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1 **Inorganic nitrogen availability alters *Eucalyptus grandis***

2 **receptivity to the ectomycorrhizal fungus *Pisolithus albus***

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4

5

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Summary

- Forest trees are able to thrive in nutrient poor soils in part because they obtain growth-limiting nutrients, especially nitrogen (N), through mutualistic symbiosis with ectomycorrhizal (ECM) fungi. Addition of inorganic N into these soils is known to disrupt this mutualism and reduce the diversity of ECM fungi. Despite its ecological impact, the mechanisms governing the observed effects of elevated inorganic N on mycorrhizal communities remain unknown.

- We address this by using a compartmentalized *in vitro* system to independently alter nutrients to each symbiont. Using stable isotopes, we traced the nutrient flux under different nutrient regimes between *Eucalyptus grandis* and its ectomycorrhizal symbiont, *Pisolithus albus*.

- We demonstrate that giving *E. grandis* independent access to N causes a significant reduction in root colonization by *P. albus*. Transcriptional analysis suggests that the observed reduction in colonization may be caused, in part, by altered transcription of microbe perception genes and defence genes. We show that delivery of N to host leaves is not increased by host nutrient deficiency but by fungal nutrient availability.

- Overall, this advances our understanding of the effects of N fertilization on ECM fungi and the factors governing nutrient transfer in the *E. grandis - P. microcarpus* interaction.

Introduction

Nutrient cycling and the health of forest ecosystems are affected by the presence of mutualistic ectomycorrhizal (ECM) fungi. Through symbiosis with tree roots, these fungi have key roles in aiding the tree gain access to growth-limiting nutrients such as nitrogen (N) in return for photosynthetically fixed carbon (C) from the tree host. ECM fungi also provide other ecosystem services such as increased sequestration of C and improved soil stability through aggregation (Rillig & Mummey, 2006; Clemmensen *et al*., 2013). While ECM relationships occur most frequently where inorganic N sources within the soil are limited and N is instead found primarily in organic forms (Read *et al*., 2004; Toljander *et al*., 2006; Lin *et al*., 2017), an increasing number of studies have shown that inorganic N enrichment in forest soils through natural causes, pollution or intentional fertilization are leading to a reduction in the level of plant root colonization by ECM fungi and a community shift in soils away from ECM fungi specialized in organic N acquisition to more generalist nitrophilic species and saprotrophs (Lilleskov *et al*., 2002; Parrent *et al*., 2006; Pardo *et al*., 2011; Morrison *et al*., 2016; Corrales *et al*., 2017; Averill *et al*., 2018). Therefore, as inorganic N availability continues to rise in forest soils (Galloway *et al*., 2004; Hietz *et al*., 2011) the negative outcomes to ecosystem services provided by ECM fungi are expected to increase (Pheonix *et al*., 2012; Field *et al*., 2014). However, a mechanism behind these observations is lacking.

One possibility for the observed reduction in ECM communities and colonization of hosts in N-rich environments is that increased nutrient availability alters the supply/demand paradigm of the mutualism, thereby disrupting fungal establishment and persistence on the root system. While nutrient exchange stands as the hallmark of ECM symbiosis, very little is known about what induces the transfer of N from the fungal symbiont to its host (Garcia *et al*.,

89   2015). There is some evidence from another lineage of mycorrhizal

90   fungi, the arbuscular mycorrhizal (AM) fungi, that exchange of

91   nutrients is based on a reciprocal rewards system, where fungal

92   delivery of nutrients to the plant host is tied to, or "rewarded" with,

93   increased C supply (Kiers *et al*., 2011; Fellbaum *et al*., 2012;

94   Fellbaum *et al*., 2014), though this may be context-dependent

95   (Stonor *et al*., 2014; Walder & van der Heijden, 2015). While little

96   correlation between N transferred by the fungus and C returns from

97   the host has been shown in ECM fungal associations (Corrêa *et al*.,

98   2008; Albarracín *et al*., 2013; Valtanen *et al*., 2014; Hortal *et al*.,

99   2017), soil N levels, particularly the availability of inorganic N, can

100 alter C/N trading dynamics (Treseder, 2004, Albarracín *et al*., 2013;

101 Näsholm *et al*., 2013; Hasselquist & Högberg, 2014). This may

102 disrupt the stability of the mutualism and lead to the observed

103 ecological outcomes. The plant may actively exclude the fungus

104 from accessing its tissues through up-regulation of defence-related

105 pathways in an effort to conserve its own C resources when N

106 availability is high. In arbuscular mycorrhizal symbioses, several

107 studies have shown that host plants actively suppress microbial

108 colonization when nutrients are plentiful (Breuillin *et al*., 2010;

109 Balzergue *et al*., 2011; Nouri *et al*., 2014; Kobae *et al*., 2016),

110 although the mechanism of this effect remains unknown (Kobae *et

111 al.*, 2016).

112

113 The main difficulty in interpreting the effects of nutrient availability

114 on the symbiosis between plant and fungal partners is that they are

115 typically grown in a common substrate, so nutrients available to one

116 partner are also available to the other. This makes it difficult to

117 determine true mechanistic cause and effect concerning how

118 symbiosis is disrupted. Using a nutrient compartment-based *in vitro*

119 system and stable isotope tracing of C and N, we remove some of

120 these limitations by giving the model ECM fungus *Pisolithus albus*

121 and its host *Eucalyptus grandis* access to independent nutrient

122 regimes. Specifically, we vary plant access to N and, independently,

123 fungal access to C. Our results from this fully factorial experiment

124 suggest that increased N nutrition to the plant alone systemically

125 induces alterations to plant-encoded microbial perception pathways

126 and increases in chemical defence pathways in a manner that

127 correlates with a reduction in the colonization of roots by the ECM

128 fungus. Further, we show that symbiotic delivery of N from the

129 fungus to the host plant leaves is not dependent on host access to

130 N. Rather, N supply to the host is increased when the fungal partner

131 is given less C. These results add to our knowledge and

132 understanding of nutrient exchange dynamics and the effect of

133 fertilization in this ecologically important relationship.

Materials and Methods

Compartment Setup and Microcosm Design

Tri-compartment 90-mm petri dishes were used to test the effect of substrate nutrition on ECM symbiosis between *P. albus* and *E. grandis* roots. The three compartments were named the 'symbiotic compartment' (SC), the 'plant-only compartment' (PC) and the 'fungus-only compartment' (FC; Fig. **1a**). The compartmental design was accomplished using a divided 90-mm petri dish to separate the SC and PC and by embedding a centrifuge tube screw cap (Edwards Co., cap capacity 1.2 mL) into the SC when media was solidifying to create the FC. Thus, all compartments were separated by solid plastic barriers to completely prevent the diffusion of nutrients from one compartment to another, allowing us to vary nutrients in such a way as to allow only plant or fungal access to a prescribed set of nutrients.

The SC contained 0.1 % glucose solidified with agar. The PC contained half strength Modified Melin- Norkrans (MMN) media with 0.1 % glucose added (Plett *et al.,* 2015). Within this medium, as we were interested in the impact of inorganic N availability, we created either a "high" N condition (10 mM total N with a $NH_4:NO_3$ at a ratio of 2.2:1 plus 1×10^{-3} mM organic N from thiamine) or a "low" N condition without added inorganic N (ammonium nitrate and diammonium phosphate were replaced with sodium hydrogen phosphate to maintain phosphorus balance; 1×10^{-3} mM N from thiamine in media). The FC contained full-strength MMN media either with 1.0 % glucose (high fungal C condition) or 0.1 % glucose (low fungal C condition). Within the FC, all inorganic N was enriched in ^{15}N ($^{15}NH_4Cl$; Sigma-Aldrich; $\geq$98 atom % ^{15}N). More detailed information on the media composition of each compartment can be found in Supporting Information Table S1. Plates were made to create all four combinations of high or low nutrients to each partner

167 to enable a fully factorial experimental design. Agar surfaces were

168 covered with sterile cellophane membranes to prevent root and

169 fungal penetration into the agar.

170

171

172 *Fungal and Plant Material*

173 Small squares (0.5 cm x 0.5 cm) of *Pisolithus albus* (isolate SI12;

174 Plett *et al*., 2015) were excised from the leading edge of a one-

175 month old colony growing on full strength MMN media (1.0 %

176 glucose). These were placed in the SC of the test plates, just below

177 the FC nutrient reservoir. They were grown for two weeks at 25°C to

178 allow for growth of the mycelium over the plastic barrier and into

179 the FC nutrient reservoir. *E. grandis* seedlings (lot 20974; CSIRO

180 seed bank), prior to addition to the microcosm, were surface

181 sterilized with 30% hydrogen peroxide for 10 minutes, rinsed three

182 times in sterile water and germinated on 1.0 % agar plates for four

183 weeks post-sterilization followed by four weeks of growth on 1/2

184 MMN plates (0.1 % glucose). Plates were sealed with Micropore tape

185 to allow gas exchange. Plants were grown in growth chambers

186 (Climatron-1260, Thermoline Scientific) with a daytime temperature

187 of 25°C and night temperature of 18°C and a 16-hour photoperiod

188 (RH: 60 %; light level: 900 μmol/m^2). As we wished to trace carbon

189 movement within our microcosm using stable isotopes, we equipped

190 the growth chamber with Sodasorb scrubbers to remove all CO_2

191 from the air and replaced it with 400 pm CO_2 depleted in ^{13}C (Aligal

192 2, Air Liquide Australia). This method depleted natural levels of

193 $^{13}CO_2$ in the air such that plant and fungal samples were sufficiently

194 different in ^{13}C isotopic abundances to trace the fungal C originating

195 from the plant (i.e. carbon with a higher ^{12}C signature would more

196 likely come from plant photosynthate while carbon with a higher ^{13}C

197 signature would come from fungal C sourced from the glucose in the

198 FC medium). The chamber air had an average atom %^{13}C of 1.0844

199 and the plants were grown in these chambers from seed to the end

200 of the experiment, giving them an atom %^{13}C ranging from 1.066-
201 1.069 (see Supporting Information Figure S2).
202
203 *Microcosm Setup and Harvest*
204 Eight-week old seedlings with branched root systems were selected
205 for the study and placed on the tri-compartment test plates with the
206 pre-grown fungus such that half of the root system was in contact
207 with the fungus in the SC, with no roots placed in or near the FC,
208 and the other half of the root system was given access to the PC.
209 Plant-only and fungus-only control plates were also produced.
210 Plates were sealed again with Micropore tape and placed in the
211 growth chambers described above at a 45° angle and rotated
212 slightly so that condensation would not pool at the interface of the
213 SC and PC sections, which would have led to unwanted nutrient
214 mixing. After 48 hours of fungal contact, three biological replicates
215 from each nutrient condition of the experiment were harvested for
216 RNA extraction and analysis. The remaining plants were left for a
217 total of two weeks of contact with the ECM fungus before
218 harvesting.
219
220 At harvest, the percentage of root tips colonized by *P. albus* was
221 recorded (number of mycorrhizal root tips/total lateral roots in
222 contact with fungus x 100%). Averages are from a total of 12
223 replicates per nutrient condition harvested over a series of three
224 independent experiments to ensure reproducibility. Leaves from
225 the plant and the mycelium of the fungus were collected for five
226 biological replicates per nutrient condition as well as three biological
227 replicates of plant-only and fungal-only controls and dried at 40°C
228 overnight. All samples for isotopic analysis originated from the same
229 experiment to reduce labelling biases between experiments. These
230 were ground up, weighed and sent for ^{13}C/^{15}N analysis by an
231 elemental analyser and isotope ratio mass spectrometry (UC Davis
232 Stable Isotope Facility, Davis, California, USA).

233

234 *Isotopic calculations*

235 Fungal and leaf C/N ratios were calculated based on the ratio of the

236 total mg C and mg N present in the dried samples as determined by

237 stable isotope analysis. Similarly, the N content of the plant leaves

238 was determined by multiplying the ratio of the mg N to mg total

239 sample (as determined from the isotopic data) by the leaf dried

240 biomass. The leaf biomass was measured for each plant after

241 harvest and drying. The percentage of total N as ^{15}N in the fungus

242 was calculated as the ratio of mg ^{15}N to mg total N multiplied by 100

243 %.

244

245 The percentage of N in the plant derived from transfer from the

246 fungus (% NDFT) was calculated according to the formula (He *et al*.,

247 2009; Tomm *et al*., 1994):

$$\%NDFT = \frac{(^{15}N_{Plant} - 0.38)}{(^{15}N_{Fungus} - 0.38)} \times 100\%$$

248

249 where $^{15}N_{Plant}$ and $^{15}N_{Fungus}$ are the atom percentages of ^{15}N in the

250 dried plant leaves and fungal mycelium as determined by stable

251 isotope analysis, respectively. The value of 0.38 represents the

252 average background atom percentage of ^{15}N found in our unlabelled

253 experimental controls. It should be noted that the calculated %

254 NDFT refers to the percent contribution of fungal N to the total N

255 pool of the plant leaves, not just that N that was acquired during the

256 two-week plant-fungal contact period.

257

258 The quantity of N received by the plant leaves via symbiosis was

259 calculated using % NDFT as above, divided by 100 to express as a

260 fraction, and multiplying it by the N content of the plant leaves in

261 micrograms.

262

263 The percentage of C acquired by the fungus via symbiosis was

264 calculated according to the formula:

$$\%C_{symbiosis} = \frac{(^{13}C_{Fungus} - 1.0958)}{(^{13}C_{Plant} - 1.0958)} \times 100\%$$

265

266 where $^{13}C_{Fungus}$ and $^{13}C_{Plant}$ are the atom percent ^{13}C values of the

267 dried fungal mycelium and plant leaves respectively, as determined

268 by stable isotope analysis. The value of 1.0958 represents the

269 average atom percent ^{13}C value for control fungi not in contact with

270 a plant. As with the plant calculations, $\%C_{symbiosis}$ represents the

271 percent contribution of plant carbon to the total mycelial C pool, not

272 just that obtained during the period of contact. Estimated

273 micrograms of carbon transferred to the fungal colonies from the

274 plant during contact were calculated using $\%C_{symbiosis}$ (divided by 100

275 to express as a fraction), and multiplying by the C content of dried

276 mycelium (as determined from the stable isotope data) and an

277 estimated biomass value of 472 μg for high C samples and 247 μg

278 for low C samples. These values were based on the average biomass

279 of control fungi not in contact with plants as it was not possible to

280 accurately record the mass of colonies in contact with plant roots as

281 the two tissue types could not be fully separated.

282

283 *Nutrient Calculation Statistical Analyses*

284 Two-way ANOVAs (unbalanced, Type III) were used to examine the

285 effect of glucose or nitrogen availability and their interaction on

286 experimental outcomes using the "car" package in R (R Core Team,

287 2016; Fox & Weisberg, 2011). Normality of the data was tested

288 using the Shapiro-Wilk normality test and homogeneity of variance

289 using the Bartlett's test in R (R Core Team, 2016). All data

290 conformed to a normalized and homogeneous distribution ($p >$

291 0.05). Any statistical outliers were removed from the dataset (based

292 on the interquartile range (IQR) method). In all instances, results

293 with a p-value of less than 0.05 were determined to be significant.

294

295 *RNA extraction and transcriptomic analysis*

296 For 48-hour time point samples, roots in the symbiotic compartment
297 were excised along with any attached fungal mycelium and snap
298 frozen in liquid nitrogen. RNA was extracted with the Qiagen
299 RNeasy plant micro kit according to manufacturer's instructions.
300 RNA for three replicates each of the high N/low C and low N/low C
301 conditions were sequenced at the Joint Genomes Institute (JGI).
302 Plate-based RNA sample prep was performed on the PerkinElmer
303 Sciclone NGS robotic liquid handling system using Illumina's TruSeq
304 Stranded mRNA HT sample prep kit utilizing poly-A selection of
305 mRNA following the protocol outlined by Illumina in their user guide:
306 http://support.illumina.com/sequencing/sequencing_kits/truseq_stra
307 nded_mrna_ht_sample_prep_kit.html, and with the following
308 conditions: total RNA starting material was 100 ng per sample and
309 10 cycles of PCR was used for library amplification. The prepared
310 libraries were quantified using KAPA Biosystem's next-generation
311 sequencing library qPCR kit and run on a Roche LightCycler 480
312 real-time PCR instrument. The quantified libraries were multiplexed
313 with other libraries, and the pool of libraries was then prepared for
314 sequencing on the Illumina HiSeq sequencing platform utilizing a
315 TruSeq paired-end cluster kit, v4, and Illumina's cBot instrument to
316 generate a clustered flow cell for sequencing. Sequencing of the
317 flow cell was performed on the Illumina HiSeq2500 sequencer using
318 HiSeq TruSeq SBS sequencing kits, v4, following a 2 x 150 indexed
319 run recipe. Raw RNA-Seq reads were filtered and trimmed using the
320 JGI QC pipeline. Using BBDuk
321 (https://sourceforge.net/projects/bbmap/), raw reads were evaluated
322 for artifact sequence by kmer matching (kmer=25), allowing 1
323 mismatch and detected artifact was trimmed from the 3' end of the
324 reads. RNA spike-in reads, PhiX reads and reads containing any Ns
325 were removed. Quality trimming was performed using the phred
326 trimming method set at Q6. Finally, following trimming, reads under
327 the length threshold were removed (minimum length 25 bases or
328 1/3 of the original read length - whichever is longer).

329

330 Filtered reads from each library were aligned to either the *E. grandis*

331 (Myberg *et al*., 2014; https://phytozome.jgi.doe.gov/pz/portal.html#!

332 info?alias=Org_Egrandis) or *P. albus* SI12

333 (https://genome.jgi.doe.gov/Pisalb1/Pisalb1.home.html)

334 reference genome using HISAT2 version 2.1.0 (Kim *et al*., 2015;

335 BAMs/ directory). Only primary hits assigned to the reverse strand

336 were included in the raw gene counts (-s 2 -p --primary options).

337 Features assigned to the forward strand were also tabulated (-s 1 -p

338 --primary options). Strandedness of each library was estimated by

339 calculating the percentage of reverse-assigned fragments to the

340 total assigned fragments (reverse plus forward hits). DESeq2

341 (version 1.18.1; Love *et al*., 2014) was subsequently used to

342 normalize data and determine which genes were differentially

343 expressed between pairs of conditions. Genes differentially

344 regulated in the plant high N condition as compared to the plant low

345 N condition were considered. Adjusted p-values were calculated by

346 incorporating a Benjamini-Hochberg FDR correction. For *E. grandis*,

347 genes differentially regulated by more than 5-fold from controls

348 were considered ($p < 0.05$) and for *P. albus*, genes differentially

349 regulated by more than 2-fold were considered ($p < 0.05$). Gene

350 annotations, gene ontology (GO) terms and signal peptide

351 probability scores were assigned to each significantly differentially

352 regulated gene based on annotation data from the online *E. grandis*

353 or *P. albus* SI12 genome resources given above.

354

355 GO term enrichment analysis was performed using the web-based

356 platform PlantRegMap (plantregmap.cbi.pku.edu.cn/go.php).

357 Significantly up- and down-regulated genes in the *E. grandis* data

358 set were assessed separately and GO terms within the Biological

359 Process aspect were considered as significantly enriched with a

360 threshold p-value of 0.01 based on a Fisher's exact test. Q-values

361 represent the adjusted p-value based on the Benjamini-Hochberg

362   method. Scatterplot visualization of results was performed using

363   the online platform ReviGo (revigo.irb.hr; Supek *et al*., 2011) using

364   default settings for a large size data set.

365

366

Results

Formation of mycorrhizal root tips was driven primarily by plant nutritional needs

The use of a tri-compartmented system to give *E. grandis* independent access to high or low plant available inorganic nitrogen and *P. albus* independent access to high or low glucose was successful (Fig. **1a**). After two weeks of symbiotic establishment between the two organisms, the percentage of mycorrhizal root tips formed in each nutrient combination was determined (Fig. **1b**). For both *E. grandis* and *P. albus,* an increase in resource availability in their respective compartments resulted in reduced root colonization, however this was only significant for the fungus when plant N was high. The lowest percentage of mycorrhizal root tips were formed when the plant had access to high N and the fungus had access to high C, while the greatest percentage of mycorrhizal root tips were formed when both had limited resources. Analysis of variance (ANOVA) demonstrates that N level to the plant was a statistically significant factor in determining the level of root colonization (Table 1).

Plant symbiotic N acquisition was fungal driven

The C/N ratio of *E. grandis* leaves was determined (Fig. **1c**). Plants grown with access to high N had lower C/N ratios in their leaves as compared to those grown without N. Whether or not the plant was associated with *P. albus* did not significantly affect the leaf C/N ratio over the time-course of the experiment. Using stable isotope labelling, we were able to determine that the plant was able to obtain N from the fungus (Fig. **1d**). The amount of plant N acquired via symbiosis (as determined by the abundance of ^{15}N in leaves) was dependent not on the N available to the plant, but on the C available to the fungus (Table 1). That is, a higher quantity of plant N was acquired from the fungus when the fungus had low access to C (Fig.

400 **1d**). Plant leaf biomass was not significantly affected by any of the
401 conditions (Supporting Information Figure S1). Plants grown without
402 access to N had significantly less total N in their leaves and thus
403 symbiotically acquired N made up a slightly larger percentage of the
404 total leaf N acquired over the lifetime of the plant, but C availability
405 to the fungus remained the only statistically significant factor in
406 explaining the percentage of plant N derived from transfer as well
407 (%NDFT; Supporting Information Figure S1). Plant N levels and
408 calculations are based on the leaf tissues only, thus we cannot
409 exclude the possibility that there is a treatment effect on N uptake
410 or partitioning between plant shoot and roots.
411
412 *Fungal acquisition of N was influenced by plant nutritional status*
413 The approximated amount of C transfer from the plant to *P. albus*
414 was not significantly different in any condition suggesting that C and
415 N transfer in this symbiosis is not tightly connected (Fig. **2a**). While
416 resource availability to either plant or fungus were not significant
417 factors in the amount of C transferred (Table 1), fungal colonies with
418 access to low C resources were smaller, and thus the C acquired
419 from the plant made up a larger percentage of the total C acquired
420 over the lifetime of the fungal colony (Supporting Information Figure
421 S2). The percentage of N as ^{15}N for each *P. albus* colony was also
422 considered (Fig. **2b**). As all N supplied to the *P. albus* colony was
423 labelled, a higher percentage of ^{15}N was indicative of greater
424 acquisition of N from the medium for a given fungal colony size.
425 Axenically grown fungal colonies (i.e. without a plant host present)
426 with access to higher levels of glucose acquired a greater
427 percentage of ^{15}N (Fig. **2b**) and were larger than those grown with
428 low levels of glucose (Supporting Information Figure S2). Fungal C/N
429 ratios did not change significantly in any condition (Fig. **2c**). The
430 addition of a plant with high N resources reduced the average
431 percentage of ^{15}N in the fungal colonies. This could be due to lower
432 acquisition from the FC or to loss of N via delivery to the plant.

433 When the added plant had access to less N resources there was a
434 significant increase in the percentage of ^{15}N in the fungus, no matter
435 how much exogenous C it was provided with. This increase in
436 fungal N is unlikely to be due to reduced transfer to the plant, as
437 this was not significantly altered by the plant N availability (Fig. **1d**),
438 nor did plant N availability affect the amount or percentage of
439 fungal C acquired from the plant. Two-way ANOVA results
440 demonstrate that both fungal C and plant N availability significantly,
441 and independently, affect the ^{15}N levels within the fungal mycelium
442 (Table 1). Thus, low N resources on the part of the plant can result
443 in a greater accumulation of N in the fungus.
444
445 *N availability altered the transcription of plant genes associated with*
446 *microbe sensing and sugar metabolism*
447 After 48-hours of contact with *P. albus*, the regulation of *E. grandis*
448 genes in roots from the symbiotic compartment and in contact with
449 the fungus was considered. A comparison of these roots from plants
450 with access to high N versus those from plants without access to N
451 revealed a set of 1,398 differentially regulated genes (760 up-
452 regulated, 638 down-regulated; $p < 0.05$; minimum of 5 times
453 differential regulation; Supporting Information Table S2). This early
454 time point was chosen to consider the differential regulation of
455 genes that may impact the eventual formation of mature
456 mycorrhizal root tips, as our results demonstrated that plant
457 available N had a significant effect on mycorrhizal root tip formation.
458 At this time point, fungal mycelium had begun to wrap around
459 lateral roots and metabolic and transcriptomic responses to contact
460 were already occurring (Wong *et al*., 2019), but no symbiotic
461 structures were formed. As these roots were in contact with the
462 fungus in the symbiotic compartment, any differentially regulated
463 genes represent a systemic effect of the nutrient status of roots
464 within the plant compartment. GO term enrichment analysis showed
465 a variety of significantly ($p < 0.01$) enriched terms in the area of

466 biological process (Fig. **3**, Supporting Information Table S3).
467 Particularly, stress response pathways were altered; with an
468 increase in expression of oxidative stress associated genes and a
469 corresponding decrease in hydrogen peroxide catabolic and redox
470 processes. Osmotic stress pathways were also up-regulated. Cell
471 wall polysaccharide associated genes were significantly up-
472 regulated, while lignin catabolism was down-regulated, pointing to a
473 potential strengthening of cell walls. There was also a shift in cell
474 communication and signalling pathways.
475
476 185 of the differentially regulated genes (95 up-regulated, 90 down-
477 regulated), representing 13.2 % of all significantly regulated genes,
478 were classified as disease resistance proteins, including leucine-rich
479 repeat (LRR) receptor kinases. Additionally, another 49 membrane
480 bound receptors (20 up-regulated, 29 down-regulated) were
481 differentially regulated. This suggests a substantial shift in the
482 network used by the roots to sense and respond to the surrounding
483 environment and may affect the ability of the plant to perceive and
484 respond to the fungus. Also of interest, 13 galactinol synthase
485 genes, one raffinose synthase and three stachyose synthase genes
486 were significantly up-regulated, suggesting increased flux into the
487 synthesis of raffinose family oligosaccharides.
488
489 *Plant responses to N availability caused transcriptomic changes in*
490 *the fungal secretome*
491 When *P. albus* was kept in a consistent nutrient environment but the
492 plant partner was given access to high N, a slight change in the
493 fungal transcriptome is seen 48-hours after contact. Overall, 404
494 genes were significantly differentially regulated in the fungus (272
495 up-regulated, 132 down-regulated; $p < 0.05$; minimum of 2 times
496 differential regulation; Supporting Information Table S4). Of these
497 genes, 17.1 % had a predicted secretion domain. Of note, some
498 nutrient transporters were down-regulated, including a nitrate

499   transporter and a phosphate permease. Two ammonium

500   transporters were also differentially regulated, one up and another

501   down. Genes for secreted proteins involved in host cell wall

502   degradation, including pectin lyases were down-regulated while five

503   heat shock proteins were up regulated. A fungal hydrophobin,

504   important in the establishment of symbiosis (Plett *et al*., 2012), was

505   also down-regulated. Overall the transcriptomic data showed a shift

506   in the production of metabolic proteins, nutrient transporters, a

507   slight increase in stress response (e.g. heat shock proteins) and a

508   change in the fungal secretome.

Discussion

The subject of what drives nutrient exchange between ECM fungi and their hosts is one of great interest within the literature (Müller *et al*., 2007; Garcia *et al*., 2015). While there is little dispute that nutrients are exchanged between the two partners during ECM symbiosis, the factors driving resource exchange are poorly understood. Understanding this is especially important given the finding that inorganic N deposition in forest soils is leading to a reduction of ECM symbiotic associations and communities in forests (Lilleskov *et al*., 2002; Parrent *et al*., 2006; Pardo *et al*., 2011; Morrison *et al.*, 2016; Corrales *et al*., 2017; Averill *et al*., 2018). This effect is not restricted to forests in the Northern Hemisphere; the decline of Eucalyptus forests in Australia has also been linked to the reduction of ECM associations caused, in part, by inorganic N addition (Horton *et al*., 2013). Our results add important information into this subject by determining the effect that N fertilization has on the plant host response to ECM fungi and how increased exogenous C-availability to the fungus affects nutrient transfer in the model interaction between *E. grandis* and *P. albus*. We have shown that at low plant N availability, *E. grandis* was able to promote colonization of its roots, compared to when N was plentiful, where it acted to reduce the number of root tips colonized (Fig. **1b**). The amount of N acquired by *E. grandis* from its symbiont, however, was, in this experiment, independent of the plants' nutritional needs (Fig. **1d**). This means that N flux was not determined by a source/sink system, nor was it based on the level of root colonization by the fungus. Neither was it dependent on C transfer from plant to fungus as this was constant in all conditions. N transfer instead appears to be controlled by the ECM fungus, with the greatest transfer of N occurring when *P. albus* has access to less exogenous C.

The symbiotic transfer of N to a host can be considered as a two-step process where N is first acquired by the ECM fungus from the

542 environment and then transferred from the fungus to the host. Our
543 results suggest that the first step of this process is influenced by the
544 nutrient status of both the ECM fungus and the plant and that N
545 uptake is dependent on the availability of nutrients in the substrate.
546 *P. albus* acquired more N from the substrate when it was given
547 plentiful C, likely to support the growth of fungal biomass rather
548 than investment in symbiosis as fewer or equivalent numbers of
549 mycorrhizal root tips were formed. This is in contrast to another
550 study using the ECM fungus *Suillus grevillei* where similar high
551 levels of glucose in an *in vitro* system resulted in greater fungal
552 aggression and host, or even non-host, root colonization (Duddridge,
553 1986). The host nutrient status also appears to influence fungal N
554 uptake: when the plant has access to plentiful N, there was a
555 significant reduction in ^{15}N scavenged by the fungus as compared to
556 when the plant had limited N resources (Fig. **2b**). As the abiotic
557 environment of the fungus had not changed, this decrease in N
558 acquisition could stem from altered resource availability at the
559 plant-fungal interface or via a communication change on the part of
560 the plant. Another study found a dependence on C availability for
561 the decomposition and uptake of soil N by ECM fungi, suggesting
562 that C supply from the plant can influence the uptake of N by the
563 fungus (Rineau *et al*., 2013). While in our experiment there was no
564 difference in net C transfer between conditions, C flux across
565 individual mycorrhizal root tips could vary. Alternatively, even at 48
566 hours post-contact, prior to the formation of a mycorrhizal root tip, N
567 transporters in the fungus were found to be significantly
568 differentially regulated in response to a plant with high N resources.
569 Thus, a transcriptional change on the part of the plant from altered
570 resource availability may be sensed by the fungus, causing the
571 alteration in its N acquisition strategy. In either case, the reduction
572 in fungal N scavenging seen when the plant has high N resources
573 may in part explain observations showing that N fertilization can
574 cause ECM fungi to halt or slow hyphal growth, have reduced

575 biomass or reduced competitiveness against other soil organisms

576 (Bidartondo *et al*., 2001; Lilleskov *et al*., 2002; Nilsson & Wallander,

577 2003; Ekblad *et al*., 2016).

578

579 While the nutrient status of both symbiotic partners play a role in

580 the process of fungal N acquisition from the environment, N delivery

581 to the host appears to be primarily under the control of the fungus,

582 based on our results. While fungal acquisition of additional N

583 resources may over a longer term result in more N transfer, over the

584 time frame of our experiment, lack of access to N on the part of the

585 plant did not result in a significant change in N delivery from the

586 fungus. This analysis does not account for the N content of plant

587 roots, and therefore we cannot exclude the possibility that N

588 partitioning or N acquisition from the fungus may be different in

589 these tissues. However, increased exogenous C availability to *P.*

590 *albus* did result in both a decrease in the amount of N transferred to

591 the plant and a trend to fewer mycorrhizal root tips formed. In

592 experiments using plant shading to alter carbon transfer to ECM

593 fungi, Hasselquist et al. (2016) found a similar result with less N

594 delivery to plant tissues when carbon transfer was high (unshaded).

595 The decrease in N transfer in our experiment was not likely

596 associated with a scarcity of N resources for *P. albus* as colonies

597 with access to high C acquired more N from the substrate and

598 maintained a similar C/N ratio to those colonies with limited access

599 to C. Overall, these results correspond well with previous field-based

600 studies showing that under low N conditions, while trees allocate

601 more C to their roots (Högberg *et al*., 2010; Corrêa *et al*., 2011), this

602 does not appear to correspond to an increase in N gain from the

603 fungus (Corrêa *et al*., 2011; Näsholm *et al*., 2013; Valtenen *et al*.,

604 2014).

605

606 In addition to altering the balance of N uptake and transfer between

607 ECM fungi and their hosts, nutrient availability affected the number

608   of mycorrhizal root tips formed. Transcriptomic analysis of genes

609   differentially regulated in *E. grandis* roots at an early time point in

610   the symbiotic interaction suggests that the addition of N altered how

611   the plant perceived and responded to the presence of *P. albus*.

612   Specifically, we found that there was a large transcriptional

613   reprogramming for a number of disease resistance proteins and

614   membrane bound receptor kinases. Disease resistance proteins,

615   including those containing LRR-RLK (leucine-rich repeat receptor-like

616   protein kinase) domains, make up a large family and are found

617   extensively in the genomes of most plants (Wu *et al.*, 2016). Their

618   function is not well understood but they are thought to act as early

619   detector networks of microbial presence and/or effectors and alter

620   signal transduction accordingly to activate plant defence pathways

621   (McHale *et al.*, 2006; Smakowska-Luzan *et al.*, 2018). Similarly,

622   membrane bound receptor proteins such as lectin receptor kinases

623   are able to sense the presence of microbial elicitors and trigger

624   downstream defence pathways (Singh & Zimmerli, 2013; Tang *et al.*,

625   2017). Thus, the large shift in transcription of these types of genes

626   may signify that the plant has altered how it perceives its external

627   environment and may change its response to the colonizing fungus.

628   Increased expression of galactinol, raffinose and stachyose synthase

629   genes, often up-regulated in response to stress or to defend against

630   a pathogen (Sengupta *et al.*, 2015), may result in lower pools of

631   simple carbohydrates available for exchange with a symbiont.

632   Generally mutualistic interactions are characterized by plant cell

633   wall softening and decreased ROS (Plett & Martin, 2017), however,

634   in our data set, N addition resulted in enrichment of genes

635   associated with plant cell wall lignification and increased ROS. Thus,

636   the addition of high levels of inorganic N to a plant causes a

637   systemic transcriptomic shift in tree roots that is overall inhospitable

638   to ectomycorrhizal colonization.

639

640   Beyond resource availability in the medium, other factors can affect

641   the level of *E. grandis* root colonization in an *in vitro* system.

642   Elevated levels of CO_2 were shown to reduce the colonization ability

643   of this same isolate of *P. albus*, while in some other related

644   *Pisolithus* isolates, elevated CO_2 increased colonization (Plett *et al.*,

645   2015). It was hypothesized that some *Pisolithus* isolates were more

646   easily able to adapt to or benefit from the plant transcriptional re-

647   programming resulting from the increase in CO_2. Thus, in a similar

648   manner, the effects of N availability on root colonization may be

649   influenced not only by the transcriptomic response of the plant to its

650   nutrient rich environment, but also by the individual fungal

651   susceptibility to that response. In another study involving the

652   interaction of *E. grandis* with the closely related *P. microcarpus*, *E.*

653   *grandis* was shown to reduce the number of root tips colonized by a

654   less cooperative symbiont (delivering less N for C than its

655   competitor) when a more cooperative competitor was present

656   (Hortal *et al.*, 2017). Transcriptomic analysis showed that plant

657   defence genes were up-regulated only in roots in contact with the

658   less cooperative symbiont. While in both the Hortal study (2017)

659   and in our present study, this altered defence signalling was

660   correlated to decreased formation of ECM root tips, it is interesting

661   to note that both C transfer to the fungus and N received in return

662   was unchanged in the earlier study. It would be of interest to

663   explore potential benefits of this defensive reaction on the part of

664   the plant if it does not improve either the costs or benefits of

665   symbiosis.

666

667   Overall, here we have significantly expanded our knowledge on how

668   nutrient trading occurs in the interaction between *E. grandis* and *P.*

669   *albus*. We have shown that plentiful nutrient resources for the plant

670   partner reduced root colonization and that, while the nutrient status

671   of *E. grandis* may influence *P. albus* to scavenge for N, the plant was

672   unable to influence the transfer of that N to its own tissues over the

673   timeline of our experiment. N transfer rather, was influenced by the

674   C needs of the fungus. It remains to be seen if this observation

675   holds in other ectomycorrhizal host pairings and on larger scales,

676   however, this substantially advances our understanding of the

677   effects of increased C/N availability on ECM fungal function in

678   natural ecosystems and assists in understanding and modelling

679   nutrient transfers in this important interaction.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

KLP, JMP and ICA designed the research. KLP and MW performed experiments. Data was analysed by KLP and VS and curated by VS, VN and IVG. Funding acquisition and project supervision was managed by ICA, JMP, FM, IVG and VN. KLP wrote the manuscript, which was reviewed by all authors.

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| Experimental Outcomes | Factors | | | | | | Number of biological replicates /condition |
| | Fungal C availability | | Plant N availability | | Fungal C x Plant N | | |
	F-ratio	P-value	F-ratio	P-value	F-ratio	P-value	
% Root tips Colonized	2.3786	0.131	12.2034	**0.001**	0.3962	0.533	9-12
Plant C/N ratio	0.4929	0.615	24.466	**1.66E-5**	0.1916	0.827	5-10
N transferred to plant leaves from symbiosis (mg)	12.9987	**0.0048**	2.9597	0.116	0.0011	0.974	3-4
C transferred to fungus via plant (μg)	0.2479	0.626	1.6728	0.217	0.0011	0.974	3-5
% of total N as ^{15}N in mycelium	13.0666	**0.003**	22.7347	**0.0003**	0.1981	0.663	3-5
Fungal C/N ratio	0.0066	0.936	0.2160	0.807	0.2310	0.795	6-10

989

990 **Table 1 Results from two-way ANOVA examining the effect of**
991 **fungal C and plant N availability and their interaction**
992 **(Fungal C x Plant N) on experimental outcomes.** Significant P
993 values (<0.05) are represented in bold type.

Figure Legends

Figure 1: Mycorrhizal root tip formation and *E. grandis* N acquisition is affected by the nutrient status of the plant and fungus. (a) Schematic of experimental set up showing the fungal-only nutrient compartment (FC), the plant-only nutrient compartment (PC) and the common symbiosis compartment (SC). (b) Percent lateral root tips colonized in *E. grandis* with (light grey bars) or without (dark grey bars) access to N in contact with *P. albus* with access to either high (10g/L) or low (1.0 g/L) glucose. (c) C to N ratio in the leaves of *E. grandis* with (light grey bars) or without (dark grey bars) access to N grown axenically or in contact with *P. albus* with access to either high (10g/L) or low (1.0 g/L) glucose. (d) Average amount (in µg) of N in the leaves of each *E. grandis* seedling (with (light grey bars) or without (dark grey bars) access to N) acquired from *P. albus* accessing either high (10g/L) or low (1.0 g/L) glucose. +/- SE.

Figure 2: *P. albus* N acquisition is affected by both the nutrient status of the plant and fungus. (a) Estimated average µg C in each *P.albus* colony that is from a plant accessing high N, or a plant without access to N and given either high (10g/L; light grey bars) or low (1.0g/L; dark grey bars) glucose. +/- SE. (b) Percentage of N in *P.albus* that is ^{15}N when grown axenically, with a plant accessing high N, or a plant without access to N and given either high (10g/L; light grey bars) or low (1.0g/L; dark grey bars) glucose. +/- SE. (c) C/N in fungal tissues grown axenically, with a plant accessing high N, or a plant without access to N and given either high (10g/L; light grey bars) or low (1.0g/L; dark grey bars) glucose. +/- SE.

Figure 3: GO term enrichment analysis identifies several enriched biological functions. (a-b) Scatterplot representation of

1027 enriched GO terms corresponding to Biological Process in genes up-

1028 regulated (a) or down-regulated (b) in *E. grandis* accessing high

1029 plant available N as compared to no plant available N after 48 hours

1030 of contact with *P. albus*. Dot sizes correlate to the number of

1031 significantly regulated genes in the data set assigned to that GO

1032 term and dots are spatially grouped based on GO term similarity.

1033 (c-d) Histogram giving the full list of enriched Biological Process GO

1034 terms for the data set and the corresponding –log10(p-value) for up-

1035 regulated (c) and down-regulated (d) genes. Significantly regulated

1036 genes show at least a five-fold change in expression ($p<0.05$; n=3).

1037 Enrichment analysis and graphical representations were generated

1038 using the PlantRegMap and ReviGo online programs respectively.

1039

1040

1041 **Supporting Information Legends**

1042

1043 **Supporting Information Figure S1: Biomass of plant leaves**

1044 **(a), total leaf N content (b), % ^{15}N in plant leaves with**

1045 **unlabeled control plant value indicated by dashed line (c),**

1046 **and percent of total plant leaf N derived from transfer (%**

1047 **NDFT) (d). +/- SE. Results from two-way ANOVAs**

1048 **(unbalanced, Type III) for the effect of carbon (C) or nitrogen**

1049 **(N) availability and their interaction (CxN) on experimental**

1050 **outcomes are indicated (significant results p<0.05).**

1051

1052 **Supporting Information Figure S2: (a) Biomass of fungal**

1053 **control colonies, +/- SE; with significant results (t-test;**

1054 **p<0.05) indicated with an asterisk (*). (b) percent of total**

1055 **fungal C obtained via symbiosis, +/- SE. Results from two-**

1056 **way ANOVAs (unbalanced, Type III) for the effect of carbon**

1057 **(C) or nitrogen (N) availability and their interaction (CxN) on**

1058 **experimental outcomes are indicated (significant results**

1059 **p<0.05). (c)**

1060 **Average %13C values for growth chamber air and media**

1061 **glucose (black bars) and leaf and fungal tissues generated**

1062 **in the experiment (light grey bars = axenic controls; dark**

1063 **grey = test samples), +/- SE.**

1064

1065 **Supporting Information Table S1: Composition of media used**

1066 **for the experiment**

1067

1068 **Supporting Information Table S2: List of differentially**

1069 **regulated genes, log2(fold change) and annotation in *E.***

1070 ***grandis* accessing high plant available N, as compared to no**

1071 **plant available N, 48 hours after contact with *P. albus*.**

1072 **Genes shown have at least a five-fold difference in**

1073 **expression (p<0.05; n=3).**

1074

1075 **Supporting Information Table S3: Complete listing of GO**

1076 **terms significantly enriched in the area of biological process**

1077 **(p<0.01) for significantly differentially regulated genes in**

1078 **the *E. grandis* dataset (p<0.05) when accessing high N, as**

1079 **compared to no N, 48 hours after contact with *P. albus*. Data**

1080 **generated by the online platform PlantRegMap.**

1081

1082 **Supporting Information Table S4: List of differentially**

1083 **regulated genes, log2(fold change), annotation and signal**

1084 **peptide probability in *P. albus* 48 hours after contact with *E.***

1085 ***grandis* accessing high plant available N as compared to no**

1086 **plant available N. Genes shown have at least a two-fold**

1087 **difference in expression (p<0.05; n=3).**

1088
1089