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An Evaluation of Organic Biostimulants as a Tool for the Sustainable Management of Viral Infections in Zucchini Plants

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Abstract: In agriculture, new and sustainable strategies are increasingly demanded to integrate the traditional management of viral diseases based on the use of virus-free propagation materials and resistant or tolerant cultivars and on the control of insect vectors. Among the possible Integrated Pest Management (IPM) approaches, organic biostimulants have shown promising results in enhancing plant tolerance to virus infections by improving plant fitness and productivity and modulating metabolic functions. In this study, the combination of two organic biostimulants, Alert D-Max and Resil EVO Q, composed of seaweed and alfalfa extracts, enzymatic hydrolysates, and micronized zeolite, was applied on the leaves and roots of zucchini squashes, both healthy and infected by zucchini yellow mosaic virus (ZYMV). Four applications were scheduled based on ZYMV inoculation timing, and plant vegetative and reproductive parameters were recorded along with the virus titre and symptom severity. The modulation of the expression of specific genes potentially involved in pattern-triggered immunity (PTI), systemic acquired resistance (SAR), and oxidative stress defence pathways was also investigated. Besides increasing the general fitness of the healthy plants, the biostimulants significantly improved the production of flowers and fruits of the infected plants, with a potential positive impact on their productivity. The repeated biostimulant applications also led to a one-tenth reduction in ZYMV titre over time and induced a progressive slowdown of symptom severity. Genes associated with SAR and PTI were up-regulated after biostimulant applications, suggesting the biostimulant-based priming of plant defence mechanisms. Due to the observed beneficial effects, the tested biostimulant mix can be an effective component of the IPM of cucurbit crops, acting as a sustainable practice for enhancing plant fitness and tolerance to potyviruses.

Keywords: zucchini yellow mosaic virus; potyvirus; Cucurbitaceae; plant fitness; plant defence pathways

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1. Introduction

Plant viruses are responsible for significant losses in agricultural crops worldwide, affecting both the quality and yield of crop products. The management of viral infections relies on preventive strategies, including virus-free propagation materials and resistant or tolerant varieties, and on the control of arthropod vectors. However, breeding challenges and the availability of resistance genes in many crops as well as the high vector efficiency represent concrete obstacles to the efficient control of virus diseases. Moreover, the use of agrochemicals against vectors is more and more discouraged due to the rise in resistant populations and its negative impact on environment and human health [1]. Indeed, regulatory frameworks are being established to achieve a reduction in the use of non-sustainable pesticides, as occurred in the European Union with the EU Sustainable Use Directive 2009/128/EC. Thus, other control and prevention approaches are being implemented in the frame of the Integrated Pest Management (IPM) of viral diseases. Among

these approaches, the use of organic-based substances, such as biopesticides, resistance inductors, biofertilizers, and biostimulants, is receiving increasing attention as a sustainable way to support traditional management strategies. This is due to the ever-increasing amount of evidence of their positive effects on plant growth and productivity as well as on plant tolerance to pathogens, including viruses [2,3].

Biostimulants are mixtures of microorganisms or organic substances, e.g., seaweed, botanicals, and biopolymers, and can be produced through several methods, such as acidification, hydrolysis, and extraction [4]. In Europe, the use and management of biostimulants in agriculture are regulated by EU regulation 1009/2019, which defines them as substances applied to plants that enhance nutrient availability in the rhizosphere, nutrition use efficiency, abiotic stress tolerance, and crop quality traits. According to this definition, any direct actions of biostimulants in adding fertility and inducing plant responses to biotic stress are excluded. However, these organic compounds can indirectly increase plant tolerance to pathogen infections and reduce the occurrence of severe symptoms by improving the fitness and defence systems of the target plant [5]. Indeed, recent studies recognize that most of the effects of seaweed extracts and other biostimulants rely on the modulation of plant metabolic pathways and not on the simple contribution to nutrient uptake [6]. Such positive effects have already been observed to act against several fungal diseases, such as downy mildew in grape and Fusarium wilt in tomato. In these case studies, the increased tolerance to pathogens of biostimulant-treated plants was associated with an increased production of phenols, flavonoids, and proteins and with the up-regulation of the expression of specific defence genes [7,8].

The regulation of plant defence pathways involved in the reaction to biotic stresses likely contributes to the mode of action of biostimulants [9]. Among these pathways, pattern-triggered immunity (PTI) is the first layer of defence which is triggered upon the perception of specific pathogen-associated molecular patterns (PAMPs) through cell surface recognition receptors. PTI activation leads to a cascade of signalling events that induce defence hormone biosynthesis, the expression of defence genes, and the physical reinforcement of cell walls [10,11]. The systemic activation of defence mechanisms in distant, uninfected plant tissues is known as systemic acquired resistance (SAR) and includes a primed state of defence-related genes, mainly those related to salicylic acid (SA) biosynthesis and responsive genes like *glutathione-s-transferase* (*GST*) [12–16]. A third defence mechanism is represented by the protection of plant tissues against reactive oxygen species (ROS) by means of the activation of ROS-scavenging enzymes, such as superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and ascorbate peroxidase (APX).

The effects of biostimulants on virus infections in plants are still poorly explored. Few single natural compounds have been observed to have a direct antiviral action, such as glucosylceramides and trichothecenes against tobacco mosaic virus (TMV) and pepper mottle virus, respectively [17,18]. The applications of complex mixtures are still at an early stage. It was recently observed that the application of seaweed extracts on tomato plants infected with tomato brown rugose fruit virus significantly improved yield and quality traits, such as the number and weight of fruit per plant, the fruit diameter, and yield per hectare [19]. In another study performed in protected conditions, the combination of two organic biostimulants induced beneficial effects on zucchini squashes (*Cucurbita pepo* L.) infected by tomato leaf curl New Delhi virus (ToLCDNV; genus *Begomovirus*, family *Geminiviridae*), a +ssDNA virus transmitted by the whitefly *Bemisia tabaci*. Biostimulant applications improved the vegetative parameters of infected plants, such as the leaf, flower, and fruit production, and enhanced the production of metabolites that are commonly involved in plant response to virus infection, such as carbohydrates, phenylpropanoids, and free amino acids [20].

Based on the encouraging results of Donati et al. [20], biostimulants with a similar composition were tested in this study against other economically important viruses of cucurbit crops: +ssRNA aphid-transmitted potyviruses (genus *Potyvirus*, family *Potyviridae*) [21,22]. The biostimulants were applied onto zucchini squashes infected by zucchini yellow mosaic virus (ZYMV), which affects several cucurbits, including pumpkin, squash, zucchini, melon,

watermelon, and cucumber [23]. The observed symptomatology in zucchini plants includes a severe mosaic and yellowing of leaves and the general stunting of the host plant. Fruits can also show symptoms, such as size reduction, twisting, and deformation, that negatively affect their marketability. Within no more than 1–2 weeks after infection, the plants stop production, facing a yield decrease of up to 90% and subsequent economic losses [24]. The management of ZYMV in *C. pepo* is currently achieved using resistant varieties and controlling aphid vectors with insecticides. However, virus resistance-breaking strains and insecticide-resistant aphid populations limit the effectiveness of such strategies that should always be used in combination with other control methods [25,26].

The biostimulants tested in this study contained a liquid extract of alfalfa (*Medicago sativa* L.), which is already known to be active against a wide range of crop pests thanks to its high content of flavonoids and saponins [27,28]. The biostimulants also contained molasses and seaweed extracts, especially brown algae, which are rich in bioactive compounds such as phytohormones, microelements, algal-specific polysaccharides, betaines, polyamines, and phenolic compounds [29]. These organic compounds were combined with an enzymatic hydrolysate of fabaceous plants, which provides free amino acids that are known to be recognized as auxin-similar signal molecules [30]. A liquid mixture of these compounds was applied at both the foliar and radical levels, in combination with micronized zeolite powder that plays an important role in water retention, and it is therefore crucial to maintain the biostimulants on the surface of the leaves and in the soil as long as possible [31]. The potential effects of these biostimulants on *C. pepo* tolerance to ZYMV infections were assessed by evaluating plant fitness and productivity and the possible activation of defence genetic pathways.

2. Materials and Methods

The effect of biostimulants on ZYMV infection in *C. pepo* was investigated by means of a series of experiments carried out in a greenhouse located at the Council for Agricultural Research and Economics—Research Centre for Plant Protection and Certification (CREA-DC)—in Rome (Italy) between spring and summer 2022.

2.1. Experimental Design

The experimental design included four treatments, healthy untreated (HU), healthy treated (HT), infected untreated (IU), and infected treated (IT) plants, composed of 12 plants each. The HT and IT plants were subjected to four biostimulant applications (T1–T4) which were scheduled based on the time of virus inoculation: 24 h before inoculation, 7 days post-inoculation (d.p.i.), 21 d.p.i., and 35 d.p.i. Foliar and root applications were carried out simultaneously.

To avoid cross-contaminations, the HU, HT, IU, and IT treatments were placed in separate boxes within the same greenhouse and were maintained at the following controlled conditions: 24 ± 1 °C, 16:8 h L:D, and 55% RH with watering as required. Three independent replicates of this experiment were performed in May, July, and August 2022; in this way, the assays were carried out on a total of 36 plants (i.e., biological replicates) per treatment. The HU, HT, IU, and IT treatments were randomly assigned to different boxes during the three independent replicates, in order to minimize possible environmental effects on the output of the experiments.

All plants were grown until 45 d.p.i., when the vegetative and reproductive parameters were recorded. During this time lapse, the leaf samplings for molecular analyses (virus titre assessment and gene expression analysis) and the symptom evaluation were performed at three intermediate time points, 8, 22, and 36 d.p.i. Such points correspond to 24 h after T2, T3, and T4, allowing for an assessment of the possible effect of each biostimulant application on fully developed plants.

2.2. Plant Material and Virus Inoculation

Zucchini seeds (*Cucurbita pepo* L. cv. Romanesco) were sown in fresh soil Completo® (Vigorplant, Fombio, Italy) using 10 cm diameter plastic pots. Plants were grown in a greenhouse under the controlled conditions described above.

The ZYMV isolate 32 from the CREA-DC collection served as the virus inoculum [32]. The isolate was regularly propagated through mechanical inoculation in zucchini plants, which tested negative for the major cucurbit viruses (watermelon mosaic virus, WMV; cucumber mosaic virus, CMV; papaya ringspot virus, PRSV; Moroccan watermelon mosaic virus, MWMV; and ToLCNDV), to exclude any cross-contaminations. Mechanical inoculation was performed as follows. ZYMV-infected young leaves were ground with phosphate buffer (1:5, *w*/*v*) in extraction bags (Bioreba AG, Reinach, Switzerland), and leaf sap (20 µL) was used to inoculate healthy plants about three days after germination: the sap was applied onto fully expanded cotyledons by rubbing them with Celite® 545 (Sigma-Aldrich Corp., St. Louis, MO, USA). After four minutes, the cotyledons were rinsed with water to eliminate the excess of infected sap and to avoid the dehydration of rubbed surfaces. The same procedure was used also to inoculate the zucchini plants used in the experimental trials.

2.3. Biostimulant Applications

The biostimulants Alert D-Max and Resil EVO Q, both marketed by ISLA Fitonutrizione (Tarquinia, Italy), were used in this study. Alert D-Max is a liquid extract of alfalfa, brown algae, and molasses with the following chemical composition: 1% organic nitrogen, 10% organic carbon, 6% potassium oxide, and 1% betaine. Resil EVO Q is an enzymatic hydrolysate of fabaceous plants containing 5% total amino acids, of which 1.5% represents free amino acids. Clinogold (ISLA Fitonutrizione, Tarquinia, Italy), a micronized zeolite powder composed of 95% clinoptililote of about max 17 µm particles, was added to the liquid mixture. These three products were applied as a unique solution on both the leaves and roots of the zucchini plants, according to the manufacturer's recommendations. For foliar application, a 0.4% Alert D-Max/0.3% Resil EVO Q/0.25% Clinogold (*v*/*v*/*w*) solution was sprayed through a nebulizer, taking care to wet both the leaf blades uniformly. For root application, 40 mL of a 5.4% Alert D-Max/5.4% Resil EVO Q/0.025% Clinogold (*v*/*v*/*w*) solution was distributed directly onto the soil of each pot.

2.4. Recording of Plant Vegetative and Reproductive Parameters

The following vegetative and reproductive parameters were recorded at 45 d.p.i. to evaluate the effect of biostimulants on plant fitness: the number of fully developed leaves, total plant biomass, number of flowers calculated as the sum of buds and developed flowers, and number of fruits > 2 cm in length. The total biomass produced by the plants (leaves, stems, and flowers) was measured as the total dry weight of all the plants from each treatment (HT, HU, IT, IU) after a dehydration process (60 °C for 48 h).

2.5. Evaluation of ZYMV Symptom Expression

The severity of ZYMV symptoms was evaluated on the zucchini leaves of IU and IT plants at 8, 22, and 36 d.p.i., using the rating scale described by Radwan et al. [33]: 0 = no symptoms; 1 = chlorotic local lesions and mild mosaic; 2 = severe mosaic; and 3 = blisters and malformations. At each time point, the score assigned to the IU and IT treatments corresponds to the value observed in >60% of IU/IT plants across the three experimental replicates (i.e., 36 plants per treatment). Concerning the fruits, their absence or reduced development did not allow for symptom evaluation at any time point.

2.6. Plant Sampling and RNA Extraction

Leaf samples for Total RNA (TRNA) extraction were collected 24 h after each biostimulant application, starting from T2. A disk from the last-expanded true leaf of each plant was taken at each time point; then, the disks from three plants per treatment were pooled

together and analysed as a single sample. Overall, four samples per treatment (HT, HU, IT, IU) were analysed at three time points in the three experimental trials. TRNA was extracted from these samples using the RNeasy Plant Mini Kit (Qiagen, Milan, Italy), and it was treated with DNase I Amplification Grade (Invitrogen Corporation, Waltham, MA, USA) following the manufacturer's instructions. RNA concentration was then measured with a NanoDrop™ spectrophotometer (ThermoFisher Scientific, Milan, Italy). The DNA-depleted RNA served as a template for both virus quantification and gene expression analyses.

2.7. Quantification of Virus Titre

Reverse transcription TaqMan® Real-Time PCR (RT-qPCR) assay was used for the relative quantification of ZYMV in the IU and IT samples using primers and probe targeting the ZYMV *coat protein* (*CP*) gene [34] (Supplementary Table S1). The housekeeping elongation factor *EF-1α* gene of *C. pepo* L. was used as an endogenous reference gene [35] (Supplementary Table S1). The RT-qPCR was carried out in a final volume of 10 µL, by adding 25–60 ng of the DNase-treated TRNA to the following mix of reagents: 2X TaqMan™ RT-PCR Mix; 40X TaqMan™ RT Enzyme Mix (TaqMan™ RNA-to-CT™ 1-Step Kit, Life Technologies, Milan, Italy); 300 nM of each primer; and 100 nM of probe. The thermal profile was the same for the amplification of both the ZYMV-*CP* and *EF-1α* genes: 15 min at 48 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C, and 1 min at 60 °C. Two technical replicates were performed for each sample, and HU plant samples served as the negative control. The RT-qPCRs were performed in a CFX96 Touch RT-PCR System (Bio-Rad, Milan, Italy). The relative virus titre was calculated by the software Bio-Rad CFX Maestro 1.1, version 4.1.2433.1219, 2017 (Bio-Rad, Milan, Italy), using the normalized expression method ($2^{-\Delta\Delta Ct}$) [36]; IU plants were considered as the control, and *EF-1α* served as the reference gene.

2.8. Analysis of Relative Expression of Defence-Related Plant Genes

The expression level of ten genes involved in the plant defence pathways of *C. pepo* L. was assessed in the HU, HT, IU, and IT plants, including six genes from the SAR pathway—*phenylalanine ammonia-lyase* (*PAL*), *pathogenesis-related gene 1* (*PR1*), *pathogenesis-related gene 2* (*PR2*), *nonexpressor of PR genes 1* (*NPR1*), *glutathione transferase* (*GST*), and *salicylic acid-binding protein 2* (*SABP2*)—three genes from the oxidative stress defence system—*peroxidase dismutase* (*POD*), *superoxide dismutase* (*SOD*), and *catalase* (*CAT*)—and one gene from the PTI pathway—*wrky transcription factor 29* (*WRKY29*). The expression level of these genes was analysed by means of a two-step SYBR Green® real-time RT-PCR assay (RT-qPCR). DNase-treated TRNA was first reverse-transcribed to cDNA. The reaction was carried out in a final volume of 18 µL, by adding 2 µL of TRNA to a mix composed of 5X first-strand buffer (Invitrogen Corporation, Waltham, MA, USA), 5 µM random primers (Promega Corporation, Madison, WI, USA), 10 µM dNTPs, and 100 U M-MLV Reverse Transcriptase (Promega Corporation, Madison, WI, USA). The reaction mix was incubated for 45 min at 42 °C and 3 min at 94 °C. cDNA was then amplified using primer pairs specific for each selected gene, which are already known to be expressed in *C. pepo* L. [37–42] (Supplementary Table S2). The housekeeping *EF-1α* gene was used as a reference [35]. The 10 µL qPCR reaction mix contained 2X SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Milan, Italy), 300 nM of each primer, and 1 µL of cDNA. The amplification thermal profile was the same for each primer pair: 3 min at 95 °C, 40 cycles at 95 °C for 10 s and at 58 °C for 30 s, and a final ramp of 0.5 °C from 65 to 95 °C for the melt curve peak analysis to assess the specificity of the SYBR Green amplification. Each sample was assayed in two technical replicates. The gene expression level was calculated according to the $2^{-\Delta\Delta Ct}$ method, by setting *EF-1α* as the reference gene and the IU plants as the control.

2.9. Statistical Analysis

Each vegetative and reproductive parameter (i.e., number of leaves, flowers, and fruits and dry biomass) was expressed as the mean value of the twelve plants representing the biological replicates of each treatment, HU, IU, HT, and IT. An analysis of variance (ANOVA) was performed among the four treatments from the three independent experimental repeats. Significantly different treatments were identified by a pairwise comparison of the means using Tukey's post hoc test.

The ΔΔCt values for the virus titre and plant gene expression for each of the three treatments (HU, HT, IT) relative to the control (IU) from the three experimental repeats were evaluated for significance by calculating the confidence intervals at $p < 0.05$ [36]. Values were considered significant, thus implying up- or down-regulation, if the fold change value (i.e., $2^{-\Delta\Delta Ct}$) was above 2.0 (up-regulation) or below 0.5 (down-regulation) and if, concurrently, the confidence interval did not comprise the value of 1 (no regulation).

All statistical analyses were performed using Rstudio (Posit team. RStudio: Integrated Development Environment for R. Posit Software, version 2024.04.2, PBC, 2024).

3. Results

Several crop traits were compared between healthy and ZYMV-infected plants, which were treated or untreated with the biostimulants Alert D-Max and Resil EVO Q. In summary, treated plants had increased growth with higher values of reproductive parameters (number of flowers and fruits) even in infected conditions. After treatment, a decrease in ZYMV titre and a progressive slowdown of symptom severity were observed in plants, together with the up-regulation of some defence genes.

3.1. Effects of Biostimulants on Plant Vegetative and Reproductive Parameters

The vegetative and reproductive parameters of the plants from the HU, IU, HT, and IT treatments were recorded at the end of each experimental trial (45 d.p.i.) and statistically analysed by an ANOVA. Concerning the vegetative parameters, the biostimulant applications had positive effects on healthy plants that showed the highest values of both leaf number and total dry weight. The infected treated plants also showed good performances, but the values of both vegetative parameters did not significantly differ from those of the untreated plants (Figure 1). In detail, the mean number of leaves produced by both healthy and ZYMV-infected plants after biostimulant applications was higher than that of the corresponding untreated plants, but this increase was significant only between the HT and IU plants (Figure 1a). The healthy treated plants also showed the highest value of the total dry weight; this value significantly differed from the values of both healthy and infected untreated plants, whereas infected treated plants showed intermediate dry biomass (Figures 1b and 2).

Significant positive effects of the biostimulants were also observed on the production of the reproductive organs of the plant, both flowers in pre- and post-anthesis and fruits. The mean number of flowers did not differ between the healthy plants, treated and untreated, and this value was also comparable to that of the infected treated plants, whereas it was significantly lower in the infected untreated plants (Figure 3a). Concerning the mean number of fruits recorded after biostimulant application, healthy plants showed the highest values, and infected plants showed numbers that were comparable to those of the healthy untreated plants and were significantly higher than those of the infected untreated plants (Figure 3b).

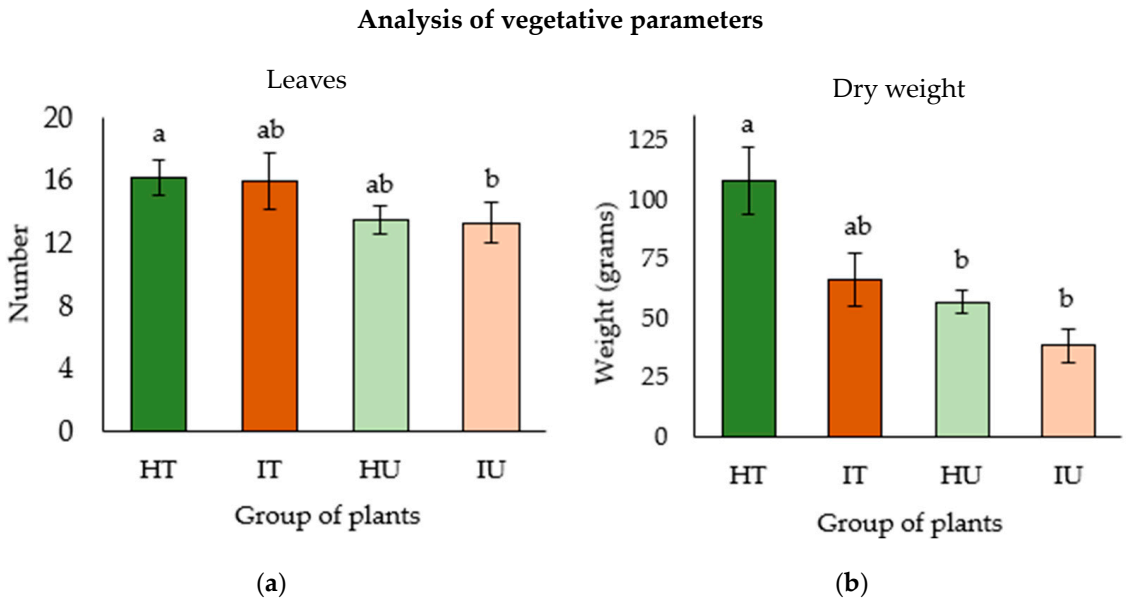

Figure 1. Analysis of vegetative parameters. (**a**) Number of leaves and (**b**) dry weight (grams) produced by healthy treated (HT), infected treated (IT), healthy untreated (HU), and infected untreated (IU) plants at 45 d.p.i. Results are expressed as mean values ± standard error (±SEM) recorded from 12 plants per treatment in three independent experimental trials. Different letters indicate statistically significant differences (Tukey test, p value < 0.05).

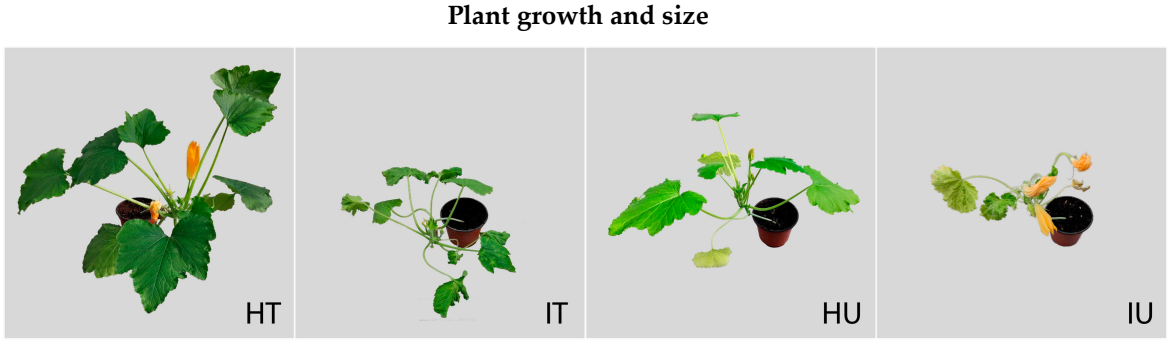

Figure 2. Plant growth and size. This figure shows 35-day old plants representative for the biomass produced within each of the four treatments: healthy treated (HT), infected treated (IT), healthy untreated (HU), and infected untreated (IU) plants.

3.2. Evaluation of Symptom Severity

Both treated and untreated infected plants did not show any symptoms at 8 d.p.i. (score = 0), when the second true leaf of the zucchini plants was fully expanded. The first symptoms were observed at 22 d.p.i., when the leaves of all the infected plants showed severe mosaic (score = 2) irrespective of biostimulant application. The untreated plants also started to show the first effects of leaf filiformism. At 36 d.p.i., the treated plants still showed leaf mosaic (score = 2), whereas the symptoms of the untreated plants worsened further, the leaves being characterized by more intense vein clearing and blister deformations (score = 3). No symptoms were observed in healthy treated and untreated plants at 36 d.p.i. (Figure 4).

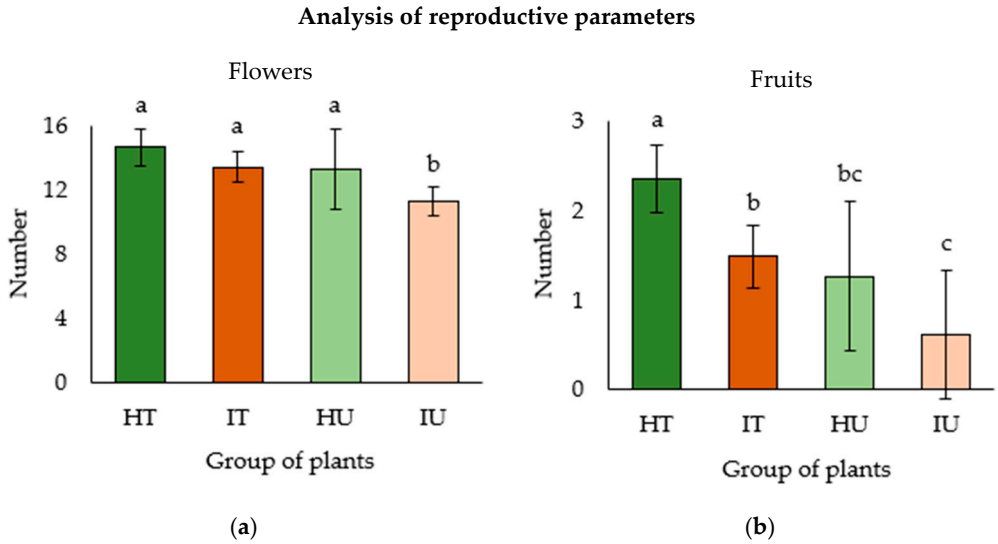

Figure 3. Analysis of reproductive parameters. Number of (**a**) flowers and (**b**) fruits produced by healthy treated (HT), infected treated (IT), healthy untreated (HU), and infected untreated (IU) plants at 45 d.p.i. Results are expressed as mean values ± standard error (±SEM) recorded from 12 plants per treatment in three independent experimental trials. Different letters indicate statistically significant differences (Tukey test, p value < 0.05).

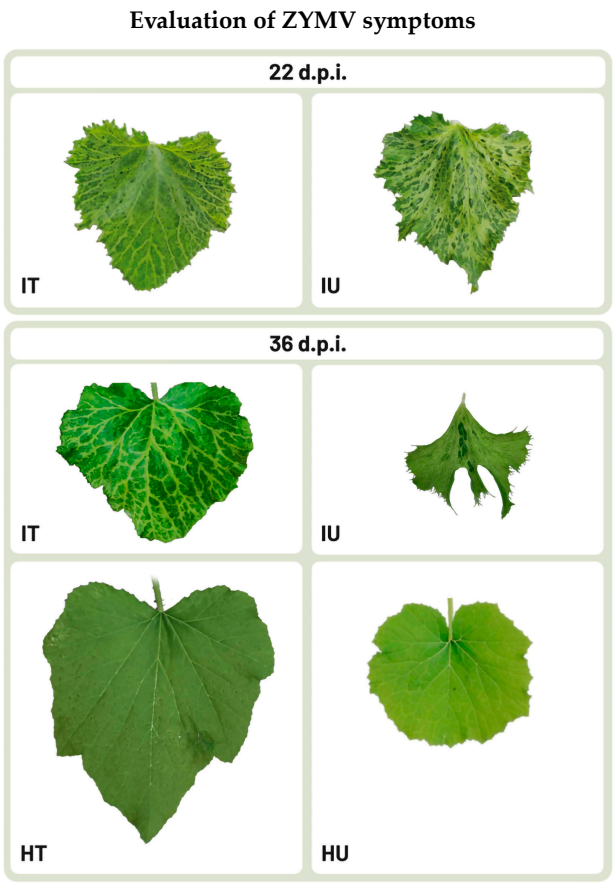

Figure 4. Evaluation of ZYMV symptoms. Symptoms observed in ZYMV-infected treated (IT) and ZYMV-infected untreated (IU) plants at 22 and 36 d.p.i. No symptoms were observed in healthy plants (healthy treated, HT, and healthy untreated, HU) at 36 d.p.i.

3.3. Effects of Biostimulants on Virus Titre

ZYMV titre was assessed by RT-qPCR in infected treated and untreated plants at 8, 22, and 36 d.p.i. The virus was detectable at 8 d.p.i., indicating that the infection occurred systemically despite the lack of symptoms. At this early stage of infection, the relative ZYMV titre value in treated plants was only slightly lower than that of the untreated ones. The ZYMV titre decreased in IT plants compared to IU plants over time, being around one-half at 21 d.p.i. and one-tenth at 35 d.p.i. when the difference became significant (Figure 5).

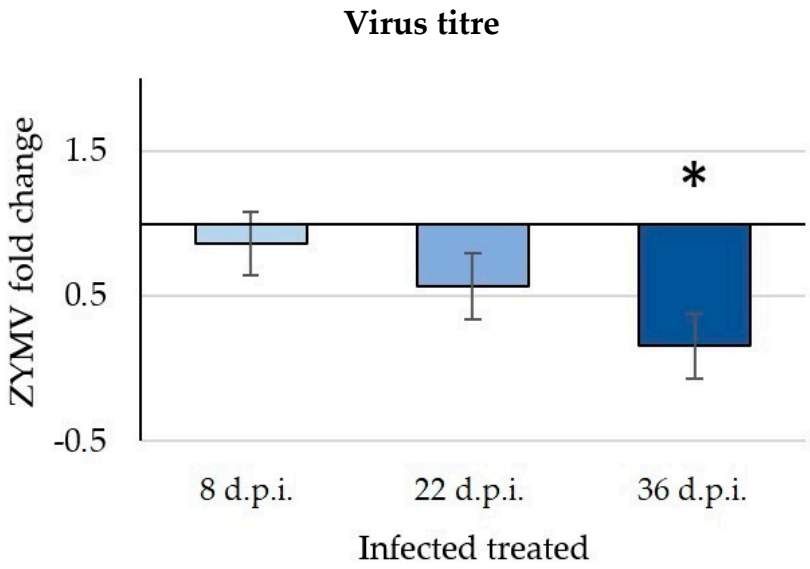

Figure 5. Analysis of ZYMV titre. Relative quantification of ZYMV at 8, 22, and 36 d.p.i. measured by using $2^{-\Delta\Delta Ct}$ method and setting ZYMV-infected untreated samples (IU) as control and *EF-1α* as reference gene (*EF-1α* expression in IU = 1). Results are expressed as mean values ± standard error (±SEM) obtained from two technical replicates of three pooled biological replicates produced in three independent experimental repeats. (*) indicates significant fold changes, i.e., values above 2.0 (up-regulation) or below 0.5 (down-regulation) with confidence interval that did not comprise value of 1 (no regulation) at $p < 0.05$.

3.4. Expression Level of Defence-Related Plant Genes

The expression of ten genes related to plant defence response (*PAL*, *PR1*, *PR2*, *NPR1*, *GST*, *SABP2*, *POD*, *SOD*, *CAT*, and *WRKY29*) was analysed at 8, 22, and 36 d.p.i. Although all the tested genes were differently expressed in all samples at every collection time, only two of them showed significant differences between the control (IU) and the infected treated plants (IT) at 36 d.p.i. At this sampling time, the expression level of the ROS-scavenging defence *POD* gene increased in both the HT and IT plants, being around twice and three times as high compared to the IU plants, respectively (Figure 6a). Nevertheless, only the relative expression of IT plants was significantly different from the control. The IT plants also showed a significant up-regulation of the *PR1* gene, which was ten-fold higher than the IU plants (Figure 6b).

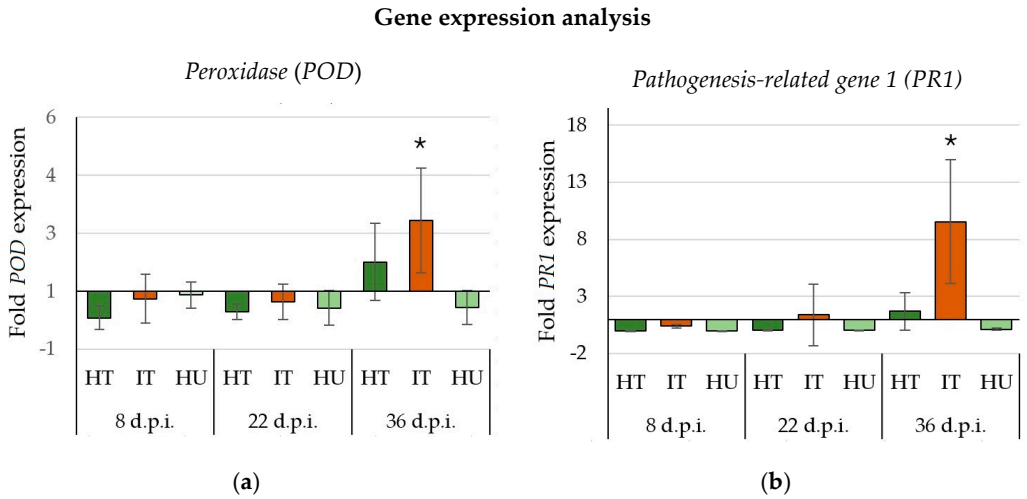

Figure 6. Gene expression analysis. Relative expression of (**a**) *peroxidase* (*POD*) and (**b**) *pathogenesis-related gene 1* (*PR1*) at 8, 22, and 36 d.p.i. measured by using $2^{-\Delta\Delta Ct}$ method and setting ZYMV-infected untreated samples (IU) as control and *EF-1α* as reference gene (*EF-1α* expression in IU = 1). Results are expressed as mean values ± standard error (±SEM) obtained from two technical replicates of three pooled biological replicates produced in three independent experimental repeats. (*) indicates significant fold changes, i.e., values above 2.0 (up-regulation) or below 0.5 (down-regulation) with confidence interval that did not comprise value of 1 (no regulation) at $p < 0.05$.

4. Discussion

Organic substances are known to induce positive effects on the growth and productivity of several crops, including cucurbits [43]. Foliar and/or soil applications of seaweed extract and humic acid substances significantly increased the fruit yield and quality of cucumber plants compared to traditional mineral fertilization and improved the response of zucchini plants to abiotic stresses, such as salinity excesses [44–48]. Few pieces of information are available about the potential impact of biostimulants on the multiple biotic stresses that cucurbits can face, such as fungal, oomycete, bacterial, and viral diseases. The beneficial effects of organic substances observed on ToLCNDV-infected zucchini squashes represents the first evidence of the potential action of biostimulants in priming cucurbit defences against pathogens [20]. Also, in our experimental conditions, these biostimulants significantly improved the fitness of healthy and ZYMV-infected zucchini plants. Indeed, after biostimulant application, both the vegetative and reproductive parameters of the healthy plants showed the highest numbers of leaves and fruits, resulting in a total biomass that was significantly higher than that of the healthy untreated plants. The increase in the number of fruits is not correlated with significant differences in the number of flowers. This could suggest a beneficial effect of the biostimulants in reducing flower dropping and/or favouring fruit setting. In the case of infected plants, the biostimulants also mitigated the impact of ZYMV on the flowers observed in the IU plants (i.e., abortion or drying or fall) and in turn improved the fruit set so that at the end of the experiments, the number of fruits in the IT plants was significantly higher than that in the IU plants and comparable to that in the healthy plants. The general well-being induced by biostimulants can support the productivity of the ZYMV-infected zucchini plants. In a large-scale perspective, field applications could contribute to maintaining the productivity of zucchini crops even in the presence of a potyvirus infection and thus reduce yield losses to economically sustainable levels. Such a kind of improvement in crop production has already been observed on ToBRFV-infected tomato plants, which showed an increase in both fruit quality and yield per hectare when treated with commercial biostimulants [19].

The physiological changes observed in ZYMV-infected zucchini squashes after biostimulant applications are also in accordance with variations in the estimates of the virus titre over time and in the transcriptomic data. The systemic circulation of ZYMV was

observed in both the treated and untreated plants, the virus being detected in new and completely expanded leaves that developed one week after inoculation. Then, the virus titre progressively decreased in the treated plants compared to the untreated ones, with the lowest value measured at the last sampling, 36 d.p.i. This trend suggests that repeated biostimulant applications could affect the replication of the virus in the host plant over time. The reduction in the ZYMV titre likely influenced the symptomatology in treated plants that showed a progressive slowdown of symptom severity. At 22 d.p.i., leaf mosaic was evident in both the treated and untreated plants, the latter also being slightly distorted. At 36 d.p.i., the untreated leaves further showed more intense vein clearing along with blistering and deformations, whereas the treated leaves maintained a symptom score comparable to that at the previous time point.

After assessing the potential effects of biostimulants on both plant fitness and virus titre, possible metabolic pathways involved in plant defence responses were investigated. No information on the transcriptional response of *C. pepo* upon the infection of potyviruses, and plant viruses in general, was available. Thus, possible target genes were selected among those involved in the response to fungi infections [42]. Ten genes involved in the SAR, PTI, and ROS defence pathways were analysed for their expression in healthy and ZYMV-infected plants with and without biostimulants. Among these, the *POD* and *PR1* genes were found to be up-regulated in infected treated plants compared to the untreated ones, providing the first insights into the gene responses to viral infection and the modulation by biostimulants. The *POD* gene encodes for one of the most important ROS-scavenging enzymes involved in the mitigation of oxidative stress [49]. Namely, this enzyme catalyses the conversion of hydrogen peroxide to water and oxygen, reduces ROS levels, and protects cellular components from oxidative damage and thus contributes to maintaining cellular integrity [50]. The *PR1* gene plays a key role in the SAR system by contributing to the synthesis and accumulation of salicylic acid [51]; moreover, it encodes for a small-sized, cysteine-rich protein that reinforces cell walls to hinder pathogen penetration [52]. For both genes, the expression levels did not significantly differ from those of the healthy untreated plants at 8 and 22 d.p.i. The increase in the transcript products was observed only from 36 d.p.i., after the last biostimulant application. At this time point, the expression of the *POD* gene increased in both healthy and infected plants, suggesting a basal priming effect of the biostimulants; nevertheless, *POD* up-regulation was particularly evident in the infected treated plants which showed transcript levels significantly higher than those of the untreated plants. The up-regulation of the *POD* and *PR1* genes in *C. pepo* was already observed in correlation with fungal diseases [8] and can now also be associated with the response to potyvirus infections. Organic-based biostimulants enhance the expression of these genes in a manner which seems to be time- and/or dose-dependent, since the up-regulation is significant only after one month and repeated applications.

Biostimulants are known to trigger plant defence priming rather than play a direct role against pathogens [5]. Also, in this study, the observed up-regulation of the *POD* and *PR1* genes could suggest a priming activity through the SAR and ROS pathways. Thus, the observed effects on plant fitness and ZYMV replication could likely be ascribable to the enhancement in plant defence response rather than to the direct inhibition of virus replication. The fact that the defence response occurs later in plant growth and after repeated applications could hamper complete prevention and control actions against infection. This delayed effect would confirm that biostimulants should not be considered as a unique solution for the complete protection of crops, but one of the possible tools to achieve an increase in productivity. Thus, the use of biostimulants should be suggested as an advantageous way to support other management strategies, both conventional and alternative ones, in the frame of IPM.

5. Conclusions

This study provided evidence of the beneficial effects of biostimulants (Alert D-Max and Resil EVO Q solution) on zucchini plants in terms of both the productivity and elicita-

tion of defence response to ZYMV infections. The positive impact of organic biostimulants on the fitness of zucchini plants was already proven in the case of abiotic stress or begomovirus infection. With the present work, this effect was also seen in plant responses to ZYMV infection, and it could potentially be extended to other potyviruses infecting cucurbits with similar infection mechanisms. These results encourage the implementation of such bioactive compounds into cucurbit production systems, as a tool for enhancing plant fitness, productivity, and tolerance to different sources of both abiotic and biotic stresses.

The economic and practical feasibility of the large-scale application of the biostimulant mix has already been ascertained since these commercial products are commonly used in greenhouse and open-field cultivations to enhance plant nutrition. Thus, the next steps will be to verify their effectiveness against potyvirus infections in field trials and to assess the long-term increase in productivity. In view of their potential large-scale performance and their very few or absent side effects, these biostimulants could be considered in the future as a useful option to develop sustainable IPM strategies and green horticultural productions.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/horticulturae10111176/s1, Table S1: Sets of primers and probes specific for ZYMV (*CP* gene) and *elongation factor* (*EF*) gene used in qPCR for virus titre analysis; Table S2: Sets of primers for targeted plant defence genes and *elongation factor* (*EF*) gene used in qPCR for relative expression of defence gene analysis.

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