Research Article

Received: 30 October 2017 Revised: 25 January 2018 Accepted article published: 25 February 2018 Published online in Wiley Online Library: 30 April 2018

(wileyonlinelibrary.com) DOI 10.1002/jsfa.8977

Evaluating a preventive biological control agent applied on grapevines against *Botrytis cinerea* and its influence on winemaking

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Abstract

BACKGROUND: This research was aimed to study the influence on grape and wine quality and on the fermentation processes of the application of a preventive biological treatment against *Botrytis cinerea* in Tempranillo Rioja grapevines. For this purpose, a biofungicide containing *Bacillus subtilis* QST713 was applied twice to the vineyard.

RESULTS: Results were compared with non-treated samples from the same vineyard and with samples treated with a chemical fungicide composed of fenhexamid. Data showed that general grape quality and spontaneous alcoholic fermentation performance were not influenced by either fungicide application. The chemical fungicide had a considerable influence on the clonal diversity of *Saccharomyces cerevisiae* species, which led to the alcoholic fermentation. Furthermore, it caused longer malolactic fermentation than with the biological fungicide and in the control. The biofungicide made malolactic fermentation 1 day shorter because the establishment of the commercial lactic acid bacteria used as a starter culture was total. After malolactic fermentation, the wines did not show significant differences in general oenological parameters.

CONCLUSION: Preventive biofungicide treatment against *B. cinerea* did not negatively influence the quality of grapes and wines and the fermentation processes. Therefore, biological control of *B. cinerea* with *B. subtilis* applied on grapevines could be advisable in oenological terms.
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Keywords: chemical fungicide; biofungicide; clonal diversity; alcoholic fermentation; malolactic fermentation

INTRODUCTION

Cultivation of grapevines is a very traditional practice in Mediterranean countries such as Spain. Indeed, it is one of the most important economic activities in some Spanish regions, such as in the 'Rioja' Qualified Designation of Origin (D.O.Ca. Rioja). Nevertheless, the economic losses caused by some fungal diseases pose a major concern for grape growers (http://www.oiv.int/es/bases-de-datos-y-estadisticas). *Botrytis cinerea* is one of those fungal diseases that affect wine growers. Furthermore, it also affects the grape and wine organoleptic qualities where necessary precautions are not taken at the correct time.[1]

Traditionally, the fight against this fungus has been carried out with chemical fungicides. Initially, fungicides formulated with copper molecules were most widely used. Some years later, those types of pesticides were thought to be responsible for hazardous effects on the environment because of their potential contamination. Trying to develop a more eco-friendly agricultural practice, other strategies, such as the employment of cover crops,[2] and other kinds of fungicides, such as fenhexamid,[3] were proposed. Despite these strategies, and even following good agricultural practices, the fungicide residues that remain on the grape surfaces would probably be transferred to the must and wines during the winemaking process.[4] This fact, along with the unexpected appearance of resistance, has been the main reason for considering the application of biological fungicides as part of the control of different crops.[1,5] Besides this, the new trend to avoid food and beverages containing chemical residues, such as copper and sulfur by-products, has meant an advance in research into new bioproducts to be employed for grapevine biocontrol strategy to improve the sustainability.[6,7]

Viticulture is a traditional field, so the treatment of grapes using biological fungicides on grapes is an emerging approach. This has meant that few studies have been carried out until now. For instance, some yeasts, such as *Candida sake*,[1] and some bacteria, such as *Bacillus subtilis*,[8] are being applied to reduce some grapevine diseases. In the case of the latter biofungicide, it can be applied on the grapevine from full bloom to only 2 or 3 days before being harvested, which makes it of interest for oenological study.

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Table 1. Average yield components ($n = 4$) of the control grapevines and of the grapevines treated with a chemical fungicide and with a biofungicide in the experimental field, with their standard deviations and the statistical assessment

Yield component	Control	Chemical fungicide	Biofungicide
Unit production (kg/vine)	4.08 ± 0.70	5.07 ± 0.6	5.02 ± 0.30
Average cluster weight (g)	342 ± 34^{a}	384 ± 28^{ab}	396 ± 19^{b}

Different superscript letters mean significant differences ($P < 0.10$) between samples.

Winemaking begins with grapes that are usually stemmed, crushed and sulfited and that contain a large population of non-*Saccharomyces* and *Saccharomyces* species. In fact, it is the *Saccharomyces cerevisiae* species that transforms grape sugars into alcohol in what is known as alcoholic fermentation (AF). During this fermentative stage, many other biological and physicochemical reactions take place. For instance, the aromatic profile changes from varietal aromas to fermentative ones, and the colour properties also vary through this first fermentation.[9] A second fermentation takes place, usually after AF, and this is referred to as malolactic fermentation (MLF). This is not strictly speaking a fermentation process but rather a biological deacidification of malic acid into lactic acid carried out by lactic acid bacteria (LAB), mainly by the species *Oenococcus oeni*. During this stage, the wine undergoes an increase in the stability of microbial and physicochemical properties such as colour.[10]

Considering all the foregoing, the winemaking process is one of the most complex microbiological, physicochemical transformations in the food industry. Trying to understand to what extent some emerging agronomic practices could influence winemaking is a very interesting area of research. For this reason, this study set out to describe the influence of the preventive application of a biofungicide to grapevines, on winemaking and wine quality in Rioja Tempranillo wines.

MATERIALS AND METHODS

Grapevine treatments

This study was performed in a *Vitis vinifera* L. cv. Tempranillo vineyard in D.O.Ca. Rioja. In order to avoid biases caused by the climatic or agronomic conditions, three treatments were applied in the same vineyard. The soil management of this vineyard was based on tillage, with approximately 3530 plants per hectare. The experimental design with randomly established blocks of four replicates per treatment was established in the vineyard. Replicates of the same treatment were in the same row, and treatments were 2.7 m. apart. Each replicate received the same agronomic management prior to treatment. The average number of plants per replicate was 25. The vineyard had no symptoms of being affected by *B. cinerea* at the beginning of the study.

Treatments were applied with an automatic knapsack sprayer. One was the control, without fungicide treatment. Another treatment, referred to as chemical fungicide, consisted of the application of a traditionally employed chemical fungicide based on fenhexamid (Teldor®, Bayer Crop Bioscience S.L.) 21 days before harvest (1.7 kg ha^{-1}). The third treatment applied with a dose of 4 kg ha^{-1} 21 days and 3 days before harvest was referred to as biofungicide. This was a biological fungicide with 5.3×10^{10} colony-forming units (CFU) per millilitre of the *B. subtilis* strain QST 713 (Serenade® Max, Bayer Crop Bioscience S.L.). Control of ripening was performed from veraison to the optimal date for harvest. Each replicate was separately harvested and vinified.

Evaluation of the grapevine yield

At the time of harvest, the yield components were evaluated in the experimental vineyard for each of the treatments, by determining the unit production (kilograms per vine) and the average cluster weight (grams).

Evaluation of the grape and must quality

To estimate the harvest date, 200 grapes were sampled at different times during the ripening process, for each treatment and replicate, in which the weight of 100 grapes, sugar concentration (Brix degrees) and pH were evaluated. At harvest, a random selection of 300 grapes per replicate was collected and weighed to obtain the average weight of 100 grapes. Then, the grapes were crushed to analyse the oenological parameters in the resulting musts. Probable alcohol, pH, titratable acidity, and potassium were analysed according to ECC official methods.[11] A spectrophotometric method was used for the determination of tartaric acid based on a complex-formation reaction with vanadate, following the Rebelein method.[12] Malic and gluconic acids were determined by an enzymatic method carried out with an automated clinical chemistry analyser (Miura One, TDI, Spain).

From each replicate, about 150 berries were collected and frozen at −20 °C to determine grape anthocyanin composition. The extraction was carried out according to Portu *et al.*[13] For a start, 50 g of each frozen grape sample was immersed into 50 mL of a methanol/water solution (mL mL^{-1}) and the pH adjusted to 2 with formic acid. The grapes were homogenized at high speed to produce a smooth paste. Then, samples were macerated in an ultrasonic bath for 10 min and were centrifuged at $5031 \times g$ at 10 °C for 10 min. Two more extractions of the resulting pellets were completed with the same volume of the solvent mixture. The final sample was frozen at −20 °C until high-performance liquid chromatography analysis. For anthocyanins analysis, 10 µL of the sample were injected into an Agilent 1260 Infinity chromatograph, equipped with a diode array detector. Separation was performed on a LiChrospher 100 RP-18 reversed-phase column (250 mm × 4.0 mm; 5 µm packing; Agilent) with precolumn LiChrospher 100 RP-18 (4 mm × 4 mm; 5 µm packing; Agilent), both at 40 °C. A flow rate of 0.63 mL min^{-1} was established. Eluents used were (A) acetonitrile/water/formic acid (3: 88.5: 8.5 v/v/v), and (B) acetonitrile/water/formic acid (50: 41.5: 8.5, v/v/v). The linear solvent gradient was as follows: 0 min, 6% B; 15 min, 30% B; 30 min, 50% B; 35 min, 60% B; 38 min, 60% B; 46 min, 6% B.[13]

Winemaking, management of fermentations and wine quality

When the grapes reached an average probable alcohol by volume (ABV) of approximately 13%, each treatment and replicate was individually harvested and vinified. The vinifications were carried out on a small scale in the experimental winery of the ICVV, in 100 L stainless steel tanks with stemmed and crushed grapes. Potassium metabisulfite was added to the samples to give a total sulfur dioxide (SO_2) concentration of 50 mg L^{-1}. These tanks were kept at a controlled temperature (25 °C) to carry out the spontaneous AF; that is, without addition of a commercial yeast starter culture. The fermentation happened with grape skins, and homogenization of

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Table 2. Average general oenological parameters and grape anthocyanins content of samples ($n = 4$) from control grapevines and from grapevines treated with a chemical fungicide and with a biofungicide at harvest moment, with their standard deviations and the statistical assessment

Oenological parameter	Control	Chemical fungicide	Biofungicide
General			
Weight of 100 grapes (g)	231 ± 11	239 ± 15	250 ± 16
Sugar (g L^{-1})	228 ± 7^{b}	217 ± 4^{a}	218 ± 3^{ab}
pH	3.38 ± 0.04	3.33 ± 0.03	3.37 ± 0.01
Total acidity (g L^{-1} tartaric acid)	6.45 ± 0.20	6.41 ± 0.30	6.40 ± 0.29
Tartaric acid (g L^{-1})	6.64 ± 0.21	6.44 ± 0.09	6.58 ± 0.21
Malic acid (g L^{-1})	3.53 ± 0.28	3.42 ± 0.34	3.52 ± 0.29
Gluconic acid (g L^{-1})	ND	ND	ND
Potassium (mg L^{-1})	1703 ± 196	1556 ± 85	1592 ± 147
Anthocyanins			
Delphinidin-3-glucoside (mg kg^{-1})	302 ± 52	292 ± 39	292 ± 22
Cyanidin-3-glucoside (mg kg^{-1})	51.1 ± 9.5	49.2 ± 15.3	50.6 ± 5.7
Petunidine-3-glucoside (mg kg^{-1})	209 ± 33	198 ± 22	199 ± 14
Peonidin-3-glucoside (mg kg^{-1})	95.4 ± 15.6	85.8 ± 21.9	93.2 ± 11.7
Malvidin-3-glucoside (mg kg^{-1})	500 ± 47	470 ± 36	489 ± 33.6
Delphinidin-3-acetylglucoside (mg kg^{-1})	22.0 ± 2.31	19.6 ± 1.3	20.0 ± 1.15
Cyanidin-3-acetylglucoside (mg kg^{-1})	3.89 ± 0.22	3.57 ± 0.30	3.69 ± 0.14
Petunidine-3-acetylglucoside (mg kg^{-1})	12.0 ± 0.9^{b}	10.7 ± 0.44^{a}	11.0 ± 0.48^{ab}
Peonidin-3-acetylglucoside (mg kg^{-1})	4.45 ± 0.25^{b}	3.85 ± 0.28^{a}	4.13 ± 0.18^{ab}
Malvidin-3-acetylglucoside (mg kg^{-1})	27.0 ± 1.0^{b}	23.2 ± 1.5^{a}	25.3 ± 1.4^{ab}
Delphinidin-3-coumarilglucoside (mg kg^{-1})	47.9 ± 4.1	44.8 ± 2.7	46.5 ± 2.5
Cyanidin-3-coumarilglucoside (mg kg^{-1})	11.4 ± 1.3	10.4 ± 1.7	10.9 ± 0.60
Petunidine-3-coumarilglucoside (mg kg^{-1})	37.2 ± 2.2	34.5 ± 2.5	35.7 ± 2.2
Peonidin-3-coumarilglucoside (mg kg^{-1})	23.3 ± 1.6	20.4 ± 1.7	22.2 ± 2.9
Malvidin-3-*cis*-coumarilglucoside (mg kg^{-1})	5.76 ± 0.40	5.81 ± 0.46	5.83 ± 0.39
Malvidin-3-*trans*-coumarilglucoside (mg kg^{-1})	137 ± 4	130 ± 15	138 ± 9
Malvidin-3-capheoilglucoside (mg kg^{-1})	3.59 ± 0.18	3.20 ± 0.26	3.39 ± 0.25
Total anthocyanins (mg kg^{-1})	1493 ± 162	1405 ± 153	1450 ± 96

Different superscript letters mean significant differences ($P < 0.10$) between samples.
ND, not detected.

skins and must was performed by punching down the cap 20 times once per day.

Alcoholic fermentation kinetics were monitored by determination of the density (g L^{-1}) decrease, as a measure of the consumption of sugars (g L^{-1}). Samples were also taken from the must to analyse their viable yeast population by plating the appropriate dilution on CGA plates, incubated at 28 °C for 48 h.[14] Then, CFU was counted daily and the population was expressed as logarithmic units per millilitre. At the time of tumultuous AF (density 1025 g L^{-1}), the clonal distribution of the *S. cerevisiae* population that performed AF was evaluated. For this purpose, ten colonies were randomly isolated from CGA plates inoculated with each replicate.[14] A restriction analysis was performed with the mitochondrial DNA following the protocol described by Pulvirenti and Giudici.[15] Mitochondrial DNA was extracted by enzymatic breakdown. The 24-h cultures were washed with sterile water and centrifuged, eliminating supernatant. Cells were incubated for 1 h with a zymolyase solution (10 mg mL^{-1}). The DNA obtained was vacuum dried and dissolved in TE (10 mmol L^{-1} Tris–HCl, 1 mmol L^{-1} EDTA). Then, samples were digested with the restriction enzyme AluI, incubating the tubes at 37 °C overnight. In order to visualize restriction patterns, electrophoresis of digested DNA was performed in a gel of 1 g of agarose in 100 mL of 0.5 × TBE buffer (45 mmol L^{-1} Tris base, 89 mmol L^{-1} boric acid, 2.5 mmol L^{-1} EDTA, pH 8.3) gel for 3.5 h at 100 V. The agarose gels stained with ethidium bromide provided the electrophoretic profiles of each isolate. These electrophoretic profiles were compared to determine the different genotypes. The conversion, normalization and further processing of profiles were carried out using InfoQuest™ FP software version 5.10; Bio-Rad.

When the wines had reached a density about 990 g L^{-1} they were pressed and fermented to dryness. The AF was completed when reducing sugars were lower than 2 g L^{-1}. Then, wines were characterized by measuring the alcoholic strength (ABV), pH, total acidity, volatile acidity, colour intensity and hue according to official ECC methods.[11] The malic, lactic and gluconic acids were determined by an enzymatic method carried out by an automated clinical chemistry analyser (Miura One, TDI, Spain) and tartaric acid by the Rebelein method.[12] Total anthocyanins were measured by decolouring using SO_2.[16] Ionized anthocyanins were determined according to Glories,[17] and the polymerization index was calculated according to Ruiz.[18] Total phenolics were determined as the total polyphenol index by spectrophotometric absorbance at 280 nm after dilution of samples. The analysis of fermentative volatile compounds in the wine was performed using the method described by Ortega *et al.*[18] with the following modifications. The extraction was carried out by mixing 4 mL of sample, 9 mL of ammonium sulfate saturated solution, 40 µL of internal standard solution (2-butanol, 4-methyl-2-pentanol, 4-hydroxy-4-methyl-2-pentanone, 2-octanol, and heptanoic acid,

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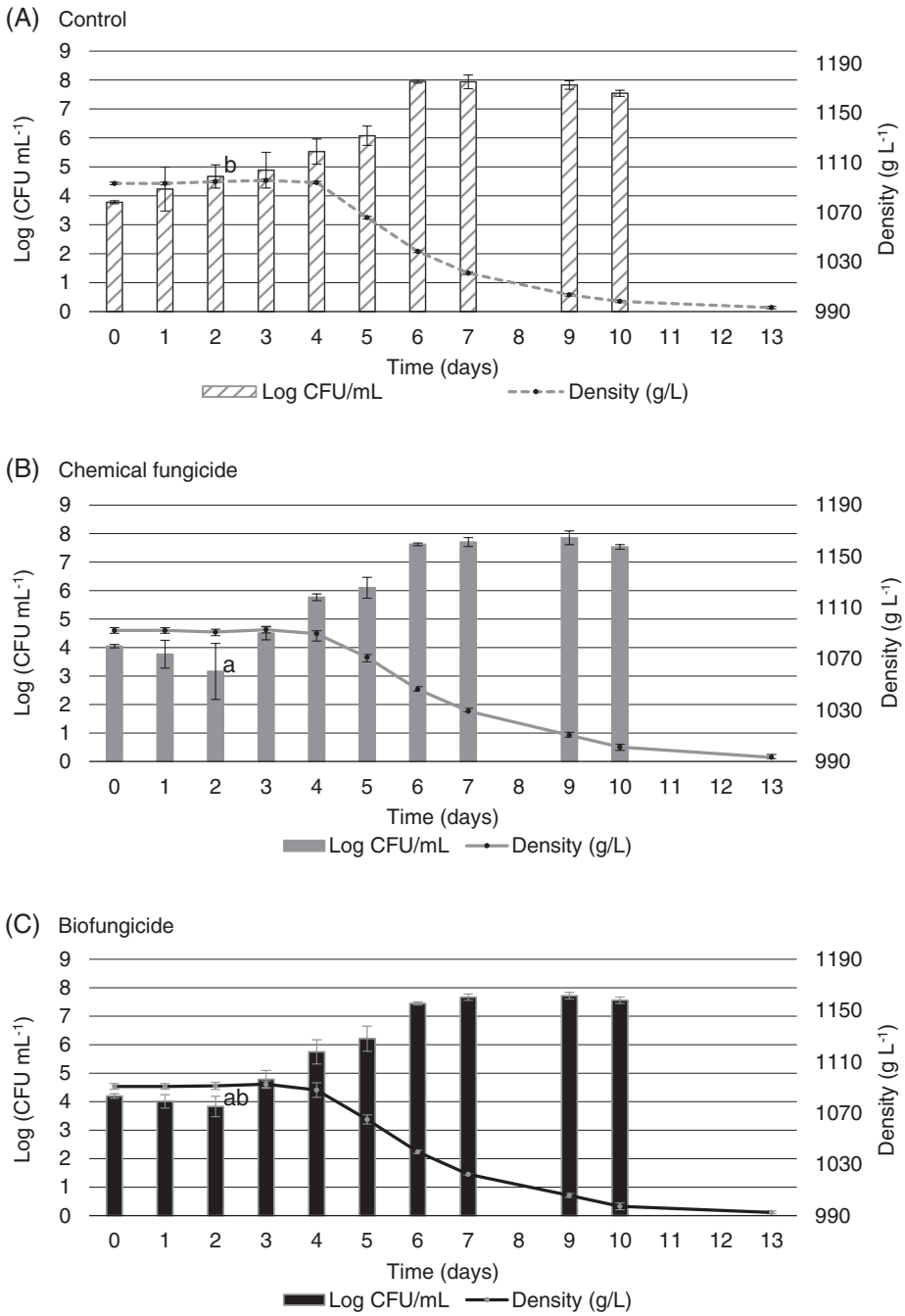

Figure 1. Evolution of average yeast population (log (CFU mL^{-1})) and of density (g L^{-1}) during the AF of the different wine samples ($n = 4$): (A) control; (B) chemical fungicide; (C) biofungicide and the statistical assessment. Error bars represented the standard deviation of both parameters for each sample. Different letters mean significant differences ($P < 0.05$) between samples. No letters mean no significant differences.

40 mg of each of them in 100 mL of ethanol) and 300 µL of dichloromethane in tubes. The tubes were shaken for 1 h at 32 × *g* and then centrifuged at 3220 × *g* for 10 min. Once the phases were separated, the dichloromethane phase was recovered. A 2 µL sample of this extract was injected in split mode onto a Hewlett-Packard (Palo Alto, CA) 6890 series II gas chromatograph equipped with an automatic injector and a Hewlett-Packard flame ionization detector. Separation was carried out with a DB-Wax capillary column (60 m × 0.32 mm i.d. × 0.5 µm film thickness; J&W Scientific, Folsom, CA, USA). The temperature program was as follows: 5 min, 40 °C; then raised up to 220 °C at a rate of 3 °C min^{-1}. The carrier gas was nitrogen at a flow rate of 3 mL min^{-1}. The injector temperature was 220 °C and the detector temperature was 280 °C.

When AF was complete, the wines were drawn off the lees and transferred to 50 L containers that were inoculated with the commercial LAB *Uvaferm alpha*® (Lallemand S.L.) to carry out the MLF, at a temperature of 20 °C. The evolution of the fermentation was controlled by periodic determination of the malic acid content (g L^{-1}). Furthermore, when malic acid had been consumed to around 60–80% of its initial amount (tumultuous MLF), the implantation control of the LAB was performed. For that purpose, the modified MRS culture media[20] was employed, and an

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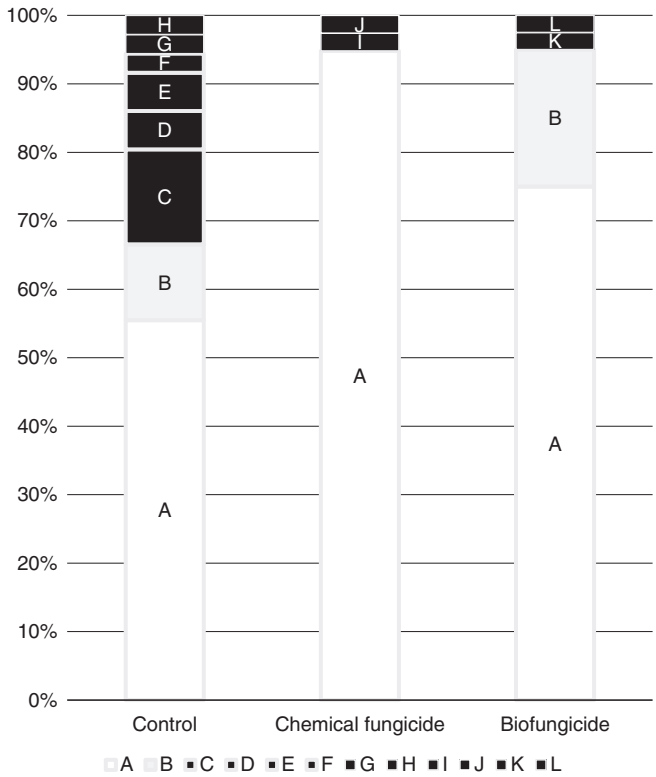

Figure 2. *Saccharomyces cerevisiae* genotypes distribution (referred to by letters A–L) of wines at tumultuous AF (density 1025 g L^{-1}) from control grapevines and from grapevines treated with a chemical fungicide and with a biofungicide ($n = 4$).

adequate dilution of samples was plated and incubated for 10 days at 30 °C in anaerobic conditions; then, five colonies were randomly isolated from plates of each replicate and were typed. The typing method was random amplified polymorphic DNA polymerase chain reaction (PCR) with the PCR specifications described by González-Arenzana *et al.*[20] After PCR, 20 mL of amplicons was run in an electrophoretic gel containing 1.4 g of agarose in 100 mL of 0.5 × TBE buffer for 2 h to 70 V. The agarose gels stained with ethidium bromide provided the electrophoretic profiles of each isolate that were processed as described earlier.

One month after MLF ended, the wines were again analysed in terms of oenological parameters, including most of the parameters described earllier for the AF.

Statistical treatment

The statistical treatment of the data obtained was done with the program SPSS for Windows, version 20.0. Analysis of variance was applied to the results to verify if there were significant differences depending on the treatments applied. In the positive case, $P \leq 0.10$, the Tukey test for the separation of means was used.

RESULTS AND DISCUSSION

This study was performed with the aim of analysing how the preventive biological control of *B. cinerea* grapevine disease would influence grape and wine quality and the winemaking process in general. For that purpose, the results obtained after spraying with a biofungicide (Serenade®) were compared with the results from vines treated with a commonly used chemical fungicide (Teldor®) and with untreated vines. In this study, no plant pathogen was detected in the vineyard (data not shown).

Table 3. Average oenological parameters after the AF of wines ($n = 4$) from control grapevines and from grapevines treated with a chemical fungicide and with a biofungicide, with their standard deviations and the statistical assessment

Oenological parameter	Control	Chemical fungicide	Biofungicide
ABV	13.1 ± 0.4	12.7 ± 0.1	12.4 ± 0.6
pH	3.55 ± 0.06^{b}	3.47 ± 0.04^{a}	3.49 ± 0.04^{ab}
Volatile acidity (g L^{-1} acetic acid)	0.28 ± 0.06	0.22 ± 0.02	0.22 ± 0.03
Total acidity (g L^{-1} tartaric acid)	8.29 ± 0.56	8.37 ± 0.18	8.20 ± 0.28
Tartaric acid (g L^{-1})	2.82 ± 0.22^{a}	3.45 ± 0.22^{b}	3.18 ± 0.10^{b}
Malic acid (g L^{-1})	3.36 ± 0.17	3.41 ± 0.44	3.52 ± 0.26
Lactic acid (g L^{-1})	0.12 ± 0.02	0.12 ± 0.02	0.12 ± 0.01
Gluconic acid (g L^{-1})	0.06 ± 0.03	0.09 ± 0.03	0.05 ± 0.03
Colour intensity	14.7 ± 0.4	14.2 ± 1.2	12.9 ± 1.5
Hue	0.40 ± 0.00^{b}	0.37 ± 0.00^{a}	0.40 ± 0.00^{b}
Total anthocyanins (mg L^{-1})	867 ± 44	828 ± 45	765 ± 96
Ionized anthocyanins (%)	15.4 ± 0.6	16.4 ± 1.1	15.7 ± 0.6
Polymerization index	1.06 ± 0.10	1.01 ± 0.20	0.95 ± 0.12
Total polyphenol index	50.8 ± 3.4	49.1 ± 3.4	46.4 ± 4.9

Different superscript letters mean significant differences ($P < 0.10$) between samples.

Initially, at the time of harvest, in which a total of 125 kg of grapes were harvested, the yield components were assessed for each one of the treatments. Table 1 shows that both fungicide treatments increased the unit production around 25% when compared with the control treatment, although the differences were not statistically significant. In addition, the average cluster weight was significantly higher (around 15%) in grapes from the biofungicide treatment when compared with the control. However, no significant differences were observed between the chemical fungicide and the other treatments. Similar results had been described in other crops affected by other fungal diseases. For instance, Cordova *et al.*[21] described a significantly higher yield in strawberry crops after the application of chemical fungicides. Similarly, Martínez[22] reported an increase in the yield of rice from 5 to 10%. Furthermore, Tedford *et al.*[23] determined that the yield increase in corn crops was enough to mitigate the investment in fungicides. In contrast, Barickman *et al.*[24] reported an increase in the yield of a pumpkin crop treated against powdery mildew with a chemical fungicide, but this increase was not observed when biofungicides were applied. As far as our study is concerned, this was the first time that a significant enhancement of the yield of healthy grapevines after being treated with chemical and biological fungicides has been reported.

The maturation control carried out with grapes to determine the optimum time of harvest indicated that weight, pH and probable alcoholic degree followed the usual pattern during the ripening process, increasing until the time of harvest (data not shown). Therefore, the agronomic evolution of the grapevine was not affected by the application of either the biofungicide or the chemical fungicide.

Results corresponding to the composition of the grape at the time of harvest are shown in Table 2. Control grapes presented

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Table 4. Average volatile compounds (mg L^{-1}) after the AF of wines ($n = 4$) from control grapevines and from grapevines treated with a chemical fungicide and with a biofungicide with their standard deviations and the statistical assessment

Aroma	Control	Chemical fungicide	Biofungicide
Alcohols			
Propanol-1	25.8 ± 4.8	24.1 ± 4.3	28.9 ± 6.6
1-Butanol	0.77 ± 0.01	0.78 ± 0.11	0.77 ± 0.12
Isobutanol	50.3 ± 6.8	45.9 ± 1.4	46.6 ± 5.0
2,3-Methyl-1-butanol	335 ± 29	331 ± 23	321 ± 35
2-Phenylethanol	53.9 ± 13.4	52.4 ± 8.6	46.1 ± 11.1
1-Hexanol	2.96 ± 0.18^{b}	2.71 ± 0.12^{a}	2.76 ± 0.07^{ab}
Methionol	2.16 ± 0.23	2.20 ± 0.23	1.82 ± 0.43
cis-3-Ethanol	0.48 ± 0.04	0.43 ± 0.03	0.46 ± 0.03
Acetates			
Isoamyl acetate	1.73 ± 0.41	1.91 ± 0.40	2.03 ± 0.39
Hexyl acetate	0.03 ± 0.01	0.02 ± 0.01	0.02 ± 0.01
2-Phenylacetate	0.17 ± 0.03	0.17 ± 0.01	0.16 ± 0.02
Esters			
Ethyl propanoate	0.09 ± 0.03^{a}	0.14 ± 0.02^{b}	0.13 ± 0.01^{b}
Ethyl-3-hydroxybutyrate	0.89 ± 0.05	0.81 ± 0.09	0.85 ± 0.05
Ethyl isobutyrate	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
Ethyl butyrate	0.28 ± 0.05	0.29 ± 0.07	0.32 ± 0.05
Ethyl hexanoate	0.46 ± 0.08	0.37 ± 0.05	0.41 ± 0.05
Ethyl octanoate	0.32 ± 0.10	0.29 ± 0.03	0.29 ± 0.05
Fatty acids			
Propanoic acid	0.59 ± 0.41	ND	ND
Isobutyric acid	4.15 ± 0.36	3.65 ± 0.87	3.87 ± 0.44
Butyric acid	3.07 ± 0.38	2.80 ± 0.24	3.03 ± 0.40
Isovaleric acid	3.42 ± 0.81	3.07 ± 0.51	3.25 ± 0.25
Hexanoic acid	2.54 ± 0.56	2.05 ± 0.27	2.14 ± 0.14
Octanoic acid	1.38 ± 0.38	1.15 ± 0.05	1.24 ± 0.03
Others			
Ethyl acetate	31.0 ± 6.5	40.4 ± 13.2	38.2 ± 5.8
Ethyl lactate	1.18 ± 0.37	1.55 ± 0.19	1.42 ± 0.23
Diethyl succinate	0.14 ± 0.05	0.12 ± 0.01	0.14 ± 0.08
Acetoin	1.50 ± 0.52^{ab}	0.93 ± 0.36^{a}	2.23 ± 0.95^{b}
Diacetyl	1.10 ± 0.28	1.24 ± 0.14	1.35 ± 0.14
Furfural	ND	ND	ND
Butyrolactone	2.07 ± 0.11	2.12 ± 0.20	2.11 ± 0.07

Different superscript letters mean significant differences ($P < 0.10$) between samples.
ND, not detected.

standard and balanced oenological parameters for Tempranillo grapes from D.O.Ca. Rioja.[25] Grapes treated with both fungicides did not show significant differences compared with the control in the average weight of 100 grapes, pH, total acidity, tartaric, malic and gluconic acids, and potassium concentration. Only the average sugar content in grapes from chemical fungicide was reduced significantly around 4% compared with the control. In contrast, no differences were established between the control sample and the sample with the biofungicide. Fenhexamid acts at the amino acid and protein synthesis level,[26] but its possible effect on sugar content decrease was not reported in a similar study carried out by Mulero *et al.*[27] Nevertheless, the biofungicide application did not produce any effect on the general grape composition.

Moreover, Table 2 shows average grape anthocyanins content of control grapes and of those from the treatments with both fungicides. As was expected, among the compounds that are responsible for the red colour in wine, malvidin-3-glucoside was the most abundant. Moreover, malvidin derivatives were the most abundant anthocyanins in the grape control, as was also shown in previous studies.[28] The application of both fungicides did not influence the content of these compounds because significant differences were not detected in the treated grapes compared with the control sample. Similar to previous studies,[28] acylated anthocyanins in the control grapes were approximately 30% of total anthocyanins. To be precise, chemical fungicide significantly decreased petunidine-3-acetylglucoside, peonidin-3-acetylglucoside and malvidin-3-acetylglucoside around 11%, 13% and 14% respectively compared with the control. However, no significant differences were observed between the chemical fungicide and biofungicide. Mulero *et al.*[27] observed a decrease in the concentration of some of the anthocyanins mentioned earlier linked to chemical fungicide application. No treatment significantly affected the total content of anthocyanins.

Few studies have been focused on the effects of fungicide application on spontaneous AF. For this purpose, the kinetics of spontaneous AF were studied with the daily density determination and viable yeast counts. As can be seen in Fig. 1, the fermentation rate was not modified by treatment with the biofungicide, being slightly slower in wines whose grapes were treated with the chemical fungicide. However, the densities began decreasing 4 days after the harvest, and their durations were for all cases 13 days. In some studies, the application of some fungicides resulted in residues that could be present during winemaking being toxic for some yeasts. Some cases in point are copper hydroxyl, sulfur, mancozeb or folpet.[29] In other cases, it has been reported that some yeasts adsorb fungicide residues, which make its toxicity decrease during winemaking.[30] Researchers such as Bizaj *et al.*[31] reported slow AF after the treatment of grapes with the fenhexamid fungicide; in contrast, others reported similar fermentation kinetics when this product was employed compared with untreated grapes.[5] Therefore, there are contradictory results regarding the influence of this chemical fungicide in the AF process. With regard to the counting of viable yeasts, except 2 days after grape incubation, there were no differences between the treatments, which shows that neither of the fungicide products was detrimental to the spontaneous development of these microorganisms responsible for the transformation of sugars from must into ethanol.

The analysis of *S. cerevisiae* genotypes that led AF (sampling in tumultuous AF, density 1025 g L^{-1}) was performed by mitochondrial DNA analysis and showed the existence of 12 distinct genotypes. The clonal distribution in each of the treatments is shown in Fig. 2. A greater clonal diversity of yeasts was observed in the control treatments (eight different clones), compared with four in the fermentations with grapes treated with the biofungicide and three in the treatment of the chemical fungicide. Consequently, the clonal diversity of the *S. cerevisiae* species in spontaneous AF decreased considerably compared with the control after the employment of the two fungicides, especially in the case of the chemical fungicide application. Other researchers have reported that the more specific fungicides applied in big doses, the lower the clonal diversity of *S. cerevisiae*,[32,33] which could be in line with these results. Generally, the impact of agriculture practices causing a decrease in diversity could be considered as

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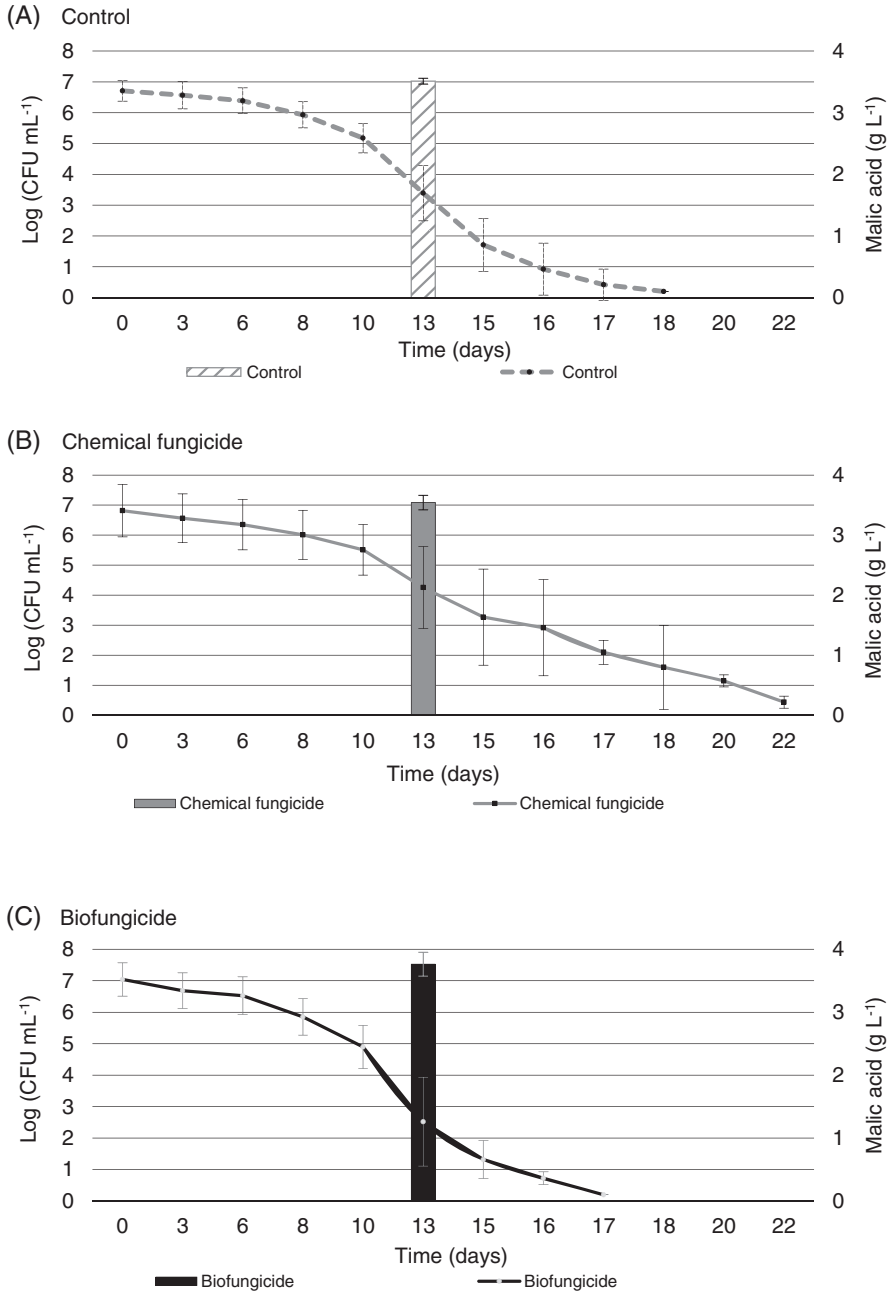

Figure 3. Average LAB population (log(CFU mL^{-1})) at tumultuous MLF (60–80% initial malic acid) and malic acid (g L^{-1}) thorough MLF of the different wine samples ($n = 4$): (A) control; (B) chemical fungicide; (C) biofungicide and the statistical assessment. Error bars represented the standard deviation of both parameters for each sample. No letters mean no significant differences.

negative.[34,35] Nonetheless, in this case, it could be positive for the starter culture establishment rate if the genotypes naturally present in wines were less adapted to the winemaking conditions. The majority clone in all the elaborations was genotype A, which represented 55.6% of the clones isolated in the fermentations of the control reservoirs, 75% in those of biofungicide and 94.9% in those corresponding to the treatment with chemical fungicide. Only one other strain (genotype B) was common in vinification of the control grapes and those treated with the biofungicide, with 11.1% and 20% respectively. The rest of the genotypes isolated were different for each of the trials. Therefore, the clonal distribution of *S. cerevisiae* in the biofungicide case was more similar to the control than to the chemical fungicide, so that a less invasive effect of the biofungicide over the *S. cerevisiae* genotypes could be inferred.

After AF, wines from the three treatments presented values of oenological parameters within the limits observed for red wines by the regulatory board of the designation[25] (Table 3). Only significant differences in pH were found because of the different concentrations of tartaric acid, which in principle could not be attributed to the treatments. The hue of wine from chemical fungicide treatment was about 8% lower than the other treatment samples. This parameter is increased in wines during ageing, but at this stage and before MLF it is not very important at this extent.[36] Although without statistical significance, it was observed that results concerning colour (colour intensity, total anthocyanins,

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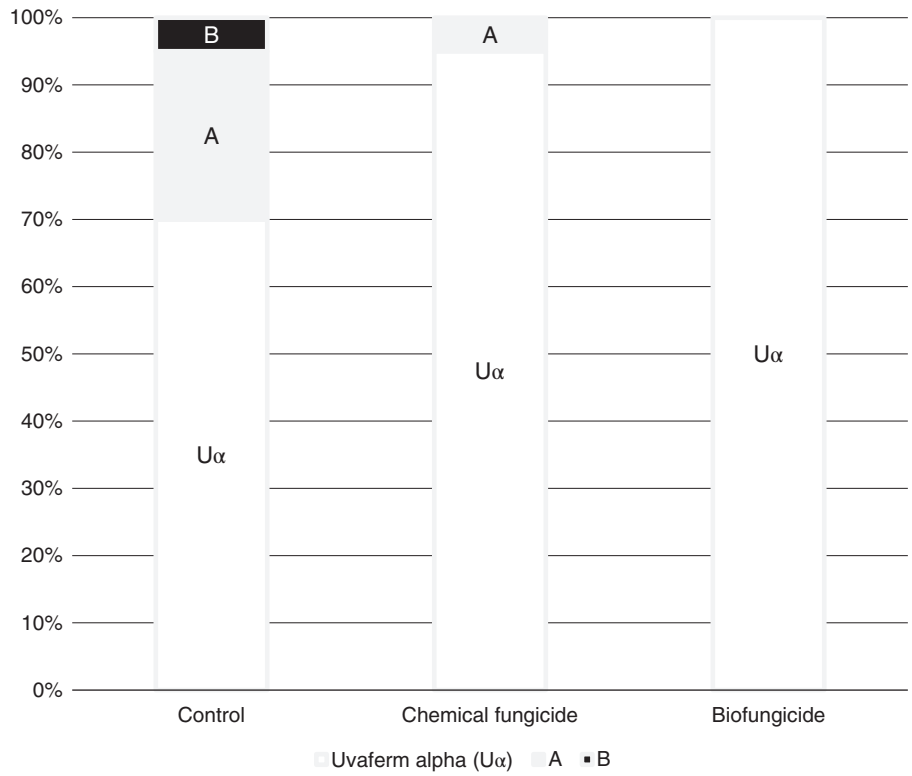

Figure 4. *Oenococcus oeni* genotypes distribution (referred as to letters Uα, A and B) of wines at each inoculated MLF (70% initial malic acid) from control grapevines and from grapevines treated with a chemical fungicide and with a biofungicide ($n = 4$).

ionized anthocyanins, polymerization index and total polyphenol index) of the control wine and wine from the chemical fungicide group were more similar than in the case of the biofungicide, which showed slightly lower values.

Thirty volatile compounds were quantified in wines (Table 4). These volatile compounds play an important role in the final quality of wine. Differences found in acetates as a consequence of treatments were reduced to 1-hexanol. The chemical fungicide significantly decreased it by around 8% compared with the control, and biofungicide reduced it by 7%, although without significance. As it is the acetates that confer herbaceous odours, fungicide treatments proved positive for wine quality. Higher alcohol acetates and esters impart fruity and floral aroma to wine,[37] and both fungicides increased ethyl propionate that confers banana and apple aroma.[38] With respect to fatty acids, compounds that can provide cheesy unfavourable aromas, no significant differences were noted between the control and treated wines. In the rest of the volatile compounds, the only significant differences found were for acetoin, with a lower content in wines that had undergone chemical fungicide treatment. This result does not have any influence on wine quality because of the high thresholds of perception.[38] Conclusively, neither fungicide caused any aromatic deviation in wines after AF. Noguerol-Pato *et al.* [39] determined that wines from grapes treated with fenhexamid had similar volatile composition to that of the control wines, which matched the current results, but no references have been found regarding the biofungicide.

The evolution of malic acid content during MLF and LAB population in tumultuous MLF is shown in Fig. 3. This shows that MLF was 4 days slower in wines whose grapes had been treated with chemical fungicide than in the control, though no significant differences were found in LAB population of samples. However, the one carried out with grapes treated with the biofungicide was 1 day faster.

Fig. 4 shows the results of implantation in tumultuous MLF. The percentage of implantation of inoculated bacteria (*Uvaferm alpha*®) was 70% in control wine, 95% in those from grapes treated with chemical fungicide and 100% in those from biofungicide. Likewise, as observed in AF, the clonal diversity of *O. oeni* was higher in the control samples, where two different genotypes were obtained as well as the commercial strain. One of the control genotypes was also isolated in fermentations from chemical fungicide. The employment of a biofungicide could have improved the establishment of the *O. oeni* starter culture and even the kinetics of the MLF. Literature reporting the impact of those chemical and biological fungicides on genotypes of *O. oeni* has not been published.

The oenological parameters of wines after MLF are shown in Table 5. Overall, significant differences were reduced to tartaric acid. The tartaric acid content was lower in wines from biofungicide-treated grapes. As the exposure to wine is after the AF, the tartaric acid content would not have been related to the fungicide treatments. After MLF, it seems that, in terms of parameters related to colour – such as colour intensity, total anthocyanins, ionized anthocyanins, polymerization index and total polyphenol index – the control wine showed slightly better values than wines from fungicide treatments. For example, colour intensity in wines from the fungicide treatment was around 9% lower than in the control. According to Briz-Cid *et al.*,[40] wine colour after the AF of grapes treated with fenhexamid was around 14% lower than the control, which was similar to the findings of this study. Nevertheless, it has been recently observed that differences in phenolic content and wine colour due to fungicides were irrelevant in comparison with the vintage effect.[41] Therefore, further research is necessary to study the influence of biofungicide over time.

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Table 5. Average oenological parameters after the MLF of wines ($n = 4$) from control grapevines and from grapevines treated with a chemical fungicide and with a biofungicide with their standard deviations and the statistical assessment

Oenological parameter	Control	Chemical fungicide	Biofungicide
ABV	13.1 ± 0.4	12.7 ± 0.1	12.4 ± 0.6
pH	3.76 ± 0.09	3.66 ± 0.05	3.67 ± 0.05
Volatile acidity (g L^{-1} acetic acid)	0.34 ± 0.04	0.31 ± 0.03	0.36 ± 0.05
Total acidity (g L^{-1} tartaric acid)	5.33 ± 0.23	5.47 ± 0.19	5.22 ± 0.17
Tartaric acid (g L^{-1})	1.81 ± 0.09^{ab}	1.91 ± 0.05^{b}	1.61 ± 0.11^{a}
Malic acid (g L^{-1})	ND	0.13 ± 0.11	0.03 ± 0.05
Lactic acid (g L^{-1})	2.53 ± 0.17	2.50 ± 0.28	2.63 ± 0.19
Colour intensity	8.38 ± 0.72	7.62 ± 0.96	7.01 ± 1.07
Total anthocyanins (mg L^{-1})	679 ± 42	611 ± 42	601 ± 58
Ionized anthocyanins (%)	16.8 ± 2.7	17.0 ± 2.0	14.6 ± 2.0
Polymerization index	1.30 ± 0.08	1.13 ± 0.14	1.10 ± 0.13
Total polyphenol index	47.8 ± 3.4	45.5 ± 2.7	45.2 ± 4.6

Different superscript letters mean significant differences ($P < 0.10$) between samples.
ND, not detected/.

CONCLUSION

Both fungicides caused an increase in the grape production in the vineyard, but did not alter either the ripening process or the AF. The chemical fungicide could be involved in a decrease of some anthocyanins in grapes and in the decrease of the genotypes of *S. cerevisiae* isolated during spontaneous AF. In the case of the biofungicide, it enhanced the establishment of the starter culture employed for performing the MLF. To sum up, the treatment of the grapes with biological fungicide did not negatively influence the quality of the grape or wine and was positive in the microbiological aspect, since it did not affect fermentative kinetics and allowed a better implantation of the bacteria inoculated to induce MLF.

ACKNOWLEDGEMENTS

The Government of La Rioja supported this work under the grant of Escribano-Viana R. BOR no. 34, 13 March 2015. We also thank the Bayer Crop Bioscience S.L. for funding support and for the application of their fungicides on the grapevines.

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