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Plant and Aphid Genotypes Modulate Legume Rhizobium-Induced Defense Against Aphids

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Abstract

Sustainable protein production is needed to ensure food security while mitigating climate change. Leguminous crops contribute to these goals by providing protein-rich seeds and improving soil fertility through nitrogen-fixing symbiosis (NFS) with rhizobia. Beyond their nutritional and agronomic benefits, legumes face major challenges from insect herbivores such as the pea aphid, *Acyrthosiphon pisum*. While NFS can enhance plant defences against aphids, the influence of plant and aphid genotypic variation on these rhizobia-mediated effects remains poorly understood. Here, we investigated how genotype interactions influence rhizobia-mediated defence priming by using two *Medicago truncatula* genotypes (A17 and R108) grown under NFS with *Sinorhizobium meliloti* 2011 or nitrate (NI) feeding conditions and infested with three clonal lines representing two pea aphid biotypes. We analysed aphid performance, plant fitness, and plant leaf expression of defence genes associated with the jasmonic acid (JA) and salicylic acid (SA) signalling pathways over a 12-day period following aphid infestation. Aphid fitness varied significantly with both plant and aphid genotypes and was further modulated by rhizobia inoculation. Expression of defence marker genes involved in SA and JA pathways was dependent on specific plant–aphid genotype combinations and was significantly modulated by rhizobia inoculation. This pattern suggests that both SA- and JA-mediated plant defences contribute to regulating aphid weight, possibly through different mechanisms or in response to different plant–aphid interactions. Our study shows that plant and aphid genotypes, rhizobia inoculation and interactions (G × G × R) are thus central components driving plant-aphid-rhizobia interaction dynamics. Our study highlights the importance of the genetic context and rhizobia symbiosis in structuring multitrophic interactions and suggests

new opportunities to optimize aphid resistance using plant-beneficial microbial associations.

1. INTRODUCTION

Legumes are an important source of protein for both humans and animals. Their capacity to establish symbiotic associations with nitrogen-fixing microbes allows them to decrease dependence on synthetic nitrogen fertilizers, contributing to climate change mitigation. However, legumes like many other crops are attacked by different pests, among which aphids represent a major threat. Aphids reduce crop productivity both by causing severe damage to plants through direct feeding or more critically, by being vectors for many plants viral diseases. Aphid species differ in their host range, some are polyphagous, feeding on different plant species, while others are restricted to a single plant family [1]. For instance, the green peach aphid *Myzus persicae* attacks more than 400 plant species, whereas the cabbage aphid *Brevicoryne brassicae* and the pea aphid *Acyrthosiphon pisum* are specialized to *Brassicaceae* and *Fabaceae* families, respectively [2,3]. Such host specialization reflects their intimate relationship with host plants during their life cycle, which involves adaptations to cope with the plant phenology, the sap nutrient composition and chemical/physical defences. However, aphid performance also depends on the interaction between its genotype or biotype and of the host-plant genotype or ecotype [4,5,6,7]. During the last decade, plant microbe, arthropod microbe and plant arthropod interactions were shown to play an essential role in the

selection, adaptation and evolution of plant–arthropod interactions, giving rise to the three-way interactions concept [8,9]. While the effects of plant and aphid genotypes have been widely studied, the role of plant rhizobia symbiosis has received less attention. Tétard-Jones et al., 2007 [10] showed that interactions between barley genotypes and aphid clones are affected by the presence or absence of rhizospheric bacteria and evidenced the importance of the [aphid]x[barley]x[rhizobacteria] genotypes on aphid fitness in the multitrophic interactions. Recently, it was also suggested that plant growth-promoting rhizobia generally have negative effects on herbivore performance or abundance, most likely through Induced Systemic Resistance (ISR) in host plants [11,12]. However, the presence of rhizobium or arbuscular mycorrhiza (AM) fungi may have positive effects on sap-sucking insects, likely due to an increase in phloem nitrogen or phosphorus levels [13] and could also negatively affect herbivory natural enemies in the field, either by changing plant attractiveness or by decreasing the nutritive quality of their host [14,15].

Medicago truncatula (barrel medic) is a model plant to explore leguminous plant biotic interactions. Multiple studies have shown that the *M. truncatula* genotype plays a critical role in determining the interaction outcome with aphids, ranging from a fully compatible to a non-compatible plant-aphid interaction [4,5,16]. For instance, the *M. truncatula* Jester ecotype provides resistance against the blue-green aphid *Acyrthosiphon kondoi* and the pea aphid *A. pisum* Harris [17,18], unlike the closely-related ecotype A17, which gives no significant resistance to aphids [19,20,21]. The variation in virulence among aphid genotypes on these different *M. truncatula* ecotypes led to the description of a molecular mechanism involving a ‘gene-for-gene’ recognition between resistance (R) genes in plants and associated avirulence (Avr) genes in aphids [5,19,75]. Similarly, variation in the induction of phytohormone-dependent plant response to aphid feeding depends on the plant and aphid genotypes. The blue green aphid induces the expression of genes associated with the salicylic acid (SA) pathway in both *M. truncatula* resistant and susceptible lines early after its attack, although with different induction kinetics [22].

In contrast, genes associated with the jasmonic acid (JA) pathway are exclusively or predominantly induced in the resistant line [22,23]. Moreover, *M. truncatula* can form an intimate nitrogen-fixing symbiosis (NFS) with the bacteria *Sinorhizobium meliloti*, which not only affects plant growth but also modulates defence responses against herbivores [24,25]. Our previous study [24], demonstrated that NFS changes *M. truncatula* response to the pea aphid (*Acyrthosphon pisum*) by affecting aphid growth and increasing the plant defence responses [24]. However, this work was conducted using a single plant and single aphid genotype. Yet, most of the previous works have examined only single plant–aphid genotype pairs, leaving the combined role of plant and aphid diversity under different symbiotic contexts largely unexplored.

Since both aphid and plant genotypes may contribute to the dynamics of these interactions, the present study aimed at unravelling genotype-dependent effects in this tripartite system based on three *A. pisum* genotypes and two *M. truncatula* ecotypes Jemalong A17 and R108. It has been demonstrated that the two *M. truncatula* ecotypes are different in their genomic structure, developmental traits, and physiological response to biotic and abiotic stresses [27,28,29,30]. We thus have quantified aphid fitness, plant biomass, and defence gene expression (SA and JA pathway markers) in a time dependent manner to test how genotype-specific interactions and rhizobia inoculation jointly shape plant–aphid dynamics. We tested the hypothesized that (i) aphid performance would vary depending on plant genotype, (ii) rhizobia symbiosis would modulate defence expression and aphid success in a plant genotype-dependent manner, and (iii) outcomes would differ among aphid genotypes due to variation in virulence. The results demonstrate the critical role of genotypic diversity in influencing the ecological fate of multitrophic interactions and enlighten the complex relationship between plant nutritional status, symbiotic associations, and aphid pressure.

2. MATERIALS AND METHODS

2.1 Plant material and growth conditions

Two ecotypes of *M. truncatula*, Jemalong A17 and R108, were used. Seeds of these ecotypes were purchased from Medicago Biological Resource Centre, INRAE, Montpellier, France. These two ecotypes were chosen because they showed different resistance levels to pea aphids: A17 carries the *RAP1* gene, which provides race-specific resistance to the pea aphid [5]. *RAP1* is ineffective against LL01 (alfalfa biotype) but effective against the pea and clover biotypes, while R108 has no resistance reported to date. Before sowing, *Medicago* seeds were sterilized as described in [31,32]. The treated seeds were placed on 0.4% agar plates in the dark at 4°C for 2 days and then at 20°C for another 2 days. After germination, six plantlets were transplanted into round pots (7.5 x 7.5 cm) filled with a vermiculite and sand mixture (2:1). All pots were transferred to a growth chamber set at 23°C (16h light) and 20°C (8h dark), with a relative humidity of 60 ± 5%, and watered with nitrogen-free medium [33]. One week after transplanting, *M. truncatula* A17 and R108 pots were divided into two groups: one group received inoculation with the *Sinorhizobium (Ensifer) meliloti* 2011 strain (named thereafter NFS for Nitrogen Fixing Symbiosis plants) [34], and the second group was supplemented once with 10 ml of 5 mM potassium nitrate (KNO_3) in water (named thereafter NI for Non-Inoculated plants) (35). For inoculation, the *S. meliloti* 2011 strain was cultured on Luria-Bertani medium supplemented with 2.5 mM $CaCl_2$ and MgSO4 (LBMC) and 200 µg/mL streptomycin for 3 days at 30°C. Subsequently, bacterial cells were grown in LBMC liquid medium for 24h, pelleted at 5000g, washed twice with sterile distilled water, and resuspended in sterile distilled water to a final optical density of 0.05 (OD 600) [33]. Each NFS plant received a 10 mL supplementation of the *S. meliloti* suspension.

2.2 Aphids rearing and mesocosm preparation for aphid infestation

Three pea aphid (*Acyrthosiphon pisum*) lines were used (Table S1): the original YR2 and T3-8V1 clones naturally harbour distinct strains of *Regiella insecticola*, and the removal of these symbionts through ampicillin treatment resulted in the creation of the YR2-amp and T3-8V1-amp (named

YR2 and T38 hereafter) lines used here [36,37]. LL01, in contrast, is naturally free of facultative symbionts. The YR2 and T38 lines are classified as "clover" biotypes, while LL01 is categorized as an "alfalfa" biotype. All aphid lines have been maintained stably for over 15 years on fava bean (*Vicia faba)*, at a temperature of 20°C under a 16:8h light/dark cycle (Fig S10). The absence of facultative symbionts was confirmed through PCR analysis [38], throughout the duration of the experiment. One week after inoculation with *S. meliloti* (allowing time to form nodules) or nitrate supplementation, NI and NFS pots (each containing six plants) were randomly assigned to different experimental groups and half of the pots were subjected to infestation with 10 first instar (L1) aphid nymphs (see Fig S10). To synchronize aphid age, 20 wingless adult females were placed on separate fava bean plants, allowing them to reproduce for 24 hours. Then, 10 nymphs (L1) from each aphid line were transferred. All plant pots were individually isolated in ventilated plastic boxes and kept in the same room at 20°C under a 16:8h light/dark photoperiod with 70% relative humidity. Aphid survival was monitored daily, and the average weight of surviving aphids was measured on day 12, just before adults began reproducing and allowing sufficient time for the plant to establish nitrogen-fixing symbiosis and mount a defence response to aphids.

2.3 Plant and aphid biomass

To assess the impact of aphid infestation on plant biomass (Fig S10), the dry weight of plant shoots was measured at 4, 8, and 12-days post-infestation (dpi). Plant shoots were dried in an 80°C oven for three days and subsequently weighed (balance accuracy ± 0.1 mg; PA214, Ohaus Corp, Parsippany, NJ, USA). The shoot dry weight of the six individual plants from three pots (total 18 plants) *per* condition was measured across three separate experiments. The development of the various pea aphid lines was assessed at 1, 4, 6, 8, and 12 days after aphid infestation (dpi). Aphid survival and their mean weight (total survived aphid weight/survived aphid number) were measured as fitness parameters. Six independent replicates were done for each time point, with distinct sets of

plants and aphids. For each condition and time point, non-aphid infested NFS and NI plants were used as controls.

2.4 Gene expression analysis by quantitative RT-qPCR

Aphids were removed from the plants using a paintbrush, and the control plants for each condition were brushed in the same way as aphid-infested plants. Six plant shoots per condition and time point were collected immediately after removal of the aphids, pooled and frozen in liquid nitrogen and stored at -80°C when necessary. Three biological replicates were prepared for each condition and time point. For RNA extraction, the frozen shoots were grinded directly in liquid nitrogen using a mortar to obtain a fine powder. Total RNAs were isolated using RNAzol® RT (Sigma), quantified (NanoDrop 2000 spectrophotometer), and analyzed on a 1.5% agarose gel electrophoresis and a Bio-analyzer chips (Agilent) to assess purity. DNA digestion (RQ1 RNAse-free DNAse) and reverse transcription (GoScript™ Reverse Transcription) were performed as described by the manufacturer (Promega). The quantitative PCR was performed using the qPCR Master Mix plus CXR (qPCR kit; Promega). Each reaction was carried out with 5 µl of a 40-fold dilution of the cDNA template and each set of specific primers (Table S2). SA defence-related genes included *Medtr2g435490*, annotated as a *pathogenesis-related protein 1* (*PR.1*) (XP_013463163.1); *Medtr1g080800*, annotated as *chitinase (PR.4)*; and *Medtr1g062630*, annotated as *thaumatin-like protein* (*PR.5*) (XM_013612651.2). JA defence-related genes included *Allene Oxide Synthase 1* (*AOS.1*) (XM_013610584.2) and *Medtr4g032865*, a *proteinase inhibitor* (PSI-1.2) (XP_013455238.1), hereafter referred to as *PI*. Real-time qPCR included a first step at 95°C for 3 min followed by 40 cycles at 95°C for 5 sec and 60°C for 30 sec (AriaMx Real-time PCR machine, Agilent). Primer efficiency was evaluated on the slope of a standard curve generated using a serial dilution of the samples. Cycle threshold values (Ct) were normalized to the average Ct of two housekeeping genes [34]: *Medtr2g436620*, which encodes the translationally-controlled tumor-like protein *MtC27* and is commonly used as a constitutive control in roots and

nodules of *M. truncatula*, and *Medtr4g109650.1* (also known as a38), which encodes an uncharacterized protein. For the qPCR experiments, three independent biological replicates were used. Each biological replicate consisted of six individual plants pooled together to minimize plant-to-plant variation. For each biological replicate, three technical replicates were included in the qPCR reactions. Gene expression levels were normalized using two housekeeping genes (Mtc27, A38), and non-aphid infested control plants were used to rescale the expression. Relative gene expression was calculated by the 2^-ΔΔCt [74] method using the RqPCRBASE package [39] with the RStudio Version 1.1.453 (https://www.rstudio.com).

2.6 Statistical analyses

For each of the nine dependent variables, the expression of the six genes, the plant dry weight, the aphid weight and survival, we used the following procedure to assess and test the interactive effect of genotypes and inoculation on their expression. We adjusted two mixed models with as fixed effect, the aphid genotype, the plant genotype, the plant inoculation, as well as the two- and three-way interactions between these variables. We also included as fixed effect the time variable (day of the experiment) as well as its two-way interactions with the aphid genotype, the plant genotype, the plant inoculation (the statistical power did not allow to assess higher order of the interactions here). We set the experimental batch and sub-batch as nested random effects. The two mixed models differ only for the time variable, which is a continuous variable in one case and a categorical variable in the other. The categorical one is better suited in case of non-linear relation with the dependent variable, but the number of parameters involved is also much higher. The best of these two models according to the Akaike Information Criterion (AIC) was used for further analysis. They were adjusted with the lme4 R package [40], and we used the DHARMa package [41] to assess concordance between data and model assumptions, including checking for overdispersion in the GLMM model. The percentage of variation explained by the retained model was assessed by the marginal pseudo-R^2 (MuMIn package) [42,43] based on the

likelihood-ratio test, with the null model containing the random effects: *marginal* pseudo-R^2 corresponds to percentage of variance explained by fixed effects only. When some significant interactions were found between the aphid genotype, plant genotype and plant inoculation, we interpreted them with post-hoc comparisons obtained by the multcomp package [44]. For each fixed effect, we estimated effect sizes using partial-η^2 [45], computed using explained sum of squares (ESS) and residual sum of squares (RSS) estimated using type 2 decomposition of variance (partial-η^2=ESS/(ESS+RSS). As stated by Cohen 1988 [46] it is a proportion, not of the total variance, but of the total from which there has been excluded the variance due to the other factor(s) and interactions. In order to ease their interpretation, we categorized these effect sizes using the thresholds proposed by [46,47] (Effect size: 0.0099 < Small < 0.0588 < Medium < 0.1379 < Large).

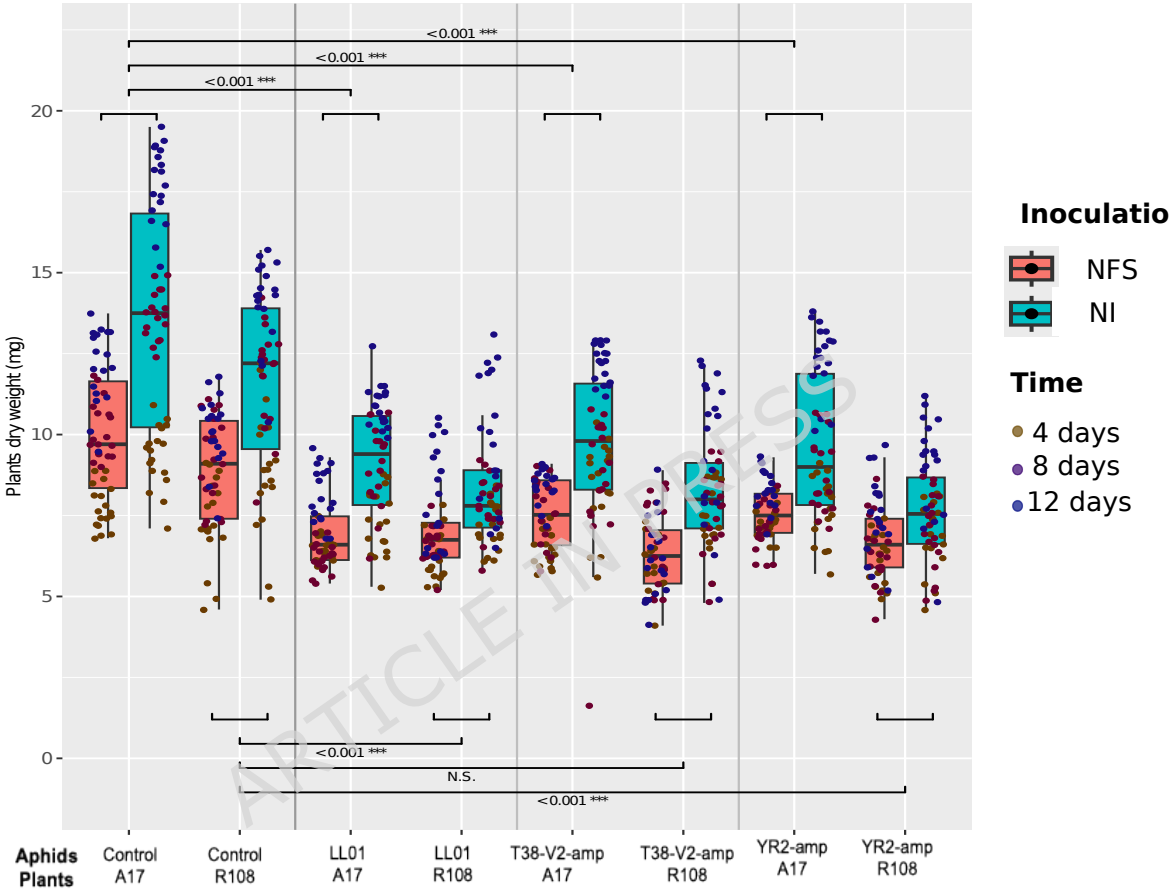
3. RESULTS

We used a three-way interaction model to explain how plant biomass, aphid fitness, and plant defence gene expression are influenced either by individual factors or by interactions among plant genotype, aphid genotype, and plant rhizobium inoculation during the time frame of the study.

3.1 Plant biomass reduction by aphids depends on plant and aphid genotype

At the start of the experiment, the shoot dry mass of the NI and NFS plants was similar (Fig S1). Over the 12 days, the dry weight of the control plants was different depending on the plant genotype (partial η^2 = 0.18, P < 0.001) and the nitrogen source (partial η^2 = 0.446, P < 0.001) (Fig 1, Table S3).

Figure 1: Aphid infestation reduces plant dry biomass in a genotype-dependent manner. Box plots show shoot dry weight of *Medicago truncatula* (genotypes A17 and R108) exposed to three pea aphid clones (LL01, YR2-amp, T38-V1-amp) under two nitrogen treatments: mineral nitrate supplementation (NI) or inoculation with *Sinorhizobium meliloti* (NFS) (n=3; each replicate is a pool of 6 plants). Boxes represent medians and interquartile ranges; Pink boxplots represent the NFS condition, blue boxplots represent the NI condition. The jitter dots represent each individual plant for dry weight measurements at discrete time points post-infestation (see time colours on the legend). Mixed model predictions for the interaction of plant inoculation x aphid genotype x plant genotype on dry weight (p=0.33; Marginal R^2 = 0.8); see text.



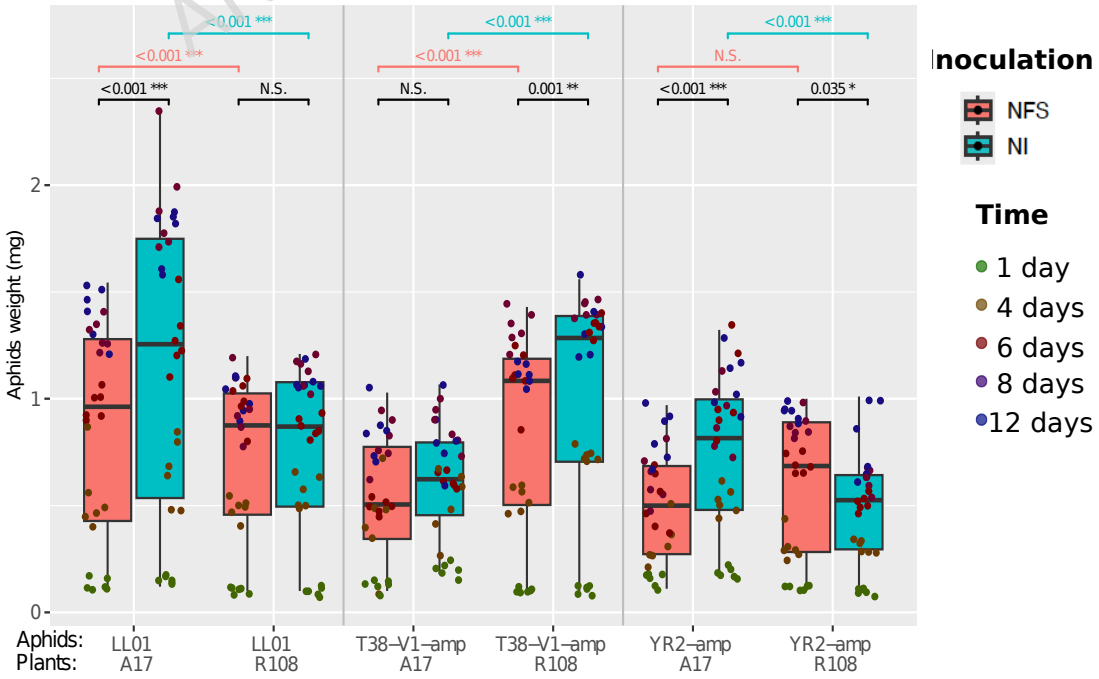
As expected, aphid infestation had a strong impact on plant dry weight (partial η^2 = 0.5, $P < 0.001$), and this was independent of the plant nutritional status (NI or NFS) (S6-9). While significant interactions were found between the plant genotype and the aphid genotype (Fig 1) (partial-η^2 =0.02, p < 0.001), aphid genotype x plant inoculation (partial-η^2 =0.068, p < 0.001) and plant genotype x plant inoculation (partial-η^2 =0.026, p < 0.001), the three-way interaction plant inoculation x aphid genotype x plant genotype was not significant (p = 0.33) (Table S3-S4). Together, these results showed that aphid infestation consistently reduced plant biomass regardless of the nitrogen source, while the scale of this reduction

was further shaped by specific plant–aphid–*S. meliloti* inoculation combinations.

3.2 Plant, aphid genotypes and plant inoculation modulate aphid weight

Aphids successfully survived and developed on both A17 and R108 *M. truncatula* genotypes regardless of the nitrogen nutritional source. However, some aphid genotypes grew better on A17 genotype and others on R108 genotype, as shown by a large effect (partial $\eta^2 = 0.32$, $P < 0.001$) on aphid weight, and by the large interaction between aphid genotype x plant genotype (partial $\eta^2 = 0.42$, $P < 0.001$) (Fig 2, Fig S5, Table 1).

Figure 2. Aphid weight over time was modulated by different *M. truncatula* genotypes and inoculation treatment. Box plots showing the mean weight of the surviving aphid (*A. pisum* clones LL01, YR2-amp, and T38-V1-amp) on two *Medicago truncatula* genotypes (A17 and R108) under two nitrogen treatment: mineral nitrate supplementation (NI in blue) and rhizobia inoculation (*Sinorhizobium meliloti*; NFS in pink). Aphid weight was measured at five independent time points (1, 4-, 6-, 8-, and 12-days post-infestation; $n = 6$ per time point). The X-axis legend indicates aphid–plant genotype combinations. Jittered points represent individual biological repeat for mean weight of the living aphids, with point colours indicating the sampling time. Mixed model predictions for the interaction of plant inoculation, aphid and plant genotype on aphid weight (Marginal $R^2 = 0.90$).



For instance, LL01 aphid genotype gained significantly higher weight on A17 than R108 (Fig 2), whereas T38 genotype performed significantly better on R108 than A17 across both nitrogen regimes (Fig 2). YR2 grew better on A17 than R108 under NI feeding (Fig 2), but this difference disappeared under NFS conditions. Plant rhizobium inoculation (NFS) had a medium effect (partial $\eta^2 = 0.13$, $P < 0.001$) on aphid weight, and interacted close to significance with aphid genotype (partial $\eta^2 = 0.017$, $P = 0.059$). For example, NFS reduced significant aphid weight in several host-aphid combinations (LL01/A17, T38-/R108, YR2/A17) (Fig 2), but had no significant effect on LL01/R108 or T38/A17 (Fig 2), and even significantly enhanced growth on YR2/R108 (Fig 2, Fig S5). The three-way interaction between aphid genotype, plant genotype, and plant inoculation had a medium effect (partial $\eta^2 = 0.095$, $P < 0.001$) (Table 1, Fig 2). Overall, these results showed that aphid fitness was strongly influenced by plant–aphid genotype interactions and further modulated by rhizobia symbiosis.

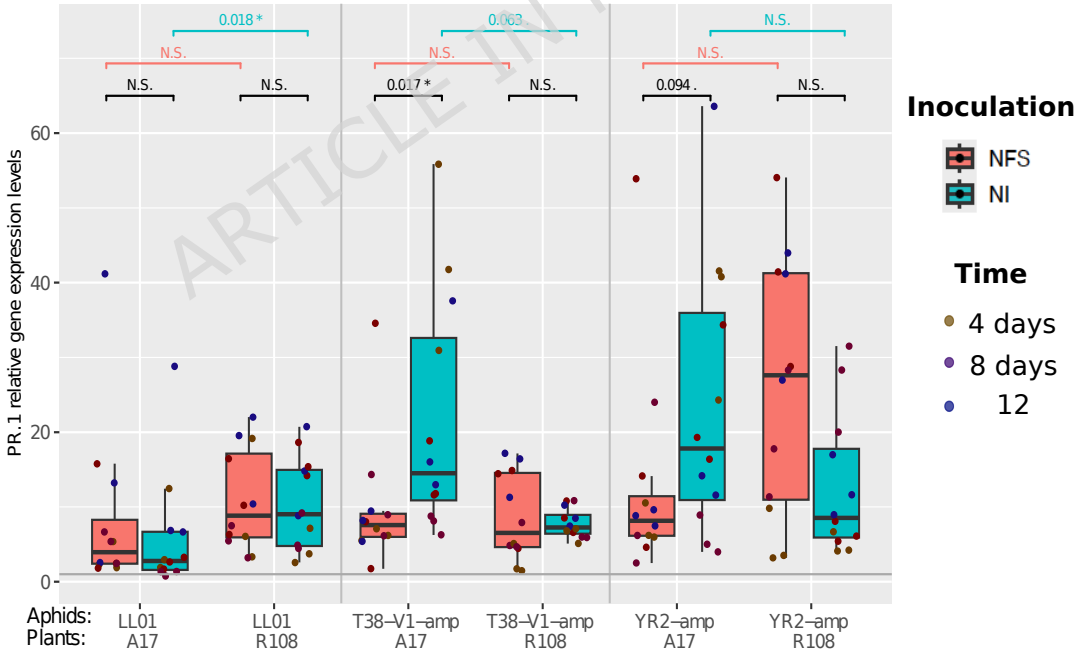
Table 1. Linear mixed-effects models assessing the effects of aphid genotype, plant genotype, inoculation with NFS or NI, and time (categorical) on aphid weight (fitness). For each factor and interaction, η2 values (effect size estimates), their interpretation (small, medium, large), and statistical significance are reported. Significance levels are indicated as follows: ***p < 0.001; **p < 0.01; *p < 0.05; .p < 0.1. Effect size interpretations follow standard thresholds (η2: small ≥ 0.01, medium ≥ 0.06, large ≥ 0.14).

Factor	**η²**	**Effect Size**	**Significance**
Aphid G.	0.32	Large	< 0.001 ***
Plant G.	0.055	Small	< 0.001 ***
Inoculation	0.13	Medium	< 0.001 ***
Time	0.79	Large	< 0.001 ***
Aphid G. × Plant G.	0.42	Large	< 0.001 ***
Aphid G. × Inoculation	0.017	Small	0.059 .
Plant G. × Inoculation	0.087	Medium	< 0.001 ***
Aphid G. × Time	0.26	Large	< 0.001 ***
Plant G. × Time	0.045	Small	0.005 **
Inoculation × Time	0.028	Small	0.053 .
Aphid G. × Plant G. × Inoc.	0.095	Medium	< 0.001 ***

3.3 Plant and aphid genotypes and inoculation treatment modulate defence gene expression

Expression levels of *PR.1*, *PR.4* and *PR.5* were influenced by both plant and aphid genotype, in a time-dependent manner (Table S4). Compared to control non-infested plants, over the time, aphid infested plants showed significantly higher gene expression levels of SA-mediated marker genes (Fig 3; S2-3).

Figure 3. *PR.1* gene expression in *M. truncatula* shoots was influenced by the duration of aphid infestation and inoculation treatment. Expression levels of the salicylic acid (SA) pathway marker gene *PR.1* in shoots of two *Medicago truncatula* genotypes (A17 and R108) infested with three aphid genotypes (LL01, YR2-amp, T38-V1-amp), under two nitrogen treatments: nitrate-fed (NI; blue) and rhizobia-inoculated (NFS; pink) (n=3; each replicate a pool of 6 plants). Expression values are rescaled relative to non-infested control plants (grey horizontal line fitted at 1), which represent basal expression levels. Box plots show median and interquartile range (IQR); dots coloured represent individual biological replicate for *PR.1* gene expression levels observations at discrete time points post-infestation. Mixed model predictions for the interaction of plant inoculation, aphid and plant genotype on *PR.1* gene expression (Marginal R^2 = 0.57).

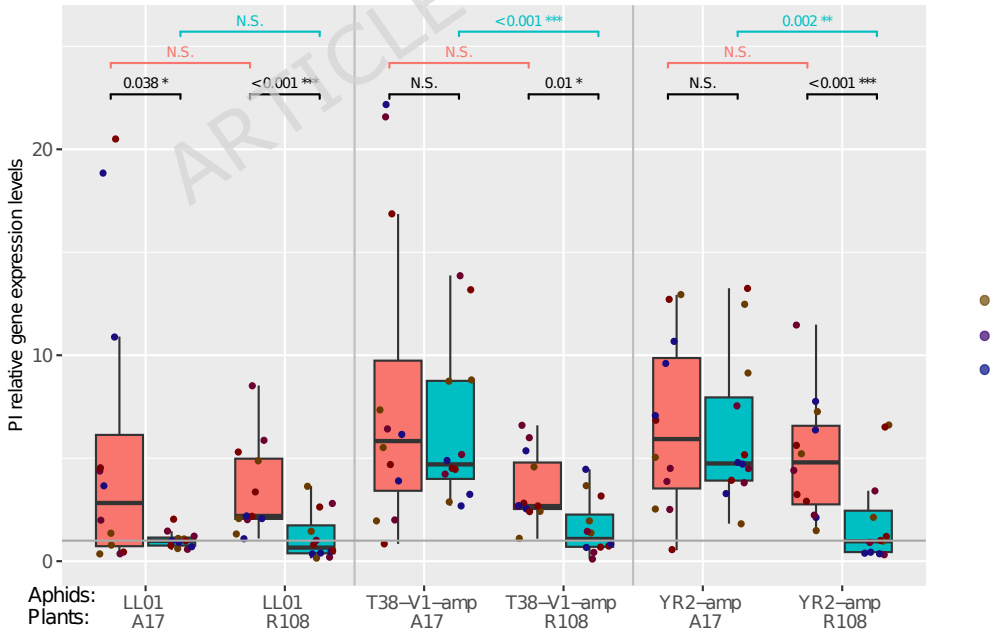


Aphid genotype had large effect on *PR.1* (Fig 3, Table 2) (partial η^2 = 0.19, $P < 0.001$) and *PR.5* expression (partial η^2 = 0.23, $P < 0.001$), while the effect on *PR4* was medium (partial η^2 = 0.065, $P = 0.015$) (Fig S2-3; Table S4). Plant genotype had a medium effect on *PR.4* (partial η^2 =

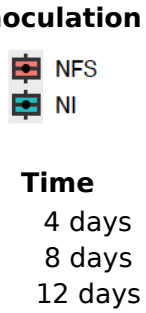
0.11, $P < 0.001$), but was non-significant for *PR.1* ($\eta^2 = 0.0038$, $P = 0.53$) or *PR.5* ($\eta^2 = 7e\text{-}4$, $P = 0.79$) (Table S4). NFS resulted in a medium effect on *PR.5* expression (partial $\eta^2 = 0.12$, $P < 0.001$), whereas its effect on *PR.1* and *PR.4* was non-significant (*PR.1* $\eta^2 = 0.0061$, $P = 0.4$; *PR.4* $\eta^2 = 0.012$, $P = 0.23$) (Table 2, Fig S2-3). The three-way interaction between aphid genotype, plant genotype, and plant inoculation significantly influenced the expression of *PR.1* ($\eta^2 = 0.08$, $P = 0.008$) but not *PR.4* and *PR.5* (Table S4, Fig S2-3).

Expression of Jasmonic acid (JA) pathway marker genes, *AOS.1* (JA biosynthesis) and protease inhibitor (*PI*) (JA responsive gene), was regulated by genotype, inoculation and time factors (Table S4, Fig 4, Fig S4).

Figure 4. The inoculation treatment and duration of aphid infestation influenced *PI* gene expression in *M. truncatula* shoots. Expression levels of the Jasmonic acid (JA) pathway marker gene *PI* in shoots of two *M. truncatula* genotypes (A17 and R108) infested with three aphid genotypes (LL01, YR2-amp, T38-V1-amp), under two nitrogen treatments: nitrate-fed (NI; blue) and rhizobia-inoculated (NFS; pink) (n=3; each replicate was a pool of 6 plants). Expression values are rescaled relative to non-infested control plants (grey horizontal line fitted at 1), which represent basal expression levels. Box plots show median and interquartile range (IQR); dots represent individual biological replicate for *PI* gene expression levels observations at discrete time points post-



infestation. Mixed model predictions for the interaction of plant inoculation, aphid and plant genotype on *PI* gene expression (Marginal $R^2 = 0.5$).



For *PI*, aphid genotype had a medium effect ($\eta^2 = 0.094$, $p = 0.002$), plant inoculation ($\eta^2 = 0.016$, $p = 0.15$) and plant genotype alone did not significantly influence *PI* expression ($\eta^2 = 0.0038$, $p = 0.5$) (Table 2, Fig 4). However, interactions between plant genotype and inoculation ($\eta^2 = 0.096$, $p < 0.001$), and between aphid genotype and plant genotype ($\eta^2 = 0.083$, $p = 0.005$), had medium effect (Table 2). Time also significantly affected *PI* expression ($\eta^2 = 0.064$, $p = 0.004$), and its interaction with plant inoculation was particularly strong ($\eta^2 = 0.11$, $p < 0.001$) (Table 2). For *AOS1*, only inoculation produced a medium effect ($\eta^2 = 0.085$, $p = 0.001$) (Table S4, Fig S4) and no significant effect of time was detected ($\eta^2 = 0.014$, $p = 0.65$). In summary, defence gene expression in both SA- and JA-pathways was differentially regulated by aphid genotype, plant genotype, and rhizobia inoculation, with several significant two-and three-way interactions detected.

Table 2. Linear mixed-effects models assessing the effects of aphid genotype, plant genotype, inoculation with NFS or NI, and time (categorical) on salicylic acid marker genes (*PR.1)* and Jasmonic acid marker genes (*PI*). For each factor and interaction, η^2 values (effect size estimates), their interpretation (small, medium, large), and statistical significance are reported. Effect size interpretations follow standard thresholds (η^2: small ≥ 0.01, medium ≥ 0.06, large ≥ 0.14). Significance levels

are indicated as follows: ***p < 0.001; **p < 0.01; *p < 0.05; p < 0.1 ·p < 0.1 (marginal significance) ; G. = genotype.

	Salicylic acid marker genes	***Jasmonic acid marker genes***
	PR.1	***PI***
Factor	**η²; Effect Size; Significance**	**η²; Effect Size; Significance**
Aphid G.	0.19; Large; < 0.001 ***	0.094; Medium; 0.002 **
Plant G.	0.0038; Negligible; 0.53	0.0038; Negligible; 0.5
Inoculation	0.0061; Small; 0.4	0.016; Small; 0.15
Time	0.11; Medium; 0.004 **	0.064; Medium; 0.004 **
Aphid G. × Plant G.	0.19; Large; < 0.001 ***	0.083; Medium; 0.005 **
Aphid G. × Inoculation	0.059; Medium; 0.031 *	0.033; Small; 0.13
Plant G. × Inoculation	0.052; Small; 0.014 *	0.096; Medium; < 0.001 ***
Aphid G. × Time	0.079; Medium; 0.14	0.013; Small; 0.45
Plant G. × Time	0.16; Large; < 0.001 ***	0.048; Small; 0.014 *
Inoculation × Time	0.092; Medium; 0.011 *	0.11; Medium; < 0.001 ***
Aphid G. × Plant G. × Inoculation	0.08; Medium; 0.008 **	0.018; Small; 0.33

4. DISCUSSION

Genetic interactions between aphids and legumes are classically viewed as determined by the genotypes of both organisms, producing either compatible or incompatible outcomes [5,48,49]. Aphid virulence appears to be a complex trait involving multiple genes that influence factors such as saliva composition, which in turn modulates plant defence responses and host compatibility [50,51]. Legume plants also form symbiotic relationships with nitrogen-fixing rhizobia, which can transiently activate early plant defence reactions that influence susceptibility to pathogens [52,53]. However, few studies have examined how these plant-rhizobia symbiosis intersect with the genotype of both plant and aphid to shape defence and aphid fitness outcomes. To investigate how this interaction (plant genotype, aphid genotype, and rhizobia symbiosis) shapes outcomes, we studied two *M. truncatula* cultivars, A17, carrying the aphid resistance QTL RAP1 [19], and R108, which lacks RAP1, and appears more susceptible to the three pea aphid lines representing different biotypes (LL01, an "alfalfa" biotype; YR2 and T38, "clover" biotypes) [5]. Our analysis focused on three key aspects: plant growth, aphid fitness, and plant defence responses during co-development of aphids and rhizobia nodules [26]. This comprehensive approach helped us to unravel how plant-rhizobia symbiosis modulates both direct plant resistance mechanisms and indirect effects on aphid success, considering the genotypic interplay across all three partners.

4.1 Genotypes and symbiotic status modify plant biomass after aphid infestation

Over the 12 days experimental time frame, the nitrate fed (NI) plants accumulated a significantly higher shoot dry biomass compared to those in rhizobia symbiosis (NFS) (Fig 1, Fig S6-9). This was expected since nitrate provides immediate and readily available nitrogen, whereas

symbiotic nitrogen fixation requires significant carbon and energy investment to establish nodules to reach the maximum availability of nitrogen compounds [26,49,54]. A strong effect of the plant genotype x inoculation was also observed, maybe due to the fact that A17 and R108 have different symbiotic effectiveness with different *Sinorhizobium* (Ensifer) inoculum [55,56]. At 21 days post inoculation, A17 has been reported to form more effective nodules with *S. meliloti* 2011 than R108 [57]. Similarly, our results show that NFS R108 plants had lower shoot biomass compared to NFS A17, which could be due to reduced nitrogen availability due to fewer or less efficient nodules. Moreover, previous studies including ours [26,58], have shown that aboveground aphid attacks can affect legume–rhizobia symbiosis by reducing nodule formation (e.g., reduction in nodule size, number, and weight, as well as dry biomass) and by downregulating the expression of genes involved in various steps of nodule development, ultimately leading to reduced nitrogen fixation [26,58]. As expected, infestation by aphid reduced shoot plant dry weight in both NI and NFS treated (Fig 1, Fig S6-9) since aphid feeding acts as a carbon sink hijacking resources from the plant [59]. Similar biomass loss independent of rhizobia status has been reported with *Aphis glycines* on soybean (Glycine max L.) [60] and is consistent with our previous work [26]. Another study, which used eight lines of *A. pisum* including LL01, showed that aphid lines differed in their ability to induce chlorosis and necrosis across A17 and R108 *M. truncatula* genotypes [5]. Chlorosis in R108 was common across multiple aphid lines, while necrosis was observed in A17 but not in R108 [5, 16]. This chlorosis associated to hypersensitive response was not quantified in our study, but chlorosis may affect photosynthesis and consequently plant growth, thus reducing the plant biomass of R108 in addition to the direct effect of aphid feeding. Here, we showed that the extent of biomass loss depended on specific plant-aphid genotype combinations, supporting that the genotype of both plant and aphid critically influences aphid-driven plant growth inhibition.

4.2 Context dependent modulation of aphid fitness by plant genotype and rhizobia

All aphid lines survived equally on both *M. truncatula* A17 and R108 genotypes but showed significant differences in aphid weight (a proxy for fitness) (Fig 2) driven by the specific plant–aphid genotype and rhizobia pairing. LL01 displayed consistently higher weight on A17 across all conditions (NFS and NI) (Fig 2), consistent with prior reports that LL01 expresses broad virulence and is specifically virulent to overcome A17 defences [5]. Conversely, when LL01 fed on R108, its fitness significantly dropped, indicating that R108 presents a stronger or more specific defence to LL01, restricting its compatibility range. T38 aphid genotype performed better on R108 than on A17, suggesting it might possess virulence traits or physiological adaptations that target the defence response present in R108, but are less effective on A17. The third genotype, YR2, had comparatively low fitness across both plant hosts, implying that it is less virulent or has a narrower host adaptation and thus, encounters robust resistance regardless of host. These results indicate how diversity of plant and aphid genotypes and gene expression allows differential exploitation of host resources, a key strategy for persistence in different environments [16,61]. Plant rhizobia inoculation further modified aphid fitness, with NFS often reducing aphid weight (LL01 on A17; T38 on R108; YR2 on A17; YR2 on R108) (Fig 2), but in some combinations (LL01 on A17; T38 on R108) inoculation improved aphid performance. These results suggest that plant symbiotic status (NFS) can sometime counterbalance the nutritional advantage of NI and that the aphid growth rate is not linked directly to the plant growth rate. These results also underscore that rhizobia inoculation does not uniformly increase host nutritional value for aphids, but instead it reshapes host quality in a genotype-dependent manner. This agrees with studies showing that rhizobia alter phloem sap amino acid composition, sugar ratios, and secondary metabolite levels, which in turn affect herbivore performance [6,63]. For instance, enhanced nitrogen assimilation via rhizobia may

increase essential amino acids in phloem sap, benefiting some aphid genotypes, while simultaneous induction of phenolics or terpenoids may reduce fitness in others. Recent work suggests that the net effect of rhizobia inoculation is conditional, producing either induced resistance or induced susceptibility depending on the host–aphid genetic pairing [13,64].

4.3 Genotype-specific and inoculation-driven modulation of plant defence responses

The plant defence signalling in response to aphid infestation was strongly influenced by plant genotype × aphid genotype (G×G) interactions, while rhizobia inoculation introduces an important layer of modulation. This dual influence shows how defence outcomes are not simply add-on, but emerge from a dynamic interplay between host genotypes and microbial partners. Previous studies have shown that aphid populations differ in their ability to induce or suppress plant defence signalling pathways based on G×G interactions with the host plant [65,66]. This aligns with our observation that aphid lines such as T38 and YR2 caused a significant downregulation of *PR.1* a marker of salicylic acid (SA)-mediated defence in *Medicago* genotype A17 (NI), but not in R108 (NI) (Fig 3). This contrast between *Medicago* genotypes suggests that they respond differently to the manipulation of defence signalling by aphid virulence factors. Such genotype × genotype (G×G) dynamics represent a molecular arms race, where host resistance traits and aphid effectors co-evolve to yield highly specific response patterns [67,68]. Importantly, these G×G outcomes were not limited to SA pathways. We also observed variable regulation of the jasmonic acid-associated *PI* gene (Fig 4), which was upregulated in R108 under NFS conditions with all aphid genotype, but only triggered by the LL01 aphid genotype in A17 (NFS). This differential *PI* regulation suggests that nodulation capacity and host–rhizobia compatibility influence the direction of defence modulation. This supports the idea that microbial partners can reprogram the defence status of plants, sometimes enhancing resistance, other times creating vulnerabilities exploitable by aphids

[67,68,69]. NFS plants showed genotype-specific shifts in the expression of both SA- and JA-related genes in agreement with the he hypothesis that symbiosis with nitrogen-fixing bacteria alters the plant immune status, either through direct modulation of hormone balances or via systemic resource reallocation [6,62,70,71]. Recently, Mbaluto & Zytynska 2025 [70] showed that Rhizobacteria inoculation suppresses aphids on barley by priming plant defence and nutritional pathways. They proposed that plant resistance was enhanced through phenylpropanoid, glutathione, and phytohormone pathways, while also promoting tolerance via improved nutrition and growth, highlighting a dynamic, multi-pathway mechanism of microbial-induced plant protection [70]. Our study provides novel insights into rhizobia and genotype-dependent plant-aphid interactions, but expanding the range of plant, aphid and rhizobia genotypes would clarify how general these patterns are. These results should be interpreted keeping in mind that additional plant and aphid genotypes will be needed to assess the generality of these interactions.

Aphid feeding typically induces SA-associated signalling, but aphids can also modulate JA/ET pathways, making defence outputs highly dependent on pathway crosstalk [11, 15, 73]. Rhizobia inoculation can further reshape these responses via systemic priming, potentially enhancing JA/ET responsiveness and thereby antagonizing SA-dependent defences, consistent with the frequently negative SA–JA interaction. Depending on the aphid and host genotype, this shift could either constrain aphid performance, if JA/ET-mediated traits are effective, or promote susceptibility, if SA-mediated defences are more important. In our study, defence marker genes displayed distinct, time-dependent expression patterns that varied with rhizobia inoculation timing, aphid infestation timing, and both plant and aphid genotype, supporting the idea that rhizobia can reconfigure the balance among SA, JA, and ET signalling during aphid attack. However, interpretation based solely on marker-gene expression remains limited, because marker genes may not reliably reflect hormone balance, pathway flux, or downstream metabolite outputs, and

pathway markers can be co-regulated by multiple signals. Therefore, while these transcriptional signatures are consistent with altered hormonal crosstalk, future work should directly quantify hormones and defence metabolites and link these measures to aphid performance and feeding behaviour to resolve causality in this multipartite interaction. Mechanistically, rhizobia colonization is known to induce cross-talk between nodulation pathways and phytohormone signalling, in particular SA, JA, and Ethylene pathways. Such cross-talk can dampen SA-dependent resistance to pathogens or herbivores while enhancing JA-dependent responses in some contexts [71,72]. In our study, the observed genotype-dependent defence shifts suggest that the inoculation effect is not uniform but instead depends on the plant host basal capacity to integrate both nodulation and defence signalling. Thus, rhizobia represent a conditional "third player" in the plant–aphid arms race, capable of swinging the defensive balance in favour of or against the plant, depending on the genetic pairing of both plant and aphid. Overall, our results reflect a broader principle of multitrophic interactions, where symbiotic microbes such as rhizobia can recalibrate plant immune response, altering the outcomes of ongoing interactions with aphids.

5. CONCLUSION

Our findings highlight that plant–aphid interactions are shaped not only by the genetic identity of the interacting species but also by the plant symbiotic context. Genotype-specific responses in both defence signalling and aphid performance underscore the ecological importance of variation within and between species. Importantly, rhizobia symbiosis emerged as a key modulator of plant defence and growth, reinforcing the idea that microbial mutualists must be integrated into ecological and evolutionary models of plant resistance. By showing that microbial associations can either enhance or constrain herbivore resistance depending on host genetic background, our findings open new avenues for exploiting beneficial symbioses to strengthen crop resilience. Targeted use of plant

genotype–microbe combinations could provide a sustainable strategy to improve plant defence against insect pests while maintaining ecosystem functions.

DECLARATIONS

Abbreviations: Not applicable

Ethics approval and consent to participate: Not applicable

Clinical trial number: Not applicable

Consent for publication: Not applicable

Availability of data and materials: The complete raw dataset and corresponding analysis scripts can be accessed at following link: https://entrepot.recherche.data.gouv.fr/dataset.xhtml?persistentId=doi:10.57745/WGBWYO

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Supplementary Figures

Figure S1: Comparison of the dry weight of the NFS and NI plants of both A17 and R108 *Medicago truncatula* genotype before aphid infestation. Box plots show shoot dry weight of *Medicago truncatula* (genotypes A17 and R108) obtained at 14 days after seeding and 7 days after Kno3 or Rhizobia treatments; either watering with a mineral nitrate solution (NI) or inoculation with *Sinorhizobium meliloti* (NFS). Boxes represent medians and interquartile ranges; Pink boxplots represent the NFS condition, while blue boxplots represent the NI condition. (n=3; each replicate a pool of 6 plants); N.S. = not significative.

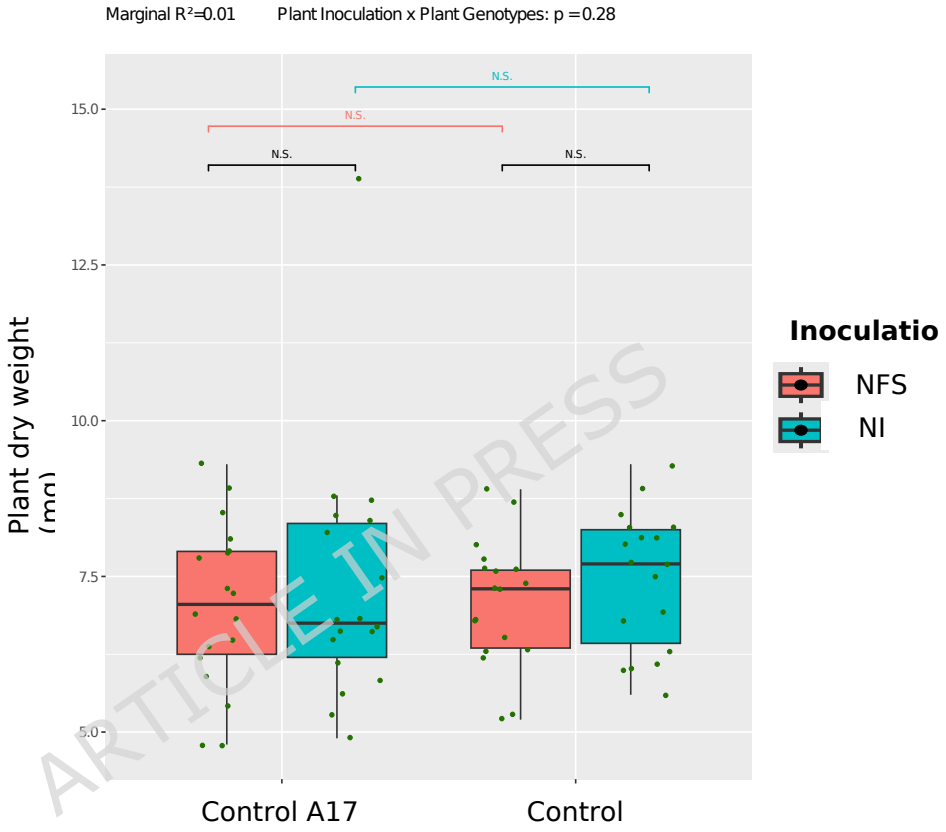


Figure S2. *PR.4* gene expression in *Medicago truncatula* shoots following aphid infestation and inoculation treatment Expression levels of the salicylic acid (SA) pathway marker gene *PR.4* in shoots of two *Medicago truncatula* genotypes (A17 and R108) infested with three aphid genotypes (LL01, YR2-amp, T38-V1-amp), under two nitrogen treatments: nitrate-fed (NI; blue) and rhizobia-inoculated (NFS; pink) (n=3 (each replicate a pool of 6 plants)). Expression values are rescaled relative to non-infested control plants (grey horizontal line), which represent basal expression levels. Box plots show median and interquartile range (IQR); dots represent individual biological replicate for *PR.4* gene expression levels measurements at discrete time points post-infestation (see time colours on the legend). Mixed model predictions for the interaction of plant inoculation, aphid and plant genotype on *PR.4* gene expression (Marginal R^2 = 0.32).

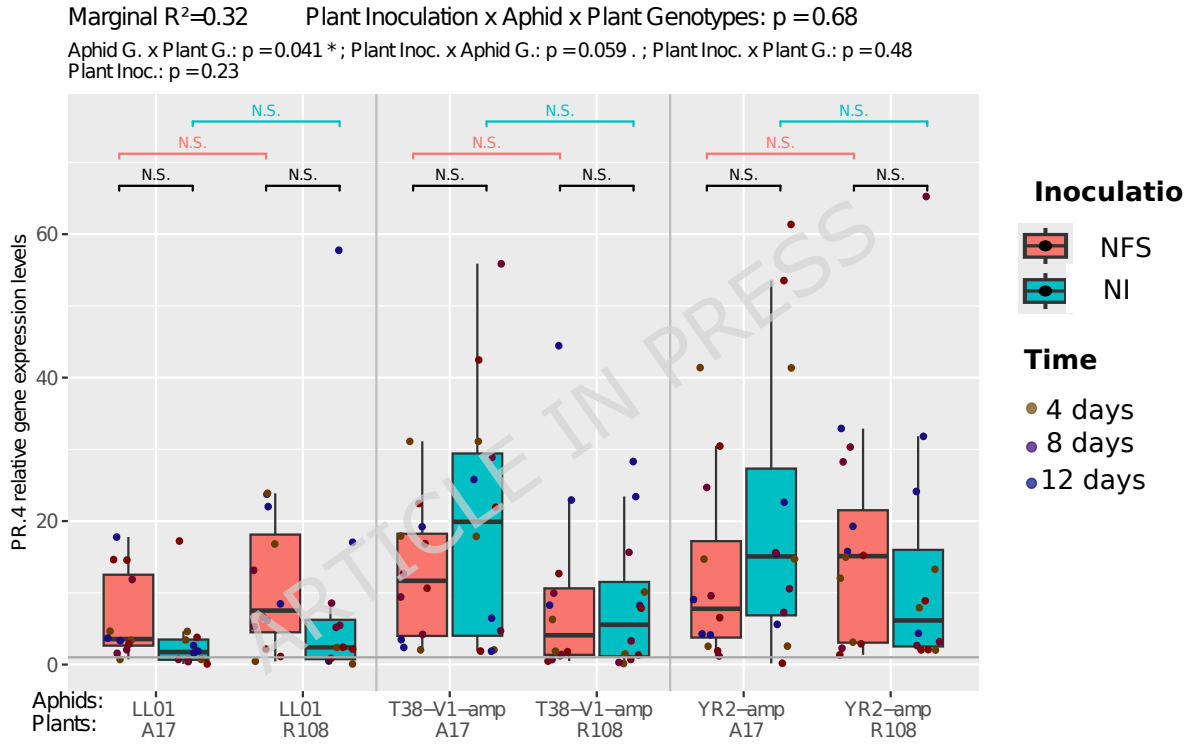


Figure S3. *PR.5* gene expression in *Medicago truncatula* shoots following aphid infestation and inoculation treatment Expression levels of the salicylic acid (SA) pathway marker gene *PR.5* in shoots of two *Medicago truncatula* genotypes (A17 and R108) infested with three aphid genotypes (LL01, YR2-amp, T38-V1-amp), under two nitrogen treatment: nitrate-fed (NI; blue) and rhizobia-inoculated (NFS; pink) (n=3; each replicate a pool of 6 plants). Expression values are rescaled relative to non-infested control plants (grey horizontal line), which represent basal expression levels. Box plots show median and interquartile range (IQR); dots coloured represent individual biological replicate for *PR.5* gene expression levels observations at discrete time points post-infestation (see time colours on the legend). Mixed model predictions for the interaction of plant inoculation, aphid and plant genotype on *PR.5* gene expression (Marginal R^2 = 0.51)

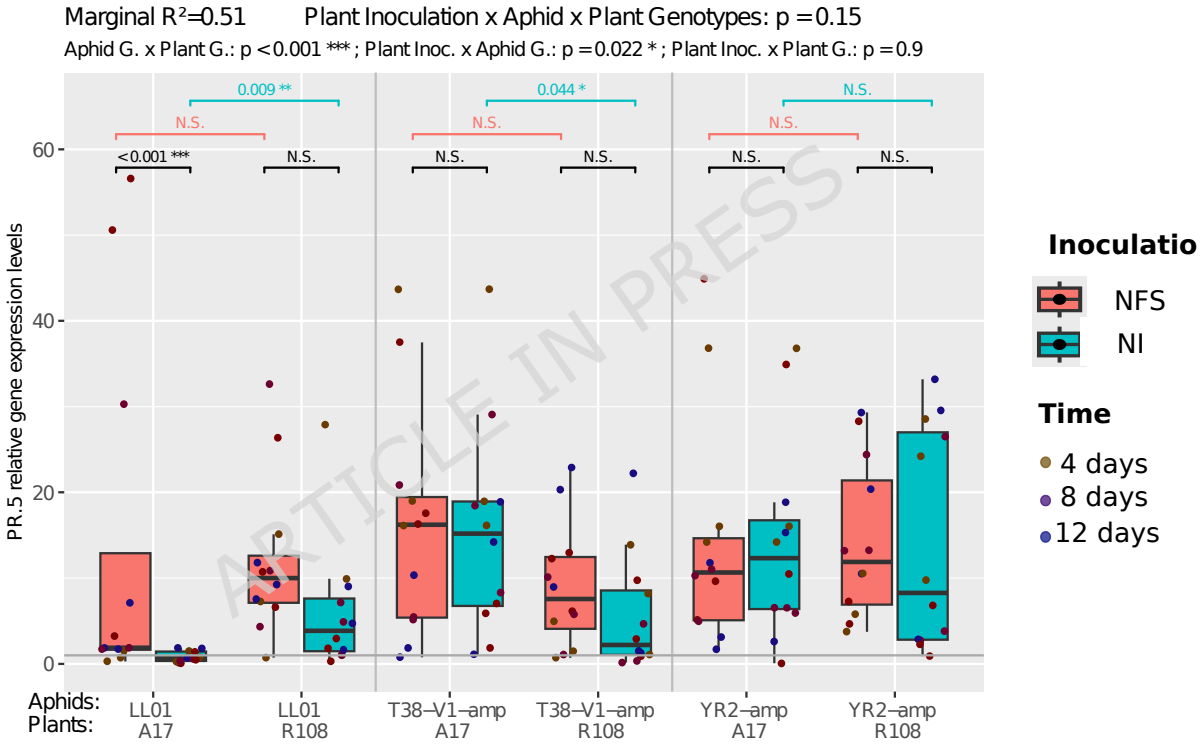


Figure S4. *AOS.1* gene expression in *Medicago truncatula* shoots following aphid infestation and inoculation treatment Expression levels of the salicylic acid (SA) pathway marker gene *AOS.1* in shoots of two *Medicago truncatula* genotypes (A17 and R108) infested with three aphid genotypes (LL01, YR2-amp, T38-V1-amp), under two nitrogen treatment: nitrate-fed (NI; blue) and rhizobia-inoculated (NFS; pink) (n=3; each replicate a pool of 6 plants). Expression values are rescaled relative to non-infested control plants (grey horizontal line), which represent basal expression levels. Box plots show median and interquartile range (IQR); dots represent individual biological replicate for *AOS.1* gene expression levels observations at discrete time points post-infestation (see time colours on the legend). Mixed model predictions for the interaction of plant ir inal R^2 = 0.3)

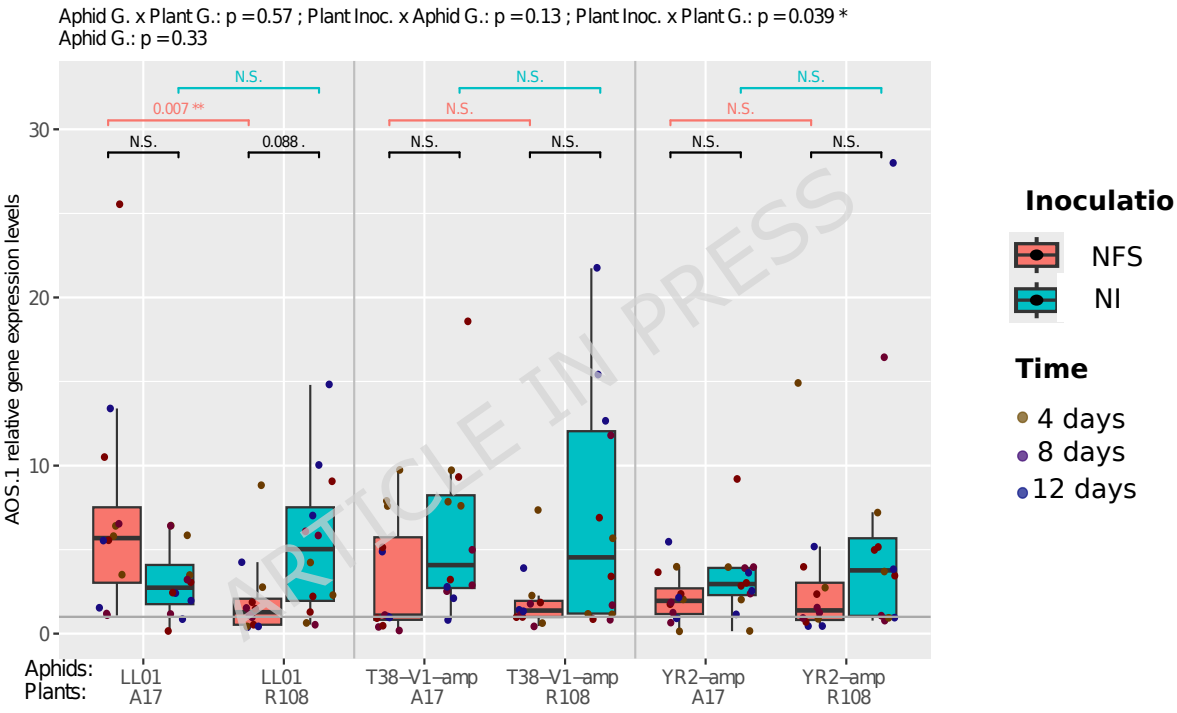
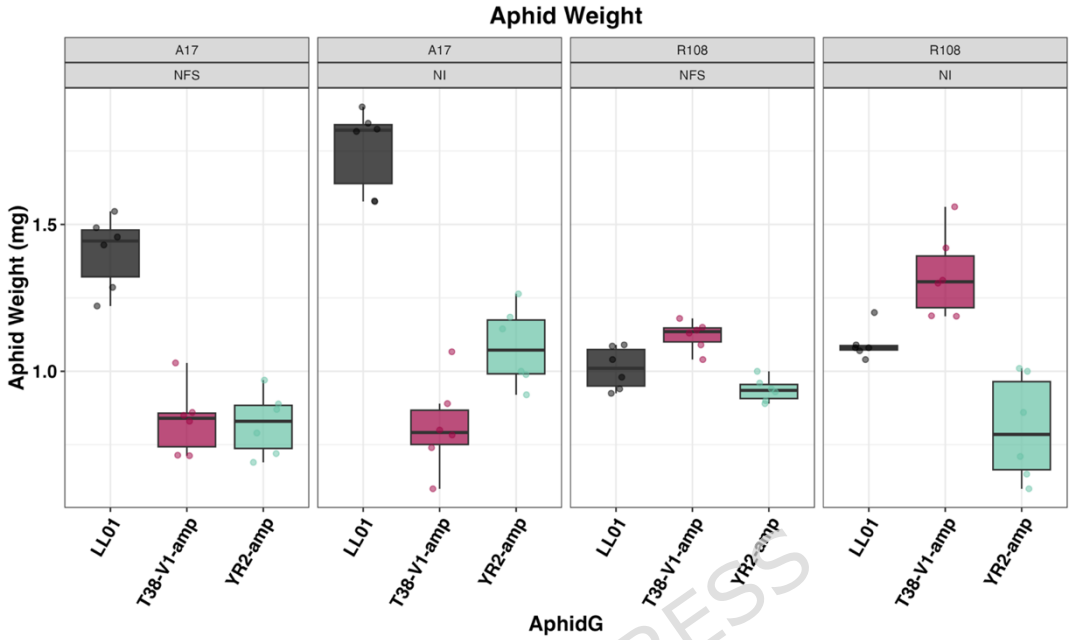
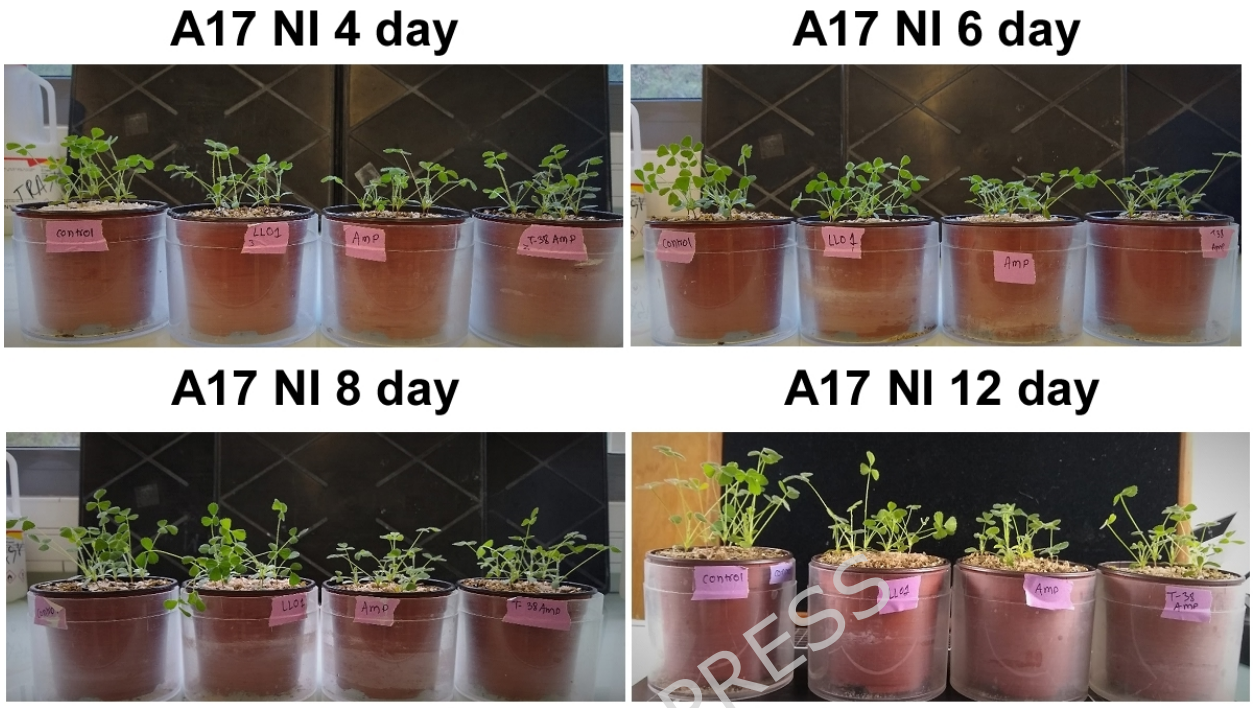


Figure S5. Adult aphid genotypes weight at 12 days post infestation on different *M. truncatula* genotypes under the two different nitrogen treatments. Box plots show the distribution of the mean weight in mg of living pea aphids at 12 days post infestation (*Acyrthosiphon pisum* clones LL01, YR2-amp, and T38-V1-amp) on two *Medicago truncatula* genotypes (A17 and R108) under two nitrogen treatment: mineral nitrate supplementation (NI) and rhizobia inoculation (*Sinorhizobium meliloti*;



NFS). (n =6) Jittered points represent individual biological repeat for mean weight of the living aphids reached at 12 days.

Figure S6: Photos of aphid-infested *M. truncatula* A17 NI plants and control plants at different time points after infestation **(A)** 4 days **(B)** 6 days **(C)** 8 days **(D)** 12 days.



pots left to right: control; LL01; YR2;T38.

Figure S7: Photos of aphid-infested *M. truncatula* A17 NFS and control plants at different time points after infestation **(A)** 4 days **(B)** 6 days **(C)** 8 days **(D)** 12 days. Pots left to right: control; LL01; YR2;T38.

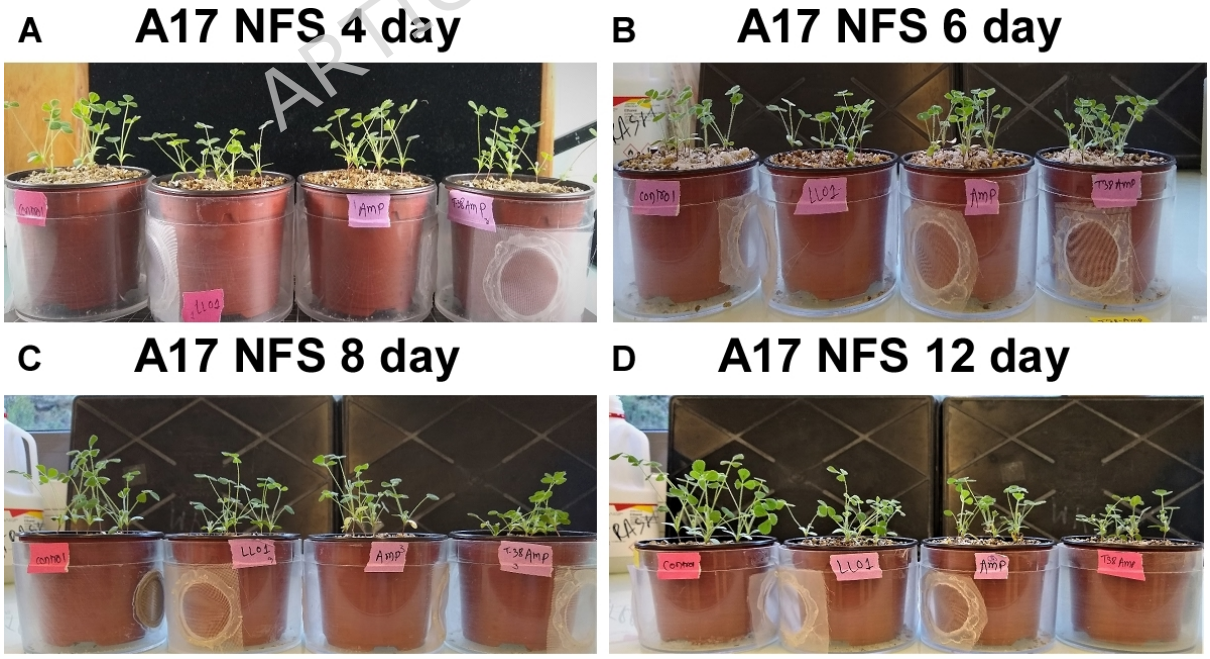


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Figure S8: Photos of *M. truncatula* aphid-infested and control R108 NI plants at different time points after infestation. **(A)** 4 days **(B)** 6 days **(C)** 8 days **(D)** 12 days. Pots left to right: control; LL01; YR2; T38.

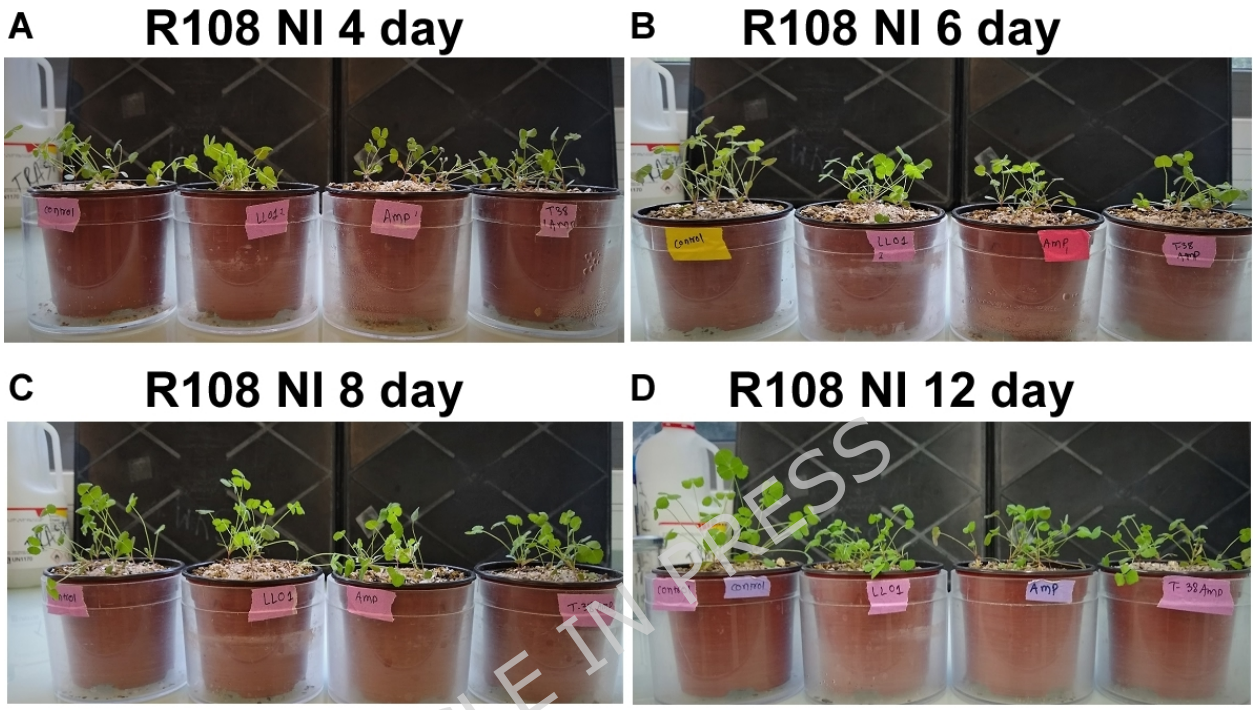


Figure S9: Photos of *M. truncatula* aphid-infested and control R108 NFS plants at different time points after infestation. **(A)** 4 days **(B)** 6 days **(C)** 8 days **(D)** 12 days. Pots left to right: control; LL01; YR2; T38.

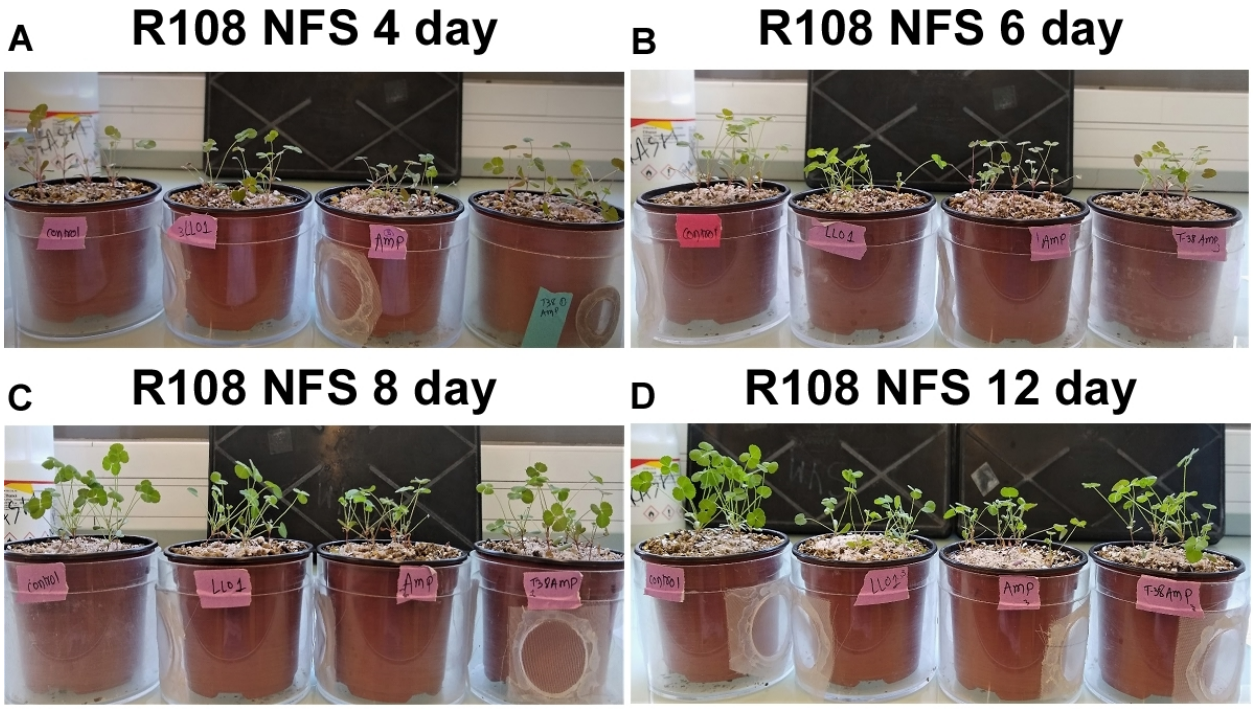
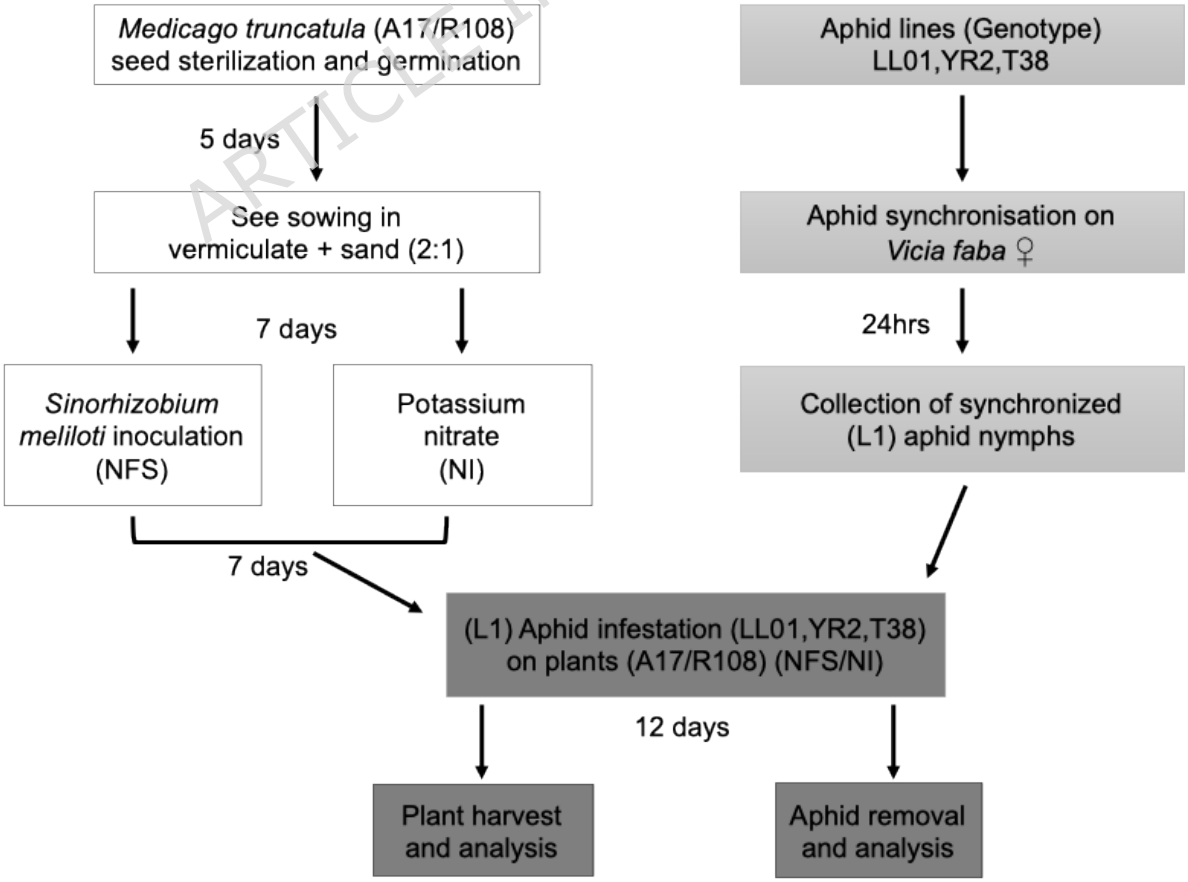


Figure S10: Experimental design, plants genotype and aphids genotype and inoculation time



Supplementary Tables

Table S1: Origin of the aphid lines

Line	Color	Plant of collection	Location	Collection date	Secondary symbiont	References
LL01	Green	Alfalfa	Lusignan, France	January 1987	none	Simon et al., 2011
YR2-Amp*	Pink	Clover	York, UK	December 2002	none	Simon et al., 2011
T3-8V1-Amp*	Green	Clover	Domagné, France	June 2003	none	Simon et al., 2011

* Aphid lines were treated with ampicillin in 2010 to remove the secondary symbionts. All lines were secondary symbionts free.

Table S2: Primer sequences and conditions for qPCR analysis. Real-time qPCR was performed as follows (AriaMx Real-time PCR machine, Agilent): 95°C for 3 min followed by 40 cycles at 95°C for 5 sec and 60°C for 30 sec. The primers efficiency was evaluated on a slope of a standard curve generated using a serial dilution of the samples. Cycle threshold values (Ct) were normalized to the average Ct of two housekeeping genes *Medtr2g436620* also named *MtC27* (the homolog *of M. sativa* translationally controlled tumour protein *Msc27*), and *Medtr4g109650*.1 also named *a38* coding for a hypothetical protein. The expression of these two genes was not affected by the treatments in our experiments. The original Ct values were obtained from the machine software (Ariamix software; Agilent).

Description	Name	Forward primer	Reverse primer	Genomic ID	References
Housekeeping gene	*Mtc27*	TGAGGGAGCAACCAAATACC	GCGAAAACCAAGCTACCATC	*Medtr2g436620*	Del Guidice et al., 2011
Housekeeping gene	*a38*	TCGTGGTGGTGGTTATCAAA	TTCAGACCTTCCCATTGACA	*Medtr4g109650*	Del Guidice et al., 2011
Pathogenesis related protein-1	*PR.1*	TTCGGGTTGGATGTGCTAAG	GGTTGAAGCTCAATGGCACT	*Medtr2g435490*	Pandharikar et al., 2020
Pathogenesis related protein-4	*PR.4*	TACTGTGCTACCTGGGAT	TGAGCTCCAGTTGCAGTATTTG	*Medtr1g080800*	This work
Thaumatin-like protein	*PR.5*	TGCCTTAGCTTTGCATTCCT	AATTTCCGCTGAGTTCGTTG	*Medtr1g062630*	This work
Proteinase Inhibitors	*PI*	TGTGGTGCAATTCTTTCAGG	ATTTTGGGGTGAGGTGTTGA	*Medtr4g032865*	Pandharikar et al., 2020
Allene Oxide Synthase	*AOS1*	GGACACCGAACTTGGACTTGAC	AAACATCCAAACGCTCTGCTTC	*Medtr1g021652*	This work

Table S3. Linear mixed-effects models assessing the effects of aphid genotype, plant genotype, inoculation with NFS or NI, and time (categorical) on aphid weight (fitness). For each factor and interaction, η^2 values (effect size estimates), their interpretation (small, medium, large), and statistical significance are reported. Effect size interpretations follow standard thresholds (η^2: small ≥ 0.01, medium ≥ 0.06, large ≥ 0.14). Significance levels are indicated as follows: ***p < 0.001; **p < 0.01; *p < 0.05; p < 0.1 (marginal significance). N.S = non-significant.

Factor	η^2	Effect Size	Significance
Aphid G.	0.5	Large	< 0.001 ***
Plant G.	0.18	Large	< 0.001 ***
Inoculation	0.45	Large	< 0.001 ***
Time	0.5	Large	< 0.001 ***
Aphid G. × Plant G.	0.02	Small	< 0.001 ***
Aphid G. × Inoculation	0.068	Medium	< 0.001 ***
Plant G. × Inoculation	0.026	Small	< 0.001 ***
Aphid G. × Time	0.18	Large	< 0.001 ***
Plant G. × Time	0.059	Medium	< 0.001 ***
Inoculation × Time	0.13	Medium	< 0.001 ***
Aphid G. × Plant G. × Inoculation	0.0041	Not specified	0.33 (N.S)

Table S4: Summary of the models testing effects of the aphid and plant genotypes and of inoculation by *S. meliloti* on the plant - aphids interaction. Each column corresponds to one explained variable describing the plant -aphids interaction, and rows give the explanatory variables and their interactions, except the last row that indicate if the time was encoded as a categorical or a continuous variable. Significance levels are indicated as follows: ***p < 0.001; **p < 0.01; *p < 0.05; p < 0.1 ·p < 0.1 (marginal significance). Effect size interpretations follow standard thresholds (η^2: small ≥ 0.01, medium ≥ 0.06, large ≥ 0.14, values < 0.01 are reported numerically to indicate negligible effects).

	Plant dry weight	**Aphids weight**	***Salicylic acid marker genes***			**Jasmonic acid marker genes**	
			PR.1	***PR.4***	***PR.5***	***PI***	***AOS.1***
Marginal R^2	**0.8**	**0.9**	**0.57**	**0.32**	**0.51**	**0.5**	**0.3**
Aphid G.	Large: 0.5; (< 0.001 ***)	Large: 0.32; (< 0.001 ***)	Large: 0.19; (< 0.001 ***)	Medium: 0.065; (0.015 *)	Large: 0.23; (< 0.001 ***)	Medium: 0.094; (0.002 **)	Small: 0.019; (0.33)
Plant G.	Large: 0.18; (< 0.001 ***)	Small: 0.055; (< 0.001 ***)	: 0.0038; (0.53)	Medium: 0.11; (< 0.001 ***)	: 7e-04; (0.79)	: 0.0038; (0.5)	: 0.0074; (0.35)
Inoculation	Large: 0.45; (< 0.001 ***)	Medium: 0.13; (< 0.001 ***)	: 0.0061; (0.4)	Small: 0.012; (0.23)	Medium: 0.12; (< 0.001 ***)	Small: 0.016; (0.15)	Medium: 0.085; (0.001 **)
Time	Large: 0.5; (< 0.001 ***)	Large: 0.79; (< 0.001 ***)	Medium: 0.11; (0.004 **)	Small: 0.052; (0.01 *)	Large: 0.14; (< 0.001 ***)	Medium: 0.064; (0.004 **)	Small: 0.014; (0.65)
Aphid G. × Plant G.	Small: 0.02; (< 0.001 ***)	Large: 0.42; (< 0.001 ***)	Large: 0.19; (< 0.001 ***)	Small: 0.05; (0.041 *)	Large: 0.18; (< 0.001 ***)	Medium: 0.083; (0.005 **)	: 0.0096; (0.57)
Aphid G. × Inoculation	Medium: 0.068; (< 0.001 ***)	Small: 0.017; (0.059 .)	Medium: 0.059; (0.031 *)	Small: 0.044; (0.059 .)	Medium: 0.064; (0.022 *)	Small: 0.033; (0.13)	Small: 0.035; (0.13)
Plant G. × Inoculation	Small: 0.026; (< 0.001 ***)	Medium: 0.087; (< 0.001 ***)	Small: 0.052; (0.014 *)	: 0.0039; (0.48)	: 0.00013; (0.9)	Medium: 0.096; (< 0.001 ***)	Small: 0.036; (0.039 *)
Aphid G. × Time	Large: 0.18; (< 0.001 ***)	Large: 0.26; (< 0.001 ***)	Medium: 0.079; (0.14)	: 0.0074; (0.63)	Medium: 0.069; (0.21)	Small: 0.013; (0.45)	Small: 0.043; (0.51)
Plant G. × Time	Medium: 0.059; (< 0.001 ***)	Small: 0.045; (0.005 **)	Large: 0.16; (< 0.001 ***)	Medium: 0.1; (< 0.001 ***)	Small: 0.053; (0.098 .)	Small: 0.048; (0.014 *)	Small: 0.037; (0.22)

Inoculation Time	Medium: 0.13; (< 0.001 ***)	Small: 0.028; (0.053 .)	Medium: 0.092; (0.011 *)	: 0.0035; (0.51)	Large: 0.18; (< 0.001 ***)	Medium: 0.11; (< 0.001 ***)	Small: 0.048; (0.13)
Aphid G. Plant G. Inoculation	: 0.0041; (0.33)	Medium: 0.095; (< 0.001 ***)	Medium: 0.08; (0.008 **)	: 0.0061; (0.68)	Small: 0.033; (0.15)	Small: 0.018; (0.33)	Small: 0.044; (0.074 .)
Encoding of the time variable	Cat.	Cat.	Cat.	Cont.	Cat.	Cont.	Cat.