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Response of White Cabbage (*Brassica oleracea* var. *capitata*) to Single and Repeated Short-Term Waterlogging

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Abstract: Climate change has a significant impact on the agricultural sector, negatively affecting plants' growth and development, with predicted strong consequences on food availability in the future. Although we are experiencing more frequent and intense heavy rainfall events, a major contributor to field flooding, there is still not much known about the impact of these events on different crops. In this study, we investigated the effects of waterlogging on a model plant white cabbage (*Brassica oleracea* var. *capitata* f. *alba*), with the aim to follow its response to both single and recurrent short-term (72-h length) waterlogging, as well as to track difference in the sensitivity between plants in different growth stages (38- and 48-day-old plants). In our 22-day experiment, settled under fully controlled conditions (16 h day/8 h night, 25 °C day/20 °C night, 60–70% relative air humidity, 250 µmol m^{-2} s^{-1} photosynthetic active radiation), with the aim to more comprehensively recognize consequences of waterlogging on plants, we measured changes in plants on multiple levels: (i) within its morphological traits (number and length of leaves, leaf area, and blade width), (ii) within chlorophyll fluorescence and multispectral traits (20 parameters), (iii) following the levels of plant stress parameters (salicylic acid, abscisic acid, proline, and total polyphenols), and (iv) following changes in the plants' elemental and mineral composition. According to our results, white cabbage was shown not to be very sensitive to waterlogging, with only plants exposed to repeated waterlogging showing signs of the congestion stress. These signs, observed in the changes of molecular stress parameters salicylic and abscisic acids, were not so clearly evident at the aboveground level. We did not observe changes in the plants' morphologies, nor their photosynthetic performance. In addition, removal of waterlogging stress resulted in complete recovery of our model plants, suggesting a prompt adaptation response of white cabbage. With the projected increased frequency of occurrence of flooding events, it will become increasingly more important to recognize crops being highly sensitive to flooding with the aim to try to adapt to the changing climate.

Keywords: white cabbage; climate change; waterlogging; morphological characteristics; stress parameters; photosynthetic ability; nutrient content

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1. Introduction

Climate change is considered one of the greatest challenges facing humanity today, inevitably impacting nearly every aspect of our environment. From a human perspective, it threatens the essentials of human health—clean air, safe drinking water, secure housing, and food security—and has the potential to undo decades of human progress [1]. Climate change has been shown to increase the frequency and intensity of extreme weather events, which will ultimately have significant economic consequences for "vulnerable sectors", including agriculture. Since cultivation of food is directly associated with weather conditions, increase in temperatures, intense heats, droughts, wildfires, storms, and heavy

rainfall will seriously affect food production, quality, and price stability. According to Ray et al. [2], the impacts are most negative in Europe, southern Africa, and Australia. Out of those extreme events, heavy rainfalls followed by flooding are projected to occur more frequently in many regions as the climate warms, including Europe [3]. Today, about 10–15% of the world's land area is affected by flooding, with an increasing trend predicted for the near future, posing a major challenge for both research and land management [4]. In developing countries, at least one-tenth (about 12 million ha) of unirrigated cropland has lost productivity due to flooding [5,6].

Of the environmental factors being strongly affected by the climate change, water shortage (drought), salinity, and extreme temperatures are those most commonly studied in different studies on plants [7], while flooding, even though severely affecting crops development and productivity, is only scarcely considered [8]. When discussing soil flooding, we should differentiate two varieties: (i) waterlogging, where only the root system in the soil is affected, and (ii) flooding, where parts or all of the shoot is also inundated [8]. Damage to plants caused in general by both flooding includes oxygen deprivation to both plant roots and the soil microbiome, later being recognized as a key component of plant health and resilience to environmental stress. Flooding leads to a switch in aerobic metabolism of plant roots to less efficient anaerobic fermentation, rapidly depleting carbohydrate reserves [9]. The soil microbiome changes during flooding, leading to intense denitrification and accumulation of ammonium and polyphenolic compounds, with some nutrients such as N and S becoming less available to plants, while others such as P, Fe, Zn, and Mn increase their availability and potentially reach toxic levels [10]. Under such conditions, carbon dioxide, molecular hydrogen, hydrogen sulfide, ethylene, and methane are produced, while the accumulation of reduced phytotoxins (Fe^{2+}; Mn^{2+}; S^{2-}; and, in high concentrations, NH_4^+) in soils retards plant growth and alters their morphological characteristics, such as a reduction in leaf size, wilting of shoots, and necrosis [9]. Low CO_2 availability in flooded leaves limits photosynthesis, and flooding causes an energy crisis in plant cells [8].

In recent years, considerable effort has been devoted to understanding plant adaptive mechanisms to abiotic stresses exacerbated by climate change. The ability of plants to survive flooding and soil oxygen deficiency is determined by an evolutionarily developed resistance to the effects of these stressors and involves a series of interrelated responses aimed at surviving periods of hypoxic and anoxic conditions and maintaining homeostasis, which is evident at multiple levels—transcriptome, proteome, and metabolome. Adaptive capabilities also include anatomical and morphological changes that can contribute to the oxygenation of plant tissues [9]. Adaptive capacity often involves a distribution of photosynthetic ability and, at the molecular level, differential expression of genes involved in the production of signaling molecules and various specialized metabolites [11]. Most crop plants are very sensitive to flooding, and dramatic yield losses occur due to flooding each year [8]. The extent of damage largely depends on the cultivar [12–14], stage of development, and climatic conditions at the time of stress exposure, as well as on the duration of stress. However, still not much is known on the effects of flooding on different crops, with most experiments being conducted on maize, barley, and soybean, and with work on wheat and rape only being started [8]. Because soil is a complex matrix, it is not clear whether the crop damage is primarily caused by anoxia or whether other stress conditions persist after waterlogging has ceased and hinder plant recovery [10]. In general, due to the complexity of the process following flooding, this area of research still remains scarcely studied.

To gain more knowledge on this topic, we settled an experiment in a growth chamber under fully controlled conditions using white cabbage plants as an experimental model. White cabbage (*Brassica oleraceae* var. *capitata* f. *alba*) belongs to the genus *Brassica*, the mustard family Brassicaceae (Cruciferae) [15], and has been shown to be very good experimental model in different studies, including those considering climate change impacts on the crops [13]. In addition, white cabbage is considered a very important crop worldwide.

It is used worldwide as food and in traditional medicine [15]. According to a report by the Food and Agriculture Organization of the United Nations (FAO), global cabbage production in 2020 was 70,862,165 tons, making it one of the most widely grown vegetables in the world [16]. At the same time, there are scarce studies on the effect of floods on white cabbage plants [17].

With the aim to more comprehensively recognize consequences of waterlogging on plants, we measured, using an array of different methods (CropReporter, LC-MS/MS, spectrophotometric analysis, etc.), changes in our model cabbage plants on four different levels: (i) plant yield and leaf morphology (effect on the plant growth), (ii) photosynthetic parameters (effect on the plant performance), (iii) stress markers (effect on plant metabolism), and (iv) micro- and macronutrient accumulation (effect on the plant nutritive status). We hypothesize that the studying of only one parameter could overlook the effect of waterlogging on different plants, depending on their previously mentioned sensitivity. Parameters that we tested in this study included (i) effects of short-term flooding on plants (72 h); (ii) effects of different frequencies of waterlogging on plant development and physiology—we subjected plants to one and two (repeated) waterlogging events; and (ii) testing sensitivity of plants at different growth stages to waterlogging. All these parameters, however, were not selected randomly but represented real field information provided by the farmers in Croatia. Data were obtained as a part of the survey conducted on the EU project PERSPIRE in collaboration with the Croatian Ministry of Agriculture [18], therefore providing our results with an additional value regarding the problems of the real farmers.

2. Materials and Methods

2.1. Materials Used for the Experimental Set-Up

Soil used for the experiment was collected from the agricultural land site situated in the proximity of Zagreb, Croatia (Jakuševec suburb, 45.756962, 16.029497). Soil was sampled in March 2021, on a field where maize (*Zea mays* L.) was the previous crop. Around 300 kg of surface soil (up to 20 cm depth) was randomly sampled from the cca 100 × 100 m size area and transferred to the laboratory where it was kept in a cold room (4 °C) until the beginning of the experiment. The soil composite sample for the experiment was prepared by thoroughly mixing the entire volume of the collected soil.

The basic chemical properties of the soil (Table 1) measured before the start of the experiment were measured according to pH (H_2O), pH (nKCl) [19], organic C (Corg) [20], total nitrogen [21], physiologically active phosphorus (P_2O_5) and potassium (K_2O) [22], and calcium carbonate content ($CaCO_3$) [23]. The soil did not receive any additional mineral fertilizer before nor during the experiment. Soil used in the study was classified as silty loam (Table 2) with 78.5% of silt and 15.0% of clay [24], while water holding capacity and water content at the wilting point [25] were 25.4 and 14.4% weight of the soil, respectively (Table 3).

Table 1. Chemical properties of the soil used in the study.

pH		%		AL-mg/100 g		%
H_2O	nKCl	Humus	N	P_2O_5	K_2O	$CaCO_3$
7.55	7.25	5.58	0.332	44.1	47.5	14.8

Table 2. Mechanical composition of the soil used in the study.

Content of Mechanical Particles in the Soil (%)					Textural Class
Coarse Sand 2.0–0.2 mm	Fine Sand 0.2–0.063 mm	Coarse Silt 0.063–0.02 mm	Fine Silt 0.02–0.002 Mm	Clay <0.002 mm	
1.7	4.8	29.5	49.0	15.0	Silty loam

Table 3. Water retention capacity of the soil used in the study.

Soil Water Retention (% Weight) at 0.33 bar [1]	15.0 bar [2]	Physiologically Active Moisture [3] % weight
25.4	14.4	11.0

[1] Water holding capacity, [2] water content at the wilting point, [3] moisture content available to the plant (the difference between the water content, expressed in mass percentages) at point [2] and point [1].

Seeds of the white cabbage (*Brassica oleracea* var. *capitata* cv. Varaždinski) were obtained by the curtesy of local farmer Jurica Cafuk (GNN 4056186869500). Seedlings were produced by sowing white cabbage seeds in the seedling propagation containers filled with substrate (Klasmann Substrat 1, Geeste, Germany). After seven days, uniformly developed seedlings were transplanted into the plastic pots (72 pots, diameter of 17 cm) filled with soil, prepared as stated above. The experiment was conducted in biological triplicates, each composed of 5 cabbage plants. Transplanted plants were grown in the greenhouse for 35 days from sowing, i.e., until the plant development stage of 3–5 leaves. After this period, plants were transferred into a growing chamber where, after several days of acclimatization, the experiment was commenced, keeping plants under the following conditions: 16 h day/8 h night regime, 25 °C day/20 °C night, 60–70% relative air humidity, and 250 μmol m^{-2} s^{-1} photosynthetic active radiation in the chamber.

2.2. Experimental Design

Randomized experimental design was used with three different waterlogging regime treatments: (1) Treatment 1: plants were waterlogged only once, at the early growth stage; (2) Treatment 2: plants were waterlogged twice, at the early and later growth stage (with 5 days of recovery time between waterlogging); and (3) Treatment 3: plants were waterlogged only once, at the later growth stage (Figure 1). To waterlog the plants, pots with transplanted plants were inserted in an extra empty wider pot protected with the plastic cover that did not allow draining of the water. In the second step, tap water was carefully poured on the soil of the inner pot until the water level reached 2–3 cm above the soil surface. The second step was repeated until the soil was saturated in the way that water level remained stable (depending on the pot, 2–3 L of water was added). In addition, the experiment included a set of control pots with transplanted plants that did not receive any waterlogging during the experiment, although in these, volumetric water content was kept stable daily (35–40%). Volumetric water content (VWC) was measured with a Theta probe ML2x sensor connected to a HH2 moisture meter (Delta-T Devices Ltd., Cambridge, UK), on the basis of mineral soil calibration. Plants were kept under waterlogging conditions for three days (72 h) and drained for two days (48 h) before sampling was conducted. Sampling was conducted at the beginning (day 0), after each waterlogging (day 5 and day 15), before waterlogging (day 10 i.e., recovery phase from the 1st waterlogging), and at the end of the experiment (day 22 i.e., full recovery phase). First, on the whole plants, being still within pots, chlorophyll fluorescence and multispectral imaging was conducted. After this, the aboveground plants were cut-off from their roots. For measurement of molecular stress parameters, five leaves were randomly selected from each triplicate and immediately frozen with liquid nitrogen. In the lab, leaf material was kept at −80 °C until being freeze-dried and homogenized into fine powder. The rest of the collected leaves were used for morphological traits measured. After the morphology measurements, the collected leaves were randomly split into two bunches used for elements and mineral composition measurements, for which half was dried and half was used fresh, depending on the analysis (details below).

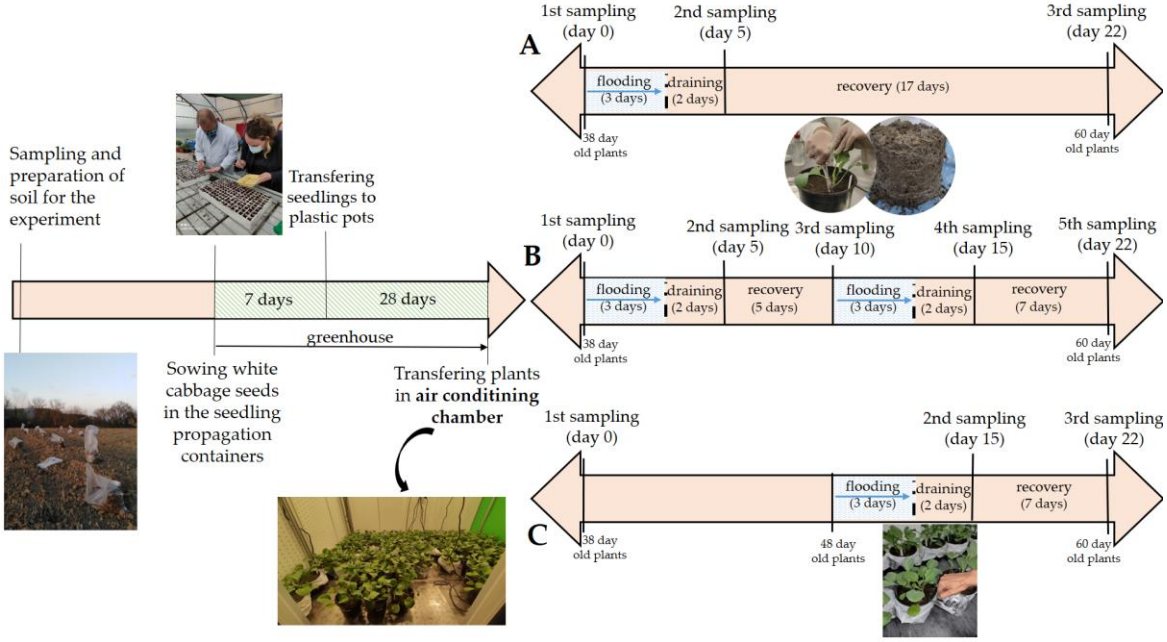

Figure 1. Experimental set-up with three different waterlogging regime treatments: Treatment 1: set-up in which the white cabbage plants were waterlogged once at the early growth stage (**A**). Treatment 2: set-up in which the white cabbage plants were waterlogged twice (**B**). Treatment 3: set-up in which the white cabbage plants were waterlogged once at the later growth stage (**C**). The experiment was conducted under controlled conditions in the growth chamber.

2.3. Plant Biomass and Morphological Characteristics

The morphological traits measured on plants were as follows: (i) mass of aerial parts, (ii) leaf length (cm), and (iii) width of the leaf blade (cm). All leaves (except for the 5 leaves immediately frozen for stress parameters analysis) sampled at each of the sampling points were scanned, and the images were analyzed using a WinFolia Pro 2016b computer image analysis system. The results of the measured morphological traits were calculated as average leaf area (cm^2), average width of the leaf blade (cm), and average leaf length (cm).

2.4. Chlorophyll Fluorescence and Multispectral Analysis

Chlorophyll fluorescence and multispectral imaging was performed on the whole plants before they were removed from the pots and cut off the roots. Analysis was performed at each of the sampling time points using CropReporter^{TM} (PhenoVation B.V., Wageningen, The Netherlands). Before the analysis, plants in the pots were dark-adapted for 30 min. For chlorophyll fluorescence excitation, 4500 µmol m^{-2} s^{-1} red LED light was used. Minimum chlorophyll fluorescence (F0) was measured after 20 µs, and maximum chlorophyll fluorescence (Fm) was measured after the saturation (800 ms). Then, plants were adapted to light under the 250 µmol m^{-2} s^{-1} LEDs for 5 min. Again, 4500 µmol m^{-2} s^{-1} red LED light was used for chlorophyll fluorescence excitation. At the onset of the saturation pulse, the steady-state fluorescence (Fs′) was measured and maximum chlorophyll fluorescence (Fm′) was measured at saturation (800 ms). After that, far-red light was switched on and minimal fluorescence yield of the illuminated plant (F0′) was estimated. From the described measurements, several fluorescence parameters were calculated: (i) maximum efficiency of PSII (Fv/Fm) [26]; (ii) the effective quantum yield of PSII (Fq′/Fm′) [27]; (iii) electron transport rate (ETR) [27]; (iv) non-photochemical quenching (NPQ) [28]; (v) coefficient of photochemical quenching (qP) [29]; (vi) coefficient of non-photochemical quenching (qN) [29]; (vii) estimation of 'open' reaction centers on the basis of a lake model (qL) [30]; (vii) quantum yield of non-regulated non-photochemical energy loss in PSII (ϕno) [31]; and (ix) quantum yield of regulated non-photochemical energy loss in PSII (ϕnpq) [31].

Multispectral imaging was performed under 250 µmol m^{-2} s^{-1} LEDs. Reflection images were collected at red (peak wavelength 640 nm), green (peak wavelength 500 nm), blue (peak wavelength 475 nm), NIR (peak wavelength 769 nm), far red (peak wavelength 710 nm), and at 730 nm and 540 nm. From the collected spectral reflectance data, different vegetation indices were calculated: Chlorophyll Index (CHI) [32] and Anthocyanin Index (ARI) [33], Normalized Differential Vegetation Index (NDVI) [34], hue (0–360°), saturation (SAT), and value (VAL).

2.5. Stress Parameter Analysis

All stress parameter analyses were conducted on the 5 leaves randomly collected at each of the sampling time-points. Leaves frozen in liquid nitrogen were freeze-dried and homogenized before analyses, as explained in the details above.

2.5.1. Proline

Proline content was determined using ninhydrin-based colorimetric assay according to [35] with some modifications. Firstly, 30 mg of the crushed freeze-dried (lyophilized) leaf material was extracted in 1 mL of 70% ethanol. To 100 µL of the extract, 1 mL of the reaction mixture was added. To obtain 50 mL of the reaction mixture, 0.5 g of ninhydrin, 30 mL of ethanoic acid, 10 mL of 96% ethanol, and 10 mL of distilled water were mixed. The reaction mixture was always prepared fresh and kept in a dark bottle. As a blank sample, 100 µL of 70% ethanol was used. Samples and blanks were heated to 95 °C for 20 min in a thermoblock. Proline contents in the samples were determined spectrophotometrically (BioSpec-1601 E, Shimadzu, Kyoto, Japan) at 520 nm. Results were calculated using a standard curve (y = 1.5234x + 0.047, R2 = 0.9988) composed of a serial dilution of the proline standard: concentrations 0.1, 0.2, 0.3, 0.4, 0.5, 1.0, and 1.5 mM, and expressed in µM L^{-1} proline mg^{-1} dw (dry weight).

2.5.2. Total Polyphenolic Content

Total phenolic content in the crushed freeze-dried (lyophilized) leaf material was determined by the Folin–Ciocalteu method adapted by Waterhouse [36]. A total of 20 µL of the plant extract, obtained as described for the proline, was mixed with 1.58 mL of dH_2O and 100 µL of the Folin–Ciocalteu reagent. After 3 s and up to 8 min, 300 µL of the saturated sodium carbonate solution was added. The mixtures were incubated for 2 h at 20 °C in the dark. Absorbance was measured using a spectrophotometer (BioSpec-1601 E, Shimadzu) at 765 nm. A total of 20 µL of 70% ethanol was used as a blank sample. The total phenolic concentration was extrapolated from the standard curve generated using serial concentrations of gallic acid (1, 0.5, 0.25, 0.125, and 0.0625 mg mL^{-1}). Results were expressed as milligrams gallic acid equivalents (mg of GA g^{-1} of dry weight).

2.5.3. Salicylic and Abscisic Acids

Sample Preparation and Analysis

Salicylic acid (SA) and abscisic acid (ABA) were purchased from Fluka, and (+)-*cis*, *trans* abscisic acid was purchased from from Duchefa-Biochemie. The internal isotope labeled standard salicylic acid-d_6 was purchased from Sigma-Aldrich, Darmstadt, Germany; (+)-*cis*, *trans* abscisic acid-d_6 from Trc. MiliQ® water (18.2 MΩ cm^{-1}; purified by MiliQ water purification system (Millipore, Bedford, MA, USA)); and HPLC gradient-grade methanol (J.T.Baker, Center Valley, PA, USA) were used with analytical-grade formic acid (FA) (Acros Organics, Geel, Belgium) for mobile phase preparation.

SA and ABA concentration in leaves were determined using LC–MS/MS analysis following a method developed in-house at the Institute Ruđer Bošković. Sample preparation included extraction of 30 mg of the crushed freeze-dried leaves in 1 mL of 10% methanol containing 1% acetic acid. A total of 40 µL of salicylic acid-d_6 and abscisic acid-d6 as internal standards (1 µg/mL) was added to each sample, followed by homogenization for 2 min at 30 Hz and then 60 min in the cold chamber. Samples were centrifuged for

10 min/13,000 rpm and the supernatant was collected and filtered (Acrodisc®, LC 13, PVDF, 0.45 µm) into vials. A total of 100 µL of the clear solution was injected into the LC column.

LC–MS/MS analysis was carried out using an Agilent Technologies 1200 series HPLC system equipped with a binary pump, a vacuum membrane degasser, an automated autosampler, and an injector interfaced with a 6420 triple quadropole mass spectrometer with an electrospray ionization source (ESI) (Agilent Technologies Inc., Palo Alto, CA, USA). The separation was performed on a Zorbax XDP C18 column (75 × 4.6 mm, 3.5 µm particle size) (Agilent Technologies Inc., Palo Alto, CA, USA). Solvents for the analysis were 0.1% formic acid (FA) in water (solvent A) and methanol (solvent B). The gradient was applied as follows: 0 min 50% A, 5–15 min 50% A-0% A, 15–17 min 0% A, 17.1–22 min 60% A. Flow rate was 0.3 mL/min.

The electrospray ionization source was operated in negative mode, and samples were detected in the multiple reaction monitoring (MRM) mode with a dwell time of 10 ms per MRM transition. The desolvation gas temperature was 350 °C with a flow rate of 6.0 L/min. The capillary voltage was 3.5 kV. The collision gas was nitrogen. The MRM transitions of precursor to product ion pairs were m/z 263-153 for ABA (quantifying ion), m/z 263-219 for ABA (qualifying ion), m/z 137-93 for SA, m/z 269-159 for ABA-d_6, and m/z 141-97 for SA-d_6. Fragmentor voltages were for ABA and ABA-d_6 100 V, and 70 V for SA and SA-d_6. Collision energy was set for SA at 15 V, for and SA-d_6 at 12 V, for ABA quantifying and ABA-d_6 at 3 V, and for ABA qualifying transition at 2 V. All data acquisition and processing were performed using Agilent MassHunter software.

Preparation of Standard and Calibrant Solutions

Stock solutions of each analyte including internal labeled standards were prepared as 1 mg mL^{-1} solutions in methanol. Stock solutions were diluted together in 10% MeOH + 0.1% FA to yield working solutions of 1 µg mL^{-1} and 100 ng mL^{-1} of each substance. A total of 100 ng mL^{-1} solution of ABA and SA in 10% MeOH + 0.1% FA was used as the QC sample. To the QC sample, we also added a mixture of isotope labeled standards ABA-d6 and SA-d6 to the final concentration 38.5 ng mL^{-1}. All standard solutions and the QC sample were stored at −20 °C.

The calibration samples were prepared from stock solutions of each analyte in 10% MeOH + 0.1% FA with the addition of the internal standard solution (40 µL of spike mixture solution ABA-d6 and SA-d6 1 µg mL^{-1}, final concentration 38.5 ng mL^{-1}). Particular calibration points were as follows: calibrant 1 ABA and SA 9.6 ng mL^{-1}, calibrant 2 ABA and SA 24 ng mL^{-1}, calibrant 3 ABA and SA 48 ng mL^{-1}, calibrant 4 ABA and SA 96 ng mL^{-1}, calibrant 5 ABA and SA 192 ng mL^{-1}, and calibrant 6 ABA and SA 480 ng mL^{-1}. A total of 5 µL of each calibrant was injected onto the LC column. The calibration curve was obtained by linear regression; the peak area ratio (analyte/internal standard) was plotted versus the analyte concentration (Figure 2). Least-squares linear regression gave Spearman correlation coefficients of r^2 = 0.9989 for ABA/ABA-d_6 (regression lines y = 0.0223 + 0.0783) and r^2 = 0.9969 for SA/SA-d_6 (regression lines y = 0.359 − 1.5305). The QC sample and instrumental blank were injected after every few runs. During analysis, all instrumental blank samples were negative, and the area of each analyte in QC samples was repeatable.

2.6. Elements and Mineral Composition Analysis

Plant leaf dry matter was determined gravimetrically by drying at 105 °C to constant mass [37]. Nitrates were determined using hot water extraction in fresh cabbage leaf samples, after which their content was determined spectrophotometrically (Evolutuion 60 S, Thermo Fisher Scientific, Waltham, MA, USA). The mineral composition of the leaves was determined in dry white cabbage leaf samples. Total nitrogen was determined by the Modified Kjeldahl method [21]. After digestion of the dry sample with concentrated HNO_3 and $HClO_4$ acids in a microwave oven (ETHOS ONE, Milestone, Latina, Italy); phosphorus was determined spectrophotometrically (Evolutuion 60 S, Thermo Fisher Scientific, USA);

potassium was determined using a flame photometer (Jenway PFP-7, London, UK); and magnesium, calcium, iron, zinc, manganese, and copper were determined by an atomic absorption spectrometer (Thermo Scientific, Solar, Oxford, UK) [38].

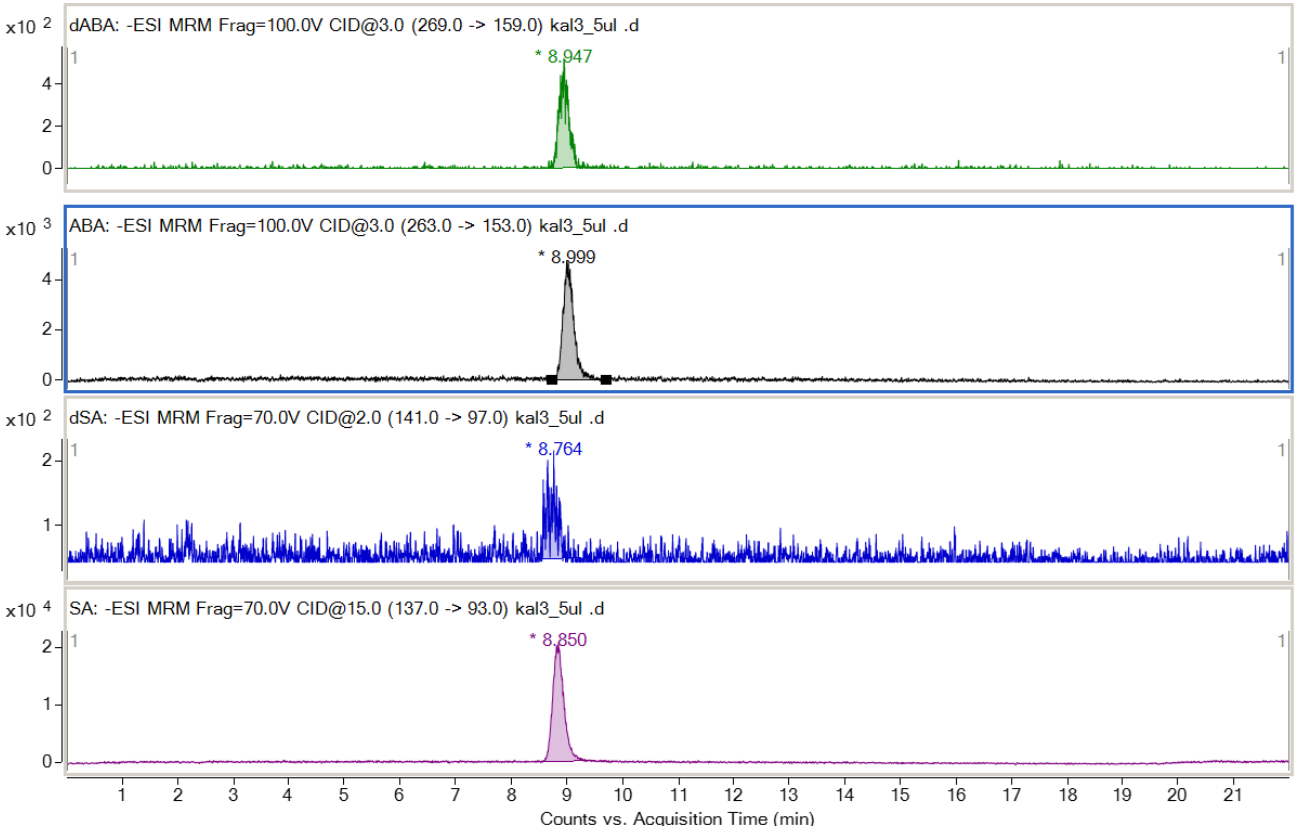

Figure 2. MRM chromatograms of calibrant 3 with ABA (black) and SA (purple) concentrations of 480 ng mL^{-1} spiked with isotopic labeled standards ABA-d_6 (green) and SA-d_6 (blue), * p value < 0.05.

2.7. Statistical Analysis

All analyses were performed in three biological replicates. Data were analyzed using the program PAST 4.1. [39]. The two-samples t-test and Mann–Whitney–Wilcoxon test were performed to find differences in the measured parameters between the control group and the waterlogged sample. The values were considered significant at $p < 0.05$ and $p < 0.01$.

3. Results

3.1. Waterlogging Effects on Plant Biomass and Morphological Characteristics

The biomass and morphological characteristics of the plants measured during the course of the experiment are presented in Table 4 as an average of biological triplicates with standard deviations. In general, no matter the sampling time-point and of the treatment considered, the mass of the waterlogged plants (mass of aerial parts in g) was not shown to be significantly different when compared to the control plants. This was also similar for other morphological traits—average leaf surface (cm^2), average leaf blade width (cm), and average leaf length (cm), which revealed low growth of plants throughout the course of the experiment (all treatments), with no statistical difference found between the waterlogged plants and the control plants. The values of the leaf surface ranged from 30.26 cm^2 minimum to 57.58 cm^2 maximum, leaf blade width from 8.27 to 15.87 cm, and leaf length ranging from 6.93 to 11.78 cm.

Table 4. Waterlogging effects on biomass and morphological characteristics of waterlogged white cabbage plants.

		Mass of Aerial Parts (g)		Average Leaf Surface (cm^2)		Average Leaf Blade Width (cm)		Average Leaf Length (cm)	
	Day 0	17.70 ± 1.25		41.80 ± 1.58		9.10 ± 0.30		10.91 ± 0.79	
		Control	Waterlogged Samples	Control	Waterlogged Samples	Control	Waterlogged Samples	Control	Waterlogged Samples
Treatment 1	After waterlogging	22.14 ± 2.79	23.22 ± 3.83	41.00 ± 4.82	38.60 ± 8.35	9.50 ± 0.30	9.50 ± 1.07	9.91 ± 0.71	9.42 ± 1.27
	At the end	26.32 ± 2.54	28.75 ± 5.03	43.50 ± 5.92	42.70 ± 5.57	10.30 ± 0.46	11.70 ± 3.62	9.40 ± 1.12	8.00 ± 0.75
Treatment 2	Before the second waterlogging	26.04 ± 3.28	23.58 ± 2.72	46.21 ± 2.94	35.15 ± 4.23 *	10.62 ± 0.43	9.69 ± 0.43	10.11 ± 1.21	8.60 ± 1.46
	After the second waterlogging	24.99 ± 2.21	25.12 ± 1.47	40.56 ± 4.32	35.85 ± 3.84	10.50 ± 0.51	9.96 ± 0.44	9.53 ± 0.32	8.64 ± 0.53
	At the end	26.32 ± 2.54	28.09 ± 2.20	43.50 ± 5.92	43.31 ± 1.99	10.30 ± 0.46	10.69 ± 0.88	9.40 ± 1.12	8.95 ± 0.36
Treatment 3	After waterlogging	24.99 ± 2.21	28.99 ± 4.65	40.56 ± 4.32	46.80 ± 5.45	10.50 ± 0.51	10.70 ± 0.73	9.53 ± 0.32	9.48 ± 0.49
	At the end	26.32 ± 2.54	27.92 ± 2.78	43.50 ± 5.92	46.70 ± 1.31	10.30 ± 0.46	10.20 ± 0.23	9.40 ± 1.12	9.70 ± 0.18

* p value < 0.05; the average values in each case are followed by the value of the standard deviation.

3.2. Waterlogging Effects on Chlorophyll Fluorescence and Multispectral Parameters

The effect of waterlogging stress on the photosynthetic performance of the white cabbage plants was evaluated by measuring various chlorophyll fluorescence parameters. This included nine chlorophyll fluorescence traits, namely, (CFT)- Fv/Fm, PSII (Fq′/Fm′), ETR, NPQ, qP, qN, qL, φno, and φnpq, and eleven multispectral traits (MST)—Red, Green, Blue, Hue, Saturation, Value, SpcGrn, FarRed, Nir, ChlIdx, and AriIdx, which are listed and explained in the Supplementary Materials (Tables S1–S4). The effect of waterlogging on the changes of these parameters was observed for only five of the abovementioned CFT traits: (i) effective quantum yield of PSII (Fq′/Fm′), (ii) electron transport rate (ETR), (iii) non-photochemical quenching (NPQ), (iv) coefficient of non-photochemical quenching (qN), and (v) quantum yield of regulated non-photochemical energy loss in PSII (ϕnpq). The results for these parameters are presented in Figure 3, for all three treatments, in the form of normalized values of selected CFT. All values represent percentages of values obtained for control plants, enabling comparison of the variables measured on different scales. Conversely, for parameters Fq′/Fm′ and ETR, a negative effect on the plants' photosynthetic performance was displayed after waterlogging in Treatment 1 as a decrease in the CFT values in this time point; for parameters NPQ, qN, and PSII (ϕnpq) a negative effect was displayed as an increase after waterlogging in the measured values (Figure 3). However, changes within these parameters were not found to be significant. Both Treatments 2 and 3 showed no clear effect of the second waterlogging and waterlogging of the plants at the later growth stage on the CFT parameter (Figure 3).

3.3. Waterlogging Effects on Plant Molecular Stress Parameters

To investigate the stress status of plants under waterlogging, four molecular stress parameters were analyzed, namely, proline, total polyphenolics, ABA, and SA content. Values shown in Table 5 are presented as an average of biological triplicates with standard deviation. Measured values indicated no changes in all four analyzed parameters in Treatment 1. However, in Treatment 2, significant changes, when compared to the control plants, were observed within three measured parameters at the time-point after the second waterlogging: decrease in proline (46.31% decrease) and ABA (53.82% decrease), and increase in SA content (104.75% increase). Finally, in Treatment 3, a significant decrease in the parameters proline (56.79% decrease) and SA (82.23% decrease) was observed after the waterlogging event. At the end of the experiment, the three mentioned parameters again reached similar values in waterlogged plants vs. control ones. Changes in the values of the fourth parameter, total polyphenolics, were observed only in Treatment 2, with

an increase observed in plants in the recovery phases—both after the first waterlogging (21.73% increase) and at the end (18.86% increase), and not after waterlogging, as expected.

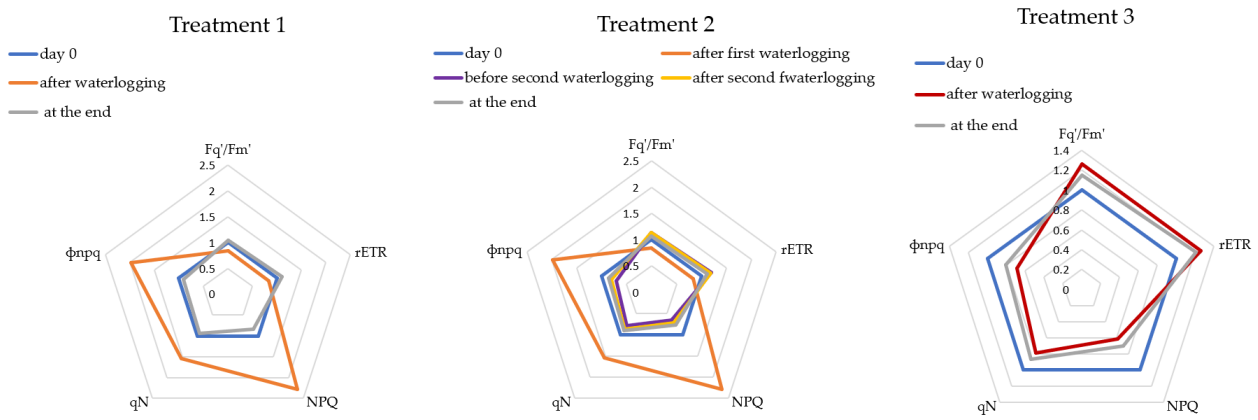

Figure 3. Spider plots obtained from photosynthetic measurements measured in 3 different treatments. Treatment 1, Treatment 2, Treatment 3. All values shown are percentages of values obtained for control plants, enabling comparison of the variables measured on different scales.

Table 5. Changes in molecular stress parameters under waterlogging.

		Proline (µmol/g DW)		Total Polyphenols (mg GAE/g DW)		ABA (ng/g DW)		SA (ng/g DW)	
	Day 0	**3.35 ± 0.03**		**17.68 ± 0.88**		**87.30 ± 16.85**		**468.21 ± 30.22**	
		Control	**Waterlogged Samples**	**Control**	**Waterlogged Samples**	**Control**	**Waterlogged Samples**	**Control**	**Waterlogged Samples**
Treatment 1	After waterlogging	5.28 ± 1.94	3.95 ± 0.87	15.82 ± 2.44	15.81 ± 0.84	206.72 ± 52.71	299.09 ± 65.20	2232.27 ± 1161.77	1167.69 ± 522.91
	At the end	3.18 ± 0.51	3.73 ± 1.51	12.75 ± 0.80	9.33 ± 2.61	184.89 ± 123.43	286.58 ± 132.00	1622.09 ± 525.56	1861.55 ± 280.73
Treatment 2	Before the second waterlogging	4.33 ± 0.94	5.96 ± 1.24	13.90 ± 0.80	16.92 ± 1.28 *	211.63 ± 39.42	203.75 ± 49.83	1054.88 ± 65.91	946.42 ± 119.46
	After the second waterlogging	8.40 ± 1.50	4.51 ± 0.90 **	12.73 ± 2.99	14.48 ± 1.24	778.88 ± 6.45	359.68 ± 40.95 *	1115.15 ± 303.97	2283.27 ± 432.92 *
	At the end	3.18 ± 0.51	3.23 ± 0.54	9.65 ± 0.67	11.47 ± 0.80 *	184.89 ± 123.43	347.47 ± 67.19	1622.089 ± 525.56	1538.58 ± 165.92
Treatment 3	After waterlogging	8.40 ± 1.50	4.77 ± 0.61 **	12.73 ± 2.99	13.57 ± 0.70	778.88 ± 6.45	309.27 ± 165.58	1115.15 ± 303.97	198.12 ± 51.22 **
	At the end	3.18 ± 0.51	3.99 ± 0.44	9.65 ± 0.67	9.75 ± 1.41	184.89 ± 123.43	149.16 ± 61.63	1622.089 ± 525.56	890.89 ± 211.33

* p value < 0.05; ** p value < 0.01. The average values in each case are followed by the value of the standard deviation.

3.4. Waterlogging Effects on the Concentration of Elements and Minerals in Plants

All data gained by measuring selected elements and minerals (nitrate, N, P, K, Ca, Mg, Fe, Zn, Mn, and Cu) in plant leaves are presented in Table S5. Measured nitrate concentration was shown to range from 30.24 to 634.66 mg NO_3 kg^{-1}, with very strong variance between biological replicates at the same sampling point. Nitrogen values ranged from 0.80 to 2.58% N DW, phosphorus from 0.15 to 0.44% P DW, potassium from 1.40 to 3.98% K DW, calcium from 92.47 to 410.83% Ca DW, magnesium from 14.75 to 68.67% Mg DW, iron from 26.97 to 95.71 mg Fe kg^{-1} DW, zinc from 9.86 to 26.85 mg Zn kg^{-1} DW, manganese from 21.42 to 78.24 mg Mn kg^{-1} DW, and copper from 1.34 to 25.80 mg Cu kg^{-1} DW.

In Treatment 1, a significant decrease was observed in four different parameters when comparing waterlogged plants to the control ones. This was seen at the time of sampling after waterlogging for the elements phosphorus (34.3% decrease), potassium (27.2% decrease), zinc (30.5% decrease), and copper (24.1% decrease). In addition, a significant increase in manganese content was observed in the waterlogged plants at the end of this treatment compared to the control plants (37.2% increase). In Treatment 2, in the recovery

time before the second waterlogging, the waterlogged plants showed a significant decrease in two parameters: magnesium by 21.9% and copper by 14.04%, compared to the control. Moreover, in Treatment 2, significant changes were observed in two different parameters at the end: an increase in calcium (22.8%) and, on the other hand, a decrease in zinc (24.2%). After the second waterlogging, only potassium showed a significant decrease (40.3%). In Treatment 3, potassium content significantly decreased in the waterlogged samples after waterlogging in the later growth stage, and zinc content also significantly decreased at the end of the experiment (24.9%).

4. Discussion

Flooding is a serious abiotic stressor that can significantly affect plant growth. Despite the fact that excessive rainfall, a major contributor to flooding, is expected to occur more frequently in the coming years, with projected high crop losses, this area of research is still poorly studied. Studies have shown that crops are sensitive to flooding and exhibit a range of morphological and metabolic changes [8]; however, they are still scarce, with often contradictory results. With the aim to provide additional data on the effects of floods on the crops, in the present study, we conducted an experiment using white cabbage as a model plant and tested its sensitivity and adaptive responses to short-term waterlogging. Even though it is an important source of food, there are an exceptionally low number of studies [17] dealing with the effects of flooding on this vegetable species. Flooding is a very complex question and can occur over different time periods and at different stages of crop growth. Therefore, our innovative approach included the selection of experimental parameters based on real field conditions experienced by our farmers in Croatia [18]. Due to climate change, in the northern continental part of Croatia, being an important agricultural area, we are experiencing more frequent excessive rainfall followed by flood on fields. According to the results of our survey conducted among Croatian farmers, excessive rainfall followed by waterlogging usually occurs in fields at the younger plant stages (before the fifth leaf), with the majority of farmers (79%) indicating that the observed floods lasted up to seven days (which is considered a "short-term flooding event") [18]. With the aim to fully address possible response of plants to waterlogging, we decided to follow plant growth parameters, changes in the plant performance, plant stress parameters and changes in plant nutritive status.

It is known that anaerobiosis occurs within a few hours after the soil is saturated with water. As Mustroph [8] summarizes, the first strategy plants adopt to avoid oxygen depletion in their flooded parts involves anatomical and morphological changes that improve gas exchange with the environment. In some plants, visible signs of deterioration begin after only 48 h of waterlogging [40]. Interestingly, in our study, after 72 h of waterlogging, we did not observe significant effects on the morphological characteristics of aboveground white cabbage plants, including the mass of aboveground parts, leaf surface, and leaf blade length and width, giving us first indications on the low sensitivity of the selected cabbage to the waterlogging stress. Although no significant effect on the leaf morphology was found in the study, there was a slight decrease in leaf area due to the reduced average leaf length, but leaf blade width increased. Lenssen et al. [41] hypothesized that waterlogged roots may act as a strong carbohydrate sink and thus inhibit investments in above-ground plant parts, which can cause leaf growth cassation. Survival under hypoxic and anoxic conditions requires two contrasting strategies, termed as low-oxygen escape and low-oxygen quiescence. In the escape strategy, plants improve leaf acclimation and aerenchyma formation, as well as increase the shoot elongation, while in the quiescence strategy, plants cease their growth and conserve carbohydrates reserves [42]. In addition, Vashist et al. [43] stated that tolerant *Arabidopsis* accessions have the capacity to dampen shoot elongation under submergence and thus conserve carbohydrates and survive longer than fast-growing plants. The noticed the response of decreased leaf length with increased width could be linked to the ethylene-caused exit of cell division and a shift to endoreduplication and differentiation [44].

As we can see from the study conducted by Casierra-Posada and Cutler [17], cabbage can show signs of deterioration under waterlogging, but probably requires more severe conditions than those settled within our experiment. In the Casierra-Posada and Cutler [17] experiment, 25 days of waterlogging significantly reduced leaf area, total dry weight, chlorophyll content, leaf area ratio, absolute growth rate, and relative growth rate of the plants. Many studies have also found that plant growth is most affected when flooding occurs at early growth stages [45–48]. Because the severity of morphological changes in plants is related to the duration of flooding and the amount of water to which plants are exposed [49], we hypothesize that our short-term waterlogging event was not severe enough to cause morphological changes in white cabbage, even when repeated or at early growth stages. The literature also indicates that submergence, where parts or all of the shoot is also under water, results in more pronounced morphological changes than waterlogging and can cause a change in leaf angle to a more upright position [50] or increase shoot growth under water to pull leaves out of the water [51]. Because white cabbage is generally considered a plant that is less sensitive to various abiotic stressors [12,13,52], it is possible that white cabbage has evolved morphophysiological adaptations that allow it to better cope with waterlogging.

Changes in the fluorescence emission of photosynthetic chlorophyll a from photosystem II (PSII) is usually one of the traits first to be shown when plants are exposed to different both biotic and abiotic stresses [53,54]. Many ecophysiological studies also suggest the Fv/Fm coefficient as an indicator of photosynthetic photoinhibition [17], which decreases when a plant is exposed to a stressor. Although in our study, slight changes in the values of the Fq′/Fm′, ETR, NPQ, qN, and ϕ npq traits indicated short-term effects of waterlogging on the plants, these values were not confirmed as statistically relevant. Measuring of the chlorophyll fluorescence and multispectral characteristics confirmed low sensitivity of our selected model plant. Although we monitored plant photochemistry through chlorophyll fluorescence measurement, for better understanding of the physiological responses of plants to waterlogging, it would be of great value to include the measurements of the gas exchange, particularly CO_2 and O_2 levels at different plant organs. The plant survival under hypoxia/anoxia highly depends on carbon metabolism (photosynthesis and respiration) adaptation [55]. Hypoxia causes fast decline in stomatal conductance, which limits CO_2 intake and inhibits photosynthesis. Hypoxia/anoxia tolerance is closely related to the carbohydrate reserves and metabolism, thus except in the production of carbohydrates, photosynthetic oxygen production is important for supporting energy gain from the respiration [55]. Mommer and Visser [56] related the higher survival of submerged plants with the increased rates of underwater photosynthesis, leading to greater carbon gain and the production of molecular oxygen.

Plants exposed to stress conditions are also known to activate many different metabolic pathways, resulting in increased levels of certain molecules recognized as plant stress parameters [57,58]. Following this hypothesis, in our experiment, we measured changes in the levels of proline, total polyphenols, ABA, and SA to determine possible effects of waterlogging on white cabbage at the metabolic level. Measurements of all four plant stress parameters showed that plants exposed to a single episode of waterlogging in the early growth stage were not stressed by waterlogging. However, repeated waterlogging (second flooding) and waterlogging at the later stage of development were found to induce a metabolic response in our plants, and the stress parameters measured showed a dependence on the treatment applied, i.e., the number of waterlogging events and the developmental stage of the plants. In previous experiments with *Brassica* plants, proline has already been reported as a very good stress marker, but mainly in the context of excessive salinity [13], drought [12], and low temperatures [59]. The trend of proline accumulation under excessive water stress is not clear in the literature, and different authors generally report different trends. Balakhnina et al. [60] reported an increase in proline content in spring canola (*Brassica napus* var. *oleifera* f. *annua* cv. Lisonne) under flooding during the first 3 days, after which proline content decreased. Teoh et al. [61] observed no changes in

proline content in banana plants under 1-day flooding. Yiu et al. [62] reported a decrease in proline content in roots of flooded onion plants. In our experiment, it was found that repeated waterlogging (second flooding) and waterlogging of the plants at the later stage of development decreased the proline content in the plants, while it increased in the control plants. At the end of the experiment, the proline content we found was comparable in all plants, regardless of the scenario, indicating that our model plant requires a short recovery period.

ABA and SA are also known to be important stress-related molecules [63]. In our experiment, both parameters showed changes when repeated waterlogging (second flooding) was included in the experiment: ABA decreased significantly, while SA increased. Similar to proline, the values measured at the end of the experiment showed that the plants recovered quickly from the stress. Kim et al. [64] also reported a decrease in ABA content in waterlogging-tolerant soybean (*Glycine max* L.) after flooding in their study and attributed this observation to an adaptation for better aerenchymal cell development. To survive flooding conditions, plants need morphological changes in their roots, and in response, the aerenchymal cells are formed, but for their development, a root cell must be detoxified and the biosynthesis of suberin must be suppressed by downregulating ABA [64]. Similar to our results, in the study by Kim et al. [64], a decrease in ABA was followed by an increase in SA. Under abiotic stress, SA regulates various plant metabolic processes and modulates the production of various osmolytes and specialized metabolites and maintains the nutrient status of the plant [63]. Increasing SA level is one of the tolerance factors for abiotic stress [64], and exogenous SA mitigates abiotic stress in various crops such as tomato [65], waxy maize [66], and soybean [67]. Our scenario, where we waterlogged the plants once but at a later stage of plant development, showed that only the levels of SA decreased under these conditions and did not increase as expected.

The third parameter, total polyphenol content, which is also commonly used as an indicator of plant stress [68,69], did not prove to be a good marker of waterlogging stress in our experiments. Similarly, Šola et al. [70] found no differences in total polyphenol content in Chinese cabbage (*Brassica rapa* ssp. *pekinensis*) treated with excess water. We found a small increase in total polyphenol content in our white cabbage plants exposed to repeated waterlogging, but not immediately after waterlogging, but with a short time delay. Because the role of polyphenols in responding to abiotic stresses, including flooding, is structure dependent, we suggest that profiling of individual polyphenolic compounds may be a better indicator, allowing us to detect differences [14].

In addition to the abovementioned parameters, we likewise did not observe any clear effect of waterlogging on the nutrient and elemental status of our plants in the experiments. Usually, lowering of the soil oxygen concentration, as occurring during waterlogging, affects nutrient uptake and changes in the levels of elements and minerals in plants. Likewise, leaching and denitrification losses of nitrogen and limited nitrogen uptake in flooded soils can lead to nitrogen deficiencies in plants, while lack of root function and movement of water and calcium in the plant can lead to calcium-related disorders in plants. Oxygen deficiency in soils has several negative effects: it alters the nitrogen pathways, reduces nutrient availability and pH [71–73], and increases the solubility of toxic metals [74,75]. Even though we did see significant impacts of waterlogging on several elements and minerals, these observations were not reproducible and therefore it was difficult to conclude their direct connection to waterlogging. The measured values indicated that short-term waterlogging stress has a minor effect on these parameters, which is consistent with other results indicating that waterlogging does not cause major damage to the aboveground parts of our model plant species. Obviously, white cabbage is a plant species that can manage short-term waterlogging, which is in fact good data for agricultural producers, especially in areas with higher predictability of spring flooding.

White cabbage, which was selected as a model for studying the effects of waterlogging on plants, proved to be quite an insensitive and adaptable plant in our experiment. Because plant response to waterlogging depends on the soil type; air temperature; and a number

of different unpredictable environmental factors such as duration, timing, and depth of waterlogging, additional studies will be required in order to draw general conclusions about white cabbage sensitivity to waterlogging. In at the same time, this study has shown us that changes in plants can sometimes be seen only at the specific levels, such as the level of specific stress metabolites, as shown in our white cabbage. This further raises the question of what might be a good but at the same time universal parameter that would allow researchers to track the effects of these extreme events on different crops. As climate change continues and waterlogging becomes an even more frequent event, similar studies on the adaptability of different crops to a changing climate will be needed. This kind of better understanding of crop response to waterlogging may allow us to propose alternative solutions to reduce waterlogging risk through improved agronomic and engineering solutions. The climate continues to evolve, and farmers need to anticipate future sensitivities and propose mitigation strategies to maintain food production under predicted climate change scenarios.

5. Conclusions

The results of our study provide the first insight into the effects of short-term flooding on white cabbage plants, which have been understudied in relation to flooding. White cabbage plants exposed to short-term waterlogging showed signs of waterlogging stress but can be classified as rather insensitive and adaptable plants. Signs of waterlogging stress were not clearly evident at the aboveground level of the plant (in both morphology and photosynthetic performance) but were so at the level of the molecules ABA and SA, which are referred to here as important waterlogging stress-related parameters. Measurements from ABA and SA showed that plants exposed to a single bout of waterlogging in the early growth stage were not stressed by waterlogging, whereas repeated waterlogging and waterlogging in the later stages of development triggered a metabolic response in plants. Elimination of waterlogging stress appears to result in complete recovery of white cabbage plants, indicating a rapid adaptation response during the recovery phase.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/agronomy13010200/s1, Table S1: Flooding effects on chlorophyll fluorescecnce traits; Tables S2 and S3: Flooding effects on multispectral traits; Table S4: List of analyzed traits with abbreviations, indication if it is measured or calculated trait, wavelength for measurement or equation for calculation, device and software and the reference if appropriate; Table S5: Flooding effects on elements and mineral composition.

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