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Direct evidence for modulation of photosynthesis by an arbuscular mycorrhiza-induced carbon sink strength

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Summary

- It has been suggested that plant carbon (C) use by symbiotic arbuscular mycorrhizal fungi (AMF) may be compensated by higher photosynthetic rates because fungal metabolism creates a strong C sink that prevents photosynthate accumulation and down-regulation of photosynthesis. This mechanism remains largely unexplored and lacks experimental evidence.
- We report here two experiments showing that the experimental manipulation of the mycorrhizal C sink significantly affected photosynthetic rates of cucumber host plants. We expected that a sudden reduction in sink strength would cause a significant reduction in photosynthetic rates, at least temporarily.
- Excision of part of the extraradical mycorrhizal mycelium from roots, and causing no disturbance to the plant, induced a sustained (10-40%) decline in photosynthetic rates that lasted from 30 min to several hours in plants that were well nourished and hydrated and in the absence of growth or photosynthesis promotion by mycorrhizal inoculation. This effect was though minor in plants growing at high (700 ppm) atmospheric CO₂.
- This is first direct experimental evidence for the carbon sink strength effects exerted by arbuscular mycorrhizal symbionts on plant photosynthesis. It encourages further experimentation on mycorrhizal source-sink relations and may have strong implications in large-scale assessments and modelling of plant photosynthesis.

Keywords: carbon (C) assimilation; elevated CO₂; gas-exchange; mycorrhiza; source-sink relations

Introduction

Arbuscular mycorrhizal fungi (AMF) have been extensively investigated because of their capacity to increase nutrient uptake and transfer, thereby improving the nutrition of their hosts (Smith & Read, 2008). However, AMF live entirely on plant carbon (C) and may use up to 20 % of plant fixed C for their own growth and functioning (Jakobsen and Rosendahl, 1990), an important counterpart of the symbiosis to date much less studied and understood than fungus-to-plant nutrient transfer (Kaschuk *et al.*, 2009). Furthermore, a ¹³C labelling experiment showed that AMF hyphae received 4.3% of the recently fixed plant C within 24 h (Tomè *et al.*, 2015). Plant C use by AMF has been suggested to be high enough to cause

growth depressions in some plants, especially during the establishment phase in roots and soil (Jakobsen, 1999; Graham, 2000; Ryan & Graham, 2002). However, it has also been hypothesized that the use of plant C by AMF may be compensated by higher photosynthetic rates in the hosts because the rapid removal of photosynthates by fungal metabolism creates a C sink strength that prevents their accumulation and down-regulation of the photosynthesis reaction (Wright *et al.*, 1998; Kaschuk *et al.*, 2009; Schweiger *et al.* 2014).

Plant photosynthesis is a complex reaction that can be limited by a large number of factors including sources (nutrients, light, water, CO₂), sinks (cells where end-products are used), and physiological conditions that are genetically defined within species for the reaction to occur, such as temperature, enzymatic activity, photosystems, etc. (Paul & Foyer, 2001; Long *et al.*, 2004). Photosynthetic rates are often higher in mycorrhizal than in non-mycorrhizal plants (Valentine *et al.*, 2001; Amaya-Carpio *et al.*, 2009; Zhu *et al.*, 2012) and this has been assumed to mainly result from improved plant nutrition, especially P nutrition, in mycorrhizal plants (Miller *et al.*, 2002; Augé *et al.*, 2016). Nevertheless, a thorough evaluation of gas-exchange, physiological and gene expression measurements showed that not only photosynthetic rates but also the entire pathway of C movement from shoots to roots was enhanced in mycorrhizal tomato plants independently from nutritional benefits (Boldt *et al.*, 2011). Similarly, at least half of the foliar metabolic changes and increased C assimilation in mycorrhizal *Plantago major* were unrelated to P levels (Schweiger *et al.*, 2014).

The alternative mechanism suggesting that the improved gas-exchange and C source-sink relationships of arbuscular mycorrhizal plants results also from the dynamic C flow from the sources aboveground to the sinks both above- and below-ground (Godbold *et al.*, 2006; Moyano *et al.*, 2007; Kaschuk *et al.*, 2009) is however much less supported by experimental evidence. Some studies have shown that increasing below-ground C sink strength by increasing the biomass or the number of root symbionts leads to further enhancement of photosynthetic rates (Gavito *et al.*, 2000, 2002; Mortimer *et al.*, 2008, 2009; Kaschuk *et al.*, 2009; Bulgarelli *et al.*, 2017). Moreover, mycorrhizas may increase plant photosynthesis and reduce plant growth depression at elevated atmospheric CO₂ (Syvertsen & Graham 1999; Gavito *et al.*, 2002; Jiffon *et al.*, 2002), and this further indicates the importance of the mycorrhizal C sink in plant source-sink relationships. C source-sink interplay in plants forming arbuscular mycorrhizal symbioses may thus have a significant effect on plant photosynthesis and C movement below-ground, which is especially relevant under future scenarios with higher availability of atmospheric CO₂ (Fatichi *et al.*, 2014, Finzi *et al.*, 2015). The mycorrhizal C sink strength and its effects on plant gas-exchange and C movement from

plant to soil are among the mycorrhizal features of potentially large environmental importance that remain mainly theoretical and await experimental evaluation (Kaschuk *et al.*, 2009). All available evidence for the effect of the AMF C sink strength on plant gas exchange and C source-sink relationships is based solely on comparisons made between mycorrhizal and non-mycorrhizal plants. This is useful yet indirect evidence for a significant effect of the mycorrhizal C sink strength on C assimilation.

We report here the results of two experiments designed to investigate if the experimental manipulation of the arbuscular mycorrhizal C sink significantly affects the photosynthetic rate of the treated plant. In the first experiment we explored two mechanisms by which the mycorrhizal symbiosis may affect photosynthesis i.e. effects of a larger C sink and of improved plant nutrition. We hypothesized that plant C use by AMF creates a C sink strength large enough to affect photosynthetic rates regardless of P level. Therefore, we expected that a sudden reduction in sink strength by detaching a major part of extraradical mycelium would cause a significant but temporary reduction in photosynthetic rates. In a second experiment we evaluated the effects of combining source and sink manipulation in photosynthesis by including a treatment with high atmospheric CO₂. We expected the mycorrhizal C sink to play a different role depending on the magnitude of sink limitation at low and high atmospheric CO₂.

Materials and methods

Soil

We used a sandy loam collected from the arable layer of an agricultural field at Tåstrup, Denmark (55.68N, 12.30E). A 1:1 mixture of the loam and quartz sand contained 8.5 mg kg⁻¹ NaHCO₃-extractable P (Olsen *et al.*, 1954) and this mixture, hereafter referred to as 'soil', was γ -irradiated (10 MeV electron beam) twice to eliminate mycorrhizal propagules (10 kGy + 15 kGy). The soil was supplied with the following nutrients as powder (mg kg⁻¹): (70) K₂SO₄, (70) CaCl₂, (20) MgSO₄.7 H₂O, (10) MnSO₄. H₂O, (2.2) CuSO₄.5H₂O, (5) ZnSO₄.7 H₂O, (0.33) CoSO₄.7 H₂O, and (0.2) Na MoO₄.2 H₂O.

Biological materials

Mycorrhizal inoculum consisted of soil, mycelium, spores and colonized subterranean clover root pieces from two-month old pot cultures of *Glomus claroideum* Schenck & Smith BEG 14. Non-mycorrhizal inoculum was obtained by autoclaving soil:root material from control

pot cultures. Subterranean clover, *Trifolium subterraneum* L. cv. Mount Barker, served as nurse plant to establish an extraradical mycelium (ERM) network in the experimental units. The experimental plant, cucumber, *Cucumis sativus* L. Aminex F1 hybrid, was planted subsequently and became rapidly connected to and colonized by the pre-established ERM. All pots received 100 ml of a filtrate from inoculum pot cultures (1:10 soil:water suspension filtered through three layers of 6 μm mesh) to restore microbiota, but excluding mycorrhizal propagules.

Model system

The model systems were established in 4 kg pots (15 cm diameter x 18 cm height, Expt 1) or 5 kg pots (22 cm diameter x 20 cm height, Expt 2). Each pot contained a large mesh bag, a small mesh bag and a test tube (Fig. 1a). Mesh openings were 25 μm that impaired root, but not mycelium, passage. The small bag contained 250 g of soil plus 100 g of the appropriate inoculum and the large bag in the centre contained 500 g soil. The test tube was filled with soil and placed 3 cm away from the central bag (Fig. 1a). The rest of the pot was filled with soil.

Clover seedlings were planted in the small lateral bag (step 1, Fig. 1a) and ERM grew from the lateral bag into the rest of pot. When ERM had built up, we cut the clover shoots and planted the experimental plant, cucumber, in the central bag (step 2, Fig. 1a). One week later, we removed the test tubes from the pots and refilled the hole with soil labelled P to confirm that the plant in the central bag had been functionally connected to the previously established ERM network (step 3, Fig. 1a). After that, a significant part of the extraradical mycelium could be detached from the cucumber host by lifting the central bags (step 4, Fig. 1b). The time line of the experiments is shown in Table 1.

Expt 1. Manipulating plant C sink strength and plant P nutritional status

The randomized block experiment had three initial treatments combining two factors, mycorrhiza and P level: mycorrhiza, high P (MHP), mycorrhiza, low P (MLP) and no mycorrhiza, high P (NMHP). A nonmycorrhizal, low P treatment was not included since those plants would become nutrient deficient and small and therefore not comparable to mycorrhizal plants. Ten replicate pots of each treatment were established and the third factor,

Lift, with two levels: lifted (L) or intact (I) was assigned randomly to five pots within each treatment later on. Pots were maintained in a walk-in growth room at 21/15°C (day/night), photosynthetic photon flux density 350-500 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Pots were rotated every week and watered by weight to maintain 70% of water holding capacity throughout the experiment. Clover plants received 25 mg N as NH_4NO_3 solution at 10 and 40 d after planting to sustain active growth.

Five wks after clover planting, spread of ERM throughout the pot was checked by taking a 1.5 x 10 cm soil core from four randomly chosen M and NM pots. The hole was refilled with soil. The ERM was extracted from soil samples and hyphal length density was determined by microscopy (Jakobsen *et al.*, 1992). Mycorrhizal pots contained three times more hyphae than non-mycorrhizal pots and were considered ready. One week later pots were randomly assigned to conform five blocks and one 1-wk old cucumber seedling was transplanted to one block per day. Soil cores were taken from all central bags and the seedlings were transplanted to fill this hole in each bag. Photosynthetic photon flux density in the room was increased to a range of 600-800 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Five days after planting, P treatments were initiated by amending central bags with 100 ml Hewitt nutrient solution containing either 5 mg P kg^{-1} (low, L) or 20 mg P kg^{-1} (high, H). This was repeated at 10 and 16 days after planting.

One week after planting, test tubes were removed from the pots and each hole was refilled with 100 g soil labelled with 10 kBq carrier-free $\text{H}_3^{32}\text{PO}_4 \text{g}^{-1}$ to confirm that the cucumber plant had become functionally connected to the ERM. A Mini 900 radiation monitor (Thermo Fisher) showed that all mycorrhizal plants contained radioactivity within four days. Three wks after planting, all plants received 50 mg N in the central compartment [to ensure that both mycorrhizal and non-mycorrhizal plants were N- sufficient]. Four wks after planting, photosynthesis in each plant was measured during 10 min at the light conditions of the growth room and working by blocks. Measurements were made on the youngest fully developed leaf using a portable gas-exchange system ((LCA-2, Analytical Development Co., Hoddeston, UK) with a Parkinson leaf chamber (PLC2 [B])). Each measurement was made at current chamber conditions (light, temperature and RH%) until reaching a stable rate. The measurements were done at seven, four and three days before imposing the ERM excision. At this stage, five of the ten pots of each treatment combination were randomly assigned to lift/no-lift and conformed five blocks.

The lift treatments were applied to one block per day, as they were planted, and repeated identically for the five blocks. Any water-stress confounding effects were prevented by watering the previous evenings and mornings. The corresponding lift and no lift plants of each treatment within the block (i.e. the mycorrhizal-high P-lift and mycorrhizal-high P-no lift) were paired and measured following a tight measuring schedule. Photosynthetic rates were measured as described above, before and after applying the lift. The central mesh bag was carefully pulled out to detach the ERM outside the bag and placed back immediately after. Photosynthetic rates were then measured every 5 min during the first 30 min, afterwards every 10 min to complete 120 min, and finally after 24 h. Two hours later we repeated this schedule with the non-mycorrhizal high-P plants and four hours later with the mycorrhizal-low P plants of the block. Pots were harvested 2 days after lifting the bags. Cucumber shoots were cut and stored in plastic bags at 4°C until measurement of leaf area, drying and weighing. The central bags were removed from the pots, roots were washed from the soil and subsampled to determine dry weight and mycorrhiza colonization; the latter was obtained by microscopy of slide-mounted roots (McGonigle *et al.*, 1990) that had been cleared and stained (Kormanik & McGraw, 1982). A subsample of dried and ground shoots was digested in nitric acid:perchloric acid (4:1) and P concentration was measured by the molybdate-blue method (Murphy & Riley, 1962) in a Technicon Autoanalyzer II (SPX Flow Technology Norderstedt, Germany). ³²P activity of the digests was measured by liquid scintillation counting (Packard, PerkinElmer, Waltham, MA, USA).

Expt 2. Manipulating C sink strength and source limitation.

The factorial experiment had two initial factors with two levels each: mycorrhiza (present, M or absent, NM) and atmospheric CO₂ level (low, 350 ppm or high, 700 ppm). Their four combinations were established from the beginning of the experiment, with 16 replicates each, and the lift factor was applied later on.

Soil was supplied with the following nutrients besides the nutrients mentioned in the soil section, to ensure no nutrient limitation (Ravnskov *et al.*, 1995; Larsen *et al.*, 1996), in mg kg⁻¹: NH₄NO₃ (86), KH₂PO₄ (44). The small lateral bag received again no nutrient addition. Lateral bags contained 300 g soil and 50 g of the appropriate inoculum. All central mesh bags in the mycorrhizal treatments received additionally 18 mg P kg⁻¹ and the non-mycorrhizal treatments 60 mg P kg⁻¹ as KH₂PO₄ that were mixed as powder to ensure non-

mycorrhizal cucumber plants had plenty of P accessible within the bag. Clover seedlings were planted in lateral bags and the sequence of activities was repeated as in Expt 1 (Table 1).

The pots were moved to growth rooms at the Risø Experimental Risk Assessment Facility (RERAF, Technical University of Denmark, Risø campus- see Ingvordsen *et al.*, 2015 for further details) set at low (350 ppm, 358 ppm measured mean concentration across the experiment) or high (700 ppm, 712 ppm measured mean concentration across the experiment) atmospheric CO₂. Each growth room was maintained at either high or low atmospheric CO₂ for a 1-wk period. Then the CO₂ concentration in the room was switched every week and plants were shifted between the two rooms to maintain the CO₂ treatments and rotated inside the rooms to minimise growth room and position differences. Plants were grown at 20°C day/ 15°C night, 14 h light, for the first 5 wk and 22°C day/18°C night and 16 h light the rest of the experiment. Photosynthetic photon flux density in the rooms increased gradually to, and decreased gradually from, a midday maximum of $750 \pm 50 \mu\text{mol m}^{-2} \text{s}^{-1}$, and 8 h at maximum light. Six weeks later soil samples were from four randomly selected mycorrhizal and nonmycorrhizal pots. Cores were taken as in Exp. 1 and ERM was on average four times higher in the mycorrhizal pots than in the non-mycorrhizal pots but there was high variation within the mycorrhizal replicates so we allowed one month more to ensure that all mycorrhizal pots had formed extensive mycelial networks. Clover plants received 20 mg N in solution to maintain active growth.

Ten weeks after planting, pots were randomly assigned to eight blocks. Preparing one block per day, clover shoots were cut from the lateral bags and two 1-wk old cucumber seedlings were planted in the central bag of each pot. One soil sample was taken from the central bags at the time of planting to estimate the amount of ERM becoming attached to the new plant, as in Exp. 1. Cucumber plants were thinned to one and fertilized with 20 mg N (M plants) or 20 mg N and 10 mg P (NM plants) in solution. When cucumber plants had two fully-developed leaves, on week 12, we made gas-exchange measurements with two automatic leaf cuvettes (CIRAS-1, PP systems, Amesbury MA, USA) that controlled CO₂ concentration. They were previously tested and gave very similar readings when clamped to the same leaf. One of the cuvettes was randomly assigned to each growth room.

Measurements were taken during 10 min in each plant at the experimental CO₂ concentration, temperature and relative humidity of the treatment but always at light saturation. After that we removed the test tube in the pots and refilled the hole with soil containing 5 kBq g⁻¹ of

carrier-free $\text{H}_3^{33}\text{PO}_4$ to confirm cucumber functional connection to the network. We registered radioactivity in most shoots with the hand-held scintillation counter two days after adding the labelled soil. Two mycorrhizal plants showing no radioactivity after four days were excluded from the experiment.

We applied the lift treatment to five blocks that were complete (we discarded four mycorrhizal pots, two pots not transferring labelled P and two with scarce mycelium development), working with one block per day, in week 13. Pots were watered the night and morning before the lift treatment to prevent water stress. Net-photosynthetic assimilation rates were measured as explained above, before lifting and every 10 min after lifting, during the first 100 min and again after 24 h. The mycorrhizal-low CO_2 -lift plant and the mycorrhizal-high CO_2 -lift plant of the block were lifted simultaneously by one person in each room at time 0 and gas-exchange measurements were made from these two plants and the corresponding mycorrhizal-low CO_2 - no lift and mycorrhizal-high CO_2 -no lift control plants following a tight measuring schedule (5 min per plant) for two hours. Two hours later we repeated this schedule with the non-mycorrhizal plants of the block. The shoots were harvested approximately 24 h after lifting the bags. Shoots were cut, leaf area was measured, and shoots were then frozen in liquid nitrogen, freeze-dried and weighed. Shoots were processed for P analysis as described in Expt 1 and total N was determined by the Kjeldahl method (Bremner & Mulvaney, 1982) in a Technicon AutoAnalyzer II. ^{33}P activity of the digests was measured by liquid scintillation counting as in Expt 1. Shoot starch and soluble sugars were measured with the anthrone method (Cerning-Beroard, 1975).

The central bags were removed from the pots, roots were washed, and subsamples taken for dry weight and mycorrhizal colonization. Samples were processed using the methods of Expt 1.

Statistical analysis

Measurements before applying excision of ERM.

We fitted linear mixed models (LMMs) to assess plant gas-exchange status before the application of the lift. For Expt 1 we evaluated photosynthetic rates in the three treatments (MHP, MLP, and NMHP), and light at leaf surface (as a covariate since these measurements were not conducted at light saturation) as fixed effects, and block and pot as random effects. The effect of pot was included since the three measurements were made from the same pot. For Expt 2, we evaluated net-photosynthetic rate at light saturation, stomatal conductance and

transpiration rate considering mycorrhiza, atmospheric [CO₂] factors and leaf temperature (covariate) as fixed effects, and block as a random effect. Models were fitted using 'lmer' function in the 'lme4' package for R (Bates *et al.*, 2015).

Temporal shifts in photosynthesis after excision of ERM.

We fitted LMMs and generalized additive mixed models (GAMMs) to analyze temporal trends of the relative photosynthetic rates after the lift. We modeled proportional changes to account for potential differences in photosynthetic rates among plants before treatment application. Block was included as a random effect on the intercept and pot was included as a random effect on both the intercept and the slope for time.

Expt 1. Given the non-linear trend in relative photosynthesis, we used GAMMs for this experiment. Two models were fitted because there was no non-mycorrhizal low P treatment in the design. The first model included time, lift and mycorrhiza effects and their interactions including data only from the high P level treatments. This allowed us to assess if lifting the bags and clamping leaves in the cuvette had an effect on photosynthetic rates of the non-mycorrhizal control. Then we fitted a second model including time, lift, P level, and their interactions to compare only the mycorrhizal treatments and assess the effect of mycelium excision for the two P levels. Both models included data for $10 \leq \text{time} \leq 120$ min and assumed normally distributed errors. Finally, we assessed lift factor effects for mycorrhizal plants one day after the lift. GAMMs were fitted using the 'gamm4' function in the homonym library for R (Wood & Scheipl, 2017).

Expt 2. We used LMMs given the clear linear trend in relative photosynthesis for this experiment. The model included Mycorrhiza, [CO₂], lift, and time factors and their interactions for photosynthetic rate data from 10 to 100 min after applying the lift. We used the same approach to analyze relative stomatal conductance and transpiration rates. We assessed lift effects for mycorrhizal plants one day after the lift to evaluate longer-term effects of mycelium excision on photosynthetic rates. LMMs were fitted using 'lmer' function in the 'lme4' package for R (Bates *et al.*, 2015).

Harvest

We fitted linear models to assess the effect of factors on variables measured only at harvest or on a specific date. Total CO₂ uptake was calculated using the photosynthetic rate measured just before applying the lift treatment and leaf area measured at harvest in Expt 2. For Expt 1,

we tested two combinations of predictors only for the high P level treatments and only for the mycorrhizal treatments, as explained above given that there was no nonmycorrhizal, low P treatment in the design. Models were fitted using the "lm" function in the "stats" library for R.

Statistical inference

We used an information-based approach for statistical inference (Anderson, 2008). For each model described above, we derived a set of models with all the possible subsets of fixed effect terms and fitted those models. Best-fit models were then selected as those whose Akaike weights (w_i) were at least one tenth of the best model's weight (w_{best}), considering they had enough evidence to be plausible (*i.e.* $\Delta AICc \approx 4.6$). Akaike weights measure the probability of a model as being the best fitted within the set of models; they are calculated from ΔAIC values and sum up to one including all models. For simplicity, we present and discuss the best-fit model for each response variable in the main text. Up to ten best-fit models and model parameters are presented in table **S1**. Model selection was done using the functions 'dredge' (for LMMs) and 'model.sel' (for GAMMs) in the 'MuMIn' library for R (Barton, 2016).

Before model selection, we performed graphical inspection of their residuals for assessing homogeneity of variance. For those variables showing a clear heteroscedasticity pattern (transpiration before lift, photosynthesis trends after lift, transpiration rate after 24 h), we re-fitted the models using the 'lme' in the 'nlme' library (Pinheiro *et al.*, 2017), which allows modeling variance through the 'varPower' function. Since during model selection, models ranking and weights were very similar to those from models fitted with 'lmer' with no heteroscedasticity correction, we present results from original 'lmer' models. All analyses were performed in R (R Core Team, 2018).

Results

Measurements before applying mycelium excision.

Photosynthetic rates had the expected positive relationship with light intensity measured at leaf surface in Expt 1, and this relation was similar in all treatments and there was no evidence for mycorrhiza or P treatment effects on photosynthetic rates (Table **S1a**). In Expt 2, cucumber plants growing at 700 ppm CO₂ had lower transpiration rates than plants at 350 ppm CO₂, but there was only weak evidence for lower stomatal conductance (Table **S1b**). Both transpiration and conductance were positively related to leaf temperature. There was no

evidence for CO₂ or mycorrhizal effects on photosynthetic rates. Therefore, in both experiments, photosynthetic rates of cucumber plants did not differ between mycorrhiza, P level, or CO₂ treatments before applying the lift treatment.

Temporal shifts in photosynthesis after mycelium excision

The lift treatment caused a reduction in photosynthetic rates of mycorrhizal plants, but not of non-mycorrhizal plants (Fig. 2), as evident from the time × mycorrhiza × lift interaction in the best-fit models of both experiments (Tables 2, 3, S1b). In Expt 1, mycorrhizal intact plants showed a slight increase whereas mycorrhizal lifted plants showed a steady reduction during the first 75 min until declining to 60% of the initial photosynthetic rate and then stayed like this until two hours after the lift (Fig. 2a). Despite some differences between individual plants, photosynthetic rates were mostly lower for lifted plants than for intact plants. In Expt 2, non-mycorrhizal plants showed a slightly decreasing trend when intact and no trend when lifted (Fig. 2d). Mycorrhizal lifted plants showed a 19% reduction immediately after mycelium excision, and afterwards slowly increasing trends yet 10-15% lower than the initial rate after two hours (Fig. 2c). In summary, there was no evidence for significant changes in the relative photosynthetic rates of non-mycorrhizal plants with or without lift, whose mean values were close to 1 throughout the experiment (Fig. 2b, d). Thus, neither clamping the leaves in the cuvette in all plants nor the disturbance by lifting the central bag of the non-mycorrhizal plants caused significant changes in photosynthesis. Therefore, the reductions observed in photosynthesis of the mycorrhizal plants with mycelium cut were not confounded by those potential disturbances.

In Expt 2, we found evidence for a differential reduction in the net-photosynthetic rates of lifted plants under both high and low CO₂ as indicated by the CO₂ × Lift interaction (Table 3), suggesting that the magnitude of the reduction depended on CO₂ level. There was an immediate 15% average reduction in low CO₂ plants and a 3% reduction in high CO₂ plants (Fig. 3). Mean values over time for intact and lifted plants were closer at high CO₂ than at low CO₂, suggesting a larger photosynthesis decline at low CO₂. However, a Time × CO₂ interaction suggested significant differences in the trajectories of photosynthetic rates during the rest of the measuring period for low and high CO₂ plants. The temporal trend was positive for low CO₂ plants and negative for high CO₂ plants (Fig. 3). Relative stomatal conductance and transpiration rate followed the same pattern after the lift treatment as relative photosynthetic rates and declined only in plants growing at low atmospheric CO₂ (Fig. 4).

The decline was more pronounced in stomatal conductance (40%) than in transpiration rate (25%). After the initial decline caused by the lift, temporal trends in stomatal conductance and transpiration rate were more affected by atmospheric CO₂ than by the mycorrhizal condition of the plants. This was evidenced by a significant T x C interaction and the lack of significant L x M, L x T, and L x T x M interactions that, in contrast, were consistently present for photosynthetic rates (Table 3).

Mycelium excision caused a reduction in photosynthetic rates also in mycorrhizal plants grown at low P in Expt 1 though of lower magnitude than in high P plants. We observed an immediate 16% average reduction in lifted plants followed by a slight additional reduction along the next two hours (Fig. 5). Best-fit models suggested the reduction did not depend on plant P level, although the trend suggested it did (Figs. 2a, 5).

The assessment of photosynthetic rates of mycorrhizal plants one day after applying the lift treatment in Expt 1 suggested no significant differences (null models as best-fit models) between intact and lifted plants grown at high or low P (Table S1c). Similarly, there was no evidence for differences in the photosynthetic rates stomatal conductance and transpiration rates of mycorrhizal lifted and intact plants of Expt 2, at low or high [CO₂], one day after the lift treatment.

Plant and fungal variables measured at harvest

For Expt 1, none of the best-fit models for non-gas exchange plant and fungal variables measured at harvest (one day after the lift treatment) included the lift factor or the interaction of lift with mycorrhiza or P level factor (Tables 4, 5, S1d). Comparing only high P plants, mycorrhizal plants had lower shoot biomass and leaf area and higher shoot P concentration, shoot P uptake, and mycorrhizal root colonization and ERM length density than non-mycorrhizal plants (Table 4). Non-mycorrhizal plants had, on the other hand, higher leaf area, shoot, root and total biomass, and shoot:root ratio than mycorrhizal plants (Table 5).

For Expt 2, most best-fit models for plant and fungal variables measured at harvest included mycorrhiza or [CO₂] factors (Table 6). The lift factor or its interactions with the other factors were not included in most models. Differences due to lift were not expected given the short time lapse (24 h) between lift application and harvest but were significant for a few variables likely from random variation since shoot:root ratio, mycorrhizal root colonization, and ERM length density would not change within one day and CO₂ uptake was calculated from photosynthetic rates measured just before the lift. Despite some CO₂ effects in shoot [N] and [P], M and NM plants reached optimal concentrations and total nutrient

uptake did not differ. Biomass and biomass allocation variables (i.e. total biomass, shoot:root ratio, specific leaf area) and starch showed the largest differences between treatment levels (Table 6). Starch was one of the variables with more pronounced differences, being considerably increased in high [CO₂] and non-mycorrhizal plants. There was only weak evidence for the accumulation of starch in plants with lift treatment and no differences in soluble carbohydrates.

Discussion

We report here evidence showing that excision of part of the ERM without disturbing the plant induced a decline in photosynthetic rates that lasted from 30 min to 120 min. The excision of ERM significantly reduced photosynthesis in plants that were well nourished and well hydrated and showed no mycorrhiza-induced promotion of growth or photosynthesis. Possible confounding effects of intrinsic differences between plants was avoided by choosing a host plant that grows better when not forming mycorrhizas (Larsen *et al.*, 1996; Ravnskov *et al.*, 1999) and by manipulating the mycorrhizal C sink and monitoring its consequences for rates of photosynthesis directly in the same plant, as opposed to comparing mycorrhizal and nonmycorrhizal plants. The results provide the first direct support to the hypothesis presented by Wright *et al.* (1998) that may explain photosynthesis stimulations observed in mycorrhizal plants in the absence of nutritional benefits and sustain the claim that maintaining high photosynthetic rates may compensate the carbon costs of arbuscular mycorrhizal fungi (Kaschuk *et al.*, 2009; Schweiger *et al.*, 2014). Our experiments represent a hitherto missing support for previous hypotheses derived from indirect evidence based on the comparison of mycorrhizal and non-mycorrhizal plants. Although our results support to the hypothesis of C compensation, we cannot prove that full compensation exists. Most likely there is a gradient that goes from no to full compensation, depending on the circumstances in which the symbiosis is established.

The decline was only temporary in all cases, as we had expected, and photosynthetic rates of most plants had recovered after 24 h when likely new C sinks began to form. New sinks could be new leaves, roots or hyphae. The photosynthesis decreases and their duration were different and more pronounced in Expt 1 than in Expt 2, likely due to lower light availability in Expt 1. Boldt *et al.* (2011) also observed smaller differences in photosynthetic rates when measuring at the growth-room light level than at light saturation. The photosynthesis decline in low P mycorrhizal plants was of smaller magnitude than in the high

P mycorrhizal plants, but the recovery seemed slower. P limitation likely increased the recovery time by constraining the mobilization of resources in those plants to sustain photosynthesis and build new sinks. Therefore, nutrients, light, and [CO₂] played their well-known role on photosynthetic rates (Long *et al.*, 2004; Bulgarelli *et al.*, 2017) but the effect of ERM excision was consistent and previously unreported. Moreover, the decline was achieved after reducing only part of the sink strength by detaching a proportion the ERM. Assuming a homogeneous distribution of ERM in the pots, which is likely since the mycelium network was previously established, we detached approximately 80% of the extraradical mycelium. This suggests that the total mycorrhizal sink strength may have larger effects on photosynthetic rates than those measured as we left intact all intraradical mycelium and the ERM inside the central bags.

Plants were maintained well-watered throughout the mycelium cut measurements to avoid confounding water stress in our evaluations after detaching part of the mycelium that likely contributed to plant water uptake. There is, however, the possibility of a “stress” signal being produced in response to the ERM excision that triggered a water stress reaction even in the absence of a real water stress condition given the observed immediate decline in stomatal conductance and especially the transpiration rates. Recent studies suggest the AM symbiosis may induce diverse local and systemic responses to drought (Bárcana *et al.*, 2015) and modulate stomatal conductance through gene regulation in the ABA pathway (Xu *et al.*, 2018). Zou *et al.* (2015) observed a slight reduction in leaf water potential but no changes in transpiration rate of trifoliolate orange 8 h after detaching part of the ERM. Those observations support the possibility of a temporary water stress reaction induced by mycelium excision similar to the disturbance reaction caused by lifting and leaf clamping that we explored by measuring also the non-mycorrhizal plants. The magnitude and duration of the photosynthetic rate drop and the fact that high CO₂ plants did not show a similar decline in gas-exchange measurements make a water stress reaction, however, an unlikely explanation for the sustained reduction in photosynthetic rates. Additionally, lift interactions with mycorrhiza and CO₂ factors were consistent in best-fit models for relative photosynthetic rates but not for relative stomatal conductance and transpiration rates. The possibility of signal induction causing reduced plant gas exchange, as an immediate response to ERM excision, deserves further study as it may have strong implications for plant functioning and development. Other plant physiological responses to mycelium excision, not as immediate as gas-exchange, are also worth investigating as late reactions. We focused on the immediate response of photosynthesis but there are other relevant functions in the host plant and the mycorrhizal

fungi that may be hampered. Nutrient transfer would likely be reduced after undergoing reallocation and depletion within the preserved mycelium network until new hyphae were formed or some areas of the mycelium network that were partially or fully closed could be reactivated (De la Providencia *et al.* 2007). Carbon and mineral nutrients allocation and reallocation within and between both symbionts after excision could be examined with labelling techniques as they may involve immediate and late reactions depending on the capacity of each organism to mobilize resources (Gavito & Olsson 2003, 2008).

Several observations support that the decline in photosynthesis was a response to a sudden reduction in sink strength: 1) The lift treatment caused only a temporary reduction in sink strength which would have recovered due to the formation of new C sinks shortly after the lift treatment; water stress would have maintained or even amplified the decline over time. 2) The photosynthetic decline was more pronounced in plants that were source limited (low CO₂) and moderately sink limited because the mycorrhizal C sink strength had been draining C and most of this drain disappeared with the lift treatment. In plants that were not source limited (high CO₂), on the other hand, photosynthesis was already sink limited and further increasing sink limitation by reducing the mycorrhizal sink strength would thus have less impact on photosynthesis. 3) Higher starch concentrations in non-mycorrhizal and especially high CO₂ plants evidenced the accumulation of unused C in those treatments and indicated those plants were highly sink limited, what gives additional support to the second point.

All plants had their roots constrained to the central bags, but non-mycorrhizal plants were larger and had allocated more biomass to shoots, especially leaves, than mycorrhizal plants. This might represent an alternative for using surplus C in the absence of the continuous C depletion by the AMF. Our model system with limited space for sink adjustments did not allow us to explore other mechanisms for new sink development in both symbionts that may replace the sink strength lost after mycelium excision but demonstrated the importance of the mycorrhizal C sink strength. The strength of source-sink gradients had been demonstrated mainly in plants connected to ERM mycelium networks where the sink strength of some plants could affect the development of others by controlling the flow of carbon and mineral nutrients (Waters & Borowicz, 1994; Walder *et al.*, 2012; Merrild *et al.*, 2013; Fellbaum *et al.*, 2014; Weremijewicz *et al.*, 2016). Research on resource biological markets linking above and belowground flows between plants and mycorrhizal fungi is rapidly expanding. Our results indicate that the effects of the mycorrhizal C sink strength on plant gas exchange extend considerably outside plant tissue. Moreover, they may help

clarifying polemic situations in which mycorrhizal fungi cause growth depressions, as observed in highly fertilized and ploughed agricultural fields, where fungal development and nutrient transfer functions are hindered and mycelium networks are continuously disturbed thereby creating a C sink that is excessive for certain crops and unlikely fully C compensated by photosynthesis stimulation (Ryan & Graham, 2002, 2018).

Demonstrating and understanding the C sink strength of the mycorrhizal symbiosis on plant photosynthetic rates is of high relevance to design adaptation programs for crops to climate change scenarios and increasing atmospheric CO₂ where multiple resource limitation may reduce or amplify the mycorrhizal stimulation of plant C assimilation. This is also critical knowledge to improve modelling responses to increasing atmospheric CO₂ and management of C sequestration in soil. Despite the current debate on how mycorrhizas should be incorporated to models predicting terrestrial ecosystem responses to increasing atmospheric CO₂ (Drake *et al.*, 2011; Terrer *et al.*, 2016, 2018; Norby *et al.*, 2017), it becomes clear that mycorrhizas should not be overlooked in such predictions (Drigo *et al.*, 2013). Our results encourage further experiments on this subject under field conditions where plants and fungi are diverse, have less resource and space constraints, and can undergo adjustments for optimal resource utilization (Ruiz-Vera *et al.*, 2017). Acknowledging the limitations of our study, we hope this first evidence will be complemented with future experiments.

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Author contributions

Financed research: IJ. Conceived research: MEG, IJ, TNM. Performed research: MEG, TNM. Conceived statistical analyses: MEG, FM. Performed statistical analyses: FM. Wrote manuscript: MEG, FM. Reviewed manuscript: MEG, IJ, TNM, FM

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Table S1. Best-fit models for all variables analyzed in Expt 1 and Expt 2.

Figure legends

Figure 1. Model system used for both experiments. (a) A pot with three compartments: (1) small lateral bag used to establish the mycelium network with clover as host plants, (2) central bag used for the cucumber plants to be connected to the established network, and (3) test tube to be removed and the space refilled with $^{32,33}\text{P}$ labelled soil to confirm functional connection of the cucumber plants to established network. (b) After completing the three steps, the central bag was lifted to detach all mycelium outside this bag (4).

Figure 2. Relative photosynthetic rates of cucumber plants measured during the first 2 h after applying the lift treatment to mycorrhizal and non-mycorrhizal plants, from Expt 1 (a, b), and Expt 2 (c, d). All plants were grown with high phosphorus (P). Thin lines represent measurements from individual plants. Thick lines are the predicted treatment values from best-fit models in each experiment.

Figure 3. Relative net-photosynthetic rates measured in Expt 2 during the first 2 h after applying the lift treatment to cucumber plants growing and measured at low (a) 350 ppm atmospheric CO_2 , and high (b) 700 ppm atmospheric CO_2 . Thin lines represent measurements from individual plants. Thick lines are the predicted treatment values from the best model.

Figure 4. Relative stomatal conductance (a, b) and transpiration rates (c, d) of cucumber plants measured in Expt 2 during the first 2 h after applying the lift treatment. Plants were grown and measured at either low (a, c) 350 ppm atmospheric CO_2 , or high (b, d) 700 ppm atmospheric CO_2 . Thin lines represent measurements from individual plants. Thick lines are the predicted treatment values from the best model.

Figure 5. Relative net-photosynthetic rates of cucumber plants measured in Expt 1 during the first 2 h after applying the lift to detach extraradical mycorrhizal mycelium to mycorrhizal plants in the low phosphorus (P) treatment. Thin lines show measured values from individual plants. Thick lines show the predicted treatment values from the best model.

Table 1. Time-line of the main events in both experiments, from the day we planted clover as initial host plant to establish a mycelium network.

Activity	Days after planting clover	
	Expt 1	Expt 2
Mycelium network established	42	66
Cutting clover from lateral bag	43	70
Planting cucumber seedlings	44-48 (One block per day from here, unless indicated)	71-75 (One block per day from here)
Introducing $^{32,33}\text{P}$	51-55	85-89
Radioactivity detected in 90% of mycorrhizal plants	55-59	87-91
Pre-lift photosynthesis measurements	72,76,77 (All blocks)	87-92
Lift treatment	80-84	95-99
Pre- and post-lift photosynthesis measurements	80-85	95-100
Harvest	82-86	96-101

Table 2. Best-fit generalized additive mixed models for Expt 1, assessing the temporal trends of relative photosynthetic rates of cucumber plants after lifting bags to detach part of the extraradical mycorrhizal mycelium.

	L	M	L×M	T×L×M	Weight
a) High P plants only	+	+	+	+	0.420
b) Mycorrhizal plants only	L	P	L×P	T×L×P	Weight
	+			+	0.598

a) Model included lift (L), mycorrhiza (M), time (T) and their interactions, only for plants under high P level. b) Model included phosphorus (P), mycorrhiza (M), time (T) and their interactions, only for mycorrhizal plants. Weights are provided for best-fit models. + indicates effects included in each model.

Table 3. Best-fit generalized linear mixed models for Expt 2, assessing relative photosynthetic rate (A_{sat}), relative stomatal conductance (g_s), and relative transpiration rate (E) of cucumber plants after lifting the central compartment to detach part of the extraradical mycorrhizal mycelium.

	C	L	M	C×L	C×M	L×M	T×C	T×L	T×M	T×C×L	T×L×M	Weight
A_{sat}	+	+	+	+		+	+	+	+		+	0.159
g_s	+	+	+	+			+					0.062
E	+	+	+	+			+	+				0.050

Initial model included atmospheric CO₂ (C), mycorrhiza inoculation (M), Lift (L), time (T) and their interactions as predictors. + indicates factor effects included in each model.

Table 4. Best-fit model results, means and standard deviations for variables measured at harvest time in Expt 1, including phosphorus (P) level (P), lift for mycelium cut (L), as predictors and only for mycorrhizal plants.

Variable	P	L	P × L	Weight	High P	Low P
Total mass (g per plant)	+			0.50	1.89(0.30)	1.96(0.41)
Root mass (g per plant)				0.41	0.37(0.05)	0.34(0.09)
Shoot mass (g per plant)	+			0.48	1.53(0.26)	1.63(0.38)
Leaf area ¹ (cm ² per plant)	+			0.73	171(29)	175(40)
Shoot [P] (mg g ⁻¹)				0.56	2.62(0.51)	1.98(0.65)
Shoot P uptake (mg per plant)				0.56	4.04(1.15)	3.10(0.87)
Mycorrhizal colonization (%)				0.46	61.1(6.8)	59.8(12.2)
Hyphal length density (m g ⁻¹)				0.58	6.52(0.91)	7.05(1.4)

Weights are provided for best-fit models. + indicates factor effects included in the model.

¹ One outlier was excluded.

Table 5. Best-fit model results, means and standard deviations for variables measured at harvest in Expt 1, including lift for mycelium cut (L), and mycorrhizal condition (M) as predictors, and only for high phosphorus (P) plants.

Variable	M	L	M×L	Weight	Mycorrhizal	Nonmycorrhizal
Total mass ¹ (g per plant)	+			0.63	1.85 (0.30)	2.17(0.41)
Root mass ¹ (g per plant)		+		0.43	0.36(0.06)	0.33(0.12)
Shoot mass (g per plant)	+			0.64	1.49(0.26)	1.84(0.37)
Leaf area (cm ² per plant)	+			0.43	165 (34)	196(31)
Shoot [P] (mg g ⁻¹)	+			0.83	2.56(0.46)	1.89(0.16)
Shoot P uptake (mg per plant)	+			0.73	3.84(1.03)	2.55(0.51)
Mycorrhizal colonization (%)	+			0.75	60.6(9.3)	0.32(0.09)
Hyphal length density (m g ⁻¹)	+			0.83	6.75(1.17)	1.60(0.57) ²

Weights are provided for best-fit models. + indicates factor effects included in the model.

¹ One outlier was excluded, ² Soil background hyphae.

Table 6. Best-fit model results for variables measured at harvest in Expt 2, including atmospheric CO₂ (C), lift (L), and mycorrhizal condition (M) as predictors.

Variable	C	L	M	C × L	C × M	Weight	LCO ₂	HCO ₂	M+	M-
Total mass (g per plant)	+		+			0.50	4.24(1.34)	6.68(2.65)	5.05(2.29)	5.87(2.55)
Root mass (g per plant)	+					0.43	0.75(0.23)	0.99(0.35)	0.86(0.33)	0.88(0.31)
Shoot mass (g per plant)	+		+			0.30	3.49(1.13)	5.69(2.34)	4.18(1.95)	4.99(2.25)
Shoot:Root	+	+	+			0.53	4.70(0.64)	5.66(0.85)	4.75(0.68)	5.60(0.88)
Asat ⁴ (μmol m ² s ⁻¹)	+	+	+			0.28	18.0(4.12)	20.3(3.20)	20.6(4.22)	17.4(3.18)
CO ₂ uptake (μmol s ⁻¹ per plant)	+		+			0.29	0.93(0.25)	1.24(0.37)	1.21(0.39)	0.95(0.24)
Leaf area (cm ² per plant)	+		+			0.25	547 (139)	654 (195)	592 (161)	641 (183)
Specific leaf area (cm ² g ⁻¹)	+					0.49	12.2(2.25)	14.9(2.92)	13.5(2.39)	13.7(3.46)
³³ P activity (Bq per plant)	+		+		+	0.55	47.1 (63.4)	264(479) ^{1,2}	300(477)	24.4(38.1) ³
Shoot [N] (mg g ⁻¹)	+		+		+	0.54	4.56(0.77)	3.01(0.75)	4.01(1.19)	3.55(0.95)
Shoot N uptake (mg per plant)						0.33	18.6(4.84)	20.1(5.86)	18.8(5.43)	19.9(5.39)
Shoot [P] (mg g ⁻¹)	+	+				0.34	5.90(0.87)	4.49(0.86)	5.17(1.16)	5.24(1.08)
Shoot P uptake (mg per plant)	+					0.43	25.6(7.35)	31.8(13.0)	26.8(10.8)	30.6(10.9)
Soluble carbohydrates (g 100 g ⁻¹)						0.40	6.91(1.25)	7.18(1.05)	7.03(1.24)	7.03(0.96)
Starch (g 100 g ⁻¹)	+		+			0.31	17.2(3.63)	32.8(4.42)	23.9(9.22)	24.3(7.94)

Mycorrhizal colonization ¹ (%)	+	+	+	0.26	42.7(10.3)	49.6(8.55)	46.1(9.90)	0 (0)
Extraradical HLD ⁴ (m g ⁻¹ soil)		+	+	0.27	6.30(2.20) ¹	6.22(2.61)	6.26(2.37)	1.19(0.56) ⁵

Means and standard deviations (n=3-6) for the most consistently significant main effects, CO₂ and mycorrhiza. Weights are provided for best-fit models. + indicates factor effects included in the model.

¹ Only for mycorrhizal plants, ²One outlier excluded, ³One root in one pot trespassed the bag and reached directly the labelled P. ⁴Hyphal length density, ⁵Soil background hyphae,









