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Green Alternatives for the Control of Fungal Diseases in Strawberry: In-Field Optimization of the Use of Elicitors, Botanical Extracts and Essential Oils

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Abstract: Finding safe and reliable alternatives to fungicides is currently one of the biggest challenges in agriculture. In this regard, this experiment investigated the effectiveness of different elicitors, botanical extracts and essential oils to control grey mold (*Botrytis cinerea*) and powdery mildew (*Podosphaera aphanis*) on strawberry plants. This trial was conducted in field conditions under a plastic tunnel with strawberry plants 'Elsanta'. A first group of strawberry plants was treated before flowering with elicitors [acibenzolar-S-Methyl–(BTH), chitosan], botanical extracts (seaweed extract, alfalfa hydrolysate) and essential oils (thyme and juniper), and grey mold incidence on flowers was evaluated (Experiment 1). Furthermore, a second group of plants was treated before (Experiment 2) and after (Experiment 3) controlled inoculation with *P. aphanis*. The results indicated that the incidence of flower infected by *B. cinerea* was reduced by approximately 50% with thyme and juniper essential oils' applications compared to the untreated control, with no significant difference observed compared to the commercial fungicide penconazole (positive control). As a consequence, the final yield of essential-oil-treated plants was +27% higher than that of non-treated plants. No significant differences emerged for other tested products against grey mold. However, gene expression analysis showed an up-regulation (>2 ÷ 5 folds as compared to control 4 days after application) of FaEDS1, FaLOX and PR gene expression (FaPR1, FaPR5, FaPR10) in leaves treated with BTH. The other natural substances tested also induced defense-related genes, albeit at a lower level than BTH. In Experiment 2, all treatments applied prior to inoculation significantly reduced the incidence and severity of powdery mildew as compared to control. At 28 days after inoculation, chitosan and thyme essential oil applications performed similarly to their positive controls (BTH and penconazole, respectively), showing a significant reduction in disease incidence (by −84 and −92%) as compared to control. Post-inoculum application of essential oils (Experiment 3) showed an efficacy similar to that of penconazole against powdery mildew. These results indicated that the tested substances could be used as alternatives to fungicides for the control of grey mold and powdery mildew in strawberry, therefore representing a valuable tool for the control of these fungal diseases under the framework of sustainable agriculture.

Keywords: *Botrytis cinerea*; *Podosphaera aphanis*; resistance inducers; defense-related genes; chitosan; seaweed extracts

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1. Introduction

Strawberry is the most important berry fruit crop produced globally with an increase of 39.4% between 2008 and 2021 [1]. In the EU, Spain (360 kiloton), Poland (162 kt), Germany

(130 kt) and Italy (117 kt) are the leading producing countries. Europe produces nearly 1.7 Kt of strawberries (21% of the world's quantity). From 2007 to 2021, the EU strawberry market increased yearly by +2.1%, reaching in 2020 ~EUR 3282 billion [2]. This growth responds to the increasing demand for red fruits, and consumers' awareness about their nutritional value and antioxidant benefits [3]. However, strawberry cultivation relies on an average of 36 sprays per year with a pesticide application of 18 kg ha^{-1}. Indeed, strawberry recurrently ranks at the top of the 'dirty dozen' (a list of 12 top pesticide-contaminated fruit/vegetables), supporting the need to develop zero-residue production methods [2,4]. Powdery mildew, caused by the obligate pathogenic fungus *Podosphaera aphanis* (Wallr.), and grey mold, the etiological agent of which is *Botrytis cinerea* Pers., are among the diseases of strawberry requiring the highest pesticide applications. Their control requires periodic application of fungicides such as penconazole, quinoxyfen, and trifloxystrobin [5–7].

Strawberry production is increasingly shifting from open fields into protected environments [8]. Unfortunately, phytosanitary problems related mainly to grey mold and powdery mildew are increasing inside the new growing environment since these diseases find conditions such as a lack of precipitation (for powdery mildew), reduced light intensities, high humidity and longer periods of temperature above 20 °C optimal [9,10].

Xenobiotic pesticides are still massively used for crop protection, with fungicides being the most widely applied chemical [11]. Indeed, despite integrated pest management being a key European cornerstone for crop production, between 2011 and 2020, pesticide sales in the EU were almost stable, around 350,000 t $year^{-1}$ [12]. Pesticides use poses serious environmental, health and social risks [13]. For example, EFSA reports that, in 2019, approx. 4% of fruits and vegetables exceeded residue limits [14]. Moreover, pesticide use may lead to a rise in pathogen resistance and contribute to the environmental resistome [6,7,15]. In Europe, the awareness of the risks of pesticides led to the development of policies for their sustainable use (Directive 2009/128/EC) and actions, such as the Farm2Fork Strategy, aiming at a reduction of 50% in pesticide use by 2030 [16].

The use of botanical extracts, essential oils and natural elicitors represents a promising and safe alternative to the use of fungicides [17–19]. Resistance inducers are considered a reliable and sustainable method for crop protection since they promote natural plant defenses against pathogens [20]. These compounds may act on systemic acquired resistance (SAR) or induced systemic resistance (ISR). Among the signaling molecules regulating plant defenses, salicylic acid (SA), jasmonic acid (JA) and ethylene play a crucial role [21]. SAR acts mainly against biotrophic pathogens such as powdery mildew and depends on SA signaling, leading to systemic expression of pathogenesis-related (PR) genes [22–24]. SA biosynthesis is activated by PAD4 (Phytoalexin Deficient 4) and EDS1 proteins (Enhanced Disease Susceptibility 1) [25,26]. Several PR proteins were identified, such as PR-1 (antifungal), PR-5 (thaumatin-like protein), and PR-10 (ribonuclease-like protein) [27,28]. JA is an important regulator involved in the ISR signaling pathway, with lipoxygenase (LOX) being a key enzyme for its biosynthesis [29–31]. JA activates the expression of genes encoding antifungal compounds such as LOX-derived oxylipins, which are synthetized in response to biotic stress [32,33]. Furthermore, plants can activate defenses with the biosynthesis of particular defensive substances (secondary metabolites) such as phenylpropanoids, isoprenoids and alkaloids [34–38].

In the absence of any biotic stress, plant defense mechanisms might be trigged through exogenous chemical elicitors (or resistance inducers), which do not directly kill the harmful organism but promote plant defense [39,40].

In the present study, we investigated different resistance inducers (acibenzolar-S-methyl, chitosan), botanicals extracts (seaweed extract, alfalfa protein hydrolysate) and essential oils (thyme and juniper). Chitosan is the deacetylated form of biopolymer chitin, which is a component of crustacean and insect exoskeletons and fungal cell walls. Chitosan was found to be able to activate L-phenylalanine ammonia-lyase (PAL) biosynthesis in different crop species, leading to a higher final accumulation of phenolic compounds with antipathogenic activity in tomato [41], papaya [42] and grape [43]. For this reason, chitosan

is often used as a plant elicitor rather than a product with a direct antimicrobial activity [44]. Seaweed extracts are a complex mixture of components such as mineral elements, amino acids, vitamins, phytohormones, betaines, sterols and polysaccharides (e.g., ulvans, agarans, carrageenans, alginates, fucans and laminarins) [45–48]. Studies have attributed to seaweed polysaccharides the role of effective elicitors of plant defense against biotic stresses [48–51]. Protein hydrolysates are defined as "mixtures of polypeptides, oligopeptides and amino acids that are manufactured from protein sources, using partial hydrolysis" [52]. They exert a biostimulating activity due to their content in plant hormones, peptides and amino acids [53–55]. Although biostimulant properties of legume-derived protein hydrolysates are well studied, few indications are present in the scientific literature regarding their eliciting action against pathogens. Essential oils have received great interest in recent years due to their antimicrobial, antiviral, biodegradable and eco-friendly properties [56–58]. Essential oils have a complex composition containing monoterpenes, sesquiterpenes and phenylpropanoids. As a consequence, resistance against them is rarely developed by pathogens [59]. In particular, *Thymus* essential oil has been demonstrated to have pronounced direct antimicrobial properties and in vitro experiments showed that this activity is mediated by two phenolic components, thymol and carvacrol [56,60,61]. The antimicrobial activity of *Juniper* essential oil, linked to some of its components such as limonene and terpinen-4-ol, has been demonstrated on fungal pathogens (e.g., *B. cinerea*) [62]. In addition to its direct antifungal activity, essential oils can have an elicitor activity against pathogens (e.g., downy mildew) on grapevine plants [63].

The aim of this work was to evaluate the efficacy of selected commercial products (elicitors and essential oils) to control grey mold and powdery mildew in strawberry plants cultivated under plastic tunnel conditions. Moreover, the impact of the tested products on the plant defense mechanism was investigated.

2. Materials and Methods

2.1. Plant Material, Growth Conditions and Tested Products

These experiments were conducted under soilless conditions within a plastic tunnel at the Laimburg Research Centre, in the municipality of Ora/Auer (46°22′ N; 11°17′ E; 237 m a.s.l.) in Alto-Adige/South Tyrol, Italy. The temperatures in the greenhouse ranged between 20–25 °C (day) and 10–15 °C (night), and relative humidity (RH) was kept at high values (Figure 1) owing to a fog system placed under the soilless structure.

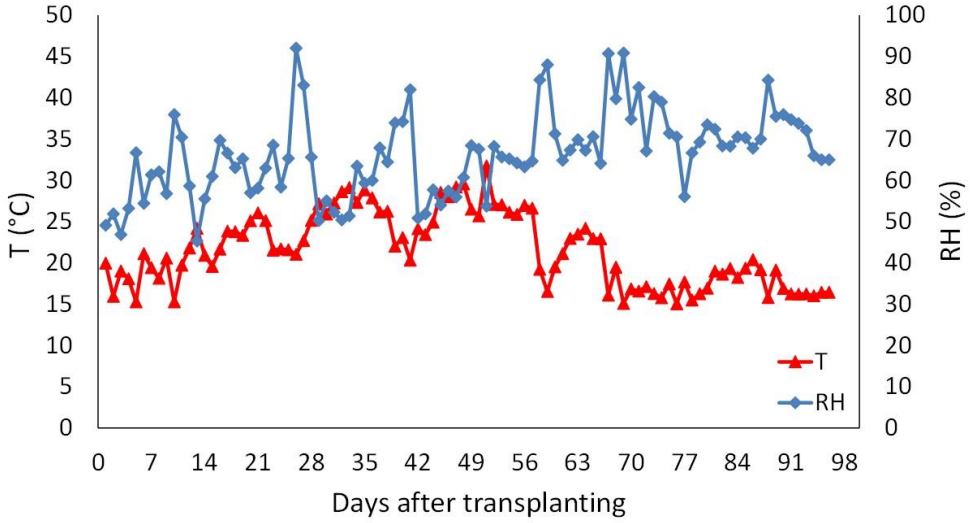

Figure 1. Climatic conditions: average daily temperature (T °C) and relative humidity (RH %) in the greenhouse during the experimental period.

Cold-stored strawberry tray plants (TP) 'Elsanta' were transplanted into individual pots (16 cm diameter) containing a mixture of natural clay, white peat, perlite and mineral fertilizer (pH 5.8, EC 0.40 dSm^{-1}, total porosity 90%, bulk density 130 kg m^{-3}; Profi Substrat, SP VM, Einheits Erde, Sinntal-Altengronau, Germany). All plants were watered daily by drip irrigation and fertilized weekly alternating between a granulated long-term NPK fertilizer (10 g per pot; Berry Fertilizer 15-8-12, Hack, Langerwehe, Germany) and a water-soluble fertilizer containing macro- and microelements (200 mL per pot at a dosage of 200 g h L^{-1}; Floral 20-20-20, Cifo, Bologna, Italy). No fungicidal applications were carried out during this experiment, whereas the acaricide (Vertimec® EC, Syngenta, Basel, Switzerland) was sprayed twice before flowering at a concentration of 0.5 mL L^{-1}.

Details on the names, abbreviations and application doses of the tested products are reported in Table 1. The fungicide Topas® and the plant defense elicitors Bion® and ChitoPlant Solution® were commercial products used as positive controls at the concentration suggested in their labels. The botanical extracts from seaweed and alfalfa were experimental products (prototypes) and were applied at concentrations previously tested for strawberry plants [64]. The essential oils from thyme and juniper were commercial products that were applied at concentrations previously found effective after in vitro spore germination essays conducted on *B. cinerea* and described in Soppelsa et al. [58]. Water was used as the negative control and in all cases, and Adesil A surfactant (Serpan, Vicenza, Italy) was added as wetting agent (0.03%) to improve the leaf coverage uniformity and solubility of different products. The products were applied as a fine mist on the upper surface of the leaves until the runoff point (50 mL per plant) using a hand sprayer before (Experiment 1) or after flowering (Experiment 2 and 3).

Table 1. Product characteristics and mode of application.

Treatment	Name	Active Ingredient	Concentration	Commercial Name
CON	Untreated	water	-	-
FUN	Fungicide	penconazole	0.25 mL L^{-1}	Topas® 200 EW, Syngenta Crop protection, Basel, Switzerland
BTH	Benzothiadiazole	acibenzolar-S-methyl	0.4 g L^{-1}	Bion® 50 WG, Syngenta Crop protection, Basel, Switzerland
CHI	Chitosan solution	chitosan	10 mL L^{-1}	ChitoPlant Solution®, Agritalia, Italy
SEA	Seaweed extract (*Ascophyllum nodosum*)	mix of components	4 g L^{-1}	Experimental product, ILSA S.p.a., Arzignano, Italy
APH	Alfalfa protein hydrolysate	mix of components	4 g L^{-1}	Experimental product, ILSA S.p.a., Arzignano, Italy
THY	Thyme essential oil (*Thymus vulgaris*)	thymol	1 mL L^{-1}	Essential oil, Vitalis Dr. Joseph, Brunico, Italy
JUN	Juniper essential oil (*Juniperus communis*)	α-pinene	1 mL L^{-1}	Essential oil, Vitalis Dr. Joseph, Brunico, Italy

2.2. Experimental Design

The research activity was subdivided into three experiments:

Experiment 1: Pre-flowering treatment

To assess the efficacy of commercial products in preventing floral infection by *B. cinerea*, a group of 66 strawberry plants was sprayed before flowering (BBCH 56—Inflorescence elongating) with a single application of the products listed in Table 1. The same plants were also sampled for gene expression analysis to investigate the molecular basis of the products' efficacy. The experimental setup was organized as a completely randomized block design with four replicates of two plants each per treatment. In addition to gene expression analysis (see Section 2.5), some parameters such as yield and fruit qualitative traits were evaluated (see Section 2.4), as well as the incidence of flowers infected by *B. cinerea* (see Section 2.3).

Experiment 2: Post-flowering treatment at pre-inoculation

A second group of 84 strawberry plants was sprayed post-flowering (BBCH 71—Receptacle protruding from sepal whorl). The plants were treated with a single application of FUN, BTH, APH and THY (Table 1). SEA was applied 2 times (second application after 3 days), while all other products were only applied one time. Seven days after treatment, the plants were artificially inoculated with *Podosphaera aphanis* (see Section 2.3). Powdery mildew assessment (Section 2.3) was conducted considering each strawberry plant as a single replication (8 plants per treatment).

Experiment 3: Post-flowering treatment at post-inoculation

This experiment aimed at investigating the potential curative effect of essential oils (THY and JUN) against *P. aphanis*. For this aim, 34 strawberry plants were sprayed with THY and JUN 24 h after inoculation. Penconazole and water were used as positive and negative control, respectively.

2.3. Disease Assessment and Inoculation Procedure of Podosphaera aphanis

Grey mold incidence was assessed in the condition of natural infection. Incidence was calculated taking into account the percentage of symptomatic flowers in each plant [65]. Powdery mildew incidence and severity were assessed after the experimental inoculation of the plants. The conidia of *P. aphanis* were collected from naturally infected, symptomatic strawberry plants 'Elsanta' obtained from a local farm. One hour before inoculation, a *P. aphanis* conidia suspension was produced by harvesting conidia by washing symptomatic strawberry leaves into a flask of 0.03% Adesil A (Serpan, Vicenza, Italy) in distilled water. Conidia concentration was quantified with a hemocytometer and adjusted to 10^5 conidia mL^{-1}, as previously reported [66] with some modifications. *P. aphanis* conidia suspension was sprayed onto the upper surface of the leaves (100 mL per plant). Approximately every 2 weeks after inoculation, powdery mildew incidence and severity were assessed. The percentage of infected leaves per plant was calculated by counting the number of healthy and infected leaves (presenting at least one powdery mildew patch per leaf) [8]. Disease severity was determined considering 12 leaves per plant (8 plants per treatment) and was evaluated with a plant image analyzer (BioLeaf—Foliar Analysis™, Federal University of Mato Grosso do Sul, Campo Grande, Brazil) and expressed as percentage of the infected area compared to the total leaf area.

2.4. Yield and Fruit Quality

Ripe strawberries (full red color) were harvested every three days, starting at 50 days after transplanting. Data from each fruit picking were used to calculate the final cumulative yield per plant (as grams $plant^{-1}$). Fruit quality was determined on four mature and homogeneous fruits per plant. The firmness (FF as kg cm^{-2}), the total soluble solids (TSS as °Brix) and the titratable acidity (TA as g L^{-1} of citric acid) were evaluated, respectively, with a penetrometer (6 mm in diameter), a portable refractometer (PAL-1, ATAGO, Tokyo, Japan) and with a titrator (TitroLine easy, SCHOTT, Mainz, Germany) by titrating strawberry pulp to pH 8.2 using 0.1 M NaOH. Extraction and quantification of phenolic compounds were carried out using a method described by Meyers et al. [67] and Wolfe et al. [68]. Briefly, phenolics were extracted from lyophilized leaves/fruits using 80% methanol acidified with H_3PO_4 and NaF. The total phenolic content was determined with the Folin–Ciocalteu colorimetric method and expressed as milligrams of gallic acid equivalents (GAE) per 100 g of dry weight (mg GAE 100 g^{-1} DW).

2.5. Gene Expression Analysis

The gene expression analysis was conducted as part of the Experiment 1 activities only (see Section 2.2) on plants treated with BTH, CHI, SEA and THY. Leaf samples were collected at 0, 48, 72, 96 and 168 h after treatment, taking two young single leaves per plant. The leaves were immersed in liquid nitrogen and stored at −80 °C. At every sampling time, two plants per treatment were considered. Each plant was sampled only once to avoid a wounding effect. Changes in expression of genes related to downstream components

of the SAR pathway (Table 2) were determined by a reverse transcription-quantitative real-time polymerase chain reaction (RT-qPCR), as described by Cellini et al. [69]. Briefly, total RNA was extracted from strawberry leaves following the manufacturer's instructions in Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, St. Luis, MI, USA). RNA was used for cDNA synthesis with reverse-transcription PCR. Specific primers were designed using Primer3Web software 4.1.0 and previously tested for specificity (Table 2). The expression of genes was measured using a StepOne Plus Real-Time PCR instrument (Thermo Fisher Scientific, Waltham, MA, USA) with a SYBR Green-based assay. Strawberry leaves pre-treated with different resistance inducers were examined for *FaPR1*, *FaPR5*, *FaPR10*, *FaEDS1* and *FaLOX* genes.

Table 2. List of primers used for RT-qPCR analysis.

Gene Name	Gene Description	Forward Primer Sequence [5′-3′]	Reverse Primer Sequence [5′-3′]
FaPR1	Pathogenesis-related protein 1	TGCTAATTCACATTATGGCG	GTTAGAGTTGTAATTATAGTAGG
FaPR5	Pathogenesis-related protein 5	CGATGCCCCTGCTTACA GTTACCCTAAGGATG	CCTCGTAATTGCTTCAA GGGCAGAACACAACC
FaPR10	Pathogenesis-related protein 10	CGAGGAATACAACTAAACCTTGCCGTCT	TACAATTTGCCACACATACACCGAAGTG
FaEDS1	Enhanced disease susceptibility 1	AAAAGAGAGACTTCAATGCCAATGTG	CTTCGTTCTTTGCGTGTCTGTAGTAGTT
FaLOX	Lipoxygenase	CGACGACGACTGGATACACCGCAGGG	GAGGTTGGCCGCTGTTTCTTGCACCGTA
FaGAPDH2	(housekeeping)	CCCAAGTAAGGATGCCCCCATGTTCG	TTGGCAAGGGGAGCAAGACAGTTGGTAG

All quantifications were normalized by the threshold cycle value (C_T) compared to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*FaGPDH2*). Gene expression analysis was expressed using the $2^{(-\Delta\Delta C_T)}$ fold change method, in which '0' fold change corresponds to 'no change'. Untreated control at the corresponding time point was used to calculate the fold changes.

2.6. Statistical Analysis

One-way analysis of variance (ANOVA) was performed to determine the significance of differences among treatments after validating the data for normal distribution (Shapiro–Wilk normality test, $p > 0.05$) and equality of variances (Bartlett's test, $p > 0.05$). For non-normal data, the Kruskal–Wallis test was applied. Data expressed in percentage were arcsin-transformed prior to the analysis of variance. In case of significance, mean separation was carried out with the least significant difference (LSD) test. All analyses were carried out in R v. 3.3.1. (R Development Core Team 2016). The results were expressed as mean ± standard error (SE).

3. Results

3.1. Efficacy against Natural Infection by Botrytis Cinerea (Experiment 1)

Natural disease incidence in untreated plants was around 50%, whereas treatment with fungicide was able to halve the percentage of infected flowers per plant compared to the control (Figure 2). An effect similar to that of the fungicide was observed in plants treated with THY and JUN (40–50% less infections in comparison to control). No significant differences were detected among other treatments (33–44% of infected flowers per plant) in comparison to untreated plants.

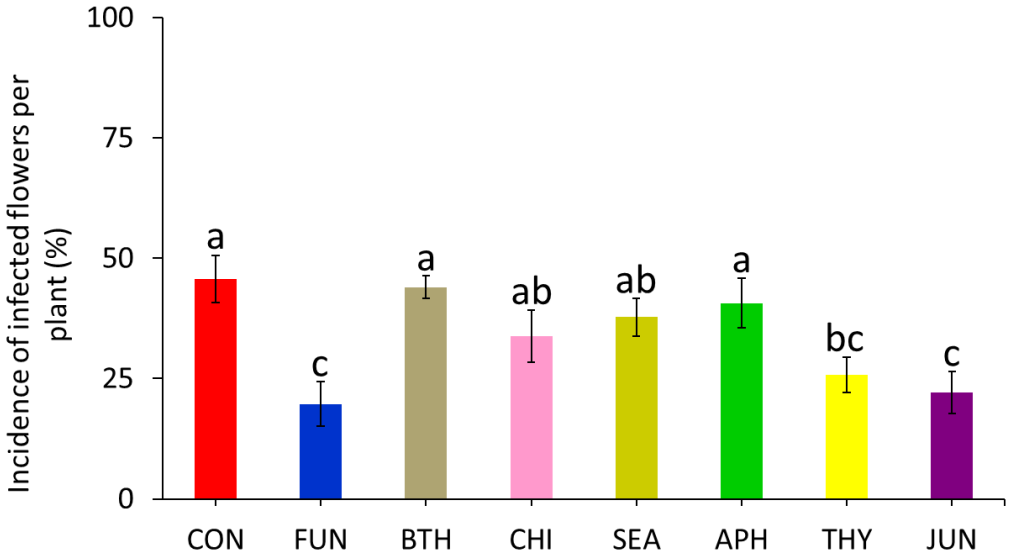

Figure 2. Incidence of flowers affected by natural infections by *Botrytis cinerea* at the end of harvest (56 days after flowering; Experiment 1). Different letters at the top of each bar indicate significant differences among treatments according to LSD test at $p < 0.05$ (mean ± SE, $n = 4$). Treatments' legend: CON, control; FUN, fungicide; BTH, benzothiadiazole; CHI, chitosan; SEA, seaweed extract; APH, alfalfa protein hydrolysate; THY, thyme essential oil; JUN, juniper essential oil.

3.2. Preventive Effects against Powdery Mildew Incidence and Severity on Leaves (Experiment 2)

Symptoms of powdery mildew on strawberry leaves started to be visible about 14 days after inoculation. Considering preventive application, the results indicated that all tested products controlled powdery mildew development on strawberry leaves (Figure 3A,B). In further detail, at 28 days after inoculation, CHI and THY applications performed a similar action to their positive controls (BTH and FUN, respectively), showing a significant incidence reduction (by −84 and −92%) as compared to control. SEA and APH treatments were shown to significantly reduce powdery mildew incidence (−42 and −58%, respectively, as compared to the control). The statistical differences among the treatments remained similar at 42 days (Figure 3A). Powdery mildew was well established in untreated plants at almost 2 months after inoculation, when 65% more of the leaves presented at least one infection site (Figure 3A). At that time, all treatments maintained an inhibitory action towards powdery mildew. In further detail, the fungal incidence in CHI-, SEA- and APH-treated plants was similar to that observed on BTH-treated plants, whereas the THY effect was analogue to that of FUN. The assessment of disease severity showed that all treatments suppressed the development of powdery mildew. Despite disease severity remaining low, with symptoms developing on less than 5% of leaf area, differences among the treatments were detected (Figure 3B). In particular, as observed for disease incidence, the application of FUN, CHI and THY was demonstrated to significantly reduce the extension of red blotches (up to 94% reduction as compared to untreated).

3.3. Curative Effects against Powdery Mildew Incidence and Severity on Leaves (Experiment 3)

Post-inoculum application of products showed the effective action of essential oils in reducing disease incidence and severity (Figure 4A,B). Although both essential oils (THY and JUN) were shown to inhibit powdery mildew development with an action similar to that of the positive control (FUN), THY seemed to have the highest inhibitory action against the pathogen, as the disease severity at 56 days was the lowest among the treatments.

3.4. Fruit Production and Quality

Plants treated with essential oils (THY and JUN) were shown to increase the final yield per plant. In the case of JUN-treated plants, the yield result was comparable to that

obtained with the fungicide treatment (around 0.160 kg plant^{-1}) (Table 3). The enhanced yield in THY- and JUN-treated plants (+23 and +31% as compared to control, respectively) was a direct consequence of the reduction in fruit losses due to infection (about 15% more than untreated fruits) (Table 3).

Treatments such as CHI and SEA were also demonstrated to significantly increase the yield (0.135 kg compared to 0.120 kg plant^{-1} in control plants). BTH- and APH-treated plants yielded the same amount as control plants. Regarding fruit weight, no significant differences were observed among the treatments (around 10–12 g fruit^{-1}) (Table 3).

A higher sugar and acidity content was observed in untreated fruits, though differences between the treated fruits were not significant (Table 3). As regards phenolic content in strawberry fruits, no significant differences were detected. FUN-treated fruits showed a tendency to increase this parameter (+24% as compared to control fruits).

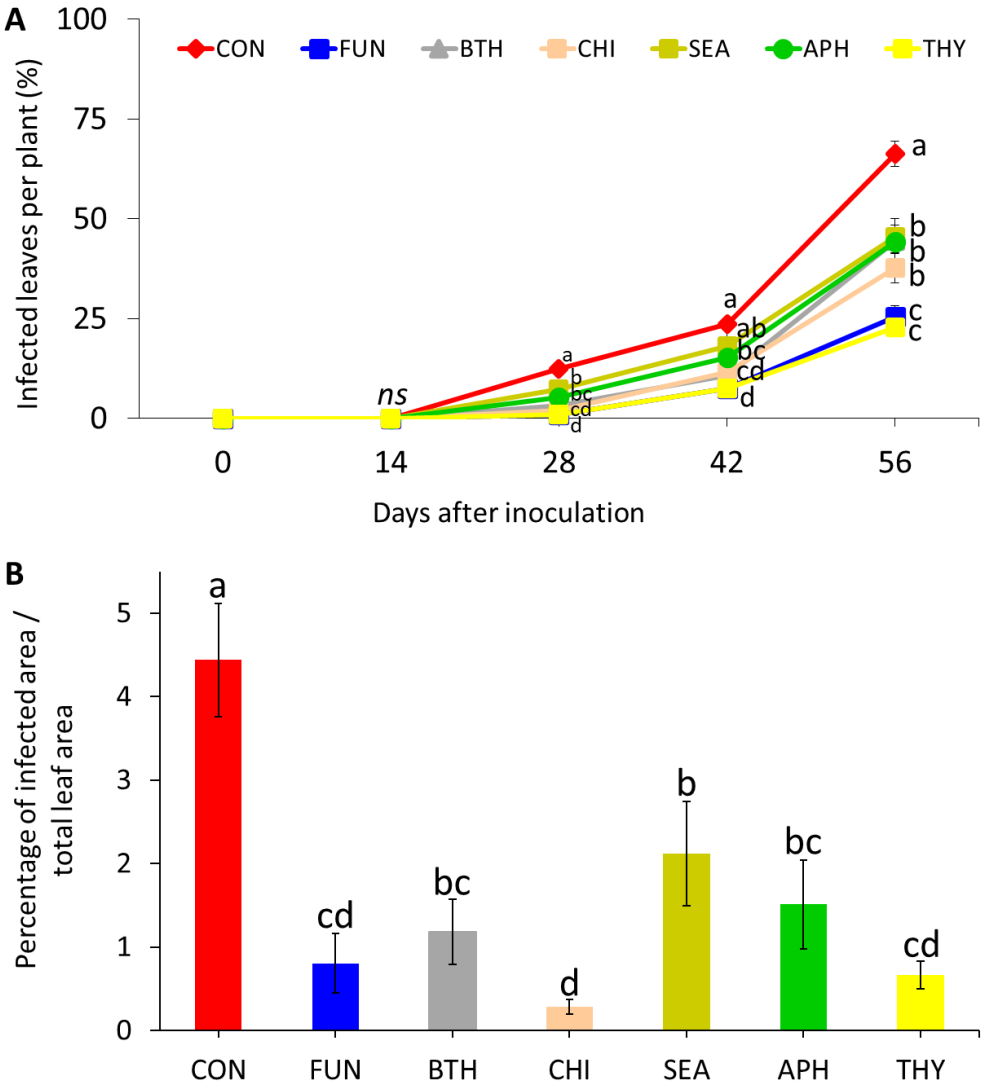

Figure 3. Powdery mildew incidence on strawberry leaves after inoculation (**A**) and severity at 56 days after inoculation (**B**). Tested products were applied 7 days before inoculation at BBCH 71 (Experiment 2). Different letters at the top of each bar indicate significant differences among treatments according to LSD test at $p < 0.05$ (mean ± SE, $n = 8$). ns: not significant.

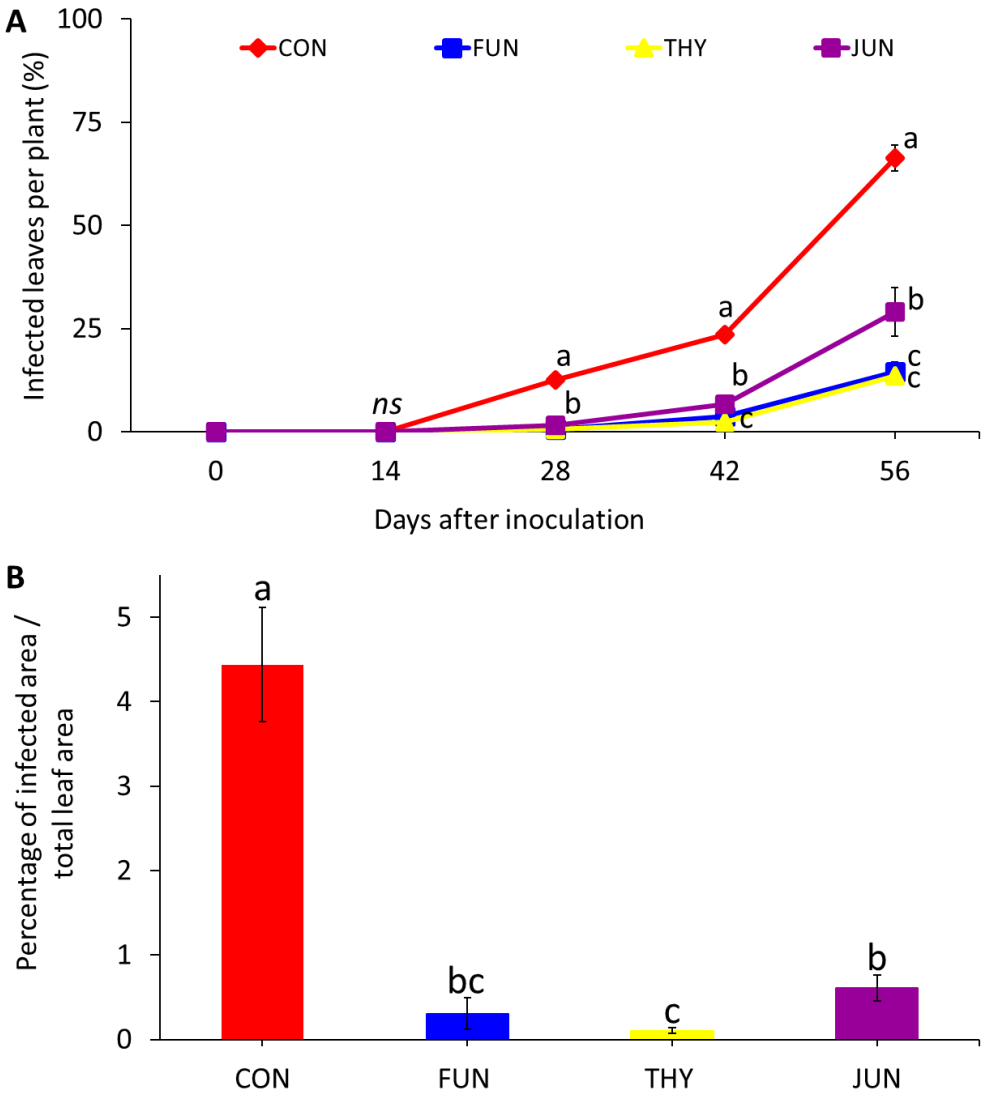

Figure 4. Powdery mildew incidence on strawberry leaves after inoculation (**A**) and severity at 56 days after inoculation (**B**). Tested products were applied 24 h after inoculation at BBCH 73 (Experiment 3). Different letters at the top of each bar indicate significant differences among treatments according to LSD test at $p < 0.05$ (mean ± SE, $n = 8$). ns: not significant.

Table 3. Yield parameters (total yield per plant, number of fruits per plant and mean fruit weight) and fruits' quality attributes as affected by tested products.

Treatment	Total Yield (kg plant^{-1})		Number Fruits Plant^{-1} (N°)		Mean Fruit Weight (g)	Total Soluble Solids (°Brix)	Titratable Acidity (g citric acid L^{-1})	Total Phenol Content (mg GAE 100 g^{-1} DW)
CON	0.120 ± 0.002	e	11.63 ± 0.71	de	10.62 ± 0.57	13.13 ± 0.27	4.29 ± 0.49	2139.09 ± 63.54
FUN	0.161 ± 0.004	a	14.00 ± 0.46	ab	11.57 ± 0.38	11.43 ± 0.52	3.75 ± 0.61	2602.84 ± 303.15
BTH	0.121 ± 0.003	de	12.00 ± 0.60	cde	10.21 ± 0.29	12.73 ± 1.06	3.48 ± 0.17	2250.58 ± 145.74
CHI	0.135 ± 0.007	c	12.38 ± 0.80	bcde	11.10 ± 0.48	11.23 ± 1.62	2.95 ± 0.33	2286.84 ± 125.48
SEA	0.133 ± 0.003	cd	13.13 ± 0.90	abcd	10.44 ± 0.72	10.68 ± 0.72	3.11 ± 0.17	2204.47 ± 92.48
APH	0.127 ± 0.005	cde	11.25 ± 0.67	e	11.54 ± 0.54	12.53 ± 0.17	3.24 ± 0.14	2431.74 ± 57.76
THY	0.148 ± 0.003	b	13.50 ± 0.63	abc	11.17 ± 0.55	11.60 ± 0.69	3.12 ± 0.24	2499.77 ± 256.05
JUN	0.157 ± 0.005	ab	14.38 ± 0.32	a	10.98 ± 0.26	11.38 ± 0.36	2.91 ± 0.14	2235.11 ± 114.87

Means ± SE within the same column followed by the same letter, do not significantly differ according to LSD test at $p < 0.05$ ($n = 4$).

3.5. Gene Expression Analysis

An up-regulation of the PR gene's expression (*FaPR1*, *FaPR5*, *FaPR10*) was observed in leaves treated with BTH (Figure 5A–C). In further detail, gene expression was highest 4 days after BTH treatment. BTH application induced a long-lasting (up to 7 days) increase in the *FaEDS1* and *FaLOX* genes' expression. The expression levels of the target genes appeared weaker in plants treated with the natural elicitors (SEA and CHI) and THY than in the positive control (BTH). As shown in Figure 5, *FaPR1*, *FaPR5*, *FaPR10* and *FaLOX* exhibited a peak of expression at 2 and 3 days after SEA application (fold change < 2). These effects completely disappeared at 4 (for *FaPR5*, *FaPR10* and *FaLOX*) and 7 days (for *FaPR1*). *FaEDS1* was not expressed after treatment. Contrary to what was observed in SEA, starting at 2 days after CHI treatment, *FaPR1*, *FaPR5*, *FaEDS1* and *FaLOX* were induced and their expression levels continued to be stable even at 7 days (except for *FaLOX*). Thyme essential oil application was demonstrated to have a low effect on the activation of defense-related genes (fold-change < 1). SEA, CHI and THY proved to enhance defense-related gene expression, even though at a lower level as compared to BTH (fold change expression < 2).

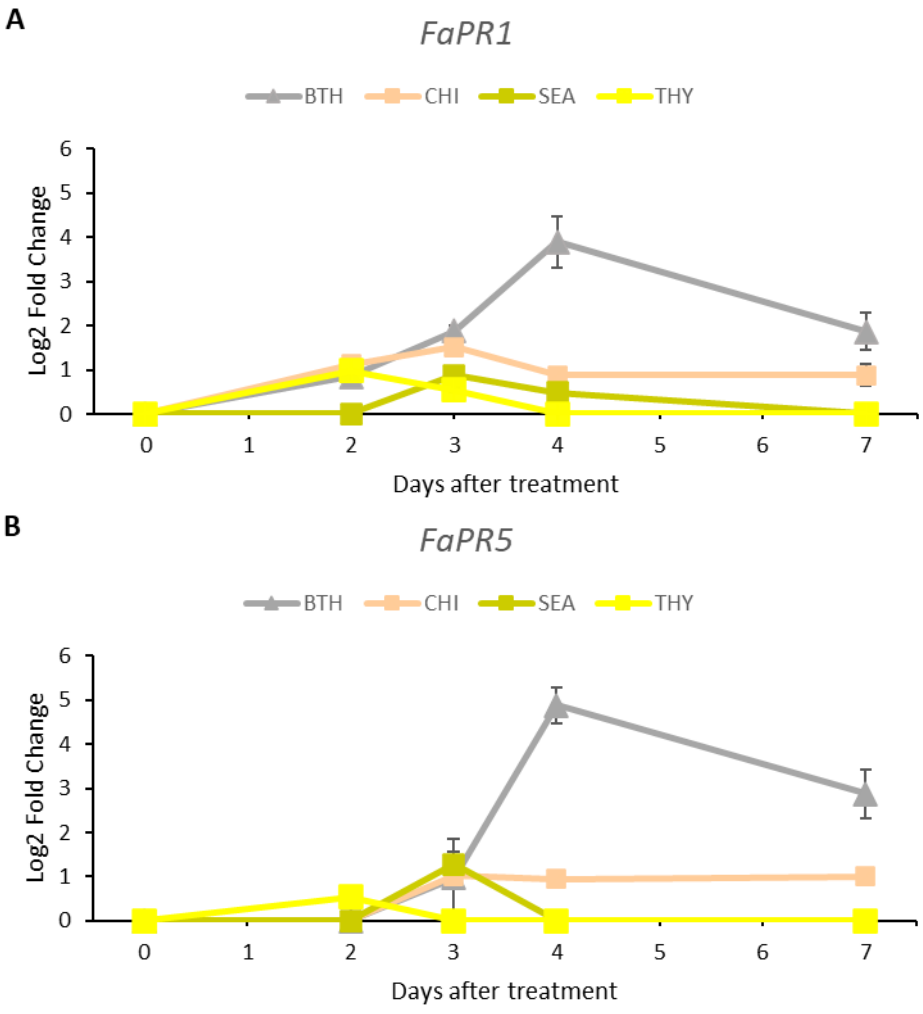

Figure 5. *Cont.*

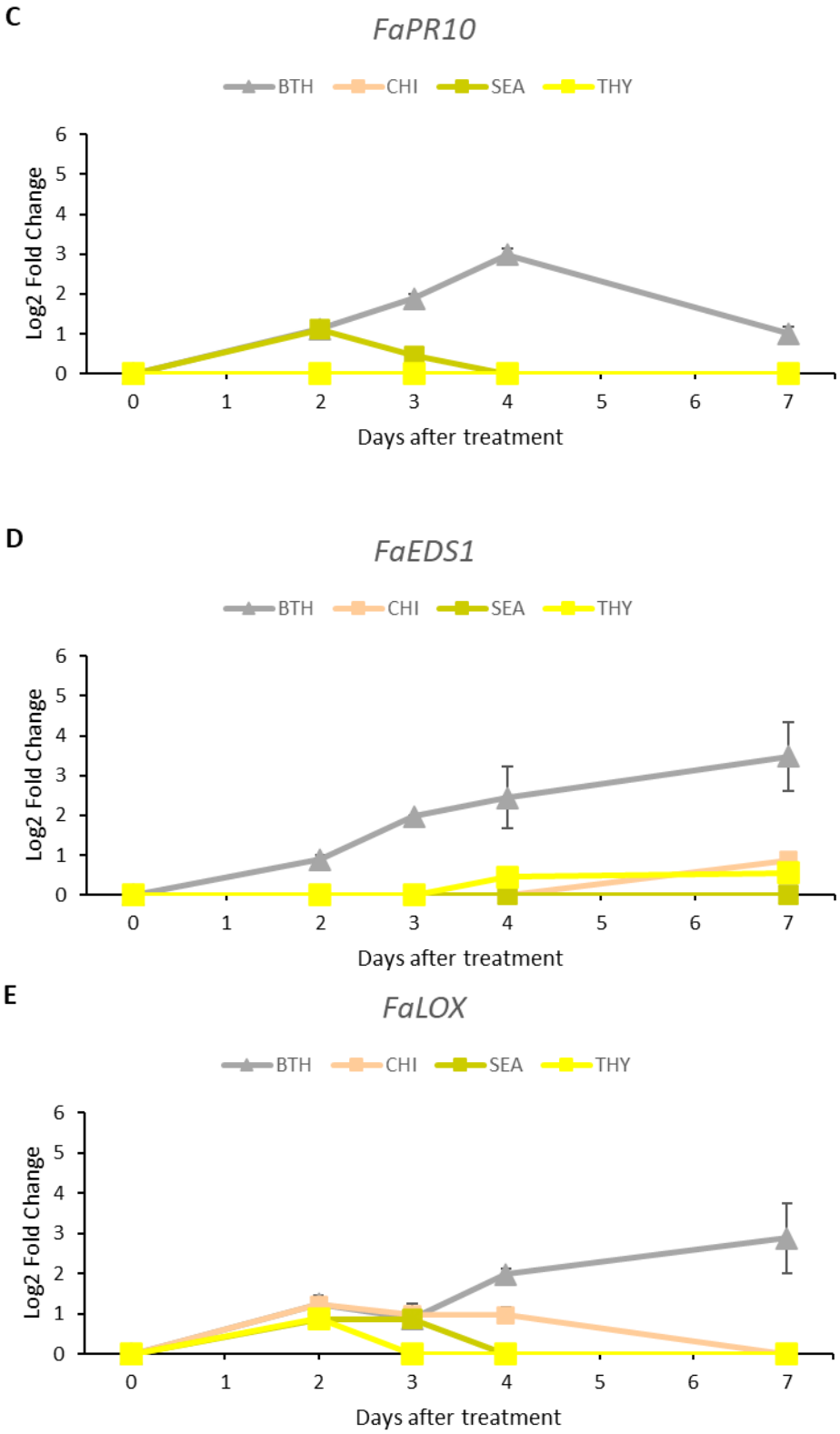

Figure 5. Expression of defense-related genes (*FaPR1* (**A**), *FaPR5* (**B**), *FaPR10* (**C**), *FaEDS1* (**D**) and *FaLOX* (**E**)) in strawberry leaves treated with benzothiadiazole, seaweed extracts, chitosan and thyme essential oil. RT-qPCR was performed using FaGPDH2 as housekeeping gene. For gene expression, we adopted the $2^{(-DDCT)}$ method, in which a '0' fold change corresponds to 'no change'. Values were normalized to the control at each time point. Data are expressed as mean ± SE.

4. Discussion

Pre-flowering applications of essential oils were the only treatments that significantly reduced the incidence of *B. cinerea* in flowers and their protective effect was comparable to that of commercial fungicide. This effect is related to the direct inhibition pathogen growth being essential oils well known for their antifungal activity [58,70,71]. In further detail, essential oils can directly inhibit the biosynthesis of chitin, β-glucans and ergosterol, alter fungal mitochondrial electron transport chain and cell division, and interfere with RNA/DNA and protein synthesis [72–77].

On the other hand, commercial products acting as resistance inducers, such as BTH, failed in protecting flowers from infection. BTH-induced resistance against diseases was observed on several plants such as tomato, *Arabidopsis*, peach, apple, strawberry and papaya [78–83]. Although our results confirmed the induction of defense responses in BTH-treated strawberry leaves (the highest level of gene expression in comparison to all tested products), the incidence of infected flowers, as well as the final yield, was not significantly different to that of the control treatment.

The lack of efficacy of BTH could be related to the timing of resistance induction. Several studies showed that the rise in plant defenses needs a minimum of 3–4 days, reaches a peak in two–three weeks and decreases to a normal level in approximately 4 weeks [84]. Indeed, in our study, a rise in defense-related genes was observed 4 days after the treatment. This indicates that in our experimental conditions, it can be assumed a timing of plant resistance similar to that found in other studies [85]. However, *B. cinerea* incidence was assessed only more than 60 days after treatment, when the BTH effect was probably already fading. Furthermore, under natural disease pressure, *B. cinerea* inoculum can build up rapidly under favorable conditions, making results less predictable. In this view, a product like essential oils, which have a dual mode of action—direct antifungal effects and induction of resistance—may result in a more consistent protective effect throughout the season.

Essential oils may also induce defense mechanisms in plant tissues, including the activation of antioxidant and defense-related enzymes and the accumulation of phenolic compounds [57,86]. A positive effect of thyme essential oil against grey mold and *Fusarium* wilt in tomato plants was observed by Ben-Jabeur et al. [60]. The authors attributed the protective action of thyme extract to a combination of a direct antifungal activity and the induction of defenses in plants.

However, in our experimental conditions, the protective effect of essential oils relies primarily on their fungicidal effects, which was also confirmed in curative treatments (Experiment 3). Indeed, application of THY did not significantly induce any defense-related gene expression in plant tissues, nor did it alter the total phenolic content (Figure 5; Table 3). The results may be related to the time required for strawberries to enhance their plant defenses or to the activation of defense mechanisms that are not associated with SAR. Concerning the induction of phenolic compounds, we measured it only on fruit that were not directly exposed to an essential oil. Furthermore, differences in phenolic compounds may be related to the induction of specific compounds rather than a generalized induction of this chemical class [87]. The reduction in flower losses by *B. cinerea* in plants treated with essential oils and fungicide could explain the increased final yield measured in those treated plants.

When considering the experiments in controlled inoculation with *P. aphanis*, the positive action of post-flowering application of elicitors and essential oils against powdery mildew infections was shown. Considering both preventive (Experiment 2) and curative actions (Experiment 3), the thyme essential oil had a protective effect against powdery mildew similar to that of the fungicide. In this experiment, thyme essential oil application resulted in a lower disease severity than in the preventive application (Experiment 2) despite the differences not being statistically significant. This finding is in agreement with studies conducted by Sturchio et al. [88] and Mostafa et al. [89], where clove, lemongrass, peppermint, rosemary, tea tree and thyme essential oils were demonstrated to be valid

fungicidal alternatives to control powdery mildew infections on *Cucurbitaceae* crops. In our experiment, chitosan, seaweed extract and alfalfa hydrolysate showed an action similar to BTH treatment against powdery mildew infection (Figure 3). The induction of resistance by chitosan application is also a well-studied topic. Being a polysaccharide, chitosan could act in the same way as described for algal polysaccharides. The accumulation of PR proteins, phytoalexins, lignin biosynthesis and callose formation was among some of the defense mechanisms induced by chitosan in treated plants [44]. As an example, grapes treated with chitosan were shown to be less susceptible to powdery mildew and to enhance phenolic concentration in berry peel [90]. Similar results were also obtained by Faoro et al. [91] with powdery mildew infection on barley.

Carrot leaves treated with seaweed extract (*Ascophyllum nodosum*) were shown to be less infected by *Alternaria* and *Botrytis* due to the activation of defense genes (e.g., *PR1*, *PR5* and *PAL*), as well as to an increased activity of defense enzymes and accumulation of phenols and phytoalexins [92]. Polysaccharides in seaweed extracts could be considered signaling molecules because they activate salicylic acid (SA) and the jasmonic acid (JA) signaling pathway, promoting the systemic acquired (SAR) and the induced systemic resistance (ISR) in treated plants [48]. Marine polysaccharides such as alginates, fucans and laminarin, which are components of the brown seaweed extract utilized, could have contributed to enhance plant defenses. The mechanism of action of alfalfa protein hydrolysate against diseases probably involves the complex biochemical composition of the hydrolysate. In further detail, two main categories of APH components such as peptides and amino acids are to be considered signaling molecules [93–95]. Cappelletti et al. [96] observed a reduced infection by powdery mildew in zucchini plants treated with different protein hydrolysates. The authors explained this result with the involvement of amino acids and peptides in the regulation of innate immune response in plants.

5. Conclusions

The effectiveness of natural products in the control of grey mold and powdery mildew was evaluated in this experiment. Essential oils showed an effect similar to that of synthetic fungicide both against *B. cinerea* and *P. aphanis,* significantly reducing the incidence and severity of infections. The highest defense-related gene expression level was induced by BTH application, even though only the incidence of powdery mildew was reduced in those treated plants. As for the other foliar applications (seaweed extract, alfalfa protein hydrolysate and chitosan), they were also demonstrated to be valid alternatives to fungicides against powdery mildew. In our experimental conditions, the effect of essential oils was primarily due to their fungicidal activity. However, in comparison to synthetic fungicides, essential oils may pose a minor risk of resistance development in the pathogen population. In fact, differently from chemical fungicides, essential oils exert several synergic antimicrobial actions [97].

Appropriate application methods (dosages, type of formulation, time and number of application) seem to be of great relevance to improve the efficiency of this group of products. Therefore, further investigations should be carried out in this direction.

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