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## Article

# Influence of Exogenous 24-Epicasterone on the Hormonal Status of Soybean Plants

Michael Derevyanchuk<sup>1,\*</sup>, Serhii Kretynin<sup>1</sup>, Yaroslava Bukhonska<sup>1</sup>, Igor Pokotylo<sup>1,3</sup>, Vladimir Khripach<sup>2</sup>, Eric Ruelland<sup>3</sup>, Roberta Filepova<sup>4</sup>, Peter Dobrev<sup>4</sup>, Jan Martinec<sup>4</sup> and Volodymyr Kravets<sup>1,\*</sup>

<sup>1</sup> V.P. Kukhar Institute of Bioorganic Chemistry and Petrochemistry, National Academy of Sciences of Ukraine, 02094 Kyiv, Ukraine; kravets.vs@gmail.com

<sup>2</sup> Institute of Bioorganic Chemistry, National Academy of Sciences of Belarus, Kuprevich Str., 5/2, 220141 Minsk, Belarus; khripach@iboch.by

<sup>3</sup> Sorbonne Universités, Génie Enzymatique et Cellulaire, UMR CNRS 7025, Université de Technologie de Compiègne, CEDEX, 60203 Compiègne, France; eric.ruelland@utc.fr

<sup>4</sup> Institute of Experimental Botany of the Czech Academy of Sciences, 16502 Prague, Czech Republic; martinec@ueb.cas.cz

\* Correspondence: Volodymyr Kravets (kravets.vs@gmail.com); Michael Derevyanchuk (derevyank@ukr.net)

**Abstract:** Brassinosteroids (BR) are key phytohormones involved in the regulation of major processes of cell metabolism guiding plant growth. In the past decades, new evidence made it clear that BRs also play a key role in the orchestration of plant responses to many abiotic and biotic stresses. In the present work we analyzed the impact of foliar treatment with 24-epicastasterone (ECS) on the endogenous content of major phytohormones (auxins, salicylic acid, jasmonic acid, abscisic acid) and their intermediates in soybean leaves 7 days following the treatment. Changes in the endogenous content of phytohormones have been identified and quantified by LC/MS. Obtained results point to a clear role of ECS in the upregulation of auxin content (indole-3-acetic acid, IAA) and downregulation of salicylic, jasmonic, and abscisic acid levels. These data confirm that under optimal conditions ECS in tested concentrations of 0.25  $\mu$ M and 1  $\mu$ M might promote growth in soybean by inducing auxin contents. Benzoic acid (a precursor of salicylic acid (SA)), but not SA itself, has also been highly accumulated under ECS treatment which indicates an activation of the adaption strategies of cell metabolism to possible environmental challenges.

**Keywords:** 24-epicastasterone; salicylic acid; auxins; hormones; soybean

## 1. Introduction

Plant hormones are biologically active compounds produced by their cells, which have a relatively complex structure and affect certain chemical molecules in the process of their metabolism. [1]

In recent years, research on the role of representatives of a rather large class of steroid hormones, brassinosteroids, in the regulation of cell metabolism at the level of signaling systems has been developing at a rather rapid rate [2-7].

BR, as a unique class of plant hormones, are involved in the regulation of plant growth and development [3,8], in particular under the influence of stress factors [9,10]. Changes in the concentration of endogenous BR [11-13] play an important role in the adaptation of plant metabolism. Exogenous BR have been shown to increase growth [14], respiration rate [15], and plant resistance, in particular under drought conditions [16] and pathogen attack [17]. BR make a strong impact on photosynthesis and the membrane properties under acclimation to temperature stress [14]. BR interact with the signaling pathways of other phytohormones [18], in particular, that of abscisic acid (ABA) [19], cytokinins [20], auxins [21-23], gibberellins (GCs) [24,25], jasmonic acid (JA) [26], ethylene [27], salicylic acid (SA) [28] and other plant hormones [29-31].

The experimental data also have shown that exogenous BR have a strong impact on endogenous BR biosynthesis. 24-epibrassinolide (EBL) at a low concentration 0.01-1  $\mu$ M could increase the BR content in the leaves of barley plants [9]. Also, EBL effectively ameliorated endogenous BR level and

plant growth suppressed by a specific inhibitor of BR biosynthetic reactions – brassinazole [9]. These studies point on complex interactions between different types of BR and other phytohormones important for a fine-tuning of plant metabolism.

To analyze the cross-interaction of brassinosteroids with other plant hormones, genetic approaches using transgenic plants and the exogenous effect of brassinosteroids on them have been widely applied [3,27,32].

Considerably less attention is paid to determining changes in hormone content *in vivo* in plants under the exogenous action of brassinosteroids [33].

The effects of BR on plant cell metabolism have been described in numerous publications, but the knowledge of its molecular mechanisms remains poorly studied. Further research opens up prospects for more efficient use of BR as an environmentally conscious regulator of plant growth and productivity [34].

The goal of the present study is to analyze the effect of exogenous 24-epicastasterone (ECS) treatment on the endogenous content of key phytohormones, in particular auxins (indole-3-acetic acid (IAA)), abscisic, salicylic and jasmonic acids content in soybean leaves. ECS is a natural brassinosteroid phytohormone, that attracts our attention mainly because it is a direct biosynthetic precursor of EBL [35], it is widely distributed in plants [36]; and plays an important role in the regulation of shoots and leaf growth [37].

Establishing the exogenous influence of the ECS on changes in the content of other hormones is another level of analysis that can provide information that will help shed more light on the complex network of cross-talk between BR and other hormones.

## 2. Results and Discussion

Indoleacetic [38], abscisic [39,40], jasmonic [41], and salicylic [42], [43] acids along with their metabolites play a key role in the regulation of plant cell metabolism as well as growth and development processes.

### 2.1. Interaction of Brassinosteroids with Auxins in Plant Cells

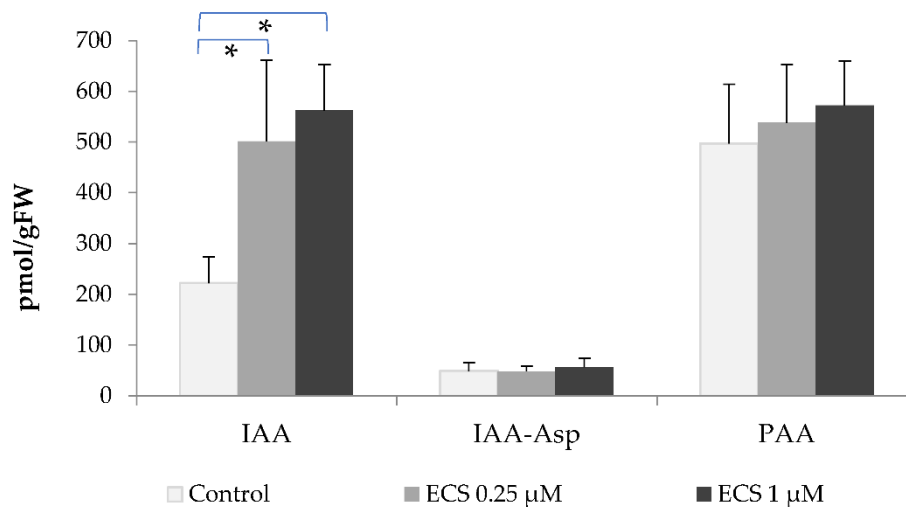
Auxins are strong inducers of plant cell metabolic reprogramming, providing rapid acceleration of their growth and development, acting locally or through distinct signaling. Yet, auxins have some inhibiting role in roots growth. IAA is one of the major and most common auxins produced by plants, which stimulates plant growth [44,45].

In our research, we detected the levels of IAA, phenylacetic acid (PAA), and IAA ester with aspartate (IAA-Asp) in soybean leaf tissues. The levels of auxins under control conditions was measured to be around 221.9 pmol/g FW for IAA, 48.5 pmol/g FW for IAA-Asp, and 497.1 pmol/g FW for PAA. Treatment of soybean plants with ECS solution (0.25  $\mu$ M, or 1  $\mu$ M) caused a strong increase in IAA content (Figure 1). In contrast, no significant changes in IAA-Asp and PAA content have been observed in soybean leaf tissues treated with the indicated concentrations of ECS (Figure 1).

PAA is a natural auxin [46] found in a number of plants [47], but to date, information on its distribution metabolism and function in plants is still limited, and our data on the response of PAA content to the effects of ECS on plants is a contribution to the study of its role in plants (Figure 1). According to our results, the content of PAA in soybean leaves exceeded more than twice the content of IAA (Figure 1), higher content of PAA compared to IAA was also found in a number of other plants among the possible explanations is higher activity and mobility in the tissues of IAA compared to PAA [47].

A significant increase in the content of IAA was earlier shown in rice treated with EBL under salinity conditions. These changes correlated with enhanced plant growth under salt stress action [48]. In contrast, no induction of IAA content by EBL was reported under optimal growth conditions [49]. In another report, brassinolide (BL) – another type of BR – promoted auxin content in *Arabidopsis* roots but inhibited auxin signaling [49]. Moreover, inhibition of BR signaling in the outer tissues of roots results in a meristem insensitivity to both BR and auxin [49]. In our study exogenous ECS under optimal conditions stimulated IAA content (Figure 1). This might point to the importance of the particular BR species and concentrations used in evoking dissimilar response in different plant

species. However, we observed no significant changes in IAA-Asp or PAA content; our data is the first observation of IAA-Asp and PAA levels in response to BR.



**Figure 1.** Influence of ECS treatment on endogenous content of auxins - indole-3-acetic acid (IAA), IAA ester with aspartate (IAA-Asp), and phenylacetic acid (PAA) in soybean leaf tissues. Plants have been sprayed twice with ECS on the 21st and 28th days after planting. On 35th days after planting soybean leaves have been harvested to determine the phytohormones content. Values are means of 8 biological samples. Experiment was repeated twice (4 variant repeats per experiment). Error bars indicate standard error of the mean (SE). \*  $p < 0.05$  (Student's  $t$ -test).

One of the key interactions between BR and auxins can be seen in their mutual effect on the regulation of gene expression. BR can regulate the expression of *YUC9* gene involved in auxin biosynthesis [49], while auxins can modulate the expression of genes involved in BR biosynthesis [50,51].

BR and auxins also interact at the cellular level. BR can promote the transport of auxins by modulating the expression of auxin transporters *PINFORMED3* (*PIN3*), *PIN4* [52] and auxin responsive genes like *IAA18*, *IAA30*, *Auxin response factor12* [53], while auxins can affect BR signaling by regulating the expression of BR receptors [54].

Via the Genevestigator array data analysis we could see that the expression level of several genes involved in auxin biosynthesis and signaling is modified in response to ECS or BL treatment in soybean (*Glycine max*) [55] (Table 1). Expression of soybean genes encoding FLAVIN-CONTAINING MONOOXYGENASE have been increased under ECS treatment (Table 1). Treatment with ECS (12 hrs), propiconazole (an inhibitor of BR biosynthesis) or combined treatment has overall decreased an expression of genes coding auxin transporters or auxin signaling components in roots of soybean (Table 1). In contrast, BL treatment in leaves strongly induced the same genes (Table 1). This indicates that a hormone concentration, its type and a site of treatment have a strong impact on the outcome of genes expression.

Auxins are involved in the metabolism reprogramming of plant cells, providing acceleration of their growth and development [56,57]. Overexpression of PIN-LIKES auxin transporters in *imp* mutants strongly influences BR signaling possibly by changing local auxin levels [58]. Auxin and BR stimulate growth and affect the elongation of the hypocotyl, but the hormone crosstalk mechanism implicated in this process is not fully clear [45,59]. BR also stimulate auxin levels important for the induction of lateral roots grown under low nitrogen stress by inducing *YUC5*, *YUC7*, and *YUC8* auxin biosynthetic genes. In BR signaling mutants *bsk3,4,7,8* and *bzr1* the expression of the above-mentioned *YUC* genes were not upregulated by low nitrogen conditions anymore. Interestingly, in *bzr1-1D* mutant plants, which have a stabilized variant of BR-dependent transcription factor BRASSINAZOLE RESISTANT 1 (BZR1) auxin genes *YUC7* and *YUC8* were upregulated constantly with no respect to low nitrogen conditions [22]. This clearly points to a close relation between BR and auxin biosynthesis on the genes level. Interactions between BR and auxins play a pivotal role in the regulation of several other key aspects of plant growth and development [58,60-62].

**Table 1.** Analysis of Genevestigator array data of BR inhibitor propiconazole, ECS and BL impact on the expression of genes involved in auxin and jasmonates biosynthesis and signaling in soybean (*Glycine max*). Values are shown in fold change of gene expression.

	propiconazole / untreated seedlings **	propiconazole / untreated roots ***	ECS / untreated roots ****	Propiconazole + ECS / propiconazole ****	propiconazole + BL / propiconazole *****	propiconazole + BL / propiconazole *****
Auxin biosynthesis genes						
Flavin-containing monooxygenase (GLYMA_10G128700) *	1.02	2	1.42	-1.95	-1.02	1.02
Flavin-containing monooxygenase (GLYMA_20G080000) *	1.01	1.16	1.37	-1.21	-1.02	-1.01
Auxin transport genes						
NPH3 domain-containing protein (GLYMA_14G102500) *	1	-1.6	-2.02	-1.31	2.3	1.33
NPH3 domain-containing protein (GLYMA_17G223000) *	-2.23	-1.25	-1.06	1.16	3.56	2.22
Protein PIN-LIKES 3 (GLYMA_03G113600) *	-1.36	-8.51	-12.89	-1.21	3.15	2.38
Auxin efflux carrier component (GLYMA_03G126000) *	1.13	-2.07	-1.77	1.3	1.77	1.02
Auxin signaling genes						
Auxin-induced protein (GLYMA_03G167400) *	1.1	1.25	-1.01	1.02	2.07	2.86
Auxin response factor (GLYMA_12G171000) *	-1.09	-2.99	-2.86	1.33	1.88	-2.17
Auxin response factor (GLYMA_12G174100) *	3.59	-2.27	1.03	1.84	-1.58	-1.21
Jasmonates biosynthesis genes						
Jasmonic acid-amido synthetase JAR1 (GLYMA_07G057900) *	-1.46	-1.57	-1.64	1.19	-1.37	-1.21
Jasmonic acid-amido synthetase JAR1 (GLYMA_16G026900) *	4.63	-1.23	-1.37	1.25	-7.85	-4

\* GLYMA\_10G128700, Panther name: INDOLE-3-PYRUVATE MONOOXYGENASE YUCCA3-RELATED, Uniprot protein: **I1LAM2**; GLYMA\_20G080000, Panther name: INDOLE-3-PYRUVATE MONOOXYGENASE YUCCA3-RELATED, Uniprot protein: **I1NEK1**; GLYMA\_07G057900, Panther name: JASMONIC ACID-AMIDO SYNTHETASE JAR1, Uniprot protein: **A0A0R4J3L2**; GLYMA\_03G167400, Panther name: AUXIN-RESPONSIVE PROTEIN IAA10-RELATED, Uniprot protein: **A0A0R0KKH0**; GLYMA\_03G126000, Panther name: AUXIN EFFLUX CARRIER COMPONENT 1, Uniprot protein: **K7KEN1**; GLYMA\_12G171000, Panther name: AUXIN RESPONSIVE FACTOR 4, Uniprot protein: **A0A0R0H664**; GLYMA\_12G174100, Panther name: AUXIN RESPONSE FACTOR 10-RELATED, Uniprot protein: **I1LTK0**; GLYMA\_16G026900, Panther name: JASMONIC ACID-AMIDO SYNTHETASE JAR1, Uniprot protein: **A0A0R0FKJ1**; GLYMA\_03G113600, Uniprot protein: **I1JMS6**; GLYMA\_17G223000, Panther name: BTB/POZ DOMAIN-CONTAINING PROTEIN NPY1, Uniprot protein: **K7MND2**; GLYMA\_14G102500, Panther name: BTB/POZ DOMAIN-CONTAINING PROTEIN NPY1, Uniprot protein: **K7M604**. Genevestigator database experiment microarray IDs – GM-00305, GM-00128. \*\* seedlings treated with propiconazole 5µM for 10 days \*\*\* roots treated with propiconazole 0.1µM for 2.5 days \*\*\*\* roots treated with propiconazole 0.01nM for 12h \*\*\*\*\* roots treated with propiconazole 0.1µM for 2d + ECS



0.01nM for 12h against roots treated with propiconazole 0.1μM for 2d \*\*\*\*\* seedlings treated with propiconazole 5μM for 10d + BL 1μM for 1h against seedlings treated with propiconazole 5μM \*\*\*\*\* seedlings treated with propiconazole 5μM for 10d + BL 1μM for 8h against seedlings treated with propiconazole 5μM.

Our results on BR-dependent upregulation of IAA levels in soybean leaves are consistent with many published research articles that indicate that ECS might stimulate the growth of soybean plants via the regulation of auxin content.

## 2.2. Interaction of Brassinosteroids with Plant Cell Stress Hormones

### 2.2.1. Interaction of Brassinosteroids with Absciscic Acid

The results of microarray studies indicate that BR and ABA provide general regulation of the expression of hundreds of different genes involved in metabolic regulation, however not much of them have overlapped [63,64]. At the same time, the key molecular mechanisms and components of signaling systems involved in these phytohormone interactions require further studies.

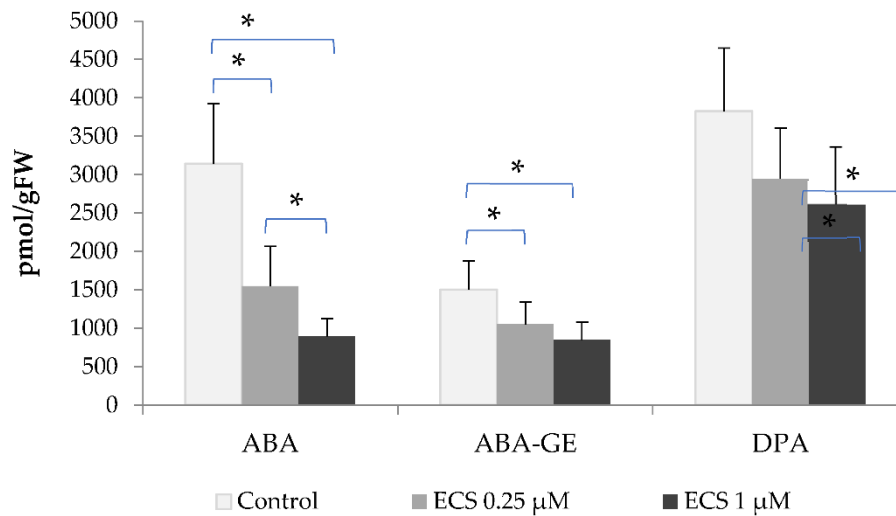
In the present study, we analyzed the contents of abscisic acid and its inactive metabolite dihydrophaseic acid (DPA) in soybean leaves. The ABA level of control plants was 3138.5 pmol/g FW, its glucose ester (ABA-GE) – 1501 pmol/g FW, and DPA – 3826.5 pmol/g FW (Figure 2). Exogenous treatment with ECS decreased ABA and ABA-GE levels in soybean leaves (Figure 2). We did not observe any significant changes in the DPA levels of the treated plants (Figure 2). BR have previously been shown to reduce ABA accumulation, resulting in the inhibition of stomatal closure induced by ABA (Ha, Shang et al. 2018). Foliar application of EBL significantly reduced the level of abscisic acid in rice plants under stress or optimal conditions [48]. Moreover, freezing conditions in tolerant barley lines itself promote the endogenous level of BR homocastaterone and decrease the ABA level [13].

BR and ABA antagonistically regulate many key developmental processes such as germination and seed maturation [65,66]. BR biosynthetic mutant, *det2-1*, and the BR signaling mutant, *bri1-1* are more sensitive to ABA inhibition of seed germination than the wild-type. These earlier observations also pointed out that the germination rate in mentioned mutants was not affected [67]. However, further investigations with BR mutants *det2-1* and *bri1-301* have found a strong reduction of seed germination in mutant and wild-type lines by exogenous ABA [64]. Overexpression of BR receptor BRI1 strongly increased the germination rate of seeds treated with ABA [53]. The involvement of BRI1-associated receptor kinase 1 (BAK1) in the regulation of ABA signaling during seed germination and primary root growth has been shown [68]. Moreover, inhibited germination of wild-type and *det2-1* lines, but not that of *bri1-301*, by ABA can be rescued by BL treatment [64]. It is known that the effects of ABA in seeds are connected mainly to the prevention of premature germination [69]. In contrast, BR attenuate the effect of ABA and promote seed germination as well as vegetative growth and development of plants [19,70]. BR antagonize ABA-mediated responses in plants through a family of transcription factors BZR1 which can reduce expression of the main ABA-signaling component ABA INSENSITIVE 5 (ABI5) [70]. Also, it has been found that BZR1 directly binds to the E-box sequences in the *ABA2* promoter region, decreasing the level of endogenous ABA in *A. thaliana* [19]. Treatment with brassinazole (Brz), a biosynthetic inhibitor of BR synthesis, increased *ABA2* expression, an effect that can be attenuated by the exogenous use of BL [19]. The results of the studies performed by J. Moon with colleagues and our results on the use of ECS in soybean (Figure 4) indicate a key role of BR in the regulation of ABA level in plants [19].

At the whole-plant level, BR and ABA can act also synergistically depending on the specific physiological process. For example, under salt stress, EBL positively regulates the expression of ABA biosynthesis genes (*OsNCED1*, *OsNCED2*, *OsNCED3*, *OsNCED4*, *OsNCED5*, *OsZEP1*) and two catabolic genes *OsBG2* and *OsABAox3* in rice [36]. Owing to these specific cell processes ABA regulates the response to environmental stresses (for complete review see [71]). ABA through the transcriptional factor ABSCISIC ACID INSENSITIVE3 (ABI3) slightly induces the BR-biosynthesis regulatory gene *OsGSR1* that evidences ABA role in the stimulation of BR production [72]. Moreover, ABA promotes the expression of *DWF4* and *CPD* - key BR-biosynthesis genes, but for effective ABA-BR crosstalk BIN2 signaling component is required [64]. Interestingly, while ABI3 promotes BR biosynthesis, BR-regulated transcription factor BES1 can directly bind another ABA transcription factor, ABSCISIC ACID INSENSITIVE5 (ABI5), which reduces ABI5 dependent genes and suppresses the ABA response [73]. This suggests that BR-ABA antagonism and synergism on the level of

hormones biosynthesis and signaling can be achieved by different transcription factors but conditions that impact these processes require further elucidation.

Seed germination is an essential stage of plant development, which is regulated by various endogenous signaling systems in interaction with environmental factors [74].



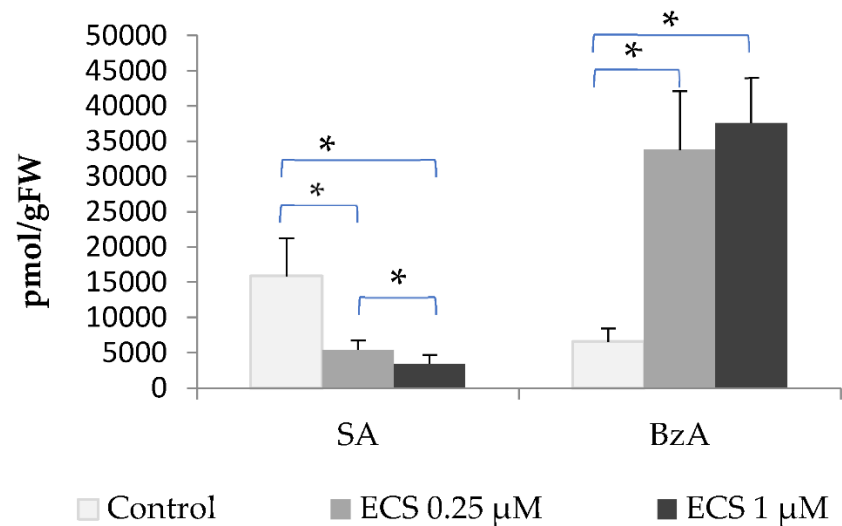
**Figure 2.** Effect of ECS on abscisic acid (ABA) and its metabolites level: ABA-glucose ester (ABA-GE) and dihydrophaseic acid (DPA). Plants have been sprayed twice with ECS on the 21st and 28th days after planting. On 35th days after planting soybean leaves have been harvested to determine the phytohormones content. Values are means of 8 biological samples. Experiment was repeated twice (4 variant repeats per experiment). Error bars indicate standard error of the mean (SE). \*  $p < 0.05$  (Student's t-test).

## 2.2.2. Effect of 24-Epicastasterone on Salicylic and Benzoic Acids Levels

In soybean plants exposed to ECS, the content of SA decreased, while the content of BzA increased significantly, and in both cases, there wasn't a significant difference between studied ECS concentrations (Figure 3).

Recent studies on hormone levels under optimal conditions observed that freezing-tolerant barley lines accumulate significant levels of SA but not BR (homocastasterone was analyzed in the paper). In contrast, under cold stress conditions tolerant lines accumulate more homocastasterone while SA content significantly drops [13]. This data is consistent with our results (Figure 3) and might point to BR and SA role in adaptation processes to abiotic stresses.

BR and SA signaling networks are known to be, at least in part, interconnected. BR can act synergistically and promote SA signaling by inactivating BR-INSENSITIVE 2 (BIN2). BIN2 was shown to phosphorylate TGA4 transcriptional factor (TF) which leads to its destabilization and prevents its functional interaction with NRP1 in mediating SA-regulated gene expression [75]. Also, EBL have been shown recently to induce SALICYLIC ACID-BINDING PROTEIN2 in wheat [53]. Moreover, because clade I TGAs (a group comprising TGA4) has a role in regulating SA biosynthesis by promoting the expression of SARD1 and CBP60g [76], BR via the reported BIN2 phosphorylation of TGA4 can thus act in SA biosynthesis control. Besides, in *Arabidopsis* lines overexpressing BRI1-associated receptor kinase 1 (BAK1) an increase in endogenous SA accumulation was reported suggesting that at least some components of the BR signaling cascade have a role in controlling SA accumulation [77].



**Figure 3.** The effect of ECS on the level of salicylic (SA) and benzoic (BzA) acids in soybean leaf tissues. Plants have been sprayed twice with ECS on the 21st and 28th days after planting. On 35th days after planting soybean leaves have been harvested to determine the phytohormones content. Values are means of 8 biological samples. Experiment was repeated twice (4 variant repeats per experiment). Error bars indicate standard error of the mean (SE). \*  $p < 0.05$  (Student's t-test).

BR also can act antagonistically with SA. Based on published data the effect of BR in controlling SA accumulation differs in plant species. Rice plants treated with BL could accumulate less SA during brown planthopper infestation; this effect was attributed to the downregulation of SA biosynthesis genes - OsPAL and OsICS1 [78]. However, latest data indicates that phenylalanine ammonia-lyase (PAL) pathway not directly leads to SA biosynthesis but have important role in regulation of SA biosynthesis [79]. Also, BL was shown to reduce the SA content in tobacco [80]. *Bsk1* plants, deficient in BR-SIGNALING KINASE1 (BSK1), accumulated less SA following infection with *G. cichoracearum* powdery mildew or *Pseudomonas syringae* pv *tomato* DC3000. Also, *bsk1* mutant resistance to pathogens was compromised [81]. These data suggest that pathogen-induced SA accumulation relies on some components of the BR signaling cascade.

SA can be produced via the isochorismate synthase (ICS) pathway (for review see [82]). In different plant species, the two pathways contribute unequally to SA biosynthesis. In *Arabidopsis*, most SA is produced via the ICS pathway independently of BzA accumulation [83]. In soybean both pathways, ICS and PAL, contribute equally to stress-induced SA accumulation [84]. The fact that an increase in BzA following ECS treatment is not translated into SA accumulation may imply that BA2H is inactivated. More so, because the pool of active SA in plants depends not only on the activity of SA biosynthetic pathways but on the rate of SA conversion to inactive metabolites (for review see [82]).

Recently, important results for understanding the pathways of SA synthesis in plants were obtained by Wu and colleagues [79], who used stable isotopes ( $^{13}\text{C}_6$ -Phe and  $^{13}\text{C}_6$ -BzA) to investigate the pathways of SA biosynthesis in *Arabidopsis*. They provided evidence that SA can be formed from benzoic acid, acting independently the phenylalanine ammonia-lyase (PAL) pathway [79].

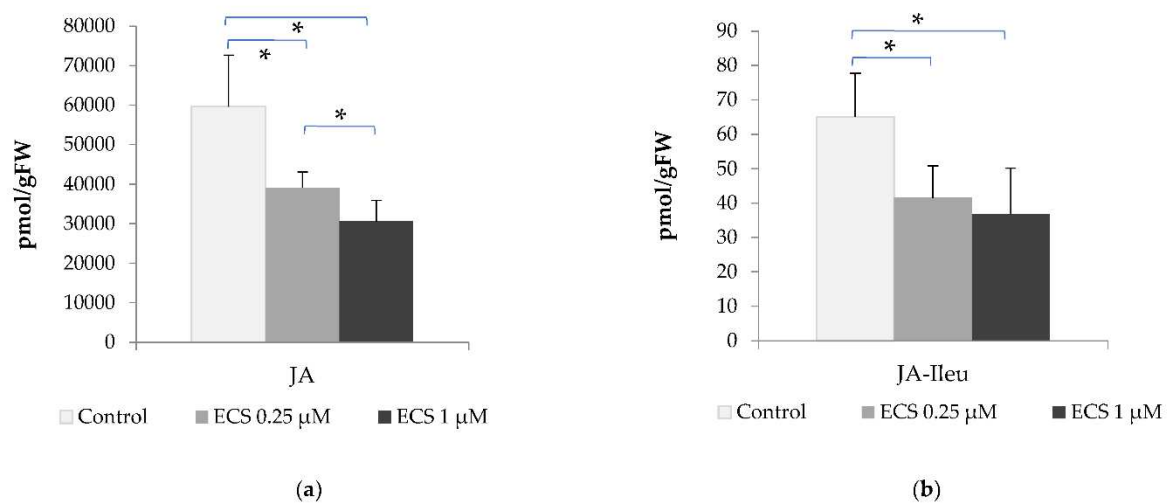
Our data on the accumulation of BzA (Figure 3) may evidence an important step in BR-SA crosstalk via regulation of SA/BzA levels. Our results suggest that ECS induces BzA levels in soybean, possibly via inactivation of BA2H. Later, BzA pool may enable rapid conversion to SA and activation of SA signaling. How SA biosynthesis is regulated by BR remains to be established.

### 2.2.3. Interaction of Brassinosteroids with Jasmonates (Jasmonic Acids and Jasmonic Acid-Isoleucine Levels)

In the present study, we analyzed the content of JA and JA conjugate with isoleucine (JA-Ile). The amino acid conjugate of isoleucine with JA is one of the common forms of JA in plant cells [85]. The content of JA in the control variant was determined in soybean leaves to be at the level of 65.03 pmol/g of FW and the JA-Ile content – 59636 pmol/g FW. Under exogenous treatment of plants with



0.25  $\mu\text{M}$  ECS, a decrease in the JA and JA-Ile content was recorded. Higher concentrations of ECS (1  $\mu\text{M}$ ) did not lead to a further decrease in the level of JA and JA-Ile (Figure 4).



**Figure 4.** The effect of ECS on the level of (a) jasmonic acid (JA) and (b) the conjugate with isoleucine amino acid (JA-Ile) in soybean leaf tissues. Plants have been sprayed twice with ECS on the 21st and 28th days after planting. On 35th days after planting soybean leaves have been harvested to determine the phytohormones content. Values are means of 8 biological samples. Experiment was repeated twice (4 variant repeats per experiment). Error bars indicate standard error of the mean (SE). \*  $p < 0.05$  (Student's t-test).

Analysis of array data on genes involved in jasmonates biosynthesis also revealed genes with modified expression in response to ECS or BL treatment [55] (Table 1). Genes which encode JASMONIC ACID-AMIDO SYNTHETASE JAR1 are highly suppressed by treatment with exogenous ECS and BL (Table 1). In contrast, propiconazole (a BR-biosynthesis inhibitor) has decreased their expression (Table 1). JASMONIC ACID-AMIDO SYNTHETASE JAR1 catalyzes the formation of JA conjugates with amino acids. These results are consistent with our data on ECS influence on JA and JA-Ile levels (Figure 4).

It has been shown previously, that mutants in BR biosynthesis or signaling pathway show higher accumulation of JA-precursor 12-oxo-phytodienoic acid [86] pointing to BR-JA antagonism in the regulation of JA content. Also, JA inhibits root growth, and expression of BR biosynthetic gene *DWF4* and lowers endogenous BR content, while exogenously added BR can attenuate JA inhibitory effects on root growth [87]. This data is also consistent with the results that we have obtained (Figure 4).

Some studies point to concentration-dependent interconnection between BR and JA antagonism/synergism switches in different parts of the plant. For example, high levels of exogenously applied EBL in rice shoots have been demonstrated to induce the expression of the JA biosynthetic gene *OsaOS2* and signaling gene *OsJAmyb* in plant roots. This also has been connected with the deterred activity of BR biosynthetic *OsDwarf* and signaling *OsBRI1* genes. In contrast, low exogenous levels of EBL inhibited *OsaOS2/OsJAmyb* genes. These changes in JA and BR gene activities correlated with increased nematode resistance in plant roots, while the low exogenous concentration of EBL decreased plant resistance to nematode attack [86].

The major portion of BR-JA antagonism depends on the common component of BR and JA pathways - BIN2 kinase. BIN2 kinase negatively regulates BR signaling by inactivating BR-dependent transcriptional factors *BRI1-EMS-SUPPRESSOR1* (*BES1*) and *BZR1*. On the opposite, BIN2 positively regulates JA signaling by phosphorylating and promoting the degradation of JAZ proteins - JA signaling repressors. BR treatment represses BIN2 activity and JA signal pathway. Gain-of-function mutations of the BIN2 promote JA signaling and also stimulate JA accumulation [88]. Rice BIN2 homolog *OsGSK2* has been shown to interact directly with a JA repressor protein *OsJAZ4* and positively regulate JA signaling and antiviral defense against rice black-streaked dwarf virus (RBSDV).

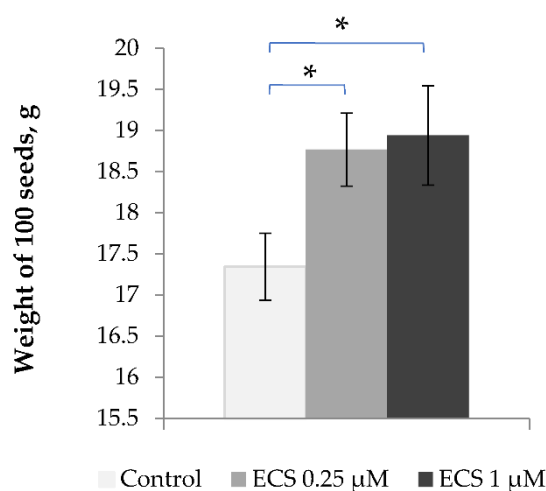
BIN2 kinase is also a part of BR-JA synergism. *OsGSK2* kinase can not only stimulate JA-signaling but also act as a negative regulator through interaction with the JA transcription factor

OsMYC2. OsMYC2 phosphorylation by OsGSK2 leads to OsMYC2 degradation and reduction of JA-mediated defense response against rice stripe virus (RSV). It has been shown that RSV suppresses BR endogenous levels to elevate the accumulation of OsGSK2 [89]. So, at least in the case of plant response to RSV, BR and JA can act synergistically - inhibition of OsGSK2 kinase by BR promotes OsMYC2 activity and JA-dependent defense response.

All the discussions above evidence an important role of BR-JA crosstalk in the regulation of plant cell metabolism. Cross-interaction between JA and BR signaling pathways might be involved in the tight balancing between plant growth and resistance. Data we obtained suggest BR-JA antagonism in soybean plant leaves under optimal conditions in response to treatment with exogenous ECS (Figure 4).

### 2.3. Effect of 24-Epicastasterone on Soybean Seed Yield

In our study we have evaluated the effect of exogenous ECS on soybean seed productivity as a long-term effect of ECS cross-hormonal interactions. We measured that the average weight of 100 seeds from control plants was corresponding to around 17.5 g, while the treatment of soybean plants with a ECS 0.25 or 1  $\mu\text{M}$  led to an increase in the seed weight to almost 19 g for 100 seeds, an increase of about 8.5% (Figure 5). Thus, the observed changes in the content of key soybean hormones induced by ECS treatment are linked with an increase in soybean seed productivity.



**Figure 5.** Influence of ECS treatment on an average weight of soybean seeds. Plants have been sprayed twice with ECS on the 21st and 28th days after planting. A batch of 100 seeds were measured. Values are means of ten biological samples. Experiment was repeated twice (10 variant repeats per experiment). Error bars indicate standard error of the mean (SE). \*  $p < 0.05$  (Student's t-test).

## 3. Materials and Methods

### 3.1. Plant Materials and Growth Conditions

Soybean seeds (*Glycine max* L. cv. Terek) were obtained from the experimental station of Poltava State Agricultural University (Ukraine). Seeds were sterilized by soaking in a 2.8% sodium hypochlorite (NaOCl) solution for 5 min and then rinsed three times with autoclaved distilled water for 2 min each, germinated on sterile filter paper, and the germinated seeds were placed in 3-liter plastic vessels filled with growth substrate mixture - Polissya soil (70%), washed sand (0.5-2.0 mm) (15%) and agrolite (15%).

Plants were grown at a constant temperature of 25°C in an artificial climate chamber (14 h light: 10 h dark) under white light provided by a continuous wide-spectrum LED (Philips) at 250  $\mu\text{mol m}^{-2} \text{s}^{-1}$  intensity. Leaves of soybean plants grown in a vegetation experiment were sprayed twice with ECS solutions (0.25 or 1  $\mu\text{M}$ ) - on 21st and 28th days after planting seedlings. On the 35th day after sowing, soybean leaves were sampled from one tier of ten different plant vessels, mixed within each variant, and four samples of 100 mg of leaf tissue were selected and frozen in liquid nitrogen for further determination of phytohormone content. Experiment have been repeated twice.

### 3.2. Treatment of Soybeans with ECS Solutions

ECS powder was synthesized in the laboratory of Volodymyr Khripach, an expert in the synthesis of different BR types and many of their derivatives. ECS dissolved in EtOH to obtain a 0.5 mM stock solution. The stock was diluted with distilled water to get 0.25 and 1  $\mu$ M final solutions used for soybean treatments. ECS concentration 0.1 and 0.25  $\mu$ M has been chosen based on preliminary experiments with BR effects on soybean and available published data on BR usage on different species.

Leaves of soybean plants grown in a vegetation experiment in plastic vessels were sprayed twice with ECS solutions (0.25 or 1  $\mu$ M) - on the 21st and 28th days after planting seedlings using a hand sprayer; control plants were sprayed with a solvent EtOH diluted with water as ECS stock. EtOH concentration in final ECS solutions or control treatments didn't exceed 0.01%.

### 3.3. Hormone Extraction and Quantification

Frozen samples (100 mg FW) were homogenized with liquid nitrogen in a mortar and pestle. The phytohormones were extracted with a cold (-20 °C) methanol/water/formic acid mixture (15/4/1, v/v) as described in [90].

Internal isotope-labeled standards (10 pmol per sample) were added for hormone analysis):  $^{13}\text{C}_6$ -IAA (Cambridge Isotope Laboratories);  $^2\text{H}_4$ -SA (Sigma-Aldrich);  $^2\text{H}_3$ -PA,  $^2\text{H}_3$ -DPA,  $^2\text{H}_5$ -ABA-GE (NRC-PBI);  $^2\text{H}_6$ -ABA,  $^2\text{H}_5$ -JA and others (Olchemim).

The extracts were passed through reversed-phase cation exchange SPE columns (Oasis-MCX, Waters) in a mixed mode (mixed phase-cation exchange). The hormone fraction containing ABA, IAA, SA, and JA was eluted with methanol. Hormone metabolites were analyzed using HPLC (Ultimate 3000, Dionex) coupled to a hybrid triple quadrupole/linear ion trap mass spectrometer (3200 Q TRAP, Applied Biosystems). Quantification of hormones was done using the isotope dilution method with multilevel calibration curves ( $R^2 > 0.99$ ). Data processing was carried out with Analyst 1.5 software (Applied Biosystems).

### 3.4. Estimation of Average Weight of Soybean Seeds

To evaluate the effect of the ECS treatment on an average weight of soybean seeds, plants were harvested by hand and pods were separated from each plant. The pods were dried in an oven at 35 °C for 24 hours to maintain homogeneous seed moisture as described by Poudel [91].

### 3.5. Statistical Analysis

P values were calculated with a two-tailed Student's t-test using Excel software. The sample size and number of independent biological repeats for each type of analysis are provided above.

## 4. Conclusions

The analysis of the mechanisms of interaction of BR with other plant phytohormones is a key process in elucidating the cooperation of hormonal signaling systems in the regulation of the growth and development of agricultural plants. Understanding the molecular mechanisms underlying this crosstalk is an important task for developing new strategies to improve plant growth and productivity. Our results showing exogenous ECS effect on the content of endogenous hormones in soybean leaves under optimal conditions point at the probable synergism of ECS with the growth-promoting hormones and its antagonism with stress hormones. However, BR synergism or antagonism with other phytohormones depends on specific conditions and might recruit different transcriptional factors; it might be inherently dependent on environmental stresses or pathogen attacks. We observed that BR can stimulate the accumulation of both growth-promoting IAA and BzA - inactive precursor of SA stress hormone - under optimal conditions that might point to efficient trading off between growth and possible stress resistance. Indeed, ECS stimulated seed yield of ECS-treated plants. We hope that the results obtained by our team will be useful to all researchers who analyze the interaction of brassinosteroids and salicylic acid, as well as its metabolites in plant cells.

Overall, the crosstalk between BR and other phytohormones is a complex and dynamic process that is still being actively studied by plant biologists.

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