agronomy

MDPI

Article

Toxicity and Preventive Activity of Chitosan, *Equisetum arvense*, Lecithin and *Salix* Cortex against *Plasmopara viticola*, the Causal Agent of Downy Mildew in Grapevine

Diego Llamazares De Miguel [1], Amaia Mena-Petite [2] and Ana María Díez-Navajas [1,*]

[1] Department of Plant Production and Protection, NEIKER-Basque Institute of Agricultural Research and Development, Basque Research and Technology Alliance (BRTA), Campus Agroalimentario de Arkaute, 01192 Arkaute, Spain
[2] Department of Plant Biology and Ecology, Faculty of Pharmacy, University of the Basque Country (UPV/EHU), 01006 Vitoria-Gasteiz, Spain
* Correspondence: adiez@neiker.eus

Abstract: Grapevine, a crop of global economic importance, is annually affected by diseases that can compromise the quality and quantity of the harvest, producing large economic losses. Downy mildew caused by *Plasmopara viticola* (Berk. & M.A. Curtis) Berl. & de Toni is one of the most important diseases in the vineyard. To fight this pathogen, winegrowers often rely on conventional chemical fungicides or copper-based formulations, whose use is determined to be reduced by the European Commission due to their environmental consequences. Hence, alternative plant protection products (PPP) in grapevine must be considered and studied. In this context, we selected several alternative commercial products, based on basic substances (BS) or low-risk active substances (LRAS), to evaluate their suitability to deal with *P. viticola*. We measured the preventive activity of the products, both *in vitro* and *in planta*, as well as their toxicity against the sporangia and zoospores of the pathogen. Results showed that four commercial products were effective against the pathogen directly and preventively, being composed of approved basic substances, more concretely, chitosan, *Equisetum arvense*, lecithins, and *Salix* cortex. Among those, the products composed of lecithins and *Salix* cortex were the most toxic and active preventively. Therefore, these basic substances should be promoted in the vineyard as an alternative to conventional treatments in order to transition to a more sustainable viticulture.

Keywords: basic substances; chitosan; *Equisetum arvense*; lecithin; *Salix* cortex; *Plasmopara viticola*; downy mildew; grapevine; plant protection products; sustainable viticulture

Citation: Llamazares De Miguel, D.; Mena-Petite, A.; Díez-Navajas, A.M. Toxicity and Preventive Activity of Chitosan, *Equisetum arvense*, Lecithin and *Salix* Cortex against *Plasmopara viticola*, the Causal Agent of Downy Mildew in Grapevine. *Agronomy* **2022**, *12*, 3139. https://doi.org/10.3390/agronomy12123139

Academic Editor: Tito Caffi

Received: 9 November 2022
Accepted: 7 December 2022
Published: 10 December 2022

1. Introduction

Grapevine, *Vitis vinifera* L., is one of the most important crops in the world, with a total surface of 7.3 million of hectares (mha) in 2021. The majority of this area is dedicated to wine production, which generated 34.3 billion EUR in the same year. Among all the wine regions, the European Union is the leader in terms of cultivated surface and accounts for 45% of the total area (3.3 mha), with the five main wine producers in Europe (France, Germany, Italy, Portugal, and Spain) generating 22.9 billion EUR [1].

Thus, given its economic importance, the vineyard needs to be protected against any potential threats, including pests and diseases. Among the diseases, downy mildew is one of the most common worldwide, caused by the oomycete *Plasmopara viticola* (Berk. & M.A. Curtis) Berl. & de Toni, an obligate biotroph affecting grapevine. Currently, the most effective strategies to control this pathogen in the vineyard are copper-based formulations and conventional fungicides, which present two main drawbacks. First, they are usually toxic to the environment and can accumulate in surface and groundwater, affecting local wildlife [2]. Secondly, their extensive use can accelerate the development of resistant

P. viticola strains, which can increase the frequency and severity of the attacks [3,4]. As a consequence, the European Commission has declared copper-based formulations as candidates for substitution in the Regulation (EU) 2018/1981 [5] and is aiming to halve the use of chemical pesticides by 2030 in accordance with the Green Deal [6], urging for the consideration of other alternative plant protection products (PPP).

Plant, seaweed, and microbial preparations are promising alternatives to the conventional products, as they contain several antifungal compounds and are usually biodegradable and non-toxic to environment [7–9]. Moreover, plant and seaweed extracts are rich in damage-associated molecular patterns (DAMPs) derived from the disruption of tissues, which are naturally produced in plants during pathogen infection and can trigger plant immunity locally [10]. On the other hand, microbial preparations provide pathogen-associated molecular patterns (PAMPs), which can also trigger plant immunity [11]. In fact, the use of DAMPs and PAMPs as PPP has been previously suggested [12], and several of them have already been demonstrated to stimulate grapevine immunity [13]. Therefore, any plant, seaweed, or microbial-based product could potentially be a source of antifungal compounds. DAMPs or PAMPs act against pathogens both directly or by stimulating the plant's natural defense. The vast number of plant, seaweed, and microorganism species offers a great opportunity for screening new alternatives to conventional fungicides.

In the European Union, some alternative PPPs are nowadays marketed as basic substances (BS), included in the Regulation (EC) No 1107/2009 [14]. BSs are non-toxic products not used or marketed mainly for plant protection, but which can have an effect against certain biotic stresses and, hence, can be considered PPP. In 2022, a total of seven BSs were approved against *P. viticola* [15]: chitosan, *Equisetum arvense* L., fructose, lecithins, *Salix* cortex, sucrose, and *Urtica* spp. According to their official approval reports, all these BSs are considered plant defense stimulators, except *Urtica* spp., which has a fungicidal nature. The same regulation of BS also holds the approval of another PPP category, low-risk active substances (LRAS), which are active substances that have a low risk to human, animal, and environmental health. Currently, three LRASs are indicated against *P. viticola* [15]: laminarin, a polysaccharide coming from brown seaweed *Laminaria digitata*, COS-OGA, an oligosaccharide composed of oligochitosans and oligopectates, and Cerevisane®, a *Saccharomyces cerevisiae* extraction.

Considering the previous information, it is apparent that the use of alternative PPPs against *P. viticola* and other diseases is supported by the European Commission. However, the real picture shows that most of Europe's and the world's vitiviniculture is still heavily dependent on chemical pesticides. In fact, according to the International Organisation of Vine and Wine, only 6% of the world's and 12% of Europe's vine area is managed organically without the use of chemical pesticides [16]. The main reason for the low use of alternative PPPs might be the scarce amount of scientific information, with only a few studies published reporting their mechanism of action and efficacy against biotic stresses. In fact, most of these products are understudied at the molecular level.

In this work, a total of four BSs have been studied: more concretely, chitosan, a naturally abundant polysaccharide present in many living organisms [17]; *Equisetum arvense*, an herbaceous perennial fern [18]; lecithin, a lipidic fraction obtained from soybean [19]; and *Salix* cortex extract, obtained from the bark of some willow species [20]. Similarly to other alternative PPPs, the literature regarding the mechanism of action of these formulations is also scarce. Chitosan is the most studied product, with many reviews analyzing its utility for managing plant diseases [21,22]. Moreover, its use against several grapevine fungal diseases [23–25] and oomycetes, such as *P. viticola*, is accepted (by stimulating the plant's natural defense [26–28]). Regarding *Equisetum arvense*, its use in grapevine has not been deeply evaluated at the molecular level. Only some works have been published demonstrating certain efficacy against grapevine fungal diseases [29,30] and against *P. viticola* [29,31]. This antifungal nature is not surprising, though, given its high concentration of flavonoids and phenols [32]. In the case of soy lecithin, its mechanism in plant protection is not known, despite being recommended for this purpose in grapevine [33]. Only very

recently, it has been reported that soy lecithin is able to modulate the expression of several defense-related genes during *P. viticola* infection [34]. Finally, concerning *Salix* cortex, some works have been published in grapevine [29,35,36], reporting a high toxicity and certain preventive activity against *P. viticola*, but its molecular mechanism remains unknown.

Therefore, in this context of lack of knowledge, our study aims to shed light on the toxicity and efficacy of some available PPPs against *P. viticola*, in order to reinforce their use in sustainable viticulture. To accomplish this, we selected several alternative commercial products that stated a defense stimulation capacity, including low-risk active substances and basic substances. Preferably, products recommended to deal with *P. viticola* were selected, including others not indicated against this pathogen. Our main objectives were (1) to evaluate the defense stimulator and preventive capacity of the formulations against *P. viticola* infection *in vitro*, (2) to know their toxicity against the pathogen's sporangia and zoospores *in vitro*, and (3) to study their efficacy at the whole-plant level.

2. Materials and Methods

2.1. Plant Material and Plasmopara Viticola Inoculum

For preventive and toxicity assays, ungrafted *V. vinifera* cv. Tempranillo RJ-78 and cv. Viura were used, obtained from pruning branches from ICVV (Logroño, Spain). For the assays used to measure the half maximal inhibitory concentration (2.5.), the same ungrafted plants of *V. vinifera* cv. Tempranillo RJ-78 were used. Finally, the whole-plant greenhouse protection assays were conducted in two clones of Tempranillo: (1) *V. vinifera* cv. Tempranillo VN-40 grafted on R-110 rootstock (Vitis Navarra, Navarra, Spain) and (2) ungrafted *V. vinifera* cv. Tempranillo RJ-78.

Pruning branches were harvested from ICVV (Logroño, Spain) and *V. vinifera* cv. Tempranillo grafted on R-110 rootstock was provided by a commercial nursery (Viveros Villanueva Vides S. L., Navarra, Spain). Pruning branches were treated with a standard fungicide solution and stored in humidity conditions at 10 °C for at least 10 days. Thereafter, they were excised into smaller pieces, and sprouting was induced by placing them in water at room temperature until the emergence of roots. Unless specified, in all cases, 3 plants were grown in 5 L pots, with enriched nutrient substrate (organic matter 90%, Sphagnum peat (160 g/L), calcium carbonate (7 g/L), NPK fertilizer (1.5 g/L) and trace elements, electrical conductivity 40 mS/m, pH 5.5–6.5; PotgrondH, Klasmann-Deilmann GmbH, Germany), with a 16 h day/8 h night photoperiod (350 umol/m^2/s; 0.51 W/m^2), and 18–25 °C room temperature, in a biosafety level 2 greenhouse and irrigated to field capacity, was used, when necessary.

P. viticola inoculum was isolated from leaf downy mildew infection spots, from a vineyard in Etxano (Biscay, Spain, 43.227865, −2.719396) in May 2020, and regularly multiplied in laboratory over detached leaves of Tempranillo and Viura varieties until use. For every inoculation, the sporangia were dissolved in commercial mineral water, and their concentration was adjusted to 2×10^4 sporangia/mL using a Thoma haematocytometer (BRAND GMBH + CO KG, Germany). Leaf discs and detached and non-detached leaves were inoculated by spraying a sporangial dilution on the abaxial side of the leaf with a manual hand sprayer.

2.2. Commercial Products

Several commercial products were selected based on their defense stimulation capacity independently of their indication against *P. viticola*, although their use against the pathogen was favored (Table 1). Distilled water was used as negative, and β-aminobutyric acid (BABA) (Sigma-Aldrich, Steinheim, Germany) was used as a positive control.

Table 1. List of the commercial products used in this study, including low-risk active substances (LRAS) and basic substances (BS).

Product	Company	Abbreviation	Composition	Category
-	Sigma-Aldrich	BABA	2 mM β-aminobutyric acid	-
Actileaf	Agrichem Bio	ACTL	Cerevisane® (*S. cerevisiae strain* LAS117) 94.1% *w*/*w*	LRAS
Activane	LIDA Plant Research	ACTV	Free aminoacids 6%	Biostimulant
Biofender Fusarum	Econatur	CHIT	Chitosan hydrochloride 1%	BS
Biofender Lectum	Econatur	LECI	Soy lecithin 25% + *E. arvense* extract 15%	Mixture of BS
Biofender Salix	Econatur	SALIX	*Salix* cortex extract 42% + chitosan hydrochloride 0.5%	Mixture of BS
Fytosave	LIDA Plant Research	FYTO	COS-OGA 1.25% *w*/*v*	LRAS
Lesoy	Idai Nature	LESOY	Soy lecithin 20%	BS
Miles	Servalesa	MILES	2.00% *w*/*v E. arvense* L.	BS
Mimetic	Idai Nature	MIME	Mn 1%, Zn 1% and *M. tenuiflora* and *Q. robur* extracts	Biostimulant
Taegro	Syngenta	TAEG	*Bacillus amyloliquefaciens strain* FZB24 13% *w*/*w*	LRAS

2.3. Preventive Effect Assay on Plasmopara Viticola

The preventive effect of the abovementioned commercial products (Table 1) against *P. viticola* was evaluated using leaf disc assays. An amount of 10 plants with 12–14 fully expanded leaves were used for each experiment. Briefly, 16 mm diameter leaf discs from the 3rd, 4th, and 5th fully expanded leaves from the apex were excised and randomly placed on humid chambers with the abaxial face up. An amount of 12 discs per time and product (total of 36 per product) were sprayed until run-off at their recommended dose (Supplementary Table S1) and incubated for 24 h. Then, remaining drops were eliminated, and 12 discs were subsequently inoculated with *P. viticola* sporangia (sp) (2×10^4 sp/mL) at different time points: 1, 2, and 4 days post treatment. During the infection process, discs were maintained at 20 °C, with 16 h of light and 8 h of darkness. The experiment was repeated three times per grapevine genotype.

2.4. Toxicity Assay on P. viticola

Only commercial products that were shown to be effective in the preventive assays were evaluated in the toxicity assays. The direct effect of the selected products on *P. viticola* was analyzed in three different ways.

Firstly, the inhibition of zoospore mobility was studied by mixing the same volume of commercial product and sporangia suspension, for 2 h, at room temperature. Both the commercial product and the sporangia suspension were prepared (double concentrated) to obtain a final mixture with the desired concentrations. The final concentration of sporangia was 2×10^4 sp/mL, and the final concentrations of the products were those recommended by the manufacturers. The number of mobile zoospores per minute per square were calculated with a Thoma haematocytometer (BRAND GMBH + CO KG, Wertheim, Germany), for all the mixtures, and compared to the control. Three independent mixtures were prepared per product, and three observations were made per mixture, having a total of 9 observations per product, in an optical microscope Nikon OPTIPHOT (Nikon, Tokyo, Japan).

Secondly, the same mixtures used to assess mobility were used to infect leaf discs to evaluate if the sporangia were able to develop the infection after being in contact with the product. An amount of 10 plants with 12–14 fully expanded leaves were used to obtain 16 mm diameter discs of the 3rd, 4th, and 5th fully expanded leaves to practice 16 mm discs. Briefly, three 10 µL drops of the mixture were deposited on the leaf discs. A total of 12 discs per mixture were inoculated. The droplets were maintained on the leaf discs for 4 h and then eliminated.

Finally, the effect of the products on moving and released zoospores was studied. For this, fresh sporangia were incubated for 2 h in water at room temperature, to induce germination and liberation of zoospores. When the release of the zoospores and their mobility was confirmed by optical microscopy, the same volume of zoospores was mixed with the product at double concentration. Mixtures were subsequently deposited on leaf discs by placing one droplet of 30 µL. The droplets were maintained on the leaf discs for 4 h to avoid the defense stimulation by BABA.

All the above-mentioned experiments were repeated three independent times in *Vitis vinifera* cv. Tempranillo RJ-78 and cv. Viura.

2.5. Half Maximal Inhibitory Concentration (IC_{50})

To further study the toxicity of the selected products, the IC_{50} value was obtained by mixing *P. viticola* sporangia with different concentrations of the commercial products at the following final concentrations: 0.1, 0.5, 1, 2, 4, and 8 mL/L for the commercial products and 0.1, 0.5, 1, 2, 4, and 8 mM for BABA. The final concentration of sporangia was adjusted to 2×10^4 sp/mL. The products and the sporangia were prepared (double concentrated) to obtain the final concentrations. Seven days after the infection, the sporulating surface was measured with ImageJ 1.53a, as described in Section 2.7 and transformed to a measure of area in mm^2 (1 mm^2 = 1,131,000 pixels).

The IC_{50} was calculated by plotting the sporulation reduction against the $\log_{10}$ of the concentrations and adjusting the values to a linear function. Using this function, the concentration corresponding to a 50% reduction was obtained. This experiment was performed three independent times in *V. vinifera* cv. Tempranillo RJ-78.

2.6. Whole-Plant Greenhouse Protection Assay

The assays started when plants had 8–10 fully expanded leaves. One plant was grown in each 5 L pot. Briefly, two product application modalities were designed, 3× and 1×. In the 3× modality, plants were sprayed 3 times at the recommended dose, separated by 1 week. In the 1× modality, just the last treatment was applied, in order to see the effect of treatment repetition in the level of protection. Two days after the last application, plants were artificially infected by a *P. viticola* suspension on the abaxial side of the 6 youngest fully expanded leaves from the apex. Thereafter, plants were maintained in a saturated environment, with a relative humidity of 90% and a temperature range of 18–22 °C, for 6 days, to induce the development of the downy mildew disease symptoms. Seven days after the infection disease, severity and the level of protection were calculated, as in Section 2.3.

2.7. Disease Severity Evaluation

For disc assays (Sections 2.3–2.5), images from infected discs were taken 7 days post infection (DPI) using a LEICA DMS1000 stereomicroscope (Leica Microsystems, Singapore) and subsequently processed with ImageJ 1.53a according to Peressotti et al. [37], with slight modifications. The disease severity reduction was obtained by comparing the average severity of the treated discs with the average severity of the negative control discs.

For greenhouse assays (Section 2.6.), the disease severity was assessed by visually estimating the percentage of sporulating surface of the 6 youngest expanded leaves in entire plant. The percentage was then transformed into a 0–6 scale to obtain the disease severity index in the leaf, similar to EPPO evaluations for the efficacy evaluation of fungicides [38], where a sporulation of 0% was given a "0" value, a sporulation smaller than 5% a "1" value, a sporulation smaller than 10% a "2" value, a sporulation smaller than 25% a "3" value, a sporulation smaller than 50% a "4" value, a sporulation smaller than 75% a "5" value, and a sporulation higher than 75% a "6" value. Those values were transformed into a disease index using the Townsend-Heuberger formula [39]:

$$\%\, of\ infection = \left(\sum (n \times v/i \times N)\right) \times 100$$

where v was the severity degree of infection, i was the highest degree of infection, n was the number of leaves presenting each infection degree, and N was the total amount of analyzed samples.

The disease severity index was used to calculate the level of protection and disease reduction by comparing that of the treated plants to that of the control.

2.8. Statistical Analysis

All statistical analyses were performed in JASP 0.16.3.0 software. Preventive, mobility, toxicity, and IC_{50} assays were analyzed by the Kruskal–Wallis test ($\alpha = 0.05$), followed by a Dunn's post hoc with Holm correction. In the preventive assays, each timepoint was analyzed in a separate test. For the greenhouse assays, data were analyzed using two-way analysis of variance (ANOVA) ($\alpha = 0.05$), followed by a Tukey's post hoc test with Holm correction.

3. Results

3.1. Preventive Assay

For the preventive assays, 11 commercial products (Table 1) were tested against downy mildew in two grapevine varieties, Viura and Tempranillo, at 1, 2, and 4 days before *P. viticola* inoculation. Seven days after the inoculation, the sporulation was measured for the control and the treatments, and a percentage of sporulation reduction was calculated. Data were not normally distributed and could not be transformed, so a Kruskal-Wallis test was used to analyze them. This analysis revealed that the reduction was only significantly affected by the commercial product ($p < 0.001$) and, to a lesser extent, by the time of application, although not significantly ($p = 0.081$). The effect of the variety was not significant ($p = 0.451$), so further analyses were made ignoring the genotype.

Among all the products tested, four products (CHIT, LECI, LESOY, and SALIX) and the control BABA showed a clear preventive protection against downy mildew at all timepoints, as reported in Figure 1. The positive control was able to inhibit sporulation by almost 100% at all timepoints, a behavior also observed with SALIX, which had a similar preventive potential of 85–95%. On the other hand, LECI and LESOY also successfully reduced sporulation, though to a lesser extent (at around 30–50%) and were not significantly different from any other product. Finally, CHIT reduced the sporulation by 10–30%, but was still more effective than other products at 1 and 2 days post treatment. The rest of the products (Table 1) never reached a 30% reduction, so they were not further investigated. Thus, four products, plus the negative (water) and positive (BABA) controls, were selected for subsequent assays: CHIT, LECI, LESOY, and SALIX. In Figure 1B, the sporulation reduction for each of the selected treatments and the different timepoints are presented.

3.2. Toxicity Assay

Firstly, to determine if the products were toxic against the pathogen, their effect on sporangial germination and the number of moving zoospores was analyzed (Figure 2). A Kruskal-Wallis test indicated that the number of mobile zoospores was affected by the product ($p < 0.001$). In fact, BABA did not inhibit the germination of sporangia, being the number of released and mobile zoospores similar to the control. The other evaluated products, however, inhibited the germination completely, and the number of observed zoospores was null, so a toxic nature could be plausible (Figure 2A).

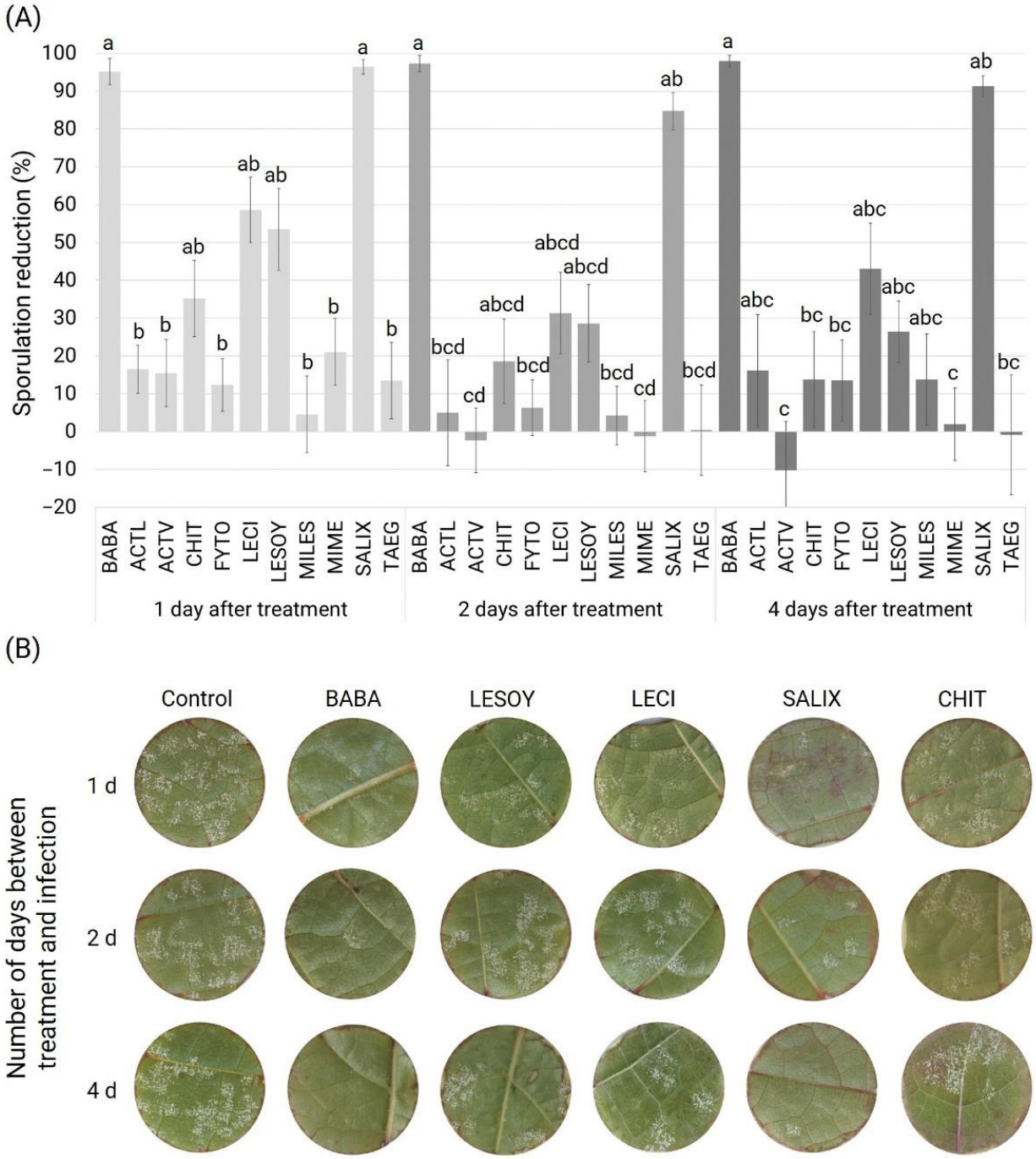

Figure 1. CHIT, LECI, LESOY, and SALIX, as the control BABA showed a good preventive protection against downy mildew, as opposed to the rest of the studied products. (**A**) Preventive activity of the commercial products against *P. viticola* (Table 1). The graph shows the average reduction of sporulation of discs infected at 1, 2 and 4 days after treatment or product application. A Dunn's post-hoc test ($\alpha = 0.05$) was performed for each timepoint to find statistical differences between products within the same timepoint, which are denoted by different small letters in the bar graph. (**B**) Visual evidence of the preventive capacity of BABA, LESOY, LECI, SALIX and CHIT seven days after pathogen inoculation. The columns show the different treatments, and the row shows the number of days between the treatment and *P. viticola* sporangia inoculation.

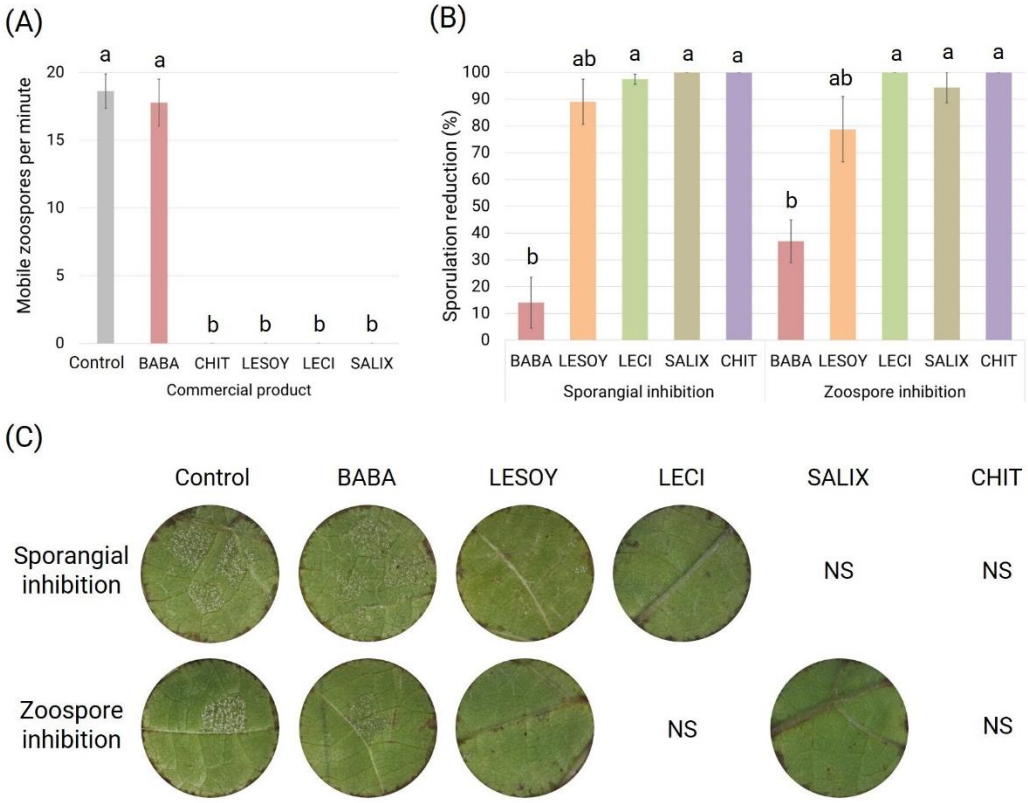

Figure 2. CHIT, LECI, LESOY, and SALIX showed a direct toxicity against *P. viticola*, both at the sporangial and zoospore level, while BABA did not affect their viability. (**A**) Mobile zoospores per minute observed when mixed with the products at the recommended dose. (**B**) Sporangial and zoospore inhibition by the products as a percentage of reduced sporulation. A Dunn's post-hoc test ($\alpha = 0.05$) was performed for both experiments to find significant differences between products, which are denoted by different small letters in the bar graphs. (**C**) Visual evidence of sporulation reduction and toxicity of the selected products on leaf discs seven days after pathogen inoculation. No sporulation (NS).

Secondly, the toxicity of the products was analyzed at the sporangia and zoospore level by measuring the sporulation reduction after the contact between sporangia and zoospores with the product. A Kruskal-Wallis test showed that the sporulation reduction was mainly explained by the commercial products ($p < 0.001$, for sporangia and zoospores) and not by the variety ($p = 0.731$ for sporangia and $p = 0.755$ for zoospores), so the variety was not taken into account. In general, BABA reduced the sporulation to a lesser extent (15% for sporangia and 35% for zoospores) than the products, which caused reductions close to 100%. As an exception, LESOY was less inhibitory than the other commercial mixtures, producing a 90% reduction at the sporangial level and 80% at the zoospore level, which indicated a lower toxicity (Figure 2B). This sporulation reduction is visually represented by leaf discs in Figure 2C, where the only two treatments that produced an evident sporulation were the control and BABA.

3.3. Half Maximal Inhibitory Concentration (IC_{50})

The toxicity was further evaluated by mixing pathogen sporangia with different concentrations of the commercial products in order to obtain the IC_{50} (Figure 3). After 2 h of direct contact, the mixtures were placed on leaf discs, and the sporulation was measured at 7 DPI using ImageJ 1.53a software. The product concentration significantly affected the sporulation, reporting a $p < 0.001$ for all the treatments.

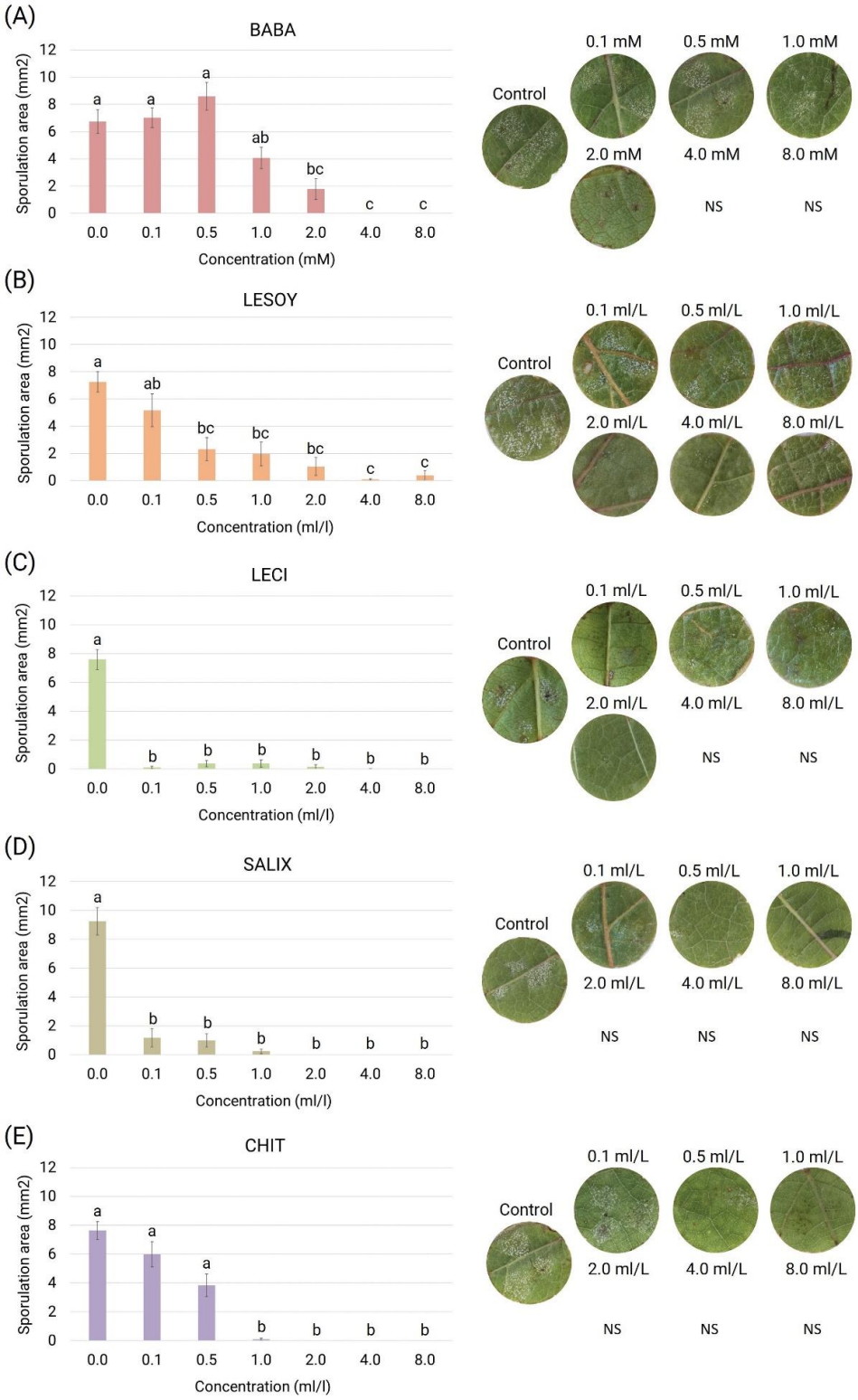

Figure 3. *P. viticola* sporangia mixed with different concentrations of the products showed a decrease in the pathogen mean sporulation area as the concentration increased. Representative photos of leaf discs are displayed seven days after infection for each product and concentration. (**A**) BABA, (**B**) LESOY, (**C**) LECI, (**D**) SALIX, and (**E**) CHIT. A Dunn's post hoc test ($\alpha = 0.05$) was performed for each product in order to find statistical differences between concentrations, which are denoted by different small letters in the bar graphs. No sporulation (NS).

Among the products, three different inhibition patterns were observed. Firstly, in the case of BABA (Figure 3A), the lowest concentrations did not affect the sporangia negatively, and no clear decrease was observed until 1 mM, but, at higher concentrations, the sporulation was inhibited. Secondly, regarding LESOY (Figure 3B) and CHIT (Figure 3E), the sporulation smoothly decreased as the concentration of the product increased. Finally, LECI (Figure 3C) and SALIX (Figure 3D) produced a sharp inhibition from the lowest concentration, which reflects a higher toxicity against *P. viticola* sporangia. For each product, the sporulation formed after the contact of the pathogen with different concentrations is presented.

From these results, their IC_{50} was calculated: 285 uL/L for CHIT and 250 uL/L for LESOY (the least toxic ones) and 0.0017 uL/L for SALIX and almost 0 uL/L for LECI (the most toxic ones). In the case of BABA, the IC_{50} was established at 1.345 mM. The graphs and formulas used for this calculation are available as supplementary material (Supplementary Figure S1).

3.4. Whole-Plant Greenhouse Protection Assay

The efficacy of the products was finally evaluated at the whole-plant level. To analyze the results, two ANOVAs were performed, one for each Tempranillo genotype. These revealed that the disease reduction was significantly affected only by the commercial product ($p < 0.001$ for both assays) and not by the modality. In fact, different levels of protections were observed for each product (Figure 4).

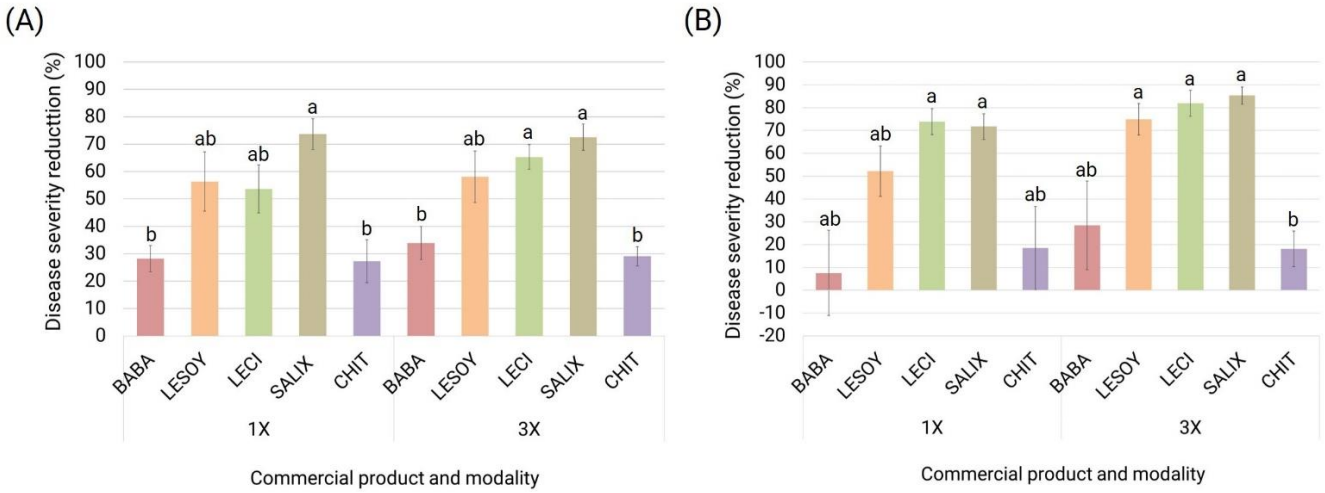

Figure 4. CHIT, LECI, LESOY and SALIX, as the control BABA showed preventive protection against downy mildew at the whole-plant level in (**A**) *Vitis vinifera* cv. Tempranillo, clone VN-40, and in (**B**) *Vitis vinifera* cv. Tempranillo, clone RJ-78. A Tukey's post hoc test ($\alpha = 0.05$) was performed for each experiment in order to find statistical differences between products and modalities (1× and 3×). Significant differences between products are denoted by different small letters in the bar graphs.

For instance, BABA and CHIT provided a low disease reduction, with values smaller than 30% in every case. LESOY and LECI, on the other hand, yielded a higher protection level and were able to reduce the disease severity from 50% and up to 80% in some cases. SALIX was the most effective product and reduced the symptoms by more than 70% in every experiment and modality, reaching values of 85%. Statistical differences were mainly observed between BABA and CHIT and the rest of the treatments, although not in all experiments and modalities. In general, no statistical differences were found between 1× and 3× modalities for any product in any assay. However, the disease reduction was higher in 3× than in 1× in most of the cases.

Finally, the effect of the clone was analyzed by performing another ANOVA. As expected, no significant differences were observed for the clones ($p = 0.515$), and a similar behavior of the products was perceived in both genotypes.

4. Discussion

In this study, we analyzed the potential of 11 commercial products to inhibit *P. viticola* infection in grapevine. This was achieved by performing a leaf disc assay *in vitro*, which is a method frequently used and accepted in the literature and is faster than whole plant assays [4]. Thereafter, the toxicity of the selected products against sporangia and zoospores was further studied *in vitro*. Finally, the efficacy of the products was evaluated at the whole-plant level in the greenhouse in order to support and complement *in vitro* results. Thus, the leaf disc assays allowed us to select four products—the most efficient ones, LESOY, LECI, SALIX, and CHIT (Table 1). Interestingly, all these formulations were composed of BS and legally indicated against *P. viticola* by the European Commission [15].

To understand the mechanism of action and potential of these products against *P. viticola*, it is of vital importance to know their origin and nature. LESOY, LECI, and SALIX are products obtained from plants, more concretely soybean and willow, whereas CHIT can be obtained from several crustaceans, insects, and fungi [17]. Normally, this type of product obtained from plants or other natural resources contains a mixture of active ingredients and displays several mechanism of actions, such as direct toxicity against the pathogen or a capacity of stimulating the defense system of vine [40]. In fact, plant extracts usually contain many secondary metabolites that can be toxic to several pathogens [41]. In the case of grapevine, many plant extracts have been proven to be toxic against *P. viticola*, as are some produced from pines, spruces, or the grapevine itself [42–45] and, in some cases, a dual toxic-stimulator effect has even been reported [46].

In our study, in order to discriminate between a direct toxic mechanism and a defense stimulation capacity, we selected a well known defense stimulator as a positive control, BABA, which has a pure defense stimulation capacity [47]. This nature was confirmed here, as we observed a very efficient preventive effect (almost by 100%) at all timepoints, but a very low toxicity, probably due to a short defense stimulation (4 h) (Figure 2). Regarding the commercial products, we also demonstrated a preventive effect as early as 96 h before infection but, unlike BABA, they displayed a high toxicity against the pathogen's sporangia and zoospores (Figure 2). Therefore, for the commercial products, a dual toxic-stimulator mechanism should be considered, not just a defense stimulator mechanism as for BABA. To our knowledge, the bibliography regarding the toxicity and defense stimulation capacity of lecithins, *Salix* cortex, and *E. arvense* against *P. viticola* is very scarce, as only chitosan has been intensely studied [26,28]. Their possible mechanism of action is analyzed in this study.

In the case of soybean lecithin, a recent review [33] supports its use as a crop protection product, but the toxic components or mechanism by which this is achieved are not described. The first reported efficacy was described long ago against tomato late blight, caused by an oomycete such as *P. viticola* [48] and, more recently, a soybean protein hydrolysate was found to be effective in controlling *P. viticola* [49]. However, this hydrolysate was mainly composed of proteins, as opposed to our products, which are rich in lipids [50].

To further understand the mechanism by which lecithins might act against *P. viticola*, it is important to know its composition. This has been frequently analyzed, reporting a mixture of phosphatides, more concretely, phosphatidylinositol, phosphatidylethanolamine, and phosphatidylserine, with the most common fatty acids (FA) being palmitic acid (16:0), stearic acid (18:0), linoleic acid (18:1), and linolenic acid (18:2) [50]. Neither the phosphatides, nor the FAs previously mentioned, have been reported to be toxic by their own against any oomycete, but saturated FAs, such as palmitic acid, display certain inhibitory capacity against plant pathogenic fungi [51]. However, the FAs present in the lecithins could act more actively via plant defense stimulation, rather than by a toxic effect. In fact, linolenic acid and its precursor linoleic acid, both present in soy lecithin, are the precursors of a wide variety of oxylipins and the plant hormone jasmonic acid (JA), which actively participate in plant defense [52]. In the case of the biotrophic microorganism *P. viticola*, salicylic acid has always been considered the main hormone orchestrating the plant response against the pathogen [53], but JA might also be involved. In fact, an early production of linolenic acid and JA could be important for the establishment of an incompatible interaction in

resistant genotypes [54–56]. Thus, lecithins could prevent *P. viticola* infection by activating oxylipins and JA-related immunity.

One of the main differences observed between the two lecithin-based commercial products was the toxicity. LESOY showed a IC_{50} of 250 µL/L and a LECI of practically 0 µL/L, which could be due to the presence of 15% of *E. arvense* or horsetail in LECI. In fact, this plant extract has an antigerminative activity against *P. viticola* zoospores, as previously demonstrated [31]. Moreover, *E. arvense* extract is rich in fatty acids, which could act as antifungal agents by entering fungal plasma membranes and destabilizing cell integrity [57,58]. Although the toxicity of both products was different, the preventive capacity was very similar (Figure 1), suggesting that *E. arvense* might have a low impact on the plant defense. In fact, one of the commercial products tested in this study (MILES) was composed solely of *E. arvense* extract and did not induce any significant preventive protection, so the preventive activity of the products could be attributed mainly to the lecithins.

Regarding *Salix* cortex, we reported a direct toxic effect against *P. viticola* sporangia with a IC_{50} of 0.0017 µL/L, indicating a very high toxicity. Contrary to BABA, LESOY, or CHIT, which were barely toxic at low quantities, the inhibition was observed from very low concentrations, as with LECI. On top of that, a very potent preventive effect was observed, similar to the control BABA. These findings concur with other studies that also found a direct toxic [29,35,36] and preventive effect [35,36] against *P. viticola*. Interestingly, the preventive effect was not found at 12 h before infection by Andreu et al., but it was observed at 48 and 4 h pre infection by Kast. In our case, the preventive activity of the extract was very potent at 24, 48, and 96 h before *P. viticola* infection, a time long enough to consider a defense stimulation capacity of SALIX.

This stimulation capacity is supported by the presence of polyphenols [29] and phenolic acids [59] in *Salix* cortex extracts, with salicylic acid (SA) being found in several willow species [60,61]. This is a key hormone in plant defense [62] and also happens to have antifungal activity [63]. In the case of *P. viticola*, resistant grapevine genotypes display a higher capacity of SA production and subsequent systemic acquired resistance (SAR) activation during the infection process [64]. In fact, the application in grapevine of the chemical analogue of SA, benzothiadiazole (BTH), activates pathogenesis-related proteins and phenylpropanoid pathway genes [65], which are typically involved in grapevine defense. Therefore, the application of a *Salix* cortex extract could help fight *P. viticola* infection in sensitive genotypes by providing external inductors of SAR.

The last product to have a notable effect against *P. viticola* was CHIT, based in chitosan, a molecule largely discussed in the literature. In our study, chitosan displayed a lower preventive activity when compared to the other products, but was still able to reduce *P. viticola* sporulation to some extent (30%). This low value contrasts with the higher preventive effect (90%) reported by Harm et al. [66], probably derived from the use of a different commercial product. We also noted that CHIT was the least toxic of the four commercial products selected, with a IC_{50} of 285 µL/L, but still inhibited sporangia germination and zoospores at the recommended dose by the manufacturer. This observation agrees with Maia et al. [67], who found a spore germination decrease of 60%.

Besides toxicity, the defense stimulator capacity of chitosan has already been discussed. Interestingly, Aziz et al. [26,28] demonstrated that, in grapevine, chitosan was able to induce several phytoalexins and to activate glucanase, chitinase, and phenylalanine-ammonia lyase (PAL) activities. In addition, the application of chitosan seems to induce the accumulation of SAs in grapevine [68]. These studies confirm that chitosan behaves as a plant defense stimulator when applied in grapevine, but also as a toxic component against *P. viticola*, as demonstrated in this work.

To support and complement all the previous *in vitro* results, whole-plant greenhouse protection assays were performed, mimicking high-risk infection conditions. Thanks to this approach, we could control and limit the climatic conditions of the chamber, reducing the variability in the efficacy of the products. Nonetheless, although these trials are always valuable, they are not sufficient for confirming the efficacy of PPP, and field experiments

are always recommended. In fact, greenhouse environments are not affected by climatic factors, such as rainfall, which can produce the runoff of PPP in field [69]. Therefore, the efficacies observed in the greenhouse might be higher than those reported in the field.

In our case, the evaluated products were efficient and able to protect whole plants in an optimal environment for the development of the pathogen (Figure 4). However, *in vitro* and whole-plant results were not always in concordance. In fact, BABA provided one of the lowest protective effects in the whole-plant assay, as opposed to *in vitro*, where it was the best treatment. On the other hand, LESOY and LECI were able to improve the disease protection observed *in vitro*. Finally, CHIT and SALIX had a similar protection level in both assays.

Regarding our four selected commercial products, very few scientific studies have reported the efficacy at the whole-plant level, and most of them have been performed in field. Lecithins are the least studied, and only one field trial has been published [19], generating a 25–30% protection against *P. viticola*, lower than our observed results of around 60–70%. In the case of SALIX, Dagostin et al. [70,71] revealed that *Salix* cortex was able to reduce *P. viticola* infection by 60% in field, close to our 70–80% observed efficacy. However, the protection was still lower than that of copper alone. Interestingly, *Salix* cortex, combined with copper, improved the efficacy of copper itself [20]. Concerning chitosan, its use in vineyard is more developed, unlike the other products. In fact, Romanazzi et al. [72] and Vitalini et al. [25] demonstrated that, in field, it was as efficient as other recognized stimulators, such as BTH, and that, when combined with copper, strongly reduced *P. viticola* infestation. In our case, we have not been able to report this efficacy, and a low protection of 20–30% was observed. Altogether, these results suggest that lecithins, *Salix* cortex, and chitosan could be very valuable substitutes or complements to conventional fungicides and copper, helping to maintain a relevant downy mildew control in vineyard.

Finally, it would be necessary to highlight the usefulness of these alternative PPPs in a climate change scenario by diversifying phytosanitary practices and increasing the resilience of the vineyard against pests and diseases. Currently, most scenarios expect a rise in global temperature [73], resulting in earlier blooming and harvest for many orchards, including grapevine [74,75]. Moreover, the frequency of *P. viticola* attacks could increase in the future, driven by higher spring temperatures in May and June, when the primary infections of the pathogen occur. In fact, the increase in temperatures will counterbalance the effect of rainfall decrease in the disease pressure, and some models predict that up to two additional phytosanitary treatments will be needed in order to reduce the outburst of primary infections [76]. However, the disease pressure and subsequent phytosanitary applications will be highly dependent on the temperature and rainfall changes expected for every specific region, with rainfall and humidity being the most limiting factor in the occurrence of primary infections [77]. In any case, considering current European policies, these additional treatments would be in conflict with the reduction of conventional fungicides imposed by the European Commission. Therefore, in this uncertain scenario, winegrowers will need to adapt and modify viticultural practices in order to improve the sanitary status of vineyards, being forced to consider alternative PPPs that will align with the European phytosanitary strategy.

5. Conclusions

In this work, we evaluated the potential of several alternative commercial products to control *P. viticola*, focusing on their direct toxicity against the pathogen and on their capacity to prevent the disease development at the whole-plant level. Among all the studied formulations, four BS-based products have stood out as the best options and have demonstrated a good preventive activity, both *in vitro* and *in planta*, and a high toxicity against the pathogen *in vitro*, indicating a double mechanism of action. To the best our knowledge, our work provides the most complete evaluation of the toxicity of chitosan, *Equisetum arvense*, lecithins, and *Salix* cortex against *P. viticola*, reporting their half maximal inhibitory concentration and describing their effect on sporangia and zoospore

viability. Nonetheless, field trials should be performed in different viticultural areas to strengthen the reliability of these results in a real disease scenario. Moreover, further genetic, physiological, and metabolic research will improve the understanding of the mechanism of action of these products in grapevine. Altogether, our results reinforce the role of chitosan, *Equisetum arvense*, lecithins, and *Salix* cortex as very useful tools towards a sustainable vitiviniculture.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12123139/s1, Table S1: Product doses used for preventive, toxicity, and whole-plant infection assays; Figure S1: Calculation of the IC_{50} of the different commercial products.

Author Contributions: Conceptualization, D.L.D.M., A.M.D.-N. and A.M.-P.; methodology, D.L.D.M.; investigation, D.L.D.M.; writing—original draft preparation, D.L.D.M.; writing—review and editing, D.L.D.M., A.M.D.-N. and A.M.-P.; supervision, A.M.D.-N. and A.M.-P.; project administration, A.M.D.-N.; funding acquisition, A.M.D.-N. Both A.M.D.-N. and A.M.-P. contributed equally to the conceptualization, editing, supervision, and review of the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Department of Economic Development, Sustainability and Environment of the Basque Government aid program for the training of young researchers and technologists in the scientific-technological and business environment of the Basque agricultural, fisheries and food sector, regulated by Decree 185 /2007, of October 23 (replaced by Decree 115/2021, of March 23) with funding number 00012-PIT2019-22. A.M.P. is part of the consolidated research group IT1682-22 of the University of the Basque Country (UPV/EHU).

Data Availability Statement: Not applicable.

Acknowledgments: We would like to thank Andrea Berdonces Bóveda for performing the IC_{50} experiments presented in this manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. International Organisation of Vine and Wine. *State of the World Vine and Wine Sector 2021*; International Organisation of Vine and Wine: Paris, France, 2022.
2. Komárek, M.; Čadková, E.; Chrastný, V.; Bordas, F.; Bollinger, J.C. Contamination of Vineyard Soils with Fungicides: A Review of Environmental and Toxicological Aspects. *Environ. Int.* **2010**, *36*, 138–151. [CrossRef]
3. Corio-Costet, M.F. Fungicide Resistance in *Plasmopara viticola* in France and Anti-Resistance Measures. In *Fungicide Resistance in Crop Protection: Risk and Management*; Thind, T.S., Ed.; CABI: Wallingford, UK, 2012; pp. 157–171. ISBN 978-1-84593-905-2.
4. Massi, F.; Torriani, S.F.F.; Borghi, L.; Toffolatti, S.L. Fungicide Resistance Evolution and Detection in Plant Pathogens: *Plasmopara viticola* as a Case Study. *Microorganisms* **2021**, *9*, 119. [CrossRef] [PubMed]
5. Commission Implementing Regulation (EU). Commission Implementing Regulation (EU) 2018/1981 of 13 December 2018 Renewing the Approval of the Active Substances Copper Compounds, as Candidates for Substitution, in Accordance with Regulation (EC) No 1107/2009 of the European Parliament and of the Council Concerning the Placing of Plant Protection Products on the Market, and Amending the Annex to Commission Implementing Regulation (EU) No 540/2011. *Off. J. Eur. Union* **2018**, *317*, 16–20.
6. Green Deal: Halving Pesticide Use by 2030. Available online: https://ec.europa.eu/eip/agriculture/en/news/green-deal-halving-pesticide-use-2030 (accessed on 5 October 2022).
7. Berthon, J.Y.; Michel, T.; Wauquier, A.; Joly, P.; Gerbore, J.; Filaire, E. Seaweed and Microalgae as Major Actors of Blue Biotechnology to Achieve Plant Stimulation and Pest and Pathogen Biocontrol—A Review of the Latest Advances and Future Prospects. *J. Agric. Sci.* **2021**, *159*, 523–534. [CrossRef]
8. Boulogne, I.; Petit, P.; Ozier-Lafontaine, H.; Desfontaines, L.; Loranger-Merciris, G. Insecticidal and Antifungal Chemicals Produced by Plants: A Review. *Environ. Chem. Lett.* **2012**, *10*, 325–347. [CrossRef]
9. Pérez, M.; Falqué, E.; Domínguez, H. Antimicrobial Action of Compounds from Marine Seaweed. *Mar. Drugs* **2016**, *14*, 52. [CrossRef]
10. Hou, S.; Liu, Z.; Shen, H.; Wu, D. Damage-Associated Molecular Pattern-Triggered Immunity in Plants. *Front. Plant Sci.* **2019**, *10*, 646. [CrossRef]
11. Henry, G.; Thonart, P.; Ongena, M. PAMPs, MAMPs, DAMPs and Others: An Update on the Diversity of Plant Immunity Elicitors. *Biotechnol. Agron. Soc. Environ.* **2012**, *16*, 257–268.

12. Quintana-Rodriguez, E.; Duran-Flores, D.; Heil, M.; Camacho-Coronel, X. Damage-Associated Molecular Patterns (DAMPs) as Future Plant Vaccines That Protect Crops from Pests. *Sci. Hortic.* **2018**, *237*, 207–220. [CrossRef]
13. Héloir, M.C.; Adrian, M.; Brulé, D.; Claverie, J.; Cordelier, S.; Daire, X.; Dorey, S.; Gauthier, A.; Lemaître-Guillier, C.; Negrel, J.; et al. Recognition of Elicitors in Grapevine: From MAMP and DAMP Perception to Induced Resistance. *Front. Plant Sci.* **2019**, *10*, 1117. [CrossRef]
14. Regulation (EC). Regulation (EC) No 1107/2009 of the European Parliament and of the Council of 21 October 2009 Concerning the Placing of Plant Protection Products on the Market and Repealing Council Directives 79/117/EEC and 91/414/EEC. *Off. J. Eur. Union* **2009**, *309*, 1–50.
15. EU Pesticides Database—Active Substances. Available online: https://ec.europa.eu/food/plant/pesticides/eu-pesticides-database/start/screen/active-substances (accessed on 5 October 2022).
16. International Organisation of Vine and Wine. *The World Organic Vineyard*; International Organisation of Vine and Wine: Paris, France, 2021.
17. Kumari, S.; Kishor, R. Chitin and Chitosan: Origin, Properties, and Applications. In *Handbook of Chitin and Chitosan*; Elsevier: Amsterdam, The Netherlands, 2020; pp. 1–33. ISBN 978-0-12-817970-3.
18. Taylor, A.; Bonafos, R.; Chovelon, M.; Parvaud, C.E.; Furet, A.; Aveline, N.; Bertrand, C.; Marchand, P.A. *Equisetum arvense* (Horsetail) Extract: The First Approved Basic Substance Allowed for EU Crop Protection. *Int. J. Bio-Resour. Stress Manag.* **2022**, *13*, 566–577. [CrossRef]
19. Marchand, P.A. Basic Substances under EC 1107/2009 Phytochemical Regulation: Experience with Non-Biocide and Food Products as Biorationals. *J. Plant Prot. Res.* **2016**, *56*, 312–318. [CrossRef]
20. Deniau, M.G.; Bonafos, R.; Chovelon, M.; Parvaud, C.E.; Furet, A.; Bertrand, C.; Marchand, P.A. Willow Extract (*Salix* Cortex), a Basic Substance of Agronomical Interests. *Int. J. Bio-Resour. Stress Manag.* **2019**, *10*, 408–418. [CrossRef]
21. Riseh, R.S.; Hassanisaadi, M.; Vatankhah, M.; Babaki, S.A.; Barka, E.A. Chitosan as a Potential Natural Compound to Manage Plant Diseases. *Int. J. Biol. Macromol.* **2022**, *220*, 998–1009. [CrossRef]
22. El Hadrami, A.; Adam, L.R.; El Hadrami, I.; Daayf, F. Chitosan in Plant Protection. *Mar. Drugs* **2010**, *8*, 968. [CrossRef] [PubMed]
23. De Bona, G.S.; Vincenzi, S.; De Marchi, F.; Angelini, E.; Bertazzon, N. Chitosan Induces Delayed Grapevine Defense Mechanisms and Protects Grapevine against *Botrytis cinerea*. *J. Plant Dis. Prot.* **2021**, *128*, 715–724. [CrossRef]
24. Buzón-Durán, L.; Langa-Lomba, N.; González-García, V.; Casanova-Gascón, J.; Martín-Gil, J.; Pérez-Lebeña, E.; Martín-Ramos, P. On the Applicability of Chitosan Oligomers-Amino Acid Conjugate Complexes as Eco-Friendly Fungicides against Grapevine Trunk Pathogens. *Agronomy* **2021**, *11*, 324. [CrossRef]
25. Vitalini, S.; Orlando, F.; Iriti, M. Field Study on the Efficacy of Plant Activators against *Plasmopara viticola*. *Plant Fungal Res.* **2020**, *2*, 2–7. [CrossRef]
26. Aziz, A.; Trotel-Aziz, P.; Dhuicq, L.; Jeandet, P.; Couderchet, M.; Vernet, G. Chitosan Oligomers and Copper Sulfate Induce Grapevine Defense Reactions and Resistance to Gray Mold and Downy Mildew. *Phytopathology* **2006**, *96*, 1188–1194. [CrossRef]
27. Garde-Cerdán, T.; Mancini, V.; Carrasco-Quiroz, M.; Servili, A.; Gutiérrez-Gamboa, G.; Foglia, R.; Pérez-Álvarez, E.P.; Romanazzi, G. Chitosan and Laminarin as Alternatives to Copper for *Plasmopara viticola* Control: Effect on Grape Amino Acid. *J. Agric. Food Chem.* **2017**, *65*, 7379–7386. [CrossRef] [PubMed]
28. Aziz, A.; Trotel-Aziz, P.; Conreux, A.; Dhuicq, L.; Jeandet, P.; Couderchet, M. Chitosan Induces Phytoalexin Synthesis, Chitinase and β-1,3-Glucanase Activities, and Resistance of Grapevine to Fungal Pathogens. In *Macromolecules and Secondary Metabolites of Grapevine and Wines*; Lavoisier, Intercept Ltd.: Paris, France, 2007.
29. Andreu, V.; Levert, A.; Amiot, A.; Cousin, A.; Aveline, N.; Bertrand, C. Chemical Composition and Antifungal Activity of Plant Extracts Traditionally Used in Organic and Biodynamic Farming. *Environ. Sci. Pollut. Res.* **2018**, *25*, 29971–29982. [CrossRef] [PubMed]
30. Langa-Lomba, N.; Buzón-Durán, L.; Martín-Ramos, P.; Casanova-Gascón, J.; Martín-Gil, J.; Sánchez-Hernández, E.; González-García, V. Assessment of Conjugate Complexes of Chitosan and Urtica Dioica or *Equisetum arvense* Extracts for the Control of Grapevine Trunk Pathogens. *Agronomy* **2021**, *11*, 976. [CrossRef]
31. La Torre, A.; Righi, L.; Iovino, V.; Battaglia, V. Evaluation of Copper Alternative Products to Control Grape Downy Mildew in Organic Farming. *J. Plant Pathol.* **2019**, *101*, 1005–1012. [CrossRef]
32. Pallag, A.; Bungau, S.; Tit, D.M.; Jurca, T.; Sirbu, V.; Honiges, A.; Horhogea, C. Comparative Study of Polyphenols, Flavonoids and Chlorophylls in *Equisetum arvense* L. Populations. *Rev. Chim.* **2016**, *67*, 530–533.
33. Jolly, M.; Vidal, R.; Marchand, P.A. Lecithins: A Food Additive Valuable for Antifungal Crop Protection. *Int. J. Econ. Plants* **2018**, *5*, 104–107. [CrossRef]
34. Llamazares-Miguel, D.; Bodin, E.; Laurens, M.; Corio-Costet, M.F.; Nieto, J.; Fernández-Navarro, J.R.; Mena-Petite, A.; Diez-Navajas, A.M. Genetic Regulation in *Vitis vinifera* by Approved Basic Substances against Downy Mildew. *BIO Web Conf.* **2022**, *50*, 03001. [CrossRef]
35. Kast, W.K. Effects of Plant Extracts on Downy Mildew of Vine-Laboratory and Field Experiments [Wirkung von Pflanzenextrakten in Labor- und Freilandversuchen Gegen Rebenperonospora]. In Proceedings of the Ecofruit—10th International Conference on Cultivation Technique and Phytopathological Problems in Organic Fruit-Growing and Viticulture, Weinsberg, Germany, 4–7 February 2002; pp. 157–162.

36. Marchand, P.A.; Isambert, C.A.; Jonis, M.; Parveaud, C.E.; Chovelon, M.; Gomez, C.; Lambion, J.; Ondet, S.J.; Aveline, N.; Molot, B.; et al. Évaluation Des Caractéristiques et de l'intérêt Agronomique de Préparations Simples de Plantes, Pour Des Productions Fruitières, Légumières et Viticoles Économes En Intrants. *Innov. Agron.* **2014**, *34*, 83–96.
37. Peressotti, E.; Duchêne, E.; Merdinoglu, D.; Mestre, P. A Semi-Automatic Non-Destructive Method to Quantify Grapevine Downy Mildew Sporulation. *J. Microbiol. Methods* **2011**, *84*, 265–271. [CrossRef]
38. EPPO. EPPO Standards—Guidelines for the Efficacy Evaluation of Plant Protection Products—PP 1/31(3) *Plasmopara viticola*. *Bull. OEPP/EPPO Bull.* **2001**, *31*, 313–317.
39. Tinivella, F.; Hirata, L.M.; Celan, M.A.; Wright, S.A.I.; Amein, T.; Schmitt, A.; Koch, E.; van der Wolf, J.M.; Groot, S.P.C.; Stephan, D.; et al. Control of Seed-Borne Pathogens on Legumes by Microbial and Other Alternative Seed Treatments. *Eur. J. Plant Pathol.* **2009**, *123*, 139–151. [CrossRef]
40. Suteu, D.; Rusu, L.; Zaharia, C.; Badeanu, M.; Daraban, G. Challenge of Utilization Vegetal Extracts as Natural Plant Protection Products. *Appl. Sci.* **2020**, *10*, 8913. [CrossRef]
41. Zaynab, M.; Fatima, M.; Abbas, S.; Sharif, Y.; Umair, M.; Zafar, M.H.; Bahadar, K. Role of Secondary Metabolites in Plant Defense against Pathogens. *Microb. Pathog.* **2018**, *124*, 198–202. [CrossRef]
42. Cohen, Y.; Wang, W.; Ben-Daniel, B.H.; Ben-Daniel, Y. Extracts of *Inula viscosa* Control Downy Mildew of Grapes Caused by *Plasmopara viticola*. *Phytopathology* **2006**, *96*, 417–424. [CrossRef]
43. Gabaston, J.; Cantos-Villar, E.; Biais, B.; Waffo-Teguo, P.; Renouf, E.; Corio-Costet, M.F.; Richard, T.; Mérillon, J.M. Stilbenes from *Vitis vinifera* L. Waste: A Sustainable Tool for Controlling *Plasmopara viticola*. *J. Agric. Food Chem.* **2017**, *65*, 2711–2718. [CrossRef]
44. Gabaston, J.; Richard, T.; Biais, B.; Waffo-Teguo, P.; Pedrot, E.; Jourdes, M.; Corio-Costet, M.F.; Mérillon, J.-M. Stilbenes from Common Spruce (*Picea abies*) Bark as Natural Antifungal Agent against Downy Mildew (*Plasmopara viticola*). *Ind. Crops Prod.* **2017**, *103*, 267–273. [CrossRef]
45. Schnee, S.; Queiroz, E.F.; Voinesco, F.; Marcourt, L.; Dubuis, P.H.; Wolfender, J.L.; Gindro, K. *Vitis vinifera* Canes, a New Source of Antifungal Compounds against *Plasmopara viticola, Erysiphe necator*, and *Botrytis cinerea*. *J. Agric. Food Chem.* **2013**, *61*, 5459–5467. [CrossRef]
46. Krzyzaniak, Y.; Trouvelot, S.; Negrel, J.; Cluzet, S.; Valls, J.; Richard, T.; Bougaud, A.; Jacquens, L.; Klinguer, A.; Chiltz, A.; et al. A Plant Extract Acts Both as a Resistance Inducer and an Oomycide Against Grapevine Downy Mildew. *Front. Plant Sci.* **2018**, *9*, 1085. [CrossRef] [PubMed]
47. Piękna-Grochala, J.; Kępczyńska, E. Induction of Resistance against Pathogens by β-Aminobutyric Acid. *Acta Physiol. Plant.* **2013**, *35*, 1735–1748. [CrossRef]
48. Misato, T.; Homma, Y.; Kō, K. The Development of a Natural Fungicide, Soybean Lecithin. *Neth. J. Plant Pathol.* **1977**, *83*, 395–402. [CrossRef]
49. Lachhab, N.; Sanzani, S.M.; Adrian, M.; Chiltz, A.; Balacey, S.; Boselli, M.; Ippolito, A.; Poinssot, B. Soybean and Casein Hydrolysates Induce Grapevine Immune Responses and Resistance against *Plasmopara viticola*. *Front. Plant Sci.* **2014**, *5*, 716. [CrossRef] [PubMed]
50. Scholfield, C.R. Composition of Soybean Lecithin. *J. Am. Oil Chem. Soc.* **1981**, *58*, 889–892. [CrossRef]
51. Liu, S.; Ruan, W.; Li, J.; Xu, H.; Wang, J.; Gao, Y.; Wang, J. Biological Control of Phytopathogenic Fungi by Fatty Acids. *Mycopathologia* **2008**, *166*, 93–102. [CrossRef]
52. Wasternack, C.; Feussner, I. The Oxylipin Pathways: Biochemistry and Function. *Annu. Rev. Plant Biol.* **2018**, *69*, 363–386. [CrossRef] [PubMed]
53. Peng, Y.; Yang, J.; Li, X.; Zhang, Y. Salicylic Acid: Biosynthesis and Signaling. *Annu. Rev. Plant Biol.* **2021**, *72*, 761–791. [CrossRef] [PubMed]
54. Ali, K.; Maltese, F.; Figueiredo, A.; Rex, M.; Fortes, A.M.; Zyprian, E.; Pais, M.S.; Verpoorte, R.; Choi, Y.H. Alterations in Grapevine Leaf Metabolism upon Inoculation with *Plasmopara viticola* in Different Time-Points. *Plant Sci.* **2012**, *191–192*, 100–107. [CrossRef] [PubMed]
55. Figueiredo, A.; Monteiro, F.; Sebastiana, M. First Clues on a Jasmonic Acid Role in Grapevine Resistance against the Biotrophic Fungus *Plasmopara viticola*. *Eur. J. Plant Pathol.* **2015**, *142*, 645–652. [CrossRef]
56. Guerreiro, A.; Figueiredo, J.; Sousa Silva, M.; Figueiredo, A. Linking Jasmonic Acid to Grapevine Resistance against the Biotrophic Oomycete *Plasmopara viticola*. *Front. Plant Sci.* **2016**, *7*, 565. [CrossRef]
57. Avis, T.J.; Bélanger, R.R. Specificity and Mode of Action of the Antifungal Fatty Acid *cis*-9-Heptadecenoic Acid Produced by *Pseudozyma flocculosa*. *Appl. Environ. Microbiol.* **2001**, *67*, 956–960. [CrossRef]
58. Bergsson, G.; Arnfinnsson, J.; Steingrímsson, Ó.; Thormar, H. In Vitro Killing of *Candida albicans* by Fatty Acids and Monoglycerides. *Antimicrob. Agents Chemother.* **2001**, *45*, 3209–3212. [CrossRef]
59. Pobłocka-Olech, L.; Krauze-Baranowska, M.; Głód, D.; Kawiak, A.; Łojkowska, E. Chromatographic Analysis of Simple Phenols in Some Species from the Genus *Salix*. *Phytochem. Anal.* **2010**, *21*, 463–469. [CrossRef] [PubMed]
60. Kammerer, B.; Kahlich, R.; Biegert, C.; Gleiter, C.H.; Heide, L. HPLC-MS/MS Analysis of Willow Bark Extracts Contained in Pharmaceutical Preparations. *Phytochem. Anal.* **2005**, *16*, 470–478. [CrossRef]
61. Ramos, P.A.B.; Moreirinha, C.; Santos, S.A.O.; Almeida, A.; Freire, C.S.R.; Silva, A.M.S.; Silvestre, A.J.D. Valorisation of Bark Lipophilic Fractions from Three Portuguese *Salix* Species: A Systematic Study of the Chemical Composition and Inhibitory Activity on *Escherichia coli*. *Ind. Crops Prod.* **2019**, *132*, 245–252. [CrossRef]

62. Ding, P.; Ding, Y. Stories of Salicylic Acid: A Plant Defense Hormone. *Trends Plant Sci.* **2020**, *25*, 549–565. [CrossRef] [PubMed]
63. da Rocha Neto, A.C.; Maraschin, M.; Di Piero, R.M. Antifungal Activity of Salicylic Acid against *Penicillium expansum* and Its Possible Mechanisms of Action. *Int. J. Food Microbiol.* **2015**, *215*, 64–70. [CrossRef] [PubMed]
64. Liu, S.; Wu, J.; Zhang, P.; Hasi, G.; Huang, Y.; Lu, J.; Zhang, Y. Response of Phytohormones and Correlation of SAR Signal Pathway Genes to the Different Resistance Levels of Grapevine against *Plasmopara viticola* Infection. *Plant Physiol. Biochem.* **2016**, *107*, 56–66. [CrossRef]
65. Burdziej, A.; Bellée, A.; Bodin, E.; Valls Fonayet, J.; Magnin, N.; Szakiel, A.; Richard, T.; Cluzet, S.; Corio-Costet, M.F. Three Types of Elicitors Induce Grapevine Resistance against Downy Mildew via Common and Specific Immune Responses. *J. Agric. Food Chem.* **2021**, *69*, 1781–1795. [CrossRef]
66. Harm, A.; Kassemeyer, H.-H.; Seibicke, T.; Regner, F. Evaluation of Chemical and Natural Resistance Inducers against Downy Mildew (*Plasmopara viticola*) in Grapevine. *Am. J. Enol. Vitic.* **2011**, *62*, 184–192. [CrossRef]
67. Maia, A.J.; Botelho, R.V.; Faria, C.M.D.R.; Leite, C.D. Ação de quitosana sobre o desenvolvimento de *Plasmopara viticola* e *Elsinoe ampelina*, *in vitro* e em videiras cv. *Isabel. Summa Phytopathol.* **2010**, *36*, 203–209. [CrossRef]
68. Prakongkha, I.; Sompong, M.; Wongkaew, S.; Athinuwat, D.; Buensanteai, N. Changes in Salicylic Acid in Grapevine Treated with Chitosan and BTH against *Sphaceloma ampelinum*, the Causal Agent of Grapevine Anthracnose. *Afr. J. Microbiol. Res.* **2013**, *7*, 557–563. [CrossRef]
69. Rial Otero, R.; Cancho Grande, B.; Arias Estévez, M.; López Periago, E.; Simal Gándara, J. Procedure for the Measurement of Soil Inputs of Plant-Protection Agents Washed off through Vineyard Canopy by Rainfall. *J. Agric. Food Chem.* **2003**, *51*, 5041–5046. [CrossRef] [PubMed]
70. Dagostin, S.; Ferrari, A.; Pertot, I. Efficacy Evaluation of Biocontrol Agents against Downy Mildew for Copper Replacement in Organic Grapevine Production in Europe. In Proceedings of the IOBC/WPRS Integrated Protection in Viticulture, Boario Terme, Italy, 20–22 October 2005; pp. 15–21.
71. Dagostin, S.; Formolo, T.; Pertot, I. Replacement of Copper in Organic Viticulture: Efficacy Evaluation of New Natural Fungicides against Downy Mildew. In Proceedings of the IOBC/WPRS Integrated Protection in Viticulture, Sicily, Italy, 25–27 October 2007; pp. 87–90.
72. Romanazzi, G.; Mancini, V.; Foglia, R.; Marcolini, D.; Kavari, M.; Piancatelli, S. Use of Chitosan and Other Natural Compounds Alone or in Different Strategies with Copper Hydroxide for Control of Grapevine Downy Mildew. *Plant Dis.* **2021**, *105*, 3261–3268. [CrossRef] [PubMed]
73. IPCC. *Global Warming of 1.5 °C: An IPCC Special Report on the Impacts of Global Warming of 1.5 °C above Pre-Industrial Levels and Related Global Greenhouse Gas Emission Pathways, in the Context of Strengthening the Global Response to the Threat of Climate Change, Sustainable Development, and Efforts to Eradicate Poverty*; Intergovernmental Panel on Climate Change: Geneva, Switzerland, 2018.
74. Gitea, M.A.; Gitea, D.; Tit, D.M.; Purza, L.; Samuel, A.D.; Bungău, S.; Badea, G.E.; Aleya, L. Orchard Management under the Effects of Climate Change: Implications for Apple, Plum, and Almond Growing. *Environ. Sci. Pollut. Res.* **2019**, *26*, 9908–9915. [CrossRef] [PubMed]
75. Droulia, F.; Charalampopoulos, I. Future Climate Change Impacts on European Viticulture: A Review on Recent Scientific Advances. *Atmosphere* **2021**, *12*, 495. [CrossRef]
76. Salinari, F.; Giosuè, S.; Tubiello, F.N.; Rettori, A.; Rossi, V.; Spanna, F.; Rosenzweig, C.; Gullino, M.L. Downy Mildew (*Plasmopara viticola*) Epidemics on Grapevine under Climate Change. *Glob. Chang. Biol.* **2006**, *12*, 1299–1307. [CrossRef]
77. Bove, F.; Savary, S.; Willocquet, L.; Rossi, V. Simulation of Potential Epidemics of Downy Mildew of Grapevine in Different Scenarios of Disease Conduciveness. *Eur. J. Plant Pathol.* **2020**, *158*, 599–614. [CrossRef]