Report on adjusting a high throughput screening tool to support water use phenotyping in forages

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Drought severely limits forage productivity. The avoidance of water deficit by increasing the capacity for water uptake or by controlling water loss are common responses. A fine interplay exists between the acquisition of water by roots in drying soil and water loss through transpiration. These two components tend to act simultaneously. The following approach and aim is therefore to provide information of shoot development, root development and water use over time of plants growing under greenhouse conditions with soil from target sites. Greenhouse studies is a vital part of phenotyping for drought conditions as allow the recording of responses that would be otherwise impossible under filed conditions. Complementary information to this document can be found in Cardoso and Rao, 2019 in the link below:

- https://cgspace.cgiar.org/handle/10568/105437

Contents

1. **Soil preparation**
   - According to type of study, soil to be used should come from a target environment, passed through a mesh of 2mm.
   - Soil can be either mixed with sand or fertilized according to specific needs, as to simulate different soil horizons.
   - Once the soil is mixed (with sand or fertilizers), basic information of texture, bulk density, witling point, field capacity, and saturated hydraulic conductivity must be included
   - Other information such as CEC, organic matter, pH and nutrient content should be included if relevant to the trial.

2. **Soil tube preparation**
   - We use a two tubes system to contain soil as well as to observe root development.

- The system consists on a transparent plastic tube, which can be punctured along its length to insert moisture probes as needed (e.g., dielectric capacitance ones) and closed at the bottom end with a punctured acrylic sheet (to allow water drainage). –

- This plastic transparent tube is in turn inserted in yellow-white polyvinyl chloride pipes (PVC) of the same height but slight greater diameter (~ 1 mm). Two plastic handles can be fitted into each one of the transparent cylinders to easily pull them up and out of the PVC pipes as needed. PVC pipes isolates transparent cylinders from sunlight and maintain a thin layer of air between transparent cylinders and PVC pipes. This set-up maintains soil temperatures around 3 °C below that of air temperature, which is similar to what recorded under field conditions.

- The system can differ in size depending on needs, but is always a longer than wider system (e.g. 120 cm length x 7.5 cm width). This is to: minimize root circling at the bottom of the pot; avoid perched water tables along the system; minimize a saturated water zone at the bottom of the contained which typically results in hypoxic conditions; allow penetration of roots into deep layers of soil profile.

- Plastic transparent tube is filled with soil to a pre-determined weight, maintaining a space free of soil to be able to irrigate the soil column from above.

3. Atmospheric conditions

- Atmospheric conditions must be recorded during the course of the experiment. The minimum required are: average temperature (day/night), average relative air humidity (day/night), average and maximum photon flux density.

4. Plant material and evaluation period

- This changes from trial to trial. Sometimes material come from seeds, other from vegetative material. It is recommended to run preliminary trials to collect information of seed vigor, seedling vigor and establishment success of material. This is minimize any effect of plant size, as to have as homogeneous plants as possible.

- Extent of evaluation period changes from plant to plant and trial to trial, but we aim to always minimize the impact of container size on plant growth and to avoid ratios of plant dry masses to pot volume larger than 2 g/l (e.g. a typical trial consists of ~ 21 days of establishment plus 28 days of evaluation of plants growing on a 5 l container).

5. Treatments

- Different types of drought can be imposed according to needs (e.g., progressive drying soil conditions, or plants kept at 60% field capacity over n number of days). This treatments are always compared to well-watered plants (plants growing on soil kept at 80-100 of filed capacity).

- A typical trial consists of a factorial combination of n genotypes by two water supply conditions (well-watered and progressive drying of soil) in at least a three-replicate complete randomized block.

6. Monitoring changes in soil moisture

- Changes in soil moisture must be recorded gravimetrically or by other means. Other means such as TDR, di-electrical capacitance are convenient since allow the insertion of probes down the soil profile. Temporal and spatial resolution of measurements aimed to monitor changes in soil moisture must be taken according to specific needs.
- At least one measurement of soil moisture before and one at the end of the evaluation period must be recorded.

7. Traits measured

- Traits measured depends on the particularity of a trial. However, for most trials we measure:

Cumulative transpired water

- The amount of evapo-transpired water is monitored by weighing each cylinder throughout the experiment every two days and prior to harvesting.
- Soil of well-watered treatment is maintained at field capacity by the addition of the same mass of water lost through evapotranspiration in a two-day period.
- The progressive drying of soil treatment is imposed by cessation of watering from the start of the experiment. Soil cylinders without plants are used to estimate evaporation of soil alone under the two levels of water supply.
- Cumulative transpired water is then calculated from the difference between evapo-transpiration and evaporation (average value of cylinders without plants).

Assessment of shoot growth and leaf rolling

- Changes in shoot area and leaf rolling throughout the experiment are estimated from digital pictures.
- A preliminary trial indicated that calculation of projected shoot areas (PSA) using two perpendicular front views (at 0 and 90 º) of grasses with different shoot architecture yield equivalent results to shoot areas estimated using three views of plants (one top view and two perpendicular front views), which in turn were positively correlated with leaf area determined destructively ($r = 0.9$, $p < 0.01$). For that reason and simplicity, most of the times, only two front views of each plant (0 and 90 º) are acquired.
- Digital pictures are captured at different days (e.g. at 0, 7, 14, 21 days….until harvest) from the onset of the experiment with a stationary digital camera (Coolpix p6000, Nikon Corporation, Japan).
- Pictures might be acquired in two time slots during the day, from 9:00 to 9:30 hrs (pre-noon) and from 11:30 to 12:00 hrs (noon).
- Pictures taken at pre-noon can be used to estimate PSA. PSA is estimated by transforming colour images into binary ones (black and white) using ImageJ software (v. 1.38, National Institutes of Health, USA) and by counting white pixels (i.e. PSA in image) out of the black background (i.e. field of view).
- Untransformed pictures taken at pre-noon and noon can be used for the visual assessment of leaf rolling at different days from the onset of the experiment. Briefly, six leaf rolling scores in plants are used as follows: 1 (no leaf rolling); 2

(some leaf rolling); 3 (severe leaf rolling; slightly wilted); 4 (severely wilted; necrosis in leaf tips); 5 (nearly dead); 6 (dead).
- Alternatively, differences in PSA of pictures taken at pre-noon and noon gives an estimate of leaf rolling at different hours, and as such sensitivity to vapour pressure deficits.

Harvest of shoot
- Plants are harvested at n days after the start of treatments. Leaves and stems are manually separated, and leaf area is measured with a leaf area meter (Li-COR 3100, Li-COR Biosciences, USA).
- The leaves and shoots are then oven dried for 96 hours at 60 ºC for determination of shoot dry mass.

Morphological characteristics of the root system
- We either measure the above destructively or non-destructively. In the last years we have shifted from washed root systems to non-washed to speed up screening processes. A common measure under both method is root length at different soil profiles. Previous research showed linear correlations at different harvests (R^2 > 0.8) between root length estimated from the visible interface of transparent cylinders and root length obtained from destructive sampling. Each method has its merits and drawbacks nonetheless.

- ***Washed root system***
  - Soil cylinders are sliced into at least five layers representing different depths from the soil surface (for example, on a 80 cm length tube: 0–0.1, 0.1–0.35, 0.35–0.6, 0.6–0.7 and 0.7–0.8 m).
  - Roots are washed free of soil with tap water and then placed in a container with few drops of wetting agent (polysorbate 20) for 10 minutes and rinsed again with tap water to remove loosened soil.
  - After that, roots of each soil profile are placed into plastic bags and stored at -20 ºC for posterior analysis.
  - For the morphological characterization, roots from each soil depth are carefully placed in an acrylic tray filled with water to minimize the overlapping of roots.
  - Dead roots and debris are removed as much as possible from the tray with tweezers and an eyedropper. Roots were then scanned to record grey images at 400 dpi with a dual scanner (EPSON Expression 1680, Japan).
  - Recorded images are processed with ImageJ software to remove background noise (i.e. soil particles of less than 0.3 mm).
  - Processed images are then used to estimate root length and average root diameter (ARD) using WinRhizo software (Regent Instruments, Canada).
  - The number of nodal roots (main roots developed from the crown) is manually counted from the recorded images.
  - Root length density (the length of roots per unit volume of soil, RLD m/m^3) at different soil depths is also calculated.
  - After scanning, roots are carefully collected to minimize loss of material and oven dried as described above to determine root dry mass.

- ***Un-washed root system***
  - This systems allows the visualization of root growth over time on the visible interface of the plastic cylinder.
  - For digital image acquisition, the transparent cylinders are temporarily removed out from the PVC pipes and placed in stand-up position under shade to minimize glare reflecting from the plastic cylinder.
  - After that, three different front views (at 0, 120, 240°) of each transparent cylinder are photographed. Images of cylinders are captured at a distance of 1.5 m using a digital camera (Coolpix p6000, Nikon, Japan) in auto mode.
  - The three different images captured from each transparent cylinder are stitched into a single one. The stitched image is then virtually sliced into sections of (60.4 cm^2 each) and each section individually analyzed as to separate live roots (roots with white to beige color), from dead roots (grey to black color) and soil (rest of colors) using histogram based segmentation in the HSB (hue-saturation-brightness) color space in ImageJ software.
  - Once separated, pixel areas of living roots are further processed into a skeleton to reduce their width to a single pixel.
  - Total length of living roots at different periods is then calculated as the sum of pixels in the set of sections, and total pixel numbers transformed into cm.
  - Mean rooting depth and maximum rooting depth are calculated as dimorphism for nodal root angles in *Brachiaria* grasses has been found.
  - Mean rooting depth is calculated as the average depth at which roots were present at the y-axis of the stitched image.

Root to shoot ratios

- The ratio of root dry mass to shoot dry mass (R/S) and the ratio of total root length to leaf area (RL/LA) are calculated on a container basis and for each grass genotype before the start and at the end of the experiment under well-watered or drought conditions.