This is a draft manucript for the MICROP MeJA concentration pilot

SUMMARY

Material and methods

References

WHERE TO PUBLISH?!

Papers listed by heart/memory/experience:

New Phytologist

Plant Cell and Enviroment

Trends in Microbiology (17.07) <https://www.sciencedirect.com/journal/trends-in-microbiology>

PNAS (12.29) - [https://www.pnas.org/](https://www.pnas.org/" \t "_blank)

ISME (10.3) - <https://www.nature.com/ismej/>

Microbiome - (11.6) - [https://microbiomejournal.biomedcentral.com/](https://microbiomejournal.biomedcentral.com/" \t "_blank)

Science of the total enviroment (7.96) - <https://www.journals.elsevier.com/science-of-the-total-environment>

mBio (7.867) - [https://journals.asm.org/journal/mbio#](https://journals.asm.org/journal/mbio)

PLOS biology (7.07) - https://journals.plos.org/plosbiology/

Molecular ecology (6.18) - [https://onlinelibrary.wiley.com/joufrnal/1365294x](https://onlinelibrary.wiley.com/joufrnal/1365294x" \t "_blank)

Frontiers in Microbiology (5.64) - <https://www.frontiersin.org/journals/microbiology>

Environmental Microbiology (5.49) - <https://sfamjournals.onlinelibrary.wiley.com/journal/14622920>  
Soil biology and biochemistry (5.29) - <https://www.journals.elsevier.com/soil-biology-and-biochemistry>

Biology and fertility of soils (5.5) - <https://www.springer.com/journal/374?gclid=CjwKCAjw-ZCKBhBkEiwAM4qfF72rOmZkBm0ybpgfnc10tJEldzRdise64Mwj4MUk4eyKBkW3If8-_BoCsxMQAvD_BwE>

applied and environmental microbiology (4.93) - <https://journals.asm.org/journal/aem>

FEMS microbial ecology( 4.19) - <https://academic.oup.com/femsec>

Plant and soil - (4.01) - <https://www.springer.com/journal/11104/?gclid=CjwKCAjw-ZCKBhBkEiwAM4qfF88g0ntjZoTn4v_jYBsz4z4U9eeFlMXtYXafxvdvDmSaYHO-L4KYaRoCF78QAvD_BwE>

Molecular Plant-Microbe Interactions (3.69) - <https://apsjournals.apsnet.org/page/mpmi/about>

Phytobiomes (3.24) - <https://apsjournals.apsnet.org/toc/pbiomes/current>

Rhizosphere (3.12) - <https://www.journals.elsevier.com/rhizosphere>

FEMS microbiology letters (2.77) <https://academic.oup.com/femsle>

Plos One (2.7) - <https://journals.plos.org/plosone/>

***Essential paper outline***

**Introduction**

* plant-insect-microbe interaction
* Holobiont
* data deluge

**Results**

* Plant growth & qPCR (show plants were stressed)
  + 1 figure with 4 pannels, raw plant biomass values [SUP?]
  + 1 figure, Cohen’s D effect size across pecies and variables
  + qPCR plot
* Beta & alpha diversity [2 figures, 3 SUP tables](Basic analysis present in any paper)
* Neutral models [3 figures, 2 sup tables]:
  + Neutral model fits (subseting community and re-ploting beta diversity)
    - PERMANOVA bootstrapping to Check artifacts
  + Above-expected differential abudances (heat tree focus on comamonadaceae)
  + Alpha diversity regression (focus on comamonadaceae)
* Differential abudance [1 fig (SUP?)](keep very short)
* Random forest [1 Fig (SUP?), 1 SUP table ](](show ASVs that matter on prediction)
* Network analysis (describe networks, ASVs with importance tags (number and families), Leave module discussion on supplementary)
* Fisher Summary (describe figure, highlight comamonadaceae)

Comamonadaceae details (show diversity and abudance over treatments)

**Discussion**

**MAIN GOAL: VALIDADE THE EXPERIMENTAL APROACH! - MeJA impacts the microbial community similarly to Oral Secretion, thus MeJA can be used in place of real insects**

**Advantages Methodological approach:**

**1) the neutral spliting-and-joining can help see subtle treatment effects ;**

**2) the neutral tree can help locate a diversity hotspot (mention rhizobiales but limit discussion)**

**2) the fisher test in a heat tree helps summarizing findings (approach to data deluge)**

**SECONDARY GOAL: HIGHLIGHT MAIN TAXA - Family Comamonadaceae are relevant to this experimental system**

**CONCLUSION: Comamonadaceae are important in insect-plant interactions**

Glossary and abbreviations

Treatments:

* Control
* MeJA 0.1mM
* MeJA 1.0mM
* *P. brassicae* OS

Species:

* *thaliana*
* *oleracea*

Plant Compartment:

* Rhizosphere / Rhizospheric communities
* Endosphere / Endospheric communities
* Rhizosphere samples
* Root samples

**Introduction**

In these neutral models, ASVs are classified as above the neutral (selected by the environment), as predicted by neutrality (the expectation is that ASVs that have many sequences should be found in many samples) or bellow expected (underdispersed, thus missing in some samples) . the slope defining the data’s fit to the model are based on a data-derived migration parameter, which quantifies the chance of samples being re-sampled from the same environment after random removal from the OUT table.

MeJA was already used by other authors to simulate insect attack. On the Brassicaceae *Cardamine cordifolia*, 1mM Jasmonic Actid was applied as a spray in the field, with 50ml per 0.25m² patch (<https://www.nature.com/articles/s41559-019-1085-x> )

**Matherial and methods**

*Experimental design and plant preparation*

Brassica oleraceae var riveira and Arabdopsisi thaliana col-0 seeds were stratified in wet filter paper and kept at 4C for 48h. stratified seeds were sown in seedling trays in 20/oct/2020 and transplanted 1 week later.Plants were harvested on 27/nov/2020 at 5 weeks old.

CFU counting: 200ul inoculation of serially diluted soil in 0.85% NaCl. Plated in LB media, amended with 50ug/L cycloheximide to suppress fungi LB: 10g Tryptone, 5g yeast extract, 5g NaCl, 15g Agar (per Liter) Ciclohexamide: 50mg/ml stock, diluted in 96% ethanol. Utilized 1 ml/L media.

*MeJA stress application*

The 1 mM and 0.1mM Methyl Jasmonate (MeJA) solutions were prepared with from a 1M stock of Methyl jasmonate 95%(Sigma 392707-5ML) diluted with ethanol 96%. The final 1L use solution has 0.1% MeJA solution in Ethanol 96% and 0.015% silwet. Control solutions received only 0.1% ethanol 96% and 0.015% Silwet. To dip the plants on MeJA solutions, first a 0.05mm mesh was placed around the base of the plants, and was left in place until the end of the experiment. Then, a plastic cover with a large central opening was placed on the surface of the pot, fully covering the internal edge of the pots and the edge of the mesh. With this the plants could be turned upside down without significant losses of soil. This allowed the aerial part of the plants to be fully submerged on the MeJA solutions for 2-3 seconds without application of MeJA on the soils. MeJA was first applied when plants were 3 weeks old, and then every 4 days thereafter until 4 applications had taken place. Plants were harvested 2 days after the last MeJA application. We utilized a Complete randomized block design with 6 blocks and 6 replicates/treatment (figure\_experimental\_design).

*RNA extraction and qPCR*

we sampled leaf tissue 3 hours after the last MeJA dipping (sampling between 2:20 and 4pm)  
For Arabdopisis, we collected 2 whole leaves from each plant, avoiding old leaves  
For Brassica, we collect 4 leaf punches (leaves 3 and 4. for the 1mM MeJA treatmentment we sampled leave 3 and the most damaged leaf (1 or 2))  
All materials were cleaned in between samples. we also chilled the leaf puncher and kept all tubes on liquid nitrogen during the sample loading process. We pre-cooled the tubes in liquid N, and kept the samples in liquid nitrogen until storage on -80C.

*Plant phenotyping*

*DNA extraction and 16S amplicon sequencing*

For harvesting, roots were gently shaken, and then stored in a 50ml falcon with 20ml of autoclaved 0.85% NaCl solution. They were then gently inverted 10 times. After the 10 inversions, roots were colected with a ethanol-cleaned tweezer to a 10ml tube with 6ml of autoclaved 1x TAE with 0.05% tween 20. The pre-washed roots (free of rhizopsheric soil) where then incubated sideways on the orbital shaker for 2 minutes, 400RPM. washed roots were then collected with a ethanol-cleaned and flame-sterilized long tweezer, and transferred to another tube (1x TAE with 0.05% tween 20.)  for additional washing. The process was repeated another time for 3 washes in total. Fully washed roots were then transfered to a 2ml tube, flash-frozen, and stored at -20.  
All 3 washes were stored on -20 for later consolidation into (2 tubes of 9ml) and centrifugation (15 min 5400 g) to generate a soil sample. up to 300ul of ressuspended pellet was used as template for soil DNA extraction. DNA extraction was performed with QIAGEN PowerSoil Pro kit. Roots in 2ml tubes were left to lyophilize for 48h, and then they were ground to dust on a painshaker for 150 sec. Powdered roots DNA was extracted with Qiagen dneasy plant pro. DNA extracts were submitted to 16S rRNA sequencing on the V3-V4 region with a Illumina MiSeq (PE300bp) at Baseclear (Leiden, the Netherlands). Libraries for root samples were prepared with PCR blockers to prevent amplification of plant DNA.

*Microbiome data pre-processing*

Adapter removal and demultiplexing of sequencing data was performed by Baseclear (Leiden, the Netherlands). Trimmomatic 0.39 was used to trim the ends of the sequences, and Cutadapt pluing in QIIME2 2021.2 was used to remove primer sequences. QIIME2 was used to apply the DADA2 pipeline for merging, denoising, and clustering of Amplicon Sequencing Variants (ASVs). A re-trained naive bayes classifier was used to classify taxonomy against the SILVA 138 SSU release with the sklearn option in QIIME2. This data was processed in the High Performance Computing Cluster Anunna (Wageningen University) and used scripts are available at <https://github.com/PedroBeschoren/WUR_HPC_Annuna>.

*Statistical analysis*

For the application of Sloan’s Neutral community models, four neutral models were generated for *B. oleraceae* rhizosphere samples, and 4 neutral models were generated for *A.thaliana* rhizosphere samples. Each model contains only the samples from a single stress treatment, but includes all samples from the data partition as a pooled source of microbes for the model’s migration parameter. This means that only a subset of highly related samples could be within the pool of microbes for selection in each treatment. Thus ASVs in the roots of *A. thaliana* do not contribute for the models of stress treatments in the rhizosphere of *B.oleraceae*. Neutral models were not constructed for root samples because of uneven sample sizes, which can affect results[cite]

To check for artefacts on the permanova based on ASVs selected by the neutral models, we performed a bootstrapping aproach. First, we select random ASVs from each treatment, instead of only selecting the ones tagged as above expected. From each treatment, we select a number of ASVs equal to the number of ASVs defined as above expected. Then, we join these ASVs together again in a same matrix and test for community similarity with a PERMANOVA. Finally we check the p, F and R2 values of these random ASVs, and compare them to the p and R2 values of the full community and of the above-expected subset. This process was repeated 100 times for each of the 2 data partitions. We utilized both CSS-normalized data and rarefied data for this test.

Random forest… Model performance was evaluated based on the model’s accuracy and Kappa, based on a 5-fold cross validated random forest (RF) repeated 100 times.

Network analysis was performed with the SpiecEasi package in R. Nodes were defined as keystone taxa if their degree and betweeness centrality, or their degree and closeness centrality, were higher the average + 1.65 standard deviations of the same metric in the network. All metrics were log-transformed prior to calculation. This aproach is similar to a one-tail test of the metric’s z distributions and is available as the function KeystoneDetector3.

Fisher test….For simplification, this analysis is unweighted. ASVs with more than one importance tag simultaneously, such as ASV\_410 from genus Nocardioides, are treated identically to an ASV that has only been tagged by a single pairwise differential abundance. Our approach currently uses rarefied data as input for the fisher tests, but sequential rarefaction could help alleviate issues generated by the rarefaction method.

**Results**

*Plant phenotype & gene expression*

Leaf dry weight and area were strongly affected by the stress treatments, with 1.0 MeJA exposure significantly reducing *B. oleraceae* biomass. For *A. thaliana*, the number of fruits of 1.0 MeJA exposed plants was significantly reduced, while the reduction of inflorescence dry weight was not significant. Nonetheless, 1.0 MeJA exposure could clearly impact plant development when compared to controls (figure\_plant\_phenotype), and necrotic spots could be observed in the surface of leaves after the first 1.0mM MeJA dipping event (figure\_plant\_pictures). These lesions were more clear in *B. oleraceae* than in *A. thaliana*. Interestingly, such lesions only occurred after the first dipping with 1.0 mM MeJA: subsequent dippings with 1.0 mM Meja did not cause further lesions. When exposing *B.oleraceae* to intermediate MeJA concentrations in another experiment (data not shown), we observed that a 0.5mM MeJA application did not cause these necrotic spots, but prevented their appearance on a subsequent dippings of 1.0mM MeJA. Finally, *B.oleraceae* leaves surface had a noticeable higher amount of wax when exposed to higher cocnentrations of MeJA

qPCR Expression of the plant genes MYC2 and LOX2, respectively located upstream and downstream of the JA-biosynthesis pathway (?), is reported on figure\_qPCR. Although treatments have high variance, MeJA 1.0 mM could clearly increase the expression of both genes in *A. thaliana*. For *B. oleraceae*, MYC2 expression was identical cross treatments, but LOX2 expression was slightly lower in control compared to any of the other treatments.

*Beta diversity for the whole community shows limited treatment effect*

As expected, the factors sample types (root and soil) and plant species (*B. oleraceae* an *A. thaliana*), explain most of the variance in the dataset, with R² of 0.16 and 0.10, respectively (SUP\_Permanova\_table\_a). These factors clearly separated the samples on the ordination space (Figure\_Beta\_diversity\_all\_samples). The effects of stress treatment are dependent on the plant species, as noted in significant interactions, but explain little variance (F = 1.841, R² = 0.04, p = 0.001). When analysed in separate, we find significant effects of stress treatment in endophtic communities of both plant species and in the rhizosphere of A. thaliana (SUP\_Permanova\_table\_b). After FDR correction, the only significant pairwise difference is between both MeJA concentrations on the endosphere of A. thaliana. (SUP\_Pairwise\_permanova\_table).

*Sloan’s Neutral models indicated taxa that separate treatments in beta diversity plots*

Rhizosphere communities from each plant species (Figure\_beta\_div\_neutral\_A) were used to construct one neutral model for each treatment. We could observe that the model fit was higher in A. thaliana (r² =0.603 ± 0.018) than in B oleraeae (r² = 0.481 ± 0.004) (Figure\_beta\_div\_neutral\_B). When the subset of above-expected taxa is evaluated in an NMDS, it is clear that they represent very different sub-communities (Figure\_beta\_div\_neutral\_C), which is supported by testing with PERMANOVA(Table\_Permanovas\_above\_neutral). All pairwise comparisons between treatments had corrected p values < 0.005 (SUP\_table\_pairwise\_comparisons\_neutral).

To generate the plots in Figure\_beta\_div\_neutral-C we first subset the taxa to include only ASVs classified as above expected (highlighted in teal on figure Figure\_beta\_div\_neutral-B). This was a subset of 377 ASVs for *A. thaliana* and 336 ASVs for *B. oleraceae*. To check whether the observed effects were due to ASV picking from within the 4 different treatments, we used a bootstrapping approach by picking random ASVs from the same samples 100 times (SUP\_Table\_Artefact\_check). Explained variance is at least 3.25 fold higher for the neutrally selected ASV set (R² = 0.656 for *A. thaliana* and 0.691 for *B. oleraceae*) than in the randomly picked ASV set (max R² = 0.202 for *A. thaliana* and 0.198 for *B. oleraceae*). Also, the F value is at least 7.24 fold higher in the neutrally selected ASV set (F = 14.152 for *A. thaliana* and 15.528 for *B. oleraceae*) than randomly picked (max F = 1.945 for *A. thaliana* and 1.729 for *B. oleraceae*). This shows that sets of randomly picked ASVs cannot nearly explain the stress treatment effects or community variance as well as the ASVs defined as above expected by the neutral model.

*Differential abundances in taxa occurring above expected values of a neutral model*

The subset of ASVs occurring above expectation of the neutral model also presented differential abundances in pairwise comparisons between treatments. This can be shown with the hierarchical taxonomies of ASVs in a large tree, with the small trees highlighting differentially abundant ASVS of each pairwise comparison in a matrix (figure Neutral\_heat\_trees). This matrix of heat trees can be interpreted by looking for (dis)similarities across rows and columns. For example, some taxa are consistently more abundant in all the treatments when compared to controls. This is the case for the genera *Nitrospira*, *Caulobacter*, *Pedobacter* and *Streptomyces* in *A. thaliana* and the genera *Bosea*, *Pelomonas*, *Sporocytophaga*, and genus 67-14 from Order olirubrobacterales in *B. oleraceae*.

In addition, hotspots of differential abundance, where different members of a supertaxon are being highlighted across different treatments, can be found in some taxons. This is the case for Family Commonadace in *B.oleracea*, as all pairwise comparisons between treatments include genera that are differentially abundant in both compared treatments. This also occurs in the Order Rhizobiales and Burkholderiales (even if we ignore members of family commonadaceae), and phylum Actinobacteriota. In A. Thaliana, Order Rhizobiales, Class Alphaproteobacteria (even when ignoring members of Order Rhizobiales), Class Gammaproteo bacteria, and Class Bacteroidia (specially members of family Chitinophagaceae) also act as hotspots of differential abundance.

However, it is possible that these taxa are very diverse in the above selected subset only because they were very diverse in the full dataset. To test for that, we fitted the taxa diversity metrics in the subset of above-neutral taxa against the diversity of the same taxa in the full dataset (figure\_alpha\_correlation\_Family, SUP\_figure\_alpha\_correlation\_Order\_Class). Class Actinobacteria, Order Rhizobiales, Order Xanthomonadales, and Family Commonadaceae are all above the confidence interval of a loess regression between the diversity of above-selected ASVs and the diversity of the complete dataset. Expect for Simpson diversity of Order Rhizobiales in *B.oleraceae*, this pattern holds for observed number of taxa, Shannon diversity, and Simpson diversity for both plant species,

*Alpha diversity*

The Shannon diversity index indicated a clear interaction between plant species and sample type (p = 0.0004, SUP\_table\_alpha\_diversity\_tests). *A.Thaliana* had higher diversity than *B.oleraceae* on soil samples, while *B.oleraceae* had more diversity than *A.Thaliana* on root samples (Figure\_alpha\_diversity). The model also indicates a significant stress treatment effect, as medians are overall lower in controls and MeJA 1.0 mM applications than Meja 0.1mM and oral secretion treatments.

*ASV Differential abundance per treatment*

Differential abundance analysis performed by deseq2 indicated 117 unique ASVs as differentially abundant across all control-treatment pairwise comparisons. Root samples clearly had more differentially abundant ASVs than soil samples, and *A.thaliana* samples had more differentially abundant ASVs than *B. oleraceae samples*. Whily sample type and plant species were separated in a bi-cluster heatmap, there was no clear pattern for groups of ASVs occurring in the same treatments (SUP\_Figure\_deseq2\_heatmap).

*Random Forest*

The Boruta R function tagged 41 ASVs as important to predict the stress treatment in the different sample partitions. We could observe that models based on entophytic communities had a higher number of ASVs classified as important when compared to rhizosphere communities. While accuracy and Kappa for the *A.thaliana* endophytic and rhizospheric models were quite similar, it was much lower in the rhizosphere communities of *B. oleraceae* when compared to endophytic communities (SUP\_Table\_RF\_output). As can be seen in the confusion matrixes, oral secretion treatments had the overall lowest prediction accuracy (SUP\_table\_confusion\_matrix). The abundance of the 41 ASVs selected by random forest can be seen in figure Figure\_rf\_ASVs. The taxonomies of these ASVs and their importance for the Boruta model are shown in SUP\_table\_rf\_taxonomies. The most common genera is Massilia (4 occurences), the most common family is Commonadaceae (9 occurences), the most common order is Burkholderiales (18 occurences). The highest mean ASV importance is found in ASVs from genus Mucilaginibacter for B. oleraceae rhizosphere; genus Leptothrix for *B. oleraceae* endosphere; genus Asticcacaulis for *A.thaliana* rhizosphere; and genus Niastella for *A.thaliana* endosphere. There were no ASVs that were important in more than one sample partition.[TO-DO: ADD ASTERISC ON BOXPLOT FIGURE, INDICATING WHO IS A COMAMONADACEAE]

*Network Analysis*

We created four co-variance networks, according sample types and plant species, which could be differentiated from random networks with the same number of nodes and edges. (SUP\_table\_random\_networks). Networks based on rhizosphere communities were more complex than network based on endosphere communities. They presented a higher number of nodes, number of edges, average degree, and maximum module size. Endosphere communities presented higher modularity, and higher ratio of positive to negative edges. The node degree of *B.oleraceae* samples presented a higher fit to power law than *A. thaliana samples*. The total number of keystone nodes, module conector nodes, module hub nodes were also higher in rhizosphere community networks than in endosphere community networks (SUP\_figure\_network\_metrics\_PCA). There was a total of 34 ASVs tagged as keystone taxa, module connectors, or module hubs for *A.thaliana* and 47 for *B.oleracea.* From the 8 keystone taxa, four were found in the rhizosphere communities of *B. oleraceae*, including ASV\_410. This ASV, from genus Nocardioides, was also classified as above neutral model predictions in all treatments and as a predictor of treatment classification by the random forest algorithm. It was the only ASV in with 3 different “tags” of importance from different methods.

*A visual summary of differential abundance, network analysis and random forest*

Differential abundance, random forest and network analysis tagged 346 different ASVs as important across the sample partitions. This pool of selected ASVs was represented in a heat tree, where we also represent fisher tests to compare proportions of each taxonomic level in the important ASV subset against the rest of the community (Fisher\_test\_relevance). The test essentially returns, after a p adjustment, whether a specific taxa is significantly more represented in the important ASV subset than on the rest of the community. For example, 8 of the 53 important ASVs in *B.olerceae* soil samples are from the genus Mucilaginibacter, while 57 out of 2499 ASVs in the non-important ASV set are from the genus Mucilaginibacter. A one-tailed fisher test indicates there is a significant difference in these proportions (padj = 0.0228) with an odds ratio of 4.18. Thus the proportion of Mucilaginibacter in the important ASV set is significantly higher than the proportion of Mucilaginibacter in the non-important ASV set. This approach allows us to summarize the output of the 3 methods across all taxonomic levels for all samples of all data partitions at a glance.

It can be observed that family Commonadaceae is highlighted as overrepresented in the important ASV sets in *B. oleraceae* roots and soils, and soils of *A. Thaliana*. We also highlight order Rhizobiales as important in roots and soils of *B. oleraceae*, and genus Flavobacterium in roots and soils of *A. thaliana*. Finally, Genus Streptomyces is very clearly highlighted as relevant in the roots of *A.thaliana*, and family Xanthobacteriaceae as important in the soils of *B. oleraceae.*

As the fisher proportions highlighted Comamonadaceae in 3 out of 4 sample partitions and this family was also highlighted by the neutral model analysis, we further analyzed it (SUP\_comamonadaceae\_diversity). The Shannon diversity of Comamonadaceae in both rhizosphere and endosphere of *B. oleraceae* was slightly higher in MeJA 0.1mM than in the controls. This is observable in the full Comamonadaceae community, Comamonadaceae occurring above expected by the neutral model, and comamonadaceae tagged as important. On the full comamonadaceae community it is also observable that the MeJA 1.0 and Oral Secretion treatments also have a slightly higher diversity than controls. despite the higher diversity, the abundance of above-neutral Commamonadace in the MeJA 0.1mM is actually lower than the controls. In *A. thaliana* we observe that MeJA 0.1mM has the highest Shannon diversity in the endosphere, even as it does not have the highest abundance. When we focus on the above-expected or important comamonadaceae in the rhizosphere, we see that control and MeJA 1.0mM have lower diversity, while MeJA 0.1mM and OS treatments both have higher diversity. In both plant species, the Shannon diversity of important Comamonadaceae in the endosphere is greatly reduced at MeJA 1.0mM, even as the abundances of comamonadaceae are very similar. While many of these differences are not statistically significant (SUP\_table\_commamonadaceae\_diversity\_and\_abundance), we had already established that this family is overly diverse in the above-expected subset and overly represented in the important ASV set.

**Discussion**

**MAIN GOAL: VALIDADE THE EXPERIMENTAL APROACH! - MeJA impacts the microbial community similarly to Oral Secretion, thus MeJA can be used in place of real insects**

**EXTRA DISCUSSION POINT:**

**1) the neutral spliting-and-joining can help see subtle effects ;**

**2) the fisher test in a heat tree helps summarizing findings**

**SECONDARY GOAL GOAL: HIGHLIGHT MAIN TAXA - Family Comamonadaceae are relevant to this experimental system**

**CONCLUSION: Comamonadaceae are important in insect-plant interactions**

* **With plant phenotyoe (necritic spots, biomass) and gene expression we could confirm that MeJA is impacting the plant.** 
  + **~~Should we include more MeJA dipping pilot data? In there we test 0.5mM MeJA & other species; it could be important~~**
* **There ave very clear community differences for the plant species ~ they are in different clades of Brassicaceae**
* **Microbial community Difference between treatments is subtle**
* **Centroid for Oral secretion treatments is between MeJA concentrations and/or control, showing it brings similar enought changes**
* **Order rhizobiales and Family commonadaceae were relevant across treatments, plant species, and sampling locations; they were diversity-enriched in the selected subsets**

*Methyl Jasmonate triggers plant defense*

The observed increase in LOX2 and MYC2 expression for A.thaliana, the occurence of necrotic spots for B. oleraceae, and reduction in plant dry weight for both species indicates that the plants were stressed by the dippings with Methy Jasmonate. This was expected. There are many studies that use MeJA as an stress inducer in different plants. Wheat (Liu et al., 2017) and Arabidopsis (Carvalhais et al., 2013) have been induced by applying MeJA in cotton balls, and then sealing the atmosphere in growth trays. Foliar MeJA sprays were used to induce stress in the pine tree *Larix olgensis* (Jiang and Yan, 2018) and the tallow tree *Triadica sebifera* was (Xiao et al., 2019). Arabidopsis has been exposed by dipping the plants in MeJA solutions in four-day intervals (Roeland L. Berendsen et al., 2018). it is well known that plants must choose between different defense pathways, more specifically Methyl jasmonate for chewer herbivores and necrotrophic phatogens and Salicilic Acid for sucker herbivores and biotrophic pathogens (Smets and Koskella, 2020). In this work we avoided foliar sprays as abaxial stomata may be unable to absorb the hormone, while dipping the plants was more convenient than sealing atmosphere in trays.

*Stress treatments were subtle on the structure of the full community but clear on the taxa occuring above neutraility*

Plant compartment and genotype effects are well known to shape microbial communities (Berendsen et al., 2012), thus the clear clustering observed in the full community (Figure\_Beta\_diversity\_all\_samples**)**  was expected. Previous studies indicate that microbial communities of MeJA-treated plants can be indistinguishable from controls in Arabidopsis (Roeland L Berendsen et al., 2018; Doornbos et al., 2011), different in endophytic but not rhizospheric or bulk soil communities (Liu et al., 2017), or different in rhizospheric communities but not on bulk soils (Carvalhais et al., 2013). We found that community differences due to the experimental stress treatment could be significant depending on plant species and sample type, but were subtle overall.[DID THIS REFERENCES TEST MEJA IN A SINGLE PLANT SP?]

However, when analysis is focused on the ASVs classified as above expected by the Sloan neutral community model (Figure\_beta\_div\_neutral b), treatment effects on microbial communities become surprisingly clear (Figure\_beta\_div\_neutral c, Pairwise\_permanova\_neutral\_table). This suggests that each treatment selects a different portion of the microbial community. Interestingly, the oral secretion treatment that acts as a positive control is clustered between both MeJA treatments for *A. thaliana*, and in between control and MeJA 0.1 treatments for *B. oleraceae*. This suggests that for *B. oleraceae* the 0.1mM MeJA treatment may accentuate the ASV selections of Oral Secretion treatment when compared to non-stressed controls. If control samples were clustered between the oral secretion and MeJA treatments we would know that they would be selecting for completely distinct microbial communities instead of an extension of observed differences. For *A. Thaliana*, it could be argued that an intermediate concentration of MeJA could be even closer to the oral secretion treatment centroid of the NMDS. Taken together, we consider that the effects of MeJA treatments on the neutrally-selected microbial communities are similar enough to the oral secretion positive control treatment to be used as a proxy for insect herbivore pressure in Brassicaceae plants. Previous work using MeJA as a proxy (Roeland L. Berendsen et al., 2018; Carvalhais et al., 2013; Doornbos et al., 2011; Liu et al., 2017) did not apply neutral community models.

The neutral model expectation is that ASVs occurring in high mean relative abundance (that is, have a large number of reads) should also have high frequency (are present in many samples) because they are neutrally or stochastically distributed. It has already been reported that the majority of ASVs fits the neutral model, from fish guts (Burns et al., 2015; Heys et al., 2022), to human lungs (Venkataraman et al., 2015), bacterioplankton (Wang et al., 2020), and geographically distant soils (Barnett et al., 2020). A deterministic process could shift the community away from neutrality (Barnett et al., 2020), with microbes more fit to the environment occurring more often than predicted by the model. These above-expected ASVs may represent “positively selected” microbes (Burns et al., 2015). Higher selection pressure in the community can potentially be seen as lower fits to the model, such as lower neutrality fit in fish gut microbes as the animal develops (Burns et al., 2015; Heys et al., 2022), alterations in bacterioplankton during cyanobacterial blooms (Wang et al., 2020), or in soil microbes in later successional stages after a disturbance (Barnett et al., 2020). In our study, *B. oleraceae* samples had a lower fit to the neutral model than *A. thaliana* samples, so selection pressures could be higher in *B. oleracea* communities.

We consider that this approach of detailing above-selected ASVs is very useful when treatment effects are subtle. Our bootstrapping approach to detect artefacts show that a same number of ASVs picked random from the same samples cannot nearly replicate the clear separation made by the neutral models. It is worth mentioning that the input data for Sloans’ neutral models should be rarefied. Rarefaction can normalize different sequencing library sizes, but it is currently under criticism and disuse (McMurdie and Holmes, 2014). While rarefaction level seems to be of little relevance in neutral models (Weiland-bra et al., 2019), approaches like repeated rarefaction (Cameron et al., 2021) could help alleviate the issue.

*“Fishing” with Fisher: 1 out of 1.111 taxa highlighted by 3 different methods*

A major challenge in microbiome analysis is addressing the complexity and diversity of microbial communities. Several analysis methods, each with their own advantages and limitations, have been developed to address this issue. Differential abundance, random forest, and network analysis, can all identify ASVs of special relevance for an experimental system. While applying different methods may help alleviate their individual drawbacks, it is still difficult to summarize their output, especially when considering the hierarchical taxonomic levels across plant species and sample types. Therefore, focusing on the 346 ASVs detected as important in these 3 methods is a challenge on its own, without tools designed to address it. Our approach was to use a statistical test to check if a taxa level has been overly represented in the list of “important” ASVs, considering the microbial community in the same dataset as a background. Once this is performed for all taxa levels in all “important” ASVs, we plot the results in a heat trees from the metacoder package, a recent tool on visualizing taxonomies of microbial communities. A relevant feature of our approach is that it is independent of the tools that tag ASVs as relevant or not. For example, network analysis could be replaces by indicator species analysis, or random forest could be substituted by genetic algorithms. As long there is a list of “important” ASVs drawn from a full microbial community, our approach can be applied to summarize results from different methods. In our data, this approach highlighted family Comamonadaceae as overly represented in them important ASV set in 3 our of 4 sample partitions, which facilitated further analysis and in depth discussion in only 1 out of the 1.111 different taxonomic groups evaluated in this report.

This visualization also gives us a taxonomic pathway: instead of stating that proteobacteria will be important in soil systems, like has been done many times before, we could find the right taxonomic level that responds to our experimental system. The Suprataxons for family Commonadaceae (order burkholderiales, class gammaproteobacteria, and phylum proteobacteria) are also all highlighted in these data partitions. However, the heat tree visualization suggests that Commonadaceae could be driving the relevance of these suprataxons in this analysis. An infrataxon to family Commonadaceae, the plant pathogenic genus *Acidovorax* (Fujiwara et al., 2022), presented a very high fold ratio in the rhizosphere communities of *b. oleraceae*. This indicates that this genera is clearly much more present in the important ASV subset than on the non-important ASV subset, and could be driving the weight given to family Commonadace in this sample partition. Genus Streptomyces is also clearly highlighted as overrepresented in the important taxa of A. thaliana roots. This genus has also been highlighted on the neutral model analysis, as above-expected Streptomyces are more abundant in the treatment conditions than in the controls.

*Family Commonadaceae was relevant across treatments, plant species, sample types, and analysis methods*

Our analysis based on neutral models indicates Family Comamonadaceae as relevant because there is always a different member in this taxa being selected by the different treatments in pairwise comparisons (**Neutral\_heat\_trees**). We also fitted the diversity of families within the above-neutral subset against the diversity of families in the full datasets (**figure\_alpha\_correlation\_shannon**), confirming that Comamonadaceae were indeed overly diverse. The summarization of differential abundance, network analysis, and random forest indicates Family Commonadaceae as over-represented in the set of ASVs tagged as important when compared to the ASVs not tagged by these methods. Note that this summarization does not consider any results from the neutral model, and is therefore both analyses are independent. Although the endosphere of *A.thaliana* does not highlight Comamonadaceae, we focus the discussion in this family.

Comamonadaceae have a very diverse physiology and live in a wide range of habitats. They have been detected in plants both as a pathogenic and as beneficial microorganism (Willems, 2014), and are have been described as enriched inside root systems (Bulgarelli et al., 2015; Edwards et al., 2015; Hacquard et al., 2015; Li et al., 2020). Members of the family have been associated to N nutrition (Cope-Selby et al., 2017; Pagé et al., 2019), heavy metal tolerance in phytoremediation (Chen et al., 2018), soil disease suppression (Li et al., 2015), suppression of phytopathogenic fungi trough negative network correlations (Durán et al., 2018), infestation of withe grubs (Geng et al., 2018), causation of fruit blotch in cucurbitaceae (Fujiwara et al., 2022), and also resistance to Fusarium wilt disease in cucurbitaceae. As environmental contexts like soil diversity levels can determine if the same strain can be detrimental or beneficial for plant development (Beschoren et al., 2020), having both pathogenic and beneficial microorganisms on the same family is unsurprising.

Comamonadaceae are also important in digestive systems of animals with poor diets (Willems, 2014), such as herbivorous insects and even algae-consuming carnivorous plants (Sirová et al., 2018). Comamonadaceae have been found to be abundant in the guts of the herbivore beetles *Dactylispa xanthospila* (Cui et al., 2021), cycad-feeding Rhopalotria furfuracea (Salzman et al., 2018), *Cryptocephalus* (Montagna et al., 2015)*,*  all life stages of the poor-diet dung beetle *Onthophagus Taurus* (Estes et al., 2013), besides larval stages of Lepidoptera *Trichoplusia ni* (Leite-Mondin et al., 2021), cycad-feeding *Chilades pandava* (Salzman et al., 2018), pyralid moths (Zhu et al., 2021), the sap-feeding white fly *Bemisia tabaci* (Santos-Garcia et al., 2020), and the springtail *Orchesella cincta* (Bahrndorff et al., 2018). Therefore, it is very likely that members of this family are actively shared and transferred between plants and herbivores, having significant roles in health and disease for both hosts. This can increase Comamonadaceae’s niche breath and dispersal, similarly to what happens in Enterobacteriaceae and Pseudomonadaceae, which are also very common and highly functional in both roots and guts (Brennan et al., 2022; Costa et al., 2014). For example*, Enterobacter ludwigii* was isolated from oral secretion of the fruitworm *Helicoverpa zea*, and its inoculation on tomato plants increased plant fruit and seed production compared to non-inoculated controls (Pan et al., 2019). A Pseudomonas strain isolated from *Plutella* moths larvae guts has shown to increase tomato growth and exhibit trains like tricalcium phosphate solubilization and production of indole 3-acetic acid (Indiragandhi et al., 2008), traits critical for plant growth promotion (Costa et al., 2014). The fact that indole 3-acetic acid is very relevant both in plant root metabolism and animal gut metabolism further drives ecological theory into holobionts, with a same bacteriam being beneficial to more than one host [adjust/complete this!]

It has already been established that soil fauna can transport microbes (Yang and van Elsas, 2018).

Note that while insect oral secretions can introduce microbes into the plants, like putative pathogenic *Pseudomonas* species (Humphrey and Whiteman, 2020), our results show that MeJA expositions can also increase diversity in this family, and RF shows their presence is not an exclusive indication of oral secretion**.** There are also other taxa highlighted as relevant in our summary and neutral approach, like Rhizobiales, that can be interacting with insect guts. Rhizobium has been found in the gut of herbivorous insects (Leite-Mondin et al., 2021), sometimes associated relevant nutritional roles like methane degradation (Montagna et al., 2015) or N nutrition in termites (Fröhlich et al., 2007) and *Plutella* moths (Indiragandhi et al., 2008).

Comamonadace abudances in soybeans are known to be affected by isoflavonoids (Pang et al., 2021). Silencing of isoflavone synthase increased the relative abudnaces of Commonadaceae (White et al., 2017), while soils treated with the isoflavone daidzein soils increased the abundances of comamonadaceae (Okutani et al., 2019).

~~Comamonadaceae originating from plants have been found in the gut of Pyralidae (chewer)~~ but not cicada (sucking) insects. a large portion of the Pyralidae gut microbiome was estimated to be sources from the host plant microbiome, which did not happen in Cicada and mammalian herbivores. The more aerobic insect gut environment could play a role in the survival of aerobic plant-associated microbes (Zhu et al., 2021)

~~The Lepidoptera~~ *~~Trichoplusia ni~~* ~~also has comamonadacea in the gut, whether it is fed with A.thaliana or tomato leaves (Leite-Mondin et al., 2021)~~

~~The guts of cycad-feeding Chilades pandava (Lepidoptera)and Rhopalotria furfuracea (Coleoptera) are partly dominated by Comamonadaceae (Salzman et al., 2018)~~

~~Presence of Comamonadaceae differentiates the gut of the whitefly Bemisia tabaci (Hemiptera) when it is feeding from the sap of watermelon (suitable host) and pepper (less suitable host) (Santos-Garcia et al., 2020)~~

~~In (Humphrey and Whiteman, 2020) putative phatogenic pseudomonas were more abundant in the phylosphere of brasiceaceease plants that were attacked by herbivores, likely because of oral secretions. Thus our oral treatment positive control may have introduce microbes we would never be able to find in the MeJA treatments~~

~~Comamonadaceae is part of the main taxa in the gut of the springtail Orchesella cincta (Bahrndorff et al., 2018)~~

~~Comamonadace abudances are affected by isoflavones  and daidzein  (Pang et al., 2021). Isoflavonoids might be inhibiting commonadaceae but increasing Xanthomonads; acidovorax is from this family and is a plant-pathogenic genus (White et al., 2017). Dadzein-treat soils increased the abundances of comamonadaceae (Okutani et al., 2019)~~

~~Comamonadaceae as more abundant in root samples than on stool samples; also shows no overlap in most abundant taxa between roots & mammal guts (Hacquard et al., 2015). Pedro has to read this more in-depth!~~

Comamonadaceae (and fusarium) are recognized by Arabidopsis immune system as it shares a motiff (SCOOP) very similar to a brassiceaceae **[ paper with details of molecular interactions has to be thoroughly read, but this might be better off in Karen’s hands]** (Hou et al., 2021)

It has been previously reported that the root endophytic communities of wheat have lower diversity when exposed to MeJA (Liu et al., 2017). Here we note that family comamonadaceae increases rather than decrease their diversity due to MeJA 0.1mM exposition.

We also note that Comamonadaceae is not overly abundant in plants or insects when compared to other families. Nonetheless it will be a key aprt of the community, as can be seen in RF and network analysis.

Plant-insect-microbiome review, by auther present in mICROPe: (Dolan et al., 2022) obligate insect endosymbiontes have smaller genomes, so this could filter out shared insect-plant holobionts. Phloem and xylem sucking insects relying on microbiomes for essential aminoacids. Generalist insects might transmit more microbes than specialists due to host range and microbe pickup. Auxin signaling is used in insect metabolism for nutrient metabolism. The review also extensively details the JA-SA balance and how this is exploited by insects and their microbes. Family comamonadaceae, however, is not highlighted at all.

Comamonadaceae seems to be quite prevalent in the gut of different insect species (Bahrndorff et al., 2018; Estes et al., 2013; Leite-Mondin et al., 2021; Santos-Garcia et al., 2020) suggesting the insect and plant could be alternating hosts, helpling on bacterial dispersal and survival. It can even be abundant in the lumen of algae-consuming aquatic carnivorous plants (Sirová et al., 2018): (“Members are considered important for the digestion of nutritionally poor diet of animal hosts”). In fact, members of this family are good competitors This is similar to what occurs in human entorobacteria found in plant roots.

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