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Improving Grape Phenolic Content and Wine Chromatic Characteristics through the Use of Two Different Elicitors: Methyl Jasmonate versus Benzothiadiazole

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ABSTRACT: Benzothiadiazole (BTH) and methyl jasmonate (MeJ) have been described as exogenous elicitors of some plant defense compounds, polyphenols among them. The objective of this study was to determine whether the application of BTH or MeJ to grape clusters at the beginning of the ripening process had any effect on the accumulation of the main flavonoid compounds in grapes (anthocyanins, flavonols, and flavanols) and the technological significance of these treatments in the resulting wines. The results obtained after a 2 year experiment indicated that both treatments increased the anthocyanin, flavonol, and proanthocyanidin content of grapes. The wines obtained from the treated grapes showed higher color intensity and total phenolic content than the wines made from control grapes. The exogenous application of these elicitors, as a complement to fungicide treatments, could be an interesting strategy for vine protection, increasing, at the same time, the phenolic content of the grapes and the resulting wines.

KEYWORDS: *grape, wine, anthocyanins, tannins, flavonols, BTH, methyl jasmonate*

INTRODUCTION

Phenolic compounds are very important in crop plants and have been the subject of a large number of studies. Three main reasons can be cited for optimizing the level of phenolic compounds in crop plants: their physiological role in plants, their technological significance for food processing, and their nutritional characteristics.

In plants, phenolic compounds contribute significantly to plant resistance against pests, pathogens, and environmental stress; they are effective as sun screens as well as antifeeding compounds; they may function as antioxidants and interact with growth regulators.[1] Moreover, some of them present antimicrobial activity and are involved in inducible resistance against pathogens, and their concentration in plant tissues may increase markedly as part of this resistance phenomenon.[2,3] This resistance process, mediated by the accumulation of endogenous salicylic acid (SA), a metabolite downstream of the biosynthetic pathway initiated by phenylalanine ammonialyase (PAL), is called systemic acquired resistance (SAR) and implies the induction of secondary metabolic pathways and the increased synthesis of products as a result of this metabolism, including phenolic compounds,[3,4] as a response to pathogen attack.

In winegrapes, the technological importance of phenolic compounds, especially flavonoids, is well-known. They are responsible for the color of wines, especially anthocyanins (colored pigments responsible for the chromatic characteristics of red wines), proanthocyanidins (responsible for the long-term stability of red wine color), and flavonols (compounds that may influence wine color through copigmentation), and some other organoleptic properties such as astringency, bitterness, and body.

Another important aspect that has been widely studied in recent years is the role of grape and wine phenolic compounds in the human diet. Many studies have suggested cardiovascular benefits, and some point to cancer chemopreventive activity and beneficial effects against other less prevalent but devastating illnesses, such as Alzheimer's disease and urinary bladder dysfunction.[5−8] Most of these beneficial functions may arise from their antioxidant action, which may occur through a combination of several distinct chemical events, including enzyme inhibition, metal chelation, hydrogen donation from suitable groups, and oxidation to a nonpropagating radical.[9−11]

Taking all this into account and although genetic factors play an important role in the phenolic compound content of grapes, several approaches have been proposed for improving the phenolic content of crop plants, in general, and winegrapes in particular. Beside genetic transformation (forbidden in most countries), a wide range of factors is able to modify the grape phenolic content, including agronomic practices, clonal selection, and those stress factors that may trigger SAR establishment.[11] However, it has been demonstrated that SAR can also be induced or enhanced by the exogenous application of natural or synthetic compounds that may have powerful effects.[12]

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Two examples of such compounds are benzothiadiazole (BTH) and methyl jasmonate (MeJ). BTH is a synthetic functional analogue of the plant endogenous hormonelike compound SA, which induces the defense genes leading to SAR establishment and an increase in phenolic production.[4] Jasmonic acid and MeJ are naturally occurring plant growth regulators that modulate chlorophyll degradation and anthocyanin biosynthesis. MeJ has been mainly implicated as a mediator in plant responses, triggered by wounding and insect feeding, and is involved in resistance against pathogens.[13]

Enzymes of the phenylpropanoid biosynthetic pathway (phenylalanine ammonia lyase and chalcone isomerase) were observed to accumulate after the application of exogenous BTH[13] and MeJ,[14] since the induction of secondary metabolite accumulation is an important stress response and jasmonates and SA (or its analogues) function as necessary signaling molecules.[15]

Both compounds have previously been used to increase both plant resistance to pathogen attacks and their phenolic compound content. In strawberries, Hukkanen et al.[16] studied the effect of BTH on the accumulation of phenolics and the improved resistance to powdery mildew. Cao et al.[17] studied the effect of BTH on the anthocyanin content and activities of related enzymes in strawberry after harvest. In grapes, studies have demonstrated an increase in anthocyanin[4] and proanthocyanidin[3] contents after the application of BTH, accompanied by increased resistance to *Botrytis* attack. Similarly, the application of MeJ to strawberries induced anthocyanin biosynthesis.[18]

Given all of these previous findings, our objective was to test, over a period of 2 consecutive years, if the treatment of vines with BTH and MeJ in the field, at the moment of véraison, affected the accumulation of the main flavonoid compounds (anthocyanins, flavonols, and flavanols) in grapes and in their resulting wines, looking forward to obtaining wines with an improved color and organoleptical characteristics.

MATERIALS AND METHODS

Plant Material and Open Field Treatments. Treatments were carried out in an experimental vineyard at Bullas (Murcia, SE, Spain) in 2009 and 2010. The study was performed on 6 year old *Vitis vinifera* L. Monastrell (syn. Mourvedre) red wine grapevines grafted onto R110 rootstock. A bilateral cordon training system trellised to a three-wire vertical system was used. Vine rows ran N-NW to S-SE, and the planting density was 3 m between rows and 1.25 m between vines. Six two-bud spurs (12 nodes) per vine were retained at pruning. The vineyard was drip-irrigated.

All treatments were applied to three replicates and were arranged in a complete randomized block design, with 10 vines for each replication. Plants were sprayed, at the beginning of veraison and 3 and 6 days after the first application, with a water suspension of BTH ([benzo-(1,2,3)-thiadiazole-7-carbothioic acid *S*-methyl ester], Sigma Aldrich, St. Louis, MO) at a concentration of 0.3 mM. MeJ (Sigma Aldrich) was applied at a concentration of 10 mM. In both treatments, Tween 80 (Sigma Aldrich) was used as a wetting agent. Control plants were sprayed with a water suspension of Tween 80 alone. When grapes reached optimum maturity, they were harvested and transported to the winery in 20 kg boxes. For chemical analysis of the polyphenolic compounds, five mature clusters per plant were randomly collected at harvest from treated and untreated grapevines. Clusters were immediately transported to the laboratory and frozen at −20 °C until analysis.

Vinifications. The grapes were crushed and destemmed and sulfited (8 g of SO_2/100 kg of grapes). The total acidity was corrected to 5.5 g/L, and selected yeasts were added (Laffort, DSM, Servian, France, 10 g of dry yeast/100 kg of grapes). All of the vinifications were conducted in triplicate, in 100 L tanks, at 25 ± 1 °C. Throughout the fermentative pomace contact period (10 days for all vinifications), the cap was punched down twice a day, and the temperature and must density were recorded. At the end of this period, the wines were pressed at 1.5 bar in a 75 L tank membrane press. Free-run and press wines were combined and stored at room temperature. One month later, the wines were racked and analyzed.

Physicochemical Determinations in Grapes. Grape analysis involved the traditional flesh measurements. Total soluble solids (°Brix) were measured using a digital refractometer (Atago RX-5000). Titratable acidity and pH were measured using an automatic titrator (Methrom, Herisau, Switzerland) with 0.1 N NaOH. Tartaric and malic acids were measured using enzymatic kits from Boehringer Mannheim GmbH (Mannhein, Germany). The methodology for carrying out these analyses is described in EEC regulation no. 2676/90.

Anthocyanins and Flavonols in Grapes and Wines. Grapes were peeled with a scalpel, and the skins were stored at −20 °C until analysis. Samples (2 g) were immersed in methanol (40 mL) in hermetically closed tubes and placed on a stirring plate at 150 rpm and 25 °C. After 4 hours, the methanolic extracts were filtered through 0.45 μm nylon filters (OlimPeak, Tecknochroma, Barcelona, Spain) and analyzed by high-performance liquid chromatography (HPLC). Samples of wines were filtered through the 0.45 μm nylon filters and directly analyzed by HPLC.

The HPLC analyses were performed on a Waters 2690 liquid chromatograph (Waters, PA), equipped with a Waters 996 diode array detector and a Lichro Cart RP-18 column (Merck, Darmstadt, Germany), 25 cm × 0.4 cm, 5 μm particle size, using as solvents HPLC-grade water plus 5% formic acid (T. J. Baker, Denventer, The Netherlands) as solvent A and HPLC-grade methanol (T. J. Baker) as solvent B, at a flow rate of 1 mL/min. Elution was performed as previously described by Bautista-Ortín et al.[19] Chromatograms were recorded at 360 (flavonols) and 520 nm (anthocyanins). Data obtained were processed using the Waters EmpowerPro software (Waters, Milford, MA).

Identification of the compounds was carried out by comparison of their UV spectra recorded with the diode array detector and those reported in the literature. An HPLC-MS analysis was conducted to confirm the identity of each peak using an LC-MSD-Trap VL-01036 liquid chromatograph-ion trap mass detector (Agilent Technologies, Waldbronn, Germany) equipped with electrospray ionization (ESI). Elution was performed in the HPLC analysis conditions described above. The heated capillary and voltage were maintained at 350 °C and 4 kV, respectively. Mass scans were measured from m/z 100 up to m/z 800. Mass spectrometry data were acquired in the negative ionization mode and processed using Data Analysis 2.1 LC/MSD Trap software (Agilent Technologies). Anthocyanins were quantified at 520 nm as malvidin 3-*O*-glucoside, using malvidin 3-*O*-glucoside chloride as external standard (Extrasynthèse, Genay, France). Flavonols were quantified at 360 nm using quercetin (Sigma Aldrich) as an external standard.

Determination of Proanthocyanidins in Grapes and Wines. The seeds and skins of 10 berries were separated from the mesocarp and rinsed with distilled−deionized water. Whole seeds and skins, previously ground to a powder with liquid nitrogen, were extracted separately in covered Erlenmeyer flasks with 10 mL of 2:1 acetone/water at room temperature for 24 h on an orbital shaker at 200 rpm. To minimize proanthocyanidin oxidation, solutions were spurged with nitrogen, and the extraction was carried out in the dark. Following extraction, the extract was concentrated under reduced pressure at 35 °C to remove acetone and then lyophilized to a dry powder. This powder was redissolved in 1 mL of methanol in a volumetric flask.

Skin and seed proanthocyanidins were determined according to the method described by Kennedy and Jones[20] with some modifications, as follows. A solution of 0.2 N HCl in methanol, containing 100 g/L phloroglucinol and 20 g/L ascorbic acid, was prepared (phloroglucinolysis reagent). The methanolic extract was reacted with the phloroglucinolysis reagent (1:1) in a water bath for 20 min at 50 °C

and then combined with 2 volumes of 200 mM aqueous sodium acetate to stop the reaction.

For wines, the samples were prepared by an optimization of the method described by Pastor del Río et al.[21] Wine (5 mL) was evaporated in a centrivap concentrator (Labconco, United States), redissolved in 3 mL of water, and then passed through a C18-SPE column (1 g, Waters), previously activated with 10 mL of methanol followed by 20 mL of water. The cartridge was washed with 20 mL of water, and the compounds of interest were eluted with 10 mL of methanol, evaporated, and then dissolved in 1 mL of methanol. Phloroglucinolysis was then carried out as described above.

HPLC analysis followed the conditions described by Ducasse et al.[22] The HPLC apparatus used was a Waters 2695 system (Waters) equipped with an autosampler system and a Waters 2996 photodiode array detector. Samples (10 μL injection volume) were injected on a Atlantis dC18 column (250 mm × 4.6 mm, 5 μm packing) protected with a guard column of the same material (20 mm × 4.6 mm, 5 μm packing) (Waters). The elution conditions were as follows: 0.8 mL/min flow rate; oven temperature, 30 °C; solvent A, water/formic acid (98:2, v/v); and solvent B, acetonitrile/solvent A (80:20 v/v). Elution began with 0% B for 5 min, linear gradient from 0 to 10% B in 30 min, and gradient from 10 to 20% in 30 min, followed by 7 min of column washing and 20 min of re-equilibration.

Proanthocyanidin cleavage products were estimated using their response factors relative to (+)-catechin, which was used as the quantitative standard. These analyses allowed the total proanthocyanidin content, the apparent mean degree of polymerization (mDP), and the percentage of each constitutive unit to be determined. The mDP was calculated as the sum of all subunits (flavan-3-ol monomer and phloroglucinol adducts, in moles) divided by the sum of all flavan-3-ol monomers (in moles).

Color Determinations in Wines. Absorbance measurements were made in a Shidmazu UV-1603 spectrophotometer (Shimadzu Deutschland GmbH) with 0.2 cm path length glass cells. The color density (CI) was calculated as the sum of absorbance at 620, 520, and 420 nm, and tint was calculated as the ratio between absorbance at 420 and 520 nm.[23] The total phenol content (TP_{wine}) and total anthocyanins were spectrophotometrically measured as described in Ribéreau Gayon et al.[24] The CIELab parameter L^* (lightness) was determined by measuring the transmittance of the wine every 10 nm from 380 to 770 nm, using the D65 illuminant and a 10° observer.

Sensory Analysis. Wines elaborated in 2010 were subjected to a sensory triangular test: Nine staff members, selected on the basis of their availability and interest in the project, were presented with three samples, two of which were identical. Samples were presented in random order in coded, clear, 125 mL official glasses. Each assessor selected the sample that he/she considered different (forced election), and they were also asked to indicate which sample was preferred. The statistical significance of the number of correct judgments versus the total number of judgments was subsequently determined. Following the normative of AENOR (UNE 87006:1992, ISO 4120:1983), assessors may not have much expertise, and so, one previous session was devoted to training in triangular analysis.

Statistical Data Treatment. Significant differences among wines and for each variable were assessed by analysis of variance (ANOVA) using Statgraphics 5.0 Plus. The LSD test was used to separate the means ($p < 0.05$) when the ANOVA test was significant.

RESULTS AND DISCUSSION

Physicochemical Composition of Grapes. Table 1 shows the physicochemical composition of control and treated grapes at the moment of harvest. Comparing the grapes from both years, the berries from 2010 were larger, with higher sugar content and higher total acidity, basically due to their higher malic acid content. Different weather conditions occurred from July to the end of September of both years (data not shown). Maximum temperatures were higher in 2009 (2−3 °C higher) and less rain fell, whereas in 2010, from July to the end of September, more rain was accumulated, especially during August (6 mm in 2009 vs 106 mm in 2010). The harvest date was September 24th in 2009 and 1 week later in 2010. The different climatic data could have influenced grape maturation and the physicochemical composition of grapes as suggested by some of the results that will be shown later on this study.

Table 1. Physicochemical Characteristics of the Grapes at the Moment of Harvest

	weight 100 berries	Brix	total acidity (g/L)	pH	g/L tartaric acid	g/L malic acid
			2009			
control	128.3 a[a]	22.6 ab	2.5 a	3.8 a	4.1 a	1.5 a
BTH	120.7 a	22.9 b	3.1 b	3.8 a	4.4 ab	1.2 a
MeJ	128.5 a	22.0 a	3.0 b	3.9 a	4.6 b	1.2 a
			2010			
control	187.1 a	23.8 a	3.7 a	3.6 a	3.9 a	2.3 a
BTH	206.1 b	25.0 ab	3.5 a	3.7 ab	4.3 a	2.3 a
MeJ	188.0 a	25.8 b	3.6 a	3.8 b	4.5 a	2.5 b

[a]Different letters in the same column indicate significant differences according to LSD test ($p < 0.05$).

With regard to the effect of the treatments, no effect was observed on berry weight in 2009. The sugar content of treated grapes did not differ from control grapes, although BTH-treated grapes presented a higher sugar content than MeJ-treated grapes. The total acidity was slightly higher ($p < 0.05$) in the grapes from both treatments as compared to control grapes. In 2010, the BTH- and MeJ-treated grapes showed a slightly higher sugar content than the control grapes (although only MeJ-treated grapes significantly differed) and similar acidity to the control grapes. BTH-treated clusters also presented larger berries, although, in general, differences were slight.

Several studies have been carried out to see whether such treatments, especially the application of BTH, have any effect on the vegetative and productive parameters of plants. BTH application has been negatively correlated with crop yield in wheat and cauliflower.[25] In beans, the results showed that seed production was slightly lower in BTH-treated plants, although differences were not significant.[26] Fumagalli et al.[2] did not detect any adverse effect in viticultural parameters following BTH treatment.

Grape Anthocyanins and Flavonols. The concentration of anthocyanins in the studied grapes is shown in Table 2. All five anthocyanins (the dihydroxylated cyanidin and peonidin 3-*O*-glucosides and the trihydroxylated delphinidin, petunidin, and malvidin 3-*O*-glucosides), together with their acylated derivatives (acetates, caffeates, and coumarates) were detected in Monastrell grapes. Malvidin 3-*O*-glucoside was the anthocyanin present at the highest concentration, although Monastrell grapes are also characterized by a relatively large concentration of dihydroxylated anthocyanis, as demonstrated in other studies.[27]

As regard flavonols (Table 3), we identified mono- (kaempferol), di- (quercetin and isorhamnetin), and trihydroxylated (myricetin and syringetin) flavonol glycosides (glucosides and glucoronides and small quantities of galactosides), although at a much lower concentration than observed for anthocyanins. Quercetin derivatives were, quantitatively, the most important flavonols in Monastrell grapes.

Table 2. Concentration of Anthocyanins in Berries Treated with BTH and MeJ

	2009			2010		
anthocyanins (μg/g skin)	control	BTH	MeJ	control	BTH	MeJ
Del[a]	660.0 a[b]	739.5 b	785.7 b	258.0 a	454.9 b	655.3 c
Cyan	436.8 a	429.0 a	476.5 a	322.2 a	639.8 c	564.9 b
Pet	1081.0 a	1280.8 b	1258.4 b	1043.0 a	1081.5 b	1629.1 c
Pn	485.9 a	515.8 a	478.4 a	485.0 a	534.4 b	823.1 c
Malv	2703.2 a	3088.5 b	2880.5 ab	2788.9 a	2875.7 b	4544.7 c
total nonacylated	5367.0 a	6053.7 b	5879.4 b	4897.1 a	5586.3 b	8217.0 c
Del Ac	48.2 a	55.6 ab	63.8 b	17.6 a	28.5 b	53.7 c
Cyan Ac	35.9 a	36.6 a	42.6 b	25.2 a	36.4 b	49.4 c
Pet Ac	76.5 a	84.6 ab	90.2 b	42.6 a	55.3 a	100.5 b
Pn Ac	45.7 a	50.2 b	50.6 b	48.1 a	83.5 b	86.1 b
Malv Ac	258.6 a	299.0 b	302.9 b	104.3 a	224.5 b	201.3 b
total Ac	464.9 a	526.0 b	550.2 b	237.8 a	428.2 b	491.0 c
Del Coum	196.8 a	239.0 c	217.9 b	73.7 a	95.9 a	149.8 b
Mal Caf	102.9 b	93.4 ab	82.8 a	44.6 a	109.5 b	192.8 c
Cyan Coum	148.6 a	165.6 b	156.8 ab	40.3 a	134.0 b	161.4 c
Pet Coum	311.0 a	375.9 b	326.3 a	37.1 a	32.7 a	67.5 b
Pn Coum	157.6 a	181.4 b	161.2 a	121.5 a	161.9 b	264.8 c
Malv Coum cis	66.6 a	82.7 c	74.9 b	21.8 a	20.5 a	85.8 b
Malv Coum trans	1073.1 a	1311.4 b	1144.1 a	269.8 a	490.4 b	799.9 c
total Coum	2056.6 a	2449.5 b	2164.1 a	564.2 a	935.4 b	1529.1 c
total acylated	2521.5 a	2975.5 b	2714.2 a	846.6 a	1473.1 b	2212.9 c
total anthocyanins (μg/g skin)	7895.6 a	9037.5 b	8594.6 b	5743.7 a	7059.4 b	10429.9 c
total anthocyanins (mg/kg grapes)	934.6 a	1072.1 b	1086.9 b	666.5 a	823.9 c	773.5 b

[a]Abbreviations: Del, delphinidin 3-*O*-glucoside; Cyan, cyanidin 3-*O*-glucoside; Pet, petunidin 3-*O*-glucoside; Pn, peonidin 3-*O*-glucoside; Malv, malvidin 3-*O*-glucoside; Ac, acetylglucosides; Cum, coumarylglucosides; and Caf, caffeate glucoside. [b]Different letters in the same row and for each year indicate significant differences according to the LSD test ($p < 0.05$).

Table 3. Concentration of Flavonols in Grape Berries Treated with BTH and MeJ

	2009			2010		
flavonols (μg/g skin)	control	BTH	MeJ	control	BTH	MeJ
M-3-glc[a]	27.1 a[b]	33.1 b	27.9 a	5.5 a	9.2 b	11.5 b
Q-3-glc	53.1 ab	57.4 b	49.0 a	18.0 a	23.7 b	38.0 c
K-3-gal	2.9 a	3.1 a	3.0 a	1.1 a	3.8 a	2.8 a
K-3-glc + S-3-glc	11.2 a	12.1 a	11.4 a	2.0 a	2.4 a	4.7 b
I-3-glc	1.3 b	1.4 b	1.0 a	1.3 a	4.9 a	4.8 a
Q-3-glcU	25.0 a	34.2 b	28.7 a	1.6 a	2.1 a	6.6 a
total flavonols (μg/g skin)	120.5 a	141.3 b	120.9 a	29.5 a	46.2 b	68.4 c
total flavonols (mg/kg grapes fresh weight)	14.2 a	16.7 b	15.4 ab	3.4 a	5.4 b	5.1 b

[a]Abbreviations: M, myricetin; Q, quercetin; K, kaempferol; I, isorhamnetin; glc, *O*-glucoside; gal, *O*-galactoside; and glcU, *O*-glucuronide. [b]Different letters in the same row and for each year indicate significant differences according to the LSD test ($p < 0.05$).

The total concentration of anthocyanins and flavonols was higher in grapes during the 2009 vintage than in 2010. This was to be expected since the higher amount of rain that occurred during 2010 would have diluted the skin phenolic content.

Both treatments increased the skin anthocyanin content both years (as compared with control grapes). The increases ranged from 8 to 14% for MeJ- and BTH-treated grapes in 2009 and were higher in 2010 (up to 81% for MeJ-treated grapes). Fumagalli et al.[2] suggested that BTH could enhance phenylalanine amonnia lyase and chalcone synthase activity since they also found an increase in grape skin anthocyanin content when grapes were treated with BTH. Similar results were found by Iriti et al.[4] Cao et al.[17,28] also found similar results in treated strawberries, where they demonstrated that the enzymes related to anthocyanin metabolism were activated by the application of BTH. It has also been demonstrated that the application of MeJ promoted an accumulation of enzymes of the phenylpropanoid pathway.[29] Mukkun and Singh[18] stated that MeJ modulates anthocyanin formation since its application in immature strawberries increased anthocyanin biosynthesis.

Flavonols are very close to anthocyanins in the biosynthetic pathway; indeed, they share most of the pathway, so that an increase in the activity of enzymes upstream in the flavonoid biosynthetic pathway may also affect the concentration of these compounds. Our results confirmed this statement since both years the BTH-treated grapes showed higher flavonol concentrations as did MeJ-treated grapes in 2010. Less information is available concerning the effect of these treatments on flavonols, and only Wang et al.[30] described an increase of flavonols in different fruits with the use of MeJ.

As with anthocyanins, the increases (expressed as percentages) were higher in 2010 than in 2009 (56 vs 17% for BTH-treated grapes and 131% for MeJ-treated grapes). Although the concentrations of flavonoids were higher in 2009 grapes, it

Table 4. Concentration and Composition of Skin Proanthocyanidins in the Grape Berries Treated with BTH and MeJ

	2009			2010		
total tannins	C[a]	BTH	MeJ	C	BTH	MeJ
μg/g of skin	3590.9 a[b]	4295.2 b	4937.7 c	2839.9 a	3401.4 b	5307.3 b
μg/berry	529.2 a	555.7 a	689.6 b	576.6 a	803.3 ab	862.2 b
mg/kg[c]	425.3 a	455.7 a	571.9 b	318.3 a	436.4 b	415.6 b
mDP	17.1 a	18.5 a	18.5 a	11.6 a	14.9 b	15.2 b
%G[d]	2.0 ab	2.2 b	1.7 a	0.9 a	0.8 a	0.8 a
%tCat	3.5 a	3.6 a	3.4 a	5.5 c	4.6 b	4.0 a
%tECat	1.9 a	1.9 a	3.0 b	3.2 c	2.1 a	2.5 b
%tECatG	0.1 b	0.1 b	0.0 a	ND	ND	ND
%extCat	1.5 ab	1.6 b	1.4 a	1.4 b	1.3 b	1.0 a
%extECat	66.7 b	66.7 b	63.5 a	63.5 a	66.0 b	64.2 ab
%extECatG	1.9 ab	2.1 b	1.6 a	0.9 a	0.8 a	0.8 a
%extEgCat	24.5 a	24.0 a	27.0 b	25.6 a	25.2 a	27.4 a

[a]C, control. [b]Different letters in the same line indicate significant differences according to the LSD test ($p < 0.05$). [c]mg/kg, mg of skin proanthocyanidins per kg of grapes (fresh weight). [d]%G, percentage of galloylation; %tCat, percentage of terminal (+)-catechin; %tECat, percentage of terminal (−)-epicatechin; %tECatG, percentage of terminal (−)-epicatechin gallate; %extCat, percentage of extension (+)-catechin; %extECat, percentage of extension (−)-epicatechin; %extEgCat, percentage of extension epigallocatechin; %extECatG, percentage of extension (−)-epicatechin gallate.

Table 5. Concentration and Composition of Seed Proanthocyanidins in the Berries Treated with BTH and MeJ

	2009			2010		
total tannins	C[a]	BTH	MeJ	C	BTH	MeJ
μg/g of seed	35679.1 a[b]	43803.6 b	38374.1 ab	26913.0 a	18839.7 a	22679.2 a
μg/berry	2971.5 a	3278.0 a	2961.0 a	1732.4 a	1546.6 a	1658.9 a
mg/kg[c]	2168.6 a	2746.6 b	2479.9 ab	963.6 a	735.8 a	797.5 a
mDP	7.4 a	7.2 a	7.1 a	7.8 a	8.3 a	8.3 a
%G[d]	14.9 b	14.2 a	14.3 a	16.1 a	16.4 a	16.3 a
%tCat	5.0 a	5.2 a	5.2 a	4.7 a	4.3 a	4.4 a
%tECat	6.3 a	6.2 a	6.2 a	4.4 a	4.0 a	4.2 a
%tECatG	2.6 a	2.6 a	2.7 a	3.7 a	3.8 a	3.7 a
%extCat	7.3 ab	7.6 b	7.1 a	7.9 a	7.7 a	7.7 a
%extECat	66.5 a	66.8 ab	67.2 b	66.9 a	67.6 a	67.4 a
%extECatG	12.3 b	11.6 a	11.6 a	3.7 a	3.8 a	3.7 a

[a]C, control. [b]Different letters in the same line indicate significant differences according to the LSD test ($p < 0.05$). [c]mg/kg, mg of seed proanthocyanidins per kg of grapes (fresh weight). [d]%G, percentage of galloilation; %tCat, percentage of terminal (+)-catechin; %tECat, percentage of terminal (−)-epicatechin; %tECatG, percentage of terminal (−)-epicatechin gallate; %extCat, percentage of extension (+)-catechin; %extECat, percentage of extension (−)-epicatechin; %extECatG, percentage of extension (−)-epicatechin gallate.

seems that the effect of treatments was more significant in 2010, especially in the grapes treated with MeJ, in which the increases ranged from 81% for anthocyanins to 131% for flavonols. The lower temperatures and higher humidity of 2010 may well have provided suitable conditions for pathogen development. Gozzo[13] stated that treatment with elicitors primed the plant to react more efficiently (especially with regard to the activation of the phenyl propanoid pathway) when challenged with a pathogen, which could explain the greater increases in anthocyanins and flavonols in the 2010 grapes.

Grape Skin and Seed Proanthocyanidins. Proanthocyanidins, commonly known as tannins, are flavonoid compounds found in grape skin and seeds. They play an important role in red wine quality since they are responsible for the complex properties related with wine mouthfeel, such as bitterness, hardness, dryness, astringency, structure, and body, and participate in reactions with wine anthocyanins, favoring wine color stability with time.

As previously observed for anthocyanins and flavonols, higher concentrations of skin proanthocyanidins were observed for all grapes (control and treated grapes) in 2009 (Table 4). Both treatments increased skin proanthocyanidins (expressed as μg/g of skin) although in 2009, only MeJ-treated grapes differed from control grapes when results were expressed as mg/kg. Again, when expressed as percentages, the increases observed in the skin proanthocyanidin concentration due to the treatments were higher in 2010 (22% for BTH-treated grapes and up to 86% for MeJ-treated grapes), again suggesting that a pathogen challenge this year may have alerted the plants to respond rapidly to this stress situation due to the treatment with the elicitors.

The degree of polymerization of these molecules (mDP), the percentage of galloylation, and percentage of composition of terminal and extension subunits have also been studied. Experimental evidence shows that mDP and the percentage of galloylation are important proanthocyanidin structural variables that have been positively correlated with wine astringency.[31,32] The values of mDP were higher in treated grapes, although differences were not significant in 2009. The percentage of galloylation did not differ from control grapes, and only small differences were observed in the percentage of

composition in tannins from control and treated grapes. This study is the first report on the effect of MeJ on grape proanthocyanidins, whereas the increase of proanthocyanidins in BTH-treated grapes was first described by Iriti et al.,[3] who found that grape proanthocyanidin levels increased by 36% after BTH treatment. These authors stated that the higher concentration of proanthocyanidins due to BTH would be part of the plant resistance mechanisms stimulated by BTH. Among other effects, proanthocyanidins inhibit the activity of the hydrolytic enzymes secreted by *Botrytis cinerea*, constraining its development and preventing tissue maceration. The increase of the mDP observed, especially in 2010, is another possible effect of the elicitors, since polymeric proanthocyanidins are reported to be more effective in inhibiting the activity of certain fungal enzymes than less polymerized proanthocyanidins.[33]

In seeds, in 2009, the tannin concentration was higher in treated grapes, whereas in 2010, the seed tannin content did not differ between treated and control grapes (Table 5).

Wine Chromatic and Sensory Characteristics. From a technological point of view, our main interest was to check whether the results observed in the treated grapes were also reflected in the corresponding wines, without forgetting that weather may have an even bigger influence on a wine's chromatic and phenolic characteristics. It was observed that wines differed each year, the color intensity, total anthocyanins (monomeric and polymeric anthocyanins that absorb at 520 nm), and total phenols being higher in 2009 wines (Table 6).

Table 6. Wine Chromatic Characteristics

	L^*	TA[a]	TP_{wine}[b]	CI[c]	tint
		2009			
control	11.1 b[d]	470.1 a	43.0 a	17.0 a	0.4 a
BTH	7.1 a	535.5 b	49.6 c	20.6 c	0.5 a
MeJ	9.7 b	519.6 b	46.0 b	18.7 b	0.4 a
		2010			
control	20.7 b	310.2 a	32.9 a	9.5 a	0.5 a
BTH	18.7 ab	341.1 ab	36.7 b	10.3 b	0.5 a
MeJ	17.4 a	369.8 b	39.0 b	10.8 b	0.6 b

[a]TA, total anthocyanidins (spectrophotometrically measured). [b]TP, total phenols (measured as optical density at 280 nm). [c]CI, wine color intensity. [d]Different letters in the same column indicate significant differences according to the LSD test ($p < 0.05$).

With regard to the effect of treatments, the positive effects of BTH and MeJ observed in the grapes were reflected in the wines. In 2009, all chromatic data were significantly higher in the wines elaborated from BTH- and MeJ-treated grapes, while L^* was significantly lower (indicating darker wines), the highest color intensity and total phenol content (TP_{wine}) being observed in wines from BTH-treated grapes. In 2010, color intensity and TP_{wine} were also significantly higher in wines from treated grapes, the wine from the MeJ-treated grapes showing the most pronounced differences from the control wine.

Also, individual wine phenolic compounds were analyzed by HPLC (Table 7). Total monomeric anthocyanins were higher in the 2009 wines than in the corresponding wines from 2010, with no differences due to treatments in 2009, whereas the anthocyanin concentration was higher (as compared to control) in wines from MeJ-treated grapes in 2010. The differences observed between spectrophotometrically measured (Table 6) and HPLC-measured (Table 7) total anthocyanins are due to the analytical method. The total content of individual monomeric anthocyanins (as quantified by HPLC) in wines does not necessarily correlate with wine absorbance at 520 nm (absorbance at which total anthocyanins are spectrophotometrically measured), since reactions with other phenolic compounds and the formation of derived pigments that also absorb at 520 nm may explain the observed differences.

The concentration of wine flavonols showed only small differences due to the grapes treatments, the differences only being significant for the wines from the BTH treated wines in 2009. Surprisingly, the concentration of tannins was higher in 2010 wines, even though their concentration in grapes was lower in 2010 than in 2009. Additionally, the percentage of galloylation was lower and the percentage of epigallocatechin subunit higher in 2010 wines as compared with 2009 wines. Because epigallocatechin is only present in the grape skins, a higher percentage of this subunit indicates a more intense extraction of skin tannins in 2010 wines.[34] One explanation of these findings might be differences in the ease with which phenolics are extracted from grape skins and seeds into musts, which may change due to climatic conditions during berry development and with the ripening stage of the grape at the moment of harvest. Extractability may increase in more mature grapes, and grapes from 2010 were more ripe than those of 2009.

With regard to the effect of treatments, wines from BTH-treated grapes showed a higher tannin content both years than control wines, as did the wine from MeJ-treated grapes in 2010. This could be of interest since a trend toward higher grade allocation (related with market value) was observed when wines had higher phenolic and tannin content.[35]

In light of the chromatic differences observed in 2009 wines between the control wine and the wines from treated grapes, it

Table 7. Concentration and Composition of Wine Flavonoids (as Determined by HPLC Analysis) Made with Grape Berries Treated with BTH and MeJ

	2009			2010		
	C[a]	BTH	MeJ	C	BTH	MeJ
total tannins (mg/L)	170.5 a[b]	270.5 b	145.9 a	224.2 a	296.7 b	256.4 b
mDP	6.6 c	5.4 b	4.2 a	5.9 b	5.2 a	5.2 a
%G[c]	5.0 a	4.5 a	6.0 b	3.1 b	2.9 a	2.9 a
%extEgCat	18.1 b	18.4 b	16.4 a	19.4 a	19.8 a	20.3 a
total anthocyanins	431.5 a	481.0 a	458.6 a	257.5 a	331.6 ab	448.9 b
total flavonols	54.5 a	68.5 b	53.4 a	51.7 a	52.6 a	54.3 a

[a]C, control. [b]Different letters in the same line indicate significant differences according to the LSD test ($p < 0.05$). [c]%G, percentage of galloylation; and %extEgCat, percentage of extension epigallocatechin.

was decided to perform a sensory triangle test with the 2010 wines to determine whether differences were discernible between wines and whether differences (if any) improved or diminished the wine sensory quality. The results of the triangle test (Table 8) demonstrated that all of the wines could be differentiated ($p < 0.05$), and a clear preference for wines elaborated from treated grapes was observed, when compared with the control wine. Although the panelist could also differentiate wines from BTH- and MeJ-treated grapes, no clear preferences were observed. Whatever the case, we have to be aware that the information on preferred samples might not be extrapolable to preferences in a wider population or under less-controlled situations.

Table 8. Results of the Triangle Test Performed by Nine Panelists in 2010 Wines

sample	no. of correct answers	preferred sample
C-BTH	9[a]	BTH (7)[b]
C-MeJ	8[a]	MeJ (7)
BTH-MeJ	7[c]	MeJ (4)

[a]Significance level, 1%. [b]The number between parentheses gives the number of panelists (among those that have correctly identified the different sample) that have chosen the indicated wine sample. Samples codes: C, control wine; BTH, wine from BTH-treated grapes; and MeJ, wine from MeJ-treated grapes. [c]Significance level, 5%.

In conclusion, the results obtained for the 2 year experiment, taking into account the differences in climatic conditions, showed that both elicitors increased grape flavonoids in both years. Although BTH and MeJ activate different signal transduction pathways to promote the plant defense mechanisms,[8] our findings mean that both of them activated phenylalanine ammonia lyase and the phenyl propanoid pathway, increasing grape phenolic content. This increase may be important for the plant since a reduced incidence and severity of gray mold infection is one of the most outstanding consequences of the use of exogenous elicitors, as demonstrated in several studies. Taking into account that MeJ is a naturally occurring plant metabolite and BTH was seen to be completely translocated and degraded in plant tissues, and therefore, no persistence or residue problems are expected,[4] both products could be considered an interesting strategy to protect the vine, as an alternative or complement to fungicide treatments, increasing, at the same time, the phenolic content of the grapes.

From a technological point of view, the results demonstrated that the wines obtained from the treated grapes showed higher color intensity and total phenolic content than the wines made from control grapes and were sensorially preferred, indicating that these treatments could be of interest to obtain wines with a deep and stable color and a potentially higher market value.

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REFERENCES

(1) Treutter, D. Managing phenol contents in crop plants by phytochemical farming and breeding. Visions and constraints. *Int. J. Mol. Sci.* **2010**, *11*, 807−857.

(2) Fumagalli, F.; Rossoni, M.; Iriti, M.; di Gennaro, A.; Faoro, F.; Borroni, E.; Borgo, M.; Scienza, A.; Sala, A.; Folco, G. From field to health: A simple way to increase the nutraceutical content of grape as shown by no-dependent vascular relaxation. *J. Agric. Food Chem.* **2006**, *54*, 5344−5349.

(3) Iriti, M.; Rossoni, M.; Borgo, M.; Ferrara, L.; Faoro, F. Induction of resistance to gray mold with benzothiadiazole modifies amino acid profile and increases proanthocyanidins in grape: Primary versus secondary metabolism. *J. Agric. Food Chem.* **2005**, *53*, 9133−9139.

(4) Iriti, M.; Rossoni, M.; Borgo, M.; Faoro, F. Benzothiadiazole enhances resveratrol and anthocyanin biosynthesis in grapevine, meanwhile improving resistance to *Botrytis cinerea*. *J. Agric. Food Chem.* **2004**, *52*, 4406−4413.

(5) Leifert, W.; Abeywardena, M. Cardioprotective actions of grape polyphenols. *Nutr. Res. (N.Y.)* **2008**, *28*, 729−737.

(6) Crozier, A.; Jaganath, I.; Clifford, M. Dietary phenolics: Chemistry, bioavailability and effects on health. *Nat. Prod. Rep.* **2009**, *26*, 1001−1043.

(7) De Pascual-Teresa, S.; Moreno, D.; García-Viguera, C. Flavanols and anthocyanins in cardiovascular health: A review of current evidence. *Int. J. Mol. Sci.* **2010**, *11*, 1679−1703.

(8) Pezzuto, J. M. Grapes and human health: A perspective. *J. Agric. Food Chem.* **2008**, *56*, 6777−6784.

(9) Leopoldini, M.; Russo, N.; Toscano, M. The molecular basis of working mechanism of natural polyphenolic antioxidants. *Food Chem.* **2011**, *125*, 288−306.

(10) Ndhlala, A.; Moyo, M.; Van Staden, J. Natural antioxidants: Fascinating or mythical biomolecules? *Molecules* **2010**, *15*, 6905−6930.

(11) Parr, A.; Bolwell, G. Phenols in the plant and in man. The potential for possible nutritional enhancement of the diet by modifying the phenol content or profile. *J. Sci. Food Agric.* **2000**, *80*, 985−1012.

(12) Scarponi, L.; Buonaurio, R.; Martinetti, L. Persistence and translocation of a benzothiadiazole derivative in tomato plants in relation to systemic acquired resistance against Pseudomonas syringe pv tomato. *Pest Manage. Sci.* **2001**, *57*, 262−268.

(13) Gozzo, F. Systemic acquired resistance in crop protection: From nature to a chemical approach. *J. Agric. Food Chem.* **2003**, *51*, 4487−4503.

(14) Repka, V. Elicitor-stimulated induction of defense mechanisms and defense gene activation in grapevine cell suspension cultures. *Biol. Plant.* **2001**, *44*, 555−565.

(15) Tassoni, A.; Fornal, S.; Franceschetti, M.; Musiani, F.; Michael, A. J.; Perry, B.; Bagni, N. Jasmonates and Na-orthovanadate promote resveratrol production in *Vitis vinifera* cv. Barbera cell cultures. *New Phytol.* **2005**, *166*, 895−905.

(16) Hukkanen, A. T.; Kokko, H. I.; Buchala, A. J.; McDougall, G. J.; Stewart, D.; Karenlampi, S. O.; Karjalainen, R. O. Benzothiadiazole induces the accumulation of phenolics and improves resistance to powdery mildew in strawberries. *J. Agric. Food Chem.* **2007**, *55*, 1862−1870.

(17) Cao, S.; Hu, Z.; Zheng, Y.; Lu, B. Effect of BTH on anthocyanin content and activities of related enzymes in strawberry after harvest. *J. Agric. Food Chem.* **2010**, *58*, 5801−5805.

(18) Mukkun, L.; Singh, Z. Methyl jasmonate plays a role in fruit ripening of "Pajaro" strawberry through stimulation of ethylene biosynthesis. *Sci. Hortic.* **2009**, *123*, 5−10.

(19) Bautista-Ortín, A. B.; Martínez-Cutillas, A.; Ros-García, J. M.; López-Roca, J. M.; Gómez-Plaza, E. Improving colour extraction and stability in red wines: the use of maceration enzymes and enological tannins. *Int. J. Food Sci. Technol.* **2005**, *40*, 1−12.

(20) Kennedy, J. A.; Jones, G. P. Analysis of proanthocyanidin cleavage products following acid-catalysis in the presence of excess phloroglucinol. *J. Agric. Food Chem.* **2001**, *49*, 1740−1746.

(21) Pastor del Rio, J. L.; Kennedy, J. A. Development of proanthocyanindins in *vitis vinifera* L. cv. Pinot noir grapes and extraction into wine. *Am. J. Enol. Vitic.* **2006**, *57*, 125−132.
(22) Ducasse, M. A.; Canal-Llauberes, R. M.; de Lumley, M.; Williams, P.; Souquet, J. M.; Fulcrand, H; Doco, T.; Cheynier, V. Effect of macerating enzyme treatment on the polyphenol and polysaccharide composition of red wines. *Food Chem.* **2010**, *118*, 369−376.
(23) Glories, Y. La coleur des vins roges. 2ème partie. Mesure, origine et interpretation. *Conn. Vigne Vin* **1984**, *18*, 253−271.
(24) Ribéreau Gayon, P.; Glories, Y.; Maujean, A.; Dubourdieu, D. *Traité d'Oenologie. 2. Chimie du Vin. Stabilisation et Traitements*; Dunod: Paris, 1998.
(25) Heil, M.; Hilpert, A.; Kaise, W.; Linsenmair, E. Reduced growth and seed set following chemical induction of pathogen defense: Does systemic acquired resistance incur allocation costs? *J. Ecol.* **2000**, *88*, 645−654.
(26) Iriti, M.; Faoro, F. Does Benzothiadiazole-induced resistance increase fitness cost in bean? *J. Plant Pathol.* **2003**, *85*, 265−270.
(27) Ortega-Regules, A.; Romero-Cascales, I.; López-Roca, J. M.; Ros-García, J. M.; Gómez-Plaza, E. Anthocyanin fingerprint of grapes: Environmental and genetic variations. *J. Sci. Food Agric.* **2006**, *86*, 1460−1467.
(28) Cao, S.; Hu, Z.; Zheng, Y.; Yang, Z.; Lu, B. Effect of BTH on antioxidant enzymes, radical-scavenging activity and decay in strawberry fruit. *Food Chem.* **2011**, *125*, 145−149.
(29) Repka, V.; Fischerova, I.; Silharova, K. Methyl jasmonate is a potent elicitor of multiple defense responses in grapevine leaves and cell-suspension cultures. *Biol. Plant.* **2004**, *48*, 273−283.
(30) Wang, S.; Zheng, W. Preharvest application of methyl jasmonate increases fruit quality and antioxidant capacity in raspberries. *Int. J. Food Sci. Technol.* **2005**, *40*, 187−195.
(31) Cadot, Y.; Minana-Castello, M. T.; Chevalier, M. Anatomical, histological, and histochemical changes in grape seeds from *Vitis vinifera* L. cv Cabernet franc during fruit development. *J. Agric. Food Chem.* **2006**, *54*, 9206−9215.
(32) Vidal, S.; Francis, I. L.; Guyot, S.; Mamet, N.; Kwiatkowski, M.; Gawel, R.; Cheynier, V.; Waters, E. The mouth-feel properties of grape and apple proanthocyanidins in a wine-like medium. *J. Sci. Food Agric.* **2003**, *83*, 564−573.
(33) Pezet, R.; Viret, O.; Perret, C.; Tabacchi, R. Latency of *Botrytis cinerea* and biochemical studies during growth and ripening of two grape berry cultivars, respectively susceptible and resistant to grey mould. *J. Phytopathol.* **2003**, *151*, 208−214.
(34) Busse-Valverde, N.; Gómez-Plaza, E.; López-Roca, J. M.; Gil-Muñoz, R.; Fernández-Fernández, J. I.; Bautista-Ortín, A. B. Effect of different enological practices on skin and seed proanthocyanidins in three varietal wines. *J. Agric. Food Chem.* **2010**, *58*, 11333−11339.
(35) Mercurio, M. D.; Dambergs, R. G.; Cozzolino, D.; Herderich, M. J.; Smith, P. A. Relationship between Red Wine Grades and Phenolics. 1. Tannin and Total Phenolics Concentrations. *J. Agric. Food Chem.* **2010**, *58*, 12313−12319.