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Clarity on frequently asked questions about drought measurements in plant physiology

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ABSTRACT

Drought, or environmental water deficit, is one of the major limiting factors affecting crop yield worldwide. Development of drought-resistant crop cultivars is a major research and development challenge. Drought-related experiments are performed usually to understand the physiological and molecular mechanisms of drought tolerance. Such experiments are also performed to develop transgenics or crop cultivars resistant to drought using physiological and molecular markers. Drought-related experiments are executed in growth chambers, growth rooms, greenhouses, wire net-houses or in research fields. However, a plethora of research publications investigating drought has experimental weaknesses and flaws with respect to the approaches used. It is, therefore, necessary for agronomists, plant breeders, plant physiologists, and molecular biologists to be aware of common pitfalls and have the minimum knowledge required for drought measurements. There are several questions that are often asked by students and professionals alike, and these questions often appear on academic social media platforms. This article summarises the questions we have been asked about drought measurements personally and those asked on academic social media platforms. It also addresses ambiguous questions arising from published literature. We aim to respond to them to the best of our knowledge in order to provide a reference point for a beginner interested in performing drought-related experiments. This article will

Abbreviations: A, net assimilation; ABA, abscisic acid; A/Ci, net assimilation/intercellular CO₂ concentration; A/E, Water use efficiency (expressed as net assimilation/transpiration); AvFw, average fresh weight; DW, dry weight; E, transpiration; FC, field capacity; F_v/F_m, maximum efficiency of photosystem II (F_v/F_m); FW, fresh weight; g_s, stomatal conductance; NPQ, non photochemical quenching; OA, osmotic adjustment; OP, osmotic potential; OPo, leaf osmotic potential calculated due to concentration effect; Opi, initial leaf osmotic potential; PAR, photosynthetically active radiation; PC, pot capacity; PEG, polyethylene glycol; PEPC, phosphoenolpyruvate carboxylase; PsbS, photosystem II 22 kDa protein; ΦPSII, efficiency of photosystem II; qP, photochemical quenching; Rubisco, ribulose-1,5, biphosphate carboxylase; RWC, relative water content; SWC, soil water content; TW, turgid weight; VPD, vapour pressure deficit; Y leaf, leaf water potential.

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only focus on drought in relation to plant physiology and will not cover the usage of the term or drought measurements in other contexts.

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Introduction

There has been growing research interest in the study of plant abiotic stresses [95]. One such stress is drought. Anthropogenic climate change is increasing the incidence and severity of soil and atmospheric water deficits across the planet [129,132], making it more and more critical to research plant and crop responses to drought. However, the approaches used to simulate drought and measure water deficit differ across labs and across disciplines [35]. A quantitative and replicable measure of water deficit requires precise determination of water potentials (Ψ_w); the lower the water potential, the stronger the water deficit stress. One powerful aspect of water potentials is their ability to link soil water availability to plant water uptake, from which plant water flow and water status can be analysed and modelled [114,123]. Nevertheless, the majority of publications concerning plant drought and water deficit stress do not measure water potential, and alternative methods and approaches are routinely used. Many are controversial, while others are gradually gaining relevance [59]. For instance, experiments performed in small pots in growth rooms are subjected to stronger edge effects and the growth conditions in such experiments are far from what is obtained or experienced in the field. In addition, there could be large temperature increases in non-ventilated greenhouses due to high levels of vapour pressure deficit (VPD) [9,41,60,61,92,115]. Thus, it may be misleading to compare data obtained from experiments performed in a growth room with those generated from greenhouses or in the field.

Over the years, young scientists, PhD, masters and undergraduate students have questioned the best methods to use for their drought research. The aim of this review is to provide some answers to the most common questions and ambiguities in the literature, as well as highlight considerations required to achieve successful water deficit measurements. First, specific questions about experimental design and methodology are addressed. Then, questions about the role of light and measurements of photosynthetic parameters in water deficit experiments are tackled. Finally, questions about water uptake and water loss with regards to root and stomatal parameters are answered.

Should the term drought or water deficit be used in experiments?

To date, it has generally been a matter of semantics which term to use, and 'drought' and 'water deficit' are often used interchangeably. Both terms are used to refer to water limitation in the course of experiments [29,38,47,56]. What has been considered important is stating clearly how water limitation was achieved and maintained. We propose that the term 'drought' be used more for environmental and agronomic situations (i.e. naturally occurring water deficit) and 'water deficit' be the preferred term used when referring to irrigation limitation and experimental treatments simulating drought. It is then up to the experimentalist to quantify the degree of water deficit [9,41,60,61,93,115]. One aspect to consider when using 'drought' or 'water deficit' is that water deficit is a more quantifiable metric than drought. As it can be measured as a difference from a saturated or well-watered state, we recommend that 'water deficit' be used in more precise terms than drought. Drought severity and duration therefore need to be defined for them to have any other meaning than a qualitative stress.

What are the main factors to consider when designing a water deficit experiment?

Experimental design for imposing water deficit treatments depends on the aim of your experiment and is largely dictated by whether you are performing closed-system, controlled environment, greenhouse, or field experiments. The onset of water deficit stress treatment also depends on what stage of drought the researcher is interested in. Is the researcher interested in seedling survival, vegetative responses, or reproductive stress such as the simulation of terminal drought? The level of severity of the water deficit should be appropriate to the species and to its ecology. For example, in water deficit-induced tree death, there is a point of no return for hydraulic failure, after which the tree is more likely to die than survive [48]; in loblolly pine (*Pinus taeda* L.) the lethal threshold was found to be at 80 % loss of hydraulic conductivity [48]. Other factors that may contribute to water deficit-induced mortality are carbon starvation and extreme light limitation [48]. Therefore, in both model and crop species, cessation of the stress, recovery, or terminal treatments need to be considered, including often overlooked agronomically relevant measurements of yield parameters [15,19].

The aim of the experiment and the stage of water deficit of interest determine the factors to consider and the tools to use for quantitation of the level of water deficit. Thus, the question about the best indicators of drought is a complex one as there are many indicators. Morphologically, leaf characteristics such as leaf area, size, weight, and colour have been used. Physiologically, relative water content, leaf water potential, leaf osmotic potential, leaf turgor potential, net assimilation (A), stomatal conductance (gs), transpiration (E), net assimilation/intercellular CO₂ (A/Ci) curve, water use efficiency (A/E), net assimilation/stomatal conductance (A/g_s) or Biomass/Water could be used as indicators [7,9,32,39,100,122]. Also, fluorescence

parameters such as the efficiency or photochemical yield of photosystem II (Φ PSII), maximum efficiency of photosystem II (Fv/Fm) and non-photochemical quenching (NPQ) are acceptable indicators of plant stress level [20,57,105]. There is growing use of indices derived from non-contact spectral measurements from reflectance and a vast literature on the quantification of xylem embolism in roots, stems and leaves in response to drought (of particular importance to tree crops, but also shown in maize and soybean, among others) [1,13,26,46,52,66,76]. Biochemically, accumulation of amino acids and soluble sugars such as proline, glycine betaine, trehalose, activities of photosynthetic enzymes such as ribulose-1,5, bisphosphate carboxylase (Rubisco), phosphoenolpyruvate carboxylase (PEPC), and others are also useful indicators [3,21,22,32].

Growth conditions and environmental factors in water deficit experiments: Do water deficit experiments in pots tell us anything about drought? Is there any point in them?

To minimise confounding factors to the water deficit response, growth limitations such as nutrients, oxygen, carbon dioxide, water, light availability, temperature, herbivores, and pathogens should be considered and if possible controlled [89]. Here we will focus on the most critical variable: water. If the experimentalist intends to perform a pot experiment in controlled environment facilities or growth rooms, it is easier to control the amount of water and substrate weight, which is one of the advantages of this approach unlike in the field. In pot experiments, water is often supplemented to reach a target pot capacity using a set temperature and irradiance [90,91]. However, experiments in controlled environment facilities usually ignore the fact that environmental factors fluctuate and are dynamic as obtained in the field and so plants may acclimate to survive or tolerate the water deficit condition [10,87,97]. A meta-analysis of plants grown in growth chambers, glasshouse and in the field showed that lab-grown plants grow faster initially, but with time are constricted morphologically and receive lower irradiance relative to glasshouse and field-grown plants [98]. Growth of plants in a greenhouse and in the field often gives a closer physiological reflection of the responses of plant species and varieties to drought than that which can be achieved in controlled environment facilities. Greenhouses and growth rooms often provide more extreme positional variation, causing strong edge effects and light gradients, compared to the field, depending on the situation. These edge effects translate to altered water deficit stress at the plant level which can be minimised by careful plot design, pot rotation, or randomisation. Also, a major issue in non-ventilated greenhouses is large temperature increases in the middle of the day, which may cause unrealistically high levels of vapour pressure deficit with consequent stomatal closure and changes in major pathways [92]. Equally, if stable isotopes are employed for tracking water use efficiency, one needs to be very careful inside greenhouses because of signal contamination of the source air isotopic signature.

Whatever data is generated using the pot approach should be considered an estimate which could be supported using leaf relative water content (RWC) and drought measuring instruments such as a gas analyser, drought meter, tensiometer, psychrometer, pressure chamber, cryoscopic osmometer, pressure probe, and soil moisture probe [68]. These instruments all have their advantages and disadvantages, with a major challenge being that they all need to be calibrated to the plant-soil system. In order to ensure that the leaves, and indeed the various plant organs, are drying at similar rates and have synchronized growth when performing a water-deficit experiment, there should be a judicious application of known required volumes of water [59].

There is also evidence that plant water status can be assessed by measuring canopy reflectance indices using a spectroradiometer [45]. There is a close relationship between the ultrasonic resonance of leaves, RWC and Ψ_w [43], thus, enabling estimation of crop water status in pot experiments and in the field [45]. In summary, as stated earlier for the previous question, the 'gold standard' for water deficit experiments depends on the aim of the experiment and is largely dictated by whether the experimentalist is performing a closed-system, controlled environment, greenhouse, or field experiments. Again, it also depends on the specific question that the researcher is interested in. In addition, care should be taken not to compare varieties of different age and when comparing varieties with different performance, as faster-growing varieties will draw water more efficiently from the substrate than slower-growing ones. Shared pot experiments can go some way to overcome these issues. Overall, water deficit experiments in pots should be performed taking into consideration confounding factors and, where possible, adequately controlled. Also, data from such experiments should be supported by those acquired using drought measuring instruments and, where possible, replicated in a greenhouse or in the field.

Do pot size, shape, material, and colour matter in drought experiments?

In many water deficit experiments, plastic or ceramic pots are used to grow plants. Pot shape, drainage, volume, material, and colour will all contribute to the rates of drying of a particular substrate and the developmental and physiological responses of the plant. The root system will grow to the volume of the pot, and therefore plant roots are almost always limited by pot size, especially when smaller pots are used [10,78,92,98]. Substrate and plants in small-sized pots tend to dry out faster than in bigger sized pots or in field conditions. Thus greater pot volume is crucial in experiments where pots will be used [10]. Smaller pots decrease plant growth by reducing photosynthetic rate per unit leaf area due to pot limitation of root growth, rather than a change in leaf morphology or biomass allocation. Larger pots, on the other hand encourage a more even plant growth. Too small and overly large pots will change experimental results. For example, if a pot is too large then the time taken to achieve sufficient water deficit may be biologically or experimentally unfeasible [97]. In addition, pot shape and height are also relevant in experiments. In the field, roots will grow deep quickly, whereas standard pots rapidly limit rooting depth. Increasingly, researchers are employing taller (and wider) pots to promote deep rooting and allow examination of vertical soil gradients more carefully.

It is noteworthy that the colour of pots can also affect the results of a water deficit experiment. Usually pot colour does not matter when conducting experiments in growth chambers or wire net-houses. Oftentimes, different coloured plastic pots are used to distinguish different sets of experiments in the same growing space. Sometimes this practice causes a huge difference in results. Black coloured pots absorb much more light and heat when exposed to direct sunlight outside and in wire net-houses, and therefore substrates will dry faster than in white or light coloured pots [102].

Growth media and substrates

The interaction of the composition of the growth medium with the plant and the environment dictates the rate of substrate drying. Evapotranspiration processes will differ by growth medium choice, and therefore careful choice of substrate is required to prevent too rapid or too slow an onset of water deficit stress conditions. The use of soil in pots (particularly small pots) as a substrate for plant growth could introduce artefacts to an experiment due to negligible aerated porosity. Low porosity prevents the even distribution and percolation of water within the potted soil, as well as reproducible water loss rates for repeatable water deficit experiments. These problems are prevented by using more homogeneous commercial substrate mixes (e.g. peat-based and peat-free composts containing inert additives such as perlite and vermiculite) which are more porous, thus allowing even drainage and water distribution [92]. In pot experiments where coarse-textured or organic-based soils are used, soil hydraulic conductivity can reduce or minimise transpiration which may lead to hypoxia, especially in shallow pots [41]. It is crucial to note that drought in the field, as mentioned earlier, is dynamic, and pot experiments in controlled environments are considered different from greenhouse or field measurements. The method used for pot measurements could be reproduced in the greenhouse or in the field. In order to maintain a particular level of water deficit, a popular method is to make use of pot capacity, which is a relative value that requires consistency to ensure reproducible results [55,91,99]. Note down pot weight (g) without substrate. Note the pot weight with the substrate and water (well-watered) (FW). Ensure sufficient replication, with a minimum of three pots per treatment. Weigh the substrate contained in the pot in an oven at 105°C to constant weight. This becomes the reference dry weight (DW).

The following formula is recommended for the estimation of Percentage Pot Capacity:

$$(FW-DW)/(AvFW-DW)*100.$$

NB: AvFW= Average FW of the replicates on Day 0 of the experiment. Pot weights should be maintained at desired target water levels (e.g. 90 %, 80 %, etc.) for a minimum of 5 days to ensure uniformity in water levels before any growth and yield measurements are taken [91]. Even when the target pot capacity has been reached, one cannot assume that a specific level of water deficit has been induced, nor does it necessarily mean that every part of the plant will experience the same level of stress [53]. Thus, when maintaining a target level of stress, the leaf water status should be measured concurrently to check that hydration in the leaf matches that of the soil. It is also important to account for changes in plant biomass over the course of an experiment, by adjusting target weights as well as by calculating specific leaf area for more reproducible growth analyses [91]. In addition to these pot methods, several other key techniques such as Relative transpiration (RT) as a function of the fraction of transpirable soil water (FTSW) have been commonly adopted to determine water status [55,91,99,107,113]. Monitoring water potentials remains the most precise way of ensuring reproducible water deficit treatments.

When is the best time to measure soil water potential?

Predawn water potential is usually the most acceptable for soil water potential [69,127]. However, in one study, the authors stated that predawn water potential may not reflect the soil water potential in woody plants and halophytes, even when well-watered [31]. Generally, it is assumed that predawn water potential equilibrates the water status accumulated by the plant overnight due to soil saturation [28]. Night-time transpiration is physiologically relevant to plants and drought responses [27,101,106], but it is generally far lower than day-time levels, and so soil water is retained. If night-time transpiration is low, predawn leaf water potential can be a useful approximation of soil water status, while daytime water potential is generally a good indicator of plant stress. However, there can be disequilibrium between soil and plants at predawn. In the field, it is actually very rare to measure values of predawn that one would expect based on plant height (for gravity correction) and soil water content. Overall, soil water potential measurement depends on the aim of the research. Is the researcher interested in unstressed or stressed plants? If it is the former, predawn is preferred, but if it is the latter, then daytime water potential should be considered.

Osmotic adjustment as a potential physiological indicator of drought tolerance. How can we measure it?

Several breeders are using osmotic adjustment (OA) in breeding programs to develop drought-tolerant crop cultivars [23]. OA can be considered an important indicator of drought resistance. There are four different methods that can be used for the measurement of OA [6]. In the first method, osmotic adjustment can be derived by applying linear regression between time-course measurements of leaf relative water content (RWC) and leaf osmotic potential (OP) of plants during the drying cycle. In this method, two linear regressions are made: one between leaf RWC and leaf OP, and a second one between leaf RWC and leaf OPo which is calculated due to concentration effect. Time-course leaf OP can be measured until RWC reaches 70 %. Beyond this, for example at a RWC of 60 %, the linear relationship between the two attributes becomes lost. OPo can be calculated as follows:

$$\text{Leaf OPo} = \text{Leaf OPi} \times [(\text{RWCi}/100)/(\text{RWC}/100)] \quad (1)$$

Where leaf OP_i is initial leaf osmotic potential in the well-watered condition, while RWC_i is leaf RWC of hydrated plants at the initial step of experiments. Plant osmotic adjustment capability is generally calculated as the difference between the two regressions, i.e.:

$$OA = \text{regression between leaf RWC and leaf OP} - \text{regression between leaf RWC and leaf OP}_0 \quad (2)$$

In the second method, OA can be estimated as the difference between leaf osmotic potential of well-watered plants and water-stressed plants. Leaf osmotic potential of non-stressed plants can be measured in the morning after last irrigation. Leaf osmotic potential of well-watered plants and water-stressed plants will then be calculated to a state of rehydration (OP₁₀₀). Leaf OP₁₀₀ (OP at rehydrated state) can be calculated as follows:

$$OP_{100} = OP[(RWC - B)/(100 - B)] \quad (3)$$

The value of RWC in this equation must be similar in all samples to ensure wilting in leaves (not leaf rolling as occurs in cereals) and generally 60 % is taken. B- a constant value of 18 %.

In the third method, OA can be estimated as the difference between leaf OP of non-stressed plants and leaf OP of stressed plants after rehydration. In this method, plants are water-stressed at RWC 60 % and then rehydrated in the evening. In the next morning, leaf OP from rehydrated water-stressed plants can be measured.

The fourth method is similar to the first method described. In this method, leaf OA can be measured by drawing a regression between RWC at a given and fixed OP which is close to leaf wilting point, such as -3.5 MPa. Higher leaf RWC shows better OA capacity at close to the wilting point [6,14,51,110].

Is the best way to qualify drought by measuring Relative Water Content?

For accuracy and reproducibility, the maintenance and measurement of water potentials across treatments is a reliable way of quantifying water deficit stress. Nevertheless, Relative Water Content, Soil Water Content and water potential measured for instance using a pressure chamber are all commonly used methods [55,91,99]. If the researcher is worried about the cost of purchasing a pressure chamber, thermocouple psychrometer, or mirror hygrometer, Relative Water Content and Soil Water Content could be used with some accuracy, but with the knowledge that a little amount of water is inevitably lost in the process [91]. One element to note here is that leaf RWC and Y leaf (leaf water potential) are related via the leaf Pressure-Volume curve. Therefore, the slope of this relationship (before and after turgor loss point) dictates how sensitive the measurement of RWC can be in the earlier versus the later stages of dehydration, relative to Y leaf [2,14].

Can polyethylene glycol (PEG) be used to induce water deficit stress?

PEG is considered an osmoticum and can be used to alter substrate water potential [44,70,131]. However, the researcher should ensure that no part of the plant root system is damaged. If a root is broken, the osmoticum will be taken up by the plant and confounds the experiment. The researcher should consider using high molecular weight PEG such as PEG 6000-8000, and not mannitol, for best results [11]. For example, mannitol has been demonstrated to induce extreme water deficit and shrinkage in maize and is unsuitable for estimation of water relations [49]. The extreme effects of mannitol were attributed to the penetrative ability of the osmoticum into tissues, unlike PEG which is a nonpenetrating osmoticum [49]. Therefore, experiments with PEG will be more reproducible in achieving defined water potentials. Although these osmotica can induce water deficit and increase our knowledge on stress physiology, they do not reflect the physiological conditions in the field [25]. However, their use counters some of the pitfalls of soil-based or compost-based drought experiments (reviewed by Lawlor [59]). For instance, coarse, fine and clay soils have wide, small and very small pores respectively. Composts that are usually used in growth room experiments have many large pores between fibres. The spaces in between pores determine the amount of water that may be held by a given soil or compost and can influence soil water content (reviewed by Lawlor [59]). This problem is countered when a non-penetrating osmoticum such as PEG is used.

In the literature, there is a disparity in the osmotic potential of PEG6000 solutions of the same percentage. How can this be explained?

As described above, water stress can be imposed on plants by the application of PEG [85]. PEG is a polymer of different sizes, such as PEG4000, PEG6000, PEG8000 [42,85]. Commercial and lab-grade products also contain molecules of different size ranges. For example, in PEG6000 the average molecular weight can range from 5500-6500 [65]. Even if the same concentration (often expressed as W/W % or W/V) is used, the osmotic potential may be different, therefore a 10 % PEG solution may give -0.3 MPa and, from other sources, it may give -0.45 MPa [75].

What is the best time to measure physiological parameters in drought-stressed leaves?

It is crucial to mention that photosynthesis and respiration vary considerably during plant development and diurnally [4,5]. Thus, it is recommended that the researcher perform a time course of gas exchange responses of their plant of interest to increasing water deficit stress. To achieve this, Infra-red Gas Analyzers (IRGAs) can be used, such as the CID Bio-Science CI-340, or the LI-COR XT-6400 and XT-6800, depending on budget and requirements. Depending on daylength and location, the plant may be most photosynthetically active at certain times of day, and therefore care should be taken to avoid gas exchange measurements when stomatal pores are closed due to circadian rhythms or high vapour pressure deficit (VPD). However, if the experiment is focussed on stress or recovery, then measuring the plant during extreme stomatal closure (e.g. high VPD) would be useful. In addition, if the focus is on survival of plants to terminal drought, night time respiration measurements will be required so that periods of negative carbon balance will be considered. There are strong

interactions between photoperiod and drought responses, particularly in crop phenology [30,37,103] and in facultative CAM plants [16,124]. Thus, there are close links between day-length and drought, and care should be taken when experiments are focused on diurnal measurements, especially if comparing across the year and seasons, in order obtain reproducible data.

The role of light and measurements of photosynthetic parameters in water deficit experiments: Do photosynthetic parameters tell us anything about differences in drought tolerance? Does drought affect the photochemical capacity of leaves?

Water stress-tolerant plants can maintain plant water status by greater absorption of water through roots, lowering water loss through transpiration and stomatal regulation [12]. Thus, rapid stomatal response to water stress is considered a drought tolerance mechanism to maintain plant water status. Stomatal conductance regulates CO₂ fixation and transpirational water loss, thereby affecting net photosynthesis and crop yield [34]. In addition, stomatal conductance has been considered a potential indicator of drought tolerance in breeding programs for a long time [3,34,116]. However, a reduction in photosynthesis due to water stress may not necessarily be associated with stomatal limitation. Some studies demonstrated that a decrease in photosynthetic rate was mainly influenced by stromal or thylakoidal reactions [54,118]. For example, while drawing relationships between a decrease in photosynthetic rate and different factors including gas exchange rate, metabolic factors in stroma and light reactions in sunflower plants under mild and severe drought stress, Tezara et al. [117] reported that a decrease in photosynthesis was associated with changes in the light reaction and ATP synthesis.

Water stress induces stomatal closure and overexcitation of the photosynthetic apparatus [94]. In order to avoid damage to the photosynthetic apparatus, excess electrons are dissipated as heat (NPQ), photochemical quenching (qP) or re-emitted as light (Chlorophyll fluorescence) [72]. In field-grown plants, the effect of drought on photochemical capacity may not be as pronounced as pot, greenhouse, or glasshouse experiments. In one study, the researchers investigated field-grown grapevines and found that drought did not induce photoinhibition in the plant, even though net assimilation and stomatal conductance were very low [36]. They also observed that there was a diversion of electrons to alternative pathways such as photorespiration [36]. In another study on field-grown beans, the researchers found that the plants avoided sun-induced photoinhibition via leaf movement or paraheliotropism [94]. They observed that the extent of sun-induced photoinhibition and leaf movement increased with water deficit [94]. These effects reduced photochemical capacity in the bean leaves as well as the D1 protein of photosystem II (PSII) [94].

Thus, the effect of drought on the photochemical capacity of leaves can be species- or cultivar- specific, or other variables may prevent clear relations between photosynthetic parameters, genotypes, and yield [96]. However, in a more controlled environment such as pot experiments in growth rooms, the level of water deficit stress may be more extreme, although light intensities may not match those of the field, thus inducing atypical responses.

Drought reduced PSII efficiency but increased NPQ? What does this imply?

Non-photochemical quenching (NPQ) is the energy dissipated as heat after the energization of PSII due to proton accumulation within the lumen [81,89,111]. Greater increases in NPQ with lower electron transport rate (ETR) may indicate that the photoprotective mechanism is in action [88,130]. NPQ is a photoprotective mechanism during drought which prevents photodamage by decreasing the generation of reactive oxygen species (ROS). This action is modulated by PsbS protein thereby enhancing quenching in the photosystems [63,81,125]. Overall, increase in NPQ implies that the plant is trying to protect itself from the negative effects of drought. The plant does this by quenching singlet excited chlorophylls to ground state, thereby dissipating excess excited energy as heat through molecular vibrations [63,81,125].

At what time of the day should Fv/Fm be taken for drought experiments?

PSII is sensitive to water stress [20,82]. Structural stability and functional activity of PSII can be measured as the quantum yield of PSII (Fv/Fm) in dark-adapted leaves and under steady-state light conditions [72]. Some researchers suggest that Fv/Fm should be measured predawn [58,112]. Time-course measurements of water-stressed plants should indicate the time point where photoinhibition occurs [62]. Under severe drought stress conditions, Fv/Fm values are significantly lower than 0.8 and indicate photoinhibition [62,72]. However, mild water stress-induced declines in photosynthesis are generally associated with stomatal closure with Fv/Fm unaffected under such condition.

What is the best light intensity to use for physiological measurements?

Similar to the response given for the previous question, it is important to perform a time-course experiment to study irradiance, photosynthetically active radiation (PAR), light absorption and utilisation by the plant of interest [62]. Such an experiment will produce a light response curve and from that curve, the peak light intensity established could be used. In one experiment with kidney beans performed in a controlled growth chamber, the authors reported that the light response curve of photosynthesis during drought matched that obtained during recovery [77]. In another drought experiment performed in the field, there was no difference in the light response curve of photosynthesis in control and drought treatments [71]. Therefore, there is a need to understand the peak light intensity for optimal interpretation of experimental results.

Researchers often measure a Rapid Light Curve (RLC) using a built-in program in fluorometers. Sometimes differences in efficiency of PSII among non-stressed and water stressed plants are not clear. What should I do next?

Photosynthetic apparatus (PSII) responds immediately to given light intensities [62]. Generally, under non-stressed and water stressed conditions, leaves are exposed to low light intensities and then increased stepwise after equilibrating 3-5 minutes up to 2000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ or higher. When the rapid light curve (RLC) shows an increase, it suggests that stomata

have not had enough time to adjust apertures to the given light intensities. Changes in intercellular CO₂ (C_i) do not occur when there are poor changes in carboxylation and PSII efficiencies [33]. To overcome this problem, slow light curve analysis should be used, particularly under water deficit conditions. It gives stomata the time to adjust and equilibrate at each light intensity level. However, it should be noted that regardless of giving stomata time to adjust to each light intensity, drought stressed plants may not reopen their stomata, in which case PSII differences are not due to different inherent photosynthetic capacities or light responses, but rather the drought effect on stomata.

Water uptake and water loss: Measurement of root and stomatal parameters

Can drought be estimated by studying plant roots alone?

Root systems absorb water needed for the maintenance of growth and demands of transpiration in plants. It has been shown that fine roots, defined as roots less than 2mm diameter, maintain the hydraulic integrity of the plant's vascular system during the early stages of water deficit [28]. When the cortical cells of fine roots are damaged, the plant's ability to tolerate drought is compromised [28]. Stomata respond rapidly to changes in VPD as well as changes in water availability in the rhizosphere, strongly influencing drought responses in plants. The relative contribution of root-derived abscisic acid (ABA) and vascular or leaf-derived ABA, to induce stomatal closure and to reprogram the plant to drought response mode, is an active area of research and remains controversial [17,67,73,74,104]. Several studies also argue that ABA does not perform this function directly, but rather electrical and hydraulic root signals do [24,108,119,128]. With this in mind, studying drought by analysing plant roots, or root-derived ABA, without concurrent leaf physiology or biochemical studies may be misleading.

Is stomatal conductance an efficient indicator of drought tolerance?

Leaf area and photosynthetic rate are important growth indicators and can be used to monitor drought [80]. Assessing photosynthetic rate is quicker and a non-invasive technique, unlike biomass or leaf area measurements. However, among non-invasive physiological indicators, measurement of stomatal conductance has a greater capacity to discriminate phenotypes [50]. Photosynthetic rate is less sensitive to drought because certain anatomical and cellular modifications occur in response to water stress to maintain photosynthetic rates at lower stomatal conductance [64]. Nonetheless, stomatal conductance can also be disadvantageous as it can be highly variable and sensitive to light and CO₂ concentration, which need to be controlled. Also, in some plants photosynthesis may be useful to discriminate phenotypes, particularly if the researcher is interested in photosynthetic responses.

Bundle sheath cells and root-derived ABA have been suggested to play regulatory roles in controlling conductance [17,84]. In drying soil, ABA is transported from the roots to leaves acting as a signal that regulates the closure of stomata [17]. However, this suggestion is currently controversial, with other studies suggesting that ABA transport from the roots may not perform this role, and rather that there is an interplay of shoot or leaf-derived ABA, electrical, and hydraulic root signals [24,108,119,128]. Nevertheless, ABA signalling continues to be a successful target for crop drought improvement with powerful potential [121]. C₃ and C₄ plants are also known to have differential sensitivity to water stress due to their different abilities to regulate oxidative stress as a result of the accumulation of ascorbic acid and glutathione [83]. Under mild water deficit stress, ABA is unaffected in both plants, but tends to be higher in C₄ relative to C₃ with increasing water deficit [83]. Overall, stomatal conductance is less sensitive and maintained longer in C₄ than in C₃ during water deficit [126].

Are cereal crops more drought tolerant than other crops?

Interestingly, cereal crops like barley, wheat, maize or rice tend to have higher ΦPSII and net assimilation relative to non-cereal crops in general and during the early stages of drought [8,18,86]. As monocot grasses, cereal crops have uniquely divergent stomatal morphologies and physiology which underpin these photosynthetic and drought responses [18]. Compared with non-monocot plant stomata, cereal crops show faster stomatal responses and are able to alter their stomatal apertures to optimise CO₂ uptake with less transpirational water loss [8,18,86]. This superior stomatal response in grasses has also been attributed to the modification of guard cell-expressed Slow Anion Channel-Associated 1 (SLAC1) involved in ABA-induced stomatal closure [109]. In dicots, ABA activates SLAC1 and induces chloride-dependent stomatal closure [40,120], however, the grass-type SLAC1 channel of barley is nitrate-sensitive, resulting in faster regulation of SLAC1 function and more rapid closure [109,120]. These differences could underlie the evolutionary success of the grasses and result in cereal crops' improved drought tolerance. Research and selection for improved drought tolerance also began earlier in cereals than in other crops [79]. Thus, apart from fast responsive stomata, other drought tolerance mechanisms adopted by cereals and other crops may include high biomass, high growth rate, delayed leaf senescence, high capacity of energy dissipation, high osmotic adjustment, deep roots, and high water uptake [79].

Is it okay to increase metabolite concentrations to increase drought tolerance?

Much attention has been given to increasing metabolite concentration in recent years. For instance, it is thought that increasing metabolites such as proline lowers the root water potential and so allows plant roots to absorb water during water deficit stress [59,91,110]. Increasing metabolite concentrations does not make for comparable studies as water loss in plants is determined by leaf area and stomatal characteristics and not by metabolic composition [59,91,110]. Also, increasing the metabolite concentrations by transgenic or molecular breeding would arguably cause major off-target effects in the metabolome and the proteome of the plants. Any increase in drought tolerance achieved might be due to the interaction of stomatal conductance with the environment and not necessarily because of metabolic adjustments [59,91,110]. This would mean an increase in the plants' leaf morphology with time, again, making a comparison between varieties difficult [59].

However, for specific species, foliar exogenous application of osmoprotectants such as sugars, sorbitol, mannitol, polyethylene glycol (PEG), and amino acids have been shown to increase their endogenous levels and drought resistance [3]. Whether osmoprotectants can be utilised in breeding remains to be seen.

Conclusions

There are many environmental and experimental factors that affect plant growth and drought responses. All these factors should be identified and controlled where possible to carry out reliable and replicable water deficit stress research. As described above, they include artefacts or unintended factors such as pot size, pot shape, pot colour, variation in substrates used, variation in the chemicals used to obtain a given osmotic potential, shading and edge effects, soil gradients in fields, emissions from heating or cooling systems, etc.. Researchers need to observe plants carefully during experimentation to timely recognise any potential anomalies. In addition, researchers need to understand the phenology and interactive behaviour of plants with their environment. Some plant species are fast-growing, and others are slow-growing. It is important to know what stage a plant should be exposed to water deficit stress (seed germination stage, seedling stage, vegetative stage). This, however, depends on the question addressed in the specific study. The physiological and morphological attributes that should be measured and the time required for optimal results should be carefully considered. Some changes occur within minutes or hours in plants in response to water stress, while other physiological and developmental changes can happen over weeks. Thus, the researcher needs to conduct time-course studies or diurnal responses of stress and recovery, and longer-term responses to progressive drought. In this review, we have approached several common questions concerning the study of drought in plant physiology. We hope that the answers provided will help early-stage researchers more thoroughly plan and systematically approach such studies. Nonetheless, there are always unanswered questions and new techniques and methodologies that arise; hence, we are open to more questions. In addition, the authors are available to be contacted through e-mail or on academic social media platforms to answer more questions.

Author contributions

CCO led the discussions and designed the initial framework. CCO, HA, MA, HB, CCCC and NPJ wrote the manuscript. All authors contributed to discussions.

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Declaration of Competing Interests

The authors wish to declare that they have no conflict of interests

CRediT authorship contribution statement

Chukwuma C. Ogbaga: Conceptualization, Writing - original draft. **Habib-ur-Rehman Athar:** Writing - review & editing. **Misbah Amir:** Writing - review & editing. **Hussan Bano:** Writing - review & editing. **Caspar C.C. Chater:** Writing - review & editing. **Nugun P. Jellason:** Writing - review & editing.

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