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Review

Methodology of Drought Stress Research: Experimental Setup and Physiological Characterization

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Abstract: Drought is one of the major stress factors affecting growth and development of plants. In this context, drought-related losses of crop plant productivity impede sustainable agriculture all over the world. In general, plants response to water deficit by multiple physiological and metabolic adaptations at the molecular, cellular and organism levels. To understand the underlying mechanisms of drought tolerance, adequate stress models and arrays of reliable stress markers are required. Therefore, in this review we comprehensively address currently available models of drought stress, based on culturing plants in soil, hydroponic or agar culture. Thereby, we critically discuss advantages and limitations of each design. We also address the methodology of drought stress characterization and discuss it in the context of real experimental approaches. Further, we highlight the trends of the methodological development in the drought stress research, i.e. complementation of conventional tests with quantification of phytohormones and reactive oxygen species (ROS), measurement of antioxidant enzyme activities, as well as comprehensive profiling of transcriptome, proteome and metabolome.

Keywords: drought stress; drought models; drought tolerance; oxidative stress; phytohormones; polyethylene glycol (PEG); stress markers

1. Introduction

Being a natural climate feature, drought occurs in almost all climate zones with varying frequency, severity and duration, being one of the most deleterious factors of environmental stress [1,2]. Indeed, even a short-term water deficit results in essential annual losses of crop yields [3,4], impeding thereby sustainable agriculture all over the world [5,6,7]. Due to the oncoming climate changes, frequency and durations of drought periods increase, making this factor one of the most important threats of the current century [8,9].

In the context of agriculture, drought is defined as a period of below-average level of precipitation [10], when the amounts of available water in the plant rhizosphere drop below the limits required for efficient growth and biomass production [11]. Such soil water deficit can be persistent in climate zones characterized by low water availability, or intermittent and unpredictable water supply during the vegetative period [12]. Because of this, drought is the major environmental

stressor, affecting plant growth and development by disruption of its water status [13]. This dramatically affects all key physiological processes, like photosynthesis, respiration and uptake of mineral nutrients [14,15]. First, drought compromises stomata function, impairs gas exchange, and leads to over-production of reactive oxygen species (ROS) and development of oxidative stress [16]. Secondly, water deficit inhibits cell division, expansion of leaf surface, growth of stem and proliferation of root cells [7]. In concert, all these factors dramatically reduce plant productivity, and might lead to death of drought-sensitive plants upon prolonged exposure to drought [17].

At the quantitative level, water deficit in the environment can be characterized by a decrease of soil water potential (Ψ_w) [18]. According to the Van't Hoff equation, it indicates a decrease in free energy of substrate water that makes water uptake from the medium under these conditions thermodynamically unfavorable and loss of water by the plant more probable. Ψ_w of 0 to -0.3 MPa are characteristic for well-watered plants, whereas the values below -0.4 MPa correspond to moderate water stress, and potentials of -1.5 to -2.0 MPa represent severe stress and permanent loss of turgor [19]. However, these values vary among species and drought model used. They are based on experience with seeds and seedlings, which are commonly more drought tolerant. Thus, in our experience, Ψ_w of -0,3 to -0,8 is more typical for an experimentally useful, i.e. recoverable, moderate drought stress in plants beyond seedling stage (v.i.). In general, leaf Ψ_w can be determined by several approaches. In the easiest, but reliable way, Ψ_w can be addressed by gravimetric method [20]. It can be also accomplished with Scholander pressure chamber and thermocouple psychrometer [21], or tensitometer [22]. Termocouple psyhrometry is one of the most popular method, which is usually accomplished with press saps or freeze-thawed leaf disks [23]. Recently, a new method for determination of Ψ_w in leaf cell apoplast, relying on the measurement of photosynthetic CO_2/H_2O gas exchange, was proposed [24].

It is important to mention, that not only the degree of Ψ_w decrease, but also its duration can affect the plant organism [25]. Therefore, water stress often develops upon minimal reduction of soil Ψ_w. To avoid this scenario, plants adopt various strategies to prevent water loss, to preserve water supply even under reduced Ψ_w, and to sustain periods of unfavorable water regimen accompanied with low water contents in tissues [10]. These drought-induced alterations can affect plant morphology, physiology and biochemistry in the degree, depending on plant species, developmental stage, as well as duration and severity of drought [4,6,7,26,27].

The main strategies, employed by plants to sustain water deficit are (*i*) drought escape, (*ii*) drought avoidance, and (*iii*) drought tolerance [28]. Generally, all these three strategies impact on the development of the state, known as drought resistance, which can be defined as ability to maintain favorable water balance and turgidity under drought conditions. In the escape strategy plants complete their life or growth cycle before the impact of drought causes harm, i.e. a seasonal response is used [4]. The strategy of drought avoidance relies on enhanced water uptake and reduced water loss, whereas drought tolerance is mediated by osmotic adjustment, extension of antioxidant capacity and development of desiccation tolerance [28]. On one hand, these strategies represent different steps of drought response (Figure 1). On another, they might indicate different climatic and ecological specializations of plant species [29]. This concept of stress avoidance and stress tolerance, proposed by Levitt [30], provides insight into plant responses to a relevant decrease of Ψ_w at the cell and organism levels [10].

As can be seen from Figure 1, the first response of the plant organism to drought as one of drought resistance strategies relies on avoiding water deficit [31]. Thereby, maintainance of tissue Ψ_w is achieved by increasing water uptake or by restricting water losses [32]. At the early steps of drought response it is mainly achieved by stomata closure, triggered by abscisic acid (ABA). However according to Muller et al. [33] roots and young leaves rapid expansion (as a major C sink) is affected earlier and more intensively than photosynthesis (C source) accordingly root growth is enhanced to provide sufficient water uptake under drought conditions. These avoidance mechanisms can secure maintaining of crop plant productivity during short-term periods of water deficiency [18]. However, this is achieved at the price of a reduced CO_2 uptake, dramatic drop in photosynthesis rate, and re-direction of assimilate transport for enhancement of root growth [33,34]. When drought persists

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during a long time, and adaptive capacities of the avoidance strategy are not sufficient for sustaining of plant growth and productivity, other mechanisms might be involved. At this step, such mechanisms, as accumulation of compatible solutes and protective proteins (i.e. so-called metabolic adjustment), cell wall hardening, ROS detoxification and metabolic changes are involved in establishment of drought tolerance [10].

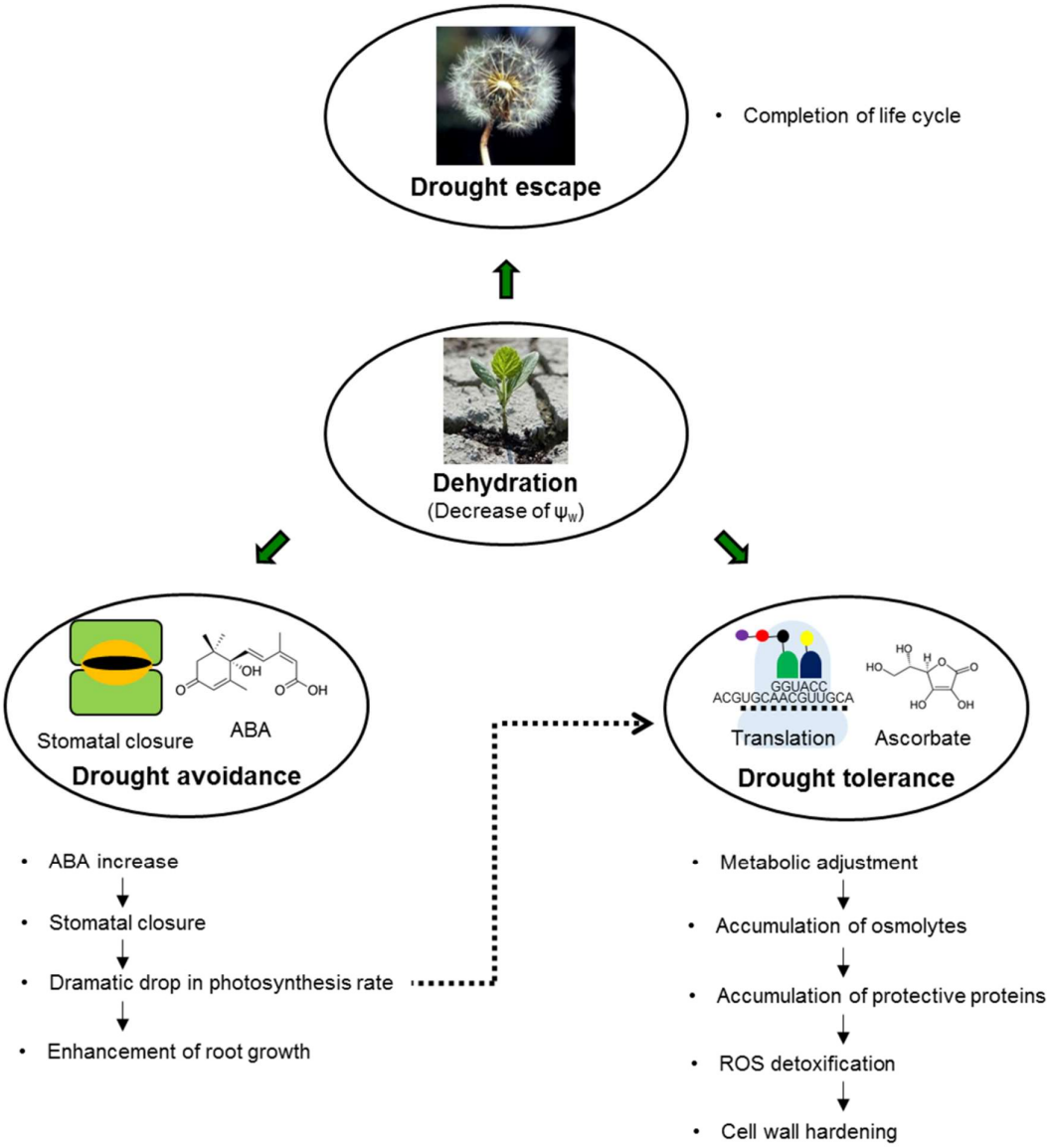

Figure 1. The main drought resistance strategies employed by plants to counter water deficit periods (drought escape, drought avoidance, and drought tolerance) and the main steps of the plant response to dehydration

Thus, plant drought resistance is a complex phenomenon requiring a global view to understand its underlying mechanisms. Obviously, the majority of the molecular events, triggered by decrease of tissue Ψ_w, cannot be unambiguously attributed solely to avoidance or tolerance strategy. Therefore, a complex multi-level regulatory network, controlling plant adaptive responses to drought stress, is required. Studies of such responses to water deficit such as stomata closure, expression of stress-specific genes, accumulation of osmolytes and up-regulation of antioxidant systems recently made considerable progress [17,35,36,37,38]. It was shown, that the mechanisms, underlying stress-resistance, are crucial for plant survival, and are associated with significant

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changes in the patterns of metabolites and proteins [10,15,35]. Hence, analysis of the changes in plant metabolome and proteome, associated with the onset of drought, might be an important step in breeding and engineering of plants with increased drought resistance [15,35], or for the development of plant protectants against drought stress [39]

Recently, Wang et al. [15] comprehensively reviewed drought-related effects on the plant proteome, including changes in signal reception and transduction, ROS scavenging, osmotic regulation, protein synthesis/turnover, modulation of cell structure, as well as carbohydrate and energy metabolism. These functional patterns of plant response to drought gave access to understanding of fine mechanisms underlying the phenomenon of stress tolerance. Apparently, for successful study of plant responses to drought stress under experimental conditions, reliable and adequate stress models are required. Accordingly, various drought model setups are established to date (Table 1). However, the available information is often complex, incomplete and inconsistent. A comprehensive literature search for drought tolerance research shows a great variability and inconsistencies in the experimental designs and methods for stress characterization [15]. Therefore, here we systematically address different experimental setups for establishment of drought stress models and consider physiological and biochemical methods for their characterization.

2. Experimental models of drought stress

Despite a large variety of available drought models, according to their basic setup, all these techniques can be classified in soil-, aqueous culture- and agar-based setups. The common feature of all drought stress models is reduction of the water potential in the substrate or medium, surrounding plant roots. However, individual methods have different applicability limitations and vary essentially in the scientific questions, which can be addressed. Therefore, all advantages and disadvantages of each individual model need to be carefully considered already at the step of experiment planning.

2.1. Soil-based drought models

The obvious advantage of this model strategy is close similarity of experimental conditions to drought, really occurring in nature and agriculture. In this case, the decrease of soil $\Psi_w$is established by gradual decline or immediate interruption of plant watering [40]. Such models adequately simulate a short-term drought, which represents the most frequent case in the European agricultural practice due to varying weather conditions [41,42]. However, difficulties in control of the substrate Ψ_w represent an essential limitation of this approach. Indeed, in this experimental setup, the severity of drought stress is determined by the rates of water evaporation from the soil surface and consumption by the plant [43]. As the rates of this processes cannot be defined by the researcher, and depend from multiple factors, reproducibility and predictability of such experiments are always questionable. Moreover, as the rates of water consumption and evaporation are relatively high, this model does not allow probing long-term drought responses, like accumulation of osmoprotective metabolites or proteins and cell wall modifications [10]. Therefore, many important aspects of plant drought tolerance and adaptation to low Ψ_w, like, for example, accumulation of osmoprotective proteins and hardening of cell walls, can be overlooked in this experimental setup, although using large and deep pots might improve this situation [10].

Despite the above mentioned problems, several improvements can be done to increase reproducibility and reliability of soil models. First of all, in this type of experiments, the size and the structure of soil particles, as well as their water capacity, should be taken into account. Thus, to achieve moderate (i.e. less severe) drought conditions, in an optimized variant of this model, plants are grown in foil-sealed vessels to prevent water evaporation from the soil surface [5]. Thereby, each pot can be equipped with a piece of tubing, inserted in soil of each vessel to facilitate re-watering of plants. Due to water supply, in this model, water deficit can be increased gradually, giving a possibility for addressing long-term plant responses to drought [44]. Moreover, stability of the water regimen can be improved by an automated irrigation system.

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Recently, Todaka et al [40] introduced an automatic irrigation system, relaying on monitoring of actual water content in soil. Using this approach, the authors proposed a drought model, able to ensure the desired values of Ψ_w (–9.8, –31.0, and –309.9 KPa). This system failed, however, to reproduce the conditions of severe dehydration. Although the optimized method described above is reliable and reproducible enough, repeated measurements of leaf and soil Ψ_w are laborious and require high amounts of plant material, which are hardly available in long-term experiments under reproducible laboratory conditions. For example, such a restriction can be critical, when mutants or transgenic plants are dealt with, in particular those, having reduced stomata density or small leaf area [45].

An elegant way to avoid this complication is culturing of mutant or transgenic plants in the same pot with the reference plants, e.g. the wild type (wt) counterparts [10]. In this case, leaf $\Psi_w$determination can be limited to the reference (or wt) plants, which are commonly more suitable for the assessment of stress markers. The obtained result can be extrapolated to the mutants. In this case, both reference or wild type and experimental or mutant plants would grow in the same medium and, therefore, exposed to the same soil Ψ_w if they are planted in a suitable scheme and position. The best way to provide a quantitative characteristic of drought stress by this approach is to complementit with a measurement of the soil Ψ_w at the end of the dehydration period.Analogously, this method can be applied to untreated and treated plants in assays for chemical drought tolerance enhancers or other phytoeffectors (v.i.) to be tested.

It is important to mention the setups, relying on inert substrate, such as vermiculite or perlite, as soil substitutes. The advantages of this approach is that the roots of experimental plants can be pulled out easily and without damage to investigate drought-related changes in water potential [46] or oxidative and metabolic responses [47] at the root level. Inert substrates are suitable for studying the effects of drought in legume-rhizobial nodule symbiosis [48]. On the other hand, their certain disadvantage is that watering unlike soil culture, is carried out not with water, but with a nutrient solution, so the impact of drought by cessation of watering plants is accompanied by the appearance of another stress factor, namely, the deficiency of mineral elements.

2.2. Drought models based on hydroponic aqueous culture

Despite of the high relevance of soil-based drought models because of their similarity to natural conditions they all have a common intrinsic limitation: the difficulty to adequately control of Ψ_w in the root microenvironment. However, this is critical when a precise definition of substrate Ψ_w is required, as in multiple or long-term experiments-comparable over months (and seasons). Therefore, the models, based on aqueous hydroponic culture with predictably decreased Ψ_w of nutritional solution, might be advantageous for such applications. The easiest way to reduce the Ψ_w of growth medium assumes decreasing its level in pots and partial exposure of roots to air, as was shown for lettuce by Koyama et al [49]. To simulate severe dehydration, plant roots can be left under air for up to eight hours [50]. Thereby, the severity of simulated drought can be defined by the duration and repetitions of such dehydration procedure. This approach is based on the fact, that Ψ_w of leaves, at least to some extent, corresponds to the index of water availability for plants, which in turn depends on water potentials of soil and plant roots [51]. Thus, affecting experimentally the Ψ_w of roots influences the Ψ_w of leaves as well. When using this approach, however, one needs to keep in mind, that dehydration degree and kinetics would strongly depend on air humidity. Further, it is necessary to remember, that in this case the plant response is dependent on root distribution (e.g. long vs. short roots).

Despite the ease of the above approach, most often desired Ψ_w values of plant rhizosphere are obtained by supplementation of nutrient solutions with osmotically active substances (osmolytes, which reduce available water), taken in calculated concentrations. This approach is based on the simulation of drought by application of osmotic stress, i.e. increase of the medium osmotic pressure in comparison to that of plant tissues [52]. Similar events occur in soil, when the water contents decrease (due to evaporation and absorption by the plant) and the concentrations of solutes grow,

resulting in increase of the osmotic component of the water potential [53]. Thus, the described setup corresponds well to natural drought. This strategy allows precise adjustment of Ψ_w and efficient monitoring of its magnitude, resulting, therefore, in high accuracy, reproducibility and inter-experimental comparability of acquired data [54]. However, when working with this kind of drought models, selection of an appropriate osmolyte requires a special attention. Thus, low molecular weight osmolytes (e.g. sugar alcohols and sodium chloride) routinely used in early studies [55] demonstrate strong negative side effects, when applied in experimental drought. Indeed, these compounds easily penetrate cell wall and plasma membrane, increasing thereby intracellular osmotic pressure and leading to plasmolysis [56]. Any salts also change ion titers and distribution in plants, affect ionic strength and trigger metabolic processes like ion transport. On the other hand, non-ionic carbohydrate-related osmolytes (e.g., sorbitol and mannitol) are readily involved in cellular metabolism themselves, and thus might directly affect results of the experiment [56], often being toxic for plants [57]. They can also increase mold growth under commonly non-sterile conditions. Because of this, the use of biologically inert polymeric osmolytes is preferable and advantageous [58]. Therefore, currently, drought stress models rely on (presumably) non-permeable high molecular weight osmolyte polyethylene glycol (PEG) with an average molecular weight of 6,000 Da or more [55,59].

It is well-documented, that PEG effectively decreases medium Ψ_w, and disrupts thereby absorption of water by plant roots [60]. In terms of this approach, 5 – 20% (w/v) [61] or even 40% (w/v) [62] PEG in growth medium enables a stabile decrease of Ψ_w during any desired period of time [63]. Importantly, PEG-based aqueous models allow the setup of recovery experiments by transfer of stressed plants to PEG-free nutrient solution or exchange of the PEG solution [10]. Therefore, PEG-based models of drought stress represent the method of choice in molecular biology and plant protectant studies and screening experiments [64]. One issue yet underexplored in the PEG model is the complexing ability of PEGs on metal ion species and thus the altered availability of the various ions for the plant. However, also under drought conditions, ion availability is changing and decreasing eventually.

One of the most promising applications of aqueous PEG-based models of osmotic stress is screening for potential drought-protective compounds. Substances that influence plant performance (without being plant protectants against biotic stress, e.g. from pathogens) in agrochemistry are defined as phytoeffectors-and include drought stress tolerance enhancers. Phytoeffectors are able to prime crop plants against a short-term drought and ensure sustaining their productivity under drought conditions with spatiotemporal control and largely independent of the crop species or variety used. Such effects were described for salicylic acid and its derivatives [65], as well as for various fungicides of the triazole [66] and imidacloprid family [67,68]. The drought-protective effects of small molecules on a plant organism are usually mediated by inhibition of enzymes, involved in plant response to stress, as it was described for poly(ADP-ribose) polymerase (PARP) in the beginning of this decade [68], although later at least direct involvement of PARP appeared doubtful [69]. If a molecular target for drought stress effects is known, and ideally the active site too, methods of computational chemistry like virtual screening and molecular docking approaches [70], allow to virtually screen thousands of structures with millions of conformers. The most promising candidates for wet lab testing can thus be identified.

For a rapid screening of such compounds, a reliable model, based on a *Lemna minor* culture, was recently developed in our group [68]. This technique (Figure 2A) relies on a microtiter plate format and assumes treatment of plants with PEG6000 or PEG8000 supplemented to the growth medium in presence and absence of potential phytoeffectors. After a 24 h of a stress period, plants are transferred to a PEG-free medium, and stress recovery is monitored for further 48 h, before the protective effect is assessed by attenuation of growth inhibition via measurement of leaf peak area increase by means of a 2D-photodocumentation visualization system.

The Lemna system has several advantages over classical spraying systems: Plants are all clones reproducing by budding, they are small and can be grown in microtiter plates (6, 12 or 24 well microtiter plate format) and under sterile conditions. The small scale allows medium throughput

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screening with small amounts of compounds. Most importantly these can be applied in a concentration dependent manner to the multi-well plate well (while spraying or dumping delivers only uncertain amounts to plants), and both root and leaf uptake is ensured. The leaves are flat and 2D phenotyping is easily done with the respective software [68]. For better reproducibility, initial root length should be unified and until termination of the experiments, plant growth should not be limited by the wells size.

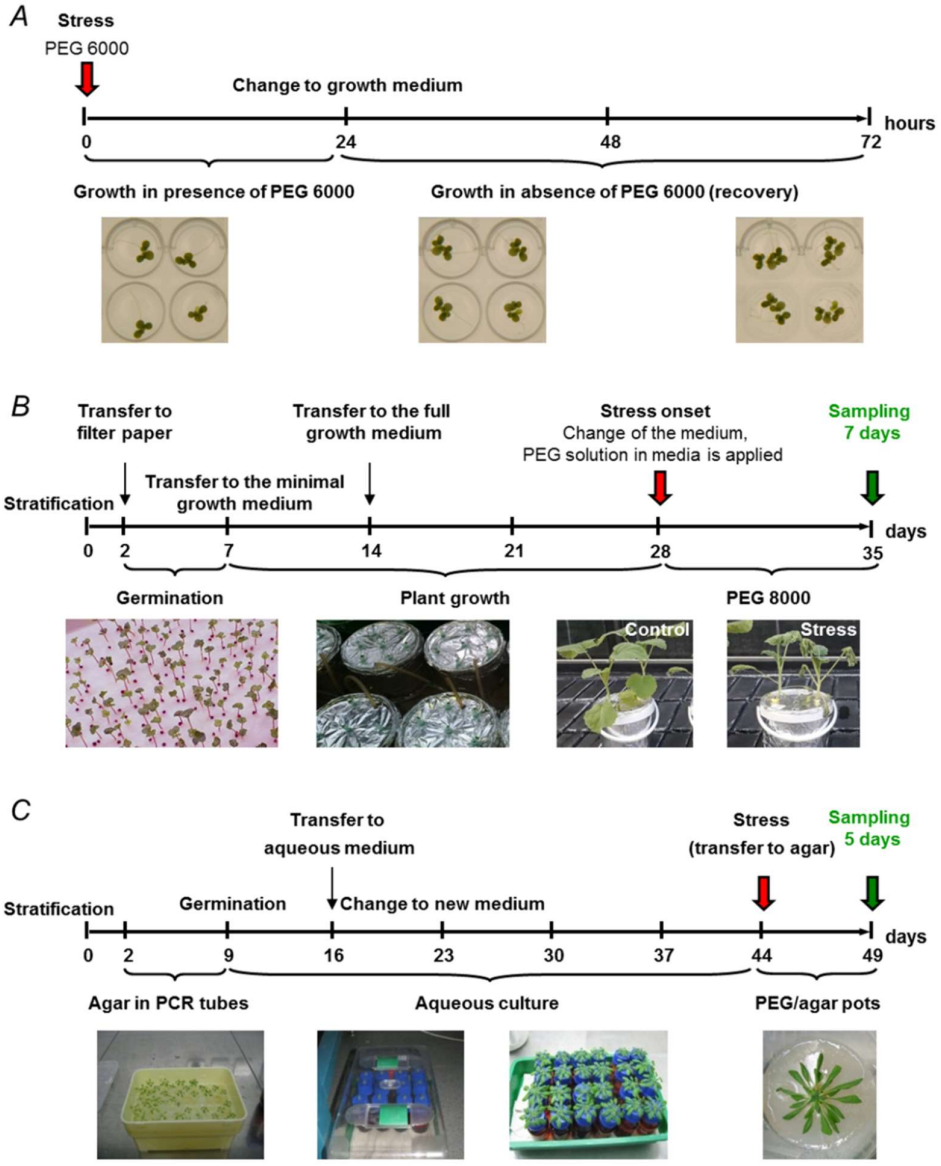

Figure 2. Experimental drought models based on osmotic stress, and established by supplementation of polyethylene glycol (PEG) to growth medium: *Lemna minor* model, established with PEG6000 supplemented to aqueous growth medium (A, [68]), *Brassica napus* model, established with aerated aqueous culture, supplemented with PEG8000 (B), and agar-based PEG infusion *Arabidopsis thaliana* model, established by overlaying solidified agar medium with PEG8000 solutions for five days(C).

Despite their wide use, PEG-based models have some intrinsic limitations, which need to be taken into account when planning experiments [63]. First, PEG-containing nutrient solutions are characterized by a high viscosity, that especially in deeper vessels compromise diffusion of oxygen

to the roots and can cause hypoxia [10]. To prevent the development of hypoxia, additional aeration needs to be provided for the plants, grown in PEG-containing medium. For this, air is continuously supplied by pumps through silicone tubes connected to the culture vessels [71]. Although this approach can be easily established for larger plants (like it was done in our lab with *B. napus*, Figure 2B [72]), small model plants, like Arabidopsis, typically grown in small vessels on large scale for highly replicated biological experiments cannot be provided with air supply individually and are typically grown under hypoxic conditions [73]. Small and flat vessels like the wells used in the *Lemna* system [68] are not prone to such problems, usually.

Another possible issue is absorption and accumulation of PEG with molecular weight of 4000 – 8000 Da in plant roots, which might result in their damage [74]. The accompanied partial root dysfunction might impact leaf dehydration in a hardly predictable way. Thus, stress responses observed in plant shoots are only partly related to osmotic stress, applied by PEG solution. The impact of PEG-related root damage on these responses is difficult to estimate, but obviously increased, when plant transfer on PEG-containing medium is accompanied with wounding of roots, which should be avoided [75].

2.3. Agar-based drought models

In general, growth of plants in agar allows avoiding or reducing the development of hypoxic state. As this is especially relevant for Arabidopsis, agar-based models are widely used in plant biology, and specifically in drought stress experiments with *Arabidopsis thaliana* seedlings [76]. Thus, van der Weele et al proposed an agar-based PEG infusion model, relying on saturation of solidified agar (filled in Petri plates) with Murashige and Skoog medium supplemented with PEG8000 during two days [77]. Unfortunately, PEG affects the solidification of agar, and therefore the direct addition to the agar medium under preparation is not advisable [73]. Because of this, generation of a desired Ψ_w of agar medium is achieved by diffusing PEG from a concentrated overlay solution into pre-formed, solidified agar. Adjusting the concentration of the overlay solution, the equilibrium in Ψ_w between aqueous overlay medium and agar is achieved after 24 h of diffusion [76]. After decantation of the PEG solution, seedlings can be transferred to the now PEG-containing agar medium (stress application), and eventually plants can be replanted to a PEG-free one after a defined treatment period (recovery).

Due to a constant character of Ψ_w, the agar-based PEG infusion model is advantageous in comparison to those based on soil or (non-aerated) aqueous culture. Thus, the Ψ_w of seedlings can achieve equilibrium with the agar medium during treatment time. Under soil drying conditions, this is impossible as soil Ψ_w changes continuously along with water evaporation and consumption by a plant. On the other hand, due to interference of PEG with root integrity [78], this equilibrium is also hardly achievable in aqueous PEG solutions (especially when PEG concentration is high). Thus, the agar-based model system currently is an ideal choice to address dehydration avoidance and mechanisms of dehydration tolerance [10]. Most commonly, PEG concentrations in the agar medium do not exceed the value needed for medium to medium-high drought stress , i.e. values of Ψ_w<-1.2 MPa [57]. However, based on the solubility of PEG8000 in water, the agar-based infusion model can be established in a broad range of overlay medium Ψ_w values from -0.47 MPa to -3.02 MPa [79].

The agar-based PEG infusion model was successfully applied to different plants and fungi [75]. Further, a similar setup (10% w/v PEG 6000 in the overlay medium) was used to probe the effect of water stress on the germination of rape oilseed (*Brassica napus*) and development of seedlings [80,81]. An essential limitation of the setup, originally proposed by Verslues and co-workers [10], was its applicability exclusively to the early steps of plant ontogenesis – seed germination and seedling development. Thus, this method was inapplicable to mature plants, and corresponding stress responses, characteristic for later stages of ontogenesis, could not be addressed in this system.

Therefore, to extend the agar-based approach to mature organisms, we modified the method of Verslues and co-workers to 5 - 7 weeks old *A. thaliana* plants [35]. This setup combined germination on agar in truncated polypropylene tubes, growth during 4 – 5 weeks in aqueous culture and

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transfer to agar medium, pre-infused with PEG8000 solutions with a polymer concentration corresponding to the targeted substrate Ψ_w (Figure 2C).

In general, our observations confirmed earlier data, indicating higher sensitivity of mature plants to drought in comparison to seedlings [10], although stress tolerance varies essentially between species. Thus, in contrast to seedlings, application of Ψ_w below -0.6 MPa led to reduced survival of plants over a period of seven days, whereas the drop of the Ψ_w to -0.4 MPa was accompanied with significant alterations in plant metabolome and proteome, indicating metabolic adjustment and changes in redox metabolism [35]. Soybean turned to be more resistant to osmotic stress, applied in an agar-based model, and successfully survived osmotic stress, applied by 8 and 16 (w/v) PEG for two weeks for both pre- and post-flowering treatments [82].

To summarize, in comparison to other setups, the agar-based PEG infusion model has two fundamental advantages. First, it provides a stable and reproducible decrease of substrate Ψ_w that cannot be achieved with the soil-based model. On the other hand, in comparison to the models based on aqueous culture, it has a higher relevance for the conditions of a real field, as it relies on a solid substrate. Secondly, the agar-based model allows precise Ψ_w setting in plant rhizosphere without accompanying hypoxia and PEG-related root toxicity. It is necessary to keep in mind, however, that this setup doesn't allow a direct extrapolation of drought effects to the field or ecosystem due to high simplicity of the model, which doesn't consider water gradient in soil and heterogeneity in terms of water holding capacity.For fast (pre-)screening of phytoeffectors, especially if only small amounts of test compounds are available, the *Lemna minor* aqueous system bears advantages [68], but must be complemented later by the solid medium methods for validation[35].

3. Physiological and biochemical characterization of drought stress

Adequate and correct application of experimental drought stress models requires their comprehensive characterization at the levels of physiology, biochemistry and molecular biology. These experiments deliver objective information on the actual functional state of the plant organism and its metabolic response to stress. This block of data is necessary to confirm the stressed state of experimental plants (i.e. development of stress response), and to estimate severity of stress-related alterations. Accordingly, a panel of physiological and biochemical markers of drought stress ideally accompanies any study, relying on modeling setups. Importantly, these markers can be used for the dynamic characterization of plant adaptive responses throughout the whole experiment, i.e. acquisition of stress kinetics. Thus, ideally, selection of the markers needs to consider all steps of drought response, starting from drought perception. It is assumed, that the drought is recognized by roots, which send a chemical message to the shoot [83]. Abscisic acid (ABA) plays the key role in this signaling [84]. This effector is synthesized in response to hydraulic signal in vascular tissues and further transported to leaf epidermis cells. Resulting stomata closure results in suppression of xylem transport, decrease of turgor and root growth arrest [37].

3.1. Water status and photosynthetic parameters as markers of drought stress

One of the first detectable symptoms of drought is dehydration of plant tissues, which is characterized with a decrease of Ψ_w and loss of leaf turgor [6]. Due to its simplicity, low time expenses and robustness, the measurement of leaf water potential prior to sunrise represents one of the most commonly used tests for this marker [85]. Although critical values of tissue water potentials are species-specific, the Ψ_w of less than -0.8 MPa are commonly recognized to be the sign of drought stress [86]. On another hand, the degree of water loss can be reliably assessed by a decrease of leaf relative water content (LRWC) [87]. In the most easy and straightforward way, this parameter can be addressed by the gravimetric method and calculation of dry weight/fresh weight ratios [87]. Despite its simplicity, this approach yields highly reproducible data. An obvious disadvantage of this method is its destructive character, i.e. consumption of plant material for each determination [85]. In this context, a non-destructive technology, based on an automatic assessment of short-wave infrared irradiation, reflected from the leaf surface, might be a good alternative [85]. Another non-destructive

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approach relies on a long-term phytomonitoring, i.e. continuous measurement of leaf transpiration, turgor, and xylem flow by means of non-damaging sensors that are attached to the plant [89].

One of the primary plant responses to dehydration is stomata closure, which aims at preventing transpiration-related water loss, and is principally essential for success of the drought avoidance strategy [90]. Similarly to dehydration itself, this parameter can be quantitatively characterized [91]. Experimentally, it can be done by the rate of gas flow through a leaf surface, or by the measuring the electrical conductivity of the water film (of constant ionic strength) on the leaf surface [92]. Therefore, stomata conductance is usually expressed in mmol/m^2/s [92]. Technically, such experiments are based on porometric measurements, i.e. determination of times required for the increase of air humidity in an isolated chamber with a leaf inside [93].

Since stomata closure disrupts the supply of parenchyma cells with carbon dioxide, drought ultimately negatively affects efficiency of photosynthesis via inhibition of carbon assimilation and light reactions of photosynthesis [5]. In the simplest way, photosynthetic activity can be addressed by quantitative determination of pigments – chlorophylls (at least chlorophyll a) and carotenoids [94]. Thereby, a decrease of chlorophyll levels is considered as a symptom of oxidative stress and may be the result of pigment photo-oxidation and chlorophyll degradation [95]. Accordingly, as was shown in a comparative screening of barley genotypes, higher chlorophyll contents were generally associated with higher drought tolerance [94,96]. This fact allows considering this indicator as an important marker of plant functional state under drought conditions.

Besides degradation of photosynthetic pigments, dehydration negatively affects the whole photosynthetic apparatus [97]. One of the most reliable markers of this phenomenon is a decrease in the activity of photosystem II (PS II) [98]. Both, relative chlorophyll contents and PS II efficiency can be easily quantified with pulse amplitude modulation (PAM) fluorometry [99,100]. Thereby, the ratio of minimum (background) and potentially maximum chlorophyll fluorescence (Fv/Fm)is interpreted as the maximum of PS II photochemical activity, and might be considered as a reliable marker of PS II photoinhibition and as one of the most important indicators of drought stress [101]. Importantly, the chlorophyll fluorescence is registered *in vivo*. Thus it does not require sampling of plant material [102]. Interestingly, in some cases, drought does not cause any alterations of PS II activity. This result, observed with potato leaves, can be explained by the impact of photochemical quenching of excess light energy by increased photorespiration [103]. It needs to be taken into account, that besides drought stress, the decrease of Fv/Fm ratio can be underlied by the onset of senescence [104].

In agreement with the described mechanisms, the features, protecting the chloroplast photosynthetic machinery from oxidative damage, might increase stress tolerance. This was illustrated in a comparative study of two *B. napus* cultivars, grown for 3 weeks in aerated aqueous nutrient medium with Ψw of -0.6 MPa (18% w/v PEG 8000) [105]. The developing stress could be recognized in both cultivars by a pronounced decrease in growth and photosynthetic parameters, including PS II activity and chlorophyll a contents. However, the cultivar with higher leaf contents of chlorophyll a and carotenoids, as well as with higher Fv/Fm ratios, demonstrated a clearly higher drought tolerance. Thus, it could be concluded, that the quantum yield of photosynthesis and the contents of chlorophyll a could be an effective selection criteria in screening for cultivars of crop plants with drought tolerance [105].

3.2 Changes in phytohormone patterns as the markers of drought stress

Plant response to environmental stress is a complex process, precisely tuned by multiple regulatory systems [12,106]. In particular, dehydration triggers activation of signal transduction cascades, including long-distance transport steps mediated by phytohormones [107]. Specifically, drought-induced stomata closure is regulated by abscisic acid (ABA) and relies on ABA-dependent signaling pathways [108]. Upon dehydration, ABA tissue contents in Arabidopsis leaves can be increased up to 30-fold [107]. In a time-course study of the drought-avoidance response, performed with Arabidopsis, early accumulation of ABA and induction of associated signaling genes coincided with a decrease in stomata conductance, as was revealed with a panel of physiological, biochemical,

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and molecular biology methods [12]. Therefore, increased levels of ABA in leaf cells represent a reliable marker of drought stress in model experiments [35].

Besides ABA, several other hormones and their interaction networks show impact on the control of stomata conductance during water deficit. Thus, auxins, cytokinins, and ethylene are prone to inhibit the ABA-mediated stomata closure mechanism, whereas brassinosteroids, isoleucinyl jasmonates, jasmonicand salicylic acids support the effects of ABA [109]. Jasmonic acid and its derivatives play a significant role in the plant responses to drought in terms of opening and closing of stomata [110], acting in an interplay with ABA and starting ABA signaling transduction [111]. In contrast to jasmonates and ABA, ethylene is involved in the stimulation of stomata opening via inhibition of NADPH oxidase in the leaves of plants, responsible for the launch of ROS-dependent stomata closure pathways [112], but ethylene also conveys senescence induction. Thus, despite their essential impact on drought response, the mentioned phytohormones have complex patterns of effects [107]. Therefore, their use as drought stress markers is hardly possible. Similarly,their potential to apply them as phytoeffectors in the field is limited. Apart from cost, bioavailabilty and stability issues, it wouldrequire an extremely balanced mixture of suitable hormones, adapted in each case to the plant species, developmental stage and status.

3.3. Metabolites as the markers of drought stress

Various abiotic stressors are known to affect profiles of plant metabolites [113]. Indeed, the phenomenon of metabolic adjustment, i.e. accumulation of osmotically active and metabolically neutral solutes, such as different sugars, amino acids (predominantly proline and glycine), betaine, polyamines, and organic acids, under drought conditions is well-documented [20]. Metabolic adjustment represents the second step of plant adaptation to drought (after stomata closure) and is critical in maintaining the water status and physiological activity of plant cells, especially during relatively short-term drought [5,114]. To address the tissue contents of drought-protective metabolites, different methodological approaches can be employed. On one hand, each group of metabolites can be analyzed individually (for example - analysis of betaine [115] and inositol [116] levels). On another hand, the whole profiles of primary metabolites can be addressed by comprehensive gas chromatography-mass spectrometry (GC-MS)-based hyphenated techniques, giving access to relative [117,118] and absolute [119] amounts of individual analytes. For the complete understanding of plant responses to drought, analysis of plant hormones and secondary metabolites can be equally essential. Thus, Ahmed et al. reported up-regulation of phenolic metabolites in the leaves of *Gossypium barbadense* L. under water deficit conditions [120]. Accordingly, Ma et al. demonstrated a drought-related increase of the expression levels of flavonoid genes and up-regulation of leaf flavonoids in *Triticum aestivum* [121].

It is important to mention, that accumulation of sugars at the background of reactive oxygen species (ROS) overproduction (usually accompanying plant response to drought) might result in enhanced formation of reactive carbonyl compounds (RCCs) and glycation of plant proteins [122,123], similar to the mechanism recently reported to occur under plant ageing [124]. Additional *in vitro* experiments with peptide and protein models showed formation of various glycoxidative modifications of lysyl and arginyl residues [125,126,127,128], prospectively with an impact on pro-inflammatory properties of glycated proteins [129]. Hence, these modifications might affect nutritional properties of plant-derived foods. Moreover, the processes of DNA damage and reparation (associated also with the PARP/PARG system [69] can impact protein glycation as well [130,131].

Remarkably, metabolic adjustment in different plants has both common and species-specific features. Thus, some osmoprotective metabolites, like glycine betaine, are specific for certain plant species, e.g. sugar beet (*Beta vulgaris*), spinach (*Spinacia oleracea*), and barley (*Hordeum vulgare*) [132], while the increase of proline content, which is apparently a crucial and the most conserved response to drought, is characteristic for a wide range of plants [133]. Obviously, such metabolites can be used as non-specific and species-specific markers of drought stress. It is important to remember, that metabolic adjustment is efficient only in a relatively short time scale, whereas, when drought persists for longer times, increased accumulation of compatible solutes can be energy and resource

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intensive for the plant. In cases of severe stress, when soil water content is largely depleted,metabolic adjustment may have only a small effect on water uptake, or even be detrimental by taking to many resources from the plants[18,134].

3.4. Protective proteins as the markers of drought stress

The long-term adaptation of plant organisms to drought is underlied by a pronounced increase in the expression of drought-specific genes, such as *Solanum tuberosum* DS2 (StDS2) [135], late embryogenesis abundant (LEA) [136]. Accordingly, biosynthesis of a broad pattern of drought-protective proteins, pre-dominantly chaperones, LEA proteins, and enzymes of anti-oxidant defense (which are referred to below in detail), is up-regulated. Chaperones form the group of proteins involved in the formation and maintenance of the native protein structure [137], and mostly represented by so-called heat shock proteins – the ubiquitous polypeptides, originally described with respect to a heat shock response, but actually involved in a broad array of stress adaptation responses [138]. Currently, special attention is paid to the role of heat shock proteins in drought tolerance [139]. Thus, Xiang et al found, that the over-expression of the heat shock protein Osnsp50.2 in rice leaf reduced water loss and increased resistance of plants to drought-related osmotic stress [140]. It was also shown, that increased expression of chaperone-like proteins ERD10 and ERD14 in *A. thaliana* cells impacted on prevention of luciferase, alcohol dehydrogenase, and citrate synthase inactivation in firefly [141].

LEA proteins represent another class of polypeptides involved in adaptation to water deficiency. These proteins were discovered more than 35 years ago in the study of embryogenesis and germination of cotton seeds [142]. The key feature of the LEA proteins, underlying their drought-protective properties, is their high hydrophylicity[143]. These molecules are known to prevent mechanical damage of mitochondria, chloroplasts and other cellular structures by forming a membrane-protecting shield, thereby preventing peroxidation of membrane lipids [111,144]. The constitutive expression level of LEA proteins can be considered as markers of drought resistance. Thus, it was shown that more LEA genes are over-expressed in drought-resistant *Gossypiumtomentosum* cultivars, in contrast to drought-sensitive ones [145].

3.5. Oxidative stress associated with drought

Water deficiency results in a disturbance of the balance between ROS (and RNS) generation and detoxification, triggering, thereby, oxidative stress, and up-regulation of ROS production under drought conditions is well-documented (comprehensively reviewed by de Carvalho et al) [146]. Due to their high reactivity, ROS are extremely toxic and are able to damage proteins, lipids, and nucleic acids [147]. Under the conditions of persisting oxidative stress, this damage can become irreversible and, finally, might lead to cell death. Indeed, excessive ROS production is a central process in response to infections too (killing the intruder cells or tissues surrounding it).

Although such ROS as singlet oxygen can be produced by the energy transfer from triplet chlorophyll to molecular oxygen [148] (Figure 3A), the main reason, underlying overproduction of cellular ROS in response to plant dehydration is the overload of the electron transport chains in chloroplasts and mitochondria due to overproduction of reduced forms of nucleotides [146,149]. Indeed, even under normal conditions, light reactions of photosynthesis are associated with a continuous ROS production [150]. Thereby, among the individual players of the chloroplast photosynthetic machinery, PS IIis the main contributor [151]. The superoxide anion radical (O_2^-) is formed on the electron acceptor side of PS II via electron leakage to molecular oxygen (Figure 3A). Due to the drought-related stomata closure and overload of electron-transport chains, the rate of this process is essentially increased [152]. The formed O_2^-can be dismutated to hydrogen peroxide (H_2O_2), which can further yield highly toxic hydroxide radical (OH^-), for example, by the Fenton reaction in the presence of certain transition metal ions [153]. On the PS II donor side, incomplete water oxidation also leads to H_2O_2 production. Dehydration affects the function of PS II, resulting in higher production of H_2O_2 and its faster transformation in OH radical [146,151].

Another important source of ROS in chloroplasts is the Mehler reaction (Figure 3A), i.e. the partial reduction of O_2 to O_2^- (with sub-sequent formation of H_2O_2) by components of the PS I – Fe-S centers and reduced ferredoxin and thioredoxin. Under stress conditions, reactions of the Calvin cycle are inhibited by lack of CO_2 due to the stomata closure. This situation provokes an over-reduction of the chloroplastic electron transport chain, which results in a higher leakage of electrons to O_2 in the Mehler reaction [146]. Importantly, the deficit of CO_2 might result in enhancement of H_2O_2 production in peroxisomes. Thereby, photorespiration is contributing over 70% of the total H_2O_2 production in C3 plants subjected to drought stress [154].

Mitochondria also can represent an important source of stress-related excess ROS. While normally approximately 1–2% of the oxygen consumed by plant mitochondria is converted to $O_2^{-\cdot}$ and H_2O_2. This increase in ROS production is mostly underlied by the complexes I and III of the mitochondrial electron transport chain, which can act as the electron donors for molecular oxygen and enhance generation of $O_2^{-\cdot}$ and H_2O_2[149]. It is assumed, that excess of NADH, produced during glycine oxidation in the photorespiratory pathway, results in an overload of the mitochondrial electron transport chain [155]. Interestingly, the activities of alternative oxidase and, probably, rotenone-insensitive NAD(P)H-dehydrogenase are involved in detoxification of ROS under these conditions and contribute, thereby, to plant drought tolerance [149].

In general, ROS production correlates well with the severity of drought stress [146]. This allows using some compounds associated with oxidative stress as the biochemical markers of drought. Thus, ROS readily attack double bonds in polyunsaturated fatty acids resulting in the formation of lipid hydroperoxides [156]. Consequently, shorter and reactive carbonyl products result from their breakdown, like e.g. malondialdehyde, known as a reliable marker of lipid oxidative damage [157,158]. The content of these compounds is increased in plant leaves under stress conditions and canbe used as drought stress markers. Similarly, H_2O_2 tissue contents are often used for an estimation of drought stress severity in plants, as this molecule represents the most stableand easily measurable form of ROS [159].

The mechanisms of plant drought tolerance necessarily include the pathways reducing ROS contents in the stressed cells. The most efficient antioxidant defense relies on the activities of specific antioxidant enzymes (Figure 3B). Thus, the enzymes of the ascorbate-glutathione cycle play a central role in detoxification of H_2O_2 under drought stress conditions [5,160]. Ascorbate peroxidase is the key antioxidant enzyme neutralizing H_2O_2 in plant cells which relies on ascorbic acid as donor of electrons [161]. The resulting dehydroascorbate can be regenerated (i.e. reduced to monodehydroascorbate) by the reaction with NADPH catalyzed by monodehydroascorbate reductase [162]. The formed toxic monodehydroascorbate is rapidly reduced to ascorbic acid by dehydroascorbate reductase, in parallel to the oxidation of glutathione to glutathione disulfide (GSSG). The subsequent regeneration of glutathione (GSH) is catalyzed by glutathione reductase, which plays a key role in maintaining the pool of reduced glutathione, required for survival under stress conditions [162,163].

The ratio of reduced to oxidized forms of ascorbate and glutathione is crucial for maintaining a favorable redox status of living cells, being an informative indicator of plant stress adaptation capacity [163]. Therefore, addressing the expression or activities of antioxidant enzymes may be an effective tool for screening different plant species and cultivars for drought tolerance. The enzymes of the ascorbate-glutathione cycle were recently considered as targets for engineering of transgenic stress-resistant plants [164].

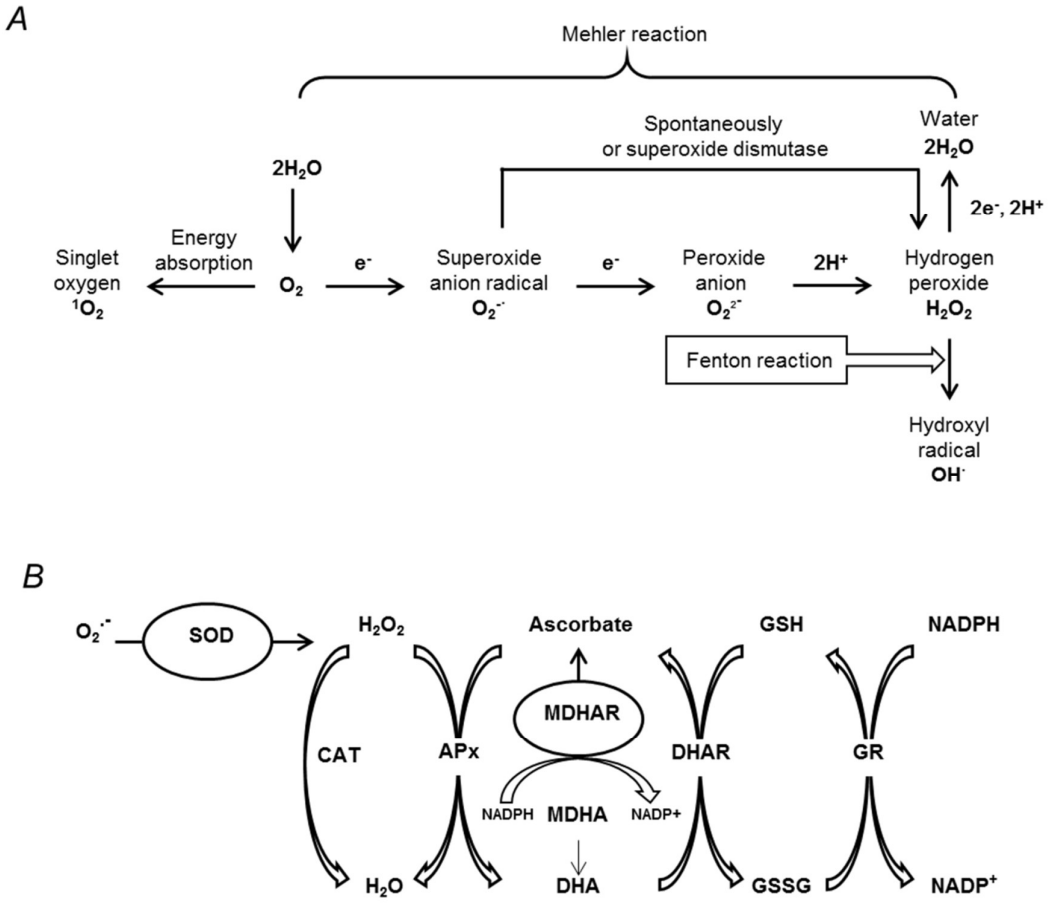

Figure 3. The main pathways of reactive oxygen species (ROS) generation in plants (A) and the major pathways of plant enzymatic antioxidant defense (B). SOD, superoxide dismutase; CAT, catalase; APx, ascorbate peroxidase; MDHA, monodehydroascorbate; MDHAR, monodehydroascorbate reductase; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; GR, glutathione reductase

4. Conclusions

The comprehensive literature survey clearly demonstrated the importance of an appropriate experimental design of reversible stress induction under reproducible and long term stable laboratory conditions. Currently, especially PEG-induced drought stress models are state of the art. Stress characterization methods include a set of standard but also species specific small molecule metabolites, and enzymes, indicative of the elucidation of drought tolerance mechanisms in plants. In this context, multiple modifications of the drought model experimental setups allow monitoring different aspects of a plant functional states, in agreement with specific objectives. Currently, the progress of studies focused on improving plant drought resistance is associated with molecular biology and "omics" techniques, eventually trying to understand and genetically or chemically influence plant responses to drought periods.

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Table 1. Overview of drought stress model setups

Species	**Drought stress model**	**Osmotically active agent**	**Ages of plant**	**Duration of stress**	**Ref.**
Arabidopsis thaliana L.	Agar system	50, 300 mmol/L mannitol	7 day	2 weeks	[165]
Arabidopsis thaliana L.	Agar system	100, 200, 300 mmol/L mannitol	8 day	1 day	[166]
Arabidopsis thaliana L.	Agar system	17% PEG 8000	2 weeks	3 day	[100]
Lemna minor L.	Hydroponic system (MTP possible)	PEG 6000 or 8000 variable conc.	Adult	add	[68]
Hordeum vulgare L.	Soil system	no	Adult	Every 15 day, until the physiological maturity	[167]
Zea mays L.	Soil system	no	Adult	Every 15 day, until the physiological maturity	[167]
Zea mays L	Hydroponic system	15% PEG 6000	5 weeks	24 hours	[168]
Populus euphratica	Soil system	no	2 months	0, 4, 8 24, 48, 96	[169]

				hours	
Solanum tuberosum L.	Agar system	Sorbitol (0.1, 0.2, 0.3 and 0.4 m) and PEG 8000 (0 %, 4.8 % and 9.6 %)	2 weeks	3 weeks	[170]
Lolium perenne L	Hydroponic system	10, 20% PEG 6000	1 weeks	4 weeks	[171]
Solanum lycopersicum L	Hydroponic system	15% PEG 8000	25 days	0, 3, 6, 24, 48 hours	[172]
Medicago sativa	Hydroponic system	15% PEG 6000	28 days	24 hours	[173]
Pistacia lentiscus	Soil system	5, 10,15, 20, 25 % PEG 6000	1,5 month	20 and 23 days	[174]
Brachypodium distachyon	Soil system	no	vegetative stage	4, 8, 12 days	[175]
Transgenic plum "Claudia verde"	Soil system	no	8 weeks	7, 15 days	[176]
Stipa purpurea	Soil system	no	trefoil stage (about 3 week growth)	7, 15 days	[177]
Saccharum spp.	Soil system	no	2 month	17 days	[178]

Hordeum vulgare L.	Hydroponic system	20 % PEG 6000	31 days	9 days	[179]
Brassica campestris ssp	Hydroponic system	60, 120 % PEG 6000	34 days	7 days	[180]
Oryza sativa L.	Soil system	no	reproductive stage	-	[181]
Cucumis sativus L	Hydroponic system	2 % PEG 6000	2 weeks	7 days	[182]

Table 2. Markers of drought stress in plants

Parameter	Growth model	Plant object	Method	Ref.
Physiological markers				
Leaf water potential, MPa	soil	Cotton (*Gossypium hirsutum* L.)	The pressure chamber technique	[183]
The relative water content, (RWC),%	soil	Potato (*Solanum tuberosum* L).	RWC (%) = [(FW - DW)/(SW - DW)] x 100, where FW, DW and SW are the fresh, dry and saturated (turgid)weights of the leaf tissues, respectively.	[184]
Stomatal conductance	soil	Tomato (*Lycopersicon esculentum* Mill.)	Abaxial stomatal conductance measurement with a diffusion porometer (AP4, Delta-T, Cambridge, UK).	[90]
Photosynthetic parameters (chlorophyll content and PSII activity)	soil	Barley (*Hordeum vulgare* L.).	Determination of leaf chlorophyll using a chlorophyll meter (SPAD-502, Minolta, Japan). Measuring of chlorophyll fluorescence with a portable fluorescence spectrometer Handy PEA (Hansatech Instruments, Norfolk, UK). Fluorescence values Fv/Fm represents the maximum quantum yield of PSII. Fv=Fm–Fo.	[96]
Biochemical markers				
Phytohormons	1.Soil	Clover (*Trifolium subterraneum* L).	1.ABA analysis in xylem sap by EL1SA (enzyme-linked immunosorbent assay)	[185]
	2.Soil	Wheat (*Triticum aestivum* L.)	2. ABA analysis on HPLC	[186]
Metabolites	soil	*Triticum spp.*	LMW drought stress-responsive metabolites in the root and leave samples of 7 wild and domesticated wheatrelatives were revealed by a gas chromatography-massspectrometry (GC-MS) based comparative metabolomicsapproach.	[187]
Protective proteins	1. Soil	1. Rice (*Oryza sativa* L).	1. Expression pattern analysis of OsHSP50.2, an HSP90 family gene	[140]
	2. Soil	2. Cotton (*Gossypium tomentosum, Gossypium hirsutum*)	2. LEA genes expression analysis and gene expression profiling	[145]
ROS and antioxidant enzymes	Water culture+ PEG6000	Wheat genotypes		[188]

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Abbreviations

2D	two-dimensional
ABA	abscisic acid
APx	ascorbate peroxidase
CAT	catalase
DHA	dehydroascorbate
DHAR	dehydroascorbate reductase
GC-MS	gas chromatography-mass spectrometry
GR	glutathione reductase
GSH	reduced glutathione
GSSG	oxidized glutathione
LEA	late embryogenesis abundant
LRWC	leaf relative water content
MDHA	monodehydroascorbate
MDHAR	monodehydroascorbate reductase
NADPH	nicotinamide adenine dinucleotide phosphate
PAM	pulse amplitude modulation
PARP	Poly (ADP-ribose) polymerase
PEG	polyethylene glycol
PS II	photosystem II
RCCs	reactive carbonyl compounds
ROS	reactive oxygen species reactive oxygen species
SOD	superoxide dismutase
St	*Solanum tuberosum*

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