploration) showed the highest ^{15}N enrichment meanwhile *Humaria* sp. and *Cenoccocum geophilum* (short-distance soil exploration)showed the lowest ^{15}N accumulation. After 18 months, they found root tips colonised by *Tomentella badia* (short- to medium-distance soil exploration) showed the highest ^{15}N accumulation compared to other ECM fungi species. This study suggested different ECM fungal species with different soil foraging strategies have different nitrogen acquisition over time. In the present study, the age of ECM tips in each group and between groups also may differ. This could influence the uptake of nitrogen and allocation of carbon. For example, Cairney & Alexander[24] observed the sinks of carbon recently fixed that were allocated to mycorrhizas of *Tylospora fibrillosa* colonising *Picea sitchensis* decreased with the age of the tips. Cairney & Alexander [25]observed young mycorrhiza tips transferred more phosphorus to the host plant compared to the old mycorrhizal root tips when directly fed with ^{32}P. This suggests a progressive reduction in nutrient transfer to the host plant as the mycorrhiza age. In the present study, with the labelling approach used, the soil was presumably labelled uniformly with ^{15}N solution. The shoots of saplings were labelled with ^{13}C at the same time, which gave equal access of ^{13}C to all saplings. Therefore, it is unlikely that accessibility to carbon and nitrogen would be a factor in determining differences in group values.

Culbin forest was chosen because the site is known to contain a rich community of ECM species [17-18] and tree growth is highly N limited [19]. The most abundant ectomycorrhiza from this site was *Cenococcum geophilum* Fr [17]. In this study, ECM root tips were harvested directly from the pine saplings collected from Culbin forest. Group 1 was likely to be an ECM root tips of *Cenococcum* sp based on its very distinctive morphology (Figures-1a, b &c: Table-1). *Cenococcum geophilum* is known to be a widely distributed ECM fungus and can be one of the most abundant in many habitats [26]. It has a wide range host with more than 200 recorded tree species as hosts from 40 genera of both angiosperms and gymnosperms[22]. It produces little external mycelium close to the surface of the mycorrhiza[17]. This ectomycorrhiza is uniformly distributed throughout the host root system[27-28,17]. Meanwhile group 6 was suspected to be ECM root tips of *Suillus* spp (Figure-1k: Table-1). These ECM root tips were very distinctive. They were tuberculate; the tips were enveloped by a dense mat of white hyphae and shaped like a small potato. It is not possible to name the other groups as the morphological characteristics were not enough to classify them into specific genera.

In future studies, it would be very useful to identify the groups of ECM tips using molecular tools. This would allow identification of the fungi and potentially their foraging strategy. Different genera have different efficiency in transferring nutrients to host plants. For example,a study conducted by Gorissen & Kuyper[12] showed that *Suillus* sp supplied more nitrogen to the host plant (*Pinus sylvestris*) than *Laccaria* l. By including a greater number of saplings would also provide a better picture in understanding the uptake of nitrogen and carbon allocation of different ECM groups.

4. Conclusion

The stable isotopes of nitrogen (^{15}N) and carbon (^{13}C) are widely used and very powerful tools to understand fungal ecology. The analysis of stable isotopes in this studyhas shown that different ECM groups have different accumulation rate of ^{15}N and ^{13}C. The analysis has also demonstrated a very strong negative correlation between ^{15}N and ^{13}C in the groups. This suggests the dynamics of carbon allocation and nitrogen acquisition may be offset, that some ECM fungi are using carbon from hosts to acquire other growth limiting nutrients (e.g. Phosphorus) which has not labelled, or that accumulation of carbon and nitrogen in ECM tips does not accurately reflect nutrient transfer processes.

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