CHAPTER 4

# Elevated and depressed $CO_2$ may have contrasting effects on host plant ability to favour high-quality symbionts over multiple generations

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**Abstract**

The symbiosis between plants and root-colonising arbuscular mycorrhizal fungi (AMF) is one of the most ecologically important and widespread examples of interspecific cooperation in the world. Plants exchange carbon for soil minerals provided by the AMF. While the relationship is generally beneficial, benefits provided to the symbiotic partner can vary and fitness effects can range from parasitic to highly mutualistic. Plants can increase the direct fitness benefits they obtain by allocating resources to high-quality fungi over lower-quality AMF. Such preferential rewarding is thought to be an important factor stabilising this ancient partnership. However, partner preferences can vary with environmental context, particularly when resource availability affects the relative value of symbiotic services. We ask how differences in atmospheric $CO_2$-levels influence root colonisation dynamics between AMF species that differ in their quality as symbiotic partners. We find that with increasing $CO_2$-conditions, the more beneficial fungal species is able to achieve a relatively higher abundance than under ambient and depressed $CO_2$-conditions. This is consistent with a scenario where higher atmospheric carbon supply enables plants to more efficiently regulate community composition. Over multiple plant generations this differentially affects the success of both species: plant hosts grown under ambient and elevated $CO_2$-conditions maintain only the more beneficial AMF species, while plants under depressed $CO_2$-conditions exclude both. These results shows how environmental context may mediate hosts' ability to regulate their symbiotic community.

## Introduction

While the evolution of costly intraspecific cooperation is commonly explained through indirect fitness benefits being directed to related individuals (kin selection) (*e.g.* Gardner *et al.*, 2011; Liao *et al.*, 2015), explaining the evolution of cooperation between species, *i.e.* mutualisms, can be difficult. In mutualistic cooperation, individuals of different species can potentially exploit the relationship by obtaining benefits without contributing to the interaction (Bao & Addicott, 1998; Bshary & Grutter, 2002a; Sachs *et al.*, 2010; Ghoul *et al.*, 2014). Therefore, because of the risk of free-riders (Ghoul *et al.*, 2014), both partners must experience direct fitness benefits in order for costly cooperation between species to arise. One way in which costly investment in a partner can be favoured is through vertical transmission of symbiotic partners because it aligns the evolutionary interests of both parties (Ewald, 1991; Herre *et al.*, 1999; Bright & Bulgheresi, 2010). Yet, we see many examples of mutualisms where partners are acquired from the environment anew each generation and cooperation still remains stable (Remy *et al.*, 1994; Denison & Kiers, 2011; Doyle, 2011; McFall-Ngai, 2014; Frommlet *et al.*, 2015; Salem *et al.*, 2015). Such horizontally transmitted symbioses can be stabilised by various forms of partner choice (Bshary & Grutter, 2002b; Gubry-Rangin *et al.*, 2010; Werner & Kiers, 2015a; Chapter 3), sanctions against cheating partners (West *et al.*, 2002b,a; Kiers *et al.*, 2003; Oono *et al.*, 2011) or preferential allocation of resources to high-quality partners that contribute more to their partners (Bever *et al.*, 2009; Adam, 2010; Kiers *et al.*, 2011; Wyatt *et al.*, 2014). All of these mechanisms have in common that they increase the relative fitness of a cooperating partner species compared to a cheater, thereby favouring investment and the provisioning of direct fitness benefits.

The resource mutualism between plants and arbuscular mycorrhizal fungi (AMF) is emerging as an important system to study the dynamics of direct fitness benefits (Schwartz & Hoeksema, 1998; Hoeksema & Schwartz, 2003; Kiers *et al.*, 2011; Verbruggen *et al.*, 2012). Plants invest in their AM fungal partners by providing them with carbon, while the fungi can benefit their hosts by providing soil minerals, primarily phosphorus (Parniske, 2008; Walder *et al.*, 2012). In this interaction, both plants and AMF can interact with multiple partners simultaneously (Giovannetti *et al.*, 2004; Montesinos-Navarro *et al.*, 2012; Walder *et al.*, 2012). There is accumulating evidence that individual host plants and fungi can detect differences in the contribution of the other partners, and preferentially allocate carbon or soil minerals to the more beneficial plant or fungus (Bever *et al.*, 2009; Kiers *et al.*, 2011; Fellbaum *et al.*, 2012, 2014; Verbruggen *et al.*, 2012). Consequentially, the mutualism can be conceptualised as a 'biological market' (Noë & Hammerstein, 1995; Schwartz & Hoeksema, 1998; Werner *et al.*, 2014c; Chapter 2), where both partners can detect direct fitness benefits obtained from the other partner and act in such a way as to maximise these benefits. This

'reciprocal preferential rewarding' is thought to stabilise the interaction by limiting the spread of cheaters (Kiers *et al.*, 2011).

While preferential allocation mechanisms can help reduce conflict and stabilise mutualistic relationships, context plays an important role in determining overall benefits to interacting partners (Hoeksema *et al.*, 2010; Werner & Kiers, 2015a). In certain environmental conditions, for instance high-phosphorus soils, AMF may have a negative fitness effect on their plant hosts (*i.e.* be parasites) (Johnson *et al.*, 1997; Johnson, 2010). Despite such variation in context determining overall benefits (and costs) of the relationship, the expectation is that hosts continue to preferentially allocate photosynthate to the AMF partner that is relatively more beneficial (Werner *et al.*, 2014c; Wyatt *et al.*, 2014). A core idea of biological markets is that trading partners should allocate resources and favour partners according to what is most beneficial in that particular exchange, *i.e.* in the specific environmental and ecological conditions locally prevalent (Noë & Hammerstein, 1995; Leimar & Hammerstein, 2010). Actors plastically respond to variable conditions, particularly to those that affect the relative value of the exchanged good or services, in an effort to maximise mutualistic benefit (Metz *et al.*, 2007; Cowden & Peterson, 2009; Fruteau *et al.*, 2009; Adam, 2010; Werner *et al.*, 2014c). In the mycorrhizal symbiosis, this raises the question if preferential allocation to higher-quality AMF is also plastic and responsive to environmental conditions, particularly to conditions that directly affect levels of exchanged resources. Alternatively, plant preference may be a fixed response, irrespective of context, for instance because plants recognise certain beneficial AMF rather than evaluating their actual contribution. If the environmental conditions shift so that one of the exchanged nutrients becomes more available, does this affect the way plant allocate resources to symbiotic partners?

We studied the effects of depressed and elevated (relative to the present ambient) $CO_2$-levels on the colonisation success of two closely related AMF species that vary in the benefits they provide (Kiers *et al.*, 2011). As obligate biotrophs, AMF can only obtain carbon from host plants (Parniske, 2008). This means they only have access to additional carbon, such as elevated $CO_2$-levels ($eCO_2$), through plant-mediated allocations. Over the last centuries, $CO_2$-levels increased considerably from an estimated 278 ppm in the 18$^{th}$ century to 390 ppm in 2011 (Hartmann *et al.*, 2013), reducing plant carbon limitation (Ainsworth & Long, 2005; Reich *et al.*, 2006) and potentially increasing AMF root colonisation and carbon allocation (Treseder, 2004; Alberton *et al.*, 2005; Drigo *et al.*, 2010, 2013; Fortuna *et al.*, 2012). Theoretically, a relative increase in carbon availability is expected to increase the value of phosphorus, selecting for increased transfer of photosynthate to AMF, and for increased reciprocal phosphorus transfer from AMF to plant. This has been predicted to favour an overall increased cooperation between plant and fungal partners (Wyatt *et al.*, 2014; Bever, 2015).

Higher plant carbon budgets could increase the plant-derived carbon available to more beneficial AMF, increasing the host's effectiveness in selecting high-quality mutualists. Consequentially, anthropogenic $CO_2$-emissions could drive a stronger and more efficient plant-AMF mutualism (Johnson *et al.*, 2013). Alternatively, carbon could become a luxury good with increasing $CO_2$-levels (Kiers & van der Heijden, 2006), and host plants could experience reduced selection to stringently allocate their photosynthate to high-quality partners (Golubski & Klausmeier, 2010). Under this scenario, high $CO_2$-levels might reduce a plant's ability to effectively structure their symbiont community. While previous work has shown shifts in AMF communities under varying $CO_2$-levels (*e.g.* from *Acaulosporaceae* and *Gigasporaceae* to *Glomeraceae* (Drigo *et al.*, 2010; Cotton *et al.*, 2015)), these studies did not test changes in specific species abundances.

Here, we studied how environmental effects mediate plant preferential allocations by modifying atmospheric $CO_2$-levels and analysing the abundance over multiple plant generations of two competing AMF species (both *Glomeraceae*) differing in the benefits they provide to plants. We expected to find a relatively higher abundance of beneficial AMF in elevated $CO_2$-conditions based on the hypothesis that increased carbon availability enables plants to more effectively favour growth of higher-quality AMF. Because such selection of high-quality partners is thought to limit the spread of less beneficial AMF (Kiers *et al.*, 2011; Werner & Kiers, 2015a), we also expected that at higher $CO_2$-levels, low-quality AMF species would be less successful at maintaining themselves in an AMF community over time. We therefore tested if the abundances of the AMF species in a mixed root community changed when grown under different $CO_2$-levels over multiple plant generations. We hypothesised that increasing $CO_2$-levels would positively affect the host's ability to mediate the fungal community to their benefit, resulting in a relative increase of the high-quality species across subsequent plant generations.

## Materials & Methods

### *Experimental Design*

We studied plant growth and intraradical AMF abundance in plants grown under depressed atmospheric $CO_2$-levels ($dCO_2$), ambient $CO_2$ ($aCO_2$) and elevated $CO_2$ ($eCO_2$) (see: *Plant growth conditions*). We inoculated *Medicago truncatula* seedlings either with monocultures of two closely related AMF species *Glomus aggregatum* or *Rhizophagus irregularis* (formerly known as *Glomus intraradices* (Krüger *et al.*, 2012)), with a 1:1 mixture of both species, or without AMF (negative control). Previous research had shown that *R. irregularis* is a higher-quality symbiont that is more beneficial to host plants, while *Glomus aggregatum* employs a less cooperative hoarding strategy rather than providing phosphorus (Kiers *et al.*, 2011). After inoculation, we grew plants for 12 weeks at their respective $CO_2$-levels, and then destructively harvested them for biomass

and fungal abundance data. We used a total of ten replicates per treatment, *i.e.* a total of 120 plants (3 $CO_2$-levels * 4 AMF-treatments). We used root material and soil from the plants inoculated with a mixed inoculum to inoculate a new generation of plants (see: *Multigenerational transfer of AMF*), which we grew at the same $CO_2$-level as previously.

### *Plant growth conditions*

We used *M. truncatula* var. Jemalong A17 (INRA, Montpellier, France). First, we scarified and sterilized seeds using 95% $H_2SO_4$ for 6.5 min, rinsing them six times in an excess of demineralised water to remove all traces of acid. The scarified seeds were cold-treated at 4°C for 4 days and then planted in autoclaved peat-based germination mix. After 10 days we washed the seedling roots with demineralised water to remove the germination mix. We then transferred the seedlings to sterilised pots (max. volume 662 ml, type MXC12, Pöppelmann, Lohne, Germany) containing autoclaved quartz sand (≥99.5% $SiO_2$). Every two weeks, we added 25 ml of Hoagland solution per pot (Hoagland & Arnon, 1950) with P content reduced to 50% of the standard solution and N content increased to 150% to favour mycorrhizal colonisation (Johnson, 2010). Plants were grown in fully controlled climate chambers at Utrecht University, under a 12/12 h day/night regime, 22/17 °C day/night temperature and 70% air humidity and were regularly watered. Light intensity during the day was 315 µmol $m^{-2}$ $s^{-1}$ (SD 14). We grew plants in separate $CO_2$-controlled climate chambers (Reftech B.V., Sassenheim, The Netherlands) which recorded the following average $CO_2$ levels during the two months of growth: depressed ($dCO_2$, 161 ppm, SD 7.5), ambient ($aCO_2$, 496 ppm, SD 58) or elevated ($eCO_2$, 743 ppm, SD 73) $CO_2$-levels.

### *AMF inoculation*

We followed the same procedure as previously described to produce AMF inocula (Werner & Kiers, 2015b; Chapter 5). We grew *in vitro* cultures of *R. irregularis* isolate 09 and G. *aggregatum* isolate 0165 on *Daucus carota* L.-transformed root organ cultures for four months, suspended the root organ cultures of AMF species in demineralised water and standardised spore densities (Engelmoer *et al.*, 2014; Werner & Kiers, 2015b), resulting in  250 AMF spores $ml^{-1}$. During planting we randomly assigned seedlings an AMF-treatment and $CO_2$-level and applied a suspension volume corresponding to 1,000 spores of *R. irregularis*, *G. aggregatum* or a 1:1 mix of both species directly to the roots. For the negative control plants we applied the same amount of demineralised water (4 ml).

### *Harvest protocol and intraradical AMF abundance*

We destructively harvested all plants 12 weeks after planting, following the same harvest protocol as described previously (Werner & Kiers, 2015b) and determining plant aboveground dry weight. We obtained three randomized root fragments subsets: one

was frozen at –20°C and used for later molecular analyses, one subset was stored in individual plastic bags at 4°C and used to inoculate a next generation of plants where applicable (in the mixed AMF treatments), while the third subset was used to determine belowground dry weight. We calculated full plant dry weight, by summing aboveground and belowground dry weights. In each of our three AMF-inoculated treatments grown under $dCO_2$, two plants had died during the experiment. We removed these from our analyses; consequentially there are only eight replicates in all $dCO_2$-conditions treated with AMF.

To determine intraradical AMF abundance, we followed the same protocol as previously described for these AMF species and host plants (Werner & Kiers, 2015b). Briefly, we used primers specific to *G. aggregatum* and *R. irregularis* mtSLU sequences. These allow us to determine AMF species-specific gene copy numbers in our DNA extract, which is a metric for the abundance of mitochondrial DNA in both species and a measure of the overall AMF abundance. This allows us to discriminate and quantify intraradical abundance of both species even in a mixed inoculum (Kiers *et al.*, 2011; Thonar *et al.*, 2012, 2014; Engelmoer *et al.*, 2014). AMF abundances as measured with this protocol have a strong positive correlation with visual AMF colonisation scoring (Werner & Kiers, 2015b), but visual identification cannot discriminate these species when colonising the same root systems as in this study. As previously, we expressed AMF abundance in copy numbers $mg^{-1}$ freeze-dried roots, correcting for DNA extraction efficiency of each sample (Engelmoer *et al.*, 2014; Werner & Kiers, 2015b).

***Multigenerational transfer of AMF***

Using the same inoculation and plant growth conditions as previously, we inoculated a new generation of *M. truncatula* seedlings using an average of 1.35 g (SD 0.23) of mycorrhizal root fragments and 61 g (SD 14.2 g) of soil from our mixed AMF treatments. This way, spores in the soil and on the mycorrhizal root fragments and AMF hyphae extending from the root fragments could colonise the new generation of plants. The transfer protocol simulates the process occurring in the field when a new generation of annual plants is recolonised by AMF from infected roots and soil spores. This allows us to study potential long-term shifts in AMF species composition in a greenhouse setting. Again, we grew plants for 12 weeks in the same controlled climate chambers before destructively harvesting them and analysing them as previously. We studied a total of three plant generations.

To determine if the transfer protocol of fungi to subsequent generations of host plants was equally efficient for both species, we performed a pilot study. Our aim was to ensure that changes in relative abundances over generations were not caused by differences in transfer efficiency, for instance due to a lower disturbance resistance of one AMF

species compared to another. The pilot experiment revealed that for both *G. aggregatum* and for *R. irregularis*, AMF abundance actually increased between two test generations (Supplementary Figure S1), with comparable increases of 17.2% (*G. aggregatum*) and 19.4% (*R. irregularis*). In these monoculture pilot studies, we found that *R. irregularis* had a significantly higher overall abundance than *G. aggregatum* ($F_{1,36}$ = 7.64, $P$ = 0.01) and that AMF abundance significantly increased between generations ($F_{1,36}$ = 41.97, $P$ <<0.01). However, we found a non-significant interaction between generation and AMF species ($F_{1,36}$ = 0.23, $P$ =0.63), statistically confirming that there were no differences in transfer efficiency between the two AMF species.

***Statistical Analysis***

We performed all our statistical analyses in R 3.2.0 (R Core Team, 2015). We first analysed full plant dry weight in the first generation to determine how $CO_2$-level and AMF-inoculation affect plant growth. We used R-package *glmulti* to build all possible linear models of the dependent variable full plant dry weight and $CO_2$-level (depressed, ambient, elevated), AMF-treatment (negative control, *G. aggregatum*, *R. irregularis* and mixed inoculation) and their interactions as independent variables and used AICc-criteria to select the best model (Calcagno & Mazancourt, 2010). We used Tukey's Honest Significant Differences tests to perform post-hoc comparisons of this model.

We then analysed AMF intraradical abundances in our initial generation to assess the effects of AMF species *(G. aggregatum* vs. *R. irregularis*), $CO_2$-level and inoculation type (monocultures containing a single AMF vs. mixed inocula). Again, we used *glmulti* and AICc-criteria to guide our model selection and fitted all possible models containing these terms and their interactions as fixed independent variables while now using the logarithm of AMF copy number $mg^{-1}$ root mass as dependent variable. We used generalised linear mixed models (GLMM) and a gamma distribution in R-package *lme4* (Bates *et al.*, 2014), because assumptions of normality and heteroscedasticity were not met. We used plant as a random effect, because in the mixed inoculum treatment we measured both AMF species on the same plant. The random effect term takes into account the resulting non-independence (Behm *et al.*, 2013; Engelmoer *et al.*, 2014; Werner & Kiers, 2015b). We used R-package *phia* to perform post-hoc tests using Holm's method to adjust for multiple comparisons (De Rosario-Martinez, 2015).

Third, we studied the AMF abundance data in our multigenerational mixed inoculum plants to determine if there was an effect of generation (first, second and third generation) or $CO_2$-level on intraradical AMF abundance. We used linear mixed models (LMMs) in *lme4* and again used *glmulti* and AICc-criteria to guide model selection, *phia* for post-hoc comparisons and plant as a random effect in our LMMs to take into account non-independence between generations.

In all our analyses, we had set AMF copy numbers that were below the limit for reliable detection to equal the detection limit (Engelmoer *et al.*, 2014; Werner & Kiers, 2015b). This means that samples below the detection limit were analysed as if the abundance was at the detection limit. This is the most conservative way to analyse our experimental design because it makes it more difficult to observe complete exclusion of an AMF from the roots,. In our analyses of multigenerational AMF abundances we observed many samples below the detection limit (41 out of 168 observations over three generations) and we therefore repeated this part of our analyses while setting AMF copy number in these samples equal to zero. We found qualitatively very similar results (Figure S2).

## Results

### *$CO_2$-level and AMF inoculation influence plant growth*

We first determined the effect of $CO_2$-levels and AMF inoculation on plant growth (Figure 1). We found that a linear model which included both $CO_2$-levels and AMF treatment, but not their interaction best explained these data. As expected, plants were smaller under $dCO_2$ (Figure 1A; $aCO_2$ mean weight 1.69 g ± S.E. 0.02 vs. $dCO_2$ mean 0.94 g ± S.E. 0.02; post-hoc: $P << 0.01$). However, in contrast to our expectations we found that plants grown under $eCO_2$-levels were slightly smaller than under $aCO_2$ ($eCO_2$ mean weight 1.57 g ± 0.03 g; $P << 0.01$).

Second, we observed that across all $CO_2$-levels plants had significantly higher biomass when inoculated with *R. irregularis* than with *G. aggregatum* (Figure 1B; p=0.04), confirming that *R. irregularis* is a higher-quality partner for the host than *G. aggregatum*. We found that inoculating the host with a mixture of both AMF resulted in a reduced plant growth compared to non-mycorrhizal plants (p=0.01), while inoculation with monocultures of *R. irregularis* (p=0.98) and *G. aggregatum* (p=0.10) did not significantly affect plant growth.

### *With increasing $CO_2$-levels, lower-quality AMF suffer in competition with high-quality AMF*

We determined the intraradical fungal abundances for plant roots when inoculated with a monoculture of either species, and with a mixed inoculum (Figure 2). In the mixed treatments, when plants have the potential for preferential allocation to a relatively higher quality AMF, we expected to find lower abundances for the lower-quality AMF *G. aggregatum*, particularly under $eCO_2$ conditions. Using AICc-criteria, we found that a model that included the main terms AMF Species, $CO_2$ and Inoculation (mixed vs. monoculture) as well as the interaction terms Species:Inoculation and $CO_2$:Inoculation best described our data. Our finding that inclusion of these two interaction terms provides a good model fit is consistent with our expectations that the effect of potential

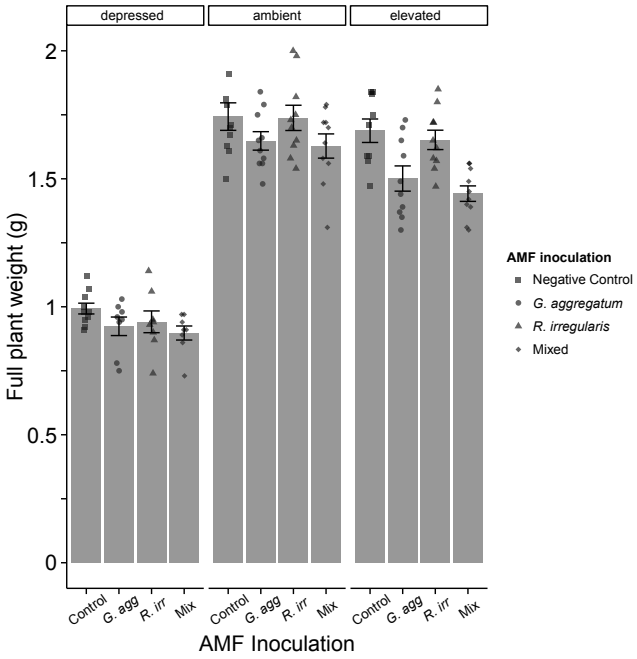


**Figure 1: Full plant weight (g) for each AMF treatment at each $CO_2$-level (± S.E.).**

Panels indicate $CO_2$-level plants were grown in.

preferential allocations (Inoculation) varies between both AMF species and between $CO_2$-conditions, while the lack of a $CO_2$:Species interactions indicates that $CO_2$-levels do not differentially affect both AMF-species.
Specifically, we wanted to know if *G. aggregatum* suffers more from being co-inoculated with *R. irregularis* than the reverse. In order to test this, we performed post-hoc tests where we compared abundance between mixed and monoculture inoculations (*i.e.* with potential for preferential rewarding and without such potential) for both AMF species at all $CO_2$-levels. We found that *R. irregularis* did not suffer from competition in $dCO_2$ ($p>0.99$), $aCO_2$ ($p>0.99$) or $eCO_2$ ($p=0.31$). In contrast we found that *G. aggregatum* suffers from competition under $aCO_2$ ($p=0.03$) and $eCO_2$ ($p<<0.001$), but not under $dCO_2$ ($p=0.29$).

This pattern is consistent with the idea that preferential allocations increase in strength with higher $CO_2$-levels. In order to further quantify this effect, we calculated Armas' relative interaction index (RII) for both AMF species at all $CO_2$-levels (Armas *et al.*, 2004). RII takes into account an organism's performance in the absence and presence of a potential competitor (or facilitator) and takes negative values for competitive

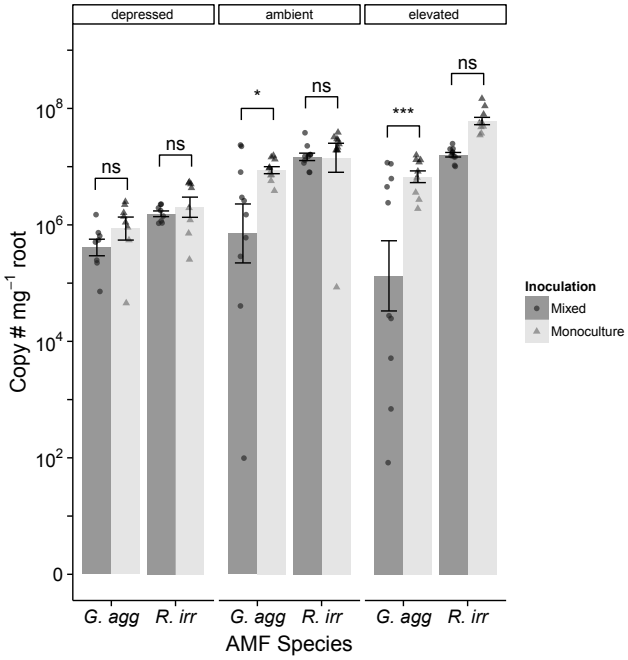


**Figure 2: Mean intraradical AMF root abundance (copy number $mg^{-1}$ dry root mass, logarithms, ± S.E.) for both *G. aggregatum* and *R. irregularis*.**
Plants were inoculated with either a monoculture containing a single AMF species or with a mixed inoculum. Panels indicate $CO_2$-levels the plants were grown under. Significant differences in abundance between mixed and monoculture inoculation are indicated for both species within each $CO_2$-level (ns: non-significant, *: P<0.05, ** P<0.01, ***P<0.001).

interactions and positive for facilitative (Armas *et al.*, 2004). We observe that for *G. aggregatum*, the average RII becomes increasingly negative with higher $CO_2$-levels (Table 1), indicating increased competition experienced from presence of the other AMF. In contrast, we observe that for *R. irregularis* average RII is zero or only slightly negative, indicating *R. irregularis* experiences no or only limited competition. These patterns (Figure 2, Table 1) suggest that *G. aggregatum* abundances are successfully reduced, particularly at $eCO_2$ but also under $aCO_2$.

Table 1: Relative Interaction Index for both AMF species.

| $CO_2$-level | $RII_{Aggregatum}$ | $RII_{Irregularis}$ |
|---|---|---|
| $dCO_2$ | -0.03 | -0.01 |
| $aCO_2$ | -0.08 | 0.0 |
| $eCO_2$ | -0.14 | -0.04 |

***Over multiple plant generations, G. aggregatum abundance is reduced below the detection limit***

We then considered these fungal abundance patterns over multiple generations, with the aim of determining if community composition became more beneficial to plants (*i.e.* relatively more *R. irregularis*) over time, and if this was affected by $CO_2$-level. In order to address this, we analysed AMF abundance for both species in the mixed treatments over three generations. We found that the lower-quality AMF is driven below the detection limit within a single generation, regardless of $CO_2$ treatment (Figure 3). In contrast, *R. irregularis* is maintained in $eCO_2$-conditions, is only slowly reduced under $aCO_2$, and is also driven below the detection limit under $dCO_2$, but only after three generations (Figure 1). Setting the AMF abundance of those samples that were below the detection limit to zero in our analysis, resulted in qualitatively the same pattern (Figure S2).

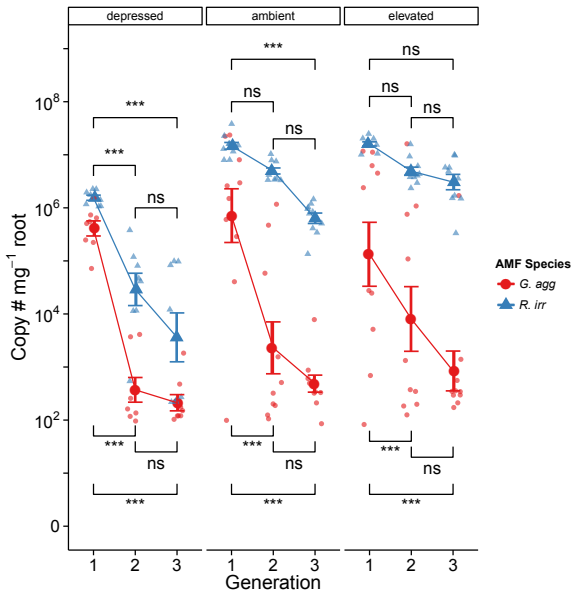


**Figure 3: Mean intraradical AMF root abundance (copy number $mg^{-1}$ dry root mass, logarithmic scale, ± S.E.) in plants inoculated with mixed inocula over three generations for both *G. aggregatum* (red) and *R. irregularis* (blue).**

Panels indicate the $CO_2$-levels plants were grown under. Asterisks indicate significance for pairwise comparisons of AMF abundances between generations for both species within each $CO_2$-level (ns: non-significant, *: P<0.05, ** P<0.01, ***P<0.001). Significances are indicated below the lines indicating abundance for *G. aggregatum*, and above the lines for *R. irregularis*.

## Discussion

Our aim was to ask if external resource availability affected how hosts mediate direct fitness benefits of two competing symbionts on a plant root system. Specifically, we wanted to determine if (i) lower-quality AMF performed worse if there was the potential for plant preferential allocations, (ii) if this was mediated in a directional way by $CO_2$-level and (iii) if this had long-term consequences for the spread of lower-quality symbionts over multiple plant generations. Our results reveal that intraradical abundance of *R. irregularis* increased with $CO_2$-level, both in mixed and monoculture treatments (Figure 2). In contrast, for the lower-quality AMF *G. aggregatum*, abundance

increased with $CO_2$ in monocultures but decreased when grown in a mixed inocula. This pattern was found under both $aCO_2$ and $eCO_2$, but not $dCO_2$ (Figure 2, Table 1), suggesting that a plant's ability to sanction low-quality partners is influenced by external conditions, specifically by $CO_2$. While we emphasise that we only studied the resulting abundance patterns and did not directly measure nutrient flows, these observations are consistent with a scenario in which plants with access to more carbon are better able to preferentially reward symbiotic partners. To definitively confirm this to be the case, future research should now directly track allocation patterns of both partners under varying $CO_2$-conditions (Fellbaum *et al.*, 2014; Pringle, 2015). However, because the symbionts can only survive on host-derived carbon, relative fungal abundances can be used as an indication of how hosts distribute the carbon over their symbionts (Kiers *et al.*, 2011). Our finding is therefore in line with theoretical predictions of a (small) positive effect of higher $CO_2$-levels on AMF cooperativity and efficient plant preferential allocations (Wyatt *et al.*, 2014; Bever, 2015), and illustrates how environmental context might affect hosts' capacity to structure their symbiotic community.

From a biological market perspective $eCO_2$-conditions are predicted to favour stronger selection by plants of partnering AMF (Wyatt *et al.*, 2014; Bever, 2015). We also studied the reverse scenario, the effect of depressed $CO_2$-conditions on symbiont community dynamics. Consistent with expectations, we found an absence of partner selection, with no evidence of preferential allocations to the high-quality AMF at $dCO_2$. Here, both AMF species experienced very similar (and small reductions) in abundance when grown in a mixture compared to in a monoculture (Figure 1), suggesting preferential allocations are not effective at $dCO_2$. Prior experiments which reduced plant carbon availability found similar patterns, namely decreased preferential allocation with increased shading (Zheng *et al.*, 2015), further confirming that reduced carbon budgets can limit the potential for plants to favour more beneficial symbiotic partners. Over long-time periods, conditions like these where there is reduced benefit of preferential allocation, might select for a loss of preferential allocation mechanisms, which may be costly to plants (Steidinger & Bever, 2014; Simonsen & Stinchcombe, 2014). In previous research we found that shading plants can also cause reduced colonisation of *G. aggregatum* in mixed inocula (Knegt *et al.*, 2014). However, in the experimental conditions used there, *G. aggregatum* did not act as a lower-quality partner (Knegt *et al.*, 2014), meaning we cannot directly interpret these results in terms of plant preferential allocations.

Combined, our current observations and previous experimental and theoretical work (Wyatt *et al.*, 2014; Zheng *et al.*, 2015; Bever, 2015), argue against the idea that plant allocation is a fixed response to partner identity but rather suggest that this is affected by environmental context. In agreement with our findings, AMF inocula from long-term FACE $CO_2$-enriched plots were found to provide more nitrogen to hosts plants,

suggesting selection for higher fitness benefits with increasing $CO_2$-levels (Gamper *et al.*, 2005). Anciently elevated $CO_2$-levels, which are thought to facilitated the evolution of the plant-AMF mutualism (Field *et al.*, 2012), may therefore also have enabled more efficient selection of high-quality AMF partners. Furthermore, recent work showed that $eCO_2$ resulted in phylogenetic clustering of AMF communities, which the authors argued was consistent with altered host selection for more beneficial AMF under $eCO_2$ (Mueller & Bohannan, 2015).

While our results are consistent with host-directed benefits to symbiont communities as a dynamic response that changes with relative value of exchanged resources, we cannot exclude that direct competition between AMF (*i.e.* not through plant mediation) plays a role. This competition could be between the intra- or extra-radical hyphae (Hepper *et al.*, 1988; Kennedy, 2010; Engelmoer *et al.*, 2014). Direct competitive interactions between fungal partners may play a role in determining fungal abundances (*e.g.* Jansa *et al.*, 2008), but because AMF are in an obligate symbiosis with the host, this direct competitive antagonism is difficult to quantify. However, in our case this would assume that direct AMF-AMF competition is affected by $CO_2$-levels, which is unlikely considering that AMF do not have direct access to environmental carbon (Parniske, 2008). It is more likely that the effects of $CO_2$-level on AMF-AMF competition are mediated by host allocation processes.

Preferential rewarding strategies are thought to stabilise mutualisms, and limit the spread of low quality partners throughout populations and over time (Oono *et al.*, 2011; Ghoul *et al.*, 2014; Steidinger & Bever, 2014; Bever, 2015). A major open question is if variation in allocation strategies will ultimately affect long-term success of competing symbionts. Our aim was to track our AMF community over multiple generations to see if shifts in community composition would occur. First, we found that overall AMF abundances decreased over three plant generations (Figure 3). This is consistent with plants at each generation trying to limit colonisation in order to minimise the negative growth effects from AMF inoculation we observed (Figure 1). Second, we find no significant change in *R. irregularis* abundance between generations under $eCO_2$ and only a very slow reduction in $aCO_2$-conditions (Figure 3), suggesting that aCO2 and particularly $eCO_2$-plants are more likely to be able to retain the high-quality AMF over longer timer periods. Third, we found that abundances of *G. aggregatum* were reduced to below the detection limit very rapidly under all $CO_2$-conditions. Although we cannot extrapolate longer than three plant generations, these patterns suggest that under higher $CO_2$-levels higher-quality symbionts are maintained, while lower-quality symbionts disappear. This suggests that the long-term patterns towards more beneficial AMF observed previously (Gamper *et al.*, 2005), may be driven by plant preferential allocations.

A limitation of our study in detecting long-term effects of $CO_2$-level on AMF dynamics is the observation that plants seem to reduce AMF colonisation in general (Figure 3). This is probably due to negative growth effects found under our laboratory conditions (Figure 1). Potentially, plants do not experience fitness benefits from AMF in our experimental conditions due to relatively short day lengths and light intensities of the growth chambers. However, one fungal partner was a better partner than the other (Figure 1) as found before (Kiers *et al.*, 2011), and thus we were able to study how the potential for host preference affected abundances. Rather than documenting an active investment every generation in the high-quality species, we found the higher-quality species was being reduced at a slower rate compared to the reduction of the low-quality species (Figure 3). We expect to find stronger patterns of preferential allocation if species or conditions utilised give both positive and negative growth effects for different AMF species. Another potential limitation of our study is that while $dCO_2$ resulted in substantial plant growth reduction, $eCO_2$ did not increase plant growth (Figure 1). This suggest that in these growth conditions, *M. truncatula* is limited by another factor than $CO_2$. One idea is that the effects of $CO_2$ on Medicago are temperature-sensitive. The closely related host plant *Medicago sativa* was found to only benefited from $eCO_2$ when temperature was also elevated (4°C increase from standard 19°C) (Aranjuelo *et al.*, 2008). Positive growth responses to $eCO_2$ would potentially reveal an even stronger shift in the AMF community to high-quality symbionts than observed here. Particularly, they would potentially reveal stronger differences between $aCO_2$ and $eCO_2$, rather than the present study, which revealed strongest effects of $CO_2$ on AMF preferential allocation when comparing $aCO_2$ and $dCO_2$ (Figures 2 and 3).

In conclusion, our results illustrate how environmental context may affect the extent to which organisms are able to optimise their symbiont community. We found that the potential for partner rewarding in *M. truncatula* is not a fixed characteristic, but responds to $CO_2$ level in a manner consistent with biological market theory (Wyatt *et al.*, 2014; Bever, 2015; Werner & Kiers, 2015a), and we found this affects the long-term success of high-quality symbionts in the community. Similar to human markets and primate biological markets (Fruteau *et al.*, 2009), changing the value of exchanged resources influences trader preferences for particular partners in a biological market of plant and microbes. This illustrates how environmental context can affect the extent to which organisms can maximise direct fitness benefits of their symbiont communities.

### Authors' Contributions

GDAW, CMJP and ETK designed research, GDAW and YZ performed the experiments, GDAW performed the analyses, GDAW and ETK wrote the manuscript.

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