Community recommendations on terminology and procedures used in flooding and low oxygen stress research

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SUMMARY

Flooding and low oxygen (O_2) stress research is a rapidly advancing area that has seen tremendous progress in the last decade, including appreciation of sensing mechanisms and a role of O_2 as a positional signal in development. However, inconsistencies in nomenclature, terminology and experimental methods hamper interpretations, heightened by technical challenges in assessing O_2 concentrations at the cellular and subcellular level. Here we present an overview of the current state of O_2 monitoring technologies and provide a unified nomenclature in flooding and low O_2 stress research. We aim to create an awareness of how experimental conditions can influence *in planta* O_2 and advocate the universal usage of the stated guidelines to promote unambiguous experimental comparisons and the reproducibility needed for addressing the major challenges in this field.

INTRODUCTION

Apart from playing a key role in important biochemical reactions, molecular oxygen (O_2) and its by-products also have crucial signalling roles in shaping plant developmental programs and environmental responses. Even under normal conditions, sharp O_2 gradients can occur within the plant when cellular O_2 demand exceeds supply, especially in dense organs such as tubers, seeds and fruits. Spatial and temporal variations in O_2 concentrations are important cues for plants to modulate development (Considine *et al.,* 2016; van Dongen & Licausi, 2015). Environmental conditions can also expand the low O_2 regions within the plant. For example, excessive rainfall can lead to partial or complete plant submergence resulting in O_2 deficiency in the root or the entire plant (Voesenek & Bailey-Serres, 2015). Climate change-associated increases in precipitation events have made flooding a major abiotic stress threatening crop production and food sustainability. This increased flooding and associated crop losses highlight the urgency of understanding plant flooding responses and tolerance mechanisms.

Timely manifestation of physiological and morphological changes triggering developmental adjustments or flooding survival strategies requires accurate sensing of O_2 levels. Despite progress in understanding how plants sense and respond to changes in intracellular O_2 concentrations (van Dongen & Licausi, 2015), several questions remain unanswered due to a lack of high resolution tools to accurately and non invasively monitor (sub)cellular O_2 concentrations. In the absence of such tools, it is therefore extremely critical for researchers in the field to be aware of how experimental conditions can influence plant O_2 levels, and thus on the importance of accurately reporting specific experimental details. This also requires a consensus on the definition of frequently used terms.

At the 15th *New Phytologist* workshop on Flooding Stress (Voesenek *et al.*, 2016), community members discussed and agreed on unified nomenclature and standard norms for low O_2 and flooding stress research. This consensus on terminology and experimental guidelines is presented here. We expect that these norms will facilitate more effective interpretation, comparison and reproducibility of research in this field. We also highlight the current challenges in noninvasively monitoring and measuring O_2 concentrations in plant cells, outlining the technologies currently available, their strengths and drawbacks, and their suitability for use in flooding and low O_2 research.

TERMINOLOGY

The inconsistent and sometimes inaccurate usage of flooding and low O_2 stress-related terms together with incomplete details regarding experimental conditions have hindered the interpretation, reproducibility and comparison of independent studies in the field. Here, we define and clarify commonly used terms used in flooding and low-O_2 related experimental conditions.

Flooding: a general term referring to excessively wet conditions, i.e. where excess water replaces gas-spaces surrounding roots and/or shoots. Flooding encompasses the following terms that describe natural events or experiments.

- **Waterlogging or soil flooding**: only the root-zone is flooded (excessive water in the soil or other rooting media).

- **Partial waterlogging or soil flooding**: partial flooding of the root-zone. Details regarding depth and extent of soil flooding should be specified.
- **Submergence**: the entire plant (root and shoot) is under water.
- **Partial submergence**: the entire root system and part of above ground organs are under water. Details regarding the submergence depth in relation to plant height or distance from soil surface should be specified.

Anaerobiosis: literally means 'life without molecular O_2'. Plants can only survive a limited time without molecular O_2. The term anaerobiosis frequently refers to the status of plants/organs exposed to a lack of O_2 for a short time, during which acclimation occurs through altered gene expression and metabolism.

Anaerobic metabolism: describes cellular energy production from carbohydrates *without* the benefit of oxidative phosphorylation and engagement of cytochrome *c* oxidase as the final electron acceptor. Anaerobic metabolism occurs when O_2 is absent and is usually associated with (but not limited to) ethanolic and/or lactate fermentation. In plants, it is also associated with accumulation of alanine and gamma-aminobutyric acid due to altered metabolite fluxes involving the tricarboxylic acid cycle among others (Narsai, *et al.*, 2011; Van Dongen & Licausi, 2015; Voesenek & Bailey-Serres, 2015). It can occur in cells within an '*anoxic core'* in tissues/organs (e.g. vascular tissues of roots (Berry & Norris, 1949; Thomson & Greenway, 1991) even under externally aerobic conditions and in densely packed tissues or organs with a low surface to volume ratio (e.g. developing fruits, tubers, seeds, meristems) (Geigenberger *et al.*, 2000; Gibbs & Greenway, 2003; Van Dongen & Licausi, 2015).

Defining –oxic conditions: The terms hypoxia and anoxia are often used interchangeably, which limits experimental reproducibility and can lead to misunderstanding of associated physiological, biochemical and molecular processes. When accurate quantification of the O_2 status of biological samples or their environment is not possible, use of -oxic terms is valid, but care should be taken when

inferring conclusions about O_2 availability from these experiments. As a guideline, we describe common -oxic words, highlighting their limitations.

- **Anoxia:** describes complete absence of O_2 in a system. This is not the same as an O_2 concentration that is too small to measure because such a condition can be maintained when the diffusive flux of O_2 into the tissue is equal to the O_2 metabolism. True anoxic conditions are unlikely to occur in plant tissues where photosynthesis and respiration are key metabolic processes (Smith & Dukes, 2013). Thus, this term should be limited to describing the *atmosphere* applied to biological samples or the environment under investigation. Most cases applying anoxic conditions involve replacing the natural atmosphere with an inert gas such as argon or nitrogen (e.g. Loreti *et al.*, 2005; Branco-Price *et al.*, 2008). Some artificial flooding treatments have also been defined as anoxic, when water was degassed prior to submergence (Baud *et al.*, 2004). In this case, however, the degassed water will not remain O_2 free unless subsequently placed in an O_2 free environment. Finally, true anoxic conditions require darkness, since the photosynthetic light reaction generates molecular O_2.
- **Normoxia:** The reference normoxic condition is often the O_2 availability in air at sea level on today's Earth, i.e. 20.95 %. However, O_2 concentrations within plant organs can be lower or higher under normoxic conditions (Van Dongen & Licausi, 2015; Pedersen *et al.*, 2016). Therefore, internal (organ/tissue/cellular) O_2 concentrations could deviate from the "normoxic environment". Under external normoxia, cells may be O_2-limited due to high metabolic activity, as in meristems (Greve *et al.*, 2003), vascular tissues of roots (Armstrong & Beckett, 1987) or due to limited diffusion in bulky tissues (Pedersen *et al.*, 2006), or tubers (Geigenberger *et al.*, 2000). O_2 levels measured in these tissues over time in the experimental system is desirable.
- **Hypoxia**: describes O_2 concentrations below normoxic without necessarily implying any impact (i.e. hypoxic treatment refers to experiments in which a plant is exposed to lower O_2 conditions than air). Hypoxia is preferably used to selectively describe O_2 concentrations below which a specific process is affected (e.g. below the critical O_2 pressure (Armstrong *et al.,* 2009) for

respiration) or a response is activated. This may imply the need for additional terms to indicate ranges of O_2 concentrations (e.g. in the field of microbiology, micro-oxic often describes 0.5 to 5% O_2 (Pessi *et al.*, 2013)). When authors use hypoxia or alternative terms to describe reduced O_2 availability, provision of precise O_2 tensions or ranges is valuable. This can include flow rates or turbulence, medium composition and temperatures for the external medium and the bulkiness, respiration rate and density of experimental tissue(s).

- **Hyperoxia/superoxia:** describe O_2 concentrations above normoxia. Hyperoxia/superoxia can result from, for instance, underwater photosynthesis and reduced outwards diffusion rate of O_2 from photosynthetic organs to the environment (Rich *et al.*, 2013; Pedersen *et al.*, 2016), or from water bodies to the atmosphere (Nikinmaa, 2014).

Although it is advisable that O_2 concentrations be described for each experimental system, authors may prefer to use -oxic conditions best suiting the study, as long as the description enables experimental replication. Detailed description of the O_2 levels assessed externally or internally (within the plant) or physical parameters that affect its availability will improve the reproducibility of observations and help design of models and meta-analyses.

THE CHALLENGE OF MONITORING OXYGEN LEVELS IN PLANTS

Flooding is a compound stress imposing changes in O_2 availability (and thus respiratory ATP production), CO_2, light, ethylene, mineral nutrients and reactive oxygen species (Voesenek & Bailey-Serres, 2015; Voesenek & Sasidharan, 2013). The severity of the stress and the response elicited depends upon genotype, developmental age of the plant, organ, tissue, and other factors including flooding depth and duration, light availability, temperature, humidity and the amount of carbohydrate storage (such as sugars, starch, lipids, protein) in cells and tissues.

It is not easy to predict what physiological changes occur in a spatial and dynamic fashion during flooding at the cellular level, especially with respect to O_2 concentration. The way in which a flooding treatment is performed will strongly influence how fast plant tissues experience low O_2 stress. Different factors, including

light levels in the water, the temperature and the volume of the water used to submerge the plants, microbial activity in the submerged soil, and O_2 concentration of the water at the beginning of the experiment will all influence how the O_2 availability to the plant changes during the treatment. Therefore, careful monitoring and reporting of the O_2 concentration around submerged plant tissue is required. Polarographic electrodes (such as the Clark-type electrode) are still most widely used for this. However, fiber-optic based sensor methods have become more popular during recent years (Rolletschek *et al.,* 2009; Ast *et al.,* 2012), since these are fast and selective. Moreover, optical sensors have the advantage that the same sensor can be used to measure molecular O_2 concentrations in solution as well as in air, and the baseline of the measurement is more stable as compared to polarographic methods which makes optical sensing more suited for long-term (days to weeks) measurements.

To avoid technical difficulties in controlling the O_2 concentration around a plant by submergence, many studies use a chamber filled with O_2-free or O_2-poor air. This has the advantage that O_2 concentrations can be changed much faster as compared to a submergence treatment, and that the actual external concentration can be controlled precisely. One should be aware, however, that a treatment with air containing little O_2 does not mimic submergence, but only changes one out of many parameters that are affected by submergence.

Apart from the importance of controlling the environmental O_2 concentration during experimental treatments, there is a strong need to obtain precise information about the plant internal O_2 concentration as well. To date, measurements of plant internal O_2 have been only accomplished via invasive means (Ast *et al.,* 2012; Ast & Draaijer, 2014; van Dongen & Licausi, 2015). Most commonly, a small sensor needle is inserted into a plant organ and O_2 concentrations are measured at the tip of the needle. The smallest needle type sensors that currently exist are based on the Clark-type sensor system (Revsbech, 1989) and commercially available sensors have a diameter of around 4 µm. These sensors are extremely fragile. More robust glass fibre-based optical sensors typically have a diameter of about 50 µm. A disadvantage of needle-type sensors is that the tissue will be damaged upon insertion, which can lead to local changes in the rate of respiratory O_2 consumption. Moreover, external O_2 is likely to

diffuse through the insertion wound into the interior of the tissue, which could lead to an overestimation of the actual *in planta* O_2 concentration.

An alternative invasive method to determine local differences or changes in O_2 concentration is by using O_2 sensitive reporter foil (Tschiersch *et al.*, 2012). Here, a special camera is used to determine O_2 concentration-dependent light emission from a special coated sensor foil that is placed on the surface of plant tissue (Jensen *et al.,* 2005). This method has been used to describe local differences in O_2 concentration of plant organs such as stems and seeds that were cut in order to access the interior tissues with the sensor foil. By doing so, these measurements allowed detection of differential O_2 consumption patterns within the plant organ (Tschiersch *et al.,* 2012). More recently, nano particles coated with a fluorescent dye have been successfully used in rhizosphere studies (Koren *et al.,* 2015). These nano particles are possible future candidates for O_2 studies at the cell level when working with large, transparent model cells such as cells of *Chara*.

To date, no method exists that enables non-invasive analysis of plant internal O_2 concentrations. The best alternative that is currently being applied makes use of reporter proteins (such as GUS, GFP or Luciferase) that are expressed under the control of low- O_2 induced promotor sequences (Gasch *et al.,* 2016). Interpretation of the expression pattern of the reporter protein allows conclusions about relative variation in the O_2 concentration between regions or through time. It will not provide, however, an exact value for the actual local concentration of O_2. Moreover, the reaction time of such reporter systems is relatively long, making it difficult to investigate rapid changes. Several other suggestions are being discussed to design alternative non-invasive molecular O_2 reporter systems. FRET (fluorescent resonance energy transfer)-based methods in which the FRET efficiency is affected by O_2-dependent protein maturation has already been applied successfully in bacterial cell cultures (Potzkei *et al.,* 2012), but there are no reports yet of the successful application of such O_2 sensors in plants.

In medical research, various non-invasive O_2 monitoring techniques are being used, including Positron Emission Tomography (PET) and nuclear magnetic resonance (NMR) technology (Roussakis *et al.,* 2015). In plants, such methods have not been reported yet to determine O_2 gradients, because of the poor resolution and because

homogenous application of the required radioisotopes or contrast agents (such as Fluorine-19 (19F)-based probes) appears difficult in plants. Further research to develop methods to determine plant internal O_2 concentrations will remain of utmost importance for the research field to develop further.

EXPERIMENTAL SYSTEMS

It is extremely important that researchers carefully detail the experimental imposition of flooding or low O_2 stress. We suggest that, in addition to details essential to any methods description, the following details specific to low O_2 and flooding studies are necessary:

Stress conditions:

- Type of flooding (waterlogging, partial or complete submergence) should include depth relative to shoot height. Investigators are encouraged to define terms used in their system, e.g. stagnant flooding. If hydroponics are used, information on aeration, O_2 status, light and medium composition are needed.
- Flooding in a natural or artificial environment should include information on light, flow, turbidity, pH, inorganic carbon concentration and temperature of the water. It is beneficial to record the rate of decline of O_2 in the soil, air and water. Soil flooding can also be documented from soil redox potential.
- Hypoxia experiments should provide details regarding the system used to achieve low O_2 conditions (and state the O_2 concentrations), including time taken to achieve the condition. Further information can include: chamber size, flow rate through the system, and details of application. The gas used to lower O_2 levels must be stated.
- In experimental setups determining O_2 flux into roots from O_2 containing bathing media, experimenters should be aware that when roots are attached to shoots, fluxes to the root can come not just from the media but also internally from the shoot (Armstrong & Armstrong, 2014).

- Recovery conditions (post-submergence or post-hypoxia) should be described, including light levels, temperature, humidity, and watering regime post-drainage. Rate of soil drainage (changes in soil water content) and changes in soil redox potential are also valuable.
- Zeitgeber time (hours after dawn) and illumination when experiments start and terminate should be mentioned.
- Plant density and orientation of growth on medium should be included.

Scoring survival: A recovery period following the removal of flooding/hypoxia/anoxia stress is essential for scoring survival (Striker, 2012). Plants should be photographed immediately before and after the treatment and at the end of the recovery period. When scoring damage, quantitative rather than qualitative data are more reproducible and can be analysed statistically (e.g., chlorophyll levels, biomass, green leaf area).

CONCLUSIONS

Careful descriptions of growth and treatment conditions, especially factors that can influence both plant external and internal O_2 concentrations are essential for clarity, reproducibility and progress in the research on plant responses to flooding and low O_2. Reporting on O_2 concentrations, whenever possible, using the most suitable, currently available methods is recommended. Ultimately, the challenge is to also achieve an understanding of the spatial and temporal dynamics of the major flooding signals O_2, ethylene, nitric oxide, reactive oxygen species and low-energy, their interactions, and how signalling modulates response from the subcellular to the whole plant level. Furthermore, the focus of many studies has been on short-term molecular signatures often under severe conditions, whereas responses associated with long-term, less severe and more chronic O_2 limitations that influence developmental plasticity deserve greater attention.

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