Interreg Italia–Österreich

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CEDRIC

BEST PRACTICE 1

Field sampling of the root system

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As part of the CEDRIC project, barley root samples were collected, from which the rhizosphere was isolated and used for subsequent molecular analyses. The field sampling provided an opportunity to apply and refine the collection methodology, which is described in detail below for the benefit of anyone interested.

1) Material preparation

- **Tools:** Sterilized shovel, stakes, tweezers, sterile gloves, sterile bags or containers (e.g., tubes, freezer bags), cleaning paper, denatured alcohol, waste collection bag, refrigerated container (with ice or ice packs) or liquid nitrogen (if required)
- **Labelling:** Prepare durable labels, permanent markers, and a notebook for notes.
- **Sterility:** All tools must be sterilized before sampling (autoclaved or sterilized with 70% ethanol)

2) Site Preparation

- Avoid collecting samples along the edges or in areas with atypical characteristics that are not representative of the entire field.
- If a large amount of material is needed, mark the sampling area with stakes or other visible markers.
- Select multiple sampling points evenly distributed to cover the entire area under study.
- Determine the correct depth for root and soil collection in advance, and maintain it consistently for all samples.

Box 1. The Root Microbiome: a Complex Ecosystem to Explore

Plants do not live in isolation within the **soil**; instead, they constantly interact with complex **microbial communities** that include **bacteria, fungi, viruses**, and other **micro- and macro**-organisms. All of these can exert a significant influence on the soil as well as on plant **physiology and health**. Some are pathogens that cause diseases, while others are beneficial: they promote plant growth, improve nutrient uptake, and increase tolerance to environmental stresses. In addition to these extremes, there are also "commensal" bacteria, which do not have an immediately evident positive or negative effect on the plant, but can nonetheless influence the balance and dynamics of the **microbial community** in the **soil** and on the **root surface.**

For these reasons, the scientific community has shown increasing interest in studying the microbiome associated with roots, particularly in the **rhizosphere** (the soil zone directly influenced by root secretions) and the **rhizoplane** (the root surface itself). However, analysing these microbial communities is challenging because a large proportion of the microorganisms present cannot be cultured in the laboratory using conventional methods. These difficulties arise from their slow growth, their requirement for specific environmental conditions, or their dependence on interactions with other microorganisms.

To overcome these limitations, researchers rely on high-throughput DNA sequencing technologies, which allow the direct analysis of microbial communities *in situ* without the need for cultivation. Such metagenomic approaches provide a more comprehensive and accurate view of microbial diversity and of the potential functions carried out by these organisms within the root ecosystem.

3) Extraction, initial cleaning, and preservation of the root sample

During sample collection, it is essential to adopt strict measures to avoid contamination: always wear gloves disinfected with denatured alcohol replace them between samples. Sterilize all tools with denatured alcohol and clean them thoroughly between each sample.

Box 2. Sampling depth: a choice guided by the study objectives

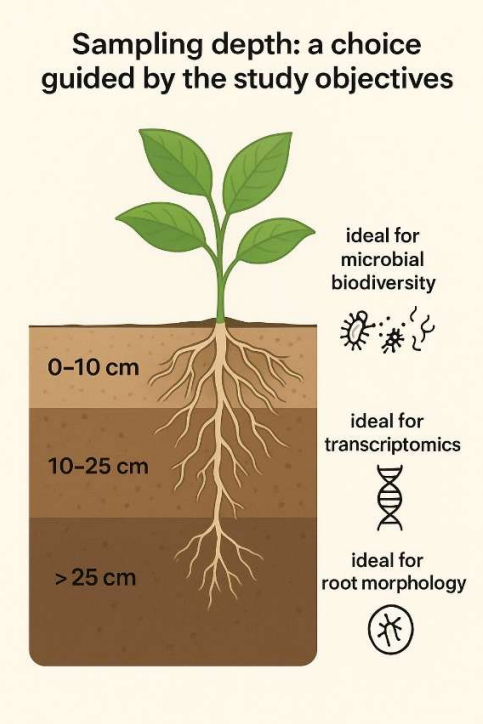
The **sampling depth** must be adjusted according to the specific objectives of the study, such as, for example, the study of **root morphology, metagenomics, transcriptomics**, the **isolation of new microorganisms**, or other research purposes.

In the case of **morphological studies**, the depth will depend on the **plant species** considered and the **structure of its root system**.

For investigations on **microbial biodiversity**, it is recommended to focus on **a depth between 0 and 25 cm**, where most of the **microbial activity associated with roots** is generally concentrated.

For studies on **plant physiology and transcriptomics**, however, it is preferable to sample the zone with the **greatest development of fine roots**, since these show **high metabolic activity** and represent the main site of interaction with the microbiota.

- Using clean gloves and a sterilized shovel, carefully dig around the plant and extract the material to be sampled.
- Remove excess soil by gently shaking the sample, leaving about 2 mm of soil attached to the roots. If necessary, a light tap at the base of the plant with a rubber mallet may be applied to help dislodge excess soil. If the residual soil layer is significantly thicker or thinner than the indicated 2 mm, it is important to record its thickness.
- The collected sample must be placed in clean bags (for example, food or freezer bags) or in tubes of suitable capacity (2, 10, 15, or 50 ml). Each sample must be labelled with a unique code to ensure its identification.



Box 3. How much to sample? Strategies to obtain representative root material

If the sampling is **conservative** and the plant must survive, collect the **minimum amount of root system** necessary for the analyses, trying to gather material from **multiple points** around the plant, including fine roots, which are **metabolically more active**.

For **morphological or architectural studies of roots**, extract the **entire root ball** using a spade, taking care to preserve the integrity of the root system. Begin digging at a suitable distance from the collar to avoid damaging the main roots. Remove the soil gradually, layer by layer, exposing the root system so as not to break or deform the roots. Extraction must be carried out **slowly and with great care**, avoiding sudden shaking that could damage fine roots. If possible, collect the entire root system and **postpone cleaning until in the laboratory**, where it can be done under more controlled conditions.

For studies aimed at analysing **microbial biodiversity associated with roots** or for **transcriptomic investigations**, it is recommended to collect **at least 500 mg of fresh roots**, preferably including the most active and colonized portions (such as fine roots).

For microorganism isolation, 3–10 grams of soil/rhizosphere per sample are generally considered adequate. After thorough homogenization, the sample can be subjected to serial dilutions and plating. It is also advisable to collect samples from multiple sites, since in the case of similar microbial species this reduces the probability of isolating multiple copies of the same strain.

- It is essential to keep the sample moist: for this purpose, it is possible to add a few millilitres of physiological solution, PBS (NaCl 0.137 M, KCl 0.0027 M, Na_2HPO_4 0.01 M, KH_2PO_4 0.0018 M), or another suitable solution when storing it.
- For samples intended for molecular analyses, it is advisable to immediately freeze the material in dry ice or liquid nitrogen, using only tubes suitable to withstand those conditions. If this procedure is not feasible in the field, temporary storage in a refrigerated container or in a polystyrene box with ice or ice packs can be used as an effective alternative to maintain sample quality. In this case, it may also be useful to employ a Soil Preservation Solution to prevent degradation and preserve the biological and molecular state of the sample in situ.
- For samples intended for microorganism isolation or for morphological and architectural studies of roots, a refrigerated container or a polystyrene box with ice or ice packs is sufficient. In this case, it is important that the refrigerant material is not placed in direct contact with the sample but is sufficiently insulated to avoid damage from freezing or excessive direct cooling.
- Thenumber of samples to be collected varies depending on the objectives of the sampling:
    - For molecular studies, such as metagenomics and transcriptomics, a minimum of 3–5 biological replicates from different plants for each treatment or condition is essential. However, a number between 5 and 10 biological replicates ensures more solid statistical validity.
    - In the case of studies on root morphology and architecture, 5–10 root biological replicates guarantee good representativeness of the population or treatment under investigation. A sampling of more than 20 roots provides greater robustness to the collected data.

- The samples collected in the field and kept at refrigerated temperature until arrival in the laboratory can be processed immediately after the cleaning phase.
  The degree of cleaning required depends on the specific objectives of the study:
    - For studies on root architecture and morphology, samples can be carefully cleaned using sterile water, to remove soil residues without damaging the structures.
    - For studies on the endosphere, meticulous cleaning followed by surface sterilization is required to eliminate external contamination.
    - The same approach applies to samples intended for physiological or transcriptomic analyses, for which it is essential to minimize the presence of exogenous contaminants.
    - For studies on the rhizosphere, samples must be treated in such a way as to allow recovery of the rhizospheric compartment, without eliminating it during cleaning.

- After cleaning, the samples can either be processed immediately, or:
    - Kept moist at 4 °C for morphological and architectural root analyses.
    - Frozen in liquid nitrogen and stored at –80 °C for molecular analyses.
    - Combined with glycerol (final concentration 21%) and stored at –80 °C if intended for microorganism (bacterial) isolation, or at 4 °C for fungal isolation, which must in any case be carried out within 48–72 hours.

Box 4. How many replicates are needed? Biological vs Technical

To obtain data with statistically significant value, it is essential to perform an adequate number of replicates. The number of replicates required varies depending on several factors, such as the natural variability of the system under study, the objectives of the research, the type of statistical analysis to be used, and the resources available. The replicates that

primarily confer significance to the results are biological replicates.

Biological replicates represent independent samples obtained from different biological units, such as plants, animals, or distinct individuals. Their purpose is to capture the natural biological variability that may exist between different subjects or experimental conditions. For example, in a study on the root microbiome, biological replicates correspond to roots collected from different plants, grown under similar or varying conditions depending on the experimental design. These replicates are essential because they allow the results to be generalized to the entire population and enable reliable statistical analyses to assess the impact of treatments or real biological conditions.

Technical replicates, on the other hand, consist of repetitions performed on the same biological sample. They are used to measure the variability introduced by experimental or laboratory processes, such as DNA or RNA extraction, PCR, library preparation for sequencing, or instrumental measurements. For example, in microbiome studies, DNA extraction from a single root can be repeated multiple times, or multiple PCR reactions can be performed on the same extract. Technical replicates are important to ensure the reliability and reproducibility of technical data, but they do not increase the biological representativeness of the study.

Metadata and Soil Coring

During field sampling, the accurate collection of metadata is essential to ensure traceability, reliability, and scientific validity of the results.

The first step is to collect enough soil, which will be used for chemical–physical analysis of the sample in the laboratory or at a specialized facility. Soil can be collected either by coring or using a spade, respecting the excavation depth used for root sampling.

Next, it is necessary to collect metadata, that is, all information describing who, what, where, when, how, and the specific details of the sampling.

It is therefore important to record:

- ☑ The name of the operator
- ☑ The plant species sampled
- ☑ The geographical coordinates of the field
- ☑ The soil conditions (dry, moist, waterlogged, etc.)
- ☑ The season and current and recent weather conditions
- ☑ Applied agricultural practices (e.g., tillage, irrigation)
- ☑ The most recent treatments applied (e.g, fertilizers, pesticides) with their respective dates
- ☑ Any other relevant observations useful for the objectives of the project

More information:

Simmons, T., Caddell, D.F., Deng, S., Coleman-Derr, D. Exploring the Root Microbiome: Extracting Bacterial Community Data from the Soil, Rhizosphere, and Root Endosphere. J. Vis. Exp. (135), e57561, doi:10.3791/57561 (2018).

Roey Angel 2020. Field sampling of root-associated microbes for DNA/RNA extraction. protocols.io https://dx.doi.org/10.17504/protocols.io.vd5e286