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# Quantifying inorganic nitrogen uptake capacity among ectomycorrhizal fungal species using MIFE™ microelectrode ion flux measurements: theory and applications

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## Abstract

A growing appreciation of the intimate association between trees and a wide diversity of mycorrhizal fungi in forest ecosystems is leading to the view that trees and their associated mycorrhizae should be considered metaorganisms or holobionts. For ectomycorrhizal associations, nitrogen (N) mobilization and uptake is a major contribution by the fungal partners. This paper reviews traditional methods of measuring N uptake by ectomycorrhizae, and describes the application of microelectrode ion flux measurement of nitrogen uptake using the MIFE™ technique to ectomycorrhizae associated with forest trees. From results obtained with microelectrode ion flux measurement thus far, we argue that plant N uptake capacity should be considered an exogenous trait related to the functional diversity among ectomycorrhizal species and communities, rather than a function of host plant root physiology, alone.

## Introduction

More than 80% of all land plant species associate with mycorrhizal fungi (Wang and Qiu 2006). These symbiotic fungi provide nutrients and water to the host plants in exchange for carbohydrates, and can improve biotic and abiotic

stress resistance. A growing appreciation of the intimate association between plants and a wide diversity of mycorrhizal fungi in forest ecosystems is shifting our perceptions, to the point where trees are no longer considered as isolated individuals, but as metaorganisms or holobionts (Dickie et al. 2015). A major challenge to our understanding of the functioning of the holobiont; however, is finding a measure of its physiological activity. Methods to measure physiological characteristics of plants and fungi, separately, are relatively well established, but measurements of the activity of the symbiotic organism are more challenging.

A key role of mycorrhizal fungi is the mobilization and acquisition of nutrients, particularly nitrogen (N) and phosphorus (P). Extraradicle mycelia occupy a larger soil volume and penetrate into finer soil pores than do plant roots, and the secretion of fungal enzymes mobilizes nutrients from organic and inorganic soil particles. While the extraradicle mycelia of both arbuscular mycorrhizae and ectomycorrhizae take up mobilized nutrients, the contrasting structural characteristics of arbuscular mycorrhizae and ectomycorrhizae have important implications for nutrient uptake pathways. Arbuscular mycorrhizae can take up nutrients through both root and fungal pathways, while ectomycorrhizae rely primarily on fungal uptake, therefore, the fungus plays a much larger role in nutrient uptake for ectomycorrhizae than for arbuscular mycorrhizae (Bücking et al. 2012). In pine roots, only 2% of the root surface is non-ectomycorrhizal, and the extraradicle mycelium represents 99% of the nutrient-absorbing surface length (Rousseau et al. 1992), therefore in ectomycorrhizae, the fungi dominate nutrient uptake of the holobiont. Compared to roots and arbuscular mycorrhizae, ectomycorrhizae are highly competitive for N and secrete a variety of hydrolytic enzymes that degrade organic polymers and allow access to organic nutrients via extraradicle mycelium (Bücking et al. 2012). In addition, the fungal mantle of ectomycorrhizae can also function in nutrient uptake and storage (Bücking et al. 2012). Overall, ectomycorrhizae are thought to make far larger contributions to plant N acquisition, in comparison to arbuscular mycorrhizae (van der Heijden et al. 2015)

Another factor that makes the study of nutrient dynamics of ectomycorrhizal (ECM) plants particularly interesting is the great diversity of

fungal partners that can participate in the symbiosis. While 65% of plant species form AM, the number of fungal species involved (*c.* 200 - 1600 species) is relatively small (Wang and Qiu 2006, Öpik et al. 2014). In contrast, only *c.* 3% of plant species form associations, but there are at least 7,750 documented ECM fungal species, and some estimates of ECM species richness range as high as 25,000 species (Rinaldi et al. 2008). These many ECM fungal species generally have a higher degree of plant host specificity than AM fungal species (Wang and Qiu 2006), but hosts vary in their receptivity. Some plants, like Douglas-fir (*Pseudotsuga menziesii* Mirb. Franco), are reputed to associate with more than 2000 ECM fungi (Trappe 1977), while others, like *Alnus,* associate with only 50 ECM fungi world-wide (Pritsch et al. 1997). It is intriguing to speculate on the potential range of functional diversity in the many different ECM partners, especially given that these community assemblages often align well with edaphic gradients associated with organic and inorganic (ammonium and nitrate) N availability (Kranabetter 2014).

Low soil N availability is one of the major factors limiting plant growth in native temperate and boreal ecosystems (Rennenberg et al. 2009), thus functional diversity in the ability of mycorrhizae to acquire N would have a significant influence on plant fitness. The forest trees that dominate cool temperate and boreal ecosystems are predominantly associated with ECM fungi, at levels of root colonization near 100%. This paper reviews traditional methods of measuring N uptake by ectomycorrhizae, and describes a more recent application of microelectrode ion flux measurement of N uptake to ectomycorrhizae associated with forest trees. To date, ion flux measurements of roots have been, for the most part, conducted with non-mycorrhizal roots, a situation far removed from the complexity of root physiology in natural environments. From results obtained with microelectrode ion flux measurement thus far, we argue that plant N uptake capacity should be considered an exogenous trait related to the functional diversity among ECM species and communities, rather than a function of host plant root physiology, alone.

## Measurement of N uptake by ectomycorrhizae

A variety of techniques have been utilized to assess total N uptake or rates of N uptake by ECM roots and fungi, with a history dating back over 100 years. The earliest studies by Frank in 1894 suggested that mycorrhizal fungi could obtain N more easily from forest humus than could higher plants (Bowen 1973). Experiments by Hatch in 1937 inferred superior uptake of N by mycorrhizal plants from large increases in plant dry weight, N content and N concentration in inoculated compared to uninoculated plants (Bowen 1973). This whole-plant technique has been used subsequently in hundreds of studies to infer superior N uptake or N utilization by ECM plants. A drawback of whole-plant studies, however, is that superior growth and nutrient status can be due to other physiological benefits of ectomycorrhizae, apart from improved N uptake. In addition, whole-plant studies assessing uptake of different N forms over periods of more than a few hours may be subject to inter-conversion of N forms by microbial activity, so we cannot confidently conclude that plants are taking up the form of N initially supplied. For logistical reasons, controlled nursery and lab studies also focus on a handful of easily culturable ECM species, resulting in limitations on our appreciation and understanding of the true range in functional traits of ECM species in natural forests.

Some early work exploring N uptake by ectomycorrhizae focused on the N uptake and N form preference of the fungal partner grown in pure culture, rather than focusing on the plant partner. Lundeberg (1970) showed that most of 27 mycorrhizal fungi grown in pure culture performed best with ammonium as the sole N source, but several grew equally well with nitrate. In more recent mycelial culture studies, ammonium has been shown to be the most readily utilized source of inorganic N (Rangel-Castro et al. 2002; Guidot et al. 2005). However, Nygren et al. (2008) demonstrated wide variation among 68 species of ECM fungi in their mycelial growth in culture with nitrate as the sole N source. Results of culture studies must be interpreted with caution, however, because the response of the ECM fungal partner, alone, may not reflect its N form preferences or uptake ability when growing in symbiosis with a host plant (Turnbull et al. 1995).

To directly measure N uptake of the ectomycorrhiza as a whole, experiments quantifying depletion of N in solution by ECM roots began in the 1960's. Carrodus (1966) found that excised beech mycorrhizae could readily absorb ammonium from ammonium chloride solution but had almost no ability to absorb nitrate. He also observed uptake of glutamic acid, aspartic acid, glutamine and aspargine by excised beech mycorrhizae (Carrodus 1966). Even earlier, Melin and Nilsson, showed ECM hyphae of intact mycorrhizal seedlings of *Pinus sylvestris* could absorb $^{15}$N-ammonium from labeled ammonium nitrate and transfer the label to different parts of the seedling (in Melin and Nilsson 1953). These authors also showed that $^{15}$N, taken up as $^{15}$N-glutamic acid by hyphae of *Boletus variegatus*, could be transferred to the roots and stems of pine seedlings (Melin and Nilsson 1953). Use of N isotopes to understand N uptake and allocation by ECM plants has broadened from enrichment studies to field-based natural abundance studies where N isotopes are used to differentiate the functional characteristics of ECM fungi (e.g. Hobbie and Högberg 2012). $^{15}$N natural abundance and enrichment studies have been used extensively to explore the sources of N used by ectomycorrhizae (reviewed in Courty et al. 2010), but isotope studies can be challenging to interpret when processing of N forms by microbes can change the N form applied in enrichment studies prior to uptake. In addition, natural abundance studies are subject to the complexities of fractionation by competing microbes and metabolic processes. Recent work using nanoscale secondary ion mass spectrometry (NanoSIMS) imaging with stable isotopes of N and carbon (C) shows potential for visualizing in situ N and C flows between soil microbes, fungi and plants (Mayerhofer et al. 2016), but this work is not yet applicable to the field.

A relatively new methodology for exploring nutrient uptake by ectomycorrhizae uses ion-selective microelectrodes. This technique can be used to measure the net flux of particular ions at the surface of tissues, including the mantle of ectomycorrhizae. Several authors have published net flux measurements of single ions at ECM surfaces (Plassard et al. 2002; Gobert and Plassard 2002, 2007; Boukcim and Plassard 2003; Li et al. 2012), but the microelectrode ion flux measurement technique (MIFE™) (Newman 2001) is the only ion-selective

microelectrode technique that can measure net fluxes of multiple ions in one position, simultaneously.

**Using the MIFE system to measure N uptake in ectomycorrhizae**

The MIFE system was developed by Dr. Ian Newman at the University of Tasmania (Newman 2001). The original concept of using ion electrochemical potentials measured at the surface of plant tissues to calculate ion net fluxes was presented by Bill Lucas (Lucas and Kochian 1986). The first application of this theory to the measurement of ion fluxes in roots was published the next year (Newman et al. 1987). Since that time, there have been over 200 papers published based on measurements made with the MIFE system on plant root, leaf and stem tissues, particularly in agricultural species such as barley, (*Hordeum vulgare* L.) wheat (*Triticum aestivum* and *T. turgidum*), broad bean (*Vicia faba* L.), canola (*Brassica napus* L.), pepper (*Capsicum chinense* Jacq.), potato (*Solanum tuberosum* L.), tomato, (*Solanum lycopersicum* L.), tobacco, (*Nicotiana tabacum* L.) and sugar beet (*Beta vulgaris* L.), but also with the model plant *Arabidopsis* and conifers. To our knowledge, we are the first to use the MIFE system to measure ion flux on ectomycorrhizae.

The theory of the MIFE system is explained in Newman (2001) and Shabala et al. (2012). Ions in solution move from areas of high electrochemical potential to low. Net ion movement can be calculated from the gradient in electrochemical potential, and the mobility and concentration of the ion in solution. The gradient can be measured by moving a microelectrode, containing a specific liquid ion exchange resin, between two positions, 10-60 µm apart, closer and farther from a tissue surface. The MIFE system can simultaneously measure net flux of up to four ions over an area of 300 $\mu m^2$, and is ideal for exploring ion net flux at the surfaces of root and ECM tissues. In our work, ECM roots are acclimated to an aerated experimental nutrient solution for 1 h before being placed in the MIFE chamber. The nutrient composition, pH, or temperature of the experimental solution can be modified, depending on the question being addressed. After acclimation, roots are placed in refreshed solution and positioned, using a microscope, so that the microelectrode tips are a few micrometers (2-20 µm)

from the region of the ECM root where measurements will be made (Figure 1). Measurement begins and the previously calibrated microelectrodes oscillate, perpendicular to the root surface, and measure ion concentrations at the two positions. Measurements are taken for a minimum of 3 min, and then net flux of each ion (nmol $m^{-2}$ $s^{-1}$) is calculated from the mean difference ion concentration, the mobility of the particular ion, the electrochemical potential gradient and the geometry of the tissue surface (Newman 2001). Using this system, we have successfully measured ion fluxes of ectomycorrhizae with smooth mantles and those with emanating hyphae by positioning the microelectrodes next to the surface of the mantle and adjusting for the radius of the root plus mantle tissues. Theoretically, the MIFE system could be used to measure ion flux on rhizomorphs or even individual hyphae, if these structures could be cultured in a way that they would not be disturbed when transferred to the MIFE system.

Figure 1. MIFE system with microelectrodes filled with ammonium-, nitrate- and proton-selective resins measuring net ion fluxes on a non-mycorrhizal root tip.

Pioneering work using ion-selective microelectrodes in a simple oscillating system to measure N flux in ECM fungi assessed nitrate net flux in *Pinus pinaster* roots associated with *Rhizopogon roseolus* or *Hebeloma cylindrosporum*. Results showed that mycorrhizal roots associated with *Rhizopogon roseolus* had a greater capacity to use nitrate than non-mycorrhizal short roots, at all nitrate concentrations (Gobert and Plassard 2002), but this enhancement of nitrate uptake was not observed to the same degree with *Hebeloma cylindrosporum* (Plassard et al. 2002). A new scanning ion-selective microelectrode technique has recently been used to measure ion fluxes in ectomycorrhizal *Populus* (Li et al. 2012), but a limitation of these studies is that fluxes of only a single ion were measured at one time. An advantage of the MIFE system is that net fluxes of up to four ions can be measured at one position, simultaneously. With this technique, we can gain a deeper understanding of ECM functional traits and N uptake response to the environment.

Recent work in our lab has applied the MIFE system to measurements of inorganic N uptake in ECM species native to the productive temperate forests of coastal British Columbia. This area is unique in comparison to many other ECM biomes because much of the landscape has naturally rich soils that supply trees with considerable inputs of mineralized N (Littke et al. 2011, Perakis and Sinkhorn 2011). Our approach has been to retrieve root systems from forest stands in the spring and fall, during peaks in fungal physiological activity, to measure net fluxes of ammonium, nitrate and protons at the mantle surface. We sample and measure N uptake capacity repeatedly over the course of a few weeks to limit the effect of root excision (all roots undergo measurements within 72 hours). An advantage of this approach over controlled-environment studies is our ability to test the native, site-adapted ECM species that populate these forest stands. To date we have tested over 40 species of ECM fungi (for methods, see Kranabetter et al. 2015). Although we have just begun to explore the potential of

the MIFE system for measuring inorganic N uptake by mycorrhizae, we have sufficient data to draw some key conclusions.

1) *Non-mycorrhizal roots cannot compete*.

Uptake of ammonium by ectomycorrhizae occurs at much higher rates than by non-mycorrhizal white root tips, demonstrating, again, for conifers, the consistent and large gains in fitness conferred by this symbiosis. Ectomycorrhizal fungi associated with Douglas-fir roots had average rates of ammonium uptake of 103 nmol $m^2$ $s^{-1}$ (ranging from 59.7 nmol $m^2$ $s^1$ for *Cortinarius hemitrichus* to 351 nmol $m^2$ $s^1$ for *Lactarius hepaticus*) (Kranabetter et al. 2015). In contrast, the average rate of ammonium uptake for non-mycorrhizal white root tips from some of the same root systems was only 5.8 nmol $m^2$ $s^1$ (unpublished data). Related conifer studies where solution pH was manipulated found non-mycorrhizal roots of Douglas-fir seedlings to only achieve maximum rates of N uptake of 58 nmol $m^2$ $s^1$ (Hawkins et al. 2008). These data support other studies in the lab and in the field showing that N uptake by ECM roots exceeds that of non-mycorrhizal roots (Wallander et al. 1997, Quoreshi and Timmer 2000) and emphasizes the competitive advantage of trees with virtually 100% root colonization by EM fungi, even in soils rich in N.

2) *Species matter*.

We find a wide range in uptake capacity for ammonium among ECM species, in large degree matching the N availability of test sites. Spring uptake of ammonium by ECM roots of Douglas-fir was greatest on a medium and rich site, compared to a poor site, and averaged over 190 nmol $m^{-2}$ $s^{-1}$ for *Tomentella* species, a ECM group common on high N soils (Kranabetter et al. 2015). The lowest uptake rates of ammonium by ECM Douglas-fir roots were by *Cortinarius* species (60 nmol $m^{-2}$ $s^{-1}$), a group common to low N soils (Kranabetter et al. 2015). Likewise, many *Cortinarius* species demonstrate significant extracellular enzyme production for the uptake of organic N, particularly under conditions of low N availability (Bödeker et al. 2014). Jones et al. (2009) also showed that differences in $^{15}N$ uptake among ECM fungi can be greater than between ECM and non-mycorrhizal plants. Our findings emphasize how uptake capacity of

inorganic N by host species is, to a large degree, an exogenous trait defined by partnerships with individual ECM species. Functional traits of ECM species related to organic N mobilization and ammonium uptake capacity are also likely to have a strong influence on the assembly of ECM fungal communities across landscapes.

To date we have focused on variation among ECM fungal species in rates of N uptake. Future work will explore variation among ECM tree species with the same fungal symbiont, to determine the degree to which host tree species control N uptake capacity. Nitrate uptake has been shown to be lower in ECM than non-mycorrhizal roots for one conifer/fungal species partnership (Boukcim and Plassard 2003), but higher in ECM roots for another species pair (Gobert and Plassard 2002), thus we will explore the top-down versus bottom-up drivers of N uptake in the plant / fungal symbiosis.

3) *Ectomycorrhizal species appear to have limited capacity for nitrate uptake*. While ammonium is generally recognized as the most readily utilized source of inorganic N by the majority of ECM fungi (Rygiewicz et al. 1984, Chalot and Plassard 2011, Clemmensen et al. 2008), we hypothesized that sites with inherently high nitrate production would be colonized by ECM species endowed with significant capacity for nitrate uptake. However, when net fluxes of ammonium and nitrate were measured simultaneously on Douglas-fir ECM species, all native fungi tested took up ammonium at higher rates than nitrate, regardless of site N availability (Kranabetter et al. 2015). In a continuation of this work on additional sites on the west coast of British Columbia, we tested nitrate uptake from forest stand on a very rich fluvial bench with high rates of in-situ production of inorganic N, particularly nitrate (Ubhi 2017). Despite the predominance of nitrate in these soils, the ECM roots collected from Douglas-fir still showed significantly greater rates of uptake of ammonium than nitrate in the MIFE measurements. Even in a second test, using a solution of pure nitrate, we found very few species had any notable uptake of nitrate with maximum uptake of only ~ 30 nmol $m^2s^{-1}$ (Ubhi 2017). A limited capacity for nitrate uptake by ECM fungi could, in part, explain the patterns in forest composition observed

over broad landscapes, where arbuscular mycorrhizal trees are thought to outcompete ECM trees on soils with high soil N, and particularly nitrate, availability (Phillips et al. 2013, Lin et al. 2017).

**Summary**

The MIFE system is a powerful tool for assessing simultaneous net fluxes of different ions over the mantle of fungal root tips, and allows elucidation of key traits that govern ECM species' function and distribution. At this time, there are no liquid ion exchange resins for organic N forms nor for phosphorus, however these may be developed in the future, which would increase the power of the system. Information on ion uptake gained with the MIFE system is a direct measure of the ability of ectomycorrhizae to take up nutrients, rather than relying upon indirect methods where nutrient uptake capacity may only be inferred. This, combined with an understanding of the extent of site colonization by the root/hyphal system, would clearly show the relative importance of ECM species niche and functional traits in soil processes and forest ecology (Bardgett et al. 2014).

From work conducted thus far with the MIFE system, we conclude that in temperate and boreal forests, where almost all trees are associated with ectomycorrhizae, N uptake capacity is an exogenous trait, driven by the mycorrhizal association. This supports the contention of Dickie et al. (2015) that a tree is not an isolated individual, but a symbiotic metaorganism whose autoecology cannot be simply reduced to the physiological traits of the host plant species. Given the great diversity of ECM species and communities that associate with relatively few tree species in cool temperate and boreal forests, we propose that the contrasting soil N-related functional traits of these diverse ECM species, along with other roles in nutrient and water uptake, facilitate tree survival and growth across a vast spectrum of distinct sites.

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# Quantifying inorganic nitrogen uptake capacity among ectomycorrhizal fungal species using MIFE™ microelectrode ion flux measurements: theory and applications

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## Abstract

A growing appreciation of the intimate association between trees and a wide diversity of mycorrhizal fungi in forest ecosystems is leading to the view that trees and their associated mycorrhizal symbionts should be considered metaorganisms or holobionts. For ectomycorrhizal associations, nitrogen (N) mobilization and uptake is a major contribution by the fungal partners. This paper reviews traditional methods of measuring N uptake by ectomycorrhizae, and describes the application of microelectrode ion flux measurement of nitrogen uptake using the MIFE™ technique to ectomycorrhizal fungi associated with forest trees. From results obtained with microelectrode ion flux measurement thus far, we argue that plant N uptake capacity should be considered an exogenous trait, related to the functional diversity among ectomycorrhizal species and communities, rather than a function of host plant root physiology, alone.

## Introduction

More than 80% of all land plant species associate with mycorrhizal fungi (Wang and Qiu 2006). These symbiotic fungi provide nutrients and water to the host plants in exchange for carbohydrates, and can improve biotic and abiotic

stress resistance. A growing appreciation of the intimate association between plants and a wide diversity of mycorrhizal fungi in forest ecosystems is shifting perceptions of plant biology, to the point where trees are no longer considered as isolated individuals, but as metaorganisms or holobionts (Vandenkoornhuyse et al. 2015, Bordenstein and Theis 2015). A major challenge in understanding the functioning of the holobiont, however, is finding a measure of its physiological activity. Methods to measure physiological characteristics of plants and fungi, separately, are relatively well established, but measurements of the activity of the symbiotic organism are more challenging.

A key role of mycorrhizal fungi is the mobilization and acquisition of nutrients, particularly nitrogen (N) and phosphorus (P). Extraradicular mycelia occupy a larger soil volume and penetrate into finer soil pores than do plant roots, and the secretion of fungal enzymes mobilizes nutrients from organic and inorganic soil particles. While the extraradicular mycelia of both arbuscular mycorrhizae and ectomycorrhizae take up mobilized nutrients, the contrasting structural characteristics of arbuscular mycorrhizae and ectomycorrhizae have important implications for nutrient uptake pathways. Arbuscular mycorrhizae can take up nutrients through both root and fungal pathways, while ectomycorrhizae rely primarily on fungal uptake, therefore, the fungus plays a much larger role in nutrient uptake for ectomycorrhizae than for arbuscular mycorrhizae (Bücking et al. 2012). In pine roots, for example, only 2% of the root surface is non-ectomycorrhizal, and the extraradicular mycelium represents 99% of the nutrient-absorbing surface length (Rousseau et al. 1992). Compared to roots and arbuscular mycorrhizae, ectomycorrhizae are highly competitive for N and secrete a variety of hydrolytic enzymes that degrade organic polymers and allow access to organic nutrients via extraradicular mycelium (Bücking et al. 2012). In addition, the fungal mantle of ectomycorrhizae can also function in nutrient uptake and storage (Bücking et al. 2012). Overall, ectomycorrhizae are thought to make far larger contributions to plant N acquisition in comparison to arbuscular mycorrhizae (van der Heijden et al. 2015)

Another factor that makes the study of nutrient dynamics of ectomycorrhizal (ECM) plants particularly interesting is the great diversity of fungal partners that can participate in the symbiosis. While 65% of plant species

form AM, the number of fungal species involved (*c.* 200 - 1600 species) is relatively small (Wang and Qiu 2006, Öpik et al. 2014). In contrast, only *c.* 3% of plant species form ectomycorrhizal associations, but there are at least 7,750 documented ECM fungal species, and some estimates of ECM species richness range as high as 25,000 species (Rinaldi et al. 2008). These many ECM fungal species generally have a higher degree of plant host specificity than AM fungal species (Wang and Qiu 2006), but hosts vary in their receptivity. Conifer forests are reputed to associate with more than 1000 ECM taxa regionally (Allen et al. 1995), while certain hosts, like *Alnus,* may associate with only a few dozen ECM species (Tedersoo et al. 2009). It is intriguing to speculate on the potential range of functional diversity in the many different ECM species, especially given that ECM communities of forest stands align well with edaphic gradients associated with increasing inorganic (ammonium and nitrate) N availability (Kranabetter 2014).

Low soil N availability is one of the major factors limiting plant growth in native temperate and boreal ecosystems (Rennenberg et al. 2009), thus functional diversity in the ability of mycorrhizae to acquire N would have a significant influence on plant fitness. The forest trees that dominate cool temperate and boreal ecosystems are predominantly associated with ECM fungi, at levels of root colonization near 100%. This paper reviews traditional methods of measuring N uptake by ectomycorrhizae, and describes a more recent application of microelectrode ion flux measurement of N uptake to ectomycorrhizae associated with forest trees. To date, ion flux measurements of roots have been, for the most part, conducted with non-mycorrhizal roots, a situation far removed from the complexity of root physiology in natural environments. From results obtained with microelectrode ion flux measurement thus far, we argue that plant N uptake capacity should be considered an expression of the interacting ECM species (i.e., an exogenous trait; Carmona et al. 2015), rather than a function of host plant root physiology, alone.

**Measurement of N uptake by ectomycorrhizae**

A variety of techniques have been utilized to assess total N uptake or rates of N uptake by ECM roots and fungi, with a history dating back over 100 years. The

earliest studies by Frank in 1894 suggested that mycorrhizal fungi could obtain N more easily from forest humus than could higher plants (Bowen 1973). Experiments by Hatch in 1937 inferred superior uptake of N by mycorrhizal plants from large increases in plant dry weight, N content and N concentration in inoculated compared to uninoculated plants (Bowen 1973). This measure of response, using the entire plant, has been used subsequently in hundreds of studies to infer superior N uptake or N utilization by ECM plants. A drawback of whole-plant studies, however, is that superior growth and nutrient status can be due to other physiological benefits of ectomycorrhizae, apart from improved N uptake. In addition, whole-plant studies assessing uptake of different N forms over periods of more than a few hours may be subject to inter-conversion of N forms by microbial activity, so it cannot be confidently concluded that plants are taking up the form of N initially supplied. For logistical reasons, controlled nursery and lab studies also focus on a handful of easily culturable ECM species, resulting in limitations on our appreciation and understanding of the true range in functional traits of ECM species in natural forests.

Some early work exploring N uptake by ectomycorrhizae focused on the N uptake and N form preference of the fungal partner grown in pure culture, rather than focusing on the plant partner. Lundeberg (1970) showed that most of 27 mycorrhizal fungi grown in pure culture performed best with ammonium as the sole N source, but several grew equally well with nitrate. In more recent mycelial culture studies, ammonium has been shown to be the most readily utilized source of inorganic N (Rangel-Castro et al. 2002; Guidot et al. 2005). However, Nygren et al. (2008) demonstrated wide variation among 68 species of ECM fungi in their mycelial growth in culture with nitrate as the sole N source. Results of culture studies must be interpreted with caution, however, because the response of the ECM fungal partner, alone, may not reflect its N form preferences or uptake ability when growing in symbiosis with a host plant (Turnbull et al. 1995).

To directly measure N uptake of the ectomycorrhiza as a whole, experiments quantifying depletion of N in solution by ECM roots began in the 1960's.

Carrodus (1966) found that excised beech mycorrhizae could readily absorb ammonium from ammonium chloride solution but had almost no ability to absorb nitrate. He also observed uptake of glutamic acid, aspartic acid, glutamine and aspargine by excised beech mycorrhizae (Carrodus 1966). Even earlier, Melin and Nilsson had shown ECM hyphae of intact mycorrhizal seedlings of *Pinus sylvestris* could absorb $^{15}$N-ammonium from labeled ammonium nitrate and transfer the label to different parts of the seedling (Melin and Nilsson 1953). These authors also showed that $^{15}$N, taken up as $^{15}$N-glutamic acid by hyphae of *Boletus variegatus*, could be transferred to the roots and stems of Scots pine seedlings (Melin and Nilsson 1953). Use of N isotopes to understand N uptake and allocation by ECM plants has broadened from enrichment studies to field-based natural abundance studies where N isotopes are used to differentiate the functional characteristics of ECM fungi (e.g. Hobbie and Högberg 2012). $^{15}$N natural abundance and enrichment studies have been used extensively to explore the sources of N used by ectomycorrhizae (reviewed in Courty et al. 2010), but isotope studies can be challenging to interpret when processing of N forms by microbes can change the N form applied in enrichment studies prior to uptake. In addition, natural abundance studies are subject to the complexities of fractionation by competing microbes and metabolic processes. Recent work using nanoscale secondary ion mass spectrometry (NanoSIMS) imaging with stable isotopes of N and carbon (C) shows potential for visualizing in situ N and C flows between soil microbes, fungi and plants (Mayerhofer et al. 2016), but this work is not yet applicable to the field.

A relatively new methodology for exploring nutrient uptake by ectomycorrhizae uses ion-selective microelectrodes. This technique can be used to measure the net flux of particular ions at the surface of tissues, including the mantle of ectomycorrhizae. Several authors have published net flux measurements of single ions at ECM surfaces (Plassard et al. 2002; Gobert and Plassard 2002, 2007; Boukcim and Plassard 2003; Li et al. 2012), but the microelectrode ion flux measurement technique (MIFE™) (Newman 2001) is the only ion-selective microelectrode technique that can measure net fluxes of multiple ions in one position, simultaneously.

**Using the MIFE system to measure N uptake in ectomycorrhizae**

The MIFE system was developed by Dr. Ian Newman at the University of Tasmania (Newman 2001). The original concept of using ion electrochemical potentials measured at the surface of plant tissues to calculate ion net fluxes was presented by Bill Lucas (Lucas and Kochian 1986). The first application of this theory to the measurement of ion fluxes in roots was published the following year (Newman et al. 1987). Since then, there have been over 200 papers published based on measurements made with the MIFE system on plant root, leaf and stem tissues, particularly in agricultural species such as barley, (*Hordeum vulgare* L.), wheat (*Triticum aestivum* and *T. turgidum*), and canola (*Brassica napus* L.).

The theory of the MIFE system is explained in Newman (2001) and Shabala et al. (2012). Ions in solution move from areas of high electrochemical potential to low. Net ion movement can be calculated from the gradient in electrochemical potential, and the mobility and concentration of the ion in solution. The gradient can be measured by moving a microelectrode, containing a specific liquid ion exchange resin, between two positions, 10-60 µm apart, closer and farther from a tissue surface. The MIFE system can simultaneously measure net flux of up to four ions over an area of 300 $\mu m^2$, and is ideal for exploring ion net flux at the surfaces of root and ECM tissues.

In our work, ECM roots are acclimated to an aerated experimental nutrient solution for 1 h before being placed in the MIFE chamber. The nutrient composition, pH, or temperature of the experimental solution can be modified, depending on the question being addressed. After acclimation, roots are placed in refreshed solution and positioned, using a microscope, so that the microelectrode tips are a few micrometers (2-20 µm) from the region of the ECM root where measurements will be made (Figure 1). Measurement begins and the previously calibrated microelectrodes oscillate, perpendicular to the root surface, and measure ion concentrations at the two positions. Measurements are taken for a minimum of 3 min, and then net flux of each ion (nmol $m^{-2}$ $s^{-1}$) is calculated from the mean difference ion concentration, the mobility of the

particular ion, the electrochemical potential gradient and the geometry of the tissue surface (Newman 2001).

Using this system, we have successfully measured ion fluxes of ectomycorrhizae with smooth mantles and those with emanating hyphae by positioning the microelectrodes next to the surface of the mantle and adjusting for the radius of the root plus mantle tissues. Theoretically, the MIFE system could be used to measure ion flux on rhizomorphs or even individual hyphae, if these structures could be cultured in a way that they would not be disturbed when transferred to the MIFE system.

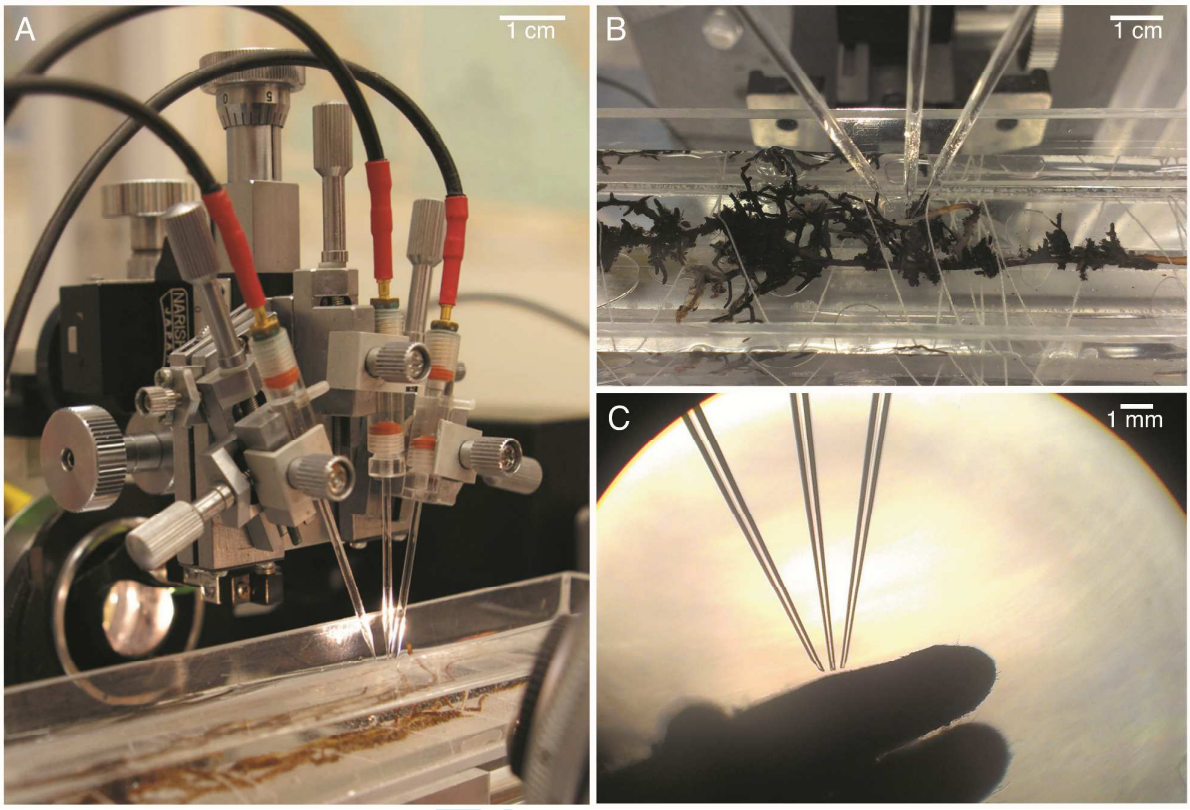


Figure 1. A) MIFE system multiple electrode mount holding three microelectrodes (filled with ammonium-, nitrate- and proton-selective resins) placed above a MIFE chamber holding roots. B) Section of coastal Douglas-fir root with secondary ECM roots immersed in a MIFE chamber, and the microelectrodes positioned above; and C) magnified view of a single ECM feeder root with microelectrodes positioned next to the fungal mantle for measurement of ion flux.

For ectomycorrhizae, pioneering work using ion-selective microelectrodes in a simple oscillating system assessed nitrate net flux in *Pinus pinaster* roots associated with *Rhizopogon roseolus* or *Hebeloma cylindrosporum*. Results showed that mycorrhizal roots associated with *Rhizopogon roseolus* had a greater capacity to use nitrate than non-mycorrhizal short roots, at all nitrate concentrations (Gobert and Plassard 2002), but this enhancement of nitrate uptake was not observed to the same degree with *Hebeloma cylindrosporum* (Plassard et al. 2002). A new scanning ion-selective microelectrode technique has recently been used to measure ion fluxes in ectomycorrhizal *Populus* (Li et al.

2012), but a limitation of these studies is that fluxes of only a single ion were measured at one time. An advantage of the MIFE system is that net fluxes of up to four ions can be measured at one position, simultaneously. With this technique, we can gain a deeper understanding of ECM functional traits and N uptake response to the environment.

Recent work in our lab has applied the MIFE system to measurements of inorganic N uptake in ECM species native to the productive temperate forests of coastal British Columbia. This area is unique in comparison to many other ECM biomes because much of the landscape has naturally rich soils that supply trees with considerable inputs of mineralized N (Littke et al. 2011, Perakis and Sinkhorn 2011). Our approach has been to retrieve root systems from forest stands in the spring and fall, during peaks in fungal physiological activity, to measure net fluxes of ammonium, nitrate and protons at the mantle surface. Root systems were immersed in high N solution (500 μM $NH_4NO_3$) to ensure $NH_4^+$ or $NO_3^-$ supply would not limit potential rates of ion uptake. We sample and measure N uptake capacity repeatedly over the course of a few weeks to minimize the effect of root excision (all roots undergo measurements within 72 hours). An advantage of this approach over controlled-environment studies is our ability to test the native, site-adapted ECM species that populate these forest stands. To date we have tested over 40 species of ECM fungi (for methods, see Kranabetter et al. 2015). Although we have just begun to explore the potential of the MIFE system for measuring inorganic N uptake by mycorrhizae, we have sufficient data to draw some key conclusions.

1) *Non-mycorrhizal roots cannot compete*.

Uptake of ammonium by ectomycorrhizae occurs at much higher rates than by non-mycorrhizal white root tips, demonstrating for conifers the consistent and large gains in fitness conferred by this symbiosis. Ectomycorrhizal fungi associated with coastal Douglas-fir (*Pseudotsuga menziesii* var. *menziesii*) roots had average rates of ammonium uptake of 103 nmol $m^2$ $s^{-1}$ (ranging from 59.7 nmol $m^2$ $s^1$ for a *Cortinarius* sp. to 351 nmol $m^2$ $s^1$ for a *Lactarius* sp.) (Kranabetter et al. 2015). In contrast, the average rate of ammonium uptake for

non-mycorrhizal white root tips from some of the same root systems was only 5.8 nmol $m^2$ $s^1$ (unpublished data). Related conifer studies where solution pH was manipulated found non-mycorrhizal roots of Douglas-fir seedlings to only achieve maximum rates of N uptake of 58 nmol $m^2$ $s^1$ (Hawkins et al. 2008). These data support other studies in the laboratory and in the field showing that N uptake by ECM roots exceeds that of non-mycorrhizal roots (Wallander et al. 1997, Quoreshi and Timmer 2000) and emphasizes the competitive advantage of trees with virtually 100% root colonization by EM fungi, even in soils rich in N.

2) *Species matter*.

We find a wide range in uptake capacity for ammonium among ECM species, in large degree matching the N availability of test sites. Spring uptake of ammonium by ECM roots of coastal Douglas-fir was greatest on a medium and rich site, compared to a poor site, and averaged over 190 nmol $m^{-2}$ $s^{-1}$ for *Tomentella* species, a ECM group common on high N soils (Kranabetter et al. 2015). The lowest uptake rates of ammonium by ECM Douglas-fir roots were by *Cortinarius* species (60 nmol $m^{-2}$ $s^{-1}$), a group common to low N soils (Kranabetter et al. 2015). Likewise, many *Cortinarius* species demonstrate significant extracellular enzyme production for the uptake of organic N, particularly under conditions of low N availability (Bödeker et al. 2014). Jones et al. (2009) also showed that differences in $^{15}N$ uptake among ECM fungi can be greater than between ECM and non-mycorrhizal plants. Our findings emphasize how uptake capacity of inorganic N by host species is, to a large degree, an exogenous trait (Carmona et al. 2015) defined by partnerships with individual ECM species. Functional diversity of the symbiont, as in this example, is essential in fully understanding the adaptive capacity of the holobiont (Vandenkoornhuyse et al. 2015, Bordenstein and Theis 2015).

3) *Ectomycorrhizal species appear to have limited capacity for nitrate uptake*.

While ammonium is generally recognized as the most readily utilized source of inorganic N by the majority of ECM fungi (Rygiewicz et al. 1984, Chalot and Plassard 2011, Clemmensen et al. 2008), we hypothesized that sites with inherently high nitrate production would be colonized by ECM species endowed

with significant capacity for nitrate uptake. However, when net fluxes of ammonium and nitrate were measured simultaneously on Douglas-fir ECM species, all native fungi tested took up ammonium at higher rates than nitrate, regardless of soil inorganic N availability (Kranabetter et al. 2015).

In a continuation of this work on additional sites on the west coast of British Columbia, we tested nitrate uptake from forest stand on a very rich fluvial bench with high rates of in-situ production of inorganic N, particularly nitrate (Ubhi 2017). Despite the predominance of nitrate in these soils, the ECM roots collected from coastal Douglas-fir still showed significantly greater rates of uptake of ammonium than nitrate in the MIFE measurements. Even in a second test, using an immersion solution of pure nitrate (500 μM $NO_3$), we found very few species had any notable uptake of nitrate, with a maximum of only ~ 30 nmol $m^2s^{-1}$ (Ubhi 2017). A limited capacity for nitrate uptake by ECM fungi could, in part, explain the broad patterns in forest composition observed over some landscapes, where arbuscular mycorrhizal trees are thought to outcompete ECM trees on soils with high soil N, and particularly nitrate, availability (Phillips et al. 2013, Lin et al. 2017).

To date we have focused on variation among ECM fungal species in rates of N uptake. Future work will explore variation among ECM tree species with the same fungal symbiont, to determine the degree to which host tree species control N uptake capacity. Nitrate uptake has been shown to be lower in ECM than non-mycorrhizal roots for one conifer/fungal species partnership (Boukcim and Plassard 2003), but higher in ECM roots for another species pair (Gobert and Plassard 2002), thus we will explore the top-down versus bottom-up drivers of N uptake in the plant/fungal symbiosis.

**Summary**

The MIFE system is a powerful tool for assessing simultaneous net fluxes of different ions over the mantle of fungal root tips, and allows elucidation of key traits that govern ECM species' function and distribution. At this time, there are no liquid ion exchange resins for organic N forms nor for phosphorus, however these may be developed in the future, which would increase the power of the

system. Information on ion uptake gained with the MIFE system is a direct measure of the ability of ectomycorrhizae to take up nutrients, rather than relying upon indirect methods where nutrient uptake capacity may only be inferred. This, combined with an understanding of the extent of site colonization by the root/hyphal system, would clearly show the relative importance of ECM species niche and functional traits in soil processes and forest ecology (Bardgett et al. 2014).

From work conducted thus far with the MIFE system, we conclude that in temperate and boreal forests, where almost all trees are associated with ectomycorrhizae, N uptake capacity is an exogenous trait, driven by the ectomycorrhizal symbiont. This supports the growing awareness among plant biologists that a tree is not an isolated individual, but a symbiotic metaorganism whose autoecology cannot be simply reduced to the physiological traits of the host plant species. Complex boreal and temperate landscapes, with a wide spectrum of site types, are often dominated by a few conifer species. We suggest these relatively simple patterns in forest composition reflect the functional plasticity of the holobiont through a symbiotic relationship with a wide array of ECM species and N-related functional traits.



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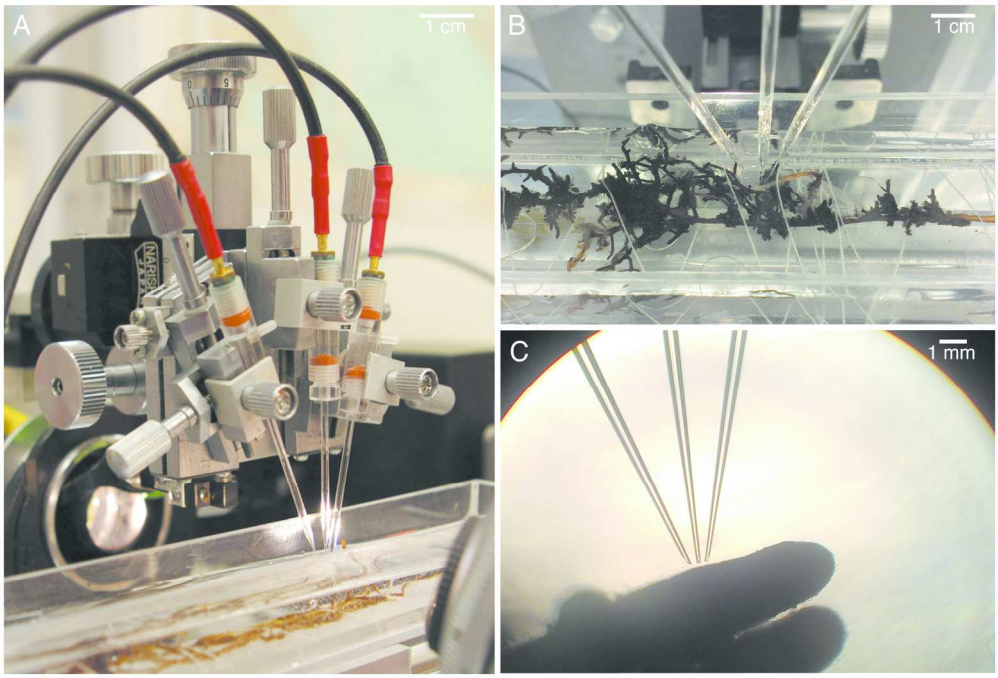


Figure 1. A) MIFE system multiple electrode mount holding three microelectrodes (filled with ammonium-, nitrate- and proton-selective resins) placed above a MIFE chamber holding roots. B) Section of coastal Douglas-fir root with secondary ECM roots immersed in a MIFE chamber, and the microelectrodes positioned above; and C) magnified view of a single ECM feeder root with microelectrodes positioned next to the fungal mantle for measurement of ion flux.

122x83mm (600 x 600 DPI)