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Enhancing Drought Tolerance in Lettuce: The Efficacy of the Seaweed-Derived Biostimulant Cytolan® Stress Applied at Different Growth Stages

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Abstract: Water stress is one of the foremost global abiotic stressors limiting agricultural productivity. Biostimulants and bioactive compounds are emerging as promising tools to enhance crop stress tolerance. This study investigates the effects of Cytolan® Stress, a novel seaweed-derived biostimulant, on the water stress tolerance of lettuce plants. Three application strategies were evaluated: priming, where the biostimulant is applied before the onset of stress to prepare the plants for adverse conditions; buffering, involving application at the onset of stress to mitigate its immediate effects; and detoxifying, where the biostimulant is applied after stress to aid in plant recovery. Biomass, stress-related parameters, antioxidant activity, osmoprotectant levels, and photosynthesis-related metrics were analyzed to elucidate its potential mechanisms of action. The results demonstrated that Cytolan® Stress in priming and buffering applications significantly improved water stress tolerance, reducing biomass loss from 45% to only 25%. Moreover, the detoxifying treatment was the most effective, as plants showed biomass values similar to those of the control plants. The biostimulant reduced oxidative stress indicators while enhancing antioxidant defenses, including ascorbate (AsA)-glutathione (GSH) cycle, antioxidant compounds, and enzyme activities. In addition, Cytolan® Stress preserved photosynthesis performance under water stress conditions. These findings highlight the potential of Cytolan® Stress to mitigate drought stress effects in lettuce, offering broader implications for crop tolerance and resilience under water-limited conditions. Further studies are recommended to explore its efficacy across different crops and stress scenarios.

Keywords: antioxidants; bioactive compounds; *Lactuca sativa*; photosynthesis; proline; water stress

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1. Introduction

Water stress and soil salinity are among the foremost global abiotic stressors affecting agricultural productivity [1,2]. Soil salinity is often exacerbated by insufficient water availability, a common condition across arid and semi-arid regions [3]. Together, these stresses impact over 100 countries and approximately 20% of the world's cultivated lands [1]. Projections suggest that by 2050, due to increasingly severe climate change effects, over 50% of arable land globally will experience water shortages, leading to substantial agricultural losses, particularly in Mediterranean and other vulnerable regions [2,4].

Water deficit, a common consequence of water stress, severely impairs plant growth and crop productivity by inducing complex morphological, physiological, and biochemical changes. Plants initially respond to water stress with rapid shoot growth inhibition (though root growth is comparatively less inhibited) and partial or complete stomatal closure, which reduces transpiration and subsequently limits CO_2 uptake, essential for photosynthesis [3,5,6]. Prolonged water stress disrupts reproductive development, leading to premature leaf senescence, wilting, and eventual leaf death. This damage largely arises from two interconnected processes: altered water relations within the plant and the excessive production of reactive oxygen species (ROS) in chloroplasts. Stomatal closure restricts CO_2; availability, causing an imbalance between electron generation and usage in photosynthesis. This disruption causes excess energy to be dissipated in Photosystem II (PSII), generating ROS such as hydrogen peroxide (H_2O_2), superoxide anions (O_2^-), and hydroxyl radicals (OH·), all of which accumulate under continued stress, posing a severe threat to cellular integrity [3,6,7]. Additionally, water stress reduces ascorbic acid levels, induces lipid peroxidation, and can result in irreversible cellular damage [6,8]. Analyzing these parameters is crucial for assessing the extent of stress a plant is experiencing and for identifying potential strategies to enhance stress tolerance and resilience.

One sustainable and environmentally friendly approach to alleviating plant stress from these conditions is the use of biostimulants. These compounds, applied externally at low concentrations, can enhance plant growth, boost tolerance to abiotic stresses, strengthen defense mechanisms against pathogens, and support reproductive development [9–12]. Among the various types of biostimulants, seaweed extracts have gained prominence for their bioactive profiles, offering a wide array of signaling molecules, phytohormones, vitamins, polysaccharides, amino acids, and essential micro- and macronutrients [13–16]. In the present study, the efficacy of Cytolan® Stress biostimulant was assayed. This product is composed of a seaweed extract but enriched with several bioactive compounds which can enhance its positive effect.

Seaweed-based biostimulants have demonstrated several beneficial effects on plant growth and tolerance. They aid in the biosynthesis of a range of nitrogenous non-protein compounds, enhance root system architecture, and improve nutrient use efficiency. They also stimulate hormonal activity within plants, contributing to improved resistance against both biotic and abiotic stresses through the production of antioxidants and osmoprotective compounds [17–20]. Specifically, under water stress conditions, seaweed biostimulants can regulate leaf gas exchange and water use efficiency (WUE), thus boosting photosynthetic performance by increasing the total chlorophyll index. Additionally, they provide osmoprotectants and reduce oxidative stress through elevated levels of enzymatic and non-enzymatic antioxidants [21–23].

Biostimulants are frequently enriched with bioactive compounds or bioregulators, which further enhance their effectiveness. Common additives include plant hormones such as auxins, gibberellins, cytokinins, brassinosteroids, abscisic acid, salicylic acid, and melatonin. Antioxidants like α-tocopherol and ascorbate, nitrogenous compounds (glycine betaine and proline), and carbohydrates and polyols (trehalose, mannitol) are also widely used. These substances play a key role in reducing oxidative stress by directly or indirectly neutralizing ROS, regenerating oxidized molecules (especially lipids and proteins), and enhancing the plant's overall antioxidant system. Furthermore, many of these compounds act as osmoprotectants, stabilizing and protecting cellular structures from oxidative damage while upregulating genes associated with stress resistance [24,25]. Due to their diverse benefits, biostimulants combined with bioactive compounds are increasingly favored, although their synergistic effects under specific abiotic stresses are still not fully understood.

Promisol S.L. has developed a new biostimulant, Cytolan® Stress, composed of the seaweed *Ascophyllum nodosum* extract enriched with bioactive compounds, including proline, mannitol, vitamins C and E, salicylic acid, and menadione sodium bisulfite. Previous studies demonstrated that this biostimulant, without menadione sodium bisulfite, promoted lettuce growth under optimal conditions [26]. The hypothesis to be tested in this study was that Cytolan® Stress application will enhance lettuce tolerance to water stress and that the time of application will affect its effect on the plant. Therefore, this study aimed to explore the effects of Cytolan® Stress application on water stress tolerance and analyze its potential action mechanisms in lettuce plants. Moreover, it evaluated the effect of different application timings: priming (application before stress), buffering (application at the start of stress), and detoxification (application after stress), to determine the most effective approach for enhancing plant tolerance and resilience.

2. Materials and Methods

2.1. Plant Material and Growth Conditions

Lettuce plants (*Lactuca sativa* cv. Maravilla de Verano) were used in this study. This cultivar was selected because it is commonly grown during the warmer months, a period when the risk of water stress is particularly high. Its widespread use in commercial production under these conditions makes it suitable for studying the effectiveness of biostimulants in mitigating water stress. Seeds were germinated and cultivated for 45 days in cell trays (3 cm × 3 cm × 10 cm) at Saliplant S.L., located in Carchuna, Granada. Subsequently, the seedlings were transferred to a growth chamber in the Department of Plant Physiology at the University of Granada, where environmental conditions were tightly controlled: relative humidity between 60–80%, temperatures of 25 °C during the day and 15 °C at night, and a photoperiod of 16 h of light and 8 h of darkness. The photosynthetic photon flux density (PPFD) was maintained at 350 µmol m^{-2} s^{-1}, as measured with an SB quantum sensor (LI-COR Inc., Lincoln, NE, USA).

Under these conditions, the plants were grown in individual pots (13 cm upper diameter, 10 cm lower diameter, 12.5 cm height, 2 L capacity) filled with a perlite-based substrate at a 1:3 ratio. Fertilization was carried out using a modified Hoagland nutrient solution tailored for lettuce cultivation and containing 4 mM KNO_3, 2 mM $Ca(NO_3)_2$, 2 mM $MgSO_4$, 1 mM KH_2PO_4, 1 mM NaH_2PO_4, 2 µM $MnCl_2$, 1 µM $ZnSO_4$, 0.25 µM $CuSO_4$, 0.1 µM Na_2MoO_4, 125 µM Fe-EDDHA, and 50 µM H_3BO_3, with the solution adjusted to a pH of 5.8. Each pot received approximately 50 mL of nutrient solution daily, ensuring that drainage did not surpass 10% of the applied volume.

2.2. Description of Treatments and Experimental Design

The water stress treatment involved reducing the nutrient solution supply by 50% (50% field capacity). The control treatment was watered at 100% field capacity. Field capacity was determined gravimetrically by saturating the growth substrate with water, allowing it to drain until no further water loss occurred, and then measuring the water content retained in the substrate. The experimental timeline was as follows: Cytolan® Stress was applied for the priming effect two days prior to water stress initiation, allowing sufficient time for the biostimulant to activate potential protective mechanisms in the plants. Water stress was then initiated, and Cytolan® Stress was applied again for the buffering effect to counteract the immediate impact of stress. The stress period lasted nine days, after which plant sampling was conducted to evaluate the priming and buffering effects, as this duration was deemed adequate to observe measurable physiological responses. Cytolan® Stress was applied for the detoxifying effect at the end of the stress period to support recovery and mitigate residual damage. Sampling for the detox effect took place ten days later to

allow enough time for the plants to exhibit recovery-related changes. The biostimulant Cytolan® Stress is composed of an alkaline extract of the seaweed *A. nodosum* to which proline, mannitol, vitamins C and E, salicylic acid, and menadione sodium bisulfite were added. The treatments were applied foliarly using a sprayer, with a control group included that did not receive the biostimulant application. The experimental design followed a completely randomized block layout, with eight plants per treatment. Each plant was grown in an individual pot and randomly positioned within the growth chamber.

2.3. Plant Sampling

As indicated in the experimental timeline, two plant samplings were conducted. The first sampling, to study the priming and buffering effects, was done at the end of the water stress period (10 days). The second sampling, to study the detox effect, occurred ten days after the stress period ended and after applying the different prototypes to the plants. All plants in each treatment group were processed immediately for further analysis.

The sampled plant material was washed and dried on filter paper to obtain fresh weight (FW). Half of the fresh samples, either immediately or after being frozen at −40 °C, were used for analysis of the following parameters: leaf area; chlorophyll *a* fluorescence (Fv/Fm, RC/ABS, PI(Abs)); photosynthetic pigments; photosynthetic efficiency (IRGA-LiCOR 6400); electrolyte leakage; relative water content; enzymatic activities of ascorbate peroxidase, glutathione reductase, and catalase; antioxidant tests (FRAP and TEAC); and concentrations of malondialdehyde (MDA), reactive oxygen species H_2O_2 and O_2^-, ascorbate and glutathione in their reduced, oxidized, and total forms, total phenols, flavonoids, anthocyanins, sucrose, proline, and glycine betaine. The other part of the plant material was dried in a forced-air oven and used to determine dry weight (DW).

2.4. Analysis of Plant Material

2.4.1. Leaf Area

Leaf area was measured using an optical reader, model LI-3000A (LI-COR Biosciences, Lincoln, NE, USA).

2.4.2. Relative Water Content

Leaf relative water content (RWC) was evaluated at the conclusion of the experiment. Leaf sections were excised, and their fresh weight (FW) was recorded promptly. The samples were then immersed in distilled water inside Petri dishes and allowed to hydrate for 4 h at room temperature under continuous light. Following this, the turgid weight (TW) was measured, and the samples were dried at 80 °C for 24 h to determine the dry weight (DW). RWC was calculated using the following formula [27]:

$$\text{RWC (\%)} = [(\text{FW} - \text{DW})/(\text{TW} - \text{DW})] \times 100$$

2.4.3. Electrolyte Leakage

Membrane stability was assessed by measuring electrolyte leakage [28]. Fresh plant material (0.3 g) was cut into pieces, washed lightly with deionized water, and placed in a test tube with 30 mL of deionized water. The tubes were vortexed for 1 min, and initial conductivity (EC1) was measured with a conductivity meter (Cond 8; XS Instruments, Carpi, Italy). The tubes were then incubated in a water bath at 100 °C for 20 min to release electrolytes and allowed to cool to room temperature. Final conductivity (EC2) was measured. Electrolyte leakage was calculated as (EC1/EC2) × 100.

2.4.4. Determination of Oxidative Markers (MDA, H_2O_2, and O_2^-)

Malondialdehyde (MDA) was measured using the trichloroacetic acid and thiobarbituric acid method according to Fu and Huang [29].

Hydrogen peroxide (H_2O_2) concentration was measured colorimetrically according to Mukherjee et al. [30].

Superoxide anion (O_2^-) concentration was analyzed by spectrophotometry following the methodology described by Barrameda-Medina et al. [31].

2.4.5. Determination of Enzyme Activities

The assay for ascorbate peroxidase (APX) and glutathione reductase (GR) activities was conducted following Rao et al. [32].

Catalase (CAT) activity was determined according to Badiani et al. [33], by analyzing H_2O_2 consumption (molar extinction coefficient 39.4 mM^{-1} cm^{-1}) at 240 nm for 3 min.

The protein concentration in enzyme extracts used for the analysis of the different enzymes in this section was determined by the Bradford method [34], using bovine serum albumin as a standard.

2.4.6. Determination of Ascorbate (AsA) and Glutathione (GSH) Forms

Total and reduced ascorbate (AsA) were assayed by the reduction of Fe^{3+} by AsA at 525 nm. Dehydroascorbic acid (DHA) was calculated as total AsA − reduced AsA [35]. Following the method based on the oxidation of DTNB (5,5-dithio-bis-2-nitrobenzoic acid), total glutathione (GSH) and oxidized GSH (GSSG) were determined at 412 nm. Reduced GSH was calculated as total GSH − GSSG [36].

2.4.7. Determination of Total Phenols, Flavonoids, and Anthocyanins

Phenols in plant samples were extracted and analyzed based on Rivero et al. [37].

Total flavonoid concentration was analyzed using the method described by Kim et al. [38] with slight modifications. The extraction method for phenols was used for flavonoids. A total of 85 µL of the methanolic phase was mixed with 340 µL H_2O and 26 µL $NaNO_2$, stirred, and left in the dark for 5 min. Then, 26 µL $AlCl_3$ was added, causing a yellow color shift, followed by 170 µL NaOH, causing a pink color shift. Samples were stirred again and kept in the dark for 15 min. Absorbance was measured at 415 nm.

Anthocyanin concentration was determined using the pH differential method according to Giusti and Wrolstad [39]. The anthocyanin concentration was calculated as:

$$[((A460 - A710) \times 449.2 \times 0.2 \times 1000)/26{,}900]$$

2.4.8. Antioxidant Capacity: FRAP and TEAC Assays

The Ferric Reducing Ability of Plasma (FRAP) assay was performed as described by Benzie and Strain [40].

The TEAC (Trolox Equivalent Antioxidant Capacity) assay was conducted following a modified protocol based on Cai et al. [41]. To generate the $ABTS^{\cdot+}$ radical, a solution of 7 mM 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was combined with 2.45 mM potassium persulfate and incubated in the dark for 16 h at room temperature. The $ABTS^{\cdot+}$ solution was then diluted with methanol until it reached an absorbance of 0.7 ± 0.02 at 734 nm. For the assay, 100 µL of leaf extract (prepared by extracting 0.5 g of leaf tissue in 10 mL methanol) was mixed with 3.9 mL of the $ABTS^{\cdot+}$ solution, incubated in the dark at room temperature for 6 min, and the absorbance was measured at 734 nm. Antioxidant capacity was quantified by comparing sample readings to a standard curve of Trolox ranging from 0 to 15 µM.

2.4.9. Determination of Soluble Sugars and Proline Concentration

To determine soluble sugars and proline, 0.5 g of plant material was homogenized in ethanol and then the methodology described by Irigoyen et al. [42] was followed to obtain soluble sugar and proline concentrations.

2.4.10. Photosynthetic Pigment Concentration

Photosynthetic pigment concentration was analyzed using Wellburn's method [43] with modifications. A total of 0.1 g of plant material was macerated in 1 mL methanol, then centrifuged at 5000× g for 5 min. Absorbance was measured at 666 nm, 653 nm, and 470 nm, using the following calculations:

$$\text{Chlorophyll } a \text{ (Chl } a\text{)} = 15.65 \times A_{666}\text{ nm} - 7.34 \times A_{653}\text{ nm}$$

$$\text{Chlorophyll } b \text{ (Chl } b\text{)} = 27.05 \times A_{653}\text{ nm} - 11.21 \times A_{666}\text{ nm}$$

$$\text{Carotenoids} = (1000 \times A_{470}\text{ nm} - 2.86 \times \text{Chl } a - 129.2 \times \text{Chl } b)/221$$

2.4.11. Chlorophyll *a* Fluorescence Analysis

Prior to measurements, plants were dark-adapted for 30 min using a specialized leaf clip. Chlorophyll a fluorescence kinetics were assessed with the Handy PEA Chlorophyll Fluorimeter (Hansatech Ltd., King's Lynn, Norfolk, UK), using red light (650 nm) at an intensity of 3000 µmol photons m^{-2} s^{-1} to induce the OJIP fluorescence phases. These phases were analyzed using the JIP test, focusing on key parameters: the maximum quantum efficiency of primary photochemistry (Fv/Fm), the performance index (PIabs), and the ratio of active reaction centers to absorbed light energy (RC/ABS) [44].

2.4.12. Gas Exchange Parameters Analysis

Gas exchange measurements were performed using an infrared gas analyzer, the LI-COR 6800 Portable Photosynthesis System (IRGA: LI-COR Inc., Lincoln, NE, USA). Intermediate leaves were positioned within the measurement cuvettes under optimal growth conditions. The instrument was pre-warmed for 30 min and calibrated prior to use. Measurements were conducted under standard cuvette conditions: photosynthetically active radiation (PAR) at 500 µmol m^2 s^{-1}, CO_2 concentration at 400 µmol mol^{-1}, leaf temperature maintained at 30 °C, and relative humidity set to 60%. Net photosynthetic rate, transpiration rate, and stomatal conductance were recorded simultaneously. Data were stored directly in the LI-COR system and analyzed using the "Photosyn Assistant" software (version 1.1). Instantaneous water use efficiency (WUE) was calculated as the ratio of the net photosynthetic rate (*A*) to the corresponding transpiration rate (*E*).

2.5. Statistical Analysis

The results were statistically evaluated using a one-way analysis of variance (ANOVA) with a 95% confidence interval. Differences between treatment means were compared using Fisher's Least Significant Difference (LSD) test at a 95% probability level. The significance levels were expressed as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; and NS not significant. All statistical analyses were carried out using Statgraphics Centurion 16.1.03 software.

3. Results

3.1. Growth-Related Parameters

Our results clearly indicate that water stress significantly reduced both above-ground biomass and leaf area, with reductions of up to 45% in fresh biomass and 43% in leaf area compared to well-watered control plants. When plants were treated with Cytolan® Stress in

a priming application (48 h before water stress initiation), a protective effect was observed. The reduction in biomass and leaf area was less pronounced compared to stressed plants without treatment, with a maximum decrease of 24% in fresh biomass and 25% in leaf area. In the buffering treatment (product application at the onset of water stress), both fresh and dry weight increased, as well as the leaf area, under water stress conditions. Finally, in the detoxifying effect (application of Cytolan® Stress after the stress period), untreated stressed plants showed the lowest values in above-ground biomass and leaf area. However, the application of Cytolan® Stress fully restored plant growth to levels comparable to control plants (Figure 1, Table 1).

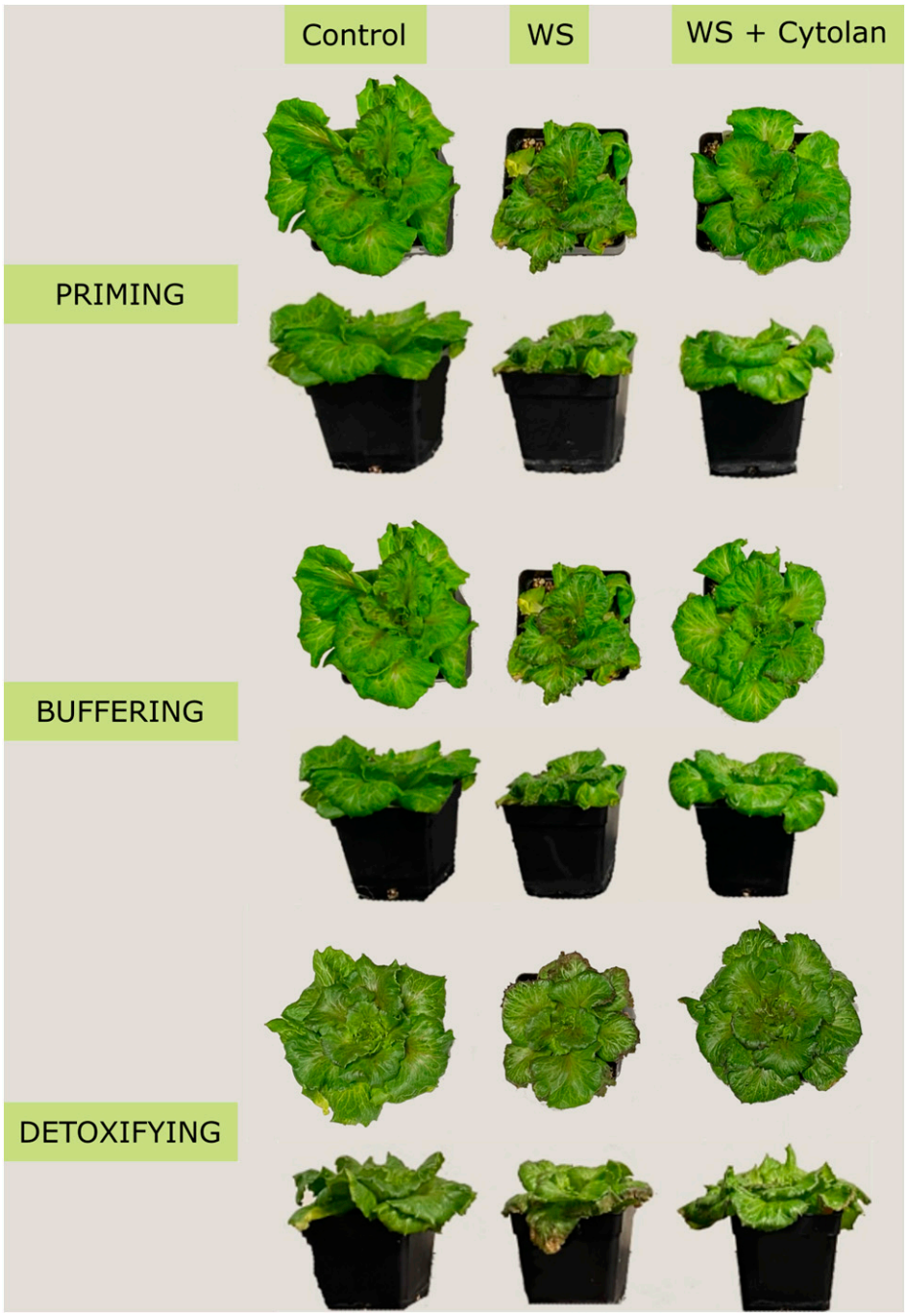

Figure 1. Photograph showing the priming, buffering, and detoxifying effects of Cytolan® Stress application on lettuce plants subjected to water stress.

Table 1. Priming, buffering, and detoxifying effects of Cytolan® Stress application on growth parameters in lettuce plants subjected to water stress.

		Fresh Weight (g Plant^{-1})	Dry Weight (g Plant^{-1})	Leaf AREA (cm^2)
Priming	Control	29.56 ± 1.43 a	2.72 ± 0.13 a	314.79 ± 4.82 a
	WS	16.34 ± 0.67 c	1.13 ± 0.04 c	178.50 ± 17.78 c
	WS + Cytolan®	22.42 ± 0.76 b	1.90 ± 0.02 b	235.00 ± 5.09 b
	p-value	***	***	***
	$LSD_{0.05}$	1.20	0.11	15.03
Buffering	Control	29.56 ± 1.43 a	2.72 ± 0.13 a	314.79 ± 4.82 a
	WS	16.34 ± 0.67 c	1.13 ± 0.04 c	178.50 ± 17.78 c
	WS + Cytolan®	23.43 ± 0.62 b	1.99 ± 0.07 b	250.23 ± 7.90 b
	p-value	***	***	***
	$LSD_{0.05}$	1.25	0.12	20.05
Detoxifying	Control	35.94 ± 0.53 a	2.79 ± 0.08 a	409.65 ± 6.27 a
	WS	28.85 ± 1.73 b	2.05 ± 0.0 b	345.65 ± 27.48 b
	WS + Cytolan®	41.93 ± 1.49 a	2.70 ± 0.06 a	395.47 ± 7.19 a
	p-value	**	***	*
	$LSD_{0.05}$	1.59	0.15	25.10

Values are means ± standard deviation (n = 9) and differences between means were compared by Fisher's Least Significant Difference (LSD) test. The levels of significance are represented as * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$). Values with different letters indicate significant differences.

3.2. Stress-Related Parameters

RWC was lowest in water-stressed plants without product application across all treatments (priming, buffering, and detox), while Cytolan® Stress application significantly improved RWC. Notably, in the detoxifying treatment, plants treated with Cytolan® Stress showed RWC levels similar to those of well-watered controls 10 days after stress (Table 2).

Table 2. Priming, buffering, and detoxifying effects of Cytolan® Stress application on LWC, EL, and oxidative stress indicators in lettuce plants subjected to water stress.

		LWC (%)	EL (%)	MDA (μMg^{-1} FW)	H_2O_2 (μg g^{-1} FW)	O_2^- (μg g^{-1} FW)
Priming	Control	87.80 ± 0.38 a	10.32 ± 0.21 ab	1.48 ± 0.16 c	16.19 ± 0.97 c	4.50 ± 0.18 c
	WS	56.70 ± 0.45 c	31.11 ± 1.44 c	5.37 ± 0.15 a	39.98 ± 1.78 a	7.99 ± 0.22 a
	WS + Cytolan®	73.02 ± 0.90 b	16.88 ± 0.19 b	3.54 ± 0.15 b	24.95 ± 1.16 b	6.80 ± 0.30 b
	p-value	***	***	***	***	**
	$LSD_{0.05}$	5.03	2.11	0.52	4.90	0.34
Buffering	Control	87.80 ± 0.38 a	10.32 ± 0.21 c	1.48 ± 0.16 c	16.19 ± 0.97 c	4.50 ± 0.18 c
	WS	56.70 ± 0.45 c	31.11 ± 1.44 a	5.37 ± 0.15 a	39.98 ± 1.78 a	7.99 ± 0.22 a
	WS + Cytolan®	71.55 ± 0.42 b	16.74 ± 0.49 b	3.55 ± 0.14 b	23.55 ± 1.12 b	6.34 ± 0.26 b
	p-value	***	***	***	***	***
	$LSD_{0.05}$	5.55	2.24	0.55	4.32	0.41
Detoxifying	Control	81.60 ± 1.45 a	12.74 ± 0.42 b	1.47 ± 0.05 c	11.64 ± 1.23 b	3.40 ± 0.13 b
	WS	71.96 ± 1.34 b	22.04 ± 0.90 a	3.74 ± 0.17 a	18.54 ± 0.53 a	6.73 ± 0.35 a
	WS + Cytolan®	79.05 ± 3.33 a	13.28 ± 0.57 b	2.14 ± 0.19 b	11.83 ± 1.57 b	3.96 ± 0.23 b
	p-value	**	**	***	**	NS
	$LSD_{0.05}$	6.21	2.59	0.64	4.95	1.95

Leaf water content (LWC), electrolyte leakage (EL), malondialdehyde (MDA). Values are means ± standard deviation (n = 9) and differences between means were compared by Fisher's Least Significant Difference (LSD) test. The levels of significance are represented as NS ($p > 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$). Values with different letters indicate significant differences.

EL was highest in untreated stressed plants, while Cytolan® Stress treatment significantly reduced leakage, with priming and buffering treatments showing the most substantial effect. In the detox treatment, EL decreased to levels comparable to control plants 10 days after stress cessation (Table 3).

Table 3. Priming, buffering, and detoxifying effects of Cytolan® Stress application on antioxidant compound concentrations and antioxidant tests in lettuce plants subjected to water stress.

		Total Phenols (mg g^{-1} FW)	Flavonoids (mg g^{-1} FW)	Anthocyanins (µg g^{-1} FW)	FRAP (mg g^{-1} FW)	TEAC (mg g^{-1} FW)
Priming	Control	0.76 ± 0.02 c	0.17 ± 0.01 c	34.09 ± 1.22 c	3.47 ± 0.07 c	3.29 ± 0.04 c
	WS	1.09 ± 0.05 b	0.24 ± 0.01 b	47.79 ± 5.53 b	4.53 ± 0.01 b	5.35 ± 0.07 b
	WS + Cytolan®	1.63 ± 0.09 a	0.36 ± 0.12 a	54.90 ± 1.27 a	5.73 ± 0.03 a	6.34 ± 0.02 a
	p-value	***	***	***	***	***
	$LSD_{0.05}$	0.15	0.03	4.87	0.28	0.17
Buffering	Control	0.76 ± 0.02 c	0.17 ± 0.01 c	34.09 ± 1.22 c	3.47 ± 0.07 c	3.29 ± 0.04 c
	WS	1.09 ± 0.05 b	0.24 ± 0.01 b	47.79 ± 5.53 b	4.53 ± 0.01 b	5.35 ± 0.07 b
	WS + Cytolan®	1.58 ± 0.01 a	0.33 ± 0.01 a	54.90 ± 1.54 a	5.47 ± 0.17 a	6.46 ± 0.03 a
	p-value	***	***	***	***	***
	$LSD_{0.05}$	0.18	0.05	4.83	0.29	0.19
Detoxifying	Control	1.67 ± 0.03 b	0.54 ± 0.12 b	73.36 ± 1.47 b	3.68 ± 0.12 a	2.68 ± 0.11 b
	WS	1.90 ± 0.03 a	0.70 ± 0.05 a	81.30 ± 3.46 a	4.22 ± 0.24 b	3.23 ± 0.06 a
	WS + Cytolan®	1.68 ± 0.07 b	0.47 ± 0.04 b	74.36 ± 1.28 b	3.34 ± 0.05 a	2.53 ± 0.09 b
	p-value	*	*	*	**	*
	$LSD_{0.05}$	0.20	0.09	6.43	0.49	0.27

Values are means ± standard deviation (n = 9) and differences between means were compared by Fisher's Least Significant Difference (LSD) test. The levels of significance are represented as * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$). Values with different letters indicate significant differences.

Considering MDA levels, they were lowest in non-stressed control plants and stressed plants treated with Cytolan® Stress, while the highest MDA values were found in untreated stressed plants (Table 2). Thus, the highest H_2O_2 and O_2^- levels were observed in water-stressed plants, whereas Cytolan® Stress treatments significantly reduced these ROS across all conditions. Notably, the detox treatment restored H_2O_2 and O_2^- concentrations to control levels 10 days post-stress (Table 2).

3.3. Antioxidant Response

Water stress significantly increased APX activity in all treatments, except in control plants, with the highest activities observed in stressed plants without Cytolan® application. Foliar Cytolan® Stress application also induced APX activity, albeit lower than in untreated stressed plants. For GR, the trend was reversed, as maximum activities were observed in the priming and buffering effects in water-stressed plants treated with Cytolan® Stress. In CAT activity, significant effects were noted only in priming and buffering treatments for stressed plants without Cytolan® Stress, showing induction of CAT. Conversely, Cytolan®-treated plants exhibited CAT activity similar to control plants. In the detox effect, Cytolan®-treated plants showed CAT values similar to non-stressed controls (Figure 2; Table S1).

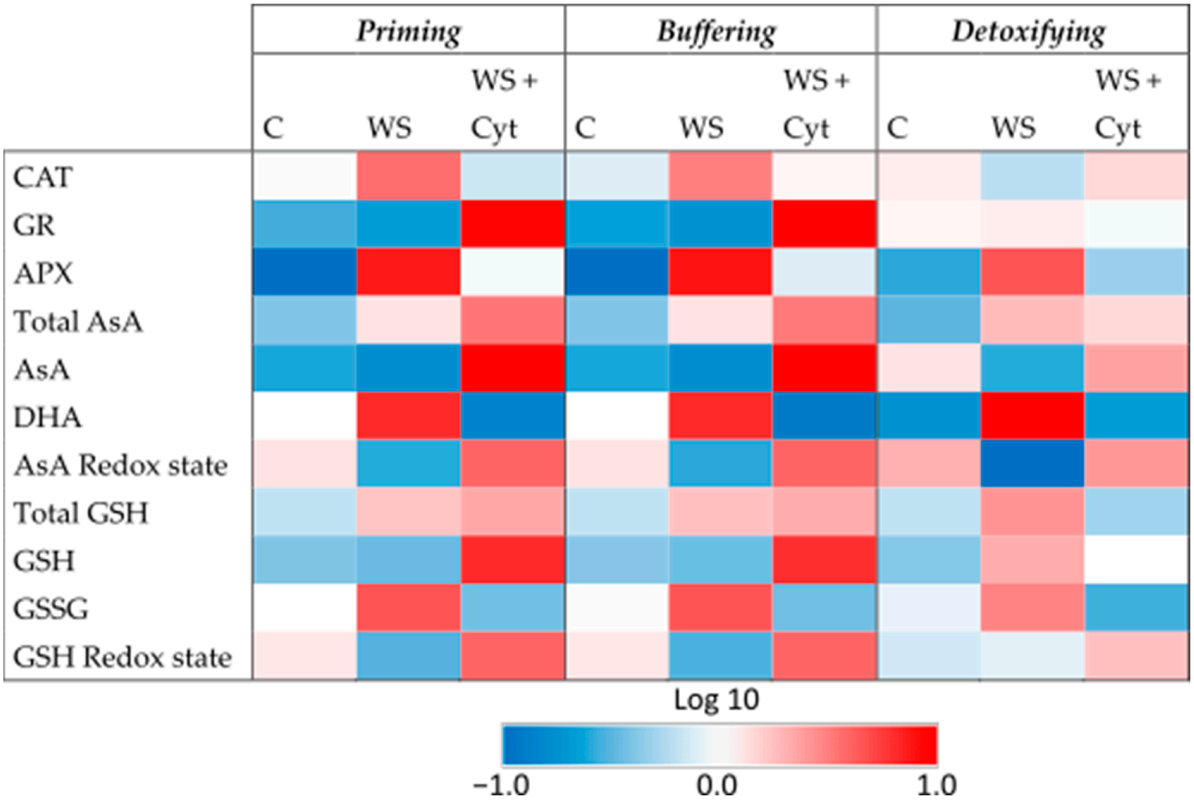

Figure 2. Heat map showing the priming, buffering, and detoxifying effects of Cytolan® Stress on antioxidant enzyme activities and AsA and GSH form concentrations in lettuce plants. Color scale refers to the logarithmic transformation (log10) of measured values (higher values are shown in red, lower values in blue, and intermediate values in white colors). To interpret the color code, refer to Supplementary Tables S1 and S2.

Both AsA and GSH total concentrations increased under water stress during priming and buffering treatments. Particularly, maximum AsA and GSH values occurred with Cytolan® Stress application under stress conditions. In stressed plants without Cytolan® Stress, minimum AsA and GSH and redox state levels, and maximum DHA and GSSG levels, were found. In contrast, foliar Cytolan® Stress application during priming and buffering produced maximum AsA and GSH and redox state levels, and minimum DHA and GSSG levels. In the detox effect, Cytolan®-treated plants displayed reduced, oxidized, and redox state values for AsA and GSH comparable to control plants. Conversely, stressed plants without Cytolan® Stress showed maximum total and oxidized AsA and GSH levels, and minimum reduced concentrations, resulting in the lowest AsA and GSH redox states (Figure 2; Table S2).

Water stress increased total phenols, flavonoids, anthocyanins, FRAP, and TEAC levels compared to control plants, but this increment was higher in plants supplied with Cytolan® Stress in priming and buffering treatments. However, plants that received Cytolan® Stress in the detoxifying treatment showed similar levels of these parameters compared to control plants (Table 3).

3.4. Osmoprotector Concentration

The highest soluble sugar, proline, and glycine betaine levels were found in water-stressed plants without the biostimulant application. Conversely, the lowest values were observed in both non-stressed control plants and water-stressed plants with Cytolan® Stress application across all studied cases (priming, buffering, and detox) (Table 4).

Table 4. Priming, buffering, and detoxifying effects of Cytolan® Stress application on some osmoprotectant compound concentrations and antioxidant tests in lettuce plants subjected to water stress.

		Soluble Sugar (mg g^{-1} FW)	Proline (µg g^{-1} FW)	Glycine Betaine (mM g^{-1} FW)
Priming	Control	7.58 ± 0.48 c	53.76 ± 7.42 c	5.15 ± 0.23 b
	WS	11.94 ± 0.43 a	154.80 ± 20.31 a	6.39 ± 0.11 a
	WS + Cytolan®	8.82 ± 0.42 b	76.35 ± 10.41 b	5.13 ± 0.25 b
	p-value	***	***	*
	$LSD_{0.05}$	1.01	10.05	0.34
Buffering	Control	7.58 ± 0.48 c	53.76 ± 7.42 c	5.15 ± 0.23 b
	WS	11.94 ± 0.43 a	154.80 ± 20.31 a	6.39 ± 0.11 a
	WS + Cytolan®	8.82 ± 0.22 b	70.76 ± 4.22 b	5.15 ± 0.32 b
	p-value	***	***	*
	$LSD_{0.05}$	1.15	15.33	0.46
Detoxifying	Control	8.71 ± 0.56 b	134.02 ± 10.44 b	3.60 ± 0.21 b
	WS	9.80 ± 0.45 a	244.22 ± 31.82 a	5.28 ± 0.21 a
	WS + Cytolan®	8.60 ± 0.27 b	147.69 ± 16.60 b	3.52 ± 0.21 b
	p-value	*	*	*
	$LSD_{0.05}$	1.04	20.56	0.41

Values are means ± standard deviation (n = 9) and differences between means were compared by Fisher's Least Significant Difference (LSD) test. The levels of significance are represented as * ($p < 0.05$) and *** ($p < 0.001$). Values with different letters indicate significant differences.

3.5. Photosynthesis Performance

The water-stressed plants without Cytolan® Stress application showed the lowest Fv/Fm values. In contrast, plants treated with Cytolan® Stress across priming, buffering, and detoxifying treatments had higher Fv/Fm values under both control and water-stressed conditions (Figure 3). Cytolan® Stress improved vitality indices linked to the photochemical phase of photosynthesis (RC/ABS and PIabs) in the three application timings (Figure 3). The lowest RC/ABS and PIabs values were seen in water-stressed plants without Cytolan® in all cases (priming, buffering, and detox), while Cytolan® application under water stress or post-stress yielded index values close to non-stressed controls (Figure 3; Table S3).

Plants grown under water stress without Cytolan® Stress showed the lowest Chl *a* and *b* concentrations across all cases, while the highest chlorophyll values in priming and buffering treatments were in non-stressed control plants (Figure 3). Cytolan® Stress application before stress (priming) or at stress onset (buffering) increased Chl *a* and *b* concentrations compared to water-stressed plants without Cytolan® Stress. However, despite these increases, Chl levels remained lower than those in non-stressed controls. In the detox effect, post-stress application of Cytolan® Stress restored Chl *a* levels to non-stressed control values. Cytolan® Stress also increased Chl *b* concentrations in stressed plants to levels similar to non-stressed controls.

Considering carotenoids, their levels were enhanced in all water-stress plants. However priming and buffering Cytolan® Stress application increased the concentration of these compounds, whereas detoxifying treatment reduced carotenoid concentration compared with stressed plants not supplied with Cytolan® Stress (Figure 3; Table S4).

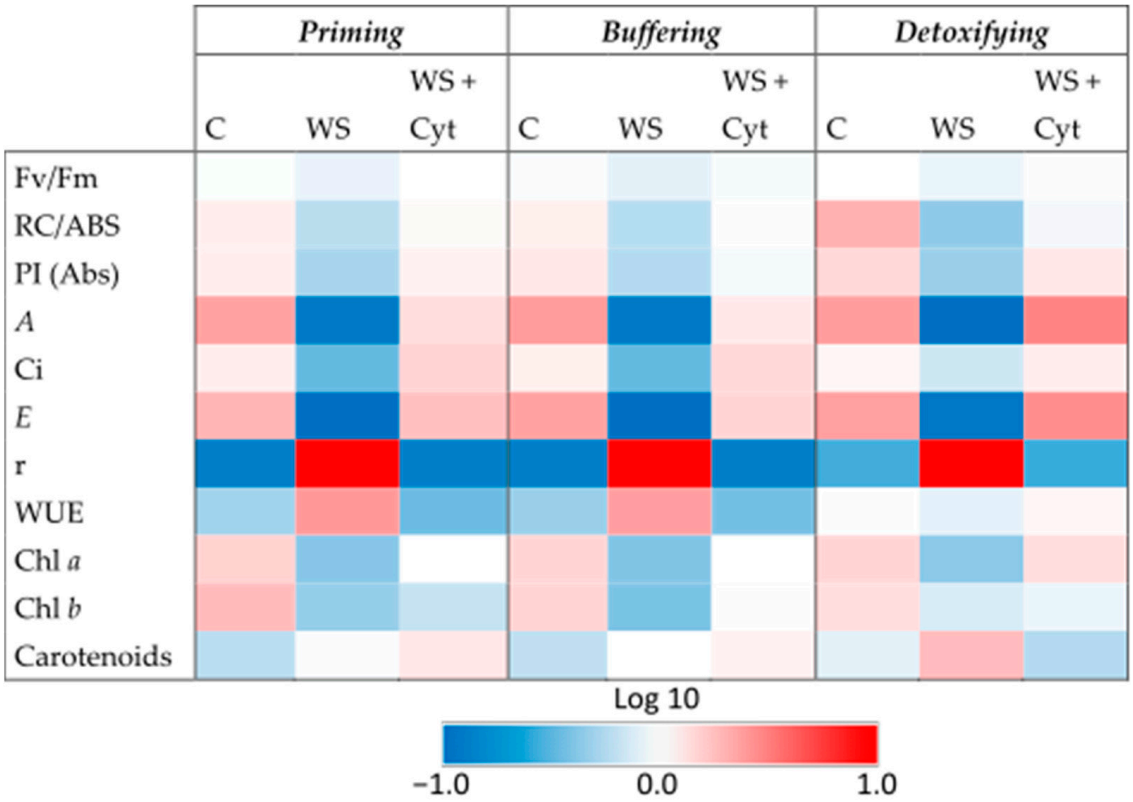

Figure 3. Heat map showing the priming, buffering, and detoxifying effect of Cytolan® Stress on photosynthesis-related parameters in lettuce plants. Color scale refers to the logarithmic transformation (log10) of measured values (higher values are shown in red, lower values in blue, and intermediate values in white colors). To interpret the color code, refer to Supplementary Table S1.

With respect to gas exchange parameters, our experiment data showed that priming, buffering, and detox applications of Cytolan® Stress enhanced *A* and *E*, and reduced r, in water-stressed plants compared to untreated stressed plants. Conversely, water-stressed plants without Cytolan® Stress application had substantially reduced *A*, Ci, and *E*, this last attributed to increased r. The WUE parameter was higher in water-stressed plants with the priming and buffering treatments, and Cytolan® Stress application reduced its values. Finally, detoxifying treatments did not show different WUE values between them (Figure 3; Table S5).

4. Discussion

Plant Growth Indicators

Water stress can adversely affect plant growth and essential physiological processes, including photosynthetic assimilation and antioxidant defense capacity [1,3,6,45,46]. Growth parameters are often reliable indicators of water stress, with specific metrics—such as biomass production and leaf area—offering insight into a plant's adaptive response to water-limited conditions. These metrics provide robust indicators for plant growth and adaptability to adverse growing conditions, such as water limitation [47,48].

In the present study, the application of Cytolan® Stress significantly increased the biomass of plants subjected to water stress. Notably, the detoxifying treatment even restored biomass to levels comparable to control plants, demonstrating its remarkable effectiveness. Other studies also found that seaweed-based biostimulants, and also the application of the bioactive compounds added to Cytolan® Stress (proline, mannitol, salicylic acid, and menadione sodium bisulfite), confer more tolerance to horticultural plants subjected to water stress [21,23,49–53]. The detoxifying treatment probably was more effective than

the priming or buffering treatments because it directly focuses on repairing accumulated stress-induced damage, such as ROS neutralization and cellular restoration. Additionally, post-stress recovery allows plants to allocate metabolic resources toward growth and repair, maximizing the impact of bioactive compounds like proline and salicylic acid. These facts are supported by studies in which seaweed-based biostimulants and osmolytes enhance antioxidant activity and support post-stress metabolic recovery [3,54].

Beyond biomass, RWC and membrane integrity—evaluated through EL percentage—are considered highly reliable indicators of a plant's ability to restore favorable hydration and tolerate water stress. Water deficit typically reduces RWC in plants sensitive to water stress, establishing it as an effective marker for identifying water-stress-tolerant genotypes [3,6,48]. In this study, the positive effect of Cytolan® Stress on RWC suggests that it acts as an intracellular water retention aid, enhancing water stress tolerance and resilience. This improved capacity for water retention provided by Cytolan® Stress could be favored by some components of the biostimulant such as menadione sodium bisulfite and proline as shown in other studies in tomato and maize [49,51]. Thus, biostimulants enhance RWC in plants under water stress. For instance, studies have shown that biostimulant applications improve RWC by enhancing osmotic adjustment and water uptake efficiency, thereby mitigating the adverse effects of drought conditions [23,55].

Under stress conditions, such as water limitation, maintaining cell membrane integrity is essential for plant survival. EL from cells is a long-established marker of cell membrane damage, particularly in the plasma membrane [3,6,48]. Here, Cytolan® Stress application, especially in the detoxifying timing, was effective in reducing the loss of electrolytes. In other studies, foliar application of biostimulants has decreased EL in sugar beet under drought conditions, indicating enhanced membrane stability [56]. Similarly, biostimulant treatments in tomato seedlings under drought stress have resulted in reduced membrane permeability, reflecting improved cell membrane integrity [57].

MDA concentration, an indicator of lipid peroxidation, reflects ROS presence, with elevated MDA suggesting excessive ROS [7]. In our study, MDA and ROS concentration results indicated that Cytolan® Stress provides protective benefits against oxidative stress by either reducing ROS generation or directly detoxifying them. Indeed, reducing ROS accumulation is critical for plant survival under water limitation, making oxidative metabolism an essential indicator of stress-induced damage [3,6,48]. Biostimulants like Cytolan® Stress are thought to mitigate oxidative stress by enhancing antioxidant defenses, thereby reducing ROS and lipid peroxidation [58,59].

To prevent damage, plants possess ROS detoxification mechanisms, among which the AsA-GSH cycle—comprising both enzymatic proteins (APX and GR) and antioxidant compounds (AsA and GSH)—is one of the most effective systems for H_2O_2 detoxification [60]. Additionally, H_2O_2 can be removed by CAT, a peroxisomal enzyme often activated under photorespiration, a symptom of stomatal closure and reduced photosynthesis rates [61]. Considering AsA-GSH cycle data, we concluded that Cytolan® Stress in priming and buffering enhanced the entire AsA-GSH cycle, promoting GSH regeneration through GR activity (converting GSSG to GSH), thereby increasing GSH availability for AsA regeneration, leading to sustained APX use. Ultimately, increased AsA and GSH redox states in stressed plants due to Cytolan® Stress application ensure greater AsA availability for APX in H_2O_2 detoxification. In other studies, components of Cytolan®, such as proline and mannitol, have been shown to aid in the osmoprotection of enzymes such as those involved in the AsA-GSH cycle [7,51]. Furthermore, the inclusion of salicylic acid could be a distinguishing factor, as it has been demonstrated to enhance the activity of key antioxidant enzymes, such as APX and GR [62]. These findings explain the low H_2O_2 concentrations in these treatments under water stress. In contrast, untreated stressed plants displayed an

unbalanced AsA-GSH cycle with low AsA and GSH redox states, indicating an inability to regenerate DHA and GSSG to reduced forms. Despite significant APX induction, these plants lacked adequate AsA for effective H_2O_2 detoxification, leading to ROS accumulation and reduced growth. Finally, Cytolan® Stress application in the detoxifying effect decreased AsA-GSH cycle activity, which suggests that post-stress Cytolan® application aids plant recovery through mechanisms other than improving the AsA-GSH cycle.

Besides the AsA-GSH cycle, plants contain non-enzymatic systems with antioxidants like phenols, flavonoids, and anthocyanins that directly detoxify ROS [63,64]. Our results suggest that Cytolan® Stress in priming and buffering effectively increased antioxidant concentrations and lettuce antioxidant capacity. The inclusion of both FRAP and TEAC tests allowed for a comprehensive assessment of the antioxidant capacity. FRAP evaluates the ability to reduce ferric ions (Fe^{3+}), providing an indication of the reducing power of the antioxidant pool [40], while TEAC measures the capacity to neutralize the $ABTS^{+}$ radical, reflecting a broader spectrum of antioxidant activity [41]. Using both methods ensured a robust evaluation of antioxidant potential, capturing different aspects of the non-enzymatic antioxidant system's capacity to detoxify ROS. Therefore, Cytolan® Stress-induced antioxidant capacity, along with AsA-GSH cycle stimulation during water stress, represents an efficient mechanism to reduce ROS accumulation and phytotoxic effects, enhancing plant adaptation under stress. In the detoxifying effect, water-stressed plants without Cytolan® Stress had the highest antioxidant levels and FRAP and TEAC values, indicating ongoing stress symptoms 10 days post-stress. However, Cytolan® Stress application in the detox effect reduced antioxidant concentrations, and FRAP and TEAC values to control levels, highlighting the product's restorative effects when applied after water stress. Other studies observed that the foliar application of some bioactive compounds present in Cytolan® Stress such as menadione sodium bisulfite, mannitol, and salicylic acid promote plant antioxidant responses and reduce oxidative stress in horticultural plants suffering water stress [50,52,53].

Compounds such as soluble sugars, proline, and quaternary ammonium compounds like glycine betaine are metabolites often considered indicators of water stress resistance, as they frequently serve osmoprotective, osmoregulatory, and antioxidant roles against ROS accumulation [65]. The increased concentration of these osmoregulatory compounds in water-stressed plants without Cytolan® application may be a mechanism to prevent excessive water loss. As we have observed, these plants showed reduced membrane integrity and the lowest LWC. Conversely, water-stressed plants with Cytolan® Stress application, which maintained higher relative leaf water content and membrane integrity, likely required less osmoprotective compound synthesis under these stress conditions. In the specific case of proline, studies have shown that under abiotic stress, reduced accumulation of this amino acid could enhance plant stress resistance. It has been found that proline degradation by proline dehydrogenase consumes O_2, reducing the likelihood of ROS generation, which may be occurring in the water-stressed treatment with Cytolan® Stress application, showing minimal ROS levels (Table 2) [7,66].

Typically, environmental stress significantly inhibits photosynthesis [6,67], and it has been shown in some plant species that biostimulant application can counteract this inhibition and restore normal growth under drought conditions [58,59]. Chl *a* fluorescence reflects the photosynthetic state of the plant and the photosynthetic changes under stress. When metabolic disruption occurs, plants emit fluorescence to dissipate excess energy, preventing damage from stress. One of the key fluorescence-derived parameters is Fv/Fm, a good indicator of plant photosynthetic performance. Healthy, minimally stressed plants typically have an Fv/Fm value around 0.85 [68]. Chl *a* fluorescence analysis also provides indices like RC/ABS, which indicates a higher proportion of active reaction centers, essen-

tial for electron transport in photosystems. Another crucial index, PIabs, reflects the overall functionality of both photosystems, with higher values indicating improved photosynthetic performance [44]. In the present study, the higher Fv/Fm values observed in plants treated with the biostimulants suggest lower fluorescence emission, and thereby lower stress. In addition, vitality indices linked to the photochemical phase of photosynthesis (RC/ABS and PIabs) suggest that Cytolan® Stress improves photochemical phase components, enhancing energy conversion efficiency. This supports the protective role of Cytolan® in mitigating water stress effects, maintaining photochemical activity similar to non-stressed control plants, particularly evident in the detox effect.

Besides Chl *a* fluorescence, other markers like photosynthetic pigments are crucial indicators of photochemical activity and its relationship to abiotic stress, especially water stress. For instance, Cáceres-Cevallos et al. [69] found that leaf Chl content is one of the most affected physiological traits under water stress, making it a reliable stress indicator. This reduction was also observed in the present study, although Cytolan® Stress, especially in the detoxifying treatment, restored Chls levels suggesting lower stress in these plants. Regarding carotenoids, these pigments serve both as accessory light-harvesting pigments and antioxidants, neutralizing ROS under stress [63]. In our study, carotenoids followed a similar trend to that of the other analyzed antioxidant compounds, indicating lower stress in plants supplied with Cytolan® Stress in the detoxifying treatment.

Under drought stress, plants limit water loss by reducing transpiration rate and closing stomata, which decreases stomatal conductance and increases resistance. This rapid stomatal closure adaptation is essential for water conservation but, if prolonged, it limits CO_2 uptake, reducing photosynthesis (especially Calvin cycle) and leaving $NADP^+$ unutilized, which eventually leads to ROS formation [7]. Studies indicate that certain biostimulants under water stress can prevent full stomatal closure, supporting photosynthesis and reducing ROS generation [21–23,58]. Our experiment data confirm that Cytolan® Stress application enhanced gas exchange parameters in water-stressed plants, likely due to improved CO_2 availability. This could boost biomass production under water stress. However, the reduction of these parameters in water-stressed plants indicates severe stomatal closure in response to water stress, improving WUE. However, despite WUE improvement, this response significantly reduced *A* and potentially increased ROS formation, explaining the marked biomass reduction in these plants. Overall, the results observed in plants supplied with Cytolan® Stress show improved photosynthetic performance. Similar results have been observed in horticultural plants subjected to water stress to which the bioactive compounds such as menadione sodium bisulfite, mannitol, and proline present in Cytolan® Stress have been applied. In these plants, a higher chlorophyll content, lower levels of Chl *a* fluorescence, and better CO_2 assimilation were observed [49,52,53], as in the present study.

5. Conclusions

Cytolan® Stress demonstrated protective and mitigating effects against drought-induced phytotoxicity in lettuce plants through several key mechanisms. First, it stimulated the AsA-GSH cycle and enhanced antioxidant defenses, effectively reducing leaf ROS levels. This led to better membrane integrity, promoting intracellular water retention. Second, Cytolan® Stress preserved photochemical activity and limited stomatal closure, enabling greater CO_2 availability, which contributed to stable Calvin cycle activity and a reduced electron transfer to oxygen, thus minimizing ROS formation. This mechanism also improved *A*, aiding biomass accumulation even under drought stress. The application of Cytolan® Stress as a priming and buffering treatment significantly reduced the toxic impact of ongoing water stress, while post-stress (detoxifying) application restored normal plant growth, eliminating all phytotoxic symptoms. These results indicate that Cytolan® Stress

can effectively counteract drought stress effects in lettuce, suggesting potential for broader applications in crop tolerance and resilience under water-limited conditions. Future research should explore its effectiveness on a wider range of crops, particularly those of agronomic importance, and investigate its performance under combined stress conditions to better understand its full potential in enhancing plant stress tolerance.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae11020157/s1, Table S1: Priming, buffering, and detoxifying effects of Cytolan® Stress application on APX, GR, and CAT activities in lettuce plants subjected to water stress; Table S2: Priming, buffering, and detoxifying effects of Cytolan® Stress application on AsA and GSH form concentrations in lettuce plants subjected to water stress; Table S3: Priming, buffering, and detoxifying effects of Cytolan® Stress application on Chl *a* fluorescence parameters in lettuce plants subjected to water stress; Table S4: Priming, buffering, and detoxifying effects of Cytolan® Stress application on photosynthetic pigment concentrations in lettuce plants subjected to water stress; Table S5: Priming, buffering, and detoxifying effects of Cytolan® Stress application on gas exchange parameters in lettuce plants subjected to water stress.

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