Early Root Herbivory Impairs Arbuscular Mycorrhizal Fungal Colonization and Shifts Defence Allocation in Establishing Plantago lanceolata

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Abstract

Research into plant-mediated indirect interactions between arbuscular mycorrhizal (AM) fungi and insect herbivores has focussed on those between plant shoots and above-ground herbivores, despite the fact that only below-ground herbivores share the same part of the host plant as AM fungi. Using Plantago lanceolata L., we aimed to characterise how early root herbivory by the vine weevil (Otiorhynchus sulcatus F.) affected subsequent colonization by AM fungi (Glomus spp.) and determine how the two affected plant growth and defensive chemistry. We exposed four week old P. lanceolata to root herbivory and AM fungi using a 2 x 2 factorial design (and quantified subsequent effects on plant biomass and iridoid glycosides (IGs) concentrations. Otiorhynchus sulcatus reduced root growth by c. 64%, whereas plant growth was unaffected by AM fungi. Root herbivory reduced extent of AM fungal colonization (by c. 61%). O. sulcatus did not influence overall IG concentrations, but caused qualitative shifts in root and shoot IGs, specifically increasing the proportion of the more toxic catalpol. These changes may reflect defensive allocation in the plant against further attack. This study demonstrates that very early root herbivory during plant development can shape future patterns of AM fungal colonization and influence defensive allocation in the plant.

Introduction

It is now accepted that terrestrial plants mediate interactions between organisms associated with them, often shaping ecosystem processes and community structure [1,2]. In particular, interactions between herbivorous insects and plant-associated fungi have become especially well studied, with numerous examples involving plant pathogens [3,4], endophytes [5,6] and symbionts [e.g. [7,8]]. Within the last group, plant-mediated effects of arbuscular mycorrhizal (AM) fungi have been shown to be beneficial, detrimental or neutral for herbivore fitness [reviewed in [9,10]], and the evolutionary outcomes have been explored [7]. In particular, it has been suggested that AM fungal-induced increases in defensive chemical concentrations lead to an increased selective advantage for plants in plant-herbivore interactions [7]. Surprisingly, current understanding of the reverse interaction (i.e. the effect of insect herbivores on AM fungi) is largely derived from studies that consider the indirect effects of above-ground insect herbivores on AM fungi, despite the two being spatially separated [9,11,12]. Given that root herbivores and AM fungi exploit the same part of the plant, either simultaneously or at different times, it seems likely that root herbivory influences AM fungal colonization. Despite this, very few studies have addressed how root herbivores and AM fungi interact, and the outcomes of these studies are mixed. For instance, AM fungi can negatively [e.g. [13,14]] or positively [e.g. [15]] affect root herbivores, whereas chewing root herbivores may stimulate [15,16], or have no effect on [e.g. [13,14]] AM fungal colonization.

In many ecosystems, root herbivores have been shown to influence the community composition of plants, above-ground herbivores and higher trophic groups [17,18]. These effects are likely related to the propensity of root herbivores to induce systemic changes in secondary metabolites in plants more frequently than shoot-feeding insects [19,20]. Indeed, low levels of root herbivory can lead to defensive ‘priming’ in the plant, resulting in reallocation of resources to defend against future herbivory [19,21,22]. AM fungi can cause similar changes in secondary metabolites throughout the plant [11,23], but no studies have yet considered the combined effect of root herbivory and AM fungi on plant secondary metabolites or whether their effects are additive, synergistic or antagonistic.

This study set out to test how root herbivory by the vine weevil (Otiorhynchus sulcatus F.) feeding on Plantago lanceolata L. affected AM fungal colonization and how this ultimately influenced plant growth and subsequent induction of secondary metabolites. Plantago lanceolata is a frequently used model system for investigating the effects of AM fungal colonisation (e.g. [24,25,26]) and herbivory (e.g. [27,28–31]), and their interaction (e.g.
[23,32,33]). *Otiorhynchus sulcatus* is a generalist root herbivore, known to attack a broad range of plant species, including *P. lanceolata* [34]. The main group of secondary metabolites in *P. lanceolata* are the iridoid glycosides [35,36], which are dominated by two compounds in particular: aucubin and catapol [37]. Catapol is a derivative of aucubin and more metabolically costly [35,36]. These compounds can have defensive roles both against herbivores [31,38] and microbes [39]. Iridoid glycoside concentrations have been shown to vary with AM fungal species in shoots of *P. lanceolata* [23,40,41] as well as with nematode root herbivory [42]. The combined effects of root herbivory and AM fungi on iridoid glycosides are unknown.

Using a factorial experiment, *P. lanceolata* plants were treated with either AM fungal inocula or sterilized AM fungal inocula, and then either exposed to a brief period of root herbivory by *O. sulcatus* or not exposed. The effects of *O. sulcatus* herbivory and AM fungi (acting alone and in combination) on plant growth and iridoid glycoside (aucubin and catapol) concentrations in the roots and shoots were then determined, as was the effect of initial *O. sulcatus* herbivory on AM fungal colonization. Based on previous research in other systems we hypothesized that early root herbivory by *O. sulcatus* would reduce plant growth, increase AM fungal colonization [15,16], and systemically increase total concentrations of iridoid glycosides in mature *P. lanceolata* [19,20]. Root herbivory has been hypothesized to increase root exudates to which AM fungi respond resulting in an increase in AM fungal abundance in roots [15,16]. In addition, we hypothesized that there would be a negative correlation between iridoid glycoside concentrations in the root, and AM fungal root colonization [40].

Materials and Methods

Study system

*Plantago lanceolata* seeds were collected from a well-established population on the grounds of the James Hutton Institute, Invergowrie, Scotland. *Otiorhynchus sulcatus* larvae used in this experiment were offspring of adults collected at night from plants and bushes surrounding the aforementioned *P. lanceolata* population. Adult vine weevils were stored in a controlled environment room at 19°C with 16:8 hours light:dark in Petri dishes. Each petri dish contained one large strawberry leaf (cut at the base of the leaf from a glasshouse stock of strawberry plants) as well as two discs of coir (Roffey Limited, Dorset, UK) in a glasshouse. Three days after the seedlings had been transplanted, 20 one-day-old *O. sulcatus* eggs were placed around each plant (c. 0.5 cm below the soil surface) in the twenty pots assigned for weevil addition (10 with sterilized and 10 with unsterilized AM fungal inocula). This approximates the typical egg density that may be laid by a colonising adult *O. sulcatus* under the prevailing environmental conditions at the site, as previously reported [44]. In a separate experiment we added vine weevil eggs to plants of the same age grown in the same soil and pots and assessed vine weevil numbers after one week by emptying pots and examining their contents under a dissecting microscope. This “extraction method” allowed us to assess whether eggs had hatched and larvae (generally a few mm long) were alive. No white vine weevil larvae were present eight days after eggs were added, but hatched eggs were observed in the soil. This indicates that likely vine weevil eggs hatched, fed, and larvae expired within eight days of addition to our experimental pots. As a result we are confident that the herbivory treatment was short in duration (especially given that in the field vine weevils feed on roots for seven months), but the results also clearly demonstrate that the effect of this herbivory on the plants was significant. The duration of herbivory was in a separate experiment we added vine weevil eggs to plants of the same age grown in the same soil and pots and assessed vine weevil numbers after one week by emptying pots and examining their contents under a dissecting microscope. This “extraction method” allowed us to assess whether eggs had hatched and larvae (generally a few mm long) were alive. No white vine weevil larvae were present eight days after eggs were added, but hatched eggs were observed in the soil. This indicates that likely vine weevil eggs hatched, fed, and larvae expired within eight days of addition to our experimental pots. As a result we are confident that the herbivory treatment was short in duration (especially given that in the field vine weevils feed on roots for seven months), but the results also clearly demonstrate that the effect of this herbivory on the plants was significant. The duration of herbivory was not deliberately manipulated, but rather was determined by the food resource available and the response of the herbivores. Two weeks following the addition of vine weevil eggs (17 days after transplantation) the length of each plant leaf was measured and all lengths added together to create the variable “total leaf length” every two weeks for 10 weeks. Two weeks after the addition of weevil *O. sulcatus* eggs, plants were fertilized with 200 ml of a simple 20-0-20 NPK solution (1 mM NH4NO3 and 5 mM KNO3), and this was then repeated every two weeks for the duration of the experiment.

While we demonstrated that *O. sulcatus* larvae could not survive longer than one week on *P. lanceolata* plants of this size, we also confirmed that no weevils had completed the larval stage (and emerged above-ground as adults) by isolating deepots in water-filled moats. Larvae do not readily come to the soil surface or move between pots, even when under duress (e.g. [45]), but any emerging adults would have fallen into the moats and been easily identified. The moats also prevented any incidental *O. sulcatus* climbing onto or between pots and foliage showed no indication of weevil herbivory.

Plants were harvested 11 weeks after transplantation. Total summed leaf lengths were recorded on the day of harvest prior to the removal of plant tissue. Above-ground tissues were removed by cutting at the base of the rosette and were flash-frozen in liquid nitrogen. Root systems were washed and care was taken to search for any vine weevil larvae present within pots before flash freezing roots. All samples were then freeze-dried, their dry weights recorded, and stored at −80°C.
AM fungal colonization was assessed by removing a random sample (<0.1 g dry weight) from each root system after freeze-drying, placing these samples into tissue cassettes, clearing with KOH, and staining with trypan blue. Slides of stained roots were analyzed using the gridline intersect method [46] at x40 magnification to score AM fungal structures, including hyphae, arbuscules, vesicles and spores in at least 100 fields of view per root system.

Iridoid glycoside analysis

Shoot and root material were analyzed for the iridoid glycosides aucubin and catalpol following a variation of the protocol of Bowers and Stamp [37] and Gardner and Stermitz [47]. Shoot and root tissues were ground using a ball mill (Mini-BeadBeater 8, BioSpec, Bartlesville, OK, USA). Sub-samples of ground shoot (0.025 g) and ground root (0.035 g) samples from plants with sufficient available biomass were extracted in methanol (HPLC grade, Rathburn Chemicals, Walkerburn, Scotland), solid particles were removed by filtration, and the extracts were evaporated at 60°C under a stream of nitrogen. One ml of an internal standard (phenyl-β-D-glucopyranoside) (0.5 mg/ml) was added to each dry sample followed by 3.0 ml H2O2, and these solutions were then subjected to three washes with pure diethyl ether to remove chlorophyll and other compounds before drying as previously. Dried samples were redissolved in 1.0 ml methanol and two replicate 100 µl aliquots were dried again before being derivatised using a 1:4 mixture of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA, Sigma Aldrich, UK) and pyridine before 1 µl was injected into an Agilent 7890A gas chromatograph with multi-mode injector operating in split mode at 275°C in a split ratio of 30:1. A Restek (Thames Restek, Buckinghamshire, UK) Rxi-1ms column [length 30 mm, internal diameter 0.25 mm, coat thickness 0.25 µm] was used with helium carrier gas delivered at 1.5 ml min⁻¹. The oven conditions were 200°C at injection, held for 1 min, before increasing at 20°C min⁻¹ to 260°C, held at this temperature for 6 min, then increased at 20°C min⁻¹ to 320°C, was and held for 10 min, giving a total run time of 23 min. The detector used was a flame ionisation detector which was operated at 320°C using nitrogen as a make-up gas. Peaks were identified by reference to the retention time of derivatized authentic aucubin and catalpol standards (Sigma Aldrich, UK).

Statistical Analyses

Data were analyzed using four statistical tests. First, to test for the effects of block, experimental treatments (AM fungal inoculation and vine weevil herbivory) and their interaction on total plant biomass, root biomass, shoot total iridoid glycoside concentrations, and the ratio of catalpol to aucubin in roots and shoots, we conducted an analysis of variance (ANOVA) in the general linear models procedure of SAS 9.2 (SAS Institute, Cary, NC). Plant biomass, root biomass, shoot total iridoid glycosides, and catalpol:aucubin in roots and shoots were log-transformed. Proportion of root length colonized by AM fungi was arcsin square root-transformed to meet the assumptions of normality. Root biomass was included as a covariate in the analysis of AM fungal colonization to control for variation in root length or biomass that could influence measures of proportional colonization. Second, we determined whether total leaf length was an acceptable proxy for plant biomass by testing for a correlation between the log-transformed variables of final total leaf length and total plant biomass. Third, we examined which factors had the greatest influence on plant biomass throughout the experiment. To test this we conducted a repeated measures ANOVA in the general linear models procedure of SAS 9.2 (SAS Institute, Cary, NC) on the five measures of total leaf length (used as proxies of plant biomass at five different time points) spanning the length of experiment. This test confirmed that changes in plant biomass were correlated with 	extit{O. sulfures} presence. Finally, to test whether AM fungal colonization was negatively correlated with root iridoid glycoside concentration or composition as previously observed by De Dyn et al [40], we conducted separate correlation analyses for plants in the two vine weevil treatments that were also in the AM fungal treatment in the correlation procedure of SAS 9.2 (SAS Institute, Cary, NC) between the log of the concentration of total iridoid glycosides or the log of the ratio of catalpol:aucubin in roots and the arsin square root-transformed proportion of root length colonized by AM fungi. SAS code for all the analyses is included in File S1.

**Results**

**Plant Biomass**

Vine weevil treatment negatively influenced the final total biomass ($F_{1,29}$ = 25.46, $p$<0.0001, Table 1, Fig. 1a) and root biomass ($F_{1,29}$ = 37.63, $p$<0.0001, Table 1, Fig. 1b) of 	extit{P. lanceolata}. However, there was no main effect of AM fungi on total plant biomass or root biomass (Table 1).

A significant positive correlation between total plant biomass and total leaf length in plants ($r$ = 0.846, $df$ = 35, $p$<0.0001) demonstrated that leaf length was an appropriate proxy for plant biomass, as has been previously demonstrated [32]. The repeated ANOVA revealed that 	extit{P. lanceolata} growth (as measured using total leaf length) was significantly affected by time ($F_{1,140}$ = 9.32, $p$<0.0001, Figure 2, Table 2), the interaction of time and vine weevil ($F_{4,140}$ = 8.97, $p$<0.0001, Table 2), and the interaction of time and vine weevil ($F_{1,140}$ = 8.97, $p$<0.0001, Table 2), but that the effect of AM fungal inoculation did not vary with time.

**AM fungal colonization**

Sterilization of AM fungal inoculum before addition to pots successfully prevented AM fungal colonization of the roots of 	extit{P. lanceolata} in the sterile treatment ($F_{1,25}$ = 325.05, $p$<0.0001, Table 3, Fig. 3). Colonization was significantly reduced by vine weevil ($F_{1,25}$ = 9.32, $p$<0.0001, Table 2) of 	extit{P. lanceolata}, and was significantly reduced by vine weevil ($F_{1,25}$ = 9.32, $p$<0.0001, Table 2), but that the effect of AM fungal inoculation did not vary with time.

**Table 1. ANOVA results for effects of AM fungal inoculation and vine weevil addition on the log-transformed values of 	extit{P. lanceolata} biomass.**

|        | Total Biomass |       |       |
|--------|---------------|-------|-------|
|        | $df$ | $F$ | $p$  | $F$ | $p$  |
| Block  | 1    | 0.32 | 0.5761 | 0.23 | 0.6333 |
| AM fungi | 1   | 0.03 | 0.8697 | 0.00 | 0.9779 |
| VW     | 1    | 25.46 | <.0001 | 37.63 | <.0001 |
| AM fungi * VW | 1 | 2.06 | 0.1611 | 1.48 | 0.2333 |
| Error  | 30   |       |       |       |       |

ANOVA results for effects of AM fungal inoculation and vine weevil addition on the log-transformed values of 	extit{P. lanceolata} total (Fig. 1a) and root (Fig. 1b) biomass. AM fungi refers to the two treatment effect of the presence of AM fungi or sterilized AM fungi within pots, while VW refers to whether 20 Vine Weevil eggs were added to pots three days after planting. Bold values indicate significant effects.

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herbivory (F\(_{1,25} = 10.08, p = 0.004\), Table 3, Fig. 3). There was a significant interaction between AM fungal colonization and vine weevil addition (Table 3) as there was no significant difference in colonization in the sterile AM fungi treatment between herbivory treatments (mean = 0 for both treatments), but the vine weevil treatment decreased colonization when both AM fungi and vine weevils were present. No adult vine weevils were recovered from the moats or during the harvest.

Iridoid glycosides

The presence of vine weevils did not alter the total concentration of iridoid glycosides in *P. lanceolata* root or shoot tissues (Table 4, Fig. 4a). AM fungi did not affect total concentration of iridoid glycosides in either shoots or roots (Table 4). Both the vine weevil treatment (F\(_{1,26} = 19.90, p = 0.0001\), Table 4, Fig. 4b), and colonization by AM fungi (F\(_{1,26} = 5.95, p = 0.0219\), Table 4, Fig. 4b) increased the ratio of catalpol to aucubin in roots, but these effects were additive, so there was no interaction between the vine weevil and AM fungal treatments. In contrast, the ratio of catalpol to aucubin in shoots only increased in the vine weevil treatment (F\(_{1,29} = 8.46, p = 0.0069\), Table 4).

AM fungal colonization and iridoid glycosides

There was no significant correlation in either the no vine weevil or vine weevil treatment between total iridoid glycoside concentration (r = -0.031, df = 9, p = 0.9374; r = -0.493, df = 5, p = 0.3991; Figure 5) and the proportion of root length colonized by AM fungi. There was also no correlation between the ratio of catalpol to aucubin in roots (r = 0.528, df = 9, p = 0.1442; r = 0.142, df = 5, p = 0.8193; Figure 5) and the proportion of root length colonized by AM fungi.

Discussion

As expected, early root herbivory by *O. sulcatus* reduced plant growth. These results are in agreement with multiple studies showing that root herbivory negatively impacts plant growth and fitness [18], although it is notable that even this very brief period of root herbivory early in the plants development continued to have detrimental effects on growth as plants matured. By the end of the experimental period plants may have compensated for root herbivory in above-ground tissues (although we did not measure reproductive output) (Figure 4), however this pattern of compensation did not occur belowground resulting in smaller plants overall.

In contrast to our hypothesis, vine weevil herbivory reduced AM fungal colonization. Although a meta-analysis has shown that shoot herbivory can have a negative influence on AM fungal colonization [48], this is the first study to demonstrate that root herbivory can negatively impact AM fungal colonization. One explanation for the reduction in AM fungal colonization could be that the loss of root tissue led to a loss of root space for AM fungi to colonize. While vine weevil herbivory did reduce root biomass, our analysis of AM fungal colonization controlled for changes in root biomass.

Figure 1. Effects of AM fungal inoculation and vine weevil addition on *P. lanceolata* a) total dry biomass (g) and b) root dry biomass. Grey bars represent the biomass of plants that received 20 vine weevil eggs (thus root herbivory) 3 days after planting, and empty bars represent plants that did not receive vine weevil eggs (and thus experienced no herbivory). AMF+ treatments (along the y-axis) represent plants that were inoculated with an unidentified *Glomus* sp. and *Glomus intraradices* (INVAM #FR121) (sold commercially by Agrauxine, Saint Evarzec, France). Error bars represent ± one standard error. See Table 1 for statistical analysis.

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Figure 2. Change in total leaf length (cm) during the experiment. Empty circles represent plants that did not receive vine weevil eggs (and thus never experienced root herbivory) while filled circles represent plants that received 20 vine weevil eggs 3 days after planting resulting in one week of root herbivory ending prior to the Week 2 measurement date. Analyses conducted within this paper show that total leaf length is correlated with total plant size, and thus graphing total leaf length shows the change in plant size in each root herbivory treatment throughout the experiment. Error bars represent ± one standard error.

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Table 2. Repeated measures ANOVA on the effect of time and AM fungal and vine weevil treatments upon log of total leaf length of *P. lanceolata*.

|              | Df | F    | P    |
|--------------|----|------|------|
| Time         | 4  | 9.32 | <.0001|
| Time*Block   | 4  | 5.31 | 0.0005|
| Time*AM fungi| 4  | 2.15 | 0.0777|
| Time*VW      | 4  | 8.97 | <.0001|
| Time*AM fungi*VW | 4  | 1.83 | 0.1258|
| Error        | 140|      |      |

Repeated measures ANOVA on the effect of time and AM fungal and vine weevil treatments upon log of total leaf length of *P. lanceolata* (a proxy for total biomass) across five time points spanning the length of the experiment (see Fig. 2). AM fungi refers to the two treatment effect of the presence of AM fungi or sterilized AM fungi within pots, while VW refers to whether 20 Vine Weevil eggs were added to pots three days after planting. Bold numbers indicate significant effects. doi:10.1371/journal.pone.0066053.t002

We hypothesized that vine weevil herbivory would result in increased total concentrations of iridoid glycosides in *P. lanceolota*. However, rather than changes in total iridoid glycoside concentrations, we observed a shift in iridoid glycoside composition in root biomass and cotyledon size [53]. Thus it is possible that De Deyn and colleagues selected for changes in plant biomass and cotyledon size [53].

However, given that AM fungi can take up to five weeks to fully colonize roots [50], the application of root herbivores in our study was virtually simultaneous with the introduction of AM fungi. As a result, timing of AM fungal and root herbivory introduction may not explain the effects of root herbivory on AM fungal colonization.

Unlike De Deyn *et al.* [40] we did not find a correlation between the proportion of root length colonized and the concentration of iridoid glycosides in *P. lanceolata* root tissues. The study by De Deyn *et al.* [40] used plants that were the result of several rounds of artificial selection for high and low levels of constitutive irioids whereas our study used plants collected from a wild population. The lack of a correlation observed in our study may be due to one of two reasons. First, there may exist a weak correlation explaining only a small part of the total variation in AM fungal colonization and iridoid glycoside concentration. As a result, without controlling for genotype within our treatments variation in AM fungal colonization and iridoid glycoside concentrations may have masked this relationship. Second, selection events can select for more than one trait (e.g. [51,52]), and previous studies of the selected lines examined by De Deyn and colleagues showed that selection on iridoid glycosides also selected for changes in plant biomass and cotyledon size [53].

We also hypothesized that vine weevil herbivory would result in increased total concentrations of iridoid glycosides in *P. lanceolata*. However, rather than changes in total iridoid glycoside concentrations, we observed a shift in iridoid glycoside composition in both root and shoot tissues. The only previous study of induction of iridoid glycosides in *P. lanceolata* compared the effects of root and shoot herbivores, and found systemic responses only in plants with constitutively low levels of iridoid glycosides in leaf tissues [42]. Studies in other systems have suggested that root herbivores often induce higher concentrations of secondary compounds in roots (reviewed in [22,54]). While root herbivory did not cause a
systemic increase in the total concentration of iridoid glycosides, it did cause significant differences in the composition of iridoid glycosides in both the roots and shoots. Specifically, vine weevil herbivory increased the ratio of catalpol to aucubin in roots and shoots, suggesting that P. lanceolata plants in this study may have deployed a more targeted anti-herbivore response [55]. In particular, increasing concentrations of the more toxic catalpol over a perhaps more costly and/or less effective systemic response (increasing overall concentrations of iridoid glycosides) could reduce future root and shoot herbivory [23,56]. This response may reflect a selective history of uncorrelated root and shoot herbivory in this population of P. lanceolata.

In contrast to the majority of previous studies, root herbivory did not lead to increases in the total concentrations of secondary chemicals in shoot tissues (reviewed in [20,22,54]). Aside from differences in secondary chemistry and phylogeny, there is no clear reason why P. lanceolata might not systemically increase all secondary compounds in response to root herbivory. This study did not differ greatly in methodology in comparison to previous studies. Root herbivory was brief, however previous studies that showed induced systemic responses followed periods of root herbivory ranging from three days [57] to eight weeks [58]. Our period of herbivory likely falls within this range. Plants in our study were ontogenetically young, only five weeks old at the time of vine weevil addition, however the ontogenetical timing of the application of root herbivores in our study was very similar to the timing of root herbivore addition in the previous study of induction of iridoid glycosides in P. lanceolata [42]. As a result, there is no clear explanation for why we did not see a systemic induction of iridoid glycosides concentrations in response to O. sulcatus herbivory in P. lanceolata.

We also observed shifts in catalpol:aucubin in the roots of plants hosting AM fungi. AM fungi have been previously shown to contribute to induced relative increases in catalpol in aboveground tissues [23]. Both aucubin and catalpol have been shown to reduce the growth and development of generalist herbivores, but smaller concentrations of catalpol are required to achieve toxic effects [59] and references therein). In this study we see that AM fungi alter constitutive levels of catalpol in root tissues, but do not influence whether or how iridoid glycoside concentrations are induced by O. sulcatus herbivory. This suggests that while there is no interaction between AM fungi and vine weevils on secondary chemical production, AM fungal influenced changes in constitutive levels of catalpol are still likely to negatively impact root herbivores. We would expect that long-term increases in

**Table 4.** ANOVA results for effects of AM fungal inoculation and vine weevil addition on the proportion of total iridoid glycosides in root and shoot biomass, and the ratio of catalpol to aucubin in root and shoot biomass.

|                      | Root Total IG | Shoot Total IG | Root Cat:Auc | Shoot Cat:Auc |
|----------------------|---------------|----------------|--------------|---------------|
|                      | df  F  p      | df  F  p      | df  F  p     | df  F  p      |
| Block                | 1  5.06 0.0328| 1  0.06 0.8063| 1  0.05 0.8308| 1  0.08 0.7754|
| AMF                  | 1  0.01 0.9058| 1  0.14 0.7094| 1  5.95 0.0219| 1  0.17 0.6832|
| Vine Weevil          | 1  2.38 0.1347| 1  0.08 0.7759| 1  19.90 0.0001| 1  8.46 0.0069|
| AMF*Vine Weevil      | 1  0.92 0.3461| 1  0.14 0.7063| 1  0.85 0.3662| 1  0.02 0.8844|
| Error                | 27 29 26 27   | 27 29 26 27   | 27 29 26 27   | 27 29 26 27   |

ANOVA results for effects of AM fungal inoculation and vine weevil addition on the log-transformed values of the proportion of total iridoid glycosides in root biomass, the proportion of total iridoid glycosides in shoot biomass, the ratio of the proportion of catalpol in root biomass to the proportion of aucubin in root biomass (root catalpol:aucubin), and the ratio of the proportion of catalpol in shoot biomass to the proportion of aucubin in shoot biomass (shoot catalpol:aucubin) (see Fig 3). AM fungi refers to the two treatment effect of the presence of AM fungi or sterilized AM fungi within pots, while VW refers to whether 20 Vine Weevil eggs were added to pots three days after planting. Bold numbers indicate significant effects.

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catalpol:aucubin will select for increased resistance to catalpol or reduced preference for AM fungal colonized plants by *O. sulcatus* larvae. The combination of the two AM fungal species did not promote host plant growth, but provided other benefits for plant fitness, namely via the promotion of iridoid glycosides in roots following herbivory. This study used two species from one phylogenetic grouping, but there is the possibility that a more diverse AM fungal community or AM fungi from different phylogenetic groups might produce different effects [9].

This is the first study to examine the potential induction of secondary compounds in response to root herbivory and AM fungi in *P. lanceolata*, and showed both shifts in the allocation of plant defense compounds both below- and above-ground in response to root herbivory, and negative effects of root herbivory on AM fungi. Changes to the strength of plant-AM fungal associations and to plant allocation to defence are likely to have far-reaching consequences for soil community diversity and ecosystem function. The picture emerging from this and other studies is of considerable diversity in the nature and strength of plant, AM fungal and herbivore responses to multitrophic interactions. Future research should focus on identifying what circumstances promote predictable ecological responses of plants, AM fungi and herbivores in multitrophic systems.

**Supporting Information**

**File S1**  SAS code for the statistical analyses within the paper. (DOCX)

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**Author Contributions**

Conceived and designed the experiments: AEB SNJ. Performed the experiments: AEB AMM BDM SC SNJ. Analyzed the data: AEB AMM BDM SNJ. Contributed reagents/materials/analysis tools: AEB AMM BDM SC SNJ. Wrote the paper: AEB AMM BDM SC SNJ.
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