Ménage-à-quatre

Combined effects of rhizobia, pea enation mosaic virus and its pea aphid vectors on broad bean biomass and hormones

M.Sc. Minor Thesis

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WAGENINGEN
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Cover photos, from left to right: *Rhizobia leguminosarum* [Credit: Dr Jeremy Burgess], Pea Enation Mosaic Virus [Credit: Melodie Putnam, Oregon State University], Pea aphid (*Acyrthosiphon pisum*) [Credit: Uni. de Barcelona]

Ménage-à-quatre

Combined effects of rhizobia, pea enation mosaic virus and its pea aphid vectors on leguminous biomass and hormones

M.Sc. Minor Thesis in Nematology NEM-80424

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Index

Abstract

Plant mutualists can play a role in the production of defences against plant antagonists. Similarly, plant antagonists can help each other by interfering with plant defences and growth, in a way that increases the fitness of antagonists. While most of the studies in different systems focus on this dual interaction of plants with their mutualists and antagonists, to date few studies have addressed the outcomes of these combined multitrophic interactions common in nature. To address this gap, we investigated the effect on biomass and hormones of fava beans while interacting with the mutualistic rhizobacteria, and the antagonistic pea enation mosaic virus (PEMV) and the pea aphid vectors. Overall, our results indicate that rhizobia led to improved plant biomass in absence of aphid herbivory and virus infection. However, this improved plant fitness was cancelled out when rhizobia-colonised plants were attacked by both antagonists. Interestingly, PEMV had an antagonistic effect on rhizobia by inducing reduction of nodule biomass, and this effect was pronounced when the plants were also attacked by aphids. This antagonistic effect on rhizobia nodulation can be explained by the high levels of salicylic acid of plants upon virus infection and virus infection combined with aphid herbivory. Additionally, PEMV infection increased jasmonic acid in the plants; however, upon the aphid attack, this hormone was reduced to levels similar to control and rhizobia-inoculated plants. This result indicates that jasmonic acid alone does not explain previous findings of the negative effects of rhizobia on aphid population and that future research is needed on the general plant metabolomic profile to elucidate the mechanisms driving this multitrophic interaction.

1. Introduction

As main primary producers on land, plants are at the centre of a dense trophic network. While some organisms establish mutualistic symbiosis with plants, others prey and parasite them [1–5]. Thus, they determine the plant's health[1–5]. These organisms vary from herbivore arthropods to bacteria and viruses, settled both above and below ground[1–5]. The organisms interacting with the plant can affect each other directly or indirectly (i.e., root symbionts affecting herbivores)[1–5]. The indirect interaction can be mediated by alteration of host plant traits, among other interactors. In addition, interactions above ground can affect those below ground and vice versa as they alter different traits of the plant [2–5].The trophic network is so complex, that most of the studies investigated only one or two organisms interacting with the plant and each other simultaneously [2,5]. Nevertheless, in an open field, several interactions co-occur. The plant responses in such a complex context cannot be directly inferred from the previous studies considering fewer interactions. As complexity increases, new plant signalling pathways are triggered. Although most signalling pathways involved in fewer interactions remain so as the complexity increases, their activation promptness and intensity changes. Additionally, crosstalk and feedback can occur among the defensive and nutrition pathways of a multi-trophic interacting plant [6–8]. Thus, experimental data about multiple interactions is

required to develop detailed knowledge of plant biotic interactions regulation. This knowledge is fundamental to design sustainable crop management strategies based on manipulation of plant symbiosis.

The mutualistic symbiosis between Leguminous and rhizobia is one of the most studied due to its benefits for agriculture. Leguminous recruit rhizobia in the rhizosphere by chemotaxis[9,10]. Together, they form a new organ, called root nodule, whose development is regulated by the cytokinin:auxin ratio and other phytohormones[9–12]. In the nodule, the rhizobia fixes atmospheric nitrogen into bioavailable ammonia for the plant . Additionally, upon formation of the nodule, the rhizobia increase the production of lateral roots by inducing auxins (IAA), therefore increasing nutrients uptake [1–3]. Moreover, rhizobia triggers Induced Systemic Resistance (ISR) via plant recognition of microbe-associated molecular patterns (e.g., chitin, flagellin and lipopolysaccharides)[13] . ISR increases plant tolerance to biotic stress, although it does not induce direct changes in defence-related gene expression in aboveground plant parts in absence of threat. Defence-related genes are systematically expressed upon biotic attack, in response to elevated levels of Jasmonic Acid (JA) [11,12]. JA functions as the main inducer of generalised defence responses to arthropods herbivores and biotrophic pathogens through formation of trichomes as well as increased synthesis of proteinase inhibitors, volatiles, alkaloids, and glucosinolates, and others[6]. Nevertheless, even upon biotic attack the JA level of rhizobia-colonised plants remains unchanged, suggesting that ISR can be based on increased sensitivity to JA rather than on increased production of JA[3]. In exchange for the increased nutrient uptake and ISR, the host plant transfers energy in the form of organic acids to the nodules [9,11–13]. When the plant is under attack by pests, the amount of nutrients available for the nodules decreases due to the trade-off between development and defence. Similarly, defence-related mechanisms can interfere with the nodule’s development.

Pea aphids *(Acyrthosiphon pisum* Harris, 1776) are one of the most studied herbivorous arthropods of leguminous, usually inflicting considerable yields loss [14]. *A. pisum* feeds on the phloem sap through its stylet[14]. This insect causes minimal cellular lysis by piercing intercellularly to the phloem sieve elements with the stylet[14]. Thus, it does not fall into the trap of releasing, and consequently hydrolysing, flavonoids and other chemical defences compartmentalised in the plant cells[14]. Additionally, as phloem-feeders, aphids activate in the plant host the salicylic acid (SA) pathway in addition to the JA pathway triggered by most arthropod herbivores[14]. The SA pathway interferes with the SA one, suppressing the plant defences against aphids [4,14]. The activation of the SA pathway to interfere with the JA one is called “Decoy hypothesis” [4–6]. Despite these elegant adaptations, a significant part of the damage caused by *A. pisum* is due to the transmission of pathogens to leguminous plants.

Pea enation mosaic virus (PEMV) is one of the main pathogens transmitted by aphids to leguminous hosts. A*. pisum* acquires PEMV feeding on the phloem sap of infected plants [7–9,11–

[17]. The PEMV Coat Protein (CP) binds membrane the N-terminal alanyl aminopeptidase in the gut of *A. pisum*, which has been identified as a receptor for uptake of PEMV required for circulative transmission in the hemocoel [18]. As circulative persistent virus, PEMV virions undergo one latent phase after acquisition [4,14]. During this phase PEMV virions circulate in the hemocoel and accumulate in the salivary glands of *A. pisum*. During the following attacks, *A. pisum* releases PEMV in the phloem through the watery saliva [19]. Consequently, PEMV spread through the plant via the phloem. PEMV infections are recognized five to seven days after inoculation by the downward curling of the leaves, followed by the appearance of chlorotic and translucent spots (mosaic) on the leaves. Besides the mosaic symptoms, PEMV can produce abaxial hyperplastic growths (enations) [20]. During infection, PEMV also alters the host plant traits to provide a more favourable host to its vector. This is accomplished by hijacking the hormonal crosstalk, to suppress the plants defences, and the nutrients, hindering plants growth in favour of aphid feeding [15,16,19,21,22].

Interestingly, it has been speculated that PEMV has negligible effect on nutritional metabolites of Fava bean (*Vicia faba* L.) plants since virus infection did not impact the plant growth, estimated by the biomass of the aboveground parts [7,8] (A.Fontana's Semester Project). However, in the mentioned study both virus-infected and uninfected plants were under aphid herbivory for twenty days. Thus, the damage caused by herbivory might have hidden the impacts on plant biomass caused by PEMV infection. A possible explanation for the negligible effect on plant biomass is the digestion of defence compounds into nutritional metabolites induced on the plant upon aphid herbivory. The suppression of the host plant defences upon aphid attack could lead to defence resources being instead allocated to growth and partially taken by virus and aphid. Although PEMV did not affect the plant biomass, sap-feeding insects and pathogens can impair the interactions of the plant with its mutualists, ultimately decreasing plant fitness [7,8,16,19,22].

Based on a previous study, the aphid vector population on PEMV-infected plants grows faster than on uninfected plants, both in numbers of generations, individuals per generation and individual mass [15,23] (A.Fontana's Semester Project). According to the "decoy hypothesis", aphids and their symbionts evolved to trigger the SA pathway to suppress JA-dependent defences, which are more effective against phloem feeders than the SA-dependent defences . However, this mechanism might have a limit, as it has been shown that a high density of aphids on uninfected plants can trigger JA-dependent defences despite the activation of SA-dependent defences.

In the multi-trophic interaction system, there is often a tug-of-war of plant altered phenotypes and its impacts on both plant mutualists and antagonists. On one hand, the induction of SA by aphid or PEMV reduces the rhizobia nodule formation, in both mass and number [7,8], because SA is a known shrinking agent of nodulation [7,8,19,22–24]. On the other hand, the root

biomass and nodulation increase under the exclusive attack of aphid [5,23]. In this case, the plant resources are re-allocates away from the herbivore and closer to nodulation regions, which protects such resources and compensates for the lost fitness by increasing nitrogen fixation [5,23]. Additionally, it has been suggested that rhizobia reduce nutrients availability and uptake of phloem-feeders, on both virus-free and virus-infected plants, by triggering JA-dependent defences [?]. Thus, rhizobia reduce the aphid population growth, potentially reducing the transmission of their carried virus [20-21]. Moreover, previous rhizobia colonisation of the host decreases the following infection rate by PEMV (A.Fontana's Semester Project). One possible explanation is that rhizobia increase the JA-dependent defences through ISR, which interferes with the SA-dependent defences hijacked by both virus and aphid vectors for suppressing the JA-dependent defences against them. The JA-signalling pathway also cross-communicates with the Abscisic Acid (ABA) signalling pathways through common transcription factors [4,6]. However, it is still unclear how rhizobia, aphid and PEMV alter host plant fitness and when collectively interacting with the plant. Analysis of biomass and phytohormones could reveal changes in nutritive and defensive strategies of the plant host induced by either rhizobia, aphid and virus.

To address this knowledge gap, this minor thesis explored the effect of rhizobia inoculation on virus-plant-vector interactions. Specifically, this thesis aimed to answer the following questions:

I. *Is there a combined effect of rhizobia, PEMV and pea aphids on plant biomass?*
   Based on the different hypotheses and previous evidence, we predict that the virus infection and aphid herbivory reduce the plant biomass while the rhizobia colonisation increases it. Possibly, the aphid attack alone increases the root biomass and nodulation at the expense of the shoot biomass. However, by triggering JA-related defences, both virus and aphid impair nodule formation, therefore decrease nodule mass and number. Nevertheless, it is still to be determined how the multi-trophic interactions among rhizobia, aphid and virus affects the plant biomass. In order to investigate these interactions, the biomass of leaf, roots and nodules of plants exposed to different combinations was assessed.

II. *Is there a combined effect of rhizobia, PEMV and pea aphids on JA, ABA and SA level in plant leaves?*
   We predict an increase in JA concentration in leaves because rhizobia triggers ISR when firstly inoculated on plants. This mechanism would partially explain the previous observed reduction of total virus-infected plants and aphid vectors on rhizobia-inoculated plants. Conversely, we hypothesise that virus infection and aphid herbivory suppress JA-dependent defences as stated on the decoy hypothesis. Thus, we predict an increase in SA concentration in leaves upon viral infection and/or herbivory.

However, it is still to be determined how the multi-trophic interactions among rhizobia, virus and aphid affects the phytohormonal crosstalk. In order to investigate it, we quantified via LC-MS the levels of JA, SA and ABA from leave extracts of plants exposed to different combinations of organisms.

Understanding how the multi-trophic interactions affect each other and the plant performance will provide a base for applied, and sustainable crops management strategies.

2. Material & Methods

2.1. Study Model

In order to address the research questions, we adopted a model that provides both agrarian and ecological relevance, and support from literature. As the model for leguminous plants, we adopted fava bean (*Vicia faba L.*), which is a universal host for pea aphids, and it is also widely cultivated as a crop for human consumption and as a cover crop. Consequently, we worked with *the Rhizobium leguminosarum* biovar. viciae (strain 3841) because it evolved mutualism with *V. faba*. The antagonists were the previously introduced pea enation mosaic virus (PEMV) and its vector, the pea aphid (*Acyrthosiphon pisum*, clone LRS1).

2.2. Plant Growth

In order to sterilise the seeds before sowing, we first treated seeds of fava beans in 10% (v/v) bleach watery solution for 10 min and then rinsed them with tap water. Subsequently, we sowed them in sterilised 1 L pots containing autoclaved potting mix (60% sand, 30% vermiculite, 10% perlite). We kept the filled pots in a climate chamber (22°C, 60% RH, 16:8 light:dark photoperiod, 400 µmol/s of light intensity). We watered the pots with 200 mL and 75 mL of deionised distilled water) after one- and two-days past sowing, respectively.

2.3. Plant Treatments

We adopted ten treatments with 12 biological replicas in this study (Fig. 1). The treatments include two controls and eight combinations of rhizobia colonisation, PEMV infection and pea aphid herbivory. The difference in treatments started from the fourth day after sowing onwards. Four days after sowing, we started fertilising the plants three times a week with 50 mL of Hoagland solution with 3321 μM of nitrates. This fertilisation ensured enough nutrients for a healthy growth without interfering with either rhizobia, PEMV or pea aphids[10]. Only one control received more nitrates in the fertiliser, the “high nitrogen control” treatment. Fifteen days after sowing, we increased the fertiliser amount of all treatments (normal and high nitrogen treatments) to 100 mL.

2.3.1. High Nitrogen Control

We set one control to discern between the effects on plants due to higher nitrogen availability, fixated by rhizobia, and the effects due to molecular interactions with rhizobia.

From four days after sowing, we watered twenty-four pots with High Nitrogen control with 50 mL of modified Hoagland solution containing 6671 μM of nitrates, instead of 3321 μM.

2.3.2. Rhizobia Inoculation

Four days after sowing, simultaneously to the increase of fertiliser in the control, we inoculated forty-eight seedlings with 0.5 mL of liquid bacterial culture of *Rhizobium leguminosarum biovar. viciae* (strain 3841). Concurrently, we poured autoclaved liquid broth, therefore without bacteria, on 84 pots. We grew the bacteria in an autoclaved mannitol-yeast broth (50 mL), incubated at 27 °C and 120 rpm for 48h. We adjusted the liquid inoculum to a cell density of 5x106 CFU/mL prior to seedling inoculation [25]. To ensure bacterial colonisation, we repeated this procedure a week later, when plants were 12-days-old .

2.3.3. Virus Infection

To infect the seedling with PEMV, we introduced 15 viruliferous second-instar spring clonal *A. pisum* (clone LRS1) on 48 plants after 10 days of sowing. Twenty-four of the forty-eight plants were already inoculated with rhizobia. Concurrently, we introduced non-viruliferous second-instar aphids on the remaining plants. To prevent the aphids from moving off the plants, we enclosed each plant with a transparent air permeable cellophane bag (220 mm width x 380 mm length; Celloclair AG, Switzerland) encircled by rubber bands. We left the aphids on the plants for 4 days to ensure virus transmission, between 9-13 days after sowing. On the thirteenth day after sowing, we removed the aphids with a painting brush and forceps. We visually assessed the absence of aphids on each plant every second day for one week. After the checking week, we removed the bags. We assessed the PEMV infection with an Enzyme-Linked Immunosorbent Assay (ELISA) test using the PEMV CP as ligand (Nano Diagnostics, AR, USA). All forty-eight plants, twelve plants per each of the four treatments including PEMV, were selected based on the ELISA-test confirmed PEMV infection.

2.3.4. Pea Aphid Herbivory

To elicit herbivory response in plants, half of the plants belonging to each treatment were individually placed into meshed cages (Figure 1; treatments: control, control fertilised with high nitrogen, rhizobia-inoculated, virus infected and, rhizobia-colonised and virus-infected plants). On each plant were released 50 second-instar non-viruliferous pea aphids. The aphids were left on the plants for 5 days, from the 23rd to the 28th after sowing. The remaining half of the plants were kept into meshed cages without aphids.

2.4. Plant harvesting

Twenty-eight days after sowing, the plants were harvested. Leaves of each plant were split from stem and branches and subsequently collected in paper bags, flash-frozen in liquid nitrogen and lyophilized for 48 h at -50°C (console freeze dryer and collector, and 0 mbar of vacuum FreeZone® 6 Litres). The roots were washed in tap water, accommodated in paper bags, flash-frozen in liquid nitrogen and also lyophilized for 48 h. Plants cross-contaminated

by the rhizobia (nodule formation) were discarded from the controls and virus-infected treatments. After the lyophilization, each sample bag was labelled with a randomised ID to prevent confirmation bias. The randomised ID was associated to the sample through a spreadsheet created with Microsoft Excel®.

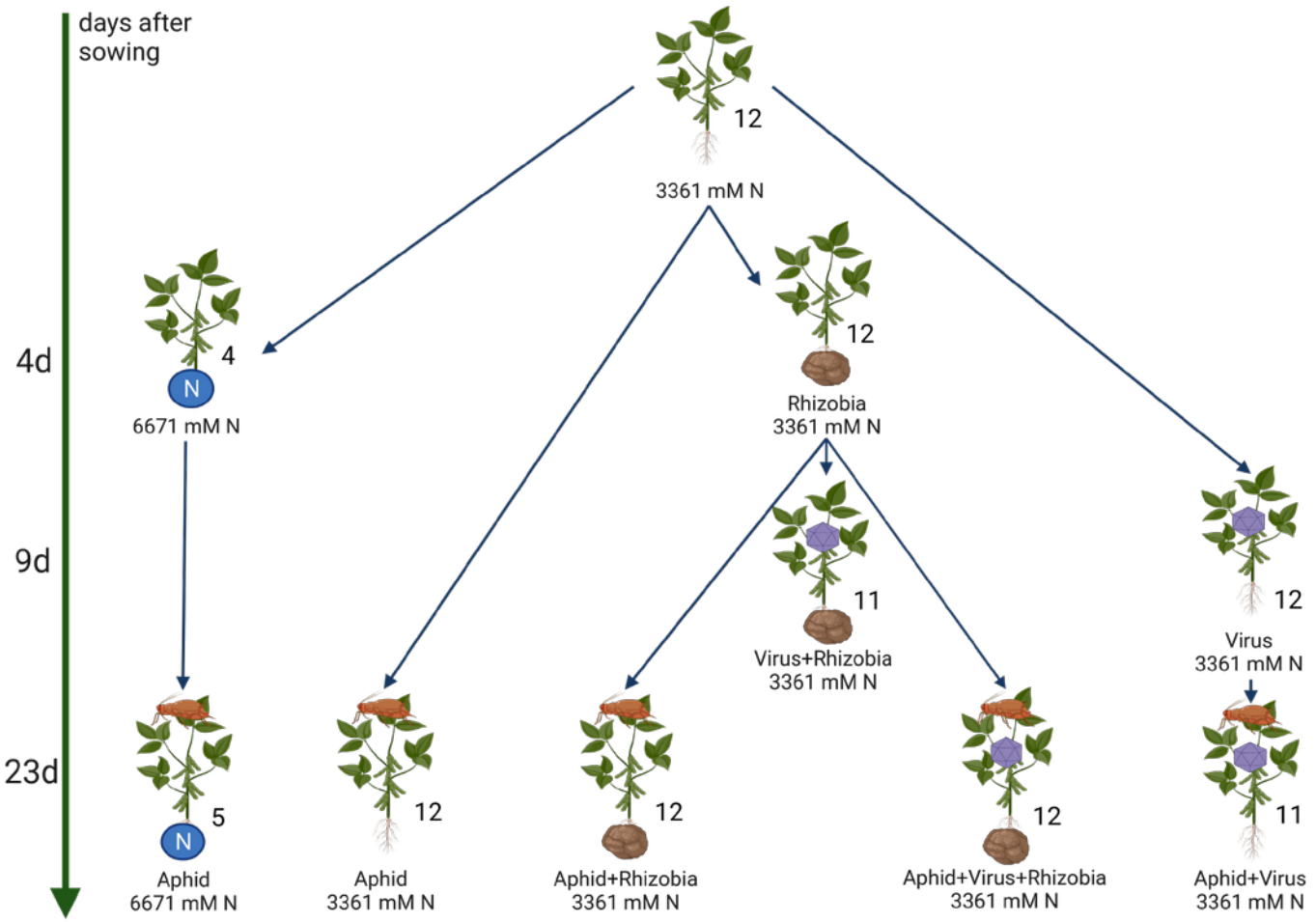


Figure 1. Plant treatments of this master thesis. Below each treatment is written the nitrogen concentration of the fertiliser and to the right is the number of replicas harvested and used. From top to bottom and left to right: 12 replicas of control fertilised with 3361 mM of nitrates, 4 replicas of control fertilised with 6671 mM of nitrates, 12 replicas colonised by rhizobia fertilised with 3361 mM of nitrates, 11 replicas colonised by rhizobia and infected with PEMV while fertilised with 3361 mM of nitrates; 12 replicas infected with PEMV while fertilised with 3361 mM of nitrates; 5 replicas attacked by pea aphids while fertilised with 6671 mM of nitrates; 12 replicas attacked by pea aphids while fertilised with 3361 mM of nitrates; 12 replicas colonised by rhizobia and attacked by pea aphids while fertilised with 3361 mM of nitrates; 12 replicas colonised by rhizobia, infected by PEMV and attacked by pea aphids while fertilised with 3361 mM of nitrates; 12 replicas infected by PEMV and attacked by pea aphids while fertilised with 3361 mM of nitrates.

2.5. Biomass recording

To assess how the different treatments affect the plant biomass, we weighted the dry biomass of leaves, roots and nodules using a Mettler Toledo AB204 balance (±0.1 mg). Additionally, we separated the nodules, counted and weighed them.

2.6. Samples processing

To prepare the leaf tissue for phytohormones extraction, we accommodated each sample into 10 mL polypropylene tubes to ground it to powder using three 3 mm round stainless-steel beads and a 2010 Geno/grinder® (SPEX® SamplePrep) at 1450 rpm for 3 to 6 min. The grinding time is approximately directly proportional to the leaf tissue mass and volume. The homogenised tissue was split into four aliquots of 9.85 ± 0.15 mg weighted into a 2 mL safe-lock Eppendorf® tube. One of the four aliquots was employed for extracting the phytohormones and the remaining tubes were stored in a freezer at -80ºC as backup and for further analysis.

2.7. Phytohormones extraction

To assess phytohormones levels in the leaves, we used a methanol extraction with internal labelled phytohormone standards as reference. We adopted Dihydro-jasmonic acid (dH-JA), 2-hydroxy-benzoic acid (d6-SA), and d6 Abscisic acid (d6-ABA) as internal quantitative standards with a final concentration of 10 ng/µL. First, we added 50 µL of the internal standard solution containing 10 ng/µL of phytohormones in methanol to the samples. Secondly, we added 750 µL of 10 mM ammonium acetate and 650 µL of absolute methanol. Thirdly, we shook the samples for 15-20 s in the Scientific Industries® Vortex Genie 2. Fourthly, we inserted the samples in a Styrofoam raft and sonicated them for 15 min in the Bandelin® Sonorex Super RK512H. Fifthly, we centrifuged the samples for 10 min at 20.000 RCF at room temperature in the Eppendorf® Centrifuge 5417R. Subsequently. We transferred 1 mL of the supernatant into a 2 mL safe-lock Eppendorf® tube. Afterwards, we dried the samples 6-8h in the Thermo Scientific® Savant SpeedVac SPD140DDA Vacuum concentrator, connected to the RVT5105 Refrigerated Vapour Trap. Consequently, we resuspended the resulting deposit in 100 µL of 0.1% (*v/v)* formic acid in distilled water. Following, the suspension was shaken for 30 s in the Scientific Industries® Vortex Genie 2. Subsequently, we placed the samples in a metal rack in a fridge at 3ºC for 10 min to make eventual fats solidify. Then, we centrifuged the samples for 10 min at 20.000 RCF at 5ºC in the Eppendorf® Centrifuge 5417R to converge remaining non-polar compounds on the tube bottom. Finally, we transferred 60 µL of each supernatant to an insert in a LC-MS glass.

2.8. LC-MS data acquisition

Following the extraction, we organised the LC-MS glass vials with insert in increasing numerical order in the racks of the Quadrupole Time-of-Flight Liquid Chromatography-Mass Spectrometry (Q-TOF LC-MS, Agilent Technologies® 1290 Infinity II) with a RRHD Zorbax Eclipse Plus-C18 column (100 mm length, 2.1 mm diameter, 1.8 µm particle size). We used a solvent gradient of 99% A (milli-Q water + 0.1% formic acid) to 99.5% B (acetonitrile + 0.1% formic acid) over 18 min with a flow rate of 0.6 ml/min. We cooled the auto-sampler to 4°C and set column temperature to 50 ˚C. Dual Agilent Jet Stream Electrospray Ionisation (Dual AJS ESI) source parameters were as follows: spray voltage (-), 3000 V; nozzle voltage (-), 1000

V; Gas temperature, 250 °C; Drying gas, 15 l/min; nebulizer, 35 psi; sheath gas temperature, 275 °C; sheath gas flow, 8 l/min. The Q-TOF LC-MS detected the mass range between 100 to 600 m/z with an acquisition rate of 6 spectra/s. We set the signal acquisition at 375.0 nm and 540.0 nm with a bandwidth of 4 nm. In the first position of each of the three used racks, we inserted a vial containing methanol to clean the syringe. We added 3 empty vials ahead of the samples to test the cleanliness of the machine and mass calibration. We estimated the concentrations of JA, SA and ABA using the software Agilent Technologies® Q-TOF Quantitative Analysis B.07.00 with a conservative approach counting the main cation produced and a range of 30 s for the retention time. Afterward, we checked each compound peak individually and its integrated area. Finally, we exported the results as a spreadsheet in Microsoft® Excel, where we normalised the estimates by the dried weight of the leaf tissue sample.

2.9. Data Analysis

(Note to self: remember referencing packages)

We performed all the statistical analyses with the software R (R Core Team, 2017) in the Integrated Development Environment (IDE) Rstudio® [28]. First, we explored the data sets by producing boxplots comparing the effects of either rhizobia, virus or aphid within each treatment and subsets of treatments using the dplyr and ggplot2 R packages [29, 30]. Subsequently, we analysed the datasets with an Aligned Rank Transform Analysis of Variance (ART ANOVA) from the ARTool and emmeans R packages [31]. We chose ART ANOVA because it is a non-parametric approach to factorial ANOVA that enables analysis of both interactions and the main effects, and the variance explained by random effects. We set rhizobia, PEMV, and pea aphids as model predictors while the fertiliser as random effect. Subsequently, we compared the effect for each treatment within the other treatments. When the single predictors or their interactions were significant, we contrasted treatments with pairwise Tukey HSD (Honestly Significant Difference) test with Sidak's adjustment using the multcomp R package. The analysis was done both including and excluding the high nitrogen control.

3. Results

3.1. Available Nitrogen increases root biomass

Contrary to our predictions, we observed only a tendency of shoot biomass increasing in plants colonised by rhizobia compared to the control (Figure 2A, ART ANOVA; $F = 2.89$, $p = 0.09$). There was still no difference in the comparison between rhizobia and the control when analysing only the top stem biomass (Figure 2B, ART ANOVA; $F = 1.83$, $p = 0.18$) or leaves biomass (Figure 2C, ART ANOVA; $F = 2.08$, $p = 0.15$). Nonetheless, we observed the predicted increase in root biomass in the comparison between plants inoculated with rhizobia and the control (Figure 3, ART ANOVA; $F = 10.66$, $p = 0.002$). All the comparison between plant

inoculated with rhizobia and those fertilised with more nitrates show no difference (Figure 1-3, ART ANOVA; $p > 0.05$)

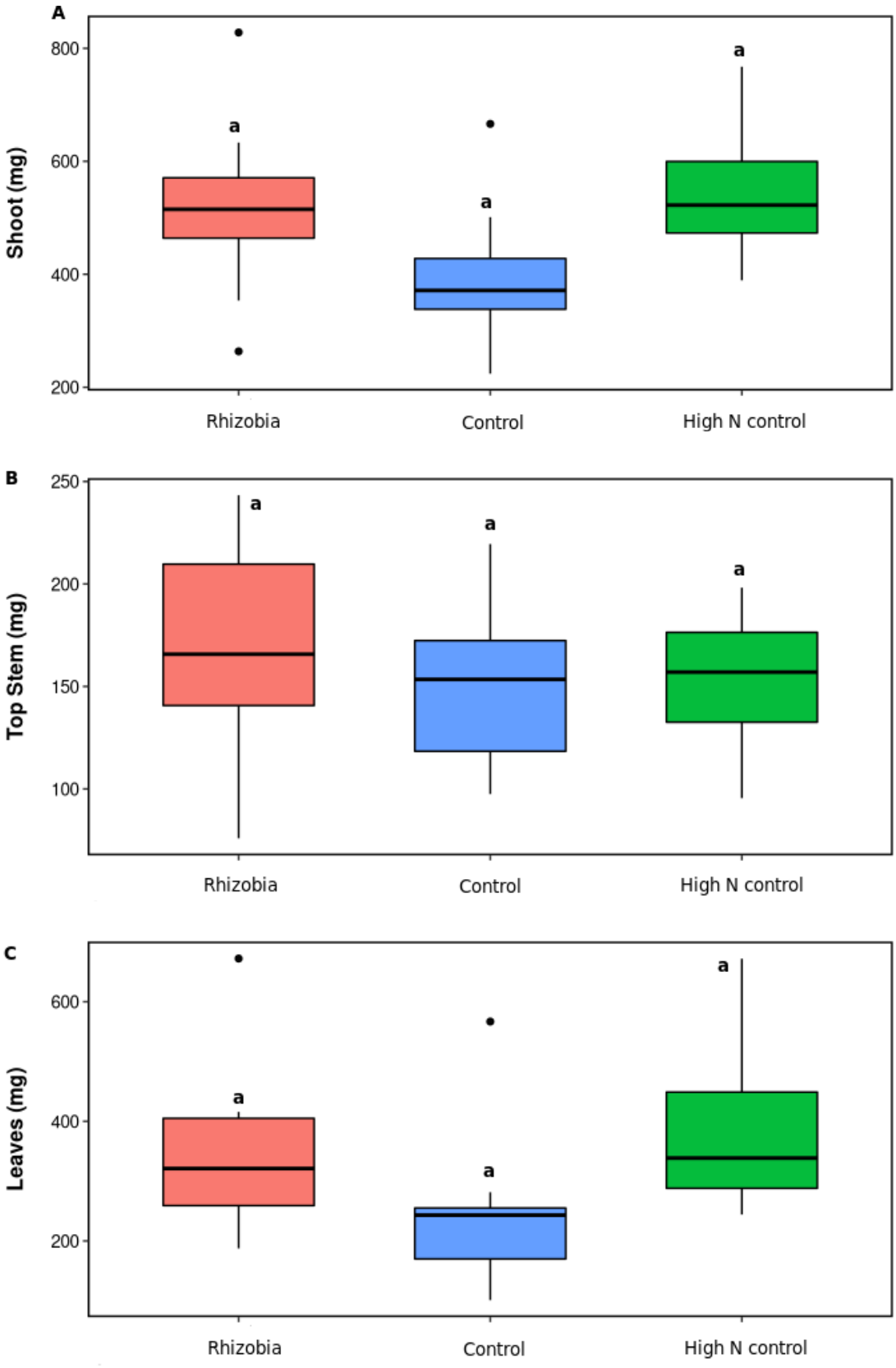


Figure 2. Shoot biomass of plants colonised by rhizobia, fertilised with high nitrogen levels and control broad beans. Comparison among (from left to right) Rhizobia (N = 12), Control (N = 12), Control

fertilised with more nitrates (N = 4) of (**A**) Shoot biomass (as sum of top stem and leaves) (**B**) Top stem biomass (**C**) Leaves biomass . Same letters do not differ according to ART Anova.

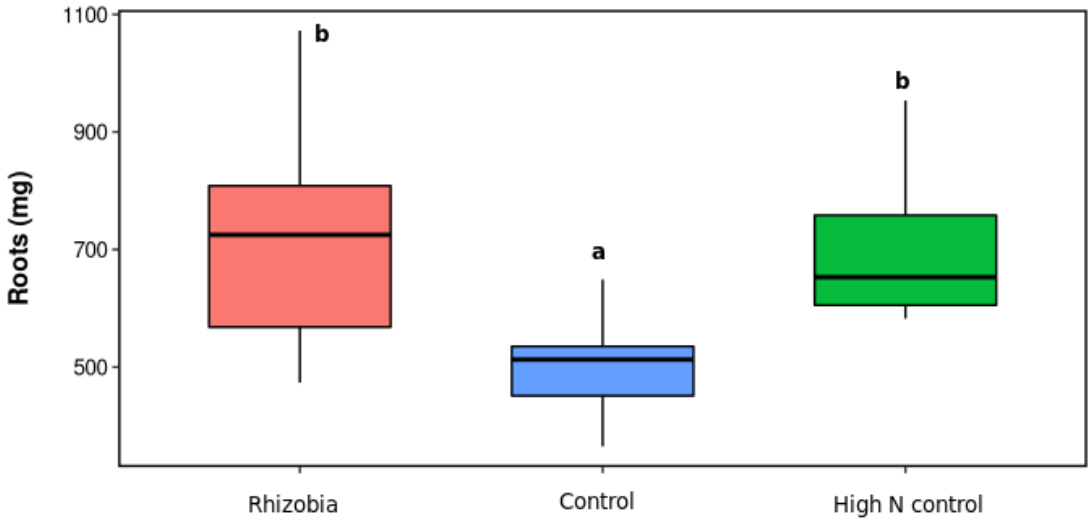


Figure 3. Root biomass of control, plants colonised by rhizobia, and plants fertilised with more nitrogen. Comparison of root biomass among Rhizobia (N = 12), Control (N = 12), Control fertilised with more nitrates (N = 4). Same letters do not differ according to ART Anova.

3.2. PEMV decreases plant biomass

As we predicted, PEMV decreased significantly both the root biomass (Figure 4, ART ANOVA; $F = 13.23$, $p < 0.01$) and the shoot biomass (Figure 5A, ART ANOVA; $F = 7.24$, $p = 0.01$) compared to the control. The effect of PEMV on shoot biomass was exclusively on the top stem (Figure 5B, ART ANOVA; $F = p < 0.01$). There is no effect of the virus on the leaves biomass (Figure 5C, ART ANOVA; $F = 0.31$, $p = 0.58$). Moreover, PEMV decreased both root (Figure 6, ART ANOVA; $F = 4.85$, $p = 0.04$) and shoot biomass (Figure 7, ART ANOVA; $F = 7.45$, $p = 0.01$) in plants colonised by rhizobia compared to uninfected plants colonised by rhizobia.

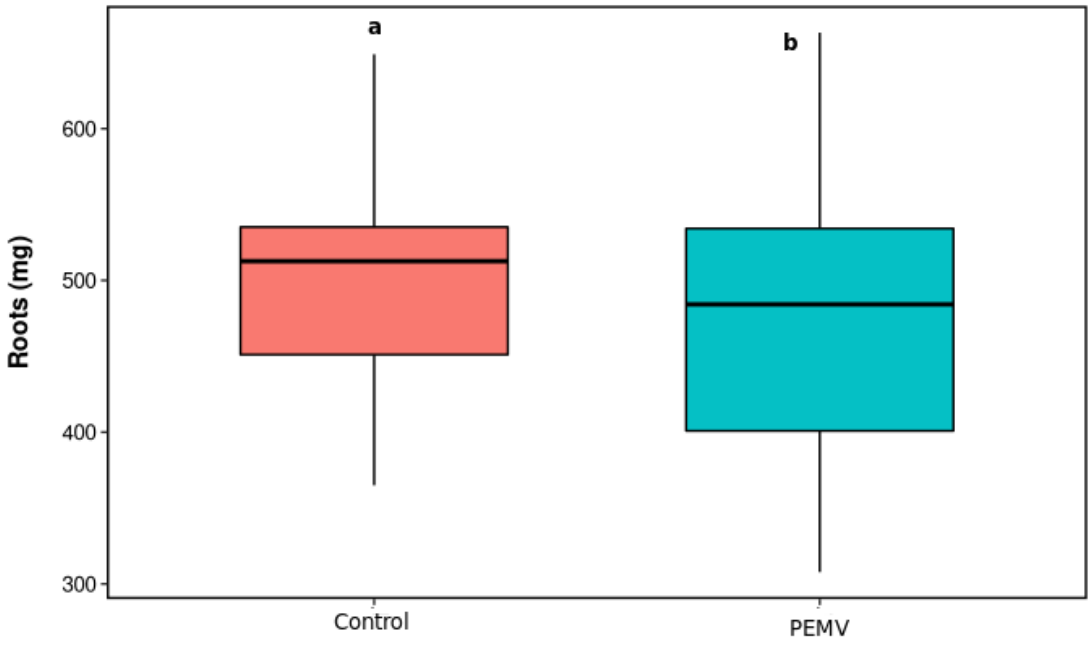
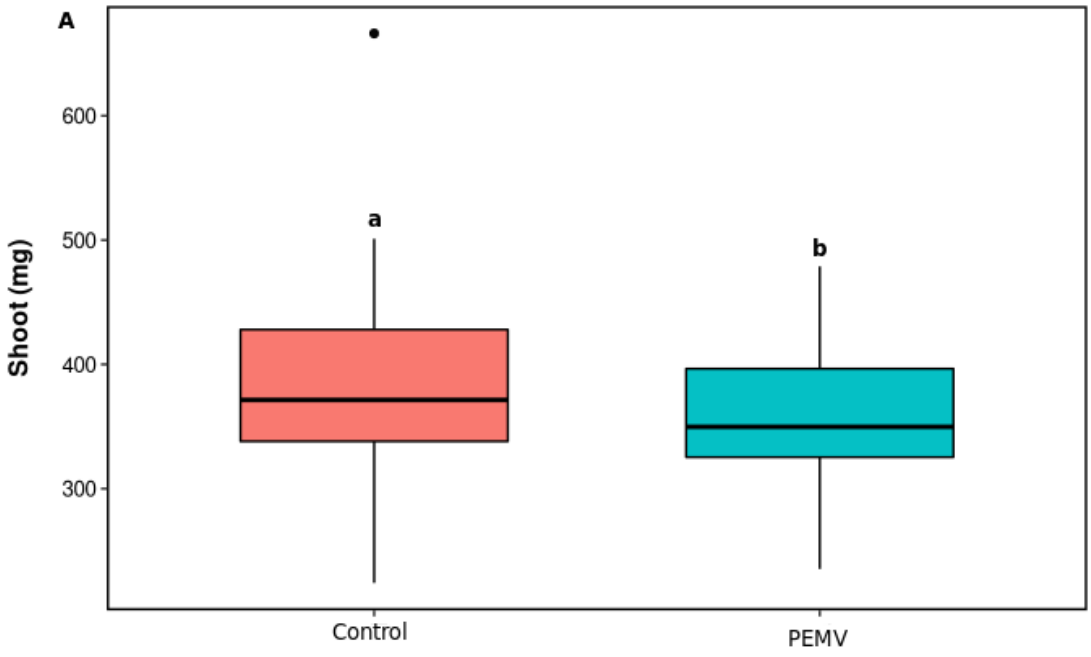


Figure 4. Root biomass of control and PEMV-infected plants. Comparison of root biomass between Control (N = 12) and PEMV-infected plants (N= 12). Same letters do not differ according to ART Anova.



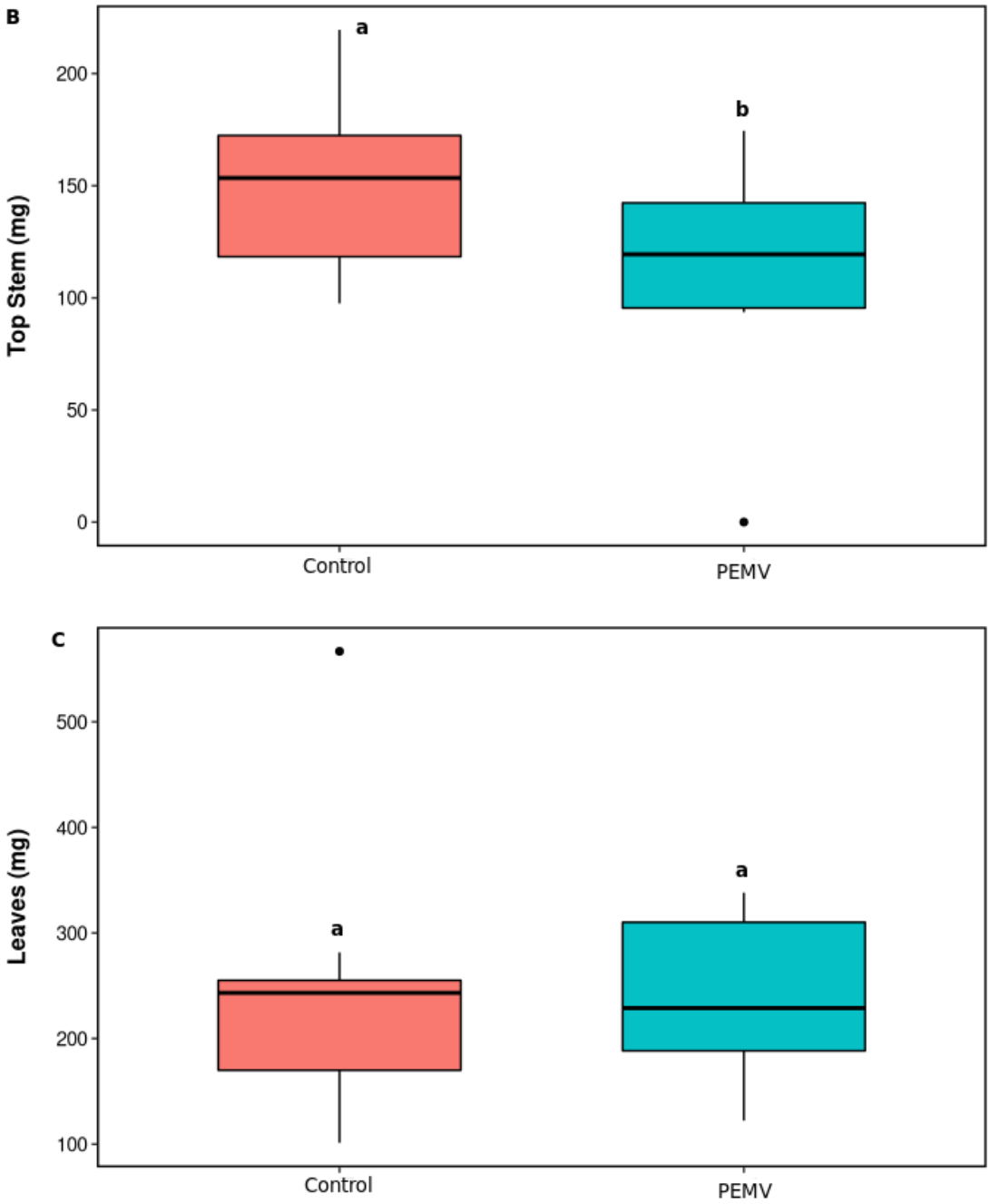


Figure 5. Shoot biomass of control and PEMV-infected plants. Comparison between Control (n = 12) and PEMV-infected plants (n= 12) of (**A**) Leaves biomass (**B**) Top stem biomass (**C**) Shoot biomass (as sum of leaves and top stem). Same letters do not differ according to ART Anova.

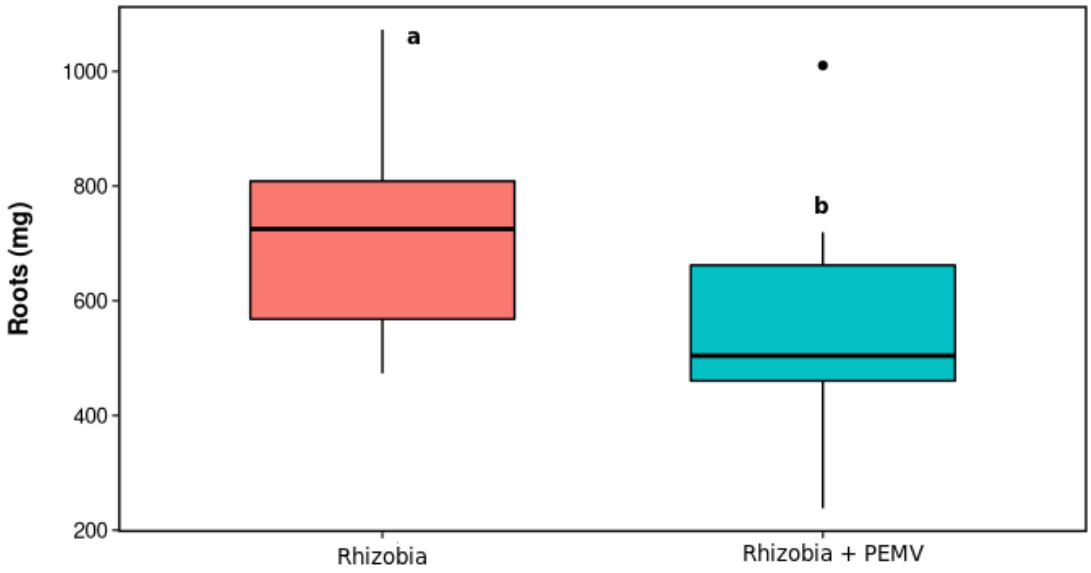
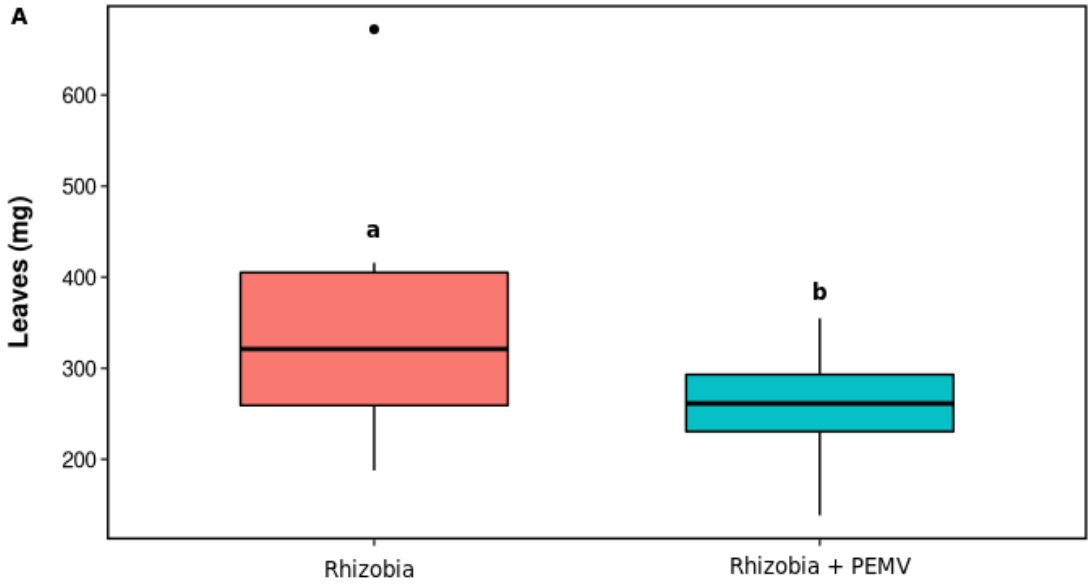


Figure 6. Root biomass of rhizobia-colonised plants and PEMV-infected rhizobia-colonised plants. Comparison between rhizobia-colonised plants (n = 12) and rhizobia-colonised plants PEMV-infected plants (n= 11) of root biomass. Same letters do not differ according to ART Anova.



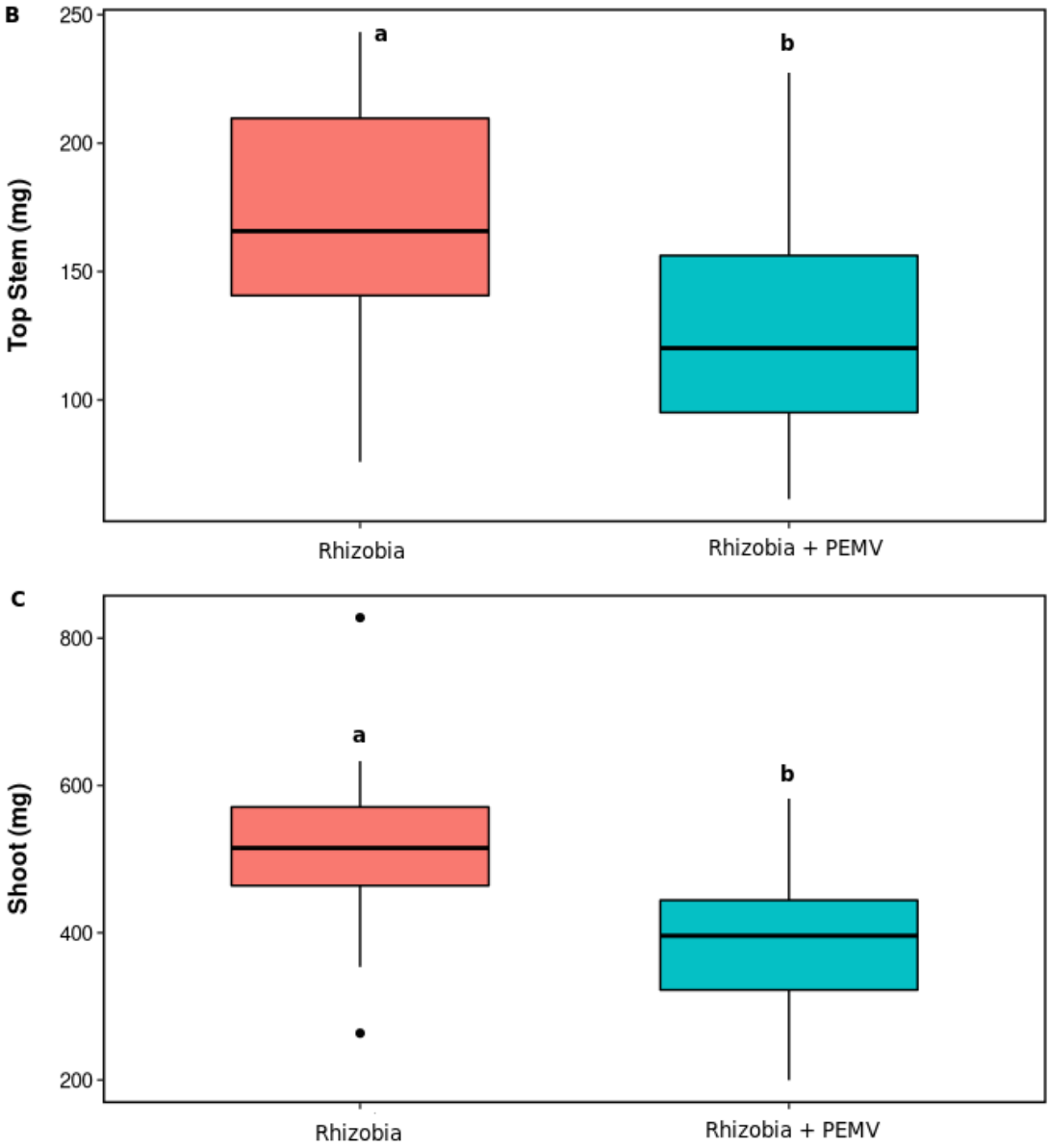


Figure 7. Shoot biomass of rhizobia-colonised plants, and PEMV-infected rhizobia-colonised plants.

Comparison between rhizobia-colonised plants (n = 12) and rhizobia-colonised plants PEMV-infected plants (n= 11) of (**A**) Leaves biomass (**B**) Top stem biomass (**C**) Shoot biomass (as sum of leaves and top stem). Same letters do not differ according to ART Anova.

3.3. Pea aphids decrease root biomass

According to prediction, the pea aphids decreased the root biomass (Figure 8, ART ANOVA, $F = 11.67$, $p < 0.01$), although it did not affect shoot biomass compared to aphid-free control plants (Figure 8, ART ANOVA, $F = 0.09$, $p = 0.77$). Additionally, when comparing rhizobia colonised plants with and without pea aphids, the herbivory did not decrease nodule weight (Figure 10B, ART ANOVA, $F = 2.90$, $p = 0.09$) nor numbers (Figure 11B, ART ANOVA, $F = 0.39$, $p = 0.54$).

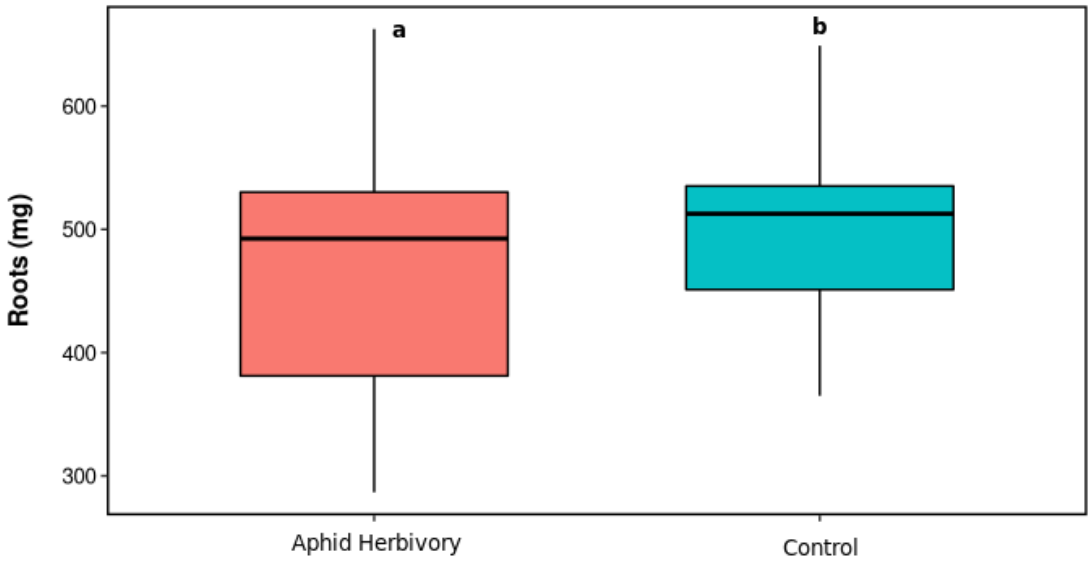


Figure 8. Root biomass of control and plant attacked by pea aphids. Comparison between plants attacked by aphid (N = 12) and control (N = 12). Same letters do not differ according to ART Anova.

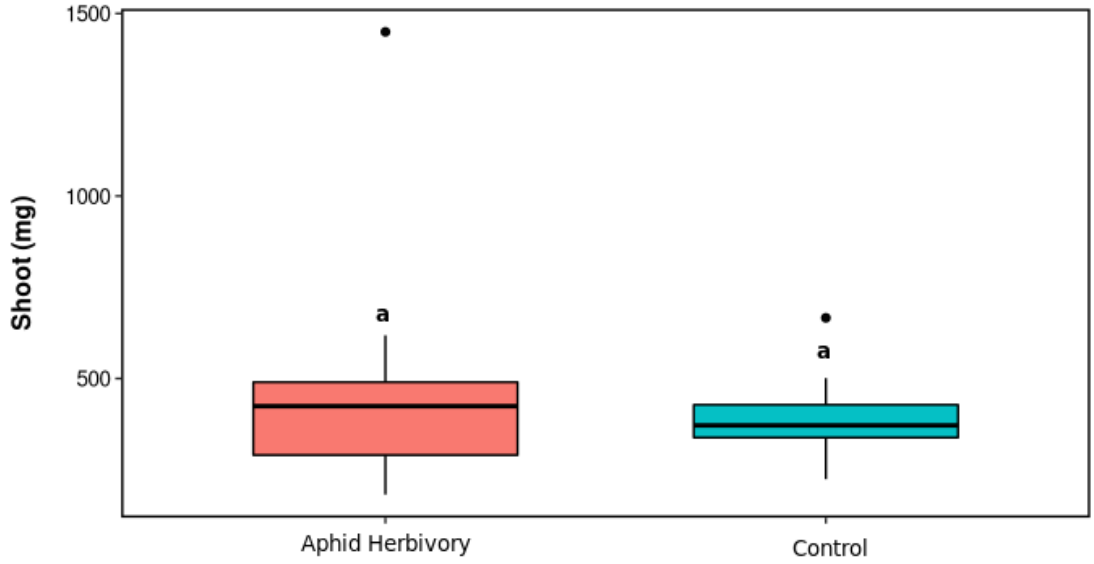


Figure 9. Shoot biomass of control and plant attacked by pea aphids. Comparison between plants attacked by aphid (N = 12) and control (N = 12). No significant difference is shown.

3.4. PEMV impairs nodule formation more than pea aphids

We observed a slight reduction in nodule biomass (Figure 10A, ART ANOVA, $F = 4.16$, $p = 0.05$) and no effects on nodule count (Figure 11A, ART ANOVA, $F = 1.32$, $p = 0.26$) when rhizobia-colonised plants were infected by PEMV. Interestingly, when the plants were additionally damaged by the aphids, we observed a stronger effect on the reduction of both nodule biomass (Figure 10B, ART ANOVA, $F = 14.18$, $p = 0.001$) and count (Figure 11B, ART ANOVA, $F = 27.67$, $p < 0.01$). However, the average nodule biomass (total nodule biomass

divided by nodule number) is unaffected by either PEMV or pea aphid (Figure 12, both p > 0.5).

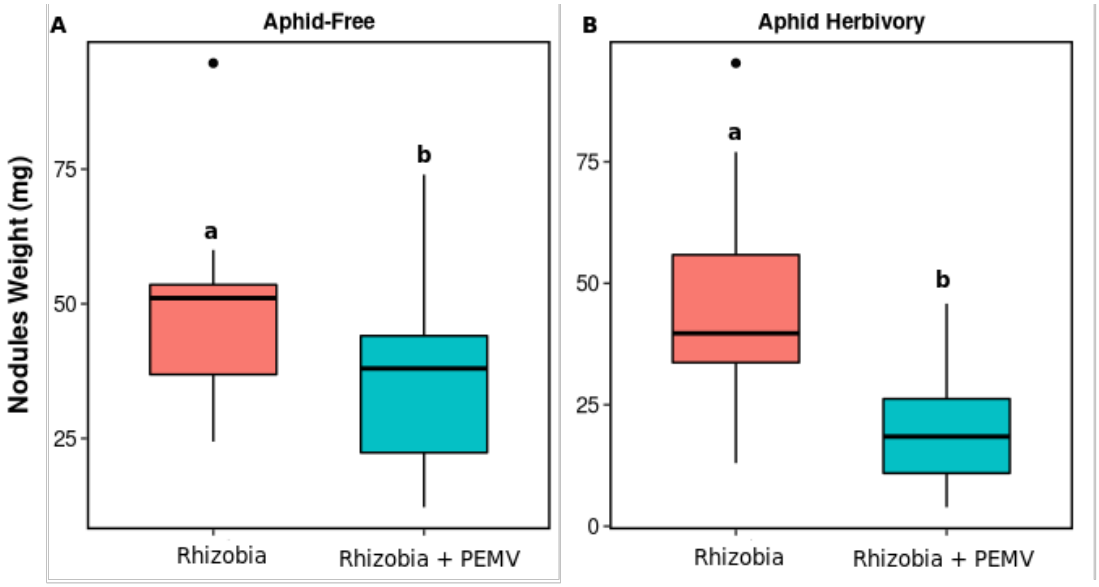


Figure 10. Nodule biomass of virus infection and aphid herbivory treatments. Comparison of nodule biomass among (from left to right) (**A**) rhizobia-colonised plants (N = 12) and rhizobia-colonised plants PEMV-infected plants (N = 11), and (**B**) rhizobia-colonised plants attacked by aphids (N = 12) and rhizobia-colonised PEMV-infected plants attacked by aphids (N = 12). Same letters do not differ according to ART Anova. **A** and **B** have different scales on the y axes.

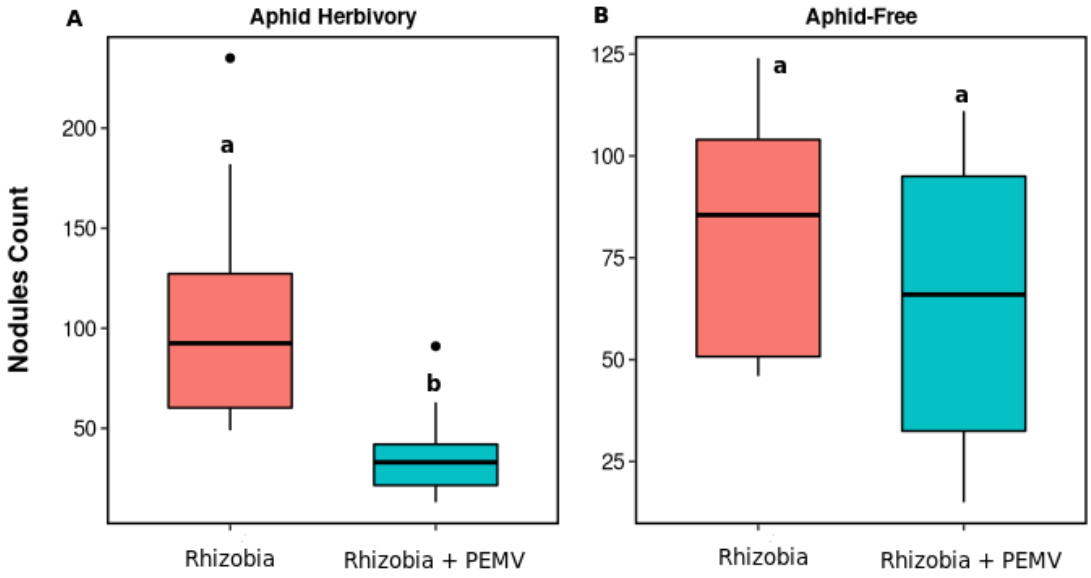


Figure 11. Nodule number under virus infection and aphid herbivory treatments. Comparison of nodule count among (from left to right) rhizobia-colonised plants attacked by aphids (N = 12), rhizobia-colonised PEMV-infected plants attacked by aphids (N = 12), rhizobia-colonised plants (N = 12) and rhizobia-colonised plants PEMV-infected plants (N = 11). Same letters do not differ according to ART Anova. **A** and **B** show different scales on the y axes.

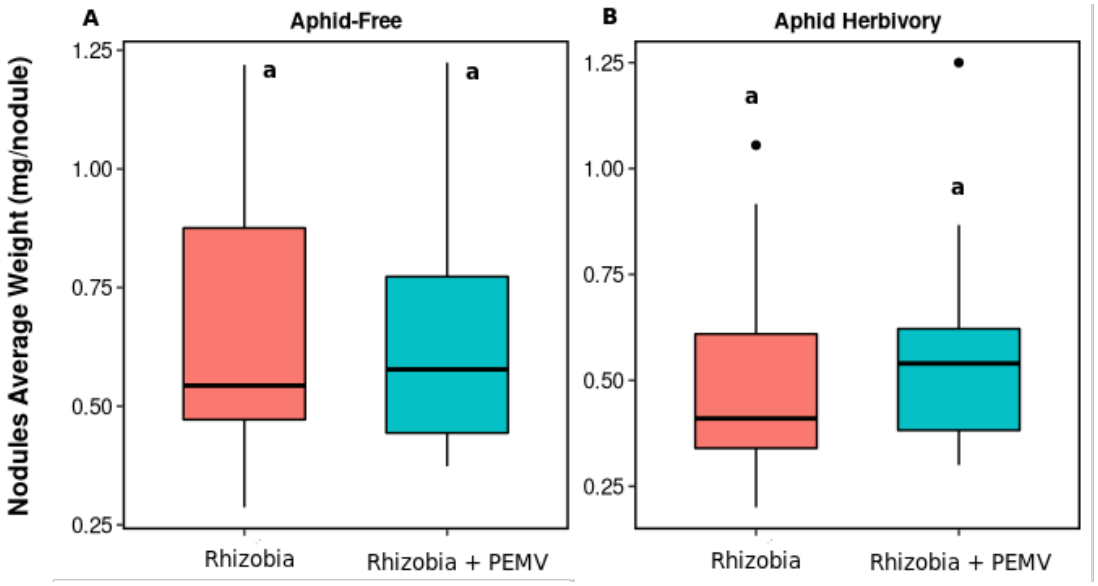


Figure 12. Nodule average biomass under virus infection and aphid herbivory treatments. Comparison of nodule average biomass among (from left to right) (**A**) rhizobia-colonised plants attacked by aphids (N = 12), rhizobia-colonised PEMV-infected plants attacked by aphids (N = 12), and (**B**) rhizobia-colonised plants (N = 12) and rhizobia-colonised plants PEMV-infected plants (N = 11). Same letters do not differ according to ART Anova.

3.5. Rhizobia affects root biomass more than PEMV and pea aphid individually

Rhizobia-colonised plants had increased root biomass when compared with the virus-infected (Figure 13A, ART ANOVA; $p < 0.01$) or aphid-damaged treatments (Figure 13B, ART ANOVA; $p < 0.01$). Additionally, we noted higher root biomass in plants colonised by rhizobia and infected by PEMV than the control (Figure 13A, ART ANOVA; $F = 4.91$, $p = 0.03$). However, we did not observe a difference in root biomass between the control and plant colonised by rhizobia, infected by PEMV and attacked by aphids (Figure 13B, ART ANOVA; $F = 0.11$, $p > 0.1$).

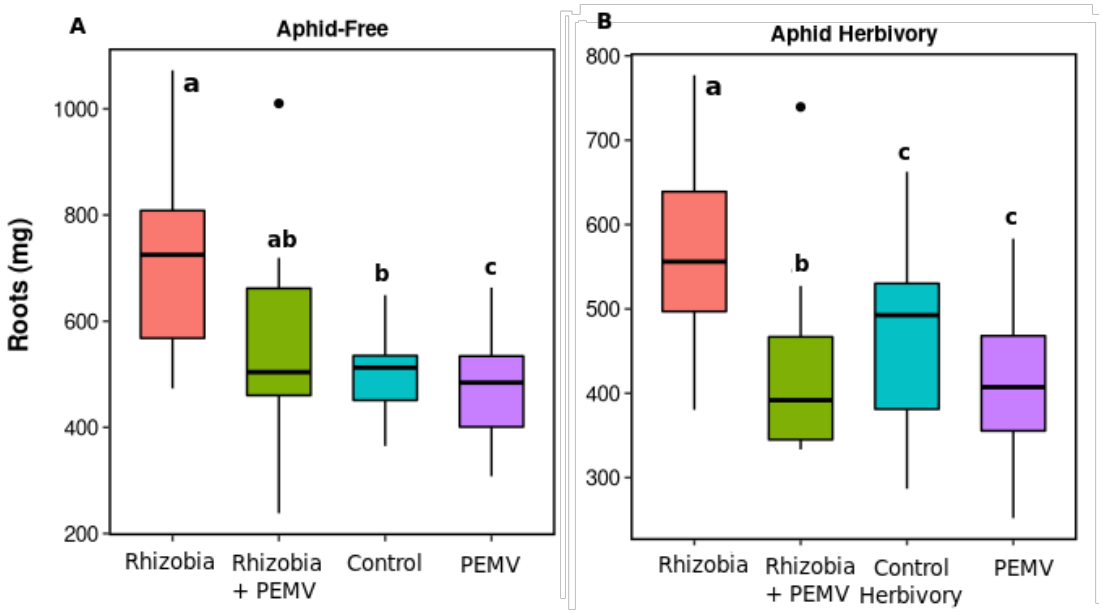


Figure 13. Roots biomass of all the treatments, except high nitrogen fertilisation. Comparison of roots biomass among (from left to right) (**A**) rhizobia-colonised plants (N = 12) and rhizobia-colonised PEMV-infected plants, control (N = 11), PEMV-infected plants (N = 12) , rhizobia-colonised plants PEMV-infected plants attacked by aphids (N = 11) and (**B**) rhizobia-colonised plants attacked by aphids, plants attacked by aphids (N = 12), and PEMV-infected plants attacked by aphids (N = 11). **A** and **B** have different scales on the y axes. Same letters do not differ according to ART Anova.

3.6. PEMV increases SA concentration in the leaf tissue

We observed an increase of SA concentration exclusively in the leaf tissue of plants infected by PEMV compared to control (Figure 14A, ART ANOVA; $F = 38.94$, $p < 0.01$). When aphid damage was added to plants infected by PEMV, we noticed only a tendency to increase SA compared to the control (Figure 14A, ART ANOVA; $F = 3.93$, $p = 0.06$). No difference was observed between the control and plants colonised by rhizobia, infected by PEMV and damaged by aphids (Figure 14B, ART ANOVA; $F = 2.89$, $p = 0.09$). No other treatment showed an increase in SA (Figure 14, ART ANOVA; $p > 0.05$).

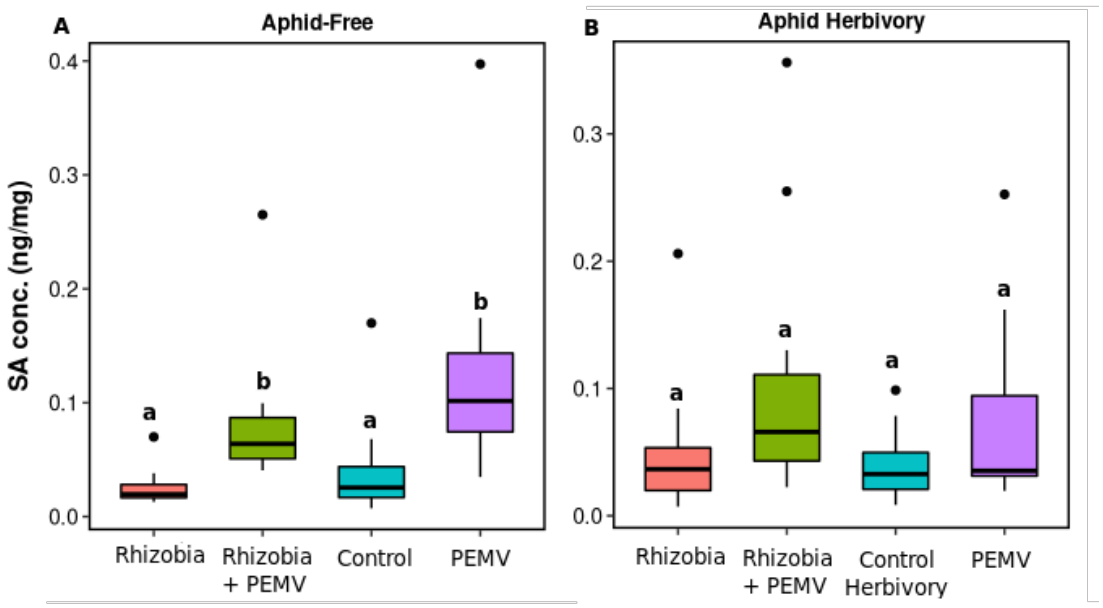


Figure 14. SA concentration (ng/mg) in leaf tissue of all the treatments, except high nitrogen fertilisation. Comparison of SA concentration (ng/mg) in leaves among (from left to right) (**A**) rhizobia-colonised plants (N = 12) and rhizobia-colonised PEMV-infected plants, control (N = 11), PEMV-infected plants (N = 12) , rhizobia-colonised plants PEMV-infected plants attacked by aphids (N = 11) and (**B**) rhizobia-colonised plants attacked by aphids, plants attacked by aphids (N = 12), and PEMV-infected plants attacked by aphids (N = 11). **A** and **B** have different scales on the y axes. Same letters do not differ according to ART Anova.

3.7. PEMV increases JA concentration in the leaf tissue

We observed an increase of JA concentration in the leaf tissue of plants infected with PEMV compared to the control (Figure 15A, ART ANOVA; $F = 11.92$, $p < 0.01$). Similarly, when plants infected by PEMV were additionally attacked by the aphids, we observed an increase of JA levels compared to control (Figure 15B, ART ANOVA; $F = 4.58$, $p = 0.03$). All other treatments did not affect JA concentration (Figure 15, ART ANOVA; $p > 0.05$).

3.8. ABA is not triggered

We did not observe any significant effect on ABA concentration in leaf tissue when all the plant treatments, in the presence and absence of aphid damage (Figure 16, ART ANOVA; $p > 0.05$).

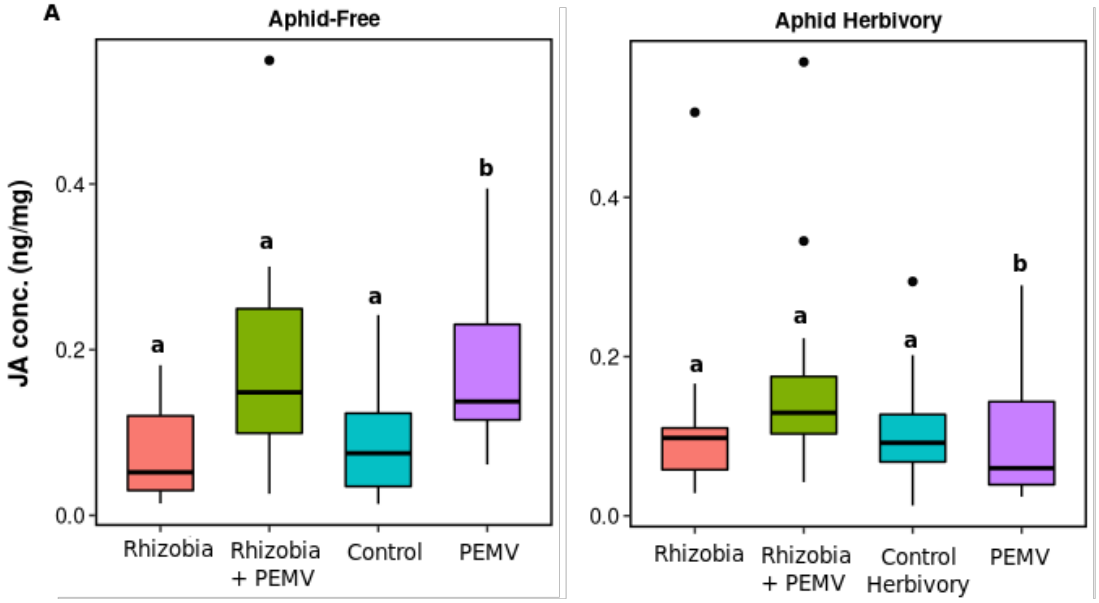


Figure 15. JA concentration (ng/mg) in leaf tissue of the treatments. Comparison of JA concentration (ng/mg) in leaves among (from left to right) rhizobia-colonised plants (N = 12) and rhizobia-colonised PEMV-infected plants, control (N = 11), PEMV-infected plants (N = 12) , rhizobia-colonised plants PEMV-infected plants attacked by aphids (N = 11) and rhizobia-colonised plants attacked by aphids, plants attacked by aphids (N = 12), and PEMV-infected plants attacked by aphids (N = 11). **A** and **B** have different scales on the y axes. Same letters do not differ according to ART Anova.

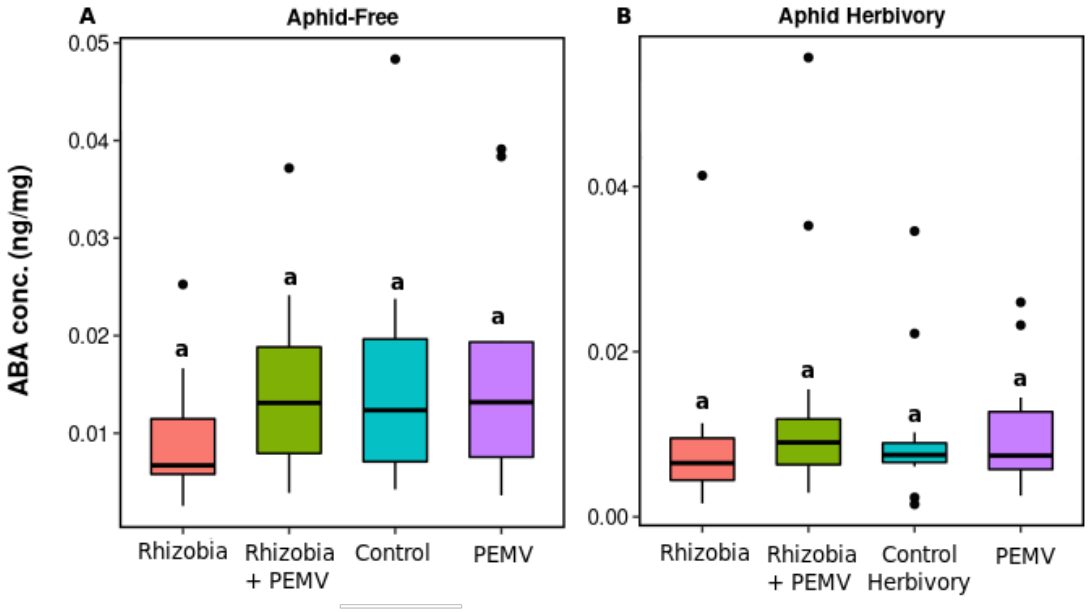


Figure 17. ABA concentration (ng/mg) in leaf tissue of all the treatments, except high nitrogen fertilisation. Comparison of ABA concentration (ng/mg) in leaves among (from left to right) (**A**) rhizobia-colonised plants (N = 12) and rhizobia-colonised PEMV-infected plants, control (N = 11), PEMV-infected plants (N = 12) , rhizobia-colonised plants PEMV-infected plants attacked by aphids (N = 11) and (**B**) rhizobia-colonised plants attacked by aphids, plants attacked by aphids (N = 12), and PEMV-infected plants attacked by aphids (N = 11). **A** and **B** have different scales on the y axes. Same letters do not differ according to ART Anova.

4. Discussion

We predicted that both rhizobia and high nitrates fertilisation would have increased the biomass of both shoot and root compared to the control. Conversely, we predicted that pea aphid and PEMV would have decreased the biomass of both shoot and root compared to the control.

As predicted, PEMV decreased both shoot and root biomass. Curiously, the PEMV effect on shoot is stronger on the top stem than leaves. This difference can be explained by the higher metabolic activity of the top stem tissue, as well as viral infection impacting less the phenotype of already developed leaves.

Surprisingly, both rhizobia and aphid did not affect the shoot biomass, despite showing the predicted effects on root biomass. In summary, rhizobia increased the root biomass while pea aphid decreased it. The surprising results might be due to the small sample size. This hypothesis is supported by the presence of several outliers and the matching observations in plants fertilised with higher concentration of nitrates. Alternatively, it could be due to secondary and primary metabolism being affected by rhizobia and aphid.

Indeed, rhizobia receive organic acids produced through photosynthesis in the shoot in exchange for the several benefits provided . Thus, the roots might increase their role as a resource sink. Concerning the effect of rhizobia on roots, we observed no significant difference ($p < 0.05$) between the increase of root biomass between plants colonised by rhizobia and the high nitrogen control. Thus, the increase in biomass can be ascribed exclusively to the increase in nitrogen availability.

A possible explanation for the negligible effect of aphids on shoot biomass is the digestion of defence compounds elicited upon aphid attack into nutritional metabolites [26]. This digestion is performed by enzymes released by the aphids in the phloem through their saliva. As an alternative explanation, the suppression of the host plant defences by the attackers could lead to defence resources being instead allocated to growth and partially taken by virus and aphid. Concerning the effect of aphids on roots and the lack of it on the shoot, we must discard the idea that there is re-allocation of nutrients from the shoot to the root. Instead, we consider that primary metabolites are diverted to the shoot to maintain foliar growth upon herbivory. Additionally, aphids feed on plant phloem, therefore sapping water, minerals, sucrose and free amino acids directed toward the roots. Finally, aphids release a proteinaceous salivary sheath in the phloem, though which it diffuses. This sheat can reduce carbon flux and initiate translocation of amino acids from roots, leaves, and sink tissues [26].

We hypothesised a mutual antagonism of rhizobia against both PEMV and pea aphids. Consequently, we measured the biomass of nodules and counted them. First, we aimed to assess an effect of PEMV and pea aphid on nodule development and growth. Secondly, we

wanted to assess if one antagonist affects the rhizobia colonisation more than the other. Finally, we planned to explore whether the average nodule biomass (biomass/number) might explain a different effect of the antagonism on nodule development and growth. We predicted a decrease in both nodule number and biomass by both PEMV and pea aphids with an additive effect of both plant antagonists combined. Thus, we predicted a higher decrease in presence of both PEMV and pea aphids. If the average nodule biomass (total nodule biomass divided by number) would have changed significantly, it could have suggested a different effect on development and growth individually. For instance, higher average nodule biomass might suggest that the antagonism affects mostly the development of the nodules, thus fewer are formed, but those already developed still grow less effected. Contrarily, lower average nodule biomass might have suggested that the antagonism affects the growth more than the development. Indeed, we observed a decrease in both number and biomass, under both PEMV infection and pea aphid herbivory. As predicted, there is an additive effect of PEMV and aphids on rhizobia colonisation of host plants, where PEMV affects rhizobia more than the pea aphid. We didn't observe a significant difference in average nodule biomass (Figure 12). Thus, our results support the PEMV antagonism against rhizobia [7,8].

We hypothesised that the impairment of nodule formation was caused by an increase of SA, a known nodule-shrinking agent, usually induced by PEMV and pea aphid attack of host plants. According to the "decoy hypothesis", the induction of SA would interfere with the JA-dependent defences that are most effective against PEMV and pea aphid. We observed such an increase of SA levels only upon infection by PEMV, even in combination with rhizobia and pea aphid. Contrary to our prediction, the pea aphid herbivory alone did not increase SA and the combination with PEMV resulted in lower levels of SA compared to PEMV-infected plants. Thus, PEMV appears to be the main antagonist against rhizobia. Additionally, the weaker effect when both PEMV and pea aphid attack might suggest the existence of a damage threshold to the decoy hypothesis. Above such threshold, the damage is too extensive to avoid triggering JA-dependent defences, thus interfering with SA. Supporting this, we observed an increase in JA when both PEMV and pea aphid attack the plant in comparison to plants only infected by PEMV. Subsequently, JA might not be the only decoy or it might not be decoy at all, but only a compound in the same pathway [17].

Concerning the antagonism against the pea aphid, a previous study showed that rhizobia colonisation reduced both the number of winged aphids and their biomass, while reducing PEMV infection rate. We hypothesised that this was due to an increase of JA as a product of ISR triggered by the rhizobia. Since JA and SA pathways interfere with each other, we predicted that rhizobia would have led to higher JA and lower SA concentrations. Interestingly, it was also hypothesised that the changes on plant phenotype that impacted aphid vectors were not necessarily driven by high JA concentration, but rather sensitivity to JA. In the current work, we observed the effect of rhizobia on neither JA nor SA, suggesting that the

altered plant phenotype detrimental to aphids can indeed be the plant sensitivity to JA levels rather than its concentration to increase. However, we did not assess the impact on the aphid population nor on the PEMV infection. Instead we based our hypothesis on a previous study focused on the aphid population. Thus, we cannot exclude that the lack of effect of JA corresponded to lack of effect on JA-dependent defences. An analysis of defence compounds and key genes in the JA-pathway is needed to exclude the absolute lack of effect of rhizobia on the JA-dependent defences. Thus, our results support the antagonism only unidirectionally, from PEMV against rhizobia. Nevertheles, based on the previous study, we hypothesise that JA is not the key phytohormone and its pathway is not the only one involved in the response against phloem-feeders.

Based on hypothesised cross-communication between the JA-signalling pathway and the ABA-signalling pathways through common transcription factors, we predicted that an effect on JA would have affected ABA. Instead, we did not observe any effect on ABA by any interaction.

In conclusion, we found that rhizobia increased root biomass while pea aphid reduced it. As expected, PEMV decreased the biomass of both plant shoots and roots. Surprisingly, rhizobia did not affect phytohormone levels. Conversely, PEMV increased both JA and SA concentration on plant leaves. As pea aphid herbivory in combination with PEMV increased JA while reducing SA, either the decoy hypothesis has a damage threshold, or the decoy target is not JA itself. Finally, despite evidence of cross-communication between JA and ABA, we found no effect on ABA levels in any plant treatment.

In order to investigate the possible lack of effect of rhizobia and pea aphid on shoot biomass, we recommend analysing the stored leaf and phloem samples with GC-MS to assess the effect of the multi-trophic interactions on metabolites, in particular sugars and amino acids. Subsequently, we suggest identifying candidate biosynthetic pathways from the results and assess the expression of key genes via qRT-PCR.

To investigate whether the antagonism between PEMV and rhizobia is mutual, we suggest using the same GC-MS to target also flavonoids and other defence compounds to assess the effects on JA-dependent defences induced by rhizobia and detrimental to aphids. Subsequently, we also suggest identifying candidate biosynthetic pathways from the results and assess the expression of key genes via qRT-PCR. Also ethylene and volatile organic compounds are likely to be involved in the plant response against aphids and viruses [24,27]. However, they would require a new experiment with different harvesting, extraction and analysis methods. Thus, a new experiment would be needed. In a new experiment, we would suggest assessing the spreading of the virus in leaves, root and nodules through ELISA using the CP as ligand or q-PCR. Finally, we recommend assessing the influence of rhizobia on the

competence of infectious aphids in transmitting PEMV by estimating PEMV titres in target plants through q-PCR.

Assessing reciprocal interaction among rhizobia, PEMV and pea aphid is fundamental for understanding the ecology of viral disease spread and crop productivity. Thus, this understanding improves crop management strategies for leguminous. Such improvements are ever more necessary due to the increasing demand of leguminous as healthy and sustainable food and fodder. As this increase in demand is not yet matched by an increase in yields, the relevance of this research increases as well.

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Supplementary Material

Biomass recordings: Excel Spreadsheet

Phytohormones concentrations: Excel Spreadsheet

Data Analysis: R markdown scripts and csv datasets