



Article

Montmorillonite Nanoclay and Formulation with *Satureja montana* Essential Oil as a Tool to Alleviate *Xanthomonas euvesicatoria* Load on *Solanum lycopersicum*

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Abstract: Bacterial spot (BS) of tomato (*S. lycopersicum*), caused by *Xanthomonas* spp., namely *X. euvesicatoria* (Xeu), is one of the major threats for the production of this crop worldwide. Developing new biocontrol solutions against this disease will allow disease management strategies to be less based on Cu compounds. Nanoclays, such as montmorillonite (NMT), have been under investigation for their antimicrobial activity, or as delivery tools/stabilizers for organic compounds, such as essential oils (EOs), that also possess antimicrobial activity against plant pathogens. This work aims to assess how the application of NMT alone or incorporating *S. montana* EO on Xeu-infected hosts (var. Oxheart) affects the shoots' redox status and antioxidant defense mechanisms. In vitro shoots, grown on Murashige and Skoog medium, were divided in two groups, Xeu-infected and uninfected (control) shoots. Shoots of each group were then treated with NMT, *S. montana* EO, EO-NMT. Results show that the NMT was able to reduce Xeu bacterial amount, while reducing ROS production and keeping the transcript levels of the defense-related genes close to those of the control. When applied to uninfected shoots, the treatments triggered the production of ROS and upregulated the phenylpropanoid and hormone pathway, which suggest that they act as defense elicitors. Globally, the results indicate that NMT has the potential to integrate BS management strategies, due to its antimicrobial activity, and that EO and/or nanoclays could be successfully employed as new disease preventive strategies, since they enhance the healthy shoots' defense, thus potentially limiting the pathogen establishment.

Keywords: *Satureja montana*; nanoclay; *Solanum lycopersicum*; bacterial spot; oxidative stress; antimicrobial natural products; phenylpropanoid pathway; hormone pathways

1. Introduction

Nowadays, phytopathogen management relies mostly on copper-based bactericides and antibiotics, and although these are affordable and easy-to-use products, with a broad-spectrum action [1,2], the continuous application of these strategies led to the appearance of resistant bacterial strains [3]. Furthermore, European Union directives forbid the use of antibiotics to control phytopathogens, and promote the reduction of copper usage, and its

replacement by natural and sustainable alternatives. Nanomaterials and nanostructures, such as the nanoclay montmorillonite (NMT), have been under investigation due to their antimicrobial activity and ability to act as carriers for antimicrobial products. Furthermore, other inorganic clays (e.g., kaolin) have been under the spotlight for their capacity of incorporating antimicrobials, while reducing the phytotoxicity of these compounds and improving their antibacterial activity [4]. However, studies on the effect of such clays on a pathosystem are sparse.

Nanoclays are inorganic porous materials of high interest for their capacity to incorporate organic compounds, namely essential oils (EOs), and for controlled release [5], with NMT being one of the most promising for EOs delivery. NMT is a phyllosilicate mineral that is composed of aluminum and silica in a laminar structure, which confers a high surface area, making it ideal for EOs' incorporation between the aluminum and the silica layers [5]. Other research has demonstrated that the active compounds of EOs and NMT form hydrogen bonds. Furthermore, the hydrophobic interactions are also important for EOs incorporation in NMT; this hydrophobicity can be altered by adding surfactants, such as Tween20[®], to the mixture by increasing the space between the layers and facilitating the intercalation of hydrophobic molecules between these layers (e.g., EOs components) [6]. These nanoclays are naturally abundant, affordable, and usually considered non-toxic [7]. They can also increase the stability and shelf-life of some active compounds and may improve the antimicrobial activity of EOs to be used in the agri-food industry [8–10]. Their antimicrobial activity, combined with their ability to act as carriers of other bioactive compounds, make NMT an outstanding candidate to be used in agriculture, namely against phytopathogens [11,12]. However, their interaction with crops and the mechanisms involved in plant defenses are not yet understood.

EOs are highly concentrated mixtures of volatile compounds with low molecular weight, extracted from several parts of plants (i.e., stems, leaves, seeds, fruits, and flowers) [13,14]. Some of these EOs have well-characterized antimicrobial properties, mainly due to the presence of phenylpropene derivatives (e.g., eugenol, methyl eugenol, anethole, estragole) and terpenoids (e.g., limonene, linalool, carvacrol, and thymol) [15,16]. The antimicrobial activity of these botanical products has been assessed mainly against food borne diseases [17–19]. Recent studies have also uncovered that the EOs present antimicrobial activity against phytopathogens, namely *Xanthomonas* spp. [20,21]. Few of the EOs have been tested yet against the *Xanthomonas* strains causing the bacterial spot disease (BS). For example, the EOs from the *Satureja* genus, namely *S. spigera* and *S. hortensis* [22,23], have revealed antimicrobial potential against those bacterial pathogens. Thus, other *Satureja* species with a high terpene and phenol content (e.g., *S. montana*) should be assessed regarding their antimicrobial potential against BS-causing *Xanthomonas* spp. For example, *S. montana* (winter savory) is highly rich in EOs [24], that are majorly composed of terpenoids (i.e., carvacrol, linalool, and thymol) and has antimicrobial activity against bacterial pathogens (e.g., *Staphylococcus aureus*) [25].

Despite all the potential of EOs to act as a viable alternative to conventional pathogen control practices, their application still requires some improvements to be made, mainly regarding the volatility of some of the EO compounds [26]. Recently, some of the carriers or delivery systems (e.g., nanocomposites) have demonstrated a reduction in EO volatilization, while increasing its solubility in aqueous solvents and improving antimicrobial activity [26,27]. Some other promising candidates, such as nanoclays, may act as EO delivery tools [28].

Xanthomonas is a bacterial genus that infects a wide variety of crops and wild plants, being responsible for diseases that cause significant economic loss [29]. Tomato (*Solanum lycopersicum* L.), one of the most important vegetable crops worldwide (FAOSTAT, 2019), is ubiquitously threatened by bacterial diseases, among which is the bacterial spot of tomato [30]. This disease is caused by four species of xanthomonads (*Xanthomonas euvesicatoria*; *X. vesicatoria*; *X. perforans*; *X. gardneri*) [31]. Among these, *X. perforans* and *X. gardneri* only cause disease in tomato, while *X. euvesicatoria* (Xeu) and *X. vesicatoria* affect other crops (e.g., peppers) [32]. BS affects the aerial parts of the plant (leaves and stems) with the

appearance of dark lesions, leaf yellowing, ultimately defoliation occurs and lesions appear on the fruit [33,34]. The *Xanthomonas* strains isolated from several crops (e.g., *Phaseolus vulgaris* and *Capsicum annum*) were reported as having resistance to several antibiotics and metals (including Cu) [35]. Hence, it is pivotal to find new natural control measures for this kind of phytopathogen.

Therefore, this work aims to explain the effects of NMT and *S. montana* EO-NMT, after 3 days of application to in vitro tomato shoots inoculated with Xeu, at a molecular and physiological levels. This study provides new knowledge on the means of NMT and EO-NMT formulations, in tomato tissues and their antimicrobial effectiveness against Xeu when applied to the leaves, ultimately shedding light on its potential application as a disease management tool in plant conditions. These data will contribute to the evaluation of the the potential of nanoclays and formulations as green, environmentally friendly alternatives for Xeu management in tomatoes. This work also elucidates the potential use of NMT as an antimicrobial or EO-nanocarrier.

2. Materials and Methods

2.1. Essential Oil and Formulation Preparation

A certified, organic essential oil from *S. montana* was purchased from Florihana Distillerie (Caussols, France), and its density at $-20\text{ }^{\circ}\text{C}$ was 1.02 g cm^{-3} . A simple EO–water emulsion was prepared by mixing *S. montana* EO ($3.92\text{ }\mu\text{L}$) with Tween20[®] ($3.63\text{ }\mu\text{L}$) up to a final concentration of 0.4 mg mL^{-1} and a final volume of 10 mL . The concentration used was selected based on our previous studies (unpublished), grounded on the minimal bactericidal concentration (MBC), which was the same concentration used here. For the EO–water emulsion, sterile MilliQ water was slowly added while vortexing at 3200 rpm (VWR, Radnor, PA, USA). For the formulation, montmorillonite (NMT) was added to a final concentration of 1% (w/v) (0.1 g) and vortexed for 2 min at 3200 rpm (VWR, Radnor, PA, USA), with a final volume of 10 mL . Finally, the ultrasonic waves were applied to the formulation using an ultrasonic cell disruptor equipped with a microtip (MicrosonTM) ($10\text{ cycles of }5\text{ s}$ with a 10 s pause between cycles at 10 W).

2.2. Shoot Culture, Inoculation and Treatments

The tomato seeds (*S. lycopersicum* var. Oxheart), purchased from a commercial supplier (Vilmorin, France), were surface disinfected with a solution of 40% (v/v) commercial bleach (Pavão©) for 20 min . The seeds were then washed three times in sterile deionized H_2O and placed in $1/2\text{ MS}$ medium (30% (w/v) sucrose; 0.7% (w/v) agar; $\text{pH} = 5.6\text{--}5.8$) for germination. Two weeks after germination, the shoots were transferred to MS media (0.5 mg L^{-1} benzyladenine (BAP); 30% (w/v) sucrose; 0.7% (w/v) agar; $\text{pH} = 5.6\text{--}5.8$) for shoot proliferation. The incubation took place in a plant-growth chamber at $24 \pm 0.5\text{ }^{\circ}\text{C}$, with a maximum photosynthetic light intensity of $250\text{ }\mu\text{mol m}^{-2}\text{ s}^{-1}$, under a $16:8\text{ h}$ (light: dark) photoperiod. Every two weeks, the shoots were used as a source of new shoots. After three months, the shoots were sub-cultured to MS medium without BAP supplementation for 7 days . Then, the shoots with a length of 10 cm were selected and randomly divided into groups for individual treatments as follows: (A) uninfected (negative control)–C; (B) infected with Xeu (positive control)–X; (C) uninfected + treated with *S. montana* EO–S; (D) infected + treated with *S. montana* EO–XS; (E) uninfected + treated with formulation–SNMT; (F) infected + treated with formulation–XSNMT; (G) uninfected + treated with NMT nanoclay–NMT; (H) infected + treated with NMT nanoclay–XNMT.

A reference strain of *X. euvesicatoria* LMG 905 was obtained from the BCCM/LMG bacterial collection (Ghent University, Belgium) and preserved at $-80\text{ }^{\circ}\text{C}$ in 30% (v/v) glycerol, at the MDE laboratory collection (FCUP, Portugal). The bacteria were cultivated in yeast extract-dextrose- CaCO_3 (YDC) medium. For infection, shoots were dipped (for 30 s) in a suspension of *X. euvesicatoria* LMG 905 in PBS (phosphate saline buffer, $\text{pH} = 7$) with an optical density at 600 nm (OD_{600}) of 0.1 A.U. After that, the shoots were transferred to new sterile boxes with MS-agar medium. Three days after the infection, the treatments were

carried out by spraying the adaxial page of the leaves with 1 mL of the treatment solution, according to the conditions explained above. Sterile MilliQ water was used for the positive (infected) and the negative controls. One day after the application of the treatments, the shoots were powdered in liquid N₂ and preserved at −80 °C for all further analysis. Two temporal and independent experiments were performed, with 10 shoots per condition.

2.3. *X. euvesicatoria* Quantification

The *X. euvesicatoria* was quantified by RT-PCR using the CFX96™ Real-Time PCR Detection System (BioRad, Hercules, CA, USA), and following the method described by Moretti et al. [36], with the following primers (xeu2.4–CTGGGAACTCATTCGCAGT, Forward; xeu2.5–TTGTGGCGCTCTTATTTCCT, Reverse), with a final product size of 208 base pairs (bp). For each reaction, 10 µL of NZYSpeedy qPCR Green Master Mix (2×), ROX plus (NZYTech™, Lisbon, Portugal), 0.8 µL of forward and reverse primer (10 mM), 0.4 µL of DEPC-treated H₂O and 8 µL of DNA template were used. The following conditions were used for the amplifications: 95 °C for 1 min followed by 40 cycles of 5 s at 95 °C and 15 s at 60 °C. The melting curve analysis was performed, beginning at 65 °C and ranging to 95 °C with an increment of 0.5 °C per each 5 s cycle. The reactions were performed in triplicate and in parallel with a standard curve that used standard dilutions from 10⁸ to 10¹ cells of Xeu LMG 905. The primer efficiencies were assessed using the Real-Time PCR miner, as described by Zhao and Fernald [37]. These calculated efficiencies were used to obtain the corrected Cqs for each amplification curve. To determine the amount of Xeu present in the shoots, the standard curve explained above ($R^2 = 0.994$) was used.

2.4. Reactive Oxygen Species' Content

2.4.1. Hydrogen Peroxide (H₂O₂) Quantification

To quantify the hydrogen peroxide, the spectrophotometric method, described by Alexieva et al. [38], was used. Briefly, 50 mg of powdered tissue was macerated in 2 mL of TCA (trichloroacetic acid) 0.1% (*w/v*), using a Mixer Mill Grinder (Retsch MM400, Haan, Germany), with a shaking frequency of 30 s^{−1} in cold conditions. The homogenate was then centrifuged at 12,000× *g* for 15 min at 4 °C, then 500 µL of supernatant were collected and mixed with 500 µL of phosphate buffer 0.1 M (pH = 7) and 1000 µL of potassium iodide (KI) 1 M. The mixture was homogenized with a vortex mixer (VWR, Radnor, PA, USA) for 5 s at 3200 rpm and incubated in the dark, at room temperature for 60 min. The absorbances of the samples were recorded at $\lambda = 390$ nm with the Multiskan™ GO plate reader spectrophotometer (Thermo Fisher, Waltham, MA, USA). A standard curve was made with known concentrations of H₂O₂, ranging between 0 and 100 µM ($R^2 = 0.993$). The results were presented as mmol of H₂O₂ per g of Fresh Weight (FW).

2.4.2. Superoxide Radical (O₂·[−]) Quantification

To semi-quantify the superoxide radical (O₂·[−]), the method described by Gajewska and Sklodowska [39] was used, with the modifications described by Costa-Santos et al. [40]. In brief, the tissue samples (50 mg) were homogenized in 2 mL of extraction buffer (phosphate buffer 0.01 M (pH = 7.8), 0.05% (*w/v*) NBT (nitroblue tetrazolium) dissolved in 100 µL of DMSO (dimethyl sulfoxide) and 10 mM sodium azide) and vortexed for 30 s at 3200 rpm (VWR, Radnor, PA, USA). The mixture was incubated for 1 h, in the dark, at room temperature. The samples were centrifuged at 13,000× *g* for 2 min at 4 °C. After centrifugation, 1.5 mL of supernatant was collected and incubated at 85 °C for 10 min in a heating block (VWR, Radnor, PA, USA). The samples were then placed at 4 °C for 10 min to stop the reaction. The absorbance (Abs) was recorded at 580 nm using the Thermo Scientific™ Multiskan™ GO Microplate Spectrophotometer (Thermo Fisher, Waltham, MA, USA). The results were presented as Abs per g of FW.

2.5. Lipid Peroxidation Assessment by Quantification of MDA

The lipid peroxidation was determined by the production of the malondialdehyde (MDA) radical, as described by Hodges et al. [41]. Succinctly, 50 mg of powdered tissue were macerated in 2 mL of TCA 0.1% (*w/v*) using a Mixer Mill Grinder (Retsch MM400, Germany) with a shaking frequency of 30 s⁻¹, in cold conditions. After centrifugation (10,000 × *g*, 10 min, 4 °C), 250 µL of the supernatant were mixed with 1000 µL of TCA 20% (*w/v*) standing for a negative control (-TBA), and another 250 µL of supernatant was added to 1000 µL of TCA 20% (*w/v*) + TBA (thiobarbituric acid) 0.5% (*w/v*) (+TBA). The samples were incubated at 95 °C for 30 min in a heating block (VWR, Radnor, PA, USA), and chilled on ice for 10 min to stop the reaction. The absorbances were recorded at 600 nm and 532 nm, using the Thermo Scientific™ Multiskan™ GO Microplate Spectrophotometer (Thermo Fisher, Waltham, MA, USA). The results were expressed as mmol of MDA equivalents (mmol MDAE) per g of FW. MDAE were calculated as follows (1).

$$\text{MDAE} = \frac{(\text{Abs}_{532}^{+TBA} - \text{Abs}_{600}^{+TBA}) - (\text{Abs}_{532}^{-TBA} - \text{Abs}_{600}^{+TBA})}{157,000} \times 10^9 \quad (1)$$

2.6. Gene Expression Analysis

To assess the expression levels of the shoots, 100 mg of powdered tissue was used for total RNA extraction, with NZYol (NZYTech™, Lisbon, Portugal), following the manufacturer's guidelines. After the RNA isolation, the samples were treated with NZY DNase I (NZYTech™, Lisbon, Portugal), according to the manufacturer's instructions and cDNA was synthesized using the NZY First-Strand cDNA Synthesis Kit (NZYTech™, Lisbon, Portugal), according to the manufacturer's instructions. cDNA was diluted in MilliQ water and stored at -20 °C until usage. Two housekeeping genes, ubiquitin (*ubi*) and tubulin (*tub*) were used to normalize the relative expression levels (Table 1). Transcripts belonging to the biotic stress defense pathways, namely, (a) carotenoid synthesis: carotenoid isomerase (*crtiso*); (b) phenylpropanoid pathway: chalcone synthase 1 (*chs1*), phenylalanine ammonia-lyase 5 (*pal5*); and (c) hormone response: oxophytodienoate reductase 3 (*opr3*), ABA aldehyde oxidase (*aao*), and aminocyclopropane carboxylate oxidase 3 (*aco3*), were quantified by RT-qPCR.

Table 1. Primer's sequence and annealing temperature of the selected genes for tomato phenylpropanoid and hormonal pathways.

Gene	Primer	Sequence	Annealing Temperature (°C)	Product Size (bp)	Reference
<i>ubi</i>	Forward	GGACGGACGTACTCTAGCTGAT	60	134	[42]
	Reverse	AGCTTTCGACCTCAAGGGTA			
<i>tub</i>	Forward	AACCTCCATTCAGGAGATGTTT	60	180	[42]
	Reverse	TCTGCTGTAGCATCCTGGTATT			
<i>crtiso</i>	Forward	GTTTGTAATCTTGGGTTTCCAGCA	60	117	[43]
	Reverse	TTGCCTTGTGGGTTTCAAGC			
<i>pal5</i>	Forward	TGGAGGAGAATTGAAGAATGCTG	60	136	[43]
	Reverse	TCCCTTTCACCACTTGTAGC			
<i>chs1</i>	Forward	ACCAACAAGGTTGCTTTGCC	60	135	[44]
	Reverse	GAGATTCACCTGGGTCCACGG			
<i>aao</i>	Forward	CCAGGCACAAACACAATCAA	60	154	[45]
	Reverse	GTCGTAAATAATATCAGACTG			
<i>opr3</i>	Forward	ATGGACTCTAATCCACTCAGCCTTG	60	152	[46]
	Reverse	TCACTGCCAAGTCTGCCTGCTTCAG			
<i>aco3</i>	Forward	GAAGGCTATGGAAGCTAATGTGG	60	104	[47]
	Reverse	ATGTGTTTAATTAGCTACGTTTG			

The RT-qPCR reactions were performed using the CFX96 Touch™ thermocycler (Bio-Rad Laboratories, Hercules, CA, USA). For each reaction, 2.5 µL of total first-strand cDNA, 10 µL of NZYSpeedy qPCR Green Master Mix (2x), ROX plus (NZYTech™, Lisbon, Portugal), 0.8 µL of forward and reverse primer (10 mM), and 5.9 µL of MilliQ water were used. The amplifications were standardized as: 95 °C for 1 min followed by 50 cycles of 5 s at 95 °C and 15 s at 60 °C. The melting curve analysis ranged from 65 °C to 95 °C with an increment of 0.5 °C per cycle of 5 s.

Real-time PCR miner [34] was used to calculate the efficiency of the primers and determine the Cq values of the amplification curves.

2.7. Hormone Extraction and Quantification

The extraction of the phytohormones 6-benzylaminopurine (BAP), abscisic acid (ABA), gibberellic acid 3 (GA3), indole-acetic-acid (IAA), jasmonic acid (JA), methyl-jasmonate (MeJA) and salicylic acid (SA) was performed as described by Van Meulebroek et al. [48]. For that, 100 mg of tissue was homogenized, by vortexing for 2 min at 3200 rpm (VWR, Radnor, PA, USA), with 1 mL of cold (−20 °C) extraction buffer composed of 75% (v/v) methanol, 20% (v/v) formic acid and 5% (v/v) MilliQ sterile water. The samples were then subjected to cold extraction, for 12 h at −20 °C. After extraction, the homogenates were centrifuged ($17,000 \times g$ for 5 min at 4 °C) and the supernatant was collected and preserved at −80 °C. Prior to quantification, 500 µL of supernatant was transferred to 30 kDa Amicon® Ultra centrifugal filter units, which were centrifuged for 10 min at $17,000 \times g$, at 4 °C. The samples were collected and filtered through a PTFE syringe filter of 0.2 µm before quantification.

The chromatographic analysis was performed in a Shimadzu LC-20 system equipped with an SPD-M20A Prominence Diode Array Detector (DAD) and a Rheodyne 7725 six-port external sample injector. A reversed phase Luna Omega Polar C18 column (150 × 4.6 mm; 5 µm, Phenomenex) with a guard column (Luna Omega Polar C18 4 × 3.0 mm, Phenomenex) was used as the stationary phase. The elution was performed in gradient mode with a mixture of 0.1% (v/v) formic acid in ultra-pure water and methanol (60:40, (v/v) for solvent A and 40:60 (v/v) for solvent B, respectively). The gradient elution program started with 0% of solvent B during 9 min. Then, solvent B increased to 100% within 0.01 min. From 9.01 to 22.0 min, the contribution of solvent B was constant. At 22.0 min, the initial conditions were re-established within 0.01 min, and held during 8.0 min to assure column equilibration. The flow rate was kept at 1.0 mL min^{−1}, the injection volume was 20 µL, and the LC column was maintained at a controlled room temperature (±2 °C). Peak detection and quantification were performed using LabSolutions software version 5.60 SP2 (Shimadzu Corporation). Gibberellic acid 3 (GA3), benzyladenine (BAP), indoleacetic acid (IAA), salicylic acid (SA), jasmonic acid (JA) and methyl jasmonate (MeJA) were detected using a 206 nm wavelength, while abscisic acid (ABA) was detected at 254 nm.

The proposed HPLC–DAD method was validated in compliance with the ICH guidelines for validation of analytical procedures [49]. The linearity and range, limits of detection (LOD) and quantification (LOQ), precision, and accuracy were evaluated. The results were expressed as ng of each hormone per g of FW. Details about experimental procedures and analytical results are included as Supplementary Material.

2.8. Statistical Analysis

The experiment was performed two times, in independent assays. In each independent assay, ten shoots per condition/treatment were used, for the analysis of each parameter. The comparisons between the treatments were made using One-way ANOVA test (Graphpad™ Prism 9, San Diego, CA, USA). For statistical comparisons, the Dunnett comparison test was used, with a confidence interval of 95%.

3. Results

3.1. *X. euvesicatoria* Quantification

The quantification of the pathogen showed that Xeu was present in all inoculated experimental conditions. The results revealed that NMT was the only treatment capable of significantly ($p < 0.05$) reducing the Xeu amount in the tomato shoots (154 times) when compared to all the other conditions (Figure 1). When the EO-water emulsion (XS) or formulation (XSNMT) were applied, no significant differences were observed in the Xeu amount, compared to control (X), 24 h after treatments.

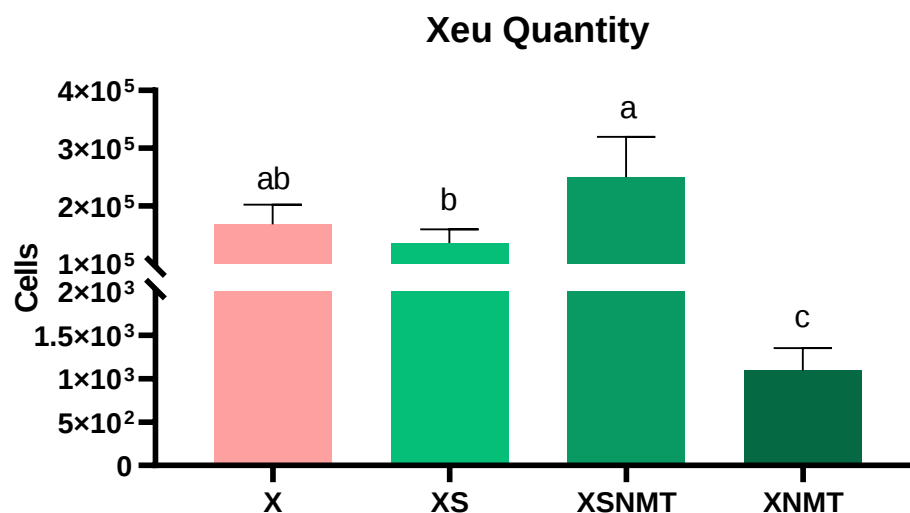


Figure 1. *X. euvesicatoria* amount in infected *S. lycopersicum* shoots, through q-PCR. Values are presented as mean $\pm$ SD ($n = 3$). Means marked with the same letter are not significantly different according to the Dunnett test ($p < 0.05$). X: infected untreated shoots; XS: infected shoots treated with *S. montana* EO; XSNMT: infected shoots treated with *S. montana* EO + NMT formulation; XNMT: infected shoots treated with NMT.

3.2. Reactive Oxygen Species' Content

The hydrogen peroxide did not show significant changes when the treatments were applied either to the uninfected tomato shoots (Figure 2a), or in the Xeu-infected ones (X), when compared to the negative control (C). However, H_2O_2 was significantly ($p < 0.05$) reduced by the application of the three treatments in the infected shoots (Figure 2b). Concerning the superoxide radical, it was significantly ($p < 0.05$) increased by the application of the treatments (EO-water emulsion; formulation; and NMT) to the uninfected shoots (Figure 2c). Similarly, the Xeu-infected leaves presented significantly higher ($p < 0.05$) $O_2^{\cdot-}$ levels compared to the uninfected control (C) (Figure 2c). The application of EO-water emulsion led to a significant ($p < 0.05$) increase in the $O_2^{\cdot-}$ content of the infected shoots (Figure 2d). However, the NMT alone, or as a carrier of EO, significantly ($p < 0.05$) reduced the superoxide radical levels of Xeu-infected tomato explants, compared to the positive control (X).

3.3. Lipid Peroxidation Assessment by Quantification of MDA

The MDA content of tomato shoots significantly ($p < 0.05$) increased when the *S. montana* EO-water emulsion was applied to the uninfected shoots (Figure 3a). However, when applied alone or as an EO carrier, the NMT led to no significant changes in the shoots' MDA content. The application of the formulation led to a significant ($p < 0.05$) decrease in the MDA content of the infected shoots compared to X (Figure 3b).

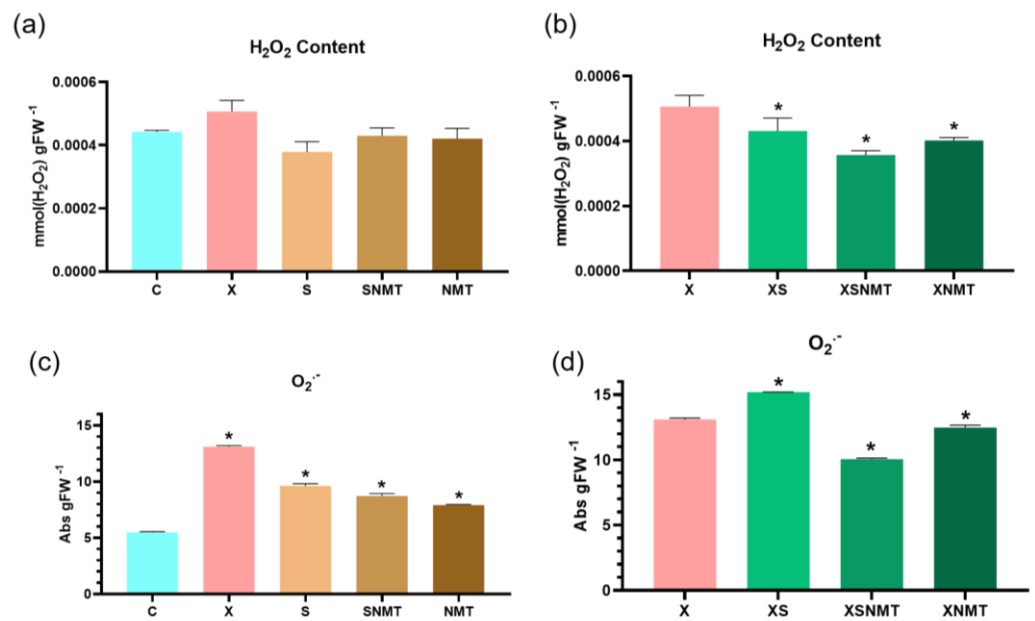


Figure 2. H_2O_2 and $\text{O}_2^{\cdot-}$ content of tomato shoots 3 days after infection and 24 h after the application of treatments. (a,c) stand for the levels of the uninfected/treated groups (S; SNMT; NMT) and the infected control (X) compared to the negative (C) control; (b,d) represent the levels on infected/treated groups (XS; XSNMT; XNMT) compared to the infected control (X). All values are presented as mean $\pm$ SD ($n = 3$). Means marked with an asterisk are statistically different from the respective control according to the Dunnett comparison ($p < 0.05$). C: uninfected untreated shoots (control); X: infected untreated shoots; S: uninfected shoots treated with *S. montana* EO; SNMT: uninfected shoots treated with *S. montana* EO + NMT formulation; NMT: uninfected shoots treated with NMT; XS: infected shoots treated with *S. montana* EO; XSNMT: infected shoots treated with *S. montana* EO + NMT formulation; XNMT: infected shoots treated with NMT.

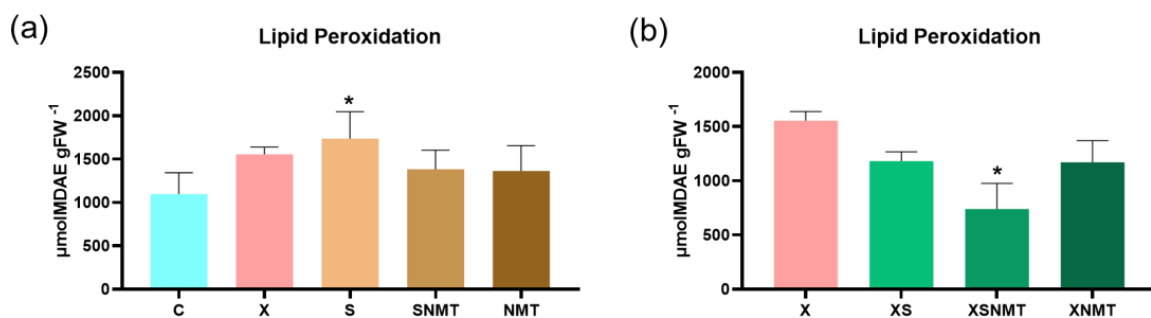


Figure 3. MDA content of *S. lycopersicum* shoots 3 days after Xeu inoculation, and 24 h after the application of treatments; (a) represents the uninfected/treated conditions (S; SNMT; NMT) and the infected/untreated control (X) compared to the uninfected/untreated control (C); (b) represents the infected/treated conditions compared to the positive control (X). Values are presented as mean $\pm$ SD ($n = 3$). Means marked with "*" are statistically different from the respective control, according to the Dunnett comparison test ($p < 0.05$). C: uninfected untreated shoots (negative control); X: infected untreated shoots (positive control); S: uninfected shoots treated with *S. montana* EO; SNMT: uninfected shoots treated with *S. montana* EO + NMT formulation; NMT: uninfected shoots treated with NMT; XS: infected shoots treated with *S. montana* EO; XSNMT: infected shoots treated with *S. montana* EO + NMT formulation; XNMT: infected shoots treated with NMT.

3.4. Gene Expression Analysis

3.4.1. Phenylpropanoid Pathway

The carotenoid pathway, namely *crtiso* was significantly ($p < 0.05$) upregulated in the Xeu-infected shoots (X), compared to the uninfected control (C) (Figure 4a). The application of each of the three treatments to the infected shoots led to a significant ($p < 0.05$) downregulation of *crtiso* (Figure 4b). As for the phenylpropanoid pathway, the application of NMT nanoclay (NMT) to the uninfected shoots led to a significant ($p < 0.05$) upregulation of *pal5* (Figure 4c,e). The *pal5* was also significantly ($p < 0.05$) upregulated by the application of the three treatments to the Xeu-infected shoots (Figure 4d). In addition, *chs1* was upregulated in the Xeu-infected shoots (X) and when the formulation/nanoclays were applied to the uninfected tomato shoots (Figure 4e). The application of the EO-water emulsion to the Xeu-infected shoots (XS) led to a significant ($p < 0.05$) downregulation of *chs1* (Figure 4f).

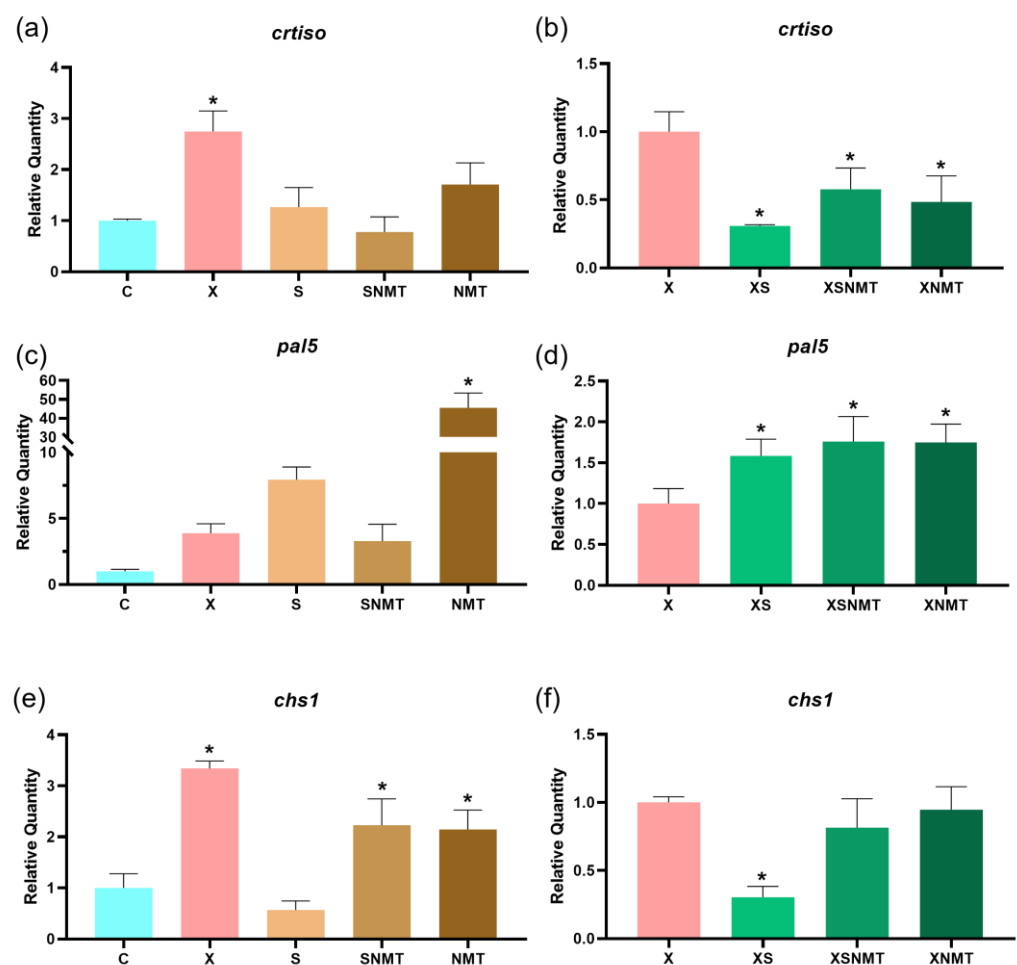


Figure 4. Expression levels of genes related to the carotenoid (*crtiso*) and the phenylpropanoid pathway (*pal5* and *chs1*), quantified by RT-qPCR. (a,c,e) represent the levels of the uninfected/treated groups, plus the positive control (X), compared to the uninfected/untreated control (C); (b,d,f) represent the levels of the infected/treated groups compared to the positive control (X). All values are presented as mean $\pm$ SD ($n = 3$). Means marked with “*” are statistically different according to the Dunnett comparison test ($p < 0.05$). C: uninfected untreated shoots; X: infected untreated shoots; S: uninfected shoots treated with *S. montana* EO; SNMT: uninfected shoots treated with *S. montana* EO + NMT formulation; NMT: uninfected shoots treated with NMT; XS: infected shoots treated with *S. montana* EO; XSNMT: infected shoots treated with *S. montana* EO + NMT formulation; XNMT: infected shoots treated with NMT.

3.4.2. Hormone Pathways

The ABA aldehyde oxidase gene (*aao*) was significantly ($p < 0.05$) upregulated in the Xeu-infected shoots, compared to the negative control ($p < 0.05$). The application of formulation to the uninfected shoots also led to a significant ($p < 0.05$) upregulation of *aao*. Contrarily, the NMT downregulated this gene in the uninfected *S. lycopersicum* shoots (Figure 5a). Both the nanoclay and formulation led to an upregulation of *aao* in the Xeu-infected shoots (Figure 5b). In the Xeu-infected shoots, *opr3* was upregulated, compared to the negative control (C). The NMT application also increased the level of *opr3* transcripts ($p < 0.05$) (Figure 5c). The application of EO-water emulsion and formulation to the Xeu-infected shoots led to a significant ($p < 0.05$) upregulation of *opr3* (Figure 5d). Finally, *aco3* was upregulated by the application of formulation and downregulated by EO-water emulsion in uninfected groups, compared to the negative control (C) (Figure 5e). The application of *S. montana* EO-water emulsion and NMT nanoclay to the Xeu-infected explants significantly downregulated *aco3* (Figure 5f).

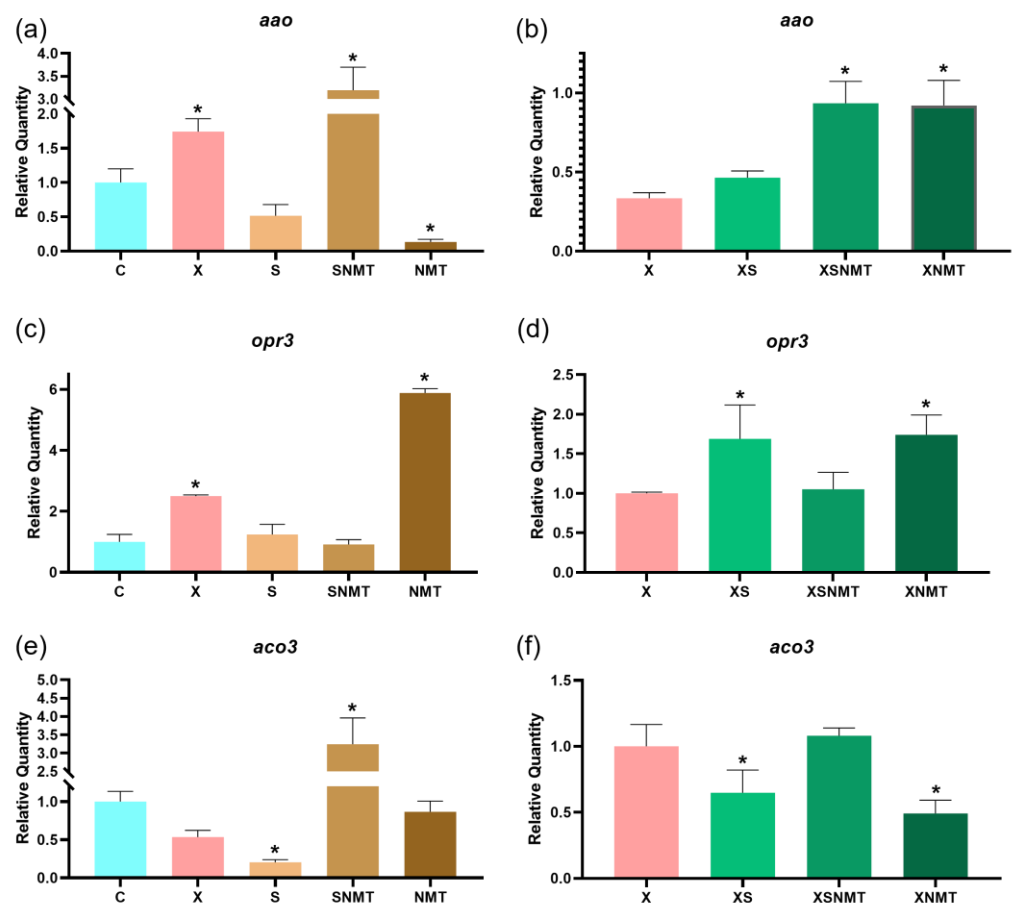


Figure 5. Expression levels of genes related to the ABA (*aao*), JA (*opr3*) and ethylene (*aco3*) pathways in tomato shoots, quantified by RT-qPCR. (a,c,e) represent the levels on uninfected/treated groups, and the positive control (X), compared to the negative control (C); (b,d,f) stand for the levels of infected/treated groups compared to the positive control (X). All values are presented as mean $\pm$ SD ($n = 3$). Values marked with an asterisk are statistically different according to the Dunnett comparison test ($p < 0.05$). C: uninfected untreated shoots; X: infected untreated shoots; S: uninfected shoots treated with *Satureja montana* EO; SNMT: uninfected shoots treated with *Satureja montana* EO + NMT formulation; NMT: uninfected shoots treated with NMT; XS: infected shoots treated with *Satureja montana* EO; XSNMT: infected shoots treated with *Satureja montana* EO + NMT formulation; XNMT: infected shoots treated with NMT.

3.5. Hormone Quantification

The validated HPLC–DAD method was applied to determine the phytohormone profile in the tomato shoots subjected to the different conditions. In this context, the levels of IAA of the uninfected shoots treated with the EO + NMT formulation increased significantly ($p < 0.05$) (Figure 6a). The application of NMT and EO + NMT formulation to the infected tomato shoots also led to significant increases in the IAA content (Figure 6b). As for GA3, ABA, JA and MeJA, no significant changes were observed in the samples compared to the respective controls (C and X) (Figure 6c–j). The levels of BAP and SA were also assessed, though they were below the minimum threshold for quantification.

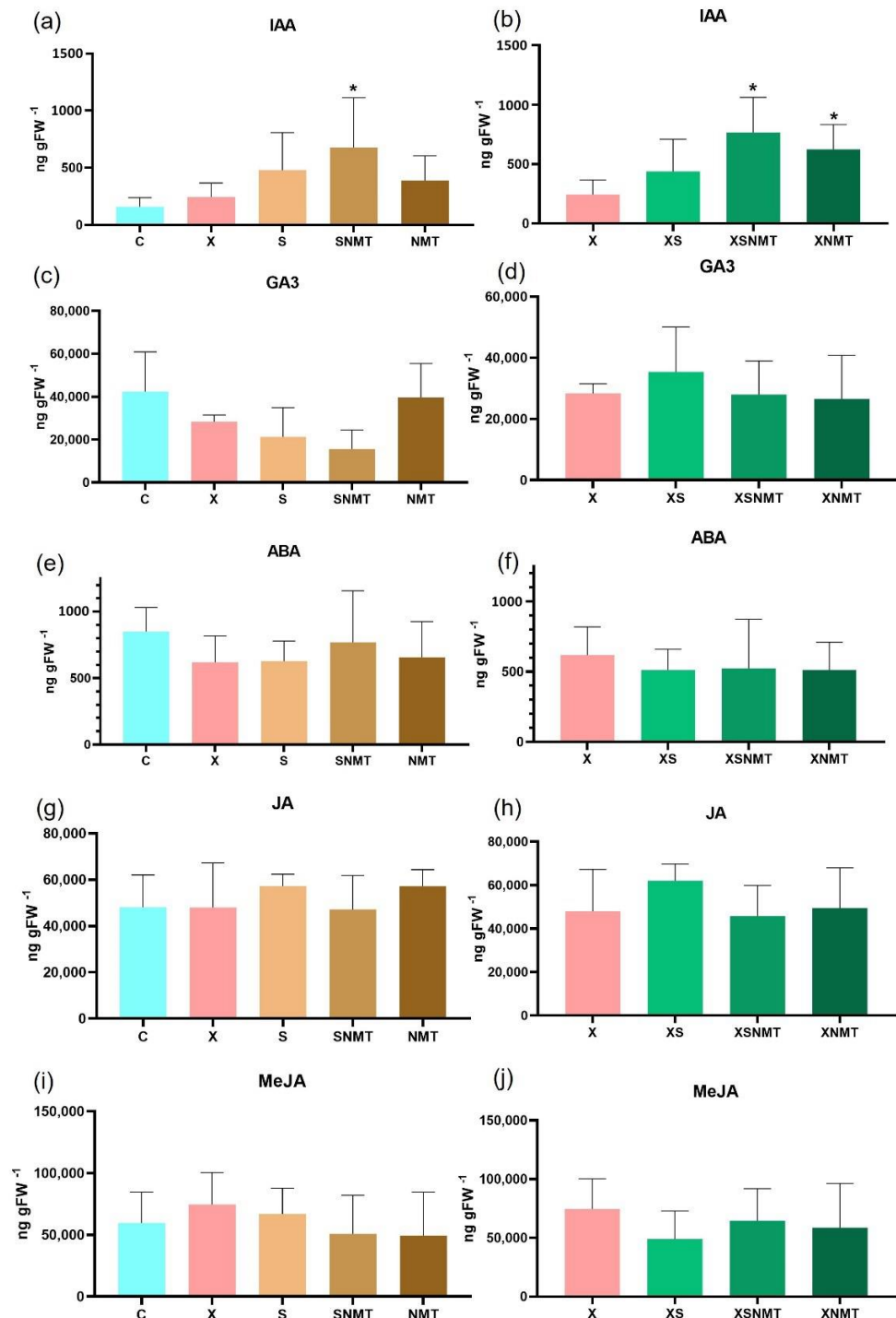


Figure 6. Phytohormone quantification of IAA (a,b); GA3 (c,d); ABA (e,f); JA (g,h); and MeJA (i,j) by

HPLC–DAD. (a,c,e,g,i) represent the levels on uninfected/treated groups and the positive control (X) compared with the negative control (C); (b,d,f,h,j) stand for the levels of infected/treated groups compared to the positive control (X). All values are presented as mean $\pm$ SD ($n = 3$). Means marked with “*” are statistically different from the respective control according to the Dunnett comparison test ($p < 0.05$). C: uninfected untreated shoots; X: infected untreated shoots; S: uninfected shoots treated with *S. montana* EO; SNMT: uninfected shoots treated with *S. montana* EO + NMT formulation; NMT: uninfected shoots treated with NMT; XS: infected shoots treated with *S. montana* EO; XSNMT: infected shoots treated with *S. montana* EO + NMT formulation; XNMT: infected shoots treated with NMT.

4. Discussion

Current strategies for BS disease management in tomato still rely on Cu-based compounds. The emergence of Cu-resistant *Xanthomonas* in the field [50] has hastened the search for viable alternatives. Furthermore, E.U. policies for biological agriculture lean towards the replacement of Cu with environmentally safe, biodegradable compounds. Therefore, NMT and formulations with other natural antimicrobial products emerge as promising tools to integrate disease management strategies, due to their antimicrobial properties [12]. However, there are some difficulties in the application of these compounds in the field, namely in the case of EOs, due to their rapid degradation and the high volatility of the organic phase [51].

The EOs of other *Satureja* spp. have already been tested against other *Xanthomonas* spp. [23]. Our results show that, 24 h after Xeu inoculation, *S. montana* EO in a water emulsion, or integrated in NMT as a formulation, was not capable of reducing the amount of Xeu on the tomato shoots; this may be due to the controlled-release rate of the EO by the NMT, that can have a significant influence on the short-term antimicrobial activity of these materials [52]. However, Oliveira-Pinto et al. [12] demonstrated the antimicrobial properties of this EO-NMT formulation in a longer-term study. Interestingly, the NMT alone was capable of significantly reducing the Xeu cell density, which is according to what was reported by Oliveira-Pinto et al. [12] regarding the antimicrobial potential of NMT against Xeu and confirms the potential of this nanoclay as a Xeu control agent in agriculture (Figure 1). The antimicrobial potential of NMT nanoclays in literature is usually associated with their combination with organic compounds or with metals (e.g., [53,54]). In agriculture, NMT has been mostly explored as a delivery tool for organic compounds, namely EOs [55,56]. However, their potential as antimicrobial agents capable of integrating plant disease management strategies remains sparsely explored. Our data clearly demonstrate that these mineral compounds are promising products acting alone (or as carriers) to controlling BS.

Our data also indicate that NMT and the formulation possess short-term antioxidant activity in the Xeu-infected shoots, reducing both H_2O_2 and superoxide radicals (Figure 2). *S. montana* EO also reduced H_2O_2 , these results being aligned with the antioxidant activities attributed to *S. montana* EOs by Miladi et al. [57]. These reductions in the ROS content of the infected shoots ultimately led to a reduction in the oxidative damage in lipids, through a reduction in the MDA content of tomato cells (Figure 3). Interestingly the application of treatments to the uninfected tomato shoots led to an increase in the superoxide content (Figure 2), which in turn resulted in a higher level of lipid damage (Figure 3). This suggests that, when applied to healthy shoots, the NMT, EO + NMT formulation, and *S. montana* EO alone might act as defense elicitors, triggering plant defense responses against bacterial pathogens. Lucas et al. [58] reported that the EOs of other species (e.g., citronella, clove, cinnamon, lemongrass, eucalyptus, thyme, and tea) might act as defense elicitors in plants. Despite remaining unclear as to the molecular mechanisms of action of these EOs, it is demonstrated here that there is an activation of the oxidative pathways. Furthermore, the increase in ROS content did not result in lipid damage when the NMT or EO + NMT formulation were applied to the uninfected shoots (Figure 3). These data show that the

NMT plays a protective role against lipid damages caused by overproduction of ROS. Further studies are required to explore the signaling and metabolic pathways triggered in response to the NMT or EO application, and their potential as stress mitigators and preventive products in plant production systems.

Concerning the regulation of the phenylpropanoid pathway, there was an upregulation of *chs1* in the Xeu-infected shoots (X). A similar response occurred when the EO + NMT formulation was applied to the uninfected shoots. The NMT upregulated both genes of this pathway (Figure 4), pointing to the pivotal role of the phenylpropanoid pathway in *Xanthomonas* infection, as reported previously by Du et al. [59], and Oliveira-Pinto et al. [43], and corroborating the hypothesis that, when applied to uninfected hosts, the NMT acts as a defense elicitor, priming the plants' immune defenses.

Xeu infection and formulation application promoted an increase in the *aao* transcripts, while the NMT reduced the same transcripts (Figure 5). Despite this downregulation of *aao*, ABA quantification revealed no changes in the ABA content on the uninfected shoots (Figure 6c), thus the downregulation of *aao* was not sufficient to suppress the whole pathway, resulting in a reduction in the ABA. However, when applied to the infected shoots, the treatments were incapable of immediately reducing *aao* levels and therefore did not suppress the ABA pathway, which is confirmed by the HPLC quantification (Figure 6d). Plants under biotic stress produce ethylene and JA, in cases of necrosis [60]. The results show that the nanoclay upregulated the *opr3* (JA pathway) which is fundamental in suppressing bacterial infection. However, the increase in JA-related transcripts was not accompanied by an accumulation of JA, or its methyl ester conjugate (MeJA), in shoots (Figure 6e,g), suggesting that some post-translational mechanism is in place, regulating the production of JA. Furthermore, GA3 (one of the most bioactive forms of gibberellic acid) levels did not suffer any significant changes by the application of treatments or pathogen presence (Figure 6i,j). This lack of GA response might be due to the fact that this hormone plays a more significant role against necrotrophic pathogens (e.g., *Erwinia amylovora*) by mediating JA/ET response [61].

The accumulation of *opr3* transcripts by the application of treatments in the infected shoots may indicate that the increases observed in the ABA pathway (Figure 5b) were not sufficient to suppress JA's role and facilitate the *Xanthomonas* infection, as reported by Long et al. [62]. Ethylene plays a key role in plants' defenses against bacterial pathogens, with its levels increasing when plants are under biotic stress [63] and low levels of ethylene are known to increase the susceptibility of *Arabidopsis thaliana* to *Erwinia carotovora* subsp. *carotovora* [64]. Furthermore, Guan et al. [65] reported that *A. thaliana* mutant lines with deficient production of ACC also presented an increased susceptibility to infection by *Pseudomonas syringae*. IAA is known to stimulate the production of ethylene in plant tissues by promoting synthesis of both ACC and ACC oxidase [66]. Thus, the significant increase in IAA levels that was observed when EO-NMT was applied to the uninfected shoots (Figure 6a), and when EO-NMT or NMT alone were applied to the infected shoots (Figure 6b), might be explained by an activation of the ethylene synthesis pathway, through ACC and ACC oxidase. However, the *aco3* transcripts were only upregulated by the application of formulation in the uninfected shoots (Figure 5c), which suggests that other transcripts of the ethylene biosynthesis pathway (*aco1*) might play a more relevant role on ethylene synthesis in response to EO application, which is currently under study.

5. Conclusions

To conclude, NMT strongly reduces the bacterial infection in tomato shoots, while decreasing the expression levels of defense-related genes, reducing ROS production, and protecting the tomato against oxidative damage, thus contributing to improve the shoots' overall health status. Moreover, other treatments (formulation/EO-water emulsion) may act as defense elicitors, enhancing the production of ROS and upregulating the hormone and phenylpropanoid pathways in the uninfected shoots. Overall, our results suggest that NMT is a promising tool for the short-term management of Xeu in tomato, while the

S. montana EO and EO + NMT formulation may act better as defense elicitors, and thus, their role as in the prevention of disease must be better explored. Further studies in plants are required to properly assess the potential of these products for field applications and to clarify the potential use of these compounds as defense elicitors.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/applnano3030009/s1>, Table S1: Analytical figures of merit obtained with the proposed HPLC-DAD methodology for the quantification of phytohormones; Table S2: Recovery values of the proposed methodology for the quantification of phytohormones in tomato leaf extracts ($n = 3$ for each concentration). Reference [67] is cited in the Supplementary Materials.

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