Title: Physiology, gene expression and metabolome of two wheat cultivars with contrasting submergence tolerance

Running title: Submergence tolerance in two wheat cultivars

Summary statement: Responses of wheat (*Triticum aestivum*) to complete submergence are not well understood as research has focused on waterlogging (soil flooding). The aim of this study was to characterize the responses of two wheat cultivars differing vastly in submergence tolerance to test if submergence tolerance was linked to shoot carbohydrate consumption as seen in rice. Growth, survival and metabolomic fingerprinting analyses revealed contrasting submergence tolerance in the two wheat cultivars in spite of similar carbohydrate consumption rates. Meanwhile, the intolerant wheat cultivar accumulated metabolites indicative of accelerated chlorophyll catabolism and lipid peroxidation over the tolerant cultivar.

Authors: Max Herzog*1, Takeshi Fukao2, Anders Winkel1, Dennis Konnerup1, Suman Lamichhane2, Jasper Benedict Alpuerto2, Harald Hasler-Sheetal3,4, Ole Pedersen1

1The Freshwater Biological Laboratory, Department of Biology, University of Copenhagen, Universitetsparken 4, 3rd floor, Copenhagen 2100, Denmark; 2Department of Crop and Soil Environmental Sciences, Virginia Tech, 1880 Pratt Drive, Blacksburg, Virginia 24061, USA; 3Nordcee, Department of Biology, University of Southern Denmark, Campusvej 55, Odense 5230, Denmark and 4VILLUM Center for Bioanalytical Sciences, University of Southern Denmark, Odense 5230, Denmark.
Physiology, gene expression and metabolome of two wheat cultivars with contrasting submergence tolerance

Abstract

Responses of wheat (Triticum aestivum) to complete submergence are not well understood as research has focused on waterlogging (soil flooding). The aim of this study was to characterize the responses of two wheat cultivars differing vastly in submergence tolerance to test if submergence tolerance was linked to shoot carbohydrate consumption as seen in rice. 18-day-old wheat cultivars Frument (intolerant) and Jackson (tolerant) grown in soil were completely submerged for up to 19 days while assessing responses in physiology, gene expression and shoot metabolome. Results revealed 50% mortality after 9.3 and 15.9 days of submergence in intolerant Frument and tolerant Jackson, respectively, and significantly higher growth in Jackson during recovery. Frument displayed faster leaf degradation as evident from leaf tissue porosity, chlorophylla and metabolomic fingerprinting. Surprisingly, shoot soluble carbohydrates, starch and individual sugars declined to similarly low levels in both cultivars by day 5, showing that cultivar Jackson tolerated longer periods of low shoot carbohydrate levels than Frument. Moreover, intolerant Frument showed higher levels of phytol and the lipid peroxidation marker malondialdehyde relative to tolerant Jackson. Consequently, we propose to further investigate the role of ethylene sensitivity and deprivation of reactive O2 species in submerged wheat.
**Key words:** Flood tolerance, gene expression, metabolomics, shoot carbohydrates, submergence, *Triticum aestivum*, wheat.

**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 & Waters 2003), where waterlogging reduces average grain yield by 43% (Herzog *et al*. 2016). In the USA, 2016 insurance pay-outs due to floods totalled US$217mn, which was 3.4-fold higher than pay-outs due to droughts and more than any other stressor alone (www.rma.usda.gov/data/cause). 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, i.e. partially submerged) the aboveground organs are under water (c.f. Sasidharan *et al*. 2017). In the present study we tested if submergence tolerance in two contrasting wheat cultivars was related to levels of shoot carbohydrates as seen in rice (*Oryza sativa*) (Singh *et al*. 2001; Das *et al*. 2005) or governed by other traits.

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) thereby hampering wheat root growth and function (Trought & Drew 1980). When floodwaters recede and tissues are re-oxygenated, reactive O₂ species (ROS) such as superoxide radicals, hydrogen peroxide or singlet O₂ 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 & Voesenek 2009). During submergence, availability of O₂ and CO₂ for aerobic respiration and photosynthesis, respectively, becomes limited, 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 & Greenway 2003).

Plants growing in flood-prone areas, i.e. terrestrial wetland plants, display traits that confer flood tolerance (Colmer & Voesenek 2009). Key traits are ‘internal aeration’ via interconnected air spaces and the formation of a barrier to radial O₂ loss (ROL) along the root base, enabling internal O₂ gas phase diffusion to submerged organs (Armstrong 1979; Colmer 2003). Traits in addition to internal O₂ transport have been classified into the Low Oxygen Quiescence Syndrome (LOQS) where plants conserve substrate by sitting through the unfavourable conditions, and the Low Oxygen Escape Syndrome (LOES) where plants elongate in order to re-establish atmospheric contact (Bailey-Serres & Voesenek 2008). The gaseous phytohormone ethylene is known to induce adaptations such as aerenchyma formation in wild wetland plants (Visser et al. 1996), wheat (Huang et al. 1997) and rice (Shiono et al. 2008).
Breeding for flood tolerant crops has resulted in high yielding rice cultivars (tolerant to waterlogging) able to withstand 14 days of submergence (Ismail et al. 2013). Several studies have documented variation in waterlogging tolerance in wheat germplasm (Van Ginkel 1991; Huang et al. 1994; Sayre et al. 1994; Collaku & Harrison 2002; Dickin et al. 2009; Hayashi et al. 2013), 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). While wheat waterlogging tolerance could have been enhanced by breeding for winter hardiness in U.K. cultivars (Dickin et al. 2009), waterlogging tolerance has to our knowledge not been specifically selected for in wheat breeding ever since a promising attempt (Setter & Waters 2003) failed due to introduction of yellow rust to Australia (Robin Wilson, pers. commun., 2017).

Meanwhile, traits conferring submergence tolerance in wheat have not yet been documented. Submergence of wheat has been observed to occur (Musgrave & Ding 1998; Winkel et al. 2017) and to decrease yields relative to waterlogging (Samad et al. 2001; Liu et al. 2016). For example, raising the level of flood water by 3 cm above the soil surface reduced wheat grain yield by 60% relative to waterlogged plants (Liu et al. 2016). The global extent of wheat partial or complete submergence has to our knowledge not been estimated, but would be a prominent risk during winter wheat dormancy when plant height is only 7-10 cm (Weaver et al. 1924) enabling a few cm of standing water to submerge large parts of the shoot. In rice, submergence tolerance is closely linked to shoot carbohydrate levels (Das et al. 2005). Hence, the aim of this study was to assess whether contrasting submergence tolerance observed in two winter wheat cultivars (tolerant Jackson and intolerant Frument) was related to levels of shoot carbohydrates, or if other traits determine wheat submergence tolerance. Cultivars Jackson and Frument were used due to their contrasting waterlogging tolerance.
Jackson has been reported as waterlogging and hypoxia tolerant (Huang & Johnson 1995; Huang et al. 1997) while Frument emerged as waterlogging intolerant following preliminary pot experiments with 3 Danish and international reference cultivars (Jackson, Chara, Nishikazekumogi; unpublished results). To evaluate submergence tolerance, the two wheat cultivars were subjected to complete submergence in a pot experiment while assessing shoot growth, plant survival, shoot metabolites (metabolomics), shoot gene expression and a range of physiological parameters in order to identify possible traits conferring submergence tolerance. We hypothesized that submergence tolerance would be related to genotype specific shoot carbohydrate consumption, prompting us to evaluate levels of soluble carbohydrates, starch and expression of genes related to carbohydrate degradation.

Materials and Methods

Plant culture

Seeds of wheat (Triticum aestivum L., cv. ‘Frument’ and ‘Jackson’) were imbibed for three hours in aerated 0.5 mM CaSO₄ and germinated for 48 hours in Petri dishes on wet paper towels in darkness at 20 °C. Three germinated seeds were sown at 10 mm depth in each of 190 round pots (height, 120 mm; diameter, 90 mm) filled with substrate (specified below) and irrigated with deionized 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 effects), part of the seedlings were thinned to one per pot before treatment. To prevent roots from exiting through the pot drainage holes, the bottom of the pots were sealed with two layers of landscape fabric. 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 (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). In a recent study using identical pots and substrate, O$_2$ 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 as in Herzog and Pedersen (2014), but with 2 mM KHCO$_3$) in a constant temperature room (20 °C, relative humidity 40-89%) served as tanks for submergence. Photosynthetic photon flux density (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. Drained controls in air grew in the same constant temperature room as the submerged plants and received the same PPFD.
Treatments commenced when all plants had a fully expanded third leaf (18 days after imbibition) by fully submerging 128 pots containing one or three plants of each cultivar. 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 carbohydrate measurements while the remaining two plants were harvested for gene expression and metabolomic analysis, respectively. The four replicates were true biological replicates since taken from separate pots from the four separate aquariums. 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 deionized 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’ (new leaf appeared during recovery) or ‘dead’ (shoot senesced, no new leaf appeared) and entire shoots (including dead leaves) were oven-dried at 60 °C for 48 h before weighing the dry mass (DM). Relative growth rate (RGR) were calculated as $\text{RGR} = (\ln W_2 - \ln W_1)/(t_2 - t_1)$, 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 8 days of treatment.

*Underwater net photosynthesis and dark respiration measurements*

Underwater net photosynthesis ($P_N$) and dark respiration ($R_D$) 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 halfway up the blade of the youngest fully expanded (third) leaf at the time of submergence. Glass cuvettes (approx. 28 mL) contained individual lamina segments in incubation medium (identical to submergence solution) and two glass beads for mixing as the cuvettes rotated on a wheel within an illuminated water bath ($P_N$, PPFD inside the vials of 1000 µmol photons m$^{-2}$ s$^{-1}$) or in darkness ($R_D$), at 20 °C.

For $R_D$, the incubation medium was initially adjusted to air equilibrium by purging with air to obtain $R_D$ at non-limiting external O$_2$ concentrations (Colmer & Pedersen 2008), while the dissolved O$_2$ concentration in the $P_N$ incubation medium was initially set at approximately 50% of air equilibrium (Pedersen *et al.* 2013). Initial concentrations of 200 µM CO$_2$ in both solutions ($P_N$ and $R_D$) was obtained by adjusting pH to 7.35 after adding 2.2 mmol KHCO$_3$ L$^{-1}$ solution. 200 µM CO$_2$ is considered an environmentally relevant CO$_2$ concentration (Colmer *et al.* 2011) and allowed direct comparison with other wheat studies (Konnerup *et al.* 2017; Winkel *et al.* 2017).

Dissolved O$_2$ concentrations in the cuvettes were measured using a calibrated mini O$_2$ optode (OP-MR, Unisense A/S, Aarhus, Denmark), connected to an optode meter (MicroOptode meter, Unisense A/S, Aarhus, Denmark). 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 and DM recorded.

Chlorophyll concentration and chlorophyll fluorescence

Chlorophyll concentrations of the lamina of the third leaf at the time of submergence (consisting of homogenized, freeze-dried leaf segments from gas film thickness, tissue porosity and gas exchange measurements) was measured following Winkel et al. (2017) after 24 h extraction in 96% ethanol.

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

Leaf gas film thickness and tissue porosity

The leaf gas film volume and tissue porosity (gas-filled volume per unit tissue volume) was measured using the “buoyancy method” on 70 mm segments of the youngest fully expanded (third) leaf lamina at time of submergence following Konnerup et al. (2017).

Shoot carbohydrate assays
The entire shoot remaining submerged following excision of the third leaf (used for measurements described above), was excised below water 5 hours into the photo period, rinsed in deionized water, blotted dry on paper towels, placed into perforated aluminium foil bags, flash-frozen in liquid nitrogen and stored at -80 °C. The harvest procedure was performed within 30-60 seconds. 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 were analyzed using the anthrone method 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. Wheat also accumulates fructan as a reserve carbohydrate (Vijn & Smeekens 1999), especially during hypoxia (Albrecht et al. 1993). For extraction of the water soluble fraction remaining after ethanol extraction, i.e. long chain fructans but not short-chained fructan, mono- and disaccharides solubilized in ethanol (Ranwala & Miller 2008), 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). For starch determination the pellet remaining from the extraction of soluble sugars was analysed following Fukao et al. (2012) using the anthrone method. 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 α-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 (Pontis 2017). 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 was harvested as described for “Shoot carbohydrate assays” 6 hours into the photo period. Shoots were freeze-dried for 72 h before shipment on silica gel and with ice packs for gene expression analysis. RNA extraction, cDNA synthesis and quantitative real time PCR (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) and published gene specific primers in the CFX Connect real-time PCR detection system (Bio-Rad). Annealing temperatures, primer sequences and their reference papers are listed in Table S1. 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 & Schmittgen 2001).

TaRP15, RNA polymerases I, II, and III, 15-kD subunit (TC265122) were used as a reference gene (Xue et al. 2008).
One entire shoot was harvested as described for “Shoot carbohydrate assays” 6 hours into the photo period and homogenized in liquid N\textsubscript{2} using mortar and pestle. Metabolites were extracted and analysed 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\textsubscript{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 untargeted liquid chromatography quadrupole time of flight mass spectrometry (LC-MS; 1290LC, 6530 QTOF MS) (both Agilent Technologies, Santa Clara, CA, USA), with data normalized to FW and internal standards following Hasler-Sheetal et al. (2015) and Hasler-Sheetal et al. (2016) with slight modifications. For determination of shoot malondialdehyde (MDA) levels we followed Yonny et al. (2017) with slight modifications. GC-MS raw data (metabolite formulas, retention times and counts) are listed in tables S2 and S3 for Jackson and Frument, respectively. Untargeted LC-MS raw data used to study relationship patterns of the non-anotated metabolites (metabolite masses, retention times, PCA loadings, 2-way ANOVA p-values and counts) are available in Supplementary Materials as a spreadsheet (Table S4). MDA raw data are presented in Table S5.

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 were ln-transformed when variances were not homogeneous, as specified in figure legends.

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 by using the amount of plants surviving upon submergence and recovery as the response variable (coded as 0, dead or 1, survived). Duration of submergence was used as the explanatory variable and survival was analysed 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 & Schemper 2002). Further details on statistical tests are given in figure legends.

**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. 1A). Survival and shoot 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 tolerant Jackson and intolerant 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. Intolerant Frument shoot RGR continued to decline faster than tolerant Jackson and thus two-way time \(\times\) cultivar ANOVA detected significant time, cultivar
and interaction effects. Importantly, when comparing cultivar growth during submergence assessed without a recovery period (i.e., harvesting shoots directly after 8 days of submergence), both cultivars exhibited similar slightly negative RGR (Fig. S1).

Plant survival also decreased significantly with the duration of complete submergence (Fig. 1B, \( P < 0.05 \), Wald Chi-square test). Intolerant Frument succumbed to submergence approximately seven days earlier than tolerant Jackson, evident from the time of submergence resulting in 50% mortality (LT\(_{50}\)) of 9.3 and 15.9 days in Frument and Jackson, respectively (Fig. 1B). Submergence duration resulting in 100% mortality also differed by seven days (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. 1B). In the following sections, we describe how this contrasting submergence tolerance is phenotypically reflected in physiological, 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 & Kende 1983; Konnerup *et al.* 2017), and leaf gas films enhance wheat submergence tolerance (Winkel *et al.* 2017). We therefore assessed if leaf gas film thickness during submergence differed between cultivars Frument and Jackson (Fig. 2A). 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.
Leaf gas film disappearance was followed by indications of submergence-induced leaf damage. Fv/FM 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. 2B), with Fv/FM 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 PN and RD rates were measured on days 0, 2, 8 and 16 of submergence (Fig. S2). In both cultivars, PN remained at initial 1.8-2.3 μmol O₂ m⁻² s⁻¹ until day 2, but approached zero on day 8. Leaf RD on day 2 was reduced to half of initial -0.45 to -0.47 μmol O₂ m⁻² s⁻¹ in both cultivars. Intolerant Frument RD rates on day 2 and 8 were 26-32% higher than tolerant Jackson but these differences were not statistically significant (Sidak’s multiple comparisons test, P > 0.05) and for PN and RD two-way ANOVA only detected significant time-effects.

In contrast to the above-mentioned factors where both cultivars responded similarly to submergence stress, tissue porosity and leaf chlorophylla in the youngest fully expanded leaf at the time of submergence declined significantly faster in intolerant Frument than in tolerant Jackson. Chlorophylla concentrations remained at initial 9.7-11.0 mg g⁻¹ DM in both cultivars until day 5, before declining faster in Frument than in Jackson (Fig. 2C) resulting in almost three-fold higher chlorophylla in tolerant 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 intolerant Frument leaves (Fig. 2D). 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 chlorophylla and leaf tissue porosity.
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.

Initial concentrations of combined ethanol and water soluble carbohydrates were significantly higher (28%) in intolerant Frument than tolerant 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. 3A). Soluble carbohydrate concentrations remained at similar levels until day 12 and 14, when they declined further in intolerant Frument resulting in shoots of tolerant Jackson containing ~30% more soluble carbohydrates, coinciding with leaf disintegration as indicated by loss in leaf porosity (Fig. 2D). 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 intolerant 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, ribose, myo-
isonitol, maltose and glucose-6-phosphate), while 1,6-anhydro-glucose first declined from day 8 (Fig. S3).

Submergence also caused starch levels to decline (Fig. S4). On day 2, Jackson and Frument contained 31% and 21% of initial starch concentrations (119-184 µmol hexose eq. g\(^{-1}\) DM), respectively, remaining at similar levels until day 8. From day 12-16 starch concentrations started to increase, especially in intolerant 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 intolerant Frument, likely explaining why starch reached highest concentrations in this cultivar. We also assessed concentrations of long-chained fructans as the water soluble carbohydrate fraction after ethanol extraction (Fig. S5). However, initial levels of long-chained fructan was less than 10% of ethanol soluble carbohydrates and remained at initial levels, thereby not indicating that the wheat shoots stored large amounts of fructan.

Neither Frument nor Jackson shoots elongated during submergence, evident from submerged and control plants exhibiting similar shoot length after 8 days of treatment (Fig. S6). 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 chlorophylla and tissue porosity loss, while responses in leaf gas film thickness, Fv/Fm, Pn, R0 and shoot length were similar amongst the two 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 untargeted 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. 4A). 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 cultivars 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. Intolerant 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. 4A). 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. S7).

Annotation of metabolic entities allowed for monitoring changes in amino acids, carbohydrates, 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. 4B). In both cultivars phytol increase coincided with chlorophylla decline in the youngest fully expanded leaf (Fig. 2C) by day 5 (Frument) and day 12 (Jackson). 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 & 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. 4C). MDA in tolerant Jackson remained close to the initial levels while in intolerant Frument MDA increased from day 12, resulting in final levels 10 times higher than initially and 11 times higher than Jackson. However, it should be noted that intolerant Frument survival rates declined by day 8 (Fig. 1) before MDA increased by day 12.

The metabolome analysis also revealed that 12 out of 17 measured amino acids (Asn, Gln, Ile, Leu, Lys, Met, Phe, Pro, Thr, Trp, 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. S8). During the first 12 days of submergence, intolerant Frument never had lower and occasionally significantly higher amino acid concentrations compared to
tolerant 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. S8). From day 14 to 16 amino acids in tolerant Jackson also generally declined, indicating that Jackson shoot tissues were increasingly degraded by that final time point. Levels of GABA also declined from day 12, being significantly higher in Frument on day 5 and 8 (Table S2 and S3).

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 qRT-PCR on days 0, 2, 5, 8 and 12 (Fig. 5). 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 intolerant Frument compared to tolerant 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 significantly (13-19%) higher in Frument than in Jackson on more than two time points ($\alpha$-AMY4-1, Fig. S9). In conclusion, during submergence intolerant Frument expressed genes encoding sucrose and fructan degrading enzymes at significantly higher levels than tolerant Jackson.
Discussion

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

Comparing growth and survival rates from the current study with those of rice (Das et al. 2009) revealed that especially tolerant Jackson can survive relatively long but faces severe shoot biomass reductions. Intolerant rice variety IR42 displayed 70%, 50% and 0% survival after 8, 10 and 12 days of winter submergence (October-November, temperature 22-24 °C), respectively, resembling Frument survival rates (Fig. 1B). Submergence tolerant rice variety FR13A exhibited 90% survival following 12 days of complete submergence, while survival in Jackson following 12 days of submergence was 100%. 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 could reflect the in comparison low waterlogging tolerance of wheat resulting in lower submergence tolerance as well. Possible traits conferring higher flooding tolerance in rice are root porosities > 35% compared to 13-22% in wheat (Colmer 2003) and a strong barrier to radial O2 loss in rice but not found in wheat (Colmer 2003).
Compared to growth during waterlogging reported in the literature, submergence-induced growth reductions recorded in the present study were higher. Wheat shoot biomass was reduced 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 d\(^{-1}\) during 8 days, Fig. S1) compared to positive shoot RGR of 0.09 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) impeding recovery growth. 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 & Drew 1982).

Intolerant Frument displayed faster leaf degradation than tolerant Jackson as evident from earlier leaf lamina chlorophyll\(a\) 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; Panda et al. 2008). This was linked to the ethylene-driven expression of \(SUB1A\), an ethylene response factor 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 intolerant Frument could be indicative of higher ethylene sensitivity or production (shown to vary between tolerant Jackson and waterlogging).
sensitive cultivar Bayles, Huang et al. (1997)) in Frument; however, further studies are needed to clarify this aspect.

Lower chlorophyll_\textsubscript{a} concentrations in intolerant Frument relative to tolerant Jackson did not result in a corresponding decline in underwater $P_\text{N}$, indicating that light harvest was not the rate-limiting step in Frument $P_\text{N}$. Although it is possible that low chlorophyll_\textsubscript{a} in Frument resulted in lower $P_\text{N}$ during recovery as seen in two rice cultivars with different chlorophyll levels after de-submergence (Alpuerto et al. 2016), this was not observed in the present study. Other factors limiting underwater $P_\text{N}$ could be damage to the photosynthetic apparatus as indicated by $F_\text{V}/F_\text{M}$ ratios of 0.70 by day 8 (Fig. 2B). Indeed, wheat underwater $P_\text{N}$ at diagnostic high external CO$_2$ (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, $F_\text{V}/F_\text{M}$ 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, $F_\text{V}/F_\text{M}$ 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. CO$_2$ limitations caused by leaf gas film loss by day 5 would also hamper $P_\text{N}$ (Verboven et al. 2014; Konnerup et al. 2017) but leaf gas film retention times did not differ between cultivars in the present study (Fig. 2A) 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 addition to the accelerated shoot disintegration observed in Frument, differences in root growth and survival could also be of importance for the ability to initiate regrowth during the recovery period. However, the status of the root system was not assessed in this study, but could be of interest considering the high waterlogging tolerance of cultivar Jackson (Huang & Johnson 1995).

Carbohydrate analysis demonstrated that the initially higher levels of soluble carbohydrates in intolerant Frument reached similar levels in both cultivars by day 2 of submergence (Fig. 3A). Frument had significantly higher levels of mRNA encoding sucrose and fructan degrading enzymes (Fig. 5), possibly explaining why soluble carbohydrates (Fig. 3A) and sucrose (Fig. 3D) declined to similar levels in Frument and Jackson by day 2 of submergence. Although intolerant Frument continued to overexpress sucrose synthases until day 12 compared to tolerant 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, fructan, glucose and fructose declines were similar in the two cultivars (Fig. 3, Fig. S4 and Fig. S5). Shoot soluble carbohydrates differed initially (day 0), but cultivars had very similar shoot carbohydrate concentrations until day 12 of submergence (when Frument leaves were infiltrated by water, Fig. 2C), thereby not indicating that different carbohydrate consumption rates explain the contrasting submergence tolerance in Frument and Jackson evident from day 8. 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). For example, shoot soluble carbohydrates were almost twice as high in tolerant FR13A than in submergence intolerant IR 4225 following 7 days of submergence (Singh et al. 2001) while being almost identical in Frument and Jackson following 8 days of submergence in the present study, indicating higher carbohydrate starvation tolerance in tolerant Jackson relative to intolerant Frument.

PCA showed that Frument metabolites changed faster relative to Jackson (Fig. 4A), 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 O₂, 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 & Perata 2009; Alpuerto et al. 2016). Moreover, Pro is also considered a powerful antioxidant (Verbruggen & 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 tolerant Jackson than intolerant Frument following submergence and recovery. A clear relationship between submergence tolerance and steady-state level of shoot carbohydrates was not evident. Only the very initial rate of 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, tolerant cultivar Jackson tolerated longer periods of low shoot carbohydrate levels than Frument. Metabolic fingerprinting revealed that the metabolome of intolerant Frument changed faster upon submergence than tolerant Jackson. These submergence-induced metabolic changes were in accordance with faster leaf senescence and leaf deterioration, as evident from leaf chlorophyll$_a$ and leaf tissue porosity data. However, leaf chlorosis did not result in lower underwater $P_N$ in intolerant Frument relative to Jackson. Elevated levels of MDA indicated that intolerant Frument experienced higher levels of ROS-inflicted membrane damage at the end of the submergence period. Greater accumulation of proline in tolerant 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.
Supplementary data

Table S1. Primer sequences and annealing temperatures.

Table S2. GC-MS raw data for wheat cultivar Jackson, including formulas and retention times.

Table S3. GC-MS raw data for wheat cultivar Frument, including formulas and retention times.

Table S4. Untargeted LC-MS raw data including masses, retention times, PCA loadings, ANOVA p-values and metabolite counts.

Table S5. Raw data of malondialdehyde (MDA) in shoots of submerged wheat cultivars Frument and Jackson.

Fig. S1. Shoot RGR during submergence (no recovery period).

Fig. S2. Leaf underwater $P_N$ and $R_D$ rates on days 0, 2, 8 and 16.

Fig. S3. GC-MS determined levels of sugars.

Fig. S4. Shoot starch levels during submergence.

Fig. S5. Shoot water soluble carbohydrate fraction after ethanol extraction.

Fig. S6. Shoot elongation during submergence.

Fig. S7. Venn diagram of untargeted LC-MS detected metabolites.

Fig. S8. GC-MS determined levels of amino acids.

Fig. S9. Relative mRNA levels of $\alpha$-amylases.
Acknowledgements

The authors would like to thank Timothy D. Colmer for valuable discussions, and Lars Iversen for statistical support. M.H., D.K. and A.W. were supported by PhD and postdoctoral fellowships from the Villum Foundation.
References


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. Plants developing new leaves during recovery were scored as surviving. (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 ($P < 0.0001$). Asterisks indicate significant difference between cultivars (Sidak’s multiple comparisons test, $P < 0.05$). Values are means ($\pm$ SD, $n = 4$-$8$). Shoot DM of Frument and Jackson controls in air at the end of recovery (0 days of submergence) were not significantly different (t-test, $P = 0.499$, $n = 6$-$7$).
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 (± SD, n = 4). Asterisks 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 < 0.0001). (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). Round symbols symbolize untreated controls in air at the end of the treatment period (day 16).
Figure 3. Shoot carbohydrates in wheat cultivars Frument and Jackson with time of submergence. The sum of ethanol and water soluble carbohydrates (A) was determined using the anthrone-method, and fructose (B), glucose (C) and sucrose (D) determined by GC-MS in Frument (open symbols) and Jackson (closed symbols). 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. Asterisks indicate significant difference between cultivars (Sidak’s multiple comparisons test, $P < 0.05$), values are means (± SD, $n = 3-4$).
Figure 4. 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 detected in shoots of submerged Frument (squares) and Jackson (triangles) wheat cultivars using untargeted LC-MS, the colour 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 Froment (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) Asterisks indicate significant difference between cultivars (Sidak’s multiple comparisons test, $P < 0.05$), values are means ($±$ SD, $n = 4$). Round symbols symbolize untreated controls in air at the end of the treatment period (day 16).
Figure 5. 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 (± SD, n = 3). Asterisks indicate significant differences between the cultivars (Sidak's multiple comparisons test, P < 0.05).