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Foliar Application of a Tagatose-Based Product Reduces Downy Mildew Symptoms through Induction of Grapevine Resistance and Anti-Oomycete Action

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Abstract: Downy mildew caused by the oomycete *Plasmopara viticola* represents one of the most devastating diseases in vineyards. Current ways to control this disease rely mainly on fungicide applications, but agro-ecological concerns have raised interest in sustainable alternative methods. Certain rare sugars, like D-tagatose, have shown efficacy in reducing various plant diseases, including grapevine downy mildew. However, the mechanism of action of D-tagatose against grapevine downy mildew is not understood. The aim of this study was to characterize the efficacy and mechanism of action of a D-tagatose-based formulated product (IFP48) against grapevine downy mildew and compare it with the correspondent active molecule, pure D-tagatose (TAG). Whereas IFP48 root treatment provided scarce protection, the leaf treatment was the most efficient, especially at the dosage of 5 g/L. In particular, IFP48 treatment directly inhibited *P. viticola* sporangia germination, upregulated the expression of defense-related genes, and increased the content of stilbene phytoalexins. Conversely, the expression of defense-related genes and the content of stilbene phytoalexins were only slightly affected by TAG, suggesting that the formulation possibly improved D-tagatose effects against downy mildew in grapevine.

Keywords: grapevine; downy mildew; D-tagatose

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1. Introduction

Grapevine (*Vitis vinifera* L.) is susceptible to several diseases that can cause significant economic losses, including downy mildew, caused by *Plasmopara viticola* (Berk. and Curt.) Berl. and de Toni, which is currently managed mainly by repetitive fungicide treatments [1–3]. Despite their efficiency, the misuse of agrochemicals has resulted in environmental pollution and public health risks [4]. To achieve the goal of the Farm to Fork Strategy of reducing the overall use of chemical pesticides by 50% in the EU, a drastic decrease in the number of fungicide applications must be accomplished in a relatively short period of time. Therefore, the European agricultural sector is looking for innovative ways to improve yields while supporting the EU's 2030 biodiversity strategy, a key pillar of the European Green Deal. Biocontrol can offer a systemic and balanced solution for a more sustainable agriculture, as a non-chemical and targeted input. To protect themselves from pathogenic threats, plants developed an immune system comprising constitutive and inducible resistance mechanisms [5]. Triggered resistance mechanisms enhance the plant capacity to defend against upcoming attacks of pests and pathogens [6–8]. Resistance induction can be assessed by analyzing various early and late defense responses, such as

oxidative burst, ion fluxes, activation of mitogen-activated protein kinases, and upregulation of defense-related genes encoding pathogenesis related (PR) proteins and enzymes involved in phytoalexin synthesis [9–11] and carbohydrate metabolism [12].

Exogenous application of certain natural compounds, including oligosaccharides derived from the cell wall of various fungi, bacteria, and host plants, or from marine algae, can be recognized by the plant and can trigger defense responses leading to increased resistance before pathogen infection [13]. The protective effect of these natural compounds can be derived from their ability to induce defense responses in plants or their direct inhibitory effect on the pathogen, or through the combination of both activities [1,14–18].

Many reports have described the importance of sugars in plant-microbe interactions and in plant resistance against pathogens [12,19–21]. This so-called sweet immunity highlighted the role of sweet endogenous saccharides as signaling molecules that can mediate faster and stronger plant defense responses after being perceived by receptors at the plasma membrane or specific intracellular locations [22–25]. The sweet immunity concept opens possibilities to induce plant resistance by exogenous sugar applications, by root, shoot, or seed treatments [12,23]. For example, elevated levels of sucrose in *Arabidopsis* tissues activated genes encoding enzymes responsible for the production of anthocyanins and secondary metabolites with protective effects [26,27]. When applied to roots, sucrose directly induced the expression of genes encoding PR proteins in rice and drastically reduced symptoms of rice blast, caused by *Magnaporthe oryzae* [28]. Similar effects have been observed with trehalose, which triggered wheat resistance against powdery mildew through papilla deposition and activation of secondary metabolic pathways [29,30], or galactinol, which triggered the defense response in tobacco leaves against *Botrytis cinerea* and *Erwinia carotovora* subsp. *carotovora* [31]. Treatment with alginate triggered phytoalexin production, phenylalanine ammonia-lyase (PAL) and peroxidase (POX) activity, and increased the resistance of rice against *M. oryzae* [32], whereas fucans caused jasmonic acid (JA)- induced resistance in tobacco against Tobacco Mosaic Virus [33]. Moreover, application of BcIEB1, a *B. cinerea* secreted protein, induced tobacco, tomato, onion, and *Arabidopsis* resistance against *B. cinerea* [34].

The *B. cinerea* glycoprotein (BcPG1) was also reported to upregulate the expression of phenylpropanoid pathway defense genes and enhance reactive oxygen species (ROS) production in grapevine [35,36]. Furthermore, cellodextrins exhibited protective effects on grapevine challenged with *B. cinerea* by inducing elevated levels of cytosolic calcium, oxidative burst, and defense-related gene expression [9]. In addition, sugar-based triggered resistance inhibited grapevine downy mildew in the case of β-1,3-glucan laminarin treatment, which led to calcium influx, alkalinization of the extracellular medium, oxidative burst, activation of two mitogen-activated protein kinases, expression of defense-related genes, and activity of chitinase and β-1,3-glucanase [10,37]. Sulfated laminarin (PS3) treatment caused a JA- and salicylic acid (SA)-dependent induction of defense responses in grapevine with enhanced H_2O_2 production, defense-related gene upregulation, callose deposition, and phenol accumulation, leading to an increased resistance against downy mildew with no direct anti-oomycete effects against *P. viticola* [10,38]. In the case of chitosan, protection from downy mildew was associated with a combination of direct anti-oomycete effects and induction of grapevine defense responses, such as the accumulation of trans-resveratrol, cis-resveratrol, ε-viniferin, and piceid in leaves [13]. Finally, treatment with oligogalacturonides (OGAs) controlled grapevine downy mildew by inducing stomatal closure as well as ROS production [39].

An increasing number of reports have shown that some rare sugars, such as D-allulose (formerly called D-psicose) and D-allose can induce defense response of different plant species and increase their resistance against various pathogens [40,41]. For example, D-allulose induced resistance in rice and citrus by upregulation of the expression of defense-related genes before pathogen inoculation [42–44]. Likewise, D-allose enhanced rice resistance against *Xanthomonas oryzae* [40,42] and tomato resistance against *Pseudomonas syringae* pv. *tomato* and *B. cinerea* [45]. D-allose triggered defense responses in rice before the

pathogen challenge by direct defense induction [40,42], whereas it primed tomato plants for the enhanced expression of *PR* genes following *B. cinerea* and *P. syringae* inoculation [45]. D-allose also induced the expression of *Arabidopsis* defense-related genes, but it did not confer protection against *Colletotrichum higginsianum* [46].

D-tagatose is another rare sugar showing high efficiency in the control of a wide spectrum of phytopathogens from the classes of oomycetes, ascomycetes, and basidiomycetes [47–50]. D-tagatose inhibited the growth of *Phytophthora infestans* [51–53] and *Hyaloperonospora arabidopsidis* [48]. Additionally, D-tagatose offered a good level of protection against cucumber powdery mildew [54] with no resistance induction in *Arabidopsis*, cucumber, or rice [48]. Thus far, D-tagatose has been reported to confer protection against downy and powdery mildews of grapevine [47–50,54], while promising significantly higher efficacy when formulated [55]. Although the great potential of this rare sugar in the control of crop pathogens is evident, further studies are required in order to better characterize and understand the mechanism of action of D-tagatose-based formulations on the specific pathosystems. The objective of this study was to investigate the efficacy and the mechanism of action of a D-tagatose-based formulated product (IFP48) to protect vines against downy mildew and to compare its effects with the correspondent active molecule, pure D-tagatose (TAG). IFP48 is currently in the process of registration on the European Union market and the authors are testing its efficiency as a biocontrol agent.

2. Materials and Methods

2.1. Plant Material and Growth Conditions

In vitro plantlets of *Vitis vinifera* cv. Chardonnay clone 7535 were produced from nodal explants on 15 mL of Murashige and Skoog (MS) medium in 25-mm culture tubes [56]. In vitro plantlets were grown in a growth chamber at a constant temperature of 26 °C with the 16/8 h (day/night) photoperiod under white light (200 µmol m^{-2} s^{-1}) and 60 ± 5% relative humidity (RH) for 8 weeks. For root treatment, six-week-old in vitro plantlets were transferred from culture tubes to Magenta boxes containing humidified soil (4600 Sorexto, Nizeray, France) and incubated for an additional week in the growth chamber before treatment. For aerial part treatment, in vitro plantlets were maintained in MS medium.

For the purposes of assessing the ability of IFP48 and TAG to protect leaves of grapevine in vitro plantlets from downy mildew (in case of both root and aerial part treatments) and for the assessment of the direct effect on *Plasmopara viticola*, 40 leaves derived from 12 in vitro plantlets were used for each tested product concentration. For the purposes of gene expression and phytoalexin analyses, 18 leaves derived from 6 in vitro plantlets were used for each condition (sampling time point). For the purposes of validating the induced resistance mechanisms (Supplementary Figure S6), 15 leaves derived from 5 in vitro plantlets were used for each product (TAG at 5 g/L, IFP48 at 5 g/L, and water control).

2.2. D-Tagatose Treatments

A D-tagatose-based formulated product (IFP48, wettable powder containing 80% D-tagatose *w*/*w*) and the pure D-tagatose (TAG) were provided by Bi-PA Nv (Biological Products for Agriculture, Londerzeel, Belgium). D-tagatose was dissolved in sterile ultrapure water (stock solution of 10 g/L), filtered through syringe filters (0.45 µm pore size; VWR International), and diluted at concentrations ranging from 1 to 10 g/L.

The ability of D-tagatose to protect the leaves from *P. viticola* was evaluated using two protocols of treatment. The first protocol (root treatment) consisted of drenching the soil of acclimated in vitro plantlets in Magenta boxes with 10 mL of formulated or pure D-tagatose at the concentrations of 1, 2, 5, and 10 g/L and water as control. After the treatment, in vitro plantlets were incubated in the growth chamber for three days before pathogen inoculation. The second protocol (aerial part treatment) consisted of dipping for two minutes in vitro plantlets in different concentrations of formulated or pure D-tagatose (1, 2, 5, and 10 g/L)

and water as control. In vitro plantlets were then incubated in the growth chamber for one day before pathogen inoculation.

2.3. Plasmopara viticola Inoculum Preparation

Plasmopara viticola was purified from infected leaves of the Chardonnay in vitro plantlets. In order to have the fresh inoculum, sporangia were propagated weekly on leaves of V. vinifera cv. Chardonnay in vitro plantlets as previously described [37]. Briefly, the inoculum was prepared by washing freshly sporulating lesions with sterile distilled water, and the suspension was adjusted to a final concentration of 1×10^5 sporangia/mL using a Malassez hemocytometer. Detached leaves were placed in glass Petri dishes on wet paper (Whatman), sprayed with sporangia suspension, and incubated in the growth chamber at 65 ± 5% RH, 24 °C, 16/8 h (day/night) photoperiod. Seven days after inoculation, leaves were transferred to darkness in a dew chamber (100% RH, 25 °C) and incubated overnight to promote pathogen sporulation.

2.4. Plasmopara viticola Inoculation and Assessment of Downy Mildew Severity

For the purposes of assessing the ability of IFP48 and TAG to protect leaves of grapevine in vitro plantlets from downy mildew, after IFP48, TAG, or water treatment (either by root or aerial part treatments), 3–4 leaves from each plantlet were detached and placed with the adaxial side on wet paper (Whatman) in glass Petri dishes and inoculated with 20 µL droplets of fresh *P. viticola* sporangial suspension (1×10^5 sporangia/mL). Leaves were then incubated in the growth chamber at 24 °C with a 16/8 h (day/night) photoperiod and 65 ± 5% RH for seven days. On the last day, leaves were incubated overnight in darkness in the dew chamber (100% RH, 25 °C) to promote pathogen sporulation.

Downy mildew severity was assessed at eight days post inoculation (dpi) on 40 leaves (from 12 plantlets) per treatment from three independent experiments by measuring the diameter of leaf surface covered by sporulation and assigning a score according to the following scale: (i) class 0, no symptoms; (ii) class I, <5 mm; (iii) class II, between 5 mm and 10 mm; (iv) class III, >10 mm [57]. Furthermore, the density of *P. viticola* sporangia was determined at 8 dpi by randomly pooling and washing five infected leaves in 1 mL sterile distilled water in a 50 mL tube. After 1 h incubation under gentle orbital shaking, suspended sporangia were counted under a light microscope using a Malassez hemocytometer and expressed as sporangia per unit of leaf fresh weight (mg).

2.5. Assessment of the Direct Effect on Plasmopara viticola

Leaves of eight-week-old in vitro plantlets were detached and placed with the adaxial side on wet paper (Whatman, Saint-Quentin-Fallavier, France) in glass Petri dishes. Leaves were sprayed with 1, 2, 5, and 10 g/L IFP48 or TAG using a manual sprayer until homogenous coverage of leaves was reached. As control, leaves were sprayed with water. Two hours later, corresponding to an incubation time insufficient for plant resistance activation [16,38], leaves were inoculated with 20 µL droplets of fresh *P. viticola* sporangial suspension (1×10^5 sporangia/mL) on the abaxial side. Leaves were incubated in the growth chamber at 24 °C with a 16/8 h (day/night) photoperiod and 65 ± 5% RH. Seven days after inoculation, leaves were incubated overnight in the dark in the dew chamber (100% RH, 25 °C) to promote pathogen sporulation. Downy mildew disease severity and the density of *P. viticola* sporangia were then scored as described above. Each experiment was performed with 40 leaves (from 12 in vitro plantlets) per treatment, and experiments were carried out Three times.

2.6. Assessment of Plant Resistance Induction against Plasmopara viticola

In the aseptic conditions under the laminar flow cabinet, IFP48 and TAG were applied by aerial part treatment as described above at 5 g/L, the most efficient low concentration. Water was used as the control treatment. At one day post-treatment (dpt), the second, third, and fourth leaves from the top were detached and washed three times in 0.001% Tween80 to

remove product residuals from leaf surfaces and discard the possible direct effects of IFP48 or TAG on the pathogen. Leaves were then placed on their adaxial side on wet Whatman paper in glass Petri dishes, then mock-inoculated or inoculated by spraying water or fresh *P. viticola* sporangial suspension (1×10^5 sporangia/mL), respectively, using a manual sprayer until homogenous coverage of leaves was reached. Samples were collected at zero, 1-, 2-, and 3- dpt from mock-inoculated leaves of IFP48-, TAG-, or water-treated plants and at 1 and 2 dpi from *P. viticola*-inoculated leaves of IFP48-, TAG-, or water-treated plants. The samples were then frozen in liquid nitrogen and stored at −80 °C until they were used for gene expression and stilbene analysis. Eighteen leaves from six in vitro plantlets were used for each condition/time point, and experiments were carried out three times. Portion of leaves treated with IFP48 and TAG at 5 g/L then washed with 0.001% Tween80 (as described above) was allocated for the purpose of validating the induced resistance mechanisms (Supplementary Figure S6). These leaves were inoculated with 20 µL droplets of fresh *P. viticola* sporangial suspension (1×10^5 sporangia/mL).

Downy mildew disease severity was assessed at 8 dpi by counting the sporangia under the light microscope as described above. Fifteen leaves (from five plants) were analyzed for each treatment, and the experiment was carried out three times. The experimental timeline is depicted below (Figure 1).

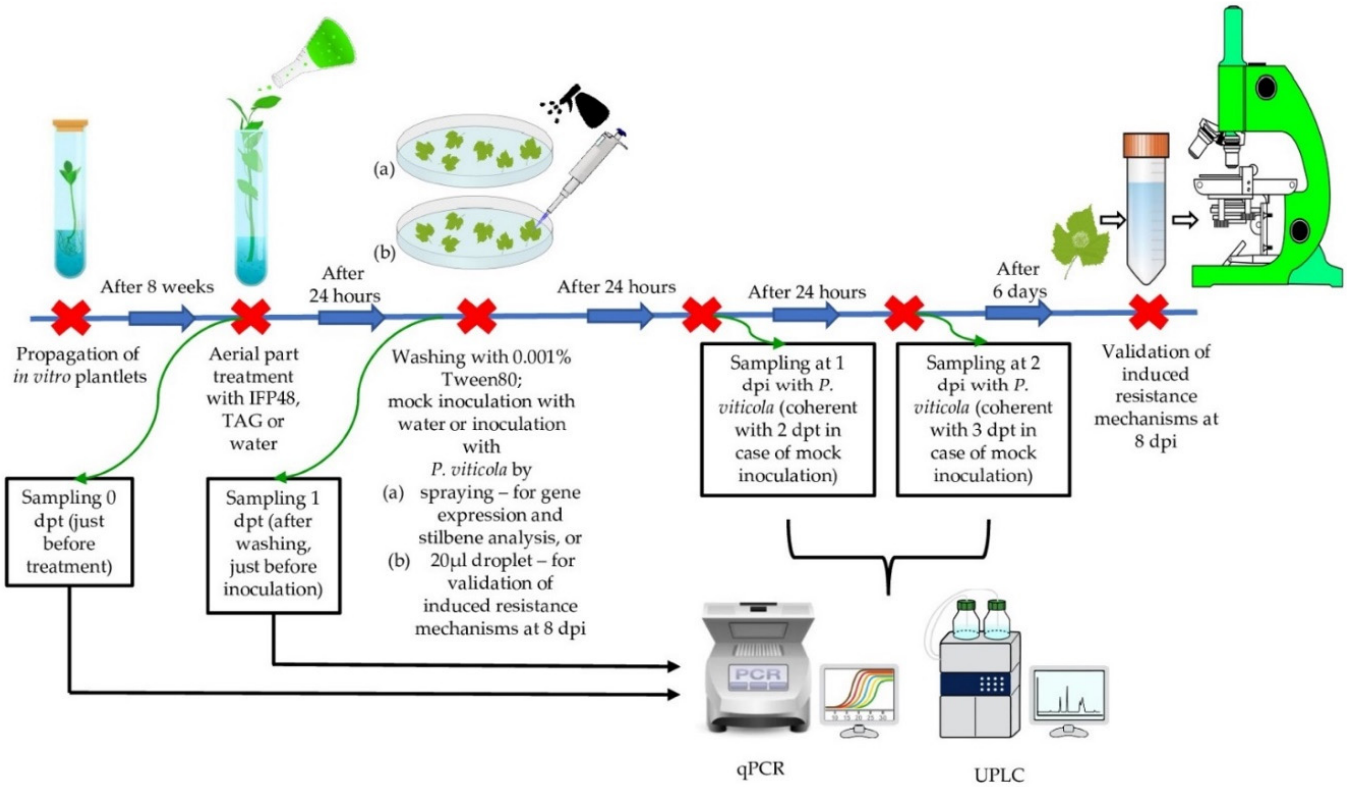

Figure 1. Experimental design used to assess the induction of plant resistance against *Plasmopara viticola*. In vitro grapevine plantlets (Chardonnay variety) were treated with either IFP48, TAG or water control (following the protocol used for aerial parts treatment) before being inoculated with *P. viticola* either by spraying or by a 20 µL drop. Finally, resistance induction was then assessed by counting spores under an 8 dpi light microscope. Sampling was performed at four different time points as described.

2.7. RNA Extraction and Gene Expression Analysis in Grapevine Leaves

Leaf samples were ground in liquid nitrogen, and total RNA was extracted from 50 mg by following the protocol of Plant RNA purification reagent according to manufacturer instructions (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). Genomic DNA was removed from extracted RNA by treatment with RQ1 RNase-free DNase (Promega,

Madison, WI, USA), and RNA quality was checked by agarose gel electrophoresis. The concentration of RNA was quantified at 260 nm using NanoDrop One (Thermo Fisher Scientific), and then adjusted to 100 ng/µL. First-strand cDNA was synthesized from 150 ng of total RNA using the Verso cDNA synthesis kit (Thermo Fisher Scientific).

The expression of seven defense-related genes was analyzed (Supplementary Table S1), including genes encoding for a pathogenesis related protein-1 (*PR-1*) and β-1,3-glucanase (*PR-2*) as markers of salicylic acid (SA) pathway [57]; a 9-lipoxygenase (*LOX9*) enzyme of the octadecanoid pathway and marker of jasmonic acid (JA) pathway [58]; a 1-aminocyclopropane carboxylic acid oxidase (*ACO*) involved in ethylene (ET) biosynthesis; a phenylalanine ammonia-lyase (*PAL*), a key enzyme of the phenylpropanoid pathway; a stilbene synthase (*STS*), responsible for the biosynthesis of resveratrol; and an acidic class IV chitinase (*PR-3*) related to JA-signaling [59,60].

The RT-PCR was performed with Absolute qPCR Mix, SYBR Green, ROX according to the manufacturer instructions (Thermo Fisher Scientific), using a CFX96 system thermocycler (Bio-Rad, Hercules, CA, USA). PCRs were carried out in 96-well plates to a final volume of 15 µL containing Absolute qPCR Mix, SYBR Green, ROX (Taq polymerase, dNTP, and SYBR Green Dye), 280 nM of forward and reverse primers, and 30-fold diluted cDNA [57]. PCR conditions were 95 °C for 10 s (denaturation) and 60 °C for 45 s (annealing/extension) for 42 cycles using two reference genes, Elongation factor 1-alpha (*EF1*) and Stress-Response Protein 60 (*60SRP*) were used as internal controls for normalization for quantitative RT-PCR analyses [57]. In order to determine the relative gene expression, fold induction formula 2-ΔΔCt was used and integrated through CFX Manager 3.0 software (Bio-Rad). The description of the formula is as follows: ΔΔCt = (Ct GI (unknown sample) − Ct GI (reference sample)) − (Ct GR (unknown sample) − Ct GR (reference sample)). Ct is cycle threshold based on the threshold crossing point of fluorescence trace of every sample; GI is the gene of interest; and GR is the reference gene. Eighteen leaves from six in vitro plantlets were used for each condition, and experiments were carried out three times.

2.8. Phytoalexin Analysis in Grapevine Leaves

Phytoalexins were extracted from 200 mg of ground leaf powder with 2 mL of a solution of methanol/water (85/15: *v*/*v*) [61]. Samples were incubated on a shaker at 800 rpm in the dark at room temperature for 2 h and centrifuged for 15 min (15,000 rpm, 4 °C). Supernatants were collected in hemolysis tubes and stored in darkness at 4 °C. Remaining pellets were suspended in 1 mL of 100% methanol, then incubated for 1 h more on the 800 rpm shaker at room temperature, and centrifuged for 15 min at 15,000 rpm and 4 °C. Supernatants were pooled together in hemolysis tubes and dried with the speed vacuum at 45 °C (Speed-Vac, Eppendorf France SAS). The remaining pellets were solubilized in 1 mL of 100% methanol (LC–MS grade) then filtered through 0.22 µm polytetrafluoroethylene (PTFE) filters into 2 mL amber vials for UPLC analysis. Analysis of stilbenic phytoalexins was performed using the ACQUITY UPLC system (Waters Corporation, Milford, MA, USA). Phytoalexins were eluted with a mixture of water and acetonitrile with 0.1% formic acid at the flow rate of 0.5 mL min^{-1}. Samples (2 µL) were injected onto an Acquity UPLC BEH C18 1.7 µm 2.1 × 100 mm column heated at 40 °C. Acquity fluorometer (Waters Corporation) was used to measure the fluorescence with an excitation wavelength of 330 nm and an emission wavelength of 375 nm. Empower 2 software (Waters Corporation) was used to analyze the signals, and stilbenic phytoalexins were quantified according to retention time and calibration with external standards [62]. Eighteen leaves from six in vitro plantlets were used for each condition, and each experiment was carried out three times.

3. Conclusions

We noticed that leaf treatment with IFP48 was the most effective, particularly at the 5 g/L, whereas the root treatment only generated a low level of protection. Specifically, IFP48 directly inhibited the germination of *P. viticola* sporangia, controlled the expression of defense-related genes, and improved the content of stilbene phytoalexins. In contrast,

the expression of defense-related genes and the amount of stilbene phytoalexins were only slightly altered by TAG, implying that the formulation may provide an improvement in the effectiveness of D-tagatose against grapevine blight.

4. Statistical Analysis

All experiments were carried out three times, with an exemption of direct effect assessment, and data were analyzed with R software version 3.6.0. One-way analysis of variance (ANOVA) Welch's test with Student's *t*-test were used to detect significant differences ($p \leq 0.05$) among treatments on downy mildew data and *Plasmopara viticola* sporangia counts. One-way ANOVA was carried out with the statistical program SPSS 20 software using post-hoc Tukey's test HSD to detect significant differences ($p \leq 0.05$) among treatments on gene expression and metabolite content data.

5. Results

5.1. IFP48 and TAG Control Grapevine Downy Mildew

IFP48 root treatment, when applied at 5 g/L, reduced the sporangia density of *P. viticola* by about 35% compared to water-treated plants (Figure 2) and affected class I and class II symptoms of disease severity (Supplementary Figure S1). When applied at 5 g/L, TAG root treatment restricted sporangia density of *P. viticola* (Figure 2) and downy mildew symptoms mainly for class II symptoms of disease severity (Supplementary Figure S1).

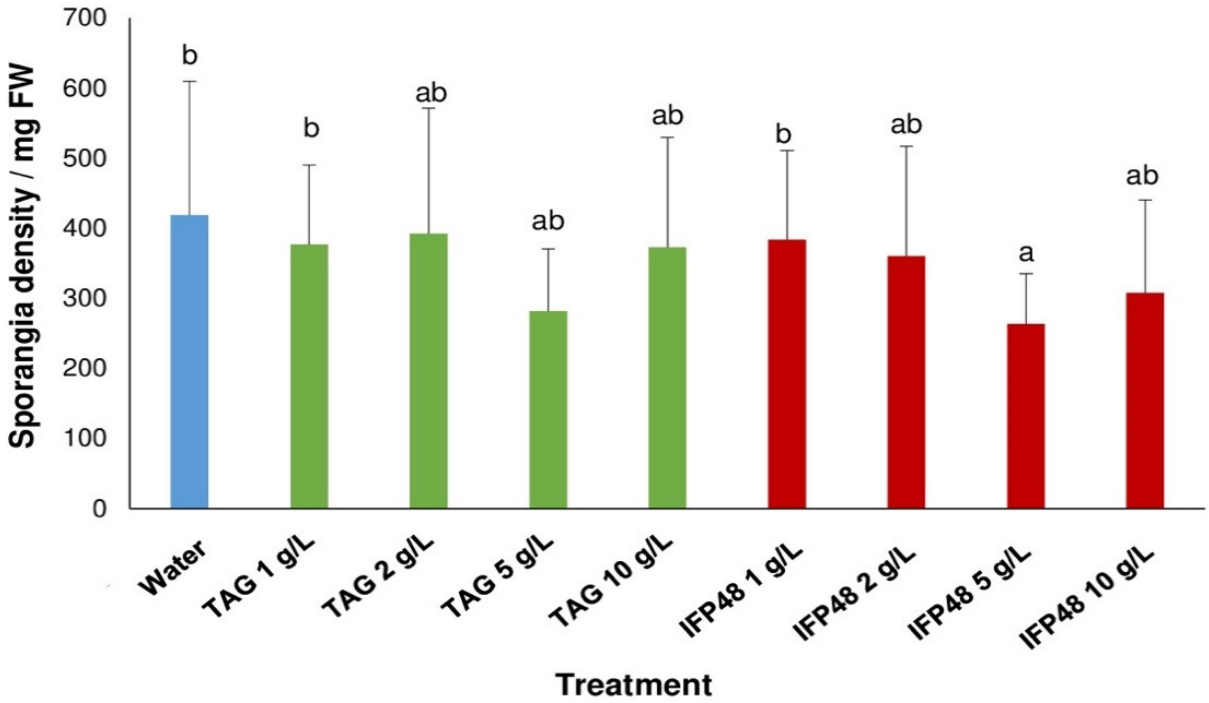

Figure 2. Downy mildew disease severity in grapevine leaves after root treatment with D-tagatose and its formulated product IFP48. Roots of in vitro-grown grapevine plantlets were treated with 1, 2, 5, and 10 g/L D-tagatose-based formulated product (IFP48) or pure D-tagatose (TAG). As control, plants were treated with water. Leaf inoculation was carried out at three days post treatment (3 dpt) and disease severity was evaluated by counting the *Plasmopara viticola* sporangia at eight days post inoculation (8 dpi). Data are means ± SD from eight plants with five leaves per plant and three independent experiments that showed similar results. Different letters indicate significant differences according to the one-way ANOVA Welch's test with Student's *t*-test ($p \leq 0.05$).

The aerial part treatment with IFP48 and TAG reduced sporangia density of *P. viticola* (Figure 3) and downy mildew severity (Supplementary Figure S2). In particular, sporangia density was comparable between IFP48 and TAG at 1, 2, and 5 g/L, whereas aerial part

treatment with 10 g/L was more effective in the case of IFP48 compared to TAG (Figure 3). Based on disease symptoms of class 0 and class I, IFP48 was more effective than TAG at 2, 5, and 10 g/L concentrations (Supplementary Figure S2). However, slight necrotic lesions were observed on the leaf edges of leaves treated with 10 g/L IFP48.

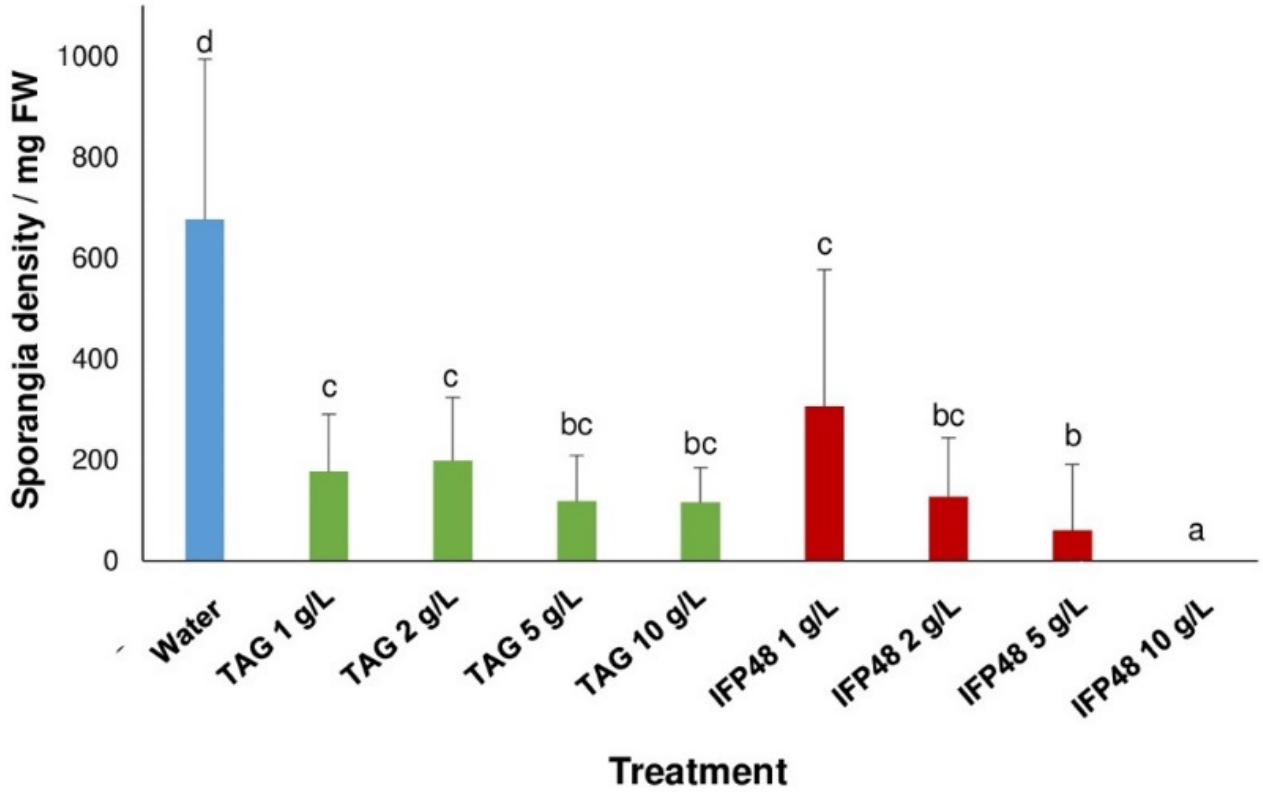

Figure 3. Downy mildew disease severity in grapevine leaves after aerial part treatment with D-tagatose and IFP48. Shoots of in vitro-grown grapevine plantlets were treated with 1, 2, 5, and 10 g/L D-tagatose-based formulated product (IFP48) or pure D-tagatose (TAG). As control, plants were treated with water. Leaf inoculation was carried out at one day post treatment (1 dpt) and disease severity was evaluated by counting the *Plasmopara viticola* sporangia at eight days post inoculation (8 dpi). Data are means ± SD from eight plants with five leaves per plant and three independent experiments that showed similar results. Different letters indicate significant differences according to the one-way ANOVA Welch's test with Student's *t*-test ($p \leq 0.05$).

5.2. IFP48 and TAG Exert a Direct Effect on P. viticola Sporangia

Spray treatment of detached leaves showed direct anti-oomycete activity of IFP48 and TAG that reduced sporangia density of *P. viticola* in a dose-dependent manner (Figure 4). Likewise, the number of asymptomatic leaves (class 0) increased, and the number of leaves which developed class III symptoms decreased with increasing concentration of IFP48 or TAG treatment (Supplementary Figure S3). A complete inhibition of *P. viticola* sporulation was observed with 5 and 10 g/L IFP48 (Figures 4 and S3). Comparing the respective concentrations, the IFP48 effect was higher than that of TAG (Figures 3 and 4). At 5 g/L, IFP48 and TAG reduced the sporangia density of *P. viticola* by 100% and 90%, respectively. Thus, the concentration of 5 g/L was selected for the further experiments due to the high efficacy by shoot application.

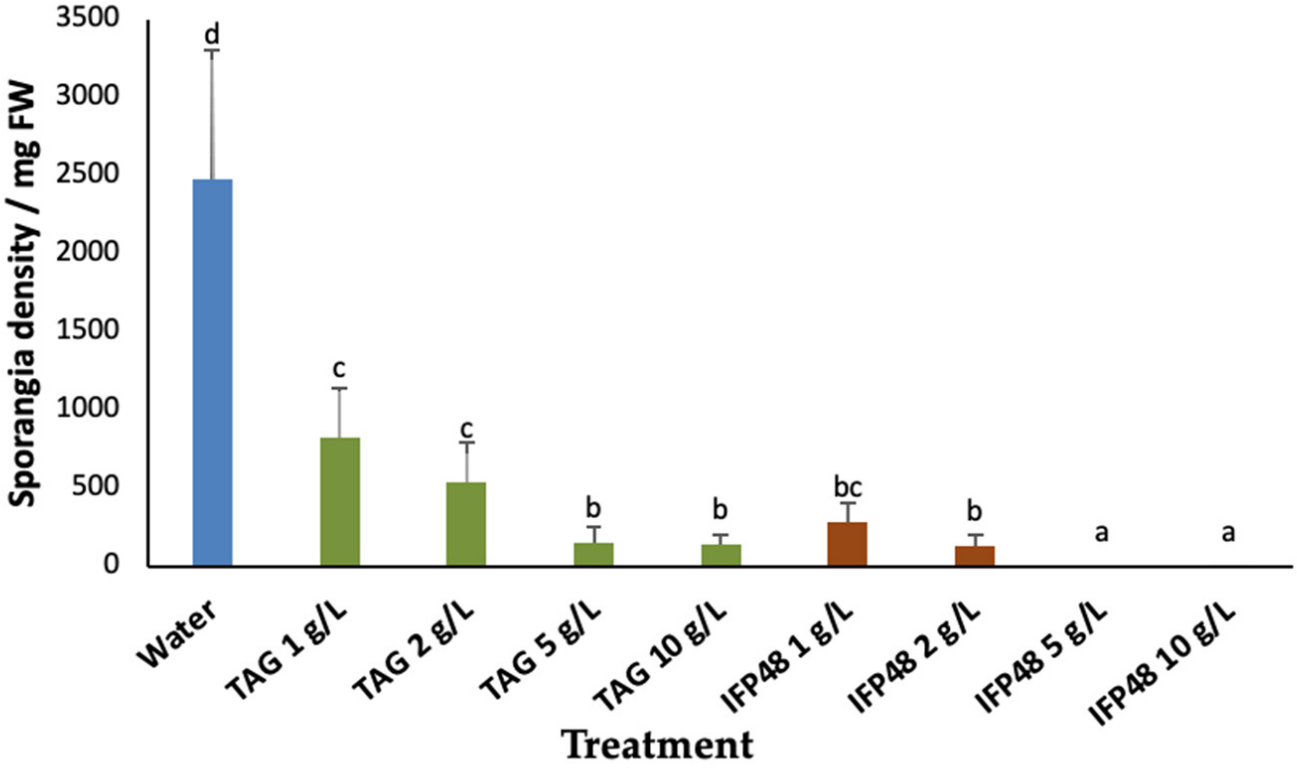

Figure 4. Direct effect of D-tagatose-based product on *Plasmopara viticola*. Detached leaves of in vitro plantlets were sprayed with 1, 2, 5, and 10 g/L D-tagatose-based formulated product (IFP48) or pure D-tagatose (TAG). As control, leaves were treated with water. Leaf inoculation was carried out at 2 h post treatment. *Plasmopara viticola* sporangia were quantified at eight days post-inoculation. Data are means ± SD from eight plants with five leaves per plant and three independent experiments. Different letters indicate significant differences according to the one-way ANOVA Welch's test with Student's *t*-test ($p \leq 0.05$).

5.3. IFP48 Upregulates Defense-Related Genes in Grapevine Plantlets before and after Pathogen Inoculation

Gene expression analysis showed that IFP48 upregulated most of analyzed genes at all-time points before and after inoculation with *P. viticola* (Figures 5 and S4). Maximum levels of gene expression were observed at 1 and 2 dpt for all genes tested. In most cases, the gene expression levels remained high after the pathogen inoculation, suggesting that IFP48-mediated resistance against *P. viticola* is partially related to the induction of defense-related genes. The expression of *ACO* (involved in ET synthesis) and *LOX9* (involved in JA pathway) were higher in IFP48-treated compared to water-treated plants at 1 and 2 dpt, but not after *P. viticola* inoculation. The expression of *PR-1* and *PR-2* (markers of SA signaling) was upregulated in response to IFP48 treatment compared to water-treated plants before and after *P. viticola* inoculation. In particular, *PR-2*, *PR-3*, *PAL*, and *STS* genes showed similar expression patterns in IFP48-treated plants, with transient upregulation at 1 dpt and further modulation at 2 dpi with *P. viticola*. For example, before the pathogen challenge, *PR-2*, *PAL*, and *STS* were transiently upregulated at 1 dpt, reaching their high expression with approximately 5-, 10-, and 8-fold increase relative to control. Thereafter, the expression of these genes gradually decreased. *PR-3* had the strongest reaction to IFP48 application. In the absence of a pathogen, accumulation of its transcripts reached over 17-fold relative to water control at 1 dpt, then it decreased to 2- and 3-fold, at 2 dpt and 3 dpt, respectively. After the inoculation with *P. viticola*, IFP48-induced *PR-3* expression remained high, but did not go higher compared to the expression before the inoculation. In contrast, TAG did not affect the expression of targeted genes compared to water-treated plants at the respective time points, with the exception of *PR-1* at 2 and 3 dpt, *PR-2* at 2 dpt, and STS at 1 dpi with *P.*

viticola, indicating a weaker and delayed effect compared to IFP48. TAG treatment did not cause any significant changes in the expression profile of *PR-3* relative to the water control. In contrast, after the inoculation, we recorded a slight TAG-induced down-regulation of *PR-3* at 1 dpi and 2 dpi time points.

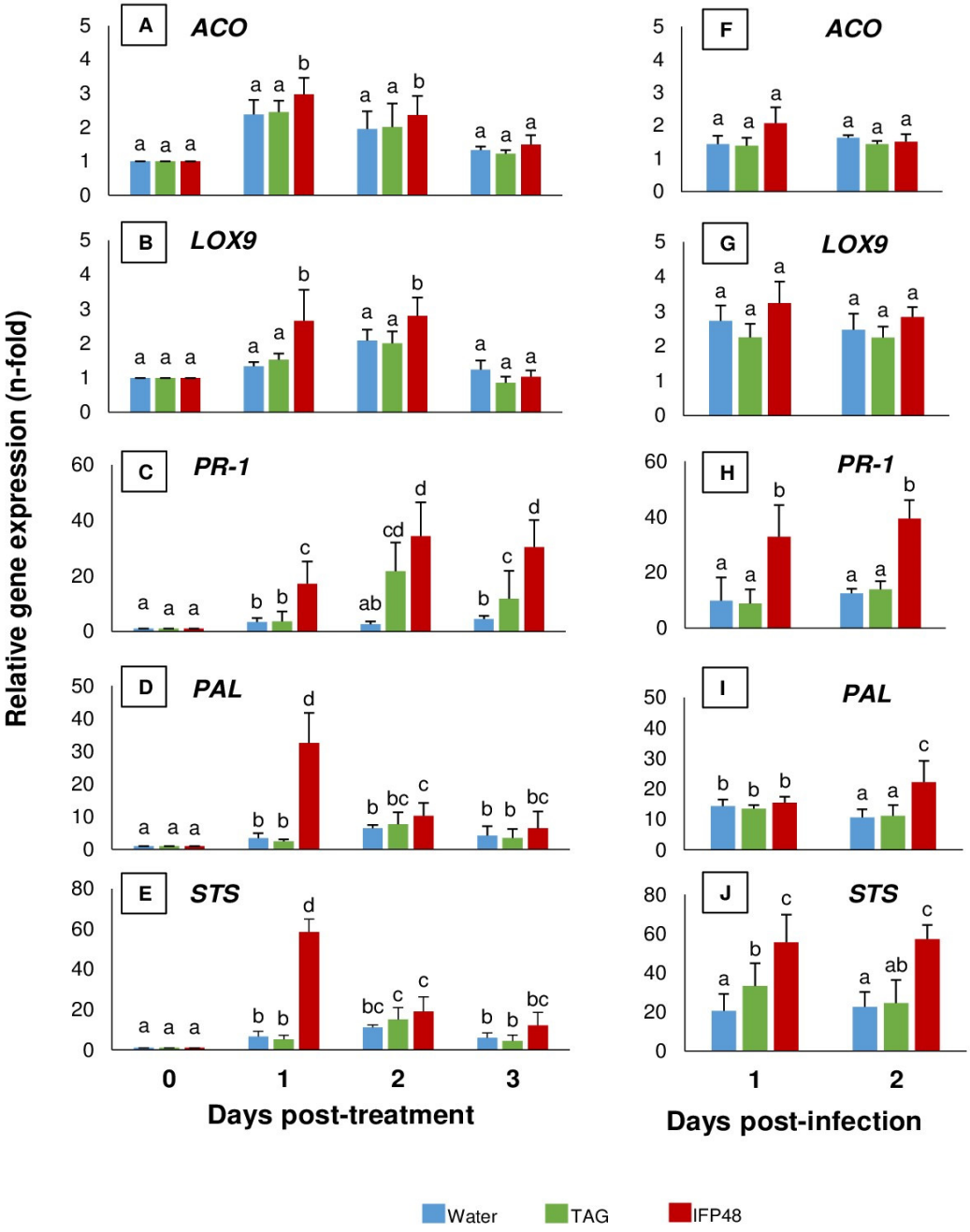

Figure 5. Effects of IFP48 and D-tagatose on relative expression of defense-related genes. Grapevine plantlets were treated with water, 5 g/L D-tagatose-based formulated product (IFP48), or 5 g/L pure D-tagatose (TAG) and leaf samples were collected before (**A**–**E**) and after *Plasmopara viticola* inoculation (**F**–**J**). The expression of genes encoding ACC oxidase (*ACO*), lipoxygenase 9 (*LOX9*), pathogenesis related 1 (*PR-1*), phenylalanine ammonia lyase (*PAL*), and stilbene synthase (*STS*) was quantified by qRT-PCR. Data are means ± SD from three independent experiments, each consisted of 18 leaves from six plantlets. Different letters indicate significant differences according to the one-way ANOVA and Tukey's test ($p \leq 0.05$).

5.4. IFP48 Increases the Accumulation of Phytoalexins in Grapevine Plantlets before and after Pathogen Inoculation

Metabolite analysis showed that IFP48 increased the content of resveratrol and its derivatives trans-piceid, δ and ε viniferin, before and after *P. viticola* inoculation (Figures 6 and S5). In particular, the content of resveratrol, trans-piceid, and viniferins was increased by IFP48 treatment at 1, 2, and 3 dpt, and it also remained high at 1 and 2 dpi with *P. viticola*. Concretely, the resveratrol content peaked 1 dpt, reaching approximately an amount over 5 µg per gram of leaf fresh weight, then slightly dropped 2 dpt and 3 dpt. The amount of resveratrol was increased in IFP48-treated leaves after the inoculation with *P. viticola* 1 dpi and 2 dpi to approximately 3 and 4 µg per gram of leaf fresh weight, respectively. The content of trans-piceid was raised to approx. 1 µg/g FW at 1 dpt, then reached 2 µg/g FW the following days (2 dpt and 3 dpt) before and after the pathogen challenge. Viniferins (ε- and δ-) showed a similar pattern. Epsilon viniferin reached approx. 0.5 µg/g FW at 1 dpt, 2 dpt, and 1 dpi time points, and approximately 1.5 µg/g FW at 3 dpt and 2 dpi, whereas delta viniferin amounts were approximately two times higher than epsilon viniferin during the whole experiment. In contrast to the content of resveratrols, which were dropping after the early peak at 1 dpt, the amount of viniferins and trans-piceid steadily grew over time, possibly indicating the conversion of resveratrols to their derivatives. Conversely, metabolite content was comparable in TAG- and water-treated leaves at the respective time points.

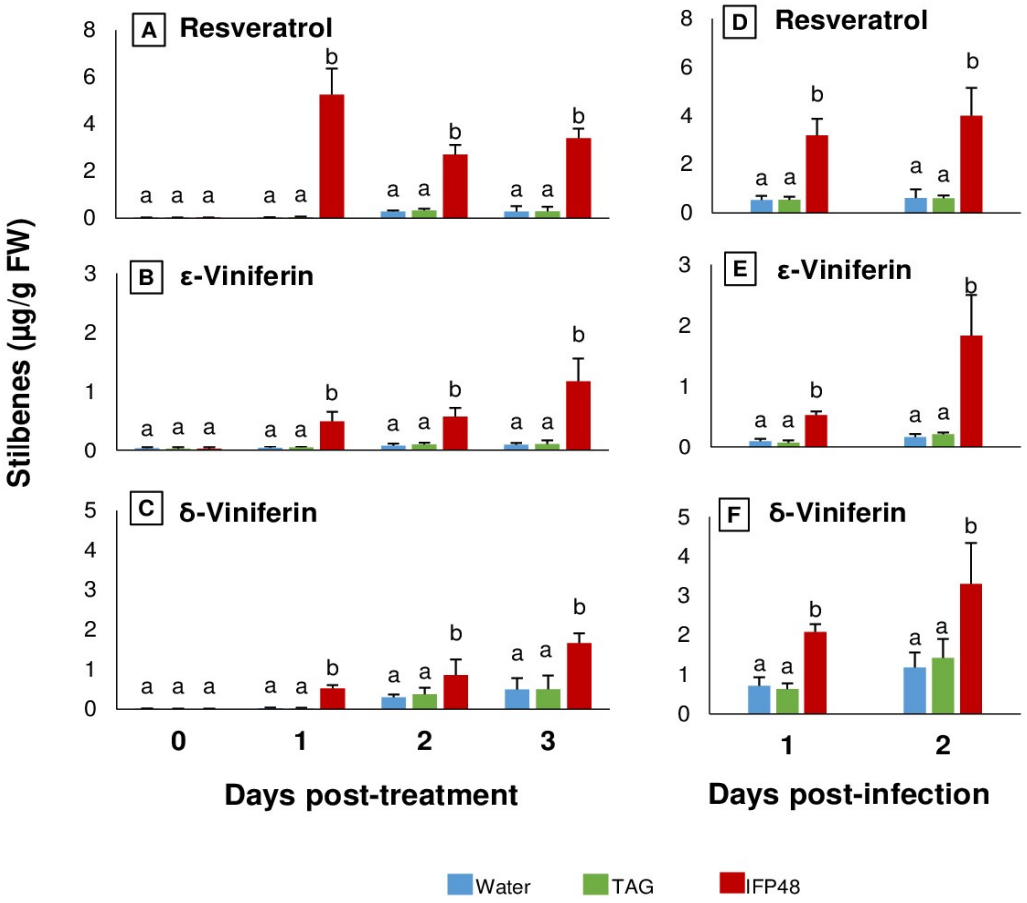

Figure 6. Effects of IFP48 and D-tagatose on resveratrol, ε-viniferin, and δ-viniferin content. Grapevine plantlets were treated with water, 5 g/L D-tagatose-based formulated product (IFP48), or 5 g/L pure D-tagatose (TAG) and leaf samples were collected before (**A**–**C**) and after *Plasmopara viticola* inoculation (**D**–**F**). Data are means ± SD from three independent experiments, each consisted of 18 leaves from 6 in vitro plantlets. Different letters indicate significant differences according to the one-way ANOVA and Tukey's test ($p \leq 0.05$).

As validation of induced resistance mechanisms, downy mildew disease severity was reduced by IFP48 treatment on Tween80-washed leaves and was comparable to water- and TAG-treated leaves (Supplementary Figure S6).

6. Discussion

Root application of rare sugars protects plants from foliar pathogens, such as D-allose [40] or D-allulose [41], against bacterial blight on rice. Likewise, D-allose application to tomato roots reduced bacterial speck and gray mold symptoms [45]. Furthermore, root application of D-tagatose reduced downy mildew and powdery mildew symptoms of cucumber leaves [54]. Although D-tagatose migration from roots to leaves was hypothesized in cucumber [54], limited protective effects were found against grapevine downy mildew after root application of IFP48, possibly due to the different pathosystem and/or treatment protocol. Conversely, leaf application of D-tagatose reduced downy mildew severity on grapevine plantlets; 5 g/L IFP48 was the optimized dosage that maximize efficacy with no necrotic lesions and phytotoxicity.

The high efficacy of IFP48 shoot application suggested multiple mechanisms of action, based on direct anti-oomycete effect and defense induction. The assessment of the direct effect of IFP48 on *P. viticola* indicated a complete protection of in vitro plantlets with 5 g/L IFP48. Anti-oomycete effects of D-tagatose have been associated to severe mitochondrial alterations, inhibition of respiration with the accumulation of ROS, alterations of sugar content and enzymatic activities in *P. infestans* [51–53]. and inhibition of glycolysis and mannan synthesis in *H. arabidopsis* [48], suggesting similar impacts on the *P. viticola* metabolism. However, mannose metabolism is not important only for oomycetes, but also for ascomycetes and basidiomycetes, supporting the broad spectrum of activity of D-tagatose against *B. cinerea* on tomato [49], *Alternaria brassicicola* on cabbage, *Rhizoctonia solani* and *Cochliobolus miyabeanus* on rice, *Colletotrichum orbiculare* on cucumber, and *Magnaporthe oryzae* on rice [48].

In addition to their direct inhibitory effects, rare sugars have been shown to induce plant resistance, such as D-allose [40,46] and D-allulose [41,44,45], by direct induction of defense responses before pathogen challenge [47]. Moreover, D-allose can also prime tomato plants for the enhanced expression of SA- and JA-responsive *PR* genes after the pathogen challenge [45]. However, when applied on cucumber, rice, and Arabidopsis, D-tagatose did not induce plant resistance [48]. Likewise, TAG did not affect defense gene modulation and phytoalexin content in grapevine plantlets before and after *P. viticola* inoculation, with the exception of a slight upregulation of *PR-1* and *PR-2* compared to water-treated plants. In contrast, IFP48 triggered grapevine resistance by a direct upregulation of defense-related genes before *P. viticola* inoculation. Moreover, expression levels of defense-related genes remained higher in IFP48-treated plants compared to water-treated plants also after *P. viticola* inoculation. The use of resistance inducers to activate JA and ET signaling pathways has been proposed as a relevant strategy to improve the grapevine resistance against downy mildew [38,58,59]. The possible activation of JA and ET pathways by IFP48 was suggested by the slight upregulation of *LOX9* and *ACO* genes, markers of the JA and ET synthesis pathways, respectively [63–65], before pathogen inoculation. Moreover, IFP48 induced the expression of *PR-1* and *PR-2*, which are classified as markers of the SA pathway [57], and *PR-3*, which may be related to JA-signaling [59,60]. Thus, our results are coherent with the assumption that the effective defense against biotrophic pathogens generally depends on the SA signaling pathway [65,66]. However, the importance of JA signaling in grapevine resistance against the downy mildew disease was also previously demonstrated [67], indicating a whole defense activation after IFP48. In more detail, *PR-3* expression showed a strong response to IFP48 treatment, and chitinases (encoded by *PR-3* genes) are well characterized as antifungal proteins [66]. Likewise, the root application of D-allulose induced the expression of several defense-related genes in rice leaves, *PR-3* included [45], as possible markers of rare sugar-triggered resistance. The mode of action of the *PR-2*-encoded enzymes relies on hydrolysis of β-1,3-glucosidic bonds of β-1,3-glucans,

which are structural building blocks of fungal and oomycete cell walls [68–70], indicating a key contribution in the IFP48-induced resistance against *P. viticola*. The upregulation of *PR-1* by IFP48 agreed with the resistance enhanced by β-amino butyric acid (BABA) and benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH) in grapevine against *P. viticola* [59]. *PR-1* and *PR-2* were also slightly modulated by TAG, confirming the SA-mediated resistance induction by D-tagatose. The appearance of leaf necrotic spots observed following the application of high concentrations of IFP48 are also in agreement with D-allose-associated SAR-like necrotic symptoms in rice leaves [42].

In addition to *PR* genes, IFP48 upregulated *PAL* and *STS* expression and upregulation of phenylpropanoid-related genes is a common strategy to improve plant resistance, as found for *PAL* in D-allulose-treated rice [45], for *PAL* and *STS* in laminarin- [37], chitosan- [13], BcPG1- [71], ergosterol- [72], β 1,3-glucan sulfate-treated [37] grapevine. As a possible consequence of defense gene modulation, IFP48 treatment increased phytoalexin content before pathogen inoculation. Phytoalexins fall into the family of stilbenes, such as resveratrol [73] and its derivatives glucoside piceid [74] and the oligomer viniferin [75]. Phytoalexins are involved in the resistance to a wide spectrum of grapevine pathogens [76,77], including *P. viticola* through interfering with zoospore release and mobility [78,79]. Our results suggest that IFP48-induced resistance against *P. viticola* is as least partially associated with the increased production of stilbenoid compounds in the leaf tissues. In particular, phytoalexin content increased in IFP48-treated plants both before and after pathogen inoculation, corroborating a direct defense induction rather than a priming state activation. Although D-allose and D-allulose led to the expression of genes involved in secondary metabolite synthesis pathways [40–42], no changes in phytoalexins production upon rare sugar treatment were measured so far. TAG did not affect the stilbene content and expression of phenylpropanoid-related genes (*PAL* and *STS*) in grapevine plantlets, suggesting that a co-formulant could improve D-tagatose efficacy against grapevine downy mildew.

Although the co-formulant included in IFP48 is protected by industrial secrecy and cannot be tested separately, the characterization of IFP48 reported in this study clarified the mechanism of action of the formulated D-tagatose against *P. viticola*. In particular, the D-tagatose-based formulated product (IFP48) reduced downy mildew symptoms by an anti-oomycete activity and by the induction of grapevine defense responses with higher efficacy compared to the pure D-tagatose. For the successful application under field conditions, rare sugars must be formulated to improve efficacy and rain resistance [12,80]. For example, co-formulants can improve D-tagatose penetration in cucumber roots and systemic accumulation in leaves [54]. A variety of co-formulants ranging from non-ionic, anionic, cationic, and amphoteric surfactants or water-soluble polymers have been proposed for D-tagatose formulation [81], and a formulation that can reduce the effective concentration of D-tagatose was announced [48]. Thus, further experiments with different D-tagatose formulations are necessary to clarify the exact contribution of co-formulants in D-tagatose efficacy under greenhouse and field conditions.

Until today, there are no published experiments with D-tagatose done in vineyards except the work of the Mochizuki group in Kagawa [48]. Authors reported that pure D-tagatose exerted protection against grapevine downy mildew, which was comparable with fungicide cyazofamid, thus additionally encouraged the prospect of using this natural compound out in the open field. We imagine that D-tagatose-based products would be best used early in the season (from April) to intercept the primary downy mildew infection. D-tagatose seems to have curative and a 7-day-long residual effects (data from pot trials with cucumber) [48], therefore, during the high *P. viticola* pressure, treatments could be done on a weekly basis. We would avoid spraying D-tagatose later in the season (perhaps conclusive with veraison phenophase (BBCH 85–75)), as its effect on necrotrophic pathogen like *B. cinerea* and yeasts, important for the later vinification processes, remains unclear until now.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12020498/s1, Figure S1: Downy mildew disease severity in grapevine leaves after root treatment with IFP48 and D-tagatose, Figure S2: Downy mildew disease severity in grapevine leaves after aerial part treatment with D-tagatose, Figure S3: Direct effect of D-tagatose-based on Plasmopara viticola, Figure S4: Effects of D-tagatose on relative expression levels of defense-related genes, Figure S5: Effects of D-tagatose on trans-piceid content, Figure S6: Downy mildew disease severity in grapevine leaves after aerial part treatment with D-tagatose; Table S1: Primers of genes used in this study.

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