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

4  
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19   **Abstract**

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

33  
34   **Introduction**

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

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

46 A key role of mycorrhizal fungi is the mobilization and acquisition of  
47 nutrients, particularly nitrogen (N) and phosphorus (P). Extraradicle mycelia  
48 occupy a larger soil volume and penetrate into finer soil pores than do plant  
49 roots, and the secretion of fungal enzymes mobilizes nutrients from organic and  
50 inorganic soil particles. While the extraradicle mycelia of both arbuscular  
51 mycorrhizae and ectomycorrhizae take up mobilized nutrients, the contrasting  
52 structural characteristics of arbuscular mycorrhizae and ectomycorrhizae have  
53 important implications for nutrient uptake pathways. Arbuscular mycorrhizae  
54 can take up nutrients through both root and fungal pathways, while  
55 ectomycorrhizae rely primarily on fungal uptake, therefore, the fungus plays a  
56 much larger role in nutrient uptake for ectomycorrhizae than for arbuscular  
57 mycorrhizae (Bücking et al. 2012). In pine roots, only 2% of the root surface is  
58 non-ectomycorrhizal, and the extraradicle mycelium represents 99% of the  
59 nutrient-absorbing surface length (Rousseau et al. 1992), therefore in  
60 ectomycorrhizae, the fungi dominate nutrient uptake of the holobiont.  
61 Compared to roots and arbuscular mycorrhizae, ectomycorrhizae are highly  
62 competitive for N and secrete a variety of hydrolytic enzymes that degrade  
63 organic polymers and allow access to organic nutrients via extraradicle  
64 mycelium (Bücking et al. 2012). In addition, the fungal mantle of  
65 ectomycorrhizae can also function in nutrient uptake and storage (Bücking et al.  
66 2012). Overall, ectomycorrhizae are thought to make far larger contributions to  
67 plant N acquisition, in comparison to arbuscular mycorrhizae (van der Heijden  
68 et al. 2015)

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

71 fungal partners that can participate in the symbiosis. While 65% of plant species  
72 form AM, the number of fungal species involved (c. 200 - 1600 species) is  
73 relatively small (Wang and Qiu 2006, Öpik et al. 2014). In contrast, only c. 3% of  
74 plant species form associations, but there are at least 7,750 documented ECM  
75 fungal species, and some estimates of ECM species richness range as high as  
76 25,000 species (Rinaldi et al. 2008). These many ECM fungal species generally  
77 have a higher degree of plant host specificity than AM fungal species (Wang and  
78 Qiu 2006), but hosts vary in their receptivity. Some plants, like Douglas-fir  
79 (*Pseudotsuga menziesii* Mirb. Franco), are reputed to associate with more than  
80 2000 ECM fungi (Trappe 1977), while others, like *Alnus*, associate with only 50  
81 ECM fungi world-wide (Pritsch et al. 1997). It is intriguing to speculate on the  
82 potential range of functional diversity in the many different ECM partners,  
83 especially given that these community assemblages often align well with edaphic  
84 gradients associated with organic and inorganic (ammonium and nitrate) N  
85 availability (Kranabetter 2014).

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

101

102 **Measurement of N uptake by ectomycorrhizae**

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

121  
122 Some early work exploring N uptake by ectomycorrhizae focused on the N  
123 uptake and N form preference of the fungal partner grown in pure culture, rather  
124 than focusing on the plant partner. Lundeberg (1970) showed that most of 27  
125 mycorrhizal fungi grown in pure culture performed best with ammonium as the  
126 sole N source, but several grew equally well with nitrate. In more recent mycelial  
127 culture studies, ammonium has been shown to be the most readily utilized  
128 source of inorganic N (Rangel-Castro et al. 2002; Guidot et al. 2005). However,  
129 Nygren et al. (2008) demonstrated wide variation among 68 species of ECM  
130 fungi in their mycelial growth in culture with nitrate as the sole N source. Results  
131 of culture studies must be interpreted with caution, however, because the  
132 response of the ECM fungal partner, alone, may not reflect its N form preferences  
133 or uptake ability when growing in symbiosis with a host plant (Turnbull et al.  
134 1995).

135

136 To directly measure N uptake of the ectomycorrhiza as a whole, experiments  
137 quantifying depletion of N in solution by ECM roots began in the 1960's.  
138 Carrodus (1966) found that excised beech mycorrhizae could readily absorb  
139 ammonium from ammonium chloride solution but had almost no ability to  
140 absorb nitrate. He also observed uptake of glutamic acid, aspartic acid,  
141 glutamine and asparagine by excised beech mycorrhizae (Carrodus 1966). Even  
142 earlier, Melin and Nilsson, showed ECM hyphae of intact mycorrhizal seedlings of  
143 *Pinus sylvestris* could absorb  $^{15}\text{N}$ -ammonium from labeled ammonium nitrate  
144 and transfer the label to different parts of the seedling (in Melin and Nilsson  
145 1953). These authors also showed that  $^{15}\text{N}$ , taken up as  $^{15}\text{N}$ -glutamic acid by  
146 hyphae of *Boletus variegatus*, could be transferred to the roots and stems of pine  
147 seedlings (Melin and Nilsson 1953). Use of N isotopes to understand N uptake  
148 and allocation by ECM plants has broadened from enrichment studies to field-  
149 based natural abundance studies where N isotopes are used to differentiate the  
150 functional characteristics of ECM fungi (e.g. Hobbie and Högberg 2012).  $^{15}\text{N}$   
151 natural abundance and enrichment studies have been used extensively to  
152 explore the sources of N used by ectomycorrhizae (reviewed in Courty et al.  
153 2010), but isotope studies can be challenging to interpret when processing of N  
154 forms by microbes can change the N form applied in enrichment studies prior to  
155 uptake. In addition, natural abundance studies are subject to the complexities of  
156 fractionation by competing microbes and metabolic processes. Recent work  
157 using nanoscale secondary ion mass spectrometry (NanoSIMS) imaging with  
158 stable isotopes of N and carbon (C) shows potential for visualizing in situ N and C  
159 flows between soil microbes, fungi and plants (Mayerhofer et al. 2016), but this  
160 work is not yet applicable to the field.

161

162 A relatively new methodology for exploring nutrient uptake by ectomycorrhizae  
163 uses ion-selective microelectrodes. This technique can be used to measure the  
164 net flux of particular ions at the surface of tissues, including the mantle of  
165 ectomycorrhizae. Several authors have published net flux measurements of  
166 single ions at ECM surfaces (Plassard et al. 2002; Gobert and Plassard 2002,  
167 2007; Boukcim and Plassard 2003; Li et al. 2012), but the microelectrode ion flux  
168 measurement technique (MIFE<sup>TM</sup>) (Newman 2001) is the only ion-selective

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

171

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

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

187

188 The theory of the MIFE system is explained in Newman (2001) and Shabala et al.  
189 (2012). Ions in solution move from areas of high electrochemical potential to  
190 low. Net ion movement can be calculated from the gradient in electrochemical  
191 potential, and the mobility and concentration of the ion in solution. The gradient  
192 can be measured by moving a microelectrode, containing a specific liquid ion  
193 exchange resin, between two positions, 10-60  $\mu\text{m}$  apart, closer and farther from  
194 a tissue surface. The MIFE system can simultaneously measure net flux of up to  
195 four ions over an area of 300  $\mu\text{m}^2$ , and is ideal for exploring ion net flux at the  
196 surfaces of root and ECM tissues. In our work, ECM roots are acclimated to an  
197 aerated experimental nutrient solution for 1 h before being placed in the MIFE  
198 chamber. The nutrient composition, pH, or temperature of the experimental  
199 solution can be modified, depending on the question being addressed. After  
200 acclimation, roots are placed in refreshed solution and positioned, using a  
201 microscope, so that the microelectrode tips are a few micrometers (2-20  $\mu\text{m}$ )



202 from the region of the ECM root where measurements will be made (Figure 1).  
203 Measurement begins and the previously calibrated microelectrodes oscillate,  
204 perpendicular to the root surface, and measure ion concentrations at the two  
205 positions. Measurements are taken for a minimum of 3 min, and then net flux of  
206 each ion ( $\text{nmol m}^{-2} \text{s}^{-1}$ ) is calculated from the mean difference ion concentration,  
207 the mobility of the particular ion, the electrochemical potential gradient and the  
208 geometry of the tissue surface (Newman 2001). Using this system, we have  
209 successfully measured ion fluxes of ectomycorrhizae with smooth mantles and  
210 those with emanating hyphae by positioning the microelectrodes next to the  
211 surface of the mantle and adjusting for the radius of the root plus mantle tissues.  
212 Theoretically, the MIFE system could be used to measure ion flux on  
213 rhizomorphs or even individual hyphae, if these structures could be cultured in a  
214 way that they would not be disturbed when transferred to the MIFE system.  
215



216

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

219

220 Pioneering work using ion-selective microelectrodes in a simple oscillating  
221 system to measure N flux in ECM fungi assessed nitrate net flux in *Pinus pinaster*  
222 roots associated with *Rhizopogon roseolus* or *Hebeloma cylindrosporum*. Results  
223 showed that mycorrhizal roots associated with *Rhizopogon roseolus* had a  
224 greater capacity to use nitrate than non-mycorrhizal short roots, at all nitrate  
225 concentrations (Gobert and Plassard 2002), but this enhancement of nitrate  
226 uptake was not observed to the same degree with *Hebeloma cylindrosporum*  
227 (Plassard et al. 2002). A new scanning ion-selective microelectrode technique  
228 has recently been used to measure ion fluxes in ectomycorrhizal *Populus* (Li et al.  
229 2012), but a limitation of these studies is that fluxes of only a single ion were  
230 measured at one time. An advantage of the MIFE system is that net fluxes of up  
231 to four ions can be measured at one position, simultaneously. With this  
232 technique, we can gain a deeper understanding of ECM functional traits and N  
233 uptake response to the environment.

234

235 Recent work in our lab has applied the MIFE system to measurements of  
236 inorganic N uptake in ECM species native to the productive temperate forests of  
237 coastal British Columbia. This area is unique in comparison to many other ECM  
238 biomes because much of the landscape has naturally rich soils that supply trees  
239 with considerable inputs of mineralized N (Littke et al. 2011, Perakis and  
240 Sinkhorn 2011). Our approach has been to retrieve root systems from forest  
241 stands in the spring and fall, during peaks in fungal physiological activity, to  
242 measure net fluxes of ammonium, nitrate and protons at the mantle surface. We  
243 sample and measure N uptake capacity repeatedly over the course of a few  
244 weeks to limit the effect of root excision (all roots undergo measurements within  
245 72 hours). An advantage of this approach over controlled-environment studies is  
246 our ability to test the native, site-adapted ECM species that populate these forest  
247 stands. To date we have tested over 40 species of ECM fungi (for methods, see  
248 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  $103 \text{ nmol m}^{-2} \text{ s}^{-1}$  (ranging from  $59.7 \text{ nmol m}^{-2} \text{ s}^{-1}$  for *Cortinarius hemitrichus* to  $351 \text{ nmol m}^{-2} \text{ s}^{-1}$  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  $5.8 \text{ nmol m}^{-2} \text{ s}^{-1}$  (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  $58 \text{ nmol m}^{-2} \text{ s}^{-1}$  (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  $190 \text{ nmol m}^{-2} \text{ s}^{-1}$  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 ( $60 \text{ nmol m}^{-2} \text{ s}^{-1}$ ), 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  $^{15}\text{N}$  uptake among ECM fungi can be greater than between ECM and non-mycorrhizal plants. Our findings emphasize how uptake capacity of

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

287

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

296

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

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

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

318

319 **Summary**

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

332

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

344

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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

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

47 A key role of mycorrhizal fungi is the mobilization and acquisition of  
48 nutrients, particularly nitrogen (N) and phosphorus (P). Extraradicular mycelia  
49 occupy a larger soil volume and penetrate into finer soil pores than do plant  
50 roots, and the secretion of fungal enzymes mobilizes nutrients from organic and  
51 inorganic soil particles. While the extraradicular mycelia of both arbuscular  
52 mycorrhizae and ectomycorrhizae take up mobilized nutrients, the contrasting  
53 structural characteristics of arbuscular mycorrhizae and ectomycorrhizae have  
54 important implications for nutrient uptake pathways. Arbuscular mycorrhizae  
55 can take up nutrients through both root and fungal pathways, while  
56 ectomycorrhizae rely primarily on fungal uptake, therefore, the fungus plays a  
57 much larger role in nutrient uptake for ectomycorrhizae than for arbuscular  
58 mycorrhizae (Bücking et al. 2012). In pine roots, for example, only 2% of the  
59 root surface is non-ectomycorrhizal, and the extraradicular mycelium represents  
60 99% of the nutrient-absorbing surface length (Rousseau et al. 1992). Compared  
61 to roots and arbuscular mycorrhizae, ectomycorrhizae are highly competitive for  
62 N and secrete a variety of hydrolytic enzymes that degrade organic polymers and  
63 allow access to organic nutrients via extraradicular mycelium (Bücking et al.  
64 2012). In addition, the fungal mantle of ectomycorrhizae can also function in  
65 nutrient uptake and storage (Bücking et al. 2012). Overall, ectomycorrhizae are  
66 thought to make far larger contributions to plant N acquisition in comparison to  
67 arbuscular mycorrhizae (van der Heijden et al. 2015)

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

71 form AM, the number of fungal species involved (c. 200 - 1600 species) is  
72 relatively small (Wang and Qiu 2006, Öpik et al. 2014). In contrast, only c. 3% of  
73 plant species form ectomycorrhizal associations, but there are at least 7,750  
74 documented ECM fungal species, and some estimates of ECM species richness  
75 range as high as 25,000 species (Rinaldi et al. 2008). These many ECM fungal  
76 species generally have a higher degree of plant host specificity than AM fungal  
77 species (Wang and Qiu 2006), but hosts vary in their receptivity. Conifer forests  
78 are reputed to associate with more than 1000 ECM taxa regionally (Allen et al.  
79 1995), while certain hosts, like *Alnus*, may associate with only a few dozen ECM  
80 species (Tedersoo et al. 2009). It is intriguing to speculate on the potential range  
81 of functional diversity in the many different ECM species, especially given that  
82 **ECM communities of forest stands** align well with edaphic gradients associated  
83 with **increasing inorganic** (ammonium and nitrate) N availability (Kranabetter  
84 2014).

85         Low soil N availability is one of the major factors limiting plant growth in  
86 native temperate and boreal ecosystems (Rennenberg et al. 2009), thus  
87 functional diversity in the ability of mycorrhizae to acquire N would have a  
88 significant influence on plant fitness. The forest trees that dominate cool  
89 temperate and boreal ecosystems are predominantly associated with ECM fungi,  
90 at levels of root colonization near 100%. This paper reviews traditional methods  
91 of measuring N uptake by ectomycorrhizae, and describes a more recent  
92 application of microelectrode ion flux measurement of N uptake to  
93 ectomycorrhizae associated with forest trees. To date, ion flux measurements of  
94 roots have been, for the most part, conducted with non-mycorrhizal roots, a  
95 situation far removed from the complexity of root physiology in natural  
96 environments. From results obtained with microelectrode ion flux measurement  
97 thus far, we argue that plant N uptake capacity should be considered an  
98 expression of the interacting ECM species (i.e., an exogenous trait; Carmona et al.  
99 2015), rather than a function of host plant root physiology, alone.

100

### 101 **Measurement of N uptake by ectomycorrhizae**

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

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

120  
121 Some early work exploring N uptake by ectomycorrhizae focused on the N  
122 uptake and N form preference of the fungal partner grown in pure culture, rather  
123 than focusing on the plant partner. Lundeberg (1970) showed that most of 27  
124 mycorrhizal fungi grown in pure culture performed best with ammonium as the  
125 sole N source, but several grew equally well with nitrate. In more recent  
126 mycelial culture studies, ammonium has been shown to be the most readily  
127 utilized source of inorganic N (Rangel-Castro et al. 2002; Guidot et al. 2005).  
128 However, Nygren et al. (2008) demonstrated wide variation among 68 species of  
129 ECM fungi in their mycelial growth in culture with nitrate as the sole N source.  
130 Results of culture studies must be interpreted with caution, however, because  
131 the response of the ECM fungal partner, alone, may not reflect its N form  
132 preferences or uptake ability when growing in symbiosis with a host plant  
133 (Turnbull et al. 1995).

134  
135 To directly measure N uptake of the ectomycorrhiza as a whole, experiments  
136 quantifying depletion of N in solution by ECM roots began in the 1960's.

137 Carrodus (1966) found that excised beech mycorrhizae could readily absorb  
138 ammonium from ammonium chloride solution but had almost no ability to  
139 absorb nitrate. He also observed uptake of glutamic acid, aspartic acid,  
140 glutamine and asparagine by excised beech mycorrhizae (Carrodus 1966). Even  
141 earlier, Melin and Nilsson **had shown** ECM hyphae of intact mycorrhizal  
142 seedlings of *Pinus sylvestris* could absorb  $^{15}\text{N}$ -ammonium from labeled  
143 ammonium nitrate and transfer the label to different parts of the seedling (Melin  
144 and Nilsson 1953). These authors also showed that  $^{15}\text{N}$ , taken up as  $^{15}\text{N}$ -glutamic  
145 acid by hyphae of *Boletus variegatus*, could be transferred to the roots and stems  
146 of **Scots** pine seedlings (Melin and Nilsson 1953). Use of N isotopes to  
147 understand N uptake and allocation by ECM plants has broadened from  
148 enrichment studies to field-based natural abundance studies where N isotopes  
149 are used to differentiate the functional characteristics of ECM fungi (e.g. Hobbie  
150 and Högberg 2012).  $^{15}\text{N}$  natural abundance and enrichment studies have been  
151 used extensively to explore the sources of N used by ectomycorrhizae (reviewed  
152 in Courty et al. 2010), but isotope studies can be challenging to interpret when  
153 processing of N forms by microbes can change the N form applied in enrichment  
154 studies prior to uptake. In addition, natural abundance studies are subject to the  
155 complexities of fractionation by competing microbes and metabolic processes.  
156 Recent work using nanoscale secondary ion mass spectrometry (NanoSIMS)  
157 imaging with stable isotopes of N and carbon (C) shows potential for visualizing  
158 in situ N and C flows between soil microbes, fungi and plants (Mayerhofer et al.  
159 2016), but this work is not yet applicable to the field.

160

161 A relatively new methodology for exploring nutrient uptake by ectomycorrhizae  
162 uses ion-selective microelectrodes. This technique can be used to measure the  
163 net flux of particular ions at the surface of tissues, including the mantle of  
164 ectomycorrhizae. Several authors have published net flux measurements of  
165 single ions at ECM surfaces (Plassard et al. 2002; Gobert and Plassard 2002,  
166 2007; Boukcim and Plassard 2003; Li et al. 2012), but the microelectrode ion flux  
167 measurement technique (MIFE™) (Newman 2001) is the only ion-selective  
168 microelectrode technique that can measure net fluxes of multiple ions in one  
169 position, simultaneously.

170

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

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

182

183 The theory of the MIFE system is explained in Newman (2001) and Shabala et al.  
184 (2012). Ions in solution move from areas of high electrochemical potential to  
185 low. Net ion movement can be calculated from the gradient in electrochemical  
186 potential, and the mobility and concentration of the ion in solution. The gradient  
187 can be measured by moving a microelectrode, containing a specific liquid ion  
188 exchange resin, between two positions, 10-60  $\mu\text{m}$  apart, closer and farther from  
189 a tissue surface. The MIFE system can simultaneously measure net flux of up to  
190 four ions over an area of 300  $\mu\text{m}^2$ , and is ideal for exploring ion net flux at the  
191 surfaces of root and ECM tissues.

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

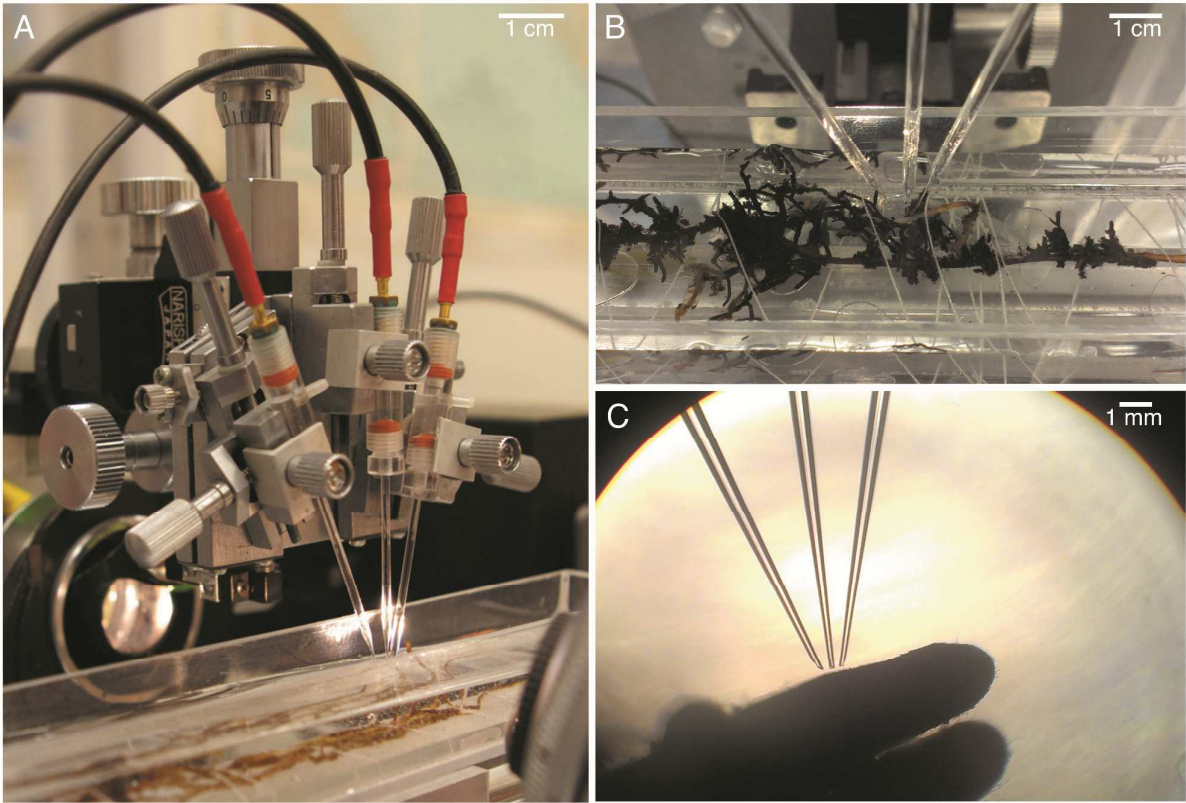
203 particular ion, the electrochemical potential gradient and the geometry of the  
204 tissue surface (Newman 2001).

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

212

Draft





213  
214

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

222

223 For ectomycorrhizae, pioneering work using ion-selective microelectrodes in a  
224 simple oscillating system assessed nitrate net flux in *Pinus pinaster* roots  
225 associated with *Rhizopogon roseolus* or *Hebeloma cylindrosporum*. Results  
226 showed that mycorrhizal roots associated with *Rhizopogon roseolus* had a  
227 greater capacity to use nitrate than non-mycorrhizal short roots, at all nitrate  
228 concentrations (Gobert and Plassard 2002), but this enhancement of nitrate  
229 uptake was not observed to the same degree with *Hebeloma cylindrosporum*  
230 (Plassard et al. 2002). A new scanning ion-selective microelectrode technique  
231 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.

237

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  $\mu\text{M}$   $\text{NH}_4\text{NO}_3$ ) to ensure  $\text{NH}_4^+$  or  $\text{NO}_3^-$  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.

256

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  $103 \text{ nmol m}^{-2} \text{ s}^{-1}$  (ranging from  $59.7 \text{ nmol m}^{-2} \text{ s}^{-1}$  for a *Cortinarius* sp. to  $351 \text{ nmol m}^{-2} \text{ s}^{-1}$  for a *Lactarius* sp.) (Kranabetter et al. 2015). In contrast, the average rate of ammonium uptake for

265 non-mycorrhizal white root tips from some of the same root systems was only  
266  $5.8 \text{ nmol m}^{-2} \text{ s}^{-1}$  (unpublished data). Related conifer studies where solution pH  
267 was manipulated found non-mycorrhizal roots of Douglas-fir seedlings to only  
268 achieve maximum rates of N uptake of  $58 \text{ nmol m}^{-2} \text{ s}^{-1}$  (Hawkins et al. 2008).  
269 These data support other studies in the **laboratory** and in the field showing that  
270 N uptake by ECM roots exceeds that of non-mycorrhizal roots (Wallander et al.  
271 1997, Quoreshi and Timmer 2000) and emphasizes the competitive advantage of  
272 trees with virtually 100% root colonization by EM fungi, even in soils rich in N.

273

## 274 2) *Species matter.*

275 We find a wide range in uptake capacity for ammonium among ECM species, in  
276 large degree matching the N availability of test sites. Spring uptake of  
277 ammonium by ECM roots of **coastal** Douglas-fir was greatest on a medium and  
278 rich site, compared to a poor site, and averaged over  $190 \text{ nmol m}^{-2} \text{ s}^{-1}$  for  
279 *Tomentella* species, a ECM group common on high N soils (Kranabetter et al.  
280 2015). The lowest uptake rates of ammonium by ECM Douglas-fir roots were by  
281 *Cortinarius* species ( $60 \text{ nmol m}^{-2} \text{ s}^{-1}$ ), a group common to low N soils  
282 (Kranabetter et al. 2015). Likewise, many *Cortinarius* species demonstrate  
283 significant extracellular enzyme production for the uptake of organic N,  
284 particularly under conditions of low N availability (Bödeker et al. 2014). Jones et  
285 al. (2009) also showed that differences in  $^{15}\text{N}$  uptake among ECM fungi can be  
286 greater than between ECM and non-mycorrhizal plants. Our findings emphasize  
287 how uptake capacity of inorganic N by host species is, to a large degree, an  
288 exogenous trait (**Carmona et al. 2015**) defined by partnerships with individual  
289 ECM species. **Functional diversity of the symbiont, as in this example, is essential**  
290 **in fully understanding the adaptive capacity of the holobiont**  
291 **(Vandenkoornhuyse et al. 2015, Bordenstein and Theis 2015).**

292

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

294 While ammonium is generally recognized as the most readily utilized source of  
295 inorganic N by the majority of ECM fungi (Rygiewicz et al. 1984, Chalot and  
296 Plassard 2011, Clemmensen et al. 2008), we hypothesized that sites with  
297 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 ( $500 \mu\text{M NO}_3$ ), we found very few species had any notable uptake of nitrate, with a maximum of only  $\sim 30 \text{ nmol m}^2\text{s}^{-1}$  (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

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

338

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

350

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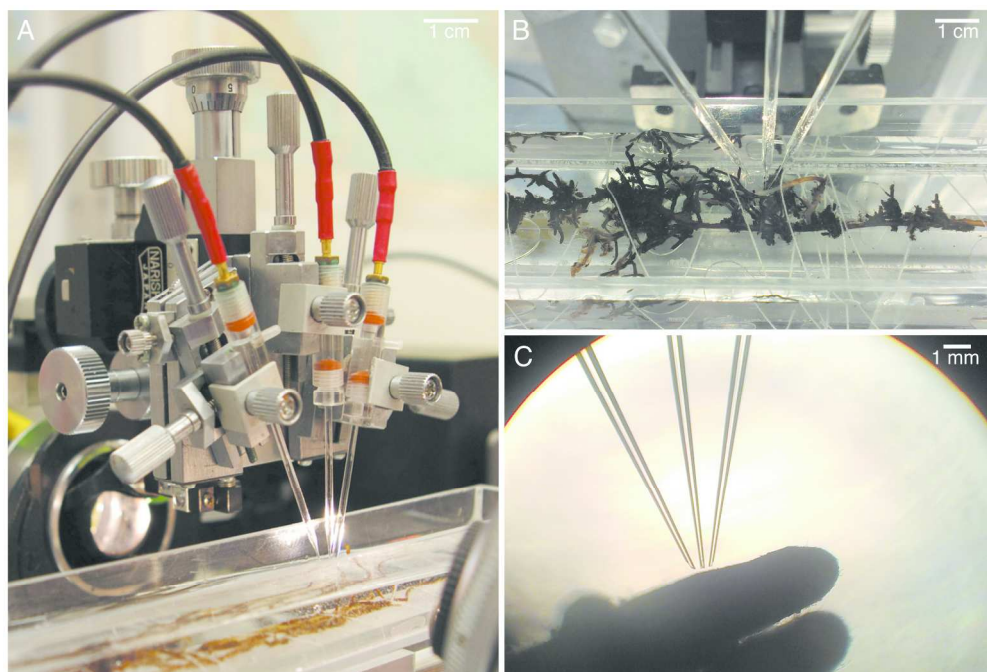


Figure 1. A) MIFE system multiple electrode mount holding three microelectrodes (filled with ammonium-, nitrate- and proton-selective resins) placed above a MIFE chamber holding roots. B) Section of coastal Douglas-fir root with secondary ECM roots immersed in a MIFE chamber, and the microelectrodes 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)