Protocol to measure LA, SLA, and LDMC

This protocol is adapted from the New Handbook for standardised measurement of plant functional traits worldwide (Pérez-Harguindeguy, et al. 2016) and the PROMETHEUS website. More details are provided in the 'detailed protocol'. We recommend reading it entirely before sampling your plant and reviewing the reference for further questions.

All further information for this project is available on the GitHub repository as well as a folder to upload your data for the project (https://github.com/BeatriceGervaisBergeron/Hyperaccumulators_functional_traits_sampling_campaign.git).

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Short Protocol

Procedure:

1. Leaves harvest	~ 5-10 min/sample
2. Leaves flesh weight measures	~ 6 min/ samples
3. Leaves scan	~ 8-10 min/samples
4. Drying biomass	~ 72 h
5. Leaves dry weight measures	~ 6 min/samples

What to collect

- Select random young but **fully expanded** and **hardened leaves** from adult plants.
- Avoid unhealthy leaves
- Sample '**sun leaves'** under relatively optimal conditions

Leaf harvest

- Collect **whole twig** sections with the leaves still attached
- place in **wet florist foam**, or place in a plastic bag with a moist paper towel (breathe into the bag) and lie flat in a cooler/icebox in the dark.
    - Ideally, the **cut end is submerged in deionized wate**r, but plastic bags are a good alternative too.
    - **xerophytic species** are better stored dry in paper bags.
    - **mildly succulent species**, try both moist and dry storage simultaneously
- Store the collected samples in a cool box or fridge (never in a freezer) in the dark
- Measure as soon as possible after collecting and rehydrating, preferably within 24 h.
    - If storage is to last for more than 24 h, low temperatures (2–6 C) are essential to avoid rotting.

Measurements

- Each leaf (**excluding the petiole**) is cut from the stem.

- The leaves are gently blotted dry with tissue paper to remove any surface water before measuring the water-saturated fresh mass.
- Weighing several tiny leaves will generally improve the accuracy of the weighing.
    - The same leaves used for fresh weight should be used for dry weight.

Leaf scan or photographs

- Place the flat leaves on a scan or board.
- Place some scale indicators in your image.
    - If scanning: Lie leaves flat on the scanner.
    - If photographing: flatten the leaves under a sheet of **Plexiglas** to avoid shadows. Photograph in high but diffuse light to avoid reflections and your own shadow. A camera mounted on a tripod, with **two lamps lighting** from different sides and no flash gives the best results.
- include a **label** with details about your leaf as well
- Scan the image in full color or grayscale and save it to your computer.
    - If photographing: download images and save them to the computer.
- Process the photo with Winfolia or with free software

Dry weight

- Place harvested material into **labeled paper bags** (same grouping as fresh weight)
- Place in a 60 °C oven for approximately three days (72h) or until weight has stabilized.
- Remove from oven and place bags into plastic bags or desiccator.
- Turn electronic balance on, open one side of the balance cabinet, and place cardboard or other non-static platform on the balance.
- Zero the scales using an empty identical bag
- Place your first sample on the scales, and wait at least 30 seconds until the scale has stabilized, record the value and remove the sample carefully.
- Repeat the last step for all samples.
- Keep plastic bags sealed between measurements to avoid the entry of moisture. Place weighed bags in a fresh plastic bag with silica gel.

Detailed protocol

Material

- Scanner attached to a computer or digital camera, computer, and whiteboard
- Image J software (shareware: http://rsbweb.nih.gov/ij/download.html) or WinFolia (with USB key)
- Circular sticker of known diameter, or object of known dimensions, for scale reference, when analyzing image (no need if using Winfolia)
- Florist foam or flash or ziplock bag with paper to keep leaves hydrated
- Coolbox and icepack
- Deionized water bottle
- Cutters and razor blades
- Plexiglass
- Two lamps (frontals?)
- Labels
- Paper bags
- Balance (ideally four digits)
- The oven going to 80°C
- Desiccators with silicate gel or rocks

Procedure:

6. Leaves harvest ~ 5-10 min/sample
7. Leaves flesh weight measures ~ 6 min/ samples
8. Leaves scan ~ 8-10 min/samples
9. Drying biomass ~ 72 h
10. Leaves dry weight measures ~ 6 min/samples

What to collect

- For robust comparisons across species, traits should be generally measured on reproductively mature, healthy-looking individuals, unless specific goals suggest otherwise.
- Individuals for measurement should be selected at random from the population of appropriate plants, or by using a systematic transect or quadrat method.
- Select the relatively young (presumable more photosynthetically active) but **fully expanded** and hardened leaves from adult plants.
- Avoid leaves with obvious symptoms of pathogen or herbivore attack, or with a substantial cover of epiphylls.
- Sample outer canopy leaves (also called '**sun leaves'**) from plants growing under relatively optimal conditions, since SLA is strongly affected by light intensity.
- We recommend collecting leaves of these species in the morning after a rain event, or a few hours after generous watering.

Leaf harvest

- We recommend collecting **whole twig** sections with the leaves still attached and not removing the leaves until just before processing.

- Harvest leaves of interest and either place in **wet florist foam**, or place in a plastic bag with a moist paper towel (breathe into the bag to increase CO2 concentration and air humidity, which will minimize transpiration water loss) and lie flat in a cooler/icebox into the dark.
    - Ideally, the samples (twigs with leaves attached) should be cut and immediately placed into test tubes or flasks, with the **cut end submerged in deionized wate**r, but plastic bags are a good alternative too.
    - The main thing is that leaves are kept cool and moist, in the dark and not allowed to shrivel up and contort.
    - Leaves in a plant press will dry and shrink somewhat, so this is a less desirable approach but can be used if there's no alternative.
    - Tissues of some **xerophytic species** (e.g. bromeliads, cacti, some species with very small, highly resinous leaves) rot very quickly when moist and warm; therefore, they are better stored dry in paper bags.
    - If in doubt (e.g. in **mildly succulent species**), and if recollecting would be difficult, try both moist and dry storage simultaneously and use the dry-stored leaves in the case of rotting of the moist-stored ones.
- Store the collected samples in a cool box or fridge (never in a freezer) in the dark, until further processing in the laboratory.
    - If no cool box is available and temperatures are high, it is better to store the samples in plastic bags without any additional moisture.
- Rehydration is preferable for most plants and essential for soft leaves (SLA higher than 10-15 m2/Kg)
    - In situations where the rehydration procedure described above cannot be applied, storage in sealed, moist plastic bags (with or without the addition of damp paper) for 12 h is an acceptable option, although generally yields approximately ~5% lower values than does the complete rehydration method.
- Measure as soon as possible after collecting and rehydrating, preferably within 24 h.
    - If storage is to last for more than 24 h, low temperatures (2–6 C) are essential to avoid rotting.
    - Xerophytic and especially succulent leaves should not be rehydrated for more than 6 h, whatever the storage or rehydration method used might be. If this process fails, we recommend collecting leaves of these species in the morning after a rain event, or a few hours after generous watering.

Measurements

- Each leaf (**excluding the petiole**) is cut from the stem.
- The leaves are gently blotted dry with tissue paper to remove any surface water before measuring the water-saturated fresh mass.
- Weighing several tiny leaves as if they were one and then dividing the weight by the number of leaves will generally improve the accuracy of the weighing.
    - The same leaves used for fresh weight should be used for dry weight.

Leaf scan or photographs

- Place the flat leaves on a scan or board
- The important thing here is to make sure there is some scale indicator in your image. A ruler works, but a circular sticker (a sticky dot or any object's pieces) of a known area is ideal.

    - If scanning: Lie leaves flat on the scanner (ensuring you have recorded which is which/location on the scanner), and spread out any lobes to ensure no overlap.
    - If photographing: you may need to flatten the leaves under a sheet of **Plexiglas** to avoid shadows. Photograph in high but diffuse light to avoid reflections and your own shadow. A camera mounted on a tripod, with **two lamps lighting** from different sides and no flash gives the best results.
    - The projected area (as in a photograph) can be measured with specialized leaf-area meters such as those from Delta-T Devices (Cambridge, UK) or LI-COR (Lincoln, Nebraska, USA).
    - If the lobes still overlap you may need to scan the leaf twice, once with the leaf intact to get the projected leaf area's perimeter and then again with the leaf cut into pieces so that there is no more overlap to get the full leaf area. Place them carefully to not damage the leaf tissue.
    - If you are to use a portable LA meter, make sure that the estimation error is not too high for your purposes, by running a preliminary check against LAs scanned in the laboratory, using a range of different leaf sizes.
- You may want to include a **label** with details about your leaf as well
- Scan the image in full color or grayscale and save it to your computer.
    - If photographing: download images and save them to the computer.
- Process the photo with Winfolia or with free software
- Freely downloadable programs are e.g. Leafarea (A. P. Askew, University of Sheffield, UK, downloadable from the Nucleo DiverSus toolbox) or, for more complex analyses including other plant organs, ImageJ (from the US National Institutes of Health; http://www.nih.gov/, accessed 22 February 2013) and GIMP (from the GNU Project; http://www.gnu.org/, accessed 22 February 2013).

Dry weight

- Place harvested material into **labeled paper bags** (petioles and laminae either separately or in the same envelope, according to the objective of the study).
    - **Grouping several tiny leaves** as if they were one and then dividing the weight by the number of leaves will generally improve the accuracy of the weighing. When converting SLA into LMA values or *vice versa*, always do so for each replicate, rather than for the average of several replicates.
- Place these in a 60 °C oven for approximately three days (72h) or until the weight has stabilized. (It is sometimes recommended at 70°C for 72h or a maximum at 80°C for 48h) The higher temperature might affect the chemistry of the leaves for further dry material analysis, so 60°C is preferred.
- Remove from oven and place bags into plastic bags. You can place more than one paper bag into a plastic bag as long as the plastic bag can still be sealed. Place a small amount of silica gel (approx 1 tablespoon per large zip lock bag) into each plastic bag.
    - If no desiccator with silica gel is available, put the samples to re-dry before weighing.
- Turn electronic balance on, open one side of the balance cabinet, and place cardboard or other non-static platform on the balance.
- Zero the scales using an empty bag that is otherwise identical to those your dried samples are stored in (e.g. bag also has been oven dried for the same period of time, and if your samples are stapled in the bags put a staple in the empty bag). Place the empty bag on the scales and press the "zero button". The scale should now read zero and is ready for measurements.

    - If the sample will not stabilize ensure it is not touching the sides of the balance cage anywhere, if it still does not stabilize, remove the sample and re-zero the scales then repeat the measurement.
- Place your first sample on the scales, and ensure it is not resting anywhere other than the measuring balance plate or cardboard platform on the measuring balance. Wait at least 30 seconds until the scale has stabilized, record the value, and remove the sample carefully.
- Repeat the last step for all samples.
- Keep plastic bags sealed between measurements to avoid the entry of moisture. Place weighed bags in a fresh plastic bag with silica gel.

Notes and troubleshooting tips

- When collecting leaves, decide if you want to include the petiole or not, and make sure this is consistent for all leaves. (Measure without the petiole, since a little part is no more of the leaf and required to be removed for pH and other chemical analysis further on)
    - ***Petioles*.** An important issue is whether or not petioles should be included in SLA measurements. The appropriate decision depends on the research question at hand. Some authors consider that the petiole is an integral part of the leaf because it is shed at abscission together with the leaf, and because it provides support and a vascular system without which the leaf cannot be displayed. Therefore, they include petioles in SLA measurements. Other authors consider that the petiole should not be included in the SLA because the main function of the petiole is the spatial positioning and hydraulic support of the leaf, thus resembling the function of the stem, whereas the main function of the leaf blade is light interception and C fixation. The fraction of leaf dry mass represented by the petiole varies from ~zero to almost 50%; therefore, the inclusion of the petiole may reduce the calculated SLA drastically. Although inclusion or not of the petiole may sometimes not be crucial within a single study, it can be a source of considerable and systematic error when comparing different studies, or even in certain same-site comparisons of species with very different leaf structures. Therefore, the best (albeit more time-consuming) option is to measure leaf blade and petioles separately, so that SLA can be calculated in both ways, thereby facilitating comparisons with other studies. When using digital images, we suggest to scan or photograph petioles and the rest of the leaves of each replicate in the same image, but in clearly different sectors, so that they can be measured together or separately according to the objectives of the study; then oven-dry and weigh petioles belonging to a replicate separately from the rest of the leaves from the same replicate. In general, make the decision that best suits your study objectives; however, specify in your publication whether petioles are included or not.
- Remember the scanner will take a mirror image of what you see on the scanner so be careful when recording plant locations on the scan screen that you note what is on your left when viewing it on the scanner will be on the right side in the scanned image.
- *Leaves of grasses and grass-like plants* Usually, only the lamina is considered, excluding the leaf sheath. However, as in the case of petioles (see Point 1 above), the decision on which measurement to take depends on the research objectives. Several species have leaves that tend to curl, or even roll up. They are generally much easier managed by cutting leaves into shorter pieces of 5-10 cm

- Heterophyllous plants In the case of species with two or more types of leaves with contrasting shapes and anatomy, such as e.g. plants with both rosette and stem leaves, collect leaves of both types in proportion to their estimated contribution to the total LA of the plant, so as to obtain a representative SLA value of the individual.

References

Pérez-Harguindeguy, N., Díaz, S., Garnier, E., Lavorel, S., Poorter, H., Jaureguiberry, P., Bret-Harte, M. S., Cornwell, W. K., Craine, J. M., Gurvich, D. E., Urcelay, C., Veneklaas, E. J., Reich, P. B., Poorter, L., Wright, I. J., Ray, P., Enrico, L., Pausas, J. G., de Vos, A. C., … Cornelissen, J. H. C. (2016). Corrigendum to : New handbook for standardised measurement of plant functional traits worldwide. *Australian Journal of Botany*, *64*(8), 715. https://doi.org/10.1071/BT12225_CO

Specific Leaf Area and Leaf Dry Matter Content. (2013, April 17). PROMETHEUS. https://prometheusprotocols.net/structure/morphology/specific-leaf-area-and-leaf-dry-matter-content/