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Garlic Extracts Nanoliposome as an Enhancer of Bioavailability of ABA and Thiamine Content and as an Antifungal Agent Against *Fusarium oxysporum* f. sp. *pisi* Infecting *Pisum sativum*

Barbara Kutasy [1,2], Géza Hegedűs [1,3], Márta Kiniczky [1], József Péter Pallos [1], Ágnes Nagy [1], István Pócsi [4,5], Klaudia Pákozdi [1,4], Máté Kállai [1], Csaba Weingart [1], Katalin Andor [1], Bettina Kovács [1] and Eszter Virág [1,6,*]

[1] Research Institute for Medicinal Plants and Herbs Ltd., Lupaszigeti Str. 4, 2011 Budakalász, Hungary; kutasy.barbara.julia@uni-mate.hu (B.K.); hegedus.geza@zek.uni-pannon.hu (G.H.); kiniczky.m@gynki.hu (M.K.); pallos.jp@gynki.hu (J.P.P.); agnes.nagy5@pte.hu (Á.N.); pakozdi.klaudia@science.unideb.hu (K.P.); kallai.m@gynki.hu (M.K.); weingart.cs@gynki.hu (C.W.); andor.k@gynki.hu (K.A.); kovacs.b@gynki.hu (B.K.)

[2] Department of Plant Physiology and Plant Ecology, Georgikon Campus, Institute of Agronomy, Hungarian University of Agriculture and Life Sciences, Festetics Str. 7, 8360 Keszthely, Hungary

[3] Department of Information Technology and Its Applications, Faculty of Information Technology, University of Pannonia, 8900 Zalaegerszeg, Hungary

[4] Department of Molecular Biotechnology and Microbiology, Institute of Biotechnology, Faculty of Science and Technology, University of Debrecen, Egyetem Sq. 1, 4032 Debrecen, Hungary; pocsi.istvan@science.unideb.hu

[5] HUN-REN-UD Fungal Stress Biology Research Group, Egyetem Sq. 1, 4032 Debrecen, Hungary

[6] Institute of One Health, Faculty of Health Science, University of Debrecen, Egyetem Sq. 1, 4032 Debrecen, Hungary

[*] Correspondence: virag.eszter@science.unideb.hu

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Abstract: *Fusarium* infections in *Pisum sativum* L. crops present a major constraint to cultivation, leading to substantial yield losses. However, effective disease management strategies, particularly the implementation of biological control methods, offer promising approaches for mitigating infection severity and limiting pathogen spread. In *P. sativum* cultivation, pathogen control is particularly challenging due to the limited penetration of pesticides into the leaves. This is attributed to the dense crystalline plate structure within the leaf cuticle, which acts as a barrier, reducing the efficacy of conventional chemical treatments. Therefore, optimizing the formulation of biopesticides and plant conditioning agents is essential to improve the absorption and bioavailability of active ingredients, ensuring more effective disease management in *P. sativum* cultivation. This study examined the exogenous effects of garlic extracts in different formulations, including EliceVakcina (liposomal formulation), Garlic-lipo (liposomal formulation), and Garlic-oil (oil-based formulation), which contained high concentrations of abscisic acid (ABA) at 6.3, 81, and 80.4 μg g^{-1}, respectively. Transcriptomic profiling, including the identification of Differentially expressed genes (DEGs) and KEGG pathway analysis of EliceVakcina-treated field samples, revealed a significant upregulation of stress- and defence-related genes, as well as pathways associated with thiamine metabolism and ABA signalling. Notably, key defence genes, including pathogenesis-related (PR1, PR2, PR4, PR5) and SnRK2, were overexpressed, indicating an enhanced stress response. HPLC-DAD analytical investigations confirmed the activation of the thiamine biosynthesis pathway, demonstrating a 14.3% increase in vitamin B1 content. Furthermore, the absence of *Fusarium* infection in the treated small-plot field cultures suggests that the tested garlic extracts formulation functions as a promising preventive biostimulant against plant fungal diseases.

Keywords: nanoparticles; biostimulants; inducers; *Fusarium*; abscisic acid; transcriptomic; pea and thiamine

1. Introduction

Over the past three decades, plant biostimulants (PBs) derived from natural materials have attracted increasing attention from farmers, commercial enterprises, and the scientific community. This interest is driven by their ability to modulate plant physiological processes, improve crop productivity, mitigate stress-induced limitations, and support sustainable environmental management [1–4]. Only a limited number of studies have examined the benefits and potential limitations of PBs in the cultivation of *Pisum sativum* L., highlighting the need for further research to optimize their application and efficacy. A commercial PB formulated with plant protein hydrolysates, amino acids, and small peptides has been shown to significantly enhance shoot length in gibberellin-deficient dwarf pea plants, demonstrating hormone-like activity [5]. Pea seeds were treated with licorice root extract, used as a PB, to enhance the plant's endogenous defence mechanisms. Compared to the control group, this pretreatment promoted seedling growth, improved photosynthetic efficiency, and strengthened antioxidant defence systems under salt stress conditions [6]. The application of single and combined soil treatments with cyanobacteria (*Spirulina platensis*) and *Trifolium alexandrinum* has the potential to improve soil fertility, enhance growth conditions, and positively influence pod development in pea fields [7]. Foliar application of moringa leaf extract on pea plants significantly enhanced growth and pod development, improved nutrient accumulation, and increased overall yield [8]. The growth-stimulatory effects of three commercially available PBs on seedling growth were examined across seven different winter pea cultivars. Asahi SL, a PB containing basic phenolic compounds, demonstrated a positive effect on seedling growth parameters. Primus B, enriched with macro- and micronutrients, specifically enhanced root development and seed germination. In contrast, high auxin phytohormone content exhibited an inhibitory effect, suppressing both stem and root growth [9]. In a related study, the effects of eight commercially available biostimulants and foliar fertilizers containing macro- and micronutrients were evaulated, on pea seed yield in field experiments conducted over three consecutive years [10]. The findings indicated an inconsistent impact on seed yield, with the variability in response closely associated with fluctuating weather conditions. These results highlight the complexity of biostimulant efficacy in field settings, emphasizing the influence of environmental factors on treatment outcomes. Two distinct PBs—one derived from plant-based sources (humic acids and fertilizers) and the other from animal protein hydrolysates (comprising L-amino acids, peptides, polypeptides, and amino acids)—were found to enhance the metabolic activity of pea plants following foliar application. However, the response to these treatments was inconsistent, non-reproducible, and largely dependent on prevailing weather conditions. Positive effects on the physiological processes of pea plants were observed in field experiments, but these benefits were evident only under favourable environmental conditions that remained stable over a three-year period [11]. To summarize, based on research on pea PBs, the development of organic PBs that can effectively protect pea plants against both abiotic and biotic stresses while positively impacting crop yield remains a challenge.

Nanotechnological approaches contribute significantly to enhancing the stability, plant uptake, and targeted delivery of biostimulants and biopesticides [12]. Recent research has increasingly focused on the use of nanotechnology-based formulations to enhance plant growth and provide effective protection against fungal pathogens, particularly *Fusarium* species. It was demonstrated that bimetallic ZnO-CuO nanoparticles synthesized through mycosynthesis significantly reduced *Fusarium oxysporum* infection in *Vicia faba*, decreasing the disease index (DI) to 22.5% and increasing protection by 74.28%, while also enhancing growth parameters and yield [13]. Similarly, they explored the antifungal efficacy of a thyme oil nanoemulsion, which effectively reduced disease severity in *Foeniculum vulgare* to 17.5% and improved plant immunity by enhancing antioxidant enzyme activities and reducing oxidative stress [14]. In another study, a synthesized silver nanoparticles were examined using *Ziziphus spina-christi* leaf extract, which showed significant antifungal activity against *F. oxysporum*, reducing disease severity to 20.8% and promoting plant defence mechanisms through increased phenolic content and antioxidant enzyme activation [15]. Additionally, Fe_2O_3 nanocomposites conjugated with humic acid and boron using gamma irradiation were investigated, which effectively suppressed *F. oxysporum*-induced wilt in *Cucumis sativus*, reducing disease severity by up to 83.33% and significantly improving morphological, biochemical, and defence-related plant traits [16]. These studies collectively underscore the potential of nanomaterials as eco-friendly, cost-effective agents for managing *Fusarium* infections, offering a sustainable approach to improving plant health, enhancing resistance, and boosting agricultural productivity. Among the various nanocarrier systems, nanoliposomes—lipid-based nanocapsules—have emerged as a promising platform for improving the efficiency and precision of active ingredient delivery within plant tissues.

The plant hormone abscisic acid (ABA) plays a crucial role in regulating various physiological processes related to growth and development, as well as mediating adaptive responses to abiotic and biotic stresses in plants [17,18]. The ABA signalling pathway serves as a central regulator within the intricate network of plant stress defence mechanisms, interacting with and modulating other signalling pathways to coordinate adaptive responses to various environmental stresses [19,20]. Although ABA is traditionally recognized as a plant growth inhibitor, numerous experimental studies have demonstrated its capacity to promote growth in both roots and shoots under certain conditions [21]. This growth-regulating role of ABA is concentration-dependent; low ABA concentrations have been shown to promote biomass accumulation, while higher concentrations typically result in growth inhibition [22–24]. ABA plays a critical role in regulating carbon metabolism and transport, thereby influencing plant growth and development. At high concentrations, ABA can mimic stress conditions, leading to the suppression of photosynthetic activity and carbon assimilation. This regulatory effect is supported by evidence from *Arabidopsis thaliana*, where the application of exogenous ABA resulted in the downregulation of genes associated with photosynthesis encoded by both the nuclear and chloroplast genomes [25,26].

Garlic is notable among herb-derived PBs for its exceptionally high endogenous ABA content. Studies have shown that ABA concentrations in garlic bulbs are 10–25 times higher than those of other key phytohormones, including cytokinin, auxin (indole-3-acetic acid, IAA), and jasmonic acid (JA) [27]. In a related study, the effects of foliar application of varying ABA concentrations (50, 100, and 150 $\mu g\ mL^{-1}$) on pea plants under water stress were investigated [28]. ABA application significantly mitigated the drought-induced reduction in shoot and root biomass. Additionally, it alleviated the decline in phytohormone levels, specifically IAA and gibberellin (GA). ABA also moderated the reduction in total chlorophyll content and stabilized the activity of key enzymes, including peroxidase (POD) and acid phosphatase (ACP), thereby supporting plant physiological functions under drought stress conditions.

Beyond cereals, *Fusarium* species also cause significant losses in legume crops. Plant extracts [29] and even endophytic fungi are used against the *Fusarium* wilt disease as eco-friendly inducers [30]. *Fusarium oxysporum* f. sp. *pisi* (Fop) is a highly destructive, soil-borne pathogen that poses a significant threat to field pea production worldwide, with the capability to infect and disseminate within the internal seed structure [31]. The infection cycle of Fop begins in the soil with the germination of spores. Following germination, the pathogenic hyphae penetrate the root tissues, subsequently invading the xylem vessels. Once inside the vascular system, the pathogen establishes itself and undergoes extensive colonization, disrupting water and nutrient transport, which can lead to wilting and plant death [32]. Notably, Fop is a major pathogen of pea, leading to severe yield reductions and a decline in crop quality. Infections caused by Fop can reduce the yields of *P. sativum* cultivars by 15–60% under severe conditions [33,34].

Several studies report that ABA exhibits antifungal activity in plants [35,36]. The upregulation of the terpenoid pathway and increased ABA content in flax following *F. oxysporum* f. sp. *lini* infection was reported [37]. This resulted in the activation of plant defence responses, including the expression of pathogenesis-related (PR) genes, cell wall remodelling, and alterations in redox status. The direct effect of ABA at concentrations of 0.1 μM, 0.5 μM, 1 μM, 10 μM, and 50 μM on the growth of *F. oxysporum* f. sp. *lini* was not observed on potato dextrose agar (PDA) for 48 h in darkness after infecting flax plants. However, the increased synthesis of ABA correlated with the resistance. The direct fungal inhibition and antifungal activity of ABA as an anti-chorismate mutant agent was reported [38].

We hypothesized that herbal extracts with high concentrations of organic compounds and ABA may mitigate *Fusarium* infections by simultaneously inhibiting pathogen growth and enhancing plant responses to various stress factors and genes involved in the plant hormone signalling network. Furthermore, this protective effect may be amplified through the application of cell-identical nanoliposomes, which are expected to improve the bioavailability and targeted delivery of the active compounds, thereby maximizing their efficacy. To validate this hypothesis, we investigated the antifungal effects of various ABA-containing PBs developed at the Research Institute for Medicinal Plants and Herbs, Ltd. (RIMPH, Ltd., Budakalász, Hungary) against Fop. Based on the observed inhibitory effects on Fop, the most effective nanoliposome formulation, ABA-enriched PBs, were selected for field trials. These field tests were further supplemented with a comprehensive global transcriptomic analysis to assess the molecular responses of treated plants.

2. Materials and Methods

2.1. Materials and Herbal Extracts

All the extracts investigated in this study were developed at RIMPH Ltd. (Budakalász, Hungary). Among these, two formulations—EliceVakcina® (formerly Elice16Indures) and Fitokondi®—are commercially available and utilized as PBs and plant conditioners. Additionally, products containing the primary active ingredient of EliceVakcina, specifically garlic bulb extract, were also examined. These included Garlic-lipo and Garlic-oil (sourced from Flavex Naturextrakte GmbH, Rehlingen-Siersburg, Germany).

The ABA content, formulation details—including nanoformulation status—and extraction methods of the tested agents are summarized in Table 1.

Table 1. Characteristics of the investigated products used in the experiments. 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 100–150 nm liposome particles. Abbr. limit of detection (LOD).

Product Name	Abbr.	Manufacturers	Formulation	Garlic Content (%)	ABA Content (μg g^{-1})	References
* EliceVakcina	E	RIMPH	nanoliposome, SC-CO_2 extract	~65	6.3 ± 1.2	[39–42]
Fitokondi	F	RIMPH	aqueous extract of herbs	~1	<LOD	[43]
* Garlic-lipo	GL	RIMPH	nanoliposome, SC-CO_2 extract	~90	80.4 ± 2.2	[44]
Garlic-oil	G	Flavex	SC-CO_2 extract	100	81.0 ± 2.4	[45]

2.2. The Toxicity Test of Garlic-Containing PBs on Four Fusarium Species

Four *Fusarium* wild-type strains were selected for susceptibility testing of ABA-containing PBs, specifically EliceVakcina, Garlic-lipo, and Garlic-oil. The selected strains included *Fusarium verticillioides*, *Fusarium graminearum*, *Fusarium oxysporum*, and *Fusarium proliferatum*. Conidiospore suspensions of these strains were preserved in 50% glycerol at −70 °C. To prepare starter inocula for growth assays, the fungal strains were cultured on Czapek-Dox agar medium supplemented with 20 g L^{-1} sucrose for 7 days at 25 °C. Following incubation, conidiospores were harvested by scraping the fungal cultures and suspending them in sterile water containing 9 g L^{-1} NaCl and 100 μL L^{-1} TWEEN-80. The resulting suspension was filtered through two layers of Miracloth (Merck-Millipore, Burlington, US) to remove hyphal fragments and debris. Spore concentrations were then quantified using a haemocytometer to ensure standardized inoculum densities. The toxicity assessment of the ABA-containing PBs was conducted using the Clinical and Laboratory Standards Institute (CLSI) broth microdilution method, employing a half-dilution series. Fungal growth inhibition was measured spectrophotometrically at 600 nm on the fourth day of incubation [46,47].

2.3. Antifungal Activity Test of Solid Agar Plate Assay

In this experiment, the antifungal activity of EliceVakcina, Garlic-lipo, and Garlic-oil extracts was evaluated against four *Fusarium* species (*F. verticillioides*, *F. graminearum*, *F. oxysporum*, and *F. proliferatum*) included in this study. The *Fusarium* strains were cultured under sporulation conditions for 7–14 days at 25 °C, with the duration adjusted based on the specific growth characteristics of each strain. A 100 μL aliquot of a spore suspension, prepared at a concentration of 5×10^6 spores mL^{-1}, was evenly distributed across the surface of PDA medium to ensure uniform inoculation. Following this, 10 μL of each plant extract was applied to a sterile filter paper disc, which was then placed at the centre of the inoculated PDA plate. The plates were incubated at 25 °C for a period of 3 days to allow for fungal growth and to assess the inhibitory effects of the tested extracts.

2.4. Inoculation of Pea Seeds with F. oxysporum f. sp. pisi

The seeds were sterilized by immersion in a 10% sodium hypochlorite solution for 30 min, followed by three thorough washes with sterile water to remove residual disinfectant. Subsequently, the seeds were soaked for 24 h in either 1% or 0.5% EliceVakcina extract, as well as 1% or 0.5% Garlic-lipo extract (~80 seeds per concentration). A separate batch of 80 seeds soaked in sterile water served as the control group. Following treatment, all seeds—including those from the control group—were washed three additional times with sterile water. Approximately 40 seeds from each concentration and the control group were then immersed in a spore suspension of Fop at a concentration of 10^4 spores mL^{-1} for 30 min to facilitate infection. Finally, the treated seeds were placed in Petri dishes lined with moist filter paper and incubated at room temperature for four days to assess germination rates and fungal infection under controlled conditions.

2.5. Nanoliposome Formulated EliceVakcina Treatments of Pea Cultures in Field

Field experiments were conducted on *P. sativum* subsp. *sativum* convar. *medullare* var. *pervicax* cultivar 'Angela' in Tata, Hungary (GPS coordinates: 47°37′58.30″ N, 18°15′54.36″ E)) [43]. The irrigated crop was sown in a randomized block design with four replicates and treated at three distinct phenological stages: BBCH16 (16-leaf stage, 21 June 2019), BBCH51 (appearance of first flower buds, 7 July 2019), and BBCH67 (main blooming, 17 July 2019). The experimental treatments consisted of different dosages of EliceVakcina—20 g ha^{-1} (E20) and 240 g ha^{-1} (E240)—as well as Fitokondi applied at a standard field dose of 4 L ha^{-1} (F). Two days following the final treatment, at the BBCH74 stage (when approximately 40% of the pods had reached their final size, with a tenderness index of 95 TE), 10 fresh leaf samples were collected from each treatment group in 4 replicates. The samples were immediately preserved in RNA Shield (Zymo Research, Irvine, CA, USA) at −70 °C and stored until further processing for RNA sequencing.

2.6. Measurements of NDVI and SPAD in EliceVakcina-Treated Field Plants

To non-destructively assess the physiological status and chlorophyll content of pea plant tissues, optical measurements were conducted in the field, including the Normalized Difference Vegetation Index (NDVI) and the Soil-Plant Analysis Development (SPAD) index. The NDVI was calculated using the formula $NDVI = (NIR - RED)/(NIR + RED)$, where NIR represents the intensity of near-infrared light and RED represents the intensity of red light [48]. The SPAD index was determined using the equation $SPAD = NIR/RED$ (Minolta, Camera, Co, Osaka, Japan). For NDVI measurements, a DJI Phantom 4 agro-drone equipped with a near-infrared camera was utilized. Aerial monitoring was conducted on the day of sample collection (19 July 2019). The captured drone images were processed into GeoTiff format using DroneDeploy software (accessed on 2 October 2023, https://www.dronedeploy.com/). This process included the creation of NDVI maps and false-color composite images for multispectral interpretation, using the standard visual RGB band range visible to the human eye. To obtain precise NDVI values, plot identification was performed by combining aerial photographs with the extraction of specific sample areas. NDVI indices were calculated using custom-developed software for near-infrared data analysis. For SPAD measurements, which reflect chlorophyll content, a portable Minolta SPAD-502 instrument (Konica Minolta Optics Inc., Osaka, Japan) was employed.

2.7. RNA-Sequencing and Differential Gene Expression Analysis of Treated Field Pea Plants

Leaf samples from plants treated with EliceVakcina at 20 g ha^{-1} (E20), EliceVakcina at 240 g ha^{-1} (E240), and Fitokondi at 4 L ha^{-1} (F) were collected following the final treatment at the BBCH74 stage. Next-generation sequencing (NGS) was conducted using the Illumina NextSeq550 platform, producing a final output ranging from 14.6 to 17.5 million single-end reads per sample. A comprehensive description of the sequencing methodology was recently reported [43]. The Trinity method [49] was employed for de novo assembly of the single-end read sets to reconstruct transcripts for each experimental group. To assess gene expression changes, transcript abundances and annotations were determined using OmicsBox v3.2 (Biobam), incorporating both control samples (C: non-treated leaves) and treated leaf samples (E20, E240, and F). The output data, including transcript abundances (CountTable) and annotations (AnnotationTable), were deposited in the Mendeley dataset (https://doi.org/10.17632/f93mjns9t6.2). Pairwise differential gene expression analysis was conducted using NOISeq v2.40.0. Differentially expressed genes (DEGs) were identified by comparing treated samples to their respective controls (C vs. E20, C vs. E240, and C vs. F). The DEG Top50 were visualized using heatmaps to highlight expression patterns.

Functional enrichment analysis was conducted using Gene Ontology (GO) annotation derived from pairwise differential expression data. Two complementary approaches were applied: Fisher's Exact Test [50] and Gene Set Enrichment Analysis (GSEA) [51]. Fisher's Exact Test was utilized to identify GO terms that were significantly over- or under-represented in the test gene set compared to a reference group. In this analysis, subsets of genes identified as upregulated or downregulated in the pairwise comparisons were used as the test sets. Pathway analysis was performed using KEGG database to gain insights into the biological pathways and molecular mechanisms associated with the DEGs. The annotated DEGs from the pairwise comparisons were directly mapped to relevant pathways, facilitating the identification of biological functions and interactions. Automated mapping and functional enrichment analyses were conducted using OmicsBox v3.2 (Bioinformatics Made Easy, BioBam Bioinformatics, 3 March 2019, accessed on 10 August 2023, https://www.biobam.com/omicsbox). This software enabled the integration of DEG results with KEGG pathways, supporting the identification of significant gene enrichments and assigning gene products to the most probable pathway candidates.

2.8. Measurements of ABA and Thiamine Content

Instrumental analysis of ABA levels in PBs, including EliceVakcina, Garlic-lipo, Garlic-oil, and Fitokondi was performed using HPLC-DAD on an Agilent 1100 series UV-VIS MWD system (Hewlett Packard, Palo Alto, CA, USA) [52]. ABA quantification was conducted using a reference standard of S-ABA assay (HPLC) > 98% (CAS: 21293-29-8, Duchefa Biochemie, Haarlem, The Netherlands). Additionally, the same HPLC system was used to measure thiamine and its metabolites in the shoot-containing pods of pea plants [53,54]. In brief, 2 g of chopped plant material was transferred into a round-bottom flask, and 25 mL of 0.05 mol L^{-1} H_2SO_4 was added. The mixture was incubated in a water bath at 85 °C for 30 min, followed by 15 min of sonication to enhance extraction. After cooling, the pH of the samples was adjusted to 4.0 using a 2.5 mol L^{-1} sodium acetate solution. Subsequently, 50 mg of clara-diastase was added, and the samples were incubated at 45–50 °C for 2 h. The treated samples were filtered through filter paper with a pore size of 2–3 μm and subjected to further analysis using HPLC equipped with an Agilent Eclipse Plus C18 column (4.6 × 250 mm, 5 μm pore size). A sample injection volume of 50 μL was used, and detection was performed at 254 nm using DAD. The reference standard for thiamine quantification was thiamine hydrochloride ≥98.0% (HPLC grade) (CAS: 67-03-8, VWR International, LLC, Radnor, PA, USA).

3. Results

3.1. Garlic Containing PBs May Inhibit Growth of Fusarium Species

Given the lack of available data on the toxic effects of supercritical carbon dioxide ($SC\text{-}CO_2$) garlic extracts on *Fusarium* species, we conducted toxicity assays using garlic extract-based PBs with high ABA concentrations. The tests were performed on four *Fusarium* species: *F. graminearum, F. oxysporum, F. proliferatum,* and *F. verticillioides* (Figure 1). The results revealed a similar response across all four *Fusarium* species, with the antifungal efficacy of the extracts ranked in the following order: Garlic-oil > Garlic-lipo >

EliceVakcina. Based on these findings, *F. oxysporum* f. sp. *pisi* was selected for subsequent experiments due to its relevance as a model organism. The outcomes from these experiments are expected to have broader applicability in other plant *Fusarium* pathogenic interactions.

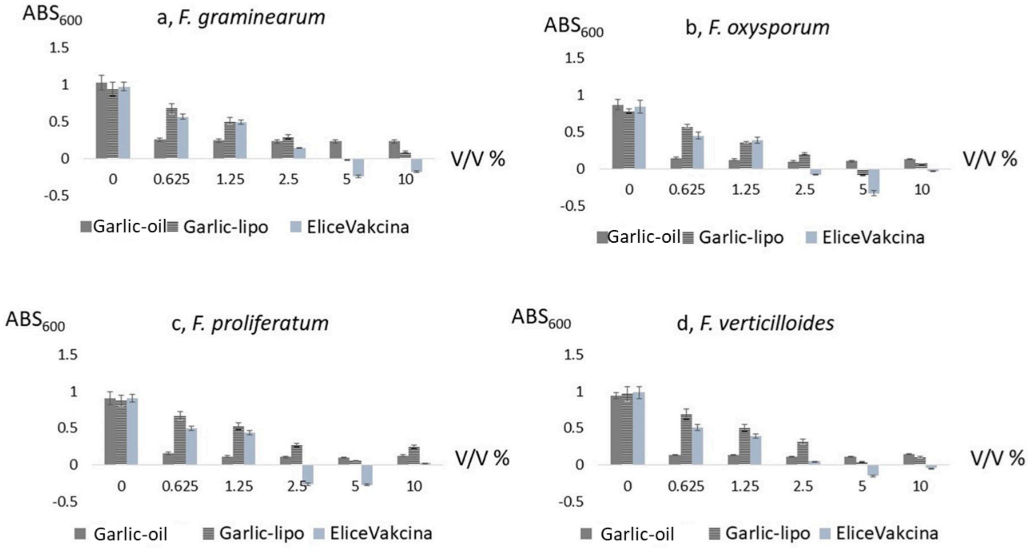

Figure 1. The inhibitory effect of ABA containing garlic PBs was investigated by a broth microdilution test on four *Fusarium* species. The half-dilution method was used for the experiment according to the CLSI standard. The figure shows the volume percentage of PBs used. The negative value may be attributed to fungal growth initiation, thereby absorbing the nanoliposomes, resulting in a decrease in liposome-derived absorbance within the system.

3.2. Antifungal Activity Test of Solid Agar Plate Assay

The four *Fusarium* species exhibited varying degrees of sensitivity to the tested herbal extracts (Figure 2). Among them, *F. proliferatum* demonstrated the highest sensitivity across all three treatments, with the most pronounced inhibition observed in response to Garlic-lipo. This formulation resulted in a substantial inhibition zone, where no sporulation was detected, indicating strong antifungal activity. In the case of *F. graminearum*, Garlic-oil exhibited the greatest inhibitory effect. The formation of conidiophores was restricted to the outer edges of the culture, suggesting a significant reduction in fungal growth. Additionally, Garlic-lipo also showed notable suppression of conidiophore development, though to a lesser extent than the Garlic-oil. For *F. verticillioides*, all three extracts inhibited mycelial growth, with the strongest antifungal effect observed for the Garlic-oil. However, sporulation inhibition was not observed for any of the treatments, indicating that while growth was suppressed, the ability to form spores remained unaffected. A similar but less pronounced inhibitory pattern was observed in Fop. Both EliceVakcina and Garlic-oil effectively inhibited mycelial growth, whereas Garlic-lipo did not produce a visible inhibition zone around the filter paper disc. Furthermore, none of the tested extracts inhibited sporulation in Fop, suggesting that while growth was affected, spore formation remained resilient to these treatments.

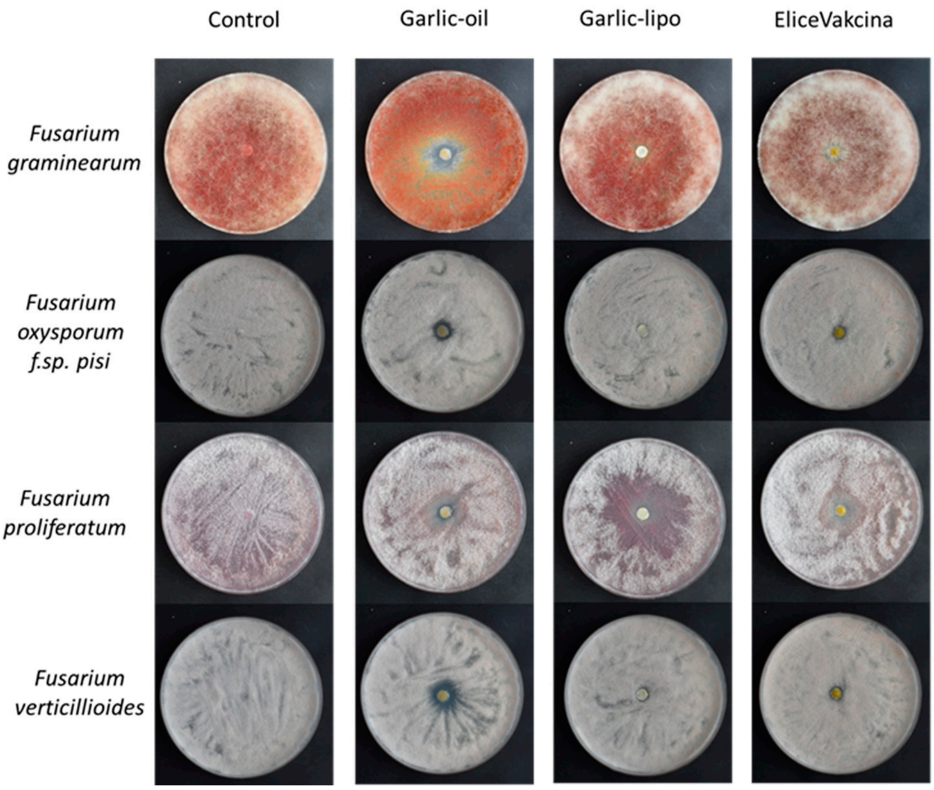

Figure 2. The four *Fusarium* species grown on PDA medium are shown in the experiment. The inhibitory effect of the herbal extract applied to the centre of the medium is observable. The *Fusarium* species exhibit varying sensitivity to the extracts.

3.3. Inoculation of Pea Seeds with Fusarium oxysporum f. sp. pisi

Nearly all seeds exposed to the Fop spore suspension became infected, and the overall infection rate remained consistent across all pretreatment groups. However, differences in infection dynamics were observed in seeds pretreated with plant extracts compared to the control. Seeds pretreated with plant extracts showed reduced discoloration in contrast to the control seeds, which developed the characteristic brownish-reddish discoloration associated with severe infection and exhibited a more aggressive infection progression. This suggests that the plant extracts may have mitigated some visual and physiological symptoms of infection, despite not preventing infection entirely. Additionally, the germination rate of infected seeds varied between treatments. Seeds treated with EliceVakcina demonstrated lower germination rates compared to those treated with Garlic-lipo (Figures 3 and 4).

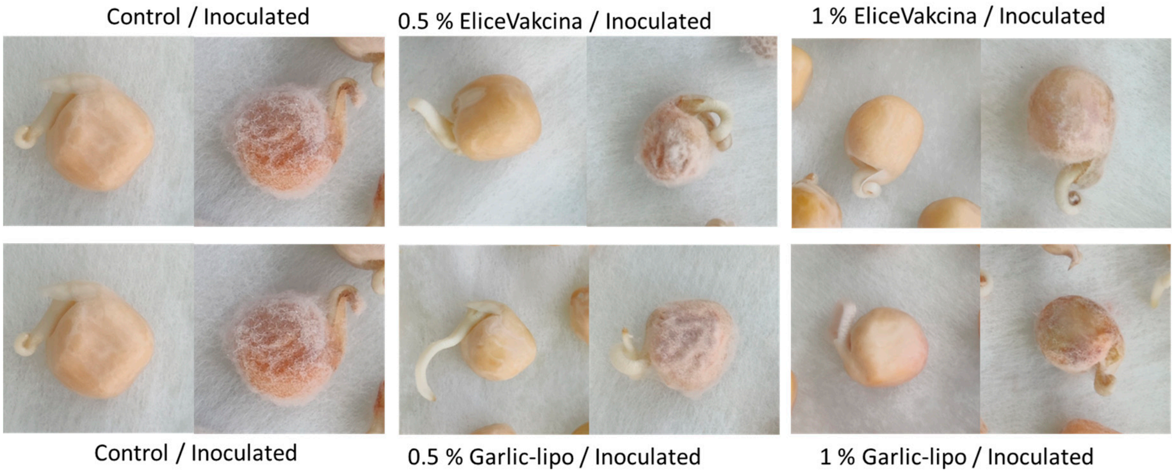

Figure 3. Seeds pretreated with EliceVakcina and Garlic-lipo were inoculated with Fop spores. The figure shows the progression of the infection after four days.

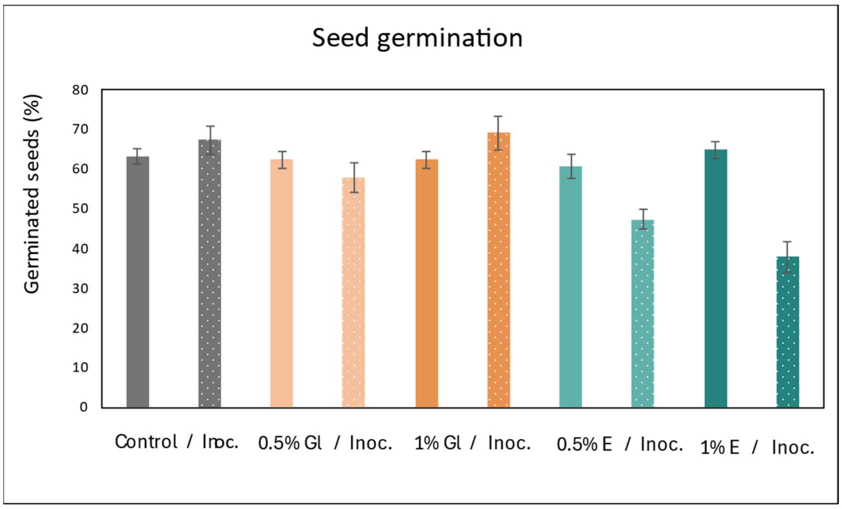

Figure 4. The figure shows the germinated seeds for each experiment. Approximately 40 seeds were used per experiment (in 3 replicates), and the percentage of germinated seeds are represented. Abbr. Inoc.—Inoculated; GL—Garlic-lipo; E– EliceVakcina.

3.4. Increased NDVI and SPAD on Field Pea Plants Treated with EliceVakcina and Fitokondi

Pea plants were treated with EliceVakcina at 20 g ha^{-1} (E20), 240 g ha^{-1} (E240), and Fitokondi at 4 L ha^{-1} (F) at three distinct phenological developmental stages. The layout of the treated plots is shown in Figure 5. To evaluate the effects of these treatments, drone monitoring, along with SPAD and NDVI measurements, were conducted during both the juvenile and adult vegetative phases (Figure 5). The NDVI measurements revealed that treatment with E20 resulted in a significant increase of 22% during the juvenile vegetative phase and 17% during the adult vegetative phase when compared to untreated control plots ($p < 0.01$). Similarly, the Fitokondi treatment led to an 18% increase during the juvenile vegetative phase and a 16% increase during the adult vegetative phase, both of which were statistically significant compared to the controls ($p < 0.01$). However, during the generative phase, treatments with E20, E240, and F did not result in significant differences in NDVI values compared to untreated controls. Furthermore, none of the treatments led to significant differences in average yield measurements, indicating that while early vegetative growth was positively influenced by the treatments, these effects did not translate into measurable yield improvements.

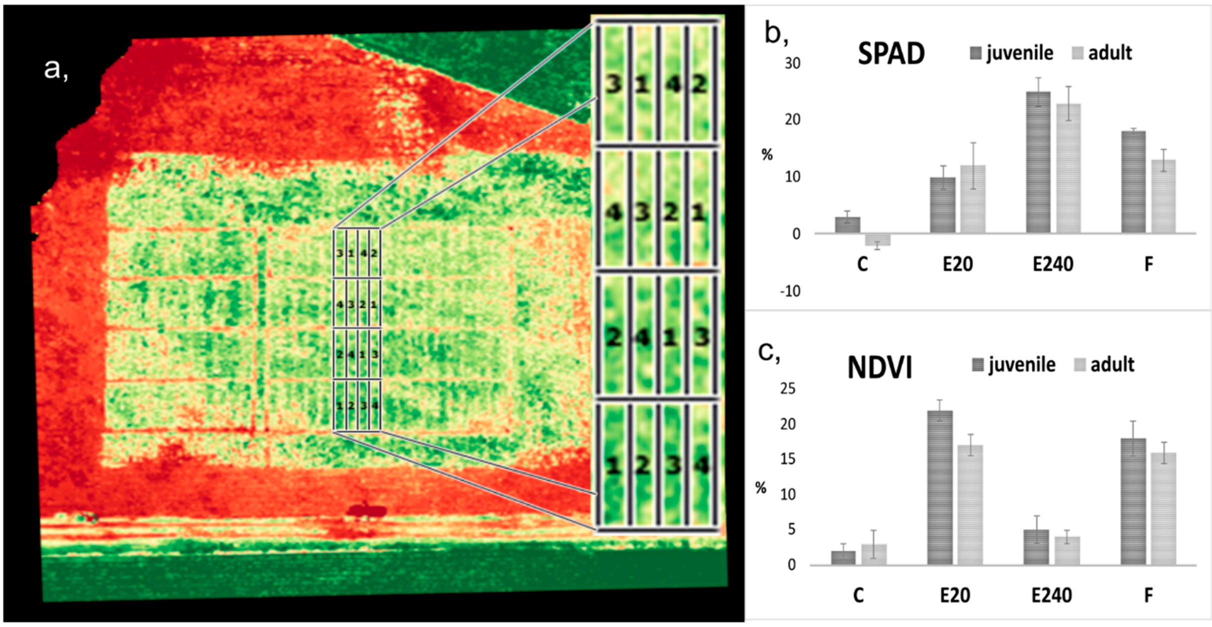

Figure 5. (**a**) Representative UAV false-colour image. Drone monitoring was conducted on the day of sample collection (adult vegetative phase). Marks of the plots: 1 (Control), 2 (EliceVakcina 20 g ha^{-1}, E20), 3 (EliceVakcina 240 g ha^{-1}, E240), and 4 (Fitokondi, F). (**b**) Measurements of SPAD and (**c**) NDVI changes in percentage. The figure illustrates also the field layout that is the basis of sample collection for RNA-seq analysis. Leaves of 10 individuals were collected randomly in each plot.

3.5. RNA-Seq Analysis of Field Pea Plants Treated by the Nanoliposome-Formulated EliceVakcina and Fitokondi

Samples of treated and non-treated pea plants were sequenced using the Illumina NextSeq550 platform, and libraries were prepared with average 15.5 M single-end reads per sample. Four libraries were deposited in the NCBI Sequence Read Archive (SRA) under the Bioproject PRJNA870114 with accession numbers SRR21124993-SRR21124996. During de novo assembly, a combined transcript dataset was built with 7513 total transcripts and 6897 total genes and was deposited in Transcriptome Shotgun Assembly (TSA) database at DBJ/EMBL/GenBank under the accession GKBF01000000. The total assembled bases (all transcripts) were 2,700,878 and detailed statistics of transcripts were published [43]. The length of transcripts was 251–2365 bp and the average length was 361 bp. Functional annotation and GO analysis were performed using the GKBF01000000 reference transcript dataset and deposited in the Mendeley database (https://doi.org/10.17632/f93mjns9t6.2).

3.6. Differential Gene Expression Analysis Revealed the Upregulation of Stress Response

DEGs were identified between E20, E240, and F treatments compared to the control (C) samples. There were order-of-magnitude differences between up- and down-regulated genes, with a higher proportion of upregulated genes observed in E20 and F treatments. Sample E240 showed the slightest transcriptomic changes (Figure 6a,b). Numerical data suggested that the transcriptomic effects of E20 and F treatments may overlap. To gain deeper insights into which gene clusters were influenced by these treatments and how these changes might impact plant immune responses, we focused on analysing defence response associated genes within the DEG Top50. The expression patterns of these genes are visualized in a heat map (Figure 6c), highlighting key differences and similarities in gene regulation between the two treatments.

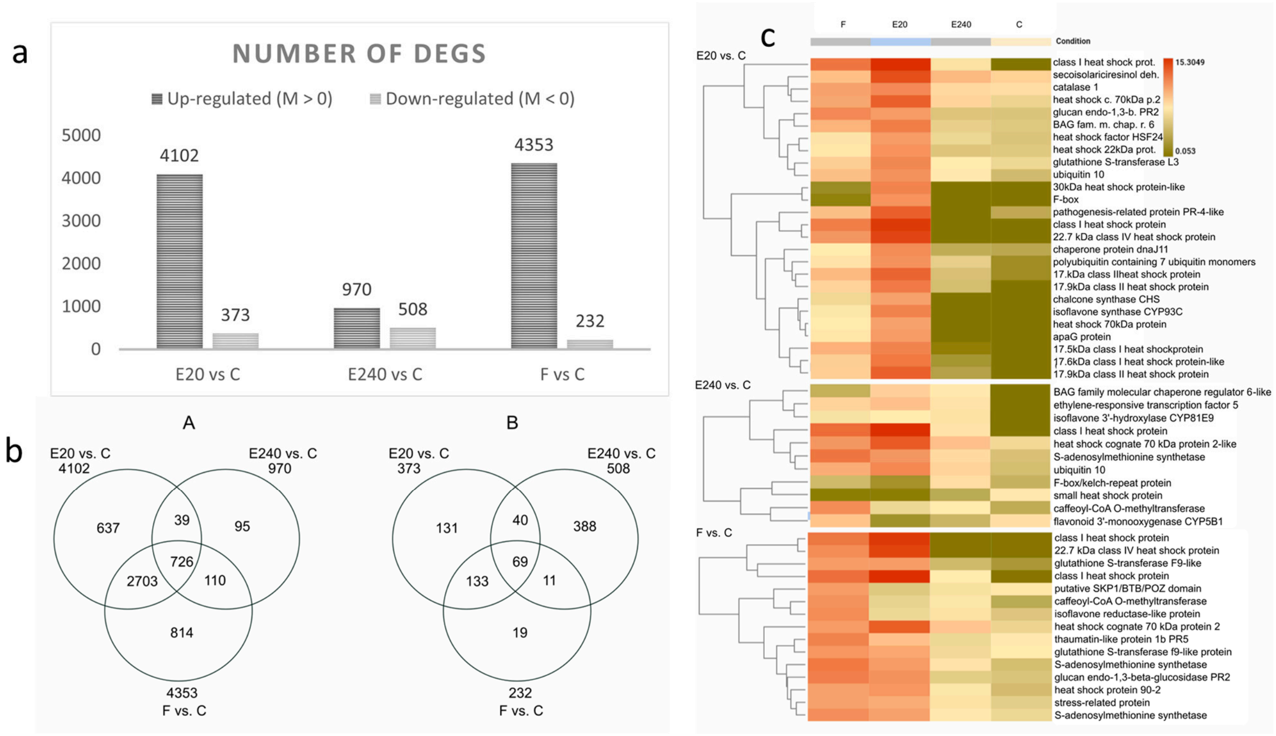

Figure 6. (**a**) The number of DEGs of the investigated sample pairs. (**b**) Venn diagrams of the up- and down-regulated DEGs among the investigated sample pairs. (**c**) Heatmap of DEG Top50 related to defence response. In pairwise DEG analysis, untreated leaves (control, C) were used as a reference, while leaves treated with EliceVakcina at 20 g ha^{-1} (E20), 240 g ha^{-1} (E240), and Fitokondi at 4 l ha^{-1} (F) were designated as test conditions.

Following PB treatments, an overrepresentation of stress response genes associated with various biological processes was observed. Notably, this included the upregulation of PRs, such as PR2 and PR4, along with multiple glutathione transferases (GSTs), which are crucial for detoxification and oxidative stress mitigation. Furthermore, genes involved in phenylpropanoid and flavonoid biosynthesis pathways exhibited significantly upregulated expression. Key genes identified included chalcone synthase (CHS), isoflavone synthase (CYP93C), isoflavone 3′-hydroxylase (CYP81E9), isoflavone reductase-like protein, and caffeoyl-CoA O-methyltransferase (CCOAOMT), all of which are implicated in plant defence and secondary metabolite production. Plant hormones play a pivotal role in coordinating stress responses and maintaining the balance between growth and defence mechanisms. Several genes involved in hormone signalling and biosynthesis were differentially expressed following PB treatment. These included the ethylene-responsive transcription factor 5 (ERF5), S-adenosylmethionine synthetase (SAM) (involved in ethylene biosynthesis), and an F-box/kelch-repeat protein, which plays a role in protein degradation and signal transduction. In addition, genes encoding heat shock proteins (HSPs), heat shock factors, and various ubiquitin-protein degradation chaperones were identified in treated samples, indicating an enhanced stress response at the protein-folding and degradation levels. Comprehensive heat maps detailing these gene expression patterns for the various treatments were previously published [43].

3.7. GO and Pathway Analysis Indicated Dose Dependency in Photosynthesis, Glutathione and Thiamine Metabolism

Enrichment analysis of pairwise DEG Top50 were performed by applying Fisher's exact test. We identified enriched GO terms that were either over- or underrepresented when comparing the reference and test groups across the three investigated samples (C vs. E20, C vs. E240, and C vs. F) (Figure 7). We discovered a significant number of up-regulated sequences associated with transmembrane transport complexes, mitochondrial respirasomes, respiratory chain complexes, and protein folding. The E20 and F treatments exhibited high similarity. Analysis of the down-regulated sequences (Figure 8) revealed a significant enrichment of chloroplast-associated functions, primarily related to photosynthetic processes. These included pathways associated with chlorophyll binding, light harvesting in photosystem I, and photosynthetic electron transport in photosystem II. These down-regulation patterns were particularly pronounced in the E20 and F treatments, suggesting a suppression of photosynthetic activity as part of the plant's stress response mechanism. In contrast, no significantly enriched GO IDs were detected for the comparison between C and E240 samples when applying the Benjamini–Hochberg (BH) adjusted *p*-value cutoff of 0.05. This lack of significant enrichment was observed in both up-regulated and down-regulated gene sets. Given the strong similarity observed between the transcriptomic effects of E20 and F treatments, a more detailed pathway analysis was conducted.

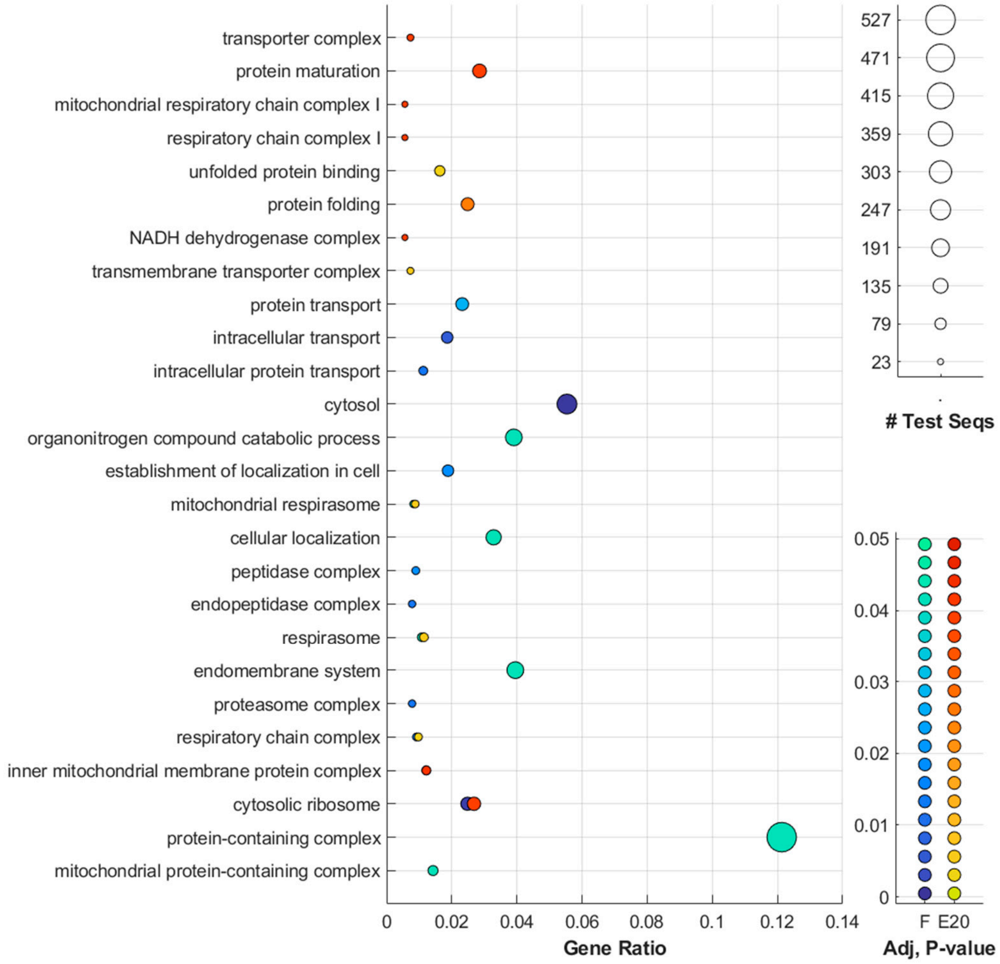

Figure 7. Fisher's exact test of up-regulated DEGs of E20 and F treatments. No enriched GO IDs were found after the test using BH adjusted *p*-value 0.05 cutoff in E240 vs. C.

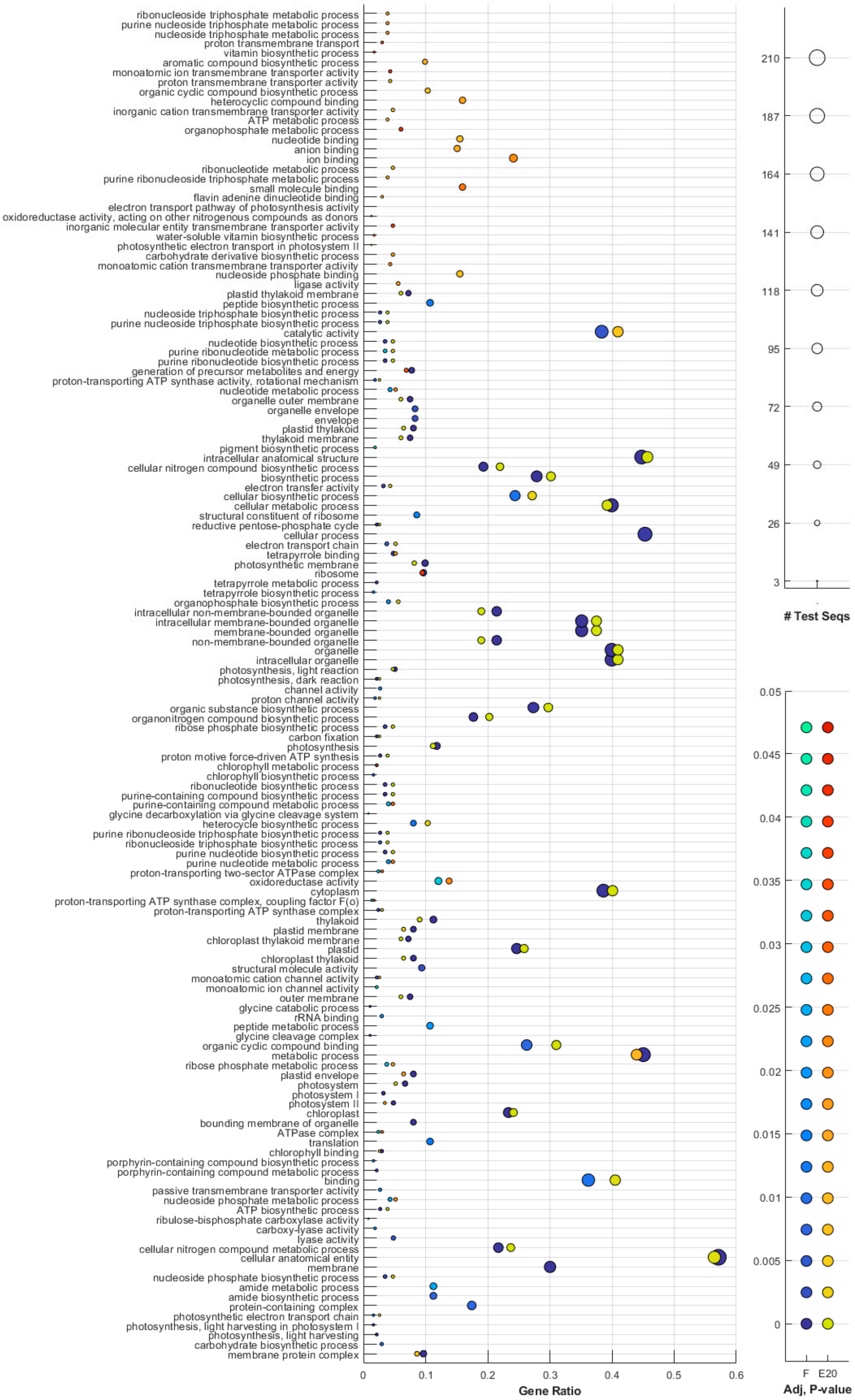

Figure 8. Fisher's exact test of down-regulated DEGs of E20 vs. C and F vs. C treatments. No enriched GO IDs were found after the test using BH adjusted *p*-value 0.05 cutoff in E240 vs. C.

The pathway analysis revealed comparable limitations in photosynthetic activity in both the E20 and F treatment groups, suggesting similar regulatory effects on photosynthesis related processes. In contrast, the E240 treatment exhibited enhanced photosynthetic activity, indicating that higher concentrations of EliceVakcina may mitigate photosynthetic restrictions observed at lower doses (Figure 9).

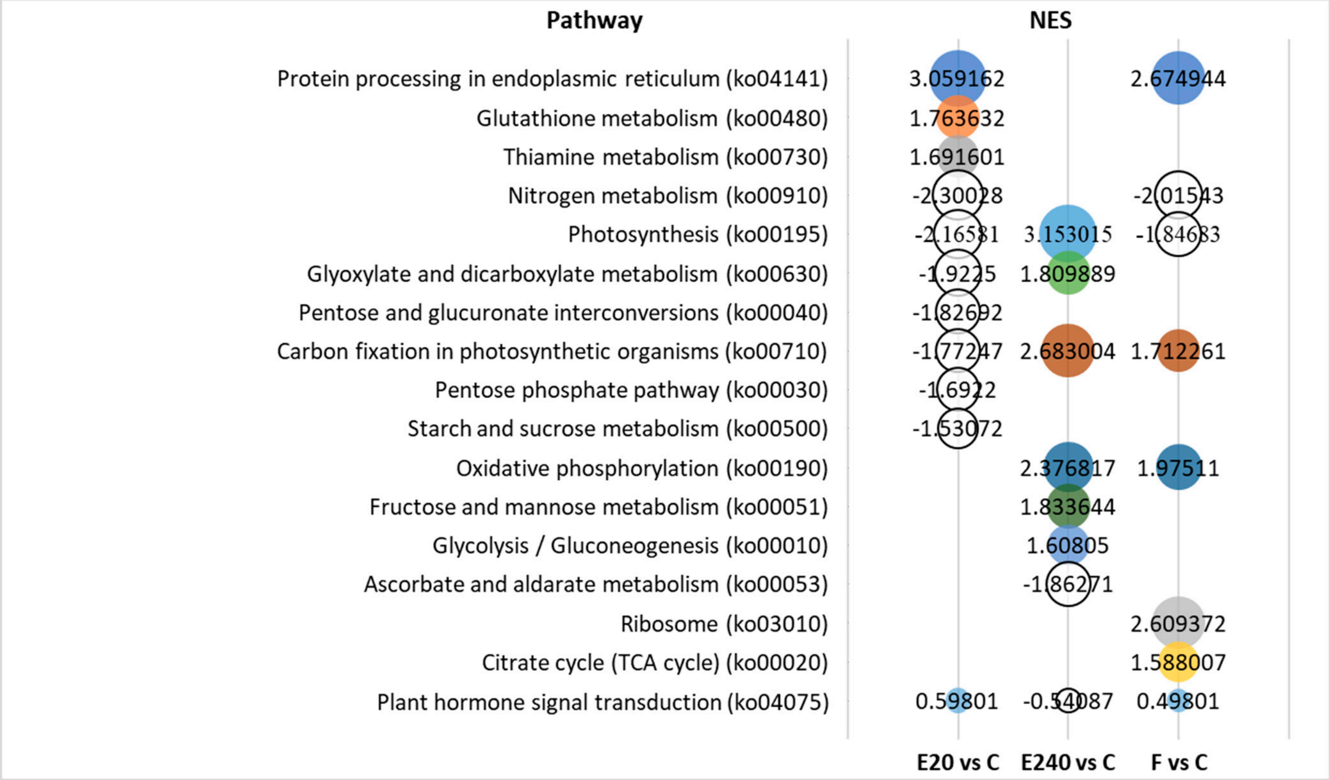

Figure 9. The KEGG pathways are categorized as BOTTOM (NES < 0, represented by empty dots) and TOP (NES > 0, depicted by coloured dots) based on Normalized Enrichment Score (NES) values. Pathways were determined based on pairwise DEGs and GSEA data alignment to the KEGG database.

Among the enriched pathways, protein processing in the endoplasmic reticulum displayed the highest Normalized Enrichment Score (NES), with notable differential expression of transcripts detected in both the E20 and F treatments. Additionally, the glutathione and thiamine metabolism pathways were uniquely upregulated in the E20 treatment, indicating a distinct activation of stress response mechanisms and metabolic priming. However, the pentose phosphate pathway, as well as starch and sucrose metabolism, were downregulated in this treatment group, suggesting a shift in carbon allocation. The E240 treatment demonstrated distinct metabolic characteristics, marked by heightened activity in fructose and mannose metabolism and glycolysis. Conversely, the ascorbate and aldarate metabolism pathways were found to be specifically downregulated in the E20 treatment, indicating a unique stress response signature at this concentration. The F treatment was characterized by significant upregulation of pathways related to ribosome biogenesis and the tricarboxylic acid (TCA) cycle, suggesting an enhancement of cellular energy production and protein synthesis. Although the plant hormone signal transduction pathway did not rank among the top NES values, it was included in the analysis due to its biological significance. Upregulation of this pathway was observed in both E20 and F treatments, highlighting its role in mediating stress and growth responses. Given the hypothesized involvement of ABA, additional analysis was conducted to investigate the expression of genes within the ABA signalling pathway.

Genes associated with Photosystem II and I, such as PsbQ, PsbS, PsaD, and PsaH, exhibited up-regulation after F treatment, while no significant changes were observed in the other two treatments. Down-regulation of the Cytochrome b6/f complex, involving the PetA gene, was observed in both F and E20 treated samples. However, it was up-regulated following E240 treatment. Photosynthetic electron transport was up-regulated in both F and E240 treatments, while no significant changes were observed in E20. F-type ATPase, involving ATPF0C, was down-regulated in both F and E20 treatments, while it was up-regulated in the E240 treatment.

The most differentially expressed sequences were associated with thiamine metabolism (ko00730) in each treatment, with only E20 being involved in the NES Top10. Numerically, we found DEGs to be enriched in 125, 52, and 128 in E20, E240, and F, respectively. Among these, up-regulation was observed in Cysteine Desulfurases (IscS), 1-Deoxy-D-Xylulose-5-Phosphate Pyruvate-Lyase (Dxs), Thiamine Phosphate Synthase, Thiamine Mono and Diphosphate—Phosphohydrolases, Phosphotransferases, and TENA_E (Aminopyrimidine Aminohydrolase). The observed upregulation of genes related to thiamine metabolism suggests that vitamin B1 content in pea plants may be enhanced following EliceVakcina treatment, particularly at the E20 dosage. This enrichment indicates a potential priming effect, enhancing the plants' capacity to respond to environmental stress. The aligned KEGG genes related to thiamine metabolism are visually represented with color-coded annotations on the KEGG pathway map (Figure 10), providing a clear visualization of the metabolic changes induced by the treatments.

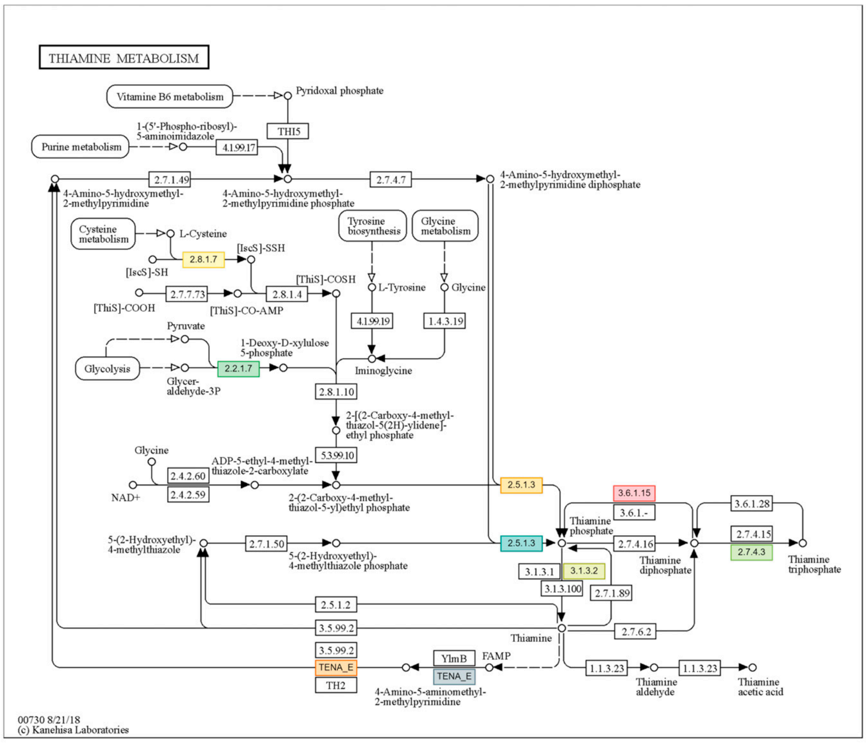

Figure 10. Pathway analysis of thiamine metabolism genes (map00730). Aligned DEGs were presented by colored boxes: cysteine desulfurases (IscS) [EC:2.8.1.7], 1-deoxy-D-xylulose-5-phosphate synthase (dxs) [EC:2.2.1.7], thiamine-phosphate pyrophosphorylase (thiE) [EC:2.5.1.3], nucleoside-triphosphatase (NTPCR) [EC:3.6.1.15], acid phosphatase (PHO) [EC:3.1.3.2], adenylate kinase (adk) [EC:2.7.4.3], and formylaminopyrimidine deformylase/aminopyrimidine aminohydrolase (TENA_E) [EC:3.5.1.- 3.5.99.-].

3.8. Strong Phytohormone Response to the Nanoliposome Formulated Garlic-Containing Elicevakcina

Given that the primary mode of action of organic PBs involves activating the plant's defence mechanisms through hormonal regulation, we investigated changes in the expression of genes associated with the plant hormone signal transduction pathway (Figure 11a). The results indicated that two treatments—E20 and F—stimulated the expression of specific genes involved in various hormonal pathways, including IAA, cytokinin, ABA, JA, and salicylic acid (SA). Conversely, a downregulation within the ABA pathway, which is typically associated with abiotic stress responses, was observed exclusively in the E240 treatment, without significant effects on other hormone-related pathways. An in silico relative gene expression analysis further validated and clarified the results of the pathway analysis by focusing on key genes involved in ABA biosynthesis and signalling. Notably, sucrose non-fermenting1-related protein kinase (SnRK2.3) exhibited significant upregulation in both E20 and F treatments, indicating an activation of ABA-related stress signalling. Additionally, the zeaxanthin epoxidase (ZEP) gene, which catalyses the initial step in ABA biosynthesis, was found to be upregulated in the E240 treatment (Figure 11b).

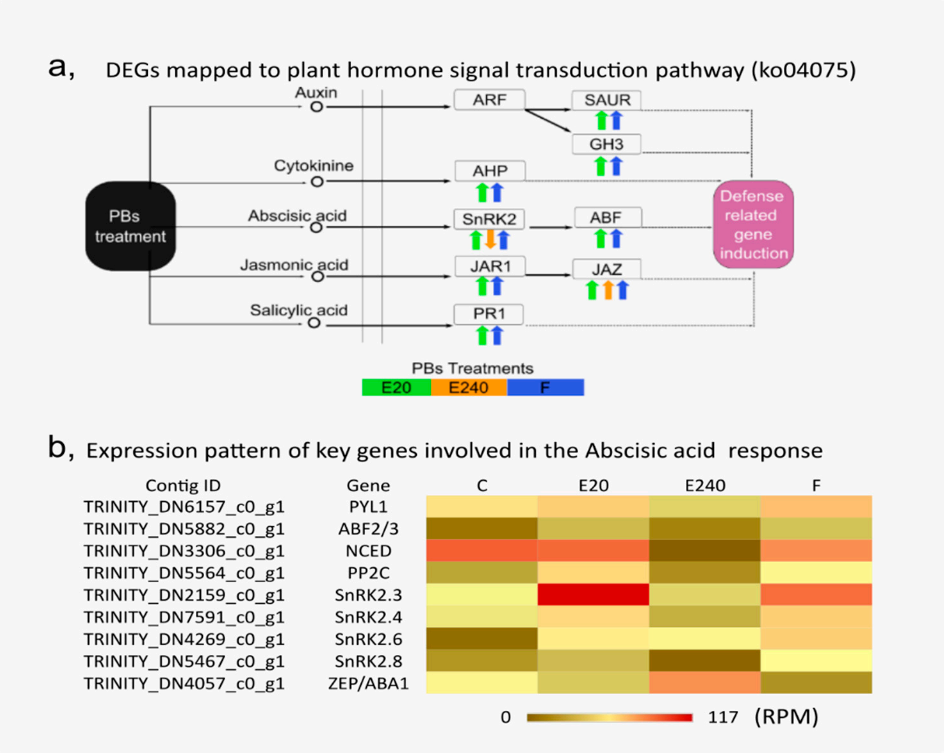

Figure 11. (**a**) Genes showing differential expressions in the plant hormone signal transduction (ko04075) pathway are involved in defence related gene induction. (**b**) In silico relative gene expression (RPM values) analysis of key genes involved in ABA biosynthesis.

3.9. Increased Thiamine Content in Field Pea Plants Treated with Nanoformulated EliceVakcina

The shoots containing pods of pea plants were treated with ElicеVakcina at a concentration of 20 g ha^{-1}, and the levels of thiamine and its metabolites were quantified using HPLC-DAD analysis. The average percentage differences calculated from peak areas were as follows: an increase of 8.0% was observed five days after treatment, and 14.3% ten days after treatment. On day 10 posttreatment, the results showed a significant increase in peak areas corresponding to the same retention time as the thiamine reference standard when compared to untreated control plants (Figure 12).

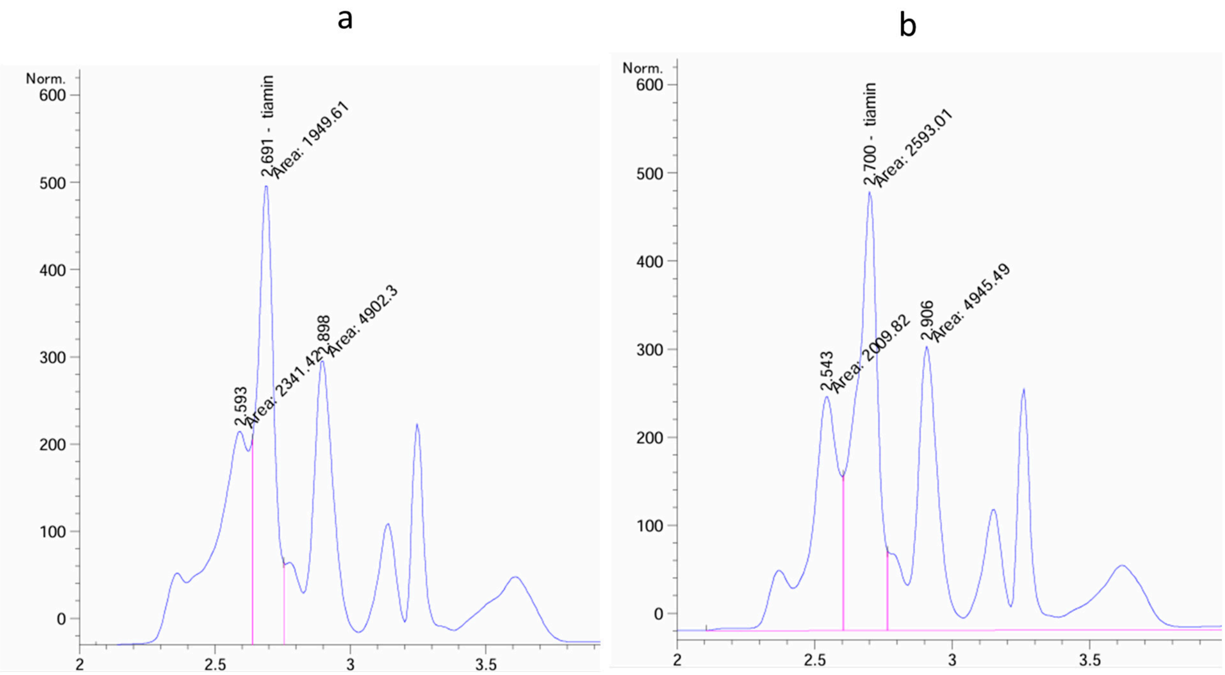

Figure 12. Representative chromatograms of HPLC-DAD measurements of thiamine in field treated pea plants. Thiamine content (peak area) of (**a**) untreated and (**b**) nanoliposome formulated EliceVakcina treated plants ten days after treatment. The average percentage differences calculated from peak area: an increase of 14.3% was observed ten days after treatment.

4. Discussion

Field protection against fungal infections caused by Fop remains a significant challenge, as no ecologically safe and effective control strategies are currently available [55]. Transcriptome analysis has proven to be a valuable tool in developing advanced plant protection methods, as it facilitates the identification of functional genes associated with resistance mechanisms in host plants and elucidates the molecular interactions involved in host pathogen dynamics. Several studies have employed transcriptomic approaches to investigate gene expression changes in response to similar pathogens, such as *Aphanomyces euteiches* and *Phytophthora pisi* infections in pea plants. These investigations have identified DEGs linked to defence-related pathways, including phenylpropanoid metabolism, hormonal signalling (involving ethylene (ET) and JA), cell wall reinforcement, and other plant immunity responses [56]. Leveraging these transcriptomic insights offers the potential for developing targeted PBs with mechanisms of action specifically designed to enhance plant defence responses. However, the effectiveness of PB treatments in peas does not always match the results observed in crops with less waxy foliage or monocotyledonous plants. This reduced efficacy is primarily due to the unique surface properties of pea leaves, which feature a dense layer of wax crystalline plates. This structural characteristic contributes to the leaves' hydrophobic nature, often referred to as "difficulty-to-wet", posing challenges for the effective application of pesticides and PBs [57].

Nano-management approaches in pea plant protection involve the application of nanotechnology to enhance pest and disease control, promote plant growth, and optimize agricultural practices. Although this field is still in its developmental stages, several nano-based strategies are being investigated for their potential in improving plant health, particularly in pea cultivation [58–61]. Despite promising advancements, the use of nanotechnology in agriculture—especially for pea plant protection—requires further research to comprehensively evaluate its long-term effects, safety considerations, and the regulatory frameworks needed for responsible implementation. Addressing these emerging concerns, the present study employed nanoliposome formulation technology to investigate its potential in enhancing plant defence mechanisms and improving overall crop health.

Certain plant based organic materials, such as herbal extracts, have demonstrated significant antifungal activity against Fop [62,63]. In vitro studies involving extracts from garlic, ginger, lemongrass, and other botanicals have shown the ability to inhibit the growth of fungal mycelia in Fop isolates obtained from pea plants [64]. The inherent antifungal potential of these natural compounds supports their development as components of ecologically safe fungicides, which could be integrated into anti-Fop strategies for sustainable field pea cultivation. Proper formulation of these herbal extracts, particularly through the inclusion of optimal concentrations of plant hormones, may enhance absorption and maximize bioavailability. In a study [65], twelve botanicals were tested against Fop, the causal agent of pea wilt, in Himachal Pradesh. Among these, garlic clove extract demonstrated complete inhibition of the pathogen, with the fungal growth rate was recorded as zero after 24 h of incubation. Building on this foundation, we developed ABA-containing botanical products designed to function as PBs in pea cultures, with additional antifungal properties targeting Fop. To assess the effects of ABA concentration and the nanoformulation of SC-CO_2 garlic extracts, we evaluated their toxicity against *Fusarium* species. The investigation involved both liposome and non-liposome formulations and tested varying garlic concentrations across four products. *F. proliferatum* exhibited significant sensitivity to all three garlic extracts. The nanoliposome-formulated Garlic-lipo exhibited the highest antifungal effectiveness against Fop. In comparison, non-liposome Garlic-oil was less effective, while EliceVakcina demonstrated even lower antifungal activity than Garlic-oil. Fitokondi showed no detectable inhibitory effect on *Fusarium* growth. Interestingly, while the Garlic-oil formulation contained 100% garlic extract, Garlic-lipo contained 90% garlic extract. Despite the lower garlic concentration, the superior effectiveness of Garlic-lipo suggests that nanoliposome formulation significantly enhances bioavailability and efficacy. This increased effectiveness may be attributed to the higher concentration of active compounds in Garlic-lipo, such as allicin, ajoene, and other sulfur-containing compounds, which are known to disrupt fungal plasma membrane functionality [66,67]. The toxicity assessment of EliceVakcina revealed detectable antifungal effects; however, fungal growth of Fop was observed across all dilutions after seven days, indicating that EliceVakcina was less toxic than Garlic-lipo. Previous studies have reported that a garlic oil exhibited potent toxicity, eliminating up to 90% of *Penicillium funiculosum* conidia in liquid media within three days [66]. The resurgence of Fop growth after seven days may be linked to the activity of hydrolase and esterase enzymes in *Fusarium* species, which are capable of degrading the active compounds in EliceVakcina, potentially reducing its effectiveness over time [68,69]. Additionally, the inherent fungistatic effect of ABA on filamentous fungi should be considered when interpreting the overall antifungal efficacy of the tested agents [38].

The results of these experiments demonstrated that the nanoliposomal formulation effectively achieved an antifungal effect against Fop under in vitro conditions. However, the application of this formulation in actual plant systems requires further investigation to validate its efficacy and safety under field conditions. It is important to emphasize that the concentration of ABA plays a critical role in determining the overall outcome of treatment. Inadequate or excessively high ABA concentrations can induce adverse effects, including dwarf growth and developmental inhibition in plants [70]. Therefore, precise optimization of ABA dosage is essential to maximize antifungal efficacy while minimizing any negative impact on plant growth and development. Future research should focus on determining the optimal concentration range for field applications to ensure both the effectiveness and safety of this treatment strategy.

It has been determined through HPLC-DAD measurements that the ABA content of Fitokondi was below the limit of detection (LOD). For EliceVakcina, the ABA content was measured at 6.3 ± 1.2 μg mL^{-1}, for Garlic-oil it was 81.4 ± 2.2 μg mL^{-1}, and for Garlic-lipo 80.0 ± 2.4 μg mL^{-1}.

A carefully optimized dose of EliceVakcina could potentially suppress fungal growth while minimizing adverse effects on seed germination.

Several studies have reported on the effects of different dosages of ABA treatment. These include low (0.1–10 nM) and high dose (100 nM) ABA treatments during the juvenile vegetative phase in *Lemna polyrhiza* [23], maize, sunflower (with a high concentration 1 mM) [24], and rice (with a high concentration 4 μM) [71]. An ABA treatment at a concentration of 1 mM was found to enhance the transport of photoassimilates from the leaves and stems to the developing grains in wheat. This improved assimilate allocation resulted in a notable increase in yield without significantly affecting the protein quality of the grains [72].

To evaluate the potential of ABA-containing formulations for plant protection, we developed field treatments using garlic extract in the nanoformulated product EliceVakcina. Fitokondi was applied as a positive control. These treatments were administered during key developmental stages of pea plants, including the juvenile, adult vegetative, and generative phases. Previous research has demonstrated the effectiveness of aqueous garlic extract as a PB in various crops, such as pepper and tomato. These studies highlighted improvements in antioxidant activity, crop quality, metabolite accumulation, and enhanced activation of the plants' defence response [73,74]. Furthermore, applications of aqueous garlic extract were shown to improve both growth and physiological conditions in *Phaseolus vulgaris* and *Vicia faba*. This improvement was associated with increased levels of phenolic compounds, carbohydrate components, free amino acids, and endogenous phytohormones such as auxins, gibberellins, and salicylates [75,76].

Despite the promising effects of garlic extract-based PBs, the specific contributions of their active hormonal components had not been thoroughly investigated. The observed physiological responses may be attributed to the high plant hormone content present in garlic extracts. Our analysis revealed that the SC-CO_2 extract contained 81 μg mL^{-1} of ABA, a key hormone involved in abiotic stress responses. ABA indirectly contributes to the plant's response to biotic stress by regulating a network of overlapping genes involved in ABA, SA, JA, and ET signalling pathways [77,78]. This complex network consists of both synergistic and antagonistic interactions that help modulate plant defence mechanisms.

Field experiments were conducted using varying doses of EliceVakcina—a low dose (20 g ha^{-1}) and a high dose (240 g ha^{-1})—alongside Fitokondi applied at 4 L ha^{-1}. Notably, no *Fusarium* infection was detected in the treated plots. To assess plant health, the NDVI was employed as a reliable physiological indicator for quantifying the proportion of healthy plant tissue, particularly in response to fungal infections such as pea fungal spot disease [79]. During field testing, SPAD and NDVI measurements obtained via remote sensing indicated a small yet statistically significant increase ($p < 0.01$) in both the adult and vegetative phases for treatments with low-dose EliceVakcina and Fitokondi. However, this positive effect was not evident during the generative phase, and no measurable improvement in yield was observed. These results suggest that the application of ABA-containing PBs can promote healthier, more infection-resistant plant stocks in pea crops. Nevertheless, this enhancement did not translate into increased yield in peas, which contrasts with previous findings in barley, where NDVI increases correlated with significant yield improvements of 27.5% and 39.9% following E20 and E240 treatments, respectively [40].

Transcriptomic analysis of field-collected samples provided valuable insights into the molecular mechanisms underlying the observed phenotypic changes. The gene expression analysis corroborated the improved plant health status in E20 and F-treated plants, as reflected by elevated NDVI values, which indicated a reduction in infected leaf surface area. Compared to the untreated control plots, the most substantial transcriptomic changes were observed in the E20 and F treatments, with 4475 and 4585 DEGs, respectively. In contrast, the E240 treatment resulted in a considerably lower number of DEGs (1478), suggesting a dose-dependent variation in transcriptomic response. The analysis revealed that E20 and F treatments shared a significant overlap of both upregulated and downregulated genes. Examination of the DEG Top50 indicated the activation of stress response pathways associated with both abiotic and biotic stress responses. Notably, genes encoding HSPs, GSTs, PR proteins, and enzymes involved in the phenylpropanoid biosynthesis pathway were significantly upregulated. The activation of PR genes in response to E20 and F treatments highlights their pivotal role in the plant's innate immune system under stress conditions. These genes are known to contribute to plant defence mechanisms against both biotic (pathogens) and abiotic (environmental) stress factors [80]. The PR2 gene encodes β-1,3-glucanase, an enzyme that hydrolyzes β-1,3-glucans, a major structural component of fungal cell walls. The enzymatic degradation of these cell walls leads to fungal cell lysis and triggers the production of phytoalexins, secondary metabolites that play a critical role in plant defence against pathogenic attacks [81]. The PR4 gene encodes a chitinase, an enzyme responsible for degrading chitin, a key polysaccharide found in fungal cell walls. By breaking down chitin, PR4 contributes significantly to the inhibition of fungal growth, particularly targeting species such as *Fusarium* and *Botrytis* [82,83]. The PR5 gene encodes a thaumatin-like protein (also known as osmotin), which exhibits well-documented antifungal properties. This protein disrupts fungal cell membranes, contributing to enhanced plant defence responses against a range of pathogens [84]. In addition to these PR genes, several GST genes were also found to be strongly induced under biotic stress conditions. Members of the GSTF (phi class) and GSTL (lambda class) gene families, such as GSTL3 and GSTF9, were significantly upregulated in the treated samples. These enzymes play crucial roles in detoxification processes and defence signalling during fungal infections [85]. Notably, GSTF9 has been identified as a key regulator of resistance to *Verticillium* wilt, highlighting its importance in enhancing plant resilience against fungal pathogens [86]. As a consequence of the E240 treatment, the upregulation of ERF5 was observed, which may play a role in regulating the molecular response to pathogen attack [87]. GO and pathway analysis revealed a reduction in cell physiological functions related to photosynthesis in the E20 and F treatments. A slight strengthening of the photosynthesis pathway was observed in the E240 treatment, which correlated with the SPAD results. In the E20 and F treatments, the Cytochrome b6/f complex (involving the PetA gene) and F-type ATPase (involving the ATPF0C gene), both involved in the photosynthesis process, were found to be downregulated or restrained. A similar effect was reported by [88], who observed that exogenous ABA treatment can lead to decreased activity of H^+-ATPase—an essential ion transporter located in the plasma membrane of plant cells. This enzyme supports the efflux of protons (H^+) from the cytoplasm, which is crucial for maintaining electrochemical gradients that drive photosynthetic processes.

Based on the NES Top10, the most prominent features of the E20 treatment were the upregulation of pathways involved in thiamine biosynthesis and protein processing in the endoplasmic reticulum. These findings suggest that treatment with PBs containing an optimized concentration of ABA may enhance the nutritional value of pea plants by stimulating the production of essential metabolites. Of particular significance is the increased activity in vitamin B1 biosynthesis, which holds notable importance for legumes such as peas—a major dietary source of thiamine. From a human health perspective, vitamin B1 plays a critical role in nerve signal transmission between the spinal cord and the brain. Additionally, it is essential for the activation of enzymes involved in energy production from carbohydrates. Beyond its metabolic role, thiamine is associated with improved memory function, protection against nerve damage, and the reduction of sugar cravings. In plants, thiamine functions as a coenzyme in several key metabolic pathways, including glycolysis, the Krebs cycle, and the pentose phosphate pathway [89]. It also plays a pivotal role in plant adaptation to various abiotic stresses, such as cold, salt, and exposure to herbicides like paraquat, as well as in responses to biotic stresses, particularly those involving oxidative stress [90]. Ref. [91] further emphasized the potential regulatory role of ABA in thiamine biosynthesis, particularly under salt and osmotic stress conditions in *A. thaliana*. These findings suggest that ABA-containing PBs could enhance stress resilience and nutritional quality in pea plants, providing a dual benefit of improved plant health and increased thiamine content, which holds significant implications for both agricultural productivity and human nutrition. The exogenous application of ABA has been shown to enhance plant responses to various environmental stimuli by activating stress-related signalling pathways [92]. Similarly, thiamine has been demonstrated to induce acquired systemic resistance (ASR) in susceptible plants, making it a promising priming agent for enhancing plant defences. Studies conducted on *A. thaliana* revealed that while thiamine did not directly trigger a robust cellular or molecular defence response under non-stress conditions, it did induce a transient expression of the PR1 gene. Upon pathogen infection, there was a sustained upregulation of PR1 and phenylalanine ammonia-lyase (PAL), accompanied by increased hydrogen peroxide accumulation and callose deposition, which are hallmarks of activated plant defence mechanisms [93]. These findings align with our results, as field-treated pea plants exhibited significant upregulation of several PR genes, including PR1, PR2, and PR4, along with the activation of various GST genes. This suggests that the ABA-containing garlic treatments effectively primed the plants' immune systems, enhancing their ability to respond to biotic stress. However, it is important to note that thiamine accumulation has been associated with adverse physiological effects, such as stunted growth and chlorophyll deficiency, as reported in previous studies [94]. Our findings corroborate these observations, as treatments with ABA-containing garlic extracts also resulted in growth inhibition and chlorophyll deficiency in pea plants. These outcomes suggest that while ABA and thiamine can effectively prime plant defences, careful optimization of treatment concentration and timing is necessary to minimize negative impacts on plant growth and development. The theoretical framework and experimental findings presented in this study provide valuable insights that may contribute to addressing the question posed by [95]. Our results demonstrated that the external induction of thiamine effectively triggered the specific metabolic processes discussed in their hypothesis. Transcriptomic analysis revealed that, in addition to the upregulation of thiamine metabolism, significant activation was observed in pathways related to amino acid metabolism, carbon fixation, starch and sucrose metabolism, and glycolysis. Moreover, an enhanced response to oxidative stress was detected, as evidenced by the upregulation of genes involved in glutathione metabolism, indicating improved cellular defence mechanisms. Despite these extensive metabolic alterations, no significant increase in yield was observed in pea plants following treatment. This outcome contrasts with findings from earlier studies, where similar treatments led to notable yield improvements in soybean and barley [40,41]. These discrepancies suggest that the effectiveness of thiamine-induced metabolic stimulation on crop yield may be species-dependent, potentially influenced by physiological and metabolic differences among plant species.

5. Conclusions

The findings of this study are significant from three key perspectives: (i) The antifungal properties of garlic PBs containing ABA could provide a preventive strategy for enhancing pea crop resilience when applied at the optimal developmental stage; (ii) The nanoliposomal formulation of garlic PBs may improve the bioavailability of active compounds such as ABA (at a measured concentration range 6.3–81 $\mu g\ g^{-1}$), potentially enhancing their efficacy. The exogenous ABA effect was proved by the high gene expression level of ABA signalling genes SnRK2 and ABF; (iii) The observed 14.3% increase in thiamine content, which may result in an improved response to external stimuli, suggests that the plant synthesizes vitamin B1 as part of a priming mechanism. This enhancement offers additional agricultural benefits, as elevated thiamine levels in pea crops may contribute to their improved nutritional value. These outcomes were achieved through the application of GMO-free technologies, including the use of high ABA-content garlic PBs in a nanoliposomal formulation, which aligns with organic farming practices. These results represent a significant advancement in chemical-free, ecologically sustainable farming methods; (iv) Thiamine, a critical micronutrient in human nutrition, is naturally present in few crops and generally in limited quantities. Given the high cost of synthetic vitamin B1 supplements, enhancing its concentration in food crops offers a more cost-effective approach. The practical application of these findings could yield substantial public health benefits by promoting the cultivation of food plants with increased thiamine content within organic farming systems. This study offers a preliminary evaluation of the plant conditioning, priming, antifungal, and vitamin B1 enhancement effects of liposomal garlic-based substances, tested under both laboratory and small-plot experimental conditions. The findings highlight the potential of these formulations for improving plant resilience and crop quality; however, further research is necessary to determine the technological feasibility of large-scale production. In addition, it is crucial to monitor crop quality parameters during field cultivation under various ecological conditions to ensure the consistency and effectiveness of the treatments across different environments. Future research efforts will focus on conducting comprehensive field trials that incorporate artificially induced *Fusarium* infections to evaluate the efficacy of these formulations under more controlled, pathogen-stressed conditions.

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Data Availability Statement: The bio project and raw reads are available in National Center for Biotechnology Information (NCBI) database under the accessions: Repository name: Plant biostimulants treatments in Pisum sativum. Data identification number: PRJNA870114. Direct link to datasets: https://www.ncbi.nlm.nih.gov/search/all/?term=PRJNA870114 Repository name: Dataset of conditioning effect of herbal extract-based plant biostimulants in pea (*Pisum sativum*) Mendeley

Data identification number (DOI): 10.17632/f93mjns9t6.2 Direct link to datasets: accessed on 20 April 2023 https://data.mendeley.com/datasets/f93mjns9t6/2.

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Conflicts of Interest: Barbara Kutasy, Géza Hegedűs, Márta Kiniczky, József Péter Pallos, Ágnes Nagy, Klaudia Pákozdi, Máté Kállai, Csaba Weingart, Katalin Andor, Bettina Kovács and Eszter Virág were employed by the company (Research Institute for Medicinal Plants and Herbs Ltd.). The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Abbreviations

ABA	Abscisic acid
ACP	Acid phosphatase
BH	Benjamini-Hochberg
C	Control, non-treated leaf
CCOAOMT	Caffeoyl-CoA O-methyltransferase
CHS	Chalcone synthase
CLSI	Clinical and Laboratory Standards Institute
CYP81E9	Isoflavone 3′-hydroxylase
CYP93C	Isoflavone synthase
DEG	Differentially expressed gene
Dxs	1-Deoxy-D-Xylulose-5-Phosphate Pyruvate-Lyase
E	EliceVakcina
E20	EliceVakcina treatment of dosage at 20 g ha^{-1}
E240	EliceVakcina treatment of dosage at 240 g ha^{-1}
ERF5	Ethylene-responsive transcription factor 5
ET	Ethylene
F	Fitokondi treatment of dosage of 4 L ha^{-1}
Fop	*Fusarium oxysporum* f. sp. *pisi*
GA	Gibberellin
GL	Garlic-lipo
GO	Gene Ontology
GSEA	Gene Set Enrichment Analysis
GST	Glutathione transferase
HPLC-DAD	High-performance liquid chromatography with diode-array detection
HSP	Heat shock protein
IAA	Indole-3-acetic acid
Inoc.	Inoculated
IscS	Cysteine Desulfurases
JA	Jasmonic acid
KEGG	Kyoto Encyclopedia of Genes and Genomes database
NDVI	Normalized Difference Vegetation Index
NES	Normalized Enrichment Score
NGS	Next-generation sequencing
NIR	Near-infrared light
PB	Plant biostimulants
POD	Peroxidase
PR	Pathogenesis-related
RED	Red light

RIMPH Ltd.	Research Institute for Medicinal Plants and Herbs Ltd.
SA	Salicylic acid
SC-CO_2	Supercritical carbon dioxide extraction
SnRK	Sucrose non-fermenting1-related protein kinase
SPAD	Soil-Plant Analysis Development
SRA	Sequence Read Archive NCBI
TCA	Tricarboxylic acid cycle
TSA	Transcriptome Shotgun Assembly NCBI
ZEP	Zeaxanthin epoxidase

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