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Research Article

Effect of foliar treatment with chitosan on phenolic composition of 'Fetească neagră' grapes and wines

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Abstract

The objective of this study was to determine the influence of chitosan treatments, applied in vineyard for plan protection, on the phenolic quality of grapes. The study was conducted for two consecutive years (2020-2021) in the wine center of Murfatlar, Romania, on 'Fetească neagră', an ancient but well-known indigenous Romanian grape variety for red wines. Chitosan is meant to offer an alternative for the classic treatment with Bordeaux mixture (BM), which represented the control variant. A combination treatment with half dose of chitosan and half dose of BM was also applied and evaluated. Chemical composition at harvest time was evaluated by measuring the content of sugar, total acidity, pH, total anthocyanin potential (A_{pH1}), anthocyanins extractable at wine pH ($A_{pH3.2}$), total polyphenol index, skin tannins, seed tannins and seeds maturity. In wines the colour parameters were determined by spectrophotometry and individual phenolic compounds by UHPLC-HRMS. In grapes, accumulation of anthocyanins increased with the total dose of chitosan applied in the vineyard, with higher values in 2020 when temperatures were higher and the rainfall values were typical for the region. Accumulation of tannins in grape skins followed a similar trend. In wine, chitosan determined a significant 72% increase in colour intensity in 2020. Among the individual phenols gallic acid was predominant, with higher values in the rainier year (2021) and significant increases determined by chitosan treatment (especially in 2021 when it increased by 97% as compared to BM treatment). Catechin and epicatechin recorded important increases in the less favourable year (2021), with confirmed increases in both years elicited by the chitosan. Quercetin and myricetin were not influenced by the chitosan treatment, but their increase was corelated with higher temperatures and inversely corelated to the amount of rainfall. Trans-resveratrol ranged between 4.3-8.0 mg L^{-1} in 2020 and 5.0-6.5 mg L^{-1} in 2021, with an important increase determined by the chitosan treatment in 2020 (89% increase compared to BM treatment).

Keywords: catechin; chitosan; epicatechin; 'Fetească neagră' cv.; gallic acid; myricetin; phenolic profile; trans-resveratrol; quercetin

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Introduction

In order to fight climate change and environmental degradation, recently, the European Commission presented a set of policy initiatives under the name of 'European Green Deal', aiming at reforming several European economic activities to become green by the year 2050 (European Commission: Brussels, 2019). For agriculture, this means working with nature in order to protect the planet and human health. Among the actions of the 'European Green Deal' the strategy called 'From Farm to Fork' (European Commission, A Farm to Fork Strategy for a fair, healthy and environmentally-friendly food system, 2020) is meant to promote a healthy and environmentally-friendly food system under fair economic conditions for all. This strategy highlights the need for the sustainability of the food production chain, by reducing the use of agrochemical compounds (fertilizers and pesticides) and, at the same time, ensuring the production of healthy, high-quality, affordable food for consumers.

In this trend, in the studies regarding the sustainable crop systems there is an increasing use of elicitors, biostimulants and plant hormones with multiple modes of action, for example, molecules that act against pests/pathogens, compounds that increase the efficiency of resources usage (such as nutrients and water) and/or substances that improve the quality characteristics of the food product. Exogenous applications of elicitors that stimulate plant defence mechanisms were so far especially studied as alternatives to conventional pathogen management (Meena *et al*., 2020) However, there is a growing number of researchers investigating the effects of elicitors on the quality characteristics of raw materials and derivate products obtained, such as grapes for wine, especially in view of improving the biosynthesis of phenolic compounds in grapes and implicitly raising the phenolic quality of wines (Niimi *et al*., 2020). Although generally it is considered that elicitors are plant- or microorganism-derived molecules able to cause an innate immune response in plant (Yang *et al*. 2021), in a broader way they can be considered as promoters of the synthesis of secondary metabolites in plants. This later case is valid for chitosan, which induces accumulation of anthocyanins in grape berries and modulates other phenolic compounds (Singh *et al*., 2020) and also enhances the antioxidant potential of the grape (Singh *et al*., 2019). Aside of their role in plant defence mechanisms (Stiller *et al*., 2021), polyphenols are also nutrients with health benefits for humans (Antoce and Stockley, 2019). Thus, this type of action promoted by chitosan can also be viewed as beneficial for the quality of the food products (grapes or wine).

Chitosan is a polymer containing units of β-1,4-D-glucosamine and units of N-acetyl-D-glucosamine, being produced by alkaline deacetylation of chitin (a polymer of N-acetyl-D-glucosamine only), which can be extracted from fungal cell walls, insects and the exoskeleton of shell fish (Arnaz *et al*., 2021). Due to its multiple cationic charges, it is able to form a semipermeable film around plant tissues, thus helping against abiotic stress (Singh *et al*., 2022) and also to inhibit several pathogens and induce defensive mechanisms in host tissues (Romanazzi *et al*., 2017). Treatments with chitosan were applied in various fruit and vegetable crops, not only for inducing defence mechanisms, but also in view of improving plant growth and physiological activities, including the use as a bio-fertilizer, thus improving product traits and facilitating their post-harvest preservation (Sharif *et al*., 2018). However, the main action for which chitosan is used in horticulture is for its demonstrated antifungal effects against powdery mildew (Ruano-Rosa *et al*., 2022) and other antimicrobial properties for which it is useful even in medical field (Abd El-Hack *et al*., 2020). According to the review of Abd El-Hack and team (2020) chitosan possesses a wide range of antibacterial activities against several Gram-positive bacteria (such as *Bacillus cereus* and *megaterium, S. aureus, Lactobacillus plantarum, brevis* and *bulgaricus, Listeria monocytogenes)* and Gram-negative bacteria *(*such as *Pseudomonas aeruginosa* and *fluorescens, Vibrio parahaemolyticus* and *cholera, Salmonella typhimurium, E. coli, Enterobacter aerogenes),* antifungal activity against molds and yeasts, such as *Botrytis cinerea, Candida lambica, Fusarium oxysporum, Rhizoctonia solani, Phomopsis asparagi* and so on.

Considering that the antimicrobial effect of chitosan is sufficiently documented and that also our treatments in the vineyard in 2020 and 2021 produced healthy grapes with no significant sign of infection (data unpublished), in this paper we concentrate on the effect of chitosan treatments on the phenolic quality of grapes and wine.

Foliar application of chitosan in vineyards was previously used to induce the accumulation of secondary metabolites of oenological interest, in order to obtain improved quality of grape berries, with enhanced antioxidant potential (Singh *et al.*, 2019) and higher anthocyanins accumulation (Singh *et al.*, 2020). In wines as well, chitosan formulations have proven their capability of enhancing antioxidant potential when compared with penconazole and methyldinocap fungicide treatments applied in the vineyard (Ruano-Rosa *et al.*, 2022).

Polyphenols from several chemical classes, alone or in combinations, have multiple beneficial health, from the most researched and demonstrated protection against cardiovascular disease (Stockley *et al.*, 2022) to diabetes, cognitive function and modulation of microbiota (Antoce and Stockley, 2019). For this reason, interest for naturally increasing the quantities of polyphenols in food products is high. Therefore, the present study focuses on the phenolic composition of 'Fetească neagră' grapes and polyphenol profile of 'Fetească negră' wines obtained from grapes from vineyards treated with chitosan or a combination of chitosan and Bordeaux mixture as compared with the treatments performed only with Bordeaux mixture, for the harvests of 2020 and 2021.

Materials and Methods

Grape cultivation

The experiments were performed in a vineyard belonging to the Research and Development Station Murfatlar cultivated with 'Fetească neagră' variety (*Vitis vinifera* L.), an autochthonous Romanian variety used for the production of quality red wines and present in various Romanian regions (Antoce and Cojocaru, 2018). In Murfatlar, the rootstock used for this variety is *Vitis berlandieri* × *Vitis riparia* Oppenheim 4 selection, clone SO_{4}-4. The vine is trained with semi-high stems and pruning done in bilateral cordons. Planting distances are 1.1 m between trunks and 2.2 m between rows.

Meteorological data were recorded during the vegetation period in both years of experiment, with an iMetos 3.3 meteorological weather station (Pessl Instruments GmbH, Weiz, Austria). The main parameters recorded were the minimum and maximum temperature and the level of rainfall.

Treatment products and application

The products used for treatments were Bordeaux mixture WDG (Cerexagri, Netherlands) with a concentration of active substance of 200 g/kg (20% w/w) metallic copper in the form of neutralized copper sulphate and KitoGreen® chitosan (Kitozyme, Belgium) from a fungal source, non-GMO, approved for use in European Union (kitogreen-agri.com).

Three experimental variants were organized as follows:

- the control variant – only with Bordeaux mixture treatments applied 12 times, dose 5 kg ha^{-1} (60 kg copper ha^{-1} $year^{-1}$);
- the chitosan variant – applied 12 times, dose 5 kg ha^{-1} (0 kg copper ha^{-1} $year^{-1}$; 60 kg chitosan ha^{-1} $year^{-1}$);
- the combination variant - Bordeaux mixture dose 5 kg ha^{-1} and chitosan dose 5 kg ha^{-1} applied 6 times (a total of 30 kg copper ha^{-1} $year^{-1}$ and 30 kg chitosan ha^{-1} $year^{-1}$).

Treatments were applied for each variant on 8 intervals of 7 vines (being equal to one row of vines), in 3 repetitions. Between each row of vines (repetitions) a buffer row of vine was left untreated.

The first treatment was applied in May (14^{th} of May 2020 and 15^{th} of May 2021, respectively), before flowering, when the shoot lengths were of about 25-30 cm. From the first treatment, for the variants with 12 treatments spraying was done once a week, while for the combination variant with 6 treatments spraying was done every other week. Last treatment was applied in July (21st of July 2020 and 22nd of July 2021, respectively).

An electric sprayer pump of 16 litres and 3 spraying nozzles was used for the treatments. Maximum spraying pressure was 6 Bar.

Wine production

'Fetească neagră' grapes were harvested in the month of September (10th of September 2020 and 13th of September 2021, respectively). Due to specific meteorological conditions, sugar concentration of the harvest differed quite much in the years of study, grapes having at harvest a total solid content (TSS) of 27.2°Brix in 2020 and 20.7°Brix in 2021. For each variant a total of about 200 kg grapes were gathered by hand. Harvested grapes were transported to the SCDVV Murfatlar winemaking station and processed into red wine in accordance to the classical maceration-fermentation method. Thus, after crushing and destemming by a small electrical destemmer-crusher the grape mash was treated with 4 g hL^{-1} SO_2, to prevent oxidation and immediate start of the fermentation, and then kept in 120 L stainless steel vessels for maceration. Mash stirring for homogenization and colour pigment extraction was done 3-4 times a day. Fermentation occurred spontaneously with the existent wild yeast, at a moderate temperature, which did not exceed 25°C at any time. After 5 days of fermentation the must was separated from the solid fragments in the mash by using a hydraulic press. The resulted grape must continue alcoholic and malolactic fermentation in glass demijohns of 25 L. Progress of malolactic fermentation was followed by measuring the content of malic acid using FTIR wine Analyser Lyza 5000 (Anton Paar, Graz, Austria). At the end of the malolactic fermentation, the wines were racked from the lees, sulphited slightly with 2 g hL^{-1} SO_2 and bottled in 0.75 L glass bottles. All wine variants were kept for maturation in bottles at 15°C for 6 months and then subjected to analysis.

Grapes analyses

The main quality parameters determined at harvest in the grape must obtained from the fresh berries are the sugar content, measured as TSS using a Smart electronic refractometer (Atago, Japan) and the total acidity, determined by titration with 0.1 M NaOH solution. The polyphenolic maturity of grapes was evaluated by the method of Glories (1984), which consists in extracting anthocyanins from the grape skin, both under conditions encountered in winemaking (pH=3.2) and under severe conditions necessary for total extraction (pH=1). The pH=3.2 solution was prepared by dissolving 5 g of tartaric acid in 800 ml of distilled water, transferring in a 1000 mL volumetric flask and then adding 22.2 ml NaOH 1 M and water up to the sign marking the volume of 1000 mL. The pH=1 solution is a 0.1 M HCl solution, obtained with 8.33 ml of 37% HCl added in 1000 mL distilled water. A volume of 50 ml of each solution is used to separately extract the anthocyanins from 50 g of the mashed grapes. Both samples were well homogenized and left 4 hours for extraction at room temperature and then filtered, thus obtaining two solutions marked as „pH_1" and „$pH_{3.2}$"., Using the differential pH method (Ribereau-Gayon *et. al*, 1976), anthocyanins were spectrophotometrically analysed in both extracts, determining the absorbance at 520 nm. A Helios Alpha UV-VIS spectrophotometer (ThermoScientific, USA) and glass cuvettes of 1 cm optical path length were used. To determine the total content of phenols, the solution extracted at wine pH ($pH_{3.2}$) was measured 280 nm, in quartz cuvettes of 1 cm.

Some indices are also measured as follows (Saint-Cricq *et al*., 1998):

- A_{pH1} = total anthocyanin potential or the anthocyanins extractable at pH = 1 (1)
- $A_{pH3.2}$ = total anthocyanins extractable at wine pH = 3.2 (2)
- EA (%) = extractability index of anthocyanins

$$\text{EA (\%)} = \left[\frac{\text{ApH1} - \text{ApH3.2}}{\text{ApH1}}\right] \times 100 \quad (3)$$

- ITP (A_{280nm}) = total polyphenol index (4)

- dpell = skin grape tannin content

$$\mathbf{dpell} = \frac{\mathbf{ApH3.2x40}}{\mathbf{1000}} \quad (5)$$

- MP (%) = seed maturity index (the contribution of seed tannins to the total polyphenols)

$$\mathbf{MP\ (\%)} = [\frac{\mathbf{A280-dpell}}{\mathbf{A280}}] \times \mathbf{100} \quad (6)$$

- dTpep = tannin content of seeds and

$$dTpep = A_{280}\text{-}dpell \quad (7)$$

Wines colour

Wine colour was determined in accordance to the OIV method by spectrophotometric measurements with the same instrument used for grape anthocyanins analyses. The main colour parameters measured were wine colour intensity (IC), which represents the sum of the values of absorbance measured at 420 nm, 520 nm and 620 nm, expressed in absorbance units (Au), and hue (H), which represents the ratio of the absorbance at 420 nm and the absorbance at 520 nm, which is dimensionless. The values of colour intensity differ greatly from one variety to another and in accordance to the time of maceration or aging, while the hue values are generally between 0.5-0.7 for young wines and are reaching a higher limit of 1.2-1.3 for aged wines (Ribéreau-Gayon, 2021). The contribution of each fundamental colour (yellow, red and blue) to the overall wine colour was calculated by dividing the absorbance value measured at 420 nm for yellow (Ye%), at 520 nm for red (Rd%) and at 620 nm for blue (Bl%) by the wine colour intensity (CI). The proportion of red colour produced by free and bound anthocyanins (dA%) was calculated using the following formula introduced by Glories (1984):

$$\mathbf{dA\%} = [1 - \frac{\mathbf{Abs\ 420+Abs620}}{\mathbf{2 \times Abs520}}] \times \mathbf{100} \quad (8)$$

For dA% the values are generally between 40 and 60, higher values showing more dominant red colour in wine (Ribéreau-Gayon, 2021).

Phenolic compounds analysis by UHPLC-HRMS

Specific phenolic compounds determination, including phenolic acids, stilbenes and flavonoids, was performed in the Liquid Chromatography Laboratory of National Research and Development Institute for Cryogenics and Isotopic Technologies using UHPLC–ESI/HRMS (ultra-high-performance liquid chromatography electrospray ionization tandem mass spectrometry) with a high-resolution Q Exactive mass spectrometer™ Focus Hybrid Quadrupole–OrbiTrap equipped with HESI, coupled to a high-performance liquid chromatograph UltiMate 3000 UHPLC (Thermo Fisher Scientific, Massachusetts, USA). The chromatographic separation of phenolic compounds was performed at 30°C on a Kinetex® C18 column (100 × 2.1 mm, 1.7 μm particle diameter), under a gradient elution of two mobile phases: A (water with 0.1% formic acid) and B (methanol with 0.1% formic acid), at a flow rate between 0.3 and 0.4 mL/min, following the gradient programme presented in Nichitoi *et al.* (2021). Full scan data in negative mode covering a scan range of m/z 75–1000 was acquired at a resolving power of 70,000 FWHM (Full Width at Half Maximum) at m/z 200. Variable data-independent analysis MS2 (vDIA) was performed at the resolution of 35,000, isolation windows and scan ranges being set as follow: 75-205 m/z, 195-305 m/z, 295-405 m/z, 395-505 m/z and 495-1000 m/z. Nitrogen was used as collision gas and auxiliary gas at a flow rate of 11 and 48 arbitrary units, respectively. The applied voltage was 2.5 kV, and the capillary temperature 320 °C. The energy of the collision cell was set at 30 eV. The data were processed using the Xcalibur software package (Version 4.1) (Thermo Fisher Scientific Massachusetts, USA).

Before analysis, the wine samples were diluted ten times with 20% methanol solution, filtered through a 0.45 μm polytetrafluoroethylene membrane and then 10 μL filtrate were injected into the chromatographic system. Quantification was performed by the external standard method, using nine-point calibration curves obtained by serial dilutions with methanol from a standard mixture of individual phenolic compounds of 10 mg L^{-1} concentration. The calibration covered a concentration range from 50 to 1750 μg L^{-1}. The calibration curves for each phenolic compound were forced through origin and a good linearity was verified for all the phenolic compounds, with calibration coefficients higher than 0.9995. Calibration curve parameters and the characteristic performances such as detection limit (LOD) and quantification limit (LOQ) are detailed in Nichitoi *et al*. (2023). In order to check the recovery factors, spiked samples were performed and the obtained results ranged between 85-103% for individual phenolic compounds. Procedural blank samples and quality control standards solutions of different concentrations were used for each set of 10 samples. The results were reported in mg L^{-1} for each individual phenolic compound.

Statistical analysis

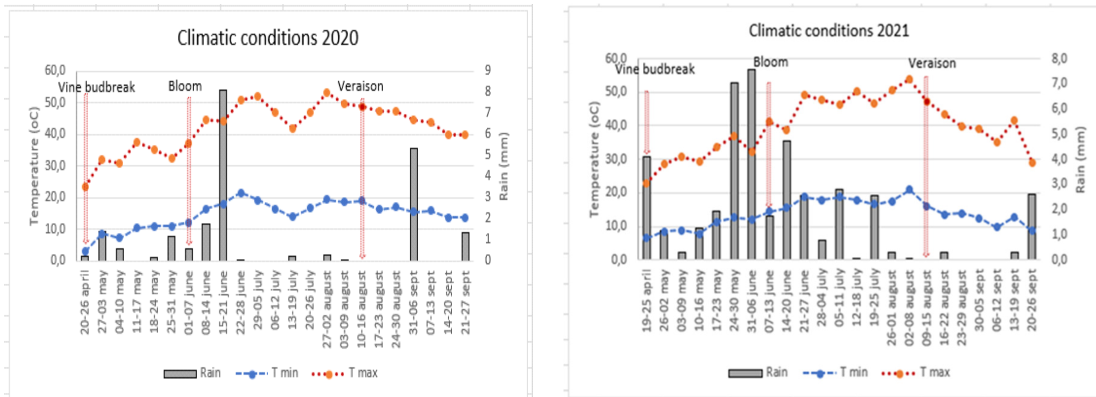
Analysis of variance (One-way ANOVA) was used to establish the significant differences induced by the treatments applied in the vineyard or the vintage year. The differences of the means were compared by a post-hoc test (Duncan multiple mean comparison test). Both analyses were performed with the software package SPSS Statistics 17.0. Other analyses and graphics were performed with Excel 2019 (Microsoft Corporation, Redmond, Washington, United States) and Origin 9.0 (Northampton, Massachusetts, USA).

Results and Discussion

Influence of chitosan treatments on grapes

The quality of grapes and wines is influenced by many factors. As regards the polyphenol accumulations, the most important parameters, that cannot be experimentally controlled, are those related to the weather conditions, fluctuations being encountered from one year to another, as the climate factors influence ripening (Xie *et al*., 2021) and polyphenols biosynthesis (Gao *et al*., 2016). Weather conditions are among the most challenging environmental factors affecting polyphenols (Teixeira *et al*. 2013) and these include sunlight, sun damage (Gambeta *et al*. 2021) or reduced UV radiation (Fernandes de Oliveira and Nieddu, 2016), temperature (Bergqvist *et al*., 2001) and available water (Castellarin *et al*., 2007).

Evolution of the main climate parameters, maximum temperature, minimum temperature and the level of rainfall, was monitored during the vegetation period (20th of April to 27th of September) and it was, as expected, dependent on the year (Figure 1). The year of 2020 was marked by daily maximum temperatures in the range of 27.1-33.9 °C and total precipitations of only 150.2 mm, while in 2021 daily maximum temperatures fluctuated in a larger interval of 24.3-33.0 °C and the rainfall level was higher, almost double, 292.9 mm. Taking into account the parameters recorded for the past years (2011-2019), the usual rainfall in the region is between 100-150 mm, with many dryer years and a few more humid years, as it was the case in 2013, a similar year with 2021.



(a) (b)

Figure 1. Main climate parameters and phenology evolution for 'Fetească neagră' grape variety at Murfatlar during the vintages 2020 (a) and 2021 (b)

The main stages of phenology – budbreak, bloom, veraison, berry development, harvest – were influenced by each year's weather conditions. Thus, although the budburst occurred in the same period of time in both 2020 and 2021 (20th – 26th of April), flowering and veraison occurred 7 days earlier in 2020 (Figure 1a), as compared to 2021 (Figure 1b).

In the studied years the climate clearly influenced some of the major grape parameters which determine the quality, such as sugar accumulation, acidity, total phenols and anthocyanins, as seen in Table 1. But, irrespective of the vintage year, the treatment with chitosan has also induced some important positive differences.

Sugar accumulation and total acidity recorded important fluctuations with the meteorological conditions. Sugar content in grapes, measured as TSS, ranged from 27.2 to 28.3°Brix in 2020 and 20.7-22.1°Brix in 2021, respectively. Total acidity varied in the range of 4.1-4.7 g L^{-1} tartaric acid in 2020 and 9.6-9.8 g L^{-1} tartaric acid in 2021. Clearly, in 2020, a year with higher maximum temperatures and less rainfall, the sugar accumulation was substantial and the acidity low (minimum acidity permitted by legislation being 3.5 g L^{-1} tartaric acid). Conversely, in 2021, a year with lower maximum temperatures and more rainfall, sugar accumulated more slowly and the acidity remained high (Table 1). These differences are only due to the weather specificities of these two years, no statistical difference being observed for the vineyard treatments applied each year.

Phenolic compound accumulation, however, depended both on the cultivation year, but also on the treatment applied in the vineyard. Phenolic compounds (phenolic acids, flavonoids, stilbenes, anthocyanins) accumulate in the grapes in various parts (skins, seeds), their content differing in quantity and quality depending on the grape variety, phase of maturity, climate, agricultural practices and treatments (Diego *et al.*, 2021).

Phenolic quality of the grapes, irrespective of the vintage year was significantly ameliorated in the variants treated with chitosan, leading to a significant increase in the total anthocyanins extractable at pH = 1, as well the anthocyanins extractable at wine pH = 3.2 (Table 1).

Our results are in good agreement with the studies of other researchers, who also demonstrated the increase in anthocyanins in some grapes and wines or the increase of other protection substances in the grape skins, as a consequence of chitosan application in vineyards (Paladines-Quezada, 2021).

Table 1. Chemical composition at harvest time of 'Fetească neagră' grapes obtained in 2020 and 2021 from experimental vineyard variants sprayed with chitosan and/or Bordeaux mixture

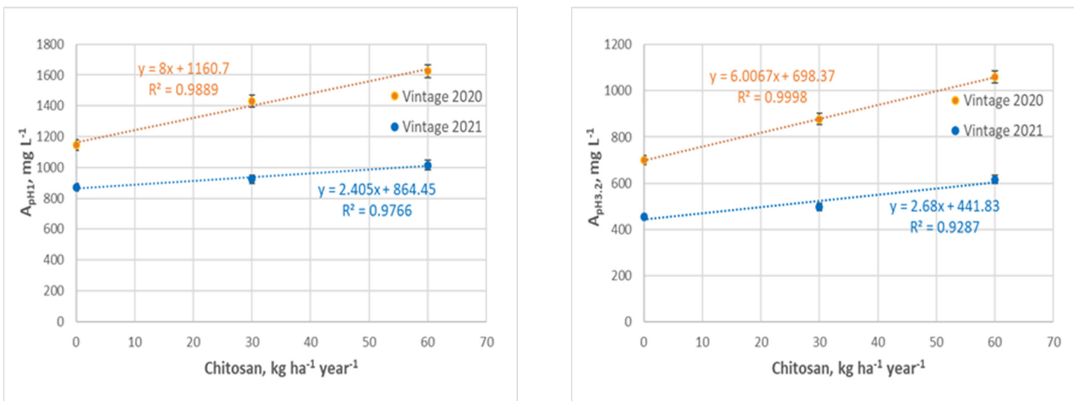
Chemical parameter	2020			2021			ANOVA		
	Bordeaux mixture (Control)	Bordeaux mixture +Chitosan	Chitosan	Control (Bordeaux mixture)	Bordeaux mixture +Chitosan	Chitosan	Treat-ment	Year	Treat-ment * Year
Content of sugar, °Brix	27.2± 2.1^{a}	28.3± 3.8^{a}	28.2 ± 1.3^{a}	20.7± 1.2^{b}	21.8±1.2^{b}	22.1±1.4^{b}	ns	***	ns
Total acidity, g L^{-1} tartaric acid	4.7± 0.3^{a}	4.3± 0.3^{a}	4.1 ± 0.5^{a}	9.8± 1.2^{b}	9.8± 0.6^{b}	9.6 ± 0.8^{b}	ns	***	ns
pH	3.4± 0.1^{ab}	3.6± 0.2^{ab}	3.8± 0.2^{a}	3.1± 0.3^{b}	3.2± 0.5^{b}	3.3± 0.2^{b}	ns	**	ns
Total anthocyanin potential (A_{pH1}), mg L^{-1}	1146.0 ± 35.8^{c}	1430.3 ± 38.1^{b}	**1626.7± 41.3^{a}**	870.9 ± 25.8^{e}	923.7 ± 27.4^{e}	**1015.2 ± 32.1^{d}**	***	***	***
Anthocyanins extractable at wine pH ($A_{pH3.2}$),mgL^{-1}	699.0 ± 20.3^{c}	877.3 ± 24.8^{b}	**1059.4 ± 27.1^{a}**	454.7 ± 14.2 f	496.5 ± 15.4^{e}	**615.5 ± 18.4^{d}**	***	***	***
AE, %	39.0±3.8^{bc}	38.7±3.0^{bc}	34.9± 2.5^{c}	47.8± 5.4^{a}	46.3±4.9^{ab}	39.4±4.2^{bc}	*	**	ns
A_{280nm} (IPT)	44.4± 3.6^{b}	**52.2± 2.8^{a}**	**58.2± 3.6^{a}**	30.0 ± 3.2^{c}	32.2± 3.9^{c}	34.2± 3.7^{c}	**	***	*
Polyphenol quality appreciated based on the total anthocyanin potential (A_{pH1})	1000-200 mgL^{-1} very good	>1200 mgL^{-1} excellent	>1200 mgL^{-1} excellent	800-1000 mgL^{-1} good	800-1000 mgL^{-1} good	1000-1200 mgL^{-1} very good	(Guerin *et al.* 2005)		
Skin tannins (dpell), g 100 ml^{-1}	28.0 ± 2.2^{c}	35.1± 2.8^{b}	**42.4 ± 3.1^{a}**	18.2± 2.2^{d}	19.9 ± 2.4^{d}	**24.6± 2.5^{c}**	***	***	*
Seed tannins (dTpep), g 100 ml^{-1}	16.4 ± 1.8^{b}	17.1 ± 2.1^{b}	15.8 ± 2.0^{b}	11.8 ± 1.5^{c}	12.3 ± 1.6^{c}	9.6 ± 1.2^{c}	**	***	*
Seeds maturity (MP), %	37.0± 2.0^{a}	39.7 ± 2.3^{a}	*27.2 ± 1.8^{b}*	39.4 ± 3.7^{a}	38.3 ± 3.5^{a}	*28.0 ± 3.1^{b}*	***	ns	ns

Average values ± standard errors (n=3). The letters in the brackets show the statistical difference among results for $p<0.05$. For the same compound, common letters for 2 or more variants show no significant difference among them. Clear significant differences induced by the chitosan are marked in the table in bold font for maximum values and italic font for minimum values.

As compared to the control treatment, the increase induced by the chitosan treatment (60 kg chitosan ha^{-1} $year^{-1}$) in the total anthocyanin potential (A_{pH1}) was 41.9% in 2020 and with 16.6% in 2021. As well, the anthocyanins extractable at wine pH ($A_{pH3.2}$) were 51.6% higher in 2020 and 35.4% higher in 2021. The increase proved to be dose-dependent (Figure 2), therefore, the combination treatment with chitosan and Bordeaux mix, which contained half the chitosan dose used in the variant treated only with chitosan, induced an intermediate anthocyanin accumulation. Correlation of the anthocyanins with the chitosan dose is linear with very good correlation coefficients for both A_{pH1} (Figure 2a) and $A_{pH3.2}$ (Figure 2b) measured in both study years. In 2020, a hotter year with less than normal rainfall, anthocyanin biosynthesis was stronger than in 2021, fact shown by the steeper slopes of the correlation equations (around 8 for A_{pH1} and 6 for $A_{pH3.2}$). This behaviour is explained by the tendency of the grapes (and other plants) to produce polyphenols, carotenoids and aroma compounds as secondary metabolites meant to counter the stress induced by higher sun radiation

(Gambetta *et al.*, 2021). In the cooler and rainier year of 2021, the anthocyanin biosynthesis was less robust, the correlation equations having smaller slopes (about 2.4 for A_{pH1} and 2.6 for $A_{pH3.2}$).

Previous studies (Silva *et al.*, 2020) revealed that the application of treatments with chitosan in the vineyard can stimulate the synthesis in grapes of secondary metabolites, such as anthocyanins, but also other phenolic compounds, and there is even evidence that treatment with chitosan can activate the key enzymes of phenylpropanoid pathways, especially phenylalanine ammonia lyase, which is the key enzyme that catalyses the first step in phenolic biosynthesis (Delaunois *et al.*, 2014). Similar results regarding the increase of phenolic compounds of grapes following the vine treatments with chitosan products were reported for the *Vitis vinifera* L. variety. Mouhtaro (Miliordos *et al.*, 2021).



(a) (b)

Figure 2. Correlation of the chitosan dose used for vine treatments and the anthocyanins accumulation in grapes: a) total anthocyanin potential (A_{pH1}); b) anthocyanins extractable at wine pH ($A_{pH3.2}$)

The total polyphenol index, ITP(A_{280nm}), recorded very high values in all cases, but especially in the year of 2020, when the weather was more favourable (between 44.4-58.2) than in 2021 (between 30.0-34.2). ITP(A_{280nm}) is considered very high when its value is over 22, high if it is between 18-22, medium if it is between 16-18, weak between 14-16 and very weak for values under 14 (El Darra 2013, citing Guerin *et al.*, 2005). In 2020 even, the accumulation of total polyphenols was significantly higher in the grapes treated with chitosan, while in 2021, a less favourable year, the treatment did not lead to significant differences.

Total phenol quality is also dependent on the quantity of the anthocyanins. In accordance to Guerin (2005) cited by El Darra (2013), the phenol quality is excellent if the level of the total anthocyanin potential (A_{pH1}) is above 1200 mg L^{-1}, very good if it is between 1000-1200 mg L^{-1}, good for 800-1000 mg L^{-1} medium for 600-800 and insufficient if it is under 600 mg L^{-1}. As we see in Table 1., polyphenol quality appreciated based on the total anthocyanin potential (A_{pH1}) for the treatment of vineyard only with chitosan was excellent in 2020 and very good in 2021, while for the treatment of vineyard only with Bordeaux mixture was just very good in 2020 and good in 2021.

Tannins, another component of the total polyphenols, accumulated significantly better in the skins of the grapes treated with chitosan, as a protection against diseases induced by the presence of this elicitor. In 'Fetească neagră', in 2020, skin tannins increased by 51% in the grapes treated with chitosan (42.4 g 100 mL^{-1}) as compared to the case of grapes treated with Bordeaux mixture (28.0 g 100 mL^{-1}). In the rainier year of 2021 the same behaviour was observed, only the increase was lower, only 24.6 g 100 mL^{-1} for the chitosan treated grapes and 18.2 g 100 mL^{-1} for the Bordeaux mixture treated grapes, that is a 35% increase when chitosan was applied. Zhang *et al.* (2020) have also determined in 'Kyoho' and 'Shine Muscat' grapes an increase in some secondary metabolites, such as resveratrol and phenols which compose the tannins (epigallocatechin gallate, catechin).

Seed tannins were not particularly affected by the treatments in the vineyard, the variation being insignificant. As expected, the vintage year induced some differences, the year with less rain and higher temperature being also more favourable to tannin accumulation. Thus, in 2020 seed tannins ranged between 15.8 and 16.4 g 100 mL^{-1}, while in 2021 they ranged from 9.6 to 12.3 g 100 mL^{-1}.

The seed maturity index also shows the good influence of the chitosan for the grape and wine quality, in both years the treatments with chitosan leading to decreased values as compared with the Bordeaux mixture treatments, representing 69% in 2020 and 71% in 2021 of the values obtained for the case of Bordeaux mixture treatments. The values reported are measured in both years at similar harvest dates, as seed maturity depends on the grape ripening, the value of this index decreasing progressively toward the harvest (Saint-Cricq de Gaulejac *et al.*, 1998). The higher the value of seed maturity index, the higher the content of tannins extractable from seeds, which means that their transfer in wines could be excessive and potentially affect the taste, by increasing the bitterness and astringency. With significantly lower seed maturity indices, 27.7% in 2020 and 28.0 in 2021, the grapes from the vines treated with chitosan are likely to produce wines with smoother tannins, which would not require longer aging, as compared to the grapes treated with Bordeaux mixture, for which the indices were 37% in 2020 and 39.4% in 2021.

Influence of chitosan treatments on wines

Wines produced from the experimental grape variants were also assessed regarding their phenolic quality. The extracted anthocyanins produced colour parameter values specific for quality wines in all samples, as shown in Table 2.

Table 2. Colour parameters of wines made in 2020 and 2021 from 'Fetească neagră' grapes produced in vineyards sprayed with chitosan and/or Bordeaux mixture

Chemical parameter	2020			2021			ANOVA		
	Bordeaux mixture (Control)	Bordeaux mixture + Chitosan	Chitosan	Bordeaux mixture (Control)	Bordeaux mixture + Chitosan	Chitosan	Treat-ment	year	Treat-ment* year
Colour intensity (CI)	7.48±0.5[bc]	9.48±1.1[b]	**12.86±1.3[a]**	6.83±1.0[c]	6.30±1.2[c]	**7.47±1.5[bc]**	**	***	*
Hue (H)	0.63±0.16[a]	0.58±0.20[a]	0.52±0.18[a]	0.75±0.17[a]	0.79±0.15[a]	0.78±0.19[a]	ns	*	ns
dA%	60.53±4.8[b]	63.82±4.5[ab]	69.02±4.3[a]	50.28±3.8[c]	49.18±3.3[c]	47.39±3.6[c]	ns	***	*
Rd%	55.88±4.4[ab]	58.02±4.0[a]	61.74±4.6[a]	50.14±3.6[b]	49.60±4.0[b]	48.73±3.3[b]	ns	***	ns
Ye%	35.29±2.7[ab]	33.76±2.4[ab]	32.04±2.1[b]	37.53±2.5[a]	38.97±2.2[a]	37.74±2.8[a]	ns	**	ns
Bl%	8.82±1.3[bc]	8.23±1.4[c]	6.22±1.1[c]	12.33±1.7[a]	11.43±1.6[ab]	13.53±2.0[a]	ns	***	*

Average values ± standard errors (n=3). The letters in the brackets show the statistical difference among results for $p<0.05$. For the same compound, common letters for 2 or more variants show no significant difference among them. Significant differences induced by the chitosan are marked in the table in bold font for maximum values. Ye =% of yellow, Rd=% of red, Bl=% of blue, dA%=flavylium cations.

Colour intensity (CI) in all wine samples was above 6 in all cases, being higher in 2020 than in the rainier year of 2021. In 2020, the treatment with chitosan lead to a significant 72% increase in the colour intensity as compared to the variant treated with Bordeaux mixture only. In 2021, chitosan also increased the colour intensity by 10%, but this increase, in accordance to ANOVA analysis, did not reach a level of significance in this year. The colour hue (H) tended to contain more red nuances when chitosan was used for the treatment (H values lower than in treatments with Bordeaux mixture), but the levels were not statistically different, irrespective of the treatment applied in the vineyard. Significant differences in hue were recorded only for the harvest year: the hue of 0.52-0.63 recorded in 2020 was brighter and made the wine look younger than the hue of 0.75-0.79 recorded in 2021, which had some more brownish shades. Even with this variation in hue from one year to another, all wines had hues specific for red young wines. Also, the proportion of red colour produced by free and bound anthocyanins (dA%), as well as the red, yellow and blue components of the colour (Rd%, Ye%, Bl%) were different in the two years of harvest due to the climatic factors, but not in accordance to the treatment. Thus, we can say that the higher content of anthocyanins induced in grapes by the chitosan treatment did not influence much the appearance of the wine colour, which is a positive thing, considering that wine appearance is one of the main traits on which customers base their wine selection.

To determine in more detail the wine quality and its potential to have more compounds with beneficial effect on health, individual phenols were also analysed. UHPLC-HRMS analysis allowed us to determine specific phenols of various chemical classes, such as phenolic acids, flavan-3-ols, flavonols and stilbenes. The compounds identified in the experimental wines are included in Table 3, for both years of study.

Table 3. Phenolic profile of wines made in 2020 and 2021 from 'Fetească neagră' grapes produced in vineyards sprayed with chitosan and/or Bordeaux mixture

Phenolic compound	2020			2021			ANOVA		
	Bordeaux mixture (Control)	Bordeaux mixture +Chitosan	Chitosan	Bordeaux mixture (Control)	Bordeaux mixture +Chitosan	Chitosan	Treat-ment	Year	Treat-ment* year
gallic acid, mg L^{-1}	18.05±0.22^{d}	18.16±0.26^{d}	**20.31±0.28^{b}**	18.16±0.19^{d}	18.72±0.20^{c}	**35.8±0.32^{a}**	***	***	***
3,4 dihydroxybenzoic acid, mg L^{-1}	3.06±0.11^{b}	2.94±0.15^{bc}	**8.36±0.21^{a}**	1.89±0.17^{d}	2.74±0.10^{c}	1.44±0.12^{e}	***	***	***
4-hydroxybenzoic acid, mg L^{-1}	1.11±0.08^{b}	1.08±0.05^{b}	**1.25±0.07^{a}**	0.6±0.05^{d}	0.75±0.07^{c}	0.64±0.09^{cd}	ns	***	*
chlorogenic acid, mg L^{-1}	0.17±0.01^{ab}	0.15±0.01^{bc}	0.18±0.02^{a}	0.13±0.02^{c}	0.10±0.02^{d}	0.13±0.01^{c}	*	***	*
syringic acid, mg L^{-1}	3.27±2.25^{a}	3.31±2.33^{a}	3.58±1.67^{a}	2.39±2.89^{c}	2.76±1.24^{b}	3.34±1.28^{a}	***	***	*
p-coumaric acid, mg L^{-1}	11.7±0.18^{c}	16.7±0.21^{a}	**12.7±0.26^{b}**	4.3±0.15^{d}	4.3±0.17^{d}	3.5±0.18^{e}	***	***	***
ferulic acid, mg L^{-1}	0.67±0.03^{b}	0.90±0.07^{a}	0.88±0.05^{a}	0.28±0.01^{c}	0.28±0.01^{c}	0.24±0.02^{c}	**	***	***
ellagic acid, mg L^{-1}	2.04±0.14^{a}	1.75±0.11^{b}	2.07±0.15^{a}	1.19±0.13^{c}	1.07±0.17^{c}	1.99±0.15^{ab}	***	***	**
abscisic acid, mg L^{-1}	0.39±0.02^{a}	0.33±0.01^{b}	0.34±0.03^{b}	0.28±0.02^{c}	0.39±0.03^{a}	0.28±0.01^{c}	**	**	***
cinnamic acid, mg L^{-1}	0.29±0.03^{c}	0.30±0.03^{c}	0.15±0.01^{d}	0.55±0.05^{b}	1.28±0.07^{a}	1.29±0.08^{a}	***	***	***
catechin, mg L^{-1}	16.91±0.30^{e}	16.16±0.25 f	**19.71±0.27^{d}**	26.32±0.33^{c}	28.62±0.30^{b}	**33.70±0.38^{a}**	***	***	***
epicatechin, mg L^{-1}	3.86±0.15^{e}	3.91±0.22^{e}	**5.15±0.26^{d}**	7.04±0.31^{c}	7.81±0.36^{b}	**13.8±0.45^{a}**	***	***	***
myricetin, mg L^{-1}	7.62±1.23^{b}	8.55±1.36^{ab}	9.91±1.28^{a}	4.25±1.09^{c}	3.48±1.02^{c}	3.66±1.05^{c}	ns	***	ns
quercetin, mg L^{-1}	7.88±1.29^{b}	10.62±1.37^{a}	11.61±1.35^{a}	0.89±0.18^{c}	1.08±0.04^{c}	1.32±0.15^{c}	**	***	*
kaempferol, mg L^{-1}	0.23±0.05^{b}	0.45±0.06^{a}	0.53±0.08^{a}	0.07±0.01^{c}	0.08±0.01^{c}	0.11±0.02^{c}	***	***	**
isorhamnetin, mg L^{-1}	0.58±0.05^{b}	0.77±0.05^{a}	0.83±0.08^{a}	0.13±0.01^{c}	0.15±0.01^{c}	0.19±0.01^{c}	***	***	**
naringin, mg L^{-1}	0.18±0.01^{b}	0.30±0.02^{a}	0.38±0.02^{a}	0.13±0.07^{b}	0.14±0.05^{b}	0.13±0.08^{b}	*	***	*
apigenin, mg L^{-1}	0.08±0.01^{ab}	0.06±0.01^{b}	0.09±0.02^{a}	0.06±0.01^{b}	0.06±0.01^{b}	0.06±0.02^{b}	ns	*	ns
pinocembrin, mg L^{-1}	0.59±0.18^{a}	0.57±0.14^{a}	0.60±0.20^{a}	0.59±0.12^{a}	0.59±0.19^{a}	0.58±0.17^{a}	ns	ns	ns
trans-resveratrol, mg L^{-1}	4.26±1.07^{b}	5.12±1.03^{b}	**8.03±1.48^{a}**	5.55±1.80^{ab}	4.97±1.21^{b}	6.48±1.64^{ab}	**	*	*

Average values ± standard errors (n=3). The letters in the brackets show the statistical difference among results for $p<0.05$. For the same compound, common letters for 2 or more variants show no significant difference among them. Clear significant differences induced by the chitosan are marked in the table in bold font for maximum values.

From the class of phenolic acids, gallic acid predominates, with values in the range of 18.05-35.80 mg L^{-1}, fact that is consistent with previous results reported for wine in general (Porgali *et al*., 2012) or for 'Fetească neagră' the variety in particular (Geana *et al*., 2011; Artem *et al*., 2021b). For both harvest years, in the wine variant obtained from grapes coming from vines treated with chitosan, the values were significantly higher than in the case of the other treatments, being 12% higher in 2020 and 97% higher in 2021 as compared to the variant from grapes coming from vines treated with Bordeaux mixture. Overall, gallic acid accumulated in higher concentrations in 2021, the year with more rain.

An increase also seems to be elicited by the chitosan in the case of flavonols content, the observed values rising in 2020 by about 17% in the case of catechin and by 33% in the case of epicatechin and even more in

2021, by 28% for catechin and 96% for epicatechin. These compounds concentrations were also influenced by the meteorological conditions of the study year, so that significant higher amounts are recorded in 2021 when it rained more (Table 3).

Two of the more prominent flavonols, quercetin and myricetin, recorded fluctuations in concentration in accordance to the year of research, but not with the treatments in the vineyard. In the dryer year, 2020, both myricetin (7.6-9.9 mg L^{-1}) and quercetin (7.9-11.6 mg L^{-1}) had higher values, while in the rainier year, 2021 myricetin concentrations were between 3.5-4.3 mg L^{-1} and those of quercetin were very low, between 0.9 to 1.3 mg L^{-1}. As quercetin is a compound with demonstrated beneficial health effects (Gökbilen *et al.*, 2022; Nutmakul, 2022; Racinowski *et al.*, 2021), this influence of the weather conditions should be noted, as the accumulation of this compound is correlated with higher temperature and less precipitations.

For many of the identified phenolic compounds (such as the fenolic acids: 3,4 dihydroxybenzoic acid, 4-hydroxybenzoic acid, chlorogenic acid, syringic acid, p-coumaric acid, ferulic acid, ellagic acid, abscisic acid, cinnamic acid, as well as flavonols: kaempferol, isorhamnetin or flavanones: naringin, apigenin, pinocembrin) the variations were not important in values, differing by a few mg L^{-1}, even though statistical analyses showed that some of the differences induced by the treatment in the vineyard or weather conditions of the year reached a level of significance.

The class of stilbenes was represented here by trans-resveratrol, a compound very well researched for its health benefits in several conditions and diseases (Thirumalaisamy *et al.*, 2022; Najafi *et al.*, 2022). Regarding the evolution of trans-resveratrol, it can be noticed that the values oscillated in the range of 4.3-8.0 mg L^{-1} in 2020 and 5.0-6.5 mg L^{-1} in 2021, significant increases from a statistical point of view being induced especially by the treatment with chitosan (89% increase in 2020 and 17% in 2021) and less by the climatic variability (Table 3).

While quercetin level cannot be influenced by the treatment with chitosan, it may be possible to increase the level of resveratrol by this treatment. Previous studies (Aziz *et al.*, 2006) have shown that in vitro chitosan oligomers with different molecular weight and degree of acetylation were able to elicit in vine leaves an accumulation of phytoalexins, such as trans- and cis-resveratrol and their derivatives, ε-viniferin and piceid. This study has also shown that chitosan applied together with copper sulphate caused a significant and strong accumulation of phytoalexins, reducing infections by *Botritis cinerea* and *Plasmopara viticola* on grapevine leaves. However, in our study, the chitosan we used applied together with the Bordeaux mixture did not lead to significant increases of trans-resveratrol.

To find out the main individual phenols characterizing the wine samples produced in a specific year or from grapes coming from vineyards in which different treatments were applied, Principal Component Analysis was performed (Figure 3).

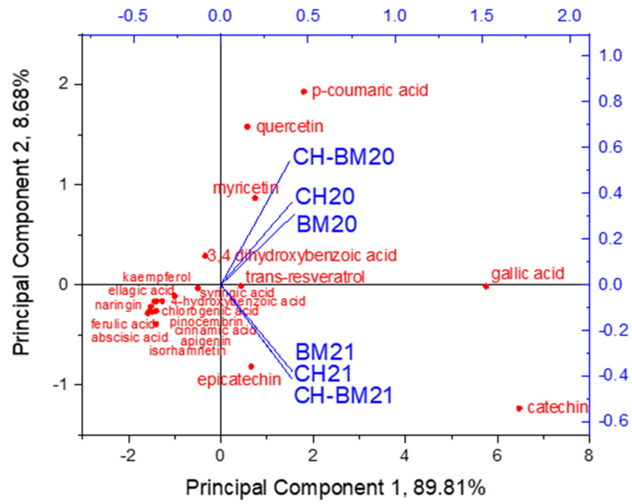


Figure 3. Principal Component Analysis of the phenolic compounds found in wines produced in 2020 and 2021 from grapes obtained from vines treated with Bordeaux mixture (BM), Chitosan (CH) or a combination of chitosan and Bordeaux mixture (CH-BM)

It can be observed that the main influence on the phenolic quality of the wine is determined by the particularities of the harvest year, samples from 2020 (BM20, CH-BM20, CH20) being clearly separated from those of 2021 (BM21, CH-BM21, CH21). The differentiation is mainly explained by the Principal Component 1, which represents 89.91% of the total variability in the experimental data. This PC1 includes most of the phenolic compounds, catechin and gallic acid having the largest weights on this axis, followed by p-coumaric acid, quercetin, myricetin and trans-resveratrol. The dryer year of 2020 was characterized especially by higher amounts of p-coumaric acid and flavonols (quercetin, myricetin) while the rainier year of 2021 is characterized by higher amounts of flavan-3-ols (catechin and epicatechin).

It can be concluded that chitosan treatment proves to be a good alternative for the use of Bordeaux mixture in viticulture, which brings too much copper in the plantations. Due to its antifungal properties, chitosan is already a popular substance for treatments in agriculture and its beneficial properties as an elicitor for protection molecules in plants (Malerba *et al*., 2015) represents a plus. As a biopolymer, chitosan is a natural product easily available, which makes its usage a viable economic solution for various purposes in agriculture (Rinaudo, 2006).

Conclusions

The results obtained in the conditions of two years with very different climatic parameters showed that treatments with chitosan in the vineyard can stimulate the synthesis of secondary metabolites in grapes, especially of phenolic compounds belonging to the anthocyanin class (red pigments), flavan-3-ols (catechin and epicatechin) and stilbenes (trans-resveratrol).

In grapes, accumulation of anthocyanins proved to be very well correlated with the total dose of chitosan applied in the vineyards, with higher values in 2020 when temperatures were higher and the rainfall values were typical for the region. Very high values of the total polyphenols (ITP index above 22) were also recorded in the case of chitosan treatments in 2020 when the weather was more favourable. The quantity of anthocyanins in the grapes was excellent in 2020 (above 1200 mg L^{-1}) and very good in 2021 (between 1000-1200 mg L^{-1}) in the case of chitosan treatment and only very good in 2020 and good in 2021 (between 800-1000 mg L^{-1}) in the case of Bordeaux mixture treatment. The accumulation of tannins in grape skins followed a similar trend, increasing in the case of chitosan treatment by 51% in 2020 and 35% as compared to the Bordeaux mixture treatment.

In wine, correlated to the accumulation of anthocyanins in grapes, colour intensity (CI) was very good (above 6) in all cases, being higher in 2020 than in the rainier year of 2021. Chitosan treatment determined a significant 72% increase in the colour intensity in 2020. Among the individual phenolic compounds determined, other than anthocyanins, gallic acid was predominant, with higher values in the rainier year (2021) and significant increases determined by the treatment with chitosan (especially in 2021 when it increased by 97% as compared to the case of Bordeaux mixture treatment). Catechin and epicatechin also recorded important increases in 2021, with confirmed increases in both years elicited by the chitosan treatment. Flavonols, represented by quercetin and myricetin, were not influenced by the chitosan treatment, but the meteorological conditions determined increases corelated with the higher temperatures and inversely corelated to the amount of rainfall. Trans-resveratrol ranged between 4.3-8.0 mg L^{-1} in 2020 and 5.0-6.5 mg L^{-1} in 2021, with an important increase determined by the chitosan treatment in 2020 (89% increase compared to Bordeaux mixture treatment).

Authors' Contributions

Conceptualization, A.O.A.; methodology, A.O.A.; investigation V.A., A.R. E.I.G. and A.O.A.; software and formal analysis A.O.A., V.A.; resources V.A., A.R. and A.O.A.; data curation, A.O.A.; writing—original draft preparation A.O.A.; graphic preparation V.A., A.O.A; writing—review and editing, A.O.A.; supervision, A.O.A.

All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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