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Research Article

Tagatose Suppresses Grapevine Powdery Mildew and Downy Mildew under Field Conditions with No Severe Impacts on Grape Must Fermentation

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Background and Aims. Grapevine is susceptible to several diseases and requires a large use of fungicides. Sustainable alternatives must be safe for humans and the environment and also should not interfere with must fermentation. The aim of this study was to implement the use of a rare sugar, tagatose, against powdery mildew and downy mildew and to assess possible side effects on *Saccharomyces cerevisiae* fermentation. *Methods and Results*. Tagatose was evaluated for the suppression of powdery mildew and downy mildew under controlled and field conditions and for its impact on *S. cerevisiae* fermentation of synthetic and grape musts. Tagatose applied at 8 kg/hareduced powdery mildew and downy mildew severity and incidence on grapevine leaves and bunches under field conditions. Tagatose caused a limited and transient slowdown of the fermentation with no negative impact on yeast viability and wine chemical composition at the end of the fermentation. *Conclusions*. Tagatose is a promising alternative for sustainable grapevine protection against powdery mildew and downy mildew with no negative impacts on the must fermentation. *Significance of the Study*. These findings pave the way for grapevine protection strategies based on the use of rare sugars as sustainable fungicides in integration with other plant protection products.

1. Introduction

Grapevine (*Vitis vinifera*) is one of the major fruit crops worldwide, and it is economically relevant both for fresh (table grapes) and processed product (wine) consumption. Most grapevine cultivars, however, are susceptible to a large spectrum of destructive diseases [1], in particular, powdery mildew caused by the biotrophic ascomycete *Erysiphe necator* Schwein (synonym *Uncinula necator* (Schw.) Burr.) [2, 3] and downy mildew caused by the biotrophic oomycete *Plasmopara viticola* (Berk. and Curt.) Berl. and De Toni [4, 5] are two of the major devastating grapevine diseases. *Erysiphe necator* colonises the epidermal layer of green tissues under warm and humid conditions and develops infected areas coated with whitish mildew symptoms [2, 3], with consequent severe losses in grape yield and quality [6]. *Plasmopara viticola* zoospores infect grapevine green tissues (leaves, tendrils, bunches, or shoots) through stomata in the presence of leaf wetness and warm temperature, and they cause green-yellow lesions on leaves (namely, "oil spots") and brown symptoms on bunches, with a consequent dramatic decrease in grape quantity and quality [4, 5].

Frequent fungicide applications (e.g., every 7–10 days during seasons and in locations with high tendency of getting infected by these diseases) are required to prevent losses due to powdery mildew and downy mildew infections [2, 5, 7–9]. For example, it was estimated that viticulture accounted for 67% of all fungicides applied to the crops in the European Union (EU) between 2001 and 2003 although it represented only 3.3% of the total European agricultural

area [10]. The overuse of pesticides raised concerns about their possible impact on human health and the environment [11], and regulations to reduce their use were implemented in several countries [12, 13], increasing the demand of sustainable alternatives. Moreover, several active substances are under scrutiny for substitution in the EU, including molecules that are currently used against powdery mildew and downy mildew (e.g., copper, metalaxyl, fluopicolide, triazoles, and quinoxyfen) [14]. The issue of finding an effective alternative against these two diseases is particularly relevant in organic production [15]. Copper, which is widely used to suppress downy mildew in organic viticulture [16], is currently allowed in the EU up to a maximum of 28 kg/ha in 7 years (EU regulation 2018/1981) [19], and further limitations in copper use are expected in the future [18, 19]. In addition, copper can inhibit *S. cerevisiae* fermentation, increase the volatile acid production [20], and decrease the concentration of higher alcohols (e.g., isoamyl alcohol) and organic acid esters (e.g., ethyl lactate) [21]. Copper residues can also modify the sugar, acid, and lipid content of grape berries [22] and negatively affect the sensory quality of wine assessed by sensory analysis [23]. Likewise, treatments with sulfur against powdery mildew can leave residues and negatively affect yeast biodiversity in spontaneous must fermentation [24] because of the selective effects of sulfur on different yeast taxa [25]. In conventional agriculture, several synthetic chemical fungicides are applied for downy mildew and powdery mildew control (e.g., ametoctradin, captan, dimethomorph, fenarimol, folpet, kresoxim-methyl, quinoxyfen, and penconazole), and they may have a negative impact on *S. cerevisiae* fermentation [24, 26–28] and on sensory wine characteristics [29, 30], indicating that their use should be concluded sufficiently in advance before harvest. Although several potential alternatives to sulfur, copper, and synthetic chemical fungicide have been studied [7, 15, 31, 32], their potential side effects on must fermentations have seldom been investigated.

Tagatose (TAG) is a monosaccharide rarely found in nature (rare sugar) [33]. Tagatose is generally recognised as "safe" by the Food and Drug Administration in the United States of America (USA) because it has no negative impact on human health, and it can be used as a low-calorie sweetener in several countries, including the United States and EU [34, 35]. Due to its limited presence in nature, the biological functions of TAG are not fully understood, and its potential application is underestimated [36–38]. The implementation, however, of cost-effective chemical and biological synthesis processes [33, 39, 40] made industrial and agricultural applications more accessible [36–38, 40]. In agriculture, TAG inhibits the growth of a wide range of phytopathogens with a negligible effect on human health and the environment [36, 38, 41]. For instance, TAG suppresses tomato and potato late blight (*Phytophthora infestans*), grey mould (*Botrytis cinerea*), brown rust (*Puccinia recondita*), rice sheath blight (*Rhizoctonia solani*), cucumber downy mildew (*Pseudoperonospora cubensis*), and powdery mildew (*Podosphaera xanthii* and *Golovinomyces cichoracearum*) [42–46]. On the grapevine, TAG reduces powdery mildew and downy mildew severity on grapevine leaves and increases the abundance of potential biocontrol microorganisms of the grapevine phyllosphere, such as *Alternaria* spp., *Aureobasidium* spp., *Exiguobacterium* spp., and *Exophiala* spp. [47]. In particular, TAG treatment directly inhibits *P. viticola* sporangia, upregulates the expression of grapevine defence-related genes, and increases the concentration of stilbene phytoalexins under controlled conditions [48, 49], indicating multiple mechanisms of action against downy mildew. Thus, the feasibility of this rare sugar as a promising sustainable fungicide should be validated under field conditions, also taking into consideration the possible impact on *S. cerevisiae* fermentation, which could be affected by the presence of TAG residues. The aim of this study was to identify the amount of TAG to be applied per hectare of a vineyard as a promising alternative to sulfur and copper against powdery mildew and downy mildew and to analyse its possible impact on must fermentation.

2. Materials and Methods

2.1. Tagatose. In all experiments, a TAG formulation was used which contained 80% (w/w) TAG as the active substance and 20% (w/w) inert coformulants (IFP48 (MCF1309) wettable powder (Kagawa University, Kagawa, Japan), Mitsui Chemicals Agro (Tokyo, Japan), and Belchim Crop Protection (Londerzeel, Belgium). Product concentration in the experiments is expressed according to the concentration of the active substance (g/L).

2.2. Efficacy Trials against Powdery Mildew and Downy Mildew under Greenhouse Conditions. Two-year-old plants (*V. vinifera* cv. Pinot Noir ENTAV 115 grafted onto Kober 5BB rootstock) were grown in 2.5 L pots containing a peat and pumice mixture (Vegetal Radic Pomice piccola, TerComposti, Calvisano, Italy) under greenhouse conditions at 25 ± 1°C with a 16:8 h light:dark photoperiod and 70 ± 10% RH for 2 months so that each plant had 12–15 leaves at the beginning of the experiment [50]. Two repeated sets of experiments were carried out against powdery mildew and downy mildew, and the treatments using 0.8, 4, 8, or 24 g/L TAG were tested based on the results of preliminary tests (data are not shown). Plants were left untreated as control (CTRL), sprayed with 5 g/L sulfur (Thiamon 80 Plus, Du Pont, Wilmington, DE, USA) or 2 g/L copper (Coprantol Hi Bio, Syngenta, Basel, Switzerland) as reference fungicides in the experiments against powdery mildew and downy mildew, respectively. Treatment was done on all leaves (15 mL per plant corresponding to 1000 L/ha) with an air compressor system (Advance, Fini, Bologna, Italy) equipped with an air spray gun working at 400 kPa pressure at the nozzle [7].

In the experiments against powdery mildew, grapevine plants were left untreated until naturally infected with *E. necator*; the experiments were started after the appearance of visible symptoms of powdery mildew [51]. Plants with comparable disease severity and incidence were selected and randomly distributed among treatments. Plants were treated and incubated under greenhouse conditions at 25 ± 1°C with

80 ± 10% RH to allow *E. necator* development. The first assessment of powdery mildew severity was carried out 7 days after incubation under greenhouse conditions. Plants received the second spray application with the respective product and were incubated for 7 days under greenhouse conditions, and the second assessment of powdery mildew severity was carried out at the end of the experiment (14 days after the first spray application) [52]. Five replicates (plants) were used for each treatment, and the experiment was carried out twice using a randomised complete block design.

In the experiments against downy mildew, plants were left to dry for 2 h under greenhouse conditions after the treatment and were subjected to 0, 10, or 30 mm of simulated rain applied with a rain simulator to give a 50 mm/h rain with a drop size similar to raindrops (0.3 to 2.5 mm), which are the intensity values of a heavy rainstorm in Northern Italy. Plants were then inoculated with *P. viticola* suspension (3×10^5 sporangia/mL) with the air compressor system (25 mL/plant) and incubated overnight in the dark at 25 ± 1°C with 99-100% RH. Plants were then kept under greenhouse conditions for 6 days and incubated overnight in the dark at 25 ± 1°C with 99-100% RH to promote *P. viticola* sporulation and to assess the disease severity [50]. Four replicates (plants) were used for each treatment, and the experiment was carried out twice using a randomised complete block design. To prepare the inoculum, a *P. viticola* population was collected in an untreated vineyard in Northern Italy (San Michele all'Adige) and maintained by weekly inoculation on greenhouse-grown plants (*V. vinifera* cv. Pinot Noir). To collect *P. viticola* sporangia for the inoculum, plants with disease symptoms were incubated overnight in the dark at 99–100% RH and 25 ± 1°C to promote pathogen sporulation. Leaves bearing freshly sporulating lesions were washed with cold (4°C) distilled water, and the concentration of the sporangia suspension was assessed with the aid of a haemocytometer and light microscope, as described by Perazzolli et al. [50].

2.3. Efficacy Trials against Powdery Mildew and Downy Mildew under Field Conditions. Two experimental vineyards located in San Michele all'Adige (Italy) and having different characteristics were used in the field experiments (Table S1). Field experiments were carried out in a randomised complete block design, and four plots, consisting of eight plants each, were used as replicates for each treatment. Meteorological data were recorded by a station nearby the two vineyards (46°11′24.1″N, 11°08′04.9″E, 203 masl).

In the efficacy trials against powdery mildew under field conditions, plants were treated with 8 kg/ha TAG (corresponding to 8 g/L optimised in the greenhouse experiments since 1000 L/ha is commonly applied to the training system used in these experiments) or were sprayed with sulfur (SUL; 4 kg/ha Thiamon 80 Plus (Du Pont, Wilmington, DE, USA)) as the reference fungicide in 2014 and 2019 (Tables S1, S2). As a control (CTRL), plants were not treated with fungicides against powdery mildew. Copper (4 L/ha Bordoflow new (Manica, Rovereto, Italy)) was used, however, to suppress downy mildew on all plots (CTRL included) in the tank mixture of the TAG and sulfur treatments. Products were dissolved in tap water and sprayed with a Solo 450 motorised backpack mist blower (Solo, Newport News, VA, USA) with a spray volume of 1000 L/ha. Plants were treated every 8–10 days according to weather conditions suitable for *E. necator* infections in agreement with commercial viticultural practice against powdery mildew in Northern Italy [9]. In 2019, plants were treated after a rainfall greater than 25–30 mm also according to Thuerig et al. [53].

In the efficacy trials against downy mildew under field conditions, plants were treated with 8 kg/ha TAG (corresponding to 8 g/L optimised in the greenhouse experiments) and left untreated as control (CTRL) or sprayed with copper (Cu, 2 kg/ha (Coprantol Hi Bio, Syngenta AG, Basel, Switzerland)) as the reference fungicide in 2014 (Tables S1, S2). In 2015, the same concentration of TAG (8 kg/ha) was applied in conjunction with copper (Table S2). In particular, TAG was sprayed before flowering (BBCH-61) [54] and after fruitset-berry growing (BBCH-73), while copper was applied (2 kg/ha (Coprantol Hi Bio)) from BBCH-61 to BBCH-73 when bunches are highly susceptible to downy mildew [53]. Thus, an additional control with plants was treated with copper only at the flowering stage and untreated (UNT) at the other phenological stages (UNT-Cu-UNT) in 2015. In both seasons, plants were treated before predicted rainfall and probable infection of *P. viticola*, according to the weather forecast (https://www.3bmeteo.com/) and DSS RIMpro-Plasmopara (https://www.rimpro.be/PlasmoparaWeb/Plasmopara.htm), in agreement with commercial viticultural practice against downy mildew in Northern Italy [7]. In 2015, plants were treated after rainfall greater than 25–30 mm also [53]. Products were dissolved in tap water and sprayed with a Solo 450 motorised backpack mist blower with a spray volume of 1000 L/ha, as described above for the experiments against powdery mildew. Plant protection products containing active substances having no effect against downy mildew were sprayed stand-alone on all plots during the season to control powdery mildew according to the disease pressure (Table S2). In particular, one and five spray applications were carried out, respectively, in 2014 (quinoxyfen, 0.25 L/ha Arius, (Dow AgroSciences, Indianapolis, IN, USA)) and in 2015 ((quinoxyfen, 0.25 L/ha Arius; spiroxamine, 1 L/ha Prosper 300 CS (Bayer, Leverkusen, Germany); penconazole, 0.4 L/ha Support 10 EC (Cheminova Agro SA, Madrid, Spain); and metrafenone, 0.25 L/ha Vivando (BASF, Ludwigshafen, Germany)).

2.4. Assessment of Disease Severity, Disease Incidence, Efficacy, and Phytotoxicity. Powdery mildew severity was assessed visually based on the proportion of infected leaf, or bunch, area covered by powdery mildew symptoms (*E. necator* sporulation or tissue necrosis), and incidence was assessed visually based on the proportion of leaves, or bunches, showing powdery mildew symptoms, according to the standard guidelines of the European and Mediterranean Plant Protection Organization (EPPO) [55]. Likewise, downy mildew severity was assessed visually based on the

proportion of infected leaf, or bunch, area covered by downy mildew symptoms (oil spots, *P. viticola* sporulation and/or necrosis), and incidence was assessed visually based on the proportion of leaves, or bunches, showing downy mildew symptoms, according to the standard EPPO guidelines [56].

In the efficacy tests under greenhouse conditions, powdery mildew severity was assessed on all treated leaves before the first treatment and then 7 and 14 days after the first treatment, while downy mildew severity was evaluated on all treated leaves 7 days after *P. viticola* inoculation. In the field experiments, the disease severity and incidence were evaluated on 60 leaves and 40 bunches selected randomly in the four central plants of each plot (replicate) every 7–12 days from the BBCH-53 to BBCH-81 (2015 and 2019) or BBCH-77 (2014).

The efficacy of each treatment was calculated as follows:

$$\text{efficacy\%} = \frac{S_C - S_T}{S_T} \cdot 100, \tag{1}$$

where S_C is the mean disease severity (%) of control plants and S_T is the disease severity (%) of treated plants. Area under the disease progress curve (AUDPC) values for disease severity on leaves was calculated as follows:

$$AUDPC = \sum_{i=1}^{n-1} \frac{(S_i + S_{i+1})(t_{i+1} - t_i)}{2}, \tag{2}$$

where S is the disease severity (%) value on leaves at time t (days), t_i is the specific number of assessments, and n is the total number of assessments.

Phytotoxicity was visually assessed by checking for discoloration and localised necrosis on leaves and bunches according to the EPPO guidelines [57].

2.5. Impact of Tagatose on Synthetic Must Fermentation. Synthetic must was prepared as described by Nehme et al. [58] and was composed of glucose (100 g/L), fructose (100 g/L), Oxoid yeast extract (1 g/L LP0021 (Thermo Fisher Scientific, Waltham, MA, USA)), $(NH_4)_2SO_4$ (2 g/L), citric acid (0.3 g/L), malic acid (5 g/L), L-tartaric acid (5 g/L), and $MgSO_4$ (0.4 g/L), KH_2PO_4 (5 g/L), and it was adjusted to pH 3.5, using 10 N NaOH. The synthetic must was filtered (0.45 μm), and an aliquot (100 mL) was then placed in a 250 mL Erlenmeyer flask and sterilised at 121°C for 15 min. Synthetic must was treated with 0.032, 0.32, 1.28, or 3.20 g/L TAG or left untreated as control (CTRL). In particular, the concentration of 0.32 g/L TAG corresponded to the estimated TAG residue in the grape must, based on the amount of TAG sprayed on the bunches under field conditions, according to the equation as follows:

$$\text{dosage} = \frac{Td \cdot i}{p \cdot r} \cdot 10^3, \tag{3}$$

where Td is the TAG concentration applied under field conditions (8 kg/ha), i is the factor that considers the treatment interception of the bunches (25%, assessed visually based on the proportion of the bunches surface with respect to leaves), p is the grape production (12500 kg/ha) under the conditions tested, and r is the mean vinification rate from grape to must (0.50 L/kg).

Saccharomyces cerevisiae (15 g/L, EC-1118 Organic (Lallemand, Montréal, QC, Canada)) was rehydrated in sterile water at 37°C under orbital shaking at 150 rpm for 20 min, and 1 mL of the *S. cerevisiae* suspension was added to each flask (150 mg/L of yeast). The synthetic must was fermented at 22 ± 1°C under orbital shaking at 100 rpm for 16 days; a randomised complete block design was carried out with three replicates (flasks) for each treatment.

2.6. Impact of Tagatose on Grape Must Fermentation. In order to prepare grape must, 40 kg of bunches were collected from three white cultivars (Pinot Gris, Chardonnay, and Gewürztraminer on 8 September 2014) and three red cultivars (Marzemino, Lagrein, and Merlot on 22 September 2014) from a vineyard subjected to integrated pest management in Northern Italy (Rovereto, 45°52′41″N 11°01′13″E, 170 masl). The vinification process started within 2 h after harvest and the berries of each cultivar were crushed and pressed separately (Figure S1). Four white musts (named W_A, W_B, W_C, and W_D) were obtained by mixing the three musts in different proportions, such as W_A by 50% Pinot Gris, 30% Chardonnay, and 20% Gewürztraminer; W_B by 30% Pinot Gris, 50% Chardonnay, and 20% Gewürztraminer; W_C by 30% Pinot Gris, 20% Chardonnay, and 50% Gewürztraminer; and W_D by 33% Pinot Gris, 33% Chardonnay, and 34% Gewürztraminer. Three red grape musts were obtained by processing each cultivar individually (R_A, Marzemino; R_B, Lagrein; and R_C, Merlot). Red grapes were crushed, soaked at 10 ± 1°C for 4 h, and pressed, according to the production of rosé wines (rosé vinification), to increase the content of phenolic compounds and inhibit microbe development (e.g., wine spoilage yeasts and acetic acid bacteria) [59].

Pectolytic enzyme (0.015 mL/L, Zymaflore P110L (Perdomini-IOC, San Martino Buon Albergo, Italy)), $K_2S_2O_5$ (80 mg/L, Winy (Esseco, San Martino di Trecate, Italy)), and bentonite (0.5 g/L (Pentagel, Perdomini-IOC)) were added to each must before the cold settling (10 ± 1°C, 36 h). Musts were racked (<20 nephelometric turbidity unit (NTU)) and the turbidity was then increased to 150–200 NTU by adding a part of the removed lees. An aliquot (6 L) of each must was left untreated as control (CTRL) and an equivalent volume (6 L) was treated with 0.32 g/L TAG according to the synthetic must fermentation results. Each must was then inoculated with 150 mg/L *S. cerevisiae* inoculum (EC-1118 Organic) and fermented in 10 L stainless steel flasks at 19 ± 1°C until depletion of sugars (<2 g/L) [60].

2.7. Chemical Analysis of Musts and Wines. Samples obtained by the synthetic must fermentation were filtered with a cellulose acetate syringe cartridge (25 mm × 0.45 μm (Alltech, Deerfield, IL, USA)), and tagatose, glucose, and fructose were quantified by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) [47, 61]. Ethanol was analysed with

the standard method of the Organization Internationale de la Vigne et du Vin (OIV) [62].

The chemical composition of musts and wines was analysed before and after grape must fermentation. A must aliquot (30 mL) was centrifuged at 4700 × g for 5 min and filtered with a cellulose acetate syringe cartridge, and the refractive index (expressed as Brix), pH, total acidity (TA), tartaric acid, malic acid, potassium, and yeast assimilable nitrogen were assessed with a WineScan FT 120 Type 77310 (Foss Electric, Hillerød, Denmark). The instrument was calibrated according to the OIV guidelines [62]. At the end of the grape must fermentation, each wine was analysed as reported by Román et al. [63], and the ethanol concentration, relative density, pH, reducing sugar, total dry extract, ashes, TA, tartaric acid, malic acid, lactic acid, volatile acidity, potassium, and glycerol were assessed with the WineScan FT 120 Type 77310 (Foss Electric).

2.8. Assessment of Fermentation Kinetics. During synthetic must fermentation, the mass [64] of each flask was assessed daily after *S. cerevisiae* inoculation using a PB400-3 technical balance (Kern & Sohn, Albstadt, Germany). During grape must fermentation, the refractive index (expressed as Brix) [65] was measured daily with an MTD-033 digital handheld refractometer (Three-In-One Enterprises, New Taipei City, Taiwan) after *S. cerevisiae* inoculation in white and red grape musts.

The flask mass of synthetic must and the refractive index of grape must were used to calculate the fermentation kinetics (%) for each time point and replicate according to the equation as follows:

$$\text{fermentation kinetics}\,(\%) = \frac{\left(x_b - x_{tp}\right)}{\left(x_b - x_e\right)} \cdot 100, \tag{4}$$

where x is the mass (for the synthetic must fermentation) or refractive index (for the grape must fermentation) at the beginning (x_b), at a specific time point (x_{tp}), and at the end (x_e) of *S. cerevisiae* fermentation.

2.9. Assessment of the Yeast Viability. Yeast viability was assessed daily by a classic plating method after *S. cerevisiae* inoculation [66]. Serial dilutions of each sample were plated on yeast potato dextrose agar, which is composed of 10 g/L of Oxoid yeast extract (LP0021 (Thermo Fisher Scientific)), 20 g/L Oxoid peptone, (Thermo Fisher Scientific) 20 g/L dextrose (Thermo Fisher Scientific), and 15 g/L Oxoid microbiological agar (Thermo Fisher Scientific). Viable yeast cells were assessed as CFU/mL 48 h after incubation at 25°C [67]. Each sample was analysed in duplicate, and $\log_{10}$-transformed CFU/mL values were calculated.

2.10. Statistical Analysis. Data were processed using STATISTICA 13.1 software (Tibco Software, Palo Alto, CA, USA).

Fermentation kinetics (%) and yeasts viable cell ($\log_{10}$ CFU/mL) data of each replicate were analysed at three key time-points of the *S. cerevisiae* fermentation, such as during 10, 50, and 90% (or last assessment, in case of yeasts viability in grape musts) of the fermentation duration in CTRL musts [68]. Normal distribution (Kolmogorov–Smirnov test, $P > 0.05$) and variance homogeneity of the data (Levene's tests, $P > 0.05$) were checked. When these conditions were satisfied, a one-way ANOVA with Tukey's test ($P \leq 0.05$) was used to detect a significant difference among treatments. In particular, data of AUDPC, disease severity, and disease incidence of field experiments, chemical parameters after synthetic must fermentation, and fermentation kinetic and yeasts viability of synthetic must fermentation were arcsin transformed as follows:

$$\text{ArcSin}\left(\sqrt{\frac{y}{100}}\right), \tag{5}$$

where y is the measured data.

When conditions for a parametric test were not satisfied, the Mann–Whitney test ($P \leq 0.05$) and Kruskal–Wallis test ($P \leq 0.05$) were used to detect a significant difference in case of pairwise or multiple comparisons, respectively. In particular, the Kruskal–Wallis test was used to demonstrate a difference ($P > 0.05$) between the two experimental repetitions of powdery mildew and downy mildew efficacy test under greenhouse conditions. Data from the two experiments were then pooled, and the Kruskal–Wallis test was used to detect a significant difference among treatments ($P \leq 0.05$). Moreover, chemical parameters after grape must fermentation, fermentation kinetics, and yeast viability of grape must fermentation were subjected to the Mann–Whitney test in order to detect a significant difference among TAG-treated and CTRL samples ($P \leq 0.05$).

3. Results

3.1. Optimal Dose of Tagatose Suppresses Powdery Mildew and Downy Mildew under Greenhouse Conditions. In the greenhouse experiments against powdery mildew, the mean disease severity on CTRL plants was between 38.5 ± 3.6% and 58.5 ± 4.4% (mean ± SE values) at the beginning (before the first spray application) and between 55.5 ± 5.8% and 78.9 ± 2.3% at the end (14 days after the first spray application) of the first and second experiments, respectively. Treatments with TAG reduced powdery mildew severity with an efficacy comparable to that of sulfur in the case of 4, 8, and 24 g/L TAG at 7 and 14 days after the first spray application (Figure 1(a)). In contrast, the reduction of powdery mildew severity after treatment with 0.8 g/L TAG was lower than that observed after sulfur treatment at both time points.

Preventive TAG treatments reduced downy mildew severity compared to CTRL plants (51.1 ± 4.4% disease severity; mean ± SE values), and the efficacy of 8 g/L TAG and 24 g/L TAG was comparable to that of copper in the absence of simulated rain (Figure 1(b)). The efficacy of 0.8 g/L TAG and 4 g/L TAG was lower than that of copper in the absence of simulated rain. Tagatose efficacy got slightly reduced ($P \leq 0.05$, Kruskal–Wallis's test) by the simulated rain, and the efficacy was comparable in plants treated with copper,

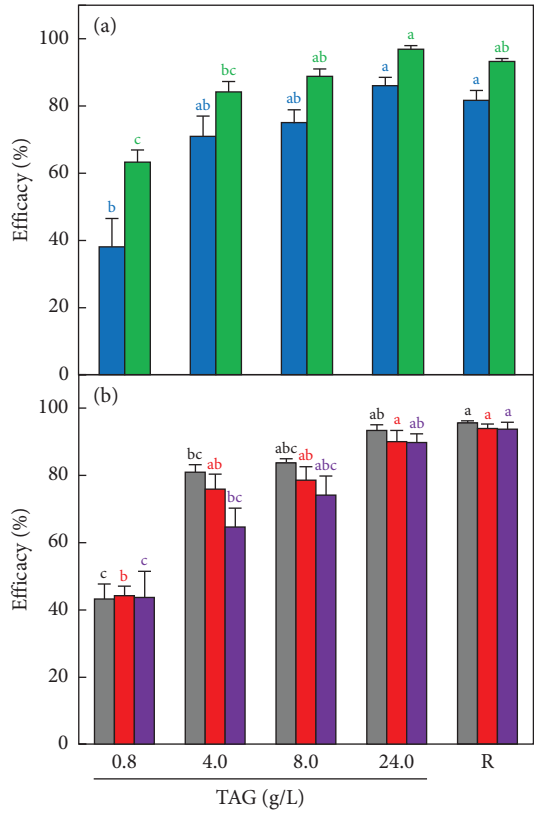


FIGURE 1: Efficacy of tagatose (TAG) against (a) powdery mildew and (b) downy mildew under greenhouse conditions. Plants left untreated as control or treated with 0.8, 4.0, 8.0, or 24.0 g/L TAG, sulfur, or copper as reference fungicides (R) against powdery mildew and downy mildew, respectively. In the efficacy test against powdery mildew, the disease severity was assessed 7 days ( ) and 14 days ( ) after the first treatment. In the efficacy test against downy mildew, plants were treated and subjected to 0 ( ), 10 ( ), or 30 mm ( ) of simulated rain before *Plasmopara viticola* inoculation. Mean and SE values of ten and eight replicates from two experiments are presented for efficacy against powdery mildew and downy mildew, respectively. For each condition (simulated rain or time point), means with the same letter are not significantly different according to the Kruskal–Wallis test ($P \leq 0.05$).

i.e., 8 g/L TAG and 24 g/L TAG in the case of 10 and 30 mm of simulated rain, respectively. As expected, copper efficacy was not affected by 10 and 30 mm of simulated rain under greenhouse conditions ($P > 0.05$, Kruskal–Wallis's test).

No phytotoxicity was recorded on TAG-treated shoots and leaves at each concentration used in the experiments under greenhouse conditions. Thus, the lowest concentration of TAG for maximum efficacy against both diseases was 8 g/L, and it was selected for field experiments.

3.2. Tagatose Suppresses Powdery Mildew and Downy Mildew under Field Conditions. Weather conditions in 2014 promoted powdery mildew development, and the disease incidence of CTRL plants was 68.8 ± 11.9 and 100.0 ± 0.0% at the last assessment (BBCH-81) on leaves and bunches, respectively (FigureS2). Weather conditions of 2019 allowed a moderate disease development; powdery mildew incidence on leaves and bunches was 14.6 ± 1.4 and 47.5 ± 8.4% on CTRL plants, respectively. In the 2014 and 2019 seasons, 14 and 15 spray applications were carried out against powdery mildew with seven and eight assessments, respectively (Table S2). Tagatose treatments reduced powdery mildew severity on leaves in 2014 and 2019 (Figure 2). In both seasons, powdery mildew severity (Figure 2) and incidence (Figure S2) on leaves of TAG-treated plants were comparable to that of SUL-treated plants. On bunches, TAG treatment reduced powdery mildew severity (Figure 2) and incidence (Figure S2) compared to that of CTRL plants, and the disease severity levels were comparable to those of SUL-treated plants. Moreover, the AUDPC of powdery mildew severity on leaves and bunches was lower on TAG-treated compared to that of CTRL plants, with the exception of the AUDPC on leaves in 2014 due to the variability among replicates, and it was comparable in TAG-treated and SUL-treated plants in both seasons (Table 1).

Weather conditions in 2014 were favourable to *P. viticola* infections, and the disease incidence was almost 100% in CTRL leaves and bunches in mid-July and the field trial was aborted early (BBCH-77; Figure S3). Downy mildew infection pressure was moderate in 2015 due to relatively dry weather conditions, and the disease incidence on CTRL leaves and bunches was 82.50 ± 6.5 and 86.9 ± 3.7% at the last assessment (BBCH-83), respectively. Tagatose treatments reduced downy mildew severity on leaves in 2015 compared to CTRL plants at the last assessment, but they did not affect downy mildew severity on leaves in 2014 at the last assessment (3). Downy mildew severity (Figure 3) and

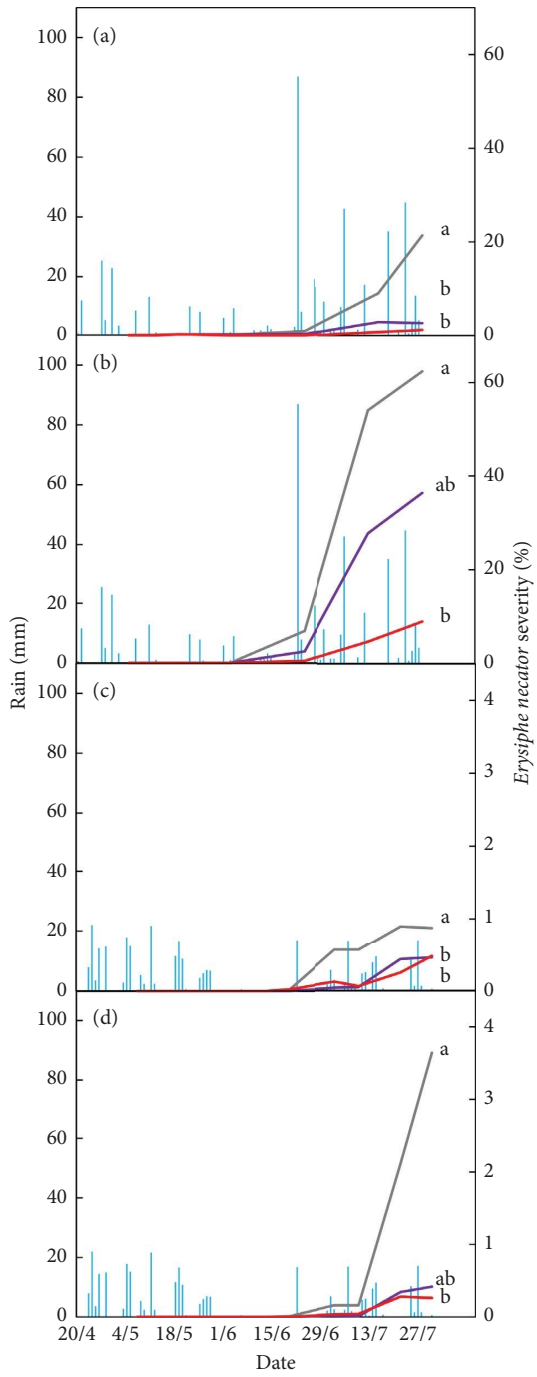


FIGURE 2: Effect of tagatose (TAG) against powdery mildew (*Erysiphe necator*) under field conditions. Powdery mildew severity was assessed on (a, c) leaves and (b, d) bunches of control plants (■) and plants treated with TAG (■) or sulfur (■) in (a, b) 2014 and (c, d) 2019. The rainfall is indicated by the vertical columns ( ). Mean and SE values of four replicates are reported for each treatment. At the last assessment, means with the same letter are not significantly different according to Tukey's test ($P \leq 0.05$).

incidence (Figure S3) on leaves of TAG-treated plants were comparable to that of Cu-treated plants and lower than that of the UNT-Cu-UNT strategy in 2015. Moreover, downy mildew severity on bunches of TAG-treated plants was comparable to that of Cu-treated plants in both seasons. TAG treatments reduced downy mildew severity on bunches in 2014 and 2015 compared to CTRL plants at the last assessment (Figure 3). The AUDPC of downy mildew severity on leaves and bunches was lower on TAG-treated plants compared to CTRL plants, with the exception of the AUDPC on leaves in 2014, and it was comparable to that of Cu-treated plants in both seasons (Table 1). In particular, 14 and 16 copper applications were carried out on Cu-treated plants with a total of 7.0 and 7.5 kg/ha of copper ions in 2014 and 2015, respectively. In 2015, four copper applications were carried out at the flowering stage in the UNT-Cu-UNT

Table 1: Area under the disease progress curve of powdery mildew and downy mildew severity on leaves and bunches of control plants and plants treated with tagatose, copper, or sulfur under field conditions.

Treatment	Powdery mildew†		Downy mildew‡	
	2014	2019	2014	2015
AUDPC on leaves				
CTRL	318.87 ± 124.88n.s.	20.80 ± 1.67a	499.93 ± 74.06a	346.40 ± 45.71a
TAG	51.29 ± 15.38n.s	5.90 ± 1.13b	320.69 ± 21.70ab	86.11 ± 14.15b
Cu	n.d.	n.d.	186.44 ± 17.63b	38.98 ± 9.29b
SUL	78.20 ± 39.18n.s.	7.28 ± 1.12b	n.d.	n.d.
UNT-Cu-UNT	n.d.	n.d.	n.d.	273.50 ± 59.39a
AUDPC on bunches				
CTRL	1584.75 ± 317.62a	40.73 ± 10.65a	707.00 ± 125.99a	229.44 ± 36.18a
TAG	405.41 ± 72.73b	4.68 ± 4.13b	228.22 ± 75.73b	32.40 ± 8.42b
Cu	n.d.	n.d.	223.52 ± 60.16b	8.89 ± 5.80b
SUL	826.59 ± 142.60ab	5.68 ± 2.06b	n.d.	n.d.
UNT-Cu-UNT	n.d.	n.d.	n.d.	188.76 ± 50.78a

Mean and standard error values of four replicates (plots of four plants each) are reported for each treatment; for each season, means with the same letter are not significantly different according to Tukey's test ($P \leq 0.05$); n.d. means treatment is not carried out in the specified season; and n.s. means not significant. †In the powdery mildew field trials, copper was applied to reduce downy mildew infection on all plants, including CTRL. ‡In the downy mildew field trials of 2015, TAG was applied in a strategy with copper at the flowering stage, while copper was applied only at the flowering stage, and plants were left untreated in the other phenological stages as an additional control. AUDPC, area under disease progress curve; CTRL, control plants; Cu, copper; SUL, sulfur; TAG, tagatose; UNT, untreated; and UNT-Cu-UNT, additional control.

strategy (Table S2) corresponding to 1.5 kg/ha of copper ions with a slight reduction of disease incidence on bunches, but no effect on disease severity on leaves and bunches were seen compared to CTRL plants.

3.3. Tagatose Slightly Affects S. cerevisiae Fermentation. The presence of 0.032 g/L TAG did not affect the fermentation kinetics (Figure 4(a)) and counts of viable yeast cells (Figure 4(b)) of synthetic must at 10, 50, and 90% of alcoholic fermentation. Likewise, the concentration of ethanol, fructose, and glucose was comparable in 0.032 g/L TAG-treated must and CTRL synthetic must at the end of alcoholic fermentation (Table 2). The presence of 0.32 g/L TAG slightly affected the fermentation kinetics at 10% and 50% of alcoholic fermentation and decreased counts of viable yeast cells at 10% of alcoholic fermentation. The fermentation kinetics and viable yeast cells, however, were comparable in 0.32 g/L TAG-treated and CTRL synthetic must at 90% of alcoholic fermentation, as well as the ethanol concentration at the end of alcoholic fermentation (Table 2). The fermentation kinetics and counts of viable yeast cells were reduced in 1.28 and 3.20 g/L TAG-treated synthetic must compared to that of CTRL synthetic must at 10, 50, and 90% of alcoholic fermentation (Figure 4), with a consequent lower concentration of ethanol and higher concentration of fructose and glucose at the end of alcoholic fermentation (Table 2).

The minimum tested concentration that caused a slight perturbation of the fermentation of synthetic must was 0.32 g/L TAG (that corresponded to the estimated TAG residues in the grape must according to the TAG amount sprayed on bunches under field conditions), and it was applied to white and red grape must before *S. cerevisiae* inoculation (Table S3). The fermentation kinetics was slightly affected by TAG in the first stages of alcoholic fermentation, and significant differences were found between 0.32 g/L TAG-treated and CTRL grape must at 10 and 50% of alcoholic fermentation of white grape must (Figure 5(a)) and at 50% of alcoholic fermentation of red grape must (Figure 5(b)). Tagatose did not affect, however, the fermentation kinetics at 90% of alcoholic fermentation and the total time needed to complete the alcoholic fermentation of the white and red grape musts. Furthermore, the counts of viable yeast cells (Figure 5) and the chemical composition of the resulting white and rosé wines were not affected by 0.32 g/L TAG (Table 3). In particular, the ethanol concentration, glucose residues, and fructose residues were comparable in 0.32 g/L TAG-treated and CTRL grape must at the end of alcoholic fermentation.

4. Discussion

Spray applications with TAG reduced powdery mildew and downy mildew symptoms on grapevine leaves and bunches in two seasons having different disease pressures. Tagatose showed dose-dependent effects against downy mildew under greenhouse conditions, and the concentration of 8 g/L (corresponding to a field application of 8 kg/ha in our conditions) reduced disease severity and incidence under field conditions comparable to that obtained with the reference fungicides (sulfur and copper). In particular, the total number of spray applications per season of TAG (less than 16) was in line with the commercial viticultural practices applied against powdery mildew [9] and downy mildew [7] in the environmental conditions of Northern Italy. A decreasing efficacy against downy mildew was, however, observed with an increasing amount of simulated rain, which was expected considering the high solubility of TAG in water [69]. The low rain fastness of TAG can also explain the low efficacy on leaves and bunches under field conditions in 2014, particularly in relation to the exceptional rain (87 mm

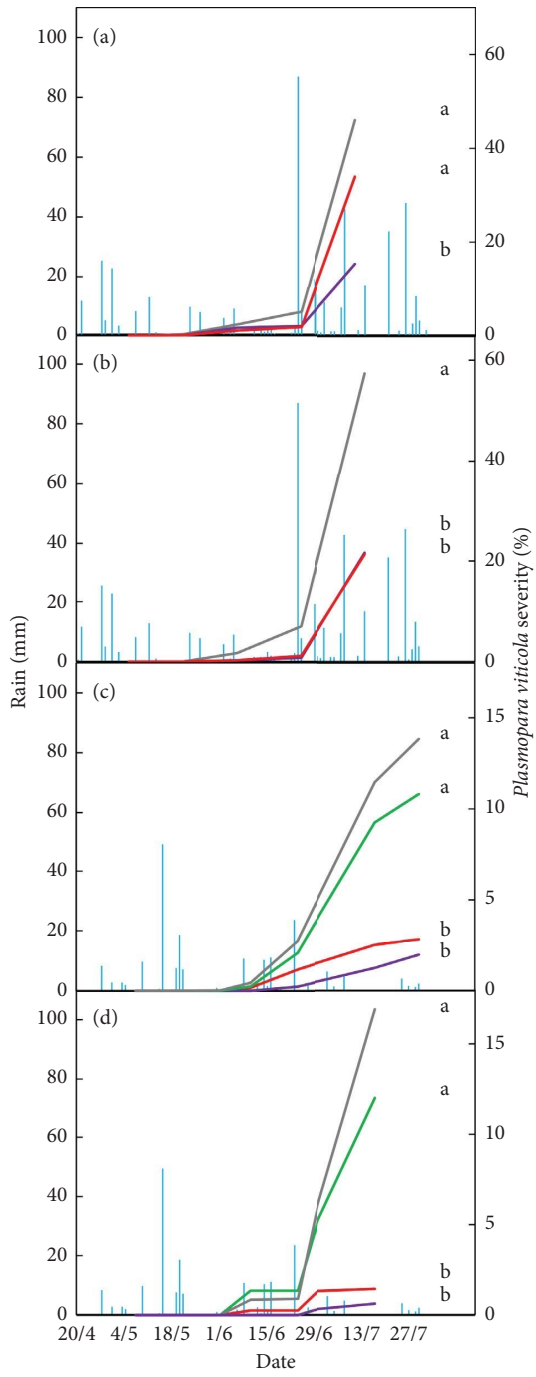
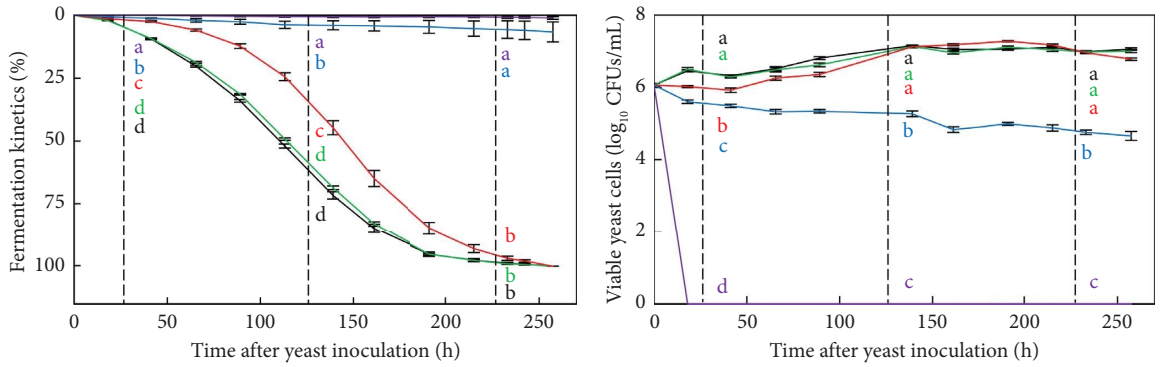


FIGURE 3: Effect of tagatose (TAG) against downy mildew (*Plasmopara viticola*) under field conditions. Downy mildew severity was assessed on (a, c) leaves and (b, d) bunches of control plants (■) and plants treated with TAG (■) or copper (■) in (a, b) 2014 and (c, d) 2015. In 2015, TAG was applied in conjunction with copper at the flowering stage, while copper was applied only at the flowering stage as an additional control (■). The rainfall (mm) is indicated by the vertical columns (|). Mean and SE values of four replicates are reported for each treatment. At the last assessment, means with the same letter are not significantly different according to Tukey's test ($P \leq 0.05$).

on 24 June 2014) that could have washed off TAG before *P. viticola* infection. The application of high TAG concentration, the increase of spray application frequency during rainy periods (according to the product costs), and/or the incorporation of suitable stickers as coformulants may further improve the efficacy of this product. The low rain fastness is a key issue in the development of alternatives to downy mildew, and several potential alternatives to synthetic fungicides and copper showed low persistence during periods of heavy rain, such as in the case of plant extracts [53]. Integrated application strategies of TAG in combination or alternation with other plant protection products are suggested to improve the efficacy in rainy periods and to reduce the risks of developing resistant races in the pathogen



(a) (b)

Figure 4: Effect of tagatose (TAG) on the fermentation of the synthetic must. (a) Profiles of fermentation kinetics and (b) viable yeast cells were assessed during alcoholic fermentation of synthetic musts treated with 0.032 (■), 0.32 (■), 1.28 (■), or 3.20 g/L (■) TAG or left untreated as control (■). Mean and SE values of three replicates are reported for each treatment. At 10, 50, and 90% of alcoholic fermentation (vertical dashed lines), means with the same letter are not significantly different according to Tukey's test ($P \leq 0.05$).

Table 2: Effect of tagatose treatment on the chemical composition of the synthetic must.

Treatment	Tagatose (mg/L)	Fructose (g/L)	Glucose (g/L)	Ethanol (%)
CTRL	n.d. (<30)e	n.d. (<0.1)c	n.d. (<0.1)c	8.50 ± 0.15a
TAG 0.032	40.00 ± 1.15d	n.d. (<0.1)c	n.d. (<0.1)c	8.45 ± 0.11a
TAG 0.32	370.00 ± 20.13c	10.63 ± 1.31b	3.00 ± 0.79b	7.98 ± 0.19a
TAG 1.28	1373.67 ± 71.91b	98.27 ± 0.55a	98.87 ± 0.70a	0.06 ± 0.00b
TAG 3.2	3226.67 ± 26.87a	95.10 ± 0.25a	96.33 ± 0.22a	0.05 ± 0.01b

Mean and SE values of the three replicates were assessed for each treatment at the end of *S. cerevisiae* fermentation; for each chemical parameter, means with the same letter are not significantly different according to Tukey's test ($P \leq 0.05$); n.d. means not detectable (in brackets the limit of quantification). CTRL is control; TAG is tagatose.

Table 3: Effect of tagatose treatment on the chemical composition of white and rosé wines.

Wine parameter	White wine		Rosé wine	
	CTRL	TAG	CTRL	TAG
Ethanol (%)	11.34 ± 0.05	11.55 ± 0.09	11.40 ± 0.19	11.27 ± 0.20
Relative density 20°C	0.99275 ± 0.00013	0.99272 ± 0.00008	0.99410 ± 0.00042	0.99421 ± 0.00037
pH	3.08 ± 0.01	3.09 ± 0.00	3.02 ± 0.08	3.05 ± 0.07
Reducing sugar (g/L)	0.3 ± 0.3	0.3 ± 0.3	1.5 ± 0.1	1.8 ± 0.2
Dry extract (g/L)	20.5 ± 0.2	21.0 ± 0.2	24.1 ± 0.8	24.1 ± 0.4
Ashes (g/L)	1.68 ± 0.03	1.65 ± 0.03	1.97 ± 0.07	1.97 ± 0.03
TA (g/L)	6.83 ± 0.17	6.75 ± 0.10	8.70 ± 0.79	8.37 ± 0.68
Tartaric acid (g/L)	2.17 ± 0.08	2.18 ± 0.10	2.04 ± 0.32	1.93 ± 0.22
Malic acid (g/L)	2.98 ± 0.10	2.94 ± 0.06	4.61 ± 0.44	4.39 ± 0.41
Lactic acid (g/L)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Volatile acidity (g/L)	0.21 ± 0.04	0.30 ± 0.04	0.35 ± 0.04	0.40 ± 0.02
Potassium (g/L)	0.72 ± 0.01	0.70 ± 0.01	0.85 ± 0.02	0.84 ± 0.01
Glycerol (g/L)	6.9 ± 0.1	7.2 ± 0.1	7.1 ± 0.2	7.5 ± 0.1

Mean and SE values of four and three replicates are reported for each treatment of white and rosé wines, respectively; for each chemical parameter, no significant difference between TAG-treated and CTRL samples was found according to Mann–Whitney's test ($P > 0.05$). CTRL, control; TAG, tagatose; TA, total acidity.

population. Multiple mechanisms of action of TAG, however, were demonstrated against *P. viticola*, such as the stimulation of leaf-associated beneficial microorganisms [47], the direct inhibitory effect on *P. viticola* sporangia, the upregulation of defence-related genes, and the increase of stilbene phytoalexin content in grapevine leaves [48, 49], suggesting low risks for the development of resistant races in pathogen populations. According to efficacy trials under field conditions and the possible mechanisms of action of TAG, the practical suggestion is to intervene with this active substance against downy mildew with preventive spray applications during the phases of low susceptibility of the plant, avoiding excessively rainy periods and integrating the plant protection strategy with products with high rain

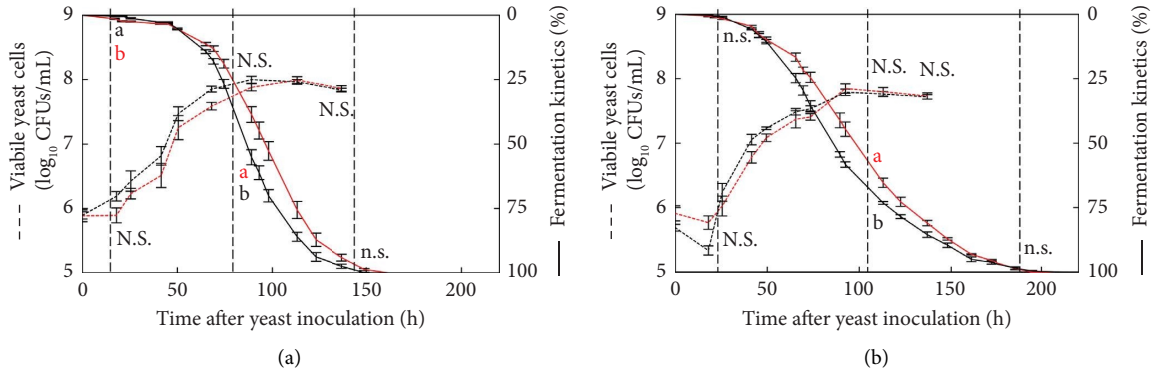


FIGURE 5: Effect of tagatose (TAG) on the fermentation of the grape must. (a) Profiles of fermentation kinetics (——) and viable yeast cells (— — —) were assessed during alcoholic fermentation of (a) white and (b) red grape must treated with 0.32 g/L TAG (——) or left untreated as control (——). Mean and SE values of four white grape musts and three red grape musts are reported for each treatment. At 10, 50, and 90% of alcoholic fermentation (vertical dashed lines), mean of viable yeast cells (uppercase) and fermentation kinetic (lowercase) with the same letter are not significantly different according to the Mann–Whitney's test ($P \leq 0.05$). n.s. means no significant difference.

fastness and high efficacy in the most critical phases. For example, TAG applied before flowering (BBCH-61) and after fruitset-berry growing (BBCH-73) in a strategy with copper during flowering (BBCH-61 to 73) improved the efficacy of the respective control (UNT-Cu-UNT strategy), with a considerable reduction of copper ions applied during the 2015 season (1.5 kg/ha in the TAG strategy compared to 7.5 kg/ha in the case of Cu-treated plants).

The problem of low rain fastness was less relevant to powdery mildew since *E. necator* infections are hindered by leaf wetness, and they are not common during rainy periods although they preferentially occur with high RH [2, 3]. Consequently, the efficacy of TAG against powdery mildew was comparable to that of sulfur in seasons characterised by a moderate (such as 2019) and a high (such as 2014) disease pressure. Copper was applied in the powdery mildew field trials to reduce downy mildew infections, and it can contribute to the reduction of *E. necator* vitality [70]. Copper was applied, however, to all plants (CTRL and SUL-treated plants included) and efficacy results supported the contribution of TAG in the reduction of powdery mildew severity and incidence on bunches and leaves. Although further studies are required to better characterise the mode of action of TAG against powdery mildew and the optimal timing for application, our efficacy results suggest that TAG could be used until the last stages of the season, thanks to its negligible effects on human health and the environment [36, 38, 41, 46]. Since TAG inhibits the growth of various microorganisms [36, 38, 46, 71, 72], the potential impact of TAG residues on *S. cerevisiae* fermentation was investigated. The fermentation of synthetic must suggested a possible inhibitory effect of TAG on *S. cerevisiae*. Grape musts, however, supplemented with the estimated maximum TAG residues (0.32 g/L, calculated according to treatments under field conditions) showed only a slight and partial slowdown of fermentation kinetics in the initial phase of alcoholic fermentation. Moreover, the fermentation slowdown observed in the initial phase was recovered during the second part of alcoholic fermentation, with no negative effects of TAG on *S. cerevisiae* viability, time needed to complete alcoholic fermentation, and the chemical composition of white and rosé wines. The limited effect of TAG on *S. cerevisiae* fermentation in grape musts can be related to the presence of nutritional factors (e.g., vitamins, lipids, and nitrogen compounds) [73] and indigenous microorganisms [74] that are known to influence *S. cerevisiae* development. The limitation of TAG effects can be related to the excess of glucose and fructose also that could compete with TAG, as previously found for the interactions between common sugars and TAG in *P. infestans* [45], although further studies are required to investigate the possible impacts of TAG with the sugar metabolism of *S. cerevisiae*. The risk of a harmful effect of TAG could be further limited on *S. cerevisiae* due to the procedures normally applied to grape must during vinification (e.g., must settling, centrifugation, and flotation) that can remove active compounds from the fermentation medium [27]. Fermentation at the laboratory scale, however, can be partially extrapolated to real conditions, [75] and further investigations are required to better characterise the effects of TAG residues on commercial wine production.

5. Conclusions

Spray applications of TAG reduced powdery mildew and downy mildew severity and incidence on grapevine leaves and bunches in two seasons having different disease pressures. Tagatose is renewable, biodegradable, safe for humans, and more sustainable than other alternatives, such as plant extracts that need plants to be cultivated and the active ingredient to be extracted. Although TAG caused a partial slowdown of fermentation kinetics in the initial phase of alcoholic fermentation, it has no negative impact on *S. cerevisiae* viability and the chemical composition of wine, suggesting that this rare sugar is a promising alternative for sustainable grapevine protection. Integration with other plant protection products, however, is suggested at the most susceptible phenological phases in case of frequent and/or intense rainfall or when the infection pressure is particularly

high. Thus, further studies on integration strategies with other plant protection products and formulations with higher rain fastness are required in order to improve TAG efficacy under field conditions.

Data Availability

The data used to support the findings are available from the corresponding author upon reasonable request.

Disclosure

The authors declare that they have no conflicts of interest.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Supplementary Materials

Figure S1: overview of the grape must fermentation process adopted to evaluate the effect of tagatose (TAG) on *Saccharomyces cerevisiae*. Before yeast inoculation, seven grape musts (four white and three red grape musts) were divided into two 6 L parts, one part was treated with 0.32 g/L TAG and the second part was left untreated which is control (CTRL). Total yeast viability and fermentation kinetics were monitored by plate counting and refractive index measurement, respectively. Chemical analyses were carried out on musts and wines before and after the alcoholic fermentation, respectively. Figure S2: effect of tagatose against powdery mildew (*Erysiphe necator*) incidence under field conditions. Powdery mildew incidence was assessed on leaves and bunches of control plants (■) and plants treated with TAG (■) or sulfur (■) in (a, b) 2014 and (c, d) 2019. The rainfall (mm) is indicated by vertical columns (|). Mean and SE values of four replicates are reported for each treatment. In the last assessment, means with the same letter are not significantly different according to Tukey's test ($P \leq 0.05$). Figure S3: effect of tagatose (TAG) against downy mildew (*Plasmopara viticola*) incidence under field conditions. Downy mildew incidence was assessed on leaves and bunches of control plants (■) and plants treated with TAG (■) or copper (■) in (a, b) 2014 and (c, d) 2015. In 2015, TAG was applied in a strategy with copper at the flowering stage, while copper was applied only at the flowering stage as an additional control (■). The rainfall is indicated by the vertical columns (|). Mean and SE values of four replicates are reported for each treatment. At the last assessment, means with the same letter are not significantly different according to Tukey's test ($P \leq 0.05$). n.s. means no significant difference. Table S1: Features of the experimental vineyards. Table S2: products were applied at the different grapevine phenological stages (BBCH-scale) against powdery mildew (seasons 2014 and 2019) and downy mildew (seasons 2014 and 2015) under field conditions. Table S3: chemical composition of grape musts before alcoholic fermentation. (*Supplementary Materials*)

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