

# Physiological traits associated with reductions in grain number in wheat and barley under waterlogging

Romina P. de San Celedonio · L. Gabriela Abeledo · Daniel J. Miralles

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## Abstract

**Aims** Negative effects of waterlogging on wheat and barley yield are expressed mainly through reductions in grain number per plant. Physiological traits associated with reductions in grain number of wheat and barley plants waterlogged at different growth stages during preanthesis were evaluated.

**Methods** Two pot experiments were carried out under contrasting environments, where wheat and barley plants were exposed to waterlogging at four different ontogenic stages, from emergence to anthesis. Physiological traits associated with grain number determination were measured at anthesis and at physiological maturity.

**Results** Waterlogging occurring during the spike growth period significantly reduced grain number per plant up to 70% in wheat and 60% in barley. Reductions in grain number per plant in wheat were mainly related

to decreases in grain number per spike, while in barley grain number reductions were related to decreases in the number of spikes. In both species waterlogging produced spike growth reductions that were associated with reductions in the number of fertile florets per spike, without effects on fruiting efficiency.

**Conclusions** The effect of waterlogging on grain number per plant differed between wheat and barley. Waterlogging reduced grain establishment in wheat by affecting the growth capacity of spikes and, consequently, reducing the number of fertile florets per spike. In barley, the main effect of waterlogging was through reductions in the number of spikes per plant, without significant changes in grain number per spike. These differences between species open ways to analyze the impact of different management practices (i.e. nitrogen fertilization, plant population) as alternative to mitigate the negative effect of waterlogging on grain yield.

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R. P. de San Celedonio · L. Abeledo · D. J. Miralles  
Cátedra de Cerealicultura, Facultad de Agronomía, Universidad de Buenos Aires, Av. San Martín 4453, C1417DSE Buenos Aires, Argentina

R. P. de San Celedonio (✉) · L. Abeledo · D. J. Miralles  
CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas), Buenos Aires, Argentina  
e-mail: romina@agro.uba.ar

R. P. de San Celedonio · D. J. Miralles  
IFEVA (Instituto de Investigaciones Fisiológicas y Ecológicas Vinculadas a la Agricultura), Av. San Martín 4453, C1417DSE Buenos Aires, Argentina

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## Introduction

The productivity of wheat and barley crops is affected by waterlogging in many farmlands worldwide (Sayre et al. 1994; Setter and Waters 2003; de San Celedonio et al. 2014b). A soil is considered waterlogged when its pores are saturated with water, which results in a severe decrease in the rate of oxygen diffusion into it (Armstrong

1979). Therefore, soil becomes hypoxic and then anoxic in a short time (Ponnamperuma 1984), affecting the root respiration and its functionality and finally the whole plant growth (de San Celedonio et al. 2017), with negative impacts on crop yield. For wheat, yield penalties as a consequence of waterlogging were estimated at ca. 43% (Herzog et al. 2016), although these reductions can vary greatly with genotype (Setter et al. 1999; Setter and Waters 2003), the crop developmental stage when waterlogging occurs (Cannell et al. 1980; Belford 1981; de San Celedonio et al. 2014a), the duration of the stress (Marti et al. 2015; Olgun et al. 2008) and interactions with the environment (de San Celedonio et al. 2014a). In barley, grain yield penalties due to waterlogging are equal or usually higher than in wheat (Cannell et al. 1984; de San Celedonio et al. 2014a), although there is genotypic variability in the sensitivity to the stress in both species (Setter et al. 1999; Setter and Waters 2003; Hoffman and Viegas 2011).

In wheat as well as in barley, it was determined that yield is most reduced when waterlogging occurs during the period immediately prior to anthesis (Sayre et al. 1994; de San Celedonio et al. 2014a), coinciding with the critical phase for yield potential determination in both crops (Fischer 1975; Arisnabarreta and Miralles 2008a). Yield variations in wheat and barley are positively and linearly related to the grain number determined per plant, which can be analyzed as the product between the number of spikes per plant and the number of grains per spike. During the period prior to anthesis, floret primordia in the spike are firstly differentiated and then a proportion of them degenerate and die in coincidence with the active growth of the spike and the stem (Kirby 1988; Arisnabarreta and Miralles 2010). The number of florets that survive defines the number of grains per spike. Moreover, the number of spikes per unit area is also defined during the period immediately prior to anthesis, as tiller mortality (Alzueta et al. 2012) is taking place simultaneously with floret death. As a result, during this period, grain number per plant, the main numerical component of grain yield, is established.

Wheat spikes have potential to differentiate up to ca. 9 floret primordia within each spikelet (González et al. 2003). However, many of them degenerate or die, and less than 4 to 5 floret primordia per spikelet reach the fertile stage at anthesis (González et al. 2005). The magnitude of floret primordia death is inversely related to the availability of resources per spike (Arisnabarreta and Miralles 2010; González et al. 2011; Ferrante et al.

2013). Therefore, the number of fertile florets per spikelet is closely and positively correlated with spike dry weight at anthesis (Miralles et al. 1998; Fischer 2007; Slafer et al. 2015). The final grain number established also depends on the efficiency with which the resources are used to set grains per unit of spike dry weight, this ratio having been named fruiting efficiency (Abbate et al. 1998; Slafer et al. 2015). Differing from wheat, the number of grains per spike in two-rowed barley is limited by the botanical structure of the spike, where only one grain per spikelet is set (García del Moral and García del Moral 1995; Arisnabarreta and Miralles 2006b). For that reason, grain number per unit area in two-rowed barley is in general related to the number of spikes more than to the grain number per spike (Alzueta et al. 2012). Grain number determination in wheat and barley in terms of number of florets has been traditionally studied under potential conditions (González et al. 2003) or under deficit conditions of water or nitrogen (Ferrante et al. 2013); however, under field conditions, plants are exposed to different kinds of stresses, such as waterlogging.

Under waterlogging conditions, the main component determining yield losses in wheat is grain number per spike (Robertson et al. 2009; de San Celedonio et al. 2014a; Marti et al. 2015). The occurrence of waterlogging during the initial phase of tillering affects the appearance of tillers, but the final number of spikes is not significantly reduced (de San Celedonio et al. 2016) due to a compensation of tiller appearance compared to the tiller mortality (Robertson et al. 2009). In the case of barley, variations in grain number per plant are mainly explained by variations in the number of spikes, due to the low plasticity of the spike to establish grains (only one grain per spikelet; García del Moral and García del Moral 1995), but grain number per spike also explains an important proportion of the variation in grain number per plant under waterlogging conditions (Cannell et al. 1984; de San Celedonio et al. 2014a). However, little is known about the traits associated with the reductions in grain number per spike in wheat and barley plants under waterlogging conditions. Understanding how physiological determinants of grain number per plant are affected during waterlogging may help us to design future strategies for breeding. In previous work it was reported that waterlogging during stem elongation of wheat affected yield by reducing spike growth before anthesis, and concomitantly reduced the number of fertile florets and grains per spike

(Marti et al. 2015). Waterlogging during the late phase of the stem elongation period reduced yield by affecting plant growth, but not the efficiency of conversion of that growth to yield (Marti et al. 2015). It is expected that waterlogging occurring during early growth stages has a lower impact on grain number determination than waterlogging during the spike growth period. Recently, it was reported that the highest effect of waterlogging on biomass accumulation occurs well after the waterlogging ceases (de San Celedonio et al. 2017). The objective of this work was to evaluate the physiological traits associated with grain number reductions in wheat and barley plants, waterlogged at different growth stages during preanthesis. The first hypothesis of this work was that waterlogging reduces grain number per spike when the stress occurs during the period of spike growth, but the greatest reduction on grain number per spike takes place when waterlogging occurs during the initial phase of spike growth, and not during the period immediately prior to anthesis. To the best of our knowledge, no studies have reported the physiological traits associated with reductions on grain number per spike in barley under waterlogging conditions during different preanthesis stages. The second hypothesis speculates that the waterlogging impact on grain number per spike would be lower in barley than in wheat, as barley plants set up yield principally by the number of spikes per plant, because of the little plasticity structure of the barley spike.

## Material and methods

### Growing conditions

Two experiments were carried out in 12 L pots (25 cm diameter × 30 cm depth) at the School of Agriculture, University of Buenos Aires, Argentina (34° 35' S, 58° 29' W) in the 2010/11 growing season. Experiment 1 (Exp 1) was carried out in a greenhouse and the sowing date was on July 2 (i.e. within the optimum range of sowing dates for the location). Experiment 2 (Exp 2) was conducted under natural conditions of radiation and temperature (field conditions) and was sown on September 6 (a late sowing date). Each pot was fertilized at sowing with 2.5 g of Triple 15 (Yara Company) fertilizer (15 N:15 P<sub>2</sub>O<sub>5</sub>:15 K<sub>2</sub>O). The plant density was 6 plants per pot and both experiments were conducted without biotic stresses.

### Experimental design and treatments

The experimental design was completely randomized. Treatments within each experiment consisted of the combination of: i) Species: Klein Chajá wheat cultivar for Exp 1, Baguette 13 wheat cultivar for Exp 2 and Scarlett barley cultivar for both experiments; ii) waterlogging conditions: a control well drained during the entire phenological cycle (Ctl) and four treatments of waterlogging applied at different growth stages before anthesis: WL 1, from emergence to beginning of tillering; WL 2, from beginning of tillering to maximum number of tillers; WL 3, from maximum number of tillers to flag leaf fully expanded and WL 4, from flag leaf fully expanded to anthesis. The wheat cultivars were different between experiments because there was not available seed of Klein Chajá at the moment of sowing Exp 2. Therefore the cultivar Baguette 13, with similar yield potential and phenology respect to Scarlett and Chajá, was selected. The selected genotypes were commercial cultivars widely used by farmers. The growth stages were determined following Zadoks et al. (1974); in the case of barley, anthesis was determined by opening spikelets and visualizing pollen release. The duration of each waterlogging treatment was of 20 days in Exp 1 and 15 days in Exp 2. The different durations of waterlogging treatments between experiments were in order to ensure the phenological stages at the beginning and ending of treatments were consistent between both experiments as phenological development was faster in Exp 2 than in Exp 1 because of the later sowing was exposed to warmer temperatures and longer daylengths. Waterlogging treatments were imposed by placing 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. After waterlogging was released, 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 and environmental conditions in each experiment please see de San Celedonio et al. (2014a).

### Measurements

At anthesis, all the spikes from 4 (Exp 1) or 3 pots (Exp 2) per treatment were sampled. In Exp 1, the number of total florets per spike visible at anthesis

and fertile florets per spike at anthesis was counted in main stem spikes using a stereomicroscope Leica MZ6 (Leica Microsystems, Wetzlar, Germany). A floret was considered fertile when it showed its style curve with stigmatic hairs differentiated (Waddington et al. 1983). After that, all the spikes of wheat and barley plants of both experiments were oven-dried at 65 °C until constant weight and then dry weight was measured. Finally, spikes were ground in a mill fitted with a 0.5 mm screen (Wiley Laboratory Mill, Philadelphia, USA) and N content was measured by Kjeldahl's micro-method analysis.

At maturity, all the plants from 8 (Exp 1) or 3 pots (Exp 2) per treatment were harvested and separated in main stem and tillers. The number of spikes from the main stem and tillers was counted and the total number of spikelets per spike and fertile spikelets per spike (i.e. spikelets with at least one grain) was determined in all spikes from main stems and 20% of the spikes from tillers. The proportion of fertile spikelets was estimated as the ratio between the number of fertile spikelets per spike and the number of total spikelets per spike. Spikes were weighed after dried at 65 °C for 72 h and then the spikes from each stratum (main stem or tiller) were threshed separately.

Average individual grain weight was measured in two sub-samples of 50 grains per each stratum. The grain number per plant was calculated from dry weight per grain and grain yield per plant, and the grain number per spike as the ratio between grain number per plant and spike number per plant. The grain number per fertile spikelet was calculated as the ratio between grain number per spike and fertile spikelet number per spike at maturity. Chaff ( $\text{g pl}^{-1}$ ) was calculated as the average spike dry weight, before threshing, minus the grain yield per spike. Fruiting efficiency ( $\text{grains g}^{-1}$ ) was considered as the ratio between grain number per plant and the chaff.

### Statistical analysis

Statistical differences between treatments were tested through analyses of variance (ANOVA) using InfoStat Professional v.1.1 (Di Rienzo et al. 2011). The mean treatment values were compared using Tukey test with significance level of 0.05. The degree of association between different variables was assessed using correlation analysis and linear regression models.

## Results

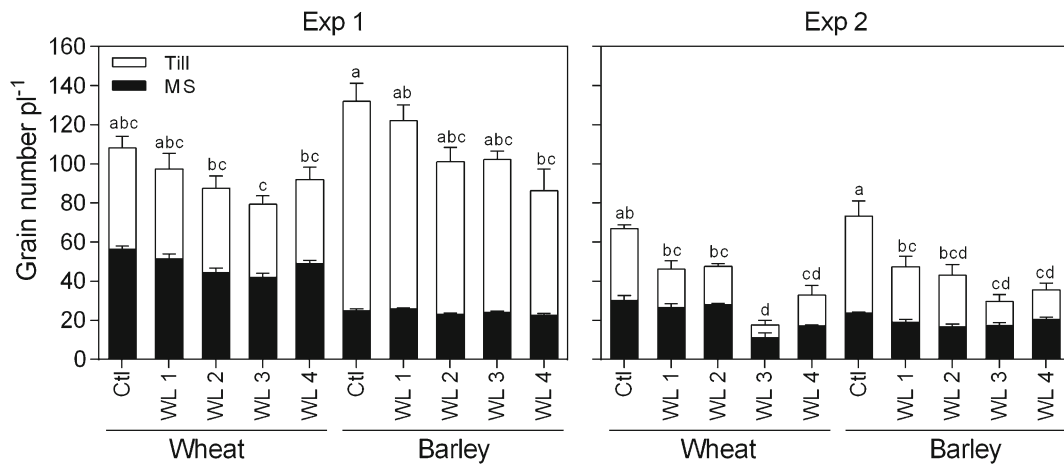
### Grain number per plant under waterlogging conditions

Waterlogging treatments significantly reduced grain number per plant in both experiments ( $p < 0.05$ ) and there was no waterlogging  $\times$  species interaction ( $p > 0.10$ ). The highest reductions of grain number per plant in wheat were observed in treatment WL 3 in both experiments, and in barley in WL 4 (Exp 1) and WL 3 (Exp 2) treatments (Fig. 1). The reductions in grain number per plant in Exp 1 were a consequence of reductions in the number of grains from the main stems in the case of wheat (WL 2 and WL 3), or from tillers in the case of barley (WL 4). In Exp 2, where the relative reductions of grain number per plant due to waterlogging were greater than in Exp 1, it was related to reductions in both grain number from the main stem and tillers in both species (Fig. 1).

There was a strong correlation between grain number per plant and grain yield for both species in both experiments (Table 1). For wheat, grain number per plant was similarly correlated to spikes per plant and grain number per spike in Exp 1, while in Exp 2 grain number per plant was strongly correlated to grain number per spike (Table 1), but not to the number of spikes per plant ( $p > 0.10$ ). In the case of barley, grain number per plant was positively related primarily to the number of spikes per plant, but also to the grain number per spike in both experiments (Table 1).

### Waterlogging reduced grain number per spike

In both experiments waterlogging  $\times$  species interactions occurred for grain number per spike ( $p < 0.01$ ). Waterlogging significantly reduced grain number per spike in wheat when treatments were applied at WL 2 and WL 3 in Exp 1 and WL 3 and WL 4 in Exp 2 (Table 2). Reductions in grain number per spike of wheat with treatment WL 3 were consistent with the reduction in the proportion of fertile spikelets at maturity in both experiments ( $p < 0.01$ ; Table 2). For Exp 1, there was also an additional reduction in the number of grains per fertile spikelet in wheat, which was also found in treatment WL 2. In the case of barley, a significant and negative effect of waterlogging was detected on the total spikelet number per spike in treatment WL 2 in Exp 1 ( $p < 0.01$ ) but the final grain number per spike in barley was not affected by any waterlogging treatment



**Fig. 1** Grain number per plant for wheat and barley exposed to waterlogging at different stages during preanthesis phases in Exp 1 (early sowing date under greenhouse; left panel) and Exp 2 (late sowing date under natural conditions; right panel). Closed bars correspond to main stem (MS) and open bars correspond to tillers (Till). Ctl, control; WL1, waterlogged from emergence to beginning of tillering; WL 2, waterlogged from beginning of tillering to

maximum number of tillers; WL3, waterlogged from maximum number of tillers to flag leaf fully expanded; WL4, waterlogged from flag leaf fully expanded to anthesis. Vertical lines within each bar represent one standard error of the mean. Different letters in each panel indicate significant differences at Tukey's test ( $p < 0.05$ )

(variations in grain number per spike in barley were due to differences between experiments) (Table 2).

In wheat, grain number per spike was most strongly related to grain number per fertile spikelet ( $R^2 = 0.87$   $p < 0.01$ ), then to chaff weight ( $R^2 = 0.82$   $p < 0.01$ ) and the number of fertile spikelets per spike ( $R^2 = 0.79$   $p < 0.01$ ), and lastly to fruiting efficiency ( $R^2 = 0.67$   $p < 0.01$ ) (Fig. 2). In the case of barley, the relation between grain number per spike and the number of fertile spikelets per spike was highly significant ( $R^2 = 0.94$   $p < 0.01$ ). Chaff weight was also related to grain number

per spike, although the correlation was weaker than that observed in wheat ( $R^2 = 0.70$   $p < 0.01$ ). For wheat, the relative effect of waterlogging on grain number per spike was in general lower than the effect on other measured traits (i.e. chaff weight, fruiting efficiency, fertile spikelets per spike and grain number per fertile spikelet). In this sense, Fig. 2 shows that values for wheat are in general below the 1:1 line. By contrast, in the case of barley the negative effect of waterlogging, in relative terms, was higher on grain number per spike than on chaff weight.

**Table 1** Correlation coefficients among grain yield per plant (GY  $\text{pl}^{-1}$ ), grain number per plant (GN  $\text{pl}^{-1}$ ), spikes per plant (Spike  $\text{pl}^{-1}$ ) and grain number per spike (GN  $\text{spike}^{-1}$ ) for wheat and

barley plants exposed to waterlogging at different stages during preanthesis in Exp 1 and Exp 2

		Exp 1			Exp 2		
		GY ( $\text{g pl}^{-1}$ )	GN $\text{pl}^{-1}$	Spike $\text{pl}^{-1}$	GY ( $\text{g pl}^{-1}$ )	GN $\text{pl}^{-1}$	Spike $\text{pl}^{-1}$
Wheat	GY ( $\text{g pl}^{-1}$ )	1			1		
	GN $\text{pl}^{-1}$	0.91 **	1		0.92 **	1	
	Spike $\text{pl}^{-1}$	0.64 **	0.70 **	1	ns	ns	1
	GN $\text{spike}^{-1}$	0.64 **	0.71 **	ns	0.88 **	0.91 **	ns
Barley	GY ( $\text{g pl}^{-1}$ )	1			1		
	GN $\text{pl}^{-1}$	0.98 **	1		0.96 **	1	
	Spike $\text{pl}^{-1}$	0.86 **	0.88 **	1	0.93 **	0.90 **	1
	GN $\text{spike}^{-1}$	0.60 **	0.61 **	ns	ns	0.62 *	ns

\*\*  $p < 0.001$ ; \*  $p < 0.05$ ; ns non-significant correlation ( $p > 0.05$ )

**Table 2** Effect of waterlogging applied at different stages during preanthesis on grain number per spike and its physiological components in wheat and barley plants for Exp 1 and Exp 2. Treatments are named as in Fig. 1

Exp	Species	Treatment	GN spike <sup>-1</sup>	Chaff (g spike <sup>-1</sup> )	Fruiting efficiency (grains g <sup>-1</sup> )	Total spikelets spike <sup>-1</sup>	Fertile spikelets (proportion)	GN fertile spikelet <sup>-1</sup>	
Exp 1	Wheat	Ctl	44.3 a	0.60 ab	74.2 bc	18.1 cd	0.87 a	2.8 a	
		WL 1	40.5 ab	0.64 a	62.9 c	16.3 d	0.86 a	2.9 a	
		WL 2	34.3 b	0.54 bc	63.5 c	18.5 c	0.78 ab	2.4 b	
		WL 3	33.9 b	0.51 c	67.8 c	18.1 cd	0.76 b	2.5 b	
		WL 4	40.2 ab	0.64 a	63.1 c	18.2 cd	0.86 a	2.6 ab	
	Barley	Ctl	18.1 c	0.22 d	85.2 ab	27.1 a	0.78 ab	0.9 c	
		WL 1	18.8 c	0.20 d	94.7 a	26.0 a	0.80 ab	0.9 c	
		WL 2	16.1 c	0.16 d	98.6 a	23.8 b	0.79 ab	0.9 c	
		WL 3	17.7 c	0.21 d	86.3 ab	26.7 a	0.78 ab	0.8 c	
		WL 4	17.5 c	0.19 d	92.5 a	27.3 a	0.72 b	0.9 c	
	Waterlogging (W)			***	***	ns	***	*	**
	Species (S)			***	***	***	***	***	***
W * S			**	***	**	***	***	**	
Exp 2	Wheat	Ctl	25.2 a	0.27 a	94.7 abc	15.2 b	0.82 a	2.0 ab	
		WL 1	18.4 abc	0.20 abc	89.0 abc	13.9 b	0.61 ab	2.1 a	
		WL 2	20.9 ab	0.26 a	81.8 bc	15.6 b	0.76 ab	1.8 abc	
		WL 3	8.7 d	0.13 bc	69.5 c	13.6 b	0.60 b	1.2 bc	
		WL 4	13.5 bcd	0.20 ab	66.0 c	15.1 b	0.58 b	1.6 abc	
	Barley	Ctl	15.9 bcd	0.14 bc	110.4 ab	22.8 a	0.73 ab	1.0 c	
		WL 1	12.7 cd	0.11 bc	118.3 a	18.6 ab	0.66 ab	1.0 c	
		WL 2	12.3 cd	0.11 bc	112.0 ab	18.0 ab	0.72 ab	1.0 c	
		WL 3	11.2 cd	0.11 c	104.6 ab	18.7 ab	0.55 b	1.1 c	
		WL 4	15.4 bcd	0.13 bc	119.6 a	21.8 a	0.69 ab	1.0 c	
	Waterlogging (W)			***	**	ns	*	***	ns
	Species (S)			**	***	***	***	ns	***
W * S			**	*	ns	ns	ns	*	

\*\*\*  $p < 0.001$ ; \*\*  $p < 0.01$ ; \*  $p < 0.05$ ; ns non-significant. For each column, mean within each experiment followed by the same letter indicates no significant differences at  $p = 0.05$

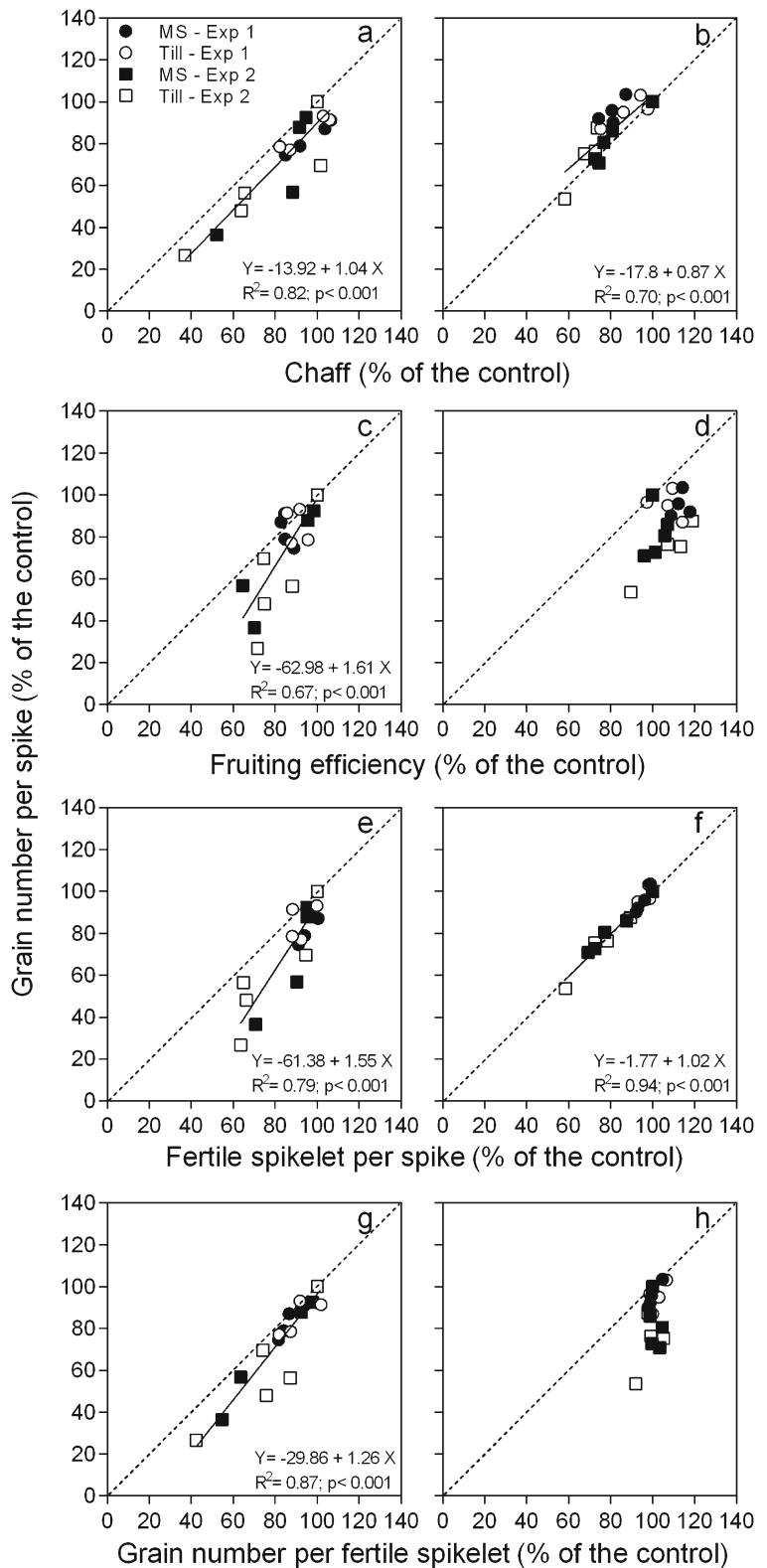
How did waterlogging affect the physiological components of grains per spike?

A significant waterlogging x species interaction was detected for spike dry weight at anthesis (SDWA) and N content per spike ( $p < 0.05$ ). The SDWA of wheat was significantly reduced in the WL 3 treatment in both experiments (Table 3), in accordance with that observed in chaff at maturity (Table 2). The amount of N in spikes at anthesis was also reduced in wheat in the WL 3 treatment in both experiments, and in the

WL 2 treatment in Exp 1. In the case of barley, the SDWA and the amount of N in spikes was reduced by WL 2 treatment in Exp 2. The differences in the amount of N in wheat spikes with WL 3 were due to the differences in biomass and also reductions in N concentration in spikes at anthesis. In Exp 1, WL 3 treatment reduced N concentration 12% in spikes, in average for wheat and barley, while in Exp 2, WL 4 increased N concentration in spikes 30% ( $p < 0.05$ ), although the SDWA and the amount of N in spikes were not affected.



**Fig. 2** Relationship between grain number per spike and chaff (a, b), fruiting efficiency (c, d), fertile spikelet number per spike (e, f) and grain number per fertile spikelet (g, h) for wheat (left panels) and barley (right panels) exposed to waterlogging at different growth stages during preanthesis. Circles correspond to Exp 1, squares to Exp 2, closed symbols are for main stems (MS) and open symbols for tillers (Till). Values are presented as percentage of the control without waterlogging of each experiment. Dotted lines represents the 1:1 relationship and solid lines indicate the fitted regressions



**Table 3** Effect of waterlogging applied at different stages during preanthesis on spike dry weight at anthesis (SDWA), N content in spikes at anthesis (N in spike) and N concentration in spikes at

anthesis ([N] in spike) in wheat and barley plants for Exp 1 and Exp 2. Treatments are named as in Fig. 1

Exp	Species	Treatment	SDWA (g spike <sup>-1</sup> )	N in spike (mg spike <sup>-1</sup> )	[N] in spike (mg gMS <sup>-1</sup> )
Exp 1	Wheat	Ctl	0.57 ab	10.36 a	18.20 a
		WL 1	0.63 a	10.54 a	17.09 ab
		WL 2	0.47 bc	7.46 b	15.88 ab
		WL 3	0.46 c	7.11 b	15.57 ab
		WL 4	0.54 abc	9.72 ab	17.82 a
	Barley	Ctl	0.22 d	3.69 c	16.45 ab
		WL 1	0.20 d	3.12 c	15.54 ab
		WL 2	0.22 d	3.40 c	15.62 ab
		WL 3	0.25 d	3.28 c	13.20 b
		WL 4	0.23 d	3.39 c	14.79 ab
Waterlogging (W)			*	**	*
Species (S)			***	***	**
W * S			***	*	ns
Exp 2	Wheat	Ctl	0.31 a	4.99 abc	16.33 ab
		WL 1	0.25 a	4.34 abc	17.54 ab
		WL 2	0.19 ab	2.91 bcd	15.29 ab
		WL 3	0.05 b	1.14 d	21.04 ab
		WL 4	0.28 a	6.20 a	22.48 a
	Barley	Ctl	0.29 a	4.55 abc	15.93 ab
		WL 1	0.16 ab	2.26 cb	14.32 b
		WL 2	0.06 b	0.90 d	16.64 ab
		WL 3	0.19 ab	3.13 bcd	16.10 ab
		WL 4	0.28 a	5.52 ab	19.51 ab
Waterlogging (W)			***	***	**
Species (S)			ns	ns	*
W * S			**	**	ns

\*\*\*  $p < 0.001$ ; \*\*  $p < 0.01$ ; \*  $p < 0.05$ ; ns non-significant. For each column, mean within each experiment followed by the same letter indicates no significant differences at  $p = 0.05$

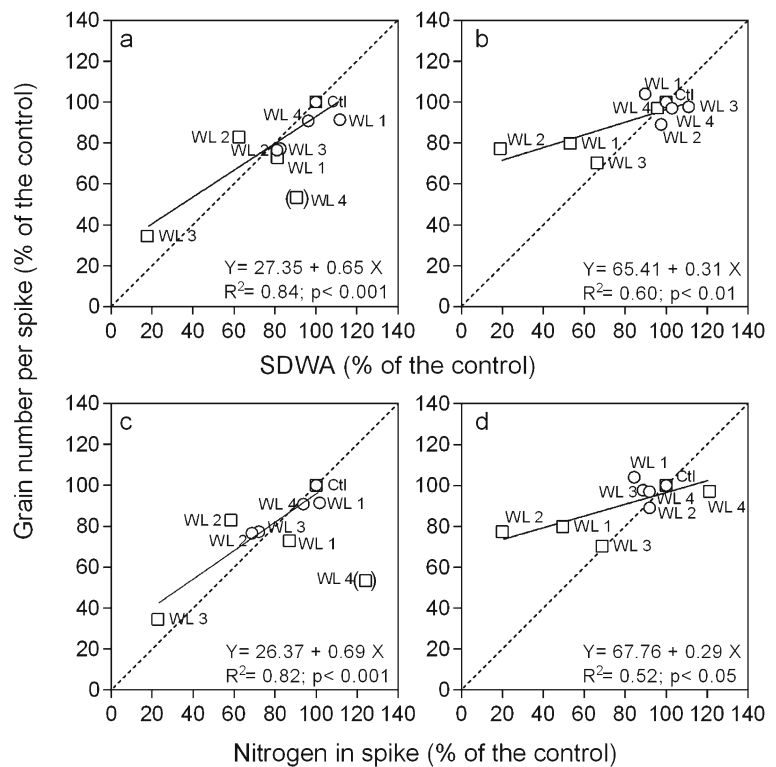
In wheat, grain number per spike was strongly related to SDWA ( $R^2 = 0.84$   $p < 0.01$ ) and to the N content in spikes at anthesis ( $R^2 = 0.82$   $p < 0.01$ ). However, in Exp 2, WL 4 treatment value was considered an outlier (external Studentized residual  $> 3$ ) in both relationships and was excluded from the fitted model (Fig. 3; see Discussion). In the WL 4 treatment in Exp 2 grain number per spike (evaluated at maturity) was more severely affected than the traits evaluated at anthesis (SDWA, nitrogen content in spike), the stage in which treatment WL 4 was ended. The N concentration in spikes was weakly and negatively related to grain number per spike in wheat ( $R^2 = 0.45$ ;  $p < 0.05$ ). In barley,

the relationship between grain number per spike and the SDWA was weaker than in wheat ( $R^2 = 0.60$   $p < 0.01$ ), and still weaker when grain number per spike was regressed with nitrogen in spikes at anthesis ( $R^2 = 0.52$   $p < 0.05$ ). The relative effect of waterlogging was higher on the SDWA than on the final grain number per spike, which can be observed in the slope of the regression ( $< 1$ ) (Fig. 3). There was no significant relationship between grain number per spike and N concentration in barley spikes ( $p > 0.10$ ).

The number of fertile florets per spike at anthesis was evaluated in main stems of wheat and barley plants in Exp 1. The total number of fertile florets



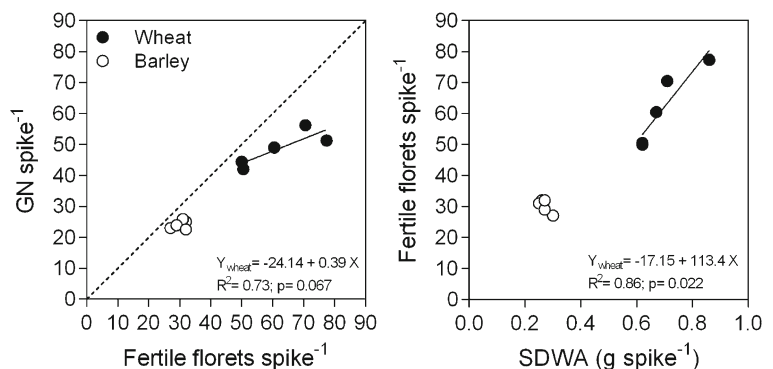
**Fig. 3** Relationship between grain number per spike (mean of the plant) and spike dry weight at anthesis (a, b), or nitrogen content in spikes at anthesis (c, d) for wheat (left panels) and barley (right panels) exposed to waterlogging at different growth stages during preanthesis. Circles correspond to Exp 1 and squares to Exp 2. Values are presented as percentage of the control without waterlogging. Exp 2 treatment WL 4 in wheat was considered an outlier point and excluded from the fitted model because it had a very high external Studentized residual ( $>3$ )



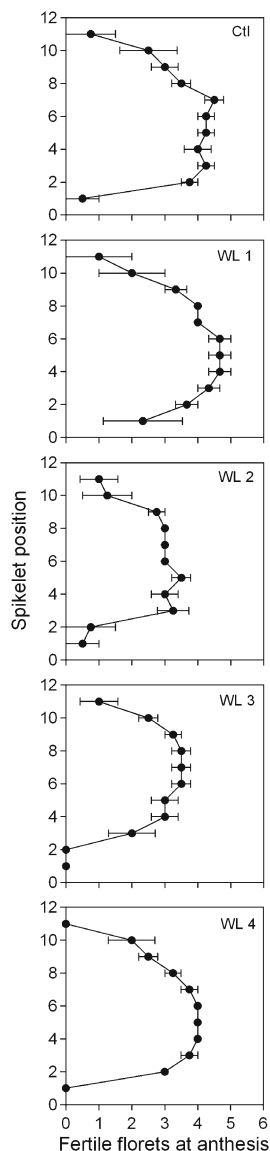
per spike was significantly reduced 30% ( $p < 0.05$ ) compared to the control in wheat by the WL 2 and WL 3 treatments, while in barley the number of fertile florets per spike was not modified by effect of waterlogging ( $p > 0.05$ ). Waterlogging did not affect grain setting (i.e the number of fertile florets that are established as grains) in any species ( $p > 0.10$ ). Thus, in wheat, grain number per spike was positively related to the number of fertile florets per spike at anthesis (Fig. 4, left panel). However, the relationship between grain number per spike and fertile florets per spike showed a slope, although positive, lower than 1

( $b = 0.39$  grains per fertile floret at anthesis) demonstrating that the greater the number of fertile florets per spike, the lower the grain setting. The number of fertile florets per spike at anthesis was positively related to the SDWA ( $R^2 = 0.82$ , Fig. 4 right panel). Conversely to that was observed in wheat, in the case of barley there was no association between grain number per spike and the number of fertile florets per spike at anthesis, nor between the number of fertile florets per spike at anthesis and the SDWA, due to the fact that there was limited variability in those traits in barley spikes.

**Fig. 4** Relationship between grain number per spike and fertile florets per spike at anthesis (left panel), and fertile florets per spike at anthesis and spike dry weight at anthesis (right panel) for main stems of wheat and barley exposed to waterlogging at different growth stages during preanthesis in Exp 1



The reduction of fertile florets per spike in wheat by waterlogging was significant in the WL 2 and WL 3 treatments but was not of the same magnitude for all spikelet positions within the spike (Fig. 5). Basal spikelets were the most severely affected by waterlogging with fertile floret reductions of 40% in WL 2 and 100% in WL 3 (sterile spikelets), compared to the control treatment. In central spikelets, fertile floret reduction was in average 25%, while in apical spikelets no reduction was observed respect to the control, due to waterlogging.



**Fig. 5** Number of fertile florets at anthesis for each spikelet position within the spike for main stem spikes of wheat exposed to waterlogging at different growth stages during preanthesis in Exp 1

## Discussion

In this study we reported negative effects of waterlogging, when applied at different stages from emergence to anthesis, on the physiological traits associated with the determination of grain number in wheat and barley plants. For wheat, it was previously reported that waterlogging during stem elongation period affected yield by affecting basic growth processes (i.e. spike growth before anthesis), with no major effects on the efficiency of conversion of that growth to yield or yield components (Martí et al. 2015). Even though it is known that the stem elongation period is critical for grain number determination, the final grain number is being established throughout the crop cycle, from seedling emergence to grain setting. In this study, we evaluate the effect of waterlogging during the whole period in which the grain number is being established (i.e. from emergence to grain setting) on physiological traits related to grain number determination in two species with different strategies to determine its grain number, as wheat and barley.

Waterlogging occurring during stem elongation (WL 2 and WL 3 in Exp1 and WL 3 and WL 4 in Exp 2) were the treatments generating a significant reduction in grain number per spike in wheat, while the treatment applied during the beginning of spike growth (WL 3) was the one that produced the highest reduction, supporting the first hypothesis of this study. In contrast to wheat, grain number per spike in barley was not affected by waterlogging. Under waterlogging conditions, previous studies showed that reductions in grain number per plant in barley were strongly associated with decreases in the number of spikes per plant (de San Celedonio et al. 2014a). Our results corroborated that in barley, independently of the environmental conditions, grain number per unit area is frequently associated with the number of spikes (Alzueta et al. 2012; de San Celedonio et al. 2014a), as the grain number per spike is limited by the structure of the spike, where each spikelet sets only one grain (García del Moral and García del Moral 1995; Arisnabarreta and Miralles 2006b). These results supported the second hypothesis of the study.

The reductions in grain number per spike, as a consequence of waterlogging, were related to reductions in the spike size, as waterlogging reduced spike dry weight at anthesis (SDWA) and at maturity (i.e. chaff weight). However, the efficiency of grain establishment per unit of spike weight (i.e. fruiting efficiency) was not

significantly reduced by waterlogging in any treatment or experiment (Table 2), similarly to that observed by Marti et al. (2015) in wheat plants waterlogged during the stem elongation period.

Although the total number of spikelets per spike was not affected by waterlogging in wheat, spikelet fertility was reduced in both experiments when waterlogging took place close to anthesis (WL 3 and WL 4). In contrast, in barley plants, the total number of spikelets per spike at maturity was reduced by the WL 2 treatment with no effect on the proportion of fertile spikelets. A possible reason for this effect on total spikelets in barley could be that the WL 2 treatment affected the spikelet differentiation and/or the floret degeneration during the early stages post-differentiation, since the treatment coincided with the stage in which spikelets are being differentiated in barley plants (Arisnabarreta and Miralles 2006a). The fact that the total number of spikelets is not determined by the appearance of a terminal spikelet, as occurs in wheat (i.e. barley spike has an indeterminate pattern of spikelet development), could have contributed to this effect. In this context, Arisnabarreta and Miralles (2010) found that reductions in N supply strongly reduced the number of florets initiated in 2- and 6-rowed barley. However, due to the way in which the number of total spikelets was measured in the present work (i.e. with the naked eye at physiological maturity), it is not possible to ensure that the negative effect of waterlogging was on spikelet differentiation or on the floret mortality, as many of the differentiated spikelets probably degenerated immediately after differentiation and were not visible at physiological maturity.

Spike dry weight at anthesis (SDWA) was a good estimator of the negative impact of waterlogging on grain establishment in wheat, as well as in barley. As widely reported in literature, the availability of resources for spike growth is directly related to the number of floret primordia that become fertile at anthesis in wheat and barley, independently of whether the resource that is being affected is radiation (Fischer 1985; Arisnabarreta and Miralles 2008b), nitrogen (Prystupa et al. 2004; Ferrante et al. 2010), or simply the capacity of growth due to an accelerated development (Miralles et al. 2000; González et al. 2003). In the present study, waterlogging affected grain establishment by means of reducing the spike growth before anthesis and consequently reducing the number of fertile florets and finally grains per spike. The grain setting, once the number of fertile florets was

determined, was not affected by waterlogging in any treatment imposed in this study.

The only exception to the high correlation found between SDWA and grain number per plant was WL4 treatment for wheat in Exp 2, which was considered an outlier in the regression. This could be the consequence of the delay in the effect of waterlogging on above-ground traits, as the most negative effect of waterlogging on above ground biomass was detected 15 or 20 days after the treatments ended (de San Celedonio et al. 2017). Therefore, in the WL 4 treatment of Exp 2, SDWA was evaluated at time that waterlogging ceased (i.e. anthesis in WL 4 treatment), but the negative effects of that treatment occurred later, and could be detected at maturity when grain number per spike was evaluated. The fact that this negative effect of waterlogging was only observed in Exp 2 could be explained by the more-stressful conditions of this experiment compared to Exp 1, as shown by the greater effects of waterlogging on grain number in Exp 2.

It has been proposed that N concentration in the spikes has a direct effect on the establishment of wheat grains (Abbate et al. 1995). Nonetheless, in the present study, no direct effect of N was found on the establishment of grains, as the relationship between grain number per spike and N concentration in spikes at anthesis was null in the case of wheat, and negative in the case of barley. Moreover, the relationship between grain number per spike and N in spike did not improve compared to when grain number per spike was plotted against SDWA (see Fig. 3). Seemingly, the effect of N on grain number per spike was mediated by the effect of N on spike growth and concomitantly spike dry weight. Thus, more N absorbed by the plant increased biomass production and its partitioning to the spikes, and was probably the driving force that determined the number of floret primordia reaching the fertile stage (Arisnabarreta and Miralles 2010).

Conforming to the fact that the main negative effect of waterlogging was through a reduction in growth capacity during the period when number of grains was being determined, it was observed that the number of fertile florets per spike was not reduced in the same proportion in all positions within the spike. Florets of lower hierarchy (i.e. those placed on basal spikelets) were the ones most negatively affected by waterlogging treatments, indicating competition for resources that primarily affected lower hierarchy organs. Similar results were found in wheat exposed to

waterlogging during stem elongation (Marti et al. 2015).

In conclusion, we found that waterlogging reduced grain establishment per spike in wheat when the stress occurred during the spike growth period, and the effect was mediated mainly by affecting basic spike growth processes. The capacity to convert spike biomass into grains in wheat was not affected by waterlogging. In barley, although spike growth was reduced, the magnitude of the effect was much less than in wheat, and the main effect of waterlogging on grain number per plant was mostly by affecting the establishment of the number of spikes and not by reducing grain number per spike. Waterlogging events prior to the beginning of stem elongation did not significantly affect the number of grains established per spike in either species.

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