Title: Leafminer attack induces plant-mediated facilitation of conspecific pupae in the soil

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Abstract

Plants and herbivores are engaged in intimate antagonistic interactions, with plants trying to mount effective defense responses and herbivores attempting to manipulate plants for their own benefit. Here we report on a new mechanism by which herbivores can facilitate their own development. We show that tomato (*Solanum lycopersicum*) leaf attack by the American serpentine leafminer *Lyriomiza trifolii* accelerates the development of conspecific pupae in the soil adjacent to the plant. This pattern was reversed in the jasmonate-signaling deficient tomato mutant *def-1*. Chemical analyses revealed that *L. trifolii* leaf attack changes the production of root volatiles in a *def-1* dependent manner. Thus, leaf-feeding herbivores can interact with their soil-dwelling pupae, and jasmonates and root volatiles likely play relevant roles in this phenomenon. This study expands the repertoire of plant-herbivore interactions to herbivory-induced modulation of metamorphosis.

Introduction

Plants are attacked by multitudes of herbivorous arthropods [1]. Upon attack, plants can alter their primary and secondary metabolism to produce toxic or anti-nutritive substances that reduce herbivore preference, performance and/or survival [2]. These chemical readjustments are controlled by plant hormonal signaling, among which the jasmonate pathway is a central regulator [3]. Some herbivores, however, have developed intricate strategies to suppress or modify induced plant responses to their own advantage [4–6]. Herbivores population dynamics are therefore strongly influenced by the interplay between the induction of plant chemical defenses and herbivores manipulation of these defenses [7–9].

Plant defense induction and manipulation can influence the interactions between the plant and different life stages of the herbivore [10]. For instance, many herbivorous species complete their life cycle in different organs of the host plant, e.g., adults feeding on shoots and conspecific larvae feeding on roots. Initial leaf herbivory by adults can elicit systemic plant responses in the roots that facilitate or inhibit subsequent larval performance [10–12]. While the impact of herbivory-induced plant defense responses on different plant-feeding life stages of conspecific and heterospecific herbivores have been studied extensively, little is known about their influence on non-feeding life stages such as the pupal stage. This is surprising considering the large number of herbivorous arthropod species (including insects belonging to the orders Diptera, Hymenoptera, Thysanoptera, Coleoptera and Lepidoptera) that pupate in the soil close to their host. Moreover, as insect pupae are immobile, their survival, i.e. adult emergence, is highly influenced by the soil habitat [13].

As drivers of soil biotic and abiotic properties and producers of biologically active metabolites, plant roots play a key role in plant-mediated interactions between leaf- and root-feeding herbivores and their life stages [14, 15]. Leaf herbivory can modulate the release of organic chemicals from roots, thus shaping the surrounding habitat and the plant-associated soil communities [16]. In particular, root emission of volatile organic compounds (VOCs) can influence the behavior of root herbivorous insects and their natural enemies [5, 16, 17] and potentially affect the physiology of the herbivore [18, 19]. Insect pupae can exchange gases through their spiracles [20, 21]. It is thus conceivable that leaf attack by herbivores may influence pupal development in the soil through systemic changes in the release of root volatile chemicals.

The American serpentine leafminer *Lyriomiza trifolii* (Burgess) (Diptera, Agromyzidae) is a polyphagous insect herbivore that causes large economic losses of ornamental plants and vegetable crops worldwide [22]. Adults of *L. trifolii* are small flies (ca. 2 mm long). Females flies feed and lay eggs inside the leaf, below the epidermis. The larvae subsequently feed within the leaf creating tunnels (mines). At the end of their third developmental stage, the larvae leave the mine and drop to the soil to pupate [23]. Due to its rapid life cycle (12-24 days), plants infested with this leafminer can generally hold several generations of this insect, which means that adults, larvae and soil-dwelling pupae simultaneously occur in the field. *L. trifolii* is an important pest of tomatoes (*Solanum lycopersicum*) and can build up to high population densities during the growing season [24]. Tomato roots are reported to produce and emit VOCs that influence the behavior of soil-living herbivores [25]. Thus, the *L. trifolii*-tomato system is an ideal model to investigate if and how plants mediate interactions between leaf-feeding herbivores and their soil-dwelling pupae. Very little is known about the interactions between *L. trifolii* and cultivated tomato at the molecular level [26]. Previous work in Arabidopsis suggests that plant resistance to *L. trifolii* mainly depends on jasmonate-regulated defense responses [27].

Here we used manipulative insect bioassays to investigate whether *L. trifolii* infestation of tomato plants affects the performance of the leafminer pupae in the soil, and whether these interactions depend on intact defense signaling through the use of a tomato mutant deficient in the biosynthesis of jasmonic acid, *defenseless-1* (*def-1*) [28]. Then, we explored whether *L. trifolii* effects on pupal development was associated with leafminer-induced and *def-1* dependent- changes in root volatile production. Our work uncovers herbivory-induced modulation of metamorphosis as a new phenomenon in plant-insect interactions.

Results

Leafminer attack facilitates pupal development in the soil

To test if aboveground infestation by the leafminer *L. trifolii* affects the performance of conspecific pupae in the soil, we infested tomato plants with *L. trifolii* and monitored pupal developmental time in the soil of infested and non-infested plants (Fig 1A). To quantify adult emergence, age-synchronized *L. trifolii* pupae were placed in Petri dishes and subsequently buried in the soil at 8 days after leafminer infestation. The Petri dishes were fitted with a permeable nylon mesh that allows for exchange of volatile chemicals between the rhizosphere and the pupae. Fourteen days after pupation, two times more adults emerged from batches of

pupae exposed to belowground volatiles from infested plants compared to non-infested plants (Fig 1B, S2 Table and S1 Fig, day 14, wild type non-infested vs infested $p \leq 0.001$, EMMeans pair-wise comparisons). This difference was not significant at the end of the emergence period (Fig 1B, S2 Table and S1 Fig, day 16, wild type non-infested vs infested $p > 0.05$, EMMeans pair-wise comparisons). Thus, *L. trifolii* leafminer infestation accelerates pupation in the soil, an effect that is most likely mediated by rhizosphere volatile chemicals.

Facilitation of pupal development is *def-1* dependent

Induced plant defenses, including those regulated by jasmonates, can mediate systemic shoot-root responses upon herbivore attack [29]. To gain insights into the potential involvement of defense signaling in leafminer induced facilitation of pupation, we evaluated the impact of leafminer infestation on pupal development in the jasmonate deficient tomato mutant *def-1*. *Def-1* plants are characterized by a reduced ability to produce jasmonic acid and mount defense responses following mechanical damage and/or herbivory [30, 31], while growth-related traits (e.g., plant height, number of leaves and dry mass) are reported to be similar to the wild type [28, 32, 33]. Compared to non-infested wild type plants, pupae developed more rapidly in the soil of non-infested *def-1* mutant plants (Fig 1BC, and S1 Fig, day 14, *def-1* non-infested vs wild type non-infested $p \leq 0.01$, EMMeans pair-wise comparisons). Strikingly, leafminer infestation reduced rather than increased adult emergence at 14 days (Fig 1C, S1 and S2 Tables, and S1 Fig, *Genotype x Herbivory*, $\chi^2 = 23.03$, df = 1, $p < 0.01$, ANOVA type II Wald chi-square tests; *def-1* non-infested vs infested $p \leq 0.001$, EMMeans pair-wise comparisons). At the end of the experiment, no differences in adult emergence were observed between infested and non-infested *def-1* plants (Fig 1C, S1 and S2 Tables and S1 Fig, *Genotype x Herbivory x Day*, $\chi^2 = 22.9$, df = 1, $p < 0.01$, ANOVA type II Wald chi-square tests day 16, *def-1* non-infested vs infested $p > 0.05$, EMMeans pair-wise comparisons). These results show that facilitation of pupal development is *def-1*- and, thus, likely jasmonate-dependent. They also demonstrate that the positive effect of leaf mining on pupal development is plant-mediated rather than directly mediated by the leafminers.

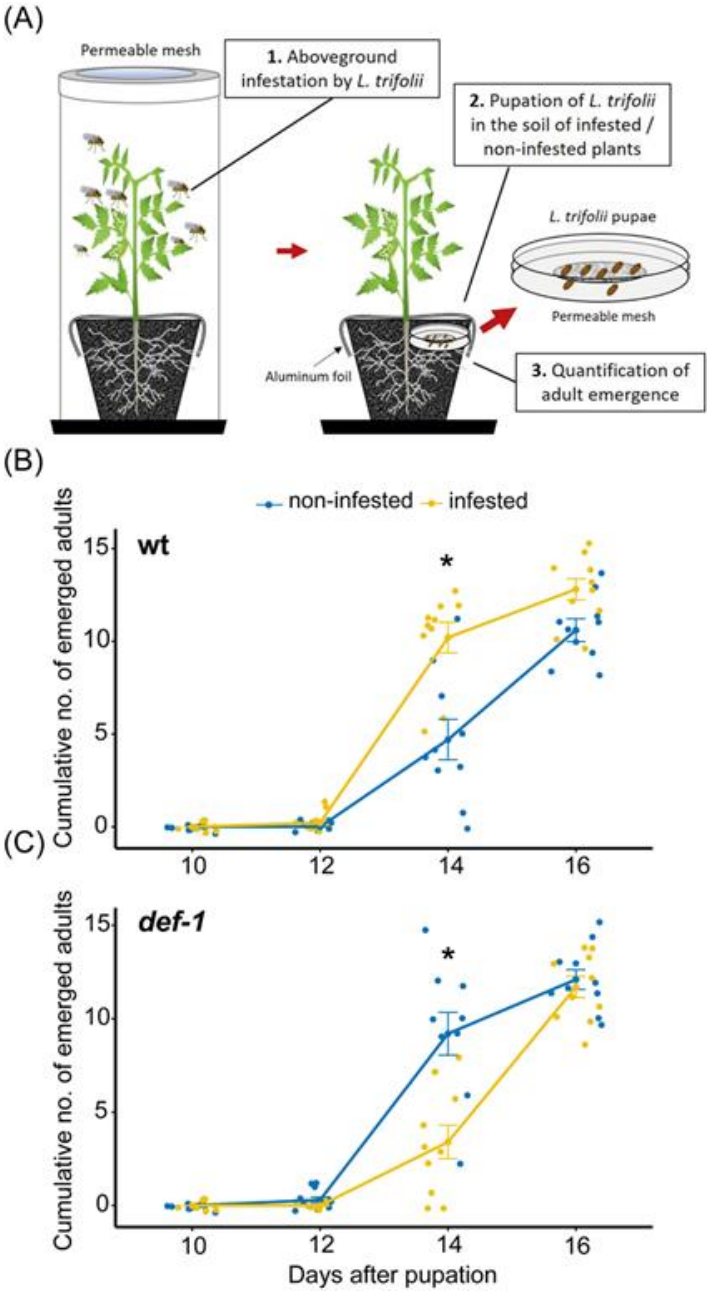

Figure 1. Leafminer attack facilitates the development of conspecific pupae in the soil in a *def-1* dependent manner. (A) Overview of the set-up to test the effect of *L. trifolii* leaf herbivory on the performance of conspecific pupae in the soil. Five-week old tomato plants were individually placed inside transparent plastic cylinders closed at one end with a lid made of insect-proof gauze and infested with seven *L. trifolii* female adult flies allowing them to feed and deposit eggs on the plants. Prior to *L. trifolii* infestation, the soil was covered with aluminum foil to separate the belowground and aboveground plant compartments. Eight days after infestation, fifteen age-synchronized *L. trifolii* pupae from an external rearing were confined to a custom-made and volatiles-permeable cage and subsequently buried into the soil of each plant. Cumulative number of *L. trifolii* adults (mean ± SEM, n = 10) that emerged from pupae exposed to belowground volatiles from (B) non-infested or *L. trifolii*-infested wild-type (wt) plants and (C) non-infested or *L. trifolii*-infested *def-1* plants were recorded every two days for a total of 16 days. Data from panels (B) and (C) were obtained from the same experiment and statistically analyzed altogether by using a negative binomial model that included plant genotype, herbivory and their interactions as fixed effects, and plant unit as the random intercept. Asterisks denote significant differences tested by pairwise comparisons of estimated marginal means with the Tukey-adjusted P value method ($P \leq 0.05$).

Facilitation of pupal development is not dependent on the severity of leaf attack

The severity of plant damage upon herbivory can influence the magnitude of systemic plant responses, and, by consequence, plant-herbivore interactions [5]. We thus tested whether leaf damage by *L. trifolii* predicts pupal development in the soil of wild type and *def-1* plants. No significant difference in the number of leaf mines was found between wild type and *def-1* plants (Fig 2A, t = 0.75, df = 17.86, p = 0.45, Student's *t*-test). Similarly, no significant correlation between the number of leaf mines and the number of emerging adults at 14 days (Fig 2B, r = -0.08, P = 0.72, Pearson) nor at 16 days (r = - 0.092, p = 0.69, Pearson) was observed. Thus, facilitation of pupal development is unlikely to be driven by the severity of leaf attack, and the different effects observed in wild type and *def-1* plants are not explained by different leaf mining activity.

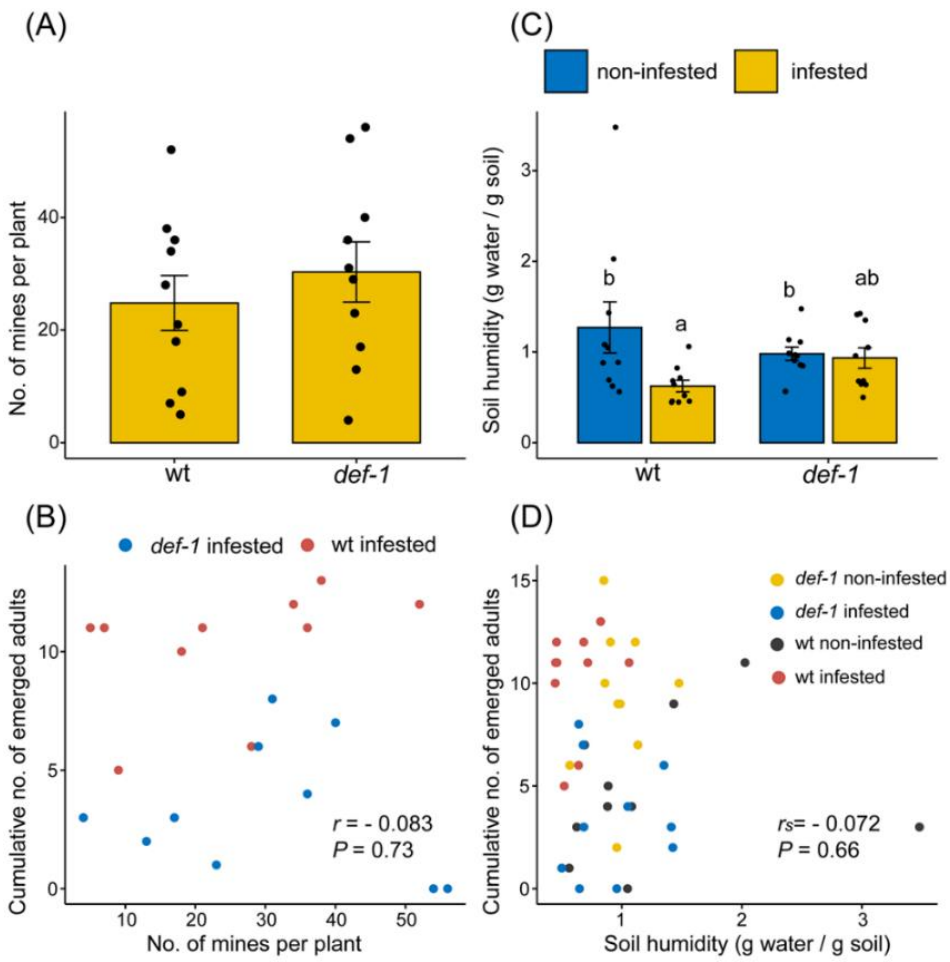

Figure 2. Facilitation of leafminer pupae in the soil is not correlated with leaf damage and soil humidity. (A) Number of mines per plant (mean ± SEM, n = 10) determined in wild-type (wt) and *def-1* plants at 24 days after *L. trifolii* infestation. Plants were individually infested with seven *L. trifolii* female adult flies allowing them to feed and lay eggs on the aboveground parts of the plant. No significant

differences were found between treatments as tested by Student's *t*-test at $P \leq 0.05$. (B) Scatter plot depicting the relationship between the number of emerged *L. trifolii* adults at 14 days after pupation and the number of mines in *L. trifolii* infested wt and *def-1* plants. The Pearson correlation coefficient (r) and P value are indicated. (C) Soil humidity (mean ± SEM, n = 10) estimated by using the gravimetric method in non-infested and *L. trifolii* infested wt and *def-1* plants at 24 days after infestation. (D) Scatter plot depicting the relationship between the number of emerged *L. trifolii* adults at 14 days after pupation and the soil humidity determined in non-infested and AG *L. trifolii* infested wt and *def-1* plants at 24 days after infestation. The Spearman's rank correlation coefficient (r_s) and P value are indicated.

Leaf attack changes soil humidity, but these changes do not explain facilitation of pupal development

Soil humidity is an important factor affecting herbivores pupal survival and adult emergence rate [34–36]. In most cases, extremely wet or dry soils can hinder adult emergence. We thus investigated whether *L. trifolii* leafminer attack changes soil humidity, and whether these changes are associated with the facilitation of pupal development. Leafminer attack significantly reduced soil humidity in wild type, but not in *def-1* plants (Fig 2C, *Genotype*, p = 0.308, *Herbivory*, $p < 0.05$, *Genotype x Herbivory*, p = 0.0511, two-way ANOVA) (Fig. 2C). Differences in soil humidity were not correlated with the number of emerging adults at 14 (Fig. 2D, r = - 0.072, p = 0.66, Spearman) nor at 16 days (r = - 0.12, p = 0.44, Spearman). While these results cannot exclude the possibility that leafminer induced changes in soil humidity may have contributed to differences in pupal developmental times, they suggest that the soil humidity in our experimental conditions does not play a major role in this process.

Leaf attack modulates root volatile profiles in a *def-1* dependent manner

Leaf herbivory can change root volatile production [15, 16], which may influence pupal development. To gain first insights into this possible mechanism underlying the observed facilitation, we measured root volatile production in infested and non-infested wild type and *def-1* plants in a separate experiment. As leafminer infestation did not affect root biomass (S1 Fig, genotype, p = 0.14, herbivory, p = 0.42, genotype x herbivory, p = 0.14, two-way ANOVA), we expressed volatiles per g of root fresh weight. We detected 18 different volatile compounds in tomato roots. Redundancy analysis revealed that plant genotype, herbivory and their interaction explained 31.8 % of the total chemical variation (Fig 3, F = 2.48, df = 3, p = 0.001, permutation F-test on the canonical R^2). In the associated score plot, the first axis explained 51.1% of the total variation explained by the variables and separated wild type plants from *def-1* mutant plants. The second axis explained 38.7 % of the total variation and separated *L. trifolii* infested from non-infested wild type plants (Fig 3A), with infested and-non infested

def-1 mutant plants clustering together in the middle. Permutation *F*-tests on individual variables confirmed this pattern and revealed significant differences in volatile profiles between plant genotypes and between infested and non-infested wild type plants, but not between infested and non-infested *def-1* plants (Fig 3A). Analysis of individual volatiles revealed that the levels of eucalyptol, 2-isopropyl-3-methoxypyrazine, 2-isobutyl-3-methoxypyrazine, cis-verbenol and 3,7-dimethyl-2,6-octadienal (Citral) were significantly higher in wild type roots compared to *def-1* roots (Fig 3B and Fig 4). Leafminer infestation slightly increased the release of methyl salicylate in both genotypes (Fig 4). Leafminer infestation suppressed the production of an unknown aldehyde in the roots of wild type plants, but not *def-1* mutant plants (Fig 4). A significant interaction between leaf infestation and genotype was also detected for (*E*)-β-caryophyllene (Fig 4), which was induced by leaf infestation in the wild type but not in *def-1* plants (although pairwise comparisons were not significant for this compound). In summary, this experiment shows that leafminer infestation changes root volatile profiles in a *def-1* dependent manner, which is compatible with the hypothesis that systemic changes in root volatiles may be responsible for the observed changes in pupal developmental times. The experiment also represents a first step towards the identification of the volatile chemicals that are responsible for this phenomenon.

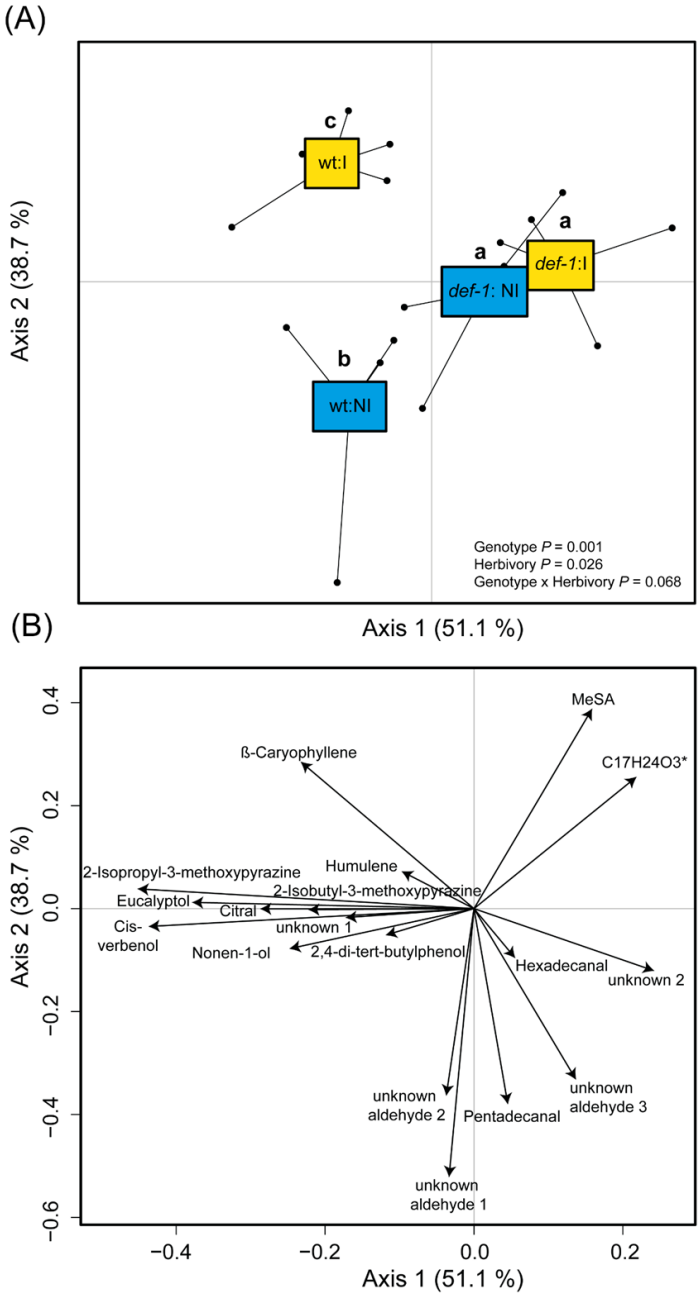

Figure 3. Leafminer herbivory modulates root volatile profiles in a *def-1* dependent manner. Redundancy analysis on tomato root volatile profiles analyzed by SPME-GC-MS in wild-type (wt) and *def-1* plants at 24 days after *L. trifolii* infestation ($n = 5$). (A) Score plot showing the first two principal axis with their explained variances in parentheses. Acronyms NI and I refers to non-infested and infested plants, respectively. The overall effects of plant genotype (G), herbivory (H), and their interaction (G x H) tested by permutation *F*-test are indicated. Different letters indicate significant differences among groups tested by permutation *F*-test pairwise comparisons corrected with the false discovery rate method at $P \leq 0.05$. (B) Loading plot displaying the contribution of each volatile compound on the separation of the treatments. Vectors length and direction denote the magnitude of their contribution and whether it is positive or negative, respectively. *The formula corresponds to 7,4-di-tert-butyl-1-oxaspiro (4-5) deca-6,9-diene-2-8-dione.

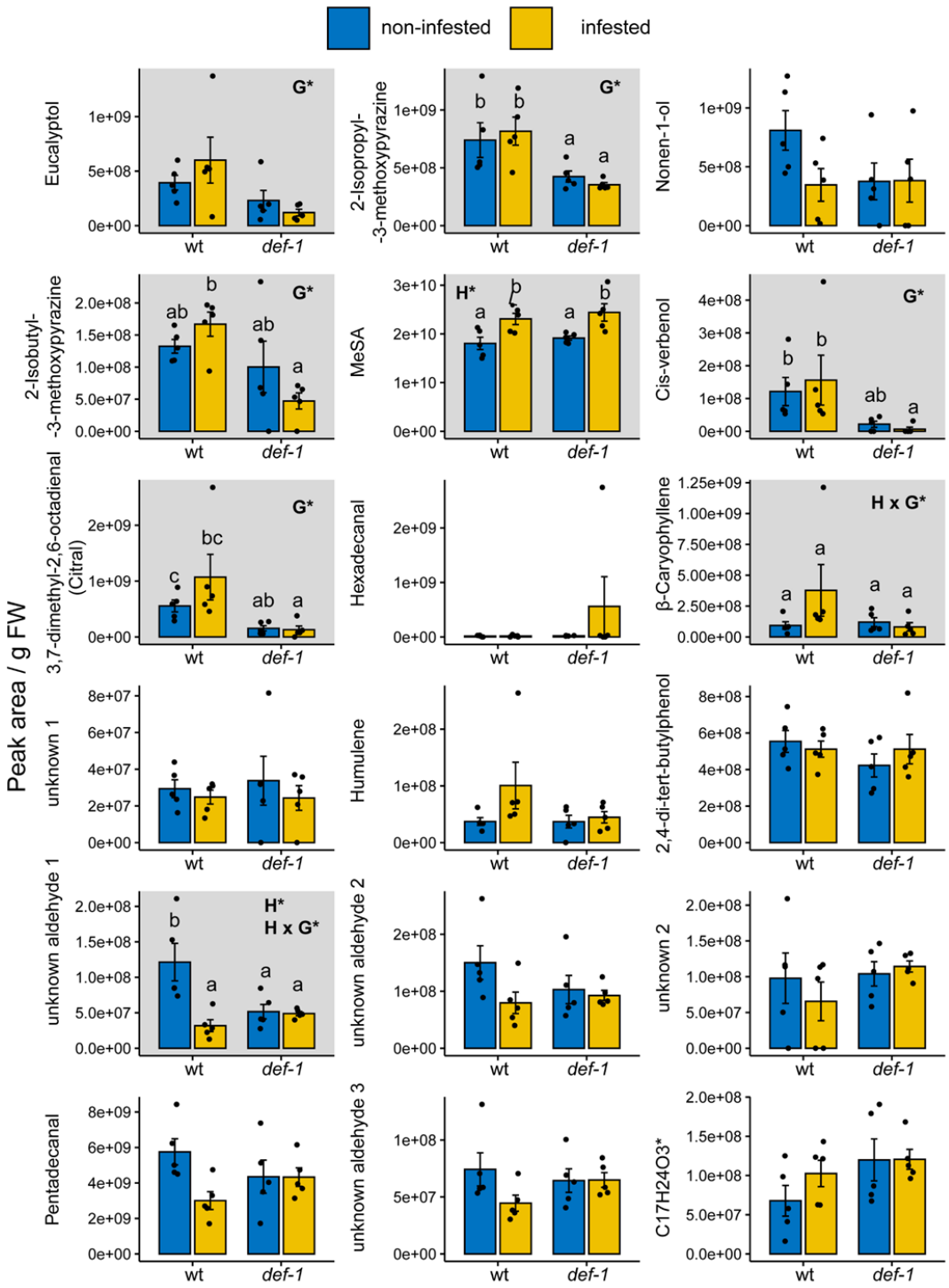

Figure 4. Leafminer herbivory affects the content of specific volatiles in tomato roots. Levels of tomato root volatiles (mean ± SEM, n = 5) detected in wild-type (wt) and *def-1* plants at 24 days after *L. trifolii* infestation. Shaded graphs highlight compounds where the effects of plant genotype (G), herbivory (H) and/or the interaction (G x H) were statistically significant as tested by two-way ANOVAs ($P \leq 0.05$). Different letters indicate significant differences among groups tested by EMMeans corrected with the false discovery rate method post hoc test ($P \leq 0.05$). *Formula corresponds to 7,4-di-tert-butyl-1-oxaspiro (4-5) deca-6,9-diene-2-8-dione.

Discussion

Plants and herbivores interact dynamically with each other, with plants trying to mount appropriate defense responses and herbivores attempting to disrupt these responses and to modulate plant metabolism to their own benefit [3]. Here, we demonstrate that leafminer attack of aboveground plant tissues triggers systemic changes in root metabolism that accelerates the development of the leafminer pupae in the soil. Below, we discuss this phenomenon from physiological and ecological points of view.

Over evolutionary time, herbivores have developed numerous strategies to feed and develop on well-defended host plants, including the systemic manipulation of plant metabolism to improve their own performance and fitness [4, 15, 37, 38]. Leaf damage by the red milkweed beetle *Tetraopes tetraophthalmus* for instance increases larval survival on the roots of the same plans [10]. Similarly, aboveground feeding by adult *Bikasha collaris* beetles on *Triadica sebifera* enhances the survival of conspecific larvae on the roots while reducing the performance of heterospecific herbivores [11, 39, 40]. Whether insect herbivores can also modify plant metabolism to directly boost pupation -a critical step in their development- has not been investigated so far. The results reported here thus represent a new mechanism by which herbivores can facilitate the performance of their offspring. Faster pupal development likely increases *L. trifolii* performance by shortening the life cycle of this species, thus potentially allowing for an increase in the number of generations per year. Furthermore, faster pupal development may reduce the risk of predation by natural enemies in the soil such as entomopathogens [41]. Both mechanisms may promote outbreaks of this pest species [42]. Further work will reveal the importance of this form of plant-mediated facilitation for *L. trifolii* population dynamics and its interactions with other herbivore species and trophic levels. Many insect herbivores pupate on plants or in the soil close to plants and are thus exposed to plant chemicals. We therefore expect plant-mediated effects of herbivory on pupation success to be common in nature.

Herbivores may modulate the performance of other plant-associated herbivore species or life stages through different mechanisms, including direct [43] and plant-mediated effects [44]. Here, we present several lines of evidence in support of the second scenario. First, the modulation of leafminer pupal development by aboveground feeding conspecifics depends on the tomato genotype, with facilitation occurring in wild type plants and suppression in *def-1* mutants. *Def-1* is deficient in induced jasmonate accumulation [28] and defense induction,

suggesting that the facilitation phenomenon observed in our study is jasmonate-dependent. This finding is in line with the central role of jasmonates in systemic signaling between leaves and roots [45, 46]. Second, leafminer attack triggers changes in soil-humidity and root volatile profiles in a *def-1* dependent manner, which is consistent with the notion that leaf attack modulates the physiology and metabolism of tomato roots in a way that facilitates pupal development in the soil. Finally, it should be kept in mind that leafminers, in contrast to chewing herbivores, essentially live within the plant and are thus unlikely to release high quantities of body odors and frass into the environment [47]. Taken together, these results strongly suggest that leafminers facilitate pupal development by inducing systemic changes in their host plant. Further experiments are required to unravel the role of jasmonates and other *def-1* dependent defense signals in this process.

What is the eco-physiological mechanism by which tomato roots of leafminer infested plants facilitate pupal development in the soil? As the pupa in our experiments did not have direct root contact, changes in root structure or endogenous root chemistry can be excluded. On the other hand, soil-dwelling pupae are known to be highly sensitive to changes in abiotic conditions, such as soil humidity [48, 49], and they may also be influenced by root exudate chemistry, including volatile and non-volatile root metabolites, which are known to modulate root herbivore behavior and performance [5, 15–17, 50]. We found that leafminer infestation significantly reduced soil humidity in wild type plants, but not in *def-1* plants. Accordingly, correlation analyses did not show any association between soil humidity and pupal development speed. Furthermore, pupal development was slower in leafminer infested than non-infested *def-1* mutants, despite the fact that soil humidity was not significantly affected by leafminer attack in this genotype. Thus, our data does not support a role of soil humidity in the systemic modulation of pupation time. Instead, we hypothesize that changes in exogenous root chemicals may be driving the observed patterns. In line with this, we observed that leafminer attack modulates root volatile production in a *def-1* dependent manner. Leafminer herbivory reduced the levels of an unknown aldehyde and slightly enhanced the accumulation of (*E*)-β-caryophyllene in wild type roots, but not in *def-1*. Although we did not find any volatiles that show a statistically significant inversion of responsiveness between the genotypes, tendencies for this phenomenon were found to occur for Eucalyptol and a isobutyl methoxypyrazine, two well-known plant aroma compounds [25, 51], with Eucalyptol having antimicrobial properties [52]. As belowground pupae can be highly susceptible to fungal diseases, the differential emission of root volatiles might modulate the microbial load of the pupa, thereby altering their

development. In addition, other belowground volatiles that were not detected in our analyses may have affected pupal development For instance, belowground ethylene emissions can be modulated by aboveground herbivory [15], making this compound a target for future analyses. Taken together, our work points to a role of systemic changes in root volatiles in mediating pupal development following leafminer attack. Further experiments with synthetic volatiles could unravel their impact on pupal development in the soil.

In conclusion, this study adds a novel facet to plant-herbivore interactions by demonstrating that leaf attack can facilitate pupal development in the soil. Plant volatiles likely play important roles in these interactions, as they are modulated by herbivore attack and readily reach non-herbivorous life stages of the herbivores, including pupa in the soil and on plants. This study represents a first step to unravel the mechanisms and ecological significance of herbivory-induced modulation of metamorphosis.

Materials and Methods

Plant and insect materials

Tomato (*Solanum lycopersicum* Mill) seeds of the jasmonate-deficient mutant *defenless-1, def-1*, and its wild-type, cv 'Castlemart', were sown in plastic trays filled with potting soil in a climate room provided with 113.6 $\mu mol \cdot m^{2} \cdot s^{-1}$ of PAR, a photoperiod of 16L:8D, 20°C, and 70% RH. Fifteen days after germination plantlets were transplanted to 13x13-cm plastic pots filled with the same potting soil.

A colony of the American serpentine leafminer *Lyriomiza trifolii* (Burgess) (Diptera: Agromyzidae) was maintained on chrysanthemum plants in a climate room at 25°C and 70% RH.

Experimental design

Five-week old wild-type (wt) and *def-1* plants were individually placed inside transparent plastic cylinders (80 cm height, 20 cm diameter) closed at one end with a lid made of insect-proof gauze (Leiss et al. 2009) (Fig. 1A). Seven *L. trifolii* female adult flies were released inside each cage. Non-infested enclosed plants served as controls. AG *L. trifolii* infested- and non-infested plants were randomly placed in a climate room provided with 113.6 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of PAR, a photoperiod of 16L:8D, 25°C and 70% RH. AG infested plants were damaged by the *L. trifolii* adult flies feeding and oviposition and, subsequently, also by developing larvae that create tunnels (mines) within the leaves. Eight days after AG infestation a custom-made

cage containing fifteen age-synchronized *L. trifolii* pupae (obtained from an external rearing maintained on chrysanthemum; see above) was buried in the soil (~1-2 cm deep) close to the stem of each plant (Fig. 1A). Custom-made cages consisted of a petri-dish sealed with parafilm whose bottom lid was perforated and covered with a permeable mesh (Fig. 1A). This system exposed the pupae to induced belowground VOCs only. Aboveground and belowground compartments of the plant were additionally separated by covering the soil with aluminum foil before *L. trifolii* infestation treatment. The number of emerged adults per cage/plant was then recorded every two days for a period of 16 days. At the end of the experiment, i.e., 24 days after infestation , the number of *L. trifolii* larvae-associated mines per plant and soil humidity were determined. In a second repetition of the experiment the effects of aboveground *L. trifolii* infestation on root dry biomass and volatiles content were determined at 24 days after infestation, i.e., 16 days after pupation (see methods below).

Soil humidity

Soil humidity was estimated using the gravimetric method [53]. A sample of fresh soil per plant was placed in a 2 ml Eppendorf tube, weighed, oven dried at 60°C for 5 days, and re-weighed. Fresh and dry soil mass (g) were calculated by subtracting the weight of empty 2 ml Eppendorf tubes. Gravimetric water content (GWC) expressed as the mass of water per mass of dry soil was then calculated as follows: GWC = [*wet soil* (*g*) – *weight dry soil* (*g*)] / *weight of dry soil* (*g*).

Root volatiles analysis

Root volatiles from *L. trifolii* infested and non-infested wt and *def-1* plants were collected and analyzed using solid-phase micro-extraction-gas chromatography-mass spectrometry (SPME-GC-MS). For this, the whole root system was sampled, washed with tap water, gently dried with filter paper, and flash-frozen in liquid nitrogen prior to analysis. Roots were then ground into a fine powder, and 100 mg was placed in a 20 ml precision thread headspace glass vial sealed with a septum of silicone/ PTFE and a screw cap made of magnetic silver with a hole (Gerstel GmbH & Co. KG). An SPME fiber (100 µm polydimethylsiloxane coating; Supelco, Bellefonte, PA, USA) was then inserted through the screw cap hole and septum for volatile collection for 40 min at 50°C. Collected volatiles were thermally desorbed for 3 min at 220°C and analyzed by GC-MS (Agilent 7820A GC interfaced with and Agilent 5977E MSD, Palo Alto, CA, USA). Desorbed volatiles were separated with split-less mode on a column (HP5-MS, 30 m, 250 µm ID, 2.5 µm film, Agilent Technologies, Palo Alto, CA, USA) with He as

carrier gas and at a flow rate of 1 mL min^{-1}. A temperature gradient of 5°C min^{-1} from 60°C (hold for 1 min) to 250°C was used. Compound identification was based on similarity to library matches (NIST search 2.2 Mass Spectral Library. Gaithersburg, MD, USA) as well as retention time and spectral comparison with pure compounds.

Statistical analysis

Data were collated in Excel and imported into R version 4.0 (R core Team, 2016) for analysis in RStudio version 1.1.463 development environment. We modeled the cumulative number of emerged adults with a negative binomial model using *glmmTMB* package [54]. Day of emergence, i.e., 12, 14 and 16 days (previous days where there were zero emerged adults for all treatments were not included in the analysis), plant genotype, herbivory and their interactions were included in the model as fixed effects, and plant unit as the random intercept. Residuals diagnosis, zero inflation and over dispersion tests were performed using the *DHARMa* package. The significance of the fixed effects from the conditional model was tested via ANOVA-type analysis (type II Wald Chi-square tests). Estimated marginal means (EMMeans), standard errors, confidence limits and significant differences between means with multiple comparison adjustments (Tukey's HSD) were estimated. Effects of herbivory, plant genotype and their interaction on soil humidity, root dry biomass, and levels of root volatile compounds were tested by two-way ANOVAs. Pairwise comparisons of EMMeans corrected with the false discovery rate method was used as post hoc test. Prior to analysis, all data were tested for normality and equal variance using the Shapiro-Wilk and Levene tests, respectively. Data that did not meet these assumptions were log10, squared root, or Tukey's Ladder of Powers transformed prior to statistical analysis. The correlation between the cumulative number of emerged adults at 14 or 16 days after pupation and (1) the number of larvae per plant or (2) the soil humidity was determined by Pearson product moment and Spearman's rank correlation tests. Principal component analysis (PCA) was conducted to explore root volatile profile differences among treatments. For this, data were first log (x+10) to correct for the presence of zeros and skewness, the means were centered to equals to zero, and the variables were scaled to have standard deviation equals to 1. To identify which factors significantly influenced the observed chemical variation in PCA we subsequently performed a redundancy analysis (RDA) followed by permutation *F*-test (999 permutations) based on the canonical R^2 [55]. Finally, we conducted permutation *F* (999 permutations) tests to assess the influence of plant genotype, herbivory and their interaction on the chemical data, as well as the differences among groups by pairwise comparisons corrected with the false discovery rate method. These

statistical analyses were performed and/or visualized using the R packages "*car*", "*olsrr*", "*rcompanion*", "*nlme*", "*emmeans*", "*ggplot2*", "*ggpbur*", "*factoextra*", *"Hotelling*" ,"*vegan*", and "*RVAideMemoire*" .

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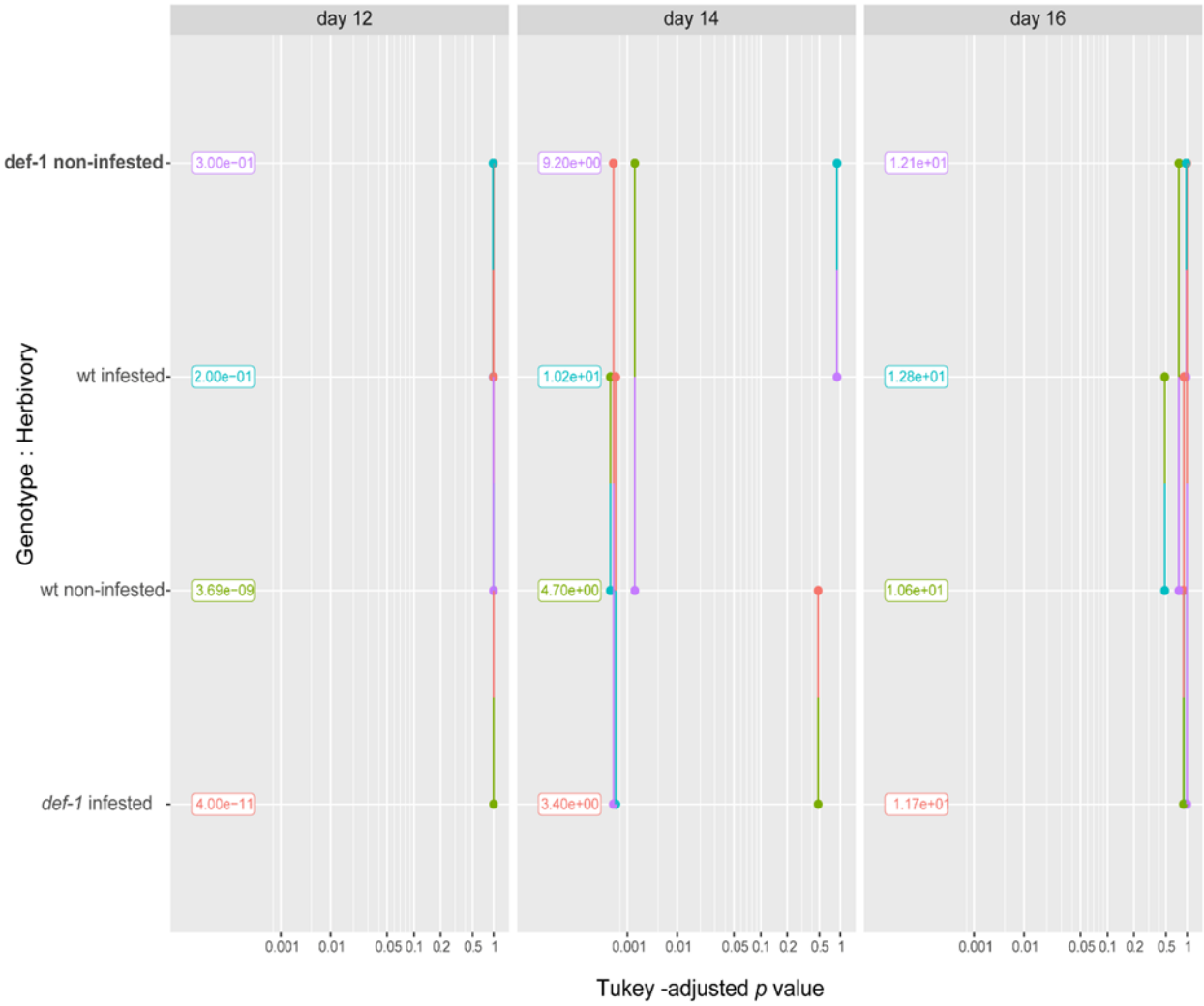
Supporting Information



S1 Fig. Plot of *p* values associated with pairwise comparisons of estimated marginal means. The effects of day of emergence, plant genotype, herbivory and their interactions, as well as the plant unit, on the cumulative number of emerged *L. trifolii* adults recorded in wild-type (wt) and *def-1* plants at 12, 14 and 16 days after pupation were modeled with a negative binomial followed by pairwise comparisons of estimated marginal means.

S1 Table. Analysis of deviance table (type II Wald Chi-square tests). The significance of the effects of day of emergence, plant genotype, herbivory and their interactions the cumulative number of emerged *L. trifolii* adults recorded in wild-type (wt) and *def-1* plants at 12, 14 and 16 days after pupation from the conditional model was tested via ANOVA-type analysis (type II Wald Chi-square tests).

Factors	Chisq	Df	Pr (>Chisq)
Day**	107.39	2	$2.2 \cdot 10^{-16}$
Genotype	0.2060	1	0.6499
Herbivory	0.41	1	0.5207
Day x Genotype	1.61	2	0.44
Day x Herbivory	0.72	2	0.69
Genotype x Herbivory**	23.03	1	$1.59 \cdot 10^{-06}$
Day x Genotype x Herbivory**	22.90	1	$1.06 \cdot 10^{-05}$

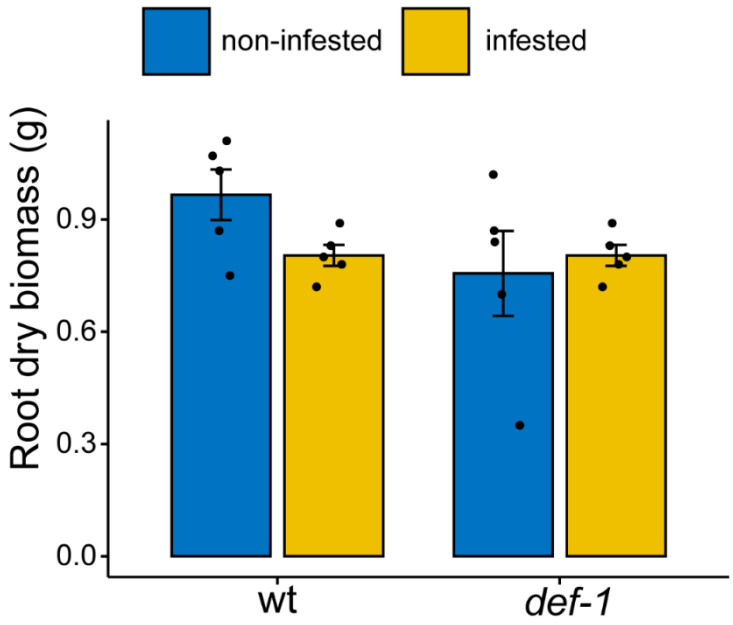
** $p < 0.001$

S2 Table. Estimated marginal means, standard errors and confidence limits.

Day	Genotype	Herbivory	Response	SE	Df	Lower CL	Upper CL
12	*def-1*	infested	$1.20 \cdot 10^{-05}$	2.00e-06	107	0.0	$1.02 \cdot 10^{50}$
14	*def-1*	Infested	3.4	5.83e-01	107	2.42	5
16	*def-1*	Infested	11.7	1.08e+00	107	9.73	14
12	wt	Infested	0.2	1.41e-01	107	0.04	1
14	wt	Infested	10.2	1.01e+00	107	8.38	12
16	wt	Infested	12.8	1.13e+00	107	10.75	15
12	*def-1*	Non-infested	0.3	1.73e-01	107	0.09	1
14	*def-1*	Non-infested	9.2	9.59e-01	107	7.47	11
16	*def-1*	Non-infested	12.1	1.10e+00	107	10.11	14
12	wt	Non-infested	0.0	1.92e-05	107	0.00	$7.58 \cdot 10^{13}$
14	wt	Non-infested	4.7	6.86e-01	107	3.51	6
16	wt	Non-infested	10.6	1.03e+00	107	8.74	13

Confidence level used: 0.95

Intervals are back transformed from the log scale



S2 Fig. Effect of *L. trifolii* herbivory on root dry biomass. Average root dry biomass (mean ± SEM, n = 5) determined in non-infested and infested wild-type (wt) and def-1 plants at 24 d after *L. trifollii* infestation.