

1 **THE BENEFITS OF MYCORRHIZAE ARE FREQUENCY-DEPENDENT: A CASE STUDY WITH A NON-**
2 **MYCORRHIZAL MUTANT OF *PISUM SATIVUM***

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20 **AUTHOR CONTRIBUTIONS**

21 GGM, FCG, JLB and CAW designed the experiment, and carried out early (unpublished) pilot studies.
22 GGM, AES, ASM and MMF carried out the experiment, and harvest. ASM and CAW measured
23 mycorrhizal colonisation. GGM performed the stable isotope analysis, analysed the data, derived the
24 model, drafted the manuscript, and all authors contributed to revisions.

25 **ABSTRACT**

26 1. Mutualisms are remarkably common in the plant kingdom. The mycorrhizal association which
27 involves plant roots and soil fungi is particularly common, and found among members of the
28 majority of plant families. This association is a resource-resource mutualism, where plants trade
29 carbon-based compounds for nutrients, such as phosphorus and nitrogen, mined by the fungi.

30 2. Evolutionary models usually assume that a mutation grants a small number of individual plants
31 the ability to associate with mycorrhizal fungi, and that this subsequently spreads through the
32 population resulting in the evolution of mutualism. This frequency-dependent hypothesis has been
33 difficult to test, because it is rare to have members of the same species that are capable and
34 incapable of forming the mutualism.

35 3. Here we describe the results of an experiment that took advantage of a mutant pea (*Pisum*
36 *sativum* L. R25) that is incapable of forming mycorrhizal (or rhizobial) associations, and differs from
37 the wildtype (*P. sativum* cv. Sparkle) by a single recessive Mendelian allele (*Pssym8*). We grew each
38 genotype either alone or in every combination of pairwise mixed- or same-genotype. We also
39 present an evolutionary matrix game, which we parameterize from the experimental ¹⁵N results,
40 that allows us to estimate the costs and benefits of the mutualism.

41 4. We find that there was no difference between R25 and WT when grown with a competitor of the
42 same genotype, but when R25 and WT compete, WT has a significant fitness advantage. From the
43 model, we estimate that the benefit in units of fitness (g pod mass) obtained from direct plant
44 nitrogen uptake is 22.2 g, and mycorrhizae increase this by only 0.6 g. The costs of plant nitrogen
45 uptake are 9.4 g, while the cost of trade with mycorrhizae is 0.1g.

46 5. From the model and experiment, we conclude that this relatively small cost-benefit ratio of the
47 mycorrhizal association is enough to drive the evolution of mutualism in frequency-dependent
48 selection. However, without the mutant R25 genotype we would not have been able to draw this
49 conclusion. This validation of frequency-dependent evolutionary models is important for continued
50 theoretical development.

51 INTRODUCTION

52 Mutualisms are remarkably common within the plant kingdom. For example, estimates
53 suggest that 80% of terrestrial plant species (92% of families) associate with soil fungi to form a
54 mutualistic mycorrhizal association (Wang & Qiu 2006). The mycorrhizal association is a resource-
55 trading mutualism where plants exchange carbon-based compounds for mineral nutrients with
56 fungi. Historically, it was thought that mycorrhizae primarily provided plants with phosphorus
57 (Sanders & Tinker 1973), but recent work has shown that arbuscular mycorrhizal fungi also transfer
58 substantial amounts of nitrogen (N) to plants from decomposed organic material (Leigh, Hodge &
59 Fitter 2009; Hodge & Fitter 2010). Nitrogen is generally the main limiting resource for terrestrial
60 plants, and thus obtaining N via mutualism has the potential to dramatically affect plant fitness
61 (Chapin, Vitousek & Vancleve 1986; Vitousek & Howarth 1991).

62 Indeed, analysis of evolutionary models suggests that mutualisms such as mycorrhizae
63 evolve when there is a fitness advantage to individuals that engage in cooperative mutualisms
64 relative to those individuals that do not engage in mutualism (Nash 1950; Axelrod & Hamilton 1981;
65 Denison *et al.* 2003). Though the details differ, these eco-evolutionary models begin with an
66 assumption that the ancestral population had no mutualism, then an individual undergoes some
67 mutation(s) that give it access to a mutualistic partner. While mutation is necessary for evolution by
68 natural selection, on its own, mutation is not sufficient to cause a stable evolutionary change. Once
69 mutation confers the mutualism ability, those mutant individuals must subsequently out-compete
70 non-mutualistic residents in the population, resulting in the mutualism spreading through the
71 population. While this is logically intuitive, and supported by a wide array of models, testing this
72 proposition is difficult because it requires a mixture of individuals from the same species that can
73 and cannot form a mutualistic association.

74 It is straightforward to assess competition among plants which are either in a mutualistic
75 relationship or are not through the use of some combination of soil sterilization or inoculation
76 experiments (Fitter 1977; Allen & Allen 1984; Moora & Zobel 1996; Hodge *et al.* 2000; Hodge 2001;

77 Hodge 2003). In general, these studies find that plant-plant competition is stronger when plants
78 associate with mycorrhizae than when they do not (Fitter 1977; Hodge 2003), or that there is no
79 difference among competing plants with or without mycorrhizae (Allen & Allen 1984; Moora & Zobel
80 1996). At first glance, this seems to call into question the evolutionary models of mutualism.
81 However, these studies assume static relationships between the strategy (e.g. mutualism vs no
82 mutualism) and fitness of an individual regardless of the strategies of its neighbours, thus neglecting
83 tests of frequency-dependent selection. The key experimental treatment required to examine the
84 frequency-dependent evolution of mutualism, which would contain a mixture of mycorrhizal and
85 non-mycorrhizal competitors from the same population, was absent from all of these studies
86 because this situation is typically not possible to experimentally create.

87 Here, we combine a frequency-dependent evolutionary matrix game of cooperation with an
88 experiment that makes use of mutant peas which cannot associate with mycorrhizae. Using a model,
89 and an experiment designed to test the model, we ask: (1) what conditions are theoretically
90 necessary for the mycorrhizal association to be an evolutionarily stable strategy (ESS)? We then test
91 the model using a unique mutant of pea (*Pisum sativum* L. R25 (*Pssym8*)) which has been shown to
92 be incapable of forming arbuscular mycorrhizal associations (Balaji *et al.* 1994; Guinel & Geil 2002).
93 Using the experiment, we ask: (2) how does mycorrhizal association across frequency-dependent
94 competitive contexts change reproductive and vegetative yields?; (3) how does the competitive
95 context change the mycorrhizal colonization of wildtype pea roots?; and (4) how does the
96 mycorrhizal association across competitive contexts alter N uptake? Finally, (5) we combine the
97 model and experiment to estimate the costs and benefits of gathering nutrients with and without
98 the mycorrhizal association by solving model equations. Combined, we illustrate how the benefits of
99 mutualism in this case were indeed frequency-dependent; moreover, without the unique mutant,
100 we would have concluded that there were no benefits to this mutualism.

101

102 **METHODS**

103 *Evolutionary game theoretic Model*

104 Evolutionary game theory is useful for examining frequency-dependent interactions.
105 Following the logic of classic evolutionary games (e.g. the prisoners' dilemma Axelrod & Hamilton
106 1981; Poundstone 1992), we derived a simple matrix game with benefits and costs that allowed us
107 to conceptualize the competition between the R25 mutant and the wildtype (WT) cv. Sparkle based
108 only on their mutualistic associations.

109 Let N_p be the nutrients that are plant-available, and let N_m be the nutrients that are fungus-
110 available and traded to the plant. We assume N_m is derived from a separate pool of organic
111 nutrients that the plants cannot access without the aid of the mycorrhizal fungus (e.g. Hodge 2001;
112 Leigh, Hodge & Fitter 2009). Similarly, let c_p be the cost associated with nutrient uptake by the plant
113 (e.g. root tissue production, ATP synthesis) and let c_m be the cost to plants of obtaining nutrients via
114 mycorrhizal association (e.g. carbohydrate or lipid trade; (Rich *et al.* 2017)). Since our goal was to
115 consider competition within our own pot experiment, the model considers competition between
116 two plants with a fixed pool of resources, but one could extend this to any number of plants by
117 replacing 2 in any model equations with x , where x is the number of individuals interacting.

118 The two pea genotypes (R25 and WT) create four possible combinations of pair-wise
119 competition. First, when two R25 mutants compete, they only have access to plant-available
120 nutrients and we assume that on average each individual plant would access half of those nutrients.
121 R25 plants only pay the cost of obtaining nutrients that are plant-available (Fig 1a). Second, when
122 R25 competes with WT, the outcome for the R25 plant is the same: they compete and obtain only
123 half the available nutrients, and pay the cost of getting those nutrients themselves (Fig 1a).
124 However, when WT competes with R25, it has access to two nutrient pools. WT competes for the
125 plant-available pool of nutrients with R25 obtaining only half on average and paying the cost of
126 obtaining those nutrients, but WT also has access to 100% of the potentially tradeable pool of
127 nutrients accessible by the fungus, and incurs the cost associated with trading for those nutrients

128 (Fig 1a). Finally, when two WT plants compete, they share both nutrient pools on average, and pay
129 both costs.

130 These interactions described above can be summarised in a two by two payoff matrix (Fig
131 1a) and by the following equations:

$$132 \quad G_{R25,R25} = \frac{N_p}{2} - c_p, \quad \text{Eqns 1}$$

$$133 \quad G_{R25,WT} = \frac{N_p}{2} - c_p,$$

$$134 \quad G_{WT,R25} = \frac{N_p}{2} + N_m - c_p - c_m, \text{ and}$$

$$135 \quad G_{WT,WT} = \frac{N_p}{2} + \frac{N_m}{2} - c_p - c_m,$$

136 Where $G_{i,j}$ is individual fitness of focal plant i competing against plant j in each of the four possible
137 pairwise interactions between R25 and WT.

138

139 *Evolutionary stable strategy definition*

140 In a matrix game, an ESS is identical to a Nash equilibrium (Maynard Smith & Price 1973;
141 Apaloo *et al.* 2014). An ESS is a strategy (or strategies) which once adopted by members of a
142 population leads to that population not being invaded by any alternative strategy (or strategies).
143 Mathematically, if we imagine that fitness is a function of strategies such that $G(v, u)$ is the fitness
144 payoff of a focal player using strategy ' v ' against a neighbour using strategy ' u ' such that $v \neq u$, then
145 v is a pure ESS if and only if: $G(v, v) > G(u, v)$ and $G(v, u) > G(u, u)$. Alternatively, u is a pure ESS
146 when the inequalities are reversed. Importantly, under this definition, mixed ESS solutions are
147 possible where the two strategies may coexist ($G(u, v) > G(v, v)$ and $G(v, u) > G(u, u)$). In
148 addition, priority effects are also possible where both strategies are ESS, but only one can occur at a
149 time depending on the history of mutation ($G(v, v) > G(u, v)$ and $G(u, u) > G(v, u)$). Importantly,
150 the ESS can theoretically exist whether or not the exact equilibrium is ever achieved in nature
151 (Maynard Smith 1982).

152

153 *Plant material*

154 To examine the effects of mycorrhizal association on competition in a frequency-dependent setting,
155 we took advantage of the pea mutant R25 which has been characterized as a non-mycorrhizal
156 mutant. The R25 mutant was obtained from a background of the cultivar 'Sparkle' and was identified
157 from a screen of gamma-radiated seeds (Markwei & LaRue 1992). Remarkably, the non-mycorrhizal
158 phenotype is controlled by a single locus (*sym8*) that exhibits simple Mendelian dominance (Balaji *et*
159 *al.* 1994). This means that the mycorrhizal and non-mycorrhizal plants, which we will use in our
160 comparison, have a constant genetic background save for a single allele difference. This is exactly
161 the type of evolutionary situation most models envision, where a rare mutant emerges and
162 potentially invades a resident population. This allows us to test evolutionary theory in a novel way.

163

164 *Fungal material*

165 The AM fungal strain used in this study was *Rhizophagus irregularis* ((Blaszk., Wubet, Renker
166 & Buscot) C. Walker & Schuessler 2010 as ['irregulare']) cv. DAOM 197918 originally obtained from
167 the Agriculture and Agri-Food Canada Glomeromycota in vitro collection (AAFC, Ottawa, ON,
168 Canada) and propagated by FCG using leek (*Allium ampeloprasum* (L.) cv. *muhlenbergii*) as a host. At
169 maturity, leek roots were assayed for mycorrhizal structures and, if present, the leek soil was stored
170 dry at 4°C.

171

172 *Experimental treatments*

173 There were four competition treatments that match the four cells of the matrix game (Fig
174 1a). Treatments included: (i) WT versus WT; (ii) WT versus R25; (iii) R25 versus WT and; (iv) R25
175 versus R25 (Fig 1b). In each competition treatment, one of the two plants was randomly designated
176 *a priori* as 'focal', and the other as 'neighbour'. With this design, neighbour plants were only present
177 to impose competition, and only the focal plants would eventually be harvested. Thus, even though
178 the R25 versus WT and WT versus R25 treatments were essentially identical, we planted both and

179 measured them independently to avoid pseudoreplication. In addition to the competition
180 treatments, there were also two controls where plants of each genotype were grown alone (Fig 1b).

181 There is some debate in the literature on the appropriate control in competition
182 experiments. Should one control for the nutrient environment (i.e. total nutrients provided per
183 plant, and soil nutrient concentration; (e.g. Gersani *et al.* 2001; McNickle & Brown 2014; Chen *et al.*
184 2015a)), or should one control for volume but not for the nutrient environment (e.g. Hess & De
185 Kroon 2007; Chen *et al.* 2015a)? Unfortunately, it is difficult to control both nutrients and pot
186 volume without confounding one of them with neighbour addition (McNickle *in press*), and thus one
187 assumption of our design was that both nutrient availability and soil nutrient concentration are
188 more important for belowground plant-plant competition than pot volume. Thus, in total there were
189 four competition treatments (all possible pairs of R25 and WT), and two no-competition controls
190 (two genotypes grown alone) replicated 15 times for a total of six treatments and 90 pots (Fig 1b).

191

192 *Greenhouse conditions*

193 All plants were grown in 15-cm diameter, 15-cm high, standard plastic pots in the Wilfrid
194 Laurier University greenhouse in Waterloo, Ontario, Canada (43°28'28.1"N, 80°31'15.2"W) from June
195 1 to July 29, 2015. Artificial light was not used yielding approximately 15h:9h light:dark at this
196 latitude and time of year. Temperature was maintained at approximately 25°C. The model assumed
197 that all interactions occurred belowground, thus screens of corrugated white plastic were erected in
198 the middle of each pot so that plants could not interact above-ground (Fig 1b). Soil was a 1:1
199 mixture of peat moss (Greenworld Garden Products, Pointe-Sapin, New Brunswick, Canada), and
200 calcined clay gravel (Turface® MVP, PROFILE Products LLC, Buffalo Grove, Illinois, USA). In addition,
201 we added to the soil, at a rate of 0.01% by volume, ground WT pea shoots which had been labelled
202 beforehand with elevated ¹⁵N by growing them with labelled ammonium nitrate. Our rationale for
203 this was that organic sources of nitrogen are known to be more readily available to the fungus than
204 to the plant (Hodge 2001; Leigh, Hodge & Fitter 2009; Hodge & Fitter 2010), and the isotopic

205 signature of the plants would let us determine nitrogen trade. This entire soil mixture was then
206 autoclaved at 120°C for 20 minutes in 10L batches.

207 Seeds of each pea genotype were surface-sterilised in 0.4% sodium hypochlorite for ten
208 minutes, rinsed three times with deionized water, and then soaked in deionized water for 18 hours
209 prior to planting. Only those seeds that had swollen and imbibed water were planted to maximize
210 germination. To inoculate pots, the sterile soil mixture described above was mixed with soil from the
211 leek trap cultures at a ratio of 1:9. Since R25 cannot form associations with mycorrhizae, all soil in
212 the experiment was inoculated with live culture as a control, even soil on which the non-mycorrhizal
213 R25 would eventually grow.

214 Nutrients were added as a mineral nutrient solution (Miracle Grow® all-purpose water
215 soluble plant food, Scotts Canada Ltd, Mississauga, Ontario, Canada). Plants were fertigated with
216 200mL of 0.5g/L nutrient solution every 7 days on Monday afternoons and then irrigated with 200mL
217 of water every 7 days on Friday mornings. Fertigation began when the plants were 14 days old; so,
218 for the first 14 days plants only received water on Mondays and Fridays. Each pot in the entire
219 experiment was placed in its own individual circular tray. The trays collected any water or nutrients
220 that drained through the pot making each pot its own closed nutrient system, and where water was
221 only lost through evaporation or transpiration. Pots were arranged in a randomized block design to
222 control for potential microclimate effects within the greenhouse. In addition, because of the screens
223 erected to block competition for light, pots were turned one quarter turn every Monday and Friday
224 to minimize the potential effects of any shading from the screens. The appropriate nutrient
225 concentration used was determined through a pilot experiment (Supplementary information).

226

227 *Harvest*

228 All plants were grown for 60 days until senescence began, and were then harvested. At
229 harvest, above ground material was sorted into shoots and fruits. These samples were dried at 60°C
230 and weighed. Below ground, fourteen 3cm-long root fragments were randomly sampled from each

231 focal plant in each pot; they were stained to assess mycorrhizal colonization as described below. In
232 the competition treatments, we could work down from the stems of each plant to ensure that we
233 collected only root fragments that belonged to focal plants. These root fragments were air-dried
234 until staining occurred. The remainder of the roots were washed on a 2mm sieve, dried and
235 weighed. For total root biomass, we attempted separation but were unable to separate the root
236 systems of the two plants.

237

238 *Estimation of mycorrhizal colonisation*

239 Dried root fragments were rehydrated in deionized water for 25 minutes in 1.5mL centrifuge
240 tubes before staining (Vierheilig *et al.* 1998). Briefly, root fragments were cleared in 10% KOH in two
241 steps (one at 95°C for 10 minutes, and the other at 95°C for 5 minutes). Cleared root fragments were
242 rinsed twice with 5% acetic acid and stained with a 1:20 (v:v) mixture of black Indian ink (Scheaffer
243 Pen and Art Supply Co., Providence, Rhode Island, USA) and 5% acetic acid at 95°C for five minutes.
244 Fragments were de-stained with 5% acetic acid for 18 hours, and vacuum-infiltrated in glycerol (30%
245 and 60%, 20 minutes each); on each slide, 7 root fragments were mounted in 60% glycerol. In total,
246 we collected 14 fragments per plant and thus there were two slides per plant for a total of 240
247 slides.

248 Mycorrhizal colonisation was scored according to the magnified intersections method
249 described in McGonigle *et al.* (1990). All intersections between the root and the eyepiece cross hair
250 were examined for colonisation, and the depth of view was adjusted to examine the entire root. At
251 each intersect we counted the number of (a) arbuscules; (b) vesicles; and (c) intra-radicular hyphae;
252 we also noted any absence of mycorrhizal structure. These data allowed us to calculate the
253 proportion of root length colonized by each of the three mycorrhizal structures.

254

255 *Stable isotope analysis*

256 Shoot material (leaves and stems) of focal plants were ground to a fine powder using a bead
257 mill (Mixer Mill MM400, Retsch GmbH, Haan, Germany). Approximately 3mg of ground tissue was
258 weighed into tin capsules (Part number 041061, Costech Analytical Technologies Inc, Valencia, CA,
259 USA). These samples were then processed by the University of California Davis Stable Isotope facility
260 using a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio
261 mass spectrometer (Sercon Ltd., Cheshire, UK). Importantly, and perhaps counter-intuitively, due to
262 fractionation of ^{15}N , lower levels of enrichment indicate more trade.

263

264 *Data analysis*

265 In general, all data were analysed using general linear mixed effects models (GLMM) in R
266 (v3.3.2) using the lme4() and lmerTest() libraries, a type III sum of squares, and the Satterthwaite
267 approximation for denominator degrees of freedom when needed (Bates 2007; R-Development-
268 Core-Team 2009). To find adequately fitting models, we first tried to adjust to alternative probability
269 distributions, and failing that, we transformed data if necessary. A separate GLMM was fit for: i)
270 shoot, fruit, and root mass; ii) percent colonisation of intraradicular hyphae, arbuscules and vesicles,
271 and; iii) ^{15}N enrichment, and N concentration as detailed below for a total of eight analyses.

272 First, analysis of biomass data included the full factorial combination of treatment (mixed
273 genotypes grown together, same genotypes grown together, or plants alone) by genotype (WT or
274 R25 mutant) with block as a random intercept. Shoot and root biomass was analysed using a
275 Gaussian error distribution. Fruit biomass required $\log(x + c)$ transformation to achieve an adequate
276 model fit using a Gaussian error distribution, where c was half the smallest fruit mass measured.
277 Significance was assessed using an F ratio test and a type III sum of squares.

278 Second, mycorrhizal colonisation was analysed with only treatment (mixed genotypes grown
279 together, same genotypes grown together, or plants alone) and microscope slide nested inside block
280 as a random intercept since two slides were separately scored per individual. Since these were count
281 data which were then used to calculate a proportion of root length colonized, we analysed them as a

282 binomial GLMM where the response variable was the proportion of root length colonised, with the
283 logistic regression weighted by the total number of observations (Harrison 2015). Significance was
284 assessed with a type III Wald's Chi, which is appropriate for binomial mixed models (Bolker *et al.*
285 2009).

286 Finally, the nitrogen data were analysed in a manner similar to the biomass data with the full
287 factorial combination of treatment (mixed genotypes grown together, same genotypes grown
288 together, or plants alone) by genotype (WT or R25 mutant) with block as a random intercept.
289 Although N concentration and ¹⁵N enrichment were both proportion data, unlike the mycorrhizal
290 data they were not based on counts, making a binomial distribution inappropriate. We thus arcsine
291 square root transformed both N variables for continuity to achieve adequate model fits with a
292 Gaussian distribution.

293

294 RESULTS

295 Model

296 From the ESS definition, and eqns 1, then we find that R25 is the ESS when:

$$297 \quad \frac{N_p}{2} - c_p > \frac{N_p}{2} + N_m - c_p - c_m, \text{ and } \frac{N_p}{2} - c_p > \frac{N_p}{2} + \frac{N_m}{2} - c_p - c_m. \quad \text{eqn 2a}$$

298 Eqn 2a can be simplified to:

$$299 \quad N_m < c_m, \text{ and } \frac{N_m}{2} < c_m. \quad \text{eqn 2b}$$

300 Similarly, WT is ESS when the opposite conditions to eqn 2a are met,

$$301 \quad N_m > c_m, \text{ and } \frac{N_m}{2} > c_m. \quad \text{eqn 3}$$

302 Interestingly, there is only one more possible solution to this matrix game, which can be
303 interpreted as alternative stable states, where both strategies are ESS, but they cannot coexist.
304 Under this solution, priority effects determine which strategy would be found within the population,
305 but only one strategy can exist at a time. This occurs under the limited range of nutrients where
306 each genotype does better in monoculture than in mixture. That is when,

307 $N_m > c_m$, but $\frac{N_m}{2} < c_m$. eqn 4

308 In general, matrix games have a fourth possible solution where the two strategies coexist
309 within a population. However, this solution is not possible in this game because it requires

310 $N_m < c_m$, but $\frac{N_m}{2} > c_m$, eqn 5

311 which is logically impossible because $N_m > \frac{N_m}{2}$.

312 Since N_m and c_m are the only relevant parameters in determining the ESS, we can
313 summarise the conditions in eqns 1-5 graphically in N_m and c_m space showing all possible
314 combinations of nutrient availability and mutualism costs and the resulting ESS solutions (Fig 1c).

315

316 *Experiment: plant biomass*

317 Biomass was analysed with the competition treatment (same genotype, mixed genotype or
318 alone) crossed with focal genotype (R25 or WT) in a full factorial design that also included block as a
319 random effect.

320 Beginning with fruit biomass, which represents life-time reproductive effort (i.e. fitness) in
321 this annual plant, there was a significant interaction between treatment and genotype (Table 1).
322 Post-hoc comparisons revealed that this interaction was driven by the WT genotype grown in
323 competition obtaining significantly more fruit biomass than plants in other treatments (Fig 2a).
324 However, when WT was grown in competition with another WT, the fruit biomass was not
325 statistically different from that of R25 nor was it different from that of either genotype grown alone
326 (Fig 2a). For shoot biomass, only the genotypes were significantly different, and the WT was larger
327 than the mutant in all treatments (Table 1, Fig 2b). For root biomass, there were no significant
328 differences among treatments or genotypes (Table 1, Fig 2c). However, roots of the two competing
329 plants could not be separated; thus in the mixed genotype competition treatments, it is difficult to
330 draw conclusions about root biomass.

331

332 *Experiment: mycorrhizal colonisation*

333 Since we confirmed that the R25 mutant is physiologically incapable of forming an
334 association with arbuscular mycorrhizal fungi because of a mutation in the *sym8* locus (Fig S3; Balaji
335 *et al.* (1994)), the analysis only included the colonisation of the WT roots across the competition
336 treatments (alone, mixed genotype, or same genotype) with block as a random effect. There were
337 no significant differences in the proportion of root length colonised by either the intraradicular
338 hyphae ($\chi^2=3.04$, $df=2$, $p=0.2181$, Fig 3a) or the arbuscules ($\chi^2=2.02$, $df=2$, $p=0.3634$, Fig 3b).
339 However, there was a significant difference in vesicles among the treatments ($\chi^2=328824.0$, $df=2$,
340 $p<0.0001$, Fig 3c). All three treatments were different where plants grown alone had the highest
341 number of vesicles, followed by plants grown in mixed genotype competition, and finally by WT
342 competing with WT plants.

343

344 *Experiment: nitrogen trade*

345 Both ^{15}N enrichment and shoot N concentration were analysed with the competition
346 treatment (same genotype, mixed genotype or alone) crossed with genotype (R25 or WT) in a full
347 factorial design with block as a random effect. For ^{15}N enrichment, the main effects of treatment
348 and genotype were statistically significant, but their interaction was not (Table 2). Thus, we plotted
349 these data for each treatment and genotype (Fig 4a), as well as for each genotype with all
350 treatments combined (Fig 4b) to show most clearly the main effects in the absence of an interaction.
351 Post-hoc tests revealed that plants that experienced competition of any type were more enriched in
352 ^{15}N than plants grown alone (Fig 4a), while the R25 mutant was more enriched than the WT (Fig 4b)
353 as expected due to fractionation during mycorrhizal trade.

354 For total N concentration, only the genotype was statistically significant, and the WT had
355 higher N content than the non-mycorrhizal mutant (Table 2, Fig 4c). This indicates that mycorrhizal
356 trade led to fitness benefits directly through increased N.

357

358 *Combining model and experiment:*

359 From our harvest data, we can use the fruit mass of these annual plants as an estimate of
360 life-time fitness payoffs (G) to solve for the parameter values required to determine the ESS. To do
361 this, we combined means according to the GLMM and post-hoc analyses (Table 1, Fig 2a) to obtain
362 the payoff matrix shown in Table 3.

363 To estimate N_p we begin with the fact that we know that the R25 mutant obtained all of its
364 N through direct uptake as N_p , while the WT obtained some N itself as N_p and some through trade
365 as N_m (Fig S3, Fig3b). The pea genotypes significantly differed in the N concentration of their tissues
366 with the WT having significantly higher N concentrations than the mutant by an average of 2.7%. We
367 assume that the other 97.3% of N in the WT tissues must have come as N_p . Thus, if we assume that
368 WT plants have the same N_p as R25 plants, then we know that:

$$369 \quad N_p + N_m = 1.027N_p \quad \text{Eqn 7a}$$

370 And therefore that

$$371 \quad N_m = 0.027N_p \quad \text{Eqn 7b}$$

372

373 Substituting mean fitness as G and $N_m = 0.027N_p$ into eqns 1 gives us the following three
374 equations with three unknowns that can be rearranged to find:

$$375 \quad 1.73 = 0.5N_p - c_p, \quad \text{Eqns 8}$$

$$376 \quad 2.23 = 0.527N_p - c_p - c_m, \text{ and}$$

$$377 \quad 1.93 = 0.5135N_p - c_p - c_m,$$

378 The solutions to this set of equations are $N_p = 22.2$ (and therefore, $N_m = 0.6$ via eqn 7b), $c_p = 9.4$,

379 and $c_m = 0.1$. From eqns 2 and 3, the mycorrhizal association is expected to evolve when $\frac{N_m}{2} > c_m$,

380 and since $\frac{0.6}{2} > 0.1$, then mycorrhizal mutualism is an ESS within our model and experimental

381 system. It might seem trivial for our model to return what we already knew: that the wildtype was

382 the ESS, and the mutant artificially created by gamma radiation was not ESS. However, the value of

383 a validated model which we have failed to reject is that it can be exported to other systems to make
384 novel predictions.

385

386 **DISCUSSION**

387 Evolutionary models suggest that mutualism evolved because it conferred an advantage to
388 mutualistic individuals over non-mutualistic individuals in a population, and thus it became the ESS
389 (Axelrod & Hamilton 1981). However, such models are not always easy to empirically validate and
390 test. The game theoretic model we presented here which was specific to the R25 mutant pea
391 system supported this classical finding. Previous studies of plant-plant competition with and without
392 mycorrhizal associations have generally found that either competing plants perform worse in the
393 presence of the fungi (Fitter 1977; Hodge 2003), or that there is no difference among competing
394 plants with or without mycorrhizal partners (Allen & Allen 1984; Moora & Zobel 1996). Indeed, if we
395 had only considered R25 vs R25 and WT vs WT treatments in our study, though the shoots of WT
396 were larger than R25, we would have also concluded that there was no fitness advantage to the
397 mycorrhizal association (Fig 2a). However, the use of the R25 mutant allowed us to examine the full
398 frequency-dependent context, and it revealed that the mycorrhizal WT had an advantage in terms of
399 reproductive output only when it competes with a non-mycorrhizal R25 mutant. When this result is
400 placed within the fitness payoff matrix of the evolutionary game, it reveals that mutualism is an ESS
401 even though there was no difference in either pure population.

402 At first, the lack of difference in fitness within the R25 vs R25 treatment and WT vs WT
403 treatments might seem like evolution should be indifferent to whether plants form mycorrhizal
404 associations, but the key to frequency-dependence is its context-dependence. Using the notation
405 from our definition of ESS, we could write this result as $G_{R25,R25} = G_{WT,WT}$, but note that this
406 comparison is not relevant to the definition of the ESS that requires either $G_{WT,WT} > G_{R25,WT}$ and
407 $G_{WT,R25} > G_{R25,R25}$, or the opposite to achieve one of the two possible pure ESSs. That is, the
408 relative fitness of either pure population is entirely irrelevant to the question of what trait should be

409 favoured by natural selection. Since the ESS is about rare mutants invading pure populations, or pure
410 populations resisting invasion (Maynard Smith & Price 1973; Maynard Smith 1982), we cannot over
411 emphasize how important the mixed genotype (i.e. R25 vs WT and WT vs R25) treatments are to
412 understanding the frequency-dependent benefits of mutualism.

413 When combined, the model and the experiment allowed us to generate estimates of the
414 costs and benefits of mycorrhizal association. These calculations express both costs and benefits in
415 units of fitness under the assumption that N uptake and fitness are correlated (Fig S1c). We
416 estimated all the model parameters, but the most interesting is to compare $\frac{N_p}{2} - c_p$ and $\frac{N_m}{2} - c_m$,
417 which represent the net-benefit obtained by the plant's own foraging behaviour versus the net
418 benefit in increments of fitness obtained from association with mycorrhizal fungi, respectively. Since
419 $\frac{N_p}{2} - c_p = 1.73$, and $\frac{N_m}{2} - c_m = 0.2$, this means that mycorrhizal plants in this experiment gained
420 approximately 11% of their reproductive output as a direct benefit of the mutualistic association
421 with mycorrhizal fungi. In the WT vs R25 treatments, this 11% benefit manifested as a significant
422 increase in reproductive output (Fig 2a). However, in the WT vs WT treatment, both plants had this
423 benefit, and so it was not apparent (Fig 2a).

424 A key caveat to these conclusions is that we assume that N_p remains unchanged in both R25
425 and WT genotypes. This requires that the plant does not adjust its own foraging activities when
426 mycorrhizae are present. We feel that this is valid because the two genotypes differed only by one
427 allele at a single locus. Another caveat is that interactions between two plants are far from a
428 population-level context. Indeed, competition among two plants is the absolute minimum design to
429 examine such questions, but a range of population densities would allow a stronger test (Hart,
430 Freckleton & Levine 2018).

431 So far, we have only discussed the plants, but there are two partners in this association and
432 it is worth considering the success of the fungal partner. Arbuscule and intraradicular hyphae
433 colonisation did not vary among treatments, but we found a significant effect of our competition
434 treatments on the colonisation of vesicles inside pea roots such that the most vesicles were

435 contained in plants grown alone, fewer in the mixed genotype competition pots, and fewer still in
436 the same genotype competition pots (Fig 3c). Vesicles are storage organs of the fungi that
437 accumulate lipids and can also become propagules upon root death (Biermann & Linderman 1983).
438 Thus, one interpretation of our data is that potential fungal propagules (an admittedly weak
439 surrogate of fungal fitness) also declined along a gradient of increasing competition intensity for the
440 plants. This might suggest that there was increased fungal competition when the fungus was
441 connected to more than one host. This could be similar to a tragedy of the commons game among
442 plants, where plants over-proliferate tissues to maximize their competitive ability (Gersani *et al.*
443 2001; McNickle & Dybzinski 2013). This over-proliferation occurs in roots, leaves and stems and,
444 while ESS maximizes competitive ability, it tends to reduce reproductive output (Dybzinski *et al.*
445 2011; McNickle *et al.* 2016). Our results may suggest that the fungal partner, acting as an extension
446 of the root system, is also increasing its number, and length, of hyphae to maximize its competitive
447 ability. This allocation of resources towards growth of the extraradicular hyphae in the soil could be
448 at the expense of vesicle differentiation, as is indicated by the reduced number of vesicles (Fig 3c).
449 The evidence for this is weak, and we have no data about the fungus behaviour outside of plant
450 roots, but we suggest this is an interesting hypothesis that requires future attention.

451 Related to this possible tragedy of the commons response in the fungi, we highlight that no
452 over-proliferation leading to a tragedy of the commons was observed for the roots. This seems to
453 be a consistent finding for pea (Meier *et al.* 2013; Chen *et al.* 2015b) (McNickle in press, but see
454 (O'Brien, Gersani & Brown 2005)).

455

456 *Conclusion*

457 Here we combined an evolutionary game theoretic model and an experiment with loss of
458 function pea mutants that could not form mycorrhizal associations to show that the benefits of
459 mycorrhizal cooperation are frequency-dependent. We used a mutant pea genotype that differs by
460 one allele from the WT to examine the full factorial evolutionary context contained in models (Fig 1).

461 First, we showed that mycorrhizal association would be the ESS when half the potential fitness
462 benefits are greater than the costs of the association (Fig 1c). Second, we showed that the
463 reproductive output of mycorrhizal plants was only significantly greater than that of non-mycorrhizal
464 plants in the frequency-dependent context of WT versus mutant (Fig 2a). Third, the only fungal
465 structures that varied across competition treatments were the vesicle storage organs, the number of
466 which declined with increasing competition intensity of plants (Fig 3c). Finally, we combined the
467 experiment and the model to calculate the costs and benefits of plant nutrient capture and plant
468 trade with the mycorrhizal fungus; we estimated that 11% of mycorrhizal plant fitness was directly
469 attributable to the mutualism. Importantly, without the R25 mutant that is incapable of forming
470 mycorrhizal associations, we would have concluded that mycorrhizae conferred no advantage. This
471 result highlights the importance of frequency-dependent selection in ecology and evolution. We
472 propose that what has been commonly called the mutualism-parasitism continuum of mycorrhizal
473 associations (Johnson, Graham & Smith 1997) might be better described as a more beneficial – less
474 beneficial continuum.

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570

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Table 1: Results of GLMM on plant growth using the lme4() and lmerTest() libraries in R.

Denominator degrees of freedom (Den df) were estimated using the Satterthwaite approximation and a Gaussian distribution. The fruit mass data were $\log(x+c)$ transformed to achieve model fit, where c was half the smallest measured value. All models included block as a random intercept. Bold with * indicates statistical significance at $p < 0.05$.

Fruit				
Factor	Num df	Den df	F	p
Treatment	2	135	2.82	0.0633
Genotype	1	135	9.63	0.0023*
Treatment X Genotype	2	135	3.84	0.0239*
Shoot				
Factor	Num df	Den df	F	p
Treatment	2	143.0	0.05	0.9555
Genotype	1	143.0	6.95	0.0093*
Treatment X Genotype	2	143.0	1.22	0.2998
Root				
Factor	Num df	Den df	F	p
Treatment	2	124.1	1.92	0.1502
Genotype	1	123.1	0.21	0.6473
Treatment X Genotype	2	123.2	0.06	0.9420

572

573

Table 2: Results of GLMM on %¹⁵N enrichment and nitrogen concentration (% mass/mass) of plant shoot material using the lme4() and lmerTest() libraries in R. Denominator degrees of freedom (Den df) were estimated using the Satterthwaite approximation. All models included block as a random effect.

% ¹⁵ N enrichment				
Factor	Num df	Den df	F	p
Treatment	2	65.7	22.28	<0.0001
Genotype	1	66.0	9.73	0.0027
Treatment X Genotype	2	65.9	0.08	0.9198

N Concentration				
Factor	Num df	Den df	F	p
Treatment	2	65.7	1.71	0.1886
Genotype	1	65.8	4.34	0.0403
Treatment X Genotype	2	65.7	1.68	0.1951

574

575

576 **Table 3:** Parameterized values for G in each interaction estimated via mean lifetime

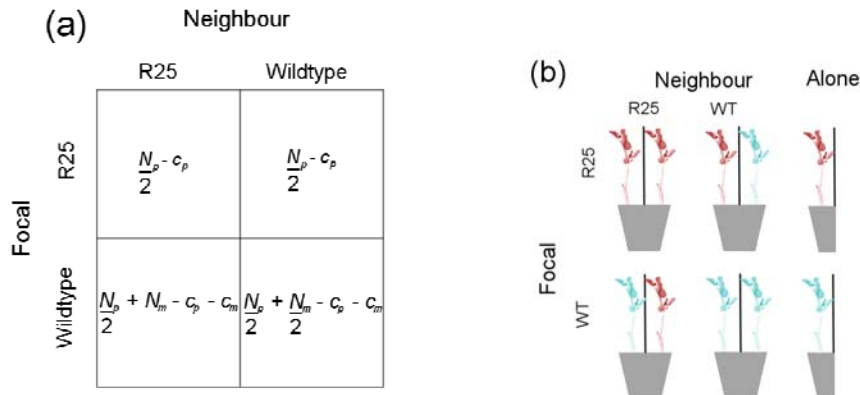
577 reproductive yield of all plants.

		Neighbour	
		R25	WT
Focal	R25	1.73 (95% CI: 1.61 – 1.85)	1.73 (95% CI: 1.61 – 1.85)
	WT	2.23 (95% CI: 1.99 – 2.47)	1.93 (95% CI: 1.76 - 2.10)

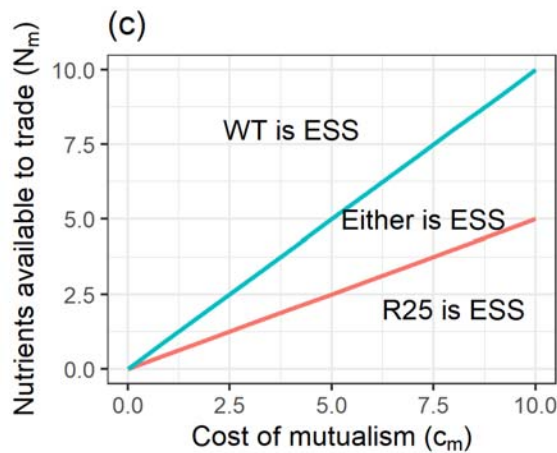
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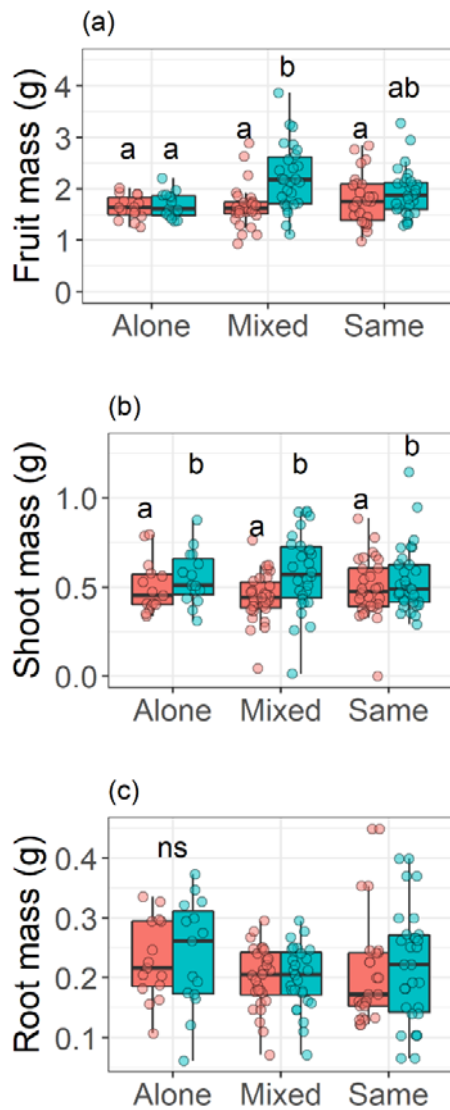
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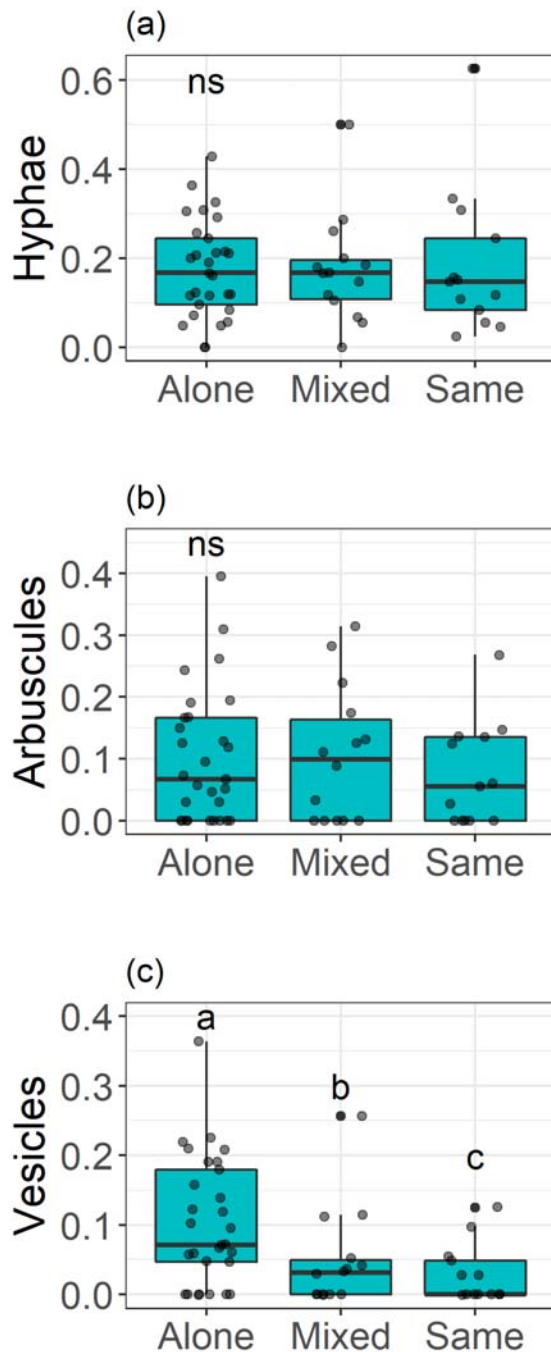
583 **FIG 1:** Basic frequency-dependent matrix game for genotype competition (a) and schematic of
 584 experimental design (b). Grey trapezoids represent pots, and black vertical lines represent
 585 corrugated plastic screens erected to prevent above ground interaction. R25 mutant plants are
 586 coloured red, and WT plants are coloured blue. There were four competition treatments
 587 representing all possible pairs of mutant and WT as either focal and neighbour as captured by the
 588 matrix game. Additionally, each plant was grown alone in pots of half the size as a per-plant nutrient
 589 control. Thus, there were six treatments in total. The ESS solutions to the matrix game for all
 590 possible values of parameter space is shown (c). The blue line represents $N_m = c_m$, and the red line
 591 represents $\frac{N_m}{2} = c_m$. When the costs of mutualism (c_m) are greater than half the benefits ($\frac{N_m}{2}$),
 592 plants should never engage in mutualism (R25 is ESS). When the costs of mutualism (c_m) are less
 593 than the maximum benefits of mutualism (N_m), then plants should always engage in mutualism (WT
 594 is ESS). In the limited set of nutrient conditions where $N_m > c_m$, but $\frac{N_m}{2} < c_m$, either strategy can
 595 be ESS depending on the history of mutations, but they cannot coexist. Instead, a priority effect will
 596 favour whichever genotype evolved first within the region and this is defined by $N_m > c_m$, but
 597 $\frac{N_m}{2} < c_m$.

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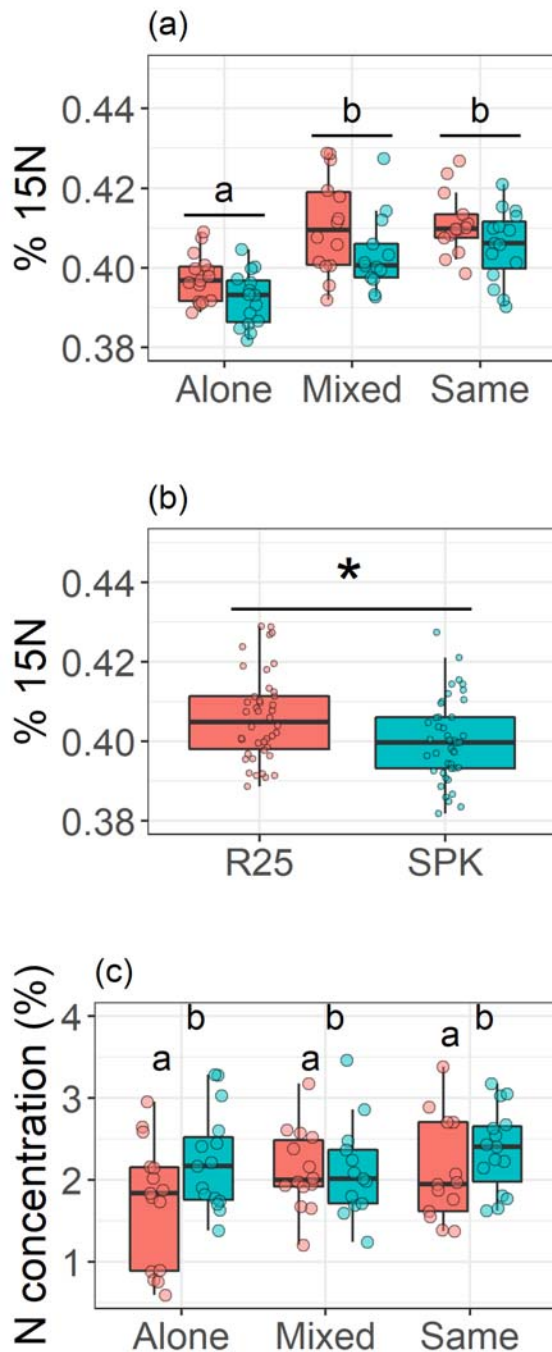
600 **FIG 2:** The fruit (a), shoot (b), and root (c) production of plants, with R25 shown in red and WT in
601 blue either grown alone, in mixed genotype competition (i.e. R25 vs WT and WT vs R25), or in same
602 genotype competition (i.e. R25 vs R25, or WT vs WT). Letters indicate significant differences in
603 means from post-hoc comparisons, while ns indicates a lack of statistical significance. The raw data
604 are plotted with a jitter around the boxplots. Note that in (c) we could not separate the roots of
605 plants in competition and so the data shown represent the roots of both plants combined, and the
606 colour represents the genotype of the focal plant even though in mixed treatments the roots of the
607 neighbour were also weighed.



608

609 **Fig 3:** The colonisation of roots as a proportion of length by the mycorrhizal structures of (a)
610 intraradicular hyphae, (b) arbuscules and (c) vesicles. Note R25 is incapable of forming mycorrhizal
611 associations (Fig S3), and so the data shown are only for WT plants. The raw data are plotted with a
612 jitter around the boxplots. Letters represent significant differences among the treatments on the x-
613 axis and ns indicates no statistical significance.

614



615

616 **FIG 4:** Shoot stable isotope enrichment across both treatments and genotypes (a), as well as just
617 among genotypes (b). The total N concentration of shoots is also shown in percent (mass/mass) (c).
618 WT is shown in blue and the mutant R25 in red. In each panel, the raw data are plotted with a jitter
619 around the boxplots. Letters or * represent significant differences among the treatments on the x-
620 axis. In panel (b), the genotypes are highlighted because the interaction between treatment and
621 genotype was not significant for %15N enrichment (Table 2), and thus pooled genotypes 15N
622 enrichment was used to parameterize the model.

623