horticulturae

MDPI

Article

Exogenous Phytohormones: Effects on Lettuce Photosynthesis, Antioxidant Response and Growth

Martynas Urbutis [1,2,*], Kristina Laužikė [1,*] and Giedrė Samuolienė [1]

[1] Lithuanian Research Centre for Agriculture and Forestry, Institute of Horticulture, Kauno Str. 30, LT-54333 Babtai, Lithuania
[2] Ikarai, Vakarų Str. 6, LT-57238 Kėdainiai, Lithuania
* Correspondence: martynas.urbutis@lammc.lt (M.U.); kristina.lauzike@lammc.lt (K.L.)

Abstract: Constantly changing environments often negatively affect yield potential. Phytohormone-based biostimulants are known for their ability to control plant development and reduce the influences of negative environmental impacts and facilitate more efficient usage of resources. The aim of this study was to evaluate the effect of phytohormone-based biostimulants on lettuce (*Lactuca sativa* L.) antioxidant and photosynthetic responses and biomass formation. Lettuce was grown in a greenhouse with supplemental lighting; a 16 h photoperiod was maintained. Ten combinations of kinetin, indole-3-acetic acid, gibberellic acid, abscisic acid and salicylic acid were applied at 12–13 BBCH. The results thereof have shown that combining growth and stress phytohormones resulted in higher biomass formation; additionally, combining two growth or two stress hormones led to antagonistic effects and reduced photosynthetic rates. Furthermore, the application of gibberellic and salicylic acid had the most positive effect on lettuce productivity. The perspective offered by this work has shown that with the manipulation of hormone concentrations, photosynthetic and antioxidant systems can be controlled, thus enabling control of yield and quality.

Keywords: *Lactuca sativa*; exogenous phytohormones; biostimulants; productivity; photosynthesis

Citation: Urbutis, M.; Laužikė, K.; Samuolienė, G. Exogenous Phytohormones: Effects on Lettuce Photosynthesis, Antioxidant Response and Growth. *Horticulturae* **2023**, *9*, 792. https://doi.org/10.3390/horticulturae9070792

Academic Editor: Miguel Guzmán

Received: 17 May 2023
Revised: 7 July 2023
Accepted: 8 July 2023
Published: 12 July 2023

1. Introduction

One of the most urgent problems in the world is a reduction in cultivated areas, which has resulted in a lack of food. Therefore, it is important to ensure a good, high-quality harvest. However, constantly changing environmental factors often negatively influence plant productivity [1]. One of the ways to maintain a stable and high-quality yield is to use phytohormone-based biostimulants. Phytohormones are known for their ability to modulate plant developmental processes, such as responses to negative factors, that may reduce yield potential. Several groups of phytohormones are known to have different effects on certain parts of plants as well as their growth and development. Cytokinins (CKs), auxins (AUXs), gibberellic acid (GA), abscisic acid (ABA) and salicylic acid (SA) control plant development through all periods of ontogenesis [2]. Physiological and molecular processes are induced by natural plant biostimulatory compounds, resulting in higher yields, better quality, and better resistance to pathogens and environmental conditions. Biostimulants based on plant hormones can also participate in the regulation and optimization of plant growth. Previous studies have shown that with the use of manipulation of exogenous hormones and their concentrations in crop growth and tillering, bud formation can be stimulated or inhibited. Liu [3] showed that the exogenous application of auxins can inhibit the growth of tiller buds in rice, while cytokinins can promote it [3]. Other studies have also demonstrated that the use of zeatin nucleosides (cytokinins) can promote the sprouting of tiller buds in maize [4]. Exogenous ABA can also affect buds by increasing the amount of endogenous ABA in the tiller nodes [5]. The exogenous application of hormones has been shown to be an effective method of regulating bud growth [6]. Plant biostimulants

can increase photosynthesis, metabolism and the response of antioxidant systems during critical growth stages.

Hormones interact with each other either by activating second messengers or through phosphorylation cascades. Hormone crosstalk and signal transduction create a complex network that can affect developmental processes and induce different responses to environmental factors. All phytohormones have regulatory roles, thus establishing antagonistic or synergistic relations with each other. Auxins and cytokinins are known to play antagonistic roles. Experiments in vitro showed that a low ratio of auxins and CKs will promote shoot formation while a high ratio of auxins and CKs will promote root formation [7]. Furthermore, CKs and GAs share diverse roles in plant development. During shoot formation, CKs are the most active; additionally, GAs participate in seed germination. Additionally, when exogenously applied, these growth regulators can change the contents of endogenous phytohormones [8]. For example, exogenously applied GAs have changed endogenous CK oxidase/dehydrogenase (CKX) activity in *Pisum sativum* [9], and this enzyme has been suggested to represent an important link between CK and GA crosstalk [10].

Wu et al. [11] have identified the mechanism of antagonistic CK and ABA crosstalk in *Gladiolus hybridus*. Corot et al. [12] demonstrated that at high PPFD (photosynthetic photon flux density) levels, ABA inhibits bud growth; while at lower PPFD levels, CKs and ABA antagonize each other, which stimulates bud growth.

Another group of stress-related phytohormones is found in SA. The literature has revealed that CKs have crosstalk with SA in a synergistic way. In damaged plants, CKs are co-regulated with SA levels [13]. It is believed that CKs up-regulate plant immunity through SA-dependent defense responses, which, in return, inhibit CK signaling. Mainly, crosstalk between CKs and SA helps plants to increase their resistance and defense responses against pathogens [14]. More than these phytohormones are included in stress regulation. For example, GAs regulate the abiotic stress responses of plants through ABA involvement. The levels of these two phytohormones regulate decisions between dormancy and germination. GAs and ABA are known to act in antagonistic ways [15]. Furthermore, an antagonistic relationship has been described between ABA and auxins. During abiotic stress conditions, crosstalk between these two phytohormones assists seed vitality, while during water stress, ABA modulates auxin transport to maintain root growth [16]. Crosstalk between phytohormones leads to the regulation of the biosynthesis of other phytohormones, which results in different plant responses.

Phytohormones such as ABA and SA are responsible for the regulation of defense and of CKs, auxins and GAs for growth-related pathways. In order for plants to survive adverse conditions, phytohormones need to crosstalk with each other [15]. Often, the response to a particular factor is achieved not by a single phytohormone but by the interaction between two or more. Additionally, hormonal crosstalk also participates in the regulation of plant development, growth and yield formation. Leaves are key plant parts that capture sunlight, synthesize important metabolites, participate in gas exchange and reactions and regulate plant growth under heterogenous conditions [17]. The main productive parts of lettuce are leaves, which, by modulating phytohormone signaling and distribution, lead to very effective adaptation to environmental variables [18]. Lettuce is a universal plant that can be grown for models and as a leafy vegetable that is rich in nutrients.

This experiment was performed with the aim to improve yield potential using a mix of two phytohormones. The chosen concentrations of phytohormones were based on previous experimental data that have not been published yet.

Advancements in plant physiology have allowed scientists to dive deeper into insights regarding plant responses. There is information in the literature about exogenous phytohormones, which regulate gene expression and explain gene crosstalk, but there is not enough information about plant metabolic and physiological responses to the application of mixtures of these phytohormones. To understand the synergistic effects of these mixtures on plant antioxidants, further investigation of photosynthetic responses and productivity has been carried out.

2. Materials and Methods

2.1. Growth Conditions

Plants were grown in 0.5 L vessels with a TERRAERDEN peat substrate (SIA Compaqpeat, Rucava, Latvia) made of upland bog; this moderately fragmented peat contained nitrogen (140–210 mg/L), phosphorus (160–240 mg/L) and potassium (180–270 mg/L) at pH 5.5–6.5 and EC ms/cm < 10. The growth took place in a greenhouse from September to October (lat. 55°, Lithuania). One vessel contained 5–7 seeds. After germination, vessels were thinned to up to 5 plants. A 16 h photoperiod of 200 µmol m^{-2} s^{-1} of supplemental light (light spectrum: 70% red, 15% blue and 15% white) (Tunsgram, Budapest, Hungary), with temperatures of +18–+23 °C by day and +10–+15 °C at night, was maintained. Lettuce (*Lactuca sativa* L. cv. Lobjoits; Green Cos, CN seeds Ltd., Cambridgeshire, UK) was sprayed with 10 different combinations of phytohormones at BBCH 12–13 (seedling stage, when the second leaf was unfolded). Treatments included a control of sprayed water; KIN + IAA; KIN + GA; KIN + ABA; KIN + SA; IAA + GA; IAA + ABA; IAA + SA; GA + ABA; GA + SA; and ABA + SA; the concentration of each phytohormone was 30 mg L^{-1}, and the amount of solution applied was based on surface area (50 mL to 0.25 m^2; 90 plants). The phytohormones were obtained from Carl Roth (Karlsruhe, Germany), Sigma-Aldrich (Darmstadt, Germany) and Alfa Aesar (Kandel, Germany). KIN—kinetin; IAA—indole-3-acetic acid; GA—gibberellic acid (as GA_3); ABA—abscisic acid; and SA—salicylic acid. Five days after application, non-destructive measurements and sample collection were performed.

2.2. Biometric Measurements

Five representative lettuce plants were selected for leaf area measurement (cm^2) with a leaf area meter (AT Delta—T Device, Cambridge, UK). The height of each plant was measured with a ruler from the ground, accounting for a millimeter of error. The dry masses of plants were determined by drying them at +70 °C for 48 h (Venti cell 222; Medcenter Einrichtungen, Gräfeling, Germany) to a constant weight.

2.3. Determination of Photosynthetic Parameters

Photosynthetic rates (P_n, µmol CO_2 m^{-2} s^{-1}), transpiration rates (E, mmol H_2O m^{-2} s^{-1}), stomatal conductances (g_s, mol H_2O m^{-2} s^{-1}) and intercellular-to-ambient CO_2 concentration (C_i/C_a) were determined from 9:00 to 12:00 a.m. using an LI-6400XT portable open-flow gas exchange system (Li-COR 6400XT Biosciences, Lincoln, NE, USA). For the measurements, the third developed leaf from each plant was chosen, and five plants were measured for one minute each. The reference air (CO_2, 400 µmol mol^{-1}), the light intensity (1000 µmol m^{-2} s^{-1}) and the flow rate of the gas pump (500 mmol s^{-1}) were set.

2.4. Chlorophyll Fluorescence Imaging Analysis

An Imaging-PAM Fluorometer M-Series MAXI-Version (Walz, Effeltrich, Germany) was used to measure the dark-adapted leaves of 3 lettuce plants over 25 min. In each plant, 4–6 areas of interest were selected, from which chlorophyll fluorescence values were measured. This instrument uses a charge-coupled device (CCD) camera (IMAGE-K, Allied Vision Technologies, Stadtroda, Germany) to capture Chl fluorescence images as a function of time and light sources as well as irradiances. Win software (Imaging PAM MAXI, ImagingWin v2.56zc) and the allocation of absorbed light energy at PSII were used to determine the effective quantum yield of photochemistry (Φ_{PSII}). The relative PSII electron transport rate (ETR); the fraction of open PSII reaction centers; and the non-photochemical quenching (NPQ), which reflects the heat dissipation of excitation energy, were also calculated [19,20].

2.5. Antioxidant Activity and Total Phenolic Content

Extracts were prepared by grinding 0.03 g (dry weight) of plant leaves and diluting them with 3 mL of 80% methanol. Each of the three biological replicates consisted of at

least five conjugated plants and was repeated in three analytical replicates. The antioxidant properties of the lettuce leaves were evaluated as follows.

The ABTS (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) radical cation was obtained by incubating 7 mM of ABTS stock solution with 2.45 mM of potassium persulfate ($K_2S_2O_8$; final concentration) and allowing the mixture to stand in the dark at room temperature for 12–16 h before use [21]. Thereafter, 20 µL of the prepared sample was mixed with 280 µL of the ABTS stock solution (diluted 1:7), and absorbance was measured after 11 min (plateau phase) at 734 nm (Spectrostar Nano, BMG Labtech microplate reader, Ortenberg, Germany). The ABTS scavenging activity of each lettuce leaf extract was calculated as the difference between the initial absorbance and that after reacting for 10 min. A calibration curve was determined using Trolox (6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid; 97% purity; Sigma-Aldrich, Burlington, MA, USA) as an external standard, with a range of concentrations from 0.1 to 0.8 mM (R^2 = 0.99). This was expressed as the ABTS (in µmol) scavenged per 1 g of dry weight (µmol g^{-1} DW).

For the DPPH (2-diphenyl-1-picrylhydrazyl) assay, a stable 126.8 µM DPPH (100% purity; Sigma-Aldrich, Burlington, MA, USA) solution was prepared in methanol [22]. Subsequently, 280 µL of the DPPH solution was transferred to a test tube and mixed with 20 µL of the lettuce leaf extract. The absorbance was scanned at 515 nm (Spectrostar Nano, BMG Labtech microplate reader, Germany) during the reaction for 16 min. The free radical scavenging capacity was expressed as the µmol of DPPH radicals scavenged per 1 g of dry weight (µmol g^{-1} DW). A calibration curve was determined using Trolox (6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid; 97% purity; Sigma-Aldrich, Burlington, MA, USA) as an external standard, with a range of concentrations from 0.1 to 0.6 mM (R^2 = 0.99).

The FRAP method is based on reducing ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}). A fresh working solution was prepared by mixing a solution of 300 mM of acetate buffer (pH 3.6) and 10 mM of TPTZ (2,4,6-tripyridyl-s-triazine) with 40 mM of HCl and 20 mM of $FeCl_3 \times 6H_2O$ at 10:1:1 (*v*/*v*/*v*) [23]. A 20 µL amount of each sample was mixed with 280 µL of the working solution and incubated in the dark for 30 min. Readings of the colored product (ferrous tripyridyl–triazine complex) were then taken at 593 nm with a Spectrostar Nano BMG Labtech microplate reader (Germany). A calibration curve was determined using $Fe_2(SO_4)_3$ (Iron (III) sulfate; 97% purity; Sigma-Aldrich, Burlington, MA, USA) as an external standard, with a range of concentrations from 0.005 to 0.5 mM (R^2 = 0.99). Antioxidant power is expressed as Fe^{2+} antioxidant capacity (Fe^{2+} µmol g^{-1} DW).

The total content of the phenolic compounds was determined as gallic acid equivalents. A 20 µL aliquot of the sample extract was mixed with 20 µL of 10% (*w*/*v*) Folin–Ciocalteu reagent and 160 µL of 1 M Na_2CO_3 solution [24]. After incubation for 20 min in the dark, absorbance was measured at 765 nm (Spectrostar Nano, BMG Labtech microplate reader, Germany). The total phenolic compound quantity in mg g^{-1} of dry weight was calculated from the calibration curve of the gallic acid (0.01–0.1 mg mL^{-1}; R^2 = 0.99).

2.6. Statistical Analysis

Statistical analysis was performed using Microsoft Excel 2016 and Addinsoft XLSTAT 2022.1 statistical and data analysis (Long Island, NY, USA). The data are presented as the means of five replicates (n = 5) linked to the sampling points. One-way analysis of variance (ANOVA) followed by Duncan's significant difference test ($p < 0.05$) for multiple comparisons was used to evaluate differences between means of measurement. Multivariate principal component analysis (PCA) and agglomerative hierarchical clustering (AHC) were performed to determine the statistical relations between the phytohormone applications used in this experiment according to the antioxidant and photosynthesis systems' responses and biometrical parameters.

3. Results

KIN + ABA increased the photosynthetic rate by up to 28% compared to the control (Figure 1a). Furthermore, GA + ABA increased the stomatal conductance by up to 42.2%

compared to the control (Figure 1b). Most treatments had no significant effects compared to the control; however, KIN + SA had significant negative effects on the photosynthetic indices. It decreased the photosynthetic rate by up to 38%, the stomatal conductance by up to 2.8 times, the intercellular CO_2 by up to 9% and the transpiration by up to 2.2 times compared to the control (Figure 1).

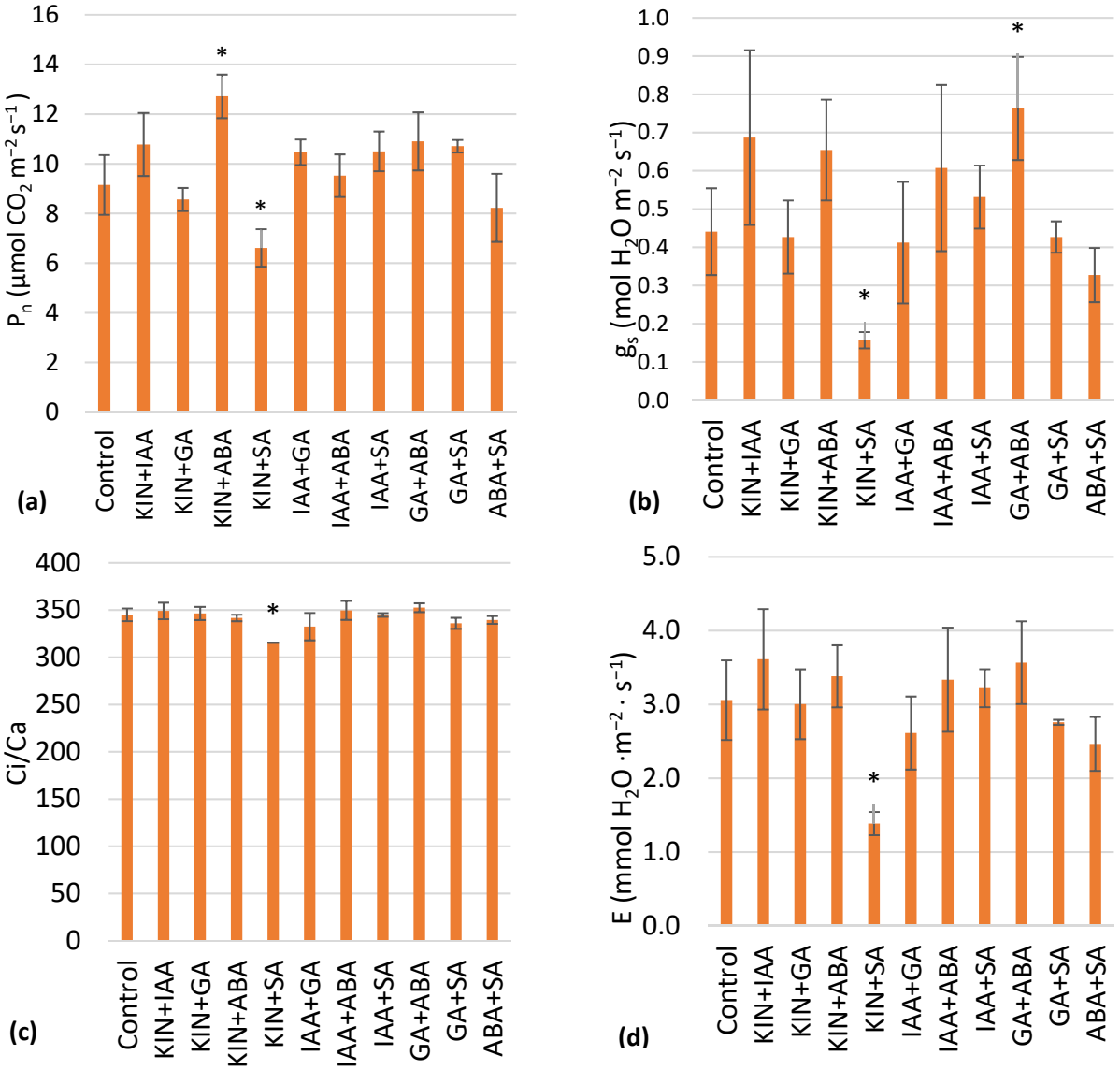


Figure 1. Effects of phytohormones on photosynthetic rate P_n (**a**); stomatal conductance, g_s (**b**); intercellular CO_2 (**c**); and transpiration, E (**d**), of lettuce leaves. Values are means ± SEs of five replicates, and * shows significant differences based on Duncan's comparison test ($p \leq 0.05$). Control—sprayed water, KIN—kinetin, IAA—indole-3-acetic acid, GA—gibberellic acid, ABA—abscisic acid, SA—salicylic acid.

The maximum quantum yield of the PSII photochemistry of the dark reactions (Fv/Fm) was negatively affected by KIN + GA (decreased by up to 4.2%) and KIN + ABA (decreased by up to 4.1%). KIN + GA also increased the Y(II) by up to 3–8% compared with the control. ABA + SA decreased the Fv/Fm by up to 5.7% (Figure 2a) and significantly decreased the Y(II) by up to 6% compared with the control (Figure 2b). With KIN + GA, KIN + ABA and KIN + SA, the same trends were determined for the ETR and Y(II); these treatments increased the ETR by up to 7.7%; 5.5% and 7.7% and the Y(II) by up to 8.2%, 6.1% and 8.2%, respectively (Figure 2c). IAA, together with KIN, GA and ABA, significantly decreased

the PS II-regulated heat emission. The NPQ significantly decreased by up to 1.5–3.5 times compared to the control, except for with the ABA + SA treatment (Figure 2d).

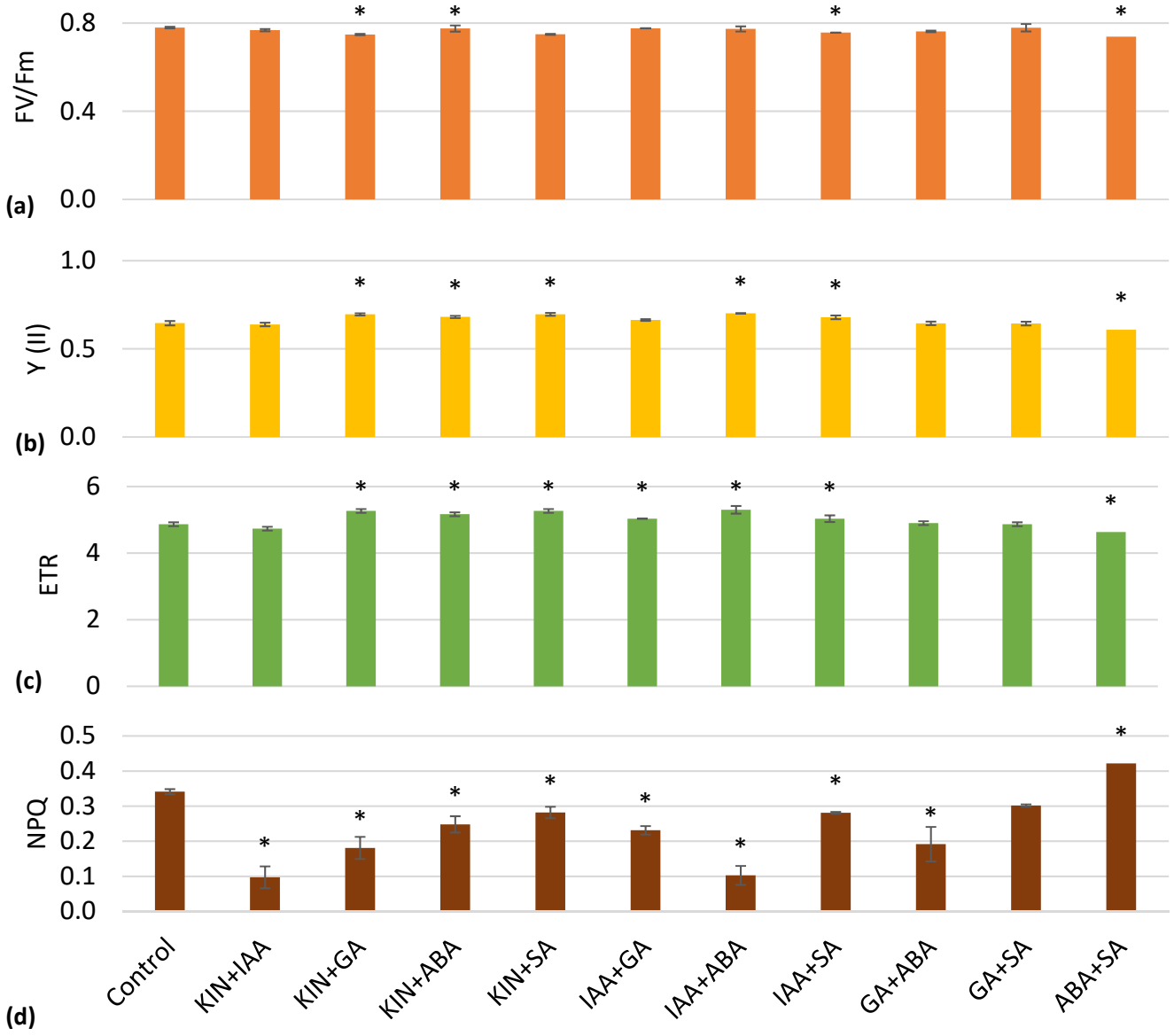


Figure 2. Effects of phytohormones on maximum photochemical efficiency of photosystem II(F_v/F_M) (**a**), photochemical quantum yield of photosystem II (Y(II)) (**b**), electron transport rate (ETR) (**c**) and non-photochemical quenching (NPQ) (**d**) of lettuce leaves. Values are means ± SEs of five replicates, and * shows significant differences based on the Duncan comparison test ($p \leq 0.05$). Control—sprayed water; KIN—kinetin; IAA—indole-3-acetic acid; GA—gibberellic acid; ABA—abscisic acid; and SA—salicylic acid.

GA + SA decreased the TPC by up to 7.7% compared to the control (Figure 3a). Treatment with phytohormone-based biostimulants had no significant effect on the radical scavenging activity of DPPH or ABTS. Furthermore, GA + SA, ABA + SA and IAA + GA significantly reduced the FRAP antioxidant activity by up to 11.4–18.1% compared to the control.

ABA + SA decreased the lettuce height by up to 19.6% compared to the control but did not have a significant impact on the leaf area or leaf numbers (Table 1). Furthermore, GA + SA increased the leaf area by up to 16.3% compared to the control. However, IAA together with GA or ABA significantly decreased the leaf area by up to 31.2–31.8% com-

pared to the control. GA + ABA and GA + SA significantly increased the fresh weight of the plant; however, no combinations showed significant effects on the dry weight compared to the control.

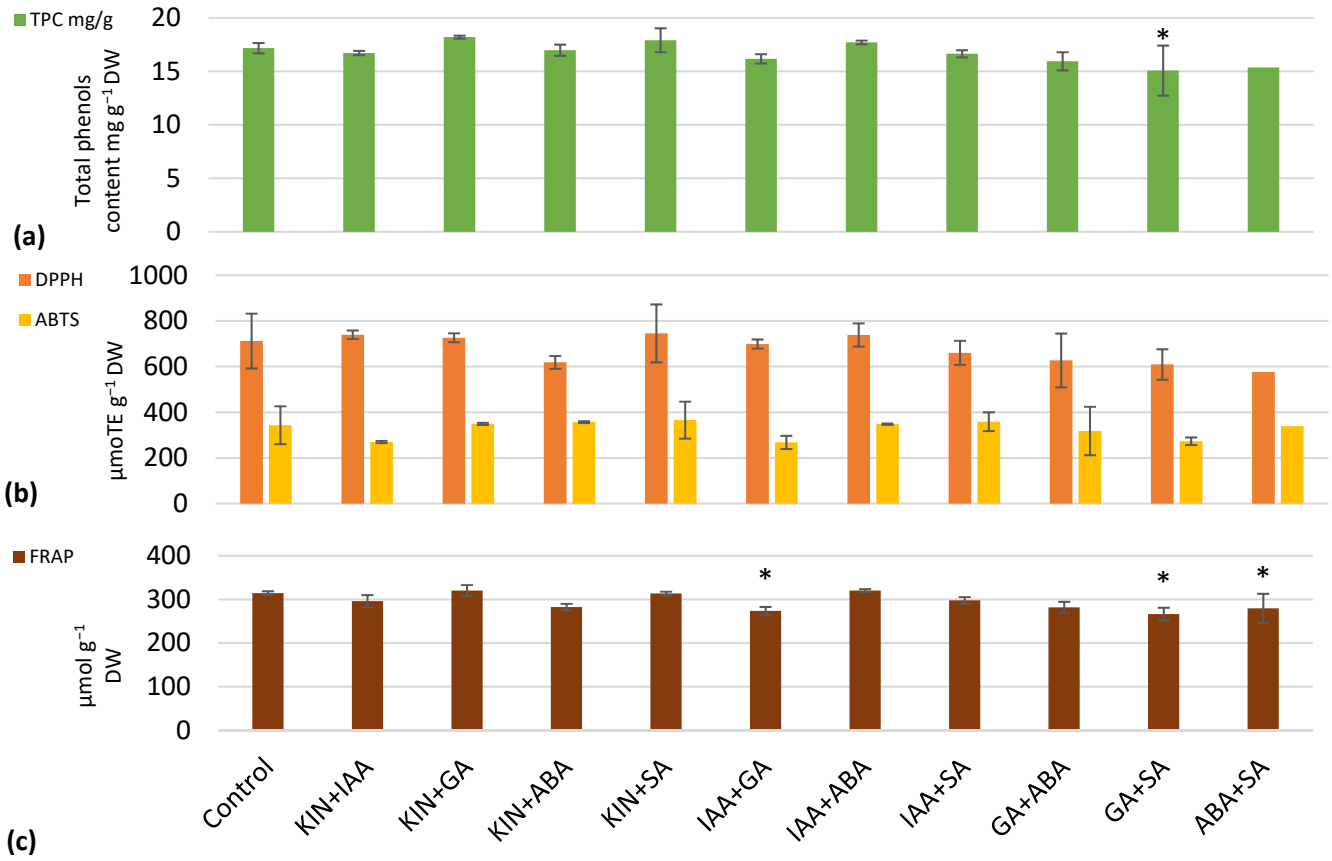
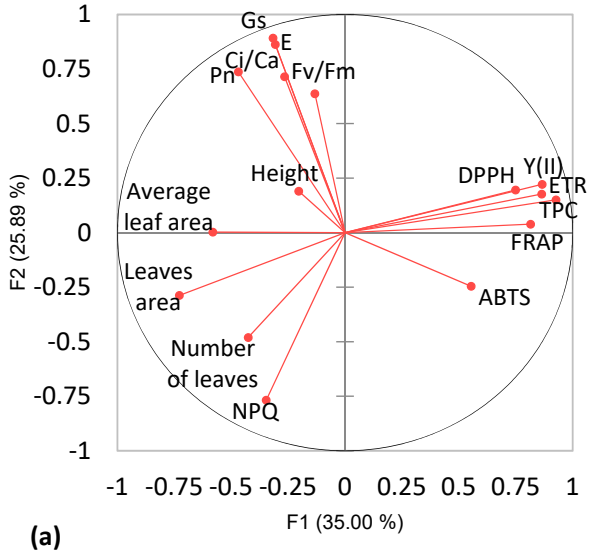


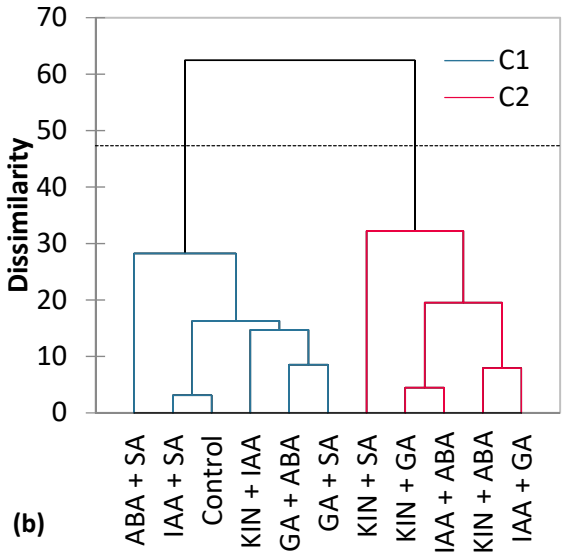
Figure 3. Effects of phytohormones on total phenol content (TPC) (**a**) and DPPH, ABTS (**b**) and FRAP (**c**) radical scavenging activity in lettuce leaves, in dry weight. Values are means ± SEs of five replicates, and * shows significant differences based on the Duncan comparison test ($p \leq 0.05$). Control—sprayed water, KIN—kinetin, IAA—indole-3-acetic acid, GA—gibberellic acid, ABA—abscisic acid, and SA—salicylic acid.

Table 1. Effects of phytohormones on lettuce height, number of leaves and total and average leaf areas. Values are means ± SEs of five replicates, and * shows significant differences based on the Duncan comparison test ($p \leq 0.05$). Control—sprayed water, KIN—kinetin, IAA—indole-3-acetic acid, GA—gibberellic acid, ABA—abscisic acid, and SA—salicylic acid.

	Lettuce Height, cm	Number of Leaves	Total Leaf Area, cm^2	Plant Fresh Weight, g	Plant Dry Weight, g
Control	12.1 ± 0.1	4.3 ± 0.6	68.0 ± 7.0	2.21 ± 0.1	0.15 ± 0.01
KIN + IAA	11.0 ± 0.9	4.7 ± 0.6	73.0 ± 7.8	2.59 ± 0.5	0.16 ± 0.03
KIN + GA	10.7 * ± 0.5	4.3 ± 0.6	58.9 ± 9.1	1.92 ± 0.2	0.14 ± 0.02
KIN + ABA	11.3 ± 1.0	4.0 ± 0.0	57.9 ± 4.6	2.20 ± 0.4	0.12 ± 0.02
KIN + SA	12.0 ± 0.7	4.3 ± 0.6	67.5 ± 5.5	2.24 ± 0.1	0.15 ± 0.03
IAA + GA	11.3 ± 0.3	4.0 ± 0.0	54.1 * ± 3.0	1.85 ± 0.2	0.12 ± 0.02
IAA + ABA	11.0 ± 0.4	4.0 ± 0.0	50.3 * ± 3.8	1.86 ± 0.3	0.12 ± 0.04
IAA + SA	11.4 ± 0.7	5.3 * ± 0.6	77.1 ± 2.4	2.63 ± 0.1	0.19 ± 0.03
GA + ABA	12.7 ± 0.3	4.0 ± 0.0	77.5 ± 5.4	2.83 * ± 0.4	0.16 ± 0.04
GA + SA	12.7 ± 1.1	5.0 ± 0.0	79.1 * ± 2.9	2.83 * ± 0.2	0.17 ± 0.02
ABA + SA	9.7 * ± 0.1	5.0 ± 0.0	75.7 ± 2.8	2.46 ± 0.3	0.18 ± 0.01

A biplot analysis of the correlations between the measured parameters showed the relationships between the individual photosynthesis and antioxidant system response parameters of lettuce under different phytohormone treatments. F1 explained 35% of the total variability, mainly affecting the photosynthetic parameters of the photochemical quantum yield of photosystem II and the electron transport rate (ETR) as well as the antioxidant parameters of the TPC, DPPH and FRAP; furthermore, F2 explained 25.89% of the total variability, affecting stomatal conductance, transpiration and non-photochemical quenching (NPQ) (Figure 4a,c).





	Height	Number of leaves	Leaves area	Average leaf area	Pn	Gs	Ci/Ca	E	Fv/Fm	Y(II)	ETR	NPQ	TPC	DPPH	ABTS	FRAP
F1	−0.204	0.425	−0.728	−0.581	−0.469	−0.316	−0.266	−0.307	−0.132	**0.866**	**0.864**	−0.347	**0.927**	**0.749**	0.554	**0.815**
F2	0.19	−0.48	−0.288	0.003	0.737	**0.892**	0.715	**0.862**	0.637	0.222	0.177	**−0.768**	0.151	0.196	−0.246	0.039

(c)

Figure 4. Correlations between measured parameters (**a**), agglomerative hierarchical clustering (AHC) (**b**) and the factor loadings (values in bold are significant according to correlation between measured parameters) (**c**) of phytohormones on different parameters in lettuce leaves.

According to the agglomerative hierarchical clustering results, all treatments came down to two main clusters (Figure 4b). Each cluster's group showed similar phytohormone effects and plant system responses to its use. The main group, together with the control, included ABA, IAA and GA in a mix with SA, as well as KIN + IAA and GA + ABA. In the second group, SA, GA and ABA were in a mix with KIN and ABA, and GA was in a mix with IAA (Figure 4b).

4. Discussion

To this date, most studies have focused on plant responses to single exogenous phytohormone treatment. The positive effect of GAs has already been observed in other experiments. It is known that treatments with GAs influence stem elongation, cell division [25], leaf expansion, induction of flowering and flower and seed formation [26]. However, ABA is considered a stress-related hormone that plays a role in water movement from root to leaf [16]. It is a key signaling molecule and important as a biomarker of oxidative stress. The results of this experiment have indicated that treatment with GA + ABA will significantly increase the fresh weight of a plant, although there is information that suggests that ABA and GA interaction is antagonistic in germination processes [27]. However, besides increased plant fresh weight, no synergistic relationship between GAs and ABA was observed after the germination processes. Furthermore, various plant hormones

communicate with SA, which is considered a defense hormone that plays roles in both local and systemic defenses in plants [28]. Alonso-Ramirez [29] found a synergic effect between endogenous SA and GAs in *Arabidopsis* at early development stages, while Xie [30] showed their antagonistic effect in barley. As discussed, biomass formation is one of the most important factors for leafy vegetables such as lettuce. In this experiment, GA + SA treatment significantly increased the plant's total leaf area compared to the control. We also recorded a positive outcome regarding plant productivity with GA and SA crosstalk, as did other authors [30]. Javed [31] found that exogenous GA application significantly influences gas exchange and chlorophyll content. Our findings show that combinations of GA with other hormones have no meaningful impacts on photosynthetic rates (Figure 1a). In addition, compared with the control, the GA + ABA application significantly increased the stomatal conductance (Figure 1b).

Auxins play a vital role in plant development. They not only participate in cell elongation, division and differentiation but also are related to signal transduction and flower development [32]. Recent studies have shown that IAA application at early development stages promotes bolting and flowering [32] and have revealed that a combination of GA and IAA will increase plant height compared to control plants or treatment with GA alone, stating that a mixture of those two groups will have a synergistic effect [32]. However, combining IAA with growth-promoting or stress-related phytohormones has not significantly influenced plant development, although IAA + GA has significantly reduced FRAP antioxidant activity. Auxins and CK play antagonistic roles, and it is well known that a low ratio of auxins and CK will promote shoot formation while a high ratio will promote root formation in vitro [7]. Our experiment revealed that an equal ratio of these phytohormones would not affect plant development significantly.

CKs comprise one of the most important groups of phytohormones and are involved in almost all development stages of plants [33]. For example, they have an important role in plant pathogenesis, and the exogenous application of CKs increases resistance to diseases [34]. Salicylic acid, however, participates in the respiration and transpiration processes and in plant responses to biotic and abiotic stress [35]. Duraid [36] found that KINs increased antioxidant enzyme activity in spinach under salt stress. SA has been found to trigger a chain of pathways by interacting with other phytohormones, such as abscisic acid, and to participate in stress mitigation [37,38]. Furthermore, synergistic crosstalk between CKs and SA has been observed. CKs have been found to up-regulate plant immunity through an SA-dependent response that inhibits CK signaling [14]. However, our results showed that KIN + SA reduced lettuce photosynthesis, as significant decreases in the photosynthetic rate, stomatal conductance, intercellular CO_2 and transpiration rate were observed (Figure 1). CKX enzyme activity has been found to be an important link between CK and GA crosstalk [10]. These two phytohormones share a diverse role in plant development, and, when exogenously applied, can change the content of endogenous phytohormones. The combination of KIN and GA significantly increased the photochemical quantum yield of photosystem II and the electron transport rate. We think that significant increases in these photochemical parameters may be attributed to KIN and its role in photomorphogenic development [39].

Thiruvengadam [40] found that phenol content significantly increased after exogenous SA and ABA treatments on Chinese cabbage. Our study revealed that in contrast to GA + SA, GA + KIN significantly increased the total phenolic content (Figure 3a). There is information in the literature [28,40] that suggests that ABA and SA increase plant antioxidant activity; help to maintain the water budget; regulate stomatal conductance, osmotic adjustment and leaf senescence; and distribute photoassimilates in Chinese cabbage [40]. In opposition to these results, ABA + SA significantly reduced the antioxidant activity of lettuce (Figure 3b).

Excessive light dissipates as heat through non-photochemical quenching [41]. Our findings show that IAA + KIN, IAA + ABA and ABA + GA shared a similar NPQ effect, which we think may be attributed to how the main role of light use efficiency is assigned to

auxin (IAA) and combinations with KIN or ABA share parallel effects, even though their mechanisms of action differ. The same tendencies were observed with GA treatments in combination with KIN or ABA. GA and IAA are known to be characterized by similar functions in plants [42]; thus, it might be stated that these two groups of phytohormones, in combination with KIN or ABA, influence the same response of non-photochemical quenching. ABA is known to play a key role in abiotic stress signaling, while SA participates in biotic stress reactions, and it is believed that these phytohormones have crosstalk with each other [43]. In general, our findings show that NPQ levels were significantly reduced with all treatments except for GA + SA, which showed no significance compared to the control. However, a significant increase in the NPQ was found with the ABA + SA treatment, which indicates that light was not used in an efficient way in that circumstance (Figure 2d). As discussed previously, treatments with ABA and SA have no positive impact on plant antioxidant activity, and during this experiment, these two phytohormones neither increased nor decreased the biomass formation of the lettuce.

The correlations between the measured parameters (Figure 4) show 60.89% total variability. F1 explained 35%, mainly affecting the photochemical quantum yield of photosystem II, the electron transport rate (ETR), the TPC, the DPPH and the FRAP; furthermore, F2 explained 25.89% of the total variability, affecting the stomatal conductance, the transpiration and the non-photochemical quenching (NPQ). For example, the GA + SA treatment significantly reduced (7.7%) the total phenol content and significantly increased the total leaf area and the fresh weight of the plant. We think that the reduced antioxidant activity of the GA + SA application resulted in the plants experiencing less stress, which enabled the lettuce to increase its yield parameters.

The use of exogenous phytohormones increases every year, and since their use in small dosages gives such results, which cannot be repeated with other methods, there is a possibility that phytohormones experience crosstalk at different levels. For example, the GA and SA treatment significantly decreased plant antioxidant activity and increased plant biomass formation in the long run. In order to achieve stable results, attention should be drawn to this treatment. However, there is not enough information about the impacts of phytohormone combinations on plant physiological and antioxidant responses. To understand their effectiveness, further research must be carried out.

5. Conclusions

Plant development depends on phytohormones and their interaction with each other. It is very important to understand the positive and negative relations of phytohormones. In our experiment, we found that combining growth-promoting and stress-related phytohormones can give positive outcomes. Significant differences were found with a GA + SA treatment, with which antioxidant activity was increased, improving biomass (total leaf and fresh weight) formation. However, other applications suppressed photosynthetic, photochemical and antioxidant responses, resulting in reduced productivity of the lettuce. This work's perspective shows that as the concentrations and selection of application time are adjusted, yield is likely to increase. Photosynthetic and antioxidant systems can be controlled with the manipulation of hormone concentrations, allowing control of yield and its quality.

Author Contributions: Conceptualization, K.L. and M.U.; methodology, K.L., M.U. and G.S.; validation, G.S.; formal analysis, K.L. and M.U.; investigation, K.L. and M.U.; data curation, K.L.; writing—original draft preparation, K.L. and M.U.; writing—review and editing, G.S.; visualization, K.L.; supervision, G.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: Not applicable.

Acknowledgments: This work was carried out within the framework of the long-term research program "Horticulture: agrobiological basics and technologies" implemented by the Lithuanian Research Centre for Agriculture and Forestry.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Cramer, G.R.; Urano, K.; Delrot, S.; Pezzotti, M.; Shinozaki, K. Effects of abiotic stress on plants: A systems biology perspective. *BMC Plant Biol.* **2011**, *11*, 163. [CrossRef] [PubMed]
2. Liu, J.; Moore, S.; Chen, C.; Lindsey, K. Crosstalk complexities between auxin, cytokinin and ethylene in Arabidopsis root development: From experiments to systems modeling and back again. *Mol. Plant* **2017**, *10*, 1480–1496. [CrossRef] [PubMed]
3. Liu, Y.; Qiangsheng, W.; Yanfeng, D.; Zhenghui, L.; Ganghua, L.; Shaohua, W. Effect of nitrogen and 6-BA on development of tillering bud and its physiological mechanism. *Acta Agron. Sin.* **2009**, *35*, 1893–1899. [CrossRef]
4. Wang, R.F.; Jiwang, Z.; Lu, P.; Shuting, D.; Peng, L.; Bin, Z. Effects of endogenous hormones on tiller development process of different maize varieties. *Sci. Agric. Sin.* **2012**, *45*, 840–847.
5. Cai, T.; Haicheng, X.; Yanping, Y.; Weibing, Y.; Dianliang, P.; Yingli, N.; Cailong, X.; Dongqing, Y.; Zhenlin, W. Mechanisms of tiller occurrence affected by exogenous IAA, GA3, and ABA in wheat with different spike-types. *Acta Agron. Sin.* **2013**, *39*, 1835–1842. [CrossRef]
6. Cai, T.; Meng, X.; Liu, X.; Liu, T.; Wang, H.; Jia, Z.; Yang, D.; Ren, X. Exogenous hormonal application regulates the occurrence of wheat tillers by changing endogenous hormones. *Front. Plant Sci.* **2018**, *9*, 1886. [CrossRef]
7. Seif El-Yazal, A.S.; Seif El-Yazal, A.M.; Dwidar, F.E.M.; Rady, M. Phytohormone crosstalk research: Cytokinin and its crosstalk with other phytohormones. *Curr. Protein Pept. Sci.* **2015**, *16*, 395–405. [CrossRef]
8. Pospíšilová, J. Participation of phytohormones in the stomatal regulation of gas exchange during water stress. *Biol. Plant.* **2003**, *46*, 491–506. [CrossRef]
9. Chow, B.; Mccourt, P. Hormone signalling from a developmental context. *J. Exp. Bot.* **2004**, *55*, 247–251. [CrossRef]
10. Todorova, D.; Vaseva, I.; Jiří, M.; Alena; Macháčková, T.; Ivana, N.; Karanov, E. Cytokinin oxidase/dehydrogenase activity as a tool in gibberellic acid/cytokinin cross talk. *Biol. Plant.* **2007**, *51*, 579–583. [CrossRef]
11. Wu, J.; Jin, Y.; Liu, C.; Vonapartis, E.; Liang, J.; Wu, W.; Gazzarrini, S.; He, J.; Yi, M. GhNAC83 inhibits corm dormancy release by regulating ABA signaling and cytokinin biosynthesis in Gladiolus hybridus. *J. Exp. Bot.* **2019**, *70*, 1221–1237. [CrossRef] [PubMed]
12. Corot, A.; Roman, H.; Douillet, O.; Autret, H.; Perez-Garcia, M.D.; Citerne, S.; Demotes-Mainard, S. Cytokinins and abscisic acid act antagonistically in the regulation of the bud outgrowth pattern by light intensity. *Front. Plant Sci.* **2017**, *8*, 1724. [CrossRef] [PubMed]
13. Masuta, C.; Tanaka, H.; Uehara, K.; Kuwata, S.; Koiwai, A.; Noma, M. Broad resistance to plant viruses in transgenic plants conferred by antisense inhibition of a host gene essential in Sadenosylmethionine- dependent transmethylation reactions. *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 6117–6121. [CrossRef] [PubMed]
14. Argueso, C.T.; Ferreira, F.J.; Epple, P.; To, J.P.C.; Hutchison, C.E.; Schaller, G.E.; Dangl, J.L.; Kieber, J.J. Two-component elements mediate interactions between cytokinin and salicylic acid in plant immunity. *PLoS Genet.* **2012**, *8*, e1002448. [CrossRef]
15. Jogawat, A. Crosstalk among phytohormone signaling pathways during abiotic stress. In *Molecular Plant Abiotic Stress: Biology and Biotechnology*; John Wiley & Sons: Hoboken, NJ, USA, 2019; pp. 209–220.
16. Latif, H.H. Physiological responses of Pisum sativum plant to exogenous ABA application under drought conditions. *Pak. J. Bot.* **2014**, *46*, 973–982.
17. Li, G.; Hu, S.; Hou, H.; Kimura, S. Heterophylly: Phenotypic plasticity of leaf shape in aquatic and amphibious plants. *Plants* **2019**, *8*, 420. [CrossRef]
18. Li, G.; Hu, S.; Zhao, X.; Kumar, S.; Li, Y.; Yang, J.; Hou, H. Mechanisms of the morphological plasticity induced by phytohormones and the environment in plants. *Int. J. Mol. Sci.* **2021**, *22*, 765. [CrossRef]
19. Moustakas, M.; Hanć, A.; Dobrikova, A.; Sperdouli, I.; Adamakis, I.D.S.; Apostolova, E. Spatial heterogeneity of cadmium effects on Salvia sclarea leaves revealed by chlorophyll fluorescence imaging analysis and laser ablation inductively coupled plasma mass spectrometry. *Materials* **2019**, *12*, 2953. [CrossRef]
20. Guidi, L.; Mori, S.; Degl'innocenti, E.; Pecchia, S. Effects of ozone exposure or fungal pathogen on white lupin leaves as determined by imaging of chlorophyll a fluorescence. *Plant Physiol. Biochem.* **2007**, *45*, 851–857. [CrossRef]
21. Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-EVANS, C. Antioxidant Activity Applying an Improved ABTS Radical Cation Decolorization Assay. *Free. Radic. Biol. Med.* **1999**, *26*, 1231–1237. [CrossRef]
22. Sharma, O.P.; Bhat, T.K. DPPH Antioxidant Assay Revisited. *Food Chem.* **2009**, *113*, 1202–1205. [CrossRef]
23. Benzie, I.F.F.; Strain, J.J. The Ferric Reducing Ability of Plasma (FRAP) as a Measure of "Antioxidant Power": The FRAP Assay. *Anal. Biochem.* **1996**, *239*, 70–76. [CrossRef] [PubMed]
24. Ainsworth, E.A.; Gillespie, K.M. Estimation of Total Phenolic Content and Other Oxidation Substrates in Plant Tissues Using Folin–Ciocalteu Reagent. *Nat. Protoc.* **2007**, *2*, 875–877. [CrossRef] [PubMed]
25. Miceli, A.; Moncada, A.; Sabatino, L. Effect of gibberellic acid on growth, yield, and quality of leaf lettuce and rocket grown in a floating system. *Agronomy* **2019**, *9*, 382. [CrossRef]
26. Khan, N.; Bano, A.; Ali, S.; Babar, M.A. Crosstalk amongst phytohormones from planta and PGPR under biotic and abiotic stresses. *Plant Growth Regul.* **2020**, *90*, 189–203. [CrossRef]
27. Cohen, P. The role of protein phosphorylation in neural and hormonal control of cellular activity. *Nature* **1982**, *296*, 613–620. [CrossRef] [PubMed]

28. Wani, A.B.; Chadar, H.; Wani, A.H.; Singh, S.; Upadhyay, N. Salicylic acid to decrease plant stress. *Environ. Chem. Lett.* **2017**, *15*, 101–123. [CrossRef]
29. Alonso-Ramirez, A.; Rodriguez, D.; Reyes, D.; Jimenez, J.A.; Nicolas, G.; Lopez-Climent, M.; Gomez-Cadenas, A.; Nicolas, C. Evidence for a role of gibberellins in salicylic acid-modulated early plant responses to abiotic stress in Arabidopsis seeds. *Plant Physiol.* **2009**, *150*, 1335–1344. [CrossRef]
30. Xie, Z.; Zhang, Z.L.; Hanzlik, S.; Cook, E.; Shen, Q.J. Salicylic acid inhibits gibberellin-induced alpha-amylase expression and seed germination via a pathway involving an abscisic-acid inducible WRKY gene. *Plant Mol. Biol.* **2007**, *64*, 293–303. [CrossRef]
31. Javed, T.; Ali, M.M.; Shabbir, R.; Anwar, R.; Afzal, I.; Mauro, R.P. Alleviation of copper-induced stress in pea (*Pisum sativum* L.) through foliar application of gibberellic acid. *Biology* **2021**, *10*, 120. [CrossRef]
32. Wang, Y.; Li, B.; Li, Y.; Du, W.; Zhang, Y.; Han, Y.; Liu, C.; Fan, S.; Hao, J. Application of exogenous auxin and gibberellin regulates the bolting of lettuce (*Lactuca sativa* L.). *Open Life Sci.* **2022**, *17*, 438–446. [CrossRef] [PubMed]
33. Hönig, M.; Plihalova, L.; Husičkova, A.; Nilser, J.; Doležal, K. Role of cytokinins in senescence, antioxidant defence and photosynthesis. *Int. J. Mol. Sci.* **2018**, *19*, 4045. [CrossRef] [PubMed]
34. Choi, J.; Huh, S.U.; Kojima, M.; Sakakibara, H.; Paek, K.H.; Hwang, I. The cytokinin-activated transcription factor ARR2 promotes plant immunity via TGA3/NPR1-dependent salicylic acid signaling in arabidopsis. *Dev. Cell* **2010**, *19*, 284–295. [CrossRef] [PubMed]
35. Rhaman, M.S.; Irman, S.; Rauf, F.; Khatun, M.; Baskin, C.C.; Murata, Y.; Hasanuzzaman, M. Seed priming with phytohormones: An effective approach for the mitigation of abiotic stress. *Plants* **2020**, *10*, 37. [CrossRef]
36. Al-Taey, D.K.; Saadoon, A.H.S.; Alazawi, S.S. Study of kinetin traetment on growth and activity of some antioxidant enzymes of spinach under salt stress. *Curr. Trends Nat. Sci.* **2021**, *10*, 457–465. [CrossRef]
37. Jia, C.; Zhang, L.; Liu, L.; Wang, J.; Li, C.; Wang, Q. Multiple phytohormone signalling pathways modulate susceptibility of tomato plants to Alternaria alternate f. sp. lycopersici. *J. Exp. Bot.* **2013**, *64*, 637–650. [CrossRef]
38. Matilla-Vazquez, M.A.; Matilla, A.J. Ethylene: Role in plants under environmental stress. In *Physiological Mechanisms and Adaptation Strategies in Plants under Changing Environment*; Springer: New York, NY, USA, 2014; Volume 2, pp. 189–222.
39. Fahad, S.; Hussain, S.; Matloob, A.; Khan, F.A.; Khaliq, A.; Saud, S.; Hassan, S.; Khan, F.; Ullah, N.; Faiq, M.; et al. Phytohormones and plant responses to salinity stress: A review. *Plant Growth Regul.* **2015**, *75*, 391–404. [CrossRef]
40. Thiruvengadam, M.; Kim, S.H.; Chung, I.M. Exogenous phytohormones increase the accumulation of health-promoting metabolites and influence the expression patterns of biosynthesis related genes and biological activity in Chinese cabbage (*Brassica rapa* spp. pekinensis). *Sci. Hortic.* **2015**, *193*, 136–146. [CrossRef]
41. Rahimzadeh-Bajgiran, P.; Munehiro, M.; Omasa, K. Relationships between the photochemical reflectance index (PRI) and chlorophyll fluorescence parameters and plant pigment indices at different leaf growth stages. *Photosynth. Res.* **2012**, *113*, 261–271. [CrossRef]
42. Jogawat, A.; Yadav, B.; Chhaya Lakra, N.; Singh, A.K.; Narayan, O.P. Crosstalk between phytohormones and secondary metabolites in the drought stress tolerance of crop plants: A review. *Physiol. Plant.* **2021**, *172*, 1106–1132. [CrossRef]
43. Marcińska, I.; Czyczlo-Mysza, I.; Skrzypek, E.; Grzesiak, M.T.; Janowiak, F.; Filek, M.; Michal, D.; Dziurka, K.; Waligorski, P.; Juzon, K.; et al. Alleviation of osmotic stress effects by exogenous application of salicylic or abscisic acid on wheat seedlings. *Int. J. Mol. Sci.* **2013**, *14*, 13171–13193. [CrossRef] [PubMed]