FULL PAPER

Antagonistic plants preferentially target arbuscular mycorrhizal fungi that are highly connected to mutualistic plants

Sofia IF Gomes[1], Miguel A Fortuna[2], Jordi Bascompte[2], Vincent SFT Merckx[3,4*]

[1] Institute of Biology, Leiden University, Leiden, the Netherlands

[2] Department of Evolutionary Biology and Environmental Studies, University of Zurich, Switzerland.

[4] Naturalis Biodiversity Center, Leiden, the Netherlands

[3] Department of Evolutionary and Population Biology, Institute for Biodiversity and Ecosystem Dynamics, University of Amsterdam, Amsterdam, the Netherlands

* corresponding author: vincent.merckx@naturalis.nl

Summary

- How antagonists – mycoheterotrophic plants that obtain carbon and soil nutrients from fungi – are integrated in the usually mutualistic arbuscular mycorrhizal networks is unknown. Here, we compare mutualistic and antagonistic plant associations with arbuscular mycorrhizal fungi and use network analysis to investigate fungal association preferences in the tripartite network.
- We sequenced root tips from mutualistic and antagonistic plants in a tropical forest to assemble the combined tripartite network between mutualistic plants, mycorrhizal fungi, and antagonistic plants. We compared the fungal ecological similarity between mutualistic and antagonist networks, and searched for modules (an antagonistic and a mutualistic plant interacting with the same pair of fungi) to investigate whether pairs of fungi simultaneously linked to plant species from each interaction type were overrepresented throughout the network.
- Antagonistic plants interacted with approximately half the fungi detected in mutualistic plants. Antagonists were indirectly linked to any of the detected mutualistic plants, and fungal pairwise ecological distances were correlated in both network types. Moreover, pairs of fungi sharing the same antagonistic and mutualistic plant species occurred more often than expected by chance.
- We hypothesize that the maintenance of antagonistic interactions is maximized by targeting well-linked mutualistic fungi, thereby minimizing the risk of carbon supply shortages.

Keywords: mycoheterotrophy, mycorrhizal networks, arbuscular mycorrhizal fungi, antagonism, mutualism

Introduction

Since the early history of life, interspecific mutualisms have been paramount in the functioning of ecosystems (Thompson, 2005; Bascompte & Jordano, 2013). Mutualisms can form complex networks of interdependence between dozens or even hundreds of species. A prime example of this 'web of life' is the 450-million-year-old mutualism between the majority of land plants and arbuscular mycorrhizal (AM) fungi (Strullu-Derrien *et al.*, 2018). In this interaction plants supply the arbuscular mycorrhizal Glomeromycotina and Mucoromycotina fungi with carbohydrates, essential for fungal survival and growth. In return, the fungi provide their host plants with mineral nutrients and water from the soil (Smith & Read, 2008; Bidartondo *et al.*, 2011). One of the key characteristics of the arbuscular mycorrhizal interaction is its low interaction specificity: a mycorrhizal plant typically associates simultaneously with multiple fungi and a mycorrhizal fungus often associates simultaneously with multiple plants (Lee *et al.*, 2013). This creates complex underground networks in which plants of different species are indirectly linked through shared arbuscular mycorrhizal fungi (Toju *et al.*, 2015; Chen *et al.*, 2017). Despite this low specificity, there is evidence that networks of plants and arbuscular mycorrhizal fungi do not assemble randomly, and effects of plant functional group (Davison et al. 2011; Sepp *et al.*, 2019), and plant or fungal evolutionary relationships (Montesinos-Navarro *et al.*, 2015; Chen *et al.*, 2017) on arbuscular mycorrhizal interaction networks have been reported.

Considerable progress has been made in dissecting the exchange of resources between plants and AM fungi, and its regulation, in recent years. Experimental work on low-diversity systems has demonstrated that control is bidirectional, and partners offering the best rate of exchange are rewarded, suggesting that AM networks consist of 'fair trade' interactions (Bever *et al.*, 2009; Kiers *et al.*, 2011). However, the importance of reciprocally regulated resource exchange is questioned, as mycorrhizas also affect plant health, interactions with other soil organisms, host-defense reactions, and suppression of non-mycorrhizal competitor plants (Walder & Van Der Heijden, 2015). Also, strictly reciprocal regulation of resource exchange does not seem to apply to all AM interactions. Some exceptional plants, for example, behave as antagonists (Selosse & Rousset, 2011; Walder & Van Der Heijden, 2015): mycoheterotrophic plants can obtain carbon from root-associated fungi and some species

have replaced photosynthesis by carbon uptake from arbuscular mycorrhizal fungi (Leake, 1994; Merckx, 2013). The mechanism underpinning carbon transfer from arbuscular mycorrhizal fungi to mycoheterotrophic plants remains unclear, but it is unlikely that resource exchange in these mycoheterotrophic associations relies on reciprocal dynamics. Hence, mycoheterotrophic plants are considered cheaters of the mycorrhizal symbiosis because they exploit the arbuscular mycorrhizal symbiosis for soil nutrients and carbon without reciprocating, to our current knowledge (Selosse & Rousset, 2011), or without apparently being sanctioned by the fungal partners (Walder & Van Der Heijden, 2015). Moreover, it has been suggested that mycoheterotrophic plants may display a truly biotrophic parasitic mode, digesting the fungus colonizing their roots (Imhof *et al.*, 2013).

Phylogenetic studies demonstrate that these cheaters evolved from mutualistic ancestors (Merckx *et al.*, 2013). Within obligate mutualisms, the critical barrier to mutualism breakdown and to the evolutionary stability of the resulting cheater species is thought to be a requirement for three-species coexistence: a cheater plant relies on a mutualistic partner – a mycorrhizal fungus – which simultaneously interacts with a mutualistic plant (Pellmyr & Leebens-Mack, 1999). In species-rich mutualisms such as the arbuscular mycorrhizal symbiosis, where multi-species coexistence is the rule, a high potential for the occurrence of these tripartite linkages is expected (Merckx & Bidartondo, 2008). Indeed, while evolution of cheating in specialized obligate mutualisms is relatively rare (Sachs & Simms, 2006), cheating in the arbuscular mycorrhizal symbiosis evolved in more than a dozen of plant clades, including over 250 species which together occur in nearly all tropical and subtropical forests (Merckx, 2013; Gomes *et al.*, 2019a).

Previous work has shown that mycoheterotrophic plants – hereafter 'antagonists' – target a subset of the mycorrhizal fungi available in the local community (e.g Bidartondo *et al.* (2002); Gomes *et al.* (2017a); Sheldrake *et al.* (2017)). Their associated fungal communities can vary in specificity (Merckx *et al.*, 2012), and in communities of co-occurring antagonists the phylogenetic diversity of their fungal associations appears to increase proportionally to their fungal overlap, a pattern which potentially responds to an ecological mechanism driven by maximizing co-occurrence and avoiding competitive exclusion among antagonistic plants.

(Gomes *et al.*, 2017b). However, whether partner choice of these antagonists is affected by mutualistic mycorrhizal interactions is currently unknown. Here, we hypothesize that antagonists preferentially associate with 'keystone' (Mills & Doak, 1993) fungi that are connected to many different mutualistic plants, since these fungi are potentially more resilient to perturbations (Bascompte & Jordano, 2007) and may be the most reliable source of carbon (Waterman *et al.*, 2013). In addition, since fungal traits play an important role in arbuscular mycorrhizal interactions – phylogenetically related AM fungi (assumed to have similar functional traits), preferentially interact with similar plant species (Chagnon *et al.*, 2015) – we hypothesize that if partner selection in tripartite networks is trait-driven we will be able to detect an influence of the phylogenetic relationships of the fungi. Here, we test these hypotheses on a combined tripartite mycorrhizal network of co-occurring antagonistic and surrounding mutualistic plants linked by shared arbuscular mycorrhizal fungi compiled by high-throughput DNA sequencing.

Materials and methods

Sampling

Since mycoheterotrophic plants are relatively rare and often have patchy distributions (Gomes *et al.*, 2019b), we sampled two 4 x 4 m subplots a few meters apart in a coastal low land plain rainforest in French Guiana (5°28'25"N 53°34'51"W) on 28 July 2014, with overlapping antagonistic species. In both subplots, roots of antagonistic plants and surrounding mutualistic plants were sampled, cleaned with water, and stored on CTAB buffer at -20°C until further processing. We found in total five antagonistic plant species: *Dictyostega orobanchoides, Gymnosiphon breviflorus* (Burmanniaceae), *Voyria aphylla, Voyriella parviflora* (Gentianaceae), and *Soridium spruceanum* (Triuridaceae). Complete individuals of antagonistic plants were dug out, and around each, 3 root tips of mutualistic plants were collected. In addition, we sampled five additional mutualistic plant root tips randomly from each quadrant of each plot aiming to better represent the local belowground mutualistic community. A detailed taxon list of antagonistic and mutualistic plant species collected can be found in Supporting Information, Table **S1**.

134 *Plant identification and fungal communities sequencing*

135 DNA was extracted from the CTAB-preserved roots with the NucleoMag96 Plant Kit
136 (Macherey-Nagel Gmbh & Co., Düren, Germany), using the KingFisher Flex Magnetic Particle
137 Processor (Thermo Fisher Scientific, Waltham, MA, USA). Mutualistic plant species were
138 identified by sequencing the markers *matK or trnL* as described in (Gomes *et al.*, 2017a). Plant
139 identification to species, genus, or family level based on BLAST against GenBank was reviewed
140 by consulting the checklist of plants in French Guiana (Berry & Weitzman, 2007). Fungal
141 communities associated with the roots of mutualistic and antagonistic plants were amplified
142 using the primers fITS7 (Ihrmark *et al.*, 2012) and ITS4 (White *et al.*, 1990) and sequenced
143 with a Personal Genome Machine (Ion Torrent; Life Technologies, Guildford, CT, USA), as
144 described in Gomes *et al.* (2017b) in two separate runs. Negative controls from the extraction
145 and the PCR reactions were included (and had zero reads). The same bioinformatics methods
146 from Gomes et al. (2017b) were used to process the raw reads from the two runs combined
147 until clustering into 97% Operational Taxonomic Units (OTUs), using USearch v.7.0 (Edgar,
148 2010). OTUs represented by less than six reads in each sample were excluded to avoid
149 spurious OTUs (Lindahl *et al.*, 2013). The taxonomical assignment of each OTU was done by
150 querying against the UNITE database (Kõljalg *et al.*, 2013). Because the antagonistic plants in
151 this study are presently only known to associate with fungi that belong to the sub-phylum
152 Glomeromycotina (Merckx *et al.*, 2012), we only retained fungal OTUs from this sub-phylum
153 in the subsequent analysis. In these analyses, we accounted for the phylogenetic relatedness
154 between fungal OTUs, due to the uncertainty of correspondence of individual OTUs with
155 taxon diversity of arbuscular mycorrhizal fungi (Flynn *et al.*, 2015), despite the fact that
156 delimitation of OTUs is not likely to interfere with ecological interpretations (Lekberg *et al.*,
157 2014). We inferred the phylogenetic relationships between the fungal OTUs following the
158 strategy of Gomes *et al.* (2017b). Briefly, we aligned the OTUs with partial reference
159 sequences and performed a phylogenetic inference in which the relationships between these
160 references were enforced based on Krüger *et al.*, (2012). Hereafter, we refer to OTUs as
161 'fungi'. The fungal communities obtained from the root samples are not necessarily similar
162 representations of the total communities of antagonists and mutualists because the sampling
163 coverage of their root systems varied: antagonistic plants are small herbs, and thus large parts
164 of their root system were collected and extracted, while for mutualistic plants, mostly forest

trees (see Results), root samples represents only a small fragment of their root system and thus the detected fungal communities associated with mutualistic plants are likely a subset of their total fungal communities.

Plant-fungal interactions

Fungal communities at the plant individual level were significantly structured by plant species identity with a minor influence of the subplot where they were collected (see Supporting Information, Methods **S1**, Figure **S1**). To contrast the interactions of antagonistic and mutualistic plants, we considered their plant-fungal interactions as a single tripartite network (Fig **1a**) by combining the fungal communities associated with individual plants from the two subplots into overall communities per plant species. By combining the data from the two subplots, we aimed to reconstruct a more robust picture of the interactions in this highly diverse rainforest while compensating for the relatively low sampling intensity (for 77 out of 220 root tips of mutualistic plants both plant identification and AM fungal community data with sufficient reads could be obtained). Plant species for which Glomeromycotina fungi were represented by less than 500 reads were excluded, resulting in removing 11 mutualistic plant species (see details in Supporting Information, Table **S1**). Moreover, because rarefying the OTU matrix has been shown to greatly increase the false positive rate of OTUs per sample (McMurdie & Holmes, 2014), we performed the subsequent analyses on 100 matrices rarefied to 844 reads per plant species, based on lowest number of reads obtained for all plant species in the observed dataset. Results are presented with mean and standard deviation values obtained from running the analyses on the rarefied matrices. To investigate fungal association patterns of antagonistic and mutualistic plants, we used incidence (binary) data, because our main interest was to determine which interactions can be established and not how abundant they are. Also, due to the difference in root sampling (see above) DNA read abundance data unlikely correspond to fungal abundances at the plant species level, in particular between plant types (antagonist and mutualists). We considered the simultaneous presence of a fungus in the roots of an antagonistic and a mutualistic plant as a potential link between both (Southworth *et al.*, 2005).

We tested for the effect of plant and fungal phylogenetic relatedness on the observed interactions in both networks. We used the fungal phylogeny described above, and the

196 phylogenetic relationships of the plants as derived from *TimeTree* (Kumar *et al.*, 2017) (see
197 details in Supporting Information, Methods **S2**). We computed Mantel test correlations
198 between the phylogenetic distance matrix and the community dissimilarity matrix in each
199 instance for antagonistic and mutualistic species individually. The phylogenetic distance
200 matrices were extracted from the phylogenetic trees of plants and fungi, and the community
201 dissimilarity matrices were calculated as the Jaccard distance on the binary interaction
202 matrices. Phylogenetic signal was calculated for the 100 rarefied matrices, and consistency of
203 significant results was assessed across multiple rarefaction depths (Supporting Information,
204 Fig. **S2**).

205 *Mutualistic and antagonistic plant-plant interactions*

206 To compare the range of fungal interactions between mutualistic and antagonistic plants, we
207 calculated their normalized degree and the phylogenetic species variability of their associated
208 fungal communities. The normalized degree of a plant species is the proportion of its
209 associated fungi out of the total possible fungi in the network (Martín González *et al.*, 2010),
210 and was calculated with the *ND* function of the *bipartite* R package (Dormann *et al.*, 2018).
211 The phylogenetic species variability (*psv*) of the fungal community associated with a plant
212 species summarizes the level to which the fungi in this community are phylogenetically
213 related (Helmus *et al.*, 2007a). When a community consists of unrelated fungi, the index
214 equals 1, indicating maximum phylogenetic variability. As relatedness increases, the index
215 approaches 0, indicating high phylogenetic specificity (Helmus *et al.*, 2007b). To explore how
216 the number of interactions relates to the phylogenetic relationships among the detected
217 fungi, we tested for a linear correlation between normalized degree and *psv* by calculating
218 the Pearson's correlation coefficient. In addition, we calculated the fungal overlap between
219 each pair of plant species as the number of fungi they share to infer plant–plant preferences
220 (Fig **1b**).

221 *Mutualistic and antagonistic fungal interactions*

222 To investigate whether fungi have similar number of interactions with plants in both
223 mutualistic and antagonistic networks, we calculated the Pearson correlation between the
224 normalised degrees of the shared fungi within each interaction type. We compared the
225 ecological similarity of the fungi shared between the two interaction type networks (i.e.
226 mutualistic and antagonistic interactions). The ecological similarity of a pair of fungi

227 represents their similarity in interactions through shared plants. We calculated similarity
228 matrices between the fungi linked to mutualistic and antagonistic plants separately, using two
229 different measures. The first measure was the Jaccard index, which corresponds to the
230 number of plant species with which both fungi interact divided by the total number of plant
231 species with which they interact, and the second was the Overlap measure: $C_{ij}/\min(d_i, d_j)$,
232 where C_{ij} is the number of shared plants between fungus *i* and *j*, and $\min(d_i, d_j)$ is the smallest
233 number of associated plants between fungus *i* and *j* (Saavedra *et al.*, 2013). The overlap
234 measure corresponds to the number of shared plant species relative to the maximum number
235 of plant species that can be shared, and takes into account the possibility that fungi have
236 differential limits in their maximum number of plant partners. We computed the Mantel test
237 correlation between the similarity matrices for the mutualistic and antagonistic interactions
238 using the two different measures. We computed the partial Mantel test correlation between
239 the similarity matrices calculated using the Jaccard and Overlap measures, controlling for the
240 phylogenetic relatedness of the shared fungi.

241

242 *Network analysis*

243 We searched for diamond-shape modules in the tripartite network (reduced to only include
244 fungi shared between both interaction types) representing a mutualistic and an antagonistic
245 plant interacting with the same pair of fungi (Fig. **2**). If the module is overrepresented
246 throughout the entire network, it can be considered a motif (Milo *et al.*, 2002; Bascompte &
247 Melián, 2005; Stouffer *et al.*, 2007). This specific motif does not consider the identity of the
248 partners *per se*, but searches for this particular topological structure. To assess whether the
249 diamond-shaped module was overrepresented relative to random expectations, we
250 randomized the original antagonistic plants – mycorrhizal fungi – mutualistic plants
251 community matrix and calculated the frequency of this diamond-shaped module in 1000
252 generated random networks. We used a null model that draws an interaction between a plant
253 and a fungal species which is proportional to the generalization level of both
254 species. Specifically, this probability is defined as the arithmetic mean of the fraction of
255 interactions of the plant and that of the fungi (null model 2 in (Fortuna & Bascompte, 2006)),
256 initially proposed in (Olesen *et al.*, 2003). Because there was an imbalanced number of plant
257 species (five antagonistic and 21 mutualistic plants), each randomized matrix resulted from

258 randomizing the antagonistic and mutualistic interactions separately, and then combined into
259 a single matrix before module search. To exclude the effects of sampling effort both in the
260 number of individuals collected (leading to roughly half of fungal reads belonged to the five
261 antagonistic species while the other half represented 21 mutualistic species) and in
262 representation of whole (antagonists) or partial (autotrophic) root systems, we repeated this
263 procedure in a set of 100 rarefied matrices where each set was resampled to multiple depths.

264 To evaluate whether the diamond-shaped module was overrepresented throughout the
265 empirical network, we calculated the standard z-score = (obs – $\overline{rand}$) / sd($\overline{rand}$), where *obs*
266 is the observed network modules and $\overline{rand}$ is the mean of the same type of modules across
267 the 1000 random networks, using a 95% confidence interval. An empiric result above the null
268 confidence interval indicates that pairs of fungi are shared between particular antagonistic
269 and mutualistic plants more often than expected by chance, reflecting a preference of
270 antagonistic plants to link to pairs of fungi that are generalists in their interactions with
271 mutualistic plants. An empirical result below the null confidence interval indicates that
272 antagonistic plants target pairs of fungi that are specialists in their interactions with
273 mutualistic plants.

274

275 **Results**

276

277 *Plant-fungal interactions*

278 We successfully extracted DNA from root tips of 32 mutualistic (123 root tips) and five
279 antagonistic (60 individuals) plant species (see Table **S1** for detailed species list). After
280 retaining samples that contained a minimum of 500 reads identified as Glomeromycotina, we
281 reduced our dataset to 21 mutualistic (77 root tips) and five antagonistic (27 individuals) plant
282 species, with a total of 365,135 reads. Mutualistic plants belonged to 24 families, including 16
283 trees, two shrubs, one herb, one vine, and one liana. We obtained 115 arbuscular mycorrhizal
284 fungi, identified as Glomeromycotina within three families Gigasporaceae (4 fungi),
285 Acaulosporaceae (14) and Glomeraceae (97). Of these, 96 OTUs (49% of total reads) were
286 present in the antagonistic network.

287 The 100 rarefied binary matrices included 26 plant species (21 mutualists and 5 antagonists)
288 and 110 ± 1.47 fungi. Of these, mutualistic networks had 100 ± 1.51 fungi and antagonistic
289 networks had 61 ± 3.14 fungi. In all the generated matrices, mutualistic and antagonistic
290 plants shared 51 ± 3.16 fungi, which represents 55% and 92% of total fungi present in
291 mutualistic and antagonistic plants, respectively (Fig. **1a**).

292 We measured a relatively low but significant phylogenetic signal of the fungal phylogeny on
293 the antagonistic (r = 0.20 ± 0.06, p = 0.003 ± 0.005) and mutualistic (r = 0.21, p = 0.001)
294 networks (see effect of rarefaction depth on phylogenetic signal in Supporting Information,
295 Fig. **S2**). We found no significant effects of plant phylogeny on any of the networks.

296

297 *Mutualistic and antagonistic plant-plant interactions*

298 The number of fungal interactions per plant species varied, with *Aspidosperma* sp. showing
299 the highest normalised degree and Sapindaceae sp. the lowest amongst the mutualistic plants
300 (Fig. **3a**). Ranked over all the plants, three antagonistic species, namely *V. aphylla*, *V.*
301 *parviflora* and *D. orobanchoides,* presented a normalised degree in the lower half of the
302 spectrum, while *G. breviflorus* and *S. spruceanum* are amongst the plants with the highest
303 number of associated fungi. We also observed a wide range of phylogenetic species variability
304 (psv) for mutualistic plants, with *Acacia* sp. and *Aspidorperma* sp. with the highest *psv* and
305 Sapindaceae sp. the lowest, throughout which, the antagonistic plants are distributed (Fig.
306 **3b**). There was a significant correlation between the normalised degree and *psv* of the
307 mutualistic plants (Pearson's correlation: r = 0.54, t = 2.80, df = 19, p = 0.011), while for the
308 antagonistic plants the number of interactions (normalised degree) does not reflect the fungal
309 diversity (*psv*) in their roots (Pearson's correlation: r = -0.29, t = -0.53, df = 3, p = 0.632).

310 Within the antagonistic plants, we observed that *S. spruceanum* shared most fungal
311 interactions with *G. breviflorus*, followed by *S. spruceanum* with *D. orobanchoides* and *V.*
312 *aphylla*, and also *G. breviflorus* with *D. orobanchoides*. We observed that antagonistic plants
313 as a group associate with fungi that are simultaneously linked to all mutualistic plants (Fig.
314 **1b**), indicating that antagonistic plants are indirectly linked to any of the mutualistic plants
315 detected in this study, which could therefore be the ultimate source of carbon for the
316 antagonistic plants. Of all the possible connections between antagonistic and mutualistic

317 plant species, *S. spruceanum* had the highest fungal overlap with *Clusia* sp., and then with
318 *Tapirira* sp. and *Protium* sp.; *G. breviflorus* with *Aspidosperma* sp. and *Protium* sp., then with
319 *Schadera* sp. and *Clusia* sp.; *D. orobanchoides* with *Tapirira* sp. and then with Fabaceae sp. 1;
320 *V. parviflora* with *Aspidosperma* sp. and then with Araceae sp., Malpighiaceae sp., and *Clusia*
321 sp; and *V. aphylla* with *Aspidosperma* sp. and then with *Paloue* sp.. These mutualistic plants
322 are also those with highest number of fungal interactions overall (Fig. **3a**). Among the
323 mutualistic plants, *Aspidosperma* sp. had the highest fungal overlap with *Protium* sp.,
324 Malpighiaceae sp. and Metteniusaceae sp., which are also among the highest ranked species
325 in terms of number of associated fungi and phylogenetic diversity (Fig. **3**).

326

327 *Mutualistic and antagonistic plant-fungal interactions*

328 We observed a significant correlation (Pearson correlation $r = 0.63$, $p < 0.001$, $t = 8.27$, $df =$
329 105) between the normalised degrees of the fungi in the antagonistic and mutualistic
330 networks (Fig. **4,** Supporting Information Fig. **S3**). Fungi present in the mutualistic network
331 but absent from the antagonistic network were generally characterized by a low normalized
332 degree, except for two *Rhizophagus* taxa which were present in the majority of mutualistic
333 plants (Fig. **4**). Moreover, we found a significant correlation of fungal ecological similarity
334 between the mutualistic and antagonistic interactions (Mantel tests: Jaccard $r = 0.20 \pm 0.04$,
335 $p = 0.002 \pm 0.002$; overlap $r = 0.24 \pm 0.04$, $p = 0.001$), and also when accounting for the
336 phylogenetic relatedness among the shared fungi (partial Mantel tests: Jaccard $r = 0.18 \pm$
337 0.003, $p = 0.018 \pm 0.04$; overlap $r = 0.22 \pm 0.04$, $p = 0.002 \pm 0.003$). We also observed that the
338 fungi with the highest normalized degrees, both in the mutualistic and antagonistic network,
339 are all members of the Glomeraceae (Fig. **4**).

340

341 *Network analysis*

342 The network analysis indicated that the presence of 2,560.39 ± 296.36 instances of the
343 diamond-shaped module in the empirical networks across the rarefied matrices was
344 overrepresented in relation to random expectations (*z-score* = 3.45 ± 0.40, $p = 0.002 \pm 0.003$).
345 The same analysis repeated for multiple rarefaction depths indicates that despite the number
346 of diamond-shaped modules increases with increasing rarefaction depth, it does not impact

347 the result that pairs of fungi share an antagonistic and a mutualistic plant more often than
348 expected by chance (Supporting Information, Fig. **S4**).

349

350 **Discussion**

351

352 We found that antagonistic plants as a group target approximately half of the fungi that are
353 potentially available, yet this subset of fungi is associated with all mutualistic plants detected
354 in this study. Thus, despite associating only with a subset of the local pool of fungi, the
355 antagonists are still indirectly linked to every mutualistic plant. Fungi not detected in the roots
356 of antagonists were generally connected to few mutualistic plants. Within the fungi that are
357 shared between the mutualistic and antagonistic networks, we detected a significant
358 ecological symmetry between the mutualistic and antagonistic interactions of the fungi: pairs
359 of fungi that interact with similar sets of mutualistic plants also interact with overlapping
360 antagonistic plants. The network analysis indicates that this pattern occurs more often than
361 expected by chance. Based on the highest fungal overlap between each antagonistic species
362 with subsets of highly connected mutualistic species, this pattern is mainly driven by an
363 antagonist preference for fungi that are well-linked to specific mutualistic plants. These
364 mutualistic plants are the ultimate sources of the carbon which antagonistic plants take up
365 from the fungi shared between mutualists and antagonists. Therefore, we suggest that the
366 observed pattern reflects a strategy in which the maintenance of antagonistic interactions is
367 maximized by targeting well-linked mutualistic fungi, thereby minimizing the risk of carbon
368 supply shortages.

369

370 *Fungal preferences of antagonistic plants*

371 We found that plant species identity had a significant influence on the fungal community
372 composition, regardless of the plant type (mutualist or antagonist), which indicates that these
373 communities are non-random subsets of the local fungal taxon pool. This supports previous
374 evidence that co-occurring plant species show differences in selectivity towards available
375 arbuscular mycorrhizal fungi (Davison *et al.*, 2011). Antagonistic plant species in particular,
376 are known to select particular groups of fungi, often a narrower range than surrounding

377 mutualistic plants (Bidartondo et al. 2002; Gomes et al. 2017). Here we observed that five co-
378 occurring antagonistic plant species collectively associate with about half of the available
379 fungal taxa (Fig. **4**). Antagonistic interactions can thus be supported by a relatively wide array
380 of arbuscular mycorrhizal taxa, as shown previously (Merckx et al. 2012, Gomes *et al.*, 2017b;
381 Sheldrake *et al.*, 2017). Although fungi from three different fungal families were detected in
382 the roots of antagonistic plants, a clear preference for Glomeraceae taxa, and *Rhizophagus*
383 *irregularis* relatives in particular, was observed. The taxa of this clade were the most
384 frequently encountered in the roots of the autotrophic plants as well (Fig. **4**). *Rhizophagus*
385 contains some of the most globally widespread and common arbuscular mycorrhizal fungi
386 (Kivlin *et al.*, 2011; Moora *et al.*, 2011; Davison *et al.*, 2015; Gomes *et al.*, 2018) although this
387 is mostly derived from studies in temperate areas. Our results indicate that the tropical
388 rainforest offers no exception to this pattern. Glomeraceae are usually not only the most
389 dominant clade in natural arbuscular mycorrhizal communities, often accounting for c. 70%
390 of all species (Montesinos-Navarro *et al.*, 2012), but they also have consistently been found
391 to include the most generalist arbuscular mycorrhizal fungi in other network studies (e.g.
392 Montesinos-Navarro *et al.,* 2012; Chagnon *et al.*, 2015; Chen *et al.*, 2017). The ability to
393 interact with many mutualistic plant species can be a potential reason for why antagonists
394 generally target Glomeraceae fungi (Merckx *et al.*, 2012; Renny *et al.*, 2017). Ecological theory
395 predicts that generalist species tend to have large distribution ranges (Brown, 1984) and,
396 consequently, are less vulnerable to (local) extinction than specialized species (Schleuning *et*
397 *al.*, 2016). Therefore, associations to generalist fungi may be advantageous for the
398 evolutionary persistence of antagonists. Furthermore, associations with multiple mutualistic
399 plant partners may increase fungal resilience to disturbance, while mediating temporal
400 fluctuations in carbon flow and interaction dynamics (Bennett *et al.*, 2013) guaranteeing
401 continuous carbon supply to the entire network without pronounced negative effects even in
402 the presence of antagonists. In addition, in the context of mycorrhizal fungi, which can be
403 linked to different plant species simultaneously (Montesinos-Navarro *et al.*, 2012), generalist
404 fungi are therefore likely to be more reliable carbon sources for antagonists. An alternative
405 and perhaps not mutually exclusive explanation for why antagonistic plants preferentially
406 target well-connected fungi may be that these fungi are less effective in detecting and
407 excluding non-mutualistic plant partners (Bruns *et al.*, 2002; Egger & Hibbett, 2004;
408 Bidartondo, 2005; Walder & Van Der Heijden, 2015).

409 *Fungal links between mutualistic and antagonistic networks*

410 We detected that pairs of fungi that interact with similar sets of mutualistic plants share links
411 with overlapping antagonistic plants. Thus, there is a high level of interaction symmetry
412 between mutualistic and antagonistic mycorrhizal networks. Also, we measured a significant
413 influence of the fungal phylogenetic relationships on both the mutualistic and antagonistic
414 interactions, showing that closely related fungi interact with similar mutualistic and
415 antagonistic plants respectively. Because, biotic interactions are mediated by functional
416 traits, and most functional traits are evolutionarily conserved, shared evolutionary history of
417 fungi can serve as a proxy for functional similarity. We therefore hypothesize that both
418 mutualistic and antagonistic interactions are shaped by evolutionary conserved functional
419 traits of the fungi. In addition, the network analysis indicated that pairs of fungi share an
420 antagonistic and a mutualistic plant more often than expected by chance. This analysis solely
421 indicates that the diamond-shaped module is overrepresented in the empirical network (Fig.
422 **2**), without reference to species degree nor to species identity. Yet, our results support that
423 the observed pattern is driven by the tendency of antagonistic plants to target fungi that are
424 well-linked to mutualistic plants (Fig. **1b**). The mutualistic plants with highest fungal overlap
425 in relation to the antagonistic plants are among those with the highest ranked degree and
426 phylogenetic species variability from the pool of detected mutualistic plant species (Fig. **3**).
427 Moreover, fungi with the highest number of interactions in the mutualistic network are also
428 among the best-connected fungi in the antagonistic network (Fig. **4**). Our findings thus reveal
429 that antagonistic plants preferentially associate with fungi that are simultaneously linked to
430 a wide range of mutualistic plants. Although many antagonistic plants share a large number
431 of fungi with the mutualistic tree *Aspidosperma* sp., the mutualistic plants with highest
432 normalised degree, each antagonistic plant species also indirectly associates with non-
433 overlapping sets of mutualistic plants, as indicated by their divergent positions in the plant-
434 plant interaction network (Fig. **2b**). This pattern suggests a potential strategy to avoid
435 competition among antagonistic plant species while targeting well connected mutualistic
436 plant species through shared fungi.

437 Our results indicate that antagonistic and mutualistic mycorrhizal networks are not linked
438 randomly but are symmetrically influenced by mutualistic and antagonistic plant identity and
439 fungal connectance. Whether these correlations are potentially influenced by spatial patterns

440 in the distribution of mutualists, antagonists, and fungi remain to be determined. Our earlier
441 work suggests that antagonistic mycorrhizal interactions can be better explained by
442 understanding plant–plant interactions through sharing fungal associations (Gomes *et al.*,
443 2017b), and indicates that antagonistic interactions may respond to an ecological mechanism
444 driven by maximizing co-occurrence and avoiding competitive exclusion among antagonists.
445 The results obtained here expand this view and suggest that not only plant-plant interactions
446 drive patterns of co-occurrence of antagonistic plants, but also the indirect interactions
447 between mutualistic and antagonistic plants through shared arbuscular mycorrhizal fungi.

448

449 *Potential sampling biases*

450 Considering that rainforests are species-rich ecosystems (e.g. (Ter Steege *et al.*, 2013)), it is
451 possible that, despite our efforts, the sampling of the belowground diversity of arbuscular
452 mycorrhizal fungi, and their plant partners remained incomplete. Furthermore, the
453 representation of roots from antagonistic and mutualistic plants was necessarily imbalanced,
454 as whole and partial root systems, respectively, were investigated. This made the use of read
455 abundances to estimate interaction strengths impossible. While other studies have addressed
456 the importance of sampling intensity, (i.e. the number of possible interactions per node which
457 directly impacts the normalised degree per species), and network size (i.e. number of nodes
458 involved) (Blüthgen *et al.*, 2007; Dormann *et al.*, 2009) on network metrics, the effect of
459 species relationships is often elusive. In our approach, we accounted for the phylogenetic
460 relationships between the fungi, as other studies have accounted for taxonomic diversity
461 (Morris *et al.*, 2014), and also we verified the consistency of our results across multiple
462 rarefactions depths, by the use of multiple rarefied matrices at each depth (Gotelli & Colwell,
463 2001). This allows us to separate the statistical patterns in our data from the influence of
464 sampling effort, both in terms of the plant species detected in the sampled roots, and in their
465 potentially incomplete number of fungal associations. Furthermore, the number of
466 interactions for mutualistic plants is moderately correlated with the phylogenetic diversity of
467 the arbuscular mycorrhizal fungi associated with these tropical trees, while for antagonistic
468 plants this correlation is not found. This further suggests that bias due to sampling effort is
469 limited. It also indicates that antagonistic plants have specialised interactions, occupying

470 particular fungal niches, which could have consequence for their occurrence and coexistence
471 (Gomes *et al.*, 2017b).

472

473 *Conclusions and future perspectives*

474 To our knowledge, our study is the first to assess how antagonistic plant species are
475 embedded in mutualistic mycorrhizal networks. We find that antagonistic plants as a group
476 interact with half of the available fungal partners, and generally target fungi that are well-
477 connected to mutualistic plants. Although antagonistic species show overlap in their fungal
478 associations, we found that they are indirectly linked to different sets of mutualistic plants
479 suggesting a potential mechanism to avoid competition by preferentially relying on different
480 carbon sources (Gomes *et al.*, 2017b). The phylogenetic relationships between the fungi,
481 likely a proxy for fungal traits, have a significant influence on these non-random tripartite
482 interactions. Therefore, we conclude that the persistence of antagonists in arbuscular
483 mycorrhizal networks is dependent on particular well-connected 'keystone' mycorrhizal
484 fungi, which provide the antagonists with carbon from a wide range of plants. Our observation
485 that the way fungi connect mutualistic and antagonistic networks is not random and that well-
486 connect fungal nodes in arbuscular mycorrhizal networks are more prone to be targeted by
487 antagonists are similar to those of (Sauve *et al.*, 2016) for a plant-pollinator-herbivore
488 network when considering binary interactions. But further research is needed to assess
489 whether this is a general feature of diffuse interactions, also when taking interaction strength
490 into account. Our study emphasises the raising awareness of considering multiple interaction
491 types simultaneously (e.g. antagonistic and mutualistic) to deepen our understanding of
492 complex biodiversity patterns (Losapio *et al.*, 2021).

493 In ectomycorrhizal systems mutualistic and achlorophyllous antagonistic plant species
494 represent opposite extremes of a continuum of interaction outcomes and several plant
495 species are able to combine photosynthesis and carbon uptake from fungi, a strategy known
496 as 'partial mycoheterotrophy' (Selosse & Roy, 2009). Recent discoveries now indicate that
497 partial mycoheterotrophy may be common among arbuscular mycorrhizal plants as well, and
498 potentially many photosynthetic understory plants are able to take up carbon from
499 associated arbuscular mycorrhizal fungi (Giesemann *et al.*, 2021). It remains to be tested

whether carbon uptake by these partially mycoheterotrophic plants occurs through sets of fungi, as suggested for fully mycoheterotrophic plant species.

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Author contributions

SG, MF, JB, and VM designed the study and collected the materials. SG performed the laboratory work and data analysis. All authors contributed to the data analysis and the writing of the manuscript.

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713 **Figures**

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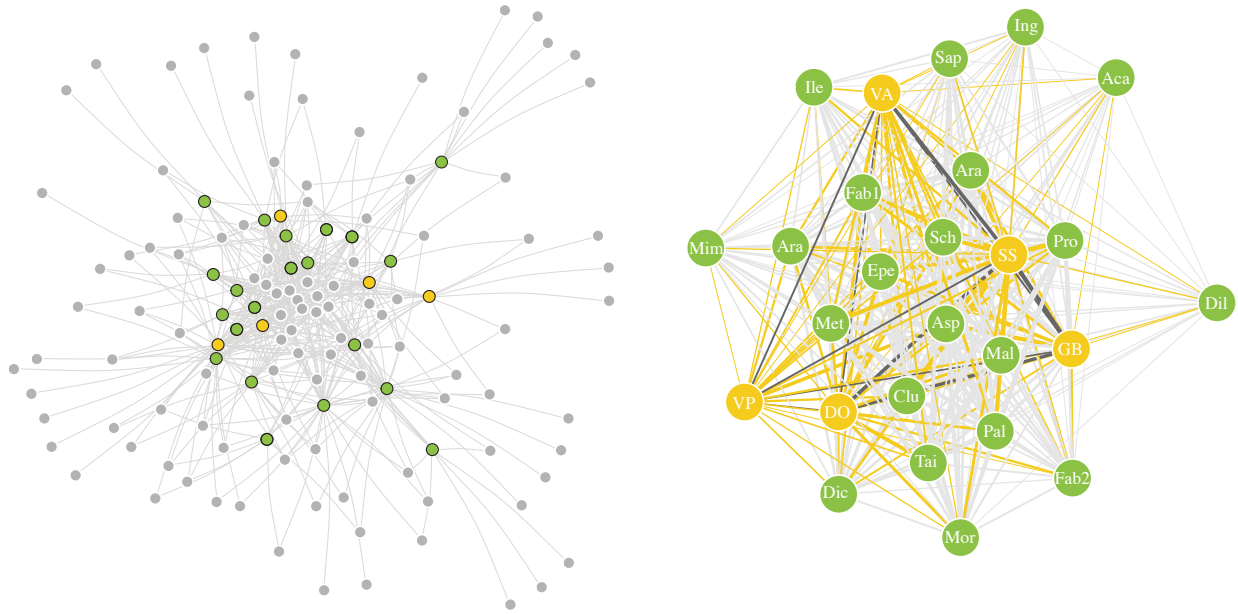

726 **Figure 1**. Tripartite arbuscular mycorrhizal interactions. (a) Visualization of the tripartite
727 network between fungi (grey) and antagonist (yellow), and mutualist (green) plants, where
728 edges represent a connection between a plant and a fungus. (b) Plant-plant fungal overlap
729 network, in which edges represent a link between plant species through shared arbuscular
730 mycorrhizal fungus. The thickness of the lines represents the interaction strength between
731 the plants (the thicker, the more fungi are shared). Yellow lines link antagonistic and
732 mutualistic plants; light grey lines link mutualistic to mutualistic plants; and dark grey lines
733 link antagonistic to antagonistic plants. Identification of autotrophic plants is indicated by the
734 first three letters of their name (see full names in Fig. 3); mycoheterotrophic plants are *S.*
735 *spruceanum* (SS), *G. breviflorus* (GB), *D. orobanchoides* (DO), *V. aphylla* (VA), and *V. parviflora*
736 (VP). In both network representations (a, b), one of the 100 rarefied matrices to a depth of
737 844 reads was used; and the Fruchterman-Reingold layout was used, in which nodes are
738 evenly distributed through the graph, where plants that share more connections are closer
739 to each other.

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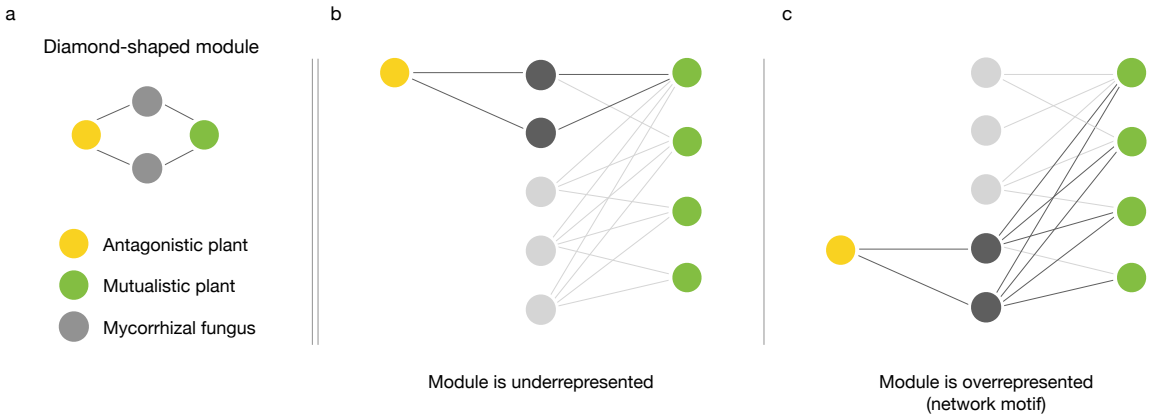

Figure 2. Diamond-shape module investigated in this paper. Representation of the module linking pairs of fungi to the same antagonistic and mutualistic plant species (a). Examples of under- (b) and overrepresentation (c) of the module in a network. When the module is overrepresented it is considered a network motif.

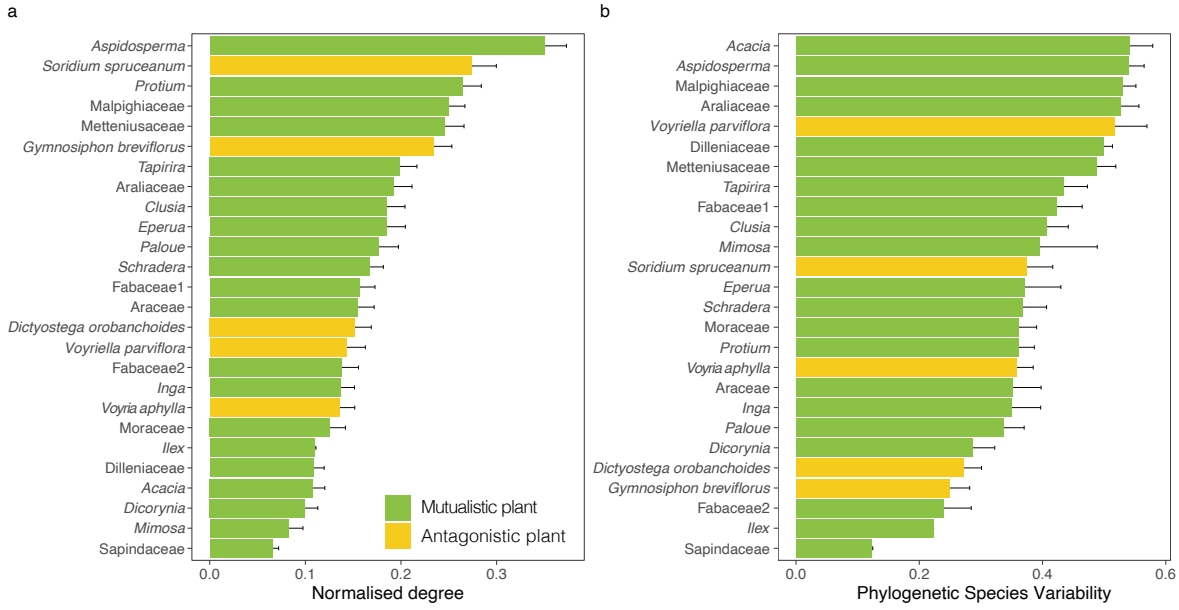

Figure 3: Mean ± SD plant normalised degree and PSV for the 21 autotrophic and five mycoheterotrophic plant species, resulted from the 100 rarefactions to a depth of 844 reads.

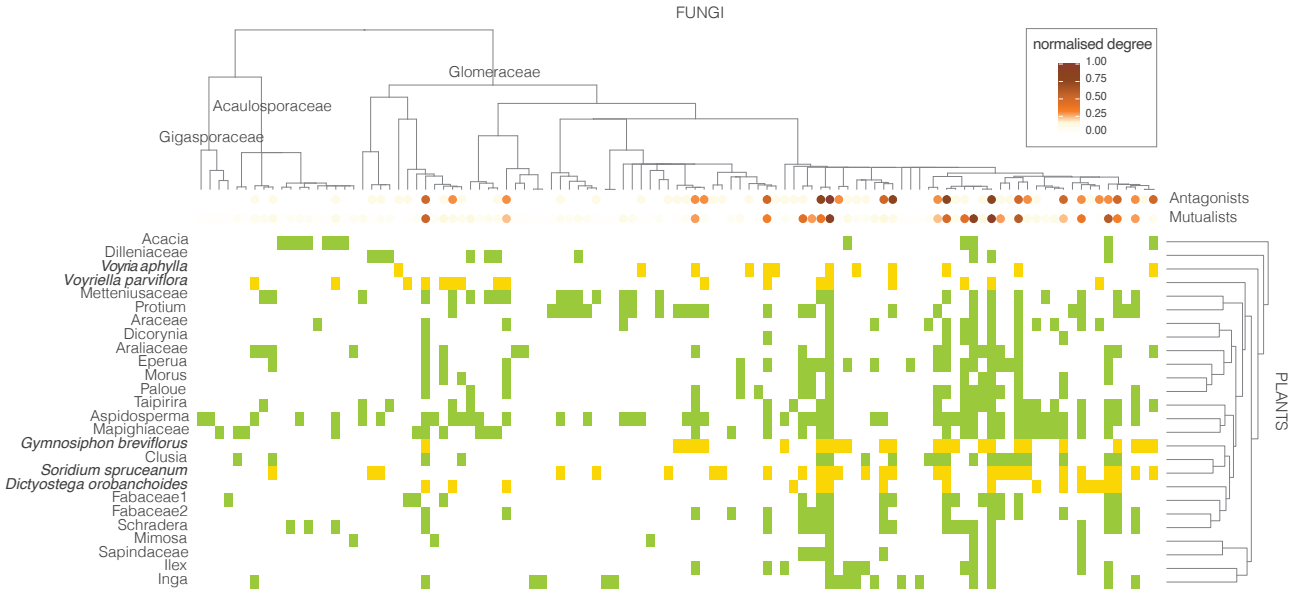

Figure 4: Plant-fungal interactions of mutualistic (green) and antagonistic (yellow) plants using a matrix rarefied to a depth of 844 reads. Phylogenetic relationships between the fungi are shown on top. Plant species are listed on the left; hierarchy clustered dendrogram based on the Bray-Curtis distance of their fungal communities is shown on the right. The intensity of the orange dots on the tips of the fungal phylogeny depicts the normalised degree (representing their interaction strength) of each fungus in each of the plant-fungal networks (i.e. in the antagonistic plants-fungi and mutualistic plants-fungi networks).