PhD Thesis

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Mechanisms of flood tolerance in wheat and rice
The role of leaf gas films during plant submergence

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Submitted on: 24 August 2017
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This thesis has been submitted to the PhD School of The Faculty of Science, University of Copenhagen
Preface

The current Ph.D. thesis represents the work of three years ranging from August 2014 to August 2017, of which one year was dedicated to teaching and course activities. The thesis was prepared at the Freshwater Biology Laboratory at the University of Copenhagen, Denmark, and was funded by the Villum Foundation. The aim of the project was to determine why wheat plants perish upon submergence. Wheat can experience floods in environments ranging from Scandinavian winter wheat fields to rice-wheat cropping systems in Asia. This thesis is based on three manuscripts with me as first author (two published, one submitted for publication) and two co-authored studies in the appendix. Emphasis was on the role of leaf gas films – air layers surrounding submerged superhydrophobic leaves – due to their importance for rice and wild wetland plant submergence tolerance. Hence, this thesis encompasses studies investigating the role of leaf gas films on wheat (and rice) submergence tolerance, but also deals with other aspects such as traits conferring waterlogging (soil flooding) tolerance. Although it may seem overly optimistic, it is my hope that this and other work will one day result in commercially available dry land crops with improved flood tolerance, allowing farmers to experience yield stability in spite of a changing climate in the decades to come.

Copenhagen, Denmark, August 2017

Max Herzog
Acknowledgements

I have many people to thank for enabling me to hand in this thesis. First of all I would like to thank my supervisor Ole Pedersen for allowing me to join the project. I know that you have felt responsible for luring me out of a permanent high school teaching position and onto the uncertain waters of science, but I am very glad you did as the last three years have been a truly great experience. I would also like to thank you for your understanding of the challenges in combining science with family life, and for you and Anja to open your home for me and my family. It is beyond what one can expect from a supervisor.

Special thanks go to Tim Colmer for invaluable scientific support, and for hosting me at the University of Western Australia. Visiting Perth was a great experience for me and my family which we will never forget. Thanks go out to other good people at UWA – ranging from my neighbour Phill driving me to work, to Imran Malik cooking the most marvellous dinner. I would also like to thank Gustavo Striker from Buenos Aires for a great collaboration – I hope to work with you again someday.

When doing this Ph.D. little was more important than the daily interaction with all the friendly people at FBL. Thank you Kathrine and Lars B. for sharing joys and frustrations of combining a Ph.D. with parenthood. Thank you Anders W. for making me laugh at even the most hopeless situations and/or datasets. Thank you Dennis for introducing me to the crazy world of science, and always helping me find The Shed, tweezers and CO₂ cylinders (etc.) at UWA. The biggest thanks to Mikkel M.Ø. and Emil K. for invaluable shrimp tank consultancy, and for trying to make me join the cross-fit team. It will never happen. Most sincere thanks to the good lab technicians Ayoe and Anne for their patience. Thank you Lars I. for statistical advice (boy, you must hear that a lot). Also thanks to Jens for all the advice on ‘røvballestatistik’, and all remaining FBL staff and students for making FBL such a nice work place.

Thanks to my parents, sister and brothers; especially for asking why I wasn’t done yet from day one. However, nobody could be more important to thank than my strong and beautiful wife Lisbet. Thank you for encouraging me to follow my heart and take on this project and for bearing with my wrinkled forehead or overly enthusiasm over drowned wheat plants. Thank you for taking care of our wonderful children when days got long and supporting my every step.
Please grow old with me. Thank you Alfred for always making me want to leave work early to read comic books and wrestle. Thank you Edith for just being, watching you grow is the best thing in the world. If you read this one day I hope you will know that you mean more to me than any piece of work will ever do – the two of you are the best I have ever made.

Max
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Abstract

Most crops are sensitive to excess water, and consequently floods have detrimental effects on crop yields worldwide. In addition, global climate change is expected to regionally increase the number of floods within decades, urging for more flood-tolerant crop cultivars to be released. The aim of this thesis was to assess mechanisms conferring rice (*Oryza sativa*) and wheat (*Triticum aestivum*) flood tolerance, focusing on the role of leaf gas films (see picture below) during plant submergence.

Reviewing the literature showed that wheat germplasm holds genetic variation towards waterlogging (soil flooding), and highlighted traits such as improved internal aeration of the root system and short term anoxia tolerance of seminal roots as conferring tolerance. However, further work on especially anoxia tolerance and genotype × environment interactions is required in order to explore the available genetic resources. Experimental work assessed the physiologic, metabolomic and genetic response of wheat subjected to complete submergence, documenting contrasting submergence tolerance between two cultivars. While both cultivars displayed similar leaf gas film retention times and carbohydrate consumption rates, results indicated that the contrasting submergence tolerance could rather be governed by tolerance to radical oxygen species or contrasting metabolic responses (other than carbohydrate consumption) to ethylene accumulation. Manipulating leaf gas film presence affected wheat and rice submergence tolerance such as plant growth and survival. However, leaf gas film retention times did not differ between 14 winter wheat cultivars, and leaf gas films did not prevent significant leaf Na\(^+\) and Cl\(^-\) intrusion, and K\(^+\) loss, during rice submergence in saline water. Due to the significant salt intrusion and low genetic variation in wheat gas film retention times, a future prominent role of leaf gas films in improving (i) wheat submergence and (ii) rice salinity tolerance was not generally supported.
Dansk resumé

Mange af menneskets vigtigste afgrøder er følsomme overfor oversvømmelser, hvilket årligt medfører store globale udbyttetab. Derudover forventes klimaforandringer indenfor årter at medføre et øget antal oversvømmelser i mange landbrugsregioner. For at sikre den fremtidige fødevareforsyning er der derfor behov for afgrøder med øget tolerance overfor abiotiske stressfaktorer såsom oversvømmelser. Formålet med denne Ph.D.-afhandling var at undersøge hvilke mekanismer der kan medføre øget oversvømmelsestolerance hos hvede (*Triticum aestivum*) og ris (*Oryza sativa*), med fokus på betydningen af bladenes ’gasfilm’ – et tyndt luftlag omkring superhydrofobe blade som dannes under neddykning (se billede på forrige side).

Et litteraturstudie over hvilke mekanismer der betinger hvedes tolerance overfor vandmættet jord (waterlogging) viste at nogle sorter er relativt tolerante, og fremhæver bl.a. intern iltrtransport via luftpæv i adventivrødder, samt røddernes anoxi-tolerance som vigtige mekanismer.

Fortolkningen af resultater på tværs af globale regioner besværliggøres dog af interaktioner imellem genotype og miljø. Derudover kræves en mere detaljeret viden om især anoxi-tolerance førend disse ressourcer kan udnyttes i planteforædlingssammenhæng. Afhandlingens eksperimentelle studier fokuserer på effekten af fuldstændig neddykning på fysiologi, genekspression og metabolisme hos ris og hvede. Heri dokumenteres bl.a. en signifikant forskel på to hvedesorters oversvømmelsestolerance. Dog var tolerancen ikke som forventet betinget af skuddets kulhydratomængde som det er kendt fra ris, eller af gasfilmens levetid, men nok nærmere regulering af fri iltradikaler, eller forskel i sorternes ethylen-sensitivitet. Manipulation af gasfilmen påvirkede vækst og overlevelse i hvede (samt vækst i ris) under neddykning, men gasfilms-levetiden blandt 14 neddykkede hvedesorter var meget ens. Derudover forhindrede gasfilmen (ved en simuleret kystnær saltvands-oversvømmelse) ikke som ventet signifikant tab af K⁺ eller indtrængen af Na⁺ og Cl⁻ i ris-blade. Grundet denne signifikante salt-indtrængen hos ris, samt den lave variation i hvedesorternes gasfilm-levetid, vurderes det ikke at gasfilmen vil have afgørende betydning for fremtidig sikring af (i) oversvømmelsestolerance i hvede og (ii) salttolerance i ris.
Thesis introduction

Floods affect ecosystems ranging from deserts to river forelands and agricultural soils (Jackson 2004). Accounting for 43% of all natural disasters recorded within a decade, floods are the most frequent natural disaster worldwide (de Guenni et al. 2005). This Ph.D.-thesis deals with the detrimental effects of floods on crop production.

Floods occur when water input exceeds the rate of infiltration, evaporation or runoff leading to flooding of the plant-soil system. Flash floods are rapidly occurring floods, often following brief torrential rain in the catchment (Brammer 1990; Adhikari et al. 2010) or failing of dam or river levees (NSSL 2017). Flash floods may be of short duration when waters run off quickly, but may also result in longer, stagnant floods in lowland areas (Setter et al. 1987). Rainwater floods are caused by heavy rain and/or poor soil drainage, while river floods occur when water levels rise above the top of river banks (Brammer 1990; Adhikari et al. 2010). Coastal floods inundate land areas when tides are higher than average, strong winds force sea water up river systems or sea waters rise due to low atmospheric pressure. Floods may be seasonal (e.g., caused by monsoon rains or snow melts) or more unexpected as due to more than average precipitation. Land use changes such as catchment deforestation, farmland drainage causing faster runoff or excess irrigation can also increase the risk of floods (Rienk et al. 2002).

Several estimates have been made as to how much land annually suffers from excess water. Some commonly used estimates are that 10% of the global land area and 20% of Europe and the Russian Federation is affected by severe soil drainage constrains (Setter & Waters 2003); 10% of all irrigated farmland suffers from waterlogging (Jackson 2004); 16% of U.S. soils limit plant production by being too wet (Boyer 1982); 15-20% of all wheat growing areas are affected by waterlogging each year (Sayre et al. 1994) and > 35% of the global rice acreage is considered flood prone (Bailey-Serres et al. 2012). Although the basis of such estimates is not always entirely clear, they indicate that excess water is a global challenge to crop production. Attempts to quantify such crop losses by reviewing historic insurance indemnity payouts in the U.S. (Boyer 1982) showed that from 1939-1978 indemnity payout due to excess water (16% of all payouts) was second only to drought (41% of all payouts). More recently, 2010-2016 floods
accounted for 25% of all indemnities, exceeding any other single stress (drought, heat, cold, pests etc.) in 4 out of 7 years (Fig. 1). Payouts due to floods averaged US$2.4bn per year, totalling US$19 billion (Fig. 1). These high indemnity payouts document that, in the U.S., floods are one of the main factors limiting crop production.

Figure 1. a) number of floods characterised as disasters (see text for definition), as monitored by the International Disaster Database at the University of Louvein in Belgium (Millennium Ecosystem Assessment 2005). b) Crop production loss due to environmental stresses from 2010-2016, based on insurance indemnities paid to farmers. Values are from the U.S. Department of Agriculture Risk Management Agency Cause of Loss Historical Data and grouped as follows: Floods (Excess moisture/precipitation/rain or floods), drought (water deficit stress), temperature (cold winter, cold wet weather, freeze, frost, heat, hot winds) and other stresses (e.g., decline in price, insects, pests, wildlife, hail, hurricanes, tornado, wind, snow and lightning). For preceding years see Boyer (1982) and Bailey-Serres et al. (2012).

In some regions floods are expected to increase in frequency and severity within the next decades due to global climate change (Parry et al. 2007). Global surveillance data indicate that the number of floods characterised as disasters (10 or more people reported killed, 100 or more people reported affected, international assistance was called or a state of emergency was declared) has increased at a constant rate since the 1940’s (Fig. 1a). However, care should be taken when interpreting these results as the number of reported floods has also increased due to improved telecommunication and better coverage of global information (de Guenni et al. 2005). As examples for the expected increase in floods, soil moisture in excess for European wheat production (already considered a persistent problem in 5 out of 13 European environmental zones) is expected to increase in the UK, the Netherlands and Denmark by 2060 (Olesen et al. 2011; Trnka et al. 2014). In addition, increased precipitation due to climate change has been projected to double crop losses from $1.5 billion to $3 billion per year in the U.S.
(Rosenzweig et al. 2002), and rising sea levels are expected to more frequently inundate large parts of Asia’s rice growing areas (Ali 1996; Wassmann et al. 2004; Sarwar & Khan 2007).

The Food and Agriculture Organization of the United Nations states that the world needs to produce 70% more food by 2050 in order to feed a world population of 9 billion (FAO 2009). To meet this goal, The World Bank Group argues that adapting Climate Smart Agriculture (encompassing production of crops more tolerant to abiotic stress) especially in developing countries will be necessary (Braimoh et al. 2016). An important step could be to improve crop flood tolerance, minimizing yield losses when agricultural soils inevitably become flooded.

**How floods affect terrestrial vegetation**

Floods are one the most dramatic environmental changes terrestrial plants can experience. The term “flooding” is used to describe excessively wet conditions encompassing “waterlogging” (soil flooding) and “submergence” situations where the above ground organs are under water (Sasidharan et al. 2017). In the following I will apply “waterlogging” when only the root zone is flooded, “submergence” when all (or part of) the shoot is submerged and “flooding” when discrimination is not necessary. The effects of waterlogging and/or submergence on terrestrial plants have been thoroughly reviewed for anoxia tolerance (Gibbs & Greenway 2003; Greenway & Gibbs 2003), waterlogging tolerance in wheat, barley and oats (Setter & Waters 2003), ethylene and O₂ signalling (Voesenek & Sasidharan 2013), underwater photosynthesis (Colmer et al. 2011), root responses (Elzenga & van Veen 2010; Sauter 2013), adaptations to submergence (Voesenek et al. 2006) and more general reviews (Bailey-Serres & Voesenek 2008; Colmer & Voesenek 2009; Striker 2012). Thus, the following sections will only provide a brief summary of the numerous challenges experienced by flooded terrestrial vegetation.

Waterlogging reduces soil O₂ levels due to the 20-30 fold lower solubility and the 10⁴ time slower diffusion of O₂ in water compared to that in air (Armstrong 1979). O₂ is therefore quickly consumed by roots and soil-microorganism when soil air-filled porosity is 10% or less (Ponnampuruma 1984), resulting in severe hypoxic or even anoxic (absence of O₂) conditions (Fig. 2a). The rate and degree of which soils turn anoxic depend on a wide range of factors, e.g., soil composition, water flow through the soil profile, temperature, biological activity etc.
Waterlogged soils experience a wide range of electrochemical changes in addition to O₂ decline (Fig. 2b). A major change is the shift to low (200 to -400 mV) redox potentials (Ponnamperuma 1984). The change from oxidative to reducing environment is caused by anaerobe microbes using oxidised soil components and organic matter as electron acceptors in their respiration, reducing soils in thermodynamic sequence (Fig. 2b). The reduction of these compounds results in the gradual disappearance of NO₃⁻, Mn⁴⁺, Fe³⁺, SO₄²⁻, CO₂ and increase in soluble NH₄⁺, Mn²⁺, Fe²⁺, S²⁻, H₂S, CH₄ (methane) and organic acids if waterlogging is prolonged (Ponnamperuma 1984). Some of these reduced compounds may have phytotoxic effects, and can enter roots and accumulate in addition to endogenously produced CO₂ and ethylene.

Waterlogging often results in low nutrient uptake by terrestrial plants due to inadequate O₂ supplies. Anaerobic energy metabolism produces some ATP, but since ATP production via glycolysis renders only 2-4 mol ATP per mol hexose compared with 24-36 mol ATP in aerated tissues, roots face energy shortage (Gibbs & Greenway 2003). Thus, during soil waterlogging the
high energy demand of growth cannot be met and root growth of terrestrial plants is arrested, leading to reduced soil exploration and reduced surface area for nutrient uptake (Elzenga & van Veen 2010). Reduced proton motive force and less negative membrane potential resulting from low ATP levels also decreases nutrient uptake per unit of root mass (Armstrong & Drew 2002; Elzenga & van Veen 2010). Anoxic steles can reduce xylem loading, resulting in reduced nutrient transport from roots to shoots (Gibbs et al. 1998; Colmer & Greenway 2011). In addition, waterlogging also alters soil nutrient dynamics. While denitrification in waterlogged soils causes losses of nitrate, P availability may increase as Fe is solubilised (Elzenga & van Veen 2010).

A paradoxical response to waterlogging is leaf wilting (Striker 2012). Both structural and functional constraints have been proposed as explanations for a disturbed water balance of shoots of waterlogged plants. A reduced root:shoot ratio is common in dryland crops when waterlogged (Huang et al. 1994b; Malik et al. 2001; Malik et al. 2002) and implies a lower water absorption surface area of roots in relation to the transpiratory surface of leaves, which together with impaired root hydraulic conductivity (Bramley & Tyerman 2010) can result in plant wilting, particularly under high evaporative demand.

Cells may also be damaged by reactive O2 species (ROS) (Blokhina et al. 2003). When water recedes and O2 re-enters the soil and plant tissues, the formation of ROS (e.g., superoxide, hydroxyl radicals, singlet O2 and hydrogen peroxide) might especially damage cell membranes. ROS production is a consequence of a low energy charge, high level of reducing equivalents and saturation of the mitochondrial electron transport chain, favouring electron leakage to O2 upon re-aeration (Blokhina et al. 2003). For details on nutrient uptake, water balance and ROS in waterlogged wheat, see Chapter 1.

The waterlogging induced stressors described above result in reduced water transport and nutrient uptake by the plant, reducing growth and yields. This is evident for wheat, with average shoot biomass, root biomass and grain yields following waterlogging showing reductions of 33%, 62%, and 43% relative to drained controls (Fig. 3), respectively.
When floodwaters rise above soil levels, shoots will also become submerged adding additional stressors to terrestrial plants (Voesenek et al. 2006). In terrestrial leaves in air, CO₂ enters leaves through open stomata, reaching chloroplasts through intracellular airspaces. Stomata are believed to close upon submergence, forcing gasses to diffuse across leaf cuticles posing much higher resistance to diffusion (Mommer et al. 2005). In addition, a thin diffusive boundary layers (DBL) of water with no turbulence but only laminar flows parallel to the tissue surface form around submerged leaves (Mommer et al. 2004; Pedersen et al. 2009), hampering CO₂ uptake for photosynthesis even further and resulting in the underwater photosynthesis ($P_N$) to be merely 9% of $P_N$ in air (mean of three species, Colmer et al. (2011)). The slow diffusion of gasses through the DBL and across cuticles, together with light reduction by turbid floodwaters, also impedes shoot uptake and/or production of O₂ required for aerobe respiration.

![Graph showing effects of waterlogging on wheat](image)

**Figure 3.** The effects on wheat of waterlogging for shoot dry mass (median = 67% of controls, n = 46), root dry mass (median = 38% of controls, n = 46) and grain yield (median = 57% of controls, n = 206) as percentage of drained controls. This summary figure was compiled from data extracted from peer-reviewed literature (see chapter 1 in this thesis). Boxes are 50% of the observations with the median shown as the horizontal line and bars are 1 and 99 percentiles; outliers are shown as ●. * denotes significant differences from 100% (Wilcoxon Signed Rank test, P < 0.0001). Data are from experiments where wheat was waterlogged in soil (either in pots or in field situations) for 7-42 days without recovery (root and shoot mass) or for 4-120 days for grain yield (most experiments with recovery).

**Adaptations in terrestrial wetland plants: what can be learnt from flood-tolerant plants?**

From the above, one might gain the impression that vigorous plant growth in water saturated soils or in water is not possible. However, nothing could be more wrong! Since
photosynthesizing macrophytes invaded land from marine habitats 400 million years ago, they have (re)colonised marine environments (e.g., seagrasses such as *Zostera*), freshwaters (rooted macrophytes) and wetlands with great success. Wetland plants show a range of adaptations, allowing tropical wetland plants to exhibit primary production rates higher than any terrestrial ecosystem (Kalff 2002). It is therefore intriguing to ask what can be learnt from these natural wetland plants to possibly improve the flood tolerance of cultivated crops. In the following, I will review the main adaptation conferring flood tolerance in wild wetland plants. Some parts of the following text have been re-written from the unpublished introduction of my master’s thesis (Herzog 2013).

Some species inhabiting flood prone areas are not truly flood-tolerant, but avoid flooding stress by completing energy-demanding periods of their lifecycle (growing, flowering, seed-setting) in dry periods, whereas flooding periods are survived by dormant life stages such as seeds, tubers and bulbs (Blom & Voesenek 1996; Bailey-Serres & Voesenek 2008). Meanwhile, flood-tolerant plants reduce flooding stress by a number of traits (Bailey-Serres & Voesenek 2008; Colmer & Voesenek 2009) enabling them to maintain a high metabolism in spite of the lowered energy harvest from underwater photosynthesis and risks of anoxia (Blom & Voesenek 1996). In the following sections adaptations to enable waterlogging and submergence tolerance, respectively, will be summarised.

*Plant adaptations to waterlogging*

As root tissues do not tolerate long term anoxia (Gibbs & Greenway 2003), acclimation to waterlogging concentrates around supplying roots with O$_2$. Waterlogging-tolerant species can form airspaces (aerenchyma) in roots and shoots upon waterlogging, ensuring low-resistance gas transport between plant organs. Many flood-tolerant species form large adventitious root systems with high porosity after waterlogging (Blom & Voesenek 1996). Aerenchyma is constitutive in rice (*Oryza sativa*), while in dryland crop species such as maize (*Zea mays*), wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*) aerenchyma does not form under drained conditions but is induced upon flooding (Yamauchi *et al.* 2013). In these crop species, root aerenchyma is of the lyzigenous type formed by programmed death and lysis of root cortical cells. Before reaching the root apex however, O$_2$ may readily be lost from roots to the soil, termed radial O$_2$ loss (ROL) (Colmer 2003).
In waterlogging-tolerant species, barriers to ROL in basal zones of the roots promote longitudinal diffusion of O$_2$ towards the root apex (Fig. 4), creating oxic conditions at these sites (Colmer 2003). This enables deeper rooting in anoxic soils (Colmer 2003). Waterlogging-sensitive species such as wheat only possess a weak ROL-barrier (Malik et al. 2011), so that O$_2$ is lost along the whole root, thereby not reaching the root apex (Colmer & Voesenek 2009).

Aerenchyma formation takes time, so that roots are likely to experience O$_2$ deficiency initially. Tolerance to short term anoxia includes maintaining ATP production via glycolysis, ensuring root functioning when aerobe respiration ceases (Bailey-Serres & Voesenek 2008). However, noting that fermentation capacity was similar in anoxia-tolerant rice and intolerant soybean, emphasis has been placed on the role of efficient energy utilization rather than ATP production per se (Atwell et al. 2015). When growing in waterlogged soils, roots may face high concentrations of various microelements such as Mn$^{2+}$ and Fe$^{2+}$. Studies have indicated that tolerance towards high concentrations of these micronutrients is an important trait in crop waterlogging tolerance (Setter et al. 2009; Shabala 2011), especially on acidic soils, with the specific action of these metabolites still remaining elusive (Shabala 2011). Tolerance to ROS requires an oxidative defence system. ROS can be detoxified by enzymes (e.g., superoxide dismutase) and antioxidants (e.g., ascorbate and glutathione). The effect of O$_2$ deficiency on the antioxidant system is species-specific (Blokhina et al. 2003), with rice and Arabidopsis showing upregulation of mRNA encoding enzymes involved in anaerobic metabolism and ROS deprivation when subjected to low O$_2$ (Bailey-Serres & Voesenek 2008).
Thesis introduction and aims

Plant adaptations to submergence

Submergence regimes differ in depth and duration, requiring different plant responses which have been categorised into two main strategies: the Low O$_2$ Quiescence Syndrome (LOQS) and the Low O$_2$ Escape Syndrome (LOES) (Bailey-Serres & Voesenek 2008; Colmer & Voesenek 2009). In the quiescence syndrome, traits concentrate around “sitting through” the unfavourable conditions. Energy (mainly from glycolysis) is spent on vital processes such as maintenance instead of growth – resulting in preservation of substrates until the floodwater recedes (Bailey-Serres & Voesenek 2008).

The LOQS differs markedly from the escape syndrome, where shoot elongation is a vital response (Bailey-Serres & Voesenek 2008). When petioles, stems or leaves elongate, the plant may be able to reestablish air contact by emerging above floodwaters. Shoot elongation (alongside other acclimations to submergence) is initiated by ethylene, a volatile plant hormone that accumulates in submerged plant tissues (Voesenek et al. 2006). A costly strategy, the escape syndrome can result in plant death if energy stores are depleted before the plant emerges. This has been illustrated in rice, where elongating cultivars show significantly lower submergence tolerance than non-elongating cultivars (Ismail et al. 2013).

Figure 4. Scheme of two contrasting radial O$_2$ loss (ROL) patterns in roots containing a weak (a) and strong (b) barrier to ROL, resulting in poor apex oxygenation in (a) as O$_2$ is lost to waterlogged and anoxic soils along the whole root. In many waterlogging-tolerant wetland plants waxy suberin depositions along the root base act as a physical barrier to ROL, resulting in an aerobic zone around the sensitive apex (b). The leaking O$_2$ detoxifies reduced soil constituents, mineralises nutrients for plant uptake and allows for deeper rooting in waterlogged soils. Figure from Striker (2012).
Some wetland plants can also form new “aquatic” leaves upon submergence, reducing their cuticle and cell wall thicknesses and reorienting chloroplasts towards the epidermis (Mommer et al. 2005). In such leaves, the diffusive resistance to entry of CO₂ and O₂ is lowered, increasing underwater photosynthesis and O₂ uptake from floodwaters at night.

Underwater photosynthesis is also enhanced by leaf gas films (Colmer & Pedersen 2008; Pedersen et al. 2009). Also termed “plant plastrons”, these ca. 50 µm thick micro layers of gas (Fig. 5) surround superhydrophobic leaf surfaces when submerged (Colmer & Pedersen 2008; Winkel et al. 2011). By enlarging the gas-water interface and possibly allowing stomata to remain open, gas exchange between plant and water is enhanced (Verboven et al. 2014). Leaf gas films have also shown to delay entry of NaCl from saline floodwater into submerged leaves (Teakle et al. 2014). However, leaf gas film retention is time-limited as leaf hydrophobicity declines with time of submergence (Teakle et al. 2014; Winkel et al. 2014). Screening 25 species of terrestrial plants under controlled laboratory conditions showed that leaf gas film retention time varied from 0 to more than 11 days, showing that gas film retention time is species specific (Winkel et al. 2016). The reason for loss of leaf hydrophobicity during submergence, and environmental factors affecting gas film collapse have not yet been resolved, but is of importance in order for submerged terrestrial plants to “stay dry under water”.

Thesis introduction and aims
Thesis aims

The overall aim of this Ph.D.-thesis was to determine why wheat plants succumb during submergence, focusing on the importance of gas films forming on submerged leaves. I attempted to achieve this by

1) Reviewing the literature on wheat flood tolerance, highlighting important traits conferring tolerance and suggesting areas for future research (Chapter 1)

2) Experimentally assessing the importance of leaf gas films during complete submergence of wheat and also rice, with the use of rice enabling me to build upon previously published rice work (Pedersen et al. 2009; Winkel et al. 2013; Winkel et al. 2014). In detail, (i) contrasting submergence tolerance in two wheat cultivars, (ii) gas film effects on ion intrusion during rice saline submergence, (iii) the effects of leaf gas film removal on wheat submergence
tolerance, (iv) variation in gas film retention times among 14 wheat cultivars and (v) reasons for leaf gas film collapse during submergence were investigated.

My hopes are that these results will help to drive research towards improving crop flood tolerance.

Work included in the current thesis


Commencing this study shortly after taking up my position as a Ph.D.-student allowed (forced) me to get acquainted with the relevant literature at an early stage. We compiled data from published studies into larger data-sets, allowing for meta-analysis of wheat responses to flooding in regard of growth, yield, nutrient uptake, photosynthetic rates, root aerenchyma formation and other physiological responses. Since research has focused on wheat waterlogging responses, chapter 1 only deals with this flooding regime. We show that wheat germplasm holds significant variation towards waterlogging, but that tolerance is affected by environmental factors (e.g., temperature, soil type) and experimental design (e.g., timing and duration of waterlogging). We highlight the importance of shoot nitrogen deficiency as main contributor to reduced wheat shoot growth upon waterlogging, while microelement toxicity appears to occur mainly in some acidic soils. Mechanisms that seem to determine wheat waterlogging tolerance are seminal root short-term anoxia tolerance (which needs to be specified), number of adventitious roots and amount of aerenchyma within these roots. Some of the suggested areas for future research are genotypic variations in ROS-tolerance, mechanisms of microelement tolerance, differences in efficiency of energy use, phloem transport and ability of plants to recovery after waterlogging.
In this interdisciplinary study we document contrasting submergence tolerance in two wheat cultivars with reputed differential waterlogging tolerance, as cultivar Jackson survived submergence approximately 7 days longer than cultivar Frument. Physiological measurements indicated that submergence induced leaf degradation (as indicated by leaf chlorophyll and leaf porosity data) several days earlier in Frument than in Jackson. Meanwhile, shoot carbohydrate levels did not seem to explain the varietal differences, although expression of genes related to carbohydrate catabolism was occasionally higher in Frument. Metabolomic analysis revealed that the two cultivars differed in concentration of the amino acid (and antioxidant) proline, peroxidation marker malondialdehyde and chlorophyll brake down product phytol suggesting that ethylene sensitivity or ROS deprivation might confer tolerance; however, more research is required to elucidate these aspects.


This experimental study was conducted during my three month change of scientific environment at the University of Western Australia, Perth. Here we focus on the potential role of leaf gas films as a physical gas barrier around rice leaves during submergence in saline waters, as shown in *Melilotus siculus*. We hypothesised that entry of Na$^+$ and Cl$^-$ and loss of K$^+$ would be delayed by the presence of leaf gas film during submergence, compared to plants with leaf gas films experimentally removed. Surprisingly, we found that in spite of leaf gas film presence, rice leaves lost substantial amounts of K$^+$ and Na$^+$ and Cl$^-$ entered tissues from the floodwater. The results indicated that in rice some leaf-water interphase was present during submergence, and also showed that leaf gas film removal diminished rice growth upon submergence. Analysing leaf surfaces using scanning electron microscopy (SEM) did not reveal any clear causes of leaf hydrophobicity decline in contrast to the results from Appendix II.
Work included in appendix


In this study our group showed that experimentally removing leaf gas films resulted in significantly shorter survival time upon complete submergence of a Danish wheat cultivar. Plants retaining gas films survived complete submergence for 13 days, while plants brushed with a detergent prior to submergence (preventing leaf gas film formation) only survived for 10 days. Underwater $P_N$ at varying light intensities showed that light reflection by leaf gas film resulted in a higher light compensation point, but that this effect was already overcome at 5% of full sunlight. This study underlines that leaf gas film removal diminishes the submergence tolerance of a dryland crop, but that leaf gas film retention time in this wheat cultivar was rather short (< 3 days).


In this study our group investigated the retention of leaf gas films during submergence by 14 wheat cultivars. Compared to a wild wetland grass, gas film retention time was relatively short due to loss of leaf hydrophobicity after 2 days of submergence. The investigated cultivars showed very little variation in regard of gas film retention time, indicating that variation for this trait in Danish wheat cultivars is low. SEM revealed that leaf cuticles were progressively covered by an unidentified substance with time of submergence, correlating with leaf hydrophobicity declines and loss of leaf gas film. The results open new questions as to why leaf gas films are lost during submergence, and indicate that in order to obtain variation in leaf gas film retention time for breeding efforts, more genetically diverse wheat genotypes should be screened.
Chapter 1: Mechanisms of waterlogging tolerance in wheat – a review of root and shoot physiology

Front page of Plant, Cell & Environment depicting partially and completely submerged winter wheat during a winter flood in Germany. Photo: Ole Pedersen
Special Issue

Mechanisms of waterlogging tolerance in wheat – a review of root and shoot physiology

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ABSTRACT

We review the detrimental effects of waterlogging on physiology, growth and yield of wheat. We highlight traits contributing to waterlogging tolerance and genetic diversity in wheat. Death of seminal roots and restriction of adventitious root length due to O2 deficiency result in low root:shoot ratio. Genotypes differ in seminal root anoxia tolerance, but mechanisms remain to be established; ethanol production rates do not explain anoxia tolerance. Root tip survival is short-term, and thereafter, seminal root re-growth upon re-aeration is limited. Genotypes differ in adventitious root numbers and in aerenchyma formation within these roots, resulting in varying waterlogging tolerances. Root extension is restricted by capacity for internal O2 movement to the apex. Sub-optimal O2 restricts root N uptake and translocation to the shoots, with N deficiency causing reduced shoot growth and grain yield. Although photosynthesis declines, sugars typically accumulate in shoots of waterlogged plants. Mn or Fe toxicity might occur in shoots of wheat on strongly acidic soils, but probably not more widely. Future breeding for waterlogging tolerance should focus on root interaeration and better N-use efficiency; exploiting the genetic diversity in wheat for these and other traits should enable improvement of waterlogging tolerance.

Key-words: adventitious roots; aerenchyma; flooding tolerance; genotypic variation; micronutrient toxicity; nitrogen deficiency; O2 deficiency; recovery ability; root anoxia tolerance; wheat (Triticum aestivum).

INTRODUCTION

Waterlogging (soil flooding), due to high rainfall, irrigation practices and/or poor soil drainage, annually affects large areas of farmlands worldwide, imposing major constraints on roots with negative impacts on crop yields (Jackson 2004). This includes wheat (Triticum aestivum), for which 15–20% of the annual crop suffers yield losses due to waterlogging (Sayre et al. 1994; Setter & Waters 2003). In the USA, annual crop insurance payouts over the past 5 years due to floods totaled more than US$2bn, second only to drought (US$3bn) as a stress (U.S. Department of Agriculture 2015). Several other regions, including those among the top 10 wheat producers (e.g. Europe and Pakistan), recently had severe floods inflicting crop damage or loss (Bailey-Serres et al. 2012). Floods are expected to increase as a consequence of climate change (Parry et al. 2007), and increased rainfall in some areas will adversely impact on wheat production (Dixon et al. 2009; Trnka et al. 2014). Increased effort will be needed to breed wheat varieties better adapted to the regionally prevailing abiotic stress factors, for example, drought or waterlogging (Trnka et al. 2014), to meet grain production needs of our increasing human population.

Waterlogging often results in anoxic (absence of O2) soils (Ponnamperuma 1972) and severe hypoxia or anoxia within roots (Armstrong 1979). Even roots with aerenchyma, which facilitates internal O2 diffusion, including in wheat (Erdmann & Wiedenroth 1986; Huang et al. 1994a), will have tissues that become severely hypoxic (Armstrong 1979; Colmer & Greenway 2011; Kotula et al. 2015). The shift in O2-deficient tissues from aerobic respiration to the low ATP-yielding fermentation results in an ‘energy crisis’ (Gibbs & Greenway 2003) and inhibition of root growth and functioning in transport of nutrients and water to the shoot (Jackson & Drew 1984; Colmer & Voesenek 2009), and eventually death of some roots. In addition to O2 deficits per se, soil redox potential declines and Mn2+ and Fe2+ and organic acids can increase in many soils (Ponnamperuma 1972). These can enter roots and accumulate in addition to endogenously produced CO2 and ethylene (e.g. for CO2, Greenway et al. 2006). Cells may also be damaged by reactive oxygen species (ROS) (Blokhina et al. 2003). Thus, when subject to soil waterlogging, roots suffer O2 deficits as well as the additional conditions summarized in the previous text; however, even O2 deficiency without the other soil chemical changes can exert severe stress on roots of dryland species, such as wheat, with consequences for the shoots (e.g. Trought & Drew 1980a).

Here, we review the physiological mechanisms conferring waterlogging tolerance in wheat by examining root and shoot responses and adaptations to low O2 stress and also the effects of the additional components of the ‘compound stress’ caused by soil waterlogging. We highlight genotypic variations where apparent.
EFFECTS OF WATERLOGGING ON ROOT GROWTH AND FUNCTIONING

Wheat root systems are reduced in size owing to growth being impeded and also damage and decay of the existing root system (Fig. 1; references in Supporting Information Table S1). The seminal root dry mass (DM) declines markedly, whereas new adventitious roots develop (Trought & Drew 1980a; Malik et al. 2001). The adventitious roots contain aerenchyma with the associated internal O2 movement to the apex enabling growth, albeit to a limited distance, into anoxic soils. The adventitious root growth does not fully compensate for loss of seminal root DM (Colmer & Greenway 2011), and so median root DM is reduced to 38% of drained controls (Fig. 1). As root growth is inhibited more than shoot growth, waterlogging reduces the median rootshoot ratio of wheat from 0.4 to 0.2 (Supporting Information Table S1). The large variation in root:shoot ratio of wheat from 0.4 to 0.2 (Supporting Information Table S1). The large variation in response of wheat to soil waterlogging is evident in our meta-analysis of published data (Fig. 1 and Supporting Information Table S1), which reflects waterlogging effects of different durations and depths, the ‘compound stress’ (i.e. O2 deficits and other changes in soil chemistry), different temperatures, soil types and plant ages/developmental stages and possible genotypic differences in responses.

Environmental parameters influencing wheat waterlogging responses

Cooler conditions result in less severe effects of waterlogging on wheat (Luxmoore et al. 1973; Trought & Drew 1982). Lower temperature results in slower O2 depletion from the soil, slower root metabolism and slower shoot growth and thus less demand for water and nutrients (Trought & Drew 1982). In some conditions (e.g. low biological activity, low temperature and mass flow of water through the soil), soil anoxia may not occur (Setter & Waters 2003). One example of the effect of soil type on the response of wheat to mid-winter waterlogging was yield being reduced by 16% in clay soil compared with 7% in a sandy soil (Cannell et al. 1980), probably caused by a faster O2 depletion during waterlogging and slower return to oxic conditions upon drainage in the clay, and as proposed by those authors a higher denitrification for the clay (Cannell et al. 1984).

Depth of waterlogging affects the degree of plant damage; for example, when the water level was at 0, 10 and 20 cm below soil surface, tillering of wheat was reduced by 62%, 45% and 24% and adventitious root main axes length per plant by 73%, 58% and 39%, respectively (Malik et al. 2001). Seminal root growth can also increase in anoxic soil zone above the water-saturated anoxic soil; for example, seminal root DM of wheat waterlogged to 10 cm below the soil surface increased by 50% as compared with plants with waterlogging to the soil surface (Malik et al. 2001). The depth and duration of waterlogging at some field sites have been described using SEW30 (sum of excess water that occurs daily in the top 30 cm soil layer; Sieben 1964). Support for such an approach for wheat is that growth and yield progressively decreased with waterlogging duration (Sharma & Swarup 1988; Malik et al. 2002; Olgun et al. 2008; Yaduvanshi et al. 2012; Marti et al. 2015), and reoccurring waterlogging periods can show additive effects (Belford 1981; Belford et al. 1985). However, limitations of using SEW30 have been pointed out (McFarlane et al. 1989; Malik et al. 2001; Setter & Waters 2003) because it does not take into account temperature, flooding frequencies, possible degrees of shoot submergence, susceptibility at different developmental stages and differential recovery responses. Although SEW30 has limitations, with the addition of temperature data (suggested by Setter et al. 2009), it provides a method to quantify and integrate durations and depths of soil waterlogging.

Although low O2 stress is the major cause of the growth reduction of wheat roots (Trought & Drew 1980c), waterlogging also results in other changes in soils that can be detrimental for roots, such as higher concentrations of Fe2+, Mn2+, ethylene, CO2 and organic acids (Ponnamperuma 1972; Ponnamperuma 1984). The soluble metal ions Fe2+ and Mn2+ can increase to potentially toxic levels (Setter et al. 1997a). High concentrations of CO2 may cause pH to decline in root cells (Greenway et al. 2006) and high ethylene can inhibit root extension (Huang et al. 1997a). Data on the effects of organic acids on wheat are lacking, but adverse effects of organic acids on especially K+ fluxes in roots of two barley (Hordeum vulgare) varieties differed in magnitude (Pang et al. 2007), so this aspect should be investigated also in wheat.

In conclusion, in order to facilitate interpretation of waterlogging experiments, details on soil type, waterlogging duration and depth, temperature and when possible other parameters such as soil O2 status and/or redox potential should be provided. In the following subsections, we consider the direct effects of O2 deficits on wheat roots, with focus on anoxia
tolerance and aerenchyma formation and internal O₂ movement. We consider physiological mechanisms contributing to waterlogging tolerance and where available highlight genotypic variation in wheat.

**O₂ deficiency adversely affects the roots of wheat**

The effect of severe root hypoxia on wheat has been evaluated by growing plants in nutrient solutions bubbled with N₂ or deoxygenated and made stagnant with 0.1% (w/v) agar to prevent convective flows (i.e. Wiengweera et al. 1997; Colmer & Greenway 2011). Wheat in N₂-flushed (Trought & Drew 1980c; Barrett-Lennard et al. 1988) or stagnant (Watkin et al. 1998) nutrient solutions for 10 to 14 d developed symptoms resembling those of plants in waterlogged soils (Trought & Drew 1980a, 1980b; Malik et al. 2001; Malik et al. 2002). The similarities were as follows: (1) reduced seminal root growth, (2) death of seminal root apical meristems, (3) growth of adventitious roots to a restricted maximum length only, (4) lower rootshoot ratio, (5) increased root porosity, (6) non-structural carbohydrate accumulation in root and shoot tissues, (7) reduced shoot nutrient concentrations and (8) early senescence of the older leaves. Recovery of growth (see Recovery from Transient Waterlogging and Grain Yield section) upon transfer of plants back to aerated nutrient solutions also somewhat mimicked plant responses to soil drainage with O₂ re-entry (Malik et al. 2001), as initially growth mainly took place in the root system rather than in the shoot, tissue sugar levels declined and adventitious roots elongated in order to restore a more balanced root:shoot ratio (Huang et al. 1994a; Huang & Johnson 1995). The effects on wheat of N₂ flushing in nutrient solution lead to the conclusion by Trought & Drew (1980c) that during early stages of waterlogging, the inadequate supply of O₂ to the roots could explain the arrest of root growth and together with reduced nutrient uptake caused by root dysfunction limits shoot growth of wheat (Trought & Drew 1980c; Buwalda et al. 1988a).

**Effects of O₂ deficiency on root respiration**

As the O₂ in roots declines, at some point so will respiration; the O₂ concentration at which respiration first declines has been termed the ‘critical O₂ pressure’ for respiration (COPR). COPR has been interpreted as being determined by the affinity of cytochrome oxidase for O₂ and its rate of consumption of O₂ and the structure of roots, which determines the diffusion of O₂ to all consumption sites, that is, to mitochondria in all cells (Berry & Norris 1949; Armstrong & Beckett 2011a). However, in the case of pea and *Arabidopsis*, it has been suggested that respiration is down-regulated as O₂ levels decline (Zabalza et al. 2009), but this view has been challenged and the difficulties of external solution measurements discussed in light of diffusion limitations to O₂ reaching interior tissues/cells (Armstrong & Beckett 2011a, 2011b). For root tissues of wheat, O₂ uptake rate was stable until reaching the COPR in the medium of 12.8 kPa for 1 mm apical tips and of 7.2 kPa for segments from 2–4 mm behind the apex (Thomson et al. 1989); there was no evidence for down-regulation of respiration. The values from Thomson et al. (1989) are based on O₂ uptake rates from solution and therefore overestimate cellular COPR (Armstrong 1979; Armstrong et al. 2009).

An alternative approach to determining COPR is the monitoring of radial O₂ loss (ROL) from intact roots in an O₂-free medium during manipulations of the shoot O₂ concentrations (Armstrong & Gaynard 1976). Using this ROL-based approach, COPR was found to be 2.1 kPa at the surface of the root elongation zone (2–7 mm behind apex) of wheat seminal roots (Thomson et al. 1990), 4.0 kPa for wheat adventitious roots (Barrett-Lennard et al. 1988) and 2.4 kPa for rice (*Oryza sativa*) adventitious roots (Armstrong & Gaynard 1976). The higher value for adventitious roots of wheat when compared with rice was suggested by the authors to be caused by lower porosity in the apex of wheat roots (i.e. diffusional limitation resulting in higher COPR). It has also been hypothesized that species with roots having large stelar diameters should have higher COPR due to a longer diffusion path (i.e. stele radius) to the innermost cells of this tissue of relatively low porosity and high rates of O₂ consumption, so that higher O₂ in the cortex would be needed to meet this demand (Armstrong et al. 2009). Cross sections of wheat adventitious roots have proportional stelar areas of 18–20% (Huang et al. 1994b; Wiengweera & Greenway 2004) compared with 5% in those of rice (McDonald et al. 2002), therefore potentially contributing to the explanation of differences in COPR between wheat and rice. Screening of wheat root stele proportions could reveal if genotypic variation exists that could then be followed up with more detailed measurements to determine COPR of potentially contrasting genotypes.

**Anoxia tolerance of wheat roots**

Without O₂ supply, respiration ceases and anaerobic energy metabolism produces some ATP. Survival in anoxia varies from hours to months for plant species and organs (Jackson & Drew 1984), with wheat roots being able to re-grow after 24 h of anoxia when hypoxically pre-treated (Waters et al. 1991a; Mustroph & Albrecht 2007). Survival of root apices, or of lateral initials, would allow seminal roots to resume growth when O₂ is again available as waterlogging recedes (Waters et al. 1991b; Goggin & Colmer 2005; Goggin & Colmer 2007; Mustroph & Albrecht 2007).

Survival of root meristems during anoxia has been assessed by resumption of root elongation upon return from anoxic to aerated solutions. Seminal roots of wheat without hypoxic acclimation die within 9 h of anoxia: 85% of roots with ‘anoxic shock’ did not resume elongation upon re-aeration (Waters et al. 1991b). By contrast, exposure to hypoxia for 15–30 h increased anoxia tolerance as 100% of seminal roots retained their elongation capacity after 24 h anoxia (Waters et al. 1991b; Goggin & Colmer 2005), or for some genotypes even up to 72 h anoxia (Goggin & Colmer 2007). The use of a hypoxic pre-treatment mimics better the gradual O₂ decline in waterlogged soils and avoids an ‘anoxic shock’ (Gibbs & Greenway 2003). During hypoxia, the activities of pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) increased by 2-fold to 4-fold and 3.5-fold to 17-fold and the rate
of ethanol production (and thereby ATP generation) in the subsequent anoxia increased 1.4–4 times (Waters et al. 1991a, 1991b). Further investigation is needed to understand wheat genotypic variation for seminal root tip survival in anoxia, the physiology of which is considered further in the following subsections.

Fermentation rates during anoxia

When suddenly exposed to anoxia, wheat seedling root tips survived for about 24 h at 15 °C and only 10 h at 25 °C (Waters et al. 1991a, 1991b). This low anoxia tolerance has been linked to the relatively low rate of ethanolic fermentation in wheat roots of 10 μmol ethanol g⁻¹ FM h⁻¹ in 5 mm root tips (Waters et al. 1991b) and 3–5 μmol ethanol g⁻¹ FM h⁻¹ in the expanded root zone; in both cases, exogenous glucose was provided as substrate (Waters et al. 1991b; Goggin & Colmer 2007). These rates are relatively low compared with that of fermentation in 5 mm root tips of maize (Zea mays) (19 μmol ethanol g⁻¹ FM h⁻¹, Saglio et al. 1988) and rice seedling shoots (22 μmol ethanol g⁻¹ FM h⁻¹, Menegus et al. 1991). However, differences between species may merely be due to differences in protein concentrations, so the relatively low fermentation rates in wheat need to be confirmed on a protein basis (Gibbs & Greenway 2003). Moreover, anoxia tolerance is determined by factors in addition to rates of ATP production during glycolysis linked to ethanol production, including the efficient utilization of the limited amount of energy available during anoxia (Atwell et al. 2015). Similar ethanolic fermentation rates (on a protein basis) in species showing very different anoxia tolerances support this view (Atwell et al. 2015). Similarly, 11 wheat genotypes did not differ for ethanolic fermentation rate (excised roots supplied with glucose) despite these showing large variation in anoxia tolerance measured as retention of seminal root elongation potential after 72 h anoxia (Goggin & Colmer 2007). Factors other than fermentation rates, for example, substrate supply, efficient use of available energy or down-regulation of energy requirements, must therefore determine genotypic differences in anoxia tolerance in wheat roots.

Wheat roots subjected to hypoxic pre-treatment, rather than to anoxic shock, had 1.4-fold to 4-fold higher ethanol production rates and greater anoxia tolerance (Waters et al. 1991b). Ethanolic fermentation in wheat roots is initially limited by low activity of PDC (Waters et al. 1991b; Albrecht et al. 2004), and hypoxic pre-treatment increased PDC activity 18-fold during 72 h of hypoxia (Albrecht et al. 2004). The increased anoxia tolerance following hypoxic pre-treatment could reflect the improved capacity for ATP production in glycolysis linked to ethanol production, as well as several other acclimations to low O₂ (cf. Greenway & Gibbs 2003).

Substrate supply

Non-structural carbohydrates (i.e. sugars) accumulate in shoots (e.g. 2-fold at 3 d of soil waterlogging) and roots (e.g. 4-fold at 6 d in N₂-flushed solution) of wheat during soil waterlogging or when in O₂-deficient nutrient solutions (Barrett-Lennard et al. 1988; Waters et al. 1991b; Albrecht et al. 1993; Huang & Johnson 1995; Malik et al. 2001, 2002; Mustroph & Albrecht 2003, 2007). During root hypoxia, fructans increased both in roots and shoots of 10-d-old wheat seedlings, which has been suggested to be an energy-efficient carbohydrate storage mechanism (Albrecht et al. 1993; Albrecht & Biemelt 1998). However, views differ on the issue of substrate supply for fermentation in anoxic wheat roots. Sugar supplies were calculated to be ample for 24 h of fermentation, but apices of anoxia-shocked roots only survived up to 9 h anoxia and exogenous glucose during anoxia resulted in five times higher retention of elongation potential (and two to three times higher ethanolic fermentation); hypoxic pre-treated roots already had high tolerance of 9 h anoxia, so there was little benefit of exogenous glucose to those roots during the same time period (Waters et al. 1991b). Loss to the medium of sugars and other metabolites, and the possibility of decreased phloem transport towards the tips, was suggested as reasons that exogenous glucose was beneficial despite the starting levels within the seminal roots (Waters et al. 1991b); such solute loss can be substantial for wheat roots in anoxia (Greenway et al. 1992). Sugar accumulation during a hypoxic pre-treatment, in addition to increased PDC and ADH activities, may increase ethanolic fermentation rates during subsequent anoxia (Albrecht et al. 2004) and enhance survival in anoxia, but even hypoxic pre-treated root tips of wheat died after 48 h anoxia (Mustroph & Albrecht 2007).

Sugar transport to wheat roots is restricted by anoxia. Sugar transport from the endosperm/shoot was reduced by 79–97% when whole seedlings were subjected to anoxia (Waters et al. 1991a). Root sugar concentrations of seedlings exposed to 72 h anoxia decreased but did not differ significantly among 11 genotypes (Goggin & Colmer 2007). Photosynthetically incorporated¹⁴C fed to shoots was detected in roots in an O₂-free nutrient solution for 4 d (Wiedenroth & Poskuta 1981), but whether ¹⁴C was at the root tips was not determined. In maize seedlings, phloem unloading to the apex was severely inhibited by anoxia (Saglio 1985). The decrease in sugar concentrations in anoxic roots contrasts with the earlier-mentioned increase in sugars in hypoxic seedling roots. Interestingly, in the 20 mm tips of adventitious roots of two varieties, which differ in waterlogging tolerance and exposed to 21 d of hypoxia, sugars were substantially higher in the tolerant variety (Huang & Johnson 1995). Whether wheat genotypes differ in phloem transport during hypoxia and the influence on waterlogging tolerance should be assessed (cf. Boru et al. 2003, but direct measurements are needed), and comparisons should ensure that tips are alive to avoid measurements on tissues that have leaked cellular contents as a result of death.

Cytoplasmic acidosis

Anoxia results in declining cytosolic pH in wheat roots, as in other plants, and if prolonged, this may lead to cell death (Ishizawa 2014). Wheat seedling root tips died within 10 h when subject to anoxia at pH 4 whereas survival was more than 90% at pH 5 and 6, indicating that low external pH would increase cytoplasmic acidosis during anoxia (Waters et al. 1991a). The
cause for the initial pH drop has been suggested to result from initial lactate production, being 1.5 \( \mu \text{mol g}^{-1} \text{FM h}^{-1} \) in wheat roots (Thomson et al. 1989; Mustroph & Albrecht 2007), but lactate production/accumulation and cytoplasmic acidification are not necessarily always correlated (Felle 2005). Moreover, maize roots show lactate efflux and a higher cytoplasmic pH after hypoxic pre-treatment (Xia & Roberts 1994). Similarly, hypoxic pre-treatment wheat roots had 2-fold higher efflux of lactate during the first 3 h of anoxia than when given anoxic shock (Mustroph & Albrecht 2007). However, the tissue concentration of lactate was only 0.5 \( \mu \text{mol g}^{-1} \) FM in entire excised wheat roots (Mustroph & Albrecht 2007), and although it would be expected to be higher in root tips, whether lactate is responsible for the observed pH decline in wheat roots remains to be determined.

Cytoplasmic pH decreases in anoxic shoots and root tips of wheat (0.8 and 0.6 pH units within 2 h, respectively) were greater than decreases of 0.5 and 0.2 pH units in shoots and roots tips of anoxia-tolerant rice (Menegus et al. 1991; Kulichikhin et al. 2007; Ishizawa 2014). Wheat cytoplasmic pH continued to decline by another 0.2 pH units so that pH was then 6.8 after 6 h anoxia in root tips (Kulichikhin et al. 2007) and 6.5 in wheat seedling shoots (Menegus et al. 1991), contrasting with rice root tips for which cytoplasmic pH stabilized at pH 7.15 during anoxia (Kulichikhin et al. 2007). After prolonged anoxia, loss of regulation of cellular pH might be a consequence of the energy shortage and reflect dying cells, rather than impaired pH regulation per se being the cause of death (Felle 2005; Atwell et al. 2015).

**Root solute loss**

Death causes cellular solute loss, but solutes may also be lost well before death occurs, for example, cation efflux due to membrane depolarization in anoxia. Wheat seedling roots lost K\(^+\), amino acids and sugars when subjected to anoxic shock and, to a lesser degree, when anoxia was preceded by a hypoxic pre-treatment (Greenway et al. 1992). Plasma membrane depolarization leads to opening of voltage-gated ion channels (Ward et al. 2009), and K\(^+\) net loss has been documented for wheat seedling roots (Greenway et al. 1992; Goggin & Colmer 2007). Investigation of genotypic variation in wheat for root K\(^+\) retention showed that the four varieties showing the most anoxia tolerance had higher tissue K\(^+\) concentrations than the two anoxia-intolerant varieties (Goggin & Colmer 2007). For wheat roots of 26-d-old plants subjected to severe hypoxia (0.23 kPa O\(_2\)) for up to 10 d, K\(^+\) loss was due to membrane depolarization rather than increased membrane permeability, as membranes under anoxia were depolarized but remained impermeable to sorbitol (Buwalda et al. 1988b). In addition to these effects of anoxia (or severe hypoxia) on solute loss from roots, organic acids in anaerobic soils can also result in membrane depolarization and K\(^+\) efflux, as demonstrated for barley roots in aerobic nutrient solution (Pang et al. 2007).

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**Aerenchyma formation in roots of wheat**

O\(_2\) movement within roots is largely determined by tissue porosity (gas volume per unit tissue volume; Armstrong 1979). Root porosity is an important trait contributing to waterlogging tolerance of wheat (e.g. Setter & Waters 2003) and other species (e.g. Justin & Armstrong 1987). The constitutive porosity of wheat roots (i.e. porosity in aerated conditions) is, like most dryland species, relatively low (median values of 2.1% and 5.2% for seminal and adventitious roots, respectively, Fig. 2, references in Supporting Information Table S3) and reflects the hexagonal pattern of cell packing in the root cortex (Trought & Drew 1980c; Xu et al. 2013) resulting in small gas-filled intercellular spaces (cf. Armstrong 1979; Malik et al. 2003). The waterlogging-induced increase in wheat root porosity results from formation of lysigenous aerenchyma in the cortex (Erdmann & Wiedenroth 1986; Huang et al. 1994b; Yamauchi et al. 2014b) resulting in 14.8% porosity (median value) of adventitious roots formed upon waterlogging (Fig. 2).

The ability of seminal roots of wheat to form aerenchyma is related to age and/or developmental stage (Supporting Information Table S3). Figure 2 shows that the four varieties showing the most anoxia tolerance had higher tissue K\(^+\) concentrations than the two anoxia-intolerant varieties (Goggin & Colmer 2007). For wheat roots of 26-d-old plants subjected to severe hypoxia (0.23 kPa O\(_2\)) for up to 10 d, K\(^+\) loss was due to membrane depolarization rather than increased membrane permeability, as membranes under anoxia were depolarized but remained impermeable to sorbitol (Buwalda et al. 1988b). In addition to these effects of anoxia (or severe hypoxia) on solute loss from roots, organic acids in anaerobic soils can also result in membrane depolarization and K\(^+\) efflux, as demonstrated for barley roots in aerobic nutrient solution (Pang et al. 2007).

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**Figure 2.** Porosity (%, gas volume per unit root volume) in seminal and adventitious roots, and aerenchyma (%, cross-sectional area) in adventitious roots, of wheat grown in aerated or drained (control) or O\(_2\)-deficient [2 studies in waterlogged soils and 12 studies in N\(_2\)-flushed/deoxygenated nutrient solutions; waterlogging (WL)] conditions. This summary figure was compiled from data extracted from peer-reviewed literature (data values, key experimental conditions and references are in Supporting Information Table S3). Boxes are 50% of the observations with the median shown as the horizontal line within the box, and bars are 1 and 99 percentiles; for porosity of seminal roots and aerenchyma of adventitious roots, the numbers of observations were insufficient for box-whiskers plots so each observation is shown with . Letters denote significant differences between control and WL (one-way ANOVA with Tukey’s multiple comparison test on log-transformed data, \( P < 0.05, n = 15–17 \) for porosity of adventitious roots and 5–7 for seminal roots); n.s. denotes no significant difference between drained and WL adventitious root aerenchyma (Mann–Whitney test, \( P = 0.556 \)). Medians are 2.1% and 5.3% porosity for control and WL seminal roots, 5.2% and 14.8% porosity for control and WL adventitious roots, and 5.5% and 20% aerenchyma for control and WL adventitious roots, respectively.
Table S3). Seedling (5- to 7-d-old) seminal roots form aerenchyma whereas older seminal roots lose this capacity (e.g. roots of 18- to 36-d-old plants; Thomson et al. 1990, 1992; Xu et al. 2013). More specifically, porosity of short, young seminal roots increased from 4–6% in aerated solution to up to 12% when in stagnant conditions whereas porosity of long, old seminal roots (>100 mm) hardly increased and was 3–7% in both conditions (Thomson et al. 1990). Interestingly, for adventitious roots in aerated solution with exogenous ethylene, shorter (100–200 mm) roots formed aerenchyma whereas longer (>300 mm) roots did not (Huang et al. 1997a). In addition to this possible decrease in responsiveness to ethylene in older/longer roots limiting aerenchyma formation, when in an anaerobic medium, the distal portions of long roots of low porosity might not receive internal O2, which would result in damage or even death of tissues also preventing the opportunity for aerenchyma to form.

In contrast to seminal roots, adventitious roots form more aerenchyma, and these newly produced roots are able to elongate at least to some extent into waterlogged soil or O2-deficient solutions (Thomson et al. 1992; Malik et al. 2002). Adventitious root constitutive porosity ranges from 3% to 7%, and during waterlogging, the porosity increases up to 11–20% by formation of aerenchyma depending on the genotype and O2 deficiency duration and treatment method (Thomson et al. 1990, 1992; Huang et al. 1997a; Wiengweera et al. 1997; Malik et al. 2001, 2002, 2003 and Supporting Information Table S3). Waterlogging-tolerant genotypes had root porosities of 20%, 14% and 11%, and waterlogging-sensitive genotypes had root porosities of 8% and 6%, after 20 d in occasionally N2-flushed nutrient solution (root types not stated, Boru et al. 2003), showing that genotypic variation for aerenchyma formation is present in wheat. The importance of this trait in potentially conferring waterlogging tolerance was illustrated in a field study using 12 genotypes, as yield as percent of controls during waterlogging was positively correlated with aerenchyma percentage of the mid-cortex (Setter 2000).

Aerenchyma formation in roots of wheat, like lyogenic aerenchyma in other species, results from the degradation of cortical cells (Erdmann et al. 1986; Huang et al. 1994b; Malik et al. 2001) via programmed cell death (PCD) as described for wheat (Jiang et al. 2010) and more generally (Evans 2004; Shiono et al. 2008; Takahashi et al. 2014). Interestingly, exposure to O2 deficiency of only the apical portion of adventitious roots is enough to trigger the development of aerenchyma along the entire main axis (Malik et al. 2003). As ethylene signalling triggers lyogenic aerenchyma formation (Steffens & Sauter 2014), including for wheat (Huang et al. 1997a; Yamauchi et al. 2014a, 2014b), possible transport of 1-aminocyclopropane-1-carboxylic acid (ACC) and/or ethylene movement from the hypoxic tip back along the roots could underpin this response (Malik et al. 2003). Exogenous ethylene (1 to 5 μL L−1) resulted in increased porosity of wheat roots in aerated nutrient solution from <5% to 18% (Huang et al. 1997a). The promoting effect of ethylene on aerenchyma formation was greatest under high doses (5 μL L−1), more pronounced for newly formed short roots (10–20 cm length) than pre-existing longer roots (30 cm length) and of higher magnitude in a waterlogging-tolerant variety than in a sensitive one (Huang et al. 1997a).

Details on mechanisms of aerenchyma formation and associated signalling in roots have been reviewed (Steffens & Sauter 2014). Recent work of interest in wheat, using seedling seminal roots, highlights the role of ROS as part of PCD in aerenchyma formation (Xu et al. 2013; Yamauchi et al. 2014b). ROS accumulation starts in the mid-cortex cells where PCD begins, accompanied with up-regulation of some genes encoding for ROS-producing enzymes (e.g. NADPH oxidase) and down-regulation of ROS-detoxifying enzymes (e.g. catalase (CAT); Xu et al. 2013). Pre-treatment of 5-d-old wheat seedlings with ACC increased aerenchyma as well as the expression of three genes encoding respiratory burst oxidase homolog (Yamauchi et al. 2014a, 2014b), which act by generating ROS, and an NADPH oxidase inhibitor partially suppressed the ACC-induced response.

Ethylene triggers other plant acclimations to flooding in addition to aerenchyma formation (reviewed by Sasidharan & Voesenek 2015); of interest here is the initiation of adventitious roots. The number of adventitious roots per wheat plant is typically reduced by hypoxia or waterlogging but proportionally less than the number of tillers per plant; therefore, adventitious root number per tiller increases (Huang et al. 1997b; Malik et al. 2001, 2002). Greater adventitious root number per tiller presumably assists the shoots to cope with the waterlogging-induced restrictions impacting on their roots (Malik et al. 2003). Wheat shows genotypic variation for the formation of adventitious roots, as 21 d of hypoxia (5% O2) decreased the number of adventitious roots in two waterlogging-sensitive varieties by 17% and 37% from aerated controls, whereas this increased by 82% in a waterlogging-tolerant variety (Huang et al. 1994a). Wheat adventitious rooting in response to ethylene is dependent on the genotype and ethylene concentration (Huang et al. 1997a). Production of adventitious roots by a waterlogging-tolerant variety increased by 17% at all ethylene concentrations (0.1, 1.0 and 5.0 μL L−1), whereas in a sensitive variety, only the lowest ethylene concentration promoted these roots (Huang et al. 1997a).

**Internal O2 supply determines root growth in waterlogged soils**

Root growth is arrested by soil waterlogging as a lack of soil O2 to support root respiration means a low energy supply cannot meet the high energy demand of growth (Elzenga & van Veen 2010). Internal O2 movement from shoots and along roots via aerenchyma can then support root extension (Armstrong 1979), but only a limited amount of O2 can reach the root apices. The restricted capacity for internal O2 movement within roots of wheat was demonstrated by the increased adventitious and seminal root extension rates when O2 around shoots was raised from 21 kPa to 80–100 kPa and thus increasing internal O2 diffusion into and along the roots (Thomson et al. 1990; Wiengweera & Greenway 2004). Maximum lengths of wheat adventitious roots in waterlogged soil (11–14 cm, Thomson et al. 1992; Malik et al. 2001) are limited by internal O2 supply to the apex owing to low to moderate porosity of tissues, high respiratory rates and/or high root ROL to the rhizosphere, all
of which in concert determine the length that roots can grow when reliant on internal O$_2$ movement to the apex (Armstrong 1979). The ‘Root-Length Model’ by Armstrong (1979) has been used to predict the maximum length of wheat roots in an anoxic medium and reliant on internal O$_2$ for seminal roots (3.4% porosity) in N$_2$-flushed nutrient solution and for adventitious roots (22% porosity) in waterlogged soil, the lengths achieved were 85% and 77% of those predicted by the model (Thomson et al. 1990, 1992; Malik et al. 2001). By comparison, rice roots reached the predicted lengths, which could be due to differences between the two species in ROL from the basal zones of roots (high in wheat and low in rice; Colmer 2003); ROL is assumed to be zero in the model. Wheat roots did reach the predicted lengths in stagnant nutrient solution where ROL is lower than in soil (Watkin et al. 1998; McDonald et al. 2001).

Root extension rates can initially be maintained in O$_2$-deficient media, but as O$_2$ concentration at the root tip decreases, extension declines at the critical O$_2$ pressure (COP$_E$) and eventually ceases (Armstrong & Webb 1985). Monitoring root extension rates and the O$_2$ concentration just behind the root apex (2–7 mm) when in anoxic medium, while manipulating O$_2$ levels around the shoot and thus at the root apex owing to internal O$_2$ movement along the roots, enables determination of the COP$_E$ (Armstrong & Webb 1985). In wheat, COP$_E$ of 5- to 7-d-old seminal roots grown in semi-stagnant solution and transferred to deoxygenated agar medium for the COP$_E$ measurements was 2.68 kPa at the root surface (Thomson et al. 1990), being 7-fold to 15-fold higher than similarly determined COP$_E$ in rice roots (0.2–0.4 kPa, Armstrong & Webb 1985). These contrasting values of COP$_E$ in wheat and rice are similar to the differences between these two species in COP$_R$ (see Effects of O$_2$ Deficiency on Root Respiration section), so that the declines in root extension presumably result from diminishing respiratory activity below the COP$_R$ (Thomson et al. 1990). The higher COP$_R$ and COP$_E$ values in wheat roots than for rice might be related to insufficient O$_2$ reaching the apex due to the low tissue porosity owing to hexagonal cell packing in wheat roots in contrast to higher tissue porosity of cubic packing of cells in rice roots.

Studies of wheat root lengths as affected by waterlogging showed a significant genotype × treatment interaction for root length density (five genotypes, Hayashi et al. 2013) and for longest adventitious root length (seven genotypes, Dickin et al. 2009), but unfortunately, root porosity and/or ROL were not measured. Maximal length was greater for adventitious roots of higher porosity for two varieties compared in stagnant nutrient solution (Watkin et al. 1998). Waterlogging for 17 d in sterilized sand reduced total length of adventitious roots to 64% of control in a variety with 30% cortical aerenchyma and to 42% of control in one with 19% cortical aerenchyma (Huang et al. 1994b). Seminal root lengths after 7 and 50 d waterlogging did not show a significant treatment × genotype interaction (seven genotypes, Dickin et al. 2009; 6 genotypes, Haque et al. 2012), which contrast with the genotypic variation for adventitious roots.

Aerenchyma formation substantially increases internal O$_2$ diffusion, while consumption of O$_2$ along the diffusion path by respiration and ROL both decrease the O$_2$ at the root tip (Armstrong 1979). Roots of many waterlogging-tolerant species, such as rice, develop a barrier to ROL in the outer cortex (Armstrong 1979; Colmer 2003). By contrast, roots of wheat have substantial ROL from basal zones (Malik et al. 2003; Malik et al. 2011; Alami et al. 2013), which decreases root growth (Thomson et al. 1992). An approach aimed at improving the tolerance of wheat to waterlogging is the transfer of the barrier to ROL from a wetland wild relative, *Hordeum marinum*, to wheat by wide hybridization and amphiploid production (Malik et al. 2011). Two *H. marinum*—wheat amphiploids had tight ROL barriers and two only moderate ROL barriers (Malik et al. 2011). This work demonstrates the potential to target specific traits from more stress-tolerant wild relatives of wheat, but the amphiploids had low fertility and thus low grain production, so further breeding would be required to potentially produce a more waterlogging-tolerant and commercially viable (high-yielding) wheat.

**How does O$_2$ deficiency affect wheat root functioning?**

**Nutrient uptake**

Arrest in root elongation upon waterlogging is pronounced in wheat, thereby leading to a reduced soil exploration and reduced surface area for uptake of nutrients (Elzenga & van Veen 2010). In addition, nutrient uptake by wheat is decreased per unit of root mass; for example, P uptake by seminal roots was 0–16% of aerated controls and by adventitious roots it was 31–73% (Barrett-Lennard et al. 1988; Kuiper et al. 1994; Wiengweera & Greenway 2004). The higher maintenance of ion uptake by the adventitious than seminal roots in O$_2$-deficient solution presumably is owing to the greater capacity for internal aeration of the adventitious roots (Colmer & Greenway 2011).

Reduced ion transport capacity in O$_2$-deficient roots low in energy is likely due to reduced proton motive force and less negative membrane potential (Elzenga & van Veen 2010). The proton motive force is generated by plasma membrane H$^+$ ATPases, but activity of these H$^+$ ‘pumps’ is reduced when adenylate energy charge declines (Armstrong & Drew 2002; Elzenga & van Veen 2010). In addition to reduced capacity for nutrient uptake by root cells, transport from roots to shoots can also be reduced by the effect of an anoxic stele to reduce xylem loading (Gibbs et al. 1998; Colmer & Greenway 2011). Cells in dense stelar tissues can experience more severe O$_2$ deficiency than epidermal and cortical cells, as has been illustrated by microelectrode profiling, for example, in excised maize roots (Gibbs et al. 1998) or intact barley adventitious roots (Kotula et al. 2015). Such conditions would also be expected in wheat roots and could reduce ion release from xylem parenchyma cells into xylem vessels (Colmer & Greenway 2011; Kotula et al. 2015).

Indeed, soil waterlogging reduces the nutrient concentrations in the shoots of wheat (Trought & Drew 1980b; Sharma & Swarup 1988; Steffens et al. 2005). Similarly, shoot nutrient concentrations are decreased when wheat is grown in N$_2$-flushed or in stagnant nutrient solutions (Trought & Drew...
1980c; Wiengweera & Greenway 2004); these results from nutrient solutions support that root hypoxia restricting respiration can reduce root nutrient uptake and/or xylem transport (Drew 1983). In addition, waterlogging also alters nutrient dynamics in soils. P availability may increase as Fe is solubilized (Elzenga & van Veen 2010). Denitrification causing losses of nitrate can occur in waterlogged soils, including soils planted with wheat (Cannell et al. 1980; Trought & Drew 1982; Belford et al. 1985; Webster et al. 1986; Hamonts et al. 2013), although to our knowledge, the amount of N lost has not been quantified for wheat fields.

**Root hydraulic conductivity**

Structural and functional constraints in O2-deficient roots have been proposed as explanations for a disturbed water balance of shoots of plants in waterlogged conditions. A reduced root:shoot ratio is common in wheat (and other dryland cereals) when waterlogged (Huang et al. 1994b; Malik et al. 2001; Malik et al. 2002) (Supporting Information Table S1) and implies a lower water absorption surface area of roots in relation to the transpiratory surface of leaves, which together with impaired root hydraulic conductivity (Bramley & Tyerman 2010) can result in plant wilting, particularly under high evaporative demand. A detailed study on water transport in wheat roots showed that short-term hypoxia (30 min at 4 kPa) reduced hydraulic conductivity of root cortical cells by 45% and transiently reduced the conductivity of entire roots, possibly due to decreased opening of aquaporins (Bramley et al. 2010). During long-term waterlogging events, root cell death, xylem blockage and changes in root anatomy, for example, for wheat, cell wall lignification (Erdmann et al. 1986) and decreased xylem diameter in adventitious roots (Huang et al. 1994b), could each affect root hydraulic conductivity (Bramley & Tyerman 2010). Leaf water potential declined for two wheat varieties after 17 d of waterlogging from −0.54 and −0.79 MPa to −1.02 and −1.10 MPa (Huang et al. 1994b). However, leaf water potential in six wheat genotypes was unaffected after 7 d in hypoxic nutrient solution (Huang et al. 1994a). Decreases in stomatal conductance of wheat in waterlogged soil or in O2-deficient nutrient solution can be substantial; 18% to 60% of controls (Trought & Drew 1980a; Huang et al. 1994b; Musgrave 1994; Malik et al. 2001; Zheng et al. 2009; Li et al. 2011; Shao et al. 2013; Wu et al. 2014) and as discussed in the Photosynthesis Decreases due to Feedback from Accumulated Sugars section can be related to reduced growth and possible feedback of high leaf sugars and not necessarily adverse plant water relations.

**Summary of effects of O2 deficiency on roots of wheat**

Soil waterlogging severely inhibits seminal root growth and nutrient uptake, whereas new adventitious roots containing aerenchyma grow and enable some nutrient uptake, albeit reduced in comparison with O2-sufficient roots. Anoxia tolerance and survival of seminal roots would presumably benefit wheat subjected to short transient waterlogging; the few available data indicate some genotypic variation in seminal root survival during up to 3 d of anoxia. Ethanol fermentation enables production of some ATP, which would be of importance, but anoxia tolerance appears to be related to additional aspects of metabolism, which requires further elucidation. Wheat genotypes differ in both the numbers of adventitious roots formed and amount of aerenchyma within these roots. Aerenchyma enhances internal movement of O2 into and along the roots, but O2 becomes limited at the apex of relatively short roots due to moderate movement and high rates of ROL from basal zones. The low porosity but high O2 demand in the stele and root tips could limit both growth and nutrient transport. Importantly, apices of many adventitious roots can survive at least for several days after root extension stops, so recovery growth can occur when transient waterlogging recedes.

**IMPACT OF WATERLOGGING ON SHOOTS**

Having considered in the previous text the direct effects of soil waterlogging on wheat roots, in this section, we examine the consequences for shoots in terms of growth, photosynthesis, sugar and nutrient status and possible toxicity of microelements.

**Waterlogging reduces wheat shoot growth**

Waterlogging generally reduces shoot growth of wheat, and median shoot DM is reduced to 67% of drained controls (Fig. 1). The high variability in the reduction of shoot DM in Fig. 1 reflects different conditions of waterlogging: (1) depth, (2) duration, (3) temperature, (4) soil type, (5) mineral nutrition regime, (6) plant age/developmental stage and (7) genotype (key experimental conditions and references are in Supporting Information Table S1). The reduction of shoot growth results from less tillering and reduced rates of leaf growth and smaller leaf size. Nitrogen (N) deficiency is one likely cause of the reduced tillering and slower growth (Belford et al. 1985; Robertson et al. 2009). The reduced rate of leaf area production, as well as early senescence of basal leaves (Trought & Drew 1980a; Malik et al. 2001; Malik et al. 2002), would reduce the area available for light interception.

Genotypic variation for shoot growth is evident for wheat in experiments with side-by-side comparisons in waterlogged soil, for example, shoot DM 70% and 40% of controls in a tolerant and sensitive variety, respectively (Huang et al. 1994b), and in hypoxic nutrient solution, for example, after 21 d, shoot DM of 46–70% of controls in six varieties (Huang et al. 1994a). A screening of 37 genotypes revealed shoot growth range of 27–63% of drained controls on an acidic soil waterlogged for 49 d (Setter et al. 2009). The yield of wheat when waterlogged has been shown to correlate with shoot DM, which is also closely related to plant tiller number (Musgrave & Ding 1998; Collaku & Harrison 2002; Hayashi et al. 2013).

Shoot DM of wheat in waterlogged soil can increase during the first few days above that of drained controls as a result of accumulation of photosynthates (Trought & Drew 1980a; Supporting Information Table S1). This accumulation of sugars

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in the shoots of wheat (Trought & Drew 1980a, 1980c; Barrett-Lennard et al. 1988; Albrecht et al. 1993; Huang & Johnson 1995; Malik et al. 2002) means that sugar production exceeds consumption/export, which is likely the consequence of a hypoxic root system demanding less substrate due to cessation of growth and/or decreased ability for phloem transport in the hypoxic roots.

Photosynthesis decreases due to feedback from accumulated sugars

Waterlogging reduces net photosynthesis (PN) in wheat, although with significant variations in both severity (Fig. 3a; references in Supporting Information Table S4) and also time of onset. We will consider separately short-term and longer-term responses to soil waterlogging of PN in wheat, as the causes of these declines in PN likely differ. Possible causes of the initial decline in PN could be stomatal closure limiting intercellular CO2 concentration (Ci), but negative feedback from carbohydrate accumulation could also decrease PN within the first days. In the longer term, N deficiency could also decrease leaf photosynthetic capacity. Numerous studies have documented that sugars accumulate in wheat shoots, stems and roots when waterlogged (Trought & Drew 1980a; Malik et al. 2001, 2002) or when in hypoxic solutions (Trought & Drew 1980a; Barrett-Lennard et al. 1988; Albrecht et al. 1993; Huang & Johnson 1995; Mustroph & Albrecht 2003, 2007), indicating that reductions in PN during short-term waterlogging are not likely to be the cause of reductions in wheat shoot growth, as tissue sugars remain high.

The different mechanisms leading to reduced PN are illustrated by correlating ΔPN [PN(waterlogged) – PN(control)] against Δ stomatal conductance [gs(waterlogged) – gs(control)], during short-term (1–3 d) and long-term (7–21 d) waterlogging of wheat (Fig. 4; references in Supporting Information Table S5). PN was significantly correlated with gs both in short-term (r = 0.79) and long-term waterlogging (r = 0.46); however, the correlation was weaker during the long-term waterlogging. Short-term effects of waterlogging on PN could be due to ‘physiological drought’ in waterlogging-sensitive species, which can even wilt, and gs would decrease to conserve water (Bramley & Tyerman 2010), resulting also in decreased intercellular CO2 (Ci) and lower PN. However, experiments on wheat (Malik et al. 2001; Wu et al. 2014) and other species (see Else et al. 2009) concluded that Ci increased upon waterlogging; that is, PN was not limited by gs. Our calculations of Ci for wheat during waterlogging on the basis of published PN and gs data (Fig. 3a,b) show that during short-term (1–3 d) waterlogging, Ci is maintained at close to that in leaves of controls (with some exceptions where values decline down to 80% of controls) and Ci increased during the long-term waterlogging to above control levels (304 and 252 μmol CO2 mol−1, respectively), supporting that in most cases, the reductions in PN for wheat in waterlogged soil were not the result of reduced Ci. Thus, declines in gs were likely a response to increased Ci. During long-term waterlogging, factors such as decreases in chlorophyll (Fig. 3c) or other components of the photosynthetic apparatus as a result of N deficiency and/or negative feedback from carbohydrate accumulation would be likely causes of reduced PN, although in some situations, possible damage to leaves potentially from

![Figure 3](http://example.com/f3.png)

**Figure 3.** Net photosynthesis (PN, a), intercellular CO2 (Ci, b) and total chlorophyll (c), in youngest leaf or flag leaf of wheat in waterlogged soil, as percent of drained controls; PN (n = 65) during 1–34 d of waterlogging, Ci (n = 23 and 24) and chlorophyll (n = 17 and 36) during short-term (1–3 d) or long-term (7–21 d) soil waterlogging. This summary figure was compiled from data extracted from peer-reviewed literature (data values, key experimental conditions and references are in Supporting Information Table S4). Boxes are 50% of the observations with the median shown as the horizontal line within the box, and bars are 1 and 99 percentiles. Median values of Ci of controls and short-term and long-term waterlogging were 251, 252 and 304 μmol mol−1, respectively. Ci was estimated as Ci = 385 μmol CO2 mol−1 (as atmospheric CO2) / (PN(gs)) (Long & Bernacchi 2003). Different letters indicate significant differences between medians of short-term versus long-term waterlogging (Mann–Whitney test, P < 0.05). ** Significant differences from 100% (Wilcoxon signed rank test, P < 0.01).
ROS or phytotoxins (Fe$^{2+}$ or Mn$^{2+}$) might also contribute. Interestingly, high nutrient application reduced the negative effect of waterlogging on PN in two experiments (Huang et al. 1994b; Wu et al. 2014). The positive effect on PN of improved mineral nutrition supports that leaf N deficiency in concert with feedback from increased sugar concentrations represses PN during waterlogging (Paul & Driscoll 1997). When measuring the effect of waterlogging on PN, $C_i$ values should be provided in order to aid interpretations.

Declines in PN when wheat is waterlogged are highly variable (Fig. 3a), and differences in environmental factors (e.g. temperature and evaporative demand) would contribute to this large variation. Studies designed to evaluate genotypic differences showed less variation, such as the following: (1) Musgrave (1994) was unable to detect a waterlogging × genotype interaction in eight genotypes, and (2) waterlogging-tolerant varieties only showed slightly higher PN rates than sensitive varieties, that is, PN relative to drained controls was 87% versus 76% and 89 versus 86% in two pairs of waterlogging-tolerant and waterlogging-sensitive varieties, respectively (Huang et al. 1994b; Zheng et al. 2009).

### Waterlogging adversely affects shoot nutrient levels in wheat

One of the first visible signs of waterlogging stress is accelerated leaf chlorosis (Fig. 3c) preceding early leaf senescence (Trought & Drew 1980a; Samad et al. 2001). The early yellowing of basal leaves of wheat during waterlogging has been linked to the remobilization of N from old to new leaves (Trought & Drew 1980b; Stieger & Feller 1994a). Our meta-analysis of previously published data (references in Supporting Information Table S6) on shoot nutrient concentrations for wheat in waterlogged soils highlights N deficiency as a key issue (Fig. 5a). Decreases in shoot nutrient concentrations with waterlogging duration are due to both decreased nutrient accumulation in the shoot (less uptake and transfer from the roots) and continued shoot growth, which results in some ‘dilution’ of the nutrients already present through increased shoot size.

The impact of nutrient deficiency on wheat in waterlogged soil, or hypoxic nutrient solutions, has been tested by increasing the availability of nutrients, and reviewed elsewhere (Colmer & Greenway 2011). Although shoot growth responds positively to increased nutrient supply, DM remained lower than aerated controls even when wheat was grown in N$_2$-flushed solutions with high nutrient concentrations (Barrett-Lennard et al. 1988), so other factors (e.g. regulation of the root:shoot ratio via hormonal changes in the plant) must also limit shoot growth of these plants with impaired root growth (Colmer & Greenway 2011).

### Possible toxicity to shoots of microelements

Observations of responses to soil waterlogging of wheat genotypes characterized as waterlogging-tolerant in Mexico (Van Ginkel et al. 1991; Boru et al. 2001) but not in Australia (Condon 1999; Setter et al. 1999) raised questions of how soil chemistry affects waterlogging tolerance in wheat (Setter et al. 2009; Yaduvanshi et al. 2012). Some authors have argued for increased evaluation of ion toxicity tolerances and breeding for tolerance to Fe$^{2+}$ and Mn$^{2+}$ to improve growth of wheat in some waterlogged soils (Setter et al. 2009; Shabala 2011; Khabaz-Saberi et al. 2012). Increased concentrations of Fe$^{2+}$ and Mn$^{2+}$ in the shoots of wheat during waterlogging have been reported (Stieger & Feller 1994a; Khabaz-Saberi et al. 2005;
Setter et al. 2009; Khabaz-Saberi et al. 2010a; Khabaz-Saberi et al. 2012), but others have failed to confirm microelement toxicity (diagnosed by shoot concentrations) prevailing in wheat during waterlogging (Trought & Drew 1980a, 1980b; Steigler & Feller 1994b; Steffens et al. 2005). Shoot concentrations of other microelements either showed negligible response to waterlogging (Cu, Zn and B) or, to our knowledge, have not been investigated for wheat (Ding & Musgrave 1995; Khabaz-Saberi et al. 2005; Setter et al. 2009). In the case of strongly acidic soils that become

Figure 5. Shoot nutrient concentrations in wheat during soil waterlogging (WL). (a) N (n = 35) and K (n = 11) and (b) P (n = 11), Mg (n = 6) and Ca (n = 11) in wheat (28 genotypes) after waterlogging in 11 different soils. In (a) and (b) boxes are 50% of the observations with the median shown as the horizontal line within the box, and bars are 1 and 99 percentiles. Red horizontal lines display the critical whole shoot value for nutrient deficiency in wheat at Feeke’s scale 5–7; plant age was 23–155 d at sampling (Snowball & Robson 1991; Reuter 1997). The median concentration of N [15.8 mg g⁻¹ dry mass (DM)] is significantly lower than the critical deficiency level (37 mg g⁻¹ DM) (Wilcoxon signed rank test, *P < 0.05; ****P < 0.0001). Data are from experiments in pots of soil or in field situations with waterlogging periods of 7–49 d and shoots sampled during waterlogging (recovery periods not included). In (c), shoot concentrations of micronutrients Mn (n = 65, median = 135, grey line; these median values were significantly lower than the toxicity threshold, P < 0.0001) and Fe (n = 65, median = 209, grey line) of wheat in drained soil and after waterlogging for 2–86 d in 16 different soils. Closed symbols indicate experiments conducted on acidic soils (pH_CaCl₂ < 4.8), open symbols indicate pH_CaCl₂ > 4.8. Different letters denote significant differences (P < 0.05) between shoot micronutrient concentrations in controls and WL conditions (Mann–Whitney test). The blue horizontal line displays the critical value for Mn toxicity in whole shoots, 37 d after sowing (Reuter 1997). Plant age was 26–70 d at sampling. No critical value for Fe toxicity could be found for wheat, but upper sufficiency level is 100 and 300 mg kg⁻¹ DM for whole shoots and young leaves, respectively (Mills et al. 1996; Reuter 1997). Mn concentration median is significantly lower than critical levels for toxicity (Wilcoxon signed rank test, P < 0.05). This summary figure was compiled from data extracted from peer-reviewed literature (data values, key experimental conditions and references are in Supporting Information Table S6).
Our meta-analysis of published shoot concentrations of Mn and Fe for wheat in waterlogged versus drained soils shows that shoot Mn concentrations did not increase significantly from several sources (Fig. 5c; references in Supporting Information Table S6); some studies even found decreases in shoot Mn (Trought & Drew 1980b; Steffens et al. 2005; Khabaz-Saberi & Rengel 2010) contrasting with others that showed Mn accumulation (Khabaz-Saberi et al. 2005; Setter et al. 2009). Mn and Fe accumulated to high levels mainly when wheat became waterlogged in strongly acidic soils (pH\textsubscript{CaCl\textsubscript{2}} = 4.1–4.8).

Possible adverse effects of microelements in shoots might be assessed from tissue concentrations where critical values for toxicity have been established for tissues and species (Reuter 1997). For wheat, Reuter (1997) lists critical values for Mn toxicity from several sources (range of 160–1100 mg kg\textsuperscript{-1} DM) but no critical value for Fe toxicity in wheat. The wide range of Mn critical toxicity values highlights the uncertainty of this approach. In our meta-analysis, we tested published shoot Mn concentrations against a toxicity threshold of 380 mg kg\textsuperscript{-1} DM, which is the toxicity value (Reuter 1997) for 37-d-old plants (Mn concentrations in Fig. 5c are from 26- to 70-d-old plants) and this value according to Reuter (1997) also summarizes the array of published tissue tests available for wheat and hence is a suitable benchmark to diagnose shoot nutrient disorders at mid-late tillering stage. Our analysis shows that the Mn toxicity threshold is only reached on very few occasions, which were for wheat when waterlogged on strongly acidic soils, and that the median of shoot Mn concentrations is significantly lower than the toxicity threshold (Fig. 5c). Interestingly, Setter et al. (2009) considered 100 mg Fe kg\textsuperscript{-1} DM to be toxic for wheat, but the basis of this value was unclear, as Reuter (1997) does not list a Fe toxicity value for wheat. Thus, shoot critical values for toxicity of Fe in wheat need to be established in order to further evaluate the shoot Fe concentrations compiled in Fig. 5c.

A strong case for the importance of microelement toxicities in wheat during 40 d of waterlogging on strongly acidic soils (pH\textsubscript{CaCl\textsubscript{2}} = 4.1–4.3) was that varieties tolerant to high Fe and Mn (and also Al) had 32–53% higher relative shoot DM compared with regular varieties (Khabaz-Saberi & Rengel 2010) and subsequently also yielded better (Khabaz-Saberi et al. 2012). However, the better shoot growth of the microelement-tolerant varieties on waterlogged acidic soils in Khabaz-Saberi et al. (2012) contrasts with the same microelement-tolerant and microelement-intolerant varieties evaluated in waterlogged acidic soils for which no difference was found (Setter et al. 2009). Here, Mn-tolerant Norquay (Khabaz-Saberi et al. 2010b) waterlogged for 49 d in a strongly acidic soil (pH\textsubscript{CaCl\textsubscript{2}} of 4.7) showed similar intermediate waterlogging tolerance as Mn-intolerant Columbus (see Fig. 2 in Setter et al. 2009), so Mn tolerance does not necessarily determine waterlogging tolerance even on acidic soil, or perhaps, other toxicities (e.g. Al or Fe) were affecting shoot growth in this study (Setter et al. 2009). That shoot Fe concentration during waterlogging can be a poor indicator of microelement stress is reflected by Fe tolerant varieties having higher Fe shoot concentrations than Fe-intolerant ones (Khabaz-Saberi & Rengel 2010; Khabaz-Saberi et al. 2012).

In summary, microelement toxicity likely impacts on wheat when waterlogged in some acidic soils, but the shoot micronutrient data available indicate that the problem is secondary to the main effect of O\textsubscript{2} deficiency impeding root growth. This conclusion is limited by the apparent lack of reliable shoot Fe critical concentrations for toxicity in wheat. Furthermore, whether tolerance to high soil microelements during waterlogging is governed by ‘exclusion’ from the roots and shoots and/or by ‘tissue tolerance’ of high concentrations within cells cannot be concluded at present and requires additional research.

**Summary**

Waterlogging reduces shoot growth of wheat, reflecting the constraints on roots. The large variation in growth reductions (Fig. 1) results from variable conditions (see Environmental Parameters Influencing Wheat Waterlogging Responses section), but also genotypic differences in tolerance as highlighted within some experiments. Waterlogging results in shoot N deficiency (Fig. 5a), which likely contributes to reduced growth. Tissue sugars often increase, despite reductions in P\textsubscript{2}O\textsubscript{5} and reduced leaf area, as sugar consumption declines owing to slow root and shoot growth. C\textsubscript{i} in leaves of waterlogged wheat is often similar to, or even exceeds, levels in drained controls, indicating non-stomatal limitations determine rates of P\textsubscript{2}O\textsubscript{5}, including possible down-regulation by high sugars. Direct adverse effects of microelements on shoot tissues appear to occur only for wheat in some acidic soils when waterlogged.

**RECOVERY FROM TRANSIENT WATERLOGGING AND GRAIN YIELD**

**Recovery of wheat seminal and adventitious root growth following waterlogging**

Most experiments investigating the effects of waterlogging on plants have not included a recovery period (Malik et al. 2002; Striker 2012), with a few exceptions and also several evaluations of crop grain yields following transient waterlogging. Recovery of wheat involves allocation of carbon to roots after waterlogging (Malik et al. 2001, 2002) and hypoxia (Araki et al. 2012b) for preferential root growth to reestablish a root: shoot ratio typical of plants in drained soil (fig. 7 in Malik et al. 2002). This preferential resource allocation to root growth would be a major reason explaining the reduced shoot growth following a period of waterlogging (Malik et al. 2002; Robertson et al. 2009). The bulk of the recovery growth is by adventitious roots (main axes and laterals) that resume extension, as seminal root apices (and much of the other tissues of seminal roots) can die within 3–4 d of waterlogging (Malik et al. 2002).

Recovery of the seminal root growth after 3 d of waterlogging resulted in structural DM during 25 d of recovery reaching a modest one-third to one-half of that of drained controls, and there was no recovery growth of seminal roots after 14 d of waterlogging (Malik et al. 2002). This was related to the death
of the apical meristems at the end of the low porosity seminal roots, and the low ability to form new lateral roots from the surviving basal portions of the seminal roots, as illustrated also by pruning of distal portions of seminal roots of 13- to 20-d-old wheat in aerated nutrient solution (Malik et al. 2002). By contrast, new laterals did form for 16-d-old wheat after 10 d growth in N₂-flushed nutrient solution and return to aerated solution (Barrett-Lennard et al. 1988). As temperature and plant age were similar, the difference could be due to quiescence of lateral initials during the period of O₂ deficiency, which was then subsequently released, or to the different genotypes used and is an interesting parameter to study further in a greater number of wheat genotypes. A study comparing seminal root lateral formation in 11 genotypes after 72 h of anoxia showed that number of laterals per cm main root varied from 0.28 to 2.61, and that most laterals were formed on roots that had lost their elongation potential during anoxia, presumably reflecting the loss of apical dominance, and thus correlated negatively with anoxia and waterlogging tolerance (Goggins & Colmer 2007). Recovery growth was also evident when six wheat genotypes were grown in hypoxic (5% O₂) nutrient solutions for 21 d followed by 7 d of re-aeration; although total length of laterals was reduced significantly in all genotypes during the hypoxia, this parameter recovered to 80% to 100% of control levels after 7 d re-aeration (Huang et al. 1994a). Recovery growth, however, did not occur for two wheat genotypes in stagnant agar solution for 14 d; root tips became flaccid and brown, and the seminal root FM did not increase upon re-aeration for 7 d (Watkin et al. 1998). The generally poor re-growth of the seminal roots of wheat highlights the importance of adventitious root growth after water subsides. This was further supported by Kuiper et al. (1994) showing that adventitious roots’ contribution to nutrient uptake increased dramatically during hypoxia and recovery, compared with the seminal root system.

In contrast to seminal roots losing their ability to re-grow within days of waterlogging, the tips of adventitious roots remained alive and resumed extension upon re-aeration after 21 d of waterlogging (Malik et al. 2002), 14 d in stagnant deoxygenated agar (Watkin et al. 1998) or 10 d in N₂-flushed nutrient solution (Barrett-Lennard et al. 1988), most likely due to a continued aeration of the roots tips via aerenchyma connected to the shoot (see Internal-O₂ Supply Determines Root Growth in Waterlogged Soils section). The continued growth/survival of the adventitious roots resulted in a greater proportion of root mass than the seminal roots after 14 d of waterlogging, and this difference was even more pronounced after 14 d of recovery (Trought & Drew 1980a).

During waterlogging or nutrient solution hypoxia, sugars (especially fructans) accumulate in wheat shoots and roots (see Substrate Supply section in Anoxia Tolerance of Wheat Roots section). When transferred back to aerated conditions from O₂-deficient nutrient solutions, wheat roots (measured for adventitious roots or for entire root systems) showed increased respiration rates compared with both continuously aerated controls and the previous anoxic period (Albrecht & Wiedenroth 1993, 1994; Huang & Johnson 1995; Boru et al. 2003; Araki et al. 2012b); for example, respiration of 20 mm adventitious root tips increased in one variety to 32% above controls following 21 d of hypoxia (Huang & Johnson 1995). The increase in respiration of roots upon return to aerated conditions is most likely due to increased biosynthesis, supported by consumption of the stored carbohydrates (Albrecht et al. 1993). Much of this substrate seems to be used upon root re-growth (Albrecht & Wiedenroth 1993; Malik et al. 2002), as root relative growth rates can be 30–40% higher after hypoxia compared with plants in continuous aeration/well-drained conditions (Barrett-Lennard et al. 1988; Malik et al. 2001). Photosynthetic rates of wheat were able to recover within 3–14 d upon soil drainage and re-entry of O₂ into the root zone (Malik et al. 2001; Shao et al. 2013), but recovery of shoot growth might be limited by N supply (see Nutrient Uptake section in How Does O₂ Deficiency Affect Wheat Root Functioning section).

### Reactive oxygen species defense systems in wheat roots during re-aeration

When water recedes and O₂ re-enters the soil and root tissues, the formation of ROS (e.g. superoxide, hydroxyl radicals, singlet oxygen and hydrogen peroxide) might especially damage cell membranes. ROS production is a consequence of a low energy charge, high level of reducing equivalents and saturation of the mitochondrial electron transport chain, favouring electron leakage to O₂ upon re-aeration (Blokhina et al. 2003). ROS can be detoxified by defense mechanisms including enzymes (e.g. superoxide dismutase (SOD)) and antioxidants (e.g. ascorbate (ASA) and glutathione (GSH)). The effect of O₂ deficiency on the antioxidant system is species-specific (Blokhina et al. 2003); in the following paragraph, the responses in wheat roots are discussed. The recovery of extension of adventitious roots near their maximum lengths (i.e. with very low O₂ at the tips during waterlogging) indicates that, if ROS increased during re-aeration, any damage was either controlled or repaired as growth resumed.

In wheat seedling roots subject to re-aeration upon 3–8 d of O₂ deficiency, reduced glutathione (GSH) and ascorbate (ASA) became oxidized to dehydroascorbate (DHA) and oxidized glutathione (GSSG), pointing to increased oxidative stress (Albrecht & Wiedenroth 1994; Biemelt et al. 1998; Blokhina et al. 2000) as did excised roots subjected to cycles of 16 h anoxia/8 h re-aeration during the third period of anoxia, defined as a GSH:total glutathione factor below 0.9 (Goggins & Colmer 2005). Duration of O₂ deprivation prior to re-aeration influences the degree of ROS production and plant response because of the following: (1) root H₂O₂ levels upon re-aeration increased with duration (2, 4 and 8 d) of the previous anoxic period (Biemelt et al. 2000), (2) three cycles of 16 h anoxia were necessary for the glutathione pool to become significantly reduced in roots (Goggins & Colmer 2005) and (3) lipid peroxidation in roots occurred after 6 d of anoxia (Albrecht & Wiedenroth 1994) in contrast to rather short (16 h) anoxia periods (Goggins & Colmer 2005). Wheat roots exposed to prolonged O₂ deficiency showed reduced, or at best similar, activity of ROS scavenging and antioxidant replenishing enzymes (glutathione reductase, GR; ascorbate peroxidase, APX; CAT; SOD) during the anoxia period (Albrecht & Wiedenroth 1994;
Biemelt et al. 1998, 2000; Goggin & Colmer 2005), with some increased activities (e.g. APX and GR activities) during re-aeration (Biemelt et al. 2000; Goggin & Colmer 2005). These experiments used sudden shifts from anoxia back to aeration, whereas soil O2 might increase more gradually upon drainage, for example, 10 d for redox potential to increase to $\geq$400 mV (Thomson et al. 1992; Malik et al. 2001). So, whether the sudden changes in controlled experiments possibly cause higher ROS formation than might occur under a slower change in O2 supply should be evaluated. Hypoxic pre-treatment prior to anoxia can induce the ROS defense system, such as a 2.5-fold increase in total glutathione whereas it declined to 50% during anoxia (Goggin & Colmer 2005). To our knowledge, ROS defense systems have not been compared among a substantial set of wheat genotypes when exposed to low O2 stress and/or re-aeration, so potential variation in this aspect remains to be assessed.

**Effects of transient waterlogging on grain yield of wheat**

Waterlogging tolerance is by agronomic definition a high grain yield under waterlogged conditions or following a transient waterlogging and could refer to absolute yields or be relative to control plants/plots in non-waterlogged conditions. Our meta-analysis of data on the effect of waterlogging on yield of wheat shows a median yield depression to 57% of controls (Fig. 1, references in Supporting Information Table S2). In some cases, a low grain yield reduction due to waterlogging (15–27% reduction of drained controls) can merely reflect low yield potential under drained conditions (e.g. 3 of 15 varieties tested by Collaku & Harrison 2002), making such genotypes of low priority for growers but potentially valuable for breeding if the tolerance has a physiological basis (Collaku & Harrison 2002). Because shoot growth is primarily affected through reduced tillering, waterlogging reduces the number of spikes per square metre (Watson et al. 1976; Cannell et al. 1984; Belford et al. 1985). However, tiller production during recovery may alleviate this effect if waterlogging does not occur too late and N is available for recovery growth (Cannell et al. 1984; Robertson et al. 2009). Besides a reduced number of spikes per square metre, waterlogging can also reduce grain number per spike and individual grain weight, all these reducing grain yield (Araki et al. 2012a; de San Celedonio et al. 2014).

The timing of waterlogging determines which yield components are affected the most in wheat. Waterlogging at early vegetative stages reduces tillering and hence spikes per plant, waterlogging at anthesis stage can reduce numbers of florets and hence grains per spike and waterlogging during grain filling can reduce weight of individual grains (Belford et al. 1985; Sayre et al. 1994; Araki et al. 2012a; Powell et al. 2012; Shao et al. 2013; de San Celedonio et al. 2014; Marti et al. 2015). A similar pattern of plant stage and the yield component affected has been reported for the effect of drought stress on wheat (Powell et al. 2012). Short waterlogging periods of 1–3 d can result in long-term detrimental effects on both growth (Malik et al. 2002) and yield of wheat (Sharma & Swarup 1988; Melhuish et al. 1991). However, under some conditions (4–12 °C), wheat can recover from prolonged waterlogging (42 to 80 d) with yields achieving 82–96% of controls (Cannell et al. 1980; Belford 1981; Belford et al. 1985). Temperature or soil type differences might explain these huge differences in responses of wheat between some studies (see Environmental Parameters Influencing Wheat Waterlogging Responses section).

Wheat might suffer N deficiency during waterlogging, which if it persists, would also be detrimental to recovery growth and grain yield (Watson et al. 1976; Cannell et al. 1980; Setter 2000; Robertson et al. 2009). Additional N fertilizer after waterlogging reduced grain yield loss by 20% for wheat in pots of soil (Robertson et al. 2009). However, if roots have suffered substantial dieback, then N application after waterlogging might not result in substantial yields (Setter 2000). Foliar spraying of N might not be practical in most situations, but wheat that received foliar N during 6 d of waterlogging after anthesis had smaller declines in grain number per spike and in individual seed weight (Wu et al. 2014).

Yield responses depend on the developmental stage when waterlogging occurs. For wheat, waterlogging was most damaging during pre-emergence, killing seeds and/or very young seedlings (Setter & Waters 2003). Effects of waterlogging on seeds or emerging seedlings are a result of complete submergence, as water near the soil surface could inundate all tissues of seeds/seedlings during emergence, including the coleoptiles; for discussion of submergence versus waterlogging, see Bailey-Serres & Voesenek (2008) and Colmer & Voesenek (2009). In contrast to rice, wheat seeds cannot germinate under anoxic conditions (Perata et al. 1992). Inability of anoxic wheat seeds to break down starch into sugars contributes to their lack of germination; provision of exogenous sugars resulted in 84% germination but still only enabled a few millimetres of root elongation and no coleoptile growth in anoxia (Perata et al. 1992).

After germination and emergence, the two other stages at which waterlogging is most detrimental for wheat yields are at the seedling stage and at anthesis (Setter & Waters 2003). Waterlogging periods of 15 to 20 d imposed on two varieties in five consecutive periods (three vegetative and two reproductive stages) along the crop life cycle with drainage and recovery after each waterlogging period showed the highest yield penalties occurred when waterlogging was applied during stem elongation to anthesis (de San Celedonio et al. 2014). However, the stage at which yield of wheat is most affected seems to depend on a combination of variety and environmental conditions. The highest yield reduction was found at post-anthesis waterlogging due to impaired grain filling caused by earlier leaf senescence and shortening of the grain filling period (Araki et al. 2012a), rather than at anthesis as described by de San Celedonio et al. (2014). Environmental effects causing higher waterlogging sensitivity at later than at early developmental stages could be higher temperatures leading to faster O2 depletion, a higher evaporative demand during the warmer late-season conditions and plant factors such as large reductions in root:shoot ratio that could occur in these older plants.

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Interestingly, a significant genotype by waterlogging timing interaction was found when analysing yield reductions in 16 spring wheat varieties waterlogged at five different developmental stages for 28–42 d. A remarkable example was for a Chinese genotype, which achieved grain yield of only 18% of control when waterlogged for 28 d at first node to mid-boot stage, but grain yield was 93% of control when waterlogged for the same duration at anthesis to grain filling stage; in several other varieties, yield reductions at these two stages were similar (e.g. 49% and 54% of controls in one variety, Sayre et al. 1994). The contrasting responses were proposed to reflect genotype breeder selection for better adapted materials to the different onsets of waterlogging that prevail in different climates, for example, late-season waterlogging in Southern China and Argentina compared with earlier waterlogging events in Mexico (Sayre et al. 1994). Chinese and Argentinean varieties better tolerated waterlogging at reproductive stages (93%, 84% and 82% of controls) compared with Mexican varieties that were superior at early stages but showed large reductions during reproductive stage waterlogging (66–88% of controls). Even though selection specifically for waterlogging tolerance may not have been conducted, tolerance may have been incorporated in the germplasm by selection for yield in the specific environments/aggregateological systems. Similarly, it has been suggested that waterlogging tolerance in winter wheats may have been indirectly selected for when breeding varieties in the UK for increased winter hardiness (Dickin et al. 2009).

Grain quality of wheat impacted by waterlogging has been considered in few studies. Waterlogging can decrease grain protein content, consistent with the earlier described shoot N deficiency (Fig. 5a), and thus affect processing quality (Olgun et al. 2008; Ashraf 2013), although this also varied between genotypes (Setter 2000). Individual grain weight, also a quality parameter, has been shown to decrease significantly (single grain weight 17% of controls) when waterlogging occurred during late developmental stages, such as during grain filling (Araki et al. 2012a; de San Celedonio et al. 2014).

**Summary**

During recovery periods, growth mainly takes places in adventitious roots as root:shoot ratio is restored, while seminal root re-growth is poor due to death of apical meristems and low formation of laterals. Root re-growth is fuelling both by sugars accumulated during waterlogging and by subsequent recovery of photosynthesis. The high substrate demand by the root system hampers shoot growth, eventually reducing grain yield. Shoot N deficiency may also cause a decline in grain yield and quality. Wheat cultivars differ in their waterlogging tolerance according to their growth stages. This is of importance to cultivation in fields prone to flooding at different times, and for design of screening and breeding strategies aimed at improvement of waterlogging tolerance in wheat.

**CONCLUSIONS AND OUTLOOK**

Root growth and physiology in wheat are adversely affected by soil waterlogging, the magnitude of which depends on genotype, plant developmental stage and the prevailing environmental conditions (especially soil type and temperature). Genotypic variation is evident for some root responses to low O₂, such as seminal root survival in short-term anoxia, adventitious rooting and amount of aerenchyma and recovery growth of lateral roots post-anoxia. Anoxia tolerance of genotypes is not related simply to ethanolic fermentation rates; further research should evaluate energy requirements for maintenance of anoxic roots and whether differences in efficiency of energy use contribute to anoxia tolerance in wheat. Ability for sugar transport via phloem to root apices during hypoxia and anoxia should also be assessed. Genotypes differ both in the numbers of adventitious roots formed and the porosity (i.e. amount of aerenchyma) and thus internal movement of O₂ into and along the roots. Even for these acclimated adventitious roots, O₂ becomes limiting at the root tip when relatively short, as porosity is moderate and O₂ is consumed in respiration and lost radially to the rhizosphere, so that growth ceases. Energy-dependent ion transport is inhibited, resulting in a restricted nutrient uptake and translocation to the shoots. Tissue sugars often increase, despite declines in photosynthesis, as demand for sugars is markedly reduced when growth is inhibited. N deficiency in shoots contributes to reduced growth during waterlogging, and would slow recovery growth. Mn or Fe toxicity can impact on wheat when waterlogged in some strongly acidic soils. Following waterlogging, adventitious root growth resumes and lateral roots possibly emerge from some of the remaining seminal roots. Ability to recover upon drainage following waterlogging is important for yield.

Identification of quantitative trait loci (QTL) associated with waterlogging tolerance in wheat (e.g. Ballesteros et al. 2014) opens up avenues, with further work, for possible marker-assisted selection and discovery of underlying genes. For submergence tolerance of rice, a very different situation to soil waterlogging of wheat with shoots in air, identification of the group VII ethylene response factor (ERFVII) transcription factors, and more specifically the SUB1A locus, has transformed rice breeding for submergence tolerance (Mackill et al. 2012). Such transcription factors regulating coordinated expressions of stress-responsive genes, if identified also for roots in waterlogged conditions, might be deployed for crop breeding (Bailey-Serres et al. 2012). Recent work using RNA interference has modulated in barley an ERFVII that is a substrate of the N-end rule pathway, with these transgenic plants showing improved waterlogging tolerance (Mendiondo et al. 2015).

We suggest three priority areas for research on traits that could contribute to breeding of more waterlogging-tolerant wheat varieties: (1) internal aeration of new adventitious roots, (2) N deficiency of the shoot and (3) recovery ability following transient waterlogging. Progress in research on root aeration in crops is worthwhile to highlight. Identification of a QTL for root porosity (as a surrogate for aerenchyma) in barley (Broughton et al. 2015) is of interest to explore also for wheat. Moreover, microarray analyses of laser micro-dissected root...
tissues of maize (Rajhi et al. 2011) and rice (Shiono et al. 2015) have identified candidate genes for aerenchyma (maize) and ROL barrier induction (rice). Use of wild relatives, such as *H. marinum* for wheat (Malik et al. 2011) and *Zea nicaraguensis* for maize (Mano et al. 2007; Abiko et al. 2012; Mano & Omori 2013), to introduce some traits (e.g. root constitutive aerenchyma and a ROL barrier) could improve waterlogging tolerance, and these wide-crosses also provide novel resources for gene discovery. Knowledge of genome sequences (e.g. wheat, IWGSC 2014) will aid translation of findings to wheat. The N deficiency in wheat owing to waterlogging could also be addressed both by seeking improved adventitious root nutrient uptake during waterlogging and possibly also targeting N-use efficiency in wheat, which is also being sought for other reasons (e.g. Jackson & Ismail 2015). Finally, much remains to be learnt regarding recovery of root growth following anoxia and waterlogging, and whole-plant recovery more generally, both of which show some apparent genotypic variation in wheat.

ACKNOWLEDGMENTS

T.D.C. acknowledges support from the Australian Research Council (DP12010482). We thank the UWA Institute of Advanced Studies for hosting O.P. as Professor-At-Large and for supporting G.S. with a Stay-fellowship to visit UWA. M.H. was supported by a PhD fellowship from the Villum Foundation.

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Table S1. Wheat growth from studies where wheat was grown in soil under waterlogged (water at soil surface unless otherwise stated) and drained conditions and recording both shoot and root dry mass (DM), allowing for root:shoot ratio calculation. Values are % reduction from controls [1-(waterlogged/drained)]. When multiple nutrient-levels were used we refer to the lowest. WL=waterlogged, DAS=Days after sowing.

Table S2. Grain yield reductions from studies where wheat was grown in soil under waterlogged (water at soil surface unless otherwise stated) and drained conditions. Values are % reduction from controls [1-(waterlogged/drained)]. When multiple nutrient-levels were used we refer to the lowest. WL=waterlogged, DAS=Days after sowing.

Table S3. Summary of average root porosity (POR) or aerenchyma (AER) in wheat roots of different varieties, plant age, growing media to induce hypoxia, hypoxia/waterlogging duration, type of root (seminal and/or adventitious-nodal).

Table S4. Reductions in photosynthetic rates calculated from studies where wheat was grown in soil under waterlogged (water at soil surface unless otherwise stated) and drained conditions. Values are % reduction from controls [1-(waterlogged/drained)]. All nutrient treatments were included. WL=waterlogged, DAS=Days after sowing, DAA=days after anthesis.

Table S5. Summary of values/range for photosynthesis (PN) and stomatal conductance (gs) in wheat under control and waterlogging conditions used for calculations in Figure 6. Information on waterlogging (WL) duration, variety, PN, gs, phenological stage during measurements and source are provided. The numbers in brackets identify the bibliographic source of each point in Figure 4.

Table S6. Shoot nutrient levels of macronutrients N, P, K, Mg, Ca (mg g\(^{-1}\) DM) and microelements Mn, Fe, Zn, Al (mg kg\(^{-1}\) DM) after soil waterlogging.
Chapter 2: Physiology, gene expression and metabolome of two wheat cultivars with contrasting submergence tolerance

Wheat subject to complete submergence in a constant temperature room at the University of Copenhagen, October 2016. Photo: Max Herzog.
Submergence tolerance in two wheat cultivars

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Abstract

Global climate change is projected to regionally increase floods on agricultural soils, reducing wheat (Triticum aestivum) yields. Responses of wheat to complete submergence are not well understood as research has focused on waterlogging (soil flooding). The aim of this study was to characterize the phenotypic responses of two wheat genotypes (cultivars) to complete submergence and to determine traits conferring submergence tolerance. 18-day-old wheat cv. Frument and Jackson grown in pots were completely submerged for up to 19 days while assessing responses in physiology, gene expression and shoot metabolome (metabolomics). Results revealed 50% mortality after 9.3 and 15.9 days of submergence in Frument and Jackson, respectively, and 3-fold higher relative growth rate in Jackson during 8 days of submergence and 25 days of recovery. Frument displayed faster leaf degradation as evident from metabolomic fingerprinting and earlier declines in leaf chlorophyll, and leaf tissue porosity. Surprisingly, shoot soluble carbohydrates, starch and individual sugars declined to similar low levels in both cultivars by day 5, in spite of Frument overexpressing genes encoding sucrose and fructan degrading enzymes relative to Jackson. Frument showed accelerated leaf chlorosis and higher levels of phytol relative to Jackson, suggesting higher ethylene sensitivity in the intolerant cultivar as seen in rice (Oryza sativa). The lipid peroxidation marker malondialdehyde increased from day 12 in Frument but not in Jackson, indicating higher reactive O2 species (ROS) inflicted damage in Frument. Our study suggests that ethylene sensitivity and ROS deprivation could be mechanisms that determine the vast differences in flood tolerance.

Introduction

Wheat (Triticum aestivum) faces flooding on estimated 15-20% of its cropping area each year, reducing growth and yield (Sayre et al., 1994; Setter and Waters, 2003), where waterlogging reduces average grain yield by 43% (Herzog et al., 2016). In the USA, 2016 insurance payouts due to floods totaled US$217mn,
which was 3.4-fold higher than payouts due to droughts and more than any other stressor alone (U.S. Risk Management Agency, 2016). Floods are expected to increase in the coming decades in major wheat production areas due to climate change, increasing demand for the development of more flood-tolerant wheat cultivars (Trnka et al., 2014). The terms “flooding” and “waterlogging” are often used interchangeably to describe excessively wet conditions, but in the following we apply “waterlogging” only when the root zone is flooded, and “submergence” when, in addition to the root system, all (or part of) the aboveground organs are under water (c.f. Sasidharan et al., 2017).

Waterlogging reduces soil O$_2$ levels due to the relatively low solubility of O$_2$ in water and the 10,000-fold slower gas diffusion in water compared to air (Armstrong, 1979). O$_2$ is quickly consumed by roots and soil microorganisms, resulting in severe hypoxic or even anoxic conditions in waterlogged soils (Ponnamperuma, 1984). Root O$_2$ deficiency hampers root growth and function of wheat (Trought and Drew, 1980). In addition to O$_2$ deficiency, various potentially toxic compounds such as Mn$^{2+}$, Fe$^{2+}$, H$_2$S and organic acids can accumulate to phytotoxic levels in waterlogged soils (Ponnamperuma, 1984). When floodwaters recede and tissues are re-oxygenated, reactive O$_2$ species (ROS) such as superoxide radicals, hydrogen peroxide or singlet O$_2$ may form and damage cell structures such as membranes, proteins and nucleic acids (Blokhina et al., 2003). When floodwaters rise above soil level, shoot tissues become submerged further increasing stress (Colmer and Voesenek, 2009). Availability of O$_2$ and CO$_2$ for aerobic respiration and photosynthesis, respectively, becomes limited during submergence, as the gasses must overcome diffusional resistances of the cuticle as well as the diffusive boundary layers (Mommer et al., 2005). Submerged terrestrial vegetation may therefore experience an ‘energy crisis’ due to low carbohydrate production and the low energy harvest in anaerobic glycolysis (Gibbs and Greenway, 2003).

Plants growing in flood-prone areas, i.e. terrestrial wetland plants, display traits that confer flood tolerance (Colmer and Voesenek, 2009). A key trait is ‘internal aeration’ via interconnected air spaces enabling internal O$_2$ gas phase diffusion to submerged organs such as roots. O$_2$ reaching submerged tissues may originate from floodwaters (Winkel et al., 2013), endogenously from underwater photosynthesis (Winkel et al., 2011; Pellegrini et al., 2017) or ‘snorkeling’ (Voesenek and Blom, 1989; Colmer and Voesenek, 2009; Herzog and Pedersen, 2014) by shoot tissues protruding into the atmosphere. Moreover, roots of most terrestrial wetland plants form a barrier to radial O$_2$ loss (ROL) reducing O$_2$ loss to anoxic soils along the roots base, ensuring adequate root tip O$_2$ for root growth (Armstrong, 1979; Colmer, 2003). The gaseous phytohormone ethylene is known to induce such adaptations in both wild wetland plants (Visser et al., 1996), wheat (Huang et al., 1997) and rice (Oryza sativa) (Shiono et al., 2008).
Breeding for flood-tolerant crops has resulted in high yielding rice cultivars able to withstand 14 days of submergence (Ismail et al., 2013). While wheat waterlogging tolerance could have been enhanced by breeding for winter hardiness in UK cultivars (Dickin et al., 2009), waterlogging tolerance is to our knowledge not specifically selected for in wheat breeding, and thus waterlogging or submergence-tolerant wheat cultivars are yet to be released. Several studies have documented variation in waterlogging tolerance in wheat germplasm (root traits: Dickin et al. (2009); Hayashi et al. (2013); shoot biomass: Huang et al. (1994); Hayashi et al. (2013); grain yield: Van Ginkel (1991); Sayre et al. (1994); Collaku and Harrison (2002)), relating to seminal root short term anoxia tolerance (allowing seminal roots to resume growth following reaeration) and formation of porous adventitious roots improving root O₂ supply and thereby nutrient uptake (Herzog et al., 2016).

Meanwhile, traits conferring submergence tolerance in wheat have not yet been documented. Submergence of wheat has been observed to occur (Musgrave and Ding, 1998; Winkel et al., 2017) and to decrease yields relative to waterlogging (Samad et al., 2001). However, the extent of partial or complete submergence of wheat has to our knowledge not been estimated, but would be a prominent risk during early stages of development when plants are still small (e.g., prolonged seedling stage in winter wheat). Hence, the aim of this study was to assess wheat genotype variations in submergence tolerance, and to determine which traits confer submergence tolerance. Two wheat cultivars with different waterlogging tolerance were subjected to complete submergence in a pot experiment while assessing plant growth, plant survival, metabolites (metabolomics), gene expression and a range of physiologic parameters in order to identify possible traits conferring submergence tolerance. We hypothesized that submergence tolerance would be related to phenotype specific carbohydrate consumption as seen in rice (Das et al., 2005), prompting us to evaluate shoot levels of soluble carbohydrates, starch, expression of genes related to carbohydrate degradation and metabolites of the primary energy metabolism.

**Results**

*Wheat cv. Frument and Jackson exhibit contrasting submergence tolerance*

Growth of wheat cultivars Frument and Jackson was strongly impaired by complete submergence, even when provided 14-25 days recovery (Fig. 1). Survival and shoot relative growth rate (RGR) declined with time of submergence, but interestingly differed significantly between cultivars (Fig. 1). Cultivar RGR already differed significantly after 8 days of submergence being 0.06 d⁻¹ and 0.02 d⁻¹ in Jackson and Frument, respectively (compared to 0.1 d⁻¹ in drained controls), relating to Jackson and Frument shoot biomass at 24% and 13% of controls in air, respectively. Frument shoot RGR continued to decline faster than...
Jackson and thus two-way time × cultivar ANOVA detected significant time, cultivar and interaction effects. Importantly, when comparing cultivar growth during submergence assessed without a recovery period (i.e., harvesting shots directly after 8 days of submergence), both cultivars exhibited similar slightly negative RGR (Fig. S1).

Figure 1. Survival and growth of 18-day-old wheat cultivars Frument and Jackson during 0-19 day complete submergence and a following 14-25 day recovery period. (A) Photos illustrating growth and survival of Jackson and Frument wheat cultivars following complete submergence. All plants were 51 days old, but had been subject to varying lengths of submergence (0-19 days) and recovery (14-25 days, depending on the duration of submergence) when the photos were taken. After 19 days of complete submergence no plants survived, hence this time point is not shown. (B) Survival of wheat cultivars with time of submergence. Symbols represent survived plants/number of desubmerged plants after 0-19 days of submergence (n = 4-8) of Frument (open symbols) and Jackson (closed symbols). The blue and green lines represent the central tendency of a logistic model fitted to Frument and Jackson survival data, respectively. Shaded areas are 95% confidence intervals. The vertical dashed line indicates 50% survival. Survival was defined as the presence of turgid, green leaf material following recovery. (C) Shoot RGR of wheat cultivars Frument (open symbols) and Jackson (closed symbols) during submergence and a following recovery period. Plant RGR was calculated from initial biomass (at the start of submergence) and biomass when all plants were 51 days old, but had been subject to varying lengths of submergence (0-19 days) and recovery (14-25 days). Two-way ANOVA showed significant time, cultivar and interaction effects (P < 0.0001). * indicate significant difference between cultivars (Sidak’s multiple comparisons test, P < 0.05). Values are means (± SE, n = 4-8). Shoot DM of Frument and Jackson controls in air at the end of recovery (0 days of submergence) were not significantly different (t-test, P = 0.499, n = 6-7).

Plant survival also decreased significantly with the duration of complete submergence (Fig. 1, P < 0.05, Wald Chi-square test). Frument succumbed to submergence approximately seven days earlier than Jackson, evident from the time of submergence resulting in 50% mortality (LT50) of 9.3 and 15.9 days in Frument and Jackson, respectively (Fig. 1). Submergence duration resulting in 100% mortality also differed by seven days
Submergence tolerance in two wheat cultivars

(12 and 19 days in Frument and Jackson, respectively). Non-overlapping 95% confidence intervals of the modeled survival curves support that cultivars differed significantly in submergence tolerance (Fig. 1). In the following sections, we describe how this contrasting submergence tolerance is phenotypically reflected in physiologic, genetic and metabolic responses of the two cultivars.

Submergence induces faster leaf degradation in Frument than Jackson

Superhydrophobic wheat leaves retain a gas film when submerged (Raskin and Kende, 1983; Konnerup et al., 2017), and leaf gas films enhance wheat submergence tolerance (Winkel 2017). We therefore assessed if leaf gas film thickness during submergence differed between cultivars Frument and Jackson (Fig. 2). In both cultivars, leaf gas film thickness declined from initial 10-24 µm to below the 3 µm detection limit by day 5 and did not recover afterwards. Consequently, leaf gas film thickness did not differ significantly between cultivars according to two-way time × cultivar ANOVA.

**Figure 2.** Gas film thickness (A), Fv/Fm (B), chlorophyll a (C) and leaf tissue porosity (D) of Frument (open symbols) and Jackson (closed symbols) wheat cultivars with time of submergence. The leaf sampled was the youngest, fully expanded leaf at time of submergence (3rd leaf). Round symbols symbolize untreated controls in air at the end of the treatment period (day 16). Values are means (± SE, n = 4), except (A) where means are ± SD (n = 4). * indicate significant difference between cultivars at single time points (Sidak’s multiple comparisons test, P < 0.05). (A) Two-way ANOVA on Ln-transformed data only showed a significant time effect (P < 0.0001) and no significant differences between cultivars in post-hoc tests. (B) Two-way repeated measures ANOVA showed a significant time effect only (P < 0.001). (C) Two-way ANOVA showed significant time, cultivar and interaction effects (P < 0.0001). (D) Two-way ANOVA showed significant time, cultivar and interaction effects (P < 0.0001).
Leaf gas film disappearance was followed by indications of submergence induced leaf damage. $F_v/F_M$ ratios measured on day 0, 5, 8 and 12 on the youngest fully expanded leaf at the time of submergence indicated increasing damage to PSII in submerged plants (Fig. 2), with $F_v/F_M$ ratios declining from initial 0.79 to 0.65-0.67 on day 12. However, two-way ANOVA detected significant time but no cultivar effect ($P = 0.2702$).

Underwater photosynthesis ($P_N$) and dark respiration ($R_D$) rates were measured on days 0, 2, 8 and 16 of submergence (Fig. S2). In both cultivars, $P_N$ remained at initial 1.8-2.3 µmol O$_2$ m$^{-2}$ s$^{-1}$ until day 2, but approached zero on day 8. Leaf $R_D$ on day 2 was reduced to half of initial -0.45 to -0.47 µmol O$_2$ m$^{-2}$ s$^{-1}$ in both genotypes. Frument respiration rates on day 2 and 8 were 26-32% higher than Jackson but these differences were not statistically significant (Sidak’s multiple comparisons test, $P > 0.05$) and for $P_N$ and $R_D$ two-way ANOVA only detected significant time-effects.

In contrast to the above-mentioned factors where both cultivars responded relatively similar to submergence stress, tissue porosity and leaf chlorophyll a in the youngest fully expanded leaf at the time of submergence declined significantly faster in Frument than in Jackson. Chlorophyll a concentrations remained at initial 9.7-11.0 mg g$^{-1}$ dry mass (DM) in both cultivars until day 5, before declining faster in Frument than in Jackson (Fig. 2) resulting in almost three-fold higher chlorophyll a in Jackson on day 12 (Sidak’s multiple comparisons test, $P < 0.0001$). Tissue porosity remained at initial 20% for the first 8 days of submergence in both cultivars, until water began to infiltrate Frument leaves (Fig. 2). In contrast, Jackson leaf porosity did not decline until day 16. Therefore, the two-way ANOVA detected significant time, cultivar and interaction effects in both leaf chlorophyll a and leaf tissue porosity.

**Shoot carbohydrates decline to similarly low levels in Frument and Jackson**

Submergence tolerance of rice has been linked to reduced underwater elongation of leaves and internodes, resulting in lower carbohydrate consumption and lower mortality in non-elongating genotypes (Das et al., 2005). In order to evaluate if the contrasting submergence tolerance observed in this study was also related to differences in cultivar elongation and carbohydrate consumption, we measured shoot length and shoot carbohydrate concentrations during submergence.
Submergence tolerance in two wheat cultivars

Figure 3. The sum of ethanol and water soluble carbohydrates (A) determined using the anthrone-method, and fructose (B), glucose (C) and sucrose (D) determined by GC-MS in shoots of wheat cultivars Frument (open symbols) and Jackson (closed symbols) with time of submergence. Round symbols symbolize untreated controls in air at the end of the treatment period (day 16). In (A) the entire shoot except for the 3rd leaf (used for other physiological measurements, see Fig. 2) was homogenized and used for analysis. Two-way time × cultivar ANOVA on Ln-transformed data showed significant time (P < 0.0001), cultivar (P = 0.0396) and interaction (P < 0.0001) effects. Time explained 91% of the variation compared to cultivar (0.5%) and interaction (5%) effects. In (B), (C) and (D) entire shoots were sampled and two-way time × cultivar ANOVA showed significant time, cultivar and interaction effects; time; time and interaction effects, respectively. * indicate significant difference between cultivars (Sidak’s multiple comparisons test, P < 0.05), values are means (± SE, n = 3-4).

Initial concentrations of combined ethanol and water soluble carbohydrates were significantly higher (28%) in Frument than Jackson (Sidak’s multiple comparisons test, P < 0.05), but on day 2 the abundance of total soluble carbohydrates was similar in the two cultivars likely due to higher sugar consumption in Frument (Fig. 3). Soluble carbohydrate concentrations remained at similar levels until day 12 and 14, when they declined further in Frument resulting in shoots of Jackson containing ~30% more soluble carbohydrates, coinciding with leaf disintegration as indicated by loss in leaf porosity. At the end of treatment (day 16), soluble carbohydrate levels in both cultivars had declined to 21-31% of initials. The periodically higher soluble carbohydrate consumption by Frument resulted in ANOVA detecting significant time, cultivar and interaction effects with factor cultivar explaining 0.5% of the variation. Shoot fructose, glucose and sucrose determined using metabolomics had declined to equally low levels in both cultivars by day 2 (Fig. 3). By day 5 these sugars had declined to ~10% of initial values and did not recover. Interestingly, cultivars did not differ significantly at any submergence time point in any of these three sugars. The significant decline, as well as the only minor cultivar differences, was also evident in other sugars (tagatose, trehalose, kestose,
Quantifying soluble carbohydrates using the colorimetric reactions with anthrone indicated that shoots continue to possess sugar reserves at 20-30% of initial levels, while metabolomics data showed that especially glucose and sucrose decline to very low levels. The anthrone method is a popular method due to its simplicity and sensitivity, but has the disadvantage of a large difference in the color intensity produced by different types of sugars (Pontis, 2017). Combined with the arbitrary units of the metabolomics method this makes comparisons with absolute numbers from the anthrone analysis (Fig. 3) challenging. In addition, carbohydrates stored in sheath and stem tissues, which were also harvested, would conceal declines to very low levels in leaf tissues.

Submergence also caused starch levels to decline (Fig. S4). On day 2, Jackson and Frument contained 31% and 21% of initial starch concentrations, respectively, remaining at similar levels until day 8. From day 12-16 starch concentrations started to increase, especially in Frument. The increasing starch concentration at such late time points is surprising, as carbohydrate production would be insignificant considering the low \( P_N \). We suggest that the increasing starch levels reflect increasing leaf blade disintegration and detachment from the shoot, causing leaf sheaths (acting as the main wheat carbohydrate storage organ, Scofield et al. (2009)) to make up most of the tissue sample resulting in seemingly higher starch concentrations. Leaf disintegration was more severe and occurred earlier in Frument, likely explaining why starch reached highest concentrations in this cultivar.
Neither Frument nor Jackson shoots elongated during submergence, evident from submerged and control plants exhibiting similar shoot length after 8 days of treatment (Fig. 4). Shoot lengths measured on day 2 and 5 confirmed that shoots did not elongate compared to drained controls (data not shown). Thus, the slight difference in cultivar carbohydrate consumption was seemingly not caused by differences in elongation response.

In conclusion, submergence-intolerant cultivar Frument displayed higher carbohydrate consumption at the early time points (0-2 days), but otherwise, levels of soluble carbohydrates and starch were similar between cultivars. Frument experienced faster chlorophyll, and tissue porosity loss, while responses in leaf gas film thickness, FV/FM, PN, R0 and shoot length were similar amongst the two cultivars.

Figure 5. Multivariate analysis (A) and selected metabolites with time of submergence (B, C) in entire shoots of wheat cultivars Frument and Jackson during 16 days of complete submergence. (A) Principal component analysis (PCA) scores plots showing PC1 vs. PC2 (top) and PC1 vs. PC3 (bottom) of 1211 metabolite entities in shoots of submerged Frument (squares) and Jackson (triangles) wheat cultivars, the color legend identifying time points. Only metabolites that passed the quality control filter are included. Day 16 controls in air clustered with the initials (data not shown). (B) Phytol in wheat cultivars Frument (open symbols) and Jackson (closed symbols) with time of submergence. Two-way time × cultivar ANOVA on Ln-transformed data showed significant time, cultivar and interaction effects. (C) Malondialdehyde (MDA) in wheat cultivars Frument (open symbols) and Jackson (closed symbols) with time of submergence. Two-way time × cultivar ANOVA on Ln-transformed data showed significant time, cultivar and interaction effects. In (B) and (C) * indicate significant difference between cultivars (Sidak’s multiple comparisons test, P < 0.05), values are means (± SE, n = 4). Round symbols symbolize untreated controls in air at the end of the treatment period (day 16).
Submergence tolerance in two wheat cultivars

Metabolomic fingerprinting reveals accelerated metabolomic changes in submerged Frument

A total of 1211 out of 74,359 mass spectral features passed our quality controls filters (present in all samples of at least one group; in 80% of the quality control samples and with a coefficient of variance < 35%) following LC-MS analysis of Frument and Jackson shoot tissues. The 1211 metabolites were used for metabolomic fingerprinting without further annotation since the high number of reproducibly detected metabolites suggests good and robust metabolome coverage (Hasler-Sheetal et al., 2016; Lindahl et al., 2017). To visualize the metabolic changes due to submergence stress, we conducted a principal component analysis (PCA) showing clear treatment and cultivar related clustering of the samples (Fig. 5). PC1 explained 38.5% of the variance separating days of submergence, while PC2 and PC3 explained 25.3% and 7.21% of the variance, respectively, with especially PC3 separating the two cultivars. Within 2 days of submergence, both genotypes had moved relative to the initials (day 0) with Frument moving furthest along PC1 and PC2, indicating a stronger metabolic response in this submergence-intolerant cultivar. Frument metabolome continued to shift faster along especially PC1, resulting in Jackson day 8 and Frument day 5 clustering in the PC1 vs. PC2 plot (Fig. 5). By the end of the experiment, Frument day 14 and day 16 had moved furthest along PC1 and PC2, clearly separating these severely degraded shoots. A VENN-diagram of the 1211 metabolites showed that the metabolic changes separating treatments in the PCA plots were driven by changes in virtually all metabolites, with time significantly affecting 1152 entities and approximately half of the metabolites showing significant time, cultivar and time × cultivar interaction effects (Fig. S5).

Annotation of metabolic entities allowed for monitoring changes in amino acids, sugars, phytol (all GC-MS) and MDA (LC-MS). Phytol results from the initial step of enzymatic chlorophyll hydrolysis by chlorophyllase (Matile et al., 1999), with two-way ANOVA showing significant time, cultivar and interaction effects (Fig. 5). In both cultivars phytol increase coincided with chlorophylla decline in the youngest fully expanded leaf (Fig. 2) by day 5 (Frument) and day 12 (Jackson). Malondialdehyde (MDA) is considered a useful indicator of lipid peroxidation (Hodges et al., 1999), as measured using thiobarbituric acid-reactive-substances (TBARS) for assessing oxidative stress in wheat and rice during submergence or hypoxia (Albrecht and Wiedenroth, 1994; Li et al., 2011; Alpuerto et al., 2016). In our study, ANOVA showed significant time, cultivar and interaction effects for shoot MDA (Fig. 5). MDA in Jackson remained close to the initial levels while in Frument MDA increased from day 12, resulting in final levels 10 times higher than initials and 11 times higher than Jackson. However, it should be noted that Frument survival rates declined before MDA increased.

The metabolome analysis also revealed that 12 out of 17 measured amino acids (Asn, Gln, Ile, Leu, Lys, Met, Phe, Pro, Thr, Thr, Tyr, Val) increased in shoots during the first 12 days of submergence, with only 5
showing decreasing or unchanged levels in both cultivars (Ala, Asp, Glu, Ser, Gly; Fig. S6). During the first 12 days of submergence, Frument never had lower and occasionally significantly higher amino acid concentrations compared to Jackson, but on day 14 and 16 all amino acid levels in Frument had dropped below Jackson levels. E.g., Pro levels were significant 5-fold higher in Jackson than Frument from day 12-16 (Sidak’s multiple comparisons test, $P < 0.05$; Fig. S6). From day 14 to 16 amino acids in Jackson also generally declined, indicating that Jackson shoot tissues were increasingly degraded by that final time point. Meanwhile, levels of GABA did not differ between cultivars (data not shown).

**Frument exhibits higher expression of genes encoding carbohydrate degrading enzymes than Jackson**

The mRNA levels of 13 genes associated with carbohydrate degradation (fructan exohydrolases, kestose exohydrolase, sucrose synthases and $\alpha$-amylases) were quantified relative to the initial (day 0) levels in Frument using quantitative real-time PCR (qRT-PCR) on days 0, 2, 5, 8 and 12 (Fig. 6). While three genes encoding fructan and kestose exohydrolases were down-regulated by submergence or remained unchanged in both cultivars (6&1-FEH, 6-FEH, 6-KEHw2), two genes encoding fructan exohydrolases were significantly upregulated in Frument compared to Jackson (1-FEHw1, 1-FEHw3) at several time points. Similar patterns were observed in sucrose synthase genes: SUS3 and SUS11 were downregulated in both cultivars, while SUS4 and SUS5 were expressed at significantly higher levels in Frument than Jackson at several time points. The expression of $\alpha$-amylase genes generally increased with time, but in contrast to genes associated with fructan and sucrose degradation, only one out of four $\alpha$-amylase genes was expressed slightly (13-19%) higher in Frument than in Jackson ($\alpha$-AMY4-1, Fig. S7). In conclusion, during submergence Frument expressed genes encoding sucrose and fructan degrading enzymes at significantly higher levels than Jackson.
Submergence tolerance in two wheat cultivars

Figure 6. Relative mRNA levels of genes associated with carbohydrate degradation. Genes are fructan exohydrolases (1FEHw1, 1FEHw3, 6-FEH, 6&1-FEH), kestose exohydrolase (6-KEHw2) and sucrose synthases (SUS3, SUS4, SUS5, SUS11), for details on primers and annealing temperatures see Table S1. Transcripts of representative genes were quantified in shoots of wheat cultivars Frument (open bars) and Jackson (closed bars) exposed to submergence for 0-12 days by quantitative real-time PCR. The relative level of each mRNA was calculated by comparison with initial (day 0) Frument. Values are means (± SE, n = 3). * indicate significant differences between the cultivars (Student’s t-test, \( P < 0.05 \)).

Discussion

Comparing submergence tolerance in two wheat cultivars revealed contrasting survival, growth, physiologic and metabolic responses during the 19 days treatment period and following 14-25 days of recovery. Frument showed accelerated structural leaf degradation, leaf chlorosis, metabolic response and elevated MDA levels compared to Jackson, while shoot carbohydrate consumption rates only differed during the initial 2 days of submergence. In the following sections we discuss these findings in relation to especially rice submergence responses.

Compared to growth during waterlogging reported in the literature, submergence induced higher growth reductions with wheat shoot biomass reductions to 29-31% of controls following 14 days of waterlogging (Malik et al., 2001; Robertson et al., 2009) and to 2-6% of controls following 14 days submergence in this study. The larger growth penalty due to submergence resulted from negative shoot RGR during submergence (-0.03 \( \text{d}^{-1} \) during 8 days, Fig. S1) compared to positive shoot RGR of 0.09 \( \text{d}^{-1} \) during 14 days of waterlogging (Malik et al., 2001) and would also have been aggravated by senesced shoot material (Winkel et al., 2017).
Submergence tolerance in two wheat cultivars

Impeding recovery growth. Moreover, while waterlogging does not lead to death of entire wheat plants (Herzog et al., 2016), mortality resulting from the additional stresses caused by complete submergence (Colmer and Voesenek, 2009) was observed following submergence for 8 days in the present study and 10 days in Winkel et al. (2017).

Comparing growth and survival rates from the current study with those of other submerged grass species revealed that especially Jackson can survive relatively long but faces severe shoot biomass reductions. Following 12 days of complete submergence and recovery, shoot biomass of tropical grasses Chloris gayana and Panicum coloratum were 21-54% of the controls (Imaz et al., 2013), compared to shoot biomass of 9% of the controls in Jackson after 12 days of submergence and 21 days of recovery. No mortality was reported in C. gayana and P. coloratum following treatment whereas mortality in Frument was 100% after 12 days. Rice submergence tolerance is very cultivar dependent. Intolerant IR42 displayed 70%, 50% and 0% survival after 8, 10 and 12 days of winter submergence (temperature 22-24 °C), respectively, and FR13A exhibited 90% survival following 12 days of complete submergence (Das et al., 2009). In the present study, Frument showed survival rates resembling submergence intolerant rice cultivar IR42 (Fig. 1). Meanwhile, survival in Jackson following 12 days of submergence was 100% compared to 90% in submergence-tolerant rice cultivar FR13A (Das et al., 2009). However, shoot biomass was severely reduced in wheat (5-17% of controls in Frument and Jackson) compared to rice (44-60% of controls in IR42 and FR13A) after 10 days of submergence and recovery (Das et al., 2009). The relatively large shoot biomass reductions in wheat compared to rice, C. gayana and P. coloratum could reflect the in comparison low waterlogging tolerance of wheat (Nishiuchi et al., 2012; Imaz et al., 2013) resulting in lower submergence tolerance as well. Possible traits conferring higher flooding tolerance in rice, C. gayana and P. coloratum are root porosities > 35% compared to 13-22% in wheat (Colmer, 2003; Imaz et al., 2013) and a strong barrier to ROL in rice but not found in wheat (Colmer, 2003). It should be noted that complete submergence of winter wheat at low temperatures during winter dormancy is likely to result in less detrimental effects as shown for waterlogging (Luxmoore et al., 1973; Trought and Drew, 1982).

Frument displayed faster leaf degradation than Jackson as evident from earlier leaf lamina chlorophyll, and leaf tissue porosity declines (Fig. 2). In rice, leaf chlorosis upon submergence is triggered by ethylene accumulation (Jackson et al., 1987), leading to lower photosynthesis (Smith et al., 1988; Winkel et al., 2014). Submergence-tolerant rice cultivars (FR13A, M202(Sub1)) had lower activity and expression of chlorophyllase due to lower ethylene sensitivity than intolerant cultivars (IR42, M202) resulting in more severe leaf chlorosis in the latter upon submergence (Smith et al., 1988; Ella et al., 2003; Fukao et al., 2006;
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Panda et al., 2008). This was linked to the ethylene-driven expression of SUB1A, an ethylene response factor (ERF) transcription factor which limits gibberellin-mediated elongation growth promoted by ethylene (Xu et al., 2006; Bailey-Serres et al., 2012). The earlier leaf chlorosis and higher levels of the chlorophyll degradation product phytol (Matile et al., 1999) in Frument could be indicative of higher ethylene sensitivity or production (shown to vary between Jackson and waterlogging sensitive cultivar Bayles, Huang et al. (1997)) in Frument; however, further studies are needed to clarify this aspect.

Lower chlorophylla concentrations in Frument relative to Jackson did not result in a corresponding decline in underwater $P_N$, indicating that light harvest was not the rate-limiting step in Frument $P_N$. Although it is possible that low chlorophylla in Frument resulted in lower $P_N$ during recovery (as seen in two rice cultivars with different chlorophyll levels after de-submergence, Alpuerto et al. (2016)), this was not assessed in the present study. Other factors limiting underwater $P_N$ could be damage to the photosynthetic apparatus as indicated by Fv/Fm ratios of 0.65-0.67 by day 12 (Fig. 2). Indeed, wheat underwater $P_N$ at diagnostic high external CO2 (2500 µM) did indicate damage to the photosynthetic apparatus after 4 days of complete submergence when rates declined to ~25% of initials (Konnerup et al., 2017). In submergence intolerant rice cultivar IR42, Fv/Fm declined to similarly low levels as in both wheat cultivars (~0.70) after 8 days of complete submergence, while remaining high in tolerant FR13A. Meanwhile, Fv/Fm did not separate rice cultivars M202 and M202(Sub1) during 3 days of submergence (Alpuerto et al., 2016) or Jackson and Frument in the present study. CO2 limitations caused by leaf gas film loss by day 5 would also hamper $P_N$ (Verboven et al., 2014; Konnerup et al., 2017) but leaf gas film retention times did not differ between cultivars in the present study (Fig. 2) or in the 14 wheat cultivars studied by Konnerup et al. (2017).

Leaf lamina tissue porosity decreased on day 12 in Frument and on day 16 in Jackson, thereby coinciding with the time point when plant survival in both cultivars reached 0% upon recovery. Leaf tissue porosity decline in submerged terrestrial plants has been interpreted as indicating structural leaf degradation (Winkel et al., 2014; Konnerup et al., 2017), i.e. loss of leaf hydrophobicity, cuticle deterioration, solute leakage and cell turgor loss allowing water to infiltrate intercellular gas filled spaces, but the sequence of events leading to porosity decrease remains to be assessed. In our experience, leaves that have lost porosity do not recover upon de-submergence resulting in senesced shoot tissues (Herzog et al., 2017; Konnerup et al., 2017; Winkel et al., 2017).
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Frument had significantly higher levels of mRNA encoding sucrose and fructan degrading enzymes (Fig. 6), explaining why the initially higher levels of soluble carbohydrates (Fig. 3) and sucrose (Fig. 3) in Frument reached similar levels in both cultivars by day 2 of submergence. Although Frument continued to overexpress sucrose synthases until day 12 compared to Jackson, sucrose remained at similar levels in the two cultivars as they had already approached zero by day 5. In addition, the patterns of shoot starch, glucose and fructose declines were similar in the two cultivars (Fig. 3 and Fig. S4). Shoot soluble carbohydrates differed initially (day 0), but cultivars had very similar shoot carbohydrate concentrations until day 12 of submergence, thereby not indicating that different carbohydrate consumption rates explain the contrasting submergence tolerance in Frument and Jackson. The similar carbohydrate consumption patterns in these two wheat cultivars contrast with rice where submergence-tolerant cultivars maintained significantly higher levels of starch and soluble carbohydrates than intolerant cultivars during submergence (Das et al., 2005; Winkel et al., 2014).

PCA showed that Frument metabolites changed faster relative to Jackson (Fig. 5), indicating that the Frument metabolome was generally more affected by submergence than Jackson. The higher levels of free amino acids in Frument than Jackson shoot tissues (up until shoot disintegration) resemble amino acid accumulation to higher levels in submergence intolerant rice cultivar M202 than tolerant M202(Sub1) during 3 days of submergence (Barding et al., 2013; Alpuerto et al., 2016). However, the current study does not support the suggestion in those two studies that, at least during prolonged submergence, differences in cultivar amino acid concentrations are linked to difference in carbohydrate consumption. Alternative explanations could be higher protein degradation and/or lower protein synthesis in Frument. During low O$_2$, pyruvate can be converted to Ala and prevent carbon loss to ethanol and lactic acid, but in the present study, Ala did not accumulate in the shoot tissues as seen in submerged rice (Barding et al., 2013; Alpuerto et al., 2016). Accumulation of amino acids, especially Pro which accumulated in Jackson but not in Frument, may also serve as osmoprotectants compensating for the loss of soluble carbohydrates during submergence or anoxia (Magneschi and Perata, 2009; Alpuerto et al., 2016). Moreover, Pro is also considered a powerful antioxidant (Verbruggen and Hermans, 2008). In the present study, MDA (a measure of cell membrane damage by ROS) indicated that Frument experienced significantly higher levels of lipid peroxidation than Jackson from day 12-16, while MDA levels in Jackson remained low even on day 16 when survival rates had started to decrease. ROS damage during submergence could occur when O$_2$ is low during the night and high during the day as seen in rice field floodwaters where diurnal pO$_2$ ranged from 5-19 kPa (Winkel et al., 2013).
Conclusion

Wheat cultivars showed contrasting submergence tolerance as evident from significantly higher survival and growth in Jackson than Frument following submergence and recovery. A clear relationship between submergence tolerance and steady-state level of shoot carbohydrates was not evident. However, the rate of initial carbohydrate consumption linked with expression of genes encoding sucrose and fructan catabolism enzymes was associated with submergence tolerance. Metabolomics analysis confirmed that cultivars experienced similarly rapid declines in shoot sugar levels, and revealed accumulation of most amino acids. Thus, cultivar Jackson tolerates longer periods of low shoot carbohydrate levels than Frument. Metabolic fingerprinting revealed that the Frument metabolome changed faster upon submergence than Jackson. These metabolic changes under submergence were in accordance with faster leaf senescence end deterioration as evident from leaf chlorophyll, and leaf tissue porosity data. However, leaf chlorosis did not result in lower underwater \( P_N \) in Frument relative to Jackson. Elevated levels of MDA indicated that Frument experienced higher levels of ROS-inflicted membrane damage at the end of the submergence period. Greater accumulation of proline in Jackson may partly contribute to the suppression of lipid peroxidation during submergence but further studies monitoring other antioxidant metabolites and enzymes are required to evaluate the ROS detoxification mechanism in tolerant and intolerant cultivars.

Materials and Methods

Plant culture

Seeds of wheat (\textit{Triticum aestivum} L., cv. ‘Frument’ and ‘Jackson’) were imbibed for three hours in aerated 0.5 mM CaSO\(_4\) and germinated for 48 hours in Petri dishes on wet paper towels in darkness at 20 °C. Cultivars Jackson and Frument were used due to their contrasting waterlogging tolerance, assuming that waterlogging and submergence tolerance could be linked. Jackson has been reported as waterlogging and hypoxia tolerant (Huang and Johnson, 1995; Huang et al., 1997) while Frument emerged as waterlogging intolerant when screening Danish (Frument, Jensen, Mariboss) and international reference cultivars (Jackson, Chara, Nishikazekumogi) for waterlogging tolerance in preliminary pot experiments.

Three germinated seeds were sown at 10 mm depth in each of 190 round pots with drainage holes (height, 120 mm; diameter, 90 mm) filled with substrate (specified below) and irrigated with deionized (DI) water. In order to obtain pots containing either three plants (providing sufficient plant material for a range of measurements, see below) or one plant (allowing for growth and survival analysis with minimum pot
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Effects), part of the seedlings were thinned to one per pot before treatment. To prevent roots from exiting through the drainage holes and into the aerated flood water, the bottom of the pots were sealed with two layers of landscape fabric (Plantex, DuPont Ltd., Hertfordshire, UK) fitted to the bottom with silicone. No roots were seen exiting the drainage holes throughout the experiment. Plants were grown September-October 2016 in Copenhagen, Denmark in a glasshouse (daytime temperature 14-25 °C, night time temperature 14-19 °C, relative humidity 20-70%) before moving to a constant temperature room for treatments.

Substrate in the pots consisted of 20 mm of washed sand at the bottom, a commercial potting mix with a dry matter content of 55-75 g l⁻¹, conductivity 3.0-5.0 mS cm⁻¹ and pH 6.0 (Pindstrup Substrate no. 2, Pindstrup Mosebrug A/S, Ryomgaard, Denmark) and a 20 mm layer of washed sand on top to reduce the flux of soil derived nutrients into the floodwater upon submergence. Each pot received 1g Osmocote slow release fertilizer (Osmocote Bloom, Everris, Geldermalsen, The Netherlands) that contained (by % mass): N, 12; P, 7.0; K, 18.0; Mg, 1.5; Fe, 0.35; Mn, 0.05; Cu, 0.045; Mo, 0.017; Zn, 0.013; B, 0.01. In a recent study using identical pots and substrate, O₂ disappeared from the soil matrix within 6-22 hours of soil flooding (Winkel et al., 2017). To control powdery mildew shoots were sprayed with a 2 g L⁻¹ sulfur solution (ECOstyle Svampefri, ECOstyle A/S, Odense, Denmark) 9, 19 and 39 days after imbibition and Flexity (Metratenon; 0.15 g L⁻¹) 15 and 41 days after imbibition.

Experimental design and treatments

The study consisted of two treatments (‘completely submerged’ and ‘controls in air’) × 2 wheat cultivars × 4-8 replicates × 0-19 days of treatment in a 2 × 2 × 7 factorial design. Four glass aquariums (length × width × height, 800 mm × 400 mm × 500 mm) filled with submergence solution (composition given below) in a constant temperature room (20 °C, relative humidity 40-89%) served as tanks for submergence. Light (PPFD of approximately 450 µmol m⁻² s⁻¹ at canopy level in the filled tanks, day/night cycle 12 h/12 h) was provided by two light panels (AkvaStabil Effektline AL 39x2, AkvaStabil, Haderslev, Denmark) per tank each holding two TL5 39 W fluorescent tubes. Drained controls in air grew in the same constant temperature room as the submerged plants and received the same PPFD, but from a different light source (Gavita Pro LEP 300, Gavita Holland BV, Aalsmeer, the Netherlands). The submergence solution was a modified Smart and Barko (1985) ‘artificial floodwater’ solution containing (in mM): CaCl₂·2H₂O, 0.62; MgSO₄·7H₂O, 0.28; KHCO₃, 2.0. The submergence solution was continuously bubbled with air and was replaced by fresh solution eight days after start of treatment.

Treatments commenced 18 days after imbibition when all plants had a fully expanded third leaf. 128 pots containing one or three plants of each genotype were fully submerged by placing pots into aquariums filled with submergence solution. Plants were randomly assigned to the four tanks, making sure that an even
distribution allowed for harvest of replicates from each tank at all time points. Additional plants subject to drained conditions served as ‘controls in air’. One pot holding three plants was harvested from each tank after 0, 2, 5, 8, 12, 14 and 16 days of submergence. One of three plants from each pot \((n = 4)\) was used for underwater net photosynthesis, dark respiration, gas film thickness, leaf tissue porosity, chlorophyll\(_a\) and shoot carbohydrates measurements while the remaining two plants were harvested for gene expression and metabolomic analysis, respectively. For growth and survival analysis, 1-2 pots holding one plant each were desubmerged from each tank for recovery \((n = 4-8)\) after 0, 8, 10, 12, 14, 16 and 19 days of submergence. Details on measurements are given in the following sections.

**Growth parameters**

Pots holding one plant were moved to empty aquariums with light panels on top and watered with DI water for 14-25 days of recovery (Striker, 2012) following submergence. Recovery duration depended on the preceding submergence event: plants submerged for only 8 days received a longer recovery period (25 days recovery) than plants submerged for 19 days (14 days recovery) in order to harvest equally old plants 51 days after imbibition. Plants were scored as ‘survived’ (green leaf tissue present) or ‘dead’ (no green leaf tissue present) and shoots were oven-dried at 60 °C for 48 h before weighing the DM. RGR were calculated as \(RGR = (\ln W_2-\ln W_1)/(t_2-t_1)\), where \(W_1\) and \(W_2\) are the initial and final weight, respectively, and \(t_1\) and \(t_2\) are the initial and final time (days), respectively. In order to determine shoot growth during submergence without a recovery period, four replicates were harvested immediately after eight days of submergence and DM recorded. Shoot length was measured on submerged and control plants randomly selected from each tank after 2, 5 and 8 days of treatment.

**Underwater net photosynthesis and dark respiration measurements**

Underwater net photosynthesis and dark respiration by lamina segments were measured using the principles described by Pedersen et al. (2013). In brief, for each replicate leaf \((n = 4)\), one lamina segment of approximately 25 mm length was taken half way up the blade of the youngest fully expanded (third) leaf at the time of submergence. Leaf tips of the youngest fully expanded leaf had prior to submergence been marked with a permanent marker to enable identification of leaves throughout the experiment. Glass cuvettes (approx. 28 mL) contained individual lamina segments in incubation medium (see submergence solution above for composition) and two glass beads for mixing as the cuvettes rotated on a wheel within an illuminated water bath \((P_n)\) or in darkness \((R_0)\), at 20 °C.
The dissolved O₂ concentration in the $P_N$ incubation medium was initially set at approximately 50% of air equilibrium, by bubbling in 1:1 volumes of N₂ and air; this reduced build-up of excess O₂ during the incubation that might otherwise have resulted in photorespiration. For $R_D$, the incubation medium was initially adjusted to air equilibrium by purging with air to obtain $R_D$ at non-limiting external O₂ concentrations (Colmer and Pedersen, 2008). Initial concentrations of 200 µM CO₂ in both solutions ($P_N$ and $R_D$) was obtained by adjusting pH to 7.35 after adding 2.2 mmol KHCO₃ L⁻¹ solution. 200 µM CO₂ is considered an environmentally relevant CO₂ concentration (Colmer et al., 2011) and allowed direct comparison with other studies on $P_N$ in submerged wheat (Konnerup et al., 2017; Winkel et al., 2017). The final solution consequently contained 200 µM CO₂, 2 mM HCO₃⁻ and 2.2 mM K⁺ with an alkalinity of 2.0 mM H⁺ equivalents L⁻¹ (Stumm and Morgan, 1996).

Following incubation with photosynthetically active radiation inside the vials of 1000 µmol photons m⁻² s⁻¹ provided by a light source (Gavita Pro LEP 300, Gavita Holland BV, Aalsmeer, Netherlands) for 60 to 90 min ($P_N$) or 120-180 min ($R_D$), dissolved O₂ concentrations in the cuvettes were measured using a mini O₂ optode (OP-MR, Unisense A/S, Aarhus, Denmark), connected to an optode meter (MicroOptode meter, Unisense A/S, Aarhus, Denmark) that was calibrated at 20.6 kPa (air bubbled DI water at 20 °C, 283.9 µmol O₂ L⁻¹) and at 0 kPa (DI water with ascorbate and KOH at 20 °C). Dissolved O₂ concentrations in cuvettes prepared and incubated in the same way as described above, but without leaf tissues, served as blanks. The projected area of leaf segments was measured by scanning the segments (bizhub C454e, Konica Minolta, Tokyo, Japan), analyzed digitally using ImageJ (Schneider et al., 2012), frozen at -20 °C, freeze-dried (Type 1102, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode, Germany) and DM recorded.

**Chlorophyll fluorescence**

Maximum photochemical quantum yield of PS II ($F_V/F_M$) was measured halfway up of the blade of the youngest fully expanded leaf at the time of submergence. Measurements were performed on the same four replicates of each cultivar subject to submergence or serving as controls in air control on day 0, 5, 8 and 12. Chlorophyll fluorescence was measured using a chlorophyll fluorometer (Junior-PAM, Heinz Walz GmbH, Effeltrich, Germany) following 20 min of dark acclimation.

**Chlorophyll concentration**

The lamina of the third leaf at the time of submergence (consisting of freeze-dried leaf segments from gas film thickness, tissue porosity and gas exchange measurements) was homogenized by cutting into max 2 × 2 mm pieces using scissors. Chlorophyll was extracted for 24 h in 96% ethanol at 20 °C in darkness,
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centrifuged at 4500 rpm for 3 min and \( A_{656} \) and \( A_{750} \) measured (Shimadzu UV-1800, Shimadzu Corp., Kyoto, Japan). Chlorophyll\(_a\) concentrations were calculated using equations of Mackinney (1941).

**Leaf gas film thickness and tissue porosity**

The leaf gas film volume (Winkel et al., 2014) and tissue porosity (gas-filled volume per unit tissue volume) was measured using the “buoyancy method” (Raskin, 1983; Thomson et al., 1990) on 70 mm segments of the youngest fully expanded (third) leaf lamina at time of submergence. The leaf segment area was measured and DM recorded as described above. Mean gas film thickness was calculated by dividing gas film volume (\( \text{mm}^3 \)) with the two-sided area (\( \text{mm}^2 \)) as wheat leaves possess gas films on both the adaxial and abaxial sides (Konnerup et al., 2017).

**Shoot carbohydrate assays**

The entire shoot remaining after excision of the third leaf, which had been used for leaf gas film thickness, tissue porosity and gas exchange measurements (described above), was excised below water, rinsed in DI water, blotted dry on paper towels, placed into perforated aluminium foil bags, flash-frozen in liquid nitrogen and stored at -80 °C. Shoots were freeze-dried and homogenized in a 2 mL Eppendorf tube using two metal beads for 20 sec on a mini bead-beater (MiniBead Beater, BioSpec Products Inc., Bartlesville, OK, USA). Shoot soluble sugars and starch were analyzed following Alpuerto et al. (2016). Ethanol soluble carbohydrates were extracted by incubating 20 mg of ground tissue in 1 mL 80% (v/v) ethanol at 80 °C for 20 min. After centrifugation (10 min at 20,800 g) the supernatant was removed and the extraction was repeated twice more. For extraction of water soluble carbohydrates the remaining pellet was re-suspended in 1 mL DI water and incubated at 80 °C for 20 min. After centrifugation (10 min at 20,800 g) the supernatant was removed and the extraction was repeated once more. Extracts containing ethanol and water-soluble carbohydrates were pooled in separate pre-weighed Eppendorf tubes and weighed for determination of extract volumes. Ethanol and water-soluble sugars were measured using the anthrone-method with glucose as the standard (Pontis, 2017). 50-200 µL extract was mixed with 200-50 µL ethanol to a final volume of 250 µL, mixed with 2.5 mL 0.2 % (w/v) anthrone solution in 96% \( \text{H}_2\text{SO}_4 \), incubated at 100 °C for 10 min, rapidly cooled and \( A_{620} \) measured on a spectrophotometer (Shimadzu UV-1800, Shimadzu Corp., Kyoto, Japan). For starch determination the pellet remaining from the extraction of soluble sugars was dried under vacuum and re-suspended in 1 mL of water containing 10 units of heat-resistant \( \alpha \)-amylase. After incubation at 95 °C for 30 min, the suspension was mixed with 25 µL 1M sodium citrate (adjusted to pH 4.8) and five units of amyloglucosidase. After incubation at 55°C for 1 h, the reaction mixture was centrifuged (30 min at 20,800 g) and glucose content in the supernatant (100-200 µL) was quantified by the anthrone method described above. Complete degradation of starch into glucose was confirmed by coloring test reaction mixtures with
Lugol’s iodine. Cultivars and time points were spread across independent extraction and measurement procedures to avoid systematic errors.

**Quantitative RT-PCR**

One entire shoot from a pot of initially three plants (of which the first was used for measurement described above) was harvested as described for “Shoot carbohydrate assays”. Shoots were freeze-dried for 72 h before shipment on silica gel and with ice packs for gene expression analysis in Blacksburg, VA, USA. Freeze-drying has been used in different plant (Román et al., 2012) and animal (Wu et al., 2012) species without loss of RNA quality or integrity. RNA extraction, cDNA synthesis and qRT-PCR were performed as described in Fukao and Bailey-Serres (2008). Total RNA was extracted using the RNeasy plant mini kit (Qiagen, Hilden, Germany). Genomic DNA was eliminated by on-column DNase treatment using a manufacturer’s protocol. Single-stranded cDNA was synthesized from 2 µg of total RNA using SuperScript IV reverse transcriptase and oligo dT primer (Thermo Scientific, Waltham, MA, USA). qRT-PCR was conducted in a 15 µL reaction using iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) in the CFX Connect real-time PCR detection system (Bio-Rad). Amplification specificity was validated by melt-curve analysis at the end of each PCR experiment. Relative transcript abundance was calculated by the comparative C_T method (Livak and Schmittgen, 2001). TaRP15, RNA polymerases I, II, and III, 15-kD subunit (TC265122) were used as a reference gene (Xue et al., 2008). Primer sequences and annealing temperatures used for qRT-PCR are listed in Table S1.

**Metabolomics**

One entire shoot was harvested as described for “Quantitative RT-PCR”, stored at -80°C and homogenized in liquid N_2 using mortar and pestle. Metabolites were extracted and analyzed as described in Hasler-Sheetal et al. (2016) with slight modifications. In brief, 50 mg homogenized shoot material was extracted (2 min in an ultrasound bath followed by 15 min on a thermo shaker both at 4°C) in 1 ml methanol/acetonitrile/water [4:4:2] at -20°C (spiked with 0.4 mg/l ^13^C_6 Sorbitol and Reserpine as internal standards). After centrifugation (19,000 g for 5 min) the metabolites in the supernatants were analyzed by gas chromatography quadrupole time of flight mass spectrometry (GC-MS; 7200 GC QTOF MS) and liquid chromatography quadrupole time of flight mass spectrometry (LC-MS; 1290LC, 6530 QTOF MS) (both Agilent Technologies, Santa Clara, CA, USA), following Hasler-Sheetal et al. (2015); Hasler-Sheetal et al. (2016) and Yonny et al. (2017) with slight modifications.
Data analysis

Data were analyzed with GRAPHPAD PRISM version 7.02 (GraphPad Software, La Jolla, CA, USA) and R
(R Core Team, 2014) for Windows statistical software. A significance level of $P < 0.05$ was used for all
analyses. Normality of distributions was confirmed by visual inspections and Shapiro-Wilk normality test,
and variance homogeneity using F-test or Brown-Forsythe test ($P > 0.05$). Data of leaf gas film thickness,
soluble carbohydrates, starch and GC-MS and LC-MS metabolites requiring transformation were ln-
transformed in order to improve variance homogeneity. Transformations were unsuccessful in improving
variance homogeneity in rates of underwater $PN$, but as sample sizes were equal making ANOVA robust to
unequal variances (Prophet Statguide, 1997; Graphpad Software Inc., 2013), we considered application of
ANOVA on untransformed data appropriate. Data sets were subject to two-way (time × cultivar) fixed factor
ANOVA and when significant main effects were found Sidak’s multiple comparisons post-hoc test was
performed. Chlorophyll fluorescence data were subject to two-way repeated measures ANOVA. Shoot
length measurements of each genotype were subject to one-way ANOVA with Tukey’s multiple
comparisons test. Shoot RGR during 8 days of submergence (no recovery) and relative mRNA levels were
analyzed using unpaired t-tests.

Using the amount of plants surviving upon submergence and recovery as the response variable (coded as 0,
dead or 1, survived), the difference in cultivar survival was tested using a linear logistic regression model, a
Generalized Linear Model (GLM) with binomial error structure and logit link function. Duration of
submergence was used as the explanatory variable and survival was analyzed for both cultivars separately.
When fitting the model we used Firth-type penalized likelihood estimation instead of maximum likelihood
estimation due to otherwise inflated standard errors caused by near separation into 0 and 100% survival in
Jackson (Heinze and Schemper, 2002).

List of Supplemental Material

Table S1. Primer sequences and annealing temperatures used in qRT-PCR.

Fig. S1. Shoot RGR of wheat cultivars Frument and Jackson during 8 days of submergence (no recovery
period).

Fig. S2. Leaf underwater photosynthesis and dark respiration of wheat cultivars Frument and Jackson with
time of submergence.
Fig. S3. GC-MS determined sugars in shoots of wheat cultivars Frument and Jackson with time of submergence.

Fig. S4. Starch in shoots of wheat cultivars Frument and Jackson with time of submergence.

Fig. S5. Metabolites detected by LC-MS in shoots of wheat cultivars Frument and Jackson during submergence, presented as Venn diagram for time, cultivar and time × cultivar interaction effects.

Fig. S6. GC-MS determined amino acids in shoots of wheat cultivars Frument and Jackson with time of submergence.

Fig. S7. Relative mRNA levels of α-amylases in shoots of wheat cultivars Frument and Jackson with time of submergence.

Acknowledgements

The authors would like to thank Timothy D. Colmer for discussing experimental procedures and data interpretation, Lars Iversen for statistical support and Victoria C. Herskov for helping with analyses of chlorophyll. M.H., D.K. and A.W. were supported by PhD and postdoctoral fellowships from the Villum Foundation.

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Chapter 3: Leaf gas films contribute to rice (*Oryza sativa*) submergence tolerance during saline floods

*Rice leaves retaining leaf gas films on the submerged leaf sections. Photo: Ole Pedersen.*
Leaf gas films contribute to rice (*Oryza sativa*) submergence tolerance during saline floods

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**ABSTRACT**

Floods and salinization of agricultural land adversely impact global rice production. We investigated whether gas films on leaves of submerged rice delay salt entry during submergence. Two-week-old plants with leaf gas films (+GF) or with gas films experimentally removed (−GF) were submerged in artificial floodwater with 0 or 50 mM NaCl for up to 16 d. Gas films were present >9 d on GF plants after which gas films were diminished. Tissue ion analysis (Na$^+$, Cl$^−$ and K$^+$) showed that gas films caused some delay of Na$^+$ entry, as leaf Na$^+$ concentration was 36–42% higher in −GF leaves than +GF leaves on days 1–5. However, significant net uptakes of Na$^+$ and Cl$^−$, and K$^+$ net loss, occurred despite the presence of gas films, indicating the likely presence of some leaf-to-floodwater contact, so that the gas layer must not have completely separated the leaf surfaces from the water. Natural loss and removal of gas films resulted in severe declines in growth, underwater photosynthesis, chlorophyll, and tissue porosity. Submergence was more detrimental to leaf $P_N$ and growth than the additional effect of 50 mM NaCl, as salt did not significantly affect underwater $P_N$ at 200 $\mu$M CO$_2$ nor growth.

Key-words: flooding; leaf Cl$^−$; leaf K$^+$; leaf Na$^+$; plant submergence tolerance; salinity; salt intrusion.

**INTRODUCTION**

Floods annually affect large areas of farmlands worldwide and cause severe crop losses when plants become submerged (Jackson 2004). Crop damage is mainly caused by the hampered gas exchange between plants and floodwater because of a 10$^5$-fold slower gas diffusion and low solubility of O$_2$ in water compared with that in air (Armstrong 1979; Voesenek et al. 2006). Paddy field rice is adapted to growth in anoxic soils and therefore is tolerant to soil waterlogging and even partial shoot submergence (Colmer et al. 2014; Kirk et al. 2014). However, only a few days of complete submergence can lead to severe damage and death of rice (Das et al. 2009), but with important differences among rice genotypes (Ismail et al. 2013). The restricted gas exchange impedes respiration and photosynthesis (also because of low light) in submerged shoots (Mommer & Visser 2005) while the consumption of soluble carbohydrates (Setter et al. 1997) further depletes tissue sugars and energy (if shoots elongate). The factors described previously contribute to damage during floods, together with desubmergence water deficits (Setter et al. 2010) and oxidative stress (Bailey-Serres & Voesenek 2008) that can result in further damage and even death.

Floodwaters may contain NaCl, and salinity is a major impediment to increasing global rice production (Negrão et al. 2011) as rice is a salt-sensitive species. For rice with shoots in air, salinity above 30 mM NaCl results in yield decreases by 12% for each ~10 mM NaCl increase (Grieve et al. 2012). Salinity imposes both an osmotic stress on the plant because of high solute concentrations outside cells, as well as ion-specific stresses caused by high Na$^+$ and Cl$^−$ concentrations in plant tissues (Munns & Tester 2008; Negrão et al. 2011). The need to improve rice salinity and submergence tolerance is further urged by climate change causing rising seawater levels and lower river flows, leading to seawater inundation of large rice growing regions such as the Vietnamese Mekong Delta (Wassmann et al. 2004). A second example is that the salinity affected areas in Bangladesh increased from about 83 million ha in 1973 to 106 million ha in 2009 (Sinha et al. 2014). Thus, efforts are being made to combine submergence tolerance and salinity tolerance in the so-called climate-smart rice (De Ocampo et al. 2013; IRRI 2016).

Rice leaves are surrounded by a gas film (initial average thickness of 50–62 $\mu$m, Pedersen et al. 2009; Winkel et al. 2013; Winkel et al. 2014) for up to 6 d during submergence in the field (Winkel et al. 2014). Presence of such gas films delayed salt entry into submerged leaves of *Melilotus siculus* (Teakle et al. 2014), but this is the only study to have evaluated this effect. Leaf gas films have been shown to enhance underwater photosynthesis of rice, dark respiration, root $pO_2$ and growth, by greatly enhancing gas exchange between leaves and floodwater (Pedersen et al. 2009; Winkel et al. 2013; Verboven et al. 2014; Winkel et al. 2014), thereby contributing to rice submergence tolerance. Our main objective was to test the effect of the presence of leaf gas films on salt entry into submerged rice leaves of *Melilotus siculus*. Unlike rice, *Melilotus siculus* is native to saline environments and is therefore well adapted to high Na$^+$ and Cl$^−$ concentrations (Teakle et al. 2014).
rice; we hypothesized that Na\(^+\) and Cl\(^-\) entry, and K\(^+\) loss, would be delayed by the presence of leaf gas films acting as an ‘insulating’ physical barrier between each leaf and saline floodwater.

**MATERIALS AND METHODS**

**Plant culture**

Seeds of rice (*Oryza sativa* L. var. Amaroo) were germinated following Mongon et al. (2014). Dehulled seeds (i.e. caryopses) were washed with dilute sodium hypochlorite (0.1%) for 30s, rinsed in deionized (DI) water and then imbibed in aerated 0.5 mM CaSO\(_4\) for 3 h. The seeds were placed on a plastic mesh floating on a 10% strength nutrient solution (for chemical composition, see below) in darkness. After 4d, the seedlings were transferred to a 25% strength nutrient solution and exposed to light. Seven days after imbibition, the seedlings were transferred to 2.2 L pots (four plants per pot) containing 100% strength nutrient solution. Plants were held individually in each of the eight holes in the lids using polyethylene foam, and the pots were covered with aluminium foil to exclude light from the root system. Eleven days after imbibition (2–3 d before submergence), roots received a hypoxic pretreatment by flushing the nutrient solution with N\(_2\) gas for 5 min. On the day before submergence, plants were transferred to 2.2 L pots (four plants per pot) containing 100% strength nutrient solution, with additional 2.5 mM NH\(_4\)NO\(_3\), made stagnant with 0.1% (w/v) agar and previously deoxygenated by flushing overnight with N\(_2\) gas.

The composition of the nutrient solution at 100% strength was as follows: KNO\(_3\), 3.75 mM; NH\(_4\)NO\(_3\), 0.625 mM (plus 2.5 mM NH\(_4\)NO\(_3\) when stagnant agar was used); KH\(_2PO_4\), 0.2 mM; MgSO\(_4\)2H\(_2\)O, 0.40 mM; Na\(_2\)O\(_3\)Si.9H\(_2\)O, 0.10 mM; CaSO\(_4\)2H\(_2\)O, 1.5 mM; KCl, 100 µM; H\(_2\)BO\(_3\), 50 µM; MnSO\(_4\), H\(_2\)O, 4.0 µM; ZnSO\(_4\),7H\(_2\)O, 4.0 µM; CuSO\(_4\),5H\(_2\)O, 1.0 µM; Na\(_2\)MoO\(_4\)2H\(_2\)O, 1.0 µM; NiSO\(_4\),7H\(_2\)O, 2.0 µM; and Fe-EDTA, 50 µM. The solution also contained 2.5 mM MES buffer, and the pH was adjusted to 6.5 using KOH. At 7–8 d after imbibition, one dose of FeSO\(_4\),7H\(_2\)O was added to each 4 L pot to a final concentration of 5.0 µM to avoid any iron deficiency in the seedlings. The nutrient solution in the pots was replaced with fresh solution every 6 d during the entire experiment and topped up with DI water as required to replace water consumed in transpiration. Plants were kept in a naturally lit, temperature-controlled (30/25 °C day/night) phytotron during October to November 2015 in Perth, Western Australia. Light in the phytotron was 741 µmol photons m\(^{-2}\)s\(^{-1}\) at midday even on a cloudy day.

**Experiment 1 – tissue ions in +GF and –GF plants during 16 d submergence in non-saline and saline (50 mM NaCl) artificial floodwaters**

Plants were grown in two batches staggered with time, owing to the limited number of cylinders in the submergence systems (described next). Submergence treatments commenced 13–15 d after imbibition when all plants had a visible fourth leaf collar. The setup of the submergence system has been described previously (Pedersen et al. 2009; Teakle et al. 2014). In short, the 2.2 L pots each containing four plants were randomly transferred to 12L clear Perspex cylinders filled with either saline (50 mM NaCl) or non-saline (0 mM NaCl) submergence solution. The basal submergence solution (artificial floodwater) contained the following: CaSO\(_4\), 2.0 mM; MgSO\(_4\), 0.25 mM; and KHCO\(_3\), 2.0 mM. The root medium in all cases was non-saline.

Cylinders filled with saline or non-saline submergence solution were connected to two separate, identical lines of aquarium pumps and ultraviolet (UV)-filters (JBL AquaCristal UV-C; JBL GmbH & Co. KG, Neuhofen, Germany). In each system of nine Perspex cylinders per line, a pH controller (JBL CO\(_2\)/pH Control; JBL GmbH & Co. KG, Neuhofen, Germany) connected to a cylinder with pressurized CO\(_2\) maintained free CO\(_2\) at 200 µM by referring to the relevant pH set points for non-saline (pH7.3, Mackereth et al. 1979) and saline (pH7.1, Pierrot et al. 2006) water. Dark plastic covered the lids of the pots and the bottom and basal sides of the cylinders, excluding light from entering basal portion of each cylinder that contained the plastic pots with the nutrient solution. Rubber-covered weights weighed down the pot in each cylinder. Plants grown in identical pots with nutrient solution and rubber weights were placed in empty cylinders (i.e. containing air) and with plastic mesh near the top of each cylinder (see below for the reason this mesh was needed especially for the submerged plants), serving as ‘emergent’ controls with shoots in air. Light in the water-filled cylinders was 8633 µmol photons m\(^{-2}\)s\(^{-1}\) at midday on a cloudy day, which was above values in air (see section ‘Plant culture’); this could be caused by filled cylinders acting as a lens thereby focusing light onto the light sensor (Walz US-SQS/ L; Heinz Walz GmbH, Effeltrich, Germany).

Before submergence, plants were either untreated, thus retaining clearly visible leaf gas films upon submergence (+GF), or the entire shoot was brushed with 0.1% (v/v) Triton X-100 (Colmer & Pedersen 2008; Pedersen et al. 2009; Winkel et al. 2013) preventing leaf gas film formation when submerged (–GF). Shoots treated with 0.1% Triton X-100 were rinsed using a separate batch of submergence solution prior to insertion of these plants into the cylinders. New leaves formed during the submergence period were brushed with 0.1% Triton X-100 and rinsed with a separate batch of submergence solution, when plants were raised out of the tanks for this process every 2 d. Plastic mesh held 20 mm below the water surface within each cylinder prevented leaf emergence into the air above the water when shoots elongated following submergence. The submergence treatment lasted 9 d for plants treated with 0.1% Triton X-100 (without leaf gas films) and 16 d for plants initially retaining leaf gas films. The shorter treatment period of the 0.1% Triton X-100 treated plants was due to the beginning of some disintegration of the leaves after 9 d of submergence (observed during a pilot experiment). Plants were harvested on days 0, 1, 2, 5, 9 and 16 of the submergence treatment. The youngest fully expanded leaves at time of submergence (leaf 4) and leaf 3 were excised and used for further analysis (the entire third leaf blade was used for tissue ion analysis;
the fourth leaf was used for measuring underwater photosynthesis, leaf gas film thickness, leaf tissue porosity, scanning electron microscopy, chlorophyll concentration and tissue ion analysis). Details of measurements are given next.

Growth

Plants were harvested for dry mass (DM) measurements on days 0, 9 and 16 of submergence treatments. Plants were separated into shoot and roots and oven dried at 60 °C for 48 h before weighing. As the experiment consisted of two different batches of plants, we calculated relative growth rates RGR = (lnW2 – lnW1)/(t2 – t1) for growth comparisons, where W1 and W2 are the initial and final weight (g), respectively, and t1 and t2 are the initial and final time (days).

Recovery was assessed following desubmergence after 9 d of submergence. Four pots each containing four plants were desubmerged and placed in empty Perspex cylinders. After 10 d with shoots again in air, the plants were scored for survival, dead and living shoot tissues were separated, samples were dried at 60 °C for 48 h, and DM was recorded.

Underwater net photosynthesis

Underwater net photosynthesis (P_{\text{N}}) was measured following the approach described in Pedersen et al. (2013). Leaf segments were incubated in a defined medium (described next) for a known time in closed transparent glass vials with gentle mixing and held at a constant temperature in light [photosynthetically active radiation (PAR) given next], after which the O2 evolution (P_{\text{N}}) by the leaf segments was measured against a blank vial lacking leaf segments. Four replicate leaves (younger fully expanded at the time of submergence from four different plants) were taken from each of the two treatments (non-saline or saline submergence). Leaf segments of 10 mm in length (projected area ~50 mm²) were excised from the top third of the lamina. Underwater P_{\text{N}} was measured at 30 °C using 25 mL glass vials with two glass beads added to provide mixing as the vials were held on a ‘turning wheel’ during incubation with PAR inside the vials of 1000 μmol photons m⁻² s⁻¹ provided from a vertically positioned light-emitting diode lamp (Valoya R300 NS1; Valoya Ltd., Helsinki, Finland) providing 94% of PAR with a colour temperature of 4800 K. Measurements were performed during the same time of day (1000–1400 h) on all days.

Following incubations of known duration (90–120 min), dissolved O2 concentration in each vial was measured using an O2 optode (Unisense OP-MR; Unisense A/S, Aarhus, Denmark) connected to an optode meter (Unisense Micro-Optode meter). The optode was calibrated at 30.0 °C in water at air equilibrium (20.6 kPa O2) and in anoxic water (0.0 kPa O2) containing 100 mM sodium ascorbate and 100 mM NaOH. Projected area of each individual leaf segment was measured using digital photos and analysis in ImageJ (Schneider et al., 2012). Samples were then immediately frozen at −20 °C, freeze-dried and DM recorded.

Leaf gas film thickness and tissue porosity

The leaf gas film volume and tissue gas-filled porosity were measured using the ‘buoyancy method’ (Raskin 1983; Thomson et al. 1990) on 50 mm segments of the fourth leaf according to Winkel et al. (2013) at room temperature. The leaf segment area was measured as described for ‘Underwater net photosynthesis’, frozen at −20 °C, freeze-dried and DM recorded. Mean gas film thickness was calculated by dividing gas film volume (mm³) by the two-sided area (mm²).

Tissue ion concentrations

In order to retrieve sufficient tissue for ion concentration analysis, the entire third leaf and the remaining ∼30–80 mm of the fourth leaf were excised from submerged plants and rinsed for 5–10 s in DI water. Leaf Na⁺, Cl⁻ and K⁺ concentrations were determined following Munns et al. (2010). In short, oven-dried (60 °C) leaf samples were extracted in 2.5–5 mL 0.5 M HNO₃ for 2 d at 25 °C. Extracts were diluted with Milli-Q water as required and analysed for Na⁺ and K⁺ (Jenway PPP7 Flame Photometer, Jenway, Essex, UK) and Cl⁻ (Slamed Chloridometer CHL 50, Slamed ING GmbH, Frankfurt, Germany). The reliability of these analyses was confirmed by taking a reference plant sample (ASPAC no. 85) with known ionic composition through the same procedures.

Chlorophyll concentration

The freeze-dried leaf segments (from underwater P_{\text{N}} measurements) were each homogenized in a 2 mL Eppendorf tube using two metal beads for 10 s on a mini bead-beater (Mini Bead Beater; BioSpec Products Inc., Bartlesville, OK, USA). Chlorophyll was extracted for 24 h in 96% ethanol, centrifuged at 9000 rpm for 3 min and chlorophyll absorbance measured at 656 and 750 nm on a spectrophotometer (Shimadzu UV-1800; Shimadzu Corp., Kyoto, Japan). Chlorophyllₐ concentrations were calculated using equations of Mackinney (1941).

Scanning electron microscopy

Leaf segments were frozen immediately after sampling and then freeze-dried. Samples were gold-coated in a sputter coater for 90 s and then analysed using a scanning electron microscope (FEI Inspect S; FEI Company, Hillsboro, OR, USA) at high vacuum mode, 12.5 kV and 500–7000 × magnification. For closer examination of wax platelets, samples were also analysed with a field emission scanning electron microscope (JEOL JSM-6335F; JEOL Ltd., Peabody, MA, USA) at 7.0 kV and 27–45,000 × magnification.

Experiment 2 – the effect of pO2 on leaf ion concentrations

To evaluate the effect of O2 supply to submerged leaves on tissue ion net uptake or loss, excised leaves were subject to 24 h incubation in darkness in saline water with pO2 set to five different levels (described next). Plants were grown to the same
Concentrations were then subtracted from the leaf type sampled from plants in experiment 1; these initial concentrations were calculated using initial tissue ion concentrations from the same experiment 1. The cut end was sealed using Vaseline. One treated (-GF) and one control (+GF) leaf were placed pairwise in four 250 mL conical flasks for each pO₂ treatment containing saline submergence solution (basal submergence solution plus 50 mM NaCl) as described previously for experiment 1; each leaf was weighed-down under the solution by a plastic-coated paper clip. Two flow controllers (Bronkhorst High-Tech B.V. series with B.V. E-5700 power supply; Bronkhorst High-Tech B.V., Ruurlo, the Netherlands) connected to a pressurized N₂ cylinder and an air pump were used to adjust pO₂ in the submergence solution to 0.01, 0.46, 1.59, 3.16 and 20.23 kPa O₂. Leaves were incubated in the dark for 24 h at 25 °C. After incubation, leaves were visually inspected for presence/absence of leaf gas films and then rinsed and analysed for tissue ion concentrations as described previously. Ion uptake rates were calculated using initial tissue ion concentrations from the same leaf type sampled from plants in experiment 1; these initial concentrations were then subtracted from the final concentrations and divided by the incubation time (24 h).

**Data analysis**

Data were analysed with GRAPHPAD PRISM version 6.07 (GraphPad Software, La Jolla, CA, USA), SYSTAT version 12.02 (Systat Software Inc., San Jose, CA, USA) and SPSS version 22 (SPSS Inc., Chicago, IL, USA) for Windows statistical software. Normally distributed data were analysed using two-way or three-way ANOVA; data requiring transformations are specified next. Variance homogeneity was confirmed by visual inspections of residual plots and Levene’s test for variance homogeneity (P > 0.05). Correlations (Fig. 4 and Supporting Information Figs S4 and S5) were analysed by calculating non-parametric Spearman rank correlation coefficients because of lack of bivariate data normality and relationships being non-linear. Significance level of P < 0.05 was used for all analyses. For ANOVA analyses, a post hoc Sidak or Tukey test was performed when significant effects were found.

Leaf gas film thickness, chlorophyll, and leaf porosity data (Fig. 1) were analysed using two-way ANOVA with ‘time’ and ‘salt’ as fixed factors (days 1–16 of treatments). Measurements performed on day 0 (initials) were excluded, and leaf gas film thickness and leaf porosity data were log and square-root transformed, respectively, in order to improve variance homogeneity. Leaf porosity variances were, however, still significantly different (Levene’s test, P = 0.033), but as sample sizes were equal making ANOVA robust to unequal variances (Prophet Statguide 1997; Graphpad Software Inc. 2013) and after visual inspection of residual plots, we considered application of ANOVA on transformed data appropriate.

Tissue ion concentrations from experiment 1 (Fig. 2) were analysed using two-way ANOVA with ‘time’ and ‘gas film’ as variables. Leaf gas film thickness, chlorophyll, and leaf porosity data (Fig. 1) were analysed using two-way ANOVA with ‘time’ and ‘salt’ as fixed factors (days 1–16 of treatments). Measurements performed on day 0 (initials) were excluded, and leaf gas film thickness and leaf porosity data were log and square-root transformed, respectively, in order to improve variance homogeneity. Leaf porosity variances were, however, still significantly different (Levene’s test, P = 0.033), but as sample sizes were equal making ANOVA robust to unequal variances (Prophet Statguide 1997; Graphpad Software Inc. 2013) and after visual inspection of residual plots, we considered application of ANOVA on transformed data appropriate.

Tissue ion concentrations from experiment 1 (Fig. 2) were analysed using two-way ANOVA with ‘time’ and ‘gas film’ as variables.
fixed factors (days 1–9 of treatments). Initials were excluded, and Na⁺ and Cl⁻ concentrations were log-transformed in order to improve variance homogeneity. Underwater \( \text{P}_N \) (Fig. 3) was analysed separately for 200 and 2000 μM free CO₂, resulting in a three-way ANOVA with ‘time’, ‘salt’ and ‘gas film’ as fixed factors. RGR (Fig. 5) and amount of dead shoot DM (Fig. 6) were analysed using two-way ANOVA with ‘salt’ and ‘gas film’ as fixed factors. Tissue ions from experiment 2 (Fig. 7 and Supporting Information Fig. S6) were analysed using two-way ANOVA with ‘gas film’ and \( \text{pO}_2 \) as fixed factors. Shoot length and tiller number (Supporting Information Table S1) were analysed using one-way ANOVA and non-parametric Kruskal–Wallis test, respectively, as transformation of tiller number was unable to ensure variance homogeneity.

RESULTS

To investigate the effects of gas films on leaves of rice submerged in saline water, we compared tissue ion concentrations and other parameters for plants retaining leaf gas films or where the gas films had been experimentally removed.
Gas films: retention duration and influence on leaf tissue ions during saline submergence

Rice leaves retained a clearly visible gas film when submerged. Initially, mean gas film thickness was 25 μm (Fig. 1). Gas film thickness declined to 18 μm (0 mM NaCl) and 13 μm (50 mM NaCl) during the first 5 d of submergence, followed by earlier loss of gas films in saline water (after day 5 and before day 9) than in non-saline water (after day 9 and before day 16). It should be noted that other studies report initial rice leaf gas film thickness ranging from 50 to 62 μm in five genotypes (Pedersen et al. 2009; Winkel et al. 2013; Winkel et al. 2014), that is, twice as thick as in the present study (Fig. 1). As one of these studies was performed on var. Amaroo (of similar age as in present study), this difference should not necessarily be interpreted as sign of genotypic variation but could result from environmental conditions (e.g. temperature during measurements, which differed with 10 °C) or different leaf sections used.

Adaxial sides of leaves showed similar macro-structures, micro-structures and nano-structures considered responsible for leaf hydrophobicity (grooves, papillae and wax platelets, respectively) prior to and after loss of gas films (Supporting Information Figs S1 and S2), with the exception of wax platelets located on papillae that showed some slight changes. These wax platelets appeared to be more rounded after loss of leaf hydrophobicity at 50 mM NaCl compared with the initials (Supporting Information Fig. S2b,c). From day 9, leaves were increasingly covered by filaments, possibly from filamentous epiphytic algae (Supporting Information Fig. S1).

To evaluate the effect of gas film removal on tissue ions, we measured Na⁺, Cl⁻ and K⁺ in the third leaf and part of the fourth leaf sampled from various plants at 4–5 time points during 9–16 d of submergence. Na⁺ uptake was substantial from day 1 even in leaves with a gas film, with tissue Na⁺ concentration having increased 4.6-fold (+GF and gas films still present) on day 5 relative to initials. Leaf Na⁺ increased even more (36–42% greater in −GF than +GF plants on days 1, 2 and 5; Fig. 2) when plants had their gas films removed prior to submergence in saline water. Consequently, two-way ANOVA showed significant gas film (and time) effects for Na⁺ (see caption of Fig. 2). Tissue Na⁺ increased to 1111 μmol Na⁺ g⁻¹ DM in −GF plants on day 9, but on this single time point, Na⁺ was not significantly higher than the 981 μmol Na⁺ g⁻¹ DM measured in plants initially possessing a gas film (note that gas films were no longer present at this sampling time). When tissue Na⁺ is expressed as a concentration in tissue water (Supporting Information Fig. S3) rather than on a DM basis, Na⁺ accumulated to 175 mM in −GF plants and 172 mM in +GF plants on day 5 at 50 mM NaCl (not significantly different).

Surprisingly, experimental removal of gas films did not significantly affect leaf tissue Cl⁻ or K⁺ concentrations of submerged plants at 50 mM NaCl (Fig. 2). On day 5 when gas films were still present on the +GF plants, both +GF and −GF leaves only retained 33% of initial K⁺ concentrations. K⁺ loss resulted in minimum tissue K⁺ of 215 μmol K⁺ g⁻¹ DM (27 mM in tissue water, −GF, day 9; Supporting Information Fig. S3). Cl⁻ concentrations had increased threefold in

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**Figure 4.** Underwater photosynthesis ($P_N$, youngest fully expanded leaf) at 200 and 2500 μM CO$_2$ of rice leaves retaining leaf gas films submerged for 1–16 d in water (containing basal ions, see Methods) with 0 (circles) or 50 (squares) mM NaCl, plotted against the corresponding leaf gas film thickness. Roots were in non-saline nutrient solution. $r$ values from non-parametric Spearman rank correlation analysis, * denoting levels of significance (levels of significance are: $P < 0.05$, $P < 0.01$, $P < 0.001$ or $P < 0.0001$ are denoted by n.s., *, **, *** or ****, respectively): 200 μM CO$_2$, $r = 0.7279$***; 2500 μM CO$_2$, $r = 0.7476$****. Values are means as presented in Figs 1 and 3.

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Experiment 1 had non-saline and saline (50 mM NaCl, ~5 dS m⁻¹) artificial floodwater, a level that allowed salinity to be imposed in one step without an ‘osmotic shock’ and 50 mM NaCl resulted in substantial Na⁺ entry into the shoot of rice at the early seedling stage (var. Amaroo; Kurniasih et al. 2013); this same variety was used in the present study. Experiment 2 investigated whether O₂ status affects ion net uptake or loss by incubating excised leaves of rice in saline (50 mM NaCl) artificial floodwater at a range of pO₂. In the first section followed, we describe the retention time of gas films and changes in leaf tissue Na⁺, Cl⁻ and K⁺ in plants with intact gas films (+GF) compared with plants with gas films removed by brushing with dilute Triton X-100 (−GF) immediately prior to submergence in artificial floodwater with 0 and 50 mM NaCl (experiment 1). We then report on leaf chlorophyll$_a$, leaf porosity, underwater $P_N$ and growth of submerged plants (experiment 1). Finally, we describe the effects of varying pO₂ on net uptake or loss of ions by submerged leaves with or without gas films (experiment 2).
both +GF and −GF leaves on day 5, with maximum tissue Cl\(^{-}\) of 719 μmol Cl\(^{-}\) g\(^{-1}\) DM on day 9 (+GF, 124 mM in tissue water; Supporting Information Fig. S3). Consequently, two-way ANOVA on leaf Cl\(^{-}\) and K\(^{+}\) concentrations only showed significant time effects, contrasting to the additional gas film effect found for leaf Na\(^{+}\) concentrations (see caption of Fig. 2).

When submerged in non-saline water, leaf tissue ions remained similar to initial levels, the only exception being Cl\(^{-}\) in +GF plants, where at the end of the treatment the tissue ions had increased almost twofold above the initial values. Hence, for plants submerged in non-saline water, −GF resulted in significantly lower tissue Cl\(^{-}\) in leaves. For control plants with shoots in air, leaf ion concentrations remained at initial values throughout the experiment (see caption of Fig. 2).

In conclusion, leaf gas films were present for at least 5 d (saline) and 9 d (non-saline) submergence and significantly delayed Na\(^{+}\) uptake, but not that of Cl\(^{-}\), and also did not prevent substantial K\(^{+}\) loss during submergence in saline water. In the following sections, we describe the effects of NaCl and submergence on the other physiological parameters measured for leaves and on plant growth and recovery upon desubmergence.

**Figure 5.** Relative growth rates (RGR) during 9 d submergence (top panel) and the following 10 d recovery period (bottom panel) in roots (a, c) and shoots (b, d) of rice plants retaining leaf gas films (+GF, open bars) or treated with 0.1% Triton X-100 and without leaf gas films (−GF, closed bars) in water (containing basal ions, see Methods) with 0 or 50 mM NaCl. Roots were in non-saline nutrient solution. Letters denote significant differences between columns (Tukey’s multiple comparisons test, \(P < 0.05\)). In all four datasets, two-way salt × GF ANOVA only detected significant effects of gas film (\(P < 0.01\)) but not salt (\(P ≥ 0.2598\)). The +GF and −GF were batches staggered with time, and the emergent control columns are therefore the mean RGR of these two columns. Values are means (±SE, \(n = 4\)).

**Figure 6.** Dead shoot tissue as percent of total shoot dry mass of rice after submergence in water (containing basal ions, see Methods) with 0 or 50 mM NaCl and a following 10 d recovery period of plants with leaf gas films (+GF, open bars) or treated with 0.1% Triton X-100 and without gas films (−GF, closed bars). Roots were in non-saline nutrient solution. Letters denote significant difference between means using a post hoc Tukey’s multiple comparisons test (\(P < 0.05\)). Two-way salt × GF ANOVA showed significant salt × GF interaction (\(P = 0.0014\)). Values are means (±SE, \(n = 4\)).

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Leaf porosity is affected by salinity and gas film removal

Gas film removal caused leaf porosity to decline substantially within the first 5 d of submergence (mean tissue porosity of 2.0% after 5 d in non-saline water; Fig. 1), compared with leaves with intact gas films under the same conditions (mean tissue porosity of 10.4%, same as initials). The adverse effects of gas film removal were stronger for plants in saline than in non-saline solution (0.0 and 11.7% porosity in −GF and +GF plants on day 5, respectively); therefore, two-way ANOVA (time × salt) showed a significant salt effect for −GF plants only. Thus, +GF plants maintained higher leaf porosity during submergence until the gas films were lost naturally.

Gas films enhance underwater gas exchange

Gas films enhanced underwater $P_N$ at 200 $\mu$M CO$_2$ (Fig. 3). At this CO$_2$ concentration, $P_N$ rates of −GF leaves were 15% of +GF leaves (when first submerged in non-saline water). The positive effect of leaf gas films on underwater $P_N$ was maintained for 5 d and then declined by day 9 as gas films diminished, with $P_N$ of +GF plants reduced to 16% (non-saline) and 7% (saline) of initials on day 16. Results of three-way ANOVA (time × salt × GF) reflected this decrease over time with significant time × salt effect at both 200 and 2500 $\mu$M CO$_2$. Elevating CO$_2$ to 2500 $\mu$M closed the gap between +GF and −GF leaves, as now, gas film removal only reduced $P_N$ to 72% of leaves with intact gas films. Increasing external CO$_2$ partially alleviated the negative effect of not possessing gas films on CO$_2$ uptake, that is, increasing external CO$_2$ can overcome the higher resistance of CO$_2$ uptake in leaves with no gas films. Plotting $P_N$ against gas film thickness (Fig. 4) revealed significant positive correlations both at low ($r = 0.73$) and high ($r = 0.75$) CO$_2$, underlining the positive effect of gas films on underwater $P_N$.

The NaCl treatment only affected $P_N$ significantly at high CO$_2$ and when gas films were removed. Under these

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conditions, \( P_N \) rates were 25% lower on average when subject to NaCl (significant on days 1, 5 and 9). Thus, three-way ANOVA (time \( \times \) salt \( \times \) GF) performed at both high and low CO\(_2\) only showed a significant salt effect at high CO\(_2\). When plotting \( P_N \) against tissue ion concentrations (Supporting Information Fig. S5), and excluding from the analysis late time points where leaves were severely damaged by submergence (characterized by low leaf chlorophyll and low leaf porosity values, see caption in Supporting Information Fig. S5), only tissue Na\(^+\) and \( P_N \) at 2500 \( \mu \)M CO\(_2\) showed a significant negative correlation \((r = -0.61)\). As no significant NaCl effect was detected for \( P_N \) at 200 \( \mu \)M CO\(_2\) \((P \geq 0.2094)\), the adverse impact on growth of submergence alone was significant whereas the 50 mM NaCl treatment during submergence had little additional effect on growth (see next section on growth analysis) in spite of \( P_N \) at high CO\(_2\) revealing some damage to the photosynthetic apparatus.

**Gas film removal significantly reduces rice growth when submerged**

Rice shoots elongated during 9 d of submergence and were 52% longer than controls in air (average of NaCl and GF treatments, Table S1). Meanwhile, submergence severely inhibited tillering, as mean tiller numbers after 9 d of submergence across NaCl and GF treatments were only 1–1.75 in submerged plants compared with 4–4.5 in controls with shoots in air (Table S1).

Complete submergence over 9 d reduced root RGR more than the reduction in shoot RGR, and gas film removal caused further reductions to growth of plants when submerged in either non-saline or saline water (Fig. 5). During 9 d of submergence in non-saline water, +GF plants maintained root and shoot RGR at 27 and 61% of controls in air, respectively, while removal of gas film resulted in root and shoot RGR of 10 and 45% of controls in air. Plants that had gas films removed also showed reduced RGR during the recovery period after desubmergence (root and shoot RGR to 60 and 55% of controls in air). By contrast, plants with intact gas films had root and shoot RGR of 98 and 84% when desubmerged relative to controls in air.

NaCl had a tendency to further decrease RGR in –GF plants (e.g. root RGR during submergence in saline water; Fig. 5a), but two-way ANOVA (salt \( \times \) GF) only detected significant gas film and no salinity effects during submergence and recovery. Nonetheless, a significant salt \( \times \) GF interaction was found when analysing the amount of shoot tissue (% of total shoot DM) that had senesced and was scored as dead after the recovery period (Fig. 6), that is, plants subjected to both dilute Triton X-100 brushing and 50 mM NaCl had lower functioning leaf area for further growth than plants only subjected to one of these treatments.

In conclusion, gas film removal had a profound effect on rice RGR during complete submergence in non-saline water and the following recovery period. On the other hand, 50 mM NaCl in the submergence solution only had limited additional effect on growth.

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**Experiment 2 – the effect of pO\(_2\) on leaf ion concentrations**

To separate the effects of gas films acting as a possible physical barrier to ion uptake/loss, and gas films resulting in higher leaf O\(_2\) status that could affect energy status and thus energy-dependent ion transport (potentially impacting K\(^+\) retention and Na\(^+\) and Cl\(^-\) ‘exclusion’ from leaves), excised leaves with and without gas films were incubated in submergence solution with 50 mM NaCl and pO\(_2\) ranging from 0.01 to 20.3 kPa (Fig. 7). Low pO\(_2\) did not seem to diminish gas films as clearly visible gas films were observed on all +GF leaves following the 24 h incubation.

Gas film removal resulted in 12% higher final tissue Na\(^+\) concentration across the pO\(_2\) range tested (Supporting Information Fig. S6); however, the difference was only significant at 0.46 kPa O\(_2\). The little difference between +GF and −GF leaf Na\(^+\) concentration was due to a high Na\(^+\) influx in both cases: average net Na\(^+\) uptake during the 24 h of submergence was 7.0 \( \mu \)mol Na\(^+\) g\(^{-1}\) DM h\(^{-1}\) in +GF and 8.7 \( \mu \)mol Na\(^+\) g\(^{-1}\) DM h\(^{-1}\) in −GF leaves (Fig. 7), so the leaf gas films acted only as a weak physical barrier as Na\(^+\) entry into +GF leaves was substantial.

Gas films on leaves acting as a rather weak barrier to tissue ion fluxes were confirmed by changes in tissue K\(^+\). Lowering pO\(_2\) to 0.01 kPa resulted in a severe loss of tissue K\(^+\) regardless of gas film presence (Fig. 7), with tissue K\(^+\) decreasing to 21% (−GF) and 25% (+GF) of leaves incubated at 20.3 kPa O\(_2\). Nonetheless, gas films did have a significant overall effect on tissue K\(^+\) according to two-way ANOVA (see caption of Fig. 7).

Interestingly, leaf Cl\(^-\) showed a significant GF \( \times \) pO\(_2\) interaction, as removal of gas films decreased tissue Cl\(^-\) by 8–16% relative to leaves retaining a gas film at 1.59–20.23 kPa O\(_2\), and increased tissue Cl\(^-\) by 20% at 0.01 and 0.46 kPa (Supporting Information Fig. S6). Although these differences in Cl\(^-\) concentrations at single pO\(_2\) levels were not significant in post hoc tests, it should be noted that in experiment 1, presence of gas films also tended to result in higher tissue Cl\(^-\) on days 5 and 9 in both non-saline (significant on day 9) and saline (not significant) water compared with leaves without gas films. These coinciding observations from two separate experiments seem to indicate a complex interaction between leaf gas films and Cl\(^-\) uptake and resulting tissue concentrations.

In conclusion, this second experiment with varying pO\(_2\) confirmed that gas films on leaves of rice apparently only act as a weak physical barrier to ion uptake/loss; a leaf-water interface, allowing for substantial K\(^+\) net loss and Na\(^+\) net uptake must have been present during the 24 h of submergence, despite the gas films being visibly present.

**DISCUSSION**

Gas film presence had the expected beneficial effects on plant growth, \( P_N \), leaf chlorophyll\(_b\), leaf porosity and shoot tissue survival for rice during submergence in saline (50 mM NaCl) water. However, the results did not support our initial hypothesis of rice leaf gas films acting as a strong physical barrier to ion uptake (Na\(^+\), Cl\(^-\)) or loss (K\(^+\)) during
submergence in saline water. Although gas film removal significantly increased Na\(^+\) uptake by plants submerged in artificial floodwater containing 50 mM NaCl, Na\(^+\) and Cl\(^-\) accumulation and K\(^+\) loss were substantial even in leaves possessing gas films (Fig. 2). Gas films acting as a rather weak physical barrier to Na\(^+\) entry were confirmed in a separate experiment (Fig. 7 and Supporting Information Fig. S6), where removal of gas films resulted in a 12% increase in leaf Na\(^+\) concentration.

Gas film retention time in the present experiment was up to 6 d longer than in the field (Winkel et al. 2014), indicating that turbid floodwaters may accelerate gas film loss. Interestingly, the gas films were retained longer by plants in the non-saline treatment as compared with those in the saline submergence (Fig. 1). Loss of gas films with time of submergence was not associated with clear structural changes of the surface of the leaf cuticle, except for wax platelets on papillae appearing more rounded (Supporting Information Figs S1 and S2). This loss of gas films without significant changes in cuticle surface structure was unexpected as leaf hydrophobicity is known to be related to the amount of wax platelets on the cuticle surface (see Neinhuis and Barthlott, 1997 for characterization of leaf hydrophobicity and the relationship with cuticle nanostructure). The recent description of the gene ‘OsHSDDI’ responsible for synthesis of epicuticular wax compounds (Zhang et al. 2016) adds new perspectives to further explore gas film retention during submergence.

### Uptake of Na\(^+\) and Cl\(^-\) and loss of K\(^+\) indicates leaf-to-water contact even with gas films present

Leaves of rice with gas films when submerged in 50 mM NaCl for 9 d had tissue ion concentrations (\(\mu\text{mol g}^{-1}\) fresh mass) of Na\(^+\) 145, Cl\(^-\) 106 and K\(^+\) 38 (data not shown), respectively, which compare with maximum concentrations of Na\(^+\) 94 and Cl\(^-\) 141 and minimum K\(^+\) 52 in the coleoptiles of rice seedlings submerged for 42–186 h at 50 mM NaCl (Kurniasih et al. 2013). The coleoptiles emerged from seeds under water and lacked gas films (Kurniasih et al. 2013). The substantial entries of Na\(^+\) and Cl\(^-\) for rice leaves with gas films when submerged in saline water at 72 and 93% of those for leaves without gas films (Fig. 2) contrast with the reduced ion entry into leaves of M. siculus with gas films of only 51 and 44% of the amounts without gas films (during the first 24 h of complete submergence, Teakle et al. 2014). These species differences in ion entry could be due to contrasting leaf morphology resulting in distinct three-dimensional (3D) structures of the gas films. The 3D tomograms of submerged Spartina anglica leaves with gas films indicated that along the leaf ridges, approximately 20% of the ridge surface is in direct contact with water (Laursiden et al. 2014). Rice also possesses plicate leaves (Wu et al. 2011) where the majority of the external gas volume is present in the deep grooves between each ridge running parallel along leaves, and presumably areas along the ridges of leaves of rice must also have some direct contact with the floodwater. With time of submergence, and with possible declines in surface hydrophobicity, these exposed patches are likely to grow in size resulting in increasingly larger interfaces (i.e. areas of direct contact) between floodwater and the leaf surface. In contrast to rice and S. anglica, M. siculus does not possess plicate leaves (see photo of submerged leaf in Teakle et al. 2014), likely resulting in much less variation in 3D structure (and thickness) of the gas film across the leaf surface. Consequently, we suggest that the differences in ion entry observed between rice (this study) and M. siculus (Teakle et al. 2014) are due to 3D structural differences in the gas layer forming the interface between cuticle and floodwater, with likely more direct leaf-to-water contact in submerged rice than in M. siculus.

Presence of significant direct leaf-to-water contact for rice leaves with gas films was supported by substantial loss of K\(^+\) from leaves submerged in water at low \(pO_2\) (Fig. 7). K\(^+\) loss during severe hypoxia or anoxia is, in the short-term, caused by depolarization of plasma membranes (Buwalda et al. 1988), leading to opening of voltage-gated ion channels (Ward et al. 2009), and during longer periods can result from deterioration or damage to membranes, as described for wheat roots (Buwalda et al. 1988; Greenway et al. 1992; Goggin & Colmer 2007). Meanwhile, the leaf K\(^+\) loss observed when submerged in saline solution at air-equilibrium \(pO_2\) (Figs 2 and 7) is most likely caused by high external Na\(^+\) known to induce K\(^+\) efflux (Shabala et al. 2006; Britto et al. 2010).

While removal of leaf gas films increased Na\(^+\) uptake by leaves of rice submerged at 50 mM NaCl, Cl\(^-\) uptake was much more similar in −GF and +GF leaves (Fig. 2). In addition, on some occasions (submerged in non-saline water in experiment 1 and at high \(pO_2\) in experiment 2), tissue Cl\(^-\) concentration was higher in +GF than in −GF leaves. We suggest that this difference in tissue Na\(^+\) and Cl\(^-\) concentrations could be caused by these ions entering the leaf in different ways: Na\(^+\) is likely to enter leaves down an electrochemical gradient, while Cl\(^-\) has to be actively taken up because of its negative charge. Rice coleoptiles submerged in 50 mM NaCl showed peak uptake of Cl\(^-\) during the initial 42–114 h of submergence (Kurniasih et al. 2013), and such Cl\(^-\) uptake can be a more rapid and less energy-demanding means to maintain cell turgor or volume than production of organic solutes (Raven 1985; Oren 1999). Energy available for Cl\(^-\) influxes via H\(^+\)−Cl\(^-\) symports and associated H\(^+\) ATPase activity required to maintain the H\(^+\) gradient across the plasma membrane (Teakle & Tyerman 2010) is likely to be higher in +GF leaves because of higher sugar levels and O\(_2\) uptake compared with −GF leaves (Peder sen et al. 2009; Winkel et al. 2013). The significant \(pO_2\) × GF interaction on leaf Cl\(^-\) concentrations in experiment 2 further supports that Cl\(^-\) uptake is altered by leaf energy status. Indeed, ion net fluxes even in the anoxia-tolerant coleoptile of rice seedlings are substantially reduced in anoxia as compared with aerated conditions for seedlings submerged in 50 mM NaCl (Kurniasih et al. 2016). Tracer experiments are needed to separate the roles of ion influx or efflux on changes in net uptake rates and leaf ion concentrations of submerged rice.

Rice subjected to 50 mM NaCl in the root medium but with shoots in air in several earlier experiments accumulated higher leaf tissue Na\(^+\) and Cl\(^-\) concentrations (Yeo & Flowers 1982; 1984; 1985; 1986) than those in the shoot tissues of rice during complete submergence in 50 mM NaCl (present study and
Gas films enhance underwater \( P_N \) and delay leaf tissue degradation of submerged rice

Leaf gas films have beneficial effects on underwater \( P_N \) of rice both in non-saline (Pedersen et al. 2009; Winkel et al. 2014) and saline (present study, Fig. 3) submergence. 3D diffusion modeling has demonstrated the enhanced leaf-floodwater gas exchange by leaves with gas films if stomata remain at least partially open during submergence (Verboven et al. 2014). The significant adverse effect of gas film removal on growth (Fig. 5) is in accordance with a previous study of submerged rice (Pedersen et al. 2009).

In addition to lowering \( P_N \), removal of leaf gas films leads to earlier leaf chlorophyll \( a \) degradation of submerged leaves (Fig. 1). This contrasts with a previous study where no difference in total leaf chlorophyll between \(+GF\) and \(-GF\) plants was found during 7 d submergence in the field (Winkel et al. 2013). We suggest that the lack of decline in chlorophyll \( a \) in \(-GF\) plants could be due to Winkel et al. (2013) sampling the youngest fully developed leaf at all time points and not beyond 7 d. In a subsequent experiment, Winkel et al. (2014) observed no chlorophyll decline until day 7 (second youngest fully expanded leaf, submerged for 13 d in the field, \(+GF\) only) consistent with declines after 9 d in the present study. Yeo and Flowers (1983) established the leaf \( Na^+ \) concentrations associated with a 50% loss of chlorophyll (LC50) for nine rice genotypes with shoots in air. However, for plants in the present study, chlorophyll \( a \) degradation was mainly caused by duration of submergence (explaining 76% of the variation in chlorophyll \( a \) according to ANOVA) rather than leaf \( Na^+ \) concentration (5% of the variation), and chlorophyll \( a \) degradation was severe even in leaves of plants submerged in non-saline water, so we refrained from calculating a LC50 in the present study. Leaf senescence is a common feature of submerged rice and has been associated with the accumulation of ethylene causing chlorophyll degradation (Jackson et al. 1987; Ella et al. 2003).

Leaf hydrophobicity has previously been suggested as an adaptation to prevent adverse effects of salt spray on leaves of some coastal plants (Ahmad & Wainwright 1976; McNeilly et al. 1987). Variation in leaf wettability and leaf \( Na^+ \) retention (upon spraying with or immersion into water containing 500 mM NaCl) was linked to distributions of three *Agrostis stolinsfera* ecotypes growing in sheltered inland habitats, seawater spray-zone or salt marshes (Ahmad & Wainwright 1976). The inland ecotype showed high wettability because of lower contact angles and shorter epicuticular waxes than the ecotypes in the salt-spray and salt marsh zones, resulting in 16 times higher \( Na^+ \) retention on the surface of leaves after 5 s immersion into saline water. Ahmad and Wainwright (1976) suggested that differences in adaxial and abaxial sides for leaf hydrophobicity in spray-zone plants but not in salt marsh plants could be an adaption to episodic inundations of the marsh plants, as inundation would affect both sides of the leaf in contrast to salt-spray. However, another low salt marsh plant from the intertidal zone (*S. anglica*) being hydrophobic only on the adaxial leaf side (Winkel et al. 2011) is not in support of two-sided leaf hydrophobicity as a general adaptation to salt water submergence, but like for rice with two-sided leaf gas films (Winkel et al. 2013), the one-sided leaf gas films on submerged *S. anglica* also benefit internal \( O_2 \) status both during the day and at night.

CONCLUSIONS

Leaf gas films contribute to rice submergence tolerance by improving underwater gas exchange, growth, internal aeration and plant sugar levels (Pedersen et al. 2009; Winkel et al. 2013). This study found that during submergence in saline water (50 mM NaCl), gas films diminished earlier than for leaves in freshwater and that rice plants possessing leaf gas films maintained higher levels of underwater \( P_N \), more growth during submergence and recovery, greater proportion of surviving shoot biomass and better maintained leaf porosity and chlorophyll \( a \). Submergence was more detrimental to leaf chlorophyll \( a \) than the additional effect of 50 mM NaCl. However, gas films on leaves of rice delayed \( Na^+ \) entry to a much smaller degree compared with leaves of *M. siculus*, which was likely due to 3D structural differences in the gas layers on these two species with probable greater leaf-to-water contact for rice. Rice has plicate leaves, like *S. anglica*, for which the ridges have some direct contact with surrounding water even when the gas film is present (Lauridsen et al. 2014). Varying \( pO_2 \) had no effect on leaf \( Na^+ \) net uptake, suggesting that the observed delay of \( Na^+ \) uptake in \(+GF\) leaves should be attributed to gas films acting as a physical barrier rather than from the possible influence of altered \( O_2 \) supply and potential improved leaf energy status.

ACKNOWLEDGMENTS

T.D.C. acknowledges support from the Australian Research Council (DP120101482). We thank the UWA Institute of Advanced Studies for hosting O.P. as Professor-at-Large. M. H., D.K. and A.W. were supported by PhD and postdoctoral fellowships from the Villum Foundation.
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© 2016 John Wiley & Sons Ltd, Plant, Cell and Environment


Received 15 August 2016; received in revised form 16 November 2016; accepted for publication 20 November 2016.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

**Figure S1.** Scanning electron microscopy micrographs of leaf surfaces of rice subject to 0–16 d submergence in non-saline (0 mM NaCl) or saline (50 mM NaCl) water (containing basal ions see Methods). Each leaf is shown at 7000 × magnification (left) showing stomata horizontal field width = 36.6 μm and 500 × magnification (right) horizontal field width = 512 μm.

**Figure S2.** Scanning electron microscopy micrographs showing wax platelets on leaf surface after loss of leaf hydrophobicity and leaf gas film disappearance (a, plants had been submerged in water with 50 mM NaCl for 9 d), and wax platelets on papillae before (b, prior to submergence) and after (c, plants had been submerged in water with 50 mM NaCl for 9 d) loss of leaf hydrophobicity and leaf gas film disappearance. Leaves are shown at 27,000 × (a) and 45,000 × (b, c) magnification (horizontal field width = 3.9 and 2.5 μm, respectively).

**Figure S3.** Leaf Na+ (a), Cl− (b) and K+ (c) concentrations in the tissue water (mM) with time of submergence in water (containing basal ions, see Methods) with 0 mM NaCl (squares) or 50 mM NaCl (circles) for rice plants with leaf gas films (+GF, open symbols) or treated with 0.1% Triton X-100 and without gas films (−GF, closed symbols). Samples were the entire third and part of the fourth leaf (see Methods). Roots were in non-saline nutrient solution. Values are means ± SE, n = 3–4 except Na+ on day 5 at 0 mM NaCl (−GF) where n = 1 due to a sampling error.

**Figure S4.** Correlations among leaf Na+ (a), Cl− (b) and K+ (c) concentrations (data from Fig. 2) and corresponding leaf chlorophyll, concentrations (data from Fig. 1b) after submergence of rice in water (containing basal ions, see Methods) with 50 mM NaCl. Leaves were either left untreated, thus retaining a leaf gas film (+GF, open symbols) or treated with 0.1% Triton X-100 (−GF, closed symbols). Roots were in non-saline nutrient solution. r values from non-parametric Spearman rank correlation analysis, * denoting levels of significance (levels of P > 0.05, P ≤ 0.05, P ≤ 0.01, P ≤ 0.001 or P ≤ 0.0001 are denoted by n.s., *, **, *, ***, ****, respectively); Na+ r = −0.5084***; Cl− r = −0.3658***; K+ r = 0.4664***.

**Figure S5.** Correlations among leaf Na+ (a), Cl− (b) and K+ (c) concentrations (data from Fig. 2) with corresponding underwater P6 (μmol O2 m−2 s−1) at 2500 μM CO2 and 50 mM NaCl (data from Fig. 3b). Leaves were either left untreated, thus retaining a leaf gas film (+GF) or treated with 0.1% Triton X-100 (−GF). Roots were in non-saline nutrient solution, and shoots were submerged in water containing NaCl treatments and basal ions (see Methods). r values from non-parametric Spearman rank correlation analysis, * denoting levels of significance (levels of P > 0.05, P ≤ 0.05, P ≤ 0.01, P ≤ 0.001 or P ≤ 0.0001 are denoted by n.s., * , **, ***, ****, respectively): Na+ r = −0.6078***; Cl− r = −0.3234 n.s.; K+ r = 0.1923 n.s. † denotes points excluded from the correlation analysis to prevent leaf deterioration with time of submergence to draw the correlation. Points were excluded when leaf both porosity and chlorophyll, was <4.5% and 8.3 mg g−1 DM, respectively, as for day 16 (+GF) and days 5 and 9 (−GF). At 200 μM, free CO2 all correlations were not significant (Spearman rank correlation analysis, P > 0.05, data not shown).

**Figure S6.** Leaf Na+ (a), Cl− (b) and K+ (c) concentrations of a youngest fully expanded leaf of rice submerged in 50 mM NaCl (containing also basal ions, see Methods) for 24 h in the dark with gas films (+GF, open symbols) or treated with 0.1% Triton X-100 and without gas films (−GF, closed symbols). During incubation, the submergence solution was maintained at 0.01, 0.46, 1.59, 3.16 and 20.23 kPa O2. * denotes significant difference between ion concentrations (Sidak’s multiple comparisons test, P < 0.05). Two-way GF × pO2 ANOVA showed a significant effect of GF on leaf Na+ (P = 0.0345) and K+ concentrations (P = 0.0091). pO2 had a significant effect on K+ concentrations (P < 0.0001). For Cl−, two-way ANOVA showed a significant pO2 × GF interaction (P = 0.0050). Values are means ± SE, n = 4.

**Table S1.** Shoot length and number of tillers of rice plants submerged in water (containing basal ions, see Methods) with 0 mM NaCl or 50 mM NaCl with leaf gas films (+GF) or treated with 0.1% Triton X-100 and without gas films (−GF). +GF and −GF plants are from different batches; hence, these have separate emergent (shoots in air) controls. Roots were in non-saline nutrient solution. Letters denote significant difference (P < 0.05) between means (±SE, n = 4) according to one-way ANOVA with Sidak’s multiple comparisons post hoc test (shoot length) or non-parametric Kruskal–Wallis with Dunn’s multiple comparisons test (number of tillers).
Conclusion and perspectives for future research

In the work of this thesis, genotypic variation towards waterlogging (Chapter 1) and submergence tolerance (Chapter 2) in wheat was established. Traits conferring waterlogging tolerance (internal aeration, seminal root short term anoxia tolerance), as well as mechanisms in need of further elucidation (e.g., anoxia tolerance, micronutrient toxicity, wheat root stele proportions, phloem transport to root tips and downregulation of energy requirements) were highlighted. In order to clarify the role of microelement toxicity in determining waterlogging tolerance, toxicity thresholds for especially Fe$^{2+}$ should be determined. Also, the location of microelements within shoot tissues (using X-ray microanalysis) would be of interest in order to determine their potential phototoxic effects. The effect of microelements on root function could be clarified using the same approach; earlier work indicated that upon waterlogging Fe formed plaques around roots but did not enter the symplast (Ding & Musgrave 1995).

Chapter 1 also highlighted that seminal root anoxia tolerance correlated to waterlogging tolerance, but was not governed by fermentation capacity or root substrate supply. A suggested follow-up on these exciting results could be to assess the ROS levels in these varieties, or investigate if energy use is more efficient in some cultivars such as regulation of cytoplasmic pH (Greenway & Gibbs 2003). In order to breed for more waterlogging-tolerant crops, the possible locations of QTL for root aerenchyma formation and ROL barrier in wheat or wild relatives (as in barley, Broughton et al. (2015)) could be of use (see Outlook for waterproofing crops – where are we at?). The N deficiency in wheat owing to waterlogging could also be addressed by seeking improved adventitious root nutrient uptake during waterlogging and possibly also targeting N-use efficiency in wheat.

Chapter 2 showed that wheat submergence tolerance differed significantly between two cultivars, however further work is needed to pinpoint traits conferring tolerance as our hypothesis that shoot carbohydrate levels could explain varietal differences was not supported by the results. The reputed waterlogging tolerance difference between the two cultivars suggests that root traits should be further studied, or that other traits such as ROS-deprivation or ethylene sensitivity could have caused the contrasting submergence tolerance. An approach similar to rice, where rice cultivars with contrasting submergence tolerance were subjected to submergence
Conclusion, perspectives and outlook

following pre-treatment with an ethylene inhibitor (1-methyl cyclopropene) could be used to evaluate this aspect (Ella et al. 2003).

Experimental removal of leaf gas film decreased wheat and rice submergence tolerance (Chapter 3, Appendix 1). However, during a saline submergence of rice, significant intrusion of ions into leaf tissues was recorded in spite of leaf gas film presence, indicating presence of a leaf-water interface and thus contrasting with delayed salt intrusion into leaves of \textit{M. siculus} retaining a leaf gas film (Chapter 3). This raises questions about the structure of leaf gas films in these two contrasting species. X-ray phase contrast micro-tomography used to visualise leaf gas films on \textit{Spartina anglica} indicated that leaf-water interfaces could exist on ridges of the plicate leaves; rice also possesses plicate leaves. An experimental setup where rice leaves with gas films are submerged into a solution containing compounds adhering to the leaf surface could reveal the presence of such an interface. Moreover, clarifying if the “unidentified substance” covering submerged leaves is a biofilm could be attempted using staining-techniques for biofilms.

Meanwhile, genotypic variation for leaf gas film retention time was not evident in Danish cultivars (Appendix II), and contrasting submergence tolerance in two wheat cultivars was not related to leaf gas film thickness or retention time (Chapter 2). Unless significant variation in gas film retention time is detected in more distinct wheat genotypes or wheat relatives, a prominent role of leaf gas films in wheat flood tolerance improvement is unlikely. When assessing the ecological significance of leaf gas films in plant communities, a correlation between presence of leaf gas films and the distribution of plant species along a flood gradient could not be established (Winkel et al. 2016). This seems to indicate that in general, leaf gas film formation during submergence is merely a side-effect of having a hydrophobic cuticle (for a number of reasons not related to submergence) rather than for improved gas exchange upon submergence \textit{per se}. Nevertheless, gas films enhance aeration and fitness for some species experiencing short and frequent submergence scenarios such as \textit{S. anglica} growing in the intertidal zone (Winkel et al. 2011). Clarifying the precise pathways of cuticle wax synthesis and their limitations during submergence could possibly also be of interest for the field of biomimicry, where scientist are partly successful in mimicking natural superhydrophobic surfaces, but failing in maintaining this characteristic for longer durations (Latthe et al. 2012).
Outlook for waterproofing crops – where are we at?

Given the genetic variation to especially submergence tolerance in rice (Das et al. 2005) and waterlogging tolerance in wheat (Chapter 1), it becomes clear that excess water can be tolerated by some crop genotypes. While the production of cultivars tolerant towards drought has been relatively successful for major crops such as maize (La Rovere et al. 2010; Cooper et al. 2014), the production of waterlogging-tolerant cultivars is still lagging behind as to my knowledge apart from rice no other commercial flood-tolerant cultivars have been produced. This might seem surprising considering the high flood-induced losses in crop production (Fig. 1), and the amount of research conducted on crop flood tolerance concluding that genetic variation for breeding efforts is present (Van Ginkel 1991; Musgrave & Ding 1998; Boru et al. 2001; Samad et al. 2001; Setter & Waters 2003; Collaku & Harrison 2005; Hayashi et al. 2013). In order to provide an overview of the status and challenges of producing flood-tolerant cultivars, the following sections review the current state in breeding for major crop (rice, wheat, soybean and maize) flood tolerance.

Rice: Serving as a promising example for the production of flood-tolerant crops, submergence tolerance was introduced into high-yielding rice cultivars in 2006 (Xu et al. 2006). Submergence-tolerant cultivars able to survive 14 days of complete submergence (Fig. 6) and with good agronomic traits were within 3 years of release grown by 4 million famers in India, Laos, Philippines and Bangladesh (Ismail et al. 2013). In the previous 28 years, several flood-resistant cultivars had been produced by conventional backcross-breeding, but had inferior grain quality and were therefore not welcomed by farmers (Septiningsih et al. 2009). The successful introduction of the Sub-1 locus conferring submergence tolerance but without significantly affecting plant morphology was achieved using marker-assisted back-crossing (Septiningsih et al. 2009; Iftekharuddaula et al. 2011). Submergence tolerance is conferred by inhibiting shoot elongation and carbohydrate depletion upon submergence, resulting in yields twice as high in submergence-tolerant compared to in intolerant cultivars (Das et al. 2005; Mackill et al. 2012). Current efforts are to improve seedling anoxia tolerance in order to promote direct seeding practices.
Figure 6. Pictures illustrating differential growth responses in wheat, rice, maize, soybean and/or their wild relatives upon flood treatments. a) Waterlogging-tolerant breeding-line (96W639-D4-13, left) and commercial wheat variety Cascades (right) after 6 weeks of waterlogging 3 weeks after sowing. From Setter and Waters (2003). b) Submergence-tolerant wheat cultivar Swarna-sub1 (right) and intolerant cultivar Swarna (left) after 10 days of submergence. Source: International Rice Research Institute. c) Flood-intolerant maize Mi29 (left) compared to wild maize relative Zea nicaraguagensis (right) during a flood. From Omori et al. (2016). d) Soybean (G. max, left) compared to wild relative (G. soja, right) upon flooding in University of Missouri field evaluations. From Valliyodan et al. (2016).

Wheat: The use of marker-assisted selection (MAS) in producing flood-tolerant wheat has been described as a great prospect for almost two decades now (Setter 2000; Setter & Waters 2003). However, to my knowledge no commercial cultivars with a reputed high waterlogging tolerance have been released using this or any other breeding approach, in spite of the notion that “three promising lines are now in the final stages of breeding evaluations” (Setter & Waters 2003). Apparently, the failure in developing a waterlogging-tolerant cultivar in this particular case was the spreading of yellow rust to Australia to which the promising lines held no resistance (Pers. Com., wheat breeder Robin Wilson, 2017).
Several studies have reported QTLs for waterlogging tolerance in wheat (Yu & Chen 2013; Ballesteros et al. 2014; Yu et al. 2014) measuring shoot and root biomass, root length, tiller number, chlorophyll content, plant height and other agronomical traits and detecting 34, 36 and 48 QTLs for waterlogging tolerance, respectively, across the genome. Such generic approaches have been criticised as insufficient, as given the breadth of QTL location, undesirable genes will inevitably be transferred during breeding attempts to move desirable genes into elite germplasm (Shabala 2011). Instead, more specific mechanism conferring waterlogging tolerance should be targeted, but this is often not achieved due to screening mostly taking place in the field where techniques used in studies on waterlogging tolerance are not applicable (Shabala 2011).

The use of *Hordeum marinum* as donor of a ROL-barrier to wheat amphiploids has often been referred to as a promising way to enhance flooding tolerance of wheat (Malik et al. 2011). However, results from a recent study challenged the potential of this approach since disomic chromosome addition lines did not possess a ROL barrier, suggesting that the genes for this trait are not located on one single chromosome (Konnerup 2016). Hopes are also that the much more advanced stage of rice genomics (owing its status as model genetic monocot) allows for the exploitation of rice genomics information for wheat improvement (Anderson 2003).

The use of molecular markers for ion toxicity/waterlogging tolerance still warrants hope to produce flood-tolerant wheat with good agronomic traits (Pers. Com., wheat breeder Robin Wilson, 2017), especially with the use of next-generation breeding tools such as high-throughput and cost-effective mapping and phenotyping becoming available for breeding (Valliyodan et al. 2016). However, future research should seek to clarify the role of micronutrient toxicity on a range of soils in order to aid breeding efforts, as some divergence seems to exist as to which traits mainly determine waterlogging tolerance.

**Maize:** Attempts to produce waterlogging-tolerant maize cultivars rely on the high waterlogging tolerance in the wild maize relative teosinte (*Zea nicaraguensis*). *Z. nicaraguensis* grows in lowland areas of Nicaragua that are frequently flooded during the rainy season and exhibits adaptations such as constitutive aerenchyma formation, a ROL-barrier and formation of adventitious roots at the soil surface. Researchers have followed a breeding strategy plan for a decade now (Mano & Omori 2015), focusing on differentiating factors determining flooding tolerance and analyzing each component separately, rather than selecting germplasm and performing genetic analysis under field conditions. This approach was chosen due to the
observations that the flood tolerance ranking of varieties was inconsistent between different researchers, and also difficult to repeat under field conditions due to interaction with environmental factors (Omori et al. 2016). Work has resulted in determination of flood tolerance trait QTL’s (mentioned above) in Z. nicaraguensis, and the production of near isogenic maize lines possessing one or more QTL’s for the given traits using marker assisted selection (Mano & Omori 2015; Omori et al. 2016). The chromosome location of a QTL for ROL-barrier has also been identified (Watanabe et al. 2017), and one of the next steps will be to pyramid these traits into maize. Expectations are that we will see the release of a flood-tolerant F1 hybrid within a few years (Omori et al. 2016).

**Soybean (Glycine max):** Soybean germplasm has been screened for waterlogging tolerance, showing that flood-intolerant lines lost twice as much yield as the flood-tolerant lines (Valliyodan et al. 2016). Comparing two genotypes with contrasting waterlogging tolerance indicated that development of aerenchyma and adventitious roots occurred faster in the flood-tolerant genotype (Valliyodan et al. 2014).

Several QTLs have been mapped for soybean flood tolerance. Some QTLs were not repeatable across years, or were tightly linked with flowering time (Githiri et al. 2006). The tight link between flowering time and flood tolerance could be explained by a longer recovery period when flowering is delayed (Githiri et al. 2006). Several other QTLs have been associated with resistance to root fungal disease Phytophthora sojae (Cornelious et al. 2005; Valliyodan et al. 2016). Apparently, a breeding program by the University of Missouri has developed three flooding lines through MAS exhibiting yields 90% of commercial checks under drained conditions, but outperforming commercial checks during flooding by up to 1 ton/hectare (Valliyodan et al. 2016). In addition, wild soybean (Glycine soja) is claimed to exhibit excellent flood tolerance, outperforming G. max (Fig. 6) and thereby indicating potential for utilizing wild relatives as in maize. However, these results and/or traits conferring higher tolerance in G. soja have not yet been identified (Valliyodan et al. 2016).

In conclusion, research in dryland crop flood tolerance is ongoing, but as the production of submergence-tolerant rice cultivars by IRRI (more than three decades of breeding efforts, Septiningsih et al. (2009)) and the production of drought-tolerant maize by CIMMYT (four decades of breeding efforts, estimated 2007-2016 research costs of $76 million, La Rovere et al. (2010)) indicate, long term commitment and investment by foundations, governments or the
private sectors are necessary. Since commercial breeders are not inclined to cover costs to run field trials for flood tolerance, globally dispersed and short term research projects without sufficient infrastructure can contribute with knowledge on plant flood responses, but are unlikely to result in major breakthroughs.
Literature cited in introduction, conclusion and outlook


Appendix I: Flood tolerance of wheat – the importance of leaf gas films during complete submergence

Photo: Ole Pedersen
Flood tolerance of wheat – the importance of leaf gas films during complete submergence

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Abstract. Submergence invokes a range of stressors to plants with impeded gas exchange between tissues and floodwater being the greatest challenge. Many terrestrial plants including wheat (\textit{Triticum aestivum} L.), possess superhydrophobic leaf cuticles that retain a thin gas film when submersed, and the gas films enhance gas exchange with the floodwater. However, leaf hydrophobicity is lost during submergence and the gas films disappear accordingly. Here, we completely submersed wheat (with or without gas films) for up to 14 days and found that plants with gas films survived significantly longer (13 days) than plants without (10 days). Plants with gas films also had less dead tissue following a period of recovery. However, this study also revealed that reflections by gas films resulted in a higher light compensation point for underwater net photosynthesis for leaves with gas films compared with leaves without ($I_C = 52$ vs $35 \mu$mol photons m$^{-2}$ s$^{-1}$ with or without gas films, respectively). Still, already at $\sim$5\% of full sunlight the beneficial effect of gas films overcame the negative under ecologically relevant CO$_2$ concentrations. Our study showed that dryland crops also benefit from leaf gas films during submergence and that this trait should be incorporated to improve flood tolerance of wheat.

Additional keywords: air film, flooding tolerance, hydrophobicity, underwater photosynthesis, underwater respiration, water repellent.

Received 7 November 2016, accepted 23 March 2017, published online 26 April 2017

Introduction

Torrential rains can result in overland floods that inundate terrestrial plants (Vervuren \textit{et al.} 2003). With the current projection on climate changes, the frequency of such flooding events is predicted to increase (Parry \textit{et al.} 2006) and already now flooding results in great annual losses in crop production (Bailey-Serres \textit{et al.} 2012). Complete submergence invokes a range of stressors to terrestrial plants of which impeded gas exchange between tissues and floodwater poses the greatest challenge (Armstrong 1979). Diffusion of gases occurs 10 000-fold faster in air compared with water so that in completely submersed plants, uptake from the floodwater of CO$_2$ for photosynthesis in light and O$_2$ for aerobic respiration in darkness is strongly diffusion limited (Pedersen and Colmer 2012). In order to sustain internal aeration, wetland plants have porous tissues where aerenchyma forms a series of interconnected air channels enabling internal gas phase diffusion of both CO$_2$ and O$_2$ (Justin and Armstrong 1987). Another common trait of roots of wetland plants is a barrier to radial O$_2$ loss (ROL) (Colmer 2003). The ROL barrier greatly reduces radial O$_2$ loss to the anoxic waterlogged soils and thereby enables aeration of the root tips so that the root can continue to grow (Waters \textit{et al.} 1989).

The slow diffusion of gases in water also greatly restricts underwater net photosynthesis ($P_N$) in aquatic plants (Madsen and Sand-Jensen 1991; Maberly and Madsen 2002) and in submerged terrestrial wetland plants (Colmer \textit{et al.} 2011). Terrestrial wetland plants lack most of the leaf acclimation traits to reduce the resistance to CO$_2$ uptake, of which the most important traits are those that reduce the resistance caused by the diffusive boundary layers (Madsen \textit{et al.} 1993). Nevertheless, as the CO$_2$ concentration of floodwaters is often several fold above air equilibrium of 10–20 mmol m$^{-3}$ (Colmer \textit{et al.} 2011), most terrestrial plants show some capacity for underwater photosynthesis although rates are generally greatly reduced compared with those in air (Colmer \textit{et al.} 2011; Winkel \textit{et al.} 2011, 2013, 2016). However, some terrestrial wetland plants possess superhydrophobic leaves and retain a thin silvery gas film on their leaves when submersed. Leaf superhydrophobicity was first established as a self-cleansing mechanism (Barthlott and Neinhuis 1997; Neinhuis and Barthlott 1997), but the leaf gas films enable species with superhydrophobic cuticles to continue photosynthesising under water at rates up to 27\% (rice, Pedersen \textit{et al.} 2009) and 28\% (\textit{Hordeum marinum} Huds., Pedersen \textit{et al.} 2010) of those in air at ecologically relevant CO$_2$ concentrations in the floodwater.
Leaf gas films have been shown to enhance submergence tolerance of completely submerged wetland plants. Leaf gas films lower the apparent resistance to gas exchange with the floodwater several fold (Colmer and Pedersen 2008; Pedersen et al. 2009; Winkel et al. 2013; Verboven et al. 2014) and although the exact mechanism has yet to be revealed, the leaf gas films resemble the ‘physical gills’ of some diving aquatic insects (Pedersen and Colmer 2012). In a study of rice, completely submerged plants with leaf gas films intact continued to grow for 7 days at rates similar to those in air, whereas plants with gas films removed ceased growth under water (Pedersen et al. 2009). Also H. marinum, a wild relative to barley, continued to grow when completely submerged when the floodwater was enriched with dissolved CO2 (200 mmol m−3) and the leaf gas films remained intact throughout the 7 days of submergence (Pedersen et al. 2010). In addition to enhancing underwater PN and thus carbohydrate production during submergence, leaf gas films have also been shown to aid internal aeration of belowground tissues in darkness of submerged terrestrial plants (Pedersen et al. 2009; Winkel et al. 2011, 2013), and so the trait is crucial for submergence tolerance of terrestrial wetland plants. More recently, it has been shown that also dryland plants possessing superhydrophobic leaf cuticles benefit from gas film formation when submerged (Winkel et al. 2016). In fact, rates of both O2 uptake in darkness and CO2 uptake and O2 production in light did not differ between five species of wetland plants and nine species of dryland plants that had leaf gas films when submerged (Winkel et al. 2016).

The wetland crop rice has been shown to greatly benefit from leaf gas films when submerged (Pedersen et al. 2009), but some dryland crops also form gas films under water e.g. wheat (Fig. 1), barley and oats (Raskin and Kende 1983). Moreover, dryland crops such as wheat can experience submergence at various stages throughout their life cycle (Fig. 1) although complete inundation is more likely at the seedling stage (Herzog et al. 2016b) but submergence tolerance of wheat has so far not been examined. This is in stark contrast to the vast literature on responses to waterlogging of wheat that reveals some tolerance to waterlogging. A recent review showed that across 17 studies, the tissue porosity of adventitious roots increased from 5.2 under drained conditions to 14.8% (median values) when waterlogged (Herzog et al. 2016b), indicating that also wheat can improve internal aeration by facilitating gas phase O2 transport to the growing root tips.

In the present study we tested the importance of leaf gas films for survival of completely submerged wheat (6–7 fully expanded leaves) by experimentally removing the gas films by brushing the leaves before submergence with a dilute detergent. The plants were submerged up to 14 days and allowed a minimum of 14 days of recovery before they were scored dead or alive and final biomass measured. During submergence, tissues were sampled to enable assessment of thickness of leaf gas films, tissue porosity of the lamina and chlorophyll

Fig. 1. A field flood event in Western Australia resulting in complete submergence of wheat (a) and a similar event in Denmark (b) where wheat is submerged late in the growth cycle. In (c), water forms perfectly spherical droplets on the surfaces of the superhydrophobic leaf cuticle (contact angle = 155.9 ± 0.7°; mean ± s.e., n = 8; see also Konnerup et al. (2017) for contact angle of additional 14 types of genotypes of wheat) and when submerged (d), a thin gas film is retained on leaf surfaces with a silvery appearance. The gas bubble (d) results from underwater photosynthesis. All photos are of wheat (Triticum aestivum) and in the case of (c) and (d), of the cultivar ‘JB Asano’.
concentration. Plants were also grown to enable mechanistic studies of the leaf gas films so that CO₂ response curves and light response curves of underwater P₅ could be established for wheat. We hypothesised that plants with initially intact gas films would survive complete submergence better than plants where the gas films had been removed, and that plants with gas films would maintain tissue integrity longer. Mechanistically, we hypothesised that leaf gas films would enhance CO₂ uptake and thus underwater P₅ within ecologically relevant CO₂ concentrations in the floodwater, but also that the reflection of light by gas films (as indicated by the silvery appearance) would be disadvantageous as limiting light levels, a novel aspect that has not previously been considered.

Materials and methods

Plant material

For mechanistic studies of CO₂ and light response of leaf segments, seeds of wheat (*Triticum aestivum* L., cv. ‘JB Asano’) were sown directly into a commercial potting mix (Pindstrup substrate no. 2, Pindstrup Mosebrug A/S) with Osmocote ‘Vegetable’ (N : P : K; 15.3 : 2.4 : 5.9) in 250 mL plastic pots. Following germination, seedlings were thinned so that each pot contained only one plant, and plants were grown in a glasshouse during August 2012 to an age of 4–5 weeks (daytime temperature 17–28°C; night-time temperature 12–18°C).

For growth and survival experiments, seeds of the same cultivar were sown directly into a commercial potting mix (Pindstrup substrate no. 2, Pindstrup Mosebrug A/S) with Osmocote (N : P : K; 15.3 : 2.4 : 5.9) in 600 mL pots with 3 cm of river sand in the bottom and on 3 cm of sand on top to reduce the flux of soil derived nutrients into the floodwater upon submergence. The beakers had drainage holes covered with two layers of fine mesh in order to constrain all roots to the pot. Following germination, seedlings were thinned so that each pot contained only one plant, and plants were grown in a glasshouse during July–August 2016 to an age of 17 days (daytime temperature 17–32°C; night-time temperature 16–26°C) experiments ended 28 days later at which point plants were 45 days old.

Growth and survival during submergence or waterlogging

Wheat occasionally becomes submerged also in well drained soils (Fig. 1) so survival during waterlogging or complete submergence was assessed over a 14 day treatment period followed by at least 2 weeks of recovery (Striker 2012). Prior to submergence, one set of plants (leaves and leaf sheaths) were brushed with 0.1% Triton X-100 (v/v) to remove hydrophobicity. Then, 160 individual 17-day-old plants were divided into five aquariums (32 in each, half with gas film removed) and completely submerged in artificial floodwater which contained (in mol m⁻³; Ca²⁺, 0.50; Mg²⁺, 0.25; Cl⁻, 1.00; SO₄²⁻, 0.25, with an alkalinity of 2 mol m⁻³) achieved by adding 200.2 g of KHCO₃ m⁻³) in a glasshouse with ambient light (PAR = 11–1151 µmol photons m⁻² s⁻¹; mid-day range, 1100–1500 hours). Floodwater O₂ and CO₂ concentrations were maintained at air equilibrium (constant partial pressures) by bubbling with atmospheric air throughout the experiment resulting in flow velocities of 0.5 to 3 cm s⁻¹ dependent on distance to air stone. Water temperature ranged from 18.8 ([O₂] = 290; [CO₂] = 16 mmol m⁻³) to 28.9°C ([O₂] = 240; [CO₂] = 12 mmol m⁻³) during the submergence period with a median value of 22.8°C ([O₂] = 269; [CO₂] = 14 mmol m⁻³). A plastic mesh was placed below the surface to prevent elongating shoots to restore atmospheric contact so that plants were always kept submerged by a minimum of 2 cm of water. At each time point, 10 individuals were desubmerged and left to drain (five with gas films initially and five with gas films removed). Another 20 individuals were waterlogged in artificial floodwater and were de-waterlogged after 5, 10 and 14 days. Furthermore 10 plants were kept under drained conditions with shoots in air (half brushed with Triton X-100, half unbrushed). Fv/Fm was measured daily to ensure that there was no adverse effect on photosynthesis in air of brushing with the detergent (see Fig S1, available as Supplementary Material to this paper). Survival was scored after plants were 45 days old and live plant material was separated from dead and both fractions were freeze-dried to obtain dry mass (DM). With this arrangement, plants submerged for 14 days had 2 weeks of recovery before scoring survival. Upon de-submergence shoot length, number of leaves and tillers were recorded.

Leaf gas film thickness and porosity

Leaf gas film thickness and leaf porosity were measured using the buoyancy method (Raskin 1983; Pedersen et al. 2009). The buoyancy of 5 cm long leaf segments from the mid lamina of the youngest fully expanded leaf was measured in de-ionised water with a 4 digit balance mounted with a hook underneath before and after brushing with 0.1% Triton X-100. Leaf gas film thickness was calculated as gas volume (m³) divided by two-times the projected area (m²); see Colmer and Pedersen (2008) for details. Leaf segments were then placed under vacuum for at least three times 5 min until all internal gas volumes were infiltrated by water, and then buoyancy was assessed again. Leaf gas film thickness and leaf porosity were measured immediately after complete submergence (initials), after 24 h and thereafter each day until the thickness of the gas layer was below detection limit (~1 µm) and for leaf porosity also after 6, 9 and 14 days of submergence.

Leaf chlorophyll concentration

Chlorophyll concentration was measured on the middle portion of what was the youngest fully expanded leaf at day 17 (day 0, start of treatment); at that time the wheat plants had 5–6 leaves. Leaf tips of the youngest fully expanded leaf were marked with a permanent marker to enable identification of leaves throughout the experiment. After harvest, leaf segments were freeze-dried for 48 h, then cut into small pieces (less than 2 mm²). Chlorophyll was extracted using 96.5% ethanol at 20°C and incubated for 24 h in darkness and then absorbance was measured at 665, 775 and 649 nm on a spectrophotometer (UV-160A, Shimadzu). Chlorophyll concentrations were calculated using equations by Wintemans and De Mots (1965).
Underwater net photosynthesis (CO2 and light response)

Underwater net photosynthesis (PN) by the lamina segments was measured using the method described by Winkel et al. (2013). In brief, for each replicate leaf (n=4), one lamina segment of ~25 mm length was taken half way up the blade of the youngest fully expanded leaf. Glass cuvettes (25 mL) contained individual lamina segments in incubation medium and two glass beads for mixing as these rotated on a wheel within an illuminated water bath, at 25°C. For CO2 response measurements, PAR was 1000 μmol photons m⁻² s⁻¹ inside the cuvettes measured using a spherical PAR sensor (US-SQS/L, Walz Heinz GmbH). Leaves were either brushed with a dilute detergent (0.1% Triton X-100) to remove hydrophobicity (without gas films) (Raskin 1983; Colmer and Pedersen 2008) or transferred directly into the artificial floodwater (see below) and served as controls (with gas films).

The artificial floodwater serving as incubation medium contained (in mol m⁻³) Ca²⁺, 0.50; Mg²⁺, 0.25; Cl⁻, 1.00; SO₄²⁻, 0.25. The dissolved O2 concentration in the incubation medium was set at ~50% of air equilibrium, by bubbling in 1 : 1 volumes of N₂ and air before adjustment of pH to obtain the desired CO₂ concentrations; this avoided build-up of excess O₂ during the measurements that might otherwise have resulted in photorespiration as described for submerged rice leaves (Setter et al. 1989). Dissolved CO₂ treatments were imposed by adding specific concentrations of KHCO₃ to the incubation medium with pH adjusted to pH 9.00–6.17 to provide a range of CO₂ concentrations from 3 to 2800 mmol m⁻³ of constant carbonate alkalinity at 2 mol m⁻³ (Stumm and Morgan 1996).

Following 60–90 min incubation, dissolved O₂ concentrations in the cuvettes were measured using a Clark-type O₂ minielectrode (OX-500, Unisense A/S), connected to a pA meter (Multimeter, Unisense A/S) and were calibrated at 20.6 kPa (air bubbled de-ionised water at 25°C) and at 0 kPa (de-ionised water with ascorbate and KOH at 25°C). Dissolved O₂ concentrations in cuvettes prepared and incubated in the same way as described above, but without leaf tissues, served as blanks. Projected area of leaf segments was measured using a laser leaf area meter (CI-202, CID Inc.).

Underwater PN as a function of light was measured as above but at a CO₂ concentration of 200 mmol m⁻³. PAR in the cuvettes was manipulated by varying the distance of the light source (GP Plus 600 W EL, Philips) or wrapping the cuvettes in neutral shading mesh resulting in a PAR gradient from 25 to 1000 μmol photons m⁻² s⁻¹ inside the cuvettes measured as above. Cuvettes wrapped in aluminium foil served as measurements for uptake of O₂ in the dark under the same mixing conditions. Vials in dark and at low irradiance (25 to 100 μmol photons m⁻² s⁻¹) were incubated up to 180 min in order to achieve sufficient differences in pO₂ between these treatments and blanks. For dark O₂ uptake, the incubation medium was initially set to air equilibrium to simulate O₂ uptake in fully aerated floodwater.

Photosynthesis in air

In order to benchmark rates of underwater photosynthesis with rates in air, PN in air by intact leaves (youngest fully expanded) with ambient (390 μmol mol⁻¹) CO₂ was measured at 25°C and PAR of 1000 μmol photons m⁻² s⁻¹, using a flow through leaf cuvette connected to an infrared gas analyser (LCI-SD, ADC). Measurements were taken from five replicate plants.

Soil pO₂

Soil pO₂ was measured at the onset of waterlogging or submergence using 4 optical O₂ needle sensors (OXF500PT, Pyroscience) connected to a four-channel meter (Firesting, Pyroscience). The optodes were positioned half way in between the stem of the plant and the rim of the pot at ~5 cm depth and the signal was logged every 1 min. using OxygenLogger (Pyroscience). Prior to measurements, O₂ optodes were calibrated at 20.6 kPa (air bubbled de-ionised water at 25°C) and at 0 kPa (de-ionised water with ascorbate and KOH at 25°C). Upon waterlogging or submergence, O₂ disappeared from the soil matrix within 6–22 h.

Data analysis

Data was graphed and analysed using GraphPad Prism 6.01 or 7.0; statistical tests used are given in table or figure captions. All tests were conducted at a probability level of 0.05 and means±s.e. are reported except in Table 1 where 95% confidence limits are also shown: 1- or 2-way ANOVAs were used as appropriate and ANOVA details are shown in Tables S1 and S2 available as Supplementary Material to this paper. A Tukey or Sidak post hoc test was applied to analyse individual time points. For survival data, a log rank (Mantel Cox) test was used to test if survival differed with and without leaf gas films and median survival time is reported. Relative growth rates were calculated assuming logarithmic growth or mortality in the time interval. CO₂ vs underwater PN was fitted using Michaelis-Menten kinetics and light vs underwater PN kinetics were fitted to a Jassby and Platt (1976) photosynthesis function. Resistance to CO₂ uptake was calculated as 1/initial slope of the CO₂ response curve and the photosynthetic light use efficiency (Φ) was calculated as the initial slope of the light response curve.

Results

Survival during complete submergence

The importance of leaf gas films for whole plant survival of complete submergence was tested by comparing control plants with plants where leaf cuticle hydrophobicity had been experimentally reduced so that gas films did not form when submerged. Plants were de-submerged at various time points ranging from 6–14 days and scored dead or alive after a recovery period of at least 14 days. Survival was defined as new growth after de-submergence and all plants were scored 28 days after the beginning of the submergence event (day 0) regardless of how long they had been submerged.

Plants with intact gas films survived 3 days longer than plants with their gas films removed. Control plants with leaf gas films initially intact survived 13 days of complete submergence but had a mortality rate of 80%, whereas after 12 days of submergence the mortality rate was 0% (all plants survived, Fig. 2). In contrast, plants with leaf gas films initially removed all survived 9 day of submergence but 80% had died after 10 days and none survived 11 days. The 3 day difference...
and leaf tissue porosity were measured initially and after 1, 2, 3 and 4 days of submergence, whereas porosity was also measured again after 6, 9 and 14 days of submergence. Gas film thickness of control plants rapidly declined so that 50% was lost already during the first 24 h of submergence (mean gas film thickness = 12 μm) and after 3 days of submergence, the thickness was below detection limit and indistinguishable from plants brushed with Triton X-100 (Fig. 3a). Leaf tissue porosity remained constant between treatments for the first 6 days of submergence with no significant effect of treatment (leaf gas film present or absent) but days of submergence (time) did show a significant effect (P<0.05, 2-way ANOVA with Sidak post hoc test); porosity values varied between 13 and 21% (Fig. 3b). After 9 days of submergence, control plants (initially with intact leaf gas films) had significantly higher leaf tissue porosity than plants with leaf gas films initially removed; 14 and 0% respectively (Fig. 3b). After 14 days of submergence, water had infiltrated the leaf tissues and tissue porosity approached 0% (Fig. 3b). Overall, 2-way ANOVA (time × gas film) showed a significant effect of time and also significant interaction effect indicating that the effect of time differed for control plants and plants with gas films initially removed (see Table S1 and caption of Fig. 3). Leaf chlorophyll concentration followed the same pattern as leaf tissue porosity with significant negative effects of time and interaction between time and gas film, but with little effect of presence or absence of leaf gas films on early time points (see Table S1 and caption of Fig. 3).

**Leaf gas film thickness, leaf tissue porosity and leaf tissue chlorophyll concentration**

During the submergence experiment, leaf gas film thickness and leaf tissue porosity were measured initially and after 1, 2, 3 and 4 days of submergence. Gas film thickness of control plants rapidly declined so that 50% was lost already during the first 24 h of submergence (mean gas film thickness = 12 μm) and after 3 days of submergence, the thickness was below detection limit and indistinguishable from plants brushed with Triton X-100 (Fig. 3a). Leaf tissue porosity remained constant between treatments for the first 6 days of submergence with no significant effect of treatment (leaf gas film present or absent) but days of submergence (time) did show a significant effect (P<0.05, 2-way ANOVA with Sidak post hoc test); porosity values varied between 13 and 21% (Fig. 3b). After 9 days of submergence, control plants (initially with intact leaf gas films) had significantly higher leaf tissue porosity than plants with leaf gas films initially removed; 14 and 0% respectively (Fig. 3b). After 14 days of submergence, water had infiltrated the leaf tissues and tissue porosity approached 0% (Fig. 3b). Overall, 2-way ANOVA (time × gas film) showed a significant effect of time and also significant interaction effect indicating that the effect of time differed for control plants and plants with gas films initially removed (see Table S1 and caption of Fig. 3). Leaf chlorophyll concentration followed the same pattern as leaf tissue porosity with significant negative effects of time and interaction between time and gas film, but with little effect of presence or absence of leaf gas films on early time points (see Table S1 and caption of Fig. 3).

**Relative growth rates, dead shoot mass and shoot elongation**

Relative growth rate (RGR) was calculated as relative increase in shoot DM from day 0 (beginning of the submergence event) to day 28 (end of recovery period) assuming exponential growth
or mortality in the period (Hunt 1982). Hence, the recovery period ranged from a minimum of 14 days for plants de-submerged after 14 days to a maximum of 28 days (control plants in air never submerged). RGR decreased significantly for plants that had been submerged for 6 days but with no significant effect of presence or absence of leaf gas films (RGR had declined 25–30% in plants with or without gas film compared with controls in air after 6 days of submergence; Table S1 and caption of Fig. 4). For plants submerged for 9 days, there was a clear but statistically insignificant trend towards control plants (initially with gas films) maintaining a higher RGR. However, after 10, 11 and 12 days of submergence, control plants had significantly higher RGR than plants with gas films removed before submergence. At these time points, plants with gas films initially removed showed negative RGR, i.e. the shoot mass after recovery was lower than at the time of submergence. After 13 and 14 days of submergence, there was no longer any significant difference between treatments since plants that initially had gas films had also died (Fig. 4a). To sum up, leaf gas film presence resulted in higher growth when submergence exceeded 6 days up until death (after 13 days). Similarly, waterlogging also significantly affected RGR, resulting in rates 24% lower than the drained controls in air (t-test, \( P < 0.0001 \), \( n = 10 \)) after 14 days of waterlogging.

At the final harvest after the recovery period, plants were separated into living or dead shoot tissues. Controls in air (never submerged but one set of plants brushed with Triton X-100) had 6.5 and 7.3% dead shoot tissues, respectively, 28 days after onset of the submergence event (plants were 45 days old). Plants submerged for 6 days had significantly more dead tissue than controls in air mainly due to time of submergence and with only little effect of presence or absence of gas films (Fig. 4b, Table S2). Control plants (initially with leaf gas films) submerged for 10–12 days had significantly less dead shoot tissues than plants with leaf gas films initially removed (Fig. 4b; plants with gas film initially removed had all died and thus consisted of all dead shoot tissues).

When plants were de-submerged at various time points during the experiment, shoot length was measured. There was no significant elongation of the shoot during the 14 days of complete submergence (shoot height at time of submergence versus shoot height after 14 days of submergence, t-test, \( P = 0.20 \), \( n = 5 \)).

**Mechanistic function of leaf gas films – CO₂ response of underwater \( P_N \)**

Underwater \( P_N \) was measured at dissolved CO₂ concentrations from 3 to 2800 mmol m⁻³ in the artificial floodwater at PAR = 1000 μmol photons m⁻² s⁻¹ (Fig. 5). Underwater \( P_N \) increased steeply in a linear fashion at CO₂ concentrations below 400 mmol m⁻³ (Fig. 5b) and reached saturation at ~1000 mmol m⁻³ with an estimated \( P_{max} \) of 5.48 μmol O₂ m⁻² s⁻¹ for leaf segments with intact gas film (Fig. 5a, Michaelis-Menten model). In contrast, the estimated \( P_{max} \) of leaf segments without gas films were significantly lower at only 1.62 μmol O₂ m⁻² s⁻¹. At 200 mmol CO₂ m⁻³, an ecological relevant floodwater CO₂ concentration (Colmer et al. 2011), underwater \( P_N \) was 6-fold higher for leaf segments with gas films than those without (Fig. 5b). The survival experiment in the present study was conducted at air equilibrium of CO₂ (~18 mmol CO₂ m⁻³) and under these conditions, the underwater \( P_N \) of

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**Fig. 3.** Leaf gas film thickness (a), leaf porosity (b) and leaf chlorophyll a concentration (c) of wheat (*Triticum aestivum*, cultivar ‘JB Asano’) during complete submergence. At day 0, plants were 17 days old, and half of the plants had the leaf hydrophobicity removed (no gas film formation when under water) with a dilute detergent before submergence. Data points are mean ± s.e. (\( n = 5 \)). Gas film thickness decreased significantly with time of submergence (1-way ANOVA) for plants with intact gas films before submergence. Leaf porosity and leaf chlorophyll a also decreased significantly with time of submergence and in both cases there was a significant time × gas film interaction (see details of ANOVA output in Table S1, available as Supplementary Material to this paper).
Control plants (initially with leaf gas films intact) was 0.22 μmol O₂ m⁻² s⁻¹ ± 0.01 or 32% higher than the P_N of plants with leaf gas films initially removed (0.16 μmol O₂ m⁻² s⁻¹ ± 0.004). For comparison, photosynthesis in air also at PAR = 1000 μmol photons m⁻² s⁻¹ and with ambient CO₂ (390 μmol mol⁻¹) was 21.6 ± 1.7 μmol O₂ m⁻² s⁻¹ (mean ± s.e., n = 4).

The reciprocal of the initial slope of underwater P_N vs dissolved CO₂ (Fig. 5b) indicates the apparent resistance to CO₂ uptake by the leaf segments. Linear regression analysis of underwater P_N in the range from 3 to 389 mmol CO₂ m⁻³ revealed an apparent resistance of 92 884 s m⁻¹ and 1 659 541 s m⁻¹ for leaf segments with and without gas films respectively (Table 1).

Mechanistic function of leaf gas films – light response of underwater P_N

Underwater P_N was also measured at a range of PAR with 200 mmol CO₂ m⁻³ in the artificial floodwater (Fig. 6). Underwater P_N increased linearly at PAR <200 μmol photons m⁻² s⁻¹ (Fig. 6b) and light saturation was reached at ~200 μmol photons m⁻² s⁻¹ for leaves with gas films and at less than 100 μmol photons m⁻² s⁻¹ for leaves with no gas films (Fig. 6b). The Jassby and Platt (1976) model predicted a P_max of 2.61 and 0.76 μmol O₂ m⁻² s⁻¹ for leaf segments with and without gas films, respectively. Rates of underwater P_N at low PAR for leaf segments with gas film were significantly lower than for leaf segments without gas...
The light compensation point was 52 \text{mol O}_2 \text{ m}^{-2} \text{ s}^{-1} for segments with and without gas films. During incubation leaf segments were exposed to 200 mmol CO_2 m^{-3} and \( P_N \) was measured as O_2 produced. (a) Each dataset was fitted to a Jassby and Platt (1976) model (\( P_{\text{max}} = 2.61 \) and 0.76 \text{mol O}_2 m^{-2} s^{-1} for segments with and without gas film, respectively, \( \alpha = 0.014 \) and 0.017 \text{mol O}_2 mol^{-1} photons for segments with and without gas film, respectively, \( I_C = 48 \) and 35 \text{mol photons m}^{-2} \text{s}^{-1} for segments with and without gas film, respectively, \( r^2 = 0.97 \) and 0.93 for segments with and without gas film respectively. Data points are mean ± s.e. (\( n = 4 \)).

![Fig. 6. Underwater net photosynthesis \((P_N)\) vs light \((PAR) = 0–1000 \text{mol photons m}^{-2} \text{s}^{-1}\), (a) 0–100 \text{mol photons m}^{-2} \text{s}^{-1}, (b) of leaf segments of wheat \((Triticum aestivum, \) cultivar 'JB Asano') with or without leaf gas films. During incubation leaf segments were exposed to 200 mmol CO_2 m^{-3} and \( P_N \) was measured as O_2 produced. (a) Each dataset was fitted to a Jassby and Platt (1976) model (\( P_{\text{max}} = 2.61 \) and 0.76 \text{mol O}_2 m^{-2} s^{-1} for segments with and without gas film, respectively, \( \alpha = 0.014 \) and 0.017 \text{mol O}_2 mol^{-1} photons for segments with and without gas film, respectively, \( I_C = 48 \) and 35 \text{mol photons m}^{-2} \text{s}^{-1} for segments with and without gas film, respectively, \( r^2 = 0.97 \) and 0.93 for segments with and without gas film respectively. Data points are mean ± s.e. (\( n = 4 \)).](image)

film up to between 50 and 100 \text{mol photons m}^{-2} \text{s}^{-1} (Fig. 6b). The light compensation point was 52 \text{mol photons m}^{-2} \text{s}^{-1} for leaves with gas films and 35 \text{mol photons m}^{-2} \text{s}^{-1} for leaves without, indicating less light captured by leaves with gas films at low light intensities also confirmed by the lower photosynthetic efficiency (\( \Phi_l \)) by leaves with gas films (0.01394 \text{mol O}_2 \text{ mol photon}^{-1}) compared with leaves with no gas film (0.0167 \text{mol O}_2 \text{ mol photon}^{-1}). However, already at around 100 \text{mol photons m}^{-2} \text{s}^{-1} (~5\% of full daylight) the improved gas exchange of leaves with gas films overcame the negative effect of light reflection by gas films.

Dark O_2 uptake (aerobic respiration, \( R_D \)) of leaf segments was significantly higher for leaf segments with gas film(s) present compared with segments without gas films demonstrating that even at atmospheric equilibrium of O_2 (258 mmol m^{-3} at 25\(^\circ\)C), the apparent resistance was too high to saturate \( R_D \) for leaf segments without gas film (\( R_D = 0.061 \) and 0.046 \text{mol O}_2 m^{-2} s^{-1} for segments with or without gas films respectively).
without gas films after 7 days of submergence. Moreover, it is also known from rice that root \( \text{O}_2 \) of completely submerged plants is strongly correlated with incident light and thus underwater \( P_N \) (Winkel et al. 2013), so even though rates of underwater \( P_N \) are modest compared with photosynthesis in air, \( \text{O}_2 \) produced in light by plants with gas films enhances internal aeration of the root system (Pedersen et al. 2009) and a healthy root system is crucial for survival following submergence or waterlogging (Herzog et al. 2013). As a consequence, we are unable to pinpoint the positive effects of leaf gas films resulting in better survival during complete submergence, i.e. carbohydrates produced in underwater photosynthesis and/or better internal aeration due to photosynthetically produced \( \text{O}_2 \) and/or \( \text{O}_2 \) taken up from the floodwater in darkness.

Plants that had leaf gas films during the first 3 days of submergence had significantly higher values of leaf porosity, leaf chlorophyll \( a \) concentration and RGR whereas the amount of dead shoot tissue was less than in plants that had leaf hydrophobicity manipulated before submergence (Figs 3, 4). As plants started reacting to submergence stress, leaf chlorophyll \( a \) declined rapidly and the leaves lost structural integrity allowing water to infiltrate intercellular gas spaces; in our experience, leaves that have started to lose chlorophyll \( a \) and tissue porosity, do not recover when de-submerged (Herzog et al. 2016a). In a similar study by Herzog et al. (2016a) on rice, where leaf hydrophobicity had also been manipulated before submergence, leaf chlorophyll \( a \) and porosity also declined faster in plants with no gas films at the time of submergence. Likewise, the loss of leaf chlorophyll \( a \) from control plants with intact gas films resembled the time course of two rice genotypes (IR42 and Swarna, sensitive to submergence) in a field flood trial where leaves also had lost 50% of the initial Chlorophyll on day 8–9 and with a somewhat parallel decline in leaf porosity (Winkel et al. 2014). Leaf porosity as well as leaf chlorophyll \( a \) concentration followed the exact same pattern for both controls with intact gas films at the time of submergence and plants with gas films removed, but shifted by 3 days, and therefore aligned well with the pattern observed for survival.

Gas films and gas exchange

The wheat cultivar ‘JB Asano’ retained leaf gas films for the first 3 days of submergence but then some changes in the leaf cuticle resulted in loss of hydrophobicity and gas films were no longer present. A screening study on gas film retention time in 14 genotypes of wheat, showed very little genotypic variation in this trait and in all cases, gas films disappeared after 3–4 days of submergence (Konnerup et al. 2017). Only the wild wetland plant, Glycera fluitans, retained a superhydrophobic cuticle for almost a week (Konnerup et al. 2017), and studies on rice (Colmer et al. 2009; Winkel et al. 2014) and H. marinum (sea barley grass, a wild relative to barley) (Pedersen et al. 2010) also showed that leaf gas films can be retained for up to 1 week of submergence. At present, however, it is not known why the cuticle loose hydrophobicity with time of submergence but one study showed that leaves of submerged wheat were covered by an unidentified substance covering the cuticle (Konnerup et al. 2017) whereas in another study, the leaves of rice did not develop such a layer but nevertheless lost leaf hydrophobicity (Herzog et al. 2016a).

Leaf gas films on wheat enabled higher underwater \( P_N \) under environmentally relevant \( \text{CO}_2 \) concentrations, and the effect of gas films on \( \text{CO}_2 \) uptake was significant both in terms of efficiency (Fig. 5b, initial slope) and capacity (Table 1). We found that rates of underwater \( P_N \) were several fold higher in leaf segments with gas film at all except for the lowest \( \text{CO}_2 \) concentrations (50 mmol m\(^{-3}\) and below). The beneficial effect of leaf gas films on underwater \( P_N \) has previously been shown for other terrestrial wetland plants such as rice (Pedersen et al. 2009) and \( P. \) australis (Colmer and Pedersen 2008). We noted that underwater \( P_N \) of wheat at low \( \text{CO}_2 \) concentrations (~20 mmol m\(^{-3}\)) in this study was similar to that of \( P. \) australis (0.22 vs 0.14 mmol m\(^{-2}\)s\(^{-1}\) respectively), whereas rice has been shown to obtain higher rates (up to 1.07 mmol m\(^{-2}\)s\(^{-1}\), Winkel et al. 2013). Although underwater \( P_N \) of wheat is only a fraction of the rate in air (21.6 mmol m\(^{-2}\)s\(^{-1}\)), we have previously shown that such low photosynthetic rates can be crucial for aeration of roots in rice (Winkel et al. 2013). If wheat possesses a similar gas pathway between roots and shoot as indicated by Malik et al. (2003), underwater photosynthesis in the presence of gas films could play an important role in internal aeration, even at low external \( \text{CO}_2 \) concentrations.

Wheat showed the same pattern of \( \text{CO}_2 \) response of underwater \( P_N \) as \( P. \) australis (Colmer and Pedersen 2008) where leaf segments without gas film never intercepted the curve of segments with gas film even at very high external \( \text{CO}_2 \) concentrations. We speculate that something restricts \( \text{CO}_2 \) and \( \text{O}_2 \) exchange across the cuticle of both wheat and \( P. \) australis and perhaps result in higher rates of photorespiration. In the present study, the resistance to \( \text{CO}_2 \) uptake was 1.6 \( \times 10^5 \) s m\(^{-1}\) for leaf segments without leaf gas film compared with the resistance of \( P. \) australis (0.5 \( \times 10^5 \) s m\(^{-1}\), Colmer and Pedersen 2008) and rice (0.3 \( \times 10^6 \) s m\(^{-1}\), Pedersen et al. 2009). Moreover in rice, the disadvantage of the missing gas film could be alleviated by high external \( \text{CO}_2 \) concentrations (Pedersen et al. 2009). Such alleviation does not take place in wheat or in \( P. \) australis indicating an even more crucial role of leaf gas films for gas exchange in completely submerged wheat.

The role of leaf gas films in light use efficiency (\( \Phi_i \)) during underwater \( P_N \) has not previously been evaluated. In accordance with our initial hypothesis, we found that gas films reflect light and therefore are disadvantageous at low light levels and the reflection results in a higher light compensation point (\( I_{c} \), Fig. 6b). We found that leaves without gas films had slightly higher light use efficiency than leaves with leaf gas films (0.017 and 0.014 mmol O\(_2\) mol\(^{-1}\) incident photons in leaves without and with gas films, respectively) but in both cases \( \Phi_i \) was in the lower range of those previously reported for aquatic angiosperms (0.012–0.058 mmol O\(_2\) mol\(^{-1}\) incident photons; Frost-Christensen and Sand-Jensen (1992)). In the former study, however, a much higher \( \text{CO}_2 \) concentration was used (500 mmol m\(^{-3}\)) in order to saturate underwater \( P_N \) whereas in the present study, we used a more environmentally relevant \( \text{CO}_2 \) concentration of 200 mmol m\(^{-3}\) (Colmer et al. 2011) and the lower \( \text{CO}_2 \) concentration could subsequently have resulted in a lower light use efficiency. In conclusion, the light use efficiency
during underwater photosynthesis of this dryland crop is not largely different from many amphibious wetland plants.

The present study shows that gas films forming on superhydrophobic leaf cuticles during submergence is a strong trait resulting in longer survival during complete submergence in the dryland crop, wheat. At present, the genetic background for cuticle structure is poorly understood but a recent study on rice suggest that at least one gene is involved in the deposition of the numerous wax platelets (Zhang et al. 2016) which is an important structural feature resulting in superhydrophobicity. At present, it is not yet known why leaf cuticles loose hydrophobicity over time when submerged but it happens in both wetland and dryland plants (Neinhuis and Barthlott 1997; Winkel et al. 2014, 2016; Konnerup et al. 2017). It is possible that with better genetic understanding of features involved in the 3D structure of the cuticle that more flood tolerant crops can be developed if the genes involved in cuticle maintenance can be overexpressed and this could lead to longer leaf gas film retention times and possibly increase the duration that wheat can survive complete submergence.

Acknowledgements

We acknowledge Timothy Colmer for constructive discussion on experimental design, Oliver Mørk for helping growing and maintaining plants as well as laboratory work and Victoria C Herskov for help with analyses of chlorophyll. The study is supported by a grant (WheatSUB) from the Villum Foundation.

References


Appendix II: Leaf gas film retention during submergence of 14 cultivars of wheat (*Triticum aestivum*)

Water drop on a superhydrophobic leaf surface. The angle between water drop and leaf surface is used to quantify leaf hydrophobicity. Photo: Dennis Konnerup
Leaf gas film retention during submergence of 14 cultivars of wheat (Triticum aestivum)

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Abstract. Flooding of fields after sudden rainfall events can result in crops being completely submerged. Some terrestrial plants, including wheat (Triticum aestivum L.), possess superhydrophobic leaf surfaces that retain a thin gas film when submerged, and the gas films enhance gas exchange with the floodwater. However, the leaves lose their hydrophobicity during submergence, and the gas films subsequently disappear. We tested gas film retention time of 14 different wheat cultivars and found that wheat could retain the gas films for a minimum of 2 days, whereas the wild wetland grass Glyceria fluitans (L.) R.Br. had thicker gas films and could retain its gas films for a minimum of 4 days. Scanning electron microscopy showed that the wheat cultivars and G. fluitans possessed high densities of epicuticular wax platelets, which could explain their superhydrophobicity. However, G. fluitans also had papillae that contributed to higher hydrophobicity during the initial submergence and could explain why G. fluitans retained gas films for a longer period of time. The loss of gas films was associated with the leaves being covered by an unidentified substance. We suggest that leaf gas film is a relevant trait to use as a selection criterion to improve the flood tolerance of crops that become temporarily submerged.

Additional keywords: air film, flooding, underwater photosynthesis, wettability.

Received 10 November 2016, accepted 31 January 2017, published online 23 March 2017

Introduction

Floods are expected to increase as a consequence of climate change (Parry 2007) and increased rainfall will affect wheat (Triticum aestivum L.) production negatively in some areas (Trnka et al. 2014). It has been estimated that 15–20\% of the 70 million ha of wheat sown each year is affected negatively by waterlogging (Sayre et al. 1994; Setter and Waters 2003). Therefore, an increased effort is needed to breed wheat cultivars that are better adapted to the regionally abiotic stress in the future climate scenarios, where some areas will experience increased rainfall and some areas increased drought (Trnka et al. 2014). Most flooding studies refer to the waterlogging scenario and use ‘flooding’ and ‘waterlogging’ interchangeably. According to Bailey-Serres et al. (2012), the term ‘flooding’ is used to describe the inundation by water of all or part of a plant, ‘waterlogging’ to describe flooding of the root system and ‘submergence’ to describe the situation where most or all aerial tissue is under water, thus we will use this terminology in the present paper.

Waterlogging results in reduction of plant growth as the soil becomes anoxic and in addition various potentially toxic compounds such as Mn\textsuperscript{2+}, Fe\textsuperscript{2+}, S\textsuperscript{2-} and carboxylic acids can accumulate (Ponnamperuma 1984) with phytotoxic effects on roots (Shahala 2011). Furthermore, nutrient uptake and the growth and yield of wheat are severely reduced when waterlogging occurs and roots suffer O\textsubscript{2} deficits (Colmer and Greenway 2011; Herzog et al. 2016b).

Root growth in anoxic or severely hypoxic soils depends on the O\textsubscript{2} that is available via diffusion from aboveground parts of the plant through aerenchyma (Armstrong 1979; Colmer 2003). The gas-filled channels of aerenchyma and other porous tissues provide an internal pathway with low resistance for O\textsubscript{2} diffusion within roots, enhancing O\textsubscript{2} movement to the root tips when in anoxic substrates (Armstrong 1979). A second feature that enhances the movement of O\textsubscript{2} to the root tip, in addition to root gas-filled porosity, is the formation of a barrier to radial O\textsubscript{2} loss (ROL) in the basal part of the roots. This feature occurs in roots of many wetland plants and diminishes ROL to the rhizosphere and can also restrict phytotoxin entry, whereas in dryland species without the barrier, most O\textsubscript{2} is lost at the basal root zone so that less O\textsubscript{2} reaches the root tip (Armstrong 1979; Colmer 2003). An approach aimed at improving the waterlogging tolerance of wheat is the transfer of the barrier to ROL from a wild relative, Hordeum marinum Huds., to wheat by wide hybridisation (Malik et al. 2011). This work has resulted in the successful development of amphiploids with a barrier to ROL but with lower yield, so further breeding is needed to produce waterlogging and commercially viable cultivars (Konnerup et al. 2017).

Submergence is a more severe type of flooding as it involves waterlogging of the root system and the shoot being submerged in floodwater. During submergence, the ~10,000-fold reduction in diffusion of gases in floodwaters limits the availability of O\textsubscript{2} and CO\textsubscript{2} for aerobic respiration and net photosynthesis (\textit{P\textsubscript{N}}),
respectively (Armstrong 1979). Plants can photosynthesise under water provided that both the light and CO₂ levels are sufficient. In air, CO₂ enters the tissue via open stomata, but under water, the stomata are assumed to close (Mommer and Visser 2005). Therefore, CO₂ first has to overcome the resistance caused by the aqueous diffusive boundary layer via slow molecular diffusion and then subsequently cross the cuticle, which adds significantly to the total resistance to CO₂ uptake (Mommer and Visser 2005). Some terrestrial wetland plants acclimate to facilitate gas exchange under water by the production of new ‘semi-aquatic’ leaves that are thin, as well as having reduced cuticles and rearrangement of chloroplasts closer to the epidermis, all resulting in lower resistance to CO₂ diffusion to chloroplasts (Mommer et al. 2005; Mommer and Visser 2005).

Many terrestrial plants have superhydrophobic leaves that result in the formation of thin gas films when immersed into water (Colmer and Pedersen 2008). The leaf gas films have been shown to increase underwater gas exchange and thus CO₂ entry to sustain underwater Pn during the day and improved internal aeration during the night (Pedersen et al. 2009; Winkel et al. 2013). Thus, retention and persistence of gas films by plants are beneficial during submergence, but factors involved in the degradation of leaf gas films during prolonged submergence requires further study. In rice, gas films were retained on submerged leaves for 4–6 days depending on genotype and the loss of gas films were strongly linked to a steep decline in underwater Pn (Winkel et al. 2014). In addition to providing the leaves with the ability to form gas films, superhydrophobic leaf surfaces are hypothesised to be an adaptation for self-cleaning and facilitate water to roll off leaves in air when it rains to prevent covering of leaves by a film of water (Neinhuis and Barthlott 1997). A water layer on a leaf surface would reduce gas exchange and thus Pn, and also increase the risk of bacteria and fungi infecting leaves (Koch and Barthlott 2009).

In wheat, superhydrophobic leaf surfaces have also been demonstrated to form gas films when submerged, and experiments on the cultivar ‘JB Asano’ showed that plants with gas films survived complete submergence significantly longer (13 days) than plants without (10 days), which could be highly relevant to improve survival of temporarily submerged wheat during field flood events (Winkel et al. 2017). This prompted us to investigate if there is any diversity of gas film retention in different wheat cultivars and what occurs when the leaves lose their hydrophobicity and subsequently the gas films. Thus, in the present study, we tested the gas film retention times during submergence of 14 cultivars of wheat, which were submerged for up to 10 days. Based on these results, we chose three wheat cultivars for more in-depth mechanistic studies consisting of underwater Pn, hydrophobicity measured as contact angles, leaf chlorophyll and scanning electron microscopy (SEM) with Glyceria fluitans included as a flooding tolerant model species.

Materials and methods

Plant material and growth conditions

Fourteen wheat (Triticum aestivum L.) cultivars were used in the study. Thirteen of the cultivars were commonly used in agriculture in northern Europe with no information on waterlogging tolerance and one was a waterlogging tolerant cultivar (Jackson) (Huang and Johnson 1995; Huang et al. 1997). Nine of the cultivars (Mariboss, Jensen, Nakskov, Torp, Gedser, Ohio, Albert, Elixir, Ambition) were supplied by Nordic Seed and four cultivars (Hereford, Substance, Benchmark and Sheriff) by Sejet Plant Breeding. Furthermore, floating sweet-grass (Glyceria fluitans (L.) R.Br.), a wild grass species which is tolerant to waterlogging and temporary submergence was included in the study as a floating tolerant model species (Mony et al. 2010).

The experiment was conducted in a constant temperature room at 15°C. Seeds (three per pot) were sown in plastic pots (diameter 60 mm, height 90 mm) with substrate. The substrate consisted of a 10 mm layer of washed sand at the bottom, then a 70 mm layer of soil with a dry matter content of 55–75 g L⁻¹, conductivity 3.0–5.0 mS cm⁻¹ and pH 6.0 (Pindstrup Substrate no. 2, Pindstrup Mosebrug A/S) and a 10 mm layer of washed sand on top; the top sand layer served to reduce nutrient release from the soil to the floodwater during submergence. Pellets of slow-release fertilizer (Osmocote Bloom) were implanted into the soil and each pot received 0.55 g fertiliser that contained (by % mass): N, 12; P, 7.0; K, 18.0; Mg, 1.5; Fe, 0.35; Mn, 0.05; Cu, 0.045; Mo, 0.017; Zn, 0.013; B, 0.01. G. fluitans was propagated by using small plants taken from tillers and potted as wheat i.e. same soil and fertilizer. The pots, which had drainage holes at the bottom, were placed on trays with 10 mm DI water, so they had access to sufficient water. Following germination, the seedlings were exposed to light (12 h photoperiod) using an artificial full spectrum light source (Gavita Pro LEP 300, Gavita Holland BV) providing photosynthetically active radiation (PAR) of ~200 μmol photons m⁻² s⁻¹ at the pot surface.

Experimental design and submergence conditions

Submergence treatments commenced when plants were 16 days old (2.0–2.5 leaf stage). One of the three seedlings in each pot was removed, so that each pot contained two seedlings of similar size. The plants were submerged in glass aquariums (length × width × height, 0.8 m × 0.4 m × 0.5 m) with artificial floodwater consisting of a modified Smart and Barko (1985) solution containing (in mM): CaCl₂·2H₂O, 0.62; MgSO₄·7H₂O, 0.28; NaHCO₃, 1.0; KCO₃, 1.2. In each aquarium, there was a pH controller (JBL CO₂/pH control) connected to a cylinder with pressurised CO₂ keeping the pH at 7.35 by bubbling with CO₂ when necessary. This maintained the free CO₂ at 200 μM, which is an environmentally relevant concentration (Colmer et al. 2011) and the solution had an alkalinity of 2.0 mM (Mackereth et al. 1978). The floodwater was circulated through UV filters (JBL AquaCristal UV-C) in order to prevent algal growth in the aquariums. The plants were submerged for 0 (non-submerged initials), 1, 2, 4, 7 or 10 days, and control plants in air were also measured on day 10. All measurements were performed on the youngest fully expanded leaf at the time of submergence (the 1st leaf).

The study consisted of two experiments (experiment 1 and 2). In experiment 1, the gas film retention times measured as gas film thickness during submergence of 14 different wheat cultivars were assessed in order to choose cultivars for experiment 2. In experiment 2, gas film thickness and more in-depth measurements consisting of underwater Pn, contact angle, chlorophyll and SEM
were undertaken on three of the 14 cultivars from experiment 1 with contrasting retention times, and *G. fluitans* was included as a flooding tolerant model species.

**Gas film thickness and leaf porosity**

Gas film volume was measured by determining buoyancy of leaf segments (60–80 mm in length) before and after gas film removal. The gas films were removed by brushing the leaves with a dilute solution (0.05% v/v) of Triton X-100 (Raskin and Kende 1983; Colmer and Pedersen 2008). The leaf segments were then vacuum infiltrated with water and buoyancy was determined again to enable calculation of tissue porosity (gas-filled volume per unit tissue volume) using the method described by Raskin (1983) and the equations as modified by Thomson *et al.* (1990). Projected area was measured for each leaf segment using digital photos and image analysis in ImageJ (Schneider *et al.* 2012). Mean gas film thickness was calculated by dividing gas film volume (mm$^3$) with the two-sided area (mm$^2$), as the leaves of both wheat and *G. fluitans* possessed gas films on both the adaxial and abaxial sides. In the present study, the detection limit of gas film thickness was ~2 μm, and therefore measurements resulting in values below 2 μm were classified as ‘gas films absent’.

**Leaf surface wettability**

Leaf surface wettability was assessed by measuring the contact angle of a 5-mm$^3$ droplet of water on the leaf surface (Breuer and Smith 1997), held flat using double-sided tape. Droplets were applied to the adaxial leaf surface, and photographed at × 90 magnification using a horizontally positioned digital microscope camera (Dino-Lite AM4013MZTL, IDCP) and the contact angle was determined by image analysis in ImageJ. The wettability of the leaf surfaces could be divided into four classes defined by their contact angle according to Koch and Barthlott (2009): superhydrophilic (contact angle <10°), hydrophilic (contact angle 10°–90°), hydrophobic (contact angle 90–150°) and superhydrophobic (contact angle >150°).

**Net photosynthesis ($P_N$) under water**

Underwater $P_N$ was measured as net O$_2$ production by leaf segments. The leaf segments were excised from the middle third of the lamina and were incubated in a defined medium (see later in this section) for a known time in closed transparent vials with gentle mixing and held at a constant temperature after which the O$_2$ evolution by the tissue was measured. Underwater $P_N$ rates ($n = 4$) were measured at 20°C using 25 mL glass vials with two glass beads added to ensure mixing as the vials rotated according to the method of Pedersen *et al.* (2013) with photosynthetically active radiation (PAR) inside the vials of 1000 μmol photons m$^{-2}$ s$^{-1}$ provided by a light source (Gavitia Pro LEP 300, Gavitia Holland BV) with a horizontal light beam. The basal incubation medium was based on the general purpose culture medium described by Smart and Barko (1985) and contained (in mM) CaCl$_2$.2H$_2$O, 0.62; MgSO$_4$.7H$_2$O, 0.28. To prepare artificial floodwater with a final concentration of 200 or 2500 μM CO$_2$ and an alkalinity of 2.0 mM (mostly bicarbonate and carbonate), we added KHCO$_3$ at 2.2 or 4.5 mM in the general purpose medium. We subsequently added known volumes of 0.1 M HCl to convert the desired portion of the HCO$_3^-$ into CO$_2$, resulting in pH values of 7.4 and 6.35 for the 200 and 2500 μM CO$_2$ respectively (Mackereth *et al.* 1978). The dissolved O$_2$ concentration in the incubation medium was set at 50% of air equilibrium (mixing solutions bubbled with N$_2$ and air in 1 : 1 volumes); this procedure was applied to reduce effects of photosynthesis (Pedersen *et al.* 2013). Vials without leaf segments served as blanks.

Following incubations of known duration (60–120 min), the dissolved O$_2$ concentration in each vial was measured using a microoptode (OP-430, fibre diameter 430 μm, Unisense A/S) connected to an optode meter (MicroOptode meter, Unisense A/S). Values of O$_2$ were logged on a computer using the software provided by Unisense (Sensor Trace Suite ver. 2.7.0). Projected area was measured as described for ‘gas film thickness’ for each individual leaf segment and underwater $P_N$ rates were calculated per one-sided leaf area. The leaf segments were then immediately frozen at −20°C and later freeze-dried for subsequent analysis of chlorophyll.

**Chlorophyll**

Chlorophyll a concentration was measured in the leaf segments used for underwater $P_N$. The freeze-dried leaf segments were each homogenised in a 2 mL Eppendorf tube using 2 metal beads for 10 s on a mini bead-beater (BioSpec Products Inc. Mini Bead Beater). Chlorophyll was extracted for 24h in 96% ethanol, centrifuged at 8000g for 3 min and chlorophyll a absorbance measured at 656 and 750 nm on a spectrophotometer (Shimadzu UV-1800). Chlorophyll a concentrations were calculated using equations by Mackinney (1941).

**SEM and analyses of SEM**

Leaf segments were frozen immediately after sampling and then freeze-dried. Samples were gold coated in a sputter coater for 90 s and then analysed using a field emission scanning electron microscope (JEOL JSM-6335F) at 7.0 kV and ×10 000 magnification or a scanning electron microscope (FEI Inspect S) at 12.5 kV and ×500 magnification. As the submergence resulted in the wax platelets on the leaves being covered by an unidentified substance, the images were analysed using ImageJ to determine the percentage of the leaf surface which was still exposed after 0 (non-submerged initials), 1, 2, 4, 7 and 10 days submergence.

**Statistical analyses**

Data were analysed by analysis of variance (ANOVA) using Type III sum of squares with the software Statgraphics XVI centurion version 16.1.11 (StatPoint Inc.). Multiple post hoc comparisons of means were performed using the Tukey’s HSD procedure at the 0.05 significance level. Data were tested for homogeneity of variance by Levene’s test. If necessary, logarithmic or square root transformations were performed to ensure homogeneity of variance, but for clarity all data are presented as untransformed. Two-tailed Pearson correlations between underwater $P_N$ at the two CO$_2$ concentrations and gas film thickness were performed using GraphPad Prism 6 (GraphPad Software Inc.).
Results

Gas film retention

In the gas film screening experiment (experiment 1), all the wheat cultivars had gas films on both sides of the leaves when submerged. The initial gas film thicknesses were in the range from 15 to 33 µm and declined with time of submergence (Fig. 1). At day 2, the gas film thicknesses were 2 to 8 µm, and by the 4th day, gas films had declined to <2 µm indicating that the gas films had been lost since the detection limit was defined to be 2 µm. The results of a 2-way ANOVA of the 14 tested cultivars showed that there was a significant main effect of time of submergence ($P < 0.0001$), but no effect of cultivar and with no significant cultivar $\times$ time of submergence interaction. This demonstrated that all the cultivars lost their gas films in a similar pattern with time of submergence. In the mechanistic experiment on the focus species (experiment 2), the three wheat cultivars Albert, Jensen and Jackson were again included and compared with G. fluviatans. For these mechanistic studies we selected the cultivar with thickest gas film (Jensen), the one with thinnest gas film (Albert) and Jackson because this cultivar has been reported to be waterlogging tolerant (Huang and Johnson 1995; Huang et al. 1997). Furthermore, we included G. fluviatans in these in-depth studies as this species has been reported to be flooding tolerant (Mony et al. 2010) and our field observations had shown that it possessed gas films when submerged under natural conditions. For the wheat cultivars, the initial gas film thickness was 24–25 µm and as in experiment 1, the gas films were lost by day 4 (Fig. 2a). For G. fluviatans, the initial gas film was 36 µm and it was maintained at this thickness until day 2, where it started to decline and was lost by day 7. In a 2-way ANOVA analysis, there were significant effects of cultivar/species and time of submergence and also a significant interaction (Table 1). The post hoc test showed that the three wheat cultivars were similar at all time points, whereas G. fluviatans had thicker gas films than wheat at day 0, 1, 2 and 4.

Leaf surface wettability

Leaf surface wettability assessed as contact angles were 152–157° initially demonstrating that all wheat cultivars and also G. fluviatans had superhydrophobic leaves before submergence (Fig. 2b). The leaves of G. fluviatans maintained superhydrophobic properties (contact angle >150°) until day 2, whereas the three wheat cultivars had lost the superhydrophobicity already on day 1 with contact angles of 130–140°. However, by day 4 the leaves of all plants had lost their hydrophobicity and were hydrophilic. The 2-way ANOVA showed significant effects of cultivar/species, time of submergence and cultivar/species $\times$ time of submergence interaction as the leaves of G. fluviatans were superhydrophilic at day 7 and day 10 with contact angles close to 0°.

The initial (Day 0) superhydrophobicity of the leaf surfaces in both wheat and G. fluviatans was caused by a dense arrangement of three-dimensional epicuticular waxes (Fig. 3). In addition, the leaf surfaces of G. fluviatans had papillae and a plicate structure, which could explain their thicker gas films and longer lasting hydrophobicity. The declines in gas film thickness and hydrophobicity measured as contact angles were associated with the leaves being covered by a layer of an unidentified substance observed using SEM (Fig. 3). The wax crystals seemed intact underneath the layer, but the three wheat cultivars were partly covered by the unidentified substance on day 2 and almost completely covered on day 4 (Figs 2c, 3). The leaves of G. fluviatans showed a delayed response and were completely covered on day 7. A plot of gas film thickness vs

Fig. 1. Leaf gas film thickness of 14 cultivars of 16 days old wheat (Triticum aestivum) with time of submergence (means ± s.e., $n=4$). At day 10, control plants in air had a gas film thickness in the range of 55–142% of the initials at day 0.
contact angle showed a distinct pattern with many points clustered around contact angles of 150° when gas films were present (Fig. 2d). Contact angles above 150° defined the leaf surfaces as superhydrophobic and the plot indicates that superhydrophobicity is necessary to have gas films thicker than 20 μm. Hydrophobic leaves (90° < θ < 150°) could retain a thin gas film below 20 μm, and hydrophilic leaves (θ < 90°) did generally not have gas films except a few points with *G. fluitans*. 

**Net photosynthesis (P_N) under water**

Underwater $P_N$ was determined at two different CO2 concentrations (200 and 2500 μM). At 200 μM CO2, $P_N$ is

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**Table 1.** Results of two-way ANOVA (F-ratios and P-values) showing the effects of cultivar/species and time of submergence (0, 1, 2, 4, 7 or 10 days) on gas film thickness, leaf porosity, contact angle, chlorophyll a and photosynthesis ($P_N$) of three wheat (*Triticum aestivum*) cultivars (Jensen, Albert and Jackson) and *Glyceria fluitans* Data are shown in Figs 2, 4 and 6.

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</tr>
<tr>
<td>Contact angle</td>
<td>11.7</td>
<td>&lt;0.0001</td>
<td>290.1</td>
</tr>
<tr>
<td>$P_N$ at 200 μM CO2</td>
<td>230.6</td>
<td>&lt;0.0001</td>
<td>441.0</td>
</tr>
<tr>
<td>$P_N$ at 2500 μM CO2</td>
<td>1.4</td>
<td>0.2389</td>
<td>242.2</td>
</tr>
<tr>
<td>Leaf porosity</td>
<td>62.0</td>
<td>&lt;0.0001</td>
<td>3.7</td>
</tr>
<tr>
<td>Chlorophyll a</td>
<td>11.0</td>
<td>&lt;0.0001</td>
<td>25.1</td>
</tr>
</tbody>
</table>

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Fig. 3. (Continued)
limited by CO₂ because of diffusion resistance from the bulk medium into the leaf and presence of a gas film is therefore beneficial as it reduces the gas exchange resistance of submerged leaves (Pedersen et al. 2009; Verboven et al. 2014). The high CO₂ concentration of 2500 μM was used to assess changes in the capacity for underwater Pₙ with time of submergence as this concentration of CO₂ was expected to saturate underwater Pₙ regardless of whether gas films were present or not. The 2-way ANOVA showed that there were significant effects of cultivar/species and time of submergence and also a significant interaction at 200 μM CO₂ (Fig. 4a; Table 1). The initial Pₙ rates at 200 μM CO₂ were 3.7–4.3 μmol O₂ m⁻² s⁻¹ for the three wheat cultivars and 5.2 μmol O₂ m⁻² s⁻¹ for G. fluitans. With time of submergence, the Pₙ rates of the wheat cultivars declined faster than G. fluitans so that at day 4, the wheat cultivars had rates in the range 0.3–0.5 μmol O₂ m⁻² s⁻¹, whereas G. fluitans had a mean rate of 1.9 μmol O₂ m⁻² s⁻¹. However, at 2500 μM CO₂ there was no main effect of cultivar/species but a significant effect of time of submergence and an interaction effect (Fig. 4b; Table 1). The initial Pₙ rates 2500 μM CO₂ were in the range 7.1–8.1 μmol O₂ m⁻² s⁻¹ with no difference between cultivar/species and all rates declined with time of submergence. However, at day 4, the Pₙ rates of the three wheat cultivars had declined to 2.3–2.9 μmol O₂ m⁻² s⁻¹ and G. fluitans had a significantly higher rate of 7.0 μmol O₂ m⁻² s⁻¹.

Correlation analyses were used to evaluate relationships between underwater Pₙ at the two different CO₂ concentrations and gas film thickness (Fig. 5). Only data points with ‘gas film present’ (gas film thickness >2 μm) were used to ensure that the correlations mainly assessed the effects of gas film thickness and not potential damages to the photosynthetic apparatus occurring after the gas films had been lost. There were positive relationships between underwater Pₙ and gas film thickness at 200 μM CO₂ for all cultivars/species (Fig. 5a), whereas at 2500 μM CO₂ there were no significant correlations between Pₙ and gas film thickness (Fig. 5b). This indicates that at the high CO₂ concentration, the gas films were less important for the capacity for underwater Pₙ as the CO₂ was not limiting and therefore the beneficial effects of the gas films on gas exchange did not enhance Pₙ.

**Leaf porosity and chlorophyll**

Initial leaf porosities were significantly higher in G. fluitans with 29% whereas the wheat cultivars had values of 15–18% porosity (Fig. 6a; Table 1). However, the porosity of G. fluitans declined during the submergence until day 7, where it was similar to the values of the wheat cultivars in the range of 16–18%. Leaf concentrations of chlorophyll a were initially 14–15 mg g⁻¹ and generally declined during submergence so at day 10, the values were 7–11 mg g⁻¹ (Fig. 6b; Table 1). The chlorophyll of G. fluitans declined faster than the wheat cultivars but at day 10, there were no significant differences between the cultivars/species.

**Discussion**

Gas film retention times during complete submergence were studied in 14 wheat cultivars and compared with the wetland plant G. fluitans. Previous studies have demonstrated that leaf gas films facilitate gas exchange with the floodwater by decreasing the resistance across the cuticle, thereby contributing to wetland and dryland crop submergence tolerance (Colmer and Pedersen 2008; Pedersen et al. 2009; Pedersen and Colmer 2012). Leaf gas films provide immediate benefits upon submergence, whereas some terrestrial wetland plants acclimate to submergence by producing new leaves which are thin and have reduced cuticles to decrease the resistance to CO₂ and O₂ diffusion.

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**Fig. 3.** Scanning electron microscopy (SEM) micrographs of adaxial leaf surfaces of three cultivars (Jensen, Albert and Jackson) of wheat (Triticum aestivum) and Glyceria fluitans with time of submergence (0, 1, 2, 4, 7 or 10 days). The scale bar is 5 μm in all rows except the top row where the scale bar is 200 μm. Larger papillae on the leaf surface of G. fluitans at the low magnification in the top row are indicated by white arrows. In addition, the leaf surface of G. fluitans was also covered by smaller papillae, and the leaves had a plicate shape.
the wettability of plant surfaces (Koch and Barthlott 2009). A high relationship between gas film thickness and leaf porosity showed that hydrophobic properties of leaf surfaces, i.e. gas film >2 µm were present. Pearson correlation analyses of underwater gas film thickness and hydrophobicity showed: For 200 µM CO₂: Jensen P=0.0030 (r²=0.60); Albert P<0.0001 (r²=0.77); Jackson P=0.0113 (r²=0.49); G. fluitans P=0.0199 (r²=0.35). For 2500 µM CO₂: Jensen P=0.2072 (r²=0.17); Albert P=0.6735 (r²=0.02); Jackson P=0.0646 (r²=0.30); G. fluitans P=0.6949 (r²=0.01).

(Mommer et al. 2005) making this strategy suitable for wetland plants that can be exposed to prolonged inundation.

The present study shows that wheat retains leaf gas films for at least 2 days when submerged (Fig. 1). The 14 tested cultivars all showed a similar pattern when gas films were lost, and we did not find cultivars that had remarkably longer gas film retention times than other cultivars. Although there were no obvious differences in gas film retention times between the cultivars, we selected three of the cultivars for more in-depth studies of their physiology when submerged. Interestingly, our results showed that G. fluitans had thicker gas films and could retain its gas films significantly longer than the wheat cultivars (Fig. 2a). The relationship between gas film thickness and hydrophobicity measured as contact angles showed that hydrophobicity was a very good proxy for whether the leaves had gas films or not (Fig. 2d).

Contact angle measurements are routinely used to characterise the wettability of plant surfaces (Koch and Barthlott 2009). A high contact angle is related to surfaces on which a water droplet forms a spherical shape and the actual contact between the droplet and the surface is very small compared with wettable surfaces. Surfaces with contact angles of >150° are classified as being extremely water-repellent or superhydrophobic (Koch and Barthlott 2009), so in the present study all wheat cultivars and G. fluitans possessed these properties at the beginning of the submergence event (Fig. 2b). Plant leaves are covered by a protective layer called the cuticle consisting of cutin and integrated and superimposed waxes which can result in hydrophobic properties. Plant waxes embedded into the cuticle are called intracuticular waxes and waxes superimposed on the surface are called epicuticular waxes (Koch and Enskat 2008). Epicuticular waxes are crystalline and can be seen on the surface as wax crystals with different shapes when investigated with SEM (Fig. 3). The classification of waxes is based on micromorphological features according to Barthlott et al. (1998). Following this terminology, waxes are termed platelets when flat crystals are connected with their narrow side to the surface like we observed in the present study in both wheat and G. fluitans (Fig. 3). This three-dimensional structure and the chemical composition of the wax crystals contribute to the
hydrophobic nature of the cuticles (Koch et al. 2008). Besides providing the leaves of some plants with a hydrophobic surface so they can form gas films when submerged, the cuticle also plays other important roles for plants. These roles include minimising water loss by transpiration, protecting against UV-radiation and self-cleaning effects as a strategy to remove surface bacteria and spores of fungi (Koch and Ensikat 2008). In wheat, it has been demonstrated by Stosch et al. (2007), that the superhydrophobic leaf cuticles function as protection against Blumeria graminis, a fungus that causes powdery mildew. The study compared intact leaves with leaves that had been wiped to remove their superhydrophobicity, and it showed that intact leaves had a significantly better removal of conidia from their surfaces. This could explain why wheat as a dryland crop could benefit from superhydrophobic leaves.

The SEM micrographs showed that all three wheat cultivars and G. fluitans possessed high densities of platelets which explained their superhydrophobicity (Fig. 3). However, G. fluitans also had papillae that would contribute to higher hydrophobicity during the initial submergence, and this could explain why G. fluitans retained gas films for at least 4 days, whereas the wheat cultivars only maintained a gas film for at least 2 days. Neinhuis and Barthlott (1997) investigated the surface structures of over 200 water-repellent plant species, and concluded that most of them possessed hierarchical surface structures formed by convex papillose epidermal cells and a very dense arrangement of three-dimensional epicuticular waxes of different shapes. Wetting of such hierarchical structures is minimised because air is trapped in cavities of the convex cell arrangements, and the hierarchical roughness enlarges the water-air interface while the solid-water interface is reduced (Bhushan and Jung 2008). The ability of superhydrophobic plant surfaces has been studied in detail for lotus (Nelumbo nucifera), where it has been reported that its persistent superhydrophobicity is attributed to the robustness of its leaf papillae in combination with their high density (Ensikat et al. 2011). Another remarkable plant in terms of hydrophobicity is Sá lvinia, which has been demonstrated to retain a gas film up to 17 days under water (Cerman et al. 2009). This extreme hydrophobicity is caused by eggbeater-shaped trichomes that are up to 2 mm long and superimposed by a layer of small hydrophobic wax crystals (Barthlott et al. 2010). In the present study, we showed that G. fluitans possessed papillae which most likely contributed to its ability to retain a gas film for 4 days, and since this species is often found in wetland habitats, it is possibly an adaptation to temporary submergence.

We showed in this study that the loss of gas films is associated with the leaves being covered by an unidentified substance. To our knowledge, this is the first time this phenomenon has been reported, but in a similar submergence study on rice, the loss of hydrophobicity was not associated with such depositions of unidentified substances (Herzog et al. 2016a). Therefore, at this point in time we can only speculate about what the causes could be. First of all, the question is whether the substance caused the gas film to disappear, or the substance formed on the leaf surfaces after the gas film had been lost. In another study, Brassica oleracea, Eucalyptus gunnii and Tropaeolum majus with different types of surface waxes, were cultivated at three different relative air humidity levels (20–30, 40–75 and 98%) (Koch et al. 2006). Plants grown at the highest humidity of 98% had decreased total wax mass and wax crystal density and showed increased leaf surface wettability, but the exact reason for this difference could not be determined. Koch et al. (2006) speculated that the differences in wax amounts at different air humidity were a consequence of different rates of wax transport from the site of synthesis through the aqueous cell wall to the exterior of the cuticle. However, it is not known how this transport occurs but it has been suggested that a co-transport of wax molecules with water serving as a solvent could be responsible for the movement of the wax molecules from the cells to the surface of the cuticles (Neinhuis et al. 2001). Thus, at an air humidity of 98% the water and wax diffusion through the cuticle would be significantly reduced since the driving force is close to zero. In our study it is also possible that the submergence of the plants resulted in lower wax movement and regeneration, because the transpiration would be greatly reduced when the plants became completely submerged.

Epicuticular wax crystals are formed by a process called self-assembly, where organic solvents are excreted from the plants and then crystallise on the cuticle surface. This self-assembly results in wax crystals but the shape and composition of these depend on several factors such as temperature, solvent and substrate (Koch and Ensikat 2008). We speculate that the self-assembly of wax crystals does not function properly when the plants are submerged as the transport of wax components to the cuticle surface might be impaired and only some of the components reach the surface. This could result in a layer of unidentified substance on the cuticle instead of new wax crystals and could cause the leaves to lose their hydrophobicity and subsequently the gas films. An alternative explanation could be that the unidentified substance formed on leaves after the gas films had disappeared, and that the substance originates from the floodwater, as organic compounds or biofilm in the water. To answer these questions, further investigations have to be conducted.

Leaf gas films have been shown to increase underwater gas exchange and thus CO2 entry to sustain higher rates of P\textsubscript{N} (Colmer and Pedersen 2008; Pedersen et al. 2009). In the present study, P\textsubscript{N} rates were measured at two CO2 concentrations. At 200 \textmu M CO2, underwater P\textsubscript{N} is limited by CO2 owing to the high resistance to diffusion from the bulk medium into the submerged leaf (Pedersen et al. 2009). Therefore, presence of gas films would be beneficial at low CO2 concentrations, whereas P\textsubscript{N} rates measured at 2500 \textmu M CO2 would not be limited by CO2. Correlation analyses showed that at 200 \textmu M CO2 there were significant positive relationships between gas film thickness and P\textsubscript{N} rates, whereas there were no correlations at 2500 \textmu M CO2 (Fig. 5). The declines with time in underwater P\textsubscript{N} at 200 \textmu M CO2 were probably due to loss of gas films as there was a strong link between decreased gas film thickness and steep declines in underwater P\textsubscript{N} during the initial 4 days of submergence for the three wheat cultivars and also G. fluitans (Figs 2a and 4a). However, at day 4 the three wheat cultivars also showed a steep decline at the ‘non-limiting’ CO2 concentrations indicating that the photosynthetic apparatus had been damaged (Fig. 4b). By contrast, G. fluitans still seemed to have well-functioning photosynthesis at day 4. At 200 \textmu M CO2, G. fluitans had significantly higher P\textsubscript{N} compared with the wheat cultivars at
all submergence durations indicating that it was generally less limited by CO$_2$. We note that $G$. flavians had similar $P_N$ rates at the two CO$_2$ concentrations at days 7 and 10, which showed that it was not limited by CO$_2$ even after the gas films had been lost suggesting that it had developed a more permeable cuticle (Fig. 4), a phenomenon observed also in new aquatic leaves of $Rumex palastris$ (Mommer et al. 2005).

We looked at leaf porosity and chlorophyll concentration to investigate whether these factors could explain the declines in $P_N$ rates during submergence (Fig. 6). Interestingly, the wheat cultivars did not show any sign of water infiltration of the tissue which would be an indication of structural degradation, whereas $G$. flavians showed declines in leaf porosity until it reached a level of 16–20% corresponding to the wheat cultivars. Concentrations of chlorophyll did show some declines but only to around 50% of the initial levels indicating that other components of the photosynthetic apparatus had been compromised. For comparison, Winkel et al. (2014) found that porosity and chlorophyll concentrations of various rice cultivars declined more drastically during a 13-day submergence period except in the submergence tolerant cultivar FR13A. This rice study was conducted at 28°C because the present study was conducted at 15°C which could probably explain why we observed a slower degradation of leaf tissue and chlorophyll. Indeed, a submergence study on the wheat cultivar ‘JB Asano’ performed at around 23°C showed a reduction in leaf chlorophyll a by 90% after 14 days of submergence (Winkel et al. 2017) indicating a strong effect of temperature on tissue degradation during submergence stress.

In summary, the present study found little diversity in the gas film retention of 14 different wheat cultivars, whereas $G$. flavians had thicker gas films and could retain its gas films significantly longer than the wheat cultivars. The SEM micrographs showed that the wheat cultivars and $G$. flavians possessed high densities of platelets which could explain their superhydrophobicity. However, $G$. flavians also had papilae that would contribute to higher hydrophobicity during the initial submergence and could explain why $G$. flavians retained gas films for 4 days, and the wheat cultivars only maintained gas films for 2 days. We have shown that the loss of gas films is associated with the leaves being covered by an unidentified substance, and to identify the origin of this substance, further investigations have to be conducted. In conclusion, the leaf gas films provide benefits upon submergence and as such is a relevant trait to use as a selection criterion to improve the flood tolerance of both wetland and dryland crops that become temporarily submerged after sudden rainfall events.

Acknowledgements

The study was supported by the Villum Foundation via the grant WheatSUB.

References


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Supporting Information to chapter 1, 2 and 3
## Supporting Information to chapter 1

### Table S1.
Wheat growth from studies where wheat was grown in soil under waterlogged (water at soil surface unless otherwise stated) and drained conditions and recording both shoot and root dry mass (DM), allowing for root:shoot ratio calculation. Values are % reduction from controls \[1-(\text{waterlogged}/\text{drained})\]. When multiple nutrient-levels were used we refer to the lowest.

$Wl =$ waterlogged, $DAS =$ Days after sowing.

<table>
<thead>
<tr>
<th>Variety</th>
<th>Soil type and temperature</th>
<th>Treatment</th>
<th>Reduction in shoot DM (%) relative to drained controls</th>
<th>Reduction in root DM (%) relative to drained controls</th>
<th>Root:shoot ratio</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jaypee and USG3209</td>
<td>Sandy loam soil. 20/18 °C.</td>
<td>WI for 28 d from 24 DAS in pots in greenhouse.</td>
<td>Jaypee: 28%. USG3209: 31%.</td>
<td>Jaypee: 37%. USG3209: 21%.</td>
<td>Controls: 0.16/0.13 (Jaypee/USG3209). WI: 0.14/0.15 (Jaypee/USG3209).</td>
<td>Ballesteros et al. (2014)</td>
</tr>
<tr>
<td>Monad</td>
<td>Silt loam soil mixed with sand. 22/10 °C.</td>
<td>WI for 22 d from 84 DAS in pots in climate chamber.</td>
<td>14%</td>
<td>55%</td>
<td>Controls: 0.7 WI: 0.37</td>
<td>Hamonts et al. (2013)</td>
</tr>
<tr>
<td>Bobwhite SH 9826</td>
<td>Granular soil and volcanic top-soil. 20/15 °C.</td>
<td>WI for 7 d 3 cm above soil level from 5 DAS in pots in greenhouse.</td>
<td>12%</td>
<td>40%</td>
<td>Controls: 0.54 WI: 0.37</td>
<td>Haque et al. (2011)</td>
</tr>
<tr>
<td>Bobwhite, Norin 61, Shiroganekomugi, Chikugoizumi, Minaminokomugi, Kinuhime</td>
<td>Granular soil and volcanic top-soil. 17-23°C.</td>
<td>WI for 7 d 3 cm above soil level from 5 DAS in pots in greenhouse.</td>
<td>-4-18% (variety range).</td>
<td>7-40% (variety range).</td>
<td>-</td>
<td>Haque et al. (2012)</td>
</tr>
<tr>
<td>Al, Mn and Fe tolerant and intolerant varieties (names not stated)</td>
<td>4 strongly acidic soils. 20/15 °C.</td>
<td>WI for 42 d from 21 DAS in pots in phytotron.</td>
<td>9-22% (variety range).</td>
<td>45-84% (variety range).</td>
<td>-</td>
<td>Khabaz-Saberi et al. (2012)</td>
</tr>
<tr>
<td>Cascades</td>
<td>Top of sandy surfaced duplex soil. 20/15 °C.</td>
<td>WI for 14 d from 21 DAS in pots in growth chamber.</td>
<td>39%</td>
<td>75%</td>
<td>Controls: 0.4 WI: 0.19</td>
<td>Malik et al. (2001)</td>
</tr>
<tr>
<td>Cascades</td>
<td>Top soil of sandy surfaced duplex soil. 20/15 °C.</td>
<td>WI for 28 d 21 DAS in pots in growth chamber. 1</td>
<td>44% after 14d wl. 72% after 28 d wl.</td>
<td>64% after 14 d wl. 86% after 28 d wl.</td>
<td>Controls: 0.33 WI 14d: 0.22</td>
<td>Malik et al. (2002)</td>
</tr>
<tr>
<td>Cascades</td>
<td>Top of sandy surfaced duplex soil. 20/15 °C.</td>
<td>WI for 21 d from 21 DAS in pots in growth chamber.</td>
<td>38%</td>
<td>74%</td>
<td>Controls: 0.58 WI: 0.25</td>
<td>Malik et al. (2003)</td>
</tr>
<tr>
<td>Chinese spring</td>
<td>Sandy duplex top soil. 20/15 °C.</td>
<td>WI for 42 d 11 DAS in pots in phytotron.</td>
<td>78%</td>
<td>91%</td>
<td>Controls: 0.4 WI: 0.17</td>
<td>Malik et al. (2011)</td>
</tr>
</tbody>
</table>
Chinese spring  | Fine grey sand topsoil. 20/15 °C. | WI for 21 d from 11 DAS in pots in phytotron. | 84% | 89% | Controls: 0.4 WI: 0.21 | McDonald et al. (2001b)
---|---|---|---|---|---|---
Wyalkatchem | Yellow sand:sandy duplex top soil mixture. 20/10 °C. | WI for 14 d from 22 DAS in pots in greenhouse. | 22% | 68% | Controls: 1.32 WI: 0.5 | Robertson et al. (2009)
---|---|---|---|---|---|---
Cappelle Deprez | Sandy soil collected from field. 14 °C. | WI for 14 d 11 DAS in cylinders. | 54% | 81% | Controls: 0.64 WI: 0.25 | Trought and Drew (1980a)
---|---|---|---|---|---|---
Cappelle Deprez | Sandy soil collected from field. 14 °C. | WI for 14 d 11 DAS in cylinders. | 40% | 72% | Controls: 0.42 WI: 0.19 | Trought and Drew (1980b)

* Authors measured structural dry mass rather than dry mass; not included in Figure 1.
Table S2. Grain yield reductions from studies where wheat was grown in soil under waterlogged (water at soil surface unless otherwise stated) and drained conditions. Values are % reduction from controls [1-(waterlogged/drained)]. When multiple nutrient-levels were used we refer to the lowest. WL = waterlogged, DAS = Days after sowing.

<table>
<thead>
<tr>
<th>Variety</th>
<th>Soil type and temperature</th>
<th>Treatment</th>
<th>Yield reduction (% reduction from controls)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non declared</td>
<td>Sandy loam. Field conditions, mean soil temperature 4-11 °C.</td>
<td>WL for 20-120 d at seedling, tillering and/or stem elongation stage in cylinders in the field.</td>
<td>7-19% depending on growth stage and duration.</td>
<td>Belford (1981)</td>
</tr>
<tr>
<td>Non declared</td>
<td>Calcerous clay soil. Field conditions, mean soil temperature 3-11 °C.</td>
<td>WL for 4, 21 or 42 d at pre-emergence, tillering or stem elongation stage in lysimeters.</td>
<td>4% at pre-emergence, 12% at tillering and 6% at stem elongation (n. s.).</td>
<td>Belford et al. (1985)</td>
</tr>
<tr>
<td>Non declared</td>
<td>Sandy loam and clay soil. Field conditions mean soil temperature 4-12 °C.</td>
<td>WL for 16, 42 or 6 d early winter, mid-winter or spring in lysimeters in the field.</td>
<td>0% (light yields) to 15% (heavy yields).</td>
<td>Cannell et al. (1980)</td>
</tr>
<tr>
<td>Claire, Deben, Xi-19</td>
<td>Fertile alluvial loam. Field conditions, mean air temperature 3-11 °C.</td>
<td>WL for 44-58 d from 64-93 DAS in lysimeters in the field in two consecutive cropping seasons.</td>
<td>20 and 24% in two consecutive years.</td>
<td>Dickin and Wright (2008)</td>
</tr>
<tr>
<td>Claire, Deben, Hereward, Riband and Xi-19.</td>
<td>Fertile alluvial loam. Field conditions, mean air temperature 3-10 °C.</td>
<td>WL for 77-86 d from 61-78 DAS in the field. Water level 10 cm below soil surface.</td>
<td>9% (mean of two years and 5 varieties).</td>
<td>Dickin et al. (2009)</td>
</tr>
<tr>
<td>Peck, UJ077296, Hill 81, Gallahad, Birch 75, Lawson, Birch 41, Braemar Velvet, M4195, Quarrion, Kellalac, Matong, Meering.</td>
<td>Loam over clay duplex soil. Field conditions.</td>
<td>Intermittent WL for 30 d or more from mid- to late-tillering in the field.</td>
<td>-3-74% (variety range).</td>
<td>Gardner and Flood (1993), summarized by Setter and Waters (2003)</td>
</tr>
<tr>
<td>Nishikazekomugi, Iwainodaichi, Shiroganekomugi, Norin 61, Uniculm.</td>
<td>Andosol. Field conditions, 3-23 °C during 2005-2010 growing seasons.</td>
<td>WL for up to 49 d from 120 DAS (jointing stage till maturity) in the field. Water level 0-5 cm below soil surface.</td>
<td>96-27% (variety and year range, see table 2 in reference).</td>
<td>Hayashi et al. (2013)</td>
</tr>
<tr>
<td>Al, Mn and Fe tolerant and intolerant varieties, only names of reference varieties stated.</td>
<td>4 strongly acidic soils. 20/15 °C.</td>
<td>WL for 42 d from 21 DAS in pots in phytotron.</td>
<td>30%/50% Al-T/Al-I 21%/41% Mn-T/Mn-l 16%/47% Fe-T/Fe-I</td>
<td>Khabaz-Saberi et al. (2012)</td>
</tr>
<tr>
<td>Yangmai 9</td>
<td>Clay soil. Field conditions.</td>
<td>WL for 7 d from 7 DAA in pots in the</td>
<td>22%</td>
<td>Li et al. (2011)</td>
</tr>
<tr>
<td>Variety</td>
<td>Soil Type</td>
<td>Field Conditions</td>
<td>Water Level</td>
<td>Notes</td>
</tr>
<tr>
<td>---------</td>
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</tr>
<tr>
<td>Yecora 70</td>
<td>Silt loam. Soil temperatures at 5, 15 and 25 °C.</td>
<td>WL for 30d from 72 DAS in pots in water bath. Flooded to a depth of 5 cm.</td>
<td>21% at 5 °C 23% at 15 °C 73% at 25 °C</td>
<td>Luxmoore et al. (1973)</td>
</tr>
<tr>
<td>Yecora 70</td>
<td>Clay loam. Field conditions, mean soil temperature 17 °C.</td>
<td>WL for 10-20 d from 101 DAS (anthesis) in the field. Flooded to a depth of 5 cm.</td>
<td>10 d: 1-2 % 20d: 16%</td>
<td>Luxmoore et al. (1973)</td>
</tr>
<tr>
<td>Oxley</td>
<td>One well drained and one waterlogging-prone duplex soil. Field conditions, 9-16 °C.</td>
<td>WL occurred on the poorly drained duplex soil by excess rainfall. Crops sown at three different dates.</td>
<td>58% reduction in well drained to poorly drained soil (mean of three sowing dates).</td>
<td>McDonald and Gardner (1987)</td>
</tr>
<tr>
<td>Coker 9877</td>
<td>Silty clay. Field conditions, mean soil temperature 25 °C.</td>
<td>WL occurred on poorly drained plots.</td>
<td>51%</td>
<td>Musgrave (1994)</td>
</tr>
<tr>
<td>Florida 303, Terral 877, Coker 9105, McNair 1003, Coker 9877, LA8564 A80-3-1-X.</td>
<td>Silty clay. Field conditions.</td>
<td>WL for 120 d from stand establishment in the field under rain shelter.</td>
<td>9-69% in 6 varieties (45% mean).</td>
<td>Musgrave and Ding (1998)</td>
</tr>
<tr>
<td>Karasu-90</td>
<td>Loamy textured soil. Field conditions.</td>
<td>WL for 5, 10, 15, 20, 25 or 50 d from flowering in PVC containers in the field.</td>
<td>4, 21, 38, 49, 74 and 90% (5, 10, 15, 20, 25 and 50 d).</td>
<td>Olgun et al. (2008)</td>
</tr>
<tr>
<td>Wyalkatchem</td>
<td>Yellow sand:sandy duplex top soil mixture. 20/10 °C.</td>
<td>WL for 14 d from 22 DAS in glasshouse.</td>
<td>32%</td>
<td>Robertson et al. (2009)</td>
</tr>
<tr>
<td>Ducula-1, Ducula-2, Ducula-3, Ducula-4, Seri 82, Pato Blanco, BR34, PF8442, Mikn Yang#11, Zhen 7843, WR89-3420, WR89-3246, 46 WR Norin, Tinamou, Ves/Myna, Prl/Sara</td>
<td>Coarse sandy clay. Field conditions.</td>
<td>WL for 48 d after emergence to mid boot or 28 d (1° node stage, mid boot, anthesis, grain filling) in the field. Water level 3-8 cm above soil surface.</td>
<td>68, 53, 70, 59, 26% at 5 different growth stages (mean of 16 varieties). Min. reduction 7%, max. 96%.</td>
<td>Sayre et al. (1994)</td>
</tr>
<tr>
<td>HD 2009</td>
<td>Poorly permeable sodic soil. Field conditions.</td>
<td>WL for 2, 4 and 6 d from 25 DAS in the field.</td>
<td>18, 29 and 47% (2, 4 and 6 d).</td>
<td>Sharma and Swarup (1988)</td>
</tr>
<tr>
<td>Arina</td>
<td>Unspecified field soil. Field conditions.</td>
<td>WL from anthesis through maturity in pots embedded in the field.</td>
<td>39%.</td>
<td>Stieger and Feller (1994)</td>
</tr>
<tr>
<td>Norin 6, Siroganegomugi</td>
<td>Field soil. Field conditions, soil temperature 7-22 °C.</td>
<td>WL for 15 d at tillering, elongation, booting or ripening stages in the field.</td>
<td>9% (mean of different growth stages, range 7-10%).</td>
<td>Suh (1978)</td>
</tr>
<tr>
<td>Variety</td>
<td>Clay soil. Field conditions.</td>
<td>WL from 7 DAA for 15 d in pots. Water level 1-2 cm above soil surface.</td>
<td>32% and 34% (variety 1 and 2).</td>
<td>Tan et al. (2008)</td>
</tr>
<tr>
<td>-------------------------------</td>
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</tr>
<tr>
<td>Yangmai 9, Yumai 34</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non declared</td>
<td>Top soil collected from upper, middle and lower valley slope (clay content increasing). Field conditions.</td>
<td>WL for 42 d from tillering stage.</td>
<td>57% (average of 3 soil types).</td>
<td>Watson et al. (1976)</td>
</tr>
<tr>
<td>Non declared</td>
<td>Sandy loam and clay. Field conditions.</td>
<td>WL in January for 21-42 d and in one year also 21 d in May, in lysimeters in three cropping seasons.</td>
<td>11% (sand, 21 d, year 1). -3% (clay, 42 d, year 2). 17% (clay, 63 d, year 3).</td>
<td>Webster et al. (1986)</td>
</tr>
<tr>
<td>HD-2009, KRL-3-4</td>
<td>Two sodic soils (pH 8.5 and 9.2). Field conditions, mean daily temperature 17 °C.</td>
<td>WL for 14 d from 21 DAS in the field. Water level 5 cm above soil surface.</td>
<td>HD-2009: 16%/34% (pH 8.5/pH 9.2). KRL-3-4: 6%/14% (pH 8.5/pH 9.2).</td>
<td>Yaduvanshi et al. (2012)</td>
</tr>
</tbody>
</table>
Table S3. Summary of average root porosity (POR) or aerenchyma (AER) in wheat roots of different varieties, plant age, growing media to induce hypoxia, hypoxia/waterlogging duration, type of root (seminal and/or adventitious-nodal).

<table>
<thead>
<tr>
<th>Variety</th>
<th>Plant age (days)</th>
<th>Growing media for inducing hypoxia</th>
<th>Treatment duration (days)</th>
<th>Root type</th>
<th>Root porosity (POR) or aerenchyma (AER) under aerated conditions</th>
<th>Root porosity (POR) or aerenchyma (AER) under hypoxic/waterlogged conditions</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chinese Spring</td>
<td>11 d</td>
<td>Stagnant nutrient solution</td>
<td>27-29 d</td>
<td>Adventitious</td>
<td>6% POR (segments of 100mm from tip)</td>
<td>19% POR (segments of 100mm from tip)</td>
<td>Alamri et al. (2013)</td>
</tr>
<tr>
<td>Gamenya</td>
<td>16 d</td>
<td>N₂ flushed nutrient solution</td>
<td>10 d</td>
<td>Seminal &amp; Adventitious</td>
<td>Not stated</td>
<td>POR 6% at 0-50 mm from root tip</td>
<td>Barrett-Lennard et al. (1988)</td>
</tr>
<tr>
<td>Jackson Coker 9835</td>
<td>14 d</td>
<td>Hypoxic nutrient solution (1 kPa O₂)</td>
<td>21 d</td>
<td>Adventitious</td>
<td>7.5% POR (segments of root axes)</td>
<td>21.5% POR (segments of root axes)</td>
<td>Huang and Johnson (1995)</td>
</tr>
<tr>
<td>Bayles Savannah</td>
<td>14 d</td>
<td>Waterlogged sand with influx of ½ strength Hoagland’s nutrient solution</td>
<td>15 d</td>
<td>Adventitious</td>
<td>Aerenchyma absent in root cortex</td>
<td>12%&lt;sup&gt;1&lt;/sup&gt; AER starting at 50mm from tip</td>
<td>Huang et al. (1994)</td>
</tr>
<tr>
<td>Cascades</td>
<td>21 d</td>
<td>Waterlogged sandy soil</td>
<td>14 d</td>
<td>Adventitious</td>
<td>4% to 8% POR along the root</td>
<td>12% to 13% POR along the root</td>
<td>Malik et al. (2001)</td>
</tr>
<tr>
<td>Cascades</td>
<td>21 d</td>
<td>Waterlogged sandy soil</td>
<td>14 d&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Adventitious</td>
<td>3% to 5% POR along the root</td>
<td>16% - 20% POR along the root</td>
<td>Malik et al. (2003)</td>
</tr>
<tr>
<td>Chinese Spring</td>
<td>11 d</td>
<td>Stagnant nutrient solution</td>
<td>21 d</td>
<td>Adventitious</td>
<td>3% POR (entire root)</td>
<td>15.9% POR (entire root)</td>
<td>Malik et al. (2011)</td>
</tr>
<tr>
<td>Chinese Spring</td>
<td>13 d</td>
<td>Stagnant nutrient solution</td>
<td>21 d</td>
<td>Seminal &amp; Adventitious</td>
<td>2.1 POR (root segments 40-50mm)</td>
<td>2.7 POR (root segments 40-50mm)</td>
<td>McDonald et al. (2001a)</td>
</tr>
</tbody>
</table>
### Supporting Information chapter 1

<table>
<thead>
<tr>
<th>Variety</th>
<th>Treatment</th>
<th>Time (d)</th>
<th>Stage</th>
<th>Adventitious</th>
<th>Morphology</th>
<th>AER</th>
<th>POR</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chinese Spring</td>
<td>Stagnant nutrient solution</td>
<td>12</td>
<td></td>
<td></td>
<td>Adventitious</td>
<td></td>
<td></td>
<td>AER 32% 10mm below root/shoot; POR 2% 3.35%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>21</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>37.3% 50 mm behind root tip; POR 1.65% 17.66%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>McDonald et al. (2001b)</td>
</tr>
<tr>
<td>Gamenya</td>
<td>Hypoxic nutrient solution</td>
<td>25</td>
<td></td>
<td></td>
<td>Seminal</td>
<td>0.8%</td>
<td></td>
<td>5.3% (root segments until 80mm from tip)</td>
</tr>
<tr>
<td></td>
<td>(0.22 kPa O(_2))</td>
<td>12</td>
<td></td>
<td></td>
<td>Adventitious</td>
<td></td>
<td></td>
<td>12.6% (root segments until 80mm from tip)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Thomson et al. (1992)</td>
</tr>
<tr>
<td>Gamenya</td>
<td>Stagnant nutrient solution</td>
<td>5-7</td>
<td></td>
<td></td>
<td>Seminal</td>
<td>3.4%</td>
<td></td>
<td>12% (entire root)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>18-23</td>
<td></td>
<td></td>
<td>Adventitious</td>
<td></td>
<td></td>
<td>6% (entire root)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>23-28</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Thomson et al. 1990</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gamenya</td>
<td>Stagnant nutrient solution</td>
<td>14</td>
<td></td>
<td></td>
<td>Adventitious</td>
<td></td>
<td></td>
<td>11.6% AER at 50 mm from tip</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>13.7% AER at 50 mm from tip</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Watkin et al. (1998)</td>
</tr>
<tr>
<td>Gamenya</td>
<td>Stagnant nutrient solution</td>
<td>16</td>
<td></td>
<td></td>
<td>Seminal</td>
<td>POR 1.3%</td>
<td></td>
<td>POR 3% 14.8%, AER 22.3%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15</td>
<td></td>
<td></td>
<td>Adventitious</td>
<td>5.2%</td>
<td></td>
<td>Wiengweera et al. (1997)</td>
</tr>
<tr>
<td>Huamai 8</td>
<td>Roots submerged in</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4% AER at 10 mm from tip and 17-18% AER at 50 mm from tip</td>
</tr>
<tr>
<td></td>
<td>distilled water</td>
<td>3</td>
<td></td>
<td></td>
<td>Seminal</td>
<td>0%</td>
<td></td>
<td>Xu et al. (2013)</td>
</tr>
<tr>
<td>Bobwhite line SH98</td>
<td>Stagnant nutrient solution</td>
<td>9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>14% AER at 10 mm from tip and 20% AER at 50 mm from tip</td>
</tr>
<tr>
<td>26</td>
<td></td>
<td>7</td>
<td></td>
<td>Adventitious</td>
<td></td>
<td></td>
<td></td>
<td>Yamauchi et al. (2014a)</td>
</tr>
<tr>
<td>Bobwhite line SH98</td>
<td>Stagnant nutrient solution</td>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3% AER at 10 mm from tip and 4% AER for the 1(^{st}) seminal root.</td>
</tr>
<tr>
<td>26</td>
<td></td>
<td></td>
<td></td>
<td>Seminal</td>
<td></td>
<td></td>
<td></td>
<td>ca. 2% AER at 10mm and 30mm from tip for the 2(^{nd}) and 3(^{rd}) seminal root.</td>
</tr>
</tbody>
</table>

1 Values correspond to aerenchyma proportion of the cortical cross sectional area (stele area was excluded, not plotted in Figure 2.

2 Plotted in Figure 3.

3 Nutrient solution with 20 μM 1-aminoacyclopropanoic acid (ACC)– not plotted in Figure 2.
### Table S4. Reductions in photosynthetic rates calculated from studies where wheat was grown in soil under waterlogged (water at soil surface unless otherwise stated) and drained conditions. Values are % reduction from controls \([1-(\text{waterlogged}/\text{drained})]\). All nutrient treatments were included. WL=waterlogged, DAS=Days after sowing, DAA=days after anthesis.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Soil type and temperature</th>
<th>Treatment</th>
<th>Leaf used for measurements</th>
<th>(P_N) reduction (% reduction from control levels)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monad</td>
<td>Silt loam soil mixed with sand. 22/10 °C.</td>
<td>WL for 22 d from 84 DAS in pots in climate chamber.</td>
<td>Flag leaf 22 d after WL, 1400 (\mu)mol photons m(^{-2}) s(^{-1}).</td>
<td>9% with additional N supply. 8% without N supply.</td>
<td>Hamonts et al. (2013)</td>
</tr>
<tr>
<td>Nishikazekomugi,</td>
<td>Andosol. Field conditions, monthly mean air temperature 4-23 °C in three consecutive cropping seasons.</td>
<td>WL for 31-34 d from jointing stage to maturity in the field. Water level 0-5 cm below soil surface.</td>
<td>Flag leaf, 1400 (\mu)mol photons m(^{-2}) s(^{-1}).</td>
<td>2008: 0%. 2009: No change for 22d, then 23% and 51%. 2010: Close to controls for 11-16 d, then 62 and 71 %.</td>
<td>Hayashi et al. (2013)</td>
</tr>
<tr>
<td>Iwainodaichi</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uniculm</td>
<td>As above.</td>
<td>As above.</td>
<td>Flag leaf, 1400 (\mu)mol photons m(^{-2}) s(^{-1}).</td>
<td>2008: No change for 8d, then 48% (22d). 2009: 70%. 2010: 63%.</td>
<td>Hayashi et al. (2013)</td>
</tr>
<tr>
<td>Daichinominori</td>
<td>As above.</td>
<td>As above.</td>
<td>As above.</td>
<td>Not within 12 d WL, then 85% after 25 d of WL.</td>
<td>Hossain et al. (2011)</td>
</tr>
<tr>
<td>Savannah</td>
<td>Sterilized fine sand. 20/15 °C.</td>
<td>WL for 17 d from 14 DAS with half (HS) or full strength (FS) Hoagland solution in pots in growth chamber. Water level 2 cm above soil surface.</td>
<td>Youngest fully expanded leaf, mid-day.</td>
<td>FS, HS: Drop by 13% within first 3 d, then stable.</td>
<td>Huang et al. (1994)</td>
</tr>
<tr>
<td>Bayles</td>
<td>As above.</td>
<td>Same.</td>
<td>Same.</td>
<td></td>
<td>Huang et al. (1994)</td>
</tr>
<tr>
<td>Yangmai 9</td>
<td>Clay soil. Field conditions.</td>
<td>WL for 7 d from 7 DAA in pots in the field. Water level 1-2 cm above soil surface.</td>
<td>Flag leaf, 1400 (\mu)mol photons m(^{-2}) s(^{-1}).</td>
<td>51%</td>
<td>Li et al. (2011)</td>
</tr>
<tr>
<td>Cascades</td>
<td>Top soil of sandy surface duplex soil. 20/15 °C.</td>
<td>WL for 14 d from 21 DAS in pots in growth chamber.</td>
<td>Youngest fully expanded leaf, 2000 (\mu)mol m(^{-2}) s(^{-1}).</td>
<td>25% decrease within 24 hours, 100% decrease after 5 d in 3rd leaf (84% in 4th leaf).</td>
<td>Malik et al. (2001)</td>
</tr>
<tr>
<td>Variety</td>
<td>Soil Type</td>
<td>Soil Conditions</td>
<td>Treatment Duration</td>
<td>Description</td>
<td>Reduction at Stages</td>
</tr>
<tr>
<td>-------------</td>
<td>--------------------</td>
<td>-----------------</td>
<td>--------------------</td>
<td>-----------------------------------------------------------------------------</td>
<td>---------------------</td>
</tr>
<tr>
<td>Yangmei 14</td>
<td>Clay soil</td>
<td>Field conditions, mean temperature 15.3 °C.</td>
<td>WL for 1-3 d at tillering, milky and booting stages in lysimeters in the field. Water level 0.5-1 cm above soil surface.</td>
<td>Top fully expanded leaves, 1000-1100 µmol photons m⁻² s⁻¹</td>
<td>12-13% reduction at tillering, n.s. at booting and milky stages after 1 day of WL.</td>
</tr>
<tr>
<td>Cappelle Deprez</td>
<td>Sandy soil collected from field. 14 °C.</td>
<td>WL for 14 d from 11 DAS in cylinders.</td>
<td>Not stated.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yannong 19</td>
<td>Alluvial top. Field conditions.</td>
<td>WL for 6 d from 3 DAA in pots in the field. Water level 0.5-1 cm above soil surface.</td>
<td>Flag leaf, 1000 µmol photons m⁻² s⁻¹.</td>
<td>15%. 7% with foliar N spray.</td>
<td></td>
</tr>
<tr>
<td>Huamai 17</td>
<td>Clay soil</td>
<td>Field conditions.</td>
<td>WL for 5 d from 7 DAA in pots in the field. Water level 1-2 cm above soil surface.</td>
<td>Flag leaf, 1200 µmol photons m⁻² s⁻¹.</td>
<td>14% after 3 d WL (n.s.).</td>
</tr>
<tr>
<td>Yangmai 12</td>
<td>As above.</td>
<td>As above.</td>
<td>Flag leaf, 1200 µmol photons m⁻² s⁻¹.</td>
<td></td>
<td>11% after 3 d WL (n.s.).</td>
</tr>
</tbody>
</table>
Table S5. Summary of values/range for photosynthesis (P_N) and stomatal conductance (g_s) in wheat under control and waterlogging conditions used for calculations in Figure 6. Information on waterlogging (WL) duration, variety, P_N, g_s, phenological stage during measurements and source are provided. The numbers in brackets identify the bibliographic source of each point in Figure 4.

<table>
<thead>
<tr>
<th>WL duration</th>
<th>Variety</th>
<th>P_N (µmol m^{-2}s^{-1}) Value or range</th>
<th>g_s (mol m^{-2}s^{-1}) Value or range</th>
<th>Phenological stage during measurements</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>Waterlogging</td>
<td>Control</td>
<td>Waterlogging</td>
</tr>
<tr>
<td>1d</td>
<td>Yangmai 14</td>
<td>24.5 – 25.20</td>
<td>22.63 – 23.30</td>
<td>0.38 – 0.45</td>
<td>0.26 – 0.32</td>
</tr>
<tr>
<td>1d</td>
<td>Yangmai 14</td>
<td>21.23 – 21.73</td>
<td>17.03 – 20.70</td>
<td>0.32 – 0.35</td>
<td>0.21 – 0.28</td>
</tr>
<tr>
<td>1d</td>
<td>Yangmai 14</td>
<td>20.73 – 23.77</td>
<td>18.55 – 21.20</td>
<td>0.29 – 0.46</td>
<td>0.22 – 0.46</td>
</tr>
<tr>
<td>1d</td>
<td>Yangmai 14</td>
<td>14.88 – 16.23</td>
<td>13.20 – 13.98</td>
<td>0.25 – 0.26</td>
<td>0.22 – 0.25</td>
</tr>
<tr>
<td>1d</td>
<td>Yangmai 9</td>
<td>18.27</td>
<td>17.70</td>
<td>0.27</td>
<td>0.26</td>
</tr>
<tr>
<td>1d</td>
<td>Cascades</td>
<td>23.34</td>
<td>17.73</td>
<td>0.38</td>
<td>0.24</td>
</tr>
<tr>
<td>1d</td>
<td>Bayles</td>
<td>20.27 – 21.76</td>
<td>24.08 – 24.82</td>
<td>0.87 – 0.88</td>
<td>0.97 – 1.03</td>
</tr>
<tr>
<td>1d</td>
<td>Savannah</td>
<td>20.81 – 21.46</td>
<td>22.99 – 23.63</td>
<td>0.86 – 0.87</td>
<td>1.0 – 1.1</td>
</tr>
<tr>
<td>2d</td>
<td>Cascades</td>
<td>23.06</td>
<td>9.38</td>
<td>0.521</td>
<td>0.136</td>
</tr>
<tr>
<td>2d</td>
<td>Yangmai 9</td>
<td>15.13</td>
<td>14.03</td>
<td>0.242</td>
<td>0.216</td>
</tr>
<tr>
<td>3d</td>
<td>Huaimai 17</td>
<td>17.5</td>
<td>15.0</td>
<td>0.20</td>
<td>0.203</td>
</tr>
<tr>
<td>3d</td>
<td>Yangmai 12</td>
<td>19.0</td>
<td>17.0</td>
<td>0.24</td>
<td>0.21</td>
</tr>
<tr>
<td>3d</td>
<td>Cascades</td>
<td>20.51</td>
<td>5.56</td>
<td>0.315</td>
<td>0.077</td>
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<tr>
<td>3d</td>
<td>Bayles</td>
<td>20.9 – 22.74</td>
<td>19.45 – 21.86</td>
<td>0.775 – 0.827</td>
<td>0.873 – 0.935</td>
</tr>
<tr>
<td>3d</td>
<td>Savannah</td>
<td>20.09 – 23.23</td>
<td>19.18 – 20.51</td>
<td>0.93 – 1.0</td>
<td>0.863 – 0.913</td>
</tr>
<tr>
<td>3d</td>
<td>Nishikazekomugi (2010)</td>
<td>20.74</td>
<td>19.7</td>
<td>0.303</td>
<td>0.298</td>
</tr>
<tr>
<td>3d</td>
<td>Uniculm (2010)</td>
<td>20.70</td>
<td>19.3</td>
<td>0.304</td>
<td>0.294</td>
</tr>
<tr>
<td>3d</td>
<td>Iwainodaichi (2010)</td>
<td>19.3</td>
<td>19.5</td>
<td>0.305</td>
<td>0.305</td>
</tr>
</tbody>
</table>

Measurements at late waterlogging (7 to 21 days)

| 7d          | Yannong 19 | 13.22 – 14.17                          | 11.25 – 13.02                          | 0.268 – 0.282                         | 0.244 – 0.265             | Post-anthesis        | Wu et al. (2014) [2]   |
| 7d          | Monad      | 23.5                                   | 18.0 – 21.3                            | 0.29                                  | 0.16 – 0.18               | Non declared         | Hamonts et al. (2012) [4]|
| 7d          | Yangmai 9  | 15.05                                  | 7.68                                   | 0.219                                 | 0.086                     | Around anthesis      | Li et al. (2011) [8]   |
| 7d          | Bayles     | 18.08 – 18.48                          | 13.34 – 16.96                          | 0.71 – 0.78                           | 0.55 – 0.68               | Tillering            | Huang et al. (1994) [7]|
| 7d          | Savannah   | 16.5 – 16.68                           | 13.78 – 15.77                          | 0.814 – 0.879                         | 0.66 – 0.71               | Tillering            | Huang et al. (1994) [7]|

Measurements at early waterlogging (1 to 3 days)
<table>
<thead>
<tr>
<th>Days</th>
<th>Cultivar/Location</th>
<th>Height ( \text{cm} )</th>
<th>Diameter ( \text{cm} )</th>
<th>weight ( \text{g} )</th>
<th>Seedling development stage</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>8d</td>
<td>Cascades</td>
<td>19.71</td>
<td>2.39</td>
<td>0.416</td>
<td>Tillering</td>
<td>Malik et al. (2001) [6]</td>
</tr>
<tr>
<td>10d</td>
<td>Cascades</td>
<td>18.96</td>
<td>3.7</td>
<td>0.393</td>
<td>Tillering</td>
<td>Malik et al. (2001) [6]</td>
</tr>
<tr>
<td>10d</td>
<td>Nishikazekomugi (2009/10)</td>
<td>16.8 – 20.89</td>
<td>15.81 – 19.7</td>
<td>0.278 – 0.292</td>
<td>Jointing-booting</td>
<td>Hayashi et al. (2013) [9]</td>
</tr>
<tr>
<td>10d</td>
<td>Uniculm (2011)</td>
<td>21.15</td>
<td>19.1</td>
<td>0.308</td>
<td>Jointing-booting</td>
<td>Hayashi et al. (2013) [9]</td>
</tr>
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<td>10d</td>
<td>Iwainodaichi (2010)</td>
<td>19.5</td>
<td>19.1</td>
<td>0.926</td>
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<td>Hayashi et al. (2013) [9]</td>
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<td>11d</td>
<td>Bayles</td>
<td>16.57 – 17.65</td>
<td>14.75 – 16.16</td>
<td>0.82 – 0.90</td>
<td>Tillering</td>
<td>Huang et al. (1994) [7]</td>
</tr>
<tr>
<td>11d</td>
<td>Savannah</td>
<td>17.04 – 18.56</td>
<td>13.98 – 16.15</td>
<td>0.88 – 0.94</td>
<td>Tillering</td>
<td>Huang et al. (1994) [7]</td>
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<tr>
<td>12d</td>
<td>Cascades</td>
<td>19.39</td>
<td>3.04</td>
<td>0.461</td>
<td>Tillering</td>
<td>Malik et al. (2001) [6]</td>
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<tr>
<td>13d</td>
<td>Bayles</td>
<td>16.96 – 17.28</td>
<td>11.81 – 15.27</td>
<td>0.665 – 0.743</td>
<td>Jointing-booting</td>
<td>Hayashi et al. (2013) [9]</td>
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<tr>
<td>13d</td>
<td>Savannah</td>
<td>15.73</td>
<td>12.67 – 13.83</td>
<td>0.766 – 0.836</td>
<td>Jointing-booting</td>
<td>Hayashi et al. (2013) [9]</td>
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<tr>
<td>13d</td>
<td>Nishikazekomugi (2009)</td>
<td>16.6</td>
<td>13.9</td>
<td>0.28</td>
<td>Jointing-booting</td>
<td>Hayashi et al. (2013) [9]</td>
</tr>
<tr>
<td>13d</td>
<td>Uniculm (2009)</td>
<td>22.3</td>
<td>21.7</td>
<td>0.29</td>
<td>Jointing-booting</td>
<td>Hayashi et al. (2013) [9]</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>Estimatively near anthesis</td>
<td>Musgrave (1994) [1]</td>
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<tr>
<td>14d</td>
<td>Bayles</td>
<td>17.12 – 18.08</td>
<td>12.21 – 13.1</td>
<td>0.741 – 0.777</td>
<td>Tillering</td>
<td>Huang et al. (1994) [7]</td>
</tr>
<tr>
<td>15d</td>
<td>Savannah</td>
<td>15.48 – 17.79</td>
<td>12.74 – 14.15</td>
<td>0.712 – 0.802</td>
<td>Tillering</td>
<td>Huang et al. (1994) [7]</td>
</tr>
<tr>
<td>15d</td>
<td>Uniculm (2010)</td>
<td>20.98</td>
<td>16.16</td>
<td>0.299</td>
<td>Jointing-booting</td>
<td>Hayashi et al. (2013) [9]</td>
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<tr>
<td>15d</td>
<td>Iwainodaichi (2010)</td>
<td>20.4</td>
<td>17.7</td>
<td>0.269</td>
<td>Jointing-booting</td>
<td>Hayashi et al. (2013) [9]</td>
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<tr>
<td>18d</td>
<td>Nishikazekomugi (2010)</td>
<td>21.94</td>
<td>18.2</td>
<td>0.298</td>
<td>Jointing-booting</td>
<td>Hayashi et al. (2013) [9]</td>
</tr>
<tr>
<td>18d</td>
<td>Uniculm (2009/10)</td>
<td>20.82 – 21.12</td>
<td>10.15 – 19.53</td>
<td>0.286 – 0.296</td>
<td>Jointing-booting</td>
<td>Hayashi et al. (2013) [9]</td>
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<tr>
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<td>Iwainodaichi (2010)</td>
<td>21.1</td>
<td>12.5</td>
<td>0.308</td>
<td>Jointing-booting</td>
<td>Hayashi et al. (2013) [9]</td>
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<tr>
<td>21d</td>
<td>Uniculm (2009)</td>
<td>11.61</td>
<td>3.84</td>
<td>0.0121</td>
<td>Jointing-booting</td>
<td>Hayashi et al. (2013) [9]</td>
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<td>Iwainodaichi (2009)</td>
<td>16.3</td>
<td>16.15</td>
<td>0.272</td>
<td>Jointing-booting</td>
<td>Hayashi et al. (2013) [9]</td>
</tr>
<tr>
<td>21d</td>
<td>Nishikazekomugi (2009)</td>
<td>19.8</td>
<td>18.45</td>
<td>0.31</td>
<td>Jointing-booting</td>
<td>Hayashi et al. (2013) [9]</td>
</tr>
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</table>
Table S6. Shoot nutrient levels of macronutrients N, P, K, Mg, Ca, (mg g⁻¹ DM) and microelements Mn, Fe, Zn, Al (mg Kg⁻¹ DM) after soil waterlogging.

<table>
<thead>
<tr>
<th>Variety</th>
<th>Soil type (if stated) and temperature (day/night or mean).</th>
<th>Treatment</th>
<th>Nutrient levels in entire shoot or leaves after waterlogging (macronutrients: mg g⁻¹ DM, microelements: mg Kg⁻¹ DM)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Claire, Deben, Hereward, and Xi-19</td>
<td>Fertile alluvial loam. Field conditions, mean air temperature 2.5-9.7 °C.</td>
<td>WI in the field for 77 and 86 d from 78 and 61 DAS. Whole shoot sampled.</td>
<td>N: 32, 34, 33, 33 (4 varieties).</td>
<td>Dickin et al. (2009)</td>
</tr>
<tr>
<td>Brookton, Cascades, Chara, Ducula-4, HD2329, Savannah</td>
<td>Strongly acidic soil (pH CaCl₂=4.5), potting mix or neutral clay soil. 14 °C.</td>
<td>WI in pots for 49 d from 21 DAS in growth cabinet. Whole shoot sampled.</td>
<td>N: 11-20 (soil type range, separate values plotted). P: 2-3 (soil type range, separate values plotted). K: 19-121 (soil type range, separate values plotted). Mg: 0.9-1.8 (soil type range, separate values plotted). Ca: 0.5-5 (soil type range, separate values plotted). Mn: 58-445 (soil type range, separate values plotted). Fe: 43-390 (soil type range, separate values plotted). Zn: 11-67 (soil type range, separate values plotted).</td>
<td>Khabaz-Saberi et al. (2005)</td>
</tr>
<tr>
<td>Mn, Fe and Al tolerant (T) and intolerant (I) lines, only names of reference varieties stated.</td>
<td>4 strongly acidic soils (pH CaCl₂=4.2-4.5). 20/15 °C.</td>
<td>WI for 42 d from 21 DAS in in pots glasshouse. Whole shoot sampled.</td>
<td>Large data set – see table 3 in reference.</td>
<td>Khabaz-Saberi et al. (2012)</td>
</tr>
<tr>
<td>Wyalkatchem</td>
<td>Yellow sand:sandy duplex top soil mixture. 20/10 °C.</td>
<td>WI for 14 d from 22 DAS (tillering) in glasshouse. Whole shoot sampled.</td>
<td>N: 15</td>
<td>Robertson et al. (2009)</td>
</tr>
<tr>
<td>Camm, Cascades, Westonia</td>
<td>Three strongly acidic soils</td>
<td>WI in pots for 49 d from 21 DAS in the field.</td>
<td>Mn: 14-146</td>
<td>Setter et al. (2009)</td>
</tr>
<tr>
<td>Location</td>
<td>Soil Type</td>
<td>Methodology</td>
<td>Nutrients</td>
<td>Sources</td>
</tr>
<tr>
<td>-----------------</td>
<td>------------------------------------</td>
<td>----------------------------------------------------------------------------</td>
<td>----------------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>Cappelle Deprez</td>
<td>Sandy soil collected from field. Grown at 14 °C; WI at 6, 10, 14 or 18 °C soil temperature.</td>
<td>WI for 14 d from 9 DAS (2 leave stage) at different temperatures in plastic cylinders in growth cabinet. Whole shoot sampled.</td>
<td>N: 10.6-14.3 (temperature range). P: 0.8-1.1 (temperature range). K: 9.1-12.2 (temperature range). Ca: 2.6-4.0 (temperature range).</td>
<td>Trought and Drew (1982)</td>
</tr>
</tbody>
</table>

1 Only 15 d plotted in Figure 5


Hossain M.A., Araki H. & Takahashi T. (2011) Poor grain filling induced by waterlogging is similar to that in abnormal early ripening in wheat in Western Japan. *Field Crops Research*, 123, 100-108.


## Supporting Information to Chapter 2

**Table S1.** Primer sequences and annealing temperatures used for qRT-PCR of genes displayed in Fig. 6 and Fig. S7.

<table>
<thead>
<tr>
<th>Enzyme name</th>
<th>Gene name</th>
<th>ID</th>
<th>Forward</th>
<th>Reverse</th>
<th>Annealing temperature (°C)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>fructan exohydrolase</td>
<td>1-FEHw1</td>
<td>AJ516025</td>
<td>CACTAGTCTCTGAAATTCACGGG</td>
<td>GCTCACCAGGTCTTTAACTAC</td>
<td>60</td>
<td>Meguro-Maoka and Yoshida (2016)</td>
</tr>
<tr>
<td>fructan 6-</td>
<td>1-FEHw3</td>
<td>AJ564996</td>
<td>AATGTGGAGAGGGGTGGGA</td>
<td>GGCTATTCTTTTCCTGCTG</td>
<td>60</td>
<td>Sharbatkhari et al. (2016)</td>
</tr>
<tr>
<td>exohydrolase</td>
<td>6-FEH</td>
<td>AM07520-5</td>
<td>CTCTGCCATTGTACATAGT</td>
<td>TCCGGACGCGTAGCCAAG</td>
<td>60</td>
<td>Meguro-Maoka and Yoshida (2016)</td>
</tr>
<tr>
<td>fructan 6&amp;1-</td>
<td>6&amp;1-FEH</td>
<td>AB089269</td>
<td>CATTTGATAGAGATCAAGCCTGAG</td>
<td>GCAACTACTTCTAAAGTCAAGAGCAG</td>
<td>60</td>
<td>Meguro-Maoka and Yoshida (2016)</td>
</tr>
<tr>
<td>exohydrolase</td>
<td>6-KEHw2</td>
<td>AB089271</td>
<td>TGAAGTAGAGCCGACGAGC</td>
<td>CATAAATGACTGACAGACTAGAGTGT</td>
<td>60</td>
<td>Meguro-Maoka and Yoshida (2016)</td>
</tr>
<tr>
<td>sucrase synthase</td>
<td>SuS3</td>
<td>TC247088</td>
<td>AAGGCTCCCACCTGCTGTTT</td>
<td>CCTGTGAAATCTTTGCTTCAAGT</td>
<td>57</td>
<td>Xue et al. (2008)</td>
</tr>
<tr>
<td>sucrase synthase</td>
<td>SuS4</td>
<td>TC272798</td>
<td>CTGGAACCCTCCTGATTCTC</td>
<td>ACATCATACATGCCCTTGT</td>
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<td>Xue et al. (2008)</td>
</tr>
<tr>
<td>sucrase synthase</td>
<td>SuS5</td>
<td>TC267682</td>
<td>AGTACACGTTCCTGCTAAACTGT</td>
<td>TCTCCTTCCCCAGTACAGA</td>
<td>59</td>
<td>Xue et al. (2008)</td>
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<td>alpha-amylase</td>
<td>α-Amy2-1</td>
<td>CK207286</td>
<td>CAAAATTTACAAAGCGCTTACGAG</td>
<td>ACTTTTACATGGAGAAGACTCTAAAGTAC</td>
<td>57</td>
<td>Barrero et al. (2013)</td>
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<tr>
<td>alpha-amylase</td>
<td>α-Amy2-8</td>
<td>X13576</td>
<td>CAGCCAGTCAGCAACTCAC</td>
<td>CATACATGATTCTGACACCC</td>
<td>55</td>
<td>Wu et al. (2013)</td>
</tr>
<tr>
<td>alpha-amylase</td>
<td>α-Amy2-54</td>
<td>X13580</td>
<td>CAGCCAGTCAGCAACTCAT</td>
<td>AGCAGAGTTGGCTTCTT</td>
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<td>Wu et al. (2013)</td>
</tr>
<tr>
<td>alpha-amylase</td>
<td>α-Amy4-1</td>
<td>CJ574929</td>
<td>CGGCTGAGACGAGATATG</td>
<td>TGGCTAATCTGCTGTGTCGTA</td>
<td>55</td>
<td>Barrero et al. (2013)</td>
</tr>
<tr>
<td>RNA polymerase I, II, III 15kD subunit</td>
<td>RP1S</td>
<td>TC265122</td>
<td>GCACAGTGCTTTGCAGATAAAG</td>
<td>GCCCTCAAGCTCAACCTA</td>
<td>60</td>
<td>Xue et al. (2008)</td>
</tr>
</tbody>
</table>
Figure S1. Shoot RGR of wheat cultivars Frument and Jackson during submergence calculated from initial biomass (day 0 of submergence) and biomass after 8 days of submergence (no recovery period). n.s. indicates no significant difference between means (t-test, $P = 0.8600$). Values are means ($\pm$ SE, $n = 4$).
Figure S2. (A) Leaf underwater photosynthesis ($P_N$) and (B) dark respiration ($R_D$) of wheat cultivars Frument (open symbols) and Jackson (closed symbols) with time of submergence at 200 µM free CO$_2$. The leaf sampled was the youngest, fully expanded leaf at the time of submergence (3$^{rd}$ leaf). Values are means (± SE, n = 4). Two-way time × cultivar ANOVA only showed a significant time effect ($P < 0.0001$) for both $P_N$ and $R_D$ and no significant cultivar effects. * indicate significant difference between cultivars (Sidak’s multiple comparisons test, $P > 0.05$). Round symbols symbolize untreated controls in air at the end of the treatment period (day 16).
Figure S3. Trajectory plots from GC-MS analysis representing the average normalized relative peak areas of sugars in shoots of wheat cultivars Frument (open symbols) and Jackson (closed symbols) with time of submergence. Round symbols symbolize untreated controls in air at the end of the treatment period (day 16). The entire shoot was homogenized and used for analysis. * indicate significant difference between cultivars (Sidak’s multiple comparisons test, \( P < 0.05 \)), values are means (± SE, \( n = 4 \)).
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Figure S4. Starch in shoots of wheat cultivars Frument (open symbols) and Jackson (closed symbols) with time of submergence. The entire shoot except for the 3rd leaf (used for other physiological measurements, see Fig. 2) was sampled. Round symbols symbolize untreated controls in air at the end of the treatment period (day 16). Two-way time × cultivar ANOVA on Ln-transformed data showed significant time ($P < 0.0001$) and cultivar ($P < 0.001$) effects; time explained 78% of the variation and cultivar 5%. * indicate significant difference between cultivars (Sidak’s multiple comparisons test, $P < 0.05$), values are means ($\pm$ SE, $n = 3-4$).
Figure S5. Metabolites detected by LC-MS in shoots of wheat cultivars Frument and Jackson during 0-16 days of complete submergence, selected by two-way ANOVA ($P < 0.05$) and presented as Venn diagram for time, cultivar and time × cultivar interaction effects. LC-MS detected 74,359 mass spectral features out of which 1211 passed our quality controls filters (present in all samples of at least one group; in 80% of the quality control samples and with a CV < 35%).
Figure S6. Trajectory plots from GC-MS analysis representing the average normalized relative peak areas of amino acids in entire shoots of wheat cultivars Frument (open symbols) and Jackson (closed symbols) with time of submergence (for legend see Fig. S3). Round symbols symbolize untreated controls in air at the end of the treatment period (day 16). * indicate significant difference between cultivars (Sidak’s multiple comparisons test, \( P < 0.05 \)), values are means (± SE, \( n = 4 \)). Levels of Ser also declined for both cultivars (data not shown).
Figure S7. Relative mRNA levels of α-amylases (α-AMY2-1, α-AMY2-8, α-AMY2-54, α-AMY4-1) in shoots of wheat cultivars Frument (open bars) and Jackson (closed bars) with time of submergence. For details on primers and annealing temperatures see Table S1. Transcripts of representative genes were quantified in shoots exposed to submergence for 0-12 days by qRT-PCR. The relative level of each mRNA was calculated by comparison with initial (day 0) Frument. Values are means (± SE, n = 3). * indicate significant differences between the cultivars (Student’s t-test, P < 0.05).

References in Table S1


Supporting Information to chapter 3

**Figure S1.** Scanning electron microscopy (SEM) micrographs of leaf surfaces of rice subject to 0-16 d submergence in non-saline (0 mM NaCl) or saline (50 mM NaCl) water (containing basal ions, see Methods). Each leaf is shown at 7000 × magnification (left) showing stomata, horizontal field width (HFW) = 36.6 µm, and 500 × magnification (right), HFW = 512 µm.
Figure S2. Scanning electron microscopy (SEM) micrographs showing wax platelets on leaf surface after loss of leaf hydrophobicity and leaf gas film disappearance (a, plants had been submerged in water with 50 mM NaCl for 9 d), and wax platelets on papillae before (b, prior to submergence) and after (c, plants had been submerged in water with 50 mM NaCl for 9 d) loss of leaf hydrophobicity and leaf gas film disappearance. Leaves are shown at 27,000 × (a) and 45,000 × (b, c) magnification (HFW = 3.9 µm and 2.5 µm, respectively).
Figure S3. Leaf Na\(^+\) (a), Cl\(^-\) (b) and K\(^+\) (c) concentrations in the tissue water (mM) with time of submergence in water (containing basal ions, see Methods) with 0 mM NaCl (squares) or 50 mM NaCl (circles) for rice plants with leaf gas films (+GF, open symbols) or treated with 0.1% Triton X-100 and without gas films (-GF, closed symbols). Samples were the entire 3\(^{rd}\) and part of the 4\(^{th}\) leaf (see Methods). Roots were in non-saline nutrient solution. Values are means (± SE, \(n = 3-4\) except Na\(^+\) on day 5 at 0 mM NaCl (-GF) where \(n = 1\) due to a sampling error).
**Figure S4.** Correlations between leaf Na\(^+\) (a), Cl\(^-\) (b) and K\(^+\) (c) concentrations (data from Fig. 2) and corresponding leaf chlorophyll\(_a\) concentrations (data from Fig. 1b) after submergence of rice in water (containing basal ions, see Methods) with 50 mM NaCl. Leaves were either left untreated thus retaining a leaf gas film (+GF, open symbols) or treated with 0.1% Triton X-100 (-GF, closed symbols). Roots were in non-saline nutrient solution. r values from non-parametric Spearman rank correlation analysis, * denoting levels of significance (levels of \(P > 0.05\), \(P \leq 0.05\), \(P \leq 0.01\), \(P \leq 0.001\) or \(P \leq 0.0001\) are denoted by n.s., *, **, ***, ****, respectively): Na\(^+\) \(r = -0.5084\)**; Cl\(^-\) \(r = -0.3658\)*; K\(^+\) \(r = 0.4664\)**.
Figure S5. Correlations between leaf Na⁺ (a), Cl⁻ (b) and K⁺ (c) concentrations (data from Fig. 2) with corresponding underwater Pₕ (µmol O₂ m⁻² s⁻¹) at 2500 µM CO₂ and 50 mM NaCl (data from Fig. 3b). Leaves were either left untreated thus retaining a leaf gas film (+GF) or treated with 0.1% Triton X-100 (-GF). Roots were in non-saline nutrient solution and shoots were submerged in water containing NaCl treatments and basal ions (see Methods). r values from non-parametric Spearman rank correlation analysis, * denoting levels of significance (levels of P > 0.05, P ≤ 0.05, P ≤ 0.01, P ≤ 0.001 or P ≤ 0.0001 are denoted by n.s., *, **, ***, ****, respectively): Na⁺ r = -0.6078**; Cl⁻ r = -0.3243 n.s.; K⁺ r = 0.1923 n.s. † denotes points excluded from the correlation analysis to prevent leaf deterioration with time of submergence to draw the correlation. Points were excluded when leaf both porosity and leaf chlorophyllₐ were < 4.5% and 8.3 mg g⁻¹ DM, respectively, as for d 16 (+GF) and d 5.
and 9 (-GF). At 200 µM free CO₂ all correlations were not significant (Spearman rank correlation analysis, P > 0.05, data not shown).

**Table S1.** Shoot length and number of tillers of rice plants submerged in water (containing basal ions, see Methods) with 0 mM NaCl or 50 mM NaCl with leaf gas films (+GF) or treated with 0.1% Triton X-100 and without gas films (-GF). +GF and –GF plants are from different batches, hence these have separate emergent (shoots in air) controls. Roots were in non-saline nutrient solution. Letters denote significant difference (P < 0.05) between means (± S.E., n = 4) according to one-way ANOVA with Sidak’s multiple comparisons post-hoc test (shoot length) or non-parametric Kruskal-Wallis with Dunn’s multiple comparisons test (number of tillers).

<table>
<thead>
<tr>
<th></th>
<th>Emergent controls</th>
<th>0 mM NaCl</th>
<th>50 mM NaCl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+GF</td>
<td>-GF</td>
<td>+GF</td>
</tr>
<tr>
<td>Shoot length (cm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>51.8 ± 3.3</td>
<td>32.5 ± 2.6</td>
<td>72 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>a</td>
<td>c</td>
<td>b</td>
</tr>
<tr>
<td>Number of tillers</td>
<td></td>
<td></td>
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<td>4.5 ± 0.6</td>
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Figure S6. Leaf Na⁺ (a), Cl⁻ (b) and K⁺ (c) concentrations of a youngest fully expanded leaf of rice submerged in 50 mM NaCl (containing also basal ions, see Methods) for 24 hours in the dark with gas films (+GF, open symbols) or treated with 0.1% Triton X-100 and without gas films (-GF, closed symbols). During incubation the submergence solution was maintained at 0.01, 0.46, 1.59, 3.16, and 20.23 kPa O₂. * denotes significant difference between ion concentrations (Sidak’s multiple comparisons test, \(P < 0.05\)). Two-way GF × pO₂ ANOVA showed a significant effect of GF on leaf Na⁺ (\(P = 0.0345\)) and K⁺ concentrations (\(P = 0.0091\)). pO₂ had a significant effect on K⁺ concentrations (\(P < 0.0001\)). For Cl⁻ two-way ANOVA showed a significant pO₂ × GF interaction (\(P = 0.0050\)). Values are means (± SE, \(n = 4\)).