# Practical measurement of generic drought adaptation-related traits

R Okono

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## Phenological traits

### Flowering date

The date when a plot reaches 50 percent flowering is recorded. To improve the quality of the data, the area to be rated can be restricted to a specific central, fully bordered part of the plot. Estimates of flowering should be recorded at least three times per week.

Under drought, lines with later flowering dates will tend to be stressed more than lines that flower early, because the stress intensity increases over time. To correct for this effect, lines can be sown with similar flowering dates in separate experiments and stress applied at the appropriate time for each experiment. Another approach is to make a statistical correction for flowering date. This can be done by using flowering date in the control as a covariate in the analysis.

### Flowering delay

Flowering delay is best expressed when the stress is severe, so it is easily seen in fields where drying occurs over a period of weeks. It is calculated as follows:

> Flowering delay = days to flowering in stress treatment – days to flowering in control treatment.

Note: Because this character is the difference between two independent measurements of flowering date, the error is generally larger for the delay than for the flowering date alone.

## Morphological traits

### Leaf rolling

Leaf rolling is scored on a scale from 1 to 5.

1 = unrolled, turgid
2 = leaf rim starts to roll
3 = leaf has a the shape of a V
4 = rolled leaf rim covers part of leaf blade
5 = leaf is rolled like an onion leaf

Note: Leaf rolling is to be measured before flowering when leaves are still more upright; leaves are less likely to roll later.

### Senescence

Senescence is scored on a scale from 0 to 10, dividing the percentage of estimated total leaf area that is dead by 10.

1 = 10% dead leaf area
2 = 20% dead leaf area
3 = 30% dead leaf area
4 = 40% dead leaf area
5 = 50% dead leaf area
6 = 60% dead leaf area
7 = 70% dead leaf area
8 = 80% dead leaf area
9 = 90% dead leaf area
10 = 100% dead leaf area

Note: Leaf senescence should be scored on two or three occasions 7–10 days apart during the later part of grain filling

### Size

In the case of traits related to the size of the plant, of an organ (leaf, spike, panicle or ear, etc), the measurement should be precisely defined (see the chapter '*Development of crop ontology for sharing crop phenotypic information*' by Shrestha et al).

## Physiological traits

### Canopy temperature

Canopy temperature is measured remotely by an infrared thermometer (IRT) which is an inexpensive device. Canopies emit long-wave infrared radiation as a function of their temperature. The IRT senses this radiation and converts it to an electrical signal, which is displayed as temperature. Using the thermometer properly is crucial to obtaining reliable data. The most important points in the protocol for using an IRT in breeding nurseries are explained below.

The correlation between canopy temperature and plant water status becomes stronger as plant water status is reduced. Therefore, measurements should be made under well-developed drought stress – typically when most of the material in the nursery presents some leaf wilting or leaf rolling at midday.

Measurements should be done at or just after the solar noon, when the plant water deficit is maximised. Since the plant water status changes over of the day, measurements on large populations must be done within about two hours.

The thermometer has a fixed angle of view (ca 2–5 degrees, depending on the model). Therefore, the size of the measured target area depends on the distance between the thermometer and the target. Distance, position and angle of measurement with respect to the viewed plot must be maintained with all plots measured.

The target must consist only of canopy leaves. Any other object in the target area, such as soil surface or panicles will result in a temperature reading which does not represent the leaf canopy temperature. Soil is generally hot and cereal inflorescences (panicles or spikes) are much warmer than leaves because they transpire very little. For this reason, screening canopy temperature measurements under drought stress can be done only after full ground cover has been attained and prior to inflorescence emergence.

Since the assessment of plant stress by canopy temperature within a breeding population is relative, atmospheric conditions during measurements should be relatively stable. Cloudy or windy conditions should be avoided. Transient cloudiness is especially difficult since it has an immediate effect on leaf temperature.

Viewing solar spectral reflectance from the canopy will not harm the instrument but may bias temperature measurement. Therefore, readings should be made with the sun behind the operator – basically similar to the rule for photography. This should be taken into account when the nursery layout is planned.

The nursery should contain a running check (control) cultivar, every 10 to 100 genotypes, depending on the case. The canopy temperature of the running check provides a basis for assessing site variability and offers a means for normalising data against such variability.

Experience shows that if work is performed carefully as outlined above, about 1.5–2.0°C can be the least significant difference (at 5 percent). If stress is sufficient and atmospheric demand for transpiration is high, genotypes may differ by up to 5–10°C on any given day, depending on the crop and the nature of the population.

Measurements should be performed several times during the drying cycle, once or twice a week, depending on the progress of stress. For each date of measurement, data can be processed in three forms: actual temperature, temperature of the genotypes as a percentage of the mean temperature of the block, and temperature of the genotypes as a percentage of the temperature of the nearest running check.

The final data by which selection is performed are usually derived from the day with the largest variation among genotypes, which is the date of maximum plant water deficit at peak stress.

***Source:***

The details above have been adapted from http://www.plantstress.com/methods/index.asp.

## Cell membrane stability

The most common application of measurement of cell membrane stability (CMS) is for heat tolerance. The general protocol detailed below involves the application of stress to the leaf after it has been subjected to hardening, followed by the measurement of electrolyte leakage using the conductimetric method.

1. The plant must be exposed to moderate heat stress for at least 24 hours before the test in order to allow for hardening. The capacity for hardening is a major component of the capacity for tolerance. Hardening can be achieved in the natural field environment if heat stress occurs, or in the greenhouse or a programmed growth chamber. Exposure of intact plants for 24 hours to 32°C (for cool season plants) or 36°C (for warm season plants) is sufficient, even at a low light level.

2. Leaf discs, pieces of leaf tissue or even whole small leaves are detached and placed in standard glass vials that can accommodate a conductivity electrode. The total area of leaf material per vial is about 15–25cm$^2$. The sample is then washed 2–3 times with deionised water. The water is drained off but samples remain wet so that they do not desiccate. At least 10 vials (samples) are prepared for each genotype. In that case, five pairs are taken from five different plants (replicates). For each pair, one vial is designated as treatment and the other as control.

3. The treatment vials are subjected to the heat stress *in vitro*. (The treatment can also involve a low temperature in the case of chilling tolerance). The vials are placed in racks and covered with plastic film so as to avoid drying the samples. Racks of vials are placed in a thermostatically

controlled water bath, ensuring that the leaf samples are completely below the level of the water surface. The temperature is set to a predetermined stress (treatment) temperature and the samples remain in the bath for 1 hour. The control vials are placed in a rack, covered with plastic film and placed at room temperature (18–25°C). The treatment temperature should be such that it will result in average population CMS values of around 50–60 percent. (This temperature will change with the species, the population and the hardening conditions). Therefore, it is necessary for some representative genotypes to be tested initially for CMS at a range of temperatures in order to determine the final treatment temperature.

4. After treatment, 20cm$^3$ of deionised water is added to each vial, making certain that all leaf material is submerged. All vials are then incubated at ca 10°C (typically, on the lowest shelf of a refrigerator) for 24h. After incubation, samples are equilibrated for 1 hour at room temperature and the conductivity of the liquid medium is measured by inserting a conductivity electrode into each vial.

5. The vials are covered with plastic film and placed in an autoclave for 15 minutes to kill the leaf tissue. The conductivity of all samples is measured again after the samples have equilibrated to room temperature.

6. Calculation:
   CMS (%) = [1 – (T1 / T2)] / [1 – (C1 / C2)] • 100
   where T1 and T2 are treatment conductivities before and after autoclaving and C1 and C2 are the respective control conductivities.

Note: When the method is adopted for assessing drought resistance as a measure of the desiccation tolerance of cell membranes, plants of all materials must be stressed to the same relative water content (RWC) of about 70 percent (depending on crop species) before being sampled into stoppered vials and brought to the laboratory. Samples should not desiccate between sampling and washing. Control samples are taken from similar leaves of well-watered plants. Once washed, 20cm$^3$ of deionised water is added to all samples and they are incubated directly for 24 hours as in step 4. The procedure then continues as described above.

***Source:***

The method above has been adapted from http://www.plantstress.com/methods/index.asp

***Additional reference:***

Tripathy JN, Zhang J, Robin S, Nguyen ThT and Nguyen HT (2000). QTLs for cell-membrane stability mapped in rice (*Oryza sativa* L) under drought stress. *Theoretical and Applied Genetics* 100:1197–1202.

## Chemical desiccation

This method is used for the evaluation of stem carbohydrate remobilisation. The procedure is described below.

1. Material for chemical desiccation should be planted in rows spaced at least 30cm apart. Such spacing allows the chemical spray to reach the lower parts of the canopy. Tests of advanced lines are planted under two treatments: desiccation and non-treated controls. The test is planted under non-stress conditions to avoid any reductions in kernel weight in the controls. It is performed by comparing the reduction in kernel weight from controls to the treated plots. The reduction in grain yield may also be used. Tested genetic materials should have a similar heading date. Otherwise the treatment will favour early accessions that will avoid the stress corresponding to chemical desiccation.

2. The spray treatment is generally applied to each genotype at 15 days after heading, or at any other common time which coincides with the onset of the exponential phase of grain filling at the specific test site. The spray may consist of solutions of magnesium chlorate, sodium chlorate or potassium iodide, all at 0.4 percent of the active ingredient. Magnesium chlorate may be difficult to purchase. The chlorates are more aggressive treatments and leaf desiccation can be seen one to two days after spraying. Potassium iodide is milder and the effect can be seen three days after treatment. With chlorates, the leaves are desiccated and bleached while, with potassium iodide, the leaves turn yellow. The spray is applied manually (usually with a back-sprayer) to the whole plant to full wetting, including the ears.

3. The percentage reduction in kernel weight due to chemical desiccation is obtained by comparing the mean kernel weight under desiccation with the mean kernel weight in the controls, for each genotype tested.

Note: The treatment can be used to evaluate advanced lines (> $F_3$) for grain filling from stem reserves. It can be used, together with grain sieving, to effect mass selection for grain filling from stem reserves in early generations.

***Source:***

The method above has been adapted from http://www.plantstress.com/methods/index.asp.

***Additional references:***

Blum A, Mayer J and Golan G (1983). Chemical desiccation of wheat plants as a simulator of post-anthesis stress. II. Relations to drought stress. *Field Crops Research* 6:149–155.

Blum A, Poyarkova H, Golan G and Mayer J (1983). Chemical desiccation of wheat plants as a simulator of post-anthesis stress. I.Effects on translocation and kernel growth. *Field Crops Research* 6:51–58.

## Leaf chlorophyll concentration

Simple hand-held instruments are now available that measure a unitless value that is directly related to chlorophyll content. These instruments determine the light attenuation at 430nm and 750nm on an area of 2 x 3mm, allowing measurement of even small leaves. One such instrument is the SPAD chlorophyll meter (Minolta Corp, Ramsey, New Jersey, USA) and the unitless number displayed is referred to as the 'SPAD chlorophyll meter reading' (SCMR). Measurements are generally done on fully expanded leaves, on the middle part of the leaf in monocotyledons, and on the leaf lamina (avoiding the mid-rib portion) in dicotyledons. SPAD readings are more stable under natural light between 10.00 and 16.00 hours. The SPAD-502 has memory space for 30 measurements. Data in the memory can be recalled or deleted at a later time, and the average value of all data in the memory can be calculated automatically.

## Mineral ash content

Leaf samples are oven dried (70°C for three days) and ground into a fine powder using a pestle and mortar or a ball mill. The procedure involves ashing of the samples by complete combustion in a muffle furnace. After recording the empty weight of a silica crucible ($W_{EC}$), a quantity of finely powdered leaf sample is placed in the crucible and its weight ($W_{CL}$) determined before placing in a muffle furnace at 600°C for 6–8 hours, until the sample is completely oxidised to ash. The crucible is cooled for a sufficient time and the weight of the crucible plus ash ($W_{CA}$) is recorded. The difference between the initial and final sample weights indicates the mineral ash content, which can be expressed either as a percentage or as g $kg^{-1}$. The ash weight ($W_A$) is calculated as $W_A = W_{CA} - W_{EC,}$ and the mineral ash content (percentage) as $(W_A / W_{CL}) \cdot 100$

## Osmotic potential

Samples consisting of three 1cm long mid-leaf segments are placed for 30 minutes at room temperature and then sealed in a thermocouple psychrometer cup (2mL) and freeze-killed at –20°C. The leaf water potential (LWP) is measured in similar leaf samples without freezing. The osmotic potential of each sample is measured by a Peltier type thermocouple psychrometer (eg, Model SC-10A, Decagon Devices, Inc, Pullman, WA) after a 2-hour equilibration period with a cooling current for 15 seconds.

***Reference:***

Brown RW and Oosterhuis DM (1992). Measuring plant and soil water potentials with thermocouple psychrometers. Some concerns. *Agronomy Journal* 84:78–86.

## Osmotic adjustment

There are four different ways to estimate osmotic adjustment in plants:

*1. Estimation of osmotic adjustment from the linear regressions of relative water content on osmotic potential, as derived from consecutive measurements during a drought stress cycle*

Two linear regressions are developed for all measurements taken during the drying cycle. One consists of the regression of the RWC on the measured osmotic potential (OP), and the other of the RWC on the calculated OP due to a concentration effect ($OP_o$). $OP_o$ is therefore an estimate of tissue OP level ascribed to the mere loss of water at each given RWC, and is calculated for each RWC data point as $OP_o = OP_i [(RWC_i / 100) / (RWC / 100)]$, where $OP_i$ is the initial OP in well-watered plants and $RWC_i$ is the initial RWC in well-watered plants. Osmotic adjustment (OA) is calculated from the two regressions as the difference between OP and $OP_o$ at an RWC of 60 percent. Natural logarithmic conversions of RWC and OP can be used to improve the linearity of the relationship

Note: This method partitions between active solute accumulation (ie, OA) and the concentration effect on OP of water loss from the tissues. However, it

is demanding on labour and plant materials. Here, it is considered as the best estimate by virtue of its extensive data acquisition along a drying cycle and its taking into account the concentration effect.

***References:***

Morgan JM (1992). Osmotic components and properties associated with genotypic differences in osmoregulation in wheat. *Australian Journal of Plant Physiology* 19:67–76.

Morgan JM (1995). Growth and yield of wheat lines with differing osmoregulative capacity at high soil water deficit in seasons of varying evaporative demand. *Field Crops Research* 40:143–152.

*2. Estimation of osmotic adjustment from the difference in osmotic potential at 100 percent relative water content between non-stressed and stressed plants.*

The OP at 100 percent relative water content ($RWC_{100}$) is calculated from a point measurement of OP and RWC at a given level of plant water deficit, with a correction for tissue apoplastic water (*B*).

The OA is calculated as the difference in the OP between non-stressed (a point measurement on the morning after last irrigation) and stressed leaves both calculated to a well-watered state ($OP_{100}$). Data for the stressed plants consist of those taken at a RWC of around 60 percent, with an LWP of about –3.5MPa, ensuring sampling at wilting in all cultivars. $OP_{100}$ is calculated as $OP_{100} = OP\ [(RWC - B) / (100 - B)]$. A value *B* of 18 percent is chosen for these calculations, as based on rice data of Turner et al (1986).

***References:***

Ludlow MM, Chu ACP, Clements RJ and Kerslake RG (1983). Adaptation of species of *Centrosema* to water stress. *Australian Journal of Plant Physiology* 10:119–130.

Ludlow MM, Santamaria FJ and Fukai S (1990). Contribution of osmotic adjustment to grain yield of *Sorghum bicolor* L (Moench) under water-limited conditions. II. Post-anthesis water stress. *Australian Journal of Agricultural Research* 41:67–78.

Turner NC, O'Toole JC, Cruz RT, Yambao EB, Ahmad S, Namuco OS and Dingkuhn M (1986). Responses of seven diverse rice cultivars to water deficit. II. Osmotic adjustment, leaf elasticity, leaf extension, leaf death, stomatal conductance and photosynthesis. *Field Crops Research* 13:273–286.

Wilson JR, Fisher MJ, Schulze ED, Dolby GR and Ludlow MM (1979). Comparison between pressure-volume and dew point hygrometry techniques for determining the water relations characteristics of grass and legume leaves. *Oecologia* 41:77–88.

*3. Estimation of osmotic adjustment from the difference in osmotic potential at 100 percent relative water content between non-stressed and stressed plants, with that of stressed plants being measured after plants or parts are rehydrated to 100 percent relative water content*

By this method, OA is calculated as the difference in measured OP between non-stressed ($OP_i$) and stressed leaves that are rehydrated. Stressed plants at an RWC of about 60 percent are irrigated in the evening and leaves are sampled the next morning for measurement of OP.

***References:***

Blum A (1989). Osmotic adjustment and growth of barley cultivars under drought stress. *Crop Science* 29:230–233.

Turner NC and Jones MM (1980). Turgor maintenance by osmotic adjustment, a review and evaluation. In: *Adaptation of plants to water and high temperature stress* (Turner NC and Kramer PJ, eds). Wiley-Interscience, New York, USA, pp 87–103.

*4. Estimation of osmotic adjustment from the linear regressions of relative water content on osmotic potential at about the point of wilting.*

The capacity for OA is estimated according to Morgan (1995) as the RWC at a given OP close to wilting (–3.5MPa). A higher RWC at a given OP indicates a relatively higher OA. This estimate is derived from the regression of RWC on OP used in Method 1, of which this method is a variation. Both methods use the same dataset for different computations.

***References:***

Chandra Babu R, Safiullah Pathan M, Blum A and Nguyen HT (1999). Comparison of measurement methods of osmotic adjustment in rice cultivars. *Crop Science* 39:150–158.

Morgan JM (1995). Growth and yield of wheat lines with differing osmoregulative capacity at high soil water deficit in seasons of varying evaporative demand. *Field Crops Research* 40:143–152.

## Root electrical capacitance

Root capacitance can be measured with a BK Precision 810A meter (Maxtec International Corp, Chicago, USA) operating at a frequency of 1kHz in the range between 200pF and 2μF. Electrical contact with the plant is established by connecting the negative electrode to the plant stem via a battery clamp at 6cm above ground level. The positive electrode is connected via a battery clamp to a copper ground rod 55cm in length inserted into the potting substrate to a depth of 15cm and positioned 5cm away from the stem base. The instrument is adjusted at the 20nF level by setting the readout to zero with the zero-adjust knob. One capacitance measurement per plant is taken at 200nF after allowing 5 seconds for the system to stabilise (ie, for the meter to reach a constant capacitance reading). High soil moisture content is essential for an accurate capacitance reading. Consequently, measurements are made after watering, on fully saturated soil and early in the morning, to prevent loss of moisture around the root systems due to evapotranspiration.

***References:***

Dalton FN (1995). *In situ* root extent measurements by electrical capacitance methods. *Plant Soil* 173:157–165.

van Beem J, Smith ME and Zobel RW (1998). Estimating root mass in maize using a portable capacitance meter. *Agronomy Journal* 90:566–570.

## Specific leaf area

The specific leaf area (SLA) is the ratio of leaf area to leaf dry weight, and is an indirect measure of leaf expansion.

## Specific leaf nitrogen

Specific leaf nitrogen (SLN) is computed by multiplying leaf nitrogen (percentage) by SLA.

## Stomatal conductance

Relatively low-cost (a few thousand US$) and easy-to-handle porometers such as the Decagon Leaf Porometer SC-1 or the Delta-T AP4 allow rapid (20–30s) measurement of leaf conductance

## Water potential

The organ to be measured is excised from the plant and is sealed in a pressure chamber with the cut edge (a petiole in the case of a broad-leaf) protruding out. Before excision, the water column in the xylem is under tension. When the water column is broken by excision of the organ, water is pulled rapidly from the xylem into the surrounding living cells under the gradient of water potential existing between cells and xylem. The chamber is then pressurised with compressed gas until the distribution of water between the living cells and the xylem conduits is returned to its initial, pre-excision, state. This can be detected visually by observing when the water returns to the open ends of the xylem conduits that can be seen in the cut surface. The pressure needed to bring the water back to its initial distribution is called the balance pressure. It is equated with whole leaf water potential. This is not absolutely correct since the balance pressure may also depend on the tissue osmotic potential which is an unknown quantity at the time of measurement. Furthermore, it is recommended that the measurement be performed with a non-transpiring leaf, and with the leaf being wrapped in a plastic bag before its excision and during measurement. However, for comparative phenotyping work where large differences are sought, these errors are of a lesser significance than with accurate physiological studies. Care must be taken to minimise the time duration between leaf excision and its measurement. Usually one person operates the chamber while another one fetches the leaf samples.

***Source:***

The description above has been adapted from http://www.plantstress.com/methods/index.asp.

## Relative water content

Topmost fully expanded leaves are sampled, unless there is interest in profiling leaves on the plant. Usually, four to six replications are taken from a single treatment or genotype. Each sample should, if possible, represent a different plant. All components of leaf water relations change during the day as irradiance and temperatures change. For no more than two hours at and after solar noon, the change is very small. This is the time window for leaf sampling, unless a daily curve of RWC is of interest.

In large broad-leaves (sunflowers, cotton, etc) leaf discs are cut from the leaves, to obtain about 5–10cm$^2$ per sample. The sample size does not have to be the same for all samples. Large veins should be avoided, and leaf discs should be large enough (around 1.5cm in diameter) so as to reduce the area of cut leaf surface/sample. Various leaf disc cutters have been designed by laboratories and may be available commercially. Alternatively a sharp cork borer can be used, cutting the leaf over a piece of dense rubber or a large rubber stopper. It is important that sampling proceed quickly.

In smaller composite leaves (groundnuts, alfalfa, clover, chickpeas) several leaflets can make up a sample quickly and conveniently. In cereals, a sample may constitute of a mid-leaf section of about 5–10cm$^2$ cut with scissors. With larger leaves such as maize or sorghum a section measuring, say, about 1 x 7cm can be cut with scissors from the area between the mid-vein and the leaf edge.

Each sample is placed in a pre-weighed airtight (possibly also oven-proof) vial. Cereal leaf sample should be placed in a vial slightly longer than the sample, with the basal part of the leaf to the bottom. Vials should immediately be placed in a cooler (at around 10–15°C) but not frozen on ice. Samples should reach the laboratory as soon as possible. This is why leaf sampling should be done quickly and it is important to enlist as much help as possible for the job.

In the laboratory, each vial is weighed to obtain the leaf sample weight (W), after which the sample is immediately hydrated to full turgidity for four hours under normal room light and temperature conditions. Some operators prefer to hydrate samples on the lower shelf of a laboratory refrigerator (at about 10°C). Samples are re-hydrated by floating on deionised water in a covered Petri dish or by receiving water into the vial to a level of 1–2cm after which the vial is capped.

After four hours, the samples are taken out of the water and are thoroughly dried of any surface moisture quickly and lightly with filter paper and immediately weighed to obtain the fully turgid weight (TW). Samples are then oven dried at 80°C for 24 hours and weighed (after being cooled down in a desiccator) to determine the dry weight. All weighing is done to the nearest mg.

The relative water content is calculated as
RWC (%) = [(W - DW) / (TW - DW)] • 100
where: W = sample fresh weight; TW = sample turgid weight; and DW = sample dry weight.

***Source:***

The description of the method above has been adapted from http://www.plantstress.com/methods/index.asp.

***Additional reference:***

Barr HD and Weatherley PE (1962). A re-examination of the relative turgidity technique for estimating water deficit in leaves. *Australian Journal of Biological Sciences* 15:413–428.