SECO Leaf Trait Protocol

John L. Godlee and Mathew Rees

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Introduction

The purpose of this protocol is to provide a common method for collecting measurements of tree leaf chemistry and leaf mass per area (LMA) from SECO plots. The protocol aims to obtain a community weighted mean of various leaf traits for each plot sampled, as well as information on the mean and variance of trait values for dominant tree species across plots within a site. Data generated using this protocol will be used to constrain models of the terrestrial carbon cycle in dry tropical vegetation.

Equipment

- Coin envelopes – one per individual tree sampled
- 6-10 mm diameter circular hole punch
- Notebook and stationery
- DBH tape measure
- Handheld GPS unit
- Large Ziploc bags
- Sealable plastic containers
- Tree cutting poles
- Silica gel – 250 g per 50 envelopes
- Two A4 Perspex plastic sheets, one white, one transparent
- Scale bar for Perspex plastic sheets
- Compact camera or smartphone

Figure 1: Example of a hole punch used to harvest leaf samples.

Contact John L. Godlee (john.godlee@ed.ac.uk) if you cannot source any of the equipment and we can try to send it by courier.

It is recommended to take extras of all field equipment, especially the hole punch, in case of breakages.

Sampling strategy

For each plot, sample the most dominant tree species, comprising ≥80% of the basal area in the plot. For each tree species, sample five individuals. Trees within each species should be sampled across a range of basal areas. For trees with multiple stems, calculate tree basal area as the sum of basal areas across all stems.

As the leaf trait sampling relies on knowing the basal area contribution of each species in the plot, tree stem data comprising at least species identity and stem diameter (DBH, diameter at breast height) must already exist.

R code is provided with this protocol to automate the identification of the dominant species per plot, and to create a stratified sample of individuals per species according to tree basal area.

Field method

Use a handheld GPS unit to record the location of each plot where leaf traits will be sampled.

Use tree cutting poles to collect 5 small branches from each sampled tree. Sample branches at different heights in the canopy. Avoid epicormic growth where possible, i.e. juvenile growth that flushes from immediately under the bark, often after a branch has broken and is regrowing.

From each branch, collect one fully expanded and undamaged leaf. Avoid diseased leaves, leaves damaged by herbivores, and senescing leaves.

For each individual, label a coin envelope with the following:

- Site name
- Plot name
- Tag ID of tree
- Species of tree
- Date collected
- Name of collector

Press leaves between perspex sheets and photograph with the scale bar and labelled coin envelope, to record leaf area. Ensure the photo is taken looking directly down onto the leaf, not at an angle. Alternatively, use a flatbed scanner connected to a laptop to speed up the process. Leaves must be photographed when fresh, as dried leaves become brittle and can roll up. A printable scale bar is provided with this protocol. Print the scale bar specifying that the image size is 4x6 inch. Double check the scale is the correct size before taking to the field. Consider laminating, printing on heavy card stock, or mounting the scale bar to make it more robust.

From each leaf, collect 10 leaf punches. Avoid prominent veins and the leaf midrib where possible. For compound leaves, collect punches across many leaflets. If the leaf is too small to collect 10 leaf punches, sample more leaves from the same branch until 10 leaf punches have been collected. Ensure all punches are the full size of the punch.

If leaves or leaflets are too small for the hole punch, sample whole leaves. Try to sample ≥3 cm^2 of leaf material per branch, across five branches per individual. Record this by adding a large '*' to the envelope.

Be sure to identify whether a leaf is a compound leaf with multiple leaflets. Sample entire leaves.

Combine leaf punches from a single individual in one coin envelope. Each envelope should contain 50 punches, 10 from each of 5 leaves.

Combine coin envelopes for a single species into a large Ziploc bag.

On the same day, combine the coin envelopes with silica gel in large Ziploc bags or a sealed plastic container to dry the leaf material. As a general rule, for up to 50 envelopes, each containing up to 10 cm^2 of leaf material, add 250 g of silica gel. Leaf material should dry within 2-3 days, after which the envelopes can be moved to another sealed container with less silica gel.

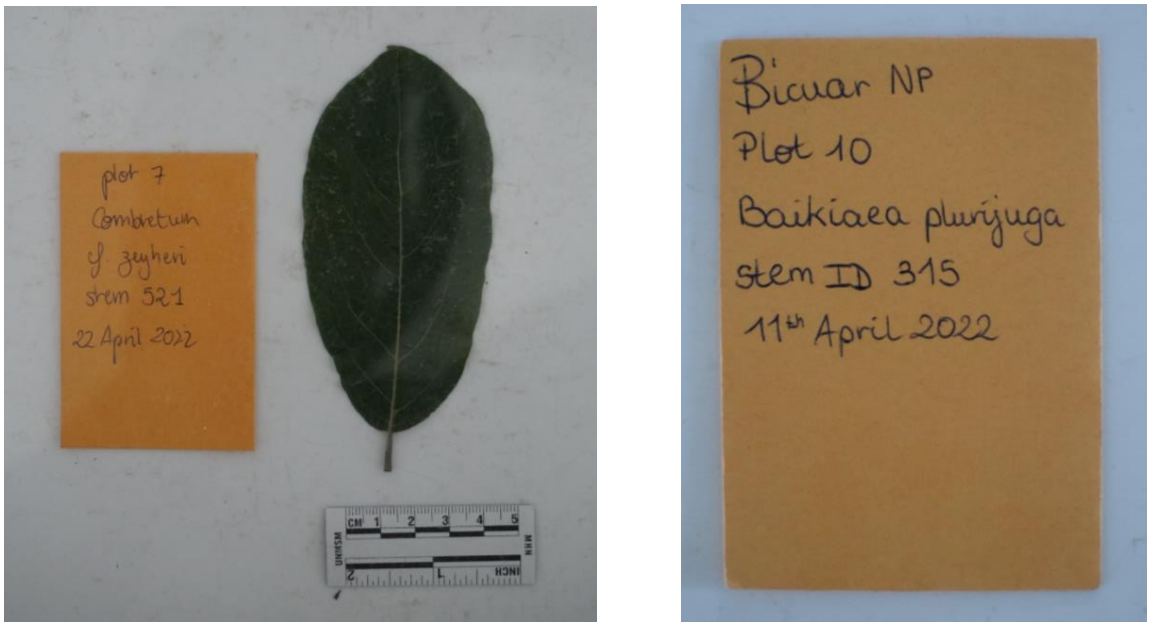

Figure 2: Example of a leaf photograph under perspex with scale bar and labelled coin envelope, to estimate leaf area. Close up of labelled coin envelope.

Figure 3: A compound leaf of Brachystegia cf. longifolia, and the same leaf with leaflets stripped to calculate leaf area.

Transport

Specimens should be sent to The University of Edinburgh for further analysis.

The address to send specimens is:

Dr. Kyle Dexter
Crew Building
Alexander Crum Brown Road
The King's Buildings
The University of Edinburgh
Edinburgh
United Kingdom
EH9 3FF

Specimens should be packaged to ensure they remain intact and sealed for the duration of the journey. Specimens should be kept in coin envelopes, with one species per Ziploc bag. Ziploc bags should be placed inside another sealed plastic container or bag, and finally inside a third sealed container. It is recommended to seal plastic containers with cable ties or duct tape.

All packages **must** be accompanied by a Letter of Authority. This letter must be contained within the package and displayed on the outside to prevent the package being opened at customs. Contact John L. Godlee (john.godlee@ed.ac.uk) for a copy of the letter.

Leaf Area Analysis

Send leaf photos to John L. Godlee (john.godlee@ed.ac.uk) for leaf area analysis or follow the protocol provided in the next section for leaf area analysis in ImageJ.

Also, see this R script (and associated sample data), which can be used to determine which trees to sample in an established plot.

100
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(cc) BY - NC - SA
http://creativecommons.org/licenses/by-nc-sa/4.0/ca/
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Using ImageJ to measure leaf area from photos or scanned images

John L. Godlee

31st May 2022

1 Installation

Install the Fiji software package from this URL: `https://imagej.net/software/fiji/`. There are versions for Windows, macOS, and Linux. Fiji is like a deluxe version of ImageJ, and includes lots of extra features. I recommend Fiji over stock ImageJ, but everything in this manual will probably work in stock ImageJ. Throughout the rest of the manual, I use "Fiji" and "ImageJ" interchangeably.

2 Scanning and photographing images

If storing leaves before scanning/photographing, try to store them flat otherwise they may roll up as they dry.

If using a scanner, set the resolution of the scanner to 300 DPI (dots per inch) for each scan, scan the whole A4 area of the scan, and save as a `.jpeg`.

Store each leaf as a separate image. Include a ruler or scale bar in the image. Make sure the leaf is flat and away from the edges of the image.

Figure 1: A good example of a scanned image of a leaf.

3 Measuring leaf area

Open Fiji, then click `File → Open...` and choose a leaf image.

First, set the scale of the image. This tells ImageJ how many pixels in the image make up one unit of measurement in the real world. To do this, first select the line tool from the tool bar and draw a straight

line of known distance across the scale bar by clicking and dragging the line across the image. Then select `Analyze → Set Scale...`. The `Distance in pixels` will be automatically filled in with the length of the line in pixels. Fill in the `Known distance` with the length on the scale that the line traverses, e.g. 4 cm. Fill in `Unit of length` with the unit of the scale, e.g. cm. `Pixel aspect ratio` can be left as 1.0, as your images will most likely have square pixels.

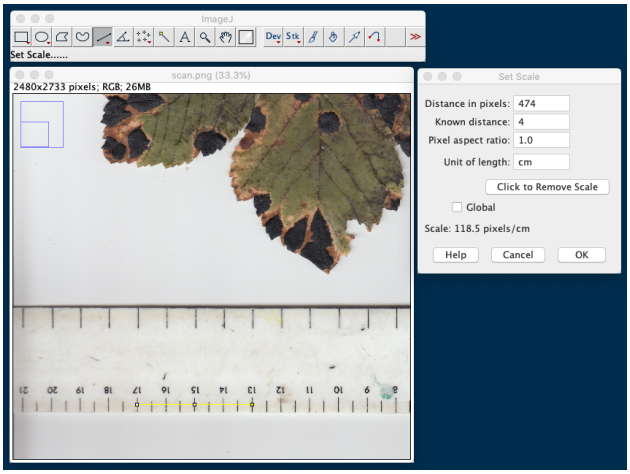

Figure 2: Example of setting the image scale using the line tool to draw a line of known length on the image.

Next, convert the image so that everything which is leaf is black and everything else is white. To do this, first convert the image to "8-bit" (grayscale) by clicking `Image → Type → 8-bit`. Then threshold the image by clicking `Image → Adjust → Threshold...`. Adjust the sliders in the `Threshold` box so the leaf is completely red, but none of the background is red. Ensure that `Dark background` is not checked, then click `Apply` and close the thresholding window. The leaf should now be black, and the background should be white. It doesn't matter if there are few small black objects such as the ruler leftover on the image, but it is important that the leaf is well defined.

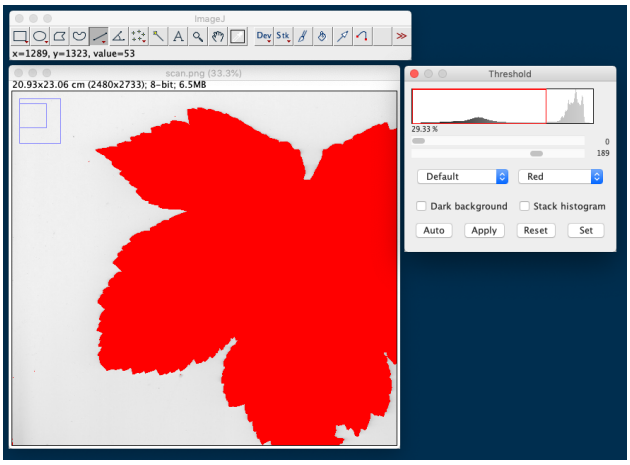

Figure 3: Example of the image binarization procedure.

To find the area of the leaf, go to `Analyze → Analyze Particles... Size (unit^2)` sets the minimum size of contiguous black areas that will be included in the analysis, by default it's set to `0-Infinity` which includes everything, but feel free to change this to something larger to remove little black areas caused by dust. The exact value will depend on the size of the leaves in the image. `Circularity` defines a range of circumference:area ratio that will be included in the analysis. Objects with a value close to 1 are completely

circular, values close to 0 are thin lines. I normally set this to `0.20-1.00`, but it will depend on the particular leaves being measured. Select `Show Outlines`. Check `Display Results`, then click `OK`.

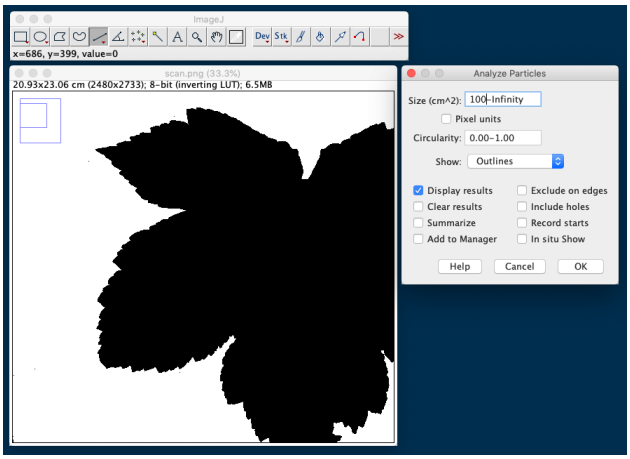

Figure 4: Example of the Analyze Particles procedure on a binarized image.

If you want to exclude certain things from the area analysis, like the petiole or the scale bar, you have two options. The first is to simply open the original image in an image editing program like Microsoft Paint or Adobe Photoshop and draw white over the objects to be excluded and use the edited image in ImageJ, ensuring that the resolution and dimensions are the same as the original image. The second is to use the `Polygon Selections` tool in ImageJ to draw a polygon around the objects of interest. Then proceed to `Analyze → Analyze Particles...`. The area calculations will only be performed inside the polygon selection.

After clicking `OK` a table is displayed which gives the area values of each black object in the image. If the image is thresholded correctly, the leaf should be by far the largest area value. If the image scale is in cm, these area values will be in cm^2. Each area value will be linked to an outlined region on the image. You can click the row in the table corresponding to the leaf and copy it, then paste into a spreadsheet.

4 Using macros

ImageJ has a built-in macro editor which can be used to write scripts to automate the process above if using scanned images with a consistent scale. Note that this method cannot be used for photos, as the scale will be different in each photo. Save the code below to a text file called `leaf_area.ijm`. Use a plain text editor to do this, not a rich text editor like Microsoft Word, and ensure that the file extension is `.ijm`, rather than `.txt`.

```
// Calculate the area of dark objects (leaves) against a white background.

// User inputs
input_path = "/Users/user/input/";
output_path = "/Users/user/output/";
dpi = 300;
min_obj_size = 0;
max_obj_size = "Infinity";
min_circ = 0.0;
max_circ = 1.0;
algorithm = "Default";
// END user inputs

list = getFileList(input_path);

for (i=0; i<(list.length); i++) {
  open(""+input_path+list[i]+"");
  file_name = getInfo("image.filename");
  px_cm = (dpi * 10) / 25.4;
  run("8-bit");
  run("Set Scale...", "distance=px_cm known=1 pixel=1 unit=cm global");
  setAutoThreshold(algorithm);
  setOption("BlackBackground", false);
  run("Convert to Mask");
  saveAs("Tiff", ""+output_path+file_name+"_binary");
  run("Analyze Particles...",
        "size=min_obj_size-max_obj_size circularity=min_circ-max_circ show=[Outlines]
    display clear");
  setOption("Display Label", true);
  saveAs("Results", ""+output_path+file_name+".csv");
  run("Clear Results");
  saveAs("Tiff", ""+output_path+file_name+"_outlines");
  image_id = getImageID();
  selectImage(image_id);
  close();
  selectWindow(""+file_name+"_binary.tif");
  close();
}
```

Code 1: ImageJ macro to calculate leaf area of many images in a directory.

Edit the script to change the `User inputs` to suit your setup. The `input_path` should point to the location of a directory on your computer which contains all the leaf images. Similarly, `output_path` should point to a directory which can be filled with thresholded images and excel files containing the results of the analysis. `min_obj_size` and `max_obj_size` define the range of object sizes to be analysed. `min_circ` and `max_circ` define the range of object circularity to be analysed. `dpi` sets the resolution of the image in DPI. The `algorithm` can also be changed. See other auto-thresholding algorithms here: `https://imagej.net/Auto_Threshold`. The script assumes that the scanned images are A4 size. If this is not the case, you will need to change the `px_cm` value to the actual ratio of image pixels per cm.

To run the macro, open ImageJ and go to `Plugins → Macros → Run...`, then select the `leaf_area.ijm` file, which you should have edited following the instructions above. Wait until the process has finished, then you should be able to extract the leaf area values from the `.csv` files created by the macro. You can use the `*_binary.tif` and `*_outlines.tif` files to check the object detection was successful.

```
# SECO: Sample dominant species across plots for traits
# John L. Godlee (john.godlee@ed.ac.uk)
# 2022-04-27

# Import example data (kilwa_fil.csv)
# This dataset includes plot id, tree id, species, and a value for basal area (ba)
dat <- read.csv("./kilwa_fil.csv")

# Define function to sample dominant species within a single plot
domSpecies <- function(x, species_name, abundance, per) {

  x_split <- split(x, x[[species_name]])

  ab_total <- sum(x[[abundance]])

  ab_per <- ab_total * per

  ab_cum <- cumsum(
    sort(
      sapply(x_split, function(y) {
        sum(y[[abundance]])
      }),
      decreasing = TRUE)
    )

  if (length(ab_cum) > 1) {
    out <- names(ab_cum)[ab_cum <= ab_per]
  } else {
    out <- names(ab_cum)
  }


  return(out)
}

# Define function to calculate rolling means
rollmean <- function(x, win) {
  n <- length(x)
  y <- x[win:n] - x[c(1, 1:(n-win))]
  y[1] <- sum(x[1:win])
  return(cumsum(y) / win)
}

# Define function to sample individuals across a range of tree sizes for one plot
rangeSample <- function(x, abundance, n) {
  # Find quantiles
  qu <- as.vector((quantile(range(x[[abundance]], na.rm = TRUE),
    seq(0,1,1/n))))

  # Get midpoint between quantiles
  qu_mid <- rev(rollmean(qu, 2))

  # For each quantile midpoint, find closest individual not already sampled
  ind <- vector()
  x <- as.data.frame(x)
  x_fil <- x

  for (i in seq_along(qu_mid)) {
    if (nrow(x_fil) > 0) {
      ind[length(ind)+1] <- rownames(x_fil)[which(abs(x_fil[[abundance]]-qu_mid[i]) ==
          min(abs(x_fil[[abundance]]-qu_mid[i])))[1]]
      x_fil <- x_fil[!rownames(x_fil) %in% ind,]
    }
  }
```

```
  return(x[ind,])
}

# Define wrapper function to sample individuals from dominant species across all plots
traitSample <- function(x, plot_id, species_name, abundance, per, n) {
  x_split <- split(x, x[[plot_id]])

  samples <- do.call(rbind, lapply(x_split, function(y) {
    dom_sp <- domSpecies(y, species_name, abundance, per)

    y_split <- split(y, y[[species_name]])[dom_sp]

    do.call(rbind, lapply(y_split, function(z) {
      rangeSample(z, abundance, n)
    }))
  }))

  rownames(samples) <- NULL

  return(samples)
}

# Run wrapper function with example data
traitSample(dat, "plot_id", "species", "ba", 0.8, 5)
```