This is a draft manucript for the MICROP MeJA concentration pilot

SUMMARY

Material and methods

References

**Matherial and methods**

*Plant and soil preparation*

Plants planted on 20/oct/2020 (MeJA concentration Pilot)*.* Plants harvested on 27/nov/2020 (MeJA concentration Pilot), at 5 weeks old. Complete randomized block design with 6 blocks and 6 replicates/treatment.

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.

*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.

*Data analysis*

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>.

**Results**

*16S amplicon sequencing*

Sequening of 16S rRNA in the V3-V4 region generated 10.6M PE reads with an average depth of 113±10.5k for 94 samples and average phred score 35.1±02. The No-template blank control only had 755 reads. 2.509M sequences of 434±11bp were kept after denoising. After removing 88 thousand plastid and mithocndrial sequences, the dataset was filtered by removing ASVs that occured less than 8 times in the dataset. This reduced the number of taxa from 7599 to 4572, but retained 99.48% of sequences. Rarefaction was performed at 8652 sequences per sample, and removed only 43 out of 4572 input taxa (figure\_rarefaction)

*Beta diversity for the whole community*

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 (Permanova\_table\_a). The effects of MeJA treatment are dependent on the plant species, but are much smaller. These effects are very clear on the ordination space, providing a clean separation of the dataset in 4 partitions (Beta\_diversity\_all\_samples). Root samples show significant interaction effects between MeJA treatments and plant species, but soil soil samples neither this interaction nor the MeJA treatments have a significant effect on microbial community composition (Permanova\_table\_b). when considering the 4 partions independently, it is cleat that MeJA effects were not a significant contribuitor to the microbial community struction in the soils of B. oelraceae (permanova\_table\_c). However, there are no significant pairwise differences between the 4 stress treatments, in none of the 4 data partitions, after FDR correction. (Pairwise\_permanova\_table)

*Detecting portions of the microbial community that are relevant to the stress response*

altouht the full community in the data partitions is barely affected by MeJA treatments, we could observe that a ASVs occurring above expected by the Sloan neutral model (sloan ref 2007) can strongly separate the different treatments of MeJA (Figure\_beta\_div\_neutral). 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 reads should be found in many samples) or bellow expected (underdispersed) . 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.

4 neutral models were generated for boleraceae soil samples, and 4 neutral models were generated for A.thaliana soil samples. Each model contains only the samples froma specific MeJA 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 microbial options for selection in each treatment (ASVs in the roots of A. thaliana do not contribute for the models of MeJA treatments in the soils of B.oleraceae). When this subset of above-expected taxa is evaluated in an NMDS, it is clear that they represent very different sub-communities. This is corroborated by high R² values and low p values for PERMANOVA on such subsets. Interestingly, the Oral secreation treatment that acts as a positive control is ploted between both MeJA treatments for A. thaliana, and in between control and MeJA 0.1 treatments for B. oleraceae. All pairwise corrected p values between p=0.0035 and p = 0.0069. This means that each treatment selects a different portion of the microbial community. Since fit to the neutral models is strongly affected by uneven sample sizes, we only applied it to soil samples.

To generate the plots in Figure\_beta\_div\_neutral-C we first made a new rarefication including only (and all) ASVs classified as above expected (highlighted in teal) on figure in Figure\_beta\_div\_neutral-B. this was a subset of (~360 and 350) ASVs, re-rarefied at XXX reads. To check wether the observed effects were due to random ASV picking from the 4 different treatments, we used a bootstrapping approach. First, we select random OTUs from each treatment, instead of selecting the ones tagged as above expected. From each treatment, we select a number of OTUs equl to the number of OTUs defined as above expected. Then, we join these OTUs together again in a same object, re-rarefy and we perform a permanova. Finally we check the p, F and R2 values of these random OTUs, 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 fo the 2 data partitions. Some p values of random draws can be significant at a <0.05 (40x for AT, 9x for BO), and minimal p value can be similar to that of the tests (1e-4). However, explained variance is ~6.48x higher for the neutrally selected than random ASVs (~0.90 VS ~0.14). also, the F value is also much higher in the neutral selected (F = 73.44 for AT and 80.94 for BO) than random draw (F max 1.42 and 1.45). This shows that the neutrally-selected ASVs can differentiate between the different treatments far better than same amount of ASVs randomly picked from the same samples.

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

Pairwise comparisons of above-expected ASV abundance across the different treatments can be seen in figure Neutral\_heat\_trees. This figure shows the hierarchical taxonomies of ASVs, and can be more easely interpreted when looking for 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. thatliana and the genera Bosea, Pelomonas, Sporocytophaga, and genus 67-14 from Order olirubrobacterales in B. oleraceae. While there are no overlap in these consistetly enriched genera over both plant species, most of them are parts of phylum Proteobacteria and Bacteroidota, with one representative of phylum Actinobacteriota in each plant species.

It can also be noticed that the 15 genera overrepresented in A.thaliana controls belong to 11 different phyla, suggesting that stress could reduce diversity of selected bacteria at a phylum level. In B.oleraceae samples, the 8 genera consistently overrepresented in controls are found in 5 different phyla.

In this figure, It is also interesting to note taxon levels acting of hotspots of differential abundance for these taxa, where different members of a supertaxon are being highlighted across different treatments. This is the case for Family Commonadace in B.oleracea, as all pairwise comparisons 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, the supertaxons Order Rhizobiales, Class Alphaproteobacteria (even when ignoring members of Order Rhizobiales), Class Gammaproteo bacteria (specially Order Burkholderiales and Family commonadeace), Class Bacteroidia (specially members of family Chitinophagaceae) also act as hotspots of differential abundance. This suggests that diversity in these taxonomic levels can selectively respond to the inflicted stress. However, it is possible that these genera act as hotspots of selection and diversity in the treatments just because they are very diverse in the soil. To test for that, we fitted the taxa diversity metrics in this subset of above-neutral taxa against the same taxa in the full dataset in a linear model (figure\_alpha\_correlation). It can be observed that Order Rhizobiales is relatively more diverse in the selected subset for both plant species. Family Chitinophagaeae is specially more diverse in A. thaliana, while Family commonadaceae is especially more diverse in B. oleraceae – even if both families seem to be relatively more diverse in both species, and none of them are part of Order Rizobiales. On the other hand, Family Oxalbacteriaceae is relatively less diverse in the selected subset compared to the full dataset, suggesting that diversity in this family plays a smaller role in MeJA responses.

*Alpha diversity*

The Shannon diversity index indicated a clear interaction between plant species and sample type (p<0.0003). A.Thaliana had higher diversity than B.oleraceae on soil samples, while B.oleraceae had more diversity than A.Thaliana on root samples. The model also indicates a significant stress treatment effect, as medians are overall lower in controls and MeJA 1.0 mM applications. This effect is more clear when testing each of the 4 data partitions individually. In this case, Stress treatments cause significant changes of Shannon diversityonly in the roots of B.oleraceae, with controls presenting less diversity than MeJA 0.1mM and Oral secretion, but similar diversity to MeJA 1.0. table alpha\_diversity\_tests shows details of these Statistical tests.

When we look only at the ASVs selected by the neutral models, MeJA 0.1mM seems to be increasing diversity for both plant species, while the diversity of control samples is also lower for both plant species.

**Discussion**

*A subset of taxa is strongly responsive to the MeJA treatments*

The above-expected subsets are expected to represent ASVs that thrive in a particular environment and are selected (although not necessarily for the benefit of the host; the environment could simply be optimal for a specific pathogen over another). It’s well understood that microbial consortia and subcommunities will have synergistic function effects as syntrophy and other metabolic routes are shared by co-evolved microorganism. we could observe on moule 12 of AT\_soil networks the positive correlation and MYX2 expression along with a 80% dominance of OTUs selected by both oral and MeJA treatments but not selected by controls. This is evidence by different methods that this subset of bacteria can have actual impacts on the MeJA response