PRO-GRACE

PROMOTING A PLANT GENETIC RESOURCE COMMUNITY FOR EUROPE

DELIVERABLE 3.1

Simplified sample collection protocols amenable to use by non-specialized personnel

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Simplified sample collection protocols amenable to use by non-specialized personnel (WP3)

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1. Contents

The Promoting a Plant Genetic Resource Community for Europe (PRO-GRACE) project is supported by the Horizon Europe programme under project number 101094738.

2. Introduction

The goal of WP3 is to provide advanced scientific services to identify and validate genomic, metabolomic, bioinformatic and phytosanitary technologies and scientific services useful for collection holders and the scientific community.
The WP3 aims to identify the metabolites to decipher plant performance and food/feed quality such as nutritional/antinutritional/toxic metabolites in crop wild relatives and wild food plants using vegetables, fruits, legumes and cereals as decided in WP4. Moreover, for the assessment of the genetic variability, WP3 aims to boost the quality of the infrastructures (genebanks, in situ plant genetic resources collections) which have limited technology capacities.

Deliverable 3.1: Simplified sample collection protocols amenable to use by non-specialized personnel. The main idea is to develop and demonstrate sample collection and shipment protocols, to simplify their usage of protocols by non-specialized personnel operating in genebanks, for the application of DNA barcoding, cytogenomic, metabolomic and phytosanitary characterization protocols. Plant sample preparation for analyses is a fundamental step in high-throughput omics strategies. Especially for plant metabolomics, quenching of hydrolytic enzymes able to affect metabolite concentrations is crucial for the accuracy of results. Since DNA is usually less labile than metabolites, most sampling and shipment procedures able to preserve the metabolome are also suitable for preventing the degradation of plant DNA or of DNA of pathogens in the plant tissue. Describing all the steps from sample collection, shipment, moving plant samples, and processing for metabolomics from a single sample allows to collect multi-omic datasets in large experimental setups (Figures 1 and 2).

3. Description of Activities

Variability among samples can emerge from various origins, encompassing both inherent biological diversity and factors associated with sample collection and storage (Gibon and Rolin 2012). Thus, sample preparation is a critical step for the isolated compounds and the accuracy of results.
Sampling can be performed as fresh frozen (liquid nitrogen) or freeze-dried (lyophilized) samples based on the intended analysis. Placing the samples in a liquid nitrogen tank to freeze them can be a simple and rapid way in metabolomics analyses. As an important factor, storage conditions must be controlled for the stability of the samples.
Plant DNA barcoding is currently a widely used tool for rapid and accurate identification of plant species or accessions within the same species. Proper classification of plant material in a genebank is an indispensable activity since the distribution worldwide of misclassified material could have very detrimental effects on research programs.

Often, plant samples must be shipped to specialized laboratories that are well-equipped for conducting genomic and metabolomic analyses, which may require cross-country shipment of plant samples. Countries have different regulations for shipping plant products such as plant samples. In general, for the import of an unprocessed plant product, a phytosanitary certificate issued by the National Plant Protection Organization of the exporting country is required.

A manuscript was prepared together by MPG, ENEA and WORLDVEG, describing sample preparation protocols for genomic/metabolomic/phytosanitary analyses, that has been submitted for publication.

4. Results

The main results related to the present deliverable as protocol has been adjusted to apply sample collection, shipment including the phytosanitary issues of moving plant samples, and processing for combined genomics and metabolomics from a single sample allowing to collect multi-omic datasets in large experimental setups. The following protocols are reported:

Sampling for DNA flow cytometry

Sampling for genomic analyses
Protocol 1: Preparation of freeze-dried leaf disks for multiplexed PCR or DArT-Seq genotyping
Protocol 2: Medium throughput preparation of pathogen-free nucleic acids for most genotyping-resequencing applications or pathogen detection
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Sampling for metabolomics analyses
Protocol 3: Preparation of freeze-dried plant material for metabolomics
Protocol 4: Preparation of fresh frozen plant material for metabolomics
Protocol 5: Preparation and shipment of metabolite extracts for metabolomic analyses
Protocol 6: Sample shipping and long-term storage

4.1 Sampling for DNA flow cytometry

Introduction

Flow cytometric estimation of DNA ploidy level and/or genome size in absolute units (pg DNA, Mbp) is performed on suspensions of intact cell nuclei. Thus, the samples delivered for the analysis must be suitable for the preparation of this type of samples. In general, fresh and not withered tissues are required. The sampled tissues must not be mechanically damaged and should be free of parasites, pathogens and epiphytes to eliminate biological contamination.

Materials

- Plastic petri dishes (5-10cm diameter depending on the size of leaves)
- Paper tissues
- Filter paper
- Distilled water
- Cooling box
- Cooling blocks
- Plastic bags
- Nursery pots for growing plantlets
- Potting soil
- Pot labels

Equipment and facilities

- Refrigerator
- Growth chamber with controlled light and temperature regime
- Greenhouse

Protocol

1. Place several layers of paper towels into a plastic petri dish (5-10cm diameter so that the leaves can fit in it); top them with a single sheet of filter paper.

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2. Moisten the paper layers with deionized H_2O.
3. Select randomly 5 different plants from each accession.
4. Collect 5 young leaves from each plant and place them immediately into a petri dish (see Note 1)
a. Samples obtained from plants cultivated under controlled conditions (i.e., growth chamber or a greenhouse) are preferred to minimize potential environmental biases due to extreme temperatures and light conditions.
b. It is possible to sample leaves from *in vitro* cultivated plantlets (i.e., in case of long-term *in vitro* storage of some clonally propagated plants). However, it is strongly advised to transfer *in vitro* grown plantlets to soil and collect leaves from small, rooted plantlets.
c. Samples collected from field-grown plants should be used only if material cultivated under controlled conditions is not available.
5. Transport the samples to flow cytometry laboratory (see Note 2)
a. Deliver the samples in as short time, as possible and avoid exposure to very high or very low temperatures during transport.
b. To avoid tissue deterioration, protect the samples from drying and high temperature during transport to a flow cytometry laboratory by placing the petri dishes into a cooling box.
c. If the samples must be sent by a courier, wrap the leaves individually in moistened paper tissue, put them in a plastic bag (remember to label the samples properly). Do not close the plastic bags air-tight - it is important to reduce the loss of humidity, but it is equally important to avoid anaerobic conditions. Sent the samples in a polystyrene box with cooling blocks by a courier (DHL, FedEx, UPS, ...) – see Note 3.

Notes

1. For more accurate results, use tissues and organs with a high proportion of cells in the G0/G1 phase of the cell cycle and with low amounts of phenolic and mucinous compounds. In most species, young not fully expanded leaves fulfil this requirement. Other tissues and organs than leaves, e.g., young stems or flower stalks, petioles, or young roots should be used as an alternative material only if leaves are not available and/or samples prepared from them are not suitable for flow cytometry.
2. If the collected fresh samples cannot be processed immediately upon delivery to a flow cytometry laboratory, they may be stored in a refrigerator. Depending on the species and time spent during a transfer to the laboratory, the samples may be stored for up to one week in a refrigerator, if adequate humidity is provided.
3. Many countries impose restrictions on importing living plant materials. It is highly recommended to check the actual import rules and requirements for permissions as well as the rules of particular couriers.

4.2 Sampling for genomic analyses

The protocols below fulfil the requirements for most types of genomic analyses, and also for nucleic acid-based detection of plant pathogens (bacteria, viruses).

Protocol 1: Preparation of Freeze-Dried Leaf Disks for Multiplexed PCR or DArT-Seq Genotyping

Leaf disks are collected in screw cap microfuge tubes, freeze-dried and shipped to a centralized laboratory/service provider for DNA extraction/ genotyping. The freeze-drying (lyophilization) method offers a robust and stable format which also facilitates easier shipment and storage. Lyophilization is used to remove moisture from various types of samples while preserving their structure (Maisl et al. 2023).

Materials

Young leaves from plant of interest

2-ml screw-cap polypropylene microfuge tubes with O-ring and frosted writing surface (e.g., Brand BR780758)

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Parafilm®
Breathe-Easier Sealing Film (e.g. VWR 10141-844)

Equipment

Freeze-drier
Refrigerated microcentrifuge

Sample preparation

1. Label the tube on the frosted surface.
2. Collect 1-2 leaf disks/plant by punching the leaves directly on the plant between the tube rim and a clean Parafilm surface held with your other hand. Twist the tube to make a clean cut. Avoid veins. The number of leaf disks varies according to the amount of tissue requested by the genotyping provider and to the plant species so it is recommended to perform preliminary tests to determine optimal amount of starting material or to consult the genotyping company. Keep on ice until step 3.
3. Centrifuge briefly to move the disks to the tube bottom.
4. Freeze the tubes containing the leaf disks in liquid nitrogen and store at -80°C until freeze-drying is performed.
5. For freeze-drying, open the tube and cover the tube opening with a piece of Breathe-Easier sealing film. If Breathe_Easier sealing film is not available, you can use Parafilm® perforated with a needle.
6. Place the samples in a tube cold rack quickly to avoid defrosting and freeze-dry overnight (times may vary according to the number of samples and efficiency of the freeze-drier).
7. Remove the sealing film and cap tightly.
8. Ship at room temperature.

Notes: *In some countries, reducing the material to powder before shipment is sufficient to comply with phytosanitary regulations. Please refer to section 6 how to check phytosanitary requirements. Grinding plant samples can be achieved by adding a tungsten bead to each tube and homogenizing it in a Mixer Mill (Retsch, model MM 400) after freeze-drying. For high throughput applications, individual tubes can be substituted by 96 deepwell polypropylene plates (eg Thermofisher 260252). In this case, leaf disks are collected using a metallic hole puncher of appropriate diameter. Plates must be sealed with air-tight polypropylene mats after freeze-drying (freeze-dried disks can stick to sealing tape and get lost).*

Protocol 2: Medium Throughput Preparation of Pathogen-Free Nucleic Acids for Most Genotyping-Resequencing Applications or Pathogen Detection

This protocol is for laboratories on a low budget that do not wish to use commercial kits or automated DNA extractors. Nucleic acids are extracted in a 96-well format using a phenol-chloroform-SDS method, precipitated with isopropanol, and shipped as a pellet. In our hands, the nucleic acids can be used for most genotyping applications, and for detection of pathogen DNA/RNA using quantitative or end-point PCR.

Materials

96-well deepwell plates (e.g. Thermofisher 260252) with polypropylene sealing mats
Collection microtubes racked 10X96 (Qiagen cat. N° 19560)
Collection microtube caps 120 x 8 (Qiagen cat. N°19566)
Isopropanol
TE buffer (Tris 10 mM / EDTA 0.1 mM pH8.0)
80% Ethanol
Phenol-chloroform (e.g., Thermofisher 17909)
Tungsten carbide beads 3 mm (e.g., Qiagen 69997)
RNase A DNase-free (e.g., Thermofisher EN0531)
Nuclease-free water (DEPC-treated or e.g., Thermofisher AM9935)

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Extraction Buffer
Tris-HCl 100 mM pH 8.0
NaCl 200 mM
EDTA 20 mM pH 8.0
Sodium dodecyl sulfate (SDS) 0,5 % (w/v)
2-mercaptoethanol 0.1% (v/v) (added fresh)

Equipment
Mixer Mill (Retsch, model MM 400)
Refrigerated high RCF (4,000-5,000 xg) microplate centrifuge
8-channel variable volume micropipettes (100-500 µL and 10-50 µL)
Benchtop plate mixer
Heating blocks

Procedure (192 samples):

1. Using a 5-mm hole puncher, collect two young leaf disks or cotyledon tissue per well in collection microtubes racked 10X96 . Enter the sample names in an Excel file for documentation.
2. Mark the collection microtubes racked 10X96 as 1 and 2, and pay attention to the orientation in order not to confuse the samples (The rack has a reference 96 well grid).
3. Freeze dry leaf material covering all the collection microtubes racked 10X 96 (Qiagen cat.N° 19560) with a bandage and a rubber band. Freeze drying is optional and allows a better tissue disruption, recovery of high molecular weight DNA and higher yield.
4. Add 1 tungsten bead to each collection microtubes racked and cover the tubes with collection microtube caps 120 x 8 (Qiagen cat. N°19566).
5. Disrupt the leaf tissue with a MM 400 homogenizer for 1 min at 25 Hz.
6. Using the 8-channel micropipette, add 400 µL extraction buffer and 100 µL phenol chloroform to each well. Seal the microtubes with collection microtube caps 120 x
7. Homogenize in MM 400 homogenizer, 2 min at 25 Herz.
8. Centrifuge 10 min at maximum speed, 4°C.
9. While centrifuging, prepare 2 clean 96-well deepwell plates on ice, numbered 1 and 2, with 250 µL isopropanol per well and 2 sealing mats, numbered 1 and 2 and with a mark on position A1.
10. Without disturbing the interface, collect 300 µL from each well using the 8-channel micropipette and transfer it to the isopropanol-filled plates (pay attention to the plate orientation).
11. Seal with a mat and mix with a benchtop plate mixer for 30 sec. Leave on ice for 20 min.
12. Centrifuge 20 min at maximum speed, 4°C.
13. Discard supernatant, blot dry on a clean paper towel and place upright on the bench.
14. Using the 8-channel micropipette, add 200 µL of 80% ethanol to each well.
15. Paying attention not to invert the mats and their orientation, seal the plates and mix with a benchtop plate mixer for 30 sec.
16. Centrifuge 10 min at maximum speed, 4°C.
17. Discard carefully the supernatant using an 8-channel micropipette.
18. Air dry the plate for 10 minutes at room temperature to allow evaporation of residual ethanol.
19. Pay attention not to invert the mats and their orientation, seal again the plates. Wrap in bubble wrap and seal with transparent packaging tape, to avoid plate breakage during transport.
20. Ship at room temperature.

<u>Upon receipt of the samples:</u>

1. Centrifuge 2 min at maximum speed to collect nucleic acid pellet at the bottom of the plate
2. <u>For DNA genotyping/resequencing or pathogen DNA detection:</u> Add 50 µL TE buffer with freshly added RNAse A (100 ng/µL) to each well. Pipette up and down 5 times. Transfer to polypropylene PCR plate. Incubate 30 min at 37°C. Seal and store at -20°C.

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3. For RNA virus detection: Add 50 µL of RNase-free water. Pipette up and down 5 times. Transfer to polypropylene PCR plate. Incubate for 10 min at 60°C. Seal and store at -20°C.
   Notes: *Since phenol-chloroform-SDS extraction inactivates all known plant pathogens, this protocol is amenable to shipment to countries with strict phytosanitary regulations without the need of a phytosanitary certificate. In our hands, it works well for most genotyping applications and for pathogens detection using quantitative or end-point PCR. For applications in which DNA quantitation is critical, DNA should not be quantified spectrophotometrically (since RNAse treatment leaves short ribonucleotides with a strong extinction coefficient at 260 nm) but using an assay based on DNA intercalating agents (e.g. Thermofisher Picogreen, cat P7589) or quantitation on an agarose gel using a known concentration DNA as reference. In case of a low number of samples, the extraction can be performed in individual 2-ml round bottom safe-lock microcentrifuge tubes (e.g. Eppendorf 030120094) for the initial homogenization step and 1.5 microcentrifuge tubes (e.g. Eppendorf 0030120086) for the subsequent steps.*

Alternate Protocol 1: DNA Extraction Using a Kit

Low throughput extraction of high-quality DNA for resequencing/ genotyping using commercial kits

Given the high cost of resequencing or GBS applications and the higher quality standards required, or in any application in which high-purity DNA is required, it is possible to use commercial kits. Below two protocols are provided for the Qiagen DNeasy Plant Mini kit and the LGC Sbeadex maxi plant kit as an example but there are several different kits in commerce that can be used for obtaining high-quality DNA. Some kits, like LGC Sbeadex maxi plant kit, are easily adaptable to high-throughput automation. Freeze-drying is required to obtain high molecular weight DNA, otherwise, fresh leaf material can be used.

Materials

2-ml safe-lock microcentrifuge tubes (e.g., Eppendorf 0030120094)
1.5 microcentrifuge tubes (e.g., Eppendorf 003012086)
Tungsten carbide beads 3 mm (e.g., Qiagen 69997)
DNeasy Plant Mini kit (Qiagen, cat. n° 69104) or Sbeadex maxi plant kit (LGC Cat. n° 41602) For Sbeadex maxi plant kit both manual and automated protocols are available.

Equipment

Mixer Mill (Retsch, model MM 400)
Refrigerated microcentrifuge
Freeze-drier (for procedure 2)

Procedure 1: DNA extraction from fresh leaf material

1. Harvest leaf material and place it (≤100 mg of young leaf for Qiagen kit or 40-120 mg for LGC kit depending on the plant species) in a 2-ml Eppendorf tube together with a tungsten carbide bead. Freeze the tube in liquid nitrogen.
2. To disrupt tissue, put the tube in a pre-cooled adapter set and grind the samples for 1 min at 25 Hz. The method of precooling the adapter set depends on the Mixer Mill accessory used (-80°C for at least two hours or liquid nitrogen).
3. Refreeze the tubes in liquid nitrogen and repeat step 2 reversing the position of the tubes within the adaptor set.
4. After the sample is ground to a fine powder, follow carefully the protocol recommended by the Manufacturer (Qiagen, DNeasy Plant handbook, Mini protocol or LGC, Sbeadex maxi plant kit).
5. If the leaf samples are not processed immediately, they can be stored at -80°C.

Procedure 2: DNA extraction from freeze-dried material

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1. Harvest leaf material in a 2-ml Eppendorf tube and freeze-dry it following the instructions in Basic Protocol 1. Weight ≤20 mg (Qiagen kit) or 10-30 mg freeze-dried (LGC kit) lyophilized tissue in a 2 ml safe lock Eppendorf tube.
2. Add a 3 mm tungsten carbide bead in the tube and grind the sample for 1 min at 25 Hz.
3. Repeat step 2 reversing the position of the tubes within the adaptor set.
4. After the sample is ground, follow carefully the protocol recommended by the Manufacturer (Qiagen, DNeasy Plant handbook, Mini protocol or LGC, Sbeadex maxi plant kit).
5. If the leaf samples are not processed immediately, they can be stored at room temperature in a bag with silica gel.

Support protocol 1: DNA quality control

Before shipment to a DNA genotyping/resequencing facility, it may be necessary to perform quality control. Usually, DNA is shipped in dry ice or, if EDTA is present (0.1 mM EDTA) it can also be shipped using ice pads if the transit time is fast (24-48 hours).

For genomic analyses, DNA isolation is a crucial step because low-quality DNA may impair the analyses (Table 1). Therefore, quality control (QC) of DNA must be performed before the analyses. Depending on the number of samples, 1-2 high quality, control samples can be added to each batch. To perform QC, usually 6 µL are required for each test (2 µL for agarose gel electrophoresis, 2 µL for nanodrop readings and 2 µL for fluorimetric determination of DNA concentration). It usually consists of the following steps:

1. Agarose (0.8% w/v) gel electrophoresis to check DNA integrity and RNA contamination. Use a high molecular weight marker like phage lambda DNA not digested and/or digested with HindIII to evaluate the size of extracted DNA. Moreover, loading different amount of the reference marker (like 25-50-100 total ng), the fluorescence can be used to have a rough estimation of the DNA concentration of the unknown samples.
2. Determination of 260/230 and 260/280 ratios by a spectrophotometer or Nanodrop (only for samples isolated with a commercial kit like DNeasy Plant Mini kit or LGC Maxi kit that eliminates oligoribonucleotides remaining after RNAse digestion). Optimal 260/280 and 260/230 ratios are respectively 1.7-2.0 and 2.0-2.2.
3. Fluorimetric determination of DNA concentration based on a DNA intercalating agent (e.g., Picogreen, Thermo Fisher Cat. No. P7589), Qubit dsDNA High Sensitivity Assay Kit (Thermo Fisher Scientific Cat. Q33230) or similar. Depending on the specific protocol/kit used and on the species, DNA concentration can range from 10-20 ng/ µL to 200 ng/µL. It is possible to increase DNA concentration by reducing elution volume.

4.1 Sampling for Metabolomics Analyses

Protocol 3: Preparation of Freeze-Dried Plant Material for Metabolomics

During the process of freeze-drying, the material is first frozen and then exposed to low pressure then subjected to a vacuum to remove the frozen water through sublimation which reduces the oxidative denaturation. Before complete drying samples are kept at low temperatures so that enzyme activity is inhibited thus enzyme-catalysed degradation is reduced.

Materials

Plant tissue (e.g., source leaves and sink leaves, stems and/or roots)
Dry ice (optional)
Liquid nitrogen
Protective glasses (goggles

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Permanent markers or stickers resistant to long-term storage at -80°C, for sample and sample set identification
Container for liquid nitrogen (e.g., Dewar)
Storage tubes or plastic bags
Freeze dryer with a vacuum pump (e.g., Christ, model Alpha 2-4)
Mixer Mill (Retsch, model MM 400, *https://www.retsch.com*)Vacuum bags
Paper tissue (e.g., Kimwipes, KIMTECH Science, 107281)
Sample storage tubes or bags to avoid sample mixture
Styrofoam shipping boxes to prevent heat shocks.

For the convenience of the freeze-drying process, the preparation steps can be summarized as:

1- Harvest appropriate plant material according to the experimental aim.
2- Immediately freeze tissue in liquid nitrogen and store it at -80°C until further processing.
3- Utilize a clean and properly working freeze-dryer (e.g., Christ, model Alpha 2-4).
4- Turn on and set up the freeze dryer with a vacuum pump according to the manufacturer`s instructions.
5- Remove the lid of the sample and quickly place it into the freeze-dryer. Place the sample on dry ice or in a container of liquid nitrogen.
   During preparation, samples must remain frozen.
   To allow water sublimation, samples must be open during the freeze-drying.
6- The freezing temperature may vary between the samples. The freezing stage includes lowering the temperature inside the freeze-dryer to freeze the sample. The product temperature must be kept below melt temperature to minimize product damage during the primary drying (sublimation) (Wolkers and Oldenhof 2015). Follow the manufacturer`s instructions to ensure the freezing temperature.
7- Depending on the used plant samples the level of dryness changes, therefore, before starting to the big experiment preliminary test is suggested.
8- After applying the test and once the desired level of dryness is achieved, gradually increase the temperature inside the freeze-dryer to atmospheric pressure.
9- Grind the sample using a mixer mill and steel balls until a fine-powdered form is obtained. Keep samples at a low temperature with liquid nitrogen or dry ice during the grinding process.
10- After lyophilization, samples can be kept in vacuum-sealed bags for long-term storage. Drying is required for some specific analyses, especially for metabolomics since water provides the medium for enzyme-mediated reactions that may cause metabolite decomposition (Kim and Verpoorte 2010). Different methods are available for drying the samples, such as ambient air drying, oven-drying, freeze-drying and trap drying (Harbourne et al. 2009). Among these methods, the most common and suitable method to dry plant material is freeze drying.

Protocol 4: Preparation of Fresh Frozen Plant Material for Metabolomics

Samples are frozen in liquid nitrogen or directly transferred to ultra-low temperature freezers as soon as after collection. Fresh frozen samples can be stored for several years in the case of being kept stable at low temperatures.

Material

The preparation of fresh frozen plant samples:

1- Harvest plant material from plants, place it inside a tube and freeze it in liquid nitrogen.
   Avoid liquid nitrogen entrance inside the tube since it can cause the tube to burst.
2- Obtain a proper grinding mill (e.g., the Retsch MM400 mill) and start grinding plant samples that are frozen in liquid nitrogen.
   Keep the grinding mill equipment cool with using liquid nitrogen while obtaining powders to avoid sample thawing.

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3- Place the sample either on dry ice or in a container of liquid nitrogen.
   During preparation, samples must remain frozen.
4- Grind frozen plant samples to a fine powder for 2 min at 25 Hz.
   Each plant material has a specific time point to become powder e.g., while leaf samples were ground with less frequency and less time fruit samples would require relatively more. If the samples do not reach to a fine powder within the suggested time point replace the tubes on liquid nitrogen to avoid sample thawing and repeat the step.
5- Label transferring tubes before cooling down in liquid nitrogen.
   Labelling of samples is important during the sample preparation from the field/greenhouse to sample processing.
6- Aliquot (50 mg) frozen ground material into 2 mL tubes.
7- For long-term storage, store material at -80°C.

Furthermore, it is recommended that all samples follow the same exact procedure before, during and after grinding and or freeze-drying since it might cause variation in the omics analysis.

Protocol 5: Preparation and Shipment of Metabolite Extracts for Metabolomic Analyses

The protocol below is adapted for shipment to countries with strict phytosanitary regulations, in which freeze-drying or pulverizing of biological materials is considered insufficient for the inactivation of possible pathogens. The introduction of a chaotropic agent like chloroform during the extraction ensures such inactivation. The protocol is adapted for the analysis of both semi-polar and non-polar metabolites but, since it involves a drying step, not for volatile ones.
Semi-polar and non-polar internal standards are added, to correct for sample loss during the procedure. It is suggested to use quality control (QC) samples for the large number of samples which then can help to treat the samples at the same way in the case of using batches. Samples must be shipped in dry ice, stored at -80°C upon arrival and analyzed as soon as possible, due to the intrinsic instability of extracts.

Materials

1.5 (conical) and 2 mL (round bottom) safe-lock microcentrifuge tubes (e.g., Eppendorf 0030120094 and 003012008620)
Samples were prepared using the instructions from Basic Protocol 3
Tungsten carbide beads 3 mm (e.g., Qiagen 69997)
LC-MS grade methanol, chloroform and water
1 M NaCl, 50 mM Tris-HCL pH 7.5
Internal standards for semi-polar (e.g., formononetin) and non-polar (e.g., alpha-tocopherol acetate) metabolites
500-µL Hamilton syringe

Equipment

Freeze-dryer
Refrigerated microcentrifuge
Vortex
Rotary shaker for microcentrifuge tubes
Rotary evaporator for microcentrifuge tubes (e.g. Thermofisher Speed-Vac or Eppendorf concentrator plus)
Analytical balance
Mixer Mill (Retsch, model MM 400)

Procedure

1. Work under dim light and on ice. Some metabolites (e.g., carotenoids) are extremely sensitive to light + oxygen at room temperature.
2. Prepare and label as many 2-ml tubes as samples.

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3. Weigh 5-25 mg freeze-dried tissue in each tube (quantity depends on the tissue and analytical method used).
4. Add a clean tungsten carbide bead to each tube.
5. Add 200 µL of methanol (HPLC grade) spiked with a fixed concentration of freshly prepared internal semi-polar standard (we use 3 ng/mL formononetin).
6. Shake for 10 min in the Mixer Mill at 30 Hz.
7. Add 400 µL chloroform (HPLC grade) spiked with a fixed concentration of the freshly prepared internal non-polar standard (we use 50 µg/mL DL-a-tocopherol acetate).
8. Shake for 10 min in Mixer Mill 300 at 30 Hz.
9. Add 200 µL Tris-NaCl buffer.
10. Vortex for 10 sec.
11. Centrifuge the samples for 10 min at a maximum speed of 4°C. Two phases should be visible. The lower one contains the non-polar metabolites and the upper one contains the semi-polar metabolites.
12. Collect 300 µL of each upper phase (semi-polar metabolites) with a Hamilton 500-µL syringe in a 1.5 ml tube without disturbing the interface. Wash the syringe with 100 % methanol between samples.
13. Collect 300 µL of each lower phase (non-polar metabolites) with a Hamilton 500-µL syringe in a 1.5 ml tube without disturbing the interface. Wash the syringe with 100 % chloroform between samples.
14. Dry completely in the dark in a rotary evaporator for microcentrifuge tubes. Dry chloroform extracts first (they dry almost immediately), then dry the semi-polar extracts. Do not mix semi-polar and chloroform extracts in the same drying batch.
15. As soon as pellets are dry, cap and store at -80°C in the dark.
16. Ship on dry ice.

Protocol 6: Sample Shipping and Long-Term Storage

Laboratories might have specific packing requirements for shipping. For instance, fresh leaf samples should be double bagged or for lyophilized samples, static electricity may cause dried tissue samples to move unexpectedly and cling to the surface (Shillito and Shan 2022). Therefore, it is suggested to consult with the laboratory if static electricity is an issue and if they have a recommended way to remedy it.
It has been shown that different ways and duration of storage can influence the stability of plant materials (Houba, Novozamsky and Van der Lee 1995). Different enzyme activities have shown distinct activities after keeping samples at -20°C and -80°C (Hartmann and Asch 2019). For a long safe storage period, we suggest keeping fresh-frozen samples at -80° for several months or years. Freeze-dried samples must be kept in vacuum-sealed bags and for short term storage silica gel packets can be placed inside the sample boxes to minimize the humidity. It is suggested to perform quality checking during storage or after shipment. Performing physicochemical or biochemical analyses at different time points during sample storage can be helpful while carrying out the quality check (Phillips et al. 2005, Fish and Davis 2003).

Materials for sample long-term storage and shipment

1. Silica gel (for the lyophilized samples).
2. Styrofoam (or any kind of proper box can be used while sending the samples safely).
3. Dry ice (for the fresh-frozen samples to avoid the sample thawing).
4. -20°C or -80°C Freezers.

1. Labelling:

- Properly label samples and print the address of the sender and recipient along with the contact information on the carriage box carefully.
- Provide crucial information to the shipping and receiving parties on the label. Additionally, include the contact information on the shipping container.
- Include all the special handling instructions, tracking number, plant species, and quantity of samples in addition indicate if the phytosanitary certificate is included.

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2. ***Packaging:***
   - Select appropriate containers: choose containers that are suitable for the type of samples being shipped, such as vials, tubes, bags or boxes.

     The fresh-frozen or fresh-frozen powdered samples are shipped on dry ice.
   - Use bubble wrap when sending the sample with dry ice which can prevent the shifting of the samples when the ice dissipates thus they can also be placed.

     Insulation materials such as styrofoam for temperature-sensitive plant materials can be used to provide thermal protection.

     Bubble wraps or tissue paper aid to prevent movement and minimize the risk of sample loss during transit.
   - Indicate the sample shipping list on the box.
   - Place lyophilized plant samples in vacuum-sealed bags so that samples do not moisturize and can be stored longer.
   - Ensure that samples are packed tightly to minimize movement and potential breakage/opening
   - Include silica gel packets inside the shipping container to minimize the potential damage caused by humidity.
3. ***Documentation***
   - Include a detailed packaging list with a description of each sample.
   - Clarify the quantity of the samples and provide all the permits and customs forms.
4. ***Shipping method***
   - Choose a suitable shipping method that aligns with the urgency of the shipment, the special requirements.
   - Keep a record of the tracking number provided by the shipping service to monitor the progress of the shipment.
   - Track the status of the shipment online and address any issues promptly.
   - Inform the recipient about the tracking number and expected delivery date of shipment.
5. ***Biosecurity***
   - Check plant health status and plant samples for any sign of pests, or diseases before packaging.
   - Check and comply with international regulations for shipping plant materials since some countries have strict rules to prevent the spread of pests and diseases.

     Pest risk usually increases with plant age and size, therefore larger plants have more potential to exposure to the diversity of pests(Eschen et al. 2017).

4.4 Phytosanitary Certificate and Import Permit:

Countries have different regulations on which plant products are regulated and which are not. In general, for the import of unprocessed plant products, a phytosanitary certificate issued by the National Plant Protection Organization of the exporting country is required. Grinding, freezing or drying are not considered as processing. Certain plants and plant products or plants originating from certain countries or regions may completely be banned from import.

1) Check whether the movement of plant samples is legally possible and whether a phytosanitary certificate or other documents are required.
National Contact points inform whether a phytosanitary certificate is required for importing plant samples: https://www.ippc.int/en/countries/all/contactpoints/. The legal environments for plant imports across countries can be found here: https://www.ippc.int/en/countries/all/legislation/.
For the EU, regulation (EU) 2016/2031 of the European Parliament and the Council regulates the import of plant material from third countries into the EU (https://eur-lex.europa.eu/legal-content/EN/TXT/HTML/?uri=CELEX:32019R2072#d1e32-216-1), and Article 13 of this regulation lists plants,

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unprocessed plant products and other objects for which a plant passport is required for their movement within the Union territory.
Professional operators can request exemptions from the national authorities from import bans or the requirement for phytosanitary certificates for official testing, scientific or educational purposes, trials, varietal selections, or breeding following the Commission Delegated Regulation (EU) 2019/829 that supplements Regulation (EU) 2016/2031. If granted, a letter of authority will be issued that must accompany shipments in lieu of a phytosanitary certificate.
In the USA, import of plant samples is regulated by Plant Protection and Quarantine (PPQ) under the authority of the Plant Protection Act plant for planting.pdf. A Controlled Import Permit (CIP) is required to import plant samples that are not intended for plating and are imported solely for destructive analysis. A containment facility may be required for importation of the regulated material. The facility will be evaluated before the permit is issued. Information and forms to apply for a CIP to import plant samples for destructive analysis can be found here: animal and plant health inspection service. The application is filed online and the CIP to import restricted or not authorized plant material is issued electronically via the ePermit system.
In Canada, movement of plants and plant parts is governed by Plant Protection Regulations (plant protection regulations). Samples for scientific research can be imported after requesting an import permit from the Canadian Food Inspection Agency (CFIA, Cadanian food inspection agency), and the permit must be obtained before the shipment arrives in Canada.

2) Check whether an additional declaration on phytosanitary certificates are required.
For the EU, Annex VII of Commission Implementing Regulation (EU) 2019/2072 lists plants, plant products and other objects, originating from third countries, and the corresponding special requirements for their introduction into the Union territory is set out. For export to third countries, the Plant Health Authority in the importing country informs about additional declarations.

3) Obtain a phytosanitary certificate for the cross border movement of plant products
The sender of the material contacts the Plant Health Authority in the exporting country and requests a phytosanitary certificate (PC). Some countries can issue electronic phytosanitary certificates in using the electronic certification tool TRACES NT (https://webgate.ec.europa.eu/tracesnt/login).

4) Make sure that a phytosanitary certificate or a Letter of Authority (please refer to (1) is accompanying the consignment.

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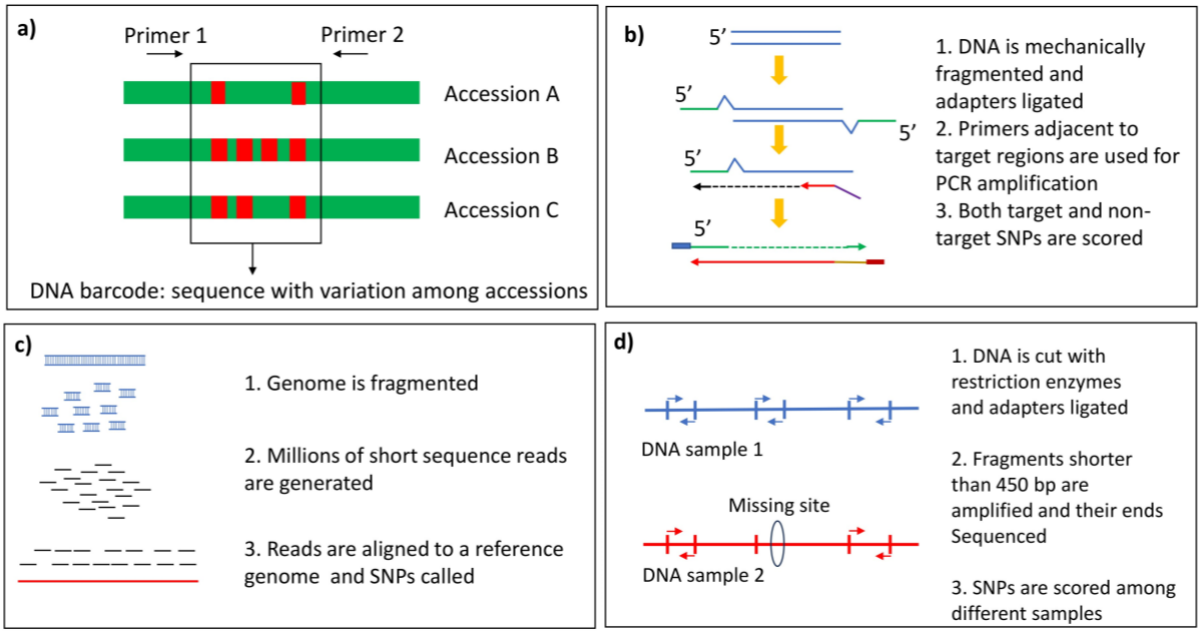

Figure 1: Schematic representation of different genotyping approaches. a) Multiplexed PCR/genotyping; b) Single Primer Extension Technology; c) Whole genome resequencing; d) Genotyping by sequencing.

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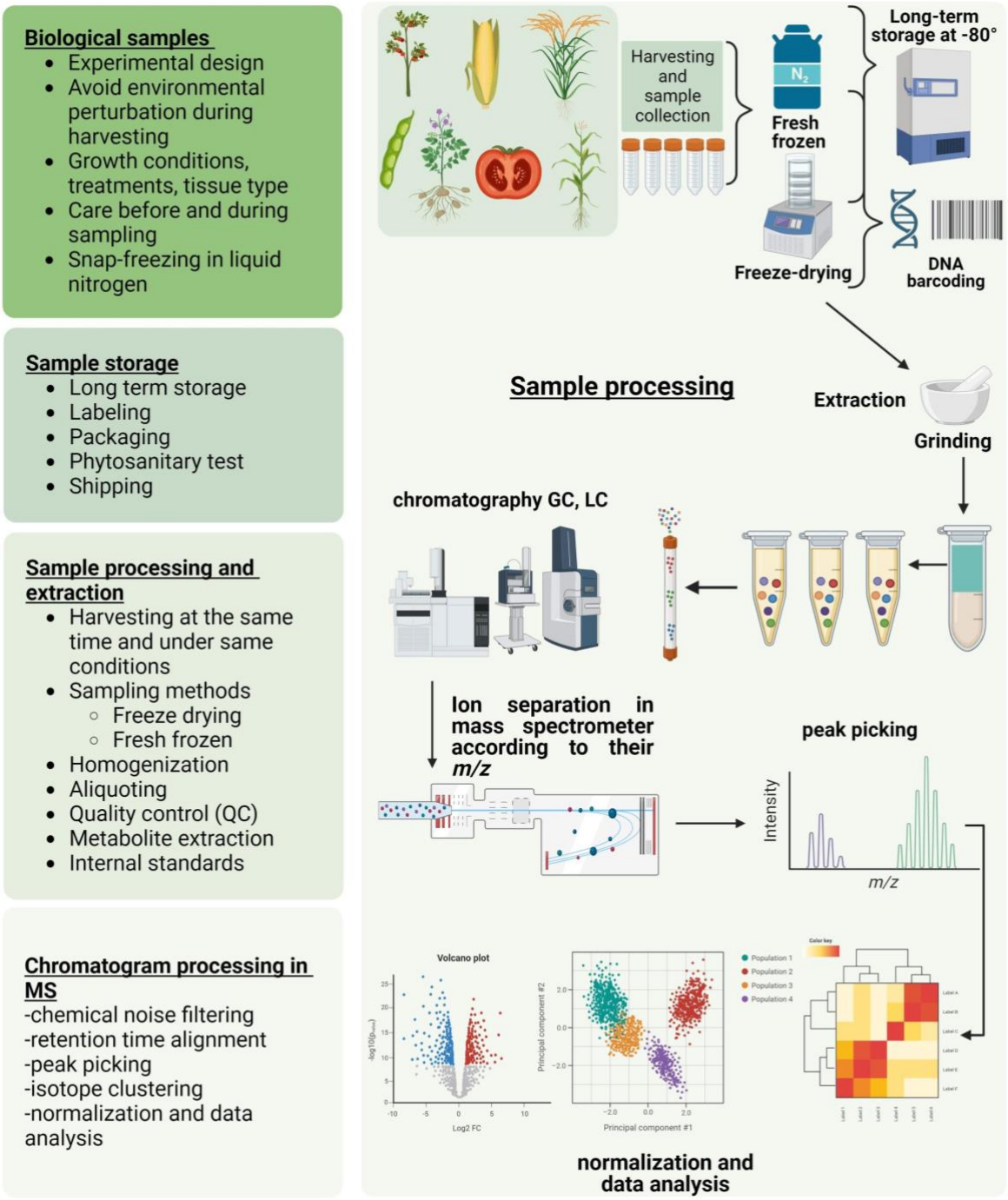

Figure 2: Schematic overview of sample processing starting from experimental design to the sample storage including metabolite extraction and metabolomics chromatogram processing (part of the figure generated by BioRender).

5. Conclusions

Freeze-drying is a standard method for post-harvest treatment of samples since it reduces their weight and volume and makes them easier to handle, store, transport, weigh, homogenize and extract with minimal degradation of the analytes. We have routinely observed a more efficient homogenization/extraction of metabolites from freeze-dried than from frozen plant tissues, as well as a higher molecular weight of the extracted DNA. Since freeze-drying occurs at low temperatures it prevents the denaturation of heat-sensitive compounds, including enzymes, vitamins, and phytochemicals. On the other hand, fresh frozen plant samples retain the biological activity of plant tissues and components, including enzymes, cellular structures and metabolites which is why it has been frequently used for enzyme activity assays and metabolic profiling studies. Although fresh-frozen plant sampling has numerous advantages, it also requires access to proper freezing equipment and storage facilities to maintain consistently low temperatures. Additionally, freezing may not be suitable for all types of plant materials; therefore, researchers should choose the sampling method that suits best their specific research plant materials.

Plant DNA barcoding is currently a widely used tool for rapid and accurate identification of plant species. Proper classification of plant material in a genebank is an indispensable activity since the distribution worldwide of misclassified material could have very detrimental effects on research programs. The protocols indicated above describe sample preparation for multiplex PCR or DArT-Seq genotyping (Protocol 1), preparation of pathogen-free nucleic acids for most genotyping-resequencing applications or pathogen detection (Protocol 2), extraction of high-quality DNA for resequencing or specific genotyping applications using commercial kits (Alternate protocol 1).

6. Deviations

Not applicable.

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