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Transient waterlogging events impair shoot and root physiology and reduce grain yield of durum wheat cultivars

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Abstract: Durum wheat (*Triticum turgidum* L. subsp. *durum* (Desf.) Husn) is a staple crop of the Mediterranean countries, where more frequent waterlogging events are predicted due to climate change. However, few investigations have been conducted on physiological and agronomic responses of this crop to waterlogging. The present study provides a comprehensive evaluation of the effects of two waterlogging durations (i.e., 14 and 35 days) on two durum wheat cultivars (i.e., Svevo and Emilio Lepido). An integrated analysis of an array of physiological, biochemical, biometric and yield parameters was performed at the end of the waterlogging events, during recovery, and at physiological maturity. Results established that effects on durum wheat varied depending on waterlogging duration. This stress imposed at tillering impaired photosynthetic activity of leaves and determined oxidative injury of the roots. These damages could not be fully recovered, subsequently slowed down tiller formation and crop growth, so depressing final grain yield. Furthermore, differences in waterlogging tolerance between cultivars were discovered. We concluded that waterlogging tolerance of durum wheat can be achieved by pyramiding the numerous physiological and biochemical parameters that confer efficient key processes (such as energy maintenance, cytosolic ion homeostasis, and ROS control and detoxification), and consequently ensure satisfying biomass and grain yield production.

Keywords: abiotic stress; antioxidants; climate change; flooding; osmoprotectans**;** reactive oxygen species; *Triticum turgidum* L. subsp. *durum*; yield.

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1. Introduction

Durum wheat (*Triticum turgidum* L. *subsp. durum* (Desf.) Husn) is one of the oldest cultivated cereals, and also plays a pivotalrole in global food security. Although the necessity of wheat grain will increase by 60%, its production might decline by 29%, because of the environmental stresses due to climate change [1]. Durum wheat is among the most widespread and economically important crops in the Mediterranean countries [2]; notwithstanding the Mediterranean environment is recognized to be extremely vulnerable to climatic changes [3]. Specifically, it has been predicted that durum wheat will be affected by more recurrent, severe, and unpredictable flooding events [4].

In rain-fed situations, flooding happens when more rain falls than the soil can absorb, or the atmosphere can evaporate. In Central Italy, excess water is likely to occur from October to April, but is more expected during the winter months (January-February), due to lower transpiration and evaporation rates of the crop. Therefore, durum wheat is more prone to excess water during the tillering stage, which is critical for tiller production and

spikelet initiation [5]. Based on the height of the water column produced, flooding can be classified as waterlogging, when water covers just the root system, or as submergence, when water also overlays the plant aerial organs [6]. In waterlogged soils, the gas diffusion through soil pores is inhibited so that the oxygen (O_2) concentration decreases rapidly, while the carbon dioxide (CO_2) and ethylene concentrations increase in the root environment [7]. A slowed O_2 influx is the main cause of injury to the roots and to the shoots they support [8]. The plants react through a series of morphological and physiological responses to the damages due to O_2 deprivation. From a physiological point of view, excess water accumulation in the root zone can induce osmotic stress and disrupt cell ion homeostasis. To cope with such stressful conditions, plants tend to accumulate compounds called osmoprotectans (such as free amino acids, non-structural carbohydrates and quaternary ammonium substances), as their accumulation may decrease the osmotic potential [9,10]. Next, the impaired root functioning under waterlogging affects the physiological responses of the shoots, particularly the carbon fixation. Waterlogging may induce partial stomatal closure that, in turn, could constrain internal CO_2 levels and limit carbon fixation [11,12]. Additionally, photosynthesis rates can also be inhibited by non-stomatal factors, e.g. oxidative injury [10] caused by reactive oxygen species (ROS) like hydrogen peroxide (H_2O_2), superoxide radicals and hydrogen radicals which impair mesophyll conductance [13] and harm photosystem II (PSII), causing cellular damage and leaf chlorosis related to chlorophyll degradation [14, 15].

In durum wheat, the O_2 deficiency caused by waterlogging has been demonstrated to prematurely senesce leaves, reduce root and shoot growth and constrain spike development, thus decreasing the final grain yield of the crop [5]. However, the effect of transient waterlogging could be somewhat compensated by subsequent recovery of the growth of roots and shoots as demonstrated in other winter cereals, like oat [16] and barley [17]. Recovery involves allocation of carbon to roots after waterlogging and hypoxia, for preferential root growth to re-establish a root to shoot ratio typical of plants of drained soils. This preferential resource allocation to root growth would be a major reason explaining the reduced shoot growth following a period of waterlogging [18]. Nevertheless, to the best of our knowledge, very few research has addressed the effects of waterlogging throughout the entire crop cycle, describing both vegetative growth and grain production. Thus, evidence on root and shoot growth during waterlogging and subsequent recovery is limited to oat, barley and common wheat [16, 17], with no confirmation existing for durum wheat.

To fill the gap of knowledge about the mechanism(s) of response of durum wheat to waterlogging and to relate the physiological responses of leaves and roots to the crop growth, recovery ability and final grain yield, the present research aimed to investigate the effects of different waterlogging durations (i.e., 14 and 35 days) at tillering on the growth and the grain yield of durum wheat, as well as to identify the main physiological traits involved in the response of roots, shoots, and leaves.

As the cultivar choice may represent a key factor in coping with waterlogging [5, 19], we compared the two durum wheat genotypes Svevo and Emilio Lepido. To the best of our knowledge the waterlogging tolerance of durum wheat cultivars currently cultivated in Italy has been previously studied only for Claudio and Svevo, which displayed very similar responses, as well [5]. In common wheat [49] Collaku and Harrison (2002) showed that high yielding genotypes were more affected by waterlogging. Thus, for the present research we selected two cultivars from those most cultivated in central Italy differing in cycle length and yielding capacity, assuming that Svevo could be less tolerant to waterlogging than Emilio Lepido due to its higher yielding capacity.

The waterlogging durations were chosen because in previous experiments we found that winter cereals exhibited grain yield reductions when waterlogging at tillering lasted for more than 16 days (barley) and 20 days (wheat and durum wheat) [5, 19, 51].

More specifically, our objectives were to assess the mechanism of response of the two durum wheat genotypes to 14 and 35 days of waterlogging at tillering, evaluating: (i) the

immediate impairment of root and shoot growth and related physiological and biochemical parameters, as well as water status; (ii) the ability to recover from the end of waterlogging up to maturity; (iii) the final grain yield.

2. Results

2.1. Meteorological Conditions

During the experiment (i.e. durum wheat cycle) total rainfall was 672 mm spread over about 80 rainy days, and mainly concentrated in the period December-February (Figure 1), as typical of the autumn-spring growing season in central Italy. Temperatures ranged from −3.1 °C to 33.9 °C (recorded in February and June, respectively), the daily mean temperature was was 10.4 °C during the waterlogging imposition, and 13.1 °C along the entire crop cycle, matching rather well with the historical data (1995-2020) for the site (13.0°C).

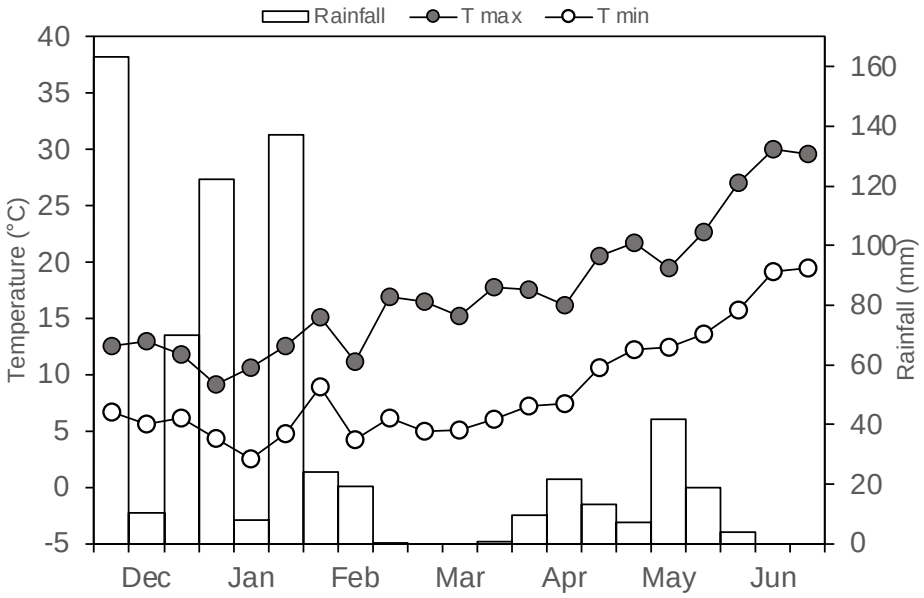

Figure 1. Air minimum (white dots) and maximum (black dots) temperatures and rainfall (bars) during the cropping season (December 2020-June 2021).

2.2. Plant phenology

Emilio Lepido started tillering only 2 days later than Svevo but the two cultivars reached flowering simultaneously (Table S1). Waterlogging slowed plant development, and plants of both cultivars waterlogged for the longest period (i.e., 35 days) reached flowering approximately one week later than controls. Conversely waterlogged and control plants of both cultivars achieved maturity concurrently.

2.3. Waterlogging immediate effects on physiological, biochemical and biometric parameters

Table 1 shows the effects of cultivar, waterlogging and their interaction on leaf and root parameters evaluated at 0, 14 and 35 days of waterlogging (DOW). At the beginning of the experiment (i.e., 0 DOW), relative water content (RWC), leaf total chlorophylls (Chl_{TOT}) and calcium ion (Ca^{2+}), leaf and root malondialdehyde (MDA), and shoot to root biomass ratio values were higher in Emilio Lepido than in Svevo, while maximum quantum efficiency of the photosystem II (PSII) photochemistry (F_v/F_m), chlorophyll a/b ratio (Chl a/b), de-epoxidation state (DEPS), leaf and root hydrogen peroxide (H_2O_2), leaf potassium ion (K^+), root Ca^{2+}, and shoot and root biomass levels were higher in Svevo (*data not shown*).

Fourteen DOW reduced CO_2 assimilation rate (A) and stomatal conductance (g_s) only in Svevo (-53 and -55%, respectively; throughout the whole text, percentages of waterlogging effects are calculated in comparison with the related controls), whereas 35 DOW reduced these parameters regardless of the cultivar (around -50%; Figure 2a,b). No waterlogging effects were reported on intercellular CO_2 concentration (C_i), and intrinsic water use efficiency (WUE_{in}) increased only in Svevo at 35 DOW (+35%; Figure 2c).

No waterlogging effects were reported on F_v/F_m. The PSII operating efficiency in light conditions (Φ_{PSII}) only decreased in Emilio Lepido at 35 DOW (-23%; Figure 2d). Differently, qP was equally reduced by both 14 and 35 DOW in both cultivars (-6%; Figure 2e), whereas non-photochemical quenching (qNP) was increased in both cultivars at 14 DOW (around +40%) and only in Emilio Lepido at 35 DOW (+40%; Figure 2f).

Similarly between cultivars, leaf water potential (Ψ_w) was not affected by waterlogging, regardless of duration, but leaf osmotic potential (Ψ_π) was reduced by 35 DOW (-17%; Figure 3a). RWC was reduced by 7% at 14 DOW and increased by 5% at 35 DOW (Figure 3b).

Total chlorophyll content was reduced by 14 DOW only in Svevo (-28%), whereas 35 DOW decreased Chl_{TOT} in both cultivars, more in Emilio Lepido than in Svevo (-47 and -31%, respectively. Figure 4a). Leaf chlorosis was also visible with the naked eye.

Total carotenoids (Car_{TOT}) were similarly reduced in both cultivars by 14 DOW (-27%) and even more by 35 DOW (-41%; Figure 4b). No waterlogging effects were reported on Chl a/b, β –carotene (β-car) and DEPS.

MDA and H_2O_2 accumulations were not observed in the leaves of both cultivars. Actually, leaf MDA levels were almost halved by both 14 and 35 DOW, and leaf H_2O_2 production was reduced of around 20% by 35 DOW (*data not shown*). Conversely, root MDA levels were increased by 14 DOW in both cultivars (almost doubled; Figure 5a), and root H_2O_2 contents were noticeably increased by 14 DOW only in Emilio Lepido (more than five-fold) and by 35 DOW in both cultivars (more than two-fold in Emilio Lepido and +63% in Svevo; Figure 5b).

Leaf K^+ content decreased by 14 DOW (-34% in both cultivars), whereas it was more decreased in Emilio Lepido than in Svevo by 35 DOW (-50 and -14%, respectively; Figure 6a). Leaf Ca^{2+} content increased only in Svevo at 14 DOW (+14%) and only in Emilio Lepido at 35 DOW (+47%; Figure 6b). Root K^+ content was reduced by 14 DOW only in Svevo (-23%), whereas it was similarly reduced by 35 DOW in both cultivars (-45%; Figure 6c). A reduction of root Ca^{2+} was observed only in Emilio Lepido at 35 DOW (-47%; Figure 6d).

The number of culms per plant was reduced in both cultivars by 14 DOW (-18 and -21% in Svevo and in Emilio Lepido, respectively), whereas it decreased only in Svevo with 35 DOW (-60%; Figure 7a). Shoot biomass was reduced in both cultivars by 14 DOW (-27%), whereas it was decreased by 35 DOW (-91% in Svevo and -33% in Emilio Lepido; Figure 7b). Conversely, although also root biomass was reduced at both 14 and 35 DOW (-62 and -86%, respectively), no differential waterlogging effects were observed between cultivars (Figure 7c). Shoot to root biomass ratio increased in both cultivars due to 14 DOW (almost doubled), whereas it increased only in Emilio Lepido due to 35 DOW (more than three-fold; Figure 7d).

The canonical discriminant analysis gave seven significant new canonical variables (Can; $P \leq 0.001$). Among the Cans, the first two (i.e., Can1 and Can2) accounted for 90.5% of the total variability (Table S1), thus indicating that the multivariate structure of the original variables (i.e., all the above-reported parameters collected at the end of waterlogging events) can be well represented by these Cans. All experimental groups were discriminated except for control and waterlogged Svevo plants at 14 DOW (Figure 8). Can1 mostly discriminated waterlogged plants of Emilio Lepido (exposed to both 14 and 35 DOW) from the others, especially from control plants of the same cultivar at the first time

of analysis. Can1 was strongly and positively correlated with qNP and root H_2O_2, while strongly and negatively correlated with Car_{TOT} and leaf K^+. Can2 mostly discriminated Svevo plants exposed to WL35 from the others, and it was strongly and positively correlated with WUE_{in} and strongly and negatively correlated with the number of culms.

Table 1. Results of two-way analysis of variance (ANOVA) for the effects of cultivar (*C*; degrees of freedom, df: 1), waterlogging (*WL*; df: 1) and their interaction (*C* × *WL*; df: 1) on physiological, water status, biochemical, and biometric parameters in wheat cultivars Emilio Lepido and Svevo subjected to 0, 14 or 35 days of waterlogging (DOW). Data are F values and *p* levels (***: $p \leq 0.001$, **: $p \leq 0.01$, *: $p \leq 0.05$, ns: $p > 0.05$). ND: not determinable (i.e., all plants having one culm per plant).

Parameter	0 DOW			14 DOW			35 DOW		
	C	*WL*	*C* × *WL*	*C*	*WL*	*C* × *WL*	*C*	*WL*	*C* × *WL*
A	0.26 ns	0.61 ns	0.04 ns	7.05 *	19.74 ***	5.78 *	0.15 ns	45.83 ***	0.56 ns
g_s	0.51 ns	0.01 ns	0.02 ns	3.54 ns	7.52 *	5.10 *	0.07 ns	42.04 ***	0.99 ns
C_i	4.38 ns	0.07 ns	0.00 ns	1.05 ns	0.46 ns	0.01 ns	3.18 ns	1.56 ns	0.20 ns
WUE_{in}	0.73 ns	0.11 ns	0.08 ns	0.07 ns	0.01 ns	4.78 *	19.86 ***	11.03 **	7.03 *
F_v/F_m	21.59 ***	0.02 ns	0.07 ns	0.03 ns	3.34 ns	0.04 ns	0.06 ns	1.19 ns	0.14 ns
Φ_{PSII}	0.26 ns	0.75 ns	0.69 ns	1.77 ns	1.07 ns	3.62 ns	24.77 ***	26.91 ***	7.62 *
qP	0.70 ns	0.07 ns	0.15 ns	0.23 ns	37.78 ***	1.42 ns	21.51 ***	9.10 *	2.11 ns
qNP	0.20 ns	0.25 ns	0.17 ns	3.43 ns	47.63 ***	0.26 ns	39.99 ***	29.36 ***	12.16 **
Ψ_w	2.40 ns	0.27 ns	0.27 ns	0.03 *	3.98 ns	0.05 ns	1.50 ns	1.50 ns	1.50 ns
Ψ_π	0.40 ns	0.42 ns	0.00 ns	0.01 ns	0.02 ns	3.05 ns	2.12 ns	18.41 **	4.18 ns
RWC	6.62 *	0.00 ns	0.15 ns	0.03 ns	5.11 *	0.11 ns	1.94 ns	5.74 *	3.75 ns
Chl_{TOT}	11.90 **	0.06 ns	0.28 ns	10.52 *	16.58 **	7.06 *	17.54 **	64.52 ***	8.65 *
Car_{TOT}	2.84 ns	0.05 ns	0.01 ns	4.64 ns	9.26 *	1.66 ns	0.00 ns	32.77 ***	4.20 ns
Chl a/b	149.67 ***	0.20 ns	0.03 ns	24.29 **	3.47 ns	4.27 ns	2.17 ns	1.92 ns	0.61 ns
β-car	4.52 ns	0.02 ns	0.00 ns	16.02 **	1.87 ns	0.45 ns	1.50 ns	1.98 ns	0.64 ns
DEPS	647.98 ***	0.01 ns	0.14 ns	2.45 ns	2.90 ns	3.99 ns	1.80 ns	3.24 ns	0.80 ns
Leaf MDA	139.95 ***	0.00 ns	0.12 ns	2.25 ns	24.71 **	1.08 ns	63.41 ***	16.66 **	4.08 ns
Leaf H_2O_2	329.04 ***	0.06 ns	0.00 ns	0.76 ns	2.38 ns	1.39 ns	369.70 ***	14.28 **	2.41 ns
Root MDA	883.67 ***	0.77 ns	0.43 ns	3.06 ns	114.77 ***	1.13 ns	60.53 ***	1.32 ns	0.82 ns
Root H_2O_2	5.68 *	0.75 ns	0.45 ns	0.86 ns	91.29 ***	42.98 ***	97.88 ***	569.87 ***	5.39 *
Leaf K^+	8.31 *	0.54 ns	1.00 ns	17.71 **	473.36 ***	3.66 ns	10.36 *	127.35 ***	44.91 ***
Leaf Ca^{2+}	29.00 ***	1.27 ns	0.72 ns	148.98 ***	26.72 ***	41.21 ***	7.09 *	37.48 ***	53.49 ***
Root K^+	1.50 ns	0.00 ns	0.68 ns	6.12 *	0.64 ns	25.33 **	34.83 ***	214.65 ***	3.13 ns
Root Ca^{2+}	57.15 ***	0.62 ns	0.01 ns	99.30 ***	1.88 ns	0.47 ns	0.81 ns	108.80 ***	69.69 ***
Culms	ND	ND	ND	0.62 ns	13.41 **	0.01 ns	10.76 *	35.98 ***	32.51 ***
Shoot biomass	25.86 ***	0.00 ns	0.00 ns	27.39 ***	24.53 **	0.16 ns	21.77 **	58.30 ***	10.09 *
Root biomass	27.08 ***	0.00 ns	0.00 ns	3.71 ns	73.85 ***	0.39 ns	3.18 ns	28.57 ***	0.27 ns
Shoot to root biomass	18.88 **	0.00 ns	0.00 ns	0.86 ns	148.43 ***	7.43 *	0.097 ns	55.97 ***	10.04 *

Parameter abbreviations: A, CO_2 assimilation rate; g_s, stomatal conductance; C_i, intercellular CO_2 concentration; WUE_{im}, intrinsic water use efficiency (i.e., A/g_s); F_v/F_m, maximum quantum efficiency of the photosystem II (PSII) photochemistry; Φ_{PSII}, PSII operating efficiency in light conditions; qP, photochemical quenching; qNP, non-photochemical quenching; Ψ_w, leaf water potential; Ψ_π, leaf osmotic potential; RWC, relative water content; Chl_{TOT}, total chlorophylls; Car_{TOT}, total carotenoids; Chl a/b, chlorophyll a/b ratio; β-car, β –carotene; DEPS, de-epoxidation state; MDA, malondialdehyde; H_2O_2, hydrogen peroxide; K^+, potassium ion; Ca^{2+}, calcium ion.

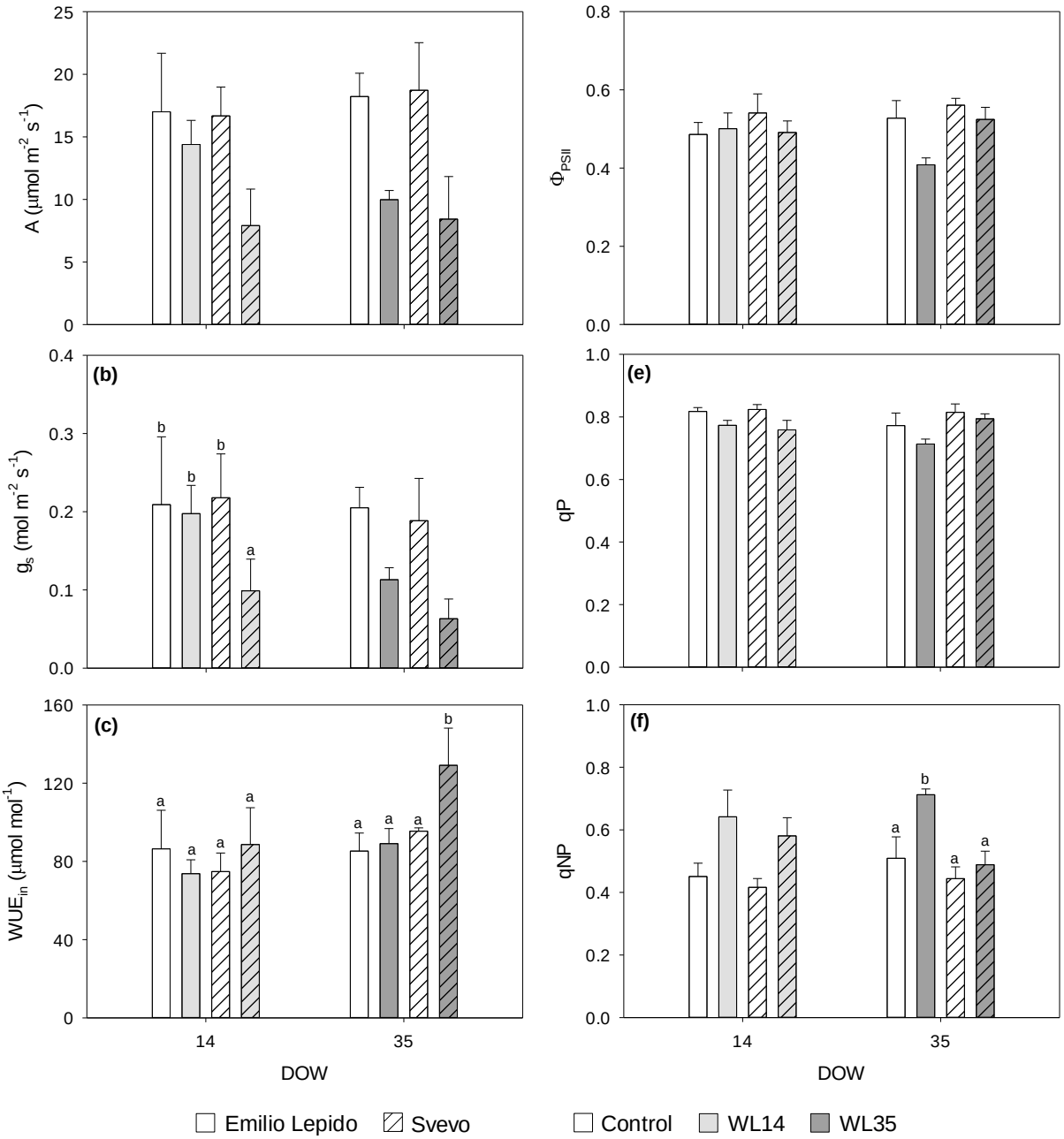

Figure 2. (**a**) CO_2 assimilation rate (A), (**b**) stomatal conductance (g_s), (**c**) intrinsic water use efficiency (WUE_{in}), (**d**) PSII operating efficiency in light conditions (Φ_{PSII}), (**e**) photochemical quenching (qP), and (**f**) non-photochemical quenching (qNP) in the durum wheat cultivars Emilio Lepido (solid) and Svevo (pattern) subjected to 0 (i.e., controls; white), 14 (i.e., WL14; light gray) or 35 (i.e., WL35; dark gray) days of waterlogging (DOW). Data are shown as mean ± standard deviation. For each waterlogging duration, in case two-way ANOVA reveals a significant cultivar × waterlogging interactive effect on the specific parameter (see Table 1), according to Tukey's *post hoc* test, different letters indicate significant differences among means ($p \leq 0.05$).

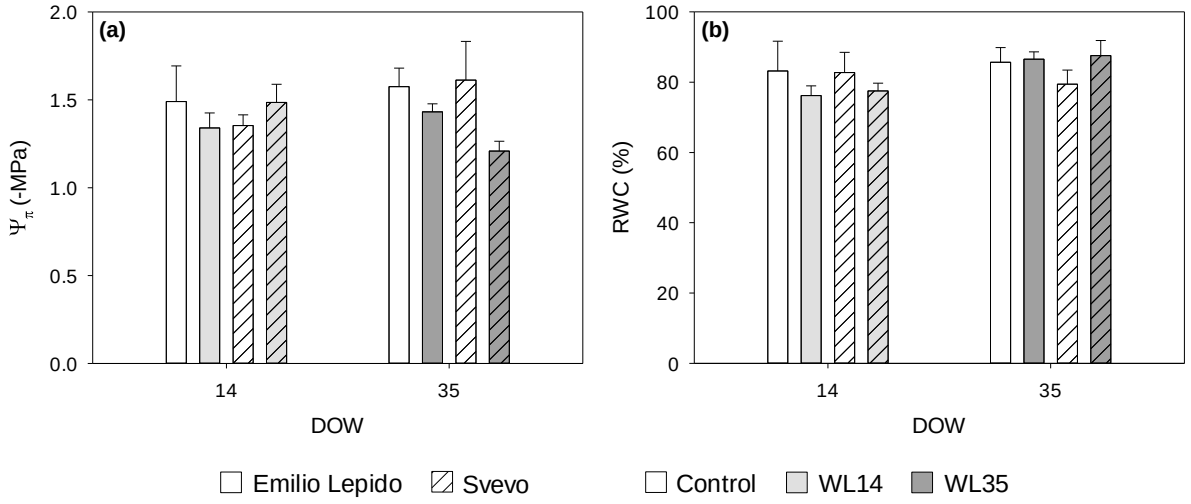

Figure 3. (**a**) Leaf osmotic potential (Ψ_π), and (**b**) leaf relative water content (RWC) of the durum wheat cultivars Emilio Lepido (solid) and Svevo (pattern) subjected to 0 (i.e., controls; white), 14 (i.e., WL14; light gray) or 35 (i.e., WL35; dark gray) days of waterlogging (DOW). Data are shown as mean ± standard deviation.

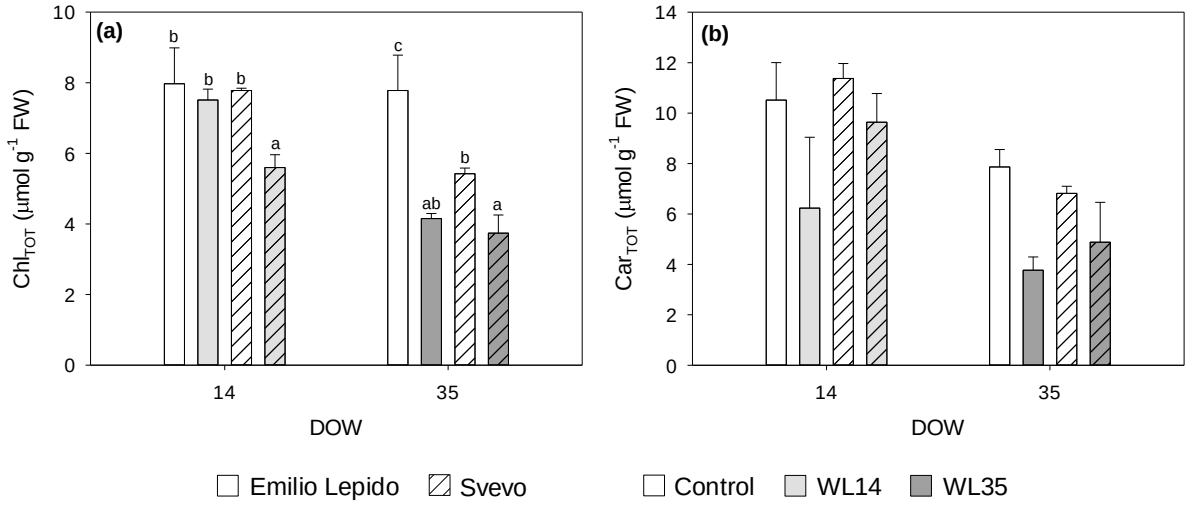

Figure 4. (**a**) Total chlorophyll (Chl_{TOT}), and (**b**) total carotenoid (Car_{TOT}) contents in leaves of the durum wheat cultivars Emilio Lepido (solid) and Svevo (pattern) subjected to 0 (i.e., controls; white), 14 (i.e., WL14; light gray) or 35 (i.e., WL35; dark gray) days of waterlogging (DOW). Data are shown as mean ± standard deviation. For each waterlogging duration, in case two-way ANOVA reveals a significant cultivar × waterlogging interactive effect on the specific parameter (see Table 1), according to Tukey's *post hoc* test, different letters indicate significant differences among means ($p \leq 0.05$). FW: fresh weight.

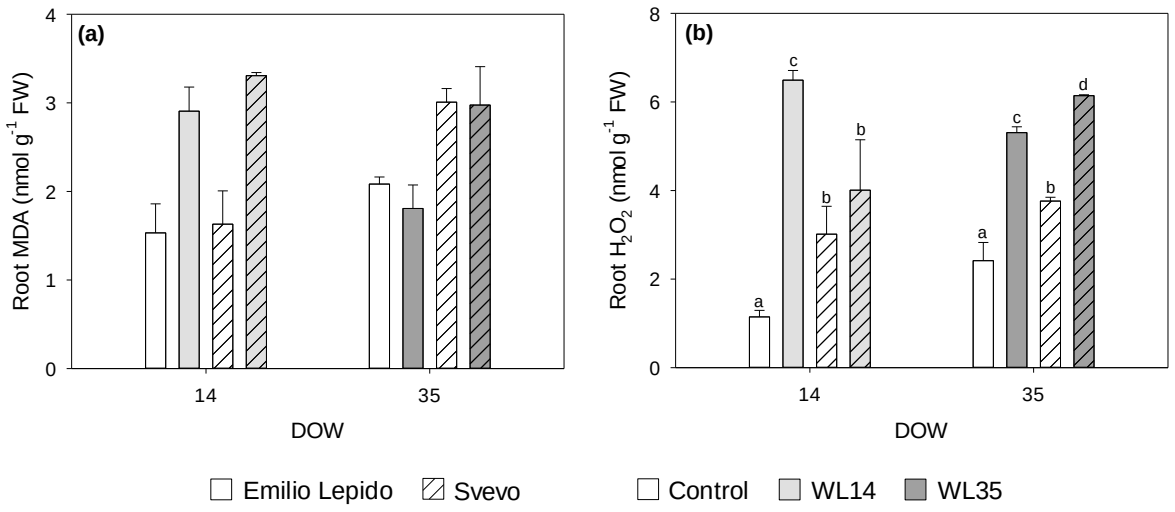

Figure 5. (**a**) Malondialdehyde (MDA), and (**b**) hydrogen peroxide (H_2O_2) contents in roots of the durum wheat cultivars Emilio Lepido (solid) and Svevo (pattern) subjected to 0 (i.e., controls; white), 14 (i.e., WL14; light gray) or 35 (i.e., WL35; dark gray) days of waterlogging (DOW). Data are shown as mean ± standard deviation. For each waterlogging duration, in case two-way ANOVA reveals a significant cultivar × waterlogging interactive effect on the specific parameter (see Table 1), according to Tukey's *post hoc* test, different letters indicate significant differences among means ($p \leq 0.05$). FW: fresh weight.

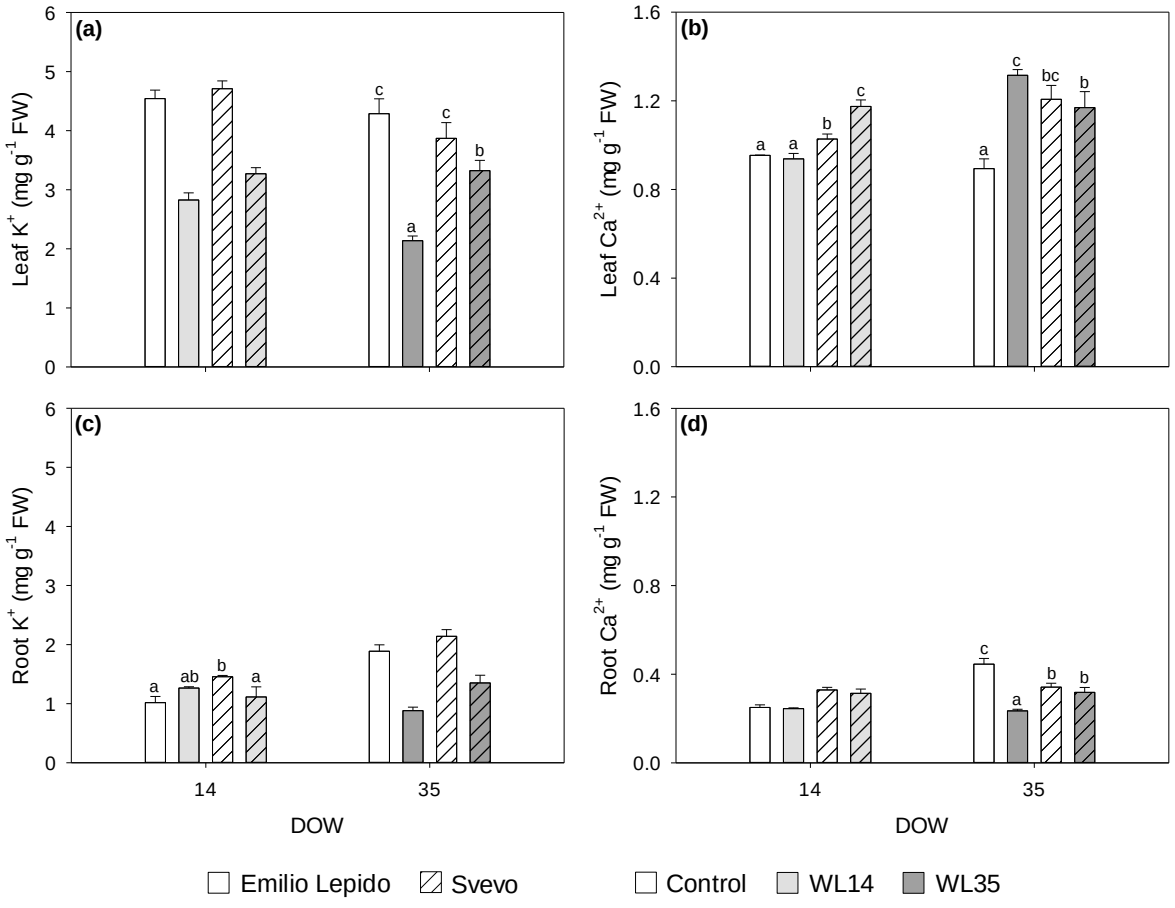

Figure 6. (**a**) Leaf K^+, (**b**) leaf Ca^{2+}, (**c**) root K^+, and (**d**) root Ca^{2+} contents of the durum wheat cultivars Emilio Lepido (solid) and Svevo (pattern) subjected to 0 (i.e., controls; white),14 (i.e., WL14; light gray) or 35 (i.e., WL35; dark gray) days of waterlogging (DOW). Data are shown as mean ± standard deviation. For each waterlogging duration, in case two-way ANOVA reveals a significant cultivar × waterlogging interactive effect on the specific parameter (see Table 1), according to Tukey's *post hoc* test, different letters indicate significant differences among means ($p \leq 0.05$). FW: fresh weight.

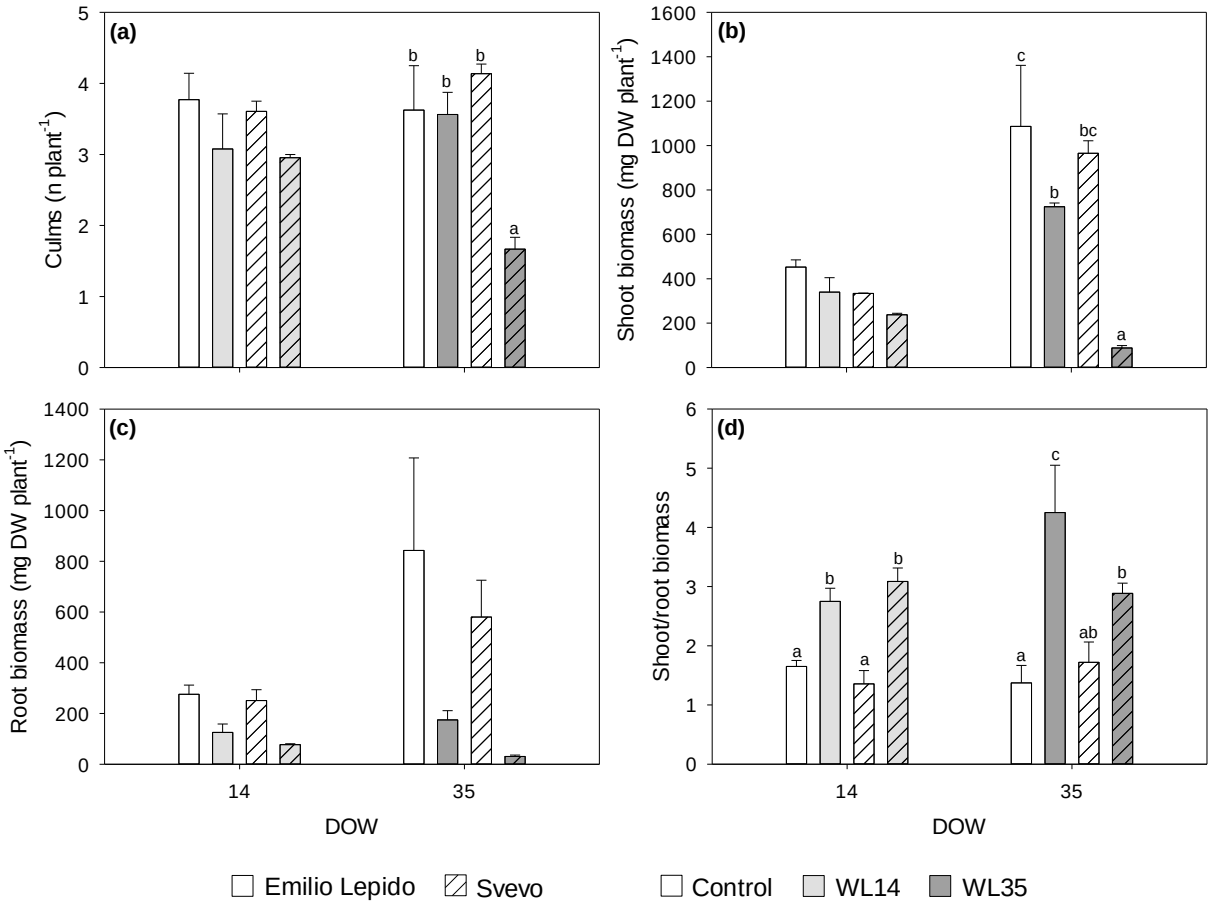

Figure 7. (**a**) Number of culms per plant, (**b**) shoot biomass, (**c**) root biomass, and (**d**) shoot to root ratio (shoot/root biomass) of the durum wheat cultivars Emilio Lepido (solid) and Svevo (pattern) subjected to 0 (i.e., controls; white), 14 (i.e., WL14; light gray) or 35 (i.e., WL35; dark gray) days of waterlogging (DOW). Data are shown as mean ± standard deviation. For each waterlogging duration, in case two-way ANOVA reveals a significant cultivar × waterlogging interactive effect on the specific parameter (see Table 1), according to Tukey's *post hoc* test, different letters indicate significant differences among means ($p \leq 0.05$). DW: dry weight. See Table 1 for parameter abbreviations.

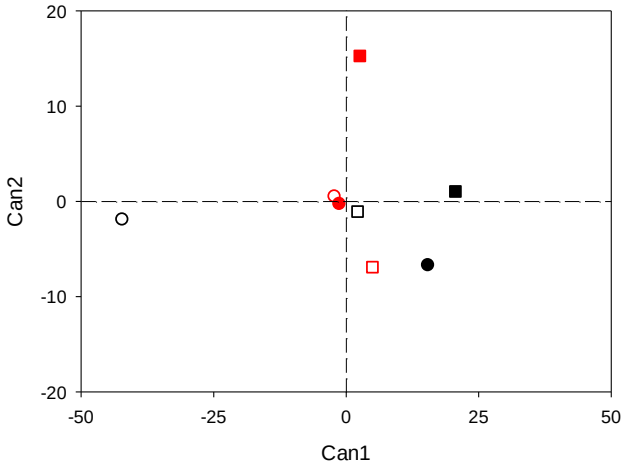

Figure 8. Discrimination of cultivar (Emilio Lepido, black; Svevo, red), waterlogging treatment (control, open; waterlogged, closed) and waterlogging duration (14 days, circle; 35 days, square) on the basis of canonical discriminant analysis applied to the full set of parameters collected at the end of waterlogging treatments on the durum wheat cultivars. The first two canonicals are shown (Can1 and Can2).

2.4. Waterlogging effects during recovery at physiological level

Table 2 shows the effects of cultivar, waterlogging and their interaction on physiological and water status parameters collected during the recovery period (i.e., 70 days from the beginning of waterlogging). No detrimental effects due to waterlogging were reported on gas exchange and chlorophyll *a* fluorescence. Actually, A increased in Emilio Lepido subjected to waterlogging for 14 days (WL14, +53%), g_s increased in both cultivars subjected to both WL14 and WL35, by around 35%.

C_i increased in both cultivars subjected only with WL35 (+7%), Φ_{PSII} and qP were higher in Emilio Lepido subjected to WL35 (+14 and +11%), and qNP was lower in Emilio Lepido subjected to WL35 (-26%) as well as in Svevo subjected to both WL14 and WL35 (-24% and -37%, respectively). Nevertheless, similarly between cultivars, WUE_{in} was lower in plants subjected to WL35 (-18%).

Table 2. Physiological parameters of the durum wheat cultivars Emilio Lepido and Svevo at recovery (70 days from the beginning of waterlogging), and previously subjected to 0, 14 or 35 days of waterlogging (C, WL14, and WL35, respectively). F values and *p* levels (***: $p \leq 0.001$, **: $p \leq 0.01$, *: $p \leq 0.05$, ns: $p > 0.05$) of two-way analysis of variance (ANOVA) for the effects of cultivar (*C*; degrees of freedom, df: 1), waterlogging (*WL*; df: 2) and their interaction (*C* × *WL*; df: 2) on parameters are shown. In case two-way ANOVA reveals a significant *C* × *WL* interactive effect on the specific parameter, according to Tukey's *post hoc* test, different letters indicate significant differences among means ($p \leq 0.05$).

Parameter	Emilio Lepido			Svevo			*ANOVA*		
	C	**WL14**	**WL35**	**C**	**WL14**	**WL35**	***C***	***WL***	***C × WL***
A	9.3±0.8 a	14.2±1.4 c	9.7±0.1 a	9.9±0.7 ab	12.5±0.2 bc	13.2±2.3 c	2.75 ns	19.83 ***	9.58 ***
g_s	0.13±0.02	0.18±0.02	0.18±0.04	0.16±0.03	0.20±0.00	0.24±0.03	13.50 **	14.29 ***	1.60 ns
C_i	262±2	251±5	285±19	275±23	278±1	289±6	7.88 *	6.88 **	1.73 ns
WUE_{in}	73±3	77±1	57±13	64±15	62±1	55±3	6.70 ns	6.10 **	1.30 ns
F_v/F_m	0.78±0.01	0.79±0.01	0.80±0.00	0.78±0.00	0.79±0.02	0.78±0.01	2.02 ns	0.77 ns	1.77 ns
Φ_{PSII}	0.56±0.01 a	0.56±0.03 a	0.64±0.01 c	0.59±0.01 ab	0.60±0.02 bc	0.62±0.03 bc	4.23 ns	21.19 ***	7.50 **
qP	0.80±0.01 ab	0.80±0.02 a	0.89±0.01 c	0.85±0.00 bc	0.85±0.01 bc	0.87±0.05 c	8.41 **	17.95 ***	6.91 **
qNP	0.46±0.00 b	0.46±0.06 b	0.34±0.02 a	0.46±0.01 b	0.35±0.04 a	0.29±0.03 a	13.39 **	34.38 ***	5.56 *

2.5. Waterlogging long-lasting effects on final grain yield

Table 3 shows the effects of cultivar, waterlogging and their interaction on biometric and yield parameters collected at physiological maturity (i.e., 125 days from the beginning of waterlogging lasted 14 or 35 days). Both Emilio Lepido and Svevo plants that had previously been subjected to WL35 showed a reduced number of culms (-29%), whereas no effects were observed on the number of spikes. Grain yield reduction was shown only in Svevo (-45 and -64% due to WL14 and WL35, respectively). Vegetative above-ground part was reduced by both WL14 and WL35 (-31 and -44%, respectively) without differences between the two cultivars, whereas the root biomass was reduced only by WL35 (-33% for Svevo and -42% for Emilio Lepido).

Table 3. Number of culms and spikes per plant (n plant^{-1}), grain yield (g plant^{-1}), and vegetative above-ground part (VAP) and root biomass (g dry weight plant^{-1}) of the durum wheat cultivars Emilio Lepido and Svevo at maturity (125 days from the beginning of waterlogging), and previously subjected to 0, 14 or 35 days of waterlogging (C, WL14 and WL35, respectively). F values and *p* levels (***: $p \leq 0.001$, **: $p \leq 0.01$, *: $p \leq 0.05$, ns: $p > 0.05$) of two-way analysis of variance (ANOVA) for the effects of cultivar (*C*; degrees of freedom, df: 1), waterlogging (*WL*; df: 2) and their interaction (*C* × *WL*; df: 2) on parameters are shown. In case two-way ANOVA reveals a significant *C* × *WL* interactive effect on the specific parameter, according to Tukey's *post hoc* test, different letters indicate significant differences among means ($p \leq 0.05$).

Parameter	Emilio Lepido			Svevo			*ANOVA*		
	C	**WL14**	**WL35**	**C**	**WL14**	**WL35**	***C***	***WL***	***C × WL***
Culms	3.2±0.3	3.3±0.3	2.4±0.2	4.0±1.2	3.1±0.3	2.6±0.1	0.34 ns	0.02 *	1.10 ns
Spikes	2.6±0.0	2.3±0.5	1.8±0.2	2.3±0.9	1.9±0.1	2.0±0.4	0.48 ns	2.61 ns	0.87 ns

Grain yield	2.5±0.1 bc	2.2±0.7 b	1.7±0.2 ab	3.3±0.3 c	1.8±0.2 ab	1.2±0.2 a	0.20 ns	27.45 ***	6.24 *
VAP biomass	5.1±0.3	3.9±1.1	3.4±0.3	5.6±0.6	3.4±0.5	2.6±0.1	0.39 ns	27.70 ***	2.26 ns
Root biomass	1.17±0.14	1.05±0.31	0.78±0.01	0.93±0.24	0.60±0.10	0.54±0.25	10.59 **	5.73 *	0.55 ns

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3. Discussion

Few studies have evaluated the impact of waterlogging on durum wheat [5, 20] and to the best of our knowledge, there is no comprehensive research on the impact of waterlogging throughout the entire crop cycle, describing responses in vegetative growth and final grain production. The present study provides a comprehensive evaluation of the mechanism of response of two cultivars of durum wheat to different waterlogging durations through an integrated analysis of an array of physiological, biochemical, biometric and yield parameters, together with water status, collected at the end of the waterlogging events, during recovery, and at maturity (i.e, BBCH 99).

Our results confirmed that a large variation in wheat responses to waterlogging exists, depending on different durations of stress conditions and on diverse genotypic sensitivity [4]. Photosynthesis decreased due to 14 DOW only in Svevo, suggesting a higher waterlogging sensitivity of this cultivar, compared with Emilio Lepido, whereas CO_2 assimilation rate was similarly impaired between cultivars by the longer 35 DOW. These photosynthetic impairments were clearly due to stomatal limitations (i.e., g_s showed the same trends as A), suggesting an isohydric behavior of both cultivars [21], while mesophyll impairments were less evident since C_i did not accumulate. The interpretation of a minor occurrence of non-stomatal limitations of photosynthesis was supported by the absence of PSII photodamage (i.e., unchanged F_v/F_m), as well as by the slight reduction of qP similarly reported between cultivars and for different waterlogging durations. No waterlogging effects on F_v/F_m (i.e., the most widely used photo-oxidative stress marker [22]) were already reported in common wheat [17]. Interesting and unexpected was the increase of WUE_{in} observed at 35 DOW only in Svevo (as also highlighted by the strong and positive correlation of this parameter with Can2, which strongly discriminated these plants from the others). This parameter is largely used in the selection of cultivars with high capacity of adaption and high yield in crop breeding projects [23, 24]. Our findings indicated that Svevo likely adopted a better strategy to regulate the use of water in attempt to cope with the longer waterlogging duration. Actually, a reduction of PSII performance (i.e., reduced Φ_{PSII}), together with an activation of the dissipation of the excess excitation energy as heat (i.e., increased qNP), were observed only in Emilio Lepido at 35 DOW (qNP, together with root H_2O_2, was positively and strongly correlated with Can1, which discriminated Emilio Lepido plants exposed to WL14 and even more those subjected to WL35 from the others), confirming that also this cultivar was not able to tolerate oxygen deprivation so long (potentially even less than Svevo at physiological level).

As paradoxical as it may sound, waterlogging often reduces water availability to plants [25]; this process is mainly caused by reduced stomatal conductance due to an increased abscisic acid accumulation [26], and reduced root hydraulic conductance [27]. Leaf RWC of both the investigated cultivars was reduced by 14 DOW, even if Ψ_w was never affected by waterlogging treatments. Conversely, leaf RWC resulted slightly increased by 35 DOW; this was likely due to an osmotic adjustment (i.e., reduced Ψ_π) adopted by the crop to maintain turgor and cell volume under such detrimental conditions. The importance of osmotic adjustment to improve drought tolerance in plants is notorious [28]; the present study confirms that this process may deserve more interest also in terms of plant responses to waterlogging [29]. Overall, the water status parameters confirmed the variation in response of durum wheat to different durations of waterlogging. On the contrary, these parameters did not highlight cultivar-specific differences, which were instead markedly pointed out by the biochemical ones.

During waterlogging, factors such as decreases in chlorophyll or other components of the photosynthetic apparatus, as a result of nitrogen deficiency and/or negative feedback from carbohydrate accumulation, have been reported as possible causes of reduced CO_2 fixation. In some conditions, disturbance to cation homeostasis (e.g., K^+ and Ca^{2+}) and possible damage of leaves from ROS or phytotoxins (e.g., Fe^{2+} or Mn^{2+}) might also contribute [4, 25, 30]. Actually, the above-mentioned impairment of the leaf gas exchange was in accordance with the overall reduction of photosynthetic pigments (i.e., Chl_{TOT} and Car_{TOT})

which play a crucial role in light harvesting for photosynthesis. The degradation of chlorophyll and carotenoids was already reported in plants exposed to waterlogging [e.g. 31], as well as to other environmental stressors [e.g. 32, 33], signifying that the chloroplast ultrastructure and photosynthetic pigments were impaired. No additional variations in leaf pigment parameters were observed due to waterlogging, indicating that leaf photoprotective mechanisms such as changing Chl a/b ratio and β-car, and increasing DEPS levels [34] have not been activated. This phenomenon was likely due to the absence of a harsh oxidative pressure induced by waterlogging at leaf level, as suggested by the above-mentioned unchanged F_v/F_m, and also confirmed by the lack of accumulation of leaf MDA (one of the major indicators of cell membrane damage) [35]. This appears a scenario completely different to the one observed at the root level.

Although it has been largely reported that roots are the plant organs mostly affected by waterlogging [4, 25], the present study pioneering demonstrated that increased oxidative pressure and accumulation of H_2O_2 occurred in the roots of waterlogged durum wheat. This outcome confirms the importance of evaluating also the belowground responses to fully elucidate the effects of waterlogging on plants. An increased lipid peroxidation was reported in the roots of both cultivars subjected to 14 DOW, although an accumulation of root H_2O_2 occurred only in Emilio Lepido. Despite root MDA accumulation was not reported at 35 DOW, a strong accumulation of H_2O_2 occurred in roots of both Emilio Lepido and Svevo subjected to the longer waterlogging (as stated above, root H_2O_2 was strongly and positively correlated with Can2, which discriminated Svevo plants exposed to WL35 from the others). Excessive MDA accumulation commonly indicates cell membrane damage, which leads to a series of negative physiological and biochemical events, including reduced photosynthesis [36]. Increased H_2O_2 production is one of the hallmarks of the low oxygen stress signal [25, 37], as well as of other stress signals [38, 39]. The elucidation of these differential responses in terms of lipid peroxidation and H_2O_2 accumulation reported between cultivars and waterlogging durations undoubtedly needs and suggests further research (the lack of root MDA increase at 35 DOW was particularly unexpected). However, this phenomenon was likely due to the activation/depression of enzymatic and non-enzymatic antioxidants, adopted by plants to regulate the stress response and signaling [4, 40]. Among antioxidants, the key role of phenylpropanoids in the response of durum wheat to waterlogging has been indicated by [10].

Such differential responses appeared also linked to cultivar- and waterlogging duration-specific regulations of membrane transporters, which were investigated at both leaf and root levels. Membrane transporters are known to play a crucial role in mediating adaptive responses to oxygen deprivation and waterlogging, especially at root level [25]. Specifically, under such detrimental conditions root K^+ uptake is commonly and markedly reduced [41, 42], so the ability of roots to maintain cytosolic K^+ homeostasis and K^+ channel functionality was named as an essential component of plant acclimation to hypoxia [43]. Conversely, hypoxia commonly induces a rapid elevation in the cytosolic Ca^{2+} concentration in plant cells [25, 44]. In addition, under waterlogging, the energy stored in roots can be reduced by inhibiting the active transport of these ions to other organs [36]. The present responses of durum wheat in terms of root K^+ contents were fully in accordance with the above-mentioned reductions of CO_2 assimilation rate observed only in Emilio Lepido at 14 DOW and in both cultivars at 35 DOW; whereas leaf K^+ contents decreased in both cultivars, regardless of waterlogging duration (leaf K^+, together with Car_{TOT}, was strongly and negatively correlated with Can1, which discriminated Emilio Lepido plants subjected to WL14 and even more those exposed to WL35 from the others) . An elevation of Ca^{2+} contents was instead observed only in leaf tissue, specifically in Svevo at 14 DOW and in Emilio Lepido at 35 DOW, indicating that waterlogging disturbed not only the mineral uptake, but also transport of ions to aerial organs that might have impaired the stomatal conductance and negatively affected the CO_2 fixation, translocation, and utilization of assimilate. Our findings corroborate those of [46], who found that stress induced production of ROS results in anomalies in several important cellular biochemical pathways/reactions.

These mechanisms operate in cellular organelles like chloroplast and mitochondria activating Ca^{2+} and K^{+} permeable cation channels at the plasma membrane; thereby they also mediate Ca^{2+} based signaling events, and K^{+} ion leakage. These outcomes not only confirm the importance of cation homeostasis in waterlogging response, but also the higher physiological sensitivity of Svevo reported at 14 DOW and the inability of both cultivars (Emilio Lepido results more sensitive in terms of WUE_{in} and PSII performance) to tolerate the longer oxygen deprivation (i.e., 35 DOW).

These differential physiological, water status and biochemical responses were only partially confirmed by biometric parameters. The different responses between cultivars at 14 DOW were not accordingly highlighted by biomass production since the number of culms, shoot and root biomass, and shoot to root biomass ratio were similarly affected in both Emilio Lepido and Svevo. In particular, waterlogging induced different biomass distribution regardless of the cultivar. Conversely, number of culms and shoot biomass indicated a higher sensitivity of Svevo at 35 DOW, suggesting that the strategy adopted by this cultivar in terms of WUE_{in} and preservation of PSII performance was not successful in terms of biomass production. Root biomass of the two cultivars was similarly impaired by 35 DOW, confirming that root dry weights significantly decrease with waterlogging longer than 20 days [5]. Yet, growth of roots and leaves are coordinated, and their relative sizes vary dynamically in response to environmental conditions, to optimize the utilization of assimilates and other resources [47]. Thus, the increased shoot to root biomass ratio of Emilio Lepido exposed to 35 DOW highlighted that, similarly to common wheat [48], also the root growth of durum wheat is inhibited more than shoot growth, as the adventitious root growth could not fully compensate for loss of seminal roots [11].

Although the detrimental effects due to waterlogging events on photosynthesis and PSII performance were no longer detectable at recovery, this phenomenon was not due to an ability of durum wheat to recover its optimal physiological functioning (it is interesting to note that at this time WUE_{in} was reduced in both cultivars previously subjected to 35 DOW), instead it was due to a mismatch between the developmental stages of control and waterlogged plants, i.e., controls were closer to maturity and thus lowered the photosynthetic process. We can thus infer that the plant growth had been slowed down by prolonged water excess, as similarly demonstrated in other winter cereals by [16, 17].

The above-mentioned damages could not be recovered and definitively compromised final biomass production and grain yield, as shown by our outcomes at physiological maturity. Grain yield of both cultivars revealed greater reduction with longer WL duration (i.e., 35 DOW), corroborating our previous results with waterlogging imposed at tillering in durum wheat that displayed differences yield losses related to waterlogging duration [5]. On the other hand, the same authors [5] also reported a significant reduction in grain yield of the durum wheat two cultivars Claudio and Svevo only when waterlogging at tillering was prolonged to more than 20 days. Our present results only partially confirmed those outcomes: this was true only for Emilio Lepido, while Svevo showed significant decrease in grain yield with both WL durations. The mean temperatures experienced throughout the 35 days of waterlogging were, in this experiment, about 10 °C, whereas they were less than 6 °C in our previous research into wheat [5]. Thus, higher temperatures during waterlogging can be responsible for the different behavior of Svevo, further confirming that effects on winter cereals can greatly vary due to meteorological conditions.

From an agronomic point of view, plant tolerance to waterlogging involves the maintenance of a relatively high grain yield under waterlogged relative to non-waterlogged conditions. Accordingly, our findings clearly showed that Emilio Lepido was more tolerant to waterlogging whereas Svevo was more sensitive even with a waterlogging duration shorter than 20 days.

To the best of our knowledge any other cultivar from Claudio and Svevo has been investigated for agronomic waterlogging tolerance [cit]. However, in common wheat high

yielding genotypes were more affected by waterlogging than lower yielding types, because they were not able to maintain high tillering as showed by [49].

Our findings corroborated their hypothesis, also for durum wheat, because Svevo was more productive in well-drained conditions and had more culm per plant, as compared to Emilio Lepido. Moreover, Svevo has been proved to have higher allocation of biomass in roots during vegetative growth and post-heading dry matter accumulation [50]. The fact that the number of culms and root biomass in Svevo were more intensely restrained by waterlogging (number of culms was positively and negatively correlated with Can2, which discriminated Svevo plants expoted to WL35 from the others), further confirmed this hypothesis.

4. Materials and Methods

4.1. Experimental site characteristics

The research was carried out from December 2020 to June 2021 at the field station of the Department of Agriculture, Food and Environment of the University of Pisa, Italy (43° 40′ N, 10° 19′ E, 1 m a.s.l). The climate of the area is hot-summer Mediterranean (Csa) with mean annual maximum and minimum daily air temperatures of 20.2 and 9.5 °C respectively, and a mean rainfall of 971 mm per year. Daily air minimum and maximum temperatures and rainfall were recorded throughout the entire period of the research by an automatic meteorological station located close to the experimental site.

*4.2. Experimental design and crop management*The experimental design consisted of two durum wheat cultivars exposed to 14 and 35 days of waterlogging (DOW) at the tillering stage, compared to well-drained controls (C). We used the two commercial cultivars Svevo and Emilio Lepido.

Svevo is a very early maturing cultivar that was released in 1996 from the genealogy CIMMYT line/Zenit and is high yielding.

Emilio Lepido is a more modern cultivar, early maturing and was released in 2011 from the genealogy Orobel//Arcobaleno/Svevo, resistant to cold temperatures. Both have a good resistance to lodging.Plants were grown in 16-L pots made from polyvinyl chloride (PVC) tubes (80 cm long and 16 cm in diameter) fitted with a PVC base. A 30-mm diameter hole was drilled in the bottom of each pot, which was fitted with a 0.9-mm mesh to contain roots and substrate loss. Pots were filled with a sandy-loam soil collected from an adjacent field that was previously cultivated with rapeseed. Main soil properties were: 55.3% sand (2 mm < ∅ < 0.05 mm), 33.8% silt (0.05 mm < ∅ < 0.002 mm), 10.9% clay (< 0.002 mm), 7.6 pH, 0.7 g kg^{-1} total nitrogen (Kjeldahl method), 4.5 mg kg^{-1} available P (Olsen method), and 68.9 mg kg^{-1} available K ($BaCl_2$-TEA method). The crop was sown on 15 December 2020, within the optimum sowing time for winter cereal production in Central Italy. After emergence, the seedlings were thinned to eight plants per pot, corresponding to 400 plants m^{-2}. Phosphorus and potassium were applied pre-planting as triple mineral phosphate and potassium sulfate, at the rates of 150 kg ha^{-1} of P_2O_5 and K_2O. Nitrogen was applied at the rate of 150 kg N ha^{-1}, and split into three applications at sowing, at pseudo-stem erection (BBCH 30), and at first node detectable (BBCH 31) as urea, in the following proportions: 30–60–60 kg N ha^{-1}. The rate of mineral N supply was the recommended value for optimal durum wheat production in Central Italy, and the adopted splitting management was proved to be an optimal mineral fertilization practice to ensure both production quantity and quality in the Mediterranean climate [51]. Throughout the experiment, phenological phases were recorded using the BBCH scale for cereals [52] to determine the timing of WL imposition, N applications and harvest. Weed control was performed by hand hoeing, and no pesticide application was needed. The crop was irrigated from flowering to maturity to prevent drought stress, with a total of 200 mm of water applied. Pots were placed outdoors and kept under drained conditions until plants reached the tillering stage (BBCH 20) on 24 February 2021, when a half of the pots were maintained in well-

drained conditions (C- controls), and the other half were exposed to waterlogging by placing pots into containers (2 × 1 × 1 m) filled with water. A layer of 1 cm of free water was maintained above the soil surface throughout the period of waterlogging, to ensure that the soil was completely saturated by water. Three replicate pots were used for all combinations of treatments.

For each cultivar, at waterlogging imposition (0 DOW – 24 February 2021) three replicate pots were harvested to determine biomass and physiological characteristics before waterlogging imposition. At the end of each period of WL - that is after two and five weeks (14 and 35 DOW) - all plants of three waterlogged pots (WL) and three well-drained pots (C) were measured for physiological and biochemical parameters (they were performed on the second and third upper and fully expanded leaves). . Other three pots per cultivar were moved from the container filled with water to drained conditions. These pots (WL pots to be measured at maturity) were supplied with the scheduled top-dressing N fertilization and kept in drained conditions until plants reached maturity. Control pots received N at the same time of the WL pots. Additional measurements of physiological and water status parameters were carried out during the recovery period, at 70 days after the beginning of WL (i.e. 56 and 35 days after the end of WL, respectively for waterlogging prolonged 14 and 35 days), to assess the water status and the physiological activities of control and waterlogged plants. At maturity, three WL and three C pots for each cultivar were harvested to assess final biomass and grain yield production.

4.3. Plant measurements

4.3.1. Leaf gas-exchange and chlorophyll *a* fluorescence

The CO_2 assimilation rate (A), stomatal conductance (g_s) and intercellular CO_2 concentration (C_i) were determined using a LI-6400 portable photosynthesis system equipped with a 2 × 3 cm chamber and a 6400-02B LED light source (Li-COR Inc., Lincoln, NE, USA), operating at 400 ppm CO_2 concentration, 25 ± 2 °C of leaf temperature, 45 ± 5 % of RH, 1.8 ± 0.2 kPa of VPD and saturating light conditions (1500 μmol m^{-2} s^{-1} PAR). Intrinsic water use efficiency (WUE_{in}) was calculated as A/g_s.

After a 40 min dark-adaptation of leaves (same used for leaf gas-exchange measurements), the maximum quantum efficiency of the photosystem II (PSII) photochemistry (F_v/F_m), the PSII operating efficiency in light conditions (Φ_{PSII}), the photochemical quenching (qP), and the non-photochemical quenching (qN) were determined by a PAM-2000 chlorophyll *a* fluorometer (Walz, Effeltrich, Germany), set as reported by [53].

4.3.2. Leaf water status

Water status parameters were determined at mid-day, according to [54]. Leaf water potential was measured using a Scholander pressure chamber (model 600 Pressure Chamber Instrument, PMS Instrument Company, Albany, NY, USA). Leaf osmotic potential was converted from osmolality (using the Van't Hoff equation) determined by a VAPRO® Vapor Pressure Osmometer (EliTech Group, Puteaux, France). Relative water content was calculated as (FW-DW)/(TW-DW) × 100, where FW is the fresh weight, TW is the turgid weight after rehydrating samples for 24 h, and DW is the dry weight after oven-drying leaves at 60 °C until constant weight.

4.3.3. Leaf pigments

Leaf pigments were determined by ultra high performance liquid chromatography (UHPLC) using a Dionex UltiMate 3000 system equipped with an Acclaim 120 C18 column (5-μm particle size, 4.6-mm internal diameter × 150-mm length) maintained into a Dionex TCC-100 column oven at 30 °C, and a Dionex UVD 170U detector (Thermo Scientific, Waltham MA, USA; [55]. Leaf material (50 mg fresh weight, FW) was homogenized in 1 mL of 100% HPLC-grade methanol and incubated overnight at 4 °C in the dark. The sample supernatants were filtered through 0.2 μm Minisart® SRT 15 aseptic filters. The pigments were eluted using 100% solvent A (acetonitrile/ methanol, 75/25, v/v) for the first

14 min to elute xanthophylls (neoxanthin, Neo; violaxanthin, Vio; antheraxanthin, Ant; lutein, Lut; zeaxanthin, Zea; in order of elution), followed by a 1.5-min linear gradient to 100% solvent B (methanol/ethylacetate, 68/32, v/v), which was pumped for 14.5 min to elute chlorophyll b (Chl b) and chlorophyll a (Chl a) and β-carotene (β-car), followed by 2-min linear gradient to 100% solvent A. The flow rate was 1 mL min^{-1}. The column was allowed to re-equilibrate in 100% solvent A for 1 min before the next injection. The pigments were detected by their absorbance at 445 nm. To quantify the pigment content, known amounts (0.003–0.5 mg ml^{-1}) of pure standards (Sigma-Aldrich, St. Louis, MO, USA) were injected into the UHPLC system and an equation correlating the peak area to pigment concentration was formulated. Chromatographic data were processed and recorded by Chromeleon Chromatography Management System software, version 7.2.10–2019 (Thermo Scientific). Total chlorophyll content (Chl_{TOT}) was calculated as Chl a + Chl b. Total carotenoid content (Car_{TOT}) was calculated as Neo + Vio + Ant + Lut + Zea + β-car, while the xanthophyll cycle pigment content (VAZ) was calculated as Vaz + Ant + Zea. The de-epoxidation state (DEPS) was calculated as (Ant + Zea)/VAZ.

4.3.4. Leaf and root lipid peroxidation and hydrogen peroxide

Lipid peroxidation was measured by the TBARS (thiobarbituric acid reactive substances) method, according to [56]. Briefly, 30 mg of leaf samples were extracted with 750 mL of 0.1% trichloroacetic acid (TCA), sonicated three times for 10 min and centrifuged at 13,000× *g* for 10 min at 4 °C. Then, 100 μL of each sample supernatant were mixed with 400 μL of 20% TCA and 0.5% thiobarbituric acid (TBA). Samples were incubated at 95 °C for 30 min, and centrifuged at 12,000× *g* for 10 min at 4 °C. The supernatant measured for absorbances at 440, 532, and 600 nm, using a fluorescence/absorbance microplate reader (Victor3 1420 Multilabel Counter, Perkin Elmer, Waltham, MA, USA). The amount of malondialdehyde (MDA) was calculated as 106 × ((A - B)/157,000), where A = (Abs 532+TBA - Abs 600+TBA) - (Abs 532-TBA - Abs 600-TBA) and B = (Abs 440+TBA - Abs 600+TBA) × 0.0571.

Hydrogen peroxide content was measured using the Amplex™ Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes, Life Technologies Corp., Carlsbad, CA, USA), according to [57]. After extraction with potassium-phosphate buffer (20 mM, pH 6.5), H_2O_2 was determined with the above-reported fluorescence/absorbance microplate reader at 530 and 590 nm for the excitation and emission of resorufin fluorescence, respectively.

4.3.5. Leaf and root cations

Leaf and root K^+ and Ca^{2+} contents were determined by Ion Chromatography (Dionex Aquion, Dionex IonPac™ CS12A, Dionex Cation Self-Regenerating Suppressor CSRS™ 300 4 mm; Sunnyvale, CA USA). According to [58], 12.5 mg FW of leaf and root tissues were suspended in 4.0 ml of HPLC-grade water, shaken for 15 min and centrifuged at 2100× *g* for 10 min. After filtration through 0.2 μm Minisart® SRT 15 aseptic filters, supernatants were eluted with 20 mM methanesulfonic acid at 1 mL min^{-1}.

4.3.6. Crop growth

At all harvesting times (0, 14 and 35 DOW), subsequently to the above-mentioned physiological measurements, plants were manually cut at ground level. After shoot removal, roots were recovered from the soil by gently washing with low flow sprinklers. The same was done at physiological maturity (BBCH 99), but additionally shoots were partitioned into culms, leaves, and spikes and spikes separated into kernels and chaff. Biomass of roots, vegetative above-ground plant parts (VAP) and grain yield were determined. For DW determination of all plant parts, the samples were oven dried at 65 °C to a constant weight.

4.4. Statistical analyses

The Shapiro-Wilk test was used to evaluate the normal distribution of data and homogeneity of variances was tested through Levene's tests, prior to analyses. The effects of

591 cultivar, waterlogging, and their interaction on the investigated parameters were assessed
592 by a two-way analysis of variance (ANOVA), using Tukey's test as the *post hoc* test. Sta-
593 tistically significant effects were considered for $p \leq 0.05$. Statistical analyses were run in
594 JMP 13.2.0 (SAS Institute Inc., Cary, NC, USA).

595 A discriminant analysis was applied to the full set of parameters collected at the end
596 of waterlogging treatments to select those that best discriminated among cultivars (Emilio
597 Lepido and Svevo), waterlogging treatment (control and waterlogged) and waterlogging
598 duration (14 and 35 days).

599

600 **5. Conclusions**

601 In conclusion, our study demonstrated that waterlogging imposed to durum wheat
602 at tillering: (i) impaired photosynthetic activity, mainly due to stomatal limitations, pig-
603 ment degradation and altered cation homeostasis; (ii) determined oxidative damage and
604 H_2O_2 accumulation in the root systems; and (iii) finally depressed the grain yield, due to
605 slowed down tiller formation and crop growth. Additionally, our results showed that gen-
606 otypic differences in waterlogging tolerance of durum wheat exist. As a matter of fact, one
607 cultivar (Emilio Lepido) was more tolerant to waterlogging than the other (Svevo). The
608 two genotypes differed not only in their immediate responses to waterlogging, but also in
609 the recovery of growth once the soil was drained. Consequently, the final grain yield of
610 the two cultivars was differently affected.

611 Therefore, our results suggest that waterlogging tolerance of durum wheat can be
612 achieved by pyramiding the numerous physiological, water status and biochemical pa-
613 rameters that confer efficient key processes such as energy maintenance, cytosolic ion ho-
614 meostasis, and ROS control and detoxification, and consequently ensure satisfying bio-
615 mass production and yield. Further research is obviously required to evaluate how the
616 investigated and other durum wheat cultivars respond to waterlogging under different
617 environmental conditions.

618

619 **Supplementary Materials:** The following are available online at www.mdpi.com/xxx/s1, Table S1:
620 Variation in physiological parameters collected in wheat cultivars Emilio Lepido and Svevo sub-
621 jected to 0, 14 or 35 days of waterlogging (C, WL14 and WL35, respectively) and then kept well-
622 watered to recover until 70 days DOW. F values and p levels (***: $p \leq 0.001$, **: $p \leq 0.01$, *: $p \leq 0.05$, ns:
623 $p > 0.05$) of two-way analysis of variance (ANOVA) for the effects of cultivar (*C*; degrees of freedom,
624 df: 1), waterlogging (*WL*; df: 2) and their interaction ($C \times WL$; df: 2) on parameters are shown. Pa-
625 rameters: A, CO_2 assimilation rate (μmol m^{-2} s^{-1}); g_s, stomatal conductance (mol m^{-2} s^{-1}); C_i, intercel-
626 lular CO_2 carbon concentration (μmol mol^{-1}); WUE_{in}, intrinsic water use efficiency (i.e, A/g_s; μmol
627 mol^{-1}); F_v/F_m, maximum quantum efficiency of the photosystem II (PSII) photochemistry; Φ_{PSII}, PSII
628 operating efficiency in light conditions; qP, photochemical quenching; qNP, non-photochemical
629 quenching.

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640 **Abbreviations:** Ant: antheraxanthin; β-car: β-carotene; Car_{TOT}: total carotenoid content; Chl a: chlo-
641 rophyll a; Chl b: chlorophyll b; Chl_{TOT}: total chlorophyll content; C_i: intercellular CO_2 concentration;

DOW: days of waterlogging; DW: dry weight; FW: fresh weight; g_s: stomatal conductance**;** HI: harvest index; Lut: lutein; MDA: malondialdehyde; Neo: neoxanthin; PSII: photosystem II; ROS: reactive oxygen species; TBA: thiobarbituric acid; TBARS: thiobarbituric acid reactive substances; TCA: trichloroacetic acid; VAP: vegetative aboveground parts; Vio: violaxanthin; WL: waterlogging. WUE_{in}: intrinsic water use efficiency**;** Zea: zeaxanthin.

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