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REGULAR ARTICLE

Differential root and shoot biomass recovery in wheat and barley with transient waterlogging during preflowering

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Abstract
Aims Wheat and barley plants exposed to waterlogging reduced their growth, but the final impact on grain yield depends on the capacity of the plant to recover after stress. The aim of this study was to evaluate shoot and root biomass accumulation in wheat and barley plants during waterlogging events applied at different stages during preflowering and after stress removal.
Methods Wheat and barley plants were waterlogged for 15–20 days at four consecutive periods during phenological cycle from emergence to flowering.
Results Waterlogging produced a delayed effect on shoot biomass, as biomass reductions were detected 20 days after waterlogging was released. The highest relative reductions of shoot biomass (60% in wheat and 68–74% in barley regarding control) occurred when waterlogging was applied early in the cycle (from emergence to tillering). Waterlogged plants showed a remarkable capacity to recover from early waterlogging (reaching similar shoot biomass as control plants at flowering), but recovery capacity decreased when waterlogging occurred later in the phenological cycle. For both species green leaf area and photosynthetic rate were reduced and water soluble carbohydrates increased when waterlogging ended, however the general trend showed values at flowering similar to the control plants. The impact of waterlogging on roots was generally higher than the one on shoots and the effect was detected immediately after treatment. The root system capacity to recover after waterlogging was lower than the one for shoots, and was higher in barley than in wheat.
Conclusions Waterlogging first damaged root biomass while effects on shoots were delayed. Shoot recovery at flowering was possible for waterlogging events previous to stem elongation, but root recovery was lower, especially for wheat.

Keywords Root biomass · Root length · Shoot biomass · Recovery · Aerenchyma formation · Photosynthesis · Water-soluble carbohydrates

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Introduction

Waterlogging is estimated to adversely affect ca. 10% of the global land area (Setter and Waters 2003). This stress

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is expected to increase in the future due to the projected tendency of precipitation to be more intense and a higher rainfall as a result of climate change (IPCC 2014). Waterlogging has a negative effect on crops mainly associated with decreases of the oxygen concentration in the soil as result of the diffusion of gases in water decreases dramatically (5000 to-10,000 times) under these conditions (Armstrong 1979). Thus, plant root and microbes respiration deplete the oxygen and soil becomes hypoxic or anoxic rapidly (Ponnamperuma 1984). Under that condition, root respiration rate decreases (Huang and Johnson 1995) and growth becomes limited (Huang et al. 1994a; Huang et al. 1997), which leads to plant death if the anaerobic condition is prolonged. Numerous studies have reported sugars accumulation in shoots and roots of wheat during waterlogging (Trought and Drew 1980; Malik et al. 2001; Malik et al. 2002) or under hypoxic solutions (Huang and Johnson 1995) this is probably due to growth reductions, especially of the roots.

Different sources of evidence showed that under waterlogging conditions seminal roots of wheat stop growing or even die (Malik et al. 2001; Malik et al. 2002). On the contrary, the formation of new aerenchymatous adventitious roots contributes to waterlogging tolerance in wheat (Colmer and Greenway 2011) and barley (Garthwaite et al. 2003; Broughton et al. 2015). Prolonged exposure to waterlogging or oxygen deficient conditions increases root porosity by the formation of aerenchyma in wheat (Thomson et al. 1990; Malik et al. 2001; Colmer and Greenway 2011; Nazemi et al. 2016) and barley (Broughton et al. 2015; Zhang et al. 2015). Depending on the type of root the effects vary due to a higher proportion of aerenchyma in adventitious than in seminal roots (Thomson et al. 1990; Huang et al. 1994b; Colmer and Greenway 2011; Herzog et al. 2016). Although waterlogged plants may produce a higher number of adventitious roots per stem (Malik et al. 2001), total root length and root dry weight per plant are significantly reduced after exposing wheat (Huang et al. 1994a; Huang and Johnson 1995; Malik et al. 2001; Malik et al. 2002) and barley plants (Broughton et al. 2015) to waterlogging.

The relative growth rate of roots in wheat is severely reduced when plants are exposed to waterlogging, while the relative growth rate of shoots is reduced in a lesser extent (Malik et al. 2001). Therefore, as it is expected, reductions on root biomass at the end of a waterlogging event are more important than those on shoot biomass, and the root:shoot ratio of waterlogged plants is reduced in relation to plants that have not suffered waterlogging (Malik et al. 2001; Araki et al. 2012b). However, different works showed that one or two weeks after waterlogging was removed, root growth rate became similar to control plants, but the negative effect of waterlogging on shoots had become higher, and plants reached similar root:shoot ratio as plants without waterlogging (Huang et al. 1994a; Huang and Johnson 1995; Malik et al. 2001; Pang et al. 2004). Once the aerated conditions are reestablished, root respiration rates increase (Huang and Johnson 1995), and roots regrowth is supported by a consumption of stored carbohydrates (Albrecht et al. 1993). The preferential resource allocation to root growth after waterlogging, to reestablish a typical root:shoot ratio of plants grown in non-waterlogged soil, has been proposed as a mechanism of recovery from transient waterlogging in wheat plants (Herzog et al. 2016). Most of the negative effects of waterlogging described above were reported in wheat or in a lesser extent, in barley plants waterlogged during early stages of the phenological cycle (before stem elongation). The effect of waterlogging and the subsequent capacity to recover when it occurs in advanced stages of development during which the most important yield components are being established (e.g. grain number per unit area), is still unknown.

Reports show that waterlogging occurring near flowering produce the highest reductions on grain yield in wheat as well as in barley (de San Celedonio et al. 2014), whereas waterlogging during early stages of development severely reduces biomass accumulation during that phase (Malik et al. 2001; Araki et al. 2012b) but produces low or even null reductions in grain yield (Setter and Waters 2003; de San Celedonio et al. 2014). This suggests that plants exposed to waterlogging during the early vegetative phase have the capacity to recover afterwards, and yield is not significantly affected (Setter and Waters 2003; de San Celedonio et al. 2014). A recent work reported that even though the tiller appearance rate was reduced when waterlogging occurred in early stages of development, both in wheat and barley, the effect was counterbalanced by a lengthening of the tillering phase, and the final tiller number and spikes per plant at maturity were similar to control plants (de San Celedonio et al. 2016). In fact, Robertson et al. (2009) found that the growth of primary tillers was severely inhibited by waterlogging in wheat, but waterlogged plants promoted the formation of

higher order tillers during the recovery period, which produced late spikes. Although that response to waterlogging can be a mechanism of the plant to recover shoot biomass after a waterlogging event in early stages of development, the recovery capacity of wheat and barley plants when it occurs throughout different stages of the phenological cycle has not been studied. Since phenology triggers changes in the root:shoot ratio during the crop cycle both in wheat and barley, and considering that the ability to recover has been suggested a as a priority area for future research, the analysis of waterlogging events impact that take place at different stages could contribute to breeding tolerant varieties (Herzog et al. 2016).

The objective of this study was to evaluate the effect of waterlogging applied at different stages of the phenological cycle (from emergence to flowering) on the growth of shoot and root systems of wheat and barley plants, and to analyze the ability of both species to recover from transient waterlogging. We hypothesized that i) the negative effect of waterlogging on the subsequent shoot growth of wheat and barley is the consequence of a previous damage to the root system, and ii) the capacity to recover from waterlogging in both species is lower when it occurs in advanced stages of development.

Material and methods

Experimental conditions

Two experiments (Exp 1 and Exp 2) were carried out using 12 L pots during 2010/11 at the School of Agronomy, University of Buenos Aires, Argentina (34° 35′ S, 58° 29′ W). Exp 1 was sown on July 2 (i.e. within the optimum range of sowing date for the location) in a greenhouse, while Exp 2 was sown on September 6 (a late sowing date) and conducted under natural field conditions with the purpose of exploring contrasting environmental conditions between both experiments. Meteorological conditions in each experiment have been described previously by de San Celedonio et al. (2014).

Experimental design and treatments

Within each experiment, treatments consisted of the combination of two factors: i) one wheat cultivar (Klein Chaja for Exp 1 and Baguette 13 for Exp 2) and one of barley (Scarlett for both experiments) and ii) five waterlogging conditions. Wheat and barley cultivars were chosen because they have similar phenology (measured as days to flowering) and high yield potential under non- waterlogging conditions. In Exp 2, the wheat cultivar was different from the one used in Exp 1 because there were not any seeds available at the moment of sowing. A cultivar with a similar yield potential and phenology as barley and the previous year wheat cultivar was selected. Waterlogging treatments consisted of a control, without waterlogging throughout the entire phenological cycle (Ctl), and the application of four consecutive periods of 20 (Exp 1) or 15 days (Exp 2) of waterlogging throughout the phenological cycle. For each experiment, waterlogging periods coincided approximately with the following stages of development: WL1, from emergence to beginning of tillering; WL 2, from beginning of tillering to maximum number of tillers; WL3, from maximum number of tillers to flag leaf fully expanded and WL4, from flag leaf fully expanded to flowering. In the case of barley, in which the true flowering occurs when the spike is inside the sheath of the flag leaf (Fernández Gómez and Wilson 2012), flowering time was determined by opening spikelets and visualizing pollen release. The experiments were arranged in a randomized design with 4 replicates in Exp 1 and 3 replicates in Exp 2 (a total of 56 pots in Exp 1 and 33 pots in Exp 2). In both experiments waterlogging was imposed by placing the pots into containers (1 m × 1 m × 0.5 m) filled with water in order to obtain 1 cm layer of free water above the surface during each waterlogging treatment period. At the end of each treatment, pots were taken out of the containers and remained without irrigation during approximately 10 days allowing them to drain freely, then they were re-watered normally. For a detailed description of the experimental set up see de San Celedonio et al. (2014).

Shoot and root biomass determinations

Plants from 4 pots per treatment were sampled at the beginning and at the end of each treatment (i.e. emergence, beginning of tillering, maximum tiller number, flag leaf fully expanded and flowering) in Exp 1. The harvest at flowering was done when each treatment reached that stage. In Exp 2, each waterlogging

treatment was harvested when treatment was released and at the end of WL 4 (all on the same day). Control pots were regularly sampled simultaneously with waterlogged pots (see Fig. 1). Biomass was separated into shoots (stems, leaves and spikes when presented) and roots. The entire root biomass on each pot was washed carefully using a 1 mm mesh sieve. Dead roots and organic matter were discarded by hand. Green leaf area was determined in each biomass sample using a leaf area meter (Li-Cor 3100, Lincoln, NE, USA). Then shoot and root biomass were dried separately for 72 h at 60 °C and dry weight was measured.

Leaf photosynthetic rate and water soluble carbohydrates determinations

Light-saturated photosynthetic rate was measured in waterlogged and control plants at the end of each waterlogging treatment in Exp 1. Measurements were taken in the youngest fully expanded leaf on the main stem using a Li-Cor 6400 photosynthesis system (Li-Cor, Lincoln, NE, USA) provided with red–blue LEDs light source (6400-02B, Li-cor) and with a 6 cm^2 chamber (3 cm × 2 cm). Gas exchange measurements were taken at 1500 μmol photon m^{-2} s^{-1} of incident photosynthetically active radiation (PAR) (400–700 nm). The CO_2 concentration incoming the chamber was set at 400 ± 2 μmol CO_2 mol^{-1} air using the system's CO_2 injector (6400–01, LI-COR). Air flow rate was 300 μmol s^{-1}.

Water-soluble carbohydrates (WSCs) content was determined on the oven dried shoots samples (stems –after the first harvest- plus sheaths). For the analysis, 0.1 g of ground sample was suspended in 10 ml of deionized water, then extracted for 90 min at 60 °C in a bath. After centrifugation at 3000 rpm for 15 min, 1 ml of the supernatant was aliquoted and mixed with 9 ml of deionized water. Determinations were made on 0.5 ml of extract using the anthrone method of Yemm and Willis (1954).

Root traits

In addition to root biomass, total root length, root diameter and aerenchyma formation were measured in Exp 1 and Exp 2 using digital images (Fernandez and Rubio 2015). Previous to oven drying the roots, a

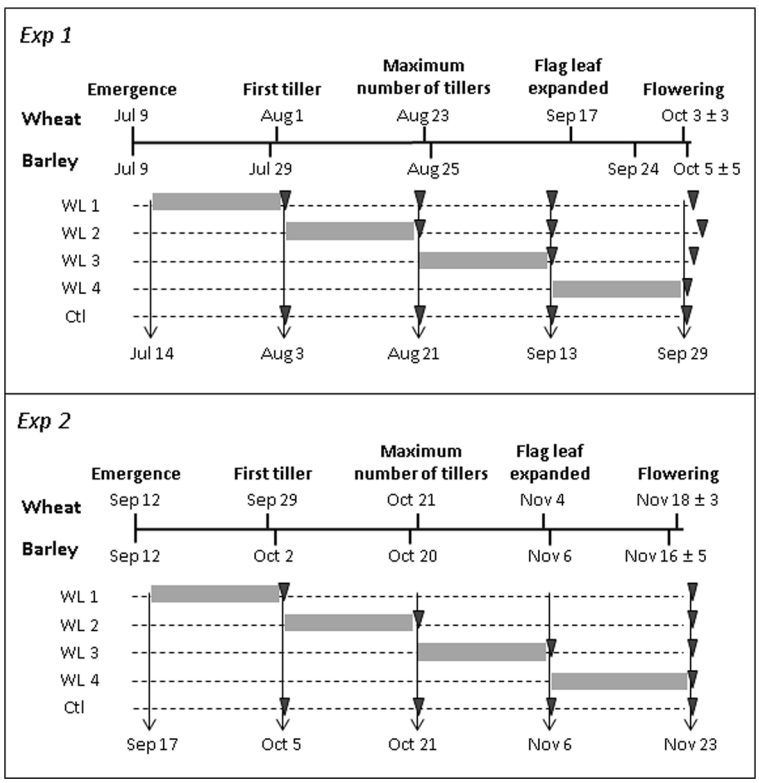

Fig. 1 Scheme indicating the moments of waterlogging treatments application throughout the plant cycle (*grey bars*) and harvest time points (*inverted triangles*) in wheat and barley for Exp 1 (*upper panel*) and Exp 2 (*bottom panel*)

representative subsample of the total root biomass per pot (i.e. from the 6 plants of each pot) was separated and digitalized at 400 dpi resolution using a dual scanner STD4800 (EPSON Perfection V700 PHOTO, Indonesia). The scanner was optimized and calibrated for root analysis by Regent Instruments Inc., Canada. Root length and root diameter were determined by analyzing digital images with WinRHIZO Pro 2012b software (Regent Instrument Inc., Canada). Total root length was calculated on a dry weight basis from the root length software outputs and the total root weight of the analyzed sample.

Aerenchyma formation in adventitious roots cortex was evaluated in Exp 1 in two plants per pot immediately after each waterlogging treatment ended and in control pots at the same time. Segments of 2 cm of each root were cut from 2 cm above the root tip in roots between 8 and 10 cm long. Transverse sections of roots were fixed in FAA, embedded in paraffin and serially cut to 10 μm width with a Minot-type rotary microtome. Sections were stained with safranin-fast green combination (Johansen 1940). The slides were photographed with a Zeiss Axioplan (Oberkochen, Germany) optical microscope and analyzed with Zeiss AxioCam ERc 5 s (Jena, Germany). Roots aerenchyma percentage was quantified as the ratio between the area occupied by aerenchyma and total cross sectional area.

Calculations

Shoot biomass, root biomass and length were expressed in g pl^{-1} (shoot biomass and root biomass) or m pl^{-1} (root length) and as the relative reductions in regard to the control without waterlogging. The relative growth rate (RGR; mg g^{-1} d^{-1}) for shoot dry weight or root dry weight were calculated for each period between samplings according to the equation of Radford (1967). The specific root length (SRL; m g^{-1}) was calculated as the ratio between root length (m pl^{-1}) and root biomass (g pl^{-1}).

Statistical analysis

Statistical differences for each waterlogging treatment compared with control plants for shoot biomass, root biomass and root length were tested using the Student's *t*-test. ANOVA was used to test for the main effects (waterlogging and species treatments) and the interaction for RGR, SRL and mean root diameter. The mean treatment values were compared by Tukey's test with significant level of 0.05. The software used for statistical analysis was InfoStat Professional v.1.1 (Di Rienzo et al. 2011).

Results

Plant phenology

In Exp 1 the emergence to flowering phase duration for the control treatment was 80 days for wheat and 83 days for barley. In Exp 2 the duration of the emergence-flowering period was shorter than in Exp 1 (67 days for wheat and 60 days for barley) as plants were exposed to more inductive conditions (i.e. longer photoperiod and warmer temperatures). Waterlogging treatments produced a delay in flowering time, especially in the WL 2 treatment, where flowering was delayed by 13–15 days in barley and by 6–10 days in wheat (for details see de San Celedonio et al. 2016).

Biomass accumulation

For the control condition, shoot biomass at flowering was similar between wheat and barley within each experiment ($p > 0.10$), but root biomass was 33% lower in barley than in wheat in both experiments ($p < 0.05$). In the control plants, shoot biomass and root biomass at flowering were on average ca. 65% lower in Exp 2 than Exp 1 for both species ($p < 0.05$; Fig. 2 and Fig. 3).

In both experiments waterlogging treatments significantly reduced shoot biomass accumulation in wheat and barley, but the response varied depending on the waterlogging treatment and the stage in which was evaluated (Fig. 2a-h for Exp 1; Fig. 3a-h for Exp 2). Immediately after the waterlogging was released there was a non-significant reduction of shoot biomass for WL 1 treatment ($p > 0.05$ for both experiments), while WL 2 reduced shoot biomass by 34% in wheat and 41% in barley ($p < 0.05$). However, shoot biomass reductions were highest 20 days after the waterlogging was released. For example, for WL 1 and WL 2 treatments in Exp 1 they were ca. 60% in wheat (Fig. 2a, b) and between 68 and 74% in barley (Fig. 2e,f). This response indicates a delay in the expression of the waterlogging effect on shoot biomass accumulation.

When shoot biomass was measured at flowering for waterlogging imposed at emergence (WL 1) and tillering (WL 2), it did not differ from control plants in any

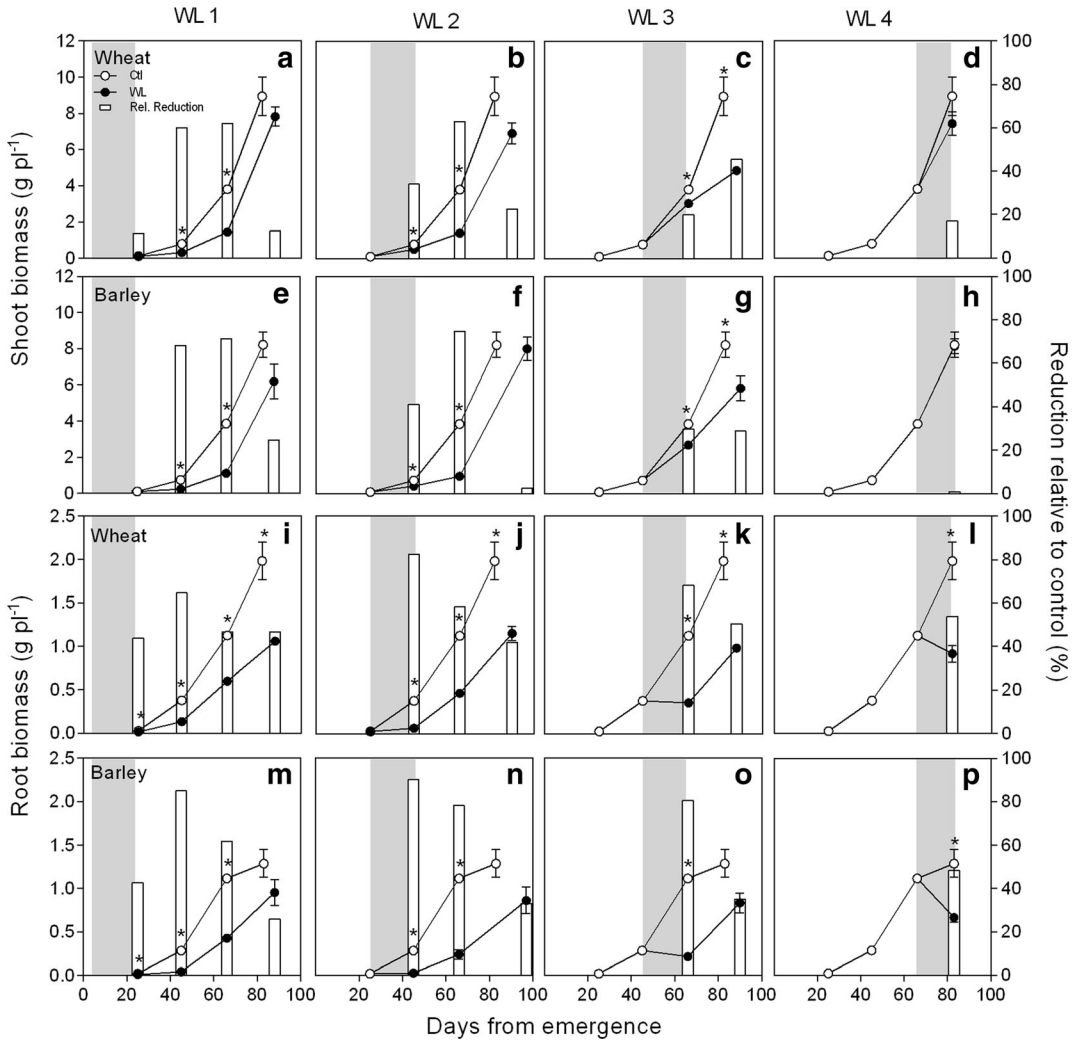

Fig. 2 Shoot (**a**-**h**) and root (**i**-**p**) biomass for wheat (**a**-**d**; **i**-**l**) and barley (**e**-**h**; **m**-**p**) exposed to waterlogging (WL; closed symbols) during different phenological periods (WL 1: waterlogged from emergence to beginning of tillering, WL 2: waterlogged from beginning of tillering to maximum number of tillers, WL 3: waterlogged from maximum number of tillers to flag leaf expanded, WL 4: waterlogged from flag leaf expanded to flowering) and a control without waterlogging (Ctl; open symbols) for Exp 1. The shadow area indicates the period of waterlogging. Circles indicate mean values in g pl^{-1} and bars indicate the relative reduction respect to the control without waterlogging (%). Error bars represent ± one standard error and are not shown when smaller than the symbol (n = 4). * indicates significant differences respect to the control at each period ($p < 0.05$)

species or experiments ($p > 0.05$), except for WL 2 for barley in Exp 2. Conversely, when waterlogging was applied at WL 3 shoot biomass at flowering was at all cases significantly lower (with the exception of barley in Exp 2, where the reduction was not significant) than that observed in the control plants due to the fact that flowering time coincided with the "delay effect" of waterlogging (Figs. 2c, g and 3c, g). Treatment WL 4 did not significantly affect shoot biomass in any species or experiment ($p > 0.05$; Fig. 2d, h and Fig. 3d, h).

At waterlogging release, reductions in root biomass ranged from 30% to 82% for wheat, and from 43% to 91% for barley, depending on the treatment and experiment ($p < 0.05$; Fig. 2i-p and Fig. 3i-p). In contrast to shoot biomass in which the highest negative effect of waterlogging was evident 20 days after the stress was released, the highest reduction in roots biomass, regarding the control was usually observed immediately after waterlogging was released. The capacity of wheat and barley to recover their root system after waterlogging

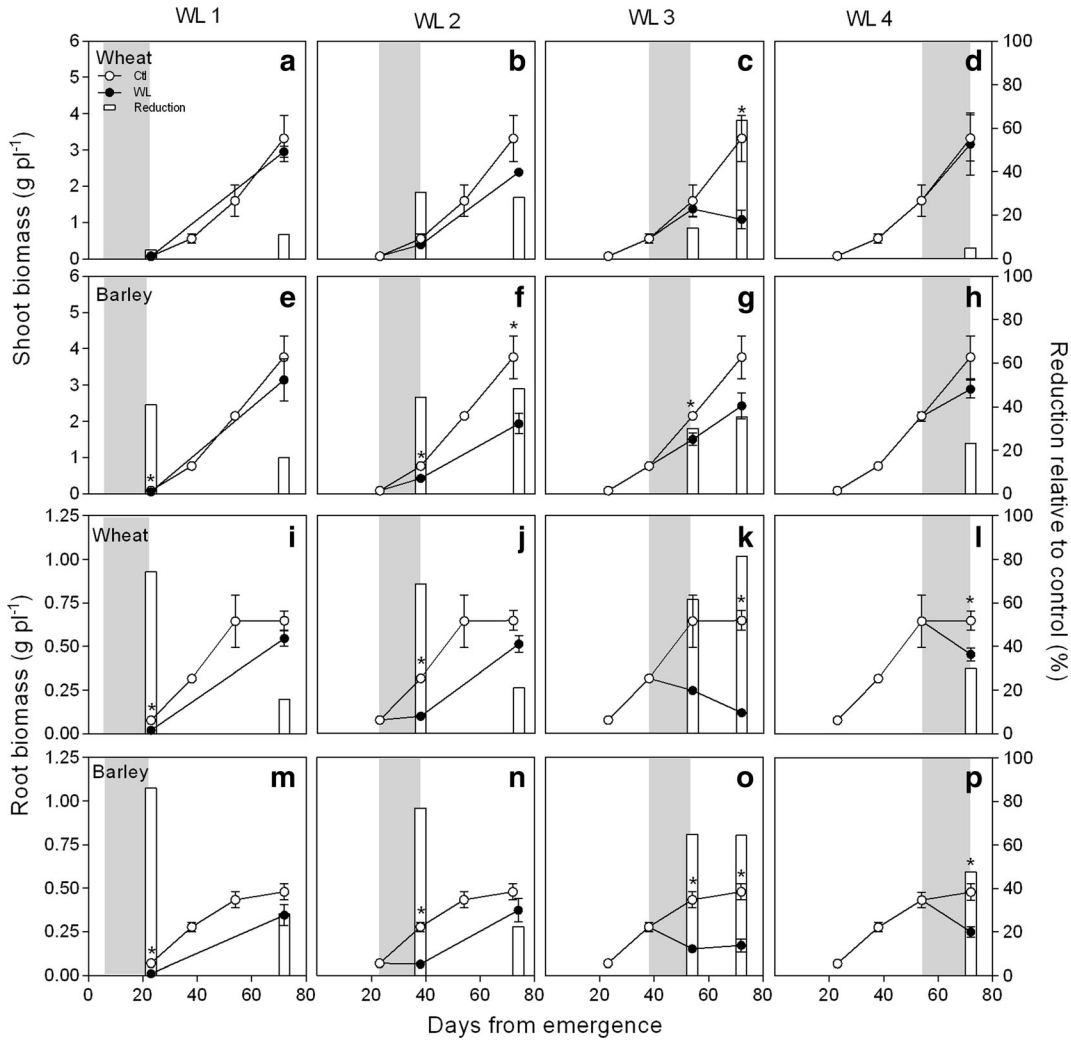

Fig. 3 Shoot (**a**-**h**) and root (**i**-**p**) biomass for wheat (**a**-**d**; **i**-**l**) and barley (**e**-**h**; **m**-**p**) exposed to waterlogging (WL; closed symbols) during different phenological periods (WL 1: waterlogged from emergence to beginning of tillering, WL 2: waterlogged from beginning of tillering to maximum number of tillers, WL 3: waterlogged from maximum number of tillers to flag leaf expanded, WL 4: waterlogged from flag leaf expanded to flowering) and a control without waterlogging (Ctl; open symbols) for Exp 2. The shadow area indicates the period of waterlogging. Circles indicate mean values in g pl^{-1} and bars indicate the relative reduction respect to the control without waterlogging (%). Error bars represent ± one standard error and are not shown when smaller than the symbol (n = 3). * indicates significant differences respect to the control at each period ($p < 0.05$)

was generally lower than the one observed for shoots. In Exp 1, root biomass (Fig. 2i-l) of wheat at flowering was ca. 50% lower than the control in all waterlogging treatments ($p < 0.05$). In Exp 2 root biomass of wheat at flowering was reduced by 81% in WL 3 and 35% in WL 4 ($p < 0.05$). Barley showed a higher root recovery than wheat after waterlogging, as plants waterlogged at early stages of development (WL 1 and WL 2) reached flowering with similar root biomass as control plants ($p > 0.05$) in both experiments (Fig. 2m-p, Fig. 3m-p).

Relative growth rate of shoots and roots

The shoot RGR did not differ between wheat and barley in any experiment ($p > 0.05$) although, this trait was significantly reduced ($p < 0.05$) due to waterlogging treatments in both species and experiments (Table 1). In Exp 1 RGR of shoots showed the highest reductions regarding control plants immediately after the release of each waterlogging treatment. In the following periods, all waterlogging treatments in Exp 1 had shown a

Table 1 Relative growth rate (RGR) of shoots and roots for different phenological periods: from emergence to beginning of tillering (Em-Till), from beginning of tillering to maximum number of tillers (Till-MNT), from maximum number of tillers to flag leaf expanded (MNT-FLE) and from flag leaf expanded to flowering (FLE-Flo) for wheat and barley (Sp) exposed to waterlogging treatments throughout the phenological cycle (WL 1: waterlogged from emergence to beginning of tillering, WL 2: waterlogged from beginning of tillering to maximum number of tillers, WL 3: waterlogged from maximum number of tillers to flag leaf expanded, WL 4: waterlogged from flag leaf to flowering, Ctl: non-waterlogged) in Exp 1 (early sowing date under greenhouse conditions; $n = 4$) and Exp 2 (late sowing date under natural conditions; $n = 3$)

			Shoot RGR (mg g^{-1} d^{-1})				Root RGR (mg g^{-1} d^{-1})			
Exp	Sp	WL	Em - Till	Till - MNT	MNT - FLE	FLE - Flo	Em - Till	Till - MTN	MTN - FLE	FLE - Flo
Exp 1	Wheat	Ctl	141.2 a	104.3 a	67.9 ab	52.4 bcd	58.05 a	144.0 a	47.1 c	34.7 abc
		WL 1	136.3 a	60.9 bc	65.6 ab	76.7 a	35.70 b	118.0 a	65.0 bc	25.7 bc
		WL 2	-	80.6 b	42.3 cd	65.9 abc	-	43.3 c	84.4 ab	37.3 ab
		WL 3	-	-	58.2 ab	21.2 e	-	-	−3.1 d	46.0 ab
		WL 4	-	-	-	41.3 de	-	-	-	−13.5 d
	Barley	Ctl	136.7 a	107.5 a	71.5 a	44.0 bcde	31.40 b	143.4 a	59.3 c	7.6 cd
		WL 1	137.5 a	51.1 c	66.8 ab	76.9 a	9.75 c	83.4 b	99.4 a	35.4 abc
		WL 2	-	78.3 b	34.2 d	67.5 ab	-	13.4 d	90.6 a	38.8 ac
		WL 3	-	-	56.0 bc	31.4 de	-	-	17.2 d	55.1 a
		WL 4	-	-	-	43.8 cde	-	-	-	−13.1 d
Exp 2	Wheat	Ctl	141.2 a	128.0 a	61.34a	38.7 a	111.0 a	93.8 a	41.2 a	−0.2 a
		WL 1	139.5 a	-	-	33.9 a	51.9 b	-	-	−9.7 a
		WL 2	-	105.4 a	-	20.0 abc	-	16.6 b	-	−12.0 a
		WL 3	-	-	54.3 a	−25.4 c	-	-	−16.2 b	−94.2 c
		WL 4	-	-	-	34.1 ab	-	-	-	−19.8 ab
	Barley	Ctl	141.5 a	143.2 a	64.2 a	29.8 ab	86.2 a	91.6 a	27.3 a	5.1 a
		WL 1	118.6 b	-	-	19.1 abc	−1.9 c	-	-	−14.4 a
		WL 2	-	104.9 a	-	−12.9 bc	-	−6.6 b	-	−10.1 a
		WL 3	-	-	41.1 a	−1.7 abc	-	-	−37.6 b	−55.8 b
		WL 4	-	-	-	16.0 abc	-	-	-	−31.3 ab

Different letters in each column indicate significant difference for Tukey's test at $p = 0.05$

remarkable recovery of the RGR, reaching values close to or even higher than control plants. In Exp 2, where RGR was measured in all cases during waterlogging and the period immediately previous to flowering, the shoot RGR recovery capacity was evident too. RGR values between flag leaf expanded and flowering of the waterlogged plants were similar to control plants (Table 1). The only exception was manifested in WL 3 treatment of wheat, in which shoot RGR were lower than those of control plants ($p < 0.05$) as the proximity of flowering time was coincident with the "delay effect" of waterlogging described above (Table 1).

The root RGR (Table 1) were also reduced by waterlogging treatments in both experiments ($p < 0.05$). In contrast to what occurred with shoot RGR, the worst effect of waterlogging for both experiments and species occurred during the waterlogging treatment and not during the recovery period, as observed for root biomass (Fig. 2). In Exp 1, wheat and barley root RGR for the WL 1 and WL 2 treatments were higher than control plants during the maximum number of tillers to flag leaf period ($p < 0.05$). At flowering, there was a significant waterlogging x species interaction ($p < 0.05$) in both experiments. For example, in Exp 1 barley root RGR measured during the flag leaf to flowering period was higher than control plants for the WL 3 treatment ($p < 0.05$). For the same period, wheat root RGR was significantly reduced by the WL 4 treatment ($p < 0.05$). In Exp 2, root RGR during the flag leaf to flowering period was significantly reduced under

treatment WL 3 in both species ($p < 0.05$), but the effect was higher in wheat than in barley.

Green leaf area, photosynthetic rate and use of reserves

Waterlogging treatments reduced green leaf area and leaf photosynthetic rate in wheat and barley, but there were differential effects in both species depending on the time when waterlogging treatments were applied (Fig. 4). In wheat, green leaf area was significantly reduced ($p < 0.05$) by waterlogging in WL 1 and WL 3 when measured immediately after both treatments were released. In barley, green leaf area was significantly reduced ($p < 0.05$) when measured at the end of WL 2, WL 3 and WL 4 treatments. When green leaf area was measured at flowering, only treatments WL 3 in wheat and WL 4 in barley produced significant reductions ($p < 0.05$), while the rest of the treatments showed values that did not differ statistically from control plants. The photosynthetic rate in wheat was reduced significantly regarding the control in WL 3 and WL 4 when measured at the end of each waterlogging treatment, while in barley significant reduction were evident in WL 1, WL 3 and WL 4. Excluding WL 4 that did not have recovery time, photosynthetic rate of all treatments at flowering time was recovered to similar values as control plants in both species ($p > 0.05$).

The culms water soluble carbohydrates (WSCs) content had increased in both species from emergence until flowering in control plants (Fig. 5). Treatments WL 1 and WL 2 increased WSCs concentration at the end of each waterlogging treatment compared to control plants in both species ($p < 0.05$). However, twenty days after waterlogging was released WSCs had decreased, showing similar (WL 1) or significantly lower values (WL 2, $p < 0.05$) regarding control plants. The WSCs accumulation were recovered after that in both treatments (Fig. 5a, b, e, f), with the exception of WL 3 in wheat that reduced WSCs concentration regarding the control ($p < 0.05$). Waterlogging treatments did not modify WSCs regarding control plants when measured at flowering. Finally, treatment WL 4 did not affect WSCs, when compared to controls, in any species ($p > 0.05$).

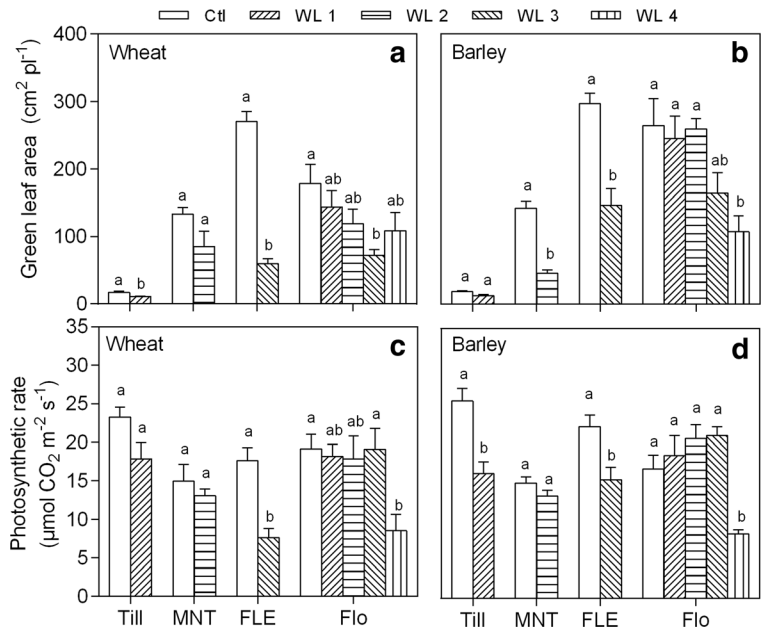

Fig. 4 Green leaf area ($cm^2\ pl^{-1}$; **a**, **b**) and leaf photosynthetic rate ($\mu mol\ CO_2\ m^{-2}\ s^{-1}$; **c**, **d**) at different ontogenic stages (beginning of tillering, Till; maximum number of tillers, MNT; Flag leaf expanded, FLE and flowering, Flo) of wheat (**a**, **c**) and barley (**b**, **d**) cultivars exposed to waterlogging at different phenological periods (WL 1: waterlogged from emergence to beginning of tillering, WL 2: waterlogged from beginning of tillering to maximum number of tillers, WL 3: waterlogged from maximum number of tillers to flag leaf expanded, WL 4: waterlogged from flag leaf expanded to flowering) and a control (Ctl) without waterlogging in Exp 1. Error bars represent ± one standard error ($n = 4$). Different letters at each growth stage indicate significant differences for Tukey's test at $p = 0.05$

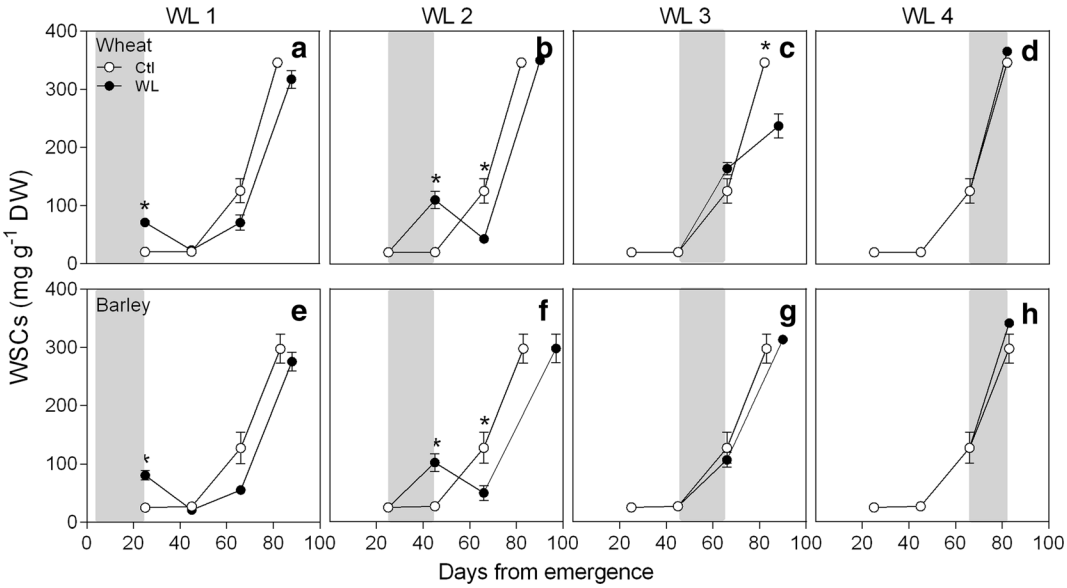

Fig. 5 Water soluble carbohydrates (WSC; mg g^{-1} dry weight) in culms (or in leaves in the first harvested sample) for wheat (**a**-**d**) and barley (**e**-**h**) exposed to waterlogging (WL; closed symbols) during different phenological periods (WL 1: waterlogged from emergence to beginning of tillering, WL 2: waterlogged from beginning of tillering to maximum number of tillers, WL 3: waterlogged from maximum number of tillers to flag leaf expanded, WL 4: waterlogged from flag leaf expanded to flowering) and a control without waterlogging (Ctl; open symbols) for Exp 1. The shadow area indicates the period of waterlogging. Error bars represent ± one standard error and are not shown when smaller than the symbol ($n = 4$). * indicate significant differences respect to the control at each period ($p < 0.05$)

Root morphology

Root length at flowering was similar in wheat and barley plants when grown under control conditions ($p > 0.10$), but was on average ca. 65% lower in Exp 2 than in Exp 1 for both species ($p < 0.05$; Fig. 6). Relative reductions of root length at the end of each waterlogging treatment ranged from 29% to 95% for barley and from 37% to 90% for wheat ($p < 0.05$; Fig. 6). The highest reductions in root length regarding control plants were observed immediately after waterlogging release in all treatments, with the exception of WL 1, in which the highest reduction was observed 20 days after the waterlogging was released, as occurred for root biomass. In Exp 1, wheat root length at flowering was reduced ca. 50% ($p < 0.05$) in all waterlogging treatments compared to the control, while in barley only treatments WL 3 and WL 4 significantly reduced root length at flowering (Fig. 6a-h). In Exp 2, wheat root length was reduced in all waterlogging treatments between 22 and 85% ($p < 0.05$). In barley, root length at flowering was reduced regarding control plants in treatments WL 2 and WL 3 ($p < 0.05$; Fig. 6i-p).

In both experiments specific root length (SRL) differed between cultivars ($p < 0.05$) for the different evaluated stages (with the exception of the stage of maximum number of tillers in Exp 1), showing barley (152 m g^{-1} in Exp 1 and 175 m g^{-1} in Exp 2) higher SRL values than wheat (112 m g^{-1} in Exp 1 and 108 m g^{-1} in Exp 2). In Exp 1, waterlogging treatments significantly reduced SRL, regarding control plants in both species at all evaluated stages before flowering (Table 2). Consistently, mean root diameters were increased by waterlogging treatments (Table 2). In Exp 2, only WL 4 treatment in barley increased significantly the SRL regarding control plants in the stages evaluated and mean root diameter was similar or higher in waterlogged plants (Table 2). The tendency to increase mean root diameter in waterlogged plants was consistent with the reduction of the proportion of thinner roots and the appearance of thicker roots (Fig. 7). For both species, more than 50% of total root length was represented by the thinnest roots (diameter < 0.2 mm). With the exception of barley in Exp 2, waterlogging treatments reduced the proportion of thinner roots (< 0.4 mm) and increased the contribution of thicker roots (> 0.4 mm) at all

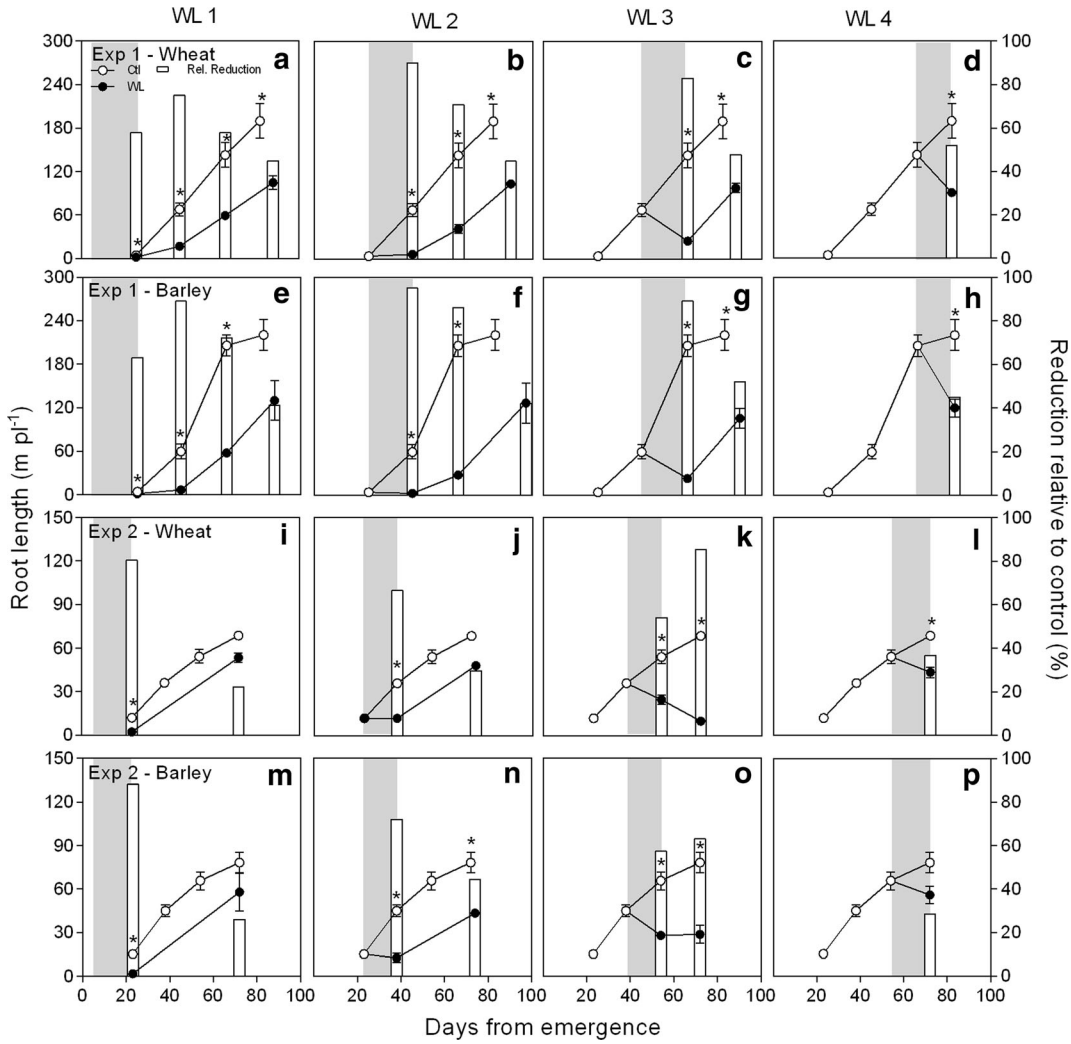

Fig. 6 Total root length for wheat (**a-d**; **i-l**) and barley (**e-h**; **m-p**) exposed to waterlogging (WL; closed symbols) during different phenological periods (WL 1: waterlogged from emergence to beginning of tillering, WL 2: waterlogged from beginning of tillering to maximum number of tillers, WL 3: waterlogged from maximum number of tillers to flag leaf expanded, WL 4: waterlogged from flag leaf expanded to flowering) and a control without waterlogging (Ctl; open symbols) for Exp 1 (a-h) and Exp 2 (i-p). The shadow area indicates the period of waterlogging. Circles indicate mean values in m pl^{-1} and bars indicate the relative reduction respect to the control without waterlogging (%). Error bars represent ± one standard error and are not shown when smaller than the symbol ($n = 3$). * indicate significant differences respect to the control at each period ($p < 0.05$)

phenological stages except for flowering, where the proportions did not change. This effect was particularly remarkable for barley in Exp 1 with treatment WL 2.

Aerenchyma formation in adventitious roots was evaluated in Exp 1 at three different stages of the wheat and barley cycle (maximum number of tillers, flag leaf and flowering) and measurements were taken immediately after waterlogging treatments finished (Fig. 8 and Table 3). Significant interaction was detected between waterlogging treatment and species at all stages evaluated ($p < 0.05$). Under non waterlogging conditions wheat and barley developed low percentages of aerenchyma in roots (between 0 and 11%), but when plants were waterlogged the volume of aerenchyma increased in wheat, reaching values between 30 and 35%, depending on the timing of waterlogging ($p < 0.05$). On the contrary, for barley, the percentages of aerenchyma of waterlogged plants were on average 8% (Table 3), although this did not vary regarding control plants ($p > 0.05$).

Table 2 Specific root length (SRL) and mean root diameter at different growth stages (beginning of tillering, Till; maximum number of tillers, MNT; flag leaf expanded, FLE; flowering, Flo) in wheat and barley (Sp) exposed to waterlogging treatments throughout the phenological cycle (WL 1: waterlogged from emergence to beginning of tillering, WL 2: waterlogged from beginning of tillering to maximum number of tillers, WL 3: waterlogged from maximum number of tillers to flag leaf expanded, WL 4: waterlogged from flag leaf to flowering, Ctl: non-waterlogged) in Exp 1 (early sowing date under greenhouse conditions) and Exp 2 (late sowing date under natural conditions)

			SRL (m g^{-1})				Diameter (mm)			
Exp	Sp	WL	Till	MNT	FLE	Flo	Till	MNT	FLE	Flo
Exp 1	Wheat	Ctl	160 ab	177 ab	126 bc	96 cd	0.29 b	0.29 b	0.28 bc	0.26 b
		WL 1	120 b	125 bc	99 bcd	99 cd	0.36 a	0.37 a	0.34 a	0.28 ab
		WL 2	-	103 c	88 cd	91 d	-	0.34 ab	0.36 a	0.30 ab
		WL 3	-	-	70 d	100 cd	-	-	0.34 a	0.32 a
		WL 4	-	-	-	100 cd	-	-	-	0.28 ab
	Barley	Ctl	200 a	208 a	185 a	172 ab	0.30 b	0.29 b	0.25 c	0.25 b
		WL 1	130 b	161 abc	138 b	134 abcd	0.30 b	0.36 a	0.32 ab	0.28 ab
		WL 2	-	111 bc	123 bc	142 abc	-	0.34 a	0.32 ab	0.26 b
		WL 3	-	-	108 bcd	127 bcd	-	-	0.33 a	0.30 ab
		WL 4	-	-	-	180 a	-	-	-	0.26 ab
Exp 2	Wheat	Ctl	157 ab	114 b	89 b	107 c	0.28 b	0.31 a	0.31 a	0.28 bc
		WL 1	119 b	-	-	99 c	0.35 a	-	-	0.32 bc
		WL 2	-	122 b	-	96 c	-	0.31 a	-	0.32 bc
		WL 3	-	-	102 b	84 c	-	-	0.35 a	0.42 a
		WL 4	-	-	-	96 c	-	-	-	0.30 bc
	Barley	Ctl	218 a	163 ab	152 a	165 b	0.26 b	0.28 b	0.27 a	0.25 c
		WL 1	175 ab	-	-	166 b	0.28 b	-	-	0.25 c
		WL 2	-	189 a	-	126 bc	-	0.28 b	-	0.28 bc
		WL 3	-	-	184 a	168 b	-	-	0.30 a	0.33 b
		WL 4	-	-	-	224 a	-	-	-	0.25 c

Different letters in each column indicate significant difference for Tukey's test at $p = 0.05$

Discussion

Effect of waterlogging on shoots as a consequence of root damage

Transient waterlogging in wheat and barley differently affected biomass accumulation depending on the phenological stage at which waterlogging was applied, and the tendency was similar for the two evaluated environments (Exp 1 and Exp 2). Waterlogging events during the leaf appearance period (WL 1, WL 2 and WL 3) significantly reduced shoot biomass between 10 and 40% regarding control plants at waterlogging release (Fig. 2 and Fig. 3), as stated in previous reports for wheat and barley (Huang et al. 1994a; Malik et al. 2001; Pang et al. 2004). However, the highest reductions in shoot biomass compared to control plants were observed after waterlogging treatments were released indicating a delayed long term effect of waterlogging on shoot growth. This effect was especially remarkable when waterlogging was applied early in the crop cycle (WL 1 and WL 2), in which reductions in shoot biomass 15 or 20 days after the end of waterlogging reached 60% in wheat and 70% in barley (Fig. 2 and Fig. 3). This delayed effect of waterlogging on growth was previously reported for wheat (Malik et al. 2001; Malik et al. 2002) and barley (Pang et al. 2004) when plants were waterlogged at early vegetative stages of 3–4 leaves, and also in wheat waterlogged during stem elongation (Araki et al. 2012a). Our results confirmed that the highest reductions in shoot biomass were evident between 2 and 3 weeks after waterlogging

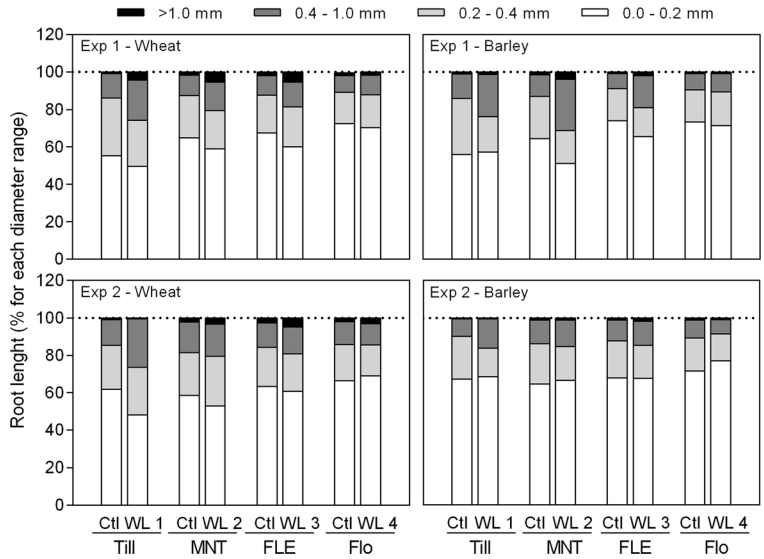

Fig. 7 Relative distribution of total root length into different ranges of root diameter (0 to 0.2 mm, 0.2 to 0.4 mm, 0.4 to 1 mm and >1 mm) at different growth stages (beginning of tillering, Till; maximum number of tillers, MNT; Flag leaf expanded, FLE and flowering, Flo) of wheat (**a**, **c**) and barley (**b**, **d**) cultivars exposed to waterlogging at different phenological periods (WL 1: waterlogged from emergence to beginning of tillering, WL 2: waterlogged from beginning of tillering to maximum number of tillers, WL 3: waterlogged from maximum number of tillers to flag leaf expanded, WL 4: waterlogged from flag leaf expanded to flowering) and a control without waterlogging in Exp 1 (**a**, **b**) and Exp 2 (**c**, **d**)

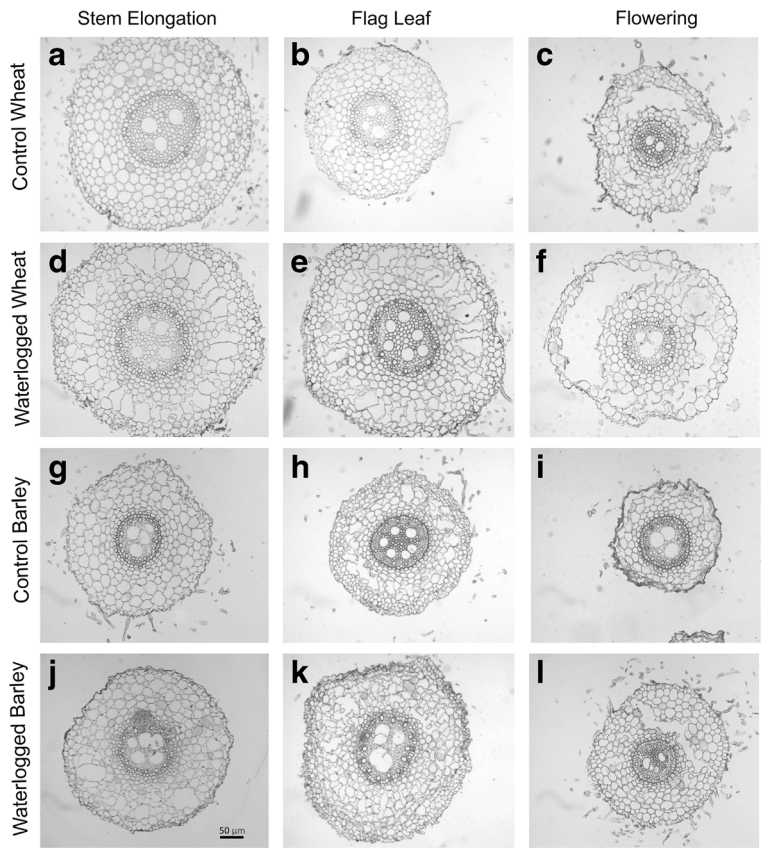

Fig. 8 Aerenchyma formation in adventitious roots of wheat (**a**-**f**) and barley (**g**-**l**) under well drained conditions (Control, **a**-**c**; **g**-**i**) and exposed to 20 days of waterlogging during different phenological periods: from beginning of tillering to maximum number of tillers (**d**, **j**), from maximum number of tillers to flag leaf expanded (**e**, **k**) and from flag leaf expanded to flowering (**f**, **l**). The cross sections were taken at the end of each waterlogging event at 20 mm from the root tip in root of between 8 and 10 cm long. Scale bar (50 μ) presented in panel **j** is applicable to all pictures

Table 3 Aerenchyma formation in adventitious roots (% of the whole root section) at different growth stages (maximum number of tillers, MNT; flag leaf expanded, FLE; flowering, Flo) for wheat and barley (Sp) exposed to different waterlogging treatments (WL) during the 20 days previous to each measurement. Data correspond to Exp 1 (early sowing date under greenhouse conditions)

Sp	Treatment	MTN	FLE	Flo
Wheat	Ctl	5.7 b	0.0 b	11.1 b
	WL	33.8 a	34.5 a	30.2 a
Barley	Ctl	1.6 a	7.9 b	2.8 b
	WL	9.6 a	5.4 b	6.5 b

Different letters in each column indicate significant difference for Tukey's test at $p = 0.05$

ended and not during the period when plants were subjected to waterlogging or immediately after. Furthermore, although the delayed negative effects of waterlogging on shoot biomass occurred in all treatments, waterlogging biggest effect was when applied during the period from beginning of tillering to maximum number of tillers.

The negative waterlogging effect on wheat and barley roots was more notorious than that on shoots, as reported for wheat (Malik et al. 2001; Malik et al. 2002; Herzog et al. 2016). Root biomass reductions in waterlogged plants reached 82% in wheat and 90% in barley (Fig. 2 and Fig. 3), while root length reductions were slightly higher, reaching 90% in wheat and 95% in barley (Fig. 6). Unlike what happened in shoots, the biggest effect of waterlogging on root system was generally observed at the moment of waterlogging release, and not during the recovery period, which is in accordance with the fact that roots are affected directly by anaerobic conditions of the soil during waterlogging (Striker 2012). The reduced shoot growth after the waterlogging period can be the consequence of the preferential resource allocation to root growth (Malik et al. 2001; Malik et al. 2002), reestablishing the shoot:root ratio (Malik et al. 2002). This reestablishment of the shoot:root ratio is a response of plants after waterlogging in order to restore water homeostasis in the new conditions of well-drained soil (Striker 2012). The delayed shoot response to waterlogging was also evident when considering the RGR, as the highest reduction in root RGR took place during the period when plants were waterlogged. As for shoots, RGR reductions in both species were in the subsequent period (Table 1). Results from the present study confirm that reductions in shoot biomass after waterlogging were a consequence of the damage to the root system when plants were waterlogged, as recently stated by Herzog et al. (2016) in a review that describes the different waterlogging tolerance mechanisms in wheat.

Photosynthesis and accumulation of carbohydrates under waterlogging

In general terms, when plants were exposed to waterlogging green leaf area and photosynthesis were reduced in both wheat and barley, in agreement with what was widely reported in the literature (see review by Herzog et al. 2016). The reduction in the photosynthetic rate could be a consequence of the negative feedback from carbohydrates acumulation that was observed in this study at all waterlogging treatments (Trought and Drew 1980; Malik et al. 2001; Malik et al. 2002). Carbohydrates accumulation in stems suggests that lower photosynthetic rate is not the cause of root and shoot growth reduction during waterlogging. On the contrary, it is possible to speculate that WSCs increased due to the lack of an active sink in plants during waterlogging, especially as a consequence of a lower root growth during that period. After waterlogging was released and plants initiated the root and shoot biomass recovery, WSCs were rapidly used and subsequently plants initiated the process of WSCs accumulation, reaching similar values as control plants at flowering (with the exception of WL 3 in wheat). The same trend was evident for green leaf area and photosynthetic rate as both were generally recovered at flowering.

Recovery of shoots and roots after waterlogging

The first response of plants to waterlogging is the decrease of root respiration rate (Huang and Johnson 1995), which affects growth and functionality of roots (Colmer and Greenway 2011). The maintenance of the root system under waterlogging conditions is crucial to keep the plant water status and photosynthetic rate, and thereby contribute to a better tolerance to waterlogging (Hayashi et al. 2013). Regarding the hypothesis that the recovery of wheat after waterlogging involves the preferential root growth regarding shoots which reestablishes the root:shoot ratio of the non-waterlogged plants (Malik et al. 2002; Herzog et al. 2016), results of our work confirmed that RGR of roots during the period subsequent to waterlogging was

higher for the waterlogged plants than for the control plants during the same period, and coincided with the lower shoot RGR of waterlogged plants. However, when considering the shoot and root biomass at the moment of flowering the reduction of root biomass regarding to control plants was higher than that of shoot biomass indicating a lower root system capacity to recover than that of the shoot biomass. Those effects were specially remarkable for wheat in Exp 1, which had reached flowering with a significantly lower root biomass than control plants at all waterlogging treatments applied after the beginning of tillering.

Most works evaluating the effect of waterlogging on plants did not explore the events occurring during the recovery period once plants were released from waterlogging (Malik et al. 2002; Pang et al. 2004; Striker 2012) as experiments are generally culminated before plants reached maturity. This kind of approximation can lead to the erroneous conclusion that the capacity of recovery for wheat and barley exposed to waterlogging during early phenological stages is low as the experiments do not continue long enough after waterlogging release, specially in crops that are harvested at maturity as wheat and barley. In our study, it was shown that despite the long term effect of waterlogging on growth, wheat and barley plants are capable of recovery and have reached shoot biomass values similar to control plants, as a consequence, no differences in shoot biomass were detected between control and waterlogged plants at flowering (Fig. 2 and Fig. 3). These results are in consonance with findings by Cannell et al. (1980) and Belford (1981), who showed a vigorous regrowth of wheat plants after waterlogging in early growth stages. The shoot biomass recovery in plants waterlogged early in the crop cycle (WL 1 and WL 2) can be partly explained by a longer period of tillers appearance (de San Celedonio et al. 2016) and the production of higher order tillers compared to plants without waterlogging (Robertson et al. 2009). Moreover, the recovery in treatments WL 1 and WL 2 was in accordance with a high shoot RGR (similar o higher than the control) during the period from flag leaf expanded to flowering, which concurs with the critical period for grain yield determination under potential conditions (Fischer 1985; Arisnabarreta and Miralles 2008) and also with the more sensitive period to waterlogging conditions (de San Celedonio et al. 2014).

The plant capacity to recover from waterlogging decreased when the stress occurred late in the cycle. Our results had shown that the lowest shoot biomass capacity to recover in wheat was when waterlogging was applied at the beginning of stem elongation (i.e. WL 3), when plants showed the highest reductions in biomass at flowering compared to control plants. This treatment was also the only one to show a significant reduction in green leaf area and a higher use of shoot carbohydrates at flowering compared to control plants (with exception of the treatment that was waterlogged immediately prior to flowering). When waterlogging occurred immediately previous to flowering (WL 4), there were not decreases in shoot biomass. However, de San Celedonio et al. (2014) reported a higher reduction in grain yield when wheat and barley plants were waterlogged immediately previous to anthesis (i.e. WL 4), confirming the lower capacity of recovery for both wheat and barley when waterlogging occurs later in the cycle. In addition, it was previously reported in de San Celedonio et al. (2014) that the magnitude of the yield loss is higher under more stressful environments such as in Exp 2 (late sowing date, with higher temperatures and evaporative demand; please see de San Celedonio et al. 2014 for details about the environmental conditions explored throughout the experiments). However, in both experiments reported in this study there were no detectable differences between wheat and barley in the shoot and root biomass relative reduction, which suggest an additional effect of waterlogging on biomass partition to grains under a more stressful environment.

The possibility to produce new tillers seems to be related to wheat and barley shoot biomass recovery capacity after waterlogging (Cannell et al. 1980; Belford 1981; Robertson et al. 2009; de San Celedonio et al. 2016). Thus, when plants are waterlogged late in the cycle, they are not able to produce new tillers and to compensate the reductions in shoot biomass caused by waterlogging. In addition, it is reasonable to assume that recovery is higher after early waterlogging because the time to recover is longer. However, this argument is not valid for roots, because their recovery capacity was low in every waterlogging event.

The different effect of waterlogging on shoot biomass and the root system measured in relative terms is summarized in Fig. 9. Relative reductions of root biomass (range from 30 to 90%) at the end of each waterlogging treatment were higher than those of shoots (range from 0 to 40%). However, during the recovery period shoot biomass reductions were similar to that of roots (near the 1:1 line). At flowering, plants showed a

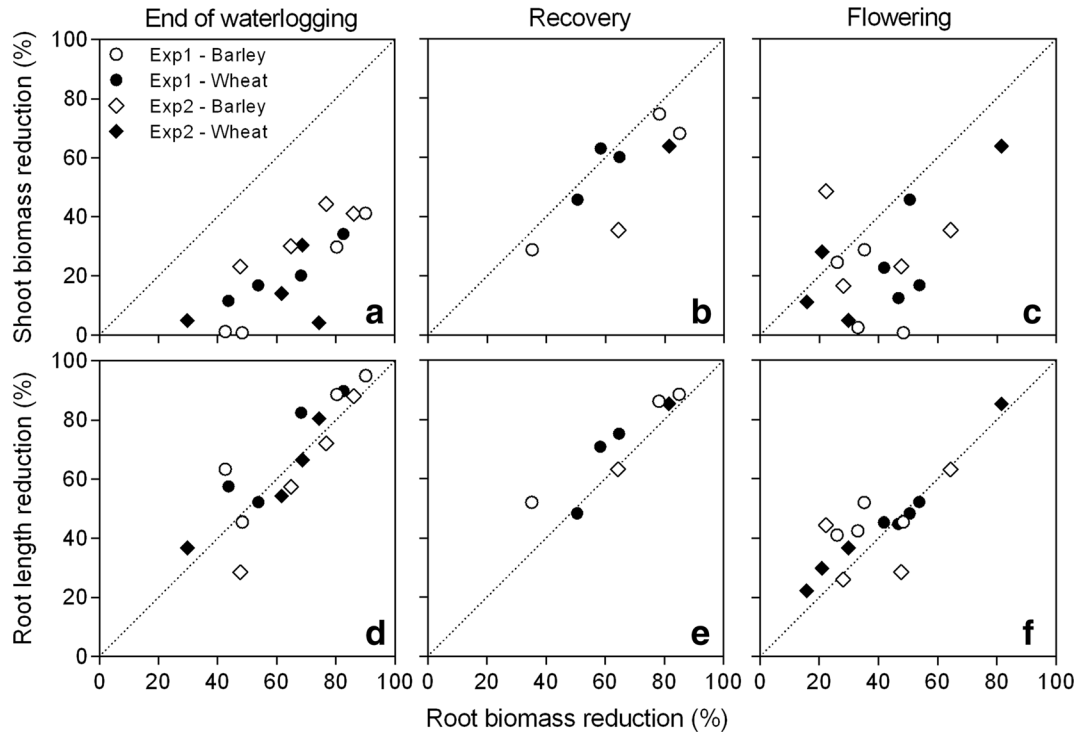

Fig. 9 Relationship between shoot biomass (**a**, **b**, **c**) or root length (**d**, **e**, **f**) reductions respect to the control (%) and root biomass reductions respect to the control (%) in wheat (closed symbols) and barley (open symbols) plants waterlogged during 15–20 at different phenological periods (from emergence to beginning of tillering, from beginning of tillering to maximum number of tillers, from maximum number of tillers to flag leaf and from flag leaf to flowering). Measurements were made at the end of each waterlogging event (**a**, **d**), 15 or 20 days after the end of waterlogging (indicated as "recovery" period, **b**, **e**) and at flowering (**c**, **f**). Dotted lines represent the 1:1 relationship

significant recovery capacity, especially for shoots, as the range of reduction was from 1 to 64%, while in root the range was from 16 to 82% (Fig. 9). Finally, there was a close relationship between reductions in root length and root biomass, and it is important to highlight that waterlogging also modified root diameter (Fig. 7).

Waterlogging affects root morphology

Root morphology and anatomy were affected by waterlogging treatments. Increases in root diameter and reductions in SRL (Table 2), together with a reduced contribution of thinner roots were evident in both species when plants were exposed to waterlogging (Fig. 7). Yamauchi et al. (2014) observed that wheat adventitious roots that emerged during stagnant conditions have thicker root diameters and larger air spaces (i.e. aerenchyma) than those emerged under aerated conditions. Thus, the observed increases in root diameter of wheat and barley after waterlogging could be a consequence of adventitious roots (of thicker diameter than preexistent roots) production during waterlogging (Malik et al. 2001, 2003), together with the death of thinner roots. Aerenchyma formation is a common response of some plants to oxygen deficiencies or waterlogging conditions (Colmer and Voesenek 2009), as oxygen supply to roots depends mainly on the oxygen transportation from shoots through aerenchyma (Armstrong 1979). In wheat (Malik et al. 2001, 2003; Jiang et al. 2010) and barley (Broughton et al. 2015; Zhang et al. 2015) aerenchyma development is induced by exposure of roots to waterlogging, and genotypes with bigger root porosity (or aerenchyma formation) tended to show a better performance under waterlogging conditions (Setter and Waters 2003). The percentage of aerenchyma in roots of the barley cultivar used in our experiment was low in both waterlogged and control plants and were consistent with values reported for barley cultivars classified as sensitive to waterlogging (Zhang et al. 2015). However, for wheat there was a significant increase of aerenchyma

for all waterlogging treatments evaluated (Table 3 and Fig. 8). The higher production of aerenchyma in wheat did not lead to a better tolerance of this species to waterlogging compared to barley when damage was evaluated at flowering. However, the production of aerenchyma in wheat could be related to a lower root biomass reduction (Fig. 2 and Fig. 3) and root length (Fig. 6) compared to barley at the end of each waterlogging treatment. Once waterlogging was removed, another mechanism appears to be involved in barley recovery, as waterlogged plants reached flowering with similar root biomass as control plants ($p > 0.05$, Fig. 3). The higher green leaf area observed in barley at flowering compared to wheat in waterlogged and control conditions (Fig. 4) suggest that the recovery of barley after waterlogging involved the maintenance of photosynthetic area, probably as a result of a higher tiller survival compared to wheat (de San Celedonio et al. 2016). However, specific experiments should be carried out in the future for a better understanding of the mechanisms involved in root recovery of both species.

In conclusion, wheat and barley root biomass was severely affected during waterlogging, but the negative effect on shoot biomass appeared later than in roots, and was a consequence of previous root damage. However, both species had shown an important capacity for shoot biomass recovery, especially when waterlogging occurred during early growth stages, and showed values at flowering that were similar to well-drained plants. Even though root RGR after waterlogging exceeded the one of control plants, the root system recovery was always lower than the shoot recovery. Moreover, the roots recovery capacity for wheat was lower than that of barley, being waterlogged wheat root biomass significantly lower than control plants at flowering.

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