



SolACE - Solutions for improving Agroecosystem and Crop Efficiency for water and nutrient use

Deliverable 2.3 Microbiome genetic and functional diversity

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1. Introduction

Soil microbiota, in particular fungi and bacteria, play a major role in soil quality and functioning, largely determining soil structure, carbon and nutrient cycling, and ultimately impacting plant performance through nutrient mineralization and mobilization, especially for N and P, root growth, plant health, and possibly increasing crop yield under stress conditions, as required to achieve food security in the coming decades. In addition to the rhizosphere microbiome, plants host an endophytic microbiome, and the composition and function of both these microbiomes strongly vary with genotypic differences in plant traits, while different plant genotypes may respond differently to beneficial microorganisms.

Focusing on three of the most important European crops (bread wheat, durum wheat and potato), the ground-breaking novelty of the SolACE project was to include such below-ground processes and related traits in the breeding strategies to be implemented throughout the project, on the one hand, and to access the benefit in a range of agroecosystem management innovations and pedo-climatic conditions combining water and N or P deficit on the other hand. This will be important to achieve high crop yields and food quality through more sustainable agricultural practices and increased resource use efficiency via the promotion of plant-microbe interactions and has so far seldom been tackled in breeding programmes.

2. Results

2.1 Assessment of response of wheat panels to AMF colonization

Bread and durum wheat panels, comprising of 250 different genotypes each, were used in field trials conducted at different locations across Europe (WP2.2). Durum wheat panels were set up by CREA at the Foggia experimental station (Southern Italy) and by INRAE-AGAP Montpellier at the Mauguio experimental station (Southern France), and bread wheat panels were set up by SYNGENTA (Levroux, France) and ARVALIS (Gréoux, Southern France). At each location, plants were either grown under a combined water and nutrient (N in this case) stress or without stress, with rigorous environmental monitoring to determine the amplitude of variation for field performance and selected above- and below-ground traits under combined realistic limitations of water and nutrients in the field. Only the field trial at Foggia (CREA) was used for this work.

2.1.1 Genotypic responses of durum wheat to AMF colonization under non-stressed field conditions

In May 2018, samples were taken at the Foggia experimental station during the grain filling growth stage of the plants. Root samples of all 250 genotypes were taken from two blocks without stress. The root systems were cleaned of soil and washed several times with a water spray gun, before being dried in the oven at 60°C. At UCLouvain, two replicates of roots of each genotype were processed, one from each block, for the quantification of AM fungal colonization. The root samples were cut into short fragments (ca. 2-cm-long) and stained with the ink method (Vierheilig et al., 1998). Briefly the root fragments were first cleared with 10% KOH (at 70°C for ca. 45 min), acidified with 1% HCl at room temperature, and stained with blue ink (for ca. 30 min). Stained roots were then stored in lactoglycerol (V:V:V – 1:1:1 – lactic acid:glycerol:H₂O) to distain and preserve until use. To quantify AMF colonization, the stained root fragments were mounted in glycerine on microscope slides and covered with microscope coverslips. The percentage of root length colonized (%RLC) was assessed microscopically under 200x magnification using the magnified intersect method (McGonigle et al., 1990). A total of ca. 100 intersects were observed per slide and the presence of AMF structures (intraradical hyphae, arbuscules, and vesicles) was recorded at each intersect.

Mean colonization rates of the 250 durum wheat genotypes in the field ranged from 3 to 50%, showing a broad mycotrophic spectrum across the 250 genotypes (Figure 1).

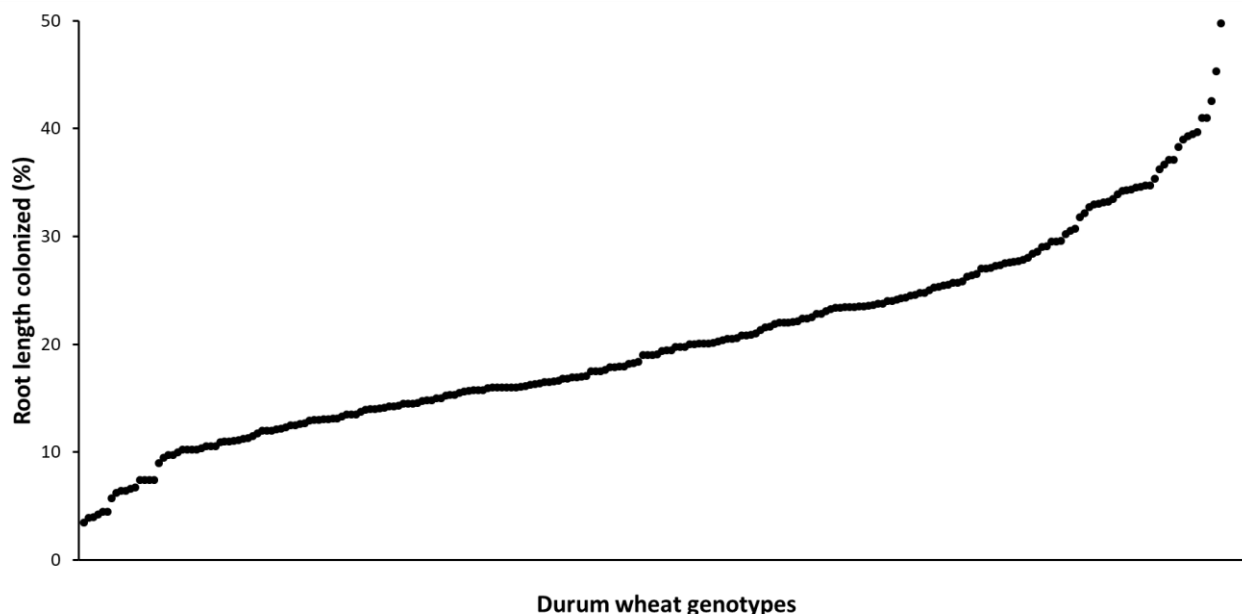


Figure 1: Mycotrophic diversity of 250 durum wheat genotypes in non-stressed blocks of the SolACE field trial at Foggia experimental station (CREA), Southern Italy

In March 2019 a selection of 15 genotypes which were representative of the whole colonization range in the field (low, medium and high) were cultivated under greenhouse conditions to validate field colonization data and to investigate the amplitude of variation in genotype response to AM fungal colonization. The selected genotypes also formed part of the SolACE shortlist of 40 genotypes (WP2.1) and included an equal distribution of drought sensitive and tolerant genotypes (Table 1). The trial consisted of two treatments, non-mycorrhized and mycorrhized (inoculated with the standard strain *Rhizophagus irregularis* MUCL 41833), and each genotype had 8 replicates (or pots) per treatment with three pseudo-replicates (or plants) per pot, resulting in a total of 720 plants. Seeds were planted in previously sterilized substrate (soil:sand, autoclaved twice at 125°C for 15 min) in 1 dm³-pots. The soil was collected from an agricultural site in the Wallonia region of Belgium, sieved through a 1 cm mesh, and mixed with sand (1:1 v:v soil:sand). The mycorrhized treatments were inoculated with ca. 1 g of maize roots previously colonized (> 80% RLC) with *R. irregularis* MUCL 41833.

Overall, mean colonization rates observed in the greenhouse were higher compared to colonization rates in the field, with mean greenhouse colonization rates ranging from 23 – 76% (Table 1). As a result, the colonization rates of the different genotypes in the field were not correlated with their corresponding colonization rates observed in the greenhouse. This can be expected because several environmental factors potentially influencing AM fungal colonization were prevalent under field conditions, but not under the controlled greenhouse conditions. Moreover, it is important to consider that the inoculum potential in the field was not controlled and different AM fungal species were naturally present to colonize the roots. However, a selection of drought tolerant genotypes which had high AMF colonization rates in the field as well as in the greenhouse could be potential candidates for cultivation under drought and nutrient stress conditions e.g. AVENTUR (SDW1008: 35% RLC in the field, and 76% RLC in the greenhouse).

Table 1: AM fungal colonization rates in the field (Foggia, Italy), and under greenhouse conditions inoculated with *Rhizophagus irregularis* MUCL 41833 (UCLouvain, Belgium), for 15 durum wheat genotypes

| Genotype | SolACE Code | Root length colonized (Mean \pm SE) | | Drought tolerance |
|----------------|-------------|---------------------------------------|------------|----------------------------------|
| | | Field | Greenhouse | |
| AW12/BIT-DP060 | SDW3011 | 3 \pm 1 | 23 \pm 6 | N.A (lowest field colonization) |
| COLOSSEO_DP087 | SDW3063 | 6 \pm 2 | 66 \pm 8 | Sensitive |
| ASTERIX | SDW2061 | 10 \pm 6 | 49 \pm 9 | Tolerant |
| BALSAMO | SDW2062 | 11 \pm 11 | 43 \pm 8 | Sensitive |
| KARUR | SDW1022 | 11 \pm 11 | 63 \pm 7 | Tolerant |
| MURANO | SDW1031 | 16 \pm 13 | 66 \pm 5 | Tolerant |
| TIZIANA | SDW2056 | 22 \pm 18 | 52 \pm 9 | Sensitive |
| RGTVOILUR | SDW1042 | 22 \pm 9 | 38 \pm 6 | Tolerant |
| BOLIDO-DP034 | SDW3012 | 23 \pm 1 | 42 \pm 9 | Sensitive |
| LGBORIS | SDW1024 | 29 \pm 16 | 67 \pm 4 | Tolerant |
| AVENTUR | SDW1008 | 35 \pm 1 | 76 \pm 4 | Tolerant |
| FURIO_CAMILLO | SDW2023 | 37 \pm 6 | 76 \pm 4 | Sensitive |
| L2574 | SDW2033 | 40 \pm 14 | 53 \pm 8 | Sensitive |
| LLOYD | SDW3027 | 41 \pm 4 | 52 \pm 8 | N.A (root system) |
| EL4X_252 | SDW4030 | 50 \pm 24 | 65 \pm 6 | N.A (highest field colonization) |

The degree to which each genotype is dependent on mycorrhizae to produce maximum growth or yield, also known as the relative mycorrhizal dependency (RMD) of the plant (Gerdemann 1975), varied among the 15 genotypes inoculated with *R. irregularis* MUCL 41833. The RMD of each genotype was determined by expressing the difference between the dry weight of the mycorrhizal plant and the dry weight of the non-mycorrhizal plant as a percentage of the dry weight of the mycorrhizal plant (Plenchette et al., 1983). Positive and negative growth responses (RMDs) were observed in the presence of *R. irregularis* MUCL 41833 across the 15 genotypes cultivated in the greenhouse (Figure 2, A). However, when investigating the relative shoot to root ratio, genotypes with a strong negative RMD had positive relative shoot to root ratios e.g. SDW4030 (EL4X_252), SDW3027 (LLOYD), SDW1042 (RGTVOILUR) (Figure 2, B). Therefore, in the presence of the AM fungus these genotypes produced less root biomass while maintaining high shoot biomass. This could explain one mechanism of the interaction between the plant and AMF, and pleads for a role of AMF in supplying nutrients (and water) to the plant. The plant needs to invest less in roots as it seems that AMF replace the roots for providing nutrients, maybe accessing pools otherwise inaccessible to roots. Therefore, AMF could be beneficial under nutrient deficient conditions, at least for some genotypes.

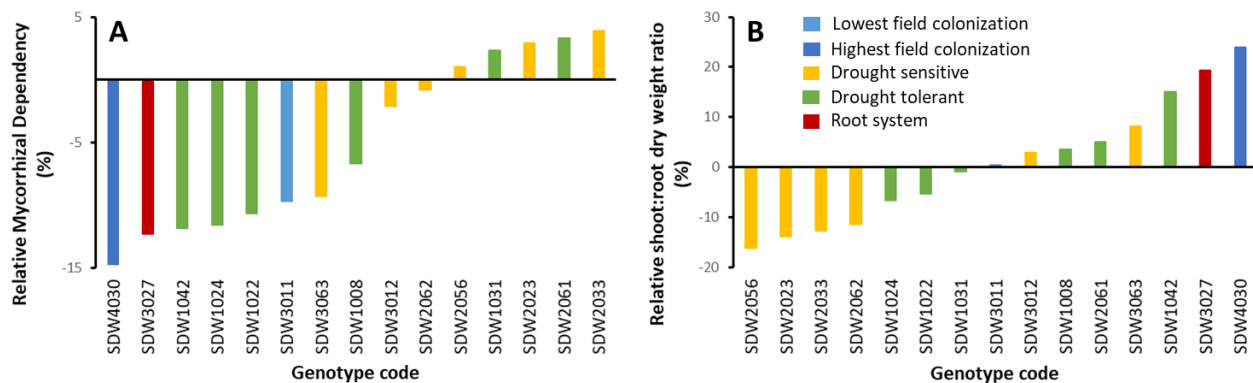


Figure 2: Durum wheat genotypic responses to AMF (*Rhizophagus irregularis* MUCL 41833) expressed as **A**) relative mycorrhizal dependency (RMD), and **B**) relative shoot to root ratio (shoot:root). The genotypes were chosen according to their root colonization levels (high to low) observed in the field during the experiment in Foggia, tolerance or sensitivity to drought and to one particular root system architecture.

The AMF colonization dataset can also be linked to root phenotype data (WP2.1) and SNP data (WP4.2) to identify root phenotype traits and genomic loci underlying the capacity of mycotrophic genotypes. This information could prove valuable for future breeding strategies. The results will be included in a publication, which is currently in preparation, and which is planned to be submitted by mid-2021.

2.2 Response of potato-associated microbiomes to combined stress and plant genotype

At the James Hutton Institute in Dundee, Scotland, a field trial was set up with ten different potato varieties (Pentland Dell, Alias, Nadine, Home Guard, Sarpo Mira, Cara, Stirling, Desiree, Inca Sun, Inca Dawn). Two treatments were imposed: i) conventional fertilizer applications (Defra RB209) with supplemental irrigation (two to three 30 min. applications per week as required) and ii) conventional fertilizer applications but without P fertilizer. Plants were either grown under a combined water and nutrient stress or without stress.

2.2.1 Microbiome analysis

In 2018 the samples were taken during tuber filling. Root and rhizosphere samples were collected in four replicates. Additional six bulk soil samples were obtained. The DNA of all samples were extracted using the DNeasy PowerSoil Kit (Qiagen, Hilden, Germany). For the bacterial microbiome the primers 799f and 1175r were used according to the AIT standard protocols for Illumina sequencing (Escobar Rodríguez et al., 2018). For the fungal community the primers ITS 1f (Gardes and Bruns, 1993) and ITS 2 (White et al., 1990) were applied. Bacterial and fungal libraries were run on the MiSeq sequencer (Illumina) with the V3.0 chemistry. The output of the Miseq run was processed using the inhouse developed bioinformatic pipeline.

The microbiome analysis revealed 1300 bacterial and 76 fungal core reproducibly occurring taxa. The microbial community of each sample was mainly explained by the habitat / compartment (root, rhizosphere and soil) (Figure 3 A, D, sample type: bacteria: $R^2=0.79$, p-value <0.001, fungi: $R^2=0.45$, p-value <0.001) but also by the applied stress (stress: bacteria: $R^2=0.09$, p-value <0.001, fungi: $R^2=0.06$, p-value <0.001). Stressed rhizosphere microbiomes had significantly reduced Shannon diversity (Figure 3, B, E) and fewer different amplicon sequence variants (ASVs) (Figure 3, C, F). In roots similar diversity effects were observed for the bacterial but not for the fungal community.

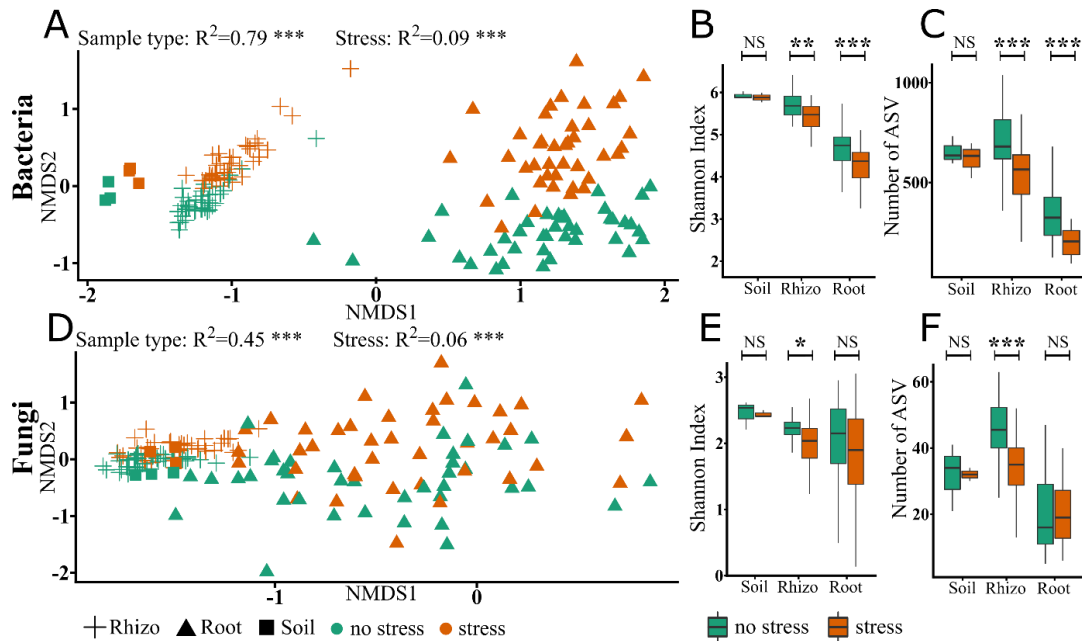


Figure 3: Microbial diversity of potato plants. Beta-diversity of the 166 potato-associated microbiota is shown in a non-metric multidimensional scaling (NMDS) ordination based on the Bray-Curtis according to sample type (shape of the symbols) and stress. 1A bacterial diversity, 1D fungal diversity. 1B Shannon diversity 1B bacteria, 1E fungal; 1C Number of bacterial ASVs, 1F Number of fungal ASVs

Figure 4 shows which part of the fungal or bacterial communities are enriched or depleted under stress conditions. For example, Actinobacteria, Sphingobacteriales and Sordariales are enriched under stress, whereas Bacteroidetes, Proteobacteria, Firmicutes and Olpidiomycolota are depleted (Figure 4).

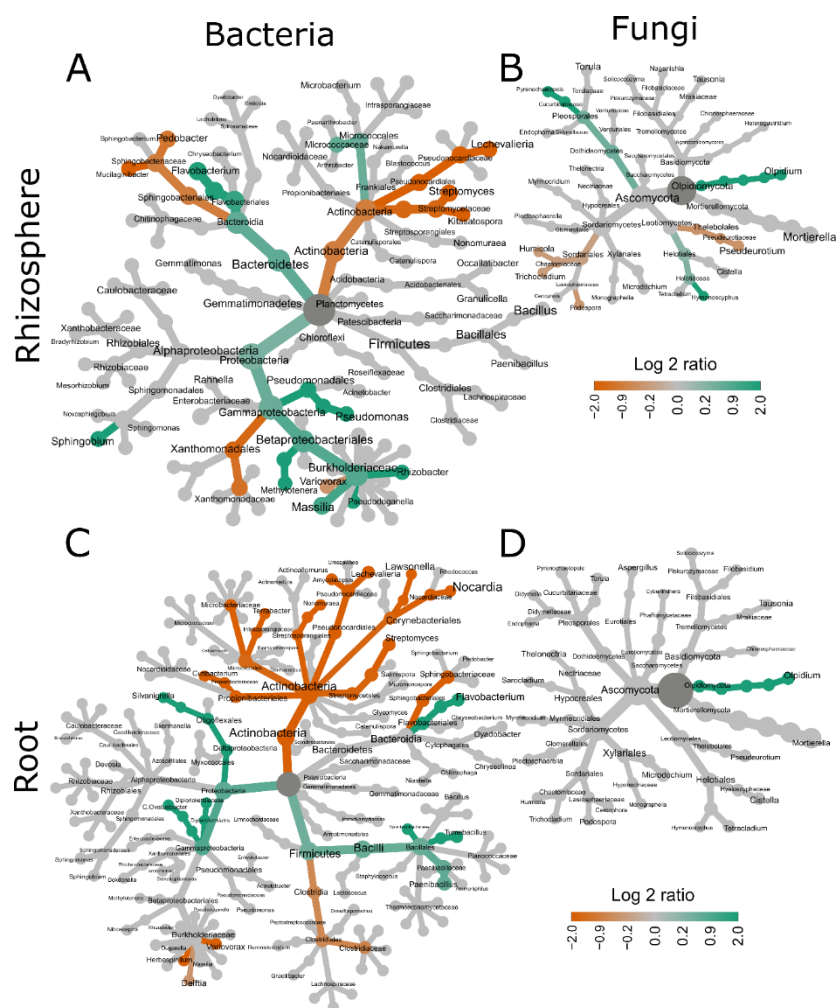


Figure 4: Differences in microbial composition. The four figures show the microbes of the (A) bacterial and (B) fungal rhizosphere as well as the (C) bacterial and (D) fungal root. Each node represents a taxonomic rank, starting from the highest rank (largest, dark grey nodes) to the genus level at the end of the branches. Colored nodes are significantly enriched (orange) or reduced (green) under stress (Wilcoxon test ≤ 0.05).

Bacterial community composition of root and rhizosphere microbiomes of the ten different genotypes did not show a clear clustering according to the performance under stress (Figure 5). Without stress all ten genotypes showed similar bacterial communities. Under the combined stress the tetraploid *Solanum tuberosum* genotypes showed similar communities but were different from the unstressed communities. The bacterial communities of the two varieties from *Solanum tuberosum* Group Phureja (Inca Sun and Inca Dawn) under stress showed a different composition as compared to those of the tetraploid varieties.

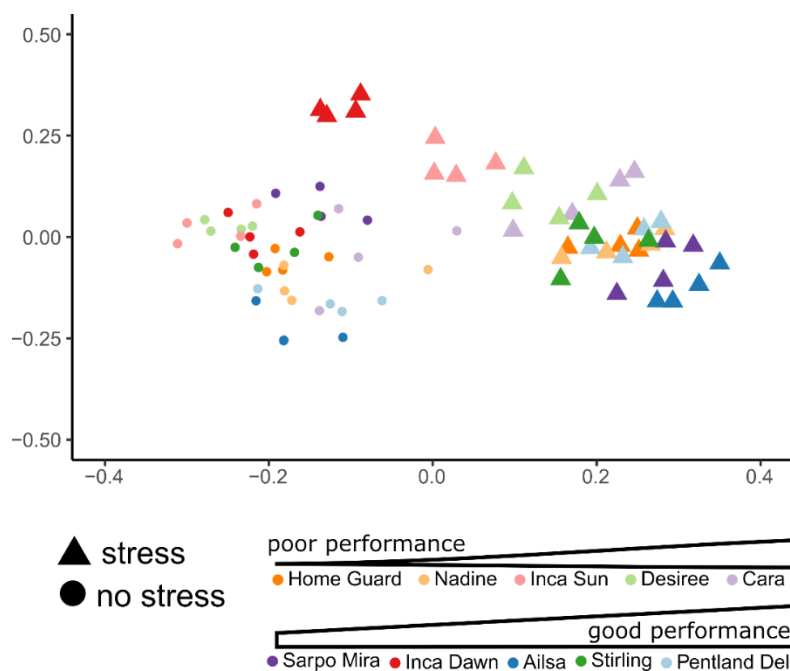


Figure 5: Bacterial composition of the ten different genotypes under stress and no stress

2.2.2 Metagenome analysis

For functional characterization of potato rhizosphere microbiomes, potato genotypes with contrasting performances under stress conditions were selected for shotgun metagenomics analysis. From cv. Désirée, a poorly performing genotype, and cv. Stirling, a well performing genotype, grown under combined stress and under no stress, rhizosphere DNA was isolated (in three replicates). Library preparation and sequencing was performed by the Vienna Biocenter Core Facility, shotgun metagenomics data were analysed at AIT using the in house developed bioinformatic pipeline. After cleaning the reads, 5572 bacterial core operational taxonomic units (OTUs), 1535 plasmid core OTUs, 7010 phage core OTUs, 285 archaeal and 50 fungal core OTUs were identified (Figure 6). Overall, the results were in line with microbiome analyses revealing significant differences of bacterial, archaeal and phage communities under stressed and unstressed conditions and pinpointing to Actinobacteria being enriched under stress conditions. Nine bacterial classes were more abundant under stress conditions and eleven bacterial classes were more abundant without stress. For the plasmids five plasmid classes were enriched under stress, whereas four were depleted.

For functional genes, which covered the genes at least by 80%, 1429 bacterial genes were found to be enriched under stress, whereas 1250 bacterial genes were more abundant under unstressed conditions. Similarly, 139 plasmid genes were enriched under stress and 129 plasmid genes enriched under no stress conditions. Looking into KEGG categories, changes were evident in functions related to N and S metabolism, production of secondary metabolites, stress responses, motility functions genes or genes involved in vitamin synthesis. For instance, the highly altered microbiomes under stress conditions showed a greater number of genes involved in the production of osmoprotectants such as trehalose or the production of vitamins such as biotin, riboflavin or tryptophan. Other changes correlated well with the depletion of Proteobacteria such as the depletion of genes involved in flagella production or glycan biosynthesis. Genes involved in environmental adaptation and detoxification (e.g. of ROS produced by stressed plants) were also more abundant under stress. For instance, an elongation factor 4 was more abundant under stress. This elongation factor is required for accurate and efficient protein synthesis under certain stress conditions. Similarly, oxidative enzymes play a role in the detoxification of ROS, plant-derived stress compounds (Figure 7).

The complete analysis for WP2.3 will be included in a publication, which is currently in preparation and is planned to be submitted at the beginning of year 2021.

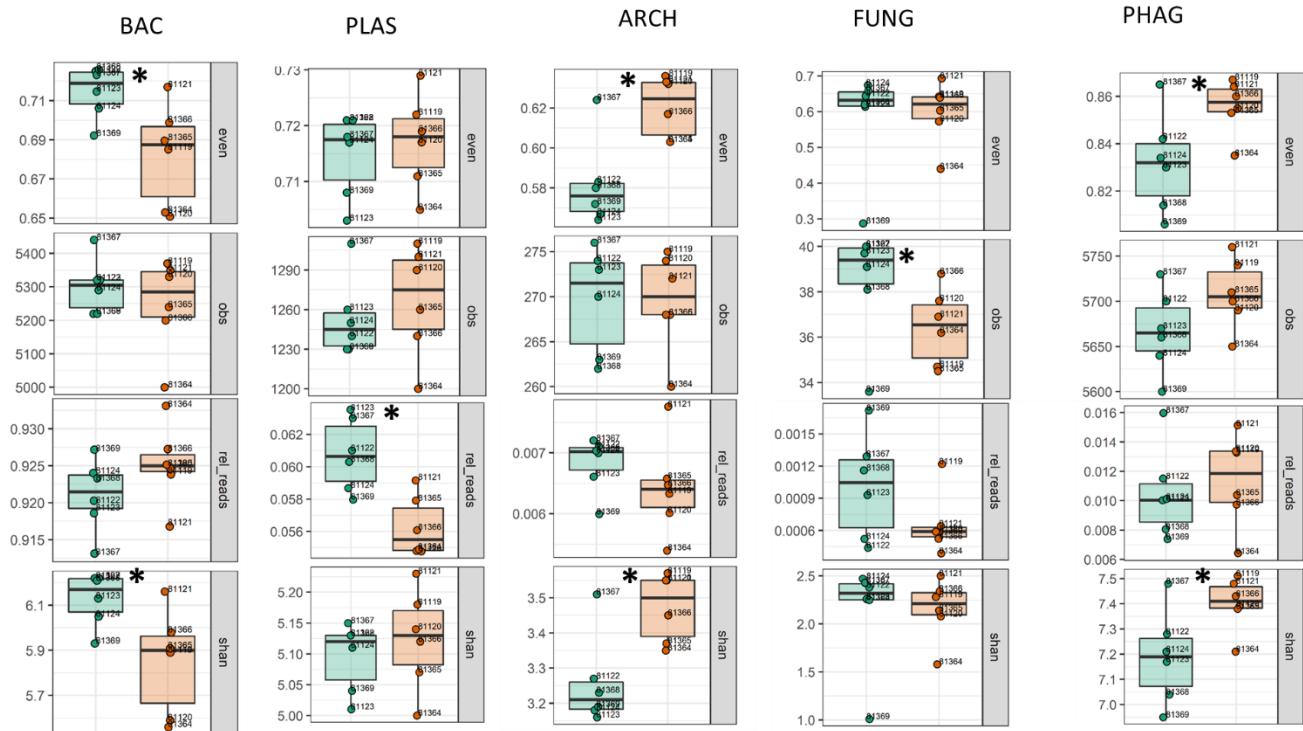


Figure 6: Abundance and diversity of microbial taxa between the stress treatments

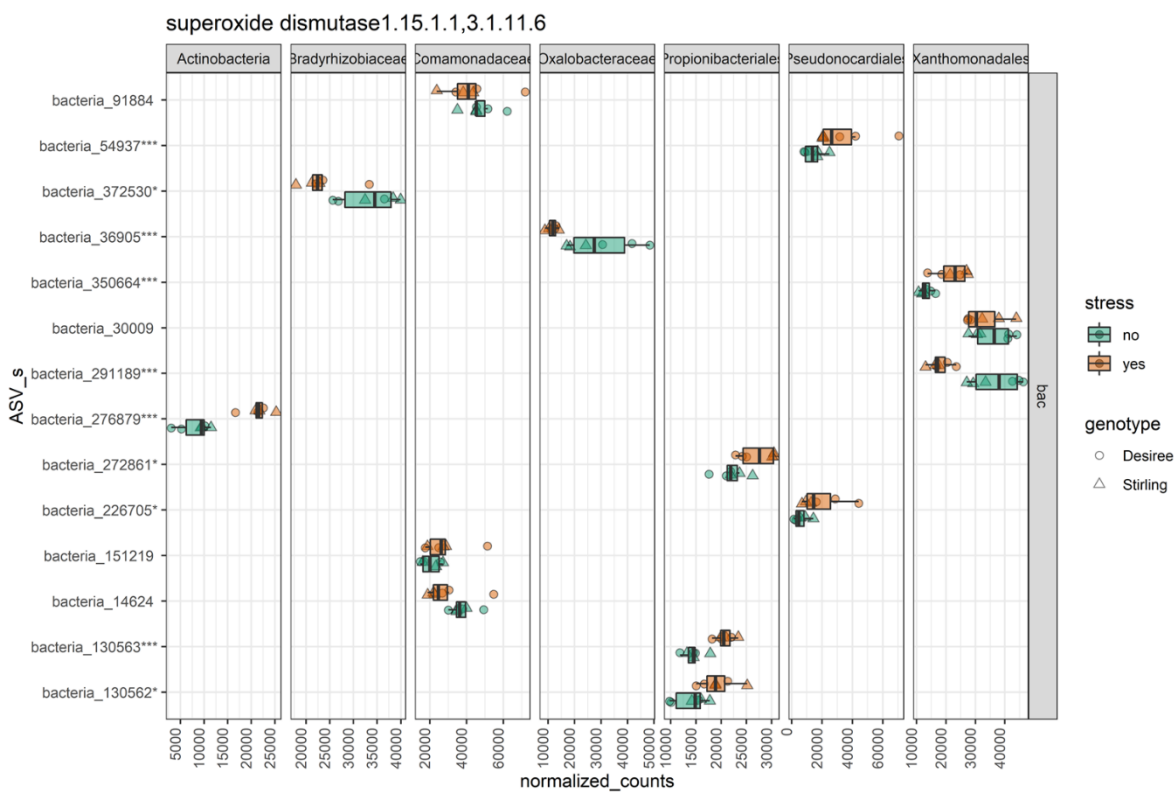


Figure 7: Abundance of superoxide dismutase in environmental adaptation in the rhizosphere of potato plants under stress or without stress associated to specific bacterial taxa

2.3 Response of bread wheat-associated microbiomes to combined stress and plant genotype

INRAE-GDEC in Clermont-Ferrand, France, set up a field trial with eleven different wheat varieties which were grown under combined water/nutrient limitation or without stress.

2.3.1 Microbiome analysis

In May 2019 AIT sampled the rhizospheres and roots of eleven bread wheat genotypes (ALBIDUM, SIROKA, BILINMIYEN96.40, BRANKA, EDWIN, BRUTA, HOKUEI, MV309-99, MUSTANG, MCGUIRE, TCI960735) that were grown under combined stress or no stress in a field trial carried out by INRAE-GDEC in Clermont-Ferrand, France. Four plant replicates per treatment were taken, each from another plot. Rhizosphere samples were processed in France and DNA was isolated using the 96-well DNeasy PowerSoil Kit (Qiagen, Hilden, Germany). Root samples were processed in Austria and DNA was isolated using the same procedure. For the analysis of bacterial communities, a two-step library preparation using the primers 799f and 1175r was used to amplify bacterial 16S rRNA genes (as described above), whereas archaeal communities were amplified using in nested approach using primers 344 F and 1041 R in the first PCR and 519 F and 860 R with the Illumina adaptors in the second PCR (Pausan et al., 2019). Besides bacterial profiling, an archaeal profiling was established and performed at the AIT. Archaeal and bacterial amplicons were sequenced on a MiSeq (Illumina, San Diego, USA) at AIT. Similar to results obtained for potato, also here the root proximity and the stress significantly influenced microbiome composition, whereas the plant genotype effect was rather minor (Figure 8).

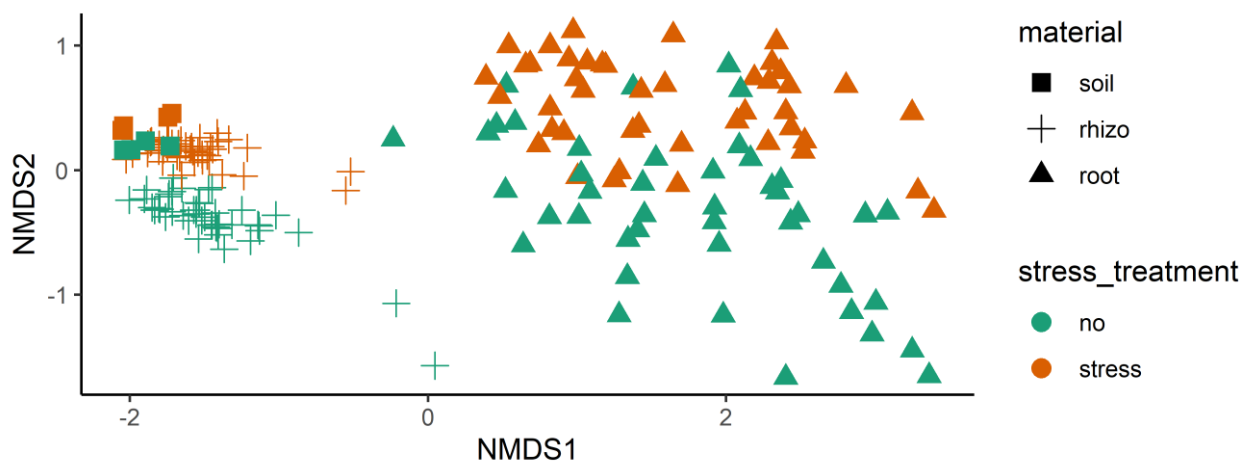


Figure 8: Bacterial beta-diversity of the 166 wheat-associated bacterial microbiota is shown in a non-metric multidimensional scaling (NMDS) ordination based on the Bray-Curtis according to sample type (shape of the symbols) and stress.

2.3.2 Metagenome analysis

Two wheat varieties were selected for the shotgun metagenomic analysis. As outlined for potato, also here a well performing genotype (MUSTANG) and a poorly performing genotype (TCI960735) were selected. Three replicates obtained from stress and no stress treatments were processed. The DNA was additionally cleaned via Amicon® Ultra Centrifugal Filters (Merck, Darmstadt, Germany) and sent to the Vienna Biocenter Core Facility for library preparation and sequencing (Illumina, NovaSeq). The data have been analysed at AIT by using the in house established bioinformatic pipeline. The first analysis showed that in total 983 Mio reads were obtained and per sample 81 Mio reads. The reads are distributed among the different kingdoms to around 11% bacteria, 0.05% to archaea, 0.05% to fungi and 0.14% to phages. Further analyses are on-going.

Results obtained within WP2.4 will be included in a publication, which is currently in preparation, and which is planned to be submitted by beginning of year 2021.

3. Conclusions

Wheat is a mycotrophic plant, for which level of colonization varies highly with genotype. This suggested a clear link between plant genetic factors and root colonization. The AMF impacts plant growth under controlled conditions as demonstrated for 15 genotypes with low to high levels of root colonization in the field. A clear link between root colonization data obtained in the field and growth promotion in the greenhouse has not been demonstrated though, probably due to the fact that in the field a population of AMF were present, while in the greenhouse only one standard AMF was considered. It is well known that plant genotype – AMF genotype matters, and it is thus not excluded that the standard strain was efficient with certain genotypes and not with others (as shown by the RMD data). Nevertheless, it is interesting to note that root to shoot ratio was decreased in the AMF-colonized plants for half of the tested durum wheat genotypes suggesting a high investment of some of the plant genotypes in the fungus relative to the root system for ensuring adequate acquisition of belowground resources. Further studies would be needed with composite populations of AMF with contrasting genotypes (sensitive and tolerant to drought) of durum wheat to clarify the role of AMF in increasing the resistance/tolerance of durum wheat to drought stress.

Generally, microbiomes were significantly impacted by drought and nutrient stress. However, the most important driver of microbiome diversity and structure was the compartment (proximity to roots) as highly different communities were found in the rhizosphere and endosphere (root) compartment. This effect was more pronounced for bacteria than for fungi. In potato, specific taxa were enriched under drought, such as taxa belonging to the Actinobacteria, Sphingobacteriales and Sordariales, and others were depleted such as Bacteroidetes, Proteobacteria and Olpidiomyota. At the community level no correlation was found with plant performance under stress, although specific indicator species were identified to correlate with plant performance under stress conditions. We also identified significant differences for mobile elements such as plasmids and phages, which play an important role in the horizontal gene transfer of genetic information but also act as important community drivers. Several microbial functions were affected by the stress treatment such as siderophore production, motility, stress response or the production of osmo-protectants and vitamins.

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5. Partners involved in the work

UCLouvain: Field sampling of wheat panels and assessment of genotypic responses in durum wheat to AMF colonization. Amplicon and library preparation of AMF.

AIT: Performed microbiome and metagenome analysis of bacteria, archaea and partly of fungi

FIBL: Field sampling of durum wheat genotypes, Foggia, Southern Italy, amplicon and library preparation of fungi in WP2.3 and WP2.4

James Hutton Institute: set up the potato trial WP2.3

INRAE-GDEC in Clermont-Ferrand: set up the wheat trial WP2.4

CREA: set up the durum wheat trial WP2.2

SYNGENTA: set up the bread wheat trial WP2.2