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ORIGINAL RESEARCH

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Exogenous melatonin application helps late-sown durum wheat to cope with waterlogging under Mediterranean environmental conditions

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Abstract

In Mediterranean countries, late-sown durum wheat (*Triticum turgidum* L. subsp. *durum*) may face waterlogging (WL) at early stages. As mitigation of waterlogging by melatonin (MT) has been poorly explored, we analyzed the effects of exogenous MT foliar application to WL-stressed durum wheat on its ecophysiological performance, growth and biomass production. Late-sown plants of a relatively tolerant cultivar (i.e., Emilio-Lepido) were subjected to two WL durations (i.e., 14 and 35 days of WL; DOW) at tillering, with or without exogenous MT application (i.e., 0 and 100 μM). Prolonged WL reduced shoot biomass (−43%), but the application of MT mitigated this detrimental effect. Waterlogging impaired photosynthesis, reducing leaf CO_2 assimilation and chlorophyll content (−61 and − 57%, at 14 and 35 DOW). In control, MT increased the photosynthetic pigments (+48%), whereas it exacerbated the decrease in photosynthesis under both WL conditions (−72%, on average). Conversely, MT reduced WL-induced oxidative damage in both shoots and roots (−25% hydrogen peroxide production), facilitating osmotic adjustments and mitigating oxidative stress. The accumulation of osmotic regulators in MT + WL plants (+140 and + 42%, in shoots and roots at 35 DOW; respectively) and mineral solutes (+140 and + 104%, on average, in shoots and roots at 14 DOW) likely mitigated WL stress, limiting the impact of oxidative stress and promoting biomass accumulation. Our results highlight the potential of MT as a bioactive compound in mitigating the adverse effects of WL on late-sown durum wheat and the importance of the complex interactions between physiological responses and environmental stressors.

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1 | INTRODUCTION

Durum wheat [*Triticum turgidum* L. subsp. durum (Desf.) Husn] is one of the earliest domesticated plant species and is now one of the most important crops of the Mediterranean basin, where it is predominantly cultivated. The water shortage during the summer is usually considered the major factor limiting yield, but excessive water can also affect cereal production (Arduini et al., 2019; Farkas et al., 2020; Monteleone et al., 2023; Pampana et al., 2016). Waterlogging (WL) is defined as the situation when water covers the root system, inhibiting gas diffusion through soil pores, hence decreasing oxygen concentration at the root system level while carbon dioxide (CO_2) and ethylene concentrations increase (Setter & Waters, 2003).

Temporary WL due to extreme rainfall has been anticipated to increase in frequency, particularly in the Mediterranean area, a critical hotspot of climate change (Boulet, 2016; Mereu et al., 2021; Noto et al., 2023). Here, the optimal time for sowing durum wheat is November, but unpredictable heavy autumn rains occur more and more frequently, not leaving the chance to run this practice; therefore, sowing may be delayed up to the second decade of February (Bassu et al., 2009). If excess spring rainfall causes WL, late-sown wheat would be at the vegetative stage (tillering), and the crop will suffer from a greater effect on shoot and root dry weight than at jointing (Ghobadi et al., 2017). The negative consequences of WL would even be exacerbated by the higher temperatures during mid-late spring compared to late winter (Arduini et al., 2019).

In Italy, the harvested area of durum wheat was about 1,300,000 ha in 2023 (ISTAT, 2024), representing a key crop for food security. However, its responses to WL have rarely been assessed, even just in terms of grain yield (Kubo et al., 2007). Our group previously found that WL lasting more than 20 days can decrease the grain yield of durum wheat by 19 to 30%, depending on the stress duration (i.e., 40 and 60 days of WL, respectively; Pampana et al., 2016). This issue was later associated with impairments of photosynthetic activity (mainly due to stomatal limitations and pigment degradations), altered cation homeostasis, and oxidative damage at the root level, although a cultivar-specific sensitivity was reported, with cv. Emilio Lepido showing higher tolerance than cv. Claudio (Cotrozzi et al., 2021).

However, several open questions remain regarding the responses of durum wheat to WL because the responses depend on crop genotype and growth stage, WL duration, soil type and climatic conditions (Tian et al., 2021). Among the various strategies to mitigate the negative effects of WL, the time of sowing and the choice of the appropriate cultivars might be paramount agronomic practices to cope with this environmental constraint (Kaur et al., 2020). Additionally, the use of eco-friendly compounds, such as microbial and non-microbial biostimulants, is also one of the most promising techniques (Arnao & Hernández-Ruiz, 2019a). In this context, melatonin (MT), an endogenic low molecular-weight indole amine (N-acetyl-5-methoxytryptamine) synthesized from the L-tryptophan (Koyama et al., 2013), has been recognized as an important signaling hormone that maintains plant physiological functioning and protects plants against abiotic stresses (Agathokleous et al., 2021). It has been proven to scavenge reactive oxygen species (ROS) and to improve the activities of the antioxidant system, protecting the photosynthetic apparatus from oxidative damage and boosting plant photosynthetic efficiency under stressful conditions, also exhibiting systemic effects (Sharma et al., 2012; Arnao & Hernández-Ruiz, 2019a). It also regulates the opening of stomata, improves chlorophyll (Chl) synthesis and RuBisCo activity, maintains membrane stability by sustaining plant water relations, and increases the expression of stress-responsive genes that support the photosynthetic machinery (Yang et al., 2022, 2023). Thus, the exogenous application of MT has been tested against drought in several plant species (e.g., Sharma and Zheng, 2019). In common wheat, it was proven to alleviate several abiotic stresses, such as drought, cold, salinity and mineral stresses (Arnao & Hernández-Ruiz, 2019b).

Conversely, few studies on the role of MT against WL stress have been published (e.g., Wang et al. 2021; Zhang et al., 2019, 2021). In common wheat, Ma et al. (2022) demonstrated that MT assisted plants in sustaining leaf photosynthesis and dry matter accumulation under WL stress, while MT application in durum wheat decreased the oxidative stress levels in response to cadmium exposure by reducing the activities of crucial enzymes involved in ROS scavenging (Aloui et al., 2024). However, to the best of our knowledge, the influence of MT has never been studied in the response of durum wheat to WL-induced stress.

Following our previous work showing that plants of durum wheat cultivar Emilio Lepido better coped with WL by means of several physio-chemical regulations, such as accumulation of osmoprotectants, antioxidants, or modifications in composition of lipids (Cotrozzi et al., 2021), here we hypothesized that exogenous MT application to WL durum wheat plants could enhance the photosynthetic performance and reduce the oxidative stress, leading the accumulation of osmolytes at leaf and root level and, concomitantly, sustaining plant growth and biomass production under WL conditions. Thus, we assumed that the most negative effects of WL conditions for durum wheat (i.e., late sowing, at tillering growth stage, and with higher temperatures) could be, at least partially, offset by the choice of a suitable cultivar and the application of an exogenous biostimulant. To test this hypothesis, late-sown (i.e., late February) plants of a relatively tolerant cultivar of durum wheat (i.e., Emilio Lepido) were subjected to two WL durations (i.e., 14 and 35 days) imposed at the tillering stage (BBCH22-23, Meier, 1997), with or without exogenous MT application.

2 | MATERIALS AND METHODS

2.1 | Experimental site and design, and crop management

The research activity was carried out from March to June 2022 at 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 area is characterized by typical Mediterranean environmental conditions, with mean annual maximum and minimum daily temperatures of 20.2 and 9.5°C, respectively, and a mean rainfall of 971 mm per year. The daily air minimum and maximum temperatures and rainfall were also recorded during the whole experiment by an automatic meteorological station placed close to the experimental site, and the phenological phases were recorded using the BBCH scale for cereals (Meier, 1997).

Durum wheat (*Triticum turgidum* L. subsp. *durum*), cultivar Emilio Lepido [released in 2011 from the genealogy Orobel/Arcobaleno/Svevo; Cotrozzi et al. (2021)], was sown on 20 February, mimicking a late sowing, at the rate of 8 seeds per pot (matching with a seeding density of 400 plants m^{-2}). Pots were made from polyvinyl chloride (PVC) pipes (80 cm in height and 16 cm in diameter – 26 L of volume), having a base with a 30 mm diameter hole to allow water drain and a 0.9 mm mesh to avoid soil and root loss. Pots were filled with a sandy-loam soil [55% sand (2 mm < ∅ < 0.05 mm), 34% silt (0.05 mm < ∅ < 0.002 mm), 11% clay (<0.002 mm)], with almost natural reaction (pH 7.6) whose main chemical and physical characteristics did not differ from those previously described by Cotrozzi et al. (2021). Pots were kept outdoors throughout the whole experiment, and around 200 mm of water was applied from flowering to maturity to prevent drought stress. Nitrogen (N) fertilization followed the most rational practice in Central Italy (Ercoli et al., 2013): a total rate of 150 kg N ha^{-1} was applied as urea and split in 30–60-60 kg N ha^{-1} at sowing, pseudo-stem erection BBCH 30; and first node (BBCH 31) detectable stages, respectively. Weed control was carried out by hand hoeing, and no crop protection products were needed.

At the tillering stage (BBCH 20), plants were exposed to the following four treatments (three replicate pots were randomly associated with each treatment): (1) no MT application and well-drained (controls), (2) MT application and well-drained (MT), (3) no MT application and WL (WL), and (4) MT application and WL (MT + WL). The MT treatment consisted in spraying once a week (at sunset) 5 mL of 100 μM MT solution (w/v in distilled water) per pot, taking care to homogenously cover all the aboveground parts of plants (control and WL pots were sprayed with a same amount of distilled water), throughout the period of stress exposure. The WL treatment was imposed by placing the pots into containers (2 × 1 × 1 m) filled with fresh water, keeping a 1 cm layer of free water above the soil surface. The number of days of WL (DOW), 14 and 35 days, were chosen according to Cotrozzi et al. (2021) as they represent two and five weeks, i.e., the less and the most lasting period possible in the area.

At WL imposition (0 DOW) and at the end of each WL treatment (i.e., 14 and 35 DOW), three pots per treatment were measured for physiological and biochemical parameters (performed on the second and third upper and fully expanded leaves), and then plants were harvested to determine biomass. Leaf material was collected, immediately frozen and stored at −80°C until biochemical analyses. Roots were retrieved from the soil with gentle water flow and immediately stored at −20°C.

2.2 | Ecophysiological measurements

2.2.1 | Leaf gas-exchange and chlorophyll a fluorescence

Net CO_2 assimilation rate (A), stomatal conductance (g_s) and intercellular CO_2 concentration (C_i) were determined using a LI-6800 portable infrared gas analyser (Li-Cor Inc.) equipped with a 2 × 3 cm chamber and a 6800-02B LED light source (Li-COR Inc.) operating at 400 μmol mol^{-1} of CO_2 concentration (ambient) and saturating light conditions (1500 μmol m^{-2} s^{-1} PAR). Temperature, relative humidity, and vapour pressure deficit inside the leaf cuvette were 25 ± 2°C, 45 ± 2%, and 1.8 ± 0.2 kPa, respectively. 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), set as reported by Cotrozzi et al. (2021).

2.2.2 | Water status

Water status parameters were determined at mid-day, according to Cotrozzi et al. (2021). Leaf water potential (Ψ_W) was measured with a Scholander-type pressure chamber (model 600, PMS Instrument) using the precautions of Turner and Long (1980). Leaf osmotic potential (Ψ_π) was determined through a VAPRO® Vapour Pressure Osmometer (ElliTech Group) and calculated by converting the osmolality using the Van't Hoff equation. Relative water content (RWC) was calculated as (FW-DW)/(TW-DW) × 100, where FW is the fresh weight, DW is the dry weight after oven-drying samples at 60°C until constant weight, and TW is the turgid weight after rehydrating samples for 24 h.

2.3 | Biochemical measurements

2.3.1 | Leaf pigment contents

Leaf photosynthetic and accessory pigments were determined according to Pisuttu et al. (2024) 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 170 U detector (Thermo Scientific). About 50 mg FW of leaf material was homogenized in 1 mL of 100% HPLC-grade methanol (MeOH) and incubated overnight at 4°C in the dark. After centrifugation at 16,000 *g* and 4°C for 15 min, supernatants were filtered through 0.2 μm Minisart® SRT aseptic

filters and injected into the UHPLC system. Pigments were eluted using 100% solvent A (acetonitrile/MeOH, 75/25, v/v) for 14 min to elute xanthophylls [neoxanthin (Neo), violaxanthin (Vio), anteraxanthin (Ant), lutein (Lut) and zeaxanthin (Zea)] followed by a 1.5 min linear gradient to 100% solvent B (MeOH/ethyl acetate, 68/32, v/v), which was pumped for 14.5 min to elute chlorophyll (Chl) *b* and *a*, and β-carotene (β-car), followed by 2 min linear gradient to 100% solvent A. The column was allowed to re-equilibrate in 100% solvent A for 1 min before the next injection. The flow rate was set at 1 mL min^{-1}. Pigments were detected by their absorbance at 445 nm. To quantify pigment contents, known amounts of pure standards (Sigma-Aldrich) 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.

2.3.2 | Leaf and root melatonin contents

Melatonin contents in leaves and roots were determined according to Vougeleka et al. (2024). Around 200 mg FW of leaf or root samples were extracted in 1 mL of chloroform and then evaporated under vacuum using a rotary vacuum concentrator (RVC 2–25 CDplus, Martin Christ Gefriertrocknungsanlagen GmbH) coupled to a refrigerated vapour trap mod. Melatonin contents were assessed with the abovementioned UHPLC system but using an UltiMate™ 3000 Fluorescence Detector (Thermo Scientific), with 280 and 350 nm as excitation and emission wavelengths. Melatonin was eluted in isocratic separation by using 100% acetonitrile/sodium phosphate dibasic (65/35, v/v) for 10 min at a 1 mL min^{-1} flow rate. Melatonin concentrations were determined by a MT standard curve (100–0.01 μg mL^{-1}).

2.3.3 | Leaf and root proline contents

Proline (PRO) was determined according to Carillo and Gibon (2011) with some modifications (Calzone et al., 2020). About 50 mg FW of leaf or root tissue were mixed with 0.5 mL of ethanol (70%, v/v), sonicated two times for 10 min at room temperature, incubated at 90°C for 20 min, and finally centrifuged at 15,000 *g* at 4°C for 20 min. About 0.1 mL of the resulting supernatants were added to 0.2 mL of a reaction mix [1% ninhydrin (w/v), acetic acid 60% (v/v) and ethanol 20% (v/v)] and solutions were further incubated at 95°C for 20 min. After cooling, samples were mixed and PRO was evaluated at 520 nm using a spectrophotometer UV–Vis 1900 (Shimadzu). The PRO concentrations were determined with a PRO standard curve (1.0–0.001 mg mL^{-1}).

2.3.4 | Leaf and root abscisic acid contents

Abscisic acid content was determined according to Mariotti et al. (2022), with minor modifications. About 200 mg FW of leaf or root samples were added to 2 mL of 80% MeOH (v/v), sonicated for 10 min at room temperature, vortexed for 1 h, and then centrifuged at 15,000 *g* at 4°C for 20 min. The supernatants were filtered and evaporated at 35°C under a vacuum. The residues were resuspended with 0.2 mL of ethyl acetate. The extract was injected into an Agilent 8890B gas chromatograph equipped with an Agilent DB-1MS (UI) capillary column (30 m × 0.25 mm, coating thickness 0.25 μm) and an Agilent 5977B single quadrupole mass detector (Agilent Technologies Inc.). Analytical conditions were as follows: the carrier gas was helium with a flow rate of 1 mL min^{-1}; the injector and the transfer line were set at 250°C; the initial column temperature was set at 90°C for 2 min, increasing to 300°C at 10°C min^{-1} for 10 min. Source and quadrupole temperatures were set at 230 and 150°C, respectively. The mass data were collected in the electron impact mode at 70 eV with a scan range of 40–500 m/z, and the ABA quantification was performed at the selected-ion monitoring mode at 190 atomic mass unit (amu) by using MassHunter Workstation (version 10.0, Agilent Technologies Inc.).

2.3.5 | Leaf soluble carbohydrate contents

Soluble carbohydrate contents (i.e., sugars) were determined according to Pisuttu et al. (2023) with slight modifications. About 50 mg FW of leaf tissue was extracted with 1 mL of 100% HPLC-demineralized water in a water bath at 60°C for 60 min. Samples were centrifuged for 15 min at 16,000 *g* at 4°C, and supernatants were filtered through 0.2 μm Minisart® SRT 15 aseptic filters. Sugars were determined by the same UHPLC system reported above equipped with a Repromer H column (9 μm particle size, 8 mm internal diameter × 300 mm length; Dr Maisch), provided with a pre-column (9 μm particle size, 8 mm internal diameter × 20 mm length; Dr Maisch), kept at 30°C. The isocratic mobile phase was 9 mM sulphuric acid, eluted at a flow rate of 1 mL min^{-1}. Sugars were detected by their absorbance at 210 nm with a differential refractometer (Shodex). To quantify sugar contents, known amounts of a pure standard were injected into the UHPLC system. An equation correlating the peak area to each soluble carbohydrate standard concentration was formulated using the software reported above. The sum of sucrose, glucose and fructose was considered as a measure of sugars.

2.3.6 | Leaf and root cation contents

Cations (i.e., K^+ and Ca^{2+}) were determined by ion chromatography (Dionex™ Aquion™ Ion Chromatography (IC) System, Waltham) equipped with a Dionex Cation Self-Regenerating Suppressor CSRS™ 300 and a 4 × 250 mm Dionex IonPac™ CS12A column Thermo

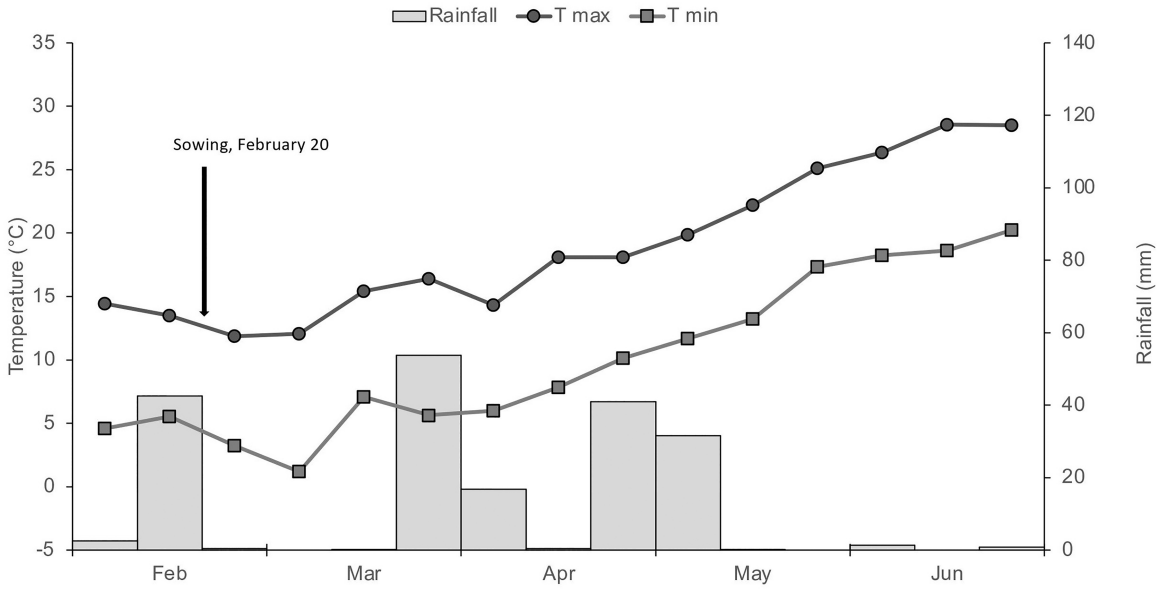


FIGURE 1 Air maximum (T max) and minimum (T min) temperatures and rainfall, recorded during the experiment (i.e., February–June 2022 for late-sown durum wheat growth cycle) in Pisa, Central Italy. Temperatures are 10-day averages, rainfall 10-day sums, data from TOS01005251 meteorological station of Regione Toscana (https://www.sir.toscana.it/).

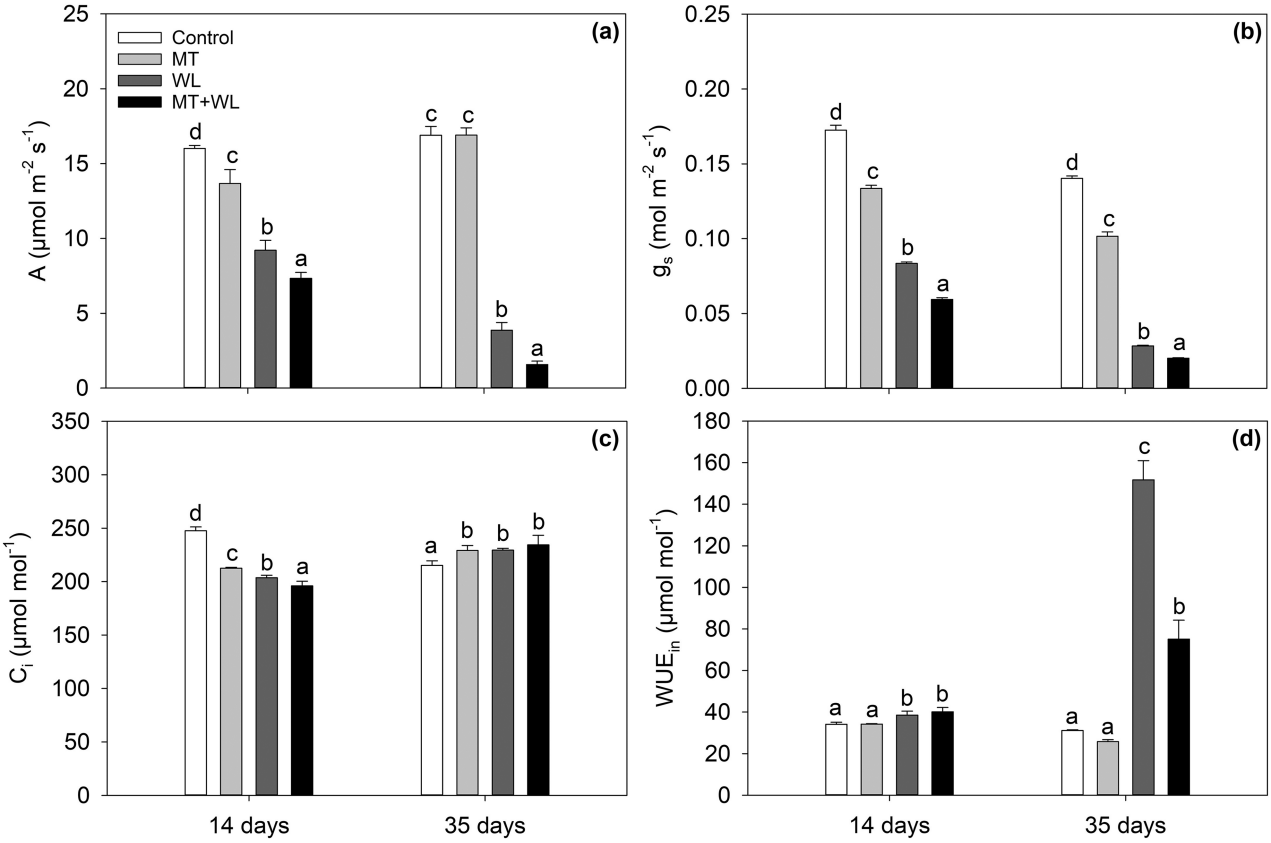


FIGURE 2 (**a**) Net CO_2 assimilation rate (A), (**b**) stomatal conductance (g_s), (**c**) intercellular CO_2 concentration (C_i) and (**d**) intrinsic water-use efficiency (WUE_{in}) in leaves of *Triticum durum* cultivar Emilio Lepido exposed to (i) no melatonin (MT) application and well-drained (control, *white*), (ii) MT foliar application and well-drained (*light gray*), (iii) no MT application and waterlogging (WL, *dark gray*), and (iv) MT foliar application and WL (MT + WL, *black*) for 14 and 35 consecutive days. Data are shown as mean ± standard deviation ($n = 4$). For each time and for those parameters for which the MT × WL interaction was significant, according to Tukey's post hoc test ($p \leq 0.05$), different letters indicate significant differences among means. For those parameters for which MT × WL interaction was not significant (Table 1), letters are not reported over bars, but the reader can refer to Table 1 and S1 for variations induced by unifactorial effects (i.e., MT and WL).

TABLE 1 Results (F-values and p-levels) of two-way analysis of variance (ANOVA) for the effects of foliar application of melatonin on physiological, biochemical and biometric parameters of *Triticum durum* cultivar Emilio Lepido after 14 and 35 consecutive days of waterlogging (DOW).

	14 DOW			35 DOW		
Parameter	**MT**	**WL**	**MT × WL**	**MT**	**WL**	**MT × WL**
A	47.29***	457.72***	5.71*	23.47***	3575.74***	23.54***
g_s	879.18***	5906.27***	49.22***	725.37***	307.41***	12379.32***
C_i	189.02***	378.37***	77.74***	11.49**	12.29**	2.59 ns
WUE_{in}	1.26 ns	46.19***	0.95 ns	159.15***	683.73***	120.25***
F_v/F_m	0.92 ns	0.02 ns	0.34 ns	4.07 ns	1076.15***	4.88*
ϕ_{PSII}	1.97 ns	15.10**	6.01*	13.67**	533.73***	9.44**
qP	73.86***	126.31***	79.20***	346.52***	11241.88***	530.53***
qNP	37.24***	930.89***	10.26***	29.46***	583.39***	37.14***
Ψ_w	1.59 ns	63.71***	39.71***	4.61 ns	485.43***	7.98*
Ψ_π	6.46*	9.79**	4.40 ns	21.01***	8.51*	63.13***
Chl_{TOT}	24.19***	69.13***	3.18 ns	51.46***	472.22***	27.04***
Chl *a*/*b*	1.63 ns	0.19 ns	4.90*	5.35*	121.27***	3.48 ns
Car_{TOT}	18.10**	68.07***	3.93 ns	32.30***	503.18***	206.63***
Leaf MT	21.75***	3.14 ns	9.69**	34.20***	50.58***	6.91*
Root MT	306.67***	0.03 ns	1.71 ns	267.96***	248.93***	149.37***
Leaf PRO	0.21 ns	1.25 ns	37.19***	195.62***	101.62***	398.35***
Root PRO	35.60***	8.31*	105.06***	5.36*	15.45**	48.00***
Leaf ABA	384.99***	3.58 ns	2.43 ns	68.38***	20.77**	100.37***
Root ABA	1066.76***	264.57***	59.78***	6079.19***	29339.08***	448.41***
Leaf Sugars	13.67**	126.47***	18.83***	35.89***	51.06***	0.68 ns
Leaf K^+	3.55 ns	269.66***	0.57 ns	77.52***	94.64***	1.77 ns
Root K^+	80.69***	0.58 ns	59.04***	4.27 ns	84.40***	0.44 ns
Leaf Ca^{2+}	161.71***	10.71*	26.30***	28.83***	40.72***	2.61 ns
Root Ca^{2+}	11.90**	95.83***	0.13 ns	3.42 ns	30.10***	4.25 ns
Leaf MDA	0.60 ns	139.00***	2.23 ns	7.84**	10.19***	7.03***
Root MDA	0.11 ns	67.99***	78.49***	108.25***	14.29**	47.29***
Leaf H_2O_2	44.98***	99.77***	3.06 ns	214.19***	276.36***	7.51*
Root H_2O_2	3.44 ns	50.22***	42.09***	41.98***	147.92***	7.54*
Shoot biomass	0.34 ns	73.74***	14.57**	16.49**	101.57***	17.71**
Root biomass	11.62**	926.75***	2.17 ns	2.89 ns	3346.18***	0.09 ns
Root to Shoot biomass	62.75***	940.31***	66.25***	13.92**	131.03***	13.29**

A: net CO_2 assimilation rate; g_s: stomatal conductance; C_i: intercellular CO_2 concentration; WUE_{in}, i.e., A/g_s: intrinsic water-use efficiency maximum quantum efficiency of the photosystem II (PSII) photochemistry (F_v/F_m); $\phi_{PSII:}$ PSII-operating efficiency in light conditions; qP: photochemical quenching; qNP: non-photochemical quenching; Ψ_w: leaf water; $\Psi_{\pi:}$ osmotic potential; $Chl_{TOT:}$ total chlorophylls; Chl *a*/*b*: chlorophyll *a*/*b* ratio; Car_{TOT}: total carotenoids; MT leaf and root melatonin; PRO: proline; ABA: abscisic acid; K^+: potassium ion; Ca^{2+} calcium ion; MDA: malondialdehyde; $H_2O_{2:}$ hydrogen peroxide.

***: $p \leq 0.001$; **: $p \leq 0.01$; *: $p \leq 0.05$; ns: $p > 0.05$.

Fisher Scientific, provided with a 4 × 50 mm Dionex IonPac™ CG12A pre-column (Thermo Scientific), according to Calzone et al. (2020). Cations were extracted by suspending 12.5 mg FW of leaf or root tissue in 4 mL of 100% HPLC-grade water, mixed for 15 min and centrifuged at 2,100 *g* for 10 min. After filtration through 0.2 µm Minisart® SRT 15 aseptic filters, the supernatants were eluted with 20 mM methansulfonic acid and the flow rate was 1 mL min^{-1}. Cation concentrations were determined by using a curve of pure mixed standards.

2.3.7 | Leaf and root lipid peroxidation and hydrogen peroxide contents

Oxidative damage was estimated by the thiobarbituric acid (TBA) reactive substances (TBARS) assay, according to Pisuttu et al. (2020), with minor modifications. Around 30 mg FW of leaf or root tissue were extracted with 750 µL of 0.1% trichloroacetic acid (TCA; w/v), sonicated three times for 10 min at room temperature, and

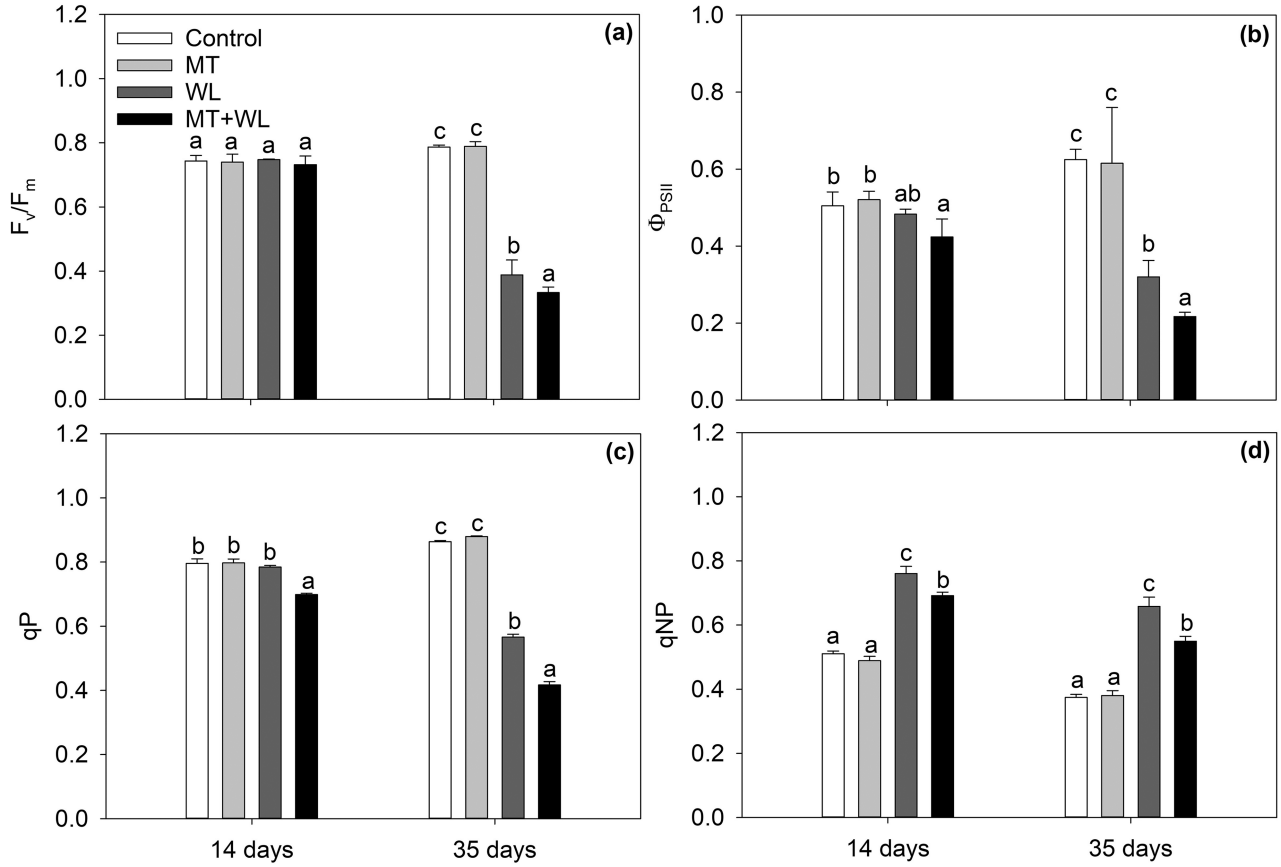


FIGURE 3 (**a**) Maximum quantum efficiency of the photosystem II (PSII) photochemistry (F_v/F_m), (**b**) PSII-operating efficiency in light conditions (ϕ_{PSII}), (**c**) photochemical quenching (qP) and (**d**) non-photochemical quenching (qNP) in leaves of *Triticum durum* cultivar Emilio Lepido exposed to (i) no melatonin (MT) application and well-drained (control, *white*), (ii) MT foliar application and well-drained (*light gray*), (iii) no MT application and waterlogging (WL, *dark gray*), and (iv) MT foliar application and WL (MT + WL, *black*) for 14 and 35 consecutive days. Data are shown as mean ± standard deviation ($n = 4$). For each time and for those parameters for which the MT × WL interaction was significant, according to Tukey's post hoc test ($p \leq 0.05$), different letters indicate significant differences among means. For those parameters for which MT × WL interaction was not significant (Table 1), letters are not reported over bars, but the reader can refer to Table 1 and S1 for variations induced by unifactorial effects (i.e., MT and WL).

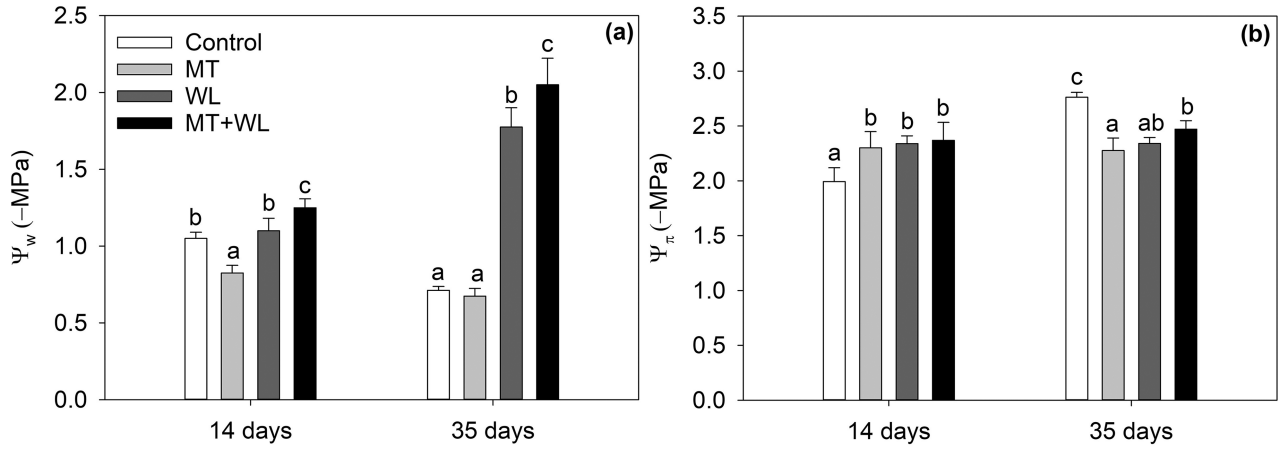


FIGURE 4 (**a**) Leaf water (Ψ_W) and (**b**) osmotic (Ψ_π) potential, in leaves of *Triticum durum* cultivar Emilio Lepido exposed to (i) no melatonin (MT) application and well-drained (control, *white*), (ii) MT foliar application and well-drained (*light gray*), (iii) no MT application and waterlogging (WL, *dark gray*), and (iv) MT foliar application and WL (MT + WL, *black*) for 14 and 35 consecutive days. Data are shown as mean ± standard deviation (n = 4). For each time and for those parameters for which the MT × WL interaction was significant, according to Tukey's post hoc test ($p \leq 0.05$), different letters indicate significant differences among means. For those parameters for which MT × WL interaction was not significant (Table 1), letters are not reported over bars, but the reader can refer to Table 1 and S1 for variations induced by unifactorial effects (i.e., MT and WL).

centrifuged at 13,000 *g* at 4°C for 10 min. Then, 100 µL of each sample supernatant was mixed with 400 µL of 20% TCA and 0.5% TBA (w/v). Samples were incubated at 95°C for 30 min, and centrifuged at 12,000 *g* and 4°C for 10 min. The absorbance of supernatants was measured at 532 nm and corrected for non-specific turbidity by subtracting the absorbance at 440 and 600 nm, using a fluorescence/absorbance microplate reader (Victor3 1420 Multilabel Counter, Perkin Elmer). The amount of malondialdehyde (MDA) was calculated as $106 \times ((A - B)/157{,}000)$, 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 (H_2O_2) content was measured using the Amplex™ Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes, Life Technologies Corp.), according to Modesti et al. (2023). About 50 mg FW of leaf or root tissue were extracted in 1 mL of potassium phosphate buffer [20 mM (w/v) pH 6.5]. The 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.

2.4 | Biometric measurements

At each sampling time, after the ecophysiological and biochemical determinations, the plants of each pot were manually cut at the ground level and vegetative aboveground plant parts' biomass determined. The pots were then opened, and the root system was separated from the soil by washing through a gentle water flow and filtering with a fine glass-fiber net (45 mesh cm^{-2}) to prevent any loss. Dry weight of all plant parts was obtained by oven-drying at 65°C to achieve a constant weight and values are reported as mg $plant^{-1}$.

2.5 | Statistical analyses

The Shapiro–Wilk test was used to evaluate the normal distribution of data and homogeneity of variances was tested by the Levene's test. At each time of analysis (i.e., 0, 14 and 35 DOW), the effects of MT, WL, and their interaction on the investigated parameters were assessed by a two-way analysis of variance (ANOVA), followed by the Tukey's post hoc test. Statistically significant effects were considered for $p \leq 0.05$. Statistical analyses were run in JMP Pro 14.0.0 (SAS Institute Inc.).

3 | RESULTS

3.1 | Meteorological conditions

During the experiment (i.e., durum wheat cycle), the total rainfall was 192 mm spread over 21 rainy days and was mainly concentrated in

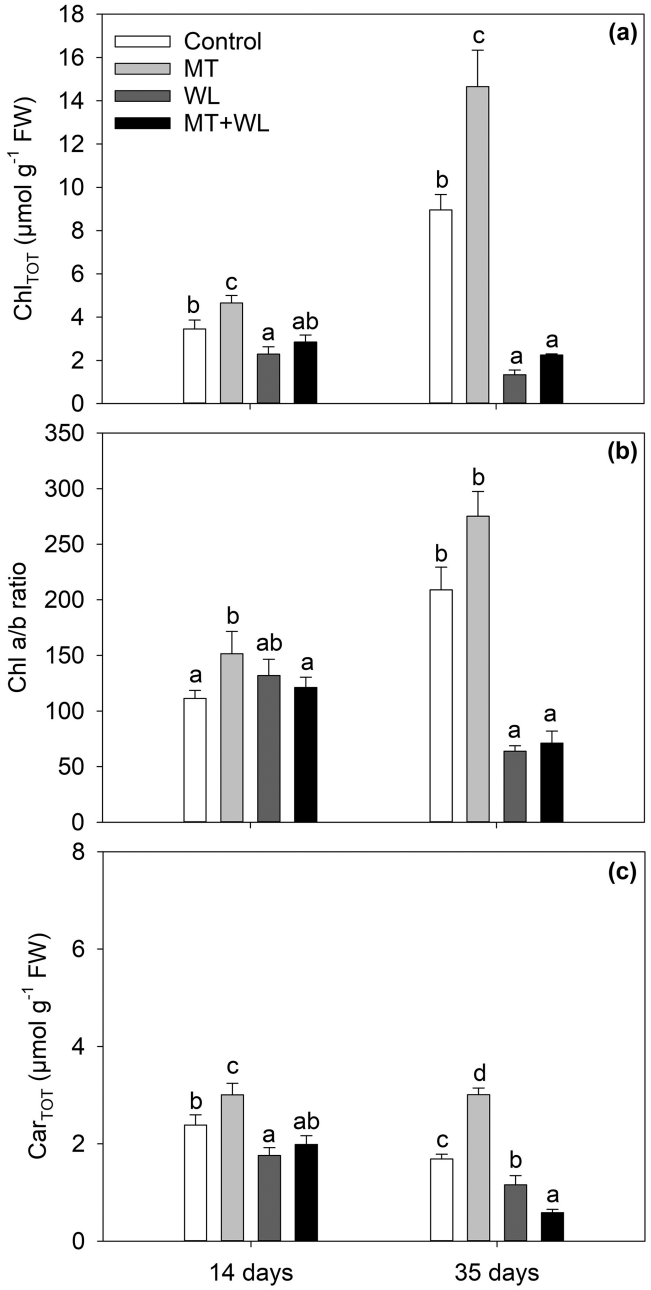


FIGURE 5 (**a**) Total chlorophyll (Chl_{TOT}), (**b**) chlorophyll *a*/*b* ratio (Chl *a*/*b*) and (**c**) total carotenoid (Car_{TOT}) in leaves of *Triticum durum* cultivar Emilio Lepido exposed to (i) no melatonin (MT) application and well-drained (control, *white*), (ii) MT foliar application and well-drained (*light gray*), (iii) no MT application and waterlogging (WL, *dark gray*), and (iv) MT foliar application and WL (MT + WL, *black*) for 14 and 35 consecutive days. Data are shown as mean ± standard deviation (n = 4). For each time and for those parameters for which the MT × WL interaction was significant, according to Tukey's post hoc test ($p \leq 0.05$), different letters indicate significant differences among means. For those parameters for which MT × WL interaction was not significant (Table 1), letters are not reported over bars, but the reader can refer to Table 1 and S1 for variations induced by unifactorial effects (i.e., MT and WL).

the period from February to early May (Figure 1), as typical in Central Italy. Temperatures ranged from 1.2 to 28.6°C (recorded in February and June, respectively), and the daily mean temperature was 14.5°C across the entire wheat growing season, slightly higher than the historical value (1995–2021) for the site (13.0°C).

3.2 | Plant phenology

Waterlogged plants for the longest period (i.e., 35 days) reached the reproductive stage about five days later than the controls (data not shown). Conversely, MT application did not affect the phenology of the crop, neither in WL nor in control plants. Plants exposed to WL showed chlorosis and early senescence of leaves, especially in the lower ones.

3.3 | Physiological parameters

At the beginning of the experiment (i.e., 0 DOW), none of the investigated physiological parameters (i.e., A, g_s, C_i, WUE_{in}, F_v/F_m, Φ_{PSII}, qP, qNP, Ψ_w and Ψ_π) showed significant differences among treatments (*data not shown*). At 14 DOW, MT induced a slight reduction in A, g_s and C_i levels by −14, −23 and − 14%, respectively (Figure 2a-c); note, throughout the whole text, the reported percentage variations are in comparison with the related controls and refer to the statistical outputs reported in Table 1. These parameters further decreased in plants exposed to WL (−42, −52 and − 17%, respectively) and even more under MT + WL (−54, −66 and − 21%, respectively). No significant changes were observed in terms of WUE_{in} values (Figure 2d). At 35 DOW, MT did not affect A and WUE_{in} levels (Figure 2a-d), while it reduced g_s by 41% (Figure 2b). Both A and g_s values decreased in WL plants (−81 and − 65%, respectively) and even more in MT + WL ones (−96 and − 70%, respectively; Figure 2a-b), whereas WUE_{in} increased more than four times in MT plants and more than two times in MT + WL ones (Figure 2d). Although a significant MT × WL interaction was not reported for C_i, both MT and WL increased this parameter (around +7%; Figure 2c, Table S1).

At 14 DOW, no significant effects were reported on F_v/F_m; ϕ_{PSII} and qP decreased only in MT + WL plants (−16 and − 12%, respectively; Figure 3a-c), while qNP increased by 49 and 35% in WL and MT + WL plants (Figure 3d). At 35 DOW, F_v/F_m, ϕ_{PSII}, and qP decreased in WL plants (−51, −49, and − 34%, respectively) and even more in MT + WL ones (−58, −65 and − 51%, respectively; Figure 3a-c), while qNP increased by 75 and 47% in WL and MT + WL plants, respectively (Figure 3d). No significant changes were thus reported for MT alone (Figure 3a-d).

At 14 DOW, Ψ_w increased in MT plants (+21%; Figure 4a), was not affected by WL alone, and decreased in MT + WL ones (−19%). Although a significant MT × WL interaction was not reported for Ψ_π, this parameter decreased by both MT and WL (around −15%; Figure 4b, Table S1). At 35 DOW, Ψ_w decreased considerably in WL plants (−101%) and even more in MT + WL ones (−187%; Figure 4a), while Ψ_π increased by 18, 15 and 11% in MT, WL and MT + WL plants, respectively (Figure 4b).

3.4 | Biochemical parameters

Similarly to physiological parameters, all the investigated biochemical ones (i.e., Chl_{TOT}, Chl *a*/*b*, Car_{TOT}, leaf and root H_2O_2, leaf and root

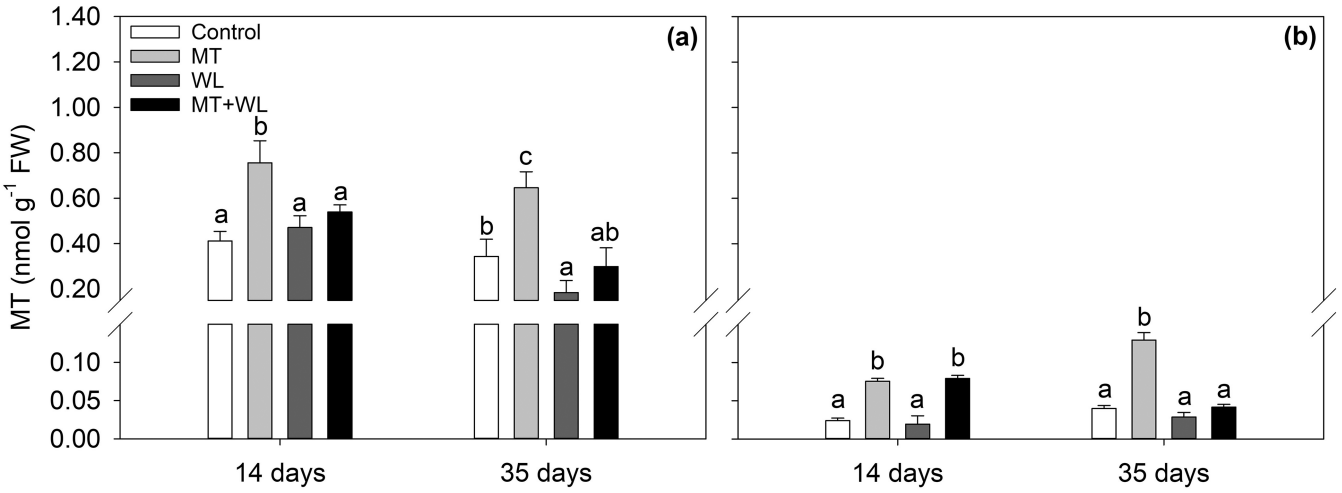


FIGURE 6 (**a**) Leaf and (**b**) root melatonin (MT) content in *Triticum durum* cultivar Emilio Lepido exposed to (i) no melatonin (MT) application and well-drained (control, *white*), (ii) MT foliar application and well-drained (*light gray*), (iii) no MT application and waterlogging (WL, *dark gray*), and (iv) MT foliar application and WL (MT + WL, *black*) for 14 and 35 consecutive days. Data are shown as mean ± standard deviation (n = 4). For each time and for those parameters for which the MT × WL interaction was significant, according to Tukey's post hoc test ($p \leq 0.05$), different letters indicate significant differences among means. For those parameters for which MT × WL interaction was not significant (Table 1), letters are not reported over bars, but the reader can refer to Table 1 and S1 for variations induced by unifactorial effects (i.e., MT and WL).

PRO, leaf and root ABA, sugars, leaf and root K^+ and Ca^{2+} ions, leaf and root MDA and H_2O_2) showed no statistical differences among treatments at the beginning of the experiment (i.e., 0 DOW; *data not shown*). At 14 DOW, Chl_{TOT}, Chl *a*/*b* and Car_{TOT} increased in MT plants (+34, +36 and + 26%, respectively), while Chl_{TOT} and Car_{TOT} decreased in WL ones (−34 and − 26%, respectively), while no changes were reported in MT + WL plants (Figure 5, Table S1). At 35 DOW, Chl_{TOT} and Car_{TOT} continued to be higher in MT plants (+63 and + 78%), while they decreased in both WL and MT + WL ones (Chl_{TOT}: around −80%; Car_{TOT}: −31 and − 65%, respectively). Chl *a*/*b* was increased by MT (+39%), while it decreased by WL (−69%), although MT × WL interaction was not significant (Figure 5b, Table S1).

At both 14 and 35 DOW, MT content increased only in MT plants, both in leaves (+84 and + 88%, respectively; Figure 6a) and in roots (+224% at 35 DOW; Figure 6b). At 14 DOW, MT content also increased in MT + WL roots, and similarly to MT ones (i.e., the MT × WL interaction was not significant) (Table S1). At 35 DOW, MT decreased by 47% in WL leaves.

At 14 DOW, PRO increased in MT and WL leaves (+23%; Figure 7a) and decreased in MT and WL roots (−20 and − 34%, respectively; Figure 7b), while it did not change in MT + WL leaves and increased in MT + WL roots (+40%). At 35 DOW, PRO decreased in MT and WL leaves (−23 and − 38%, respectively), while severely increased in MT + WL ones (Figure 7a); only a slight increase in MT plants was reported at root level (+59%; Figure 7b). Regardless of MT treatment, WL decreased ABA in leaves (−21%; Figure 7c, Table S1) at 14 DOW, while it decreased by 22 and 14% in roots under MT and MT + WL, respectively, and increased by 21% under WL (Figure 7d). At 35 DOW, leaf ABA content decreased under WL (−15%) and increased under MT + WL (+34%; Figure 7c), while it increased by 26, 68 and 112% in roots of MT, WL and MT + WL plants, respectively (Figure 7d).

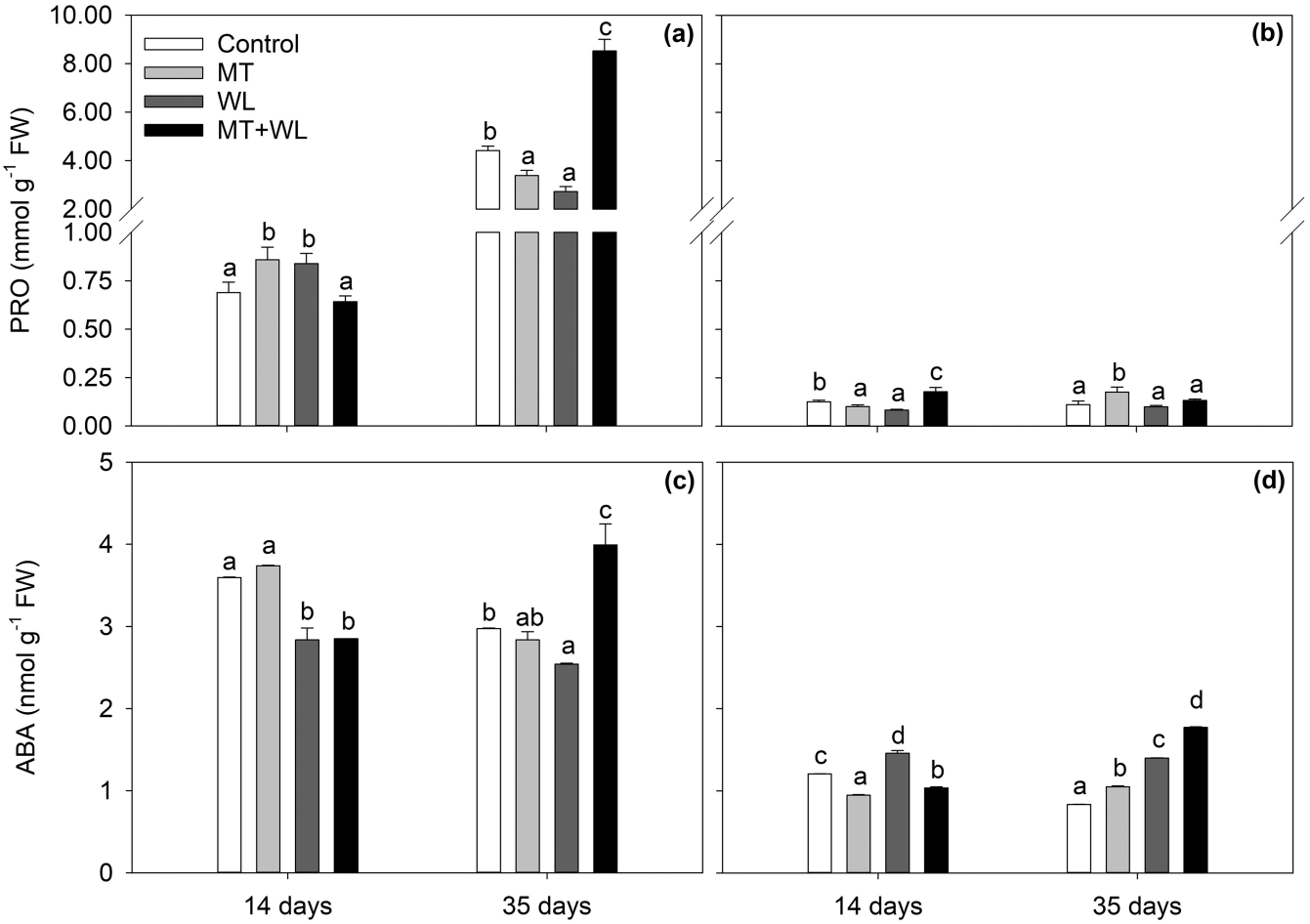


FIGURE 7 (**a, c**) Leaf and (**b, d**) root proline (PRO; **a-b**) and abscisic acid (ABA; **c-d**) contents in *Triticum durum* cultivar Emilio Lepido exposed to (i) no MT application and well-drained (controls, *white*), (ii) MT foliar application and well-drained (MT, *light gray*), (iii) no MT application and waterlogging (WL, *dark gray*), and (iv) MT foliar application and waterlogging (MT + WL, *black*) for 14 and 35 consecutive days. Data are shown as mean ± standard deviation (n = 4). For each time and for those parameters for which the MT × WL interaction was significant, according to Tukey's post hoc test ($p \leq 0.05$), different letters indicate significant differences among means. For those parameters for which MT × WL interaction was not significant (Table 1), letters are not reported over bars, but the reader can refer to Table 1 and S1 for variations induced by unifactorial effects (i.e., MT and WL).

At 14 DOW, sugars content was by 63 and 30% augmented in WL and MT + WL leaves and, even though a significant MT × WL interaction was not stated on leaf sugars at 35 DOW, it increased by 38% in MT and WL leaves (on average), and by 77% in MT + WL ones (Figure 8, Table S1).

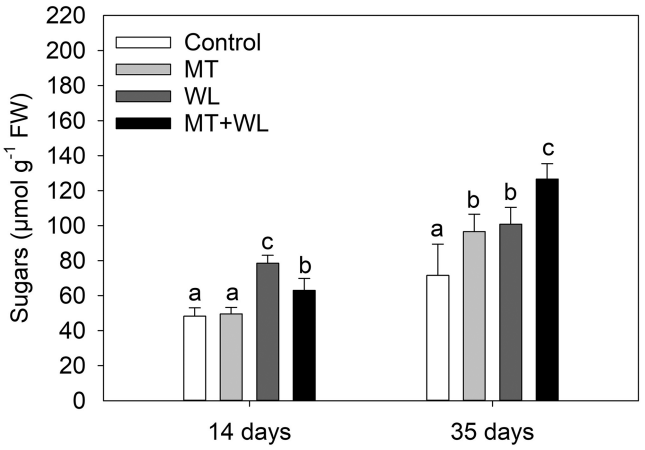


FIGURE 8 Leaf sugars content in *Triticum durum* cultivar Emilio Lepido exposed to (i) no melatonin (MT) application and well-drained (control, *white*), (ii) MT foliar application and well-drained (*light gray*), (iii) no MT application and waterlogging (WL, *dark gray*), and (iv) MT foliar application and WL (MT + WL, *black*) for 14 and 35 consecutive days. Data are shown as mean ± standard deviation (n = 4). For each time and for those parameters for which the MT × WL interaction was significant, according to Tukey's post hoc test ($p \leq 0.05$), different letters indicate significant differences among means. For those parameters for which MT × WL interaction was not significant (Table 1), letters are not reported over bars, but the reader can refer to Table 1 and S1 for variations induced by unifactorial effects (i.e., MT and WL).

Although a significant MT × WL interaction was never reported on leaf K^+, this parameter was decreased by WL at 14 DOW (−45%) and by both MT and WL at 35 DOW (around −45%, as average; *data not shown*). Root K^+ content was lowered by 52% in WL plants, while it ws boosted by 73% in MT + WL ones at 14 DOW. At 35 DOW, it decreased under both WL and MT + WL (around −45%), despite the absence of significant MT × WL interaction (Figure 9a, Table S1). Leaf Ca^{2+} content increased in MT and MT + WL plants at 14 DOW (+38%, on average; Figure 9b), while it decreased in WL ones (−57%). Although a significant MT × WL interaction was not reported, at 35 DOW, Ca^{2+} only increased in WL leaves (+38%) (Table S1), whereas root Ca^{2+} was increased by MT and WL at 14 DOW (−89%, as average), and decreased by WL at 35 DOW (−33%; *data not shown*).

At 14 DOW, although a MT × ML significant interaction was not reported for leaf MDA and H_2O_2, WL reduced both these parameters (−72 and − 31%, respectively; Figure 10a, c; Table S1), and MT augmented leaf H_2O_2 (+13%) (Table S1); at 35 DOW, MDA increased mostly in WL leaves (+57%), but also in MT + WL ones (+21%), while H_2O_2 grew under WL (+28%) and decreased under MT (−17%) (Table S1). At root level, MDA declined in MT plants at 14 DOW (−38%), while it increased in MT + WL at both times of analysis (+38 and + 54% at 14 and 35 DOW, respectively; Figure 10b). At 14 DOW, root H_2O_2 greatly increased under WL (+64%) but also under MT and MT + WL (+23%, on average; Figure 10d). Similar responses were reported at 35 DOW, even if root H_2O_2 did not change in MT plants (+64 and + 24%, under WL and MT + WL, respectively).

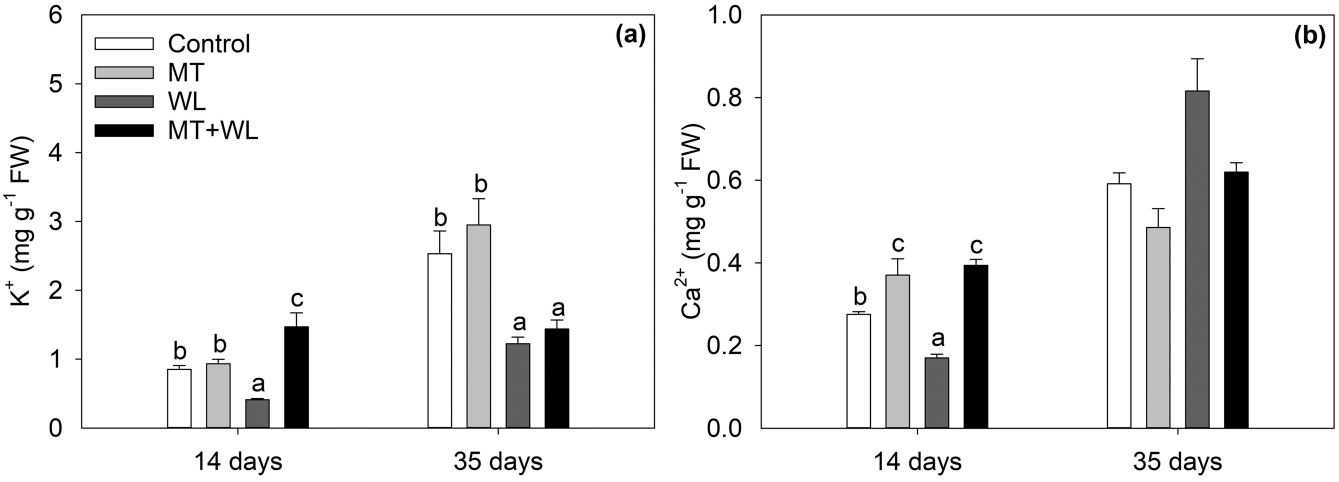


FIGURE 9 (**a**) Root potassium (K^+) and (**b**) leaf calcium (Ca^{2+}) content in *Triticum durum* cultivar Emilio Lepido exposed to (i) no melatonin (MT) application and well-drained (control, *white*), (ii) MT foliar application and well-drained (*light gray*), (iii) no MT application and waterlogging (WL, *dark gray*), and (iv) MT foliar application and WL (MT + WL, *black*) for 14 and 35 consecutive days. Data are shown as mean ± standard deviation (n = 4). For each time and for those parameters for which the MT × WL interaction was significant, according to Tukey's post hoc test ($p \leq 0.05$), different letters indicate significant differences among means. For those parameters for which MT × WL interaction was not significant (Table 1), letters are not reported over bars, but the reader can refer to Table 1 and S1 for variations induced by unifactorial effects (i.e., MT and WL).

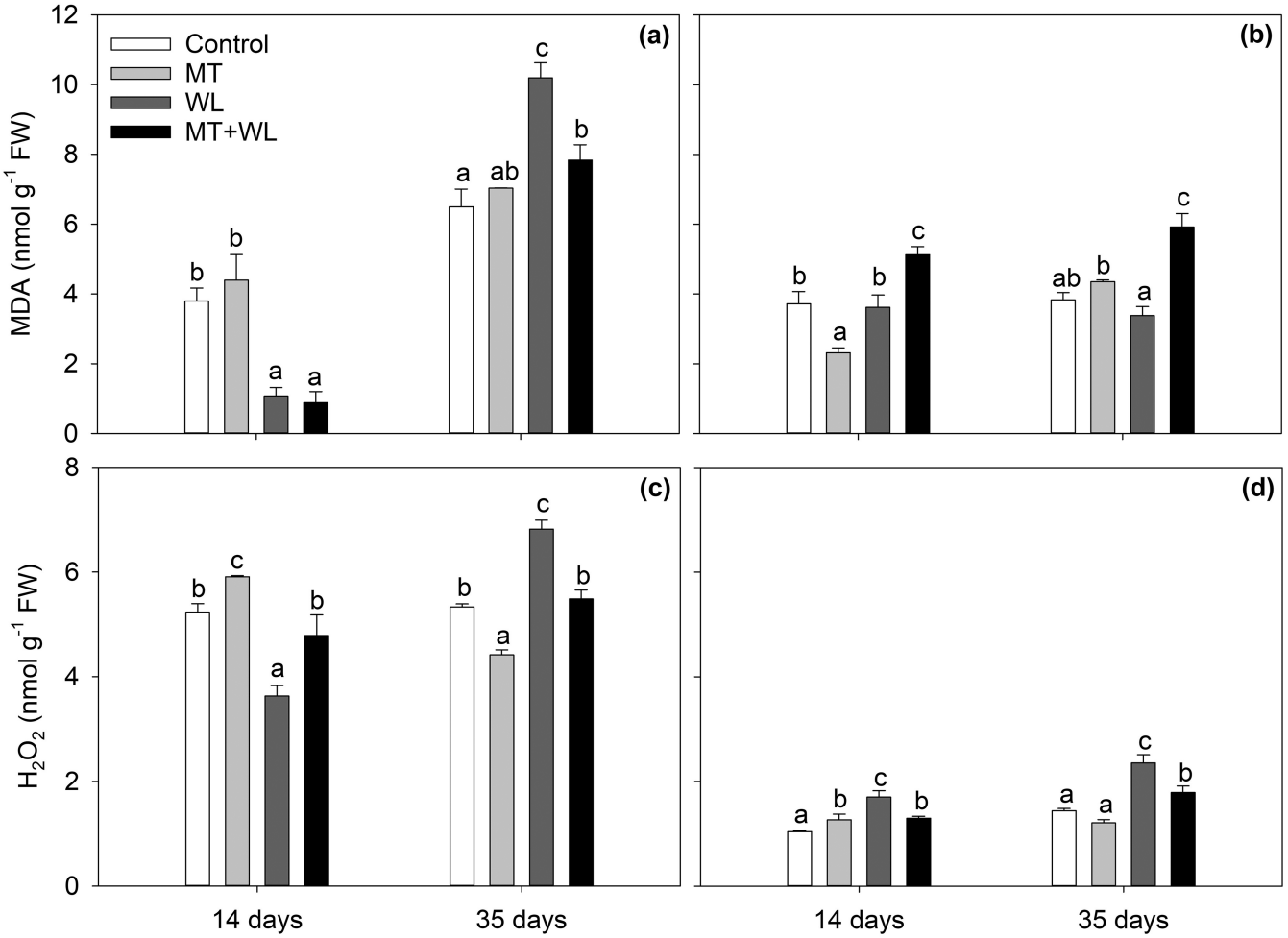


FIGURE 10 (**a, c**) Leaf and (**b, d**) root malondialdehyde by-product (MDA; **a, b**) and hydrogen peroxide (H_2O_2, **c, d**) content in *Triticum durum* cultivar Emilio Lepido exposed to (i) no melatonin (MT) application and well-drained (control, *white*), (ii) MT foliar application and well-drained (*light gray*), (iii) no MT application and waterlogging (WL, *dark gray*), and (iv) MT foliar application and WL (MT + WL, *black*) for 14 and 35 consecutive days. Data are shown as mean ± standard deviation (n = 4). For each time and for those parameters for which the MT × WL interaction was significant, according to Tukey's post hoc test ($p \leq 0.05$), different letters indicate significant differences among means. For those parameters for which MT × WL interaction was not significant (Table 1), letters are not reported over bars, but the reader can refer to Table 1 and S1 for variations induced by unifactorial effects (i.e., MT and WL).

3.5 | Biometric parameters

At 0 DOW, none of the biometric parameters analyzed (i.e., shoot DW, root DW, root-to-shoot ratio) showed statistical differences among the plant treatments. At 14 DOW, shoot and root (despite the effects of MT and WL appeared to be independent of each other for this parameter) DW were higher in MT plants (+14% on average; Figure 11a, b; Table S1). Waterlogging, irrespectively to MT exogenous application (i.e., in both WL and MT + WL treatments), decreased the biomass of shoots and roots by −23 and − 76%, as an average over the two treatments. Similarly, the shoot-to-root ratio was strongly reduced in WL and in MT + WL plants (−75 and − 64%, respectively; Figure 11c). At 35 DOW, shoot and root DW did not change in MT plants, while they declined by −43 and − 18%, in WL and MT + WL plants, respectively, in shoots and even more in roots (−82%, on average). Therefore, the root-to-shoot ratio fell by −66 and − 79%, respectively in WL and MT + WL.

4 | DISCUSSION

4.1 | Prolonged waterlogging at tillering reduced shoot and root biomass, but melatonin application mitigated this detrimental effect

During the early stages of WL, as well as when prolonged, the inadequate supply of oxygen to the roots could explain the interruption of root growth, as already observed in other studies in other cereals like common wheat, barley, maize, oat, and rice (Pan et al., 2021; Arduini et al., 2019). Specifically, the decreased root-to-shoot ratio of WL plants is due to the inhibition of seminal root growth, which is partially replaced by adventitious ones, able to elongate at least to some extent into WL soil or oxygen-deficient solutions (Thomson et al., 1992; Malik et al., 2002; Tong et al., 2021). As a result of the impaired root functions (Pais et al., 2022) and the inability of plants to

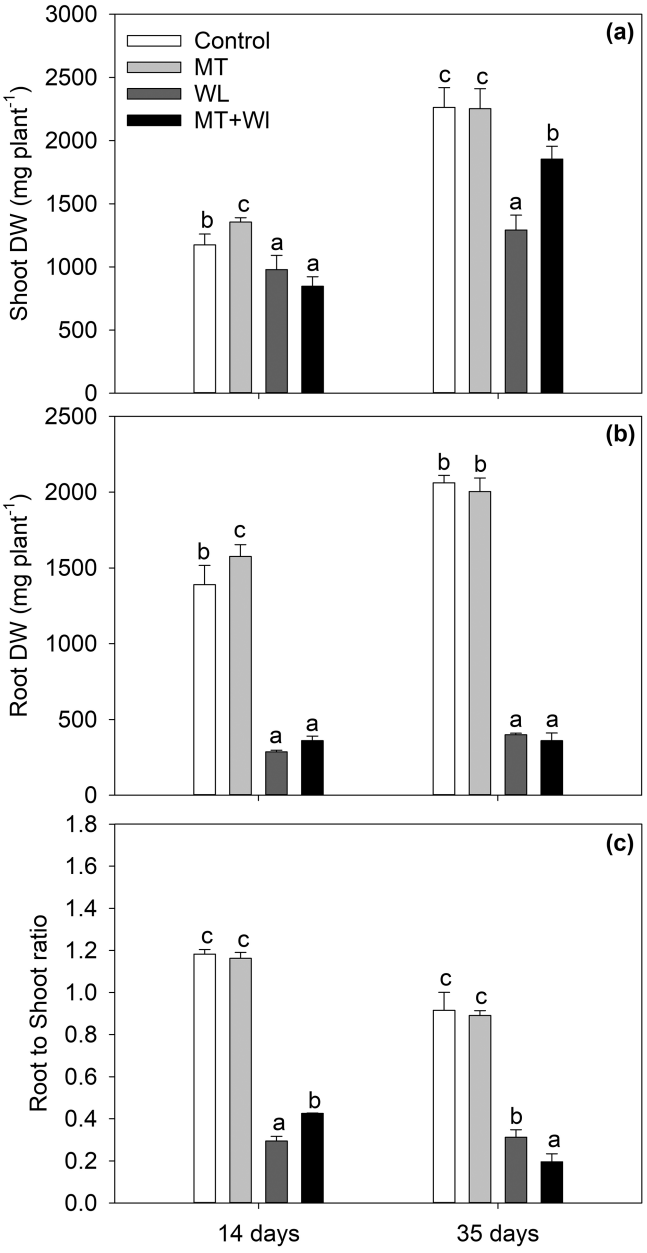


FIGURE 11 (**a**) Shoot and (**b**) root biomass and (**c**) root-to-shoot ratio in *Triticum durum* cultivar Emilio Lepido exposed to (i) no melatonin (MT) application and well-drained (control, *white*), (ii) MT foliar application and well-drained (*light gray*), (iii) no MT application and waterlogging (WL, *dark gray*), and (iv) MT foliar application and WL (MT + WL, *black*) for 14 and 35 consecutive days. Data are shown as mean ± standard deviation (n = 4). For each time and for those parameters for which the MT × WL interaction was significant, according to Tukey's post hoc test ($p \leq 0.05$), different letters indicate significant differences among means. For those parameters for which MT × WL interaction was not significant (Table 1), letters are not reported over bars, but the reader can refer to Table 1 and S1 for variations induced by unifactorial effects (i.e., MT and WL).

cope with the WL stress, a reduction in shoot physiological performance occurred in WL plants, leading to an overall reduction of aboveground biomass.

Interestingly, during the application to control plants (i.e., not waterlogged), MT was adsorbed from the leaves, also increasing the levels in the root apparatus, supporting the shoot growth and the root biomass development soon (i.e., at 14 DOW; Teng et al., 2022). Actually, leaf MT content did not increase under WL conditions, unexpectedly, likely due to an interaction of MT with other signaling molecules (Ahmad et al., 2023).

Melatonin also mitigated the effects of WL at 35 DOW and preserved the C-reserves, allowing the development of shoots and preparing the plants to face the reproductive stages.

Conversely, the translocation of MT to the root apparatus did not occur under WL conditions and the dramatic effects due to the hypoxic conditions were not avoided at root level, as confirmed by the reduced root biomass. Although positive effects of MT were previously reported, a larger impact is commonly observed when MT is distributed directly to the roots, regardless the application procedure and target (e.g., leaves, seeds or roots in soil systems; Agathokleous et al., 2021).

4.2 | Melatonin treatment did not mitigate waterlogging-induced photosynthetic performance of cv. Emilio Lepido at tillering stage

As early as at 14 DOW, WL induced the reduction of leaf CO_2 assimilation due to stomatal closure and the reduction of photosynthetic and accessory pigments. Concomitantly, a slight decrease of C_i and an increase of WUE_{in} was observed. However, photosystems' activity was not altered due to the activation of an efficient protective mechanism of energy dissipation (i.e., higher qNP and consumption of Car_{TOT}). At 35 DOW, photosynthetic activity remained very low (together with g_s), while WUE_{in} increased further, suggesting a lack of WL-induced reduction of root hydraulic conductivity. Specifically, the decline of F_v/F_m ratio and qP suggested that PSII reaction centers were closed and photoinhibition occurred. The qNP values rose, meaning that the light level absorbed exceeded the capacity of electron transport, and Car_{TOT} decreased, indicating that the mechanism adopted as photoprotection was unable to avoid the damages to photosystems. Different authors (e.g., Voesenek et al., 2006; Ren et al., 2016) reported that under prolonged WL conditions, the enzyme activities related to photosynthesis were inhibited and the Chl synthesis ability of leaves declined, leading to leaf senescence and all related symptoms such as yellowing. Otherwise, WL can lead to changes at the membrane level, with structural impacts that affect components of cell wall and plasma membranes and may reflect severe membrane damage and low survival ability (Barickman et al., 2019).

Interestingly, MT sprayed in the absence of WL induced a stomatal closure, which also caused a temporary reduction of photosynthetic activity (only) at 14 DOW. However, the early increase of Chl_{TOT} (with a major contribution of Chl *a*) and Car_{TOT} contents avoided the non-stomatal impairments, which allowed optimal photosystem functions. However, despite the positive effects of MT

observed in plants not exposed to WL at 14 DOW, its application exacerbated the decrease of photosynthesis caused by WL, mainly due to stomatal limitations (i.e., lower values of g_s). At 35 DOW, non-stomatal limitations contributed to the worsening of the photosynthetic activity as demonstrated by (i) the inhibition of photosynthetic electron transport, (ii) PSII functionality with direct consequences to the integrity of photosystems and chloroplasts structures, and (iii) Chls and Cars content reduction. Overall, these outcomes suggest that even was reported as somewhat tolerant to WL by Cotrozzi et al. (2021). In the present research, cv. Emilio Lepido was not able to cope with this abiotic stress when occurring so late at tillering and under higher temperatures. Furthermore, the MT treatment lost its effectiveness in sustaining the photosynthetic apparatus of WL plants.

4.3 | Melatonin application reduced waterlogging-induced oxidative damages by activating a vigorous osmotic adjustment

Both hydric and biochemical regulations of cv. Emilio Lepido subjected to WL were found to be highly compromised, independently from the duration of the WL exposure. At 14 DOW, osmotic imbalance due to variations in organic and inorganic osmolytes occurred between leaves (PRO increase, ABA reduction, sugars increase and Ca^{2+} reduction) and roots (i.e., decrease of PRO, and ABA accumulation, K^+ decrease). Osmotic adjustments were observed also at 35 DOW (i.e., a general decrease of PRO and ABA contents in the leaves and its accumulation in the roots, leaf sugars and leaf Ca^{2+} increase, reduction of root K^+), twinned by water imbalance (i.e., decrease of Ψ_w and increase of Ψ_π). In addition, the H_2O_2 accumulation observed in the whole plant indicated the presence of oxidative stress, further confirmed by MDA accumulation in the leaves, but lipid peroxidation never occurred at the root level; once applied, MT could potentially cause a temporary stress to the targeted tissues and concentrations above optimal could be toxic to the plant (Colombage et al., 2023).

Acting as an osmotic regulatory substance in plants (Vougeleka et al., 2024), MT enables the maintenance of ion homeostasis and growth regulation (Chen et al., 2020), as demonstrated by the reduced Ψ_π, unvaried root K^+ and increased Ca^{2+} leaf levels, and optimum interaction between organic osmotic regulators (e.g., PRO and ABA), which can together increase the cell fluid concentration, generally enhancing plant tolerance to the stress. Melatonin has been found to induce WL tolerance in winter wheat (Ma et al., 2022) by sustaining aerobic respiration in root tissues and protecting leaf photosynthesis from oxidative damage through efficient repression of the ROS burst and consequent mitochondria degradation (Ma et al., 2022). Similarly, MT significantly improved the WL tolerance and alleviated the stress damage of maize (Altaf et al., 2022), apple (Zheng et al., 2017), kiwifruit (Huo et al., 2022), and reprogrammed polyamine and ethylene metabolism in alfalfa (Zhang et al., 2019).

However, in our experiment, when applied at foliar level during WL imposition, MT only partially protected plants from the stress. At 14 DOW, despite the activation of a recurring osmotic adjustment (i.e., balancing of PRO and ABA between leaves and roots, accumulation of ions and soluble sugars), Ψ_w and Ψ_π decreased, H_2O_2 accumulation and lipid peroxidation occurred in the root apparatus. The membrane injuries observed could also be attributed to the programmed cell death and degradation frequently occurring in cortical cells of plant root under hypoxia, producing tissue cavities and leading to aerenchyma formation, deputed to the exchange of oxygen from non-waterlogged tissue to the root system, and the discharge of CO_2 and toxic volatile substances from WL tissue (Pan et al., 2021). At 35 DOW, the concomitant leaf accumulation of PRO, ABA (also in the roots) and sugars represented the main attempt to face the dramatic consequences of WL stress. Indeed, at this time, even if Ψ_w decreased even more, the accumulation of compatible organic solutes and the flow of mineral solutes (which remained unvaried at both leaf and root levels) facilitated the osmotic adjustments (Akula et al., 2018), thus comprising the impact of oxidative stress and limiting the loss of biomass by ensuring a considerable development of aboveground plant parts, as effectively observed at the end of the experiment (i.e., considerable biomass development and contained MDA accumulation, in comparison to WL plants). Specifically, PRO is a proteinogenic amino acid that plays a highly beneficial role in plants exposed to environmental stresses as an osmolyte, a free radical scavenger, a carbon and nitrogen reserve, and a signaling molecule (Szabados & Savouré, 2010; Hayat et al., 2012), while ABA can have a positive role in the modulation of plant responses to hypoxia and the ability to recover during the post-hypoxic period (Pan et al., 2021). High ABA levels can lead to stomatal closure and high WUE_{in} in plants (Lamarque et al., 2020; Cardoso et al., 2020), increasing water status and cell turgor, thus potentially resulting in higher grain filling (Moura et al., 2023). Specifically, under prolonged WL conditions, carbohydrate deprivation due to reduced photosynthesis and aerobic respiration induced the plants to obtain energy from the initial carbohydrate reserves. This may be a crucial factor in tolerance to hypoxia/anoxia, typical to WL-resistant genotypes, as the plant utilizes its stored glucose reserves to maintain metabolic activity under anaerobic conditions (Fukao et al., 2019; Sharma and Zheng, 2019), favoring better growth and biomass accumulation (Sairam et al., 2009, 2011; Takahashi et al., 2018). On the other hand, the accumulation of soluble sugars such as sucrose, glucose, and fructose in stressed plants, as also observed in the current experiment, could enhance WL tolerance by preventing oxidative stress and stabilizing plasma membranes and biomolecules (Sairam et al., 2009, 2011; Kuai et al., 2014; Barickman et al., 2019).

5 | CONCLUSIONS

In this pioneering study on durum wheat exposed to WL, exogenous MT applied directly on the leaves preserved the aboveground plant organs and, thanks to an efficient osmotic regulation mechanism, the oxidative stress was confined, and an acceptable biomass development was reached despite the impairment of photosynthetic

functions. Conversely, the limited accumulation of MT at the root level did not protect the belowground plant parts from WL, mainly resulting in reduced development of root biomass. Melatonin seems especially effective on the organs in which it is applied. However, no matter the application modality, melatonin should protect the part exposed to the stress (here roots) and not only the organs where it was applied (here shoots) to obtain a more impacting positive effect. Field-trials are mandatory to validate which distribution mode (e.g., preventive application, in soil distribution) would better perform from a farmer's point of view when durum wheat has been sown later at the end of winter. Further studies are necessary to elucidate also how environmental conditions, time and length of WL exposure, and other agronomic practices could influence the effectiveness of MT application in durum wheat.

AUTHOR CONTRIBUTIONS

Cristina Nali: Conceptualization, Supervision, Writing – review & editing. **Claudia Pisuttu**: Formal analysis, Investigation, Writing – original draft. **Daniele Antichi**: Supervision. **Elisa Pellegrini**: Conceptualization, Resources, Supervision, Writing – review & editing. **Giacomo Lorenzini**: Supervision, Writing – review & editing. **Giuseppe Quaratiello**: Formal analysis, Investigation, Writing – original draft. **Lorenzo Cotrozzi**: Conceptualization, Supervision, Writing – review & editing. **Mariagrazia Tonelli**: Investigation, Methodology. **Silvia Pampana**: Conceptualization, Formal analysis, Investigation, Data curation, Supervision, Writing – original draft, Writing – review & editing. **Samuele Risoli**: Data curation, Investigation, Writing – original draft.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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