Drought adaptation mechanisms should guide experimental design

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The mechanism, or hypothesis, of how a plant might be adapted to drought should strongly influence experimental design. For instance, an experiment testing for water conservation should be distinct from a damage tolerance evaluation. Here we define four new, general mechanisms for plant adaptation to drought, so that experiments can be more easily designed based upon the definitions. A series of experimental methods are suggested along with appropriate physiological measurements related to the drought adaptation mechanisms. The suggestion is made that the experimental manipulation should match the rate, length, and severity of soil water deficit needed to test the hypothesized type of drought adaptation mechanism.

18 **What are drought, stress and damage?**

19 Drought may be defined from the perspective of precipitation, soil or plant water status, or
 20 availability of human water supply [1]. Accordingly, the ideal definition for comparative
 21 physiological studies would be independent of the characteristics of a particular plant, but related
 22 to the local environment. If drought is independent of the plant, then it can be uniformly applied
 23 to any genotype or species present in an environment. Thus, we define *drought as a decrease in*
 24 *water inputs into an agro/ecosystem over time, sufficient to result in soil water deficit* [2]. This
 25 definition encompasses many forms of drought such as rainfall anomalies, irrigation failure or
 26 annual dry seasons. Whether drought affects a plant is determined by the plant characteristics
 27 and environment. Soil water deficit (SWD) is the key variable that links soil water with plant
 28 physiology, and is defined as a decrease in the available soil water i.e. water losses are greater
 29 than inputs. Alternative definitions of drought are often based upon soil water limiting plant
 30 uptake [3]. However these definitions are not useful when comparing genotypes/species where
 31 SWD can vary due to differences plant leaf area, roots and physiology. Thus, we have chosen to
 32 replace the specific term ‘soil water deficit’ for ‘drought’ in the definitions of drought
 33 **adaptations** (see Glossary).

34 Stress may be defined as a negative change in the physiology of a plant away from a reference
 35 state, due to the action of an external stress factor or internal stress (see [4-6] for more
 36 discussion). In physics, stress refers to an external factor such as temperature while strain is the
 37 response of the material [7]. However, physiology literature generally uses stress in reference to
 38 physiological responses [4]. Therefore, we prefer to define *stress factors* as external and *stresses*
 39 as physiological responses. Note that physiological responses to daily ‘normal’ variation in
 40 potential stress factors such as light and temperature should not be termed stress, but as a part of

the reference state [5]. Drought induced damage is a negative stress that would persist for some time after SWD has ceased. Damage may be recoverable or irrecoverable, and in response to these deviations from unstressed conditions, acclimation or adaptation occurs.

New definitions of mechanisms for dealing with drought

General terms used as objectives or mechanisms in drought research are: drought or stress tolerance, drought resistance, etc. (Figure 1). These terms are poorly specific of a plant characteristic or the drought phenomenon, therefore, demonstrating that these mechanisms occur in a genotype/species is difficult. For instance, the manner in which ‘drought tolerance’ is typically applied in the literature could mean that a plant tolerates stress, or tolerates damage or that it avoids water stress, thereby tolerating drought.

The basic terminology provided by the highly cited Levitt [7, 8]: drought escape, avoidance and tolerance was not widely adopted with only ‘drought tolerance’ widely used (Figure 1). Other excellent reviews provide examples of drought adaptation [2, 9-11], but in practice little link is generally made between the proposed adaptation and experimental manipulation. Interestingly, literature associated with plant biology tends to focus on ‘stress tolerance’ rather than ‘water conservation’, the greater focus of ecology and crop related literature (Figure 1).

Clearly, nuanced definitions of plant mechanistic response to drought are needed to drive directed approaches to experimental design. Here we propose four terms representing a hierarchy of adaptation mechanisms (Figure 2, Key Figure; Figure 3: diagnostic graphs of mechanisms; see also Online Supplemental Information Figure S1, a flow chart). For instance, a plant may avoid SWD despite a lack of water inputs (termed *soil water deficit avoidance*; Figure 2). Examples include plants that explore deeper soils [12], or have slow root growth, leaving water for later in

the season [13, 14], plants that conserve water through lower leaf area or transpiration [15-17] or match **phenology** to the wet season [18].

Plants that avoid SWD may have distinct physiological mechanisms from plants that encounter SWD but avoid physiological stress through **osmotic adjustment**, water storage in organs or root isolation from soil [19] (termed *stress avoidance*; Figure 2). These mechanisms require specialized adaptations like succulence [19] or are temporary, as osmotic adjustment can only allow access to limited volumes of soil water [20].

Mechanisms that allow plants to tolerate SWD can include *damage avoidance* by preventing stresses from resulting in damage. Damage avoidance may also have a buffering effect, where a given stress level leads to less damage (Figure 3). Examples include changing leaf orientation away from light [21], heat dissipation of excess absorbed energy by PSII [22], the Mehler reaction or photorespiration [23, 24], and altered root to shoot ratios [25]. Damage avoidance is the preferred term as a similar term, stress tolerance, is commonly used in a general manner.

Finally, plants may tolerate damage through recovery mechanisms (*damage tolerance*). On a daily time scale, recovery is important as nighttime allows recovery of many processes. Refilling of embolized xylem vessels [26] and growth of new conductive tissue [27] are also examples of damage tolerance.

The value of defining a putative drought adaptation mechanism prior to undertaking research is that this objective can be used to design experiments that control the factor affecting the mechanism of interest; an experiment could vary damage to assess damage tolerance, or it could vary water input to assess a plant's ability to conserve water and avoid SWD.

Difficulties with new (and old) definitions of drought adaptations

The mechanism by which a plant is apparently adapted to drought may depend upon the particular physiological process considered. For instance, plants that avoid stress (negative water potentials) by isolating roots from the drying soil [19] would lead to an increase in another stress – stomatal limitations to photosynthesis. Similarly, changing leaf orientation avoids photosynthetic damage, but also decreases transpiration and affects SWD. Consequently, it is not appropriate to label a plant as having one adaptation mechanism, but many, depending upon the physiological system of interest.

Most mechanisms of drought adaptation lead to tradeoffs that ultimately cause a decrease in potential yield. Responses that result in stomatal closure, decreased leaf growth, decreased light interception, all decrease photosynthesis, and growth. For instance, a greater root to shoot ratio may avoid negative water potentials (stress), but also limits the plant's ability to capture light and carbon (leading to damage to yield).

Regardless of the adoption of these new definitions of drought adaptation, the main point of this article remains - drought experiments need to be explicitly designed with mechanisms of drought adaptation in mind. What follows are explanations of appropriate experiments and measurements for testing comparative drought adaptation mechanisms.

Experimental design based upon mechanisms

The experiments highlighted in Box 1 are often used as comparative tests of drought adaptations.

In general, the following rule can be applied: *an experiment should match the degree of manipulation of SWD, stress and damage to the nature of the mechanism being tested.* For instance, testing of genotypes for *soil water deficit avoidance* versus *stress avoidance* mechanisms should be approached differently. The initial term can imply that two genotypes

differ in how fast available water is used i.e. that genotypes access separate volumes of soil and thus are able to conserve water for future use. The appropriate pot or field experiment for this type of investigation would separate the genotypes in pots or in the field by border rows of the same genotype to prevent competition. In natural ecosystems competition may be appropriate. The metric of success should be the resulting conservation of accessible soil water. Alternative mechanisms of SWD avoidance include differences in root exploration and phenology, and should be tested in other manners (e.g. see also Online Supplemental Information Figure S1).

Stress avoidance implies that for the same SWD, one genotype would not show stress responses. The appropriate manipulation is to equalize soil moisture contents between genotypes. Thus, controlled SWDs through the use of shared pots, close field plantings, or regulated dry-downs appear more appropriate experiments (Box 1). These experimental designs account for differences in growth and leaf area affecting dry-down rates, but are comparatively rarely employed (especially shared pots). While controlled SWD experiments allow comparison of genotypes at similar stress levels, they largely preclude comparisons of SWD avoidance as SWD is equalized.

Should SWD, stress and damage be measured in an environmental context?

Variation of soil moisture can typically be used to result in the stress and damage levels needed to test if genotypes avoid damage, or recover differentially (Box 1; Figure 3). However, the development of SWD, stress, damage and recovery are functions of the plant and environment. For instance, plant water status is determined by the combination of water availability (supply) and physiology, phenology and the leaf to air **evaporative gradient** (demand). Thus, a distinction could be made between experiments that investigate the function of a particular

mechanism versus experiments that test how that mechanism performs in an environmental context. Studies of mechanism can be performed in more controlled environments so that environmental variation independent to drought is minimized, small differences are cleanly detectable and the experiment is repeatable.

However, drought in the context of the natural environment exists alongside multi-dimensional variation in environmental factors. Thus, it is unlikely that drought mechanisms studied in the absence of other environmental stress factors will be easily extendable to a functional context. In contextual studies, the level of drought should be considered relative to variation in other environmental conditions (temperatures, evaporative demand, light) and the length of exposure to drought (pots - days versus field - weeks) [28]. While daily variation in environment may be minimal for controlled environments, common garden and field experiments are likely to have different temperature and light conditions for stages of SWD progression. Thus, environmental variation may confound experiments where genotypes reach high stress intensity on different days, and therefore controlled experimental designs listed in Box 1 may be preferable in variable environments. If genotypes have the same SWD while exposed to stress factors and sufficient physiological variables are measured then stress avoidance, damage avoidance and damage tolerance can likely be distinguished. Damage is more likely to occur in field experiments where development of stress can take weeks and environmental conditions are more severe. Thus, experiments testing for stress and damage should expose plants to environmental variation, and match the rate of SWD imposition and duration to what the plant would experience in a sustained field dry down.

Measures of soil water, stress and damage

The list of variables that measure stress and damage is likely infinite; we simply point out literature dealing with typical variables used in ecophysiology [29-31].

Soil water. Soil moisture is the key factor that is manipulated in many drought experiments, but three variables are needed to represent moisture: soil water content, **matric potential** and **hydraulic conductivity**, and these are dependent upon soil type [29, 31]. Matric potential typically limits water extraction, although soil water content is the easiest to measure and control (e.g. by weighing). Typical media used in pot experiments, coarse textured or organic-based soils, make it difficult to control moisture. In such soils, soil hydraulic conductivity can limit transpiration at even moderate matric potentials [32]. Finer textured soils can lead to hypoxia, particularly in shallow pots [33], and pots have problems with wetting, nutrients and salinity [10, 34]. Soil mixtures, fritted clay, deep pots and draining wicks may allow pot experiments with soils that are more realistic of natural dry-downs [35, 36].

Stress. Stomatal conductance provides an easy, integrated measure of physiological response of plants to SWD. However, environmental variation needs to be accounted for [17], or standardized as stomata respond to light and evaporative demand. Plant transpiration is easily measured in pots by weighing [37, 38]. Inter-day variation in environment, particularly evaporative demand, can be accounted for using the normalized transpiration ratio to fraction of transpirable soil water [39] or plant available water [40] protocols. Leaf transpiration measured in gas exchange chambers is not representative of leaves prior to measurement, with stomatal conductance the preferred measure [41]. Dark-adapted maximum photochemical efficiency of PSII (F_v/F_m) is a useful indicator of past stress in leaves and acclimation. Low F_v/F_m can represent damage avoidance due to associated upregulation of excess energy dissipation processes [22]. F_v/F_m depression is sustained and may decrease photosynthetic rates and thus

represent damage too. Leaf relative water contents can be measured to represent drought stress [42], but have some technical problems [43, 44]. Stem and leaf water potentials are used for measurements of plant stress using a number of established, rapid techniques [45-49]. Coding of wilting stages are useful rapid measurements [45, 50]. Leaf orientation/rolling and compensatory growth may indicate stress and damage avoidance responses [21], but decrease photosynthesis and transpiration. Leaf/canopy temperature is determined by the energy balance – a function of weather and plant characteristics including stomatal conductance [51]. Consequently, canopy temperature depression relative to air is often used as a proxy for severe stomatal closure while removing effects of air temperature. Other environmental factors can be accounted for using the temperature-based crop water stress index [52, 53]. Leaf elongation, internode expansion, growth and other turgor mediated processes are also representative of stress [54]. However, sustained turgor effects on growth may be considered damage. Changes in respiration, protein synthesis, nutrient uptake all may represent a SWD response (stress) or lead to long-term negative effects (damage).

Damage. Photosynthesis is a vital component of yield/growth potential, but is difficult to assign as stress or damage because CO₂ exchange results from many processes: carboxylation, photorespiration and respiration, and stomatal, diffusional and metabolic limitations [55]. However, determination of sustained biochemical limitations [56] could be used as an indication of damage [57]. Lower photosynthesis also decreases available carbohydrates, which may be unrecoverable, and thus is a form of damage. PSII quantum efficiency and estimated electron transport rate are similarly affected by many processes, and in particular vary greatly with light intensity [58, 59]. Chlorophyll bleaching and loss of membrane integrity are other examples of damage [60]. Low stem and leaf hydraulic conductance can limit recovery of leaves after re-

watering [61]. Leaf senescence and reproductive failure represent damage as an unrecoverable opportunity cost. All physiological damages are likely to have effects on growth expressed as productivity, biomass, relative growth rate and yield.

Concluding remarks

How many drought papers have you read that simply withhold water from plants regardless of the mechanism being tested? Here we contend that manipulations used to impose drought should match the hypothesized mechanisms of adaptation, and give clear examples of such experimental protocols. We also redefine drought adaptation mechanisms so that they are more specific of physiology and relate more easily to experimental design. The most important suggestion is that the chosen experimental design matches the level of drought intensity, speed of stress development and duration specified by the hypothesized mechanism. Future research could focus on improving experimental protocols to investigate drought adaptations, particularly in the field (see Outstanding Questions).

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Figure Legends

Figure 1. Use of terms related to drought adaptations in scientific literature by discipline. The numbers of papers are results are from Google Scholar searches for the listed terms combined with +”drought” +”plant” and either +“Plant Biology”, +“Crop”, or +“Ecology”. Terms promoted by Levitt [7, 8] and those proposed here are indicated. Citations and patents were excluded.

Key Figure 2. A new set of definitions for mechanisms of crop/plant drought adaptation. In this scheme, the response mechanisms of plants are a function of the interaction of the environment with physiology. Four new terms are proposed that relate to increasing levels of drought intensity and distinct physiological mechanisms underlying the adaptations. The four terms are not commonly used and thus avoid any confusion with current terms: WUE, drought tolerance etc.

Figure 3. Suggested diagnostic experimental manipulations for testing the presence of a specific physiological drought adaptation mechanism. Plants that have the hypothesized mechanism of adaptation to drought (+trait) are shown in comparison to a control plant. The x-axis is the variable that should be controlled, and y-axis the measured response variable. For an adequate experiment both variables need to be measured. Appropriate damage or stress related variables could be used in these tests, but note that many physiological variables report function, which is proportional to 1-stress or 1-damage.

Box 1. Examples of comparative drought experiment designs

General experiments

Natural dry downs: un-manipulated SWD experiments require measurements of SWD, stress or damage so that genotypes/species can be compared.

Soil water deficit avoidance

Uncontrolled soil dry down: genotypes are grown in separate soil volumes, watering is ceased and monitoring of SWD and plant water status is performed until stress occurs. Experiments could account for different leaf areas, pot dry-down rates matched to field data [57] and, in the field, many row plots limiting competition with adjacent plots [62].

Deficit irrigation: application of irrigation based upon supplying a percentage of the **potential evapotranspiration** (ET_o), however the actual ET is likely to vary with genotype. Thus, it is difficult to test other mechanisms of drought adaptation without measurements of SWD, stress and damage.

Root depth: altered patterns of water extraction and root length density with depth can be monitored with rhizotron tubes, deep pots or tube experiments [63, 64].

Stress avoidance experiments

Shared pot/soil volume: by growing genotypes in the same pot or field soil volume they are exposed to similar SWDs [65, 66]. Above-ground competition can be limited by choosing an appropriate control genotype (e.g. short, non-climbing), or limiting the duration of growth and providing a growth barrier. In the field, small plot sizes (one row, or interspersed planting) and probably controlled depth irrigation (e.g. drip) are needed to ensure that the genotypes share the same soil volume, but root depth variation and competition for light are complications in the field.

Regulated pot water deficit: Regulation of the rate of SWD can be achieved through other mechanisms including: daily watering of pots to maintain soil water contents/weights similar between treatments [57]. Dry-down rates can be equalized by planting grass in pots to equalize leaf area/transpiration per pot [67]. Small pots relative to leaf area may lead to undesirable, large daily fluctuations in soil water content.

Damage avoidance or damage tolerance experiments

Osmotic stress: membrane impermeable compounds (e.g. polyethylene glycol) can be used to establish levels of osmotic stress that are equivalent to SWD's, but have the potential for hypoxia due to high viscosity, toxic impurities and require measurements of osmotic potential. Alternatively, membrane permeable osmolytes (e.g. NaCl) can be used, with additional salinity/ionic effects [68].

Other: the controlled dry-down experiments described above may be suitably adapted to vary stress or damage, and *natural dry-downs* or other experiments may achieve variation in measured stress and damage, with loss of control.

Figure1

Figure3