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Report

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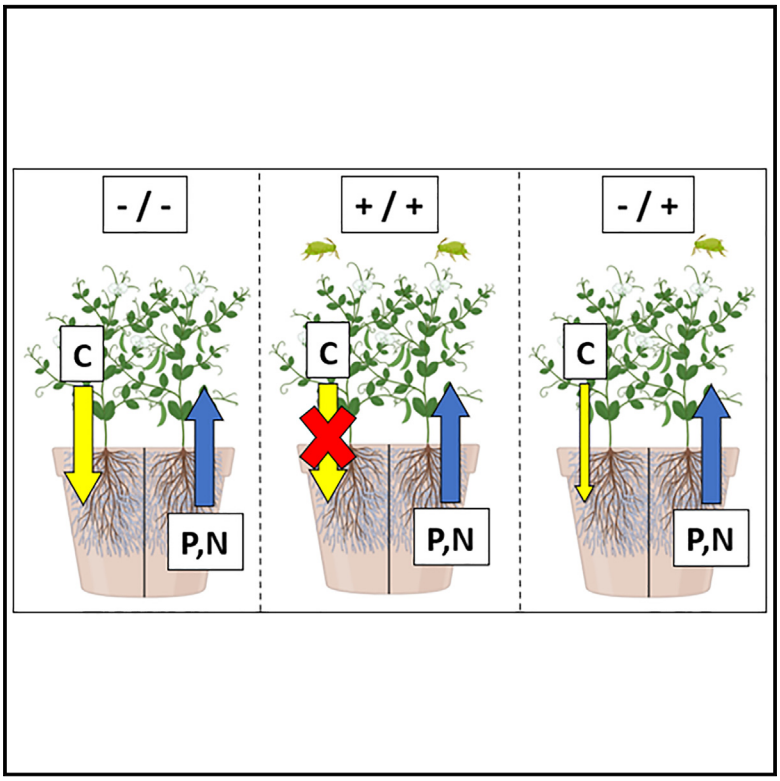
Herbivore-driven disruption of arbuscular mycorrhizal carbon-for-nutrient exchange is ameliorated by neighboring plants

Graphical abstract



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In brief

Durant et al. show that mycorrhizal nutrient exchange involves community-wide interactions between multiple players. Loss of carbon inputs into mycorrhizal fungi caused by aphid herbivory of one plant can be compensated for by aphid-free neighbors, demonstrating the responsiveness and resilience of mycorrhizal plant communities to biological stressors.

Highlights

- We assessed neighbors' impact on mycorrhizal function in plant-aphid interactions
- When both plants were aphid infested, carbon supply to mycorrhizal fungi decreased
- If only one plant was infested, carbon supply was partially restored
- Mycorrhizal nutrient exchanges are better understood as community-wide interactions

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Herbivore-driven disruption of arbuscular mycorrhizal carbon-for-nutrient exchange is ameliorated by neighboring plants

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SUMMARY

Arbuscular mycorrhizal fungi colonize the roots of most plants, forming a near-ubiquitous symbiosis[1] that is typically characterized by the bi-directional exchange of fungal-acquired nutrients for plant-fixed carbon.[2] Mycorrhizal fungi can form below-ground networks[3–6] with potential to facilitate the movement of carbon, nutrients, and defense signals across plant communities.[7–9] The importance of neighbors in mediating carbon-for-nutrient exchange between mycorrhizal fungi and their plant hosts remains equivocal, particularly when other competing pressures for plant resources are present. We manipulated carbon source and sink strengths of neighboring pairs of host plants through exposure to aphids and tracked the movement of carbon and nutrients through mycorrhizal fungal networks with isotope tracers. When carbon sink strengths of both neighboring plants were increased by aphid herbivory, plant carbon supply to extraradical mycorrhizal fungal hyphae was reduced, but mycorrhizal phosphorus supply to both plants was maintained, albeit variably, across treatments. However, when the sink strength of only one plant in a pair was increased, carbon supply to mycorrhizal fungi was restored. Our results show that loss of carbon inputs into mycorrhizal fungal hyphae from one plant may be ameliorated through inputs of a neighbor, demonstrating the responsiveness and resilience of mycorrhizal plant communities to biological stressors. Furthermore, our results indicate that mycorrhizal nutrient exchange dynamics are better understood as community-wide interactions between multiple players rather than as strict exchanges between individual plants and their symbionts, suggesting that mycorrhizal C-for-nutrient exchange is likely based more on unequal terms of trade than the "fair trade" model for symbiosis.

RESULTS AND DISCUSSION

In nature, most plants form symbioses with mycorrhizal fungi. The most widespread mycorrhizal associations are formed between >70% of land plants and fungi belonging to the fungal phylum Glomeromycota, and together these symbiotic associations are known as arbuscular mycorrhizas.[1] Plant communities may form networks of interacting mycorrhizal fungi beneath the soil, comprising extensively branched extraradical fungal hyphae, which can fuse together via anastomosis.[3–6] Arbuscular mycorrhizal (AM) partnerships are characterized by the bi-directional exchange of plant-fixed carbon (C) for fungal-acquired nutrients (nitrogen [N] and phosphorus [P]), providing nutritional benefits to both partners and playing a critical role in the structure and function of wider ecosystems.[2] Mycorrhizal networks—either as interconnected or interacting entities—may act as bridges between neighboring plants, potentially allowing various signaling molecules to be transmitted between neighbors.[7–9] This could be particularly important when plants are exposed to abiotic and/or biotic stresses because interplant signaling through an AM fungal network can enhance disease resistance and stress tolerance of neighboring plants.[7,9] The broader impacts of such stressors on the exchange of plant-fixed carbon for AM fungal-acquired soil nutrients between

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neighboring plants remain unknown and represent a critical knowledge gap in our understanding of the nutrient exchange dynamics governing the symbiosis and the role of interacting mycorrhizas in ecosystem structure, function, and resilience.

The nutritional function of AM fungi is most accurately considered as a dynamic continuum, spanning parasitism through mutualism,[10] with the functional significance of specific relationships being dependent upon a wide range of interacting biotic and abiotic factors.[11] These include plant and fungal identity, interactions with pests or pathogens, soil nutrient availability, and atmospheric CO_2.[12,13] Resource exchanges between AM symbionts are often discussed in terms of reciprocity with an individual plant, with more "generous" partners being "rewarded" with nourishment by their symbiont, whereas "uncooperative" symbionts face nutritional sanctions.[14–17] However, models of reciprocal resource exchange typically do not account for interactions between AM fungi that may connect neighboring plants. This is a critical consideration given that nutrients and carbon may be distributed among plant and soil communities via interacting or common mycorrhizal networks (CMNs).[5,18] As such, the nutritional outcomes of mycorrhizal symbioses need to be considered in terms of interactions between neighboring AM plants and extraradical AM fungal hyphae, particularly where additional interactions with other organisms, such as herbivores, represent competitive pressure for plant C resources.

Aphid herbivores acquire C and other nutrients directly from their host plants by piercing their stylets into the sieve elements of plants and sucking C-rich sap from the phloem.[19] Aphids also activate plant defense signaling pathways,[20] which can decrease local photosynthetic rates, reducing the relative C source strength of the plant.[21–24] Aphid herbivory induces interplant signaling via mycorrhizal networks,[25,26] acting as an "early warning system" to neighboring plants by inducing changes in the volatile organic compound (VOC) profiles of both infested and neighboring uninfested plants, resulting in a community-wide enhanced defense response.[25] Because aphids reproduce rapidly, enhanced defense via signaling across interacting mycorrhizas could be beneficial in reducing infestation of neighboring plants, providing advantages to the plants and AM fungi involved.

Plants colonized by aphids transfer much less C to their AM fungal partners than uninfested plants, while the supply of AM fungal-acquired soil nutrients to the host plant is maintained.[27] However, the use of an individual plant host for the AM fungi, which has often been the case in laboratory experiments, may have forced obligately biotrophic AM fungi to form a sub-optimal partnership, which does not reflect the dynamics of AM symbioses in natural ecosystems, where a suite of host plants, with and without pests, may provide a range of options for C inputs.[28] It is unknown whether a reduction in C allocation to mycorrhizas by infested plants (as per Charters et al.[27]) could affect nutrient transfer from AM fungi to neighboring plants. It is possible that neighboring plants contribute C to support potentially interconnected mycorrhizal hyphae, thereby offsetting the reduced C inputs by the infested plant and ensuring mycorrhizal nutrient supply to all plants is maintained.

Our lack of understanding of the role of interacting extraradical mycorrhizal fungal hyphae in mediating functional responses of AM symbioses across neighboring plants represents a significant knowledge gap, especially given the near-ubiquity of mycorrhizal associations in nature. In particular, the role of AM fungi in supporting and mediating plant community C dynamics, as well as the role of individual associations versus interacting and potentially interconnected networks of extraradical AM fungal hyphae in regulating resource exchange in plant-AM symbioses, is largely unknown. We used isotope tracers and aphid herbivores (*Acyrthosiphon pisum*) to investigate the role of interacting AM fungal hyphae in mediating carbon-for-nutrient exchange dynamics between neighboring AM symbionts with competing pressures for plant C resources.

In all results, "−/−" refers to treatments in which neither neighboring plant was exposed to aphids, and "+/+" refers to treatments where both neighboring plants were exposed to aphids. "−/+" refers to the treatment in which only one plant was exposed to aphids.

Fungal colonization and density

AM fungal colonization ranged between 19% and 28% in the roots of all plants, with colonization being significantly higher ($p = 0.002$; $df = 3$; $F = 5.436$; one-way ANOVA) in roots of plants in pots containing neighboring pairs of plants that were not exposed to aphids (−/− treatment) than in plants within treatments with aphids (+/+ and −/+) (Table S1). There was no difference in AM fungal colonization between aphid treatments (Table S1). The mean soil AM hyphal length densities ranged from 0.9 to 1.6 m hyphae g^{-1} soil across treatments, and, again, there were no differences between treatments (Table S2; $p = 0.434$; $df = 8$; chi-squared = 8; Kruskal-Wallis).

Plant biomass

Aphid treatment had no effect on shoot biomass (Figure 1A; $p = 0.845$; $df = 3$; $F = 0.272$; one-way ANOVA), but root biomass was significantly greater for plants in +/+ treatments compared with plants in the −/− treatment (Figure 1B; $p = 0.026$; $df = 3$; $F = 3.277$; one-way ANOVA, Tukey HSD test). Aphid load did not differ between treatments (Figure S1; $p = 0.8766$; $df = 35.316$; $t = -0.154$; Student's t test); therefore, it was removed as a factor from subsequent analyses.

AM fungi-to-plant ^{33}P and ^{15}N transfer

Across all treatments, there was a large amount of variation (including replicates with no ^{33}P transfer being evident) with no significant differences between shoot ^{33}P content and concentrations, regardless of aphid infestation status of neighboring plants (Figures 2A and 2B; $p = 0.106$, $df = 1$, $F = 2.912$; and $p = 0.0837$, $df = 1$, $F = 3.165$, respectively; Table S3). When comparing shoot ^{33}P content and concentration of neighboring plants within treatments, the aphid status of the neighboring plant only significantly affected shoot ^{33}P content and concentration of the other plant when both plants were exposed to aphids (Figures 2A and 2B; $p = 0.017$ and $p = 0.004$, respectively; Table S4; linear mixed-effect model). When at least one plant in a pair was uninfested (−/− and −/+), ^{33}P content and concentration did not differ significantly between neighboring plants (Table S3; linear-mixed effect model).

For all treatments, ^{15}N content and concentrations were also highly variable, but there were no notable trends between aphid treatments (Figures 2C and 2D; $p = 0.236$, $df = 1$, $F = 1.456$; and

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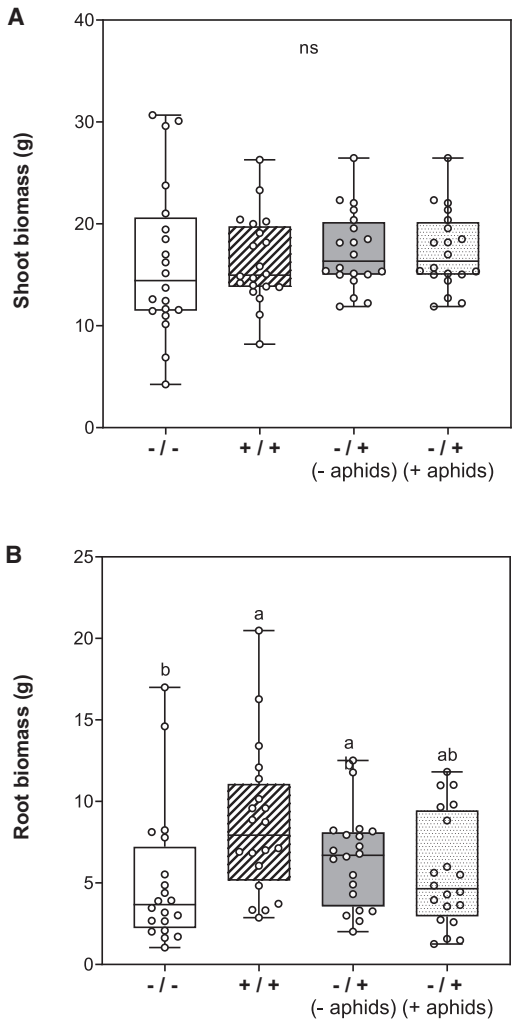

Figure 1. Plant biomass

(A) Shoot biomass and (B) root biomass. White boxes represent systems where both plants were unexposed to aphids, lined boxes represent systems where both plants were exposed to aphids, gray boxes represent the unexposed plants in systems where one plant was exposed and one was unexposed to aphids, and dotted bars represent exposed plants in that same system. Boxes extend from the 25th to the 75th percentile. Middle lines represent median values, and whiskers extend to minimum and maximum data points (n = 20 with data points coming from all individual plants, i.e., not paired with neighbors). Different letters indicate significant differences between treatment means (where $p < 0.05$, Tukey HSD tests). "ns" denotes a non-significant interaction. Figure S1

$p = 0.335$, df = 1, $F = 0.954$, respectively; Table S3). Additionally, shoot ^{15}N content and concentration of neighbors did not significantly differ based on aphid status of either plant (Figures 2C and 2D; Table S4; linear mixed-effect model).

Carbon transfer from plants to AM fungi

Plants within the +/+ treatment did not transfer a detectable amount of C to their AM fungal networks in our experiments, showing that aphid herbivory represents a substantial C sink. In contrast, −/− treatment transferred a substantial amount of C to extraradical mycorrhizal networks, amounting to >100 times more C than plants in the −/+ treatment (Figure 3A, $p = 0.0001$; df = 2; chi-squared = 11.14; Kruskal-Wallis), representing around 0.15% of total carbon that was fixed by the plants during the labeling period (Figure 3B, $p \leq 0.0001$; df = 2; chi-squared = 12.10; Kruskal-Wallis). Together, plants in the −/+ treatment transferred less recently fixed C to their fungal networks than those in the −/− treatment (Figure 3A; $p = 0.050$; Kruskal-Wallis, one-way ANOVA), representing around 0.01% of the total carbon fixed by the plants during the labeling period (Figure 3B). Wholly uninfested plants retained less C in their shoots compared with when aphids were present but similar amounts of C in their roots (Figures S2A and S2B). Aphids assimilated similar amounts of fungal C in both treatments in which they were applied (Figure S2C).

Our experiments demonstrate that, when there is competition for plant C resources, in this case via aphid herbivory, the allocation of plant-fixed C to extraradical AM fungal hyphae is reduced, supporting previous observations in individual plant-fungal experimental systems.[27] The impact of this reduction in C allocation on plant access to and assimilation of mycorrhizal-acquired nutrients was mediated to some extent across neighboring plants, potentially via interactions between interacting AM fungi or CMNs. When both neighboring plants were exposed to aphids, the extraradical AM fungal mycelium received very little plant-derived C and returned relatively little ^{15}N and ^{33}P to either host plant. When only one plant of the plants in a pair was exposed to aphids, an intermediate amount of C was transferred to the AM fungal mycelium, whereas both plants were supplied with AM fungal-acquired ^{33}P and ^{15}N. Interestingly, even more nutrients were supplied to the plant pairs in this treatment compared with those in the completely aphid-free treatment, which provided the extraradical AM fungal hyphal mycelia with the highest total amount of C. These results are not related to % root colonization by AM fungi, which, taken together, runs counter to expectations that mycorrhizal mutualisms are governed only by a "biological markets" style of exchange dynamics.[14] Instead, our data demonstrate that there is greater complexity in C-for-nutrient exchange dynamics in mycorrhizal symbioses than is apparent in highly simplified experimental systems. This is particularly pertinent when the functional significance of mycorrhizas across plant communities and multi-trophic interactions with other organisms are taken into consideration.

No differences in AM fungal nutrient transfer to host plants across herbivory treatments

Across all aphid treatments, plants in the neighboring pairs assimilated mycorrhizal-acquired ^{33}P and ^{15}N, although the amounts acquired were highly variable within treatments. This finding is in line with previous observations[27] in single plant systems in which there were similarly no significant differences in AM fungal-acquired ^{33}P assimilation by host plants that were either exposed or unexposed to aphid herbivory. It is possible that such variation reflects the isotope tracing techniques used; such measurements can only ever provide a snapshot of the C and nutrient dynamics in complex biological systems at a single given time point and should be viewed with this important consideration in mind. Additionally, plants may also acquire soil nutrients directly through direct or indirect pathways, e.g.,

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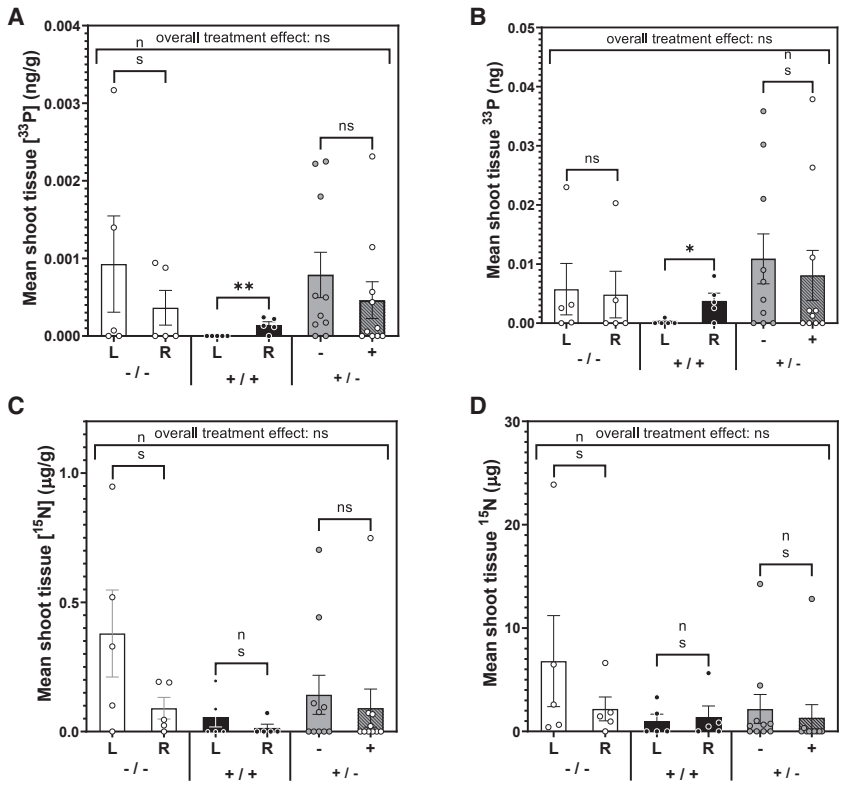

Figure 2. AM fungi-to-plant ^{33}P and ^{15}N transfer

(A) Mean shoot ^{33}P concentration, (B) mean shoot ^{33}P content, (C) mean shoot ^{15}N concentration, and (D) mean shoot ^{15}N content with neighboring plants separated. −/− denotes systems where both plants were not exposed to aphids; +/+ denotes systems where both plants were exposed to aphids; +/− denotes systems where one plant was exposed to aphids (gray bars represent the unexposed plants in those systems, whereas striped bars represent the aphid-exposed plants; L and R indicate left and right plants in neighboring plant pairs). (A–C) n = 5 for all treatments except −/+: − aphids and −/+: + aphids, where n = 10. Error bars represent standard error of the mean. Asterisks indicate significant differences between treatment means (where $p < 0.05$, linear mixed-effect model), and ns denotes non-significance.

See also Tables S3 and S4.

roots and/or alternative microbial cycling, although this is controlled for by our experimental design. Despite this, when aphid-free plants were paired with aphid-exposed neighbors in our experiments, there was a trend for both plants to assimilate greater amounts of ^{33}P via AM fungi compared with when both plants were aphid-free (Figures 2A and 2B), despite AM fungal colonization being greater in the roots of uninfested plant pairs (Table S1). As such, it is important to note that the degree to which plant roots are colonized by AM fungi does not appear to link directly to AM fungal function in terms of ^{33}P transfer to the plants in our experiments. The trend for plants with aphid-exposed neighbors to generally assimilate more mycorrhizal-acquired ^{33}P than aphid-free pairs may reflect a greater demand for these nutrients by the plant hosts, potentially for initiation of plant defense responses involving P.[29] These include phosphate-containing compounds, such as trehalose phosphate.[29–31] Upregulation of these and related defenses would drive the increase in sink strength for P in aphid-exposed plants, potentially leading to mycorrhizal-acquired P accumulating in plant tissues.

We observed similar trends (albeit not significant) for AM fungal-acquired ^{15}N assimilation in host plants, with variability within treatments. In our experiments, there was a trend for the assimilation of ^{15}N in aphid-free plants to be lower when the neighboring plant was exposed to aphids than when the neighbor was aphid-free. Although not significant, our results suggest that aphid herbivory has the potential to hinder N assimilation by neighboring host plants, and not just for the individuals directly subjected to resource pressure. Interestingly, the low ^{15}N assimilation of aphid-infested plants contrasts with findings that aphids assimilate ^{15}N from AM fungi via host plants, acting as nitrogen sinks.[32] Similar to P, N is often involved in plant defense chemistry; for example, jasmonic acid-isoleucine conjugate plays an important role in plant defense against *A. pisum* in some plants, although this role does not appear to be ubiquitous.[33] In our experiments, however, it appears that aphid-exposed plants did not require a greater N supply, potentially reflecting a reduction of jasmonic acid-isoleucine conjugate precursors, such as 12-oxo phytodienoic acid.[33]

Neighboring plants mediate aphid impacts on mycorrhizal carbon allocation

Plants exposed to aphid herbivory dramatically reduced C supply to AM fungal partners, supporting findings from past experiments.[27] This reduction occurred regardless of whether the neighboring plant was exposed to aphids or not (Figure 3). This observation was consistent when both neighboring plants were infested by aphids, despite the root biomass being significantly greater than the other treatments (Figure 1B), potentially reflecting host plants holding C in their roots rather than exporting it to AM fungi. However, root C accumulated during the 24-h ^{14}C-labeling period was similar across treatments, despite there being greater root biomass when both neighbors were exposed to aphids. This is likely due to the nature of isotope tracing methods, which represent only a brief snapshot of C dynamics at a given point in time versus plant C allocation patterns across lifecycles.

The aphid loads of plants within and between each treatment were not significantly different from each other (Figure S1); therefore, this factor is unlikely to have played a role in driving the changes we observed in C or nutrient transfer. Where they were applied, aphids assimilated similar amounts of recently fixed C from host plants (Figure S2C), which could have affected the availability of C resources for transfer to AM fungi. In line with this, we found that C acquisition by AM fungi when aphids were present in the system decreased (Figure 3A). It is possible that the aphid-exposed plants in all pairings reduced C transfer to

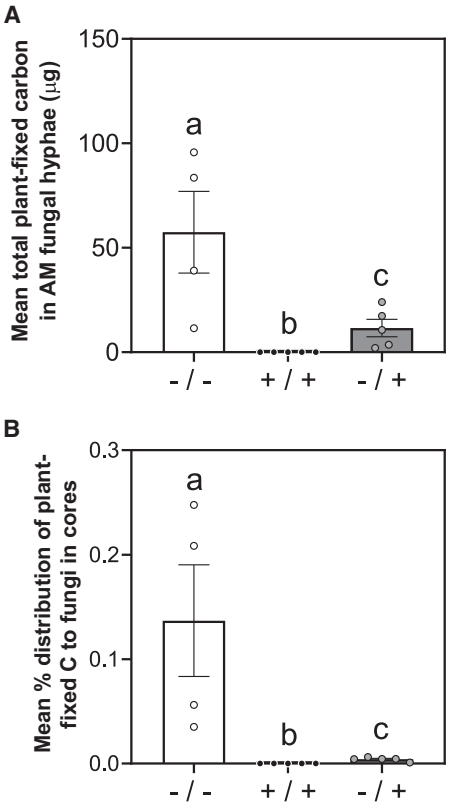

Figure 3. Carbon transfer from plants to AM fungi

(A) Mean total plant-fixed carbon in extraradical AM fungal hyphae (μg) and (B) mean % distribution of plant-fixed C to AM fungi within cores during the experiment. Error bars represent SEM. Different letters indicate significant differences between treatment means (where $p < 0.05$, Mann-Whitney U tests) ($n = 4$ for −/−; $n = 5$ for +/+ and −/+.) See also Figure S2. Mean concentration of C in (a) shoot tissues; (b) root tissues; and (c) aphids (μg/g).

extraradical AM fungal mycelium as a result of aphid-induced decreased rates of photosynthesis,[21–24] although the shoots of plants exposed to aphids contained more recently fixed C than those that were not exposed to aphids (Figure S2A), suggesting a withholding of resources rather than physiological impairment. As such, it is possible that plant C allocation to extraradical AM fungal mycelium, whether the fungi were simply interacting or had formed a CMN, was redistributed toward defense responses in aphid-exposed plants.

Because many defense compounds are C-rich, such as those in the salicylic acid defense pathway (reviewed by Thompson and Goggin[20]), this could result in an overall reduction in the amount of host plant C available to support AM fungal partners. Interplant signaling via mycorrhizal networks is involved in plant defense responses against aphids.[7,9] C-containing[25,26] VOCs are emitted by uninfested plants when they are connected by a CMN to neighboring plants infested with aphids.[26] As such, up-regulation of defense pathways in response to aphid exposure (or perceived exposure via the CMN) could conceivably be an important mechanism by which the amount of C available for allocation to AM fungal symbionts is reduced, a model first put forward by Babikova et al.[25] Thus, the presence of aphids in our experiments likely decreased the C source strength of the exposed plant, making the aphid-free plant the primary C source for AM fungal mycelium within the pots.

When only one plant was exposed to aphids, the extraradical AM fungal hyphae received more recently photosynthetically fixed carbon than when neighboring plants were both subjected to aphid herbivory, but less than when both plants were not exposed to aphids (Figure 3). Given the dramatic reduction in C allocation to AM fungal hyphae when both host plants were exposed to aphids, our observation that an intermediate amount of C (~6-fold reduction compared with when both plants are uninfested) is supplied to the mycorrhizal mycelia suggests that the aphid-free plant within the pot may have contributed the bulk of C to extraradical AM fungal hyphae within the soil, potentially offsetting some of the C limitation imposed by aphid herbivory of the neighboring plant. As mentioned previously, uninfested plants neighboring infested plants still potentially incur an aphid-derived C cost. This could include the production and release of defensive VOCs[26] upon detection of signals transmitted by the infested neighbor,[25] potentially explaining why there is not a proportional 50% reduction in C transfer to AM fungi by plants in the −/+ treatment compared with those in the −/− treatment.

The intermediate supply of C to external AM fungal hyphae may have contributed to the maintenance of nutrient flow to host plants we observed, contrasting with a wholly "reciprocal-rewards"-based model of mutualism. Through this evolutionarily stabilizing mechanism of mutualism,[14] host plants may "reward" specific, so-called generous fungal partners with more C compared with fungi that do not supply soil nutrients. In turn, "cooperative" fungal symbionts supply mineral nutrients preferentially to the plant hosts that supply them with adequate C resources.[15,34,35] Our data partially support this model of mutualism: we observed that more AM-acquired nutrients are supplied to the plant hosts that supply extraradical mycorrhizal mycelium with C (i.e., aphid-free plants) than plants that do not contribute as much (i.e., aphid-exposed plants). However, our data also show that even in treatments in which both neighboring plants were exposed to aphids and very little C was transferred to mycorrhizal fungi, the AM fungi continued to supply ^{33}P and ^{15}N tracers to the plants, albeit in a smaller amount. This could be a result of the development of smaller, less extensive extraradical mycelia or even the formation of discrete, competing mycorrhizal networks, owing to the reduced resources available to the AM fungi. However, given that there were no differences observed between or within aphid treatments in terms of % root colonization or soil AM fungal hyphal lengths, this seems unlikely.

In our experiments, in the −/+ treatment in which the mycorrhizal mycelium appears to have been largely supported through C contributions of the aphid-free plants, both plants within the network (i.e., aphid-exposed and aphid-free) received equivalent amounts of AM fungal-acquired nutrients (Figure 4). The amount of mycorrhizal-mediated ^{33}P and ^{15}N received by these plants was greater than it was in aphid-free treatments in which plant hosts supplied nearly twice as much C to extraradical mycorrhizal mycelium (Figure 4). This observation runs counter to what might be expected from a reciprocal reward model of mutualism. As such, our data perhaps align more closely with an "unequal terms of trade" model.[36] Through this model, plants

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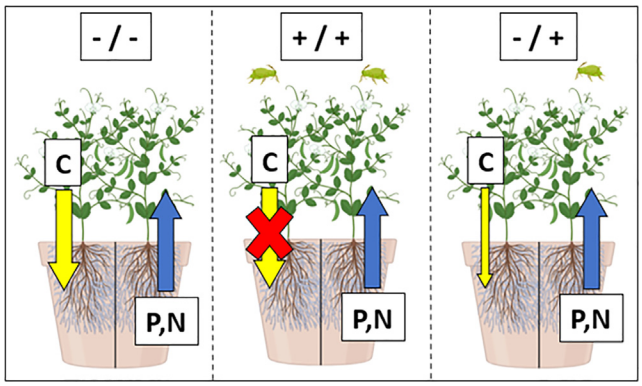

Figure 4. C-for-nutrient exchange under no aphid infestation, total aphid infestation, and partial aphid infestation

Yellow arrows denote C allocation from plants to AM fungi and blue arrows denote P and N allocation from AM fungi to plants. The thickness of arrows represents the strength of nutrient transfer. Red cross indicates absence of C transfer.

may derive equivalent benefit from but contribute unequally to shared mycorrhizal networks depending on resource supply and availability, underlining the importance of community-wide interactions on resource availability.

Wider perspectives

Our results demonstrate that the quantity of nutrients supplied to individual plants within communities by AM fungi is not dependent on the amount of C supplied to AM fungi by individual, one-on-one interactions. Instead, the net flow of C into AM fungal hyphae across plant communities is more important for nutrient release from AM fungi. In our experiments this resulted in plants that supplied little or no C (i.e., those exposed to aphids) to AM fungi being rewarded with P and N, potentially because of C inputs from neighboring aphid-free plants. This suggests nutrient transfer may not necessarily be based on specific exchanges of C for P and N between individuals but is instead driven more by source and sink strengths across plant communities. Similarly, AM fungi do not appear to discern which plant host contributes the most C. Instead, providing that C is made available by the plant community, mycorrhizal-acquired P and N appear to be drawn to host plants according to relative P and/or N demand. Such demand is likely to be governed by species-specific requirements, as well as the specific demands of biotic and abiotic interactions.

Our experiments demonstrate the potential importance of plant and fungal neighbors in influencing carbon-for-nutrient exchange dynamics between specific AM fungal and plant partners, with the impacts of interactive parties being mediated across the wider community. Given that the degree to which AM symbiosis is nutritionally mutualistic can be highly species specific or even genotype specific,[12,13] future research should investigate the role of plant identity within plant and associated fungal communities. It is possible that shared mycorrhizal fungi ameliorate the effects of competition for plant resources on mycorrhizal nutrient assimilation and that this ability is affected by diversity in plant communities, with related impacts on plant competitiveness and community structure and function.[37] Developing a greater understanding of the role of shared and competing mycorrhizal mycelia in mediating plant community nutrient dynamics, particularly when plants are under biotic or abiotic stress, and the relative intensity or load of the stress, will therefore be critical in achieving broader conservation and restoration targets in the future.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead contact
  - Materials availability
  - Data and code availability
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
  - Plant material and growth conditions
  - Aphid culture conditions
- METHOD DETAILS
  - Experimental treatments
  - Construction of aphid clip cages
  - AM fungal colonization of roots and soil
  - Aphid load (see Figure S1)
  - Determination of carbon for nutrient exchange between plants and AM fungal partners
  - Determination of carbon for nutrient exchange between plants and AM fungal partners
  - Plant harvest and sample analysis
  - Equations
- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.cub.2023.05.033.

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AUTHOR CONTRIBUTIONS

E.D., S.M.S., and K.J.F. conceived and designed the experiment. E.D., G.A.H., and N.H. performed the experiment, harvest, and sample analyses. E.D., D.Z.C., D.J., and K.J.F. analyzed and interpreted the results. E.D. wrote the first draft of the manuscript. All authors contributed to the development and editing of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research. One or more of the authors of this paper self-identifies as a gender minority in their field of research. While citing references scientifically relevant for this work, we also actively worked to promote gender balance in our reference list.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Acetic acid - glacial	VWR	8187552500
^{33}P-phosphoric acid	Hartmann Analytic	FF-01
Lactic acid 90%	Acros	189870010
^{14}C-sodium bicarbonate	Perkin Elmer	NEC086H001MC
Potassium Hydroxide	Acros	134060010
Ethanol	Sigma	32221
Pelikan Brilliant Black ink	N/A	N/A
Poly(vinyl alcohol)	Sigma	363146
Glycerol	Acros	158920025
Sulphuric acid	VWR	20700.323
Emulsify-safe	Perkin Elmer	6013389
Ammonium Molybdate.4H2O	Generon	AB0067
Ascorbic acid	Sigma	A92902
Sodium Hydroxide	Fisher	BP359–500
Sodium diHydrogen Orthophosphate.2H2O	Fisher	S/3760/53
CarbonCount	Meridian Biotechnologies	CT/10
CarbonTrap	Meridian Biotechnologies	CC/10
Hydrogen Peroxide 35%	Acros	202460010
Experimental models: Organisms/strains		
Acyrthosiphon pisum	Dr. Elizabeth Duncan, University of Leeds	N/A
Pisum sativum cv. Meteor	Premier Seeds Direct	PEA10
Rhizophagus irregularis	PlantWorks, UK	N/A
Software and algorithms		
GraphPad Prism v9.0	GraphPad Software	https://graphpad.com
R v4.2.1	R	http://R-project.org
R Studio 2022.07.01	RStudio	https://rstudio.com

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Katie Field (k.j.field@sheffield.ac.uk).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- All data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Plant material and growth conditions

Pairs of dwarf peas (*Pisum sativum*) were grown in a substrate of a ratio of 1:1 acid-washed silica sand to M3 compost (East Riding Horticulture; Sutton Upon Derwent, UK) in a total of 40 x 22.2cm diameter pots (80 plants), containing 5L unsterilized substrate

(Figure S1). 100g of commercial AM fungal inoculum (*Rhizophagus irregularis*; Plantworks, UK) was mixed into the substrate of each pot. *R. irregularis* was chosen due to its broad host range and near-ubiquity in nature. All substrate was unsterilized and was sourced from the same producer, the same fungal inoculant and seeds from the same sources were used across treatments, and all pots were maintained in the same controlled environment glasshouse. As such, soil microbial communities were likely consistent across treatments.

Nylon mesh with 35μm pore size was fastened down the middle of each 5L pot using hot glue to ensure neighboring plant roots were separated while permitting the fungal network to extend through (Figure S3). Two 35μm nylon mesh-covered, windowed-PVC cores were inserted into the pot.[38] Cores were filled with the same substrate as the pots, and one was placed on either side of the central mesh partition at time of planting. Seeds were planted outside of the cores in the total bulk soil and placed equidistant from all cores. 2 seeds were planted on either side of the mesh (4 seeds in total per pot) in case of non-germinating seeds. Where necessary, 1-2 plants were weeded out after 2 weeks so that only one plant was left on either side of the mesh (2 plants in total per pot). The mesh covering the core windows permitted AM fungal hyphal ingrowth but prevented plant root penetration, thereby creating AM fungal hyphal-only zones inside the cores within the pots. In each pot, one of the mesh covered cores remained static throughout the experiment, preserving hyphal connections between the core contents and plant roots (Figure S4). The other core was regularly rotated gently to sever the AM fungal hyphal connections between plant roots and the core contents. A third mesh covered core was filled with glass wool and inserted into one pot of each treatment group to allow monitoring of below-ground respiration by the extraradical mycelium of *R. irregularis* throughout the ^{14}C labelling period. Plants were maintained at 18°C during the day and 16°C during the night at 60% humidity for a 16-hour day in glasshouse conditions. Plants were grown inside insect rearing tents (Watkins & Doncaster, Leominster, UK) with 4 pots per tent for the entire experimental period (~12 weeks). Subsamples of soil (~5g) from the cores and the pot were sampled prior to aphid treatment to ensure AM fungal networks had formed throughout the pot and was confirmed by ink staining according to the methods of Brundrett et al.[39]

Aphid culture conditions

Pea aphid cultures (*Acyrthosiphon pisum*) were maintained on broad beans (*Vicia faba*) using the same plant growth conditions as experimental plants for 8 weeks and moved to dwarf pea plants of the same variety used in the experiment (*P. sativum*) 4 weeks before application to experimental plants to ensure generational adaptation of aphids to the experimental plants.

METHOD DETAILS

Experimental treatments

Paired plants were split into three treatment groups; the first where neither of the plants were exposed to aphids (- / - treatment), the second where both plants were exposed to aphids (+ / + treatment), and the third where one plant in the pot was exposed to aphids while the neighboring plant was aphid-free (+ / - treatment) (Figure S1). For the + aphid treatments, 5 adult aphids were placed on the bottom (abaxial surface) of a fully expanded leaf on the first branch of 10-week-old plants using clip cages and left on until harvest (18 days). Clip cages were assembled by using 35μm nylon mesh to ensure an equal exposure to herbivory across plants and to prevent movement of aphids between plants. For plants that were not exposed to aphids, empty clip cages were placed on the same leaves to control for potential impacts of cage effects.

Construction of aphid clip cages

Double pronged metal alligator clips were heated over a flame and held open so that the ends of each prong rested in the middle of the sides of 11mm thick clear plastic rings (2 rings per clip, one ring per prong). Once heated sufficiently, clips were pressed against the sides of the rings so that the clips melted the rings, and they were fastened together. Next, 35μm pore size nylon mesh was hot glued onto the outsides of both plastic rings, while upholstery foam rings were hot glued onto the insides. To attach clip cages to the plants, wooden toothpicks were taped on the top of wooden skewers, which were fastened to the outside of the pots. Aphid clip cages were placed on the toothpicks, with the toothpick inserted into holes on the ends of the alligator clips of the clip cages. The clip cages were then clipped onto a fully expanded leaf on the first branch of 10-week-old plants, either containing aphids or not.

AM fungal colonization of roots and soil

At the end of the experiment, above- and below-ground biomass were separated. Root samples for each plant (i.e. two plants per pot) were cleared in 10% KOH at 70°C for 50 minutes, stained using acidified ink for 1 hour, and de-stained in 1% acetic acid for 3 days. Roots were then mounted on microscope slides using polyvinyl lacto-glycerol (PVLG) and set overnight at 60°C. Under 100x magnification, the frequency and intensity of AM colonization in the root system and intensity of colonization in the root fragments were measured and estimated using equations from Trouvelot et al.[40] (see also Table S1).

Fungal hyphae were extracted from the soil by taking 4-5g of bulk substrate from each pot and mixing in 500ml distilled water. After decanting into 200ml and stirring for 30 seconds, 10ml was extracted and two 5ml samples were subsequently filtered through 0.45μm membrane filters and stained with ink vinegar stain. From this, AM fungal hyphal lengths per pot were determined using the gridline-intersection methodology at 100 x magnification with 50 fields of view[41] (see also Table S2).

Aphid load (see Figure S1)

At harvest, aphids were removed from the infested plants and stored in their clip cages at -20°C. Aphid load (N g^{-1}) was calculated for the infested treatments by dividing the final aphid abundance (N) within the clip cage by the shoot biomass (g).[42]

Determination of carbon for nutrient exchange between plants and AM fungal partners

24 hours after aphid exposure, one core in each pot was injected with 100 μl of a labelling solution, containing 1MBq ^{33}P-orthophosphate (111TBq $mmol^{-1}$ Sp Act, total of 0.297ng ^{33}P; Hartmann Analytic, Brunswick, Germany) and ^{15}N-ammonium chloride (1mg/ml; Sigma Aldrich, UK) via a pierced capillary tube (as in Field et al.[43]).

In half of the pots for each treatment, cores containing isotope tracers were gently rotated through 90° immediately after isotope introduction to sever the AM fungal hyphal connections between the core contents and the plants (Figure S4). Cores containing isotopes in the remaining half of the pots were left static to preserve hyphal connections while the non-labelled cores were rotated to control for any effects of disturbance. Core rotations were conducted every 48 hours throughout the labelling period. By monitoring and comparing the isotope tracer uptake to plants where tracers were applied to rotated cores versus those applied to static cores, we can control for nutrient tracer uptake via passive diffusion or alternative microbial processes. Following tracer application, plant material was monitored daily for tracer uptake using a hand-held Geiger monitor.

Determination of carbon for nutrient exchange between plants and AM fungal partners

Sixteen days after ^{33}P and ^{15}N introduction, the tops of the cores were sealed using anhydrous lanolin and plastic caps and pots were enclosed in gastight chambers.[27] At the beginning of the 16-hour photoperiod, a 0.925MBq pulse of $^{14}CO_2$ was liberated in the headspace of plants by injecting 2ml 90% lactic acid into a cuvette containing 25μl $Na^{14}CO_3$ (1.62GBq $mmol^{-1}$ Sp Act; Perkin Elmer, USA). To record the drawdown of $^{14}CO_2$ by plants, 1 mL of chamber headspace gas was immediately sampled and again every four hours for the 16-hour photoperiod. To measure respiration and flux of ^{14}C belowground, gas samples were taken through the glass wool core and every two hours thereafter throughout the labelling period. Gas samples were injected into gas-evacuated vials containing the carbon trapping chemical Carbo-Sorb and liquid scintillant Permafluor (Perkin Elmer, UK) in equal volumes. Sample radioactivity was determined via liquid scintillation counting (TriCarb 3100TR liquid scintillation analyzer; Isotech). At the end of the 16-hour photoperiod, 4ml of 2M KOH was injected into the vial caps of the substrate filled cores to capture any remaining $^{14}CO_2$.

Plant harvest and sample analysis

After ^{14}C labelling was completed, polythene bags were removed from all pots and aphid clip cages were removed and stored at -20°C until subsequent ^{33}P, ^{15}N, and ^{14}C analysis. Substrate filled cores, bulk substrate, and plants were removed and separated into shoots, roots, bulk substrate, rotated core substrate, and static core substrate. A 5-15g sample of each bulk substrate replicate was taken and stored at -20°C for hyphal length quantification. Roots were cleaned with tap water and then root and shoot samples were stored at -80°C. Sub-samples of roots were also taken for % root length colonization by AM fungi and stored in 70% EtOH (v/v) at room temperature. Remaining plant and substrate material was stored at -20°C and subsequently freeze-dried along with the aphid samples. Biomass (dry weight) was measured before analyzing for ^{15}N, ^{33}P, and ^{14}C.

To determine ^{15}N transfer from fungus to plant, plant material (including control plant material not labelled with ^{15}N for background detection) was weighed in 3-5mg samples into tin capsules (Sercon) and ^{15}N content quantified by continuous-flow isotope ratio mass spectrometry (PDZ 2020 IRMS; Sercon). Air was used as the reference standard and the IRMS detector was regularly calibrated to commercially available reference gases. ^{15}N uptake by plants was calculated using Equation 1.

Freeze-dried plant material was homogenized using a mill (Waring Commercial) and 100mg samples of shoot, root, and substrate samples were weighed out in triplicate and stored in acid washed test tubes. ^{33}P content of plant, soil, and aphid samples was determined by digesting samples in 1ml of concentrated sulphuric acid at 350°C for 15 minutes. 100μl of hydrogen peroxide was subsequently added to each sample before they were returned to the digest block and heated until colorless. Digests were then diluted to 10ml with dH_2O. Liquid scintillation (TriCarb 3100TR liquid scintillation analyzer; Isotech) was used to quantify ^{33}P activity in each sample. 2ml of each digest solution was added to 10ml of Emulsify-safe scintillant (Perkin Elmer, USA) and ^{33}P content was determined (Equation 2).

^{14}C within plant, substrate, and aphid samples was quantified through sample oxidation (307 Packard Sample Oxidizer, Isotech) and liquid scintillation. 50mg of freeze-dried shoot, root, bulk substrate, rotated core substrate, and static core substrate material were weighed in duplicate into Combusto-cones (Perkin Elmer, USA). The total C fixed by plants ($^{12}CO_2$ and $^{14}CO_2$) transferred to the AM fungi or assimilated by aphids was determined using Equations 3 and 4. By subtracting the amount of plant-fixed C present within the rotated cores from the amount present within the static cores, movement of ^{14}C into the core via diffusion or alternative microbial C cycling processes is accounted for Thirkell et al.[12] and total plant-fixed C transferred to AM fungi may be determined. This value was then scaled up by the mass of soil in the pot to give total fungal C (μg) across the AM fungal networks.

Equations

The following equations were used in calculating plant and fungal ^{33}P, ^{15}N, and ^{14}C content.

^{15}N content

$$M_{Ex} = \left(\frac{[At_{lab} - At_{cont}]}{100}\right)\left(M\left[\frac{\%N}{100}\right]\right) \quad \text{(Equation 1)}$$

Where MEx = mass excess of the isotope (g); Atlab = atom percentage of the isotope in labelled microcosm; Atcont = atom percentage of the isotope in paired control microcosm; M = biomass of sample (g); and % N = percentage of the nitrogen.[44]

^{33}P content

$$M^{33}P = \left(\frac{\left[\frac{cDPM}{60}\right]}{SAct}\right) MwtDF \quad \text{(Equation 2)}$$

Where $M^{33}P$ = mass of ^{33}P (mg); cDPM = counts as disintegrations per minute; SAct = specific activity of the course (Bq mmol-1); DF = dilution factor; and Mwt = molecular mass of P.[45]

Total C content

$$T_{pf} \text{ or } T_{pa} = \left(\left[\frac{A}{A_{sp}}\right] m_a\right) + (P_r \times m_c) \quad \text{(Equation 3)}$$

Where T_{pf} or T_{pa} = total C transferred from plant to fungus or assimilated by aphids (g); A = radioactivity of the tissue sample (Bq); A_{sp} = specific activity of the source (Bq Mol^{-1}); m_a = atomic mass of ^{14}C; P_r = proportion of the total ^{14}C label supplied present in the tissue; and m_c = mass of C (g) in the CO_2 present in the labelling chamber, from the ideal gas law (adapted from Cameron et al.[46]).

$$M_{cd} = M_{cd}\left(\frac{PVcd}{RT}\right) \therefore m_c = m_{cd} \times 0.27292 \quad \text{(Equation 4)}$$

Where m_{cd} = mass of CO_2 (g); M_{cd} = molecular mass of CO_2 (44.01 g mol^{-1}); P = pressure (kPa); V_{cd} = volume of CO_2 in the chamber (0.003m3); m_c = mass of unlabelled C in the labelling chamber (g); M= Molar mass (12.011 g); R = universal gas constant (J K^{-1} mol^{-1}); T = absolute temperature (K); m_c = mass of C (g) in the CO_2 present in the labelling chamber, where 0.27292 is the proportion of C in CO_2 on a mass fraction basis.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistics were performed with R[47] and GraphPad Prism 9.0.0 for Windows.[47] Graphs were made using GraphPad Prism 9.0.0 for Windows.[47]

For fungal colonization, one-way ANOVAs and Tukey post hoc tests were performed on the dataset. For fungal density, data did not satisfy assumptions of the one-way ANOVA, regardless of transformations. Therefore, Kruskal-Wallis tests were performed, followed by Mann-Whitney U tests. One-way ANOVAs were performed on plant biomass datasets followed by Tukey HSD tests where $P<0.05$, and a Student's t-test was used to analyze aphid load for + / + and + / - treatments. For plant P and N data, to compare neighboring plants within treatment groups, linear mixed effect models were performed and included fixed effects for the aphid status of each plant. To assess the overall treatment effect (i.e., - / - vs. + / + vs. + / -), analyses were conducted at the level of individual plants using linear mixed effect models. Linear mixed effect models were conducted using R,[48] specific methods and code are detailed in the supplemental information. The model included fixed effects for the aphid status of each plant, the aphid status of its neighbor, and their interaction. A random intercept for pot was included to control for non-independence among pairs of plants from the same pot. Lastly, fungal C data did not satisfy assumptions of the one-way ANOVA, regardless of transformations, so Kruskal-Wallis tests were performed followed by Mann-Whitney U tests. One outlier was removed from - / - as it was greater than two standard deviations from the mean. Statistical details of experiments are included in the results and discussion and corresponding figure legends. Outputs of statistical tests are included in the supplemental information (Tables S1–S4). In all tests, n = number of individual plants unless indicated otherwise.