Funded by the Horizon 2020 Framework Programme of th

EXCALIBUR

D1.6 Report on initial soil microbial populations

(Deliverable 1.6)

Acronym: EXCALIBUR

Grant Agreement: 817946

Horizon 2020 Programme

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Work Package	WP1
Deliverable	1.6
Due date	M 17 (30/11/2021)
Submission date	28-01-2022
Dissemination level	Public, fully open
Deliverable lead	NHM
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Abstract	The aim of this document is assess initial soil microbial biodiversity was assessed using culture-independent and culture-dependent approaches.
Keywords	Baseline assessment, soil, bacteria, archaea, arbuscular mycorrhizal fungi, 16S rRNA gene, ITS, quantitative PCR, colony-forming units, most probable methodologies

1. Background

The initial soil microbial biodiversity was assessed using culture-independent and culture-dependent approaches as part of Task 1.4 (Assessment of initial soil microbial populations). Microorganisms are of particular importance for carbon and nutrient cycling in soil environments. These processes performed by microorganisms include decomposition of organic biomass and, together with earthworms, the generation of bioavailable nutrients. The richness and relative abundance of microbial communities can be affected by physical and chemical characteristics of soils, vegetation and geographic location. Plants interact with microbes in the soil, which can affect plant growth, health, and adaptation to stressful environments.

The findings of Task 1.4 will form baseline information for the biodiversity dynamics assessments and the bioinoculant treatments (biocontrol and biofertilizers) in relation to the three model horticultural crop plants tomato, strawberry and apples, the application of organic and conventional management practices, as well as the location of experimental trials in multiple pedoclimate zones that will be carried out in WP3-5. Culture and culture-independent approaches provided complimentary information of soil microbial populations. Culture-independent methodologies enable profiling microbial communities directly from DNA without the need to be able to grow the microorganisms in the laboratory. These approaches allow to assess taxa with high and low abundances, as well as enable comparisons without the need to use growth media that might create selective growth conditions and biases. Quantitative PCR (qPCR) allows to determine the number of copies of specific genes in the soil. For the delivery of the assessment of the initial soil population, qPCR was completed for functional genes that contribute to the nitrogen cycle such as bacteria and archaea specific ammonia monooxygenase A (*amoA*), as well as nitrite reduction (*nirK*) for soils from experimental fields that will be used for biofertilizers trials.

Culture-dependent methodologies were applied to obtain information on how many microorganisms are viable in soil which is impossible with molecular methods. For the delivery of the task, we estimated the number of viable heterotrophic bacteria and fungi by way of colony-forming units (CFU), as well as determined the concentration and richness of arbuscular mycorrhizal fungi (AMF) using Most Probable Number (MPN) methodology. The combination of molecular and microbiological results will be important to define a multi-criteria model that will constitute the reference framework to confront with the new proposed strategy for management of microbial-based plant nutrition and protection.

2. Documentation of delays

Issues out of the control of the Excalibur WP leader, task leader and project partners occurred in 2021 that led to several analyses not being completed by the 30 November 2021. The microbiological analyses such CFU and qPCR took longer than expected due the delay of shipping of samples and availability of laboratory staff. The high throughput sequencing analyses were delayed due longer than expected shipping times for consumables, ad hoc maintenance of servers and in house MiSeq Illumina sequencing facility, as well as delay in processing time of samples by external sequencing companies. The Excalibur task and WP leaders have communicated the issues and discussed mitigation. The delays will not have an impact on other tasks, milestones and deliverables in WP1 to WP6.

3. Characterisation of initial soil microbial populations using Bacteria and Archaea 16S rRNA and fungal Internal transcribed spacer (ITS) region high throughput sequencing

The 16S rRNA gene and the ITS region were selected because they have been shown to provide good coverage of bacteria and archaea and fungal phyla respectively. There are also large reference sequence databases available for taxonomic assignment of the sequences.

3.1. Material and Methods

Soil sample collection and preparation

For the DNA analysis, 4 composite soil samples per trial were collected before the beginning of the trials. The bulk soil subsamples for the composite samples were collected from 10 areas within the field where the plants will be planted following the guidelines (D 1.2, section 2). Following the guidelines (D 1.2, section 2) 10 subsamples were collected and combined in a clean bucket to make 1 composite sample and be split into different containers for the different analysis including the DNA and microbiological analyses. If plants were already growing in the field at the beginning of the trial, 2-3 soil subsamples were collected from near the roots of plants from 4 plots before the start of the trials, and allowed to generate a composite soil sample that is made from 10 subsamples. The soil samples were sieved and stored according to the instructions in guidelines for sampling methods D 1.2 and analytical method D 1.3.

Polymerase chain reaction (PCR), purification and quantification of PCR-products, and Illumina MiSeq sequencing

DNA extractions were performed using E.Z.N.A soil kit following the manufacturer's instructions (E.Z.N.A.®, Kit Soil DNA**,** Omega Bio-tek). The DNA quantity was evaluated by Nanodrop and Qubit following the manufacturer's instructions, and the DNA quality was determined using Nanodrop based 260:280 and 260:230 ratios. Low quality and yields DNA extracts (less than 12ng/μl) were purified using the Amicon Ultra 30K 0.5 ml Centrifugal Filters (EMD Millipore). Taxonomic gene markers were amplified using PCR Bollmann-Giolai et al (2020) using specific primers to amplify the bacterial and archaea 16S rRNA gene (Apprill et al. 2015; Parada et al. 2016), and fungi Internal transcribed spacer (ITS) region (White et al. 1990). The pooled purified 16S rRNA gene PCR products were sequenced at the Natural History Museum sequencing facility using an Illumina MiSeq platform (2 x 250 cycles, Illumina, San Diego, CA) and the ITS PCR products were sequenced by Novogene following company's standard protocols.

16S rRNA gene and fungal ITS processing and statistical analyses

For the bioinformatics analyses, the sequences were demultiplexed based on the indices using QIIME 2.0 (https://qiime2.org/). Paired-end reads are merged to obtain full length amplicon sequence reads and trimmed for quality control. DADA2 was used to denoise sequences and generation amplicon sequence variants (ASVs) which refers to unique DNA sequences detected in the samples. The taxonomic assignment of ASV was done with SILVA (1.8) for 16S rRNA gene and UNITE (8.2) was used for fungal ITS. Statistical analyses were performed in Qiime and RStudio. The 16S rRNA gene sequencing was completed for all soil samples and about 75% of samples for fungal specific ITS sequencing. Delays were due longer than expected shipping time for consumables, ad hoc maintenance of servers that affected the use of the in house MiSeq Illumina platform as well as delays of sequencing of amplicons by external sequencing companies. The delays will not have an impact on the tasks, deliverables and milestones in the other WPs.

3.2. Results

Baseline assessment of bacteria and archaea 16S rRNA gene and fungal ITS populations in soils for Excalibur trials

The 16S rRNA gene high throughput assessment identified diverse bacteria and archaea populations in all experimental field sites. The chao1 index was determined and showed considerable variation across trial sites, crop types and management practises (Figure 1). Bacteria dominated soil communities. Proteobacteria, Actinobacteria, Acidobacteriota, Planctomycetota, Bacteriodota, Chloroflexi and Verrucomicrobiota were the bacterial phyla with the highest relative abundance in the soil samples. The Crenarchaeota were the archaeal phylum with the highest relative abundance (Figure 2). The soil communities from different countries were compared. Weighted Unifrac PCOA analysis at 16S rRNA ASV level suggested that some trial sites from different countries varied in their 16S rRNA gene populations such as Poland, Denmark, and England, but the majority of sites did not show a clear separation by country (Figure 3). There were

no striking differences observed in the community assemblages from trial sites in different pedoclimate zones (Figure 4), and sites that will be used for apple, tomato and strawberry crop trials (Figure 5).

The fungal ITS populations were evaluated using high throughput assessment and diverse communities were identified in all samples. The median chao1 index was higher for sites for IPM management thank organic practices (Figure 6). The fungal groups with the highest relative abundance belonged to Ascomycota, Basidiomycota and Dothideomycetes. Other fungi belonged to Rozellomycota, Mucoromycota, Zoopagomycota, Mortierellomycota, Chytridomycota, Olpidiomocyta, and Glomeromycota (Figure 7). Weighted Unifrac PCOA analysis at ASV level for soil fungal ITS population did not identify clear differences between trial sites that will be used for different crop type (Figure 8). A comparison by pedoclimate zone, suggested that soil fungal populations from Continental and Atlantic trial sites showed considerable overlap, and were different from soils from Mediterranean sites (Figure 9). A comparison by country of the sites suggested that fungal populations were similar in sites from Slovenia and Italy (Mediterranean pedoclimate zone) and distinct to other sites. Sites from Poland and England also showed considerable overlap, whereas the remaining fungal populations from Germany, France and Austria showed more variability (Figure 10).
The analysis allowed to characterize the baseline microbial populations using high throughput sequencing. The evaluation identified that the soils are comprised of diverse bacteria, archaea and fungal assemblages, and it was possible to evaluate the relationship between community composition, pedoclimate zone, crop type and horticultural management practices. The findings will be used in the other work packages to evaluate the influence of bioinoculants on the soil microbial diversity and plant microbiomes interactions. It will help to gain a better understanding on how soil microbial populations and beneficial microorganisms improve the plant growth and health. The results obtained from the assessment of native (baseline) microbial biodiversity in these trials will allow to define a multi-criteria model that will constitute the reference framework to confront with the new proposed strategy for management of microbial-based plant nutrition and protection.

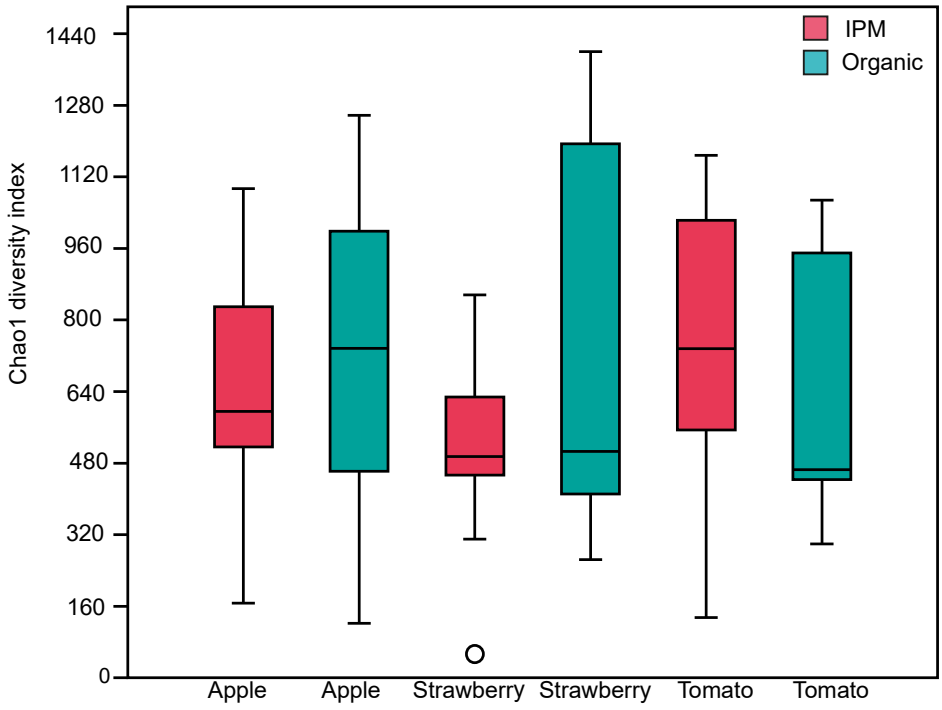

Figure 1. Chao1 diversity index for soil 16S rRNA gene communities at sites that will be used for apple, strawberry and tomato trials. Colour code represents the field management practices.

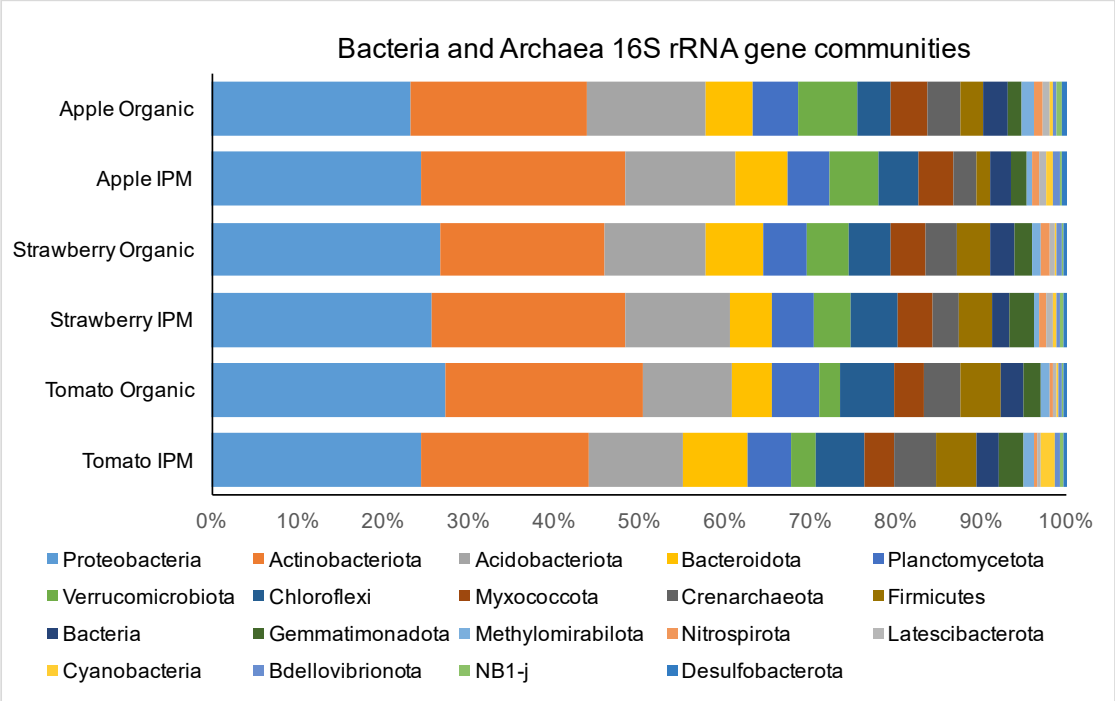

Figure 2. The 20 most abundant 16S rRNA gene Bacteria and Archaea phyla detected from soils at sites that will be used for apple, strawberry and tomato trials.

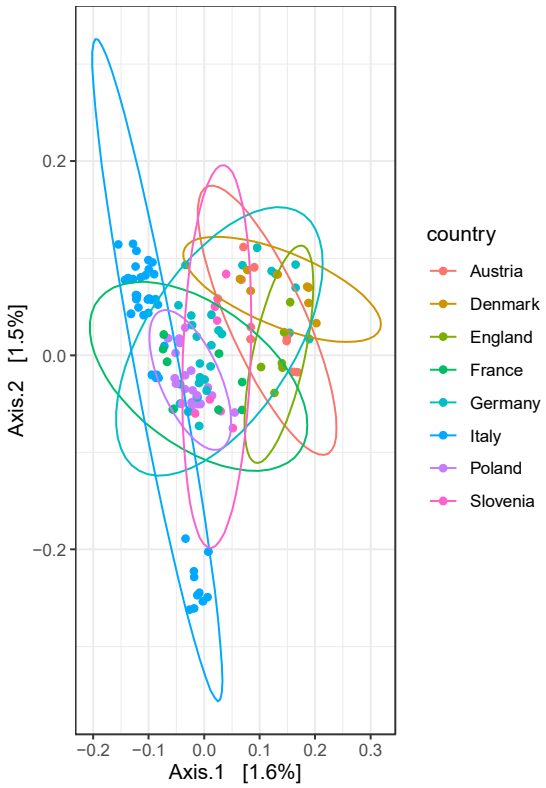

Figure 3. Weighted Unifrac PCOA analysis for soil 16S rRNA gene population. Colours represent countries. Ellipses represent 95% confidence intervals.

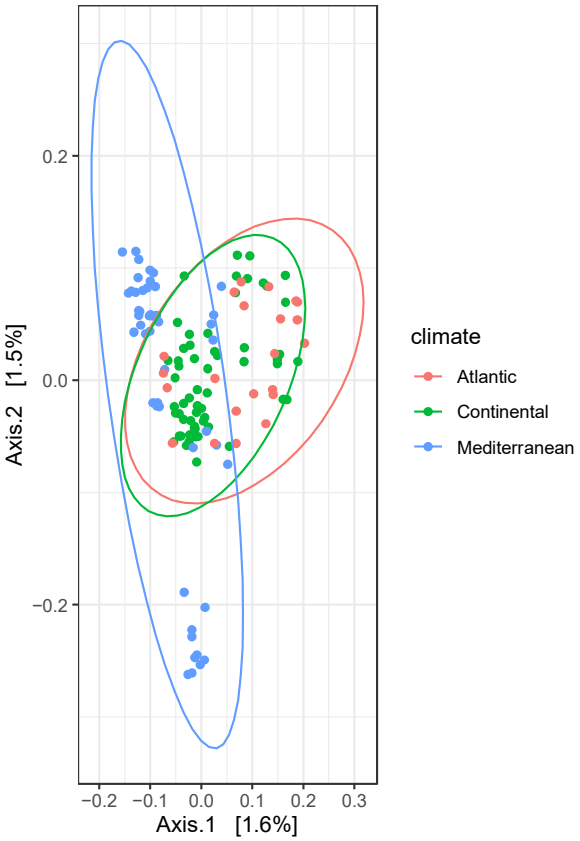

Figure 4. Weighted Unifrac PCOA analysis for soil 16S rRNA gene population. Colours represent pedoclimate zones. Ellipses represent 95% confidence intervals.

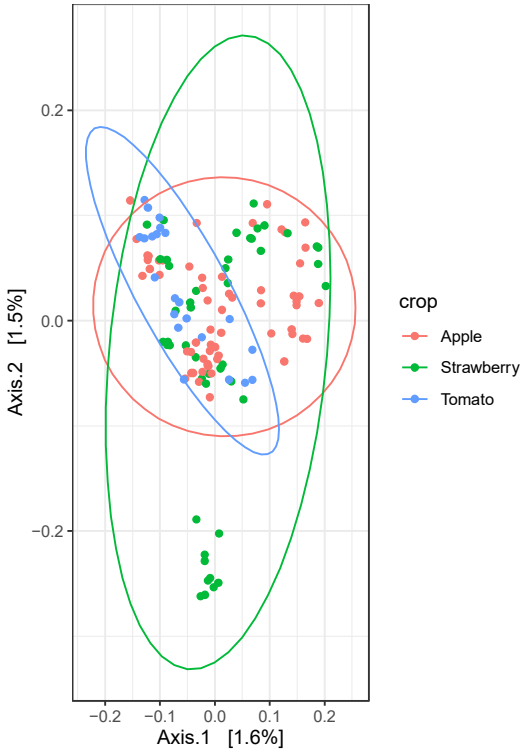

Figure 5. Weighted Unifrac PCOA analysis for soil 16S rRNA gene population. Colours represent crops. Ellipses represent 95% confidence intervals.

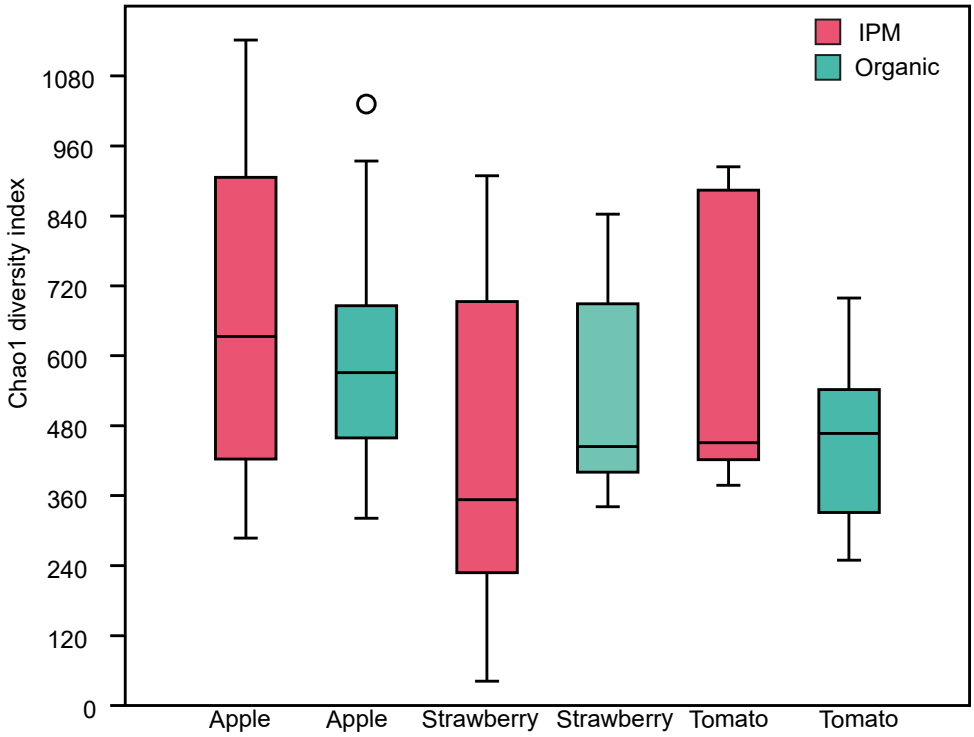

Figure 6. Chao1 diversity index for fungal communities based on ITS regions. Colour code represents the management practices.

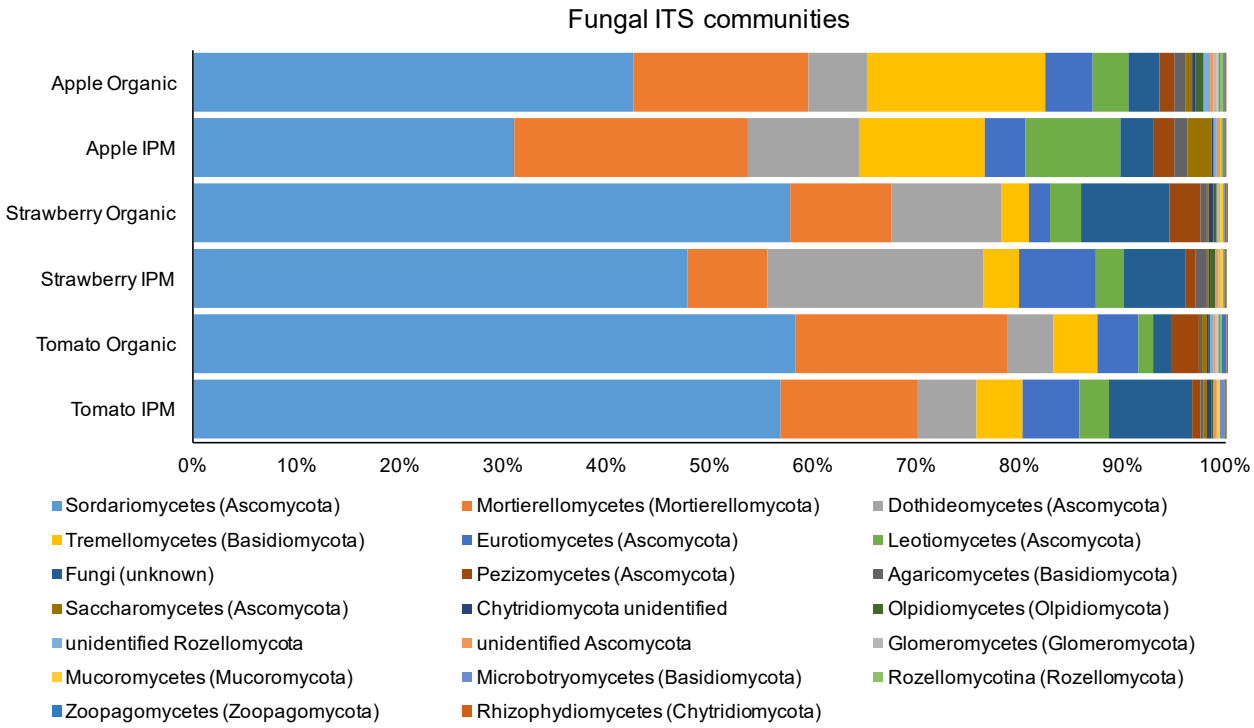

Figure 7. The 20 most abundant ITS fungal orders in soils from sites that will be used for apple, strawberry and tomato trials.

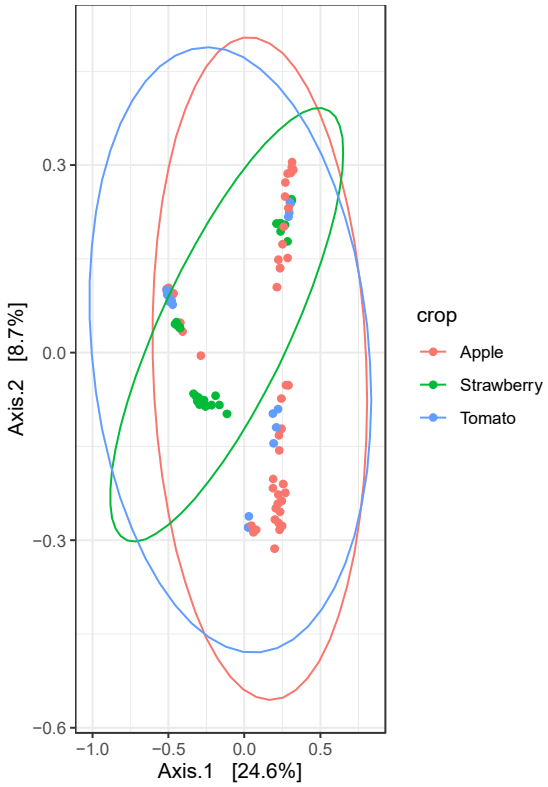

Figure 8. Weighted Unifrac PCOA analysis for soil fungal ITS population. Colours represent crops. Ellipses represent 95% confidence intervals.

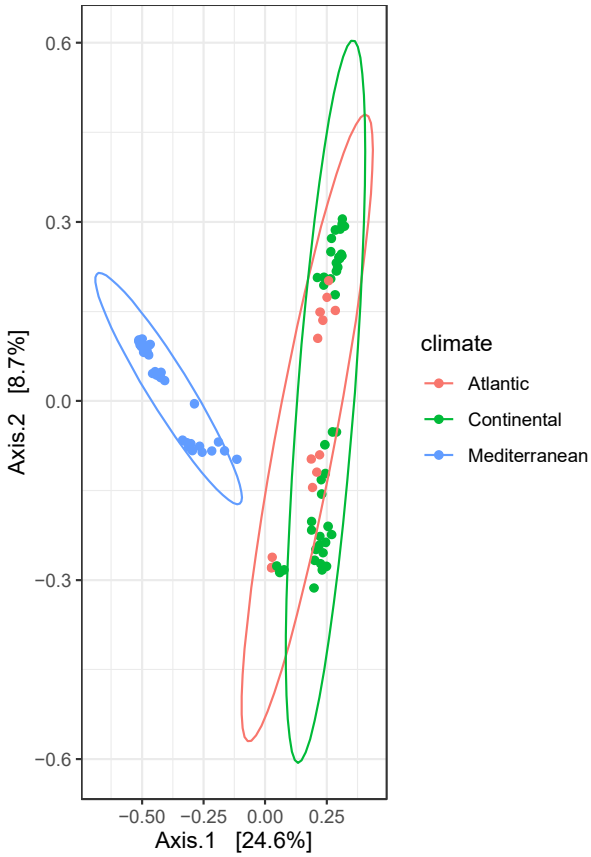

Figure 9. Weighted Unifrac PCOA analysis for soil fungal ITS population. Colours represent pedoclimate zones. Ellipses represent 95% confidence intervals.

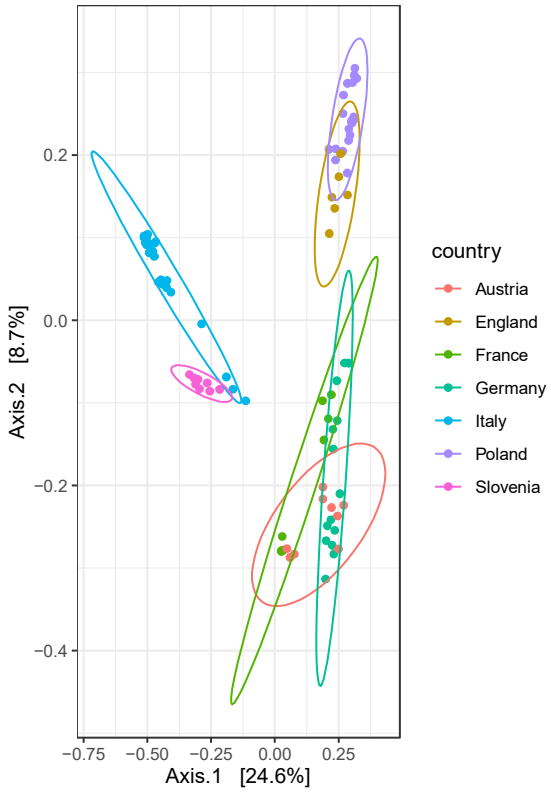

Figure 10. Weighted Unifrac PCOA analysis for soil fungal ITS population. Colours represent countries. Ellipses represent 95% confidence intervals.

4. Quantification of gene copy number of functional genes in the nitrogen cycle in the soils using qPCR

Functional genes for enzymes important in the nitrogen cycle were quantified in the soil using qPCR for the Excalibur experimental field sites. The *amoA* gene, encoding the a-subunit of the Ammonia monooxygenase A, is widely used to investigate nitrification (Levy-Booth et al., 2014; Schauss et al., 2009). It is possible to distinguish *amoA* by bacterial and archaea communities due to its strongly conserved nucleotide sequence (Norton et al., 2002). The conversion of nitric oxide and nitrous oxide is performed by the nitrite reductase and the enzyme is well represented by the *nirK* gene (Cu-containing) (Levy-Booth et al., 2014).

4.1. Material and Methods

The qPCR protocol was performed with minor modifications as described in ISO standard 17601 (ISO, 2016) as outlined in detail in the guidelines (D1.3, section 1.2.3.). It is the procedure used to set-up and perform quantitative PCR (qPCR) to quantify the abundance of functional groups from soil extracted DNA. The soils were sampled, transported, pre-processed, stored and shipped as described in the general method (Guidelines for soil sampling – D6). The DNA extraction were performed as described above. Primer pairs as reported by the literature are listed in Table 1. All qPCR assays of target gene of interest (GOI) are performed in duplicates on each template at the dilution showing no inhibition of Taq polymerase and on duplicates on plasmid standard DNA dilutions. Negative controls made of molecular grade water are measured in duplicates to confirm no unspecific amplification. Samples obtained by partners hosting field trials were equally distributed between CREA, TUG, NIAB and UNITO. The focus was on the biofertilizer trials to see the gene abundances of the selected genes of interests initially. On-going analyses will cover the quantification of genes encoding the nitrite reduction (nitrite reductase *nirS* gene), dinitrogen fixation (dinitrogen reductase, *nifH* gene) and the nitrogen regulatory protein nrfA (*nrfA* gene). The delays

were due to essential staff being on sick leave for extended time period. The delays will not have an impact on the tasks, deliverables and milestones in the other WP.

Table 1: Overview of primer pairs used for amplifications of specific genes of interest in Excalibur.

Amplicon	Forward primer	Reverse primer	Length of amplicon	Annealing temperature
amoA	amoA-1F	amoA-2R	490	60
	GGG GTT TCTACT GGT GGT	CCC CTC KGS AAA GCC TTC TTC		
nirK	nirK876	nirK1040	165	58
	ATY GGC GGV CAY GGC GA	GCC TCG ATC AGR TTR TGG TT		
amoA archaea	amoA-1F	amoA-2R		
	STA ATG GTC TGG CTT AGA CG	GCG GCC ATC CAT CTG TAT GT		
nirS	niS2F	nirS3R	164	55
	TAC CAC CCS GAR CCG C GC GT	GCC GCC GTC RTG VAG GAA		
nifH	nifHF	nifHR	457	50
	AAAGGYGGWATCGGYAARTCCA CCAC	TTGTTSGCSGCRTACATSGCCAT CAT		
nrfA	nrfAF2aw	nrfA7R1	269	52
	CAR TGY CAY GTB GAR TA	TWN GGC ATR TGR CAR TC		

4.2. Results

The presented results cover the quantification of Bacterial *amoA*, Archaeal *amoA* and the *nirK* using qPCR in soils from experimental fields for apples and strawberries and organic and IPM management practices in Germany, Italy, Austria and Poland. The Bacterial *amoA* gene was detected in all sites and there was considerable variability between the observed copy number ranging from a median 2.5 to 7 log10 copies per gram of soil (Figure 10). The majority of soils from apple orchards in Germany had lower abundance than field sites for apples, strawberry and tomato crop trials in Austria, Italy and Poland. The Archaeal *amoA* ranged from median value of 5 to 8 log10 copies per gram of soils and soils from apple orchard in Germany tended to have lower copy number of Archaeal *amoA* per gram of soil (Figure 11). There was no clear distinction between sites for organic and IPM management practises for Bacterial and Archaeal *amoA* across soils. The *nirK* gene were detected with a median value of 5-7.5 log10 copies per gram of soil (Figure 12).

The qPCR results were also compared across crop type irrespective of experimental field site and country (Figure 13, Figure 14, Figure 15). The comparison suggested that median copy numbers of Bacterial *AmoA* of 5.1 log10 per gram of soil across the three crops and two management types. There was more variation for the Archaeal *amoA*, and the median copy number was lower for the sites with organic than the IPM management practice. The *nirK* had a lower copy number per gram of soil in strawberries than for apples and tomatoes. Due to limited number of samples, it was not possible at this stage to compare management practices.

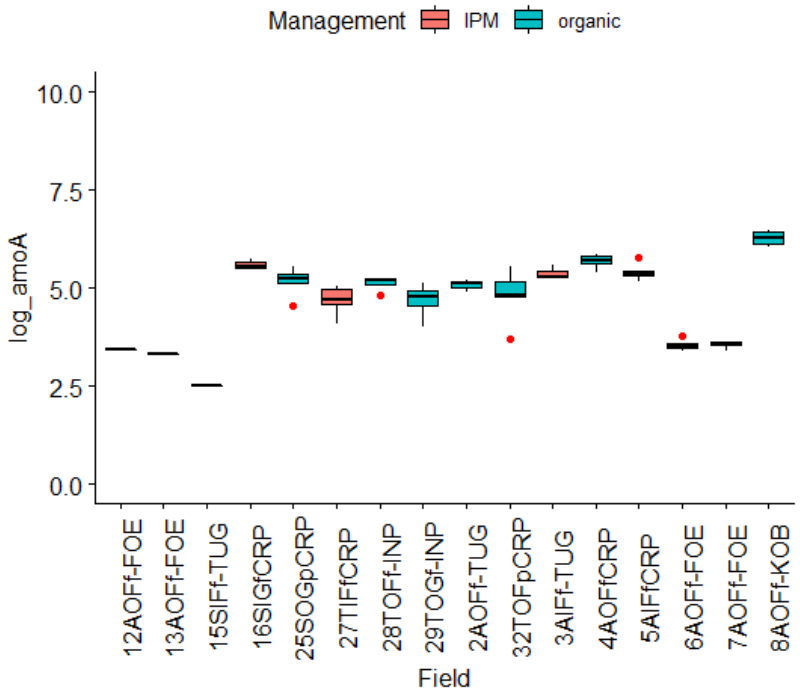

Figure 10: Baseline assessment of Archaeal Ammonia monooxygenase, *amoA*, gene copy number per gram of soil in different EXCALIBUR fields. Colour code represents the field management practices. Outliers are shown in red.

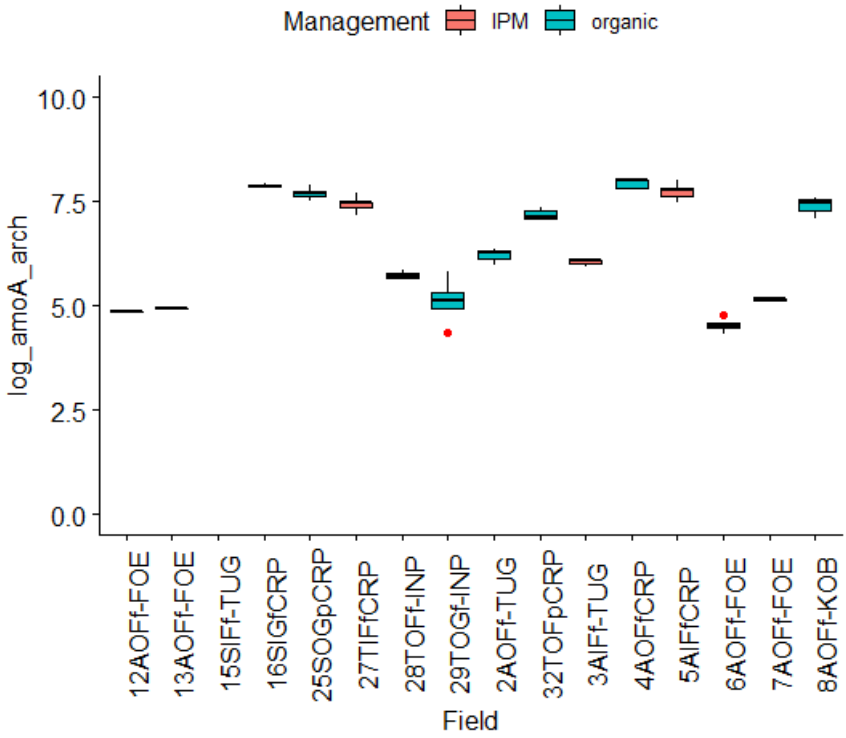

Figure 11: Baseline assessment of Bacterial Ammonia monooxygenase, *amoA*, gene copy number per gram of soil in different EXCALIBUR fields. Colour code represents the field management practices. Outliers are shown in red.

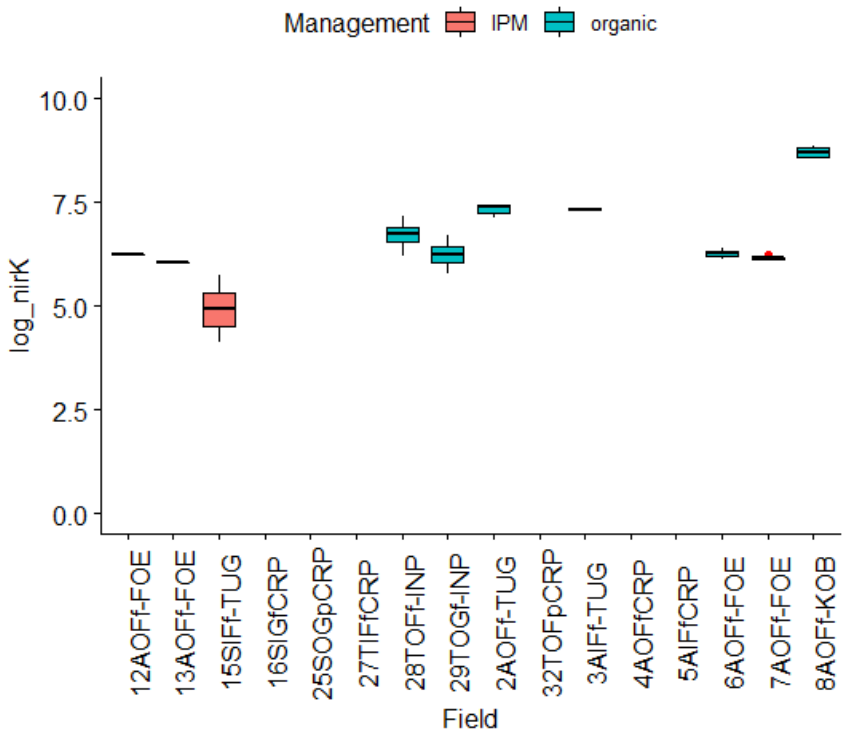

Figure 12: Baseline assessment of nitrate reductase, *nirK*, gene copy number per gram of soil in different EXCALIBUR fields. Colour code represents the field management practices.

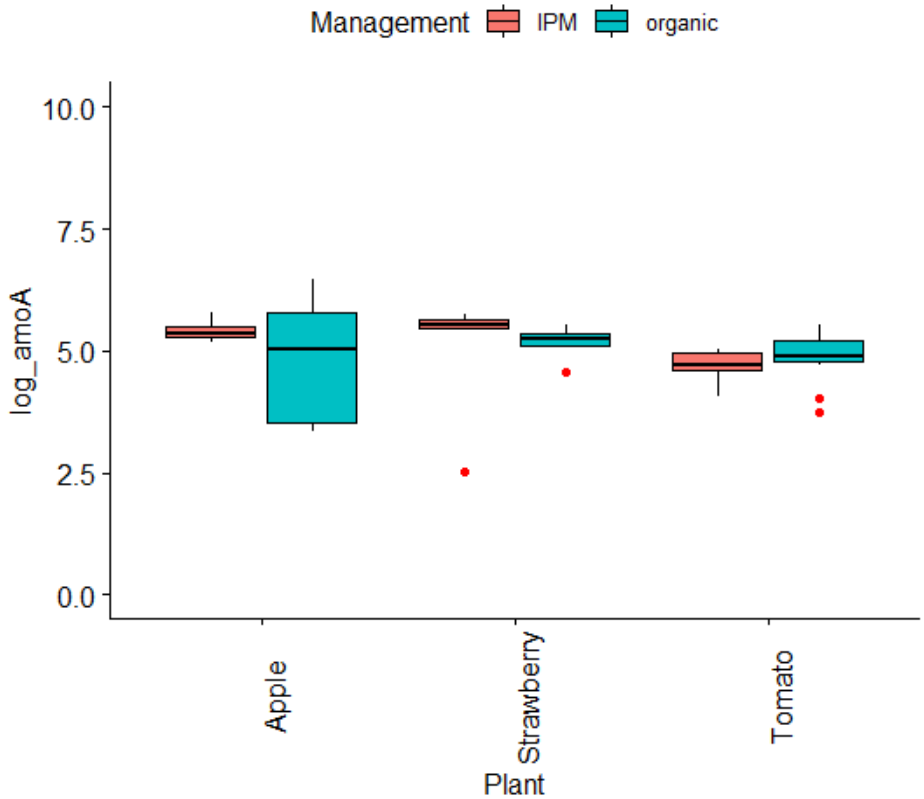

Figure 13. Baseline assessment of Bacterial Ammonia monooxygenase, *amoA*, gene copy number per gram of soil for different crop types. Colour code represents the field management practices. Outliers are shown in red.

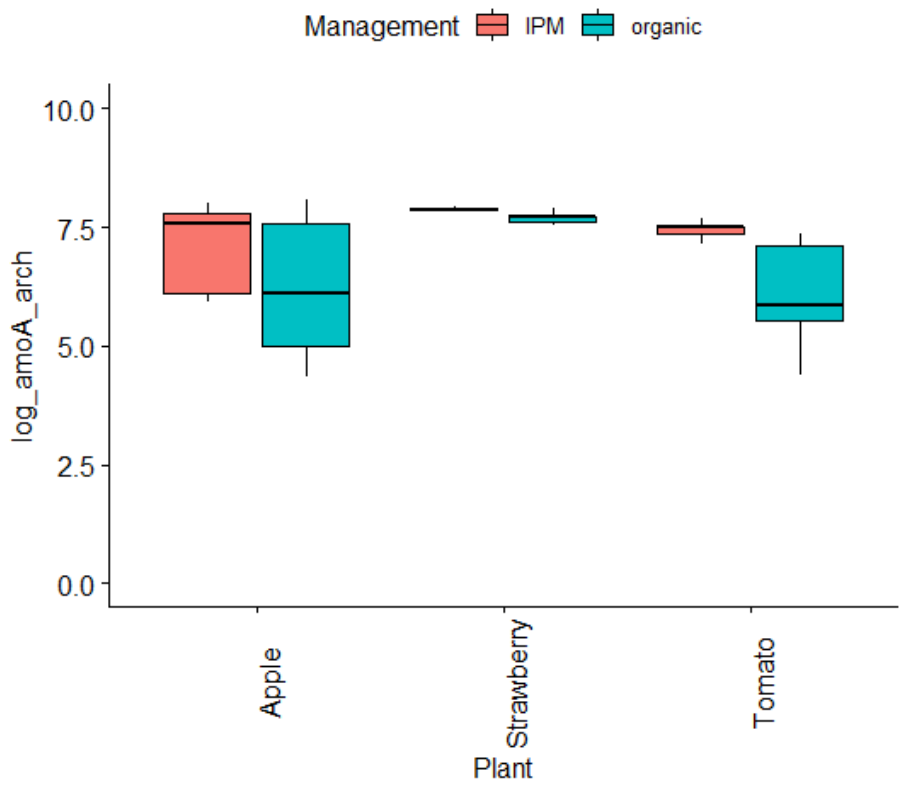

Figure 14. Baseline assessment of Archaeal Ammonia monooxygenase, *amoA*, gene copy number per gram of soil for different crop types. Colour code represents the field management practices.

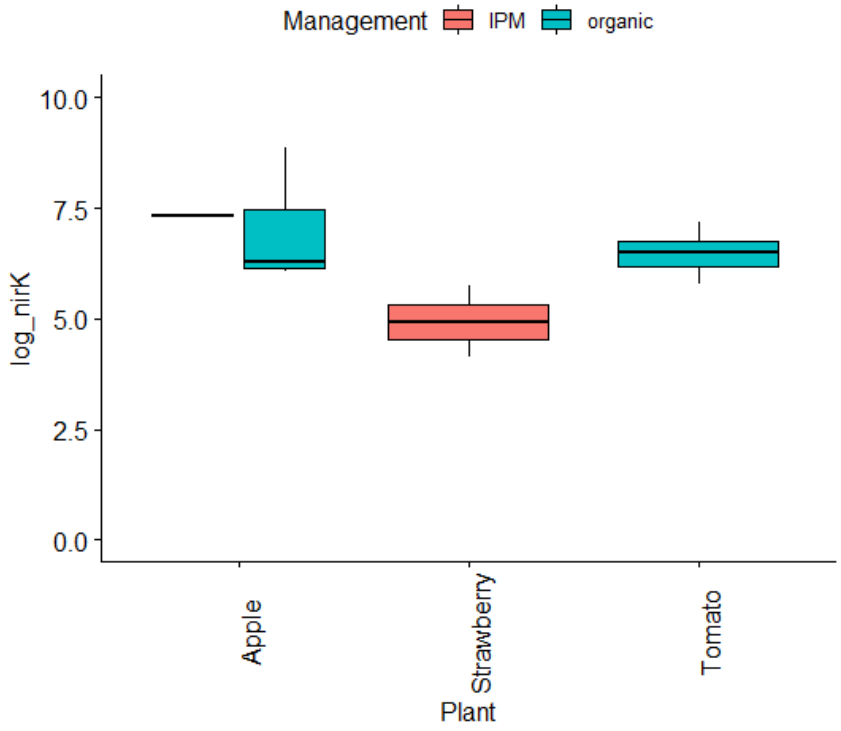

Figure 15. Baseline assessment of nitrate reductase, *nirK* gene copy number per gram of soil for different crop types. Colour code represents the field management practices.

5. Quantification of soil bacteria and fungi using colony forming units

Microbiological evaluation of soils for the baseline assessment were performed by colony forming units (CFU) of bacterial and fungal counts are presented for a selection of trial sites.

5.1. Material and Methods

For each soil sample, one gram of soil was suspended in 9 ml of sterile PBS (Phosphate-buffered saline) or physiological solution (0.85% NaCl) and vortexed thoroughly. From this stock solution, serial dilutions (1:10) were performed up to 10^{-7}. Aliquots of 100 µl from each dilution were plated in duplicates on each culture media and incubated at room temperature upside down.

For bacterial counts, aliquots were spread-plated on R2A containing cycloheximide (20µg/mL) to prevent fungal growth and counted after 24 and 48h. Plates were incubated longer to check if no additional colonies appear.

For fungal counts, aliquots were spread-plated on PDA (containing 24 g/L of potato dextrose broth + 14 g/L of agar or use directly 39g/L of potato dextrose agar or according to manufacturer instructions) containing streptomycin sulfate (50 µg/mL), penicillin G sodium salt (100 µg/mL) and tetracycline hydrochloride salt (10 µg/mL) to prevent bacterial contaminations. Plates were first counted after 48h and incubated longer to check for additional growth. For the evaluation, only plates with CFU (colony forming units) between 30-300 were counted. If more plates/dilutions were countable, mean values were calculated. Number of CFU per gram soil were calculated according to the formula below:

$$N_c \times Df \times 10 \times 9 = N_{CFU}/g \text{ soil}$$

N_c = Number of colonies
Df = Dilution factor
N_{CFU} = Number of colony forming units

5.2. Results

The assessment of colony forming unit (CFU) allowed the determination of viable bacteria and fungi associated with the soils that will be used for a range of trials with different bioinoculants. Results re presented for soil samples that were collected for the baseline assessment for trial sites that will be used of IPM and organic management practises for strawberries and apples. The bacterial CFU per gram of soil were higher than fungal CFU for all sites (Figure 16 and Figure 17). A total of 10^5 to 10^8 bacteria CFU and 10^3 to 10^5 fungal CFU per gram soils were identified from the soil samples. Experimental field sites for strawberry crops tended to have a higher CFU than soils from apple orchards.

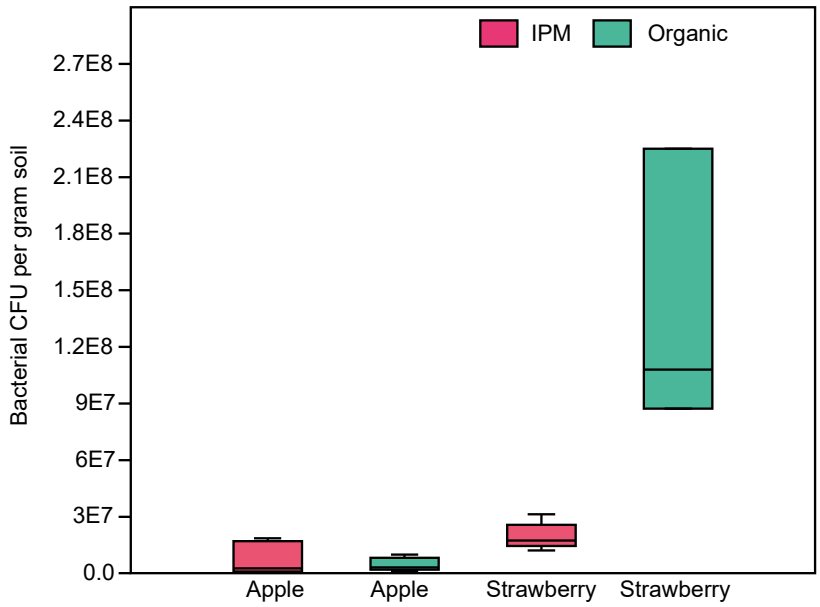

Figure 16: Overview of bacterial CFU counts for soils for sites that will be used for strawberry and apple crops and IPM and organic trials.

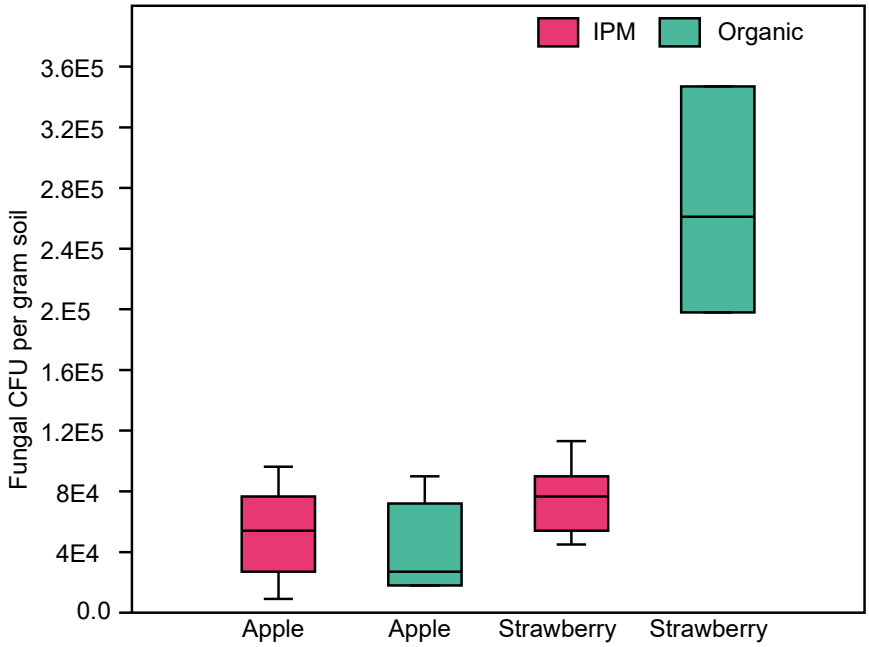

Figure 17. Overview of fungal CFU counts for soils for sites that will be used for strawberry and apple trials

6. Assessment of Arbuscular Mycorrhizal Fungi (AMF) concentration in soils before the start of the trials (AMF)

AMF are fungi that form a mutualistic symbiosis with the roots of plants, and they can enhance the update of nutrients from the soil as well as provide other benefits to the host plant. For baseline data, AMF concentration of soils were evaluated for soils from experimental trial sites in Austria, Denmark, France, Poland, Italy, Slovenia, Germany and the UK.

6.1. Material and Methods

AMF was assessed using the Most Probably Number (MPN) methodology. In brief, four dilutions of each soil were prepared at 1, 10, 100, and 1000 in sterile substrate and 5 replicates per dilution per soil. The dilutions were added to growth media with lucerne seedlings. After 5 weeks of incubations the samples were stained according to the technique described by Phillips and Hayman (1970) and MPN of mycorrhizal fungal propagules was estimated using Tables 100-1 and 100-2 in Cochran (1950). This technique allowed evaluating of the number of active propagules of AMF in the soil with propagule corresponding to a part or structure of a fungus (i.e. hyphae, spores, arbuscules), that is capable of developing to anew fungi organisms. The soils were assigned to poor (0-1000 propagules/kg soil), moderately poor (1000-5000 propagules/kg soil), moderately rich (5000-10000 propagules/kg soil) and rich (>10000 propagules per soil) in AMF propagules richness based on the number of AMF propagules per kg soil, as shown in Figure 18.

6.2. Results

These results will allow to determine the concentration and richness of AMF in soils before the beginning of trials (Figure 19). At the beginning of each trial, most of soils can be defined as “poor” in AMF (less than 1000 propagules/kg soil). Two or less trial sites classified as moderately poor, moderate rich or rich in AMF propagules. Soils with moderately and moderately rich AMF richness were only determined from trials that will be used for used for strawberries. The data will be important for WP4 because the same analysis will be performed at the end of the trials and compared with the baseline data to evaluate if the bioinoculant treatments have influenced the AMF in the soils. In addition, the colonization rate of AMF inside the roots will be evaluated during the trials and at the end of the trials as AMF can also be affected by type of crop plants and edaphic conditions in addition to the bioinoculants.

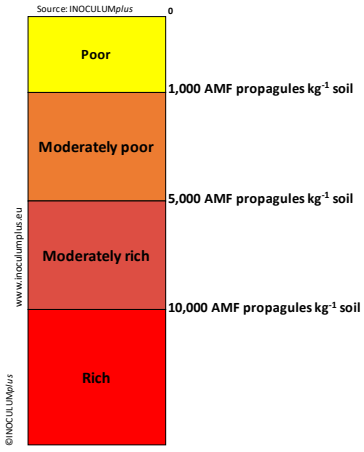

Figure 18. AMF propagules richness was grouped into four classes such as poor, moderately poor, moderately rich and rich based on number of propagules per kg soil.

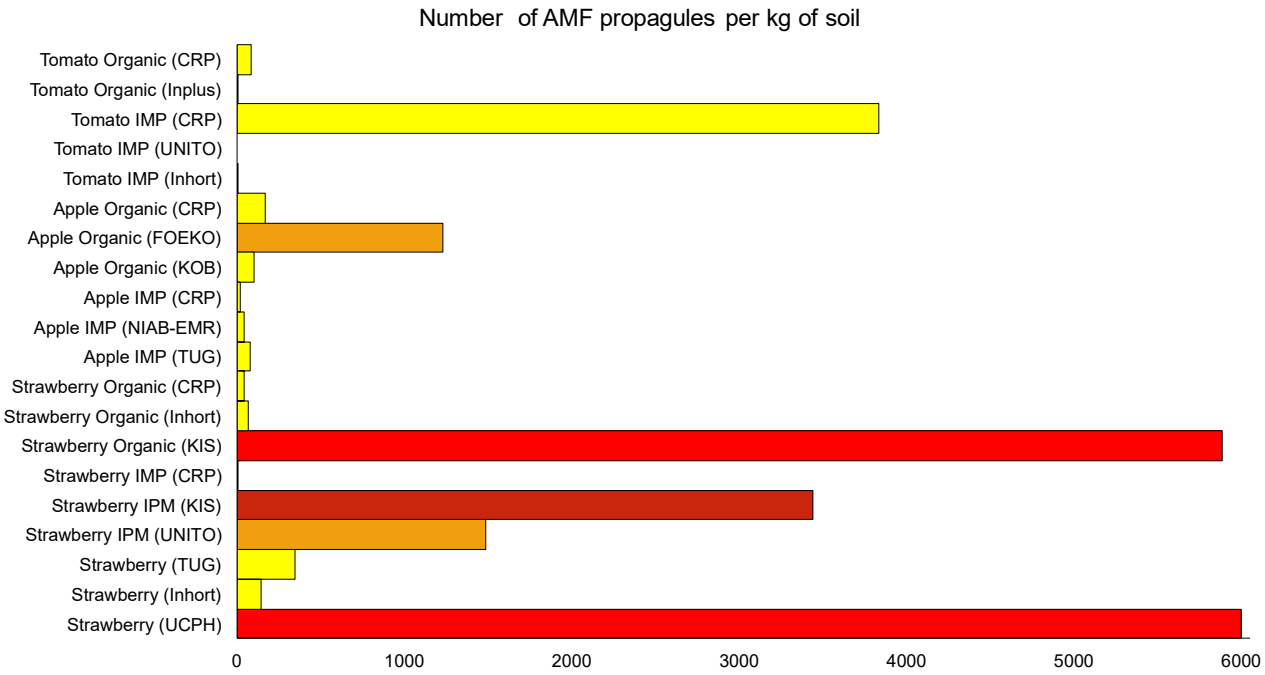

Figure 19. Average number of AMF propagules richness per kg of soil in selected trials sites that will be used for tomato, apple and strawberry trials. Colour code represents AMF richness as shown in Figure 18.

7. Conclusions

The analysis allowed to characterize the baseline microbial populations using culture-independent approaches such as marker gene high throughput sequencing to determine the composition and richness of microbial communities and detect and quantify genes implicated in the nitrogen cycle using qPCR. The microbiology assessments showed the presence of viable bacteria and fungi. The evaluation identified that the soils are comprised of diverse bacteria, archaea and fungal assemblages. The baseline data also showed that microbial populations vary across experimental trial sites and communities can be affected by pedoclimate zone, crop type and horticultural management practices. The findings highlight to importance to understand the soil microbiology at each of the experimental trial site, and importance of in-depth characterization soil microbiology. The findings will be used in the other work packages to evaluate the influence of bioinoculants on the soil microbial diversity and plant microbiomes interactions. It will help to gain a better understanding on how soil microbial populations and beneficial microorganisms improve the plant growth and health. The results obtained from the assessment of native (baseline) microbial biodiversity in these trials will allow to define a multi-criteria model that will constitute the reference framework to confront with the new proposed strategy for management of microbial-based plant nutrition and protection. The findings will also be published in a peer-reviewed publications and disseminated to the research community at national and international conferences.

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