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EXCALIBUR

Exploiting the multifunctional potential of belowground biodiversity in horticultural farming

Deliverable 1.2

COMMON GUIDELINES FOR SAMPLING METHODS

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Authors: Eligio Malusà (INHORT)

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Abstract	The aim of these guidelines is to standardize soil sampling procedures performed during the EXCALIBUR project. They are adopted by the EXCALIBUR project and followed by the partners during the field trials (under both open-field and protected conditions). The document is part of a comprehensive series including guidelines for all the analyses foreseen in EXCALIBUR. These guidelines have been developed to suit the conditions and goals foreseen in the project activities. Therefore, in case of other research scopes, they should undergo a revision process to adapt to the specific cases.
Keywords	Soil sampling procedures, soil storage, soil transport

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1. EXECUTIVE SUMMARY

The aim of these experimental guidelines is to standardize soil sampling procedures performed during the EXCALIBUR project. They are adopted by the EXCALIBUR project and followed by the partners in performing the field trials (under both open and protected conditions).

The document is part of a comprehensive series including guidelines for all the analyses foreseen by the project activities. These guidelines have been developed to suit the conditions and goals foreseen in the project activities. Therefore, in case of other research scopes, they should undergo a revision process to adapt to the specific cases.

2. PROCEDURES FOR SOIL SAMPLING AND PREPARATION

2.1 Glossary in brief

- Soil sample: the sample to be analyzed in laboratory, obtained through the procedures described below in order to have a sample representative of the native soil from a given field/treatment.
- Soil subsample: each sample unit taken in the field, according to the soil sampling strategy adopted.
- Composite sample: the sample obtained by pooling the subsamples collected in each field; each subsample must contribute the same amount to the composite.
- Soil aliquots: are distinct aliquots of the soil sample, to be sent to the different laboratories.
  [from D. L. Sparks (Editor) 1996. Chapter I - Sampling, in: Methods of Soil Analysis – Part 3 Chemical Methods.]
- Field: is the whole field of the trial (orchard, plantation). Plot is the part of the field that contains a repetition of the treatment.

2.2 General aspects

The main criteria to be considered about soil sampling is to assure the highest homogeneity of the sample and representativeness of the field soil.

The following features should be taken into consideration as general remarks to be considered while reading the following instructions:

a) In order to maximize the consistency of the results, all the partners working on each trial will analyze the “same” soil samples. Thus, a **unique soil sample** (with its replicates) shall be collected in a sufficient amount to carry out all the different analyses foreseen by all the interested partners: chemical, physical, biochemical, molecular, microbiological and for microfauna. Therefore, the collected soil sample will be subdivided in several aliquots, each having a sufficient amount of soil necessary for the specific analysis of the involved partners.

b) The samples necessary for the initial analyses (WP1) could be done on a bare soil, in case the crop is still not present on the field. However, for samples to be collected on the field trials (WP3) hosting plants or other trials carried out in other WPs (including WP1 apple fields already available), the "bulk soil that is in the vicinity of the root system" should be collected. This definition could be used also in our reports/publications. In fact, even though this is not a clear definition as for the "rhizospheric" soil, we believe that a practical approach, particularly in case of the apple root system, should be considered to collect a sufficient amount of such soil.

c) In general, when sampling bare soil (WP1) it is important to collect it from soil (field) that has been "cleaned" from residues of previous crops, to avoid, particularly for biochemical, microbiological and molecular analyses, a possible bias in the results due to the residual organic matter. This means that the soil should have been tilled before sampling.

d) The methods for taking the soil samples on which the mesofauna collection and some of the physical analyses will be carried out will be described in further specific instructions within the relevant analytical methods, since they require a specific methodology. However, the same principle of collection in the vicinity of the plants root system applies also to these samples.

e) The weight of each replicate of the laboratory sample shall be at least **2 kg**, to allow performing all planned analyses (WP1). This amount derives from the following analysis requirements:
1) Chemical analyses: about 500 g
2) Physical analyses: about 500 g
3) Nematodes community analyses: about 500 g
4) Biochemical analyses: about 250 g
5) Microbiological analyses: about 100 g
6) AMF analyses (Most probable Number protocol): about 500 g
7) Molecular analyses: about 10-50 g
For other analyses, the sample weight will depend on the kind and number of analyses to be performed.

f) Considering that, even if remote, it may be possible that samples to be analyzed by another partner can be damaged or lost during shipping from the sampling partner, it is suggested to collect and prepare all samples in a double quantity and send just half of them. When the receiving lab will confirm to have well received the samples, then it will be possible to discharge the other half kept by the sending partner. In the unlikely event of losing/damaging the first parcel, the second half can be sent.

g) Sampling for WP1 tasks shall include 4 laboratory samples (replicates) for the initial characterization of the field. Sampling for WP3 and other WPs tasks shall include at least 3 replicates (better 4) per treatment. In case of not homogeneous soil, a higher number of replicates could be considered.

The laboratory soil sample (i.e. the sample to be delivered to the laboratory/es for the different analyses) shall be put into a paper or plastic bag, including a label written with a pencil, with the sample code as proposed in the following table:

crop	**treatement**	**sample type**	**replicate**	**partner-field**	**date**
Apple = A *Strawberry = S* *Tomato = T*	*Untreated = U* *ORG = A* *IPM = B* *ORG+IPM = C*	*Rhizosphere = R* *Bulk = B* *Plant = P*	*from 1 to 5*	*number of the Partner (e.g. 1= CREA, 2=INHORT, etc.) and of the field trial*	*ddmmyyyy*

For example: the sample code of an untreated soil rhizospheric sample collected in the experimental trial of Skierniewice (POLAND), hosting apple under organic management should be: AUR1_2-1_07102019.

2.3 General soil sampling procedure

Sampling is performed with an auger, collecting at least 10 subsamples at a 0-30 cm depth (normally of about 50-100 g each) per treatment/plot (see Figure 1 below). If an auger is not available, the subsample may be collected using a spade. These subsamples, the same amount in weight each, will be pooled (see below) to give a composite sample.

Generally, the upper layer (0-5 cm), i.e. the mulch layer, litter layer, stone layer and crust at the soil surface, is removed before sampling.

Depending on the crop, subsamples can be collected as follows:

- using the Z (or W) shape pattern in case of **bare soil** (WP1): subsamples are collected along a line that resembles the Z (or W) on the whole field/plot (see Fig. 2a).
- **near the root system** (WP3, and WP1 for already established apple orchards): collecting in the vicinity of the roots, possibly from two opposite sides of the plant (see Fig. 2b).

The soil subsamples are collected into a clean bucket, which must be cleaned before another sample is collected. The overall composite sample (mix of the subsamples) is than homogenized (mixed) in the bucket or on a clean plastic sheet. The total amount of soil is then spread on the plastic sheet and littles stones, organic parts (e.g. plant residues, roots, etc.) and visible animals shall be removed.
Further on, (in the field or later on a table/lab bench) the soil is spread on a plastic sheet and arranged to form a rectangle with approximatively constant thickness. The area is divided by two diagonals and two opposite portions are mixed and homogenized, spread again on the plastic sheet and divided by diagonals until reaching the required size of final sample. The procedure shall be repeated in order to split the sample in more replicates (laboratory samples) as specified below.
If possible, the sample should be sieved (mesh 2 mm) before the final amount is ready for storage or delivery. In case a sieve is not available, the removal of visible organic debris or little stones and sample homogenization has to be done manually.

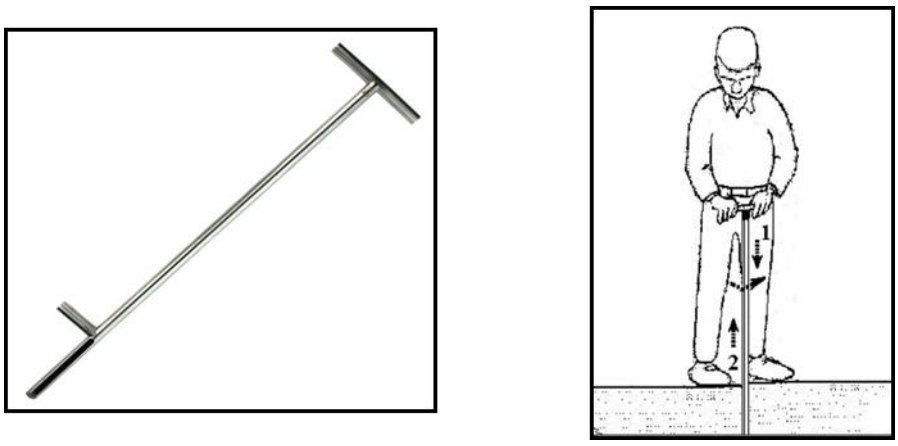

Figure 1 - Soil auger and method of collecting a sub-sample

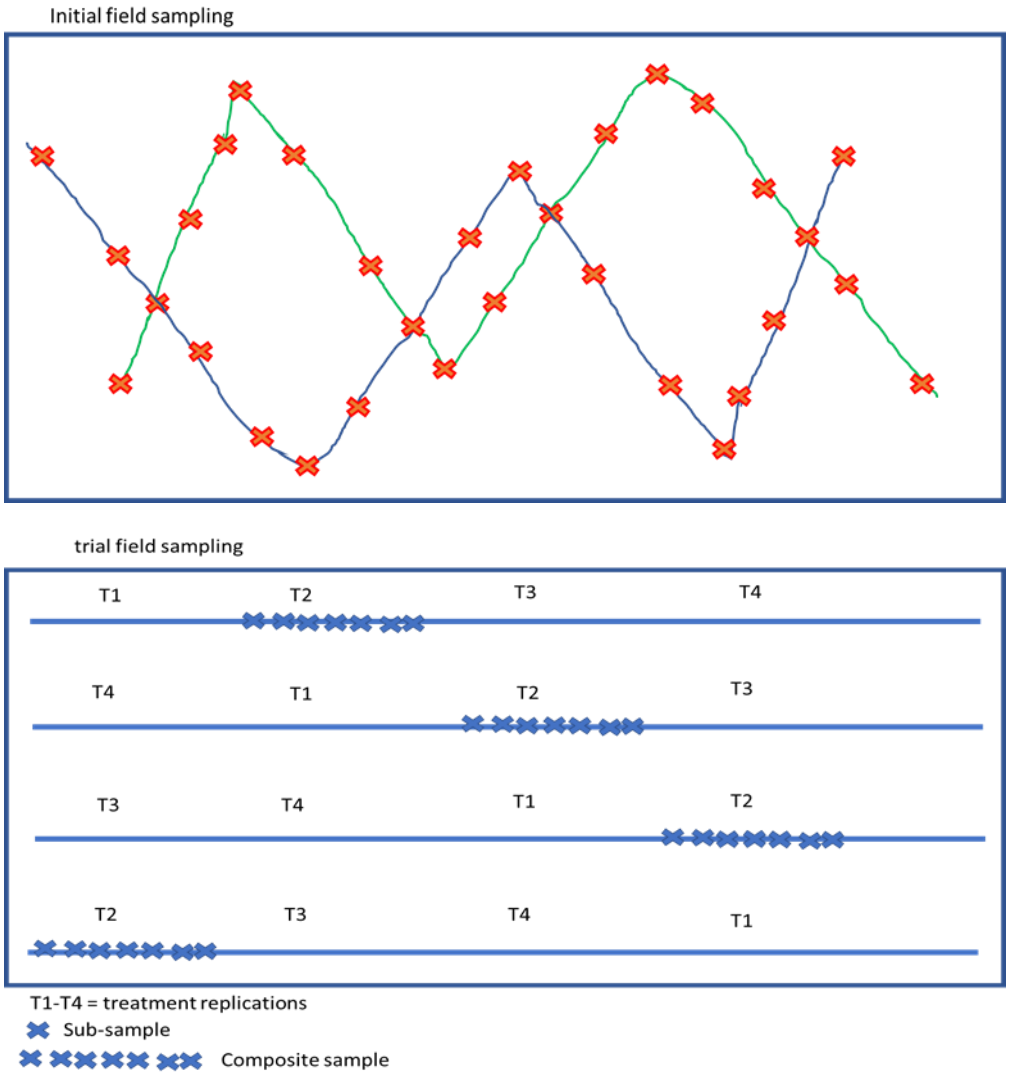

Figure 2 - Above (a): sampling scheme for bare soil; Below (b): sampling scheme for the soil close to the roots

2.4 Storage and delivery of soil samples

In case of chemical and basic physical analyses, the soil can be transported and stored under room temperature. Delivery to the laboratory shall occur as rapidly as possible, within a few days at the latest. Storage shall be done in a shadowed, well aerated place.

In case of nematodes analysis, the samples should be stored in a cold and dark place and analyzed within a day. In case of delivery to other labs, the sample should be kept at 4°C also during the transport and delivered as soon as possible.

In case of all other analyses (biochemical, microbiological and molecular), the soil must be stored at -20°C if not used immediately.

However, in case of AMF analyses, samples can be stored at room temperature if delivery to the laboratory occurs within a few days at the latest. If not, samples must be stored at 4°C.

The delivery of samples to other laboratories shall be organized assuring that the samples are maintained in the same storage conditions set after sampling.

2.5 Soil pre-processing

At the moment of its use a re-conditioning process must be applied on each sample: thawing of samples that were previously frozen must be done with special care.

For the analyses of microbial activity (e.g., soil respiration), a thawing period of one week at 4°C and another three days at 20°C are recommended (if necessary, a shortened thawing period of one day at 20°C may also be suitable).

For DNA analyses, the thawing period should be as short as possible to avoid degradation processes.

Freezing the samples can change the water-holding capacity. Therefore, water-holding capacity should be determined before and after thawing.

3. SPECIFIC INSTRUCTIONS FOR SAMPLES FOR MOLECULAR ANALYSES

Soil samples destined to DNA extraction and, for some selected case studies, to RNA extraction must be taken as subsamples of the same laboratory sample used to measure the other parameters (i.e. enzymatic activities, chemical or physical analyses, etc.).

Soil samples destined to DNA extraction will contemplate biological replicates: 4 samples per treatment (the same used for the measurement of other biological parameters). The 4 samples should have soil chemical and physical properties well within the average of all samples taken from one treatment and destined to soil chemical and physical analyses. They must not have extreme properties in order to avoid outliers.

Soil aliquots destined to DNA and RNA extraction must be sieved (<2 mm) to homogenize the sample and reduce potential contamination with plant and animal material. High clay and/or moisture content, however, can inhibit effective sieving. In this case the removal of visible organic debris and sample homogenization must be done manually. Once homogenized, soil samples need to be stored until further processing, the storage conditions must be chosen carefully. The homogenization, sieving and collection in dedicated containers can be done in the field when possible, or at the laboratory. In any case the storage and eventual shipping need to be done according to the same procedures, which are described below.

3.1 Storage of soil samples for DNA extraction

Recommended procedure

DNA extraction procedure can start from either fresh soil or (more feasibly) from frozen soil. The best option is to put samples immediately at -20°C or lower. Since most microbial cells burst during the freeze-thaw cycle that occurs when samples are extracted, a single freeze-thaw cycle is desirable, in order to obtain reproducible amounts of DNA. Therefore, soil samples for DNA extraction should be stored at -20°C, already sieved, homogenized and weighted in sterile DNAse free vials (example: 2ml screw-cap, cryogenic tubes, sterile, DNase-free).

As most labs may not have the facilities to freeze soil in the field, we propose a method where samples will be taken, put on ice in the field (i.e. into a thermo-stable shipping box like the Styrofoam with enough wet ice packs to keep temperature at about 4°C during shipping) sieved or hand homogenized upon arrival, aliquoted to 10 g in individual tubes and frozen to -20°C at the arrival. In this way we avoid the destruction of DNA inside the soil.

Each vial must be univocally labelled with cold-resistant writings/stickers.

3.2 Collection/storage of soil samples for RNA extraction.

Recommended procedure

For collection, transport and storage of soils needed for total RNA extraction, it is recommended to use the LifeGuard™ Soil Preservation Solution which is commercialized by Qiagen. This product efficiently protects nucleic acids from degradation in soil samples preventing RNase and DNase activity.

1) Weight 2.5 g of soil in a 15 ml RNase and DNase free Tube (i.e. a 15ml screw-cap cryogeny tube) and add 6 ml of LifeGuard™ Soil Preservation Solution (1 g of soil requires 2.5 ml of solution – you can add the solution to the tubes in sterility, before going to the field). If you cannot weight soil in the field use a volume of soil as a reference (a 5 ml tube, or equivalent).

2) Vortex or gently mix by hand the soil and the solution to obtain a mixture.

3) Store the soil in the LifeGuard™ Soil Preservation for one month at -20°C (2 weeks at 4°C or 1 week at room temperature).

4) Shipping can be performed at this stage. At 4°C, using boxes with ice packs like in eluted DNA shipping.

5) When you are ready for the total RNA extraction, samples can be slowly thawed at 4°C, if kept at -20, then centrifuged at 2500 x g for 5 min at 4°C to remove the solution and collect the soil to be further processed.

Reagents

LifeGuard Soil Preservation (from Qiagen): For the ambient temperature stabilization of microbial RNA in soil.

(https://www.qiagen.com/us/shop/sample-technologies/protein/stabilization-and-fixation/lifeguard-soil-preservation/#orderinginformation)

Materials and equipment

- 2ml screw-cap, cryogenic tubes sterile, RNase-free, DNase-free, (i.e. from BRAND, code 114841, or Sigma-Aldrich screw-cap TPP® 2.0 ml cryotubes, code Z760951)
- Cold resistant small labels and/or adhesive tapes
- Permanent-ink pen or labels with cold resistant glue (try before use)
- Sterile 15 ml screw-cap tubes RNase-free, DNase-free (if used for shipping be sure the closure is safe for liquids).
- Box container for 2ml vials (and for 15 ml vials in case you ship soil for RNA extraction)
- Styrofoam boxes
- wet ice packs
- Parafilm
- -20°C freezer
- Precision scale (4 decimals)
- A field scale (to weight soil for RNA extraction, 2.5 g/6 ml LifeGuard solution)
- Gloves suitable for RNase and DNase free lab.

3.3 References

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