Thesis of Doctoral (Ph.D.) Dissertation

Evaluation of the Biostimulant Activity of Liposome-Formulated Herbal Extracts in Major Field Crops

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Debrecen
2025

1. BACKGROUND AND OBJECTIVES OF THE DOCTORAL DISSERTATION

In the cultivation of economic crops, it is crucial to protect the plant against pests, to support the plant's response to abiotic and biotic stresses, and thus to maintain the quantitative and qualitative parameters of the crop. Using plant biostimulants (PBs), even plant-based biostimulants can contribute effectively to these objectives of integrated crop production and protection, which aim to prevent the emergence of pests or reduce their levels below economic thresholds while reducing the environmental impact (Rouphael and Colla, 2020; Yakhin et al., 2017).

Nanotechnological formulations offer an entirely novel approach to improve PB stability, penetration and even delivery to the target site in the plant organism (Abdollahdokht et al., 2022; Manchikanti, 2019; Nuruzzaman et al., 2019). Lipid-based nanocapsules include nanoliposomes composed of non-toxic biodegradable phospholipids forming single or multilayered vesicles (Campbell, 1983; Jogaiah et al., 2021). Although widely used to encapsulate drugs, enzymes, vitamins, and bioactive plant extracts (Chawda et al., 2017; Emami et al., 2016), their use for agricultural applications is less explored.

Our experiments tested the Elice16® product family, EliceVakcina and Garlic-lipo plant conditioners developed by the Research Institute for Medicinal Plants and Herbs, Ltd. (RIMPH, Ltd., Budakalász, Hungary). Different supercritical carbon dioxide (SC-CO_2) medicinal plant extracts are encapsulated in a nano-sized liposome of plant-derived lipids. According to published experience, exogenous abscisic acid (ABA) treatments stimulate plant responses to biotic or abiotic stresses and increase yield parameters (Aroca et al., 2008; Chan, 2012; Travaglia et al., 2010). It is also important to note that ABA and chaperone signaling pathways are central regulators of plant defense against stress (Bulgakov et al., 2019).

The focus of the studies was *in silico* analyses, genome-wide transcriptomic profiling was performed. These analyses revealed the altered defense processes in plants due to treatments, which is a gap, as there are few publications on transcriptomic analysis (González-Morales et al., 2021). We used a workflow of transcriptomic analyses that allows us to follow significant changes in biosynthetic pathways, the dynamics of these changes, and changes in gene-level expression.

During my PhD work, I have set the following scientific studies as my objectives:

1. Development of PBs consisting of nano liposome formulated herbal extracts (Garlic-lipo and EliceVakcina) with high ABA content.
2. Investigation of antifungal activity of the produced PBs.
3. Demonstration of the effect of Garlic-lipo treatment by genome-wide transcriptomic profiling.
4. Demonstration of the effect of EliceVakcina treatment by genome-wide transcriptomic profiling.
5. Comparative analysis of the effects of the formulated plant biostimulants on phytohormone signaling and plant defense response pathways.

2. MATERIAL AND METHODS

2.1. The plant-based biostimulants

EliceVakcina is a Nébih-approved (National Food Chain Safety Office) nanoliposome-formulated plant conditioner containing 11 herbal extracts. The extracted method was SC-CO_2 extraction, the main ingredient being garlic (*Allium sativum* L.) clove extract. The other formulation, laboratory named Garlic-lipo, is under development and is a single component SC-CO_2 garlic extract. Its formulation is similar to that of EliceVakcina liposome. In our experiments, we used the unformulated SC-CO_2 garlic extract as Garlic-oil, indicating the characteristic of the substance. Fitokondi, also included in the experiment as a positive control, is a Nébih-approved plant conditioner containing aqueous extracts of medicinal plants, but its formulation is not liposomal (Table 1).

Table 1. Characteristics of the investigated products used in the experiments.

Product name	Formulation	Garlic content (%)	ABA content (µg g^{-1})
*EliceVakcina	nanoliposome, SC-CO_2 extract	~65	6.3± 1.2
*Garlic-lipo	nanoliposome, SC-CO_2 extract	~90	80.4± 2.2
Garlic-oil	SC-CO_2 extract	100	81.0± 2.4
Fitokondi	aqueous extract of herbs	~1	<LOD

ABA concentrations were determined using high-performance liquid chromatography with diode-array detection (HPLC-DAD). The abbreviation SC-CO_2 refers to supercritical carbon dioxide extraction. *PBs with nano formulation containing 100-200 nm liposome particles. Abbr. limit of detection (LOD)

The extracts were encapsulated in 100-200 nm multilamellar vesicles using an active trapping technique (Mayer et al., 1986). Electrostatic stabilization (Zeta potential), dispersion properties (polydispersity index-PDI) and nanoparticle size distribution were measured by dynamic light scattering using a Zetasizer Ultra instrument. The size of liposomes was also determined by transmission electron microscopy (TEM). The ABA content of the extracts was detected by high-performance liquid chromatography with diode-array detection (HPLC-DAD) measurement.

2.2. The antifungal effects of plant-based biostimulants

The biostimulant effect of extracts was tested on *Fusarium* species under laboratory conditions (*F. verticilloides* (FGSC 7600)*, F. graminearum* (FGSC 9075)*, F. oxysporum* (FGSC 9935) - Fungal Genetics Stock Center (FGSC), Kansas State University, Manhattan, US) és *F. proliferatum* (NRRL 62905 - Joint Genome Institute (JGI), University of California, Berkeley, US). The toxicity test, determining the Minimal Inhibitory Concentration (MIC), was performed according to the Clinical and Laboratory Standards Institute (CLSI) microdilution method using half dilution from 10 V/V%. In addition, the antifungal activity test of solid agar plate assay was also used (Perczak et al., 2019; Pfaller et al., 2000). In the experiment, spore suspensions of 7–14 days were used, according to the characteristics of the different *Fusarium* species. The concentration of spores was adjusted to 5×10^6 spores/ml, then 100 µl was inoculated onto the surface of Potato Dextrose Agar (PDA - Merck KGaA, Darmstadt, Germany) medium. After that, 10 µl of plant extract was added to a sterile filter paper disc placed in the middle of the medium. Incubation was carried out at 25°C for three days.

The formulations' effect on pea seeds' germination capacity was tested. We also investigated whether the treatment alters the germination of seeds infected with *Fusarium* spore suspensions. The seeds were soaked in 0.5-1% EliceVakcina and Garlic-lipo extract for 24 hours. For the infection test, a spore suspension of *F. oxysporum* with a concentration of 10^4 spores/ml was used for 30 minutes. The treated seeds were placed on wet filter paper and incubated at room temperature for four days.

2.3. Treatments of plant-based biostimulators

The effect of Garlic-lipo (240 g/ha) treatments was investigated under greenhouse conditions by spraying on the leaves of *Triticum aestivum* 'Cellule' plants at the early vegetative stage. For molecular biological studies, leaves were collected before treatment (control – 0 min) and 15 min, 24 h and 48 h after treatment in three biological replicates.

Small plot experiments were carried out with *Pisum sativum subsp. sativum convar. medullare var. pervicax* 'Angela', *Brassica napus* 'GK Mécses' and *Glycine max* 'ES Director' plants. During vegetation, three applications of the EliceVakcina were made at doses of 20 and 240 g/ha at different developmental stages (juvenile/mature vegetative and reproductive phases). Leaf samples were taken for molecular biological analyses two days after the treatments. Thus, for transcriptome analysis, *P. sativum* and *B. napus* were sampled once during the reproductive

phase. *G. max* plants were sampled three times during the cultivation (juvenile/mature vegetative and reproductive phase), but unfortunately, no libraries were made of the samples taken in the generative phase.

2.4. Effects of plant-based biostimulants using bioinformatics analyses

Samples were sequenced by the Illumina NextSeq550 platform, and next-generation sequencing (NGS) libraries were prepared using single-end reads. Using *de novo* assembly, a combined transcript dataset was gained that was examined using pairwise differential expression genes (DEG). Numerical analysis of DEG allowed for comparing the gene pools of two samples collected after different treatments. Then, the contigs of the 50 genes with the highest significant difference in expression (Top50 DEG) were annotated. Functional annotation and Gene Ontology (GO) analyses were performed according to GO terms and used as the basis for pathway analysis (Kyoto Encyclopedia of Genes and Genomes Pathway analysis - KEGG Pathway analysis). Time-course expression analysis revealed the dynamics of changes in biosynthetic and signaling pathways. The processes highlighted in the analyses were further investigated, and the expression levels of the individual genes involved were determined by Reads Per Million mapped read (RPM) calculation. The values of the genes with outstanding expression levels were validated by reverse transcription real-time quantitative PCR (Reverse transcription-quantitative polymerase chain reaction - RT-qPCR) analysis.

3. RESULTS AND DISCUSSION

3.1. Development of plant biostimulants consisting of liposomal herbal extracts formulated with high ABA content

The liposomal properties of the plant-based biostimulants showed stability, as reported in the literature (Hanachi et al., 2022). EliceVakcina and Garlic-lipo formulations showed particle sizes of 233 and 149.2 nm, PDI of 0.1733 and 0.13035, and Zeta potential of -40.66 to -40.53 mV, respectively. Furthermore, the size and structure of the prepared liposomes were also characterized by TEM (Figure 1).

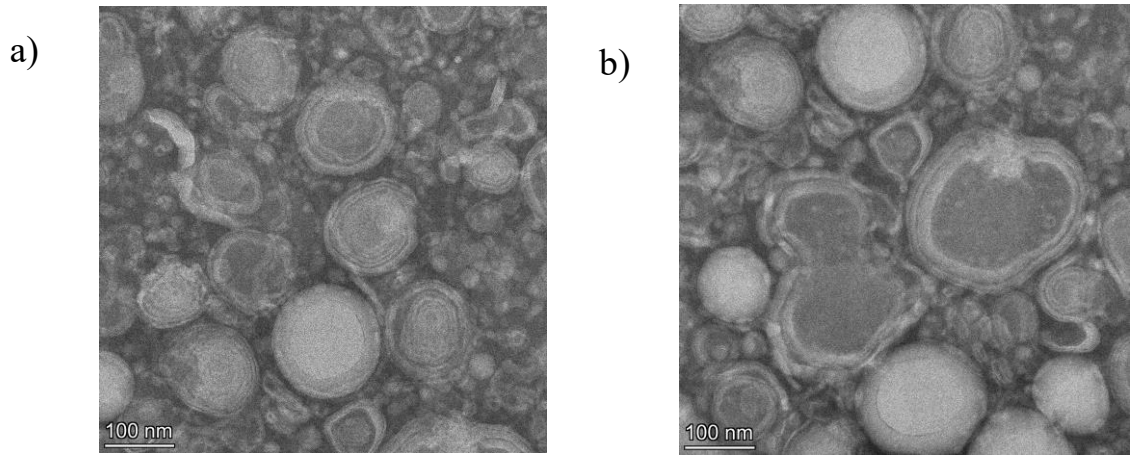

Figure 1. TEM images of the structure of (a) EliceVakcina, (b) Garlic-lipo liposomes. The multilamellar liposomes are ~100-200 nm in size

Garlic extract has a higher ABA content than other phytohormones (Arif et al., 2019). According to our HPLC-DAD measurements, ABA levels were 81.4±2.2 µg/g for Garlic-lipo extract, 6.3±1.2 µg/g for EliceVakcina partially containing garlic extract, and 80.0± 2.4 µg/g for Garlic-oil without liposome formulation. Fitokondi does not contain ABA hormone. However, according to the HPLC-DAD measurements, the four plant-based biostimulants did not contain thiamine.

3.2. Investigation of antifungal activity of the plant biostimulants

The use of garlic extracts as a PB against *Fusarium* species has been published (Hayat et al., 2016; Pinilla et al., 2019). In the microdilution toxicity test, preparations containing garlic extracts inhibited the growth of *Fusarium graminearum, F. oxysporum, F. proliferatum* and *F. verticilloides* species, with all four species showing similar responses to the extracts. According to the absorbance measurement, a significant difference was observed when using the 0.625 V/V% dose, with Garlic-lipo (-0.1612) being the most effective, followed by EliceVakcina (0.11755), and finally Garlic-oil (0.62745). When using a higher volume percentage, at 2.5-10 V/V% measurements, there was no significant difference in the inhibitory effect of formulated and unformulated garlic extract on the growth of *Fusarium* species.

The antifungal activity test using the solid agar plate assay was conducted, revealing that the four *Fusarium* species exhibited different sensitivities to the tested herbal extracts (Figure 2). The most sensitive to all three extracts was *F. proliferatum*, which reacted strongly to the Garlic-lipo treatment. This resulted in an extensive zone of inhibition where no spore formation was observed. All three extracts, with varying degrees of sensitivity, inhibited the mycelium formation of the other species. In the case of *F. graminearum*, the highest inhibition was caused by Garlic-oil, as conidiophores were only observed on the edge of the medium. In addition, Garlic-lipo has significantly reduced mycelium growth. The mycelium growth F. *verticillioides* was inhibited by all three extracts, but the highest sensitivity was triggered by Garlic-oil treatment. A similar phenomenon can be observed in *F. oxysporum*, though to a lesser extent.

The germination capacity of pea seeds was tested by treating them with a 0.5-1% solution of EliceVakcina and Garlic-lipo. Both biostimulators reduced germination capacity on uninfected seeds. However, the 1% Garlic-lipo treatment with the *F. oxysporum* spore system is facilitated.

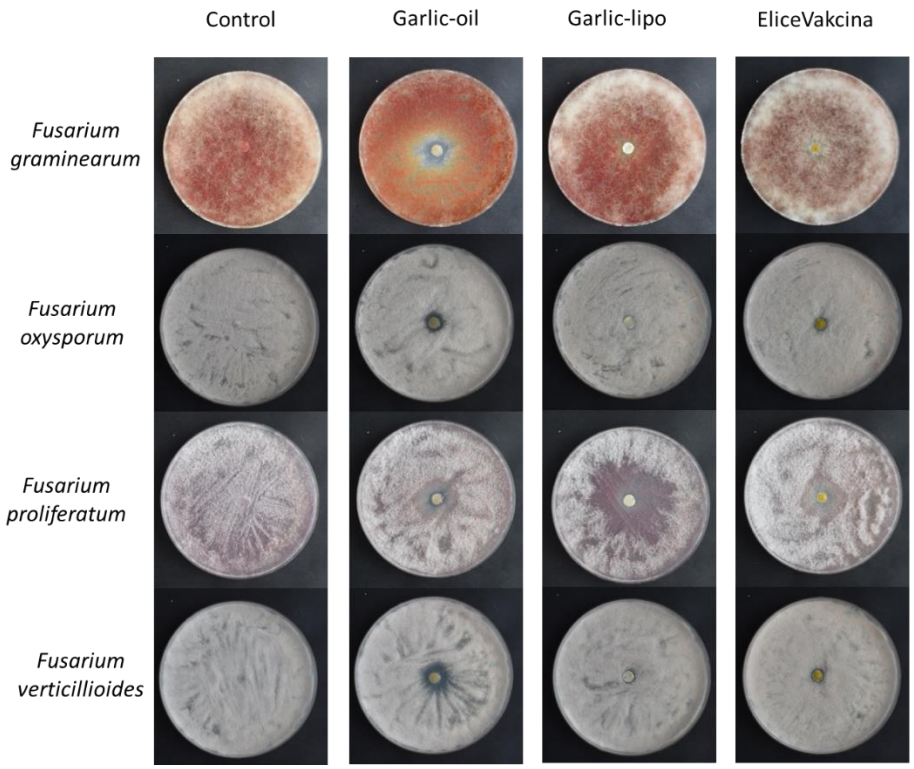

Figure 2. The four *Fusarium* species grown on PDA medium are shown in the experiment. The inhibitory effect of the herbal extract applied to the centre of the medium is observable. The *Fusarium* species exhibit varying sensitivity to the extracts

3.3. Large-scale genome wide transcriptional profiling experiment of the Garlic-lipo PB focusing on plant's physiological process in greenhouse experiments

In the greenhouse experiments, samples were taken two days after *T. aestivum* plants were treated with Garlic-lipo to determine the dynamics of changes (15 min, 24 h, 48 h). Top50 DEG analysis revealed changes in the expression levels of genes related to the ABA biosynthesis and signaling pathway (9-cis-epoxycarotenoid dioxygenase 3 (NCED3), early ABA-inducible protein 22 (HVA22), U-box domain-containing protein 19 (PUB19), late embryogenesis abundant protein 31 (LEA31)), the pathogenesis-related (PR1-5) proteins, and related transcription factors (TF) (homeobox-leucine zipper protein 22/24 (HOX22/24), zinc finger protein12 (ZAT12), R1/2-type myeloblastosis S1 (MYBS1), dehydration-responsive element-binding protein 1G (DREB1G)) (Figure 3). As a result of the treatment, the expression of PR genes (9764-1603 contigs) and stress-induced genes (2345-214) increases significantly.

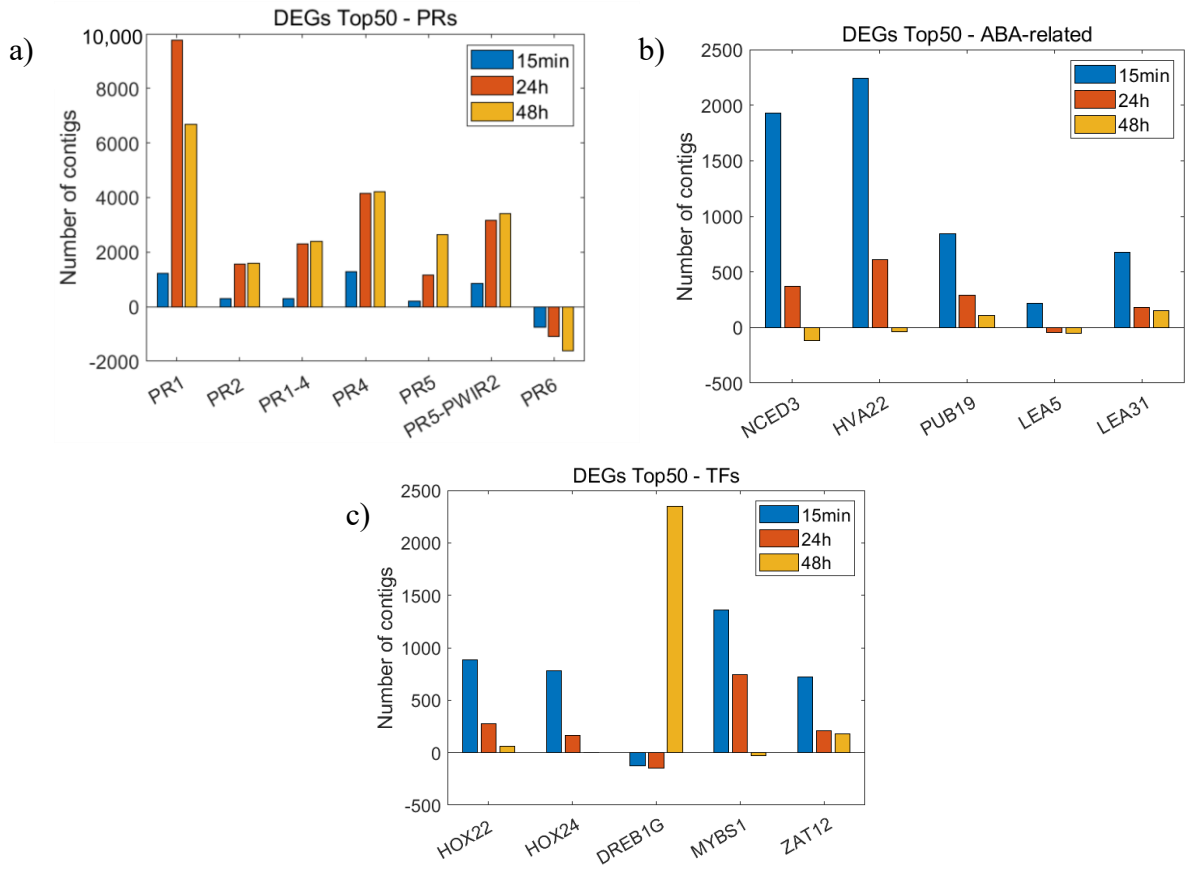

Figure 3. The determination of the top 50 differentially expressed genes (DEGs) *in silico* is represented in three sampling times. The numerical analysis of DEGs in a pairwise comparison of two times was examined using OmixBox.BioBam based on the RSEM and edgeR programs. These quantitative statistical methods to evaluate the significance of individual genes were implemented to examine the changes in the expression levels of (a) PR genes (control vs. 48 h), (b) ABA pathway-related genes (control vs. 15 min), and (c) TF genes (control vs. 15 min)

RT-qPCR validated gene expression levels determined by DEG analysis. PR1-5 showed increased expression levels 24 and 48 hours after treatment. Similar changes in expression levels of ABA pathway-related genes and TFs were also detected during validation, as predicted by *in silico* analysis. These genes generally showed maximum expression levels in 15 min and 24 h samples. Only the DREB1G TF was highly expressed in the 48 h sample by DEG analysis, which was confirmed by RT-qPCR (Figure 4).

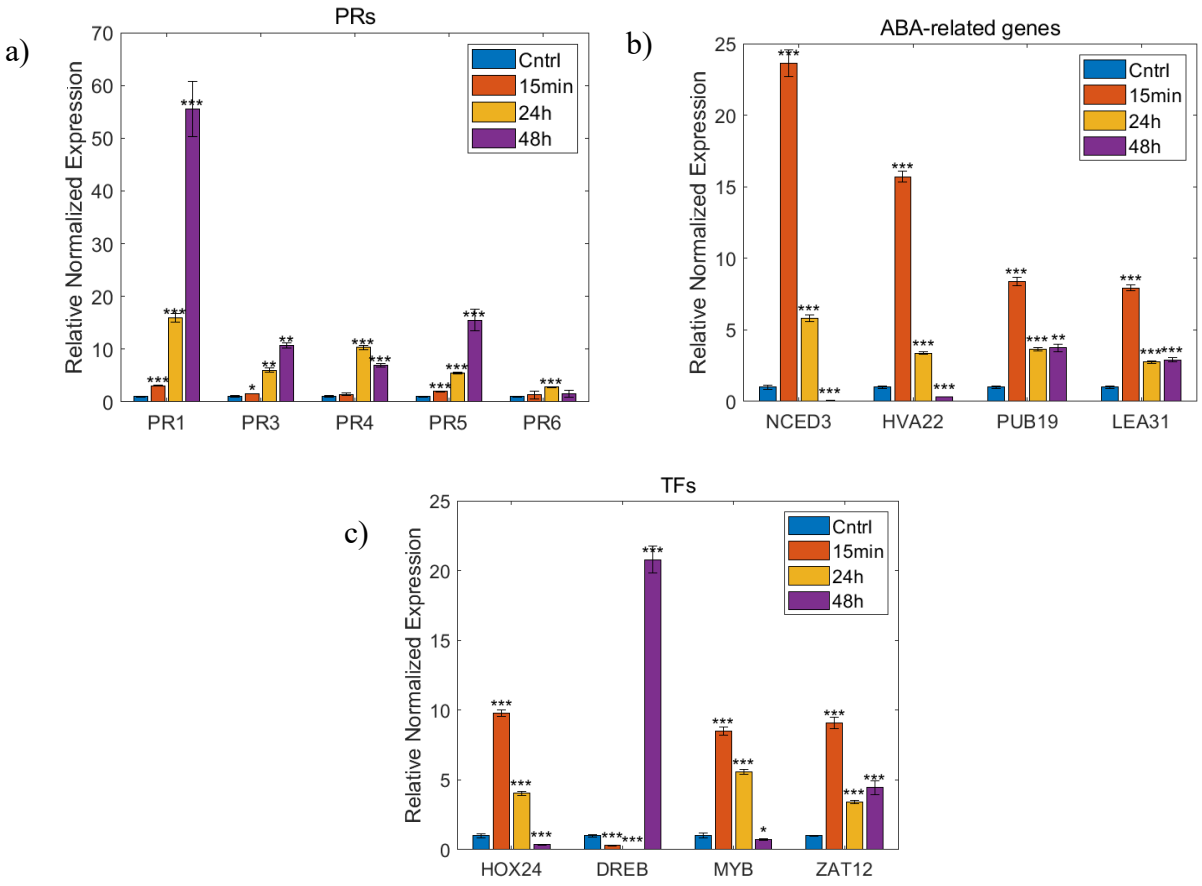

Figure 4. Results of the real-time PCR reactions performed at three sampling timepoints compared to control. The changes in the expression levels of (a) PR genes, (b) ABA pathway-related genes, and (c) TF genes. The values are represented as mean ± SEM (n = 4). $p < 0.05$ vs. Control, $p < 0.05$ vs. treated group. One asterisk indicates statistically significant difference between the means of a treated sample set compared to the mean of the control sample set to 5%; two asterisks indicate statistically significant difference to 1%; three asterisks indicate statistically significant difference to 0.1%

The results of garlic lipo treatment of *T. aestivum* plants showed that the activity of genes involved in thiamine metabolism, glutathione metabolism, and phenylpropanoid biosynthesis was the most significant. Among the plant signaling pathways, the mitogen-activated protein kinase (MAPK) signaling, the plant hormone signal transduction, and the plant-pathogen interaction showed increased expression levels. Plant hormone signal transduction processes were further investigated using RPM calculation. Our results showed that the activity of genes involved in ABA and jasmonic acid (JA) signal transduction and biosynthesis processes increased after treatments of Garlic-lipo.

To gain more information about the physiological processes in wheat after PB treatment, the samples were examined using time-course expression analysis. Measuring expression change is an effective tool for evaluating data from RNA-seq technology, specifically identifying genes whose expression shows a significant difference between experimental groups. Using Transcriptome Shotgun Assembly (TSA) and CountTable data, we tested and filtered 5287 contigs, which were classified into nine clusters.

The nine clusters were divided into four groups: (a) the expression level gradually increased, (b) the expression level gradually decreased, (c) the expression levels increased initially and then decreased, (d) the expression level was initially decreased and then returned to the original level. The word clouds are based on GO analysis, representing the biological process by summing the annotated genes of each group; the letter size depends on the sequence number (Figure 5).

The group of genes with progressively increasing expression levels (cluster 1) are mainly related to defense response pathways, which are glutathione metabolism, phenylpropanoid, flavonoid and terpenoid biosynthesis, plant-pathogen interaction, MAPK signaling pathway and cytochrome P450 metabolism pathways.

Clusters of genes with progressively decreasing expression levels (clusters 3, 4, 6, and 7) were associated with cellular-level biosynthetic and metabolic processes and showed down-regulation.

Groups of genes with initially elevated and then reduced expression levels (clusters 2, 8, and 9) are involved in various transport and translation processes.

A group of genes with initially reduced expression levels, then returning to their original levels (cluster 5), are involved in transcription and metabolic processes.

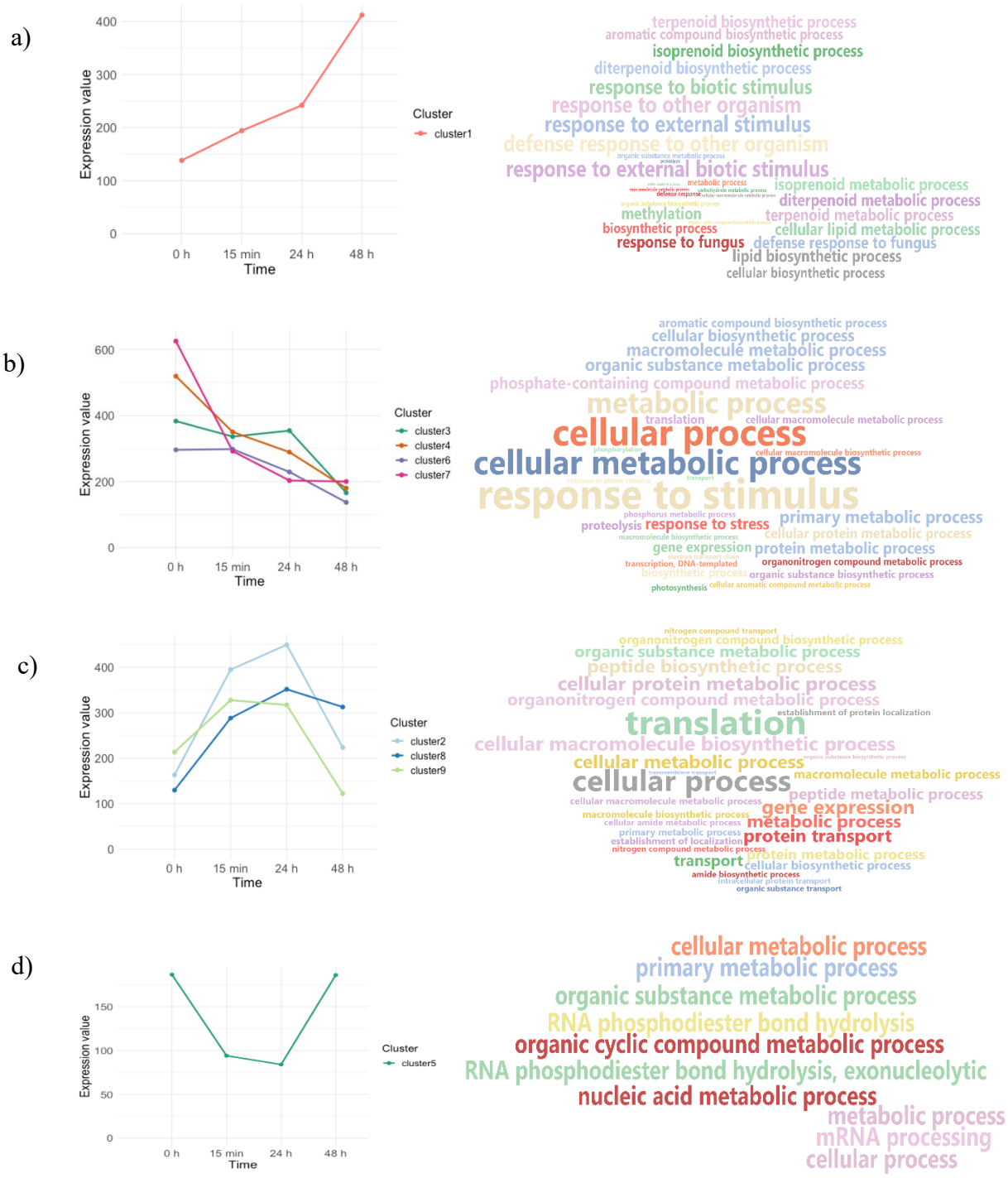

Figure 5. Time-course expression alterations. Graph showing the median level expression (significance level 0.05; R-squared cutoff 0.7) of each cluster of genes across times. WordClouds represent Gene Ontology (GO) term (biological process) summing annotated genes of each group, the font size depends on the sequence count. Genes with significantly different expression levels were classified into 9 clusters according to the dynamics of change: (a) expression level gradually was increased, (b) gradually decreased, (c) initially increased and then reduced, (d) initially decreased and then returned to its original level

3.4. Large-scale genome wide transcriptional profiling experiment of the EliceVakcina PB focusing on plant's physiological process in small plot experiments

In the greenhouse experiment, we demonstrated that sampling two days after treatment is a suitable time to study the processes involved in the plant defense response. Therefore, we sampled only at this time in the small plot experiments with *P. sativum, B. napus,* and *G. max* treated with EliceVakcina.

Top50 DEG analysis of *P. sativum* samples showed the activity of genes involved in the stress response at both low (Figure 6) and high doses. As observed in the wheat samples, PR2/4, glutathione metabolism genes, genes associated with flavonoid biosynthesis (chalcone synthase (CHS), isoflavone synthase (CYP93C), isoflavone 3'-hydroxylase (CYP81E9), caffeoyl-CoA methyltransferase (CCOAOMT)), cell defense catalase (CAT), and several heat shock proteins (HSP) were overexpressed.

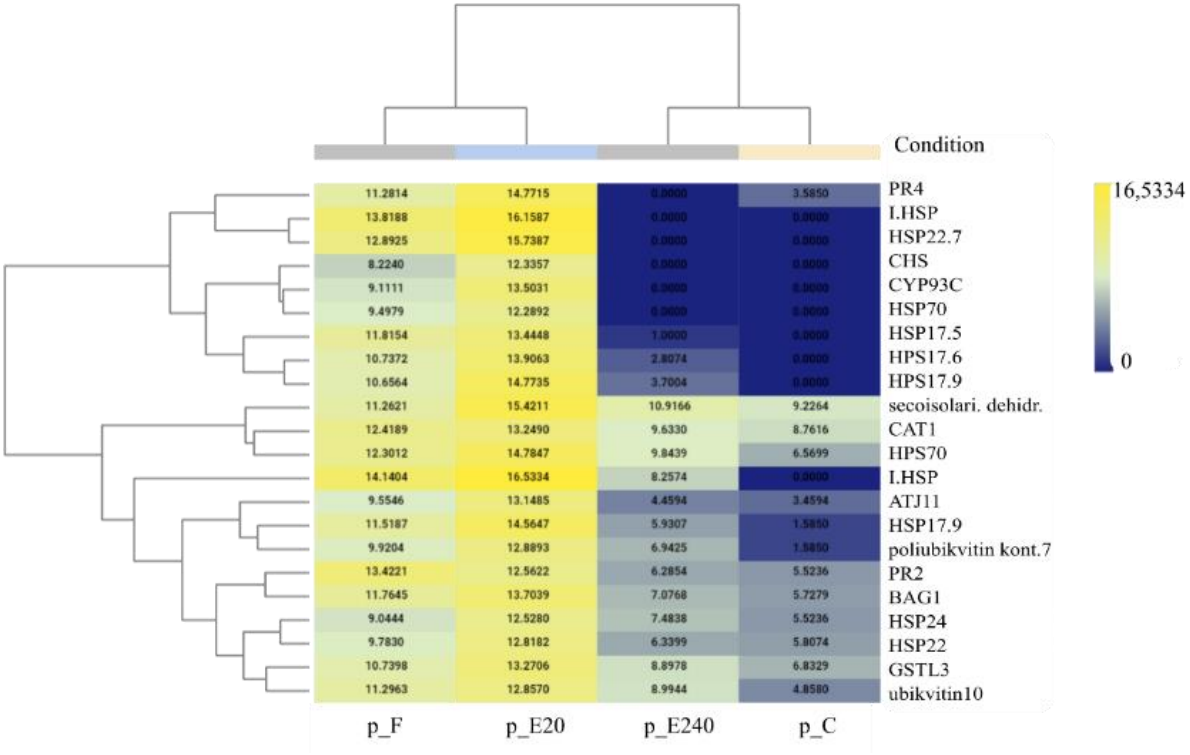

Figure 6. *P. sativum* Top50 DEG heatmap (p_C vs. p_E20), where untreated is the reference (control C) and EliceVakcina 20 g/ha (E20) is the treated sample compared. The gene expression levels of the EliceVakcina 240 g/ha (E240) and Fitokondi 4 l/ha (F) treated samples are also shown for the gene expression levels found in the analysis

The KEGG pathway analyses showed that the biosynthetic pathways involved in the plant stress response showed a high level of differentiation after treatments. Glutathione metabolism, phenylpropanoid biosynthesis, vitamin B1 metabolism, and carotenoid biosynthesis were highlighted. Among the signal transduction pathways, MAPK signaling, plant hormone signal transduction and plant-pathogen interaction genes showed high expression levels. In *P. sativum* samples, RPM analysis of plant hormone signal transduction after EliceVakcina treatment showed high activity of ABA-dependent genes, JA pathway genes, and salicylic acid (SA) pathway genes.

In *B. napus* samples, ABA-related enzymes with altered activity as a result of treatment were detected by Top50 DEG analysis, such as LEA46, protein phosphatases 2C3 (PP2C3), dehydrin Rab18 (RAB18) and low-temperature-induced 65 kDa protein (LTI65). Furthermore, we observed an overexpression of another stress-related plant hormone, JA-related proteins (UDP-glycosyltransferase 74D1 (UGT74D1), indol-3-yl-methylglucosinolate hydroxylase (CYP81F1)). Furthermore, the genes for S-adenosylmethionine decarboxylase proenzyme 3 (SAMDC3), involved in polyamine synthesis and enzymes involved in ubiquitination, showed high activity (Figure 7).

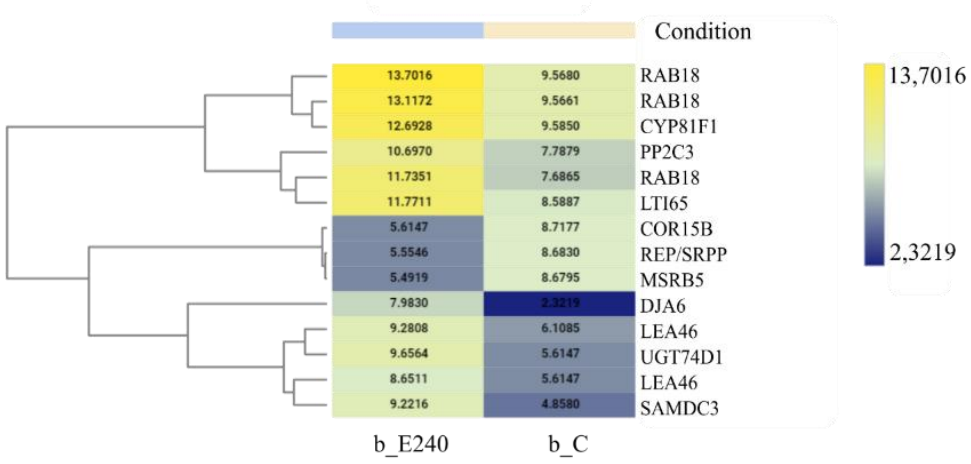

Figure 7. *B. napus* Top50 DEG heatmap detail comparing high-dose treatment with untreated sample: b_C vs. b_E240

Pathway analysis was also investigated in *B. napus* plants after high-dose treatment with EliceVakcina. The processes involved in plant stress defense responses, glutathione metabolism, plant-pathogen interaction, plant hormone signal transduction, MAPK signaling, thiamine metabolism, and phenylpropanoid biosynthesis showed significant differences. The RPM analysis of plant hormone signal transduction, genes related to ABA, JA signaling and

biosynthesis pathway showed high expression changes. In contrast, the activity of SA-dependent genes was partially observed.

G. max plants were treated at the juvenile vegetative stage with EliceVakcina at 20 and 240 g/ha. Top50 DEG analysis showed different stress response genes activity (Figure 8): cytochrome P450 82A3 (CYP82A3) involved in the catalysis of the redox reaction, heavy metal-associated isoprenylated plant protein 26 (HIPP26), as well as the nitrogen deficiency stress-responsive urea proton symporter (DUR3), the bacterial attack-responsive zinc-finger protein WRKY48 TF, the SA plant hormone-related AAA-ATPase, and the auxin (AUX)-related WALLS ARE THIN1 (WAT1) gene. The ABA-dependent galactinol synthase 2 (GOLS2) and PUB19, as well as stress response genes (ABC transporter C family member 4 (ABCC4), UDP-glycosyltransferase 79A6 (FG2)) associated with transport processes, also showed increased activity.

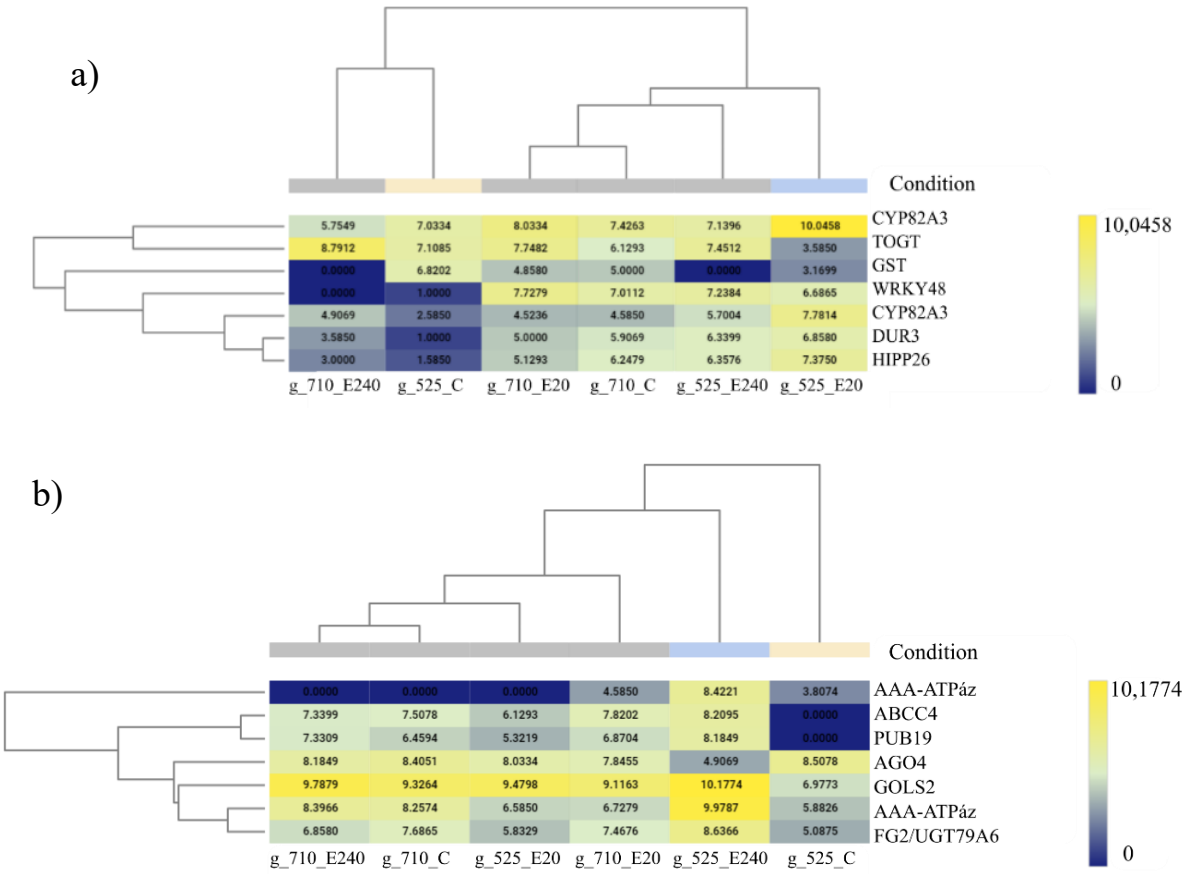

Figure 8. *G. max* Top50 DEG heatmap comparing a) low and b) high-dose treatment with the untreated sample: g_525_C vs g_525_E20. Data for other samples are also shown (g_525_E240, g_710_E20, g_710_E240, g_710_C)

The results of treatment of *G. max* plants with EliceVakcina showed elevated expression levels of genes involved in glutathione metabolism and phenylpropanoid biosynthesis processes compared to the control. As in the previous samples, soy also showed activation of genes involved in vitamin B1 metabolism. Among the signal transduction pathways, MAPK signaling, plant hormone signal transduction, and plant-pathogen interaction activity were markedly increased in samples collected after treatments. RPM data of plant hormone signal transduction gave few hits for genes in the ABA pathway. However, the measured data showed up-regulation for genes related to the JA and SA pathways in many cases.

3.5 The effect of PBs on phytohormone signaling and plant defense response pathways

After reviewing all *in silico* analyses, it can be concluded that Garlic-lipo treatment may activate genes that affect plant development and stress defense mechanisms, indicative of the strong regulation of ABA signaling and the biosynthetic pathway. The synthesis of the plant hormone ABA plays a key role in plant development and adaptive stress responses (Cutler et al., 2010). Although the ABA hormone is generally considered a plant growth inhibitor, several experiments have demonstrated its ability to dose-dependently enhance growth in both roots and shoots (Humplík et al., 2017; Zhang and Davies, 1990). Also, a potential regulatory role of ABA in thiamine biosynthesis under salt and osmotic stress conditions has been detected (Rapala-Kozik et al., 2012). ABA is also a central molecule in other signaling processes in plant stress (Bulgakov et al., 2019). We detected the activity of PR genes in samples treated with Garlic-lipo. PR genes encompassing systemic acquired resistance (PR1, PR2 and PR5) and local acquired resistance (PR3, PR4) showed overexpression. In addition, KEGG pathway analysis of treated samples detected several up-regulated genes involved in thiamine metabolism, plant hormone signal transduction, plant-pathogen interaction, phenylpropanoid, terpenoid and stilbenoid biosynthesis, and glutathione metabolism. These pathways result in the stress-protective response, disease resistance, abiotic and biotic stress response, detoxification, and promotion of plant development (Figure 9).

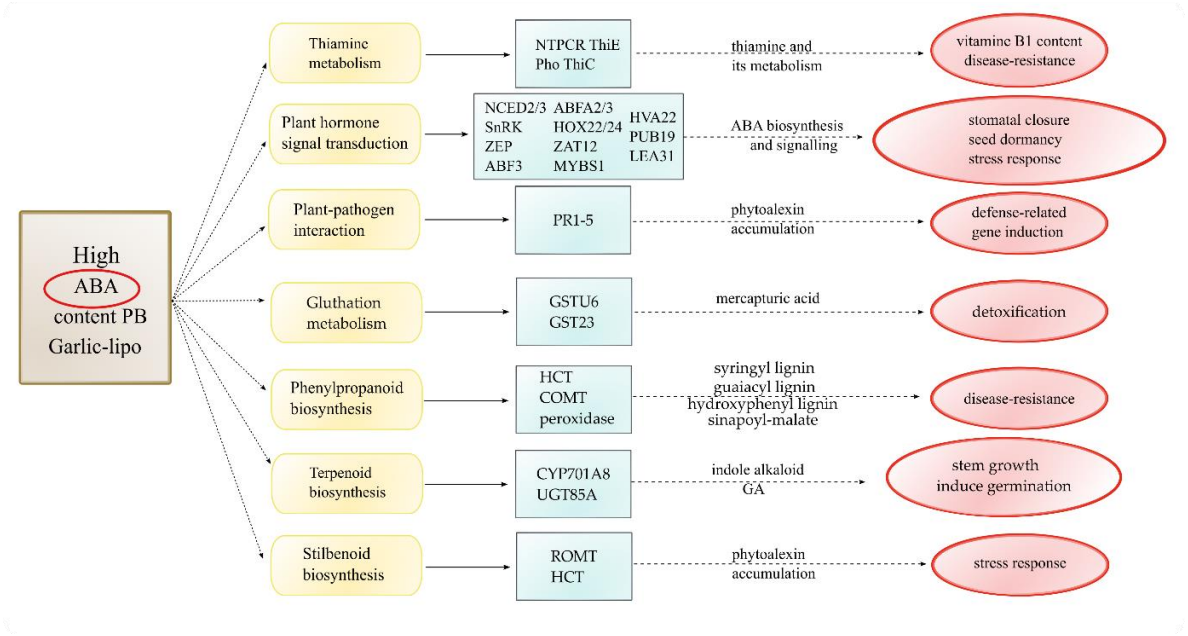

Figure 9. Summary of the effect of Garlic-lipo treatment in wheat based on KEGG pathway analysis. Activated pathways are in yellow, genes in blue, and processes in red boxes

Overall, in the small plot experiments, vitamin B1 metabolism, glutathione metabolism, plant hormone signaling (ABA, JA, SA), and plant-pathogen interaction pathways were observed to be active in all three cultures after treatment with high-ABA plant-based biostimulants (Figure 10).

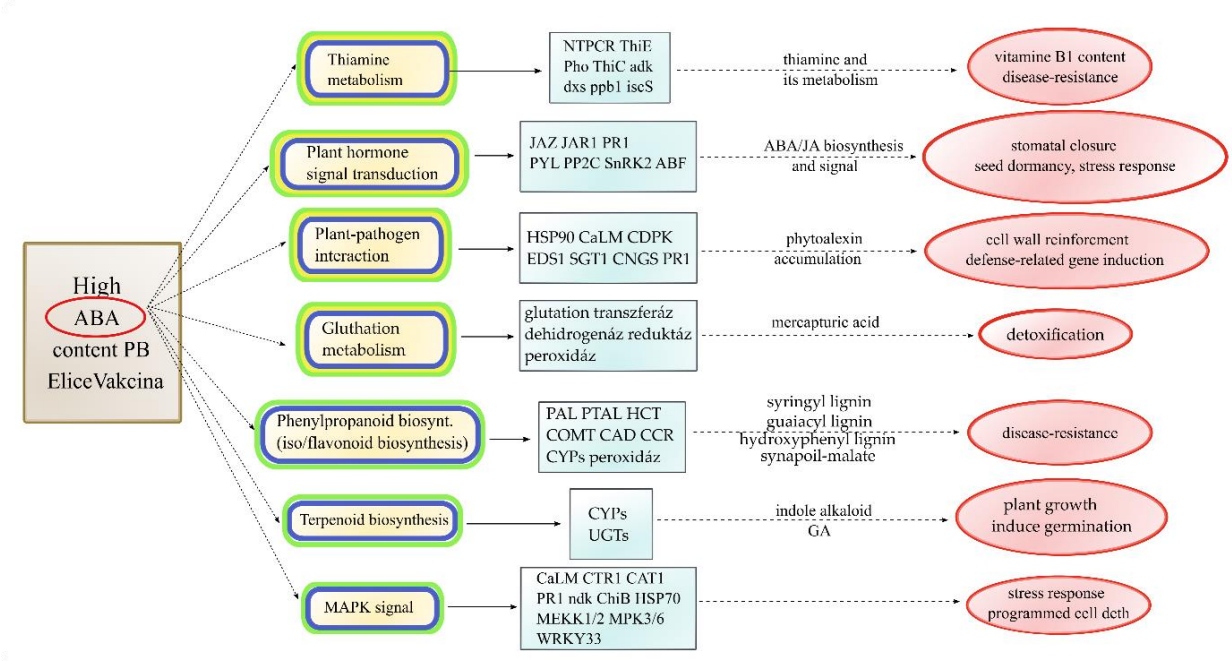

Figure 10. Summary of the effect of EliceVakcina treatment in the three small plot experiments based on KEGG pathway analysis. Activated pathways are shown in yellow, genes in blue, and processes in red boxes. Colours bordering the pathway boxes indicate the crop in which the pathway is active: green - pea, yellow - rapeseed, blue - soybean

Several publications have described that ABA plays a potential central regulatory role during plant stress in abiotic stress tolerance (Sah et al., 2016), thiamine biosynthesis (Rapala-Kozik et al., 2012) and plant hormone function through endogenous accumulation. According to pathway analyses, thiamine metabolism showed high activity. Therefore, the thiamine and its metabolites content of pea shoots containing a pea crop after treatment with EliceVakcina 20 g/ha were measured using HPLC-DAD (Figure 11). According to the chromatogram, the area under the standard signal detecting thiamine and its metabolites in the shoot increased 8% at 5 days and 14.3% at 10 days after treatment.

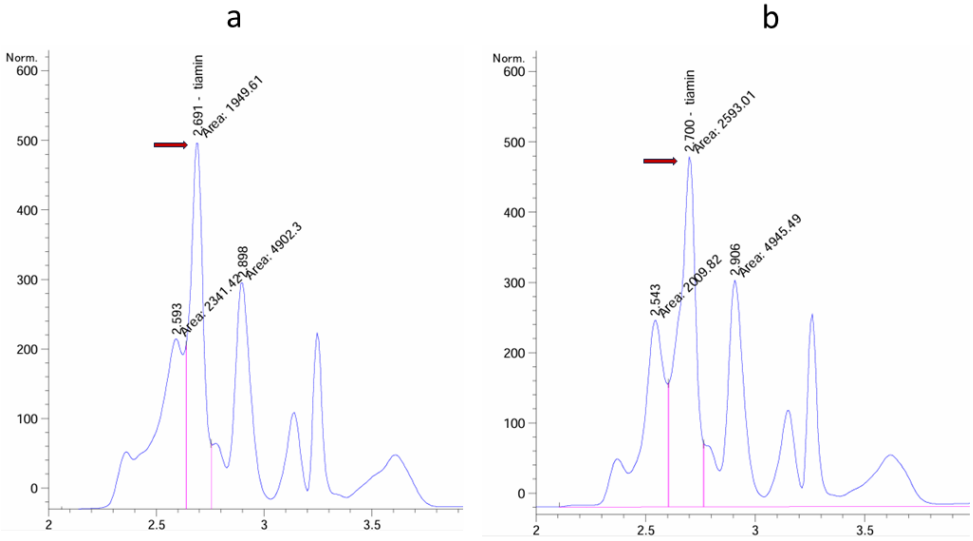

Figure 11. Representative chromatograms of HPLC-DAD measurements of thiamine in field treated pea plants. Thiamine content (peak area) of (a) untreated and (b) nanoliposome formulated EliceVakcina treated plants ten days after treatment

Together, ABA and AUX regulate many aspects of plant growth and development, seed germination, primary root growth (Sun and Li, 2014); stomatal germination regulation in interaction with ethylene (ET) (Harrison, 2012); plant defense and gene expression with jasmonates (Anderson et al., 2004). In our analyses, we detected correlations with the literature descriptions.

However, we also found differences between the three treated plant species. Only after treating pea and soybean plants was the MAPK cascade, phenylpropanoid biosynthesis, heat shock proteins, SA hormone, and PR gene activity enhanced. ABA is also integrated with other signaling pathways through its complex signaling network (Kumar et al., 2019). The MAPK

cascade involves several ABA-dependent responses such as seed germination, guard cell signaling and antioxidant defense (Jammes et al., 2009; Xing et al., 2008; Zhang et al., 2012).

ABA also plays a role in the defense strategy against biotic stress factors such as fungi. Plants produce antioxidant secondary metabolites (phenylpropanoids, terpenoids), which are substrates of ABA, promoting its synthesis. Increased synthesis of ABA correlates with *Fusarium oxysporum* resistance as a positive regulator of the plant's early response to infection (Boba et al., 2020). The response to pathogens involves the production of PR proteins, including glucanases, chitinases, peroxidases, and pectinase inhibitors. It also leads to changes in the cell wall, particularly by methyl esterification and lignification of pectin, forming a physical barrier to fungal growth (Wojtasik et al., 2011). The SA hormone plays a critical role during pathogen infection, reducing the susceptibility of plants to pathogen infection (Di et al., 2016).

With all this knowledge, it can be assumed that PB treatment with an appropriate ABA concentration increases plants' abiotic and biotic stress tolerance and nutritional value.

4. NEW SCIENTIFIC RESULTS

1. Development and characterization of liposomal biostimulants

Two novel liposomal formulations of supercritical carbon dioxide herbal extracts, EliceVakcina and Garlic-lipo, were produced and bioassayed. Both formulations were found to contain high levels of abscisic acid, quantified as 81.4 ± 2.2 µg/g in Garlic-lipo and 6.3 ± 1.2 µg/g in EliceVakcina.

2. Antifungal activity against *Fusarium* spp.

The antifungal activity of liposomal abscisic acid-rich biostimulants such as EliceVakcina and Garlic-lipo was demonstrated against plant-pathogenic *Fusarium* species, including *F. oxysporum, F. graminearum, F. verticillioides*, and *F. proliferatum*. At a concentration of 0.625 v/v%, absorbance at 600 nm (ABS_{600}) was measured as 0.11755 for EliceVakcina and –0.1612 for Garlic-lipo, indicating strong growth inhibition.

3. Preventive activation of plant stress defense pathways

It was shown that both EliceVakcina and Garlic-lipo exert preventive effects on stress-related defense pathways in plants. These effects were demonstrated across taxonomically diverse plant species (*Triticum aestivum, Pisum sativum, Brassica napus* and *Glycine max*), supporting the broad applicability of these biostimulants.

4. Upregulation of pathogenesis-related and stress-inducible genes in wheat

A significant upregulation of pathogenesis-related genes (9764–1603 contigs) and stress inducible genes (2345–214 contigs) was observed in *Triticum aestivum* plants following treatment with Garlic-lipo, a garlic-derived nanoliposomal extract containing high levels of abscisic acid.

5. Stimulation of vitamin B1 pathway in field crops

The biostimulant EliceVakcina was shown to enhance environmental stress resistance in field crops such as *Pisum sativum, Brassica napus*, and *Glycine max* by activating the vitamin B1 biosynthetic pathway. The number of differentially expressed sequences related to thiamine biosynthesis (*P. sativum*: 125; *B. napus*: 57; *G. max*: 54) exceeded those associated with classical phytohormone regulation (*P. sativum*: 11; *B. napus*: 8; *G. max*: 7), suggesting a previously unrecognized mechanism of action.

6. Vitamin B1 enrichment in pea

In *P. sativum* plants, treatment with a high abscisic acid - content biostimulant led to the upregulation of vitamin B1 biosynthesis genes and was accompanied by a 14.3% increase in thiamine content 10 days after application.

5. RESULTS THAT CAN BE USED IN PRACTICE

The practical application of the studied plant-based biostimulants in agriculture is beneficial in several areas:

1. Improving crop quality and content: EliceVakcina increases the amount of vitamin B1 in peas, which is beneficial from a nutritional and physiological point of view.

2. Increasing stress tolerance: Biostimulants activate ABA-dependent signaling pathways, thus helping plants overcome abiotic (drought, heat stress) and biotic (pathogens) stress. Biostimulants can be used as exogenous ABA treatment, which activates other signaling pathways through ABA-dependent signaling pathways, enhancing the plant's stress tolerance.

3. Fungicidal effect: Liposome-formulated garlic extracts effectively inhibit the growth of *Fusarium* species, reducing crop loss. According to the microdilution toxicity test, the growth of *F. graminearum, F. oxysporum, F. proliferatum* and *F. verticilloides* species was suppressed. Of the fungal species tested, *F. proliferatum* showed the most significant inhibition of spore formation, responding particularly strongly to Garlic-lipo treatment, showing an extensive zone of inhibition.

4. More efficient active ingredient delivery: Nanoliposome technology increases the stability of active ingredients, reduces evaporation and enhances absorption, so a smaller amount of biostimulant preparation may be sufficient to achieve the desired effect. This formulation helps to avoid low water solubility and oxidation caused by the environment. The release rate of bioactive substances becomes controllable by temperature, pH, the ionic strength of the release medium, and biotic and/or abiotic stressors. This is a novel approach to the delivery of herbal extracts with negative physicochemical properties related to bioactive components, which has a place in agricultural use.

5. Broad applicability: Experiments have shown positive effects in several crops (wheat, peas, rapeseed, soybean), so the technology can be integrated into several production systems.

6. Overall, the use of biostimulants can contribute to sustainable agriculture, reduce the use of chemical pesticides, and increase crop safety.

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7. PUBLICATIONS ON THE SUBJECT OF THE DISSERTATION

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Subject: PhD Publication List

Candidate: Barbara Kutasy
Doctoral School: Kálmán Kerpely Doctoral School
MTMT ID: 10048758

List of publications related to the dissertation

Foreign language scientific articles in international journals (6)

1. **Kutasy, B.**, Kiniczky, M., Decsi, K., Kálmán, N., Hegedűs, G., Alföldi, Z. P., Virág, E.: 'Garlic-lipo'4Plants: liposome-Encapsulated Garlic Extract Stimulates ABA Pathway and PR Genes in Wheat (Triticum aestivum).
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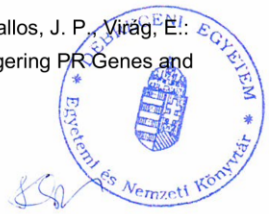

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DOI: http://dx.doi.org/10.1016/j.heliyon.2023.e13954

Total IF of journals (all publications): 41,066
Total IF of journals (publications related to the dissertation): 12

The Candidate's publication data submitted to the Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

18 February, 2025