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Benzothiadiazole Enhances Resveratrol and Anthocyanin Biosynthesis in Grapevine, Meanwhile Improving Resistance to *Botrytis cinerea*

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Pre-harvest multiple treatments of grapevine (cv. Merlot) with the plant activator benzothiadiazole (BTH, 0.3 mM) enhanced *trans*-resveratrol content in berries by about 40%. An even more striking effect was observed on anthocyanin synthesis, particularly on malvidine 3-glucoside, malvidine 3-(6-*O*-acetyl)glucoside and malvidine 3-(6-*O*-*p*-coumaroyl)glucoside, whose amounts were more than doubled. These data were obtained with a novel and time-saving HPLC method, set up for the simultaneous detection of stilbenes and anthocyanins, using an RF-10Axl fluorimetric detector instrument, with excitation at 330 nm and emission at 374 nm, and a SPD-Avp UV detector with absorption at 520 nm. Furthermore, BTH treatments induced systemic acquired resistance in grapevine, as assessed by inoculating clusters from treated and untreated plants with *Botrytis cinerea*. Disease severity, estimated according to the percentage of infected berries per cluster, was significantly reduced in grapes from BTH-treated plants. These results indicate that BTH treatments, besides improving the content of two important classes of nutraceuticals, with their well-known antioxidant, antitumoral, and phytoestrogenic activities, could be exploited in vineyard to protect grape against gray mould infection, thereby limiting an excessive use of fungicides

KEYWORDS: Anthocyanins; resveratrol; BTH; HPLC; *Botrytis cinerea*; *Vitis vinifera*; nutraceuticals; systemic acquired resistance; phytoalexins.

INTRODUCTION

Plants produce a great variety of secondary metabolites. The importance of this chemical diversity is linked to defense traits, against both biotic and abiotic stresses. With regard to resistance against pathogens, these compounds are classified as constitutive phytoanticipins or inducible phytoalexins, whose concentration, in plant tissues, increases strongly in response to microbial challenge or chemical treatments (reviewed in refs *1* and *2*).

Phenylpropanoids form a major class of secondary metabolites, being ubiquitous in plant kingdom. They are derived from phenylalanine produced via the shikimic acid pathway and exhibit a distinctive chemical structure. Phenylalanine deamination to cinnamic acid (**Figure 1**), catalyzed by phenylalanine ammonia-lyase (PAL), is the first step of phenylpropanoid pathway. Flavonoids and stilbenes, also referred to as polyphenolics, are distinguished from simple phenolics, in that their synthesis originates from a branching point of the above pathway. Indeed, the enzymes chalcone synthase (CHS) and stilbene synthase (STS) convert a phenylpropanoid structure, such as the *p*-coumaroyl-CoA, into intermediates, incorporating three additional C_2 (acetate) units. The resulting polyketide chains evolve into more stable forms with the formation of a new aromatic ring, and give rise either to precursors of different subclasses of flavonoids, including the anthocyanidins, or to the stilbenes (see **Figure 1**) (reviewed in ref *3*).

Trans-resveratrol (3,4′,5-trihydroxystilbene) is a phytoalexin involved in both constitutive and inducible plant defense mechanisms. This compound is present in leaves and berry skin of most grape cultivars (Vitaceae), as well as in a great number of plants, either Monocotyledons (Gramineae and Liliaceae) or Dicotyledons (Moraceae and Leguminoseae) (*1*). Moreover, though resveratrol synthesis decreases at the véraison, the initial step of grape ripening, the compound has been found at significant levels in red wine (*4*).

The synthesis of resveratrol plays a pivotal role in resistance mechanisms of certain plants against fungal infection. It has been shown that the rapidity and the amount of resveratrol synthesis are positively correlated with resistance of grapevine cultivars to gray mould, caused by *Botrytis cinerea* (*5*). In vineyards, gray mould is particularly detrimental, affecting both grape yield and wine quality. The latter is degraded through conversion of sugars into glycerol and gluconic acid (*6*). *B.*

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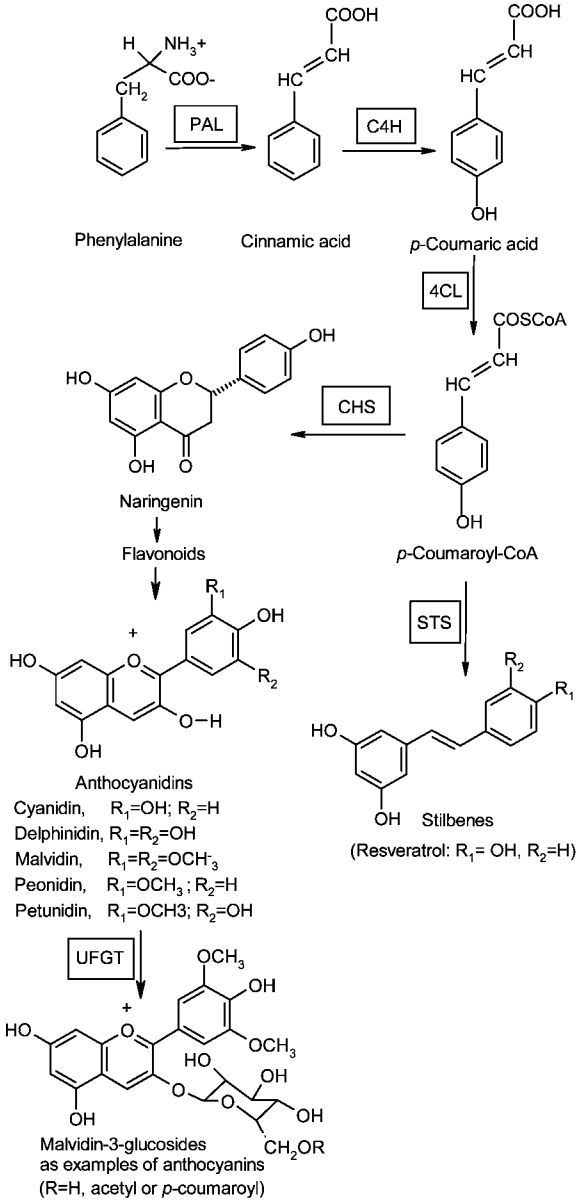

Figure 1. Stilbenes and anthocyanins biosynthesis through the phenylpropanoid pathway: PAL, phenylalanine ammonia-lyase; C4H, cinnamate-4-hydroxylase; 4CL, 4-coumarate CoA-ligase; CHS, chalcone synthase; STS, stilbene synthase; UFGT, UDP glucose-flavonoid 3-*O*-glucosyltransferase.

cinerea can also infect at low temperatures, causing important economic losses, either in preharvest or postharvest crops (*7*). For the above reasons, many efforts have been made, in the past decades, to improve grapevine resistance to this disease.

Recent pieces of evidence suggest that resveratrol may have an array of biological activities in medicine and nutrition. Moderate red wine consumption reduces the risk of coronary heart disease. Cardioprotective and hypocholesterolemic effects are due to inhibition of low-density lipoprotein (LDL) oxidation, platelet aggregation and coagulation. Resveratrol has also a phytoestrogenic activity, competing with estradiol for estrogen receptor. Besides its antioxidant, antimutagenic, and antiinflammatory activity, it inhibits cellular events associated with tumor initiation, promotion, and progression, being particularly useful in chemoprevention (*8*−*11*). However, it is still under debate whether the cardioprotective effect of grape and wine is due to the resveratrol, ethanol, or other antioxidant compounds present, such as tannins and flavonoids. In particular, grape and red wine contain many of these compounds, and they are some of the components of the Mediterranean diet, which includes foodstuffs rich in antioxidants and unsatured fats, with cardioprotective effects as well. Moreover, some of the cardioprotective effects of poliphenolics have been demonstrated only in vitro, and hence, their in vivo antioxidant power has not been definitively estimated yet (*10*, *12*).

Among flavonoids, anthocyanidins are the most prominent pigments in grape skin (*13*). Their conjugated derivatives, anthocyanins, are mainly bound to sugars, hydroxycinnamates, and organic acids. These water soluble pigments produce blue, red, and purple hues, conferring color to flowers, fruits, vegetables, and other plant organs, such as leaves, stems, seeds, and roots (*14*). They play different physiological roles, as pollinator attractants, in seed dispersal and in plant protection from environmental stresses. Hence, they took a key role in plant coevolution with an array of other organisms (*15*).

The most common anthocyanidins, in higher plants, are cyanidin, petunidin, peonidin, pelargonidin, delphinidin, and malvidin. The differences among these six aglycons are due to the number and position of their hydroxyl groups and to their methylation degree. However, the anthocyanins of *Vitis vinifera* sp. are structurally based upon five aglycons only (**Figure 1**), as pelargonidin derivatives have never been isolated from grape (*16*, *17*).

Anthocyanin biosynthesis starts at véraison and continues during the ripening phase, accumulating in berry skin. Several agroecological factors, such as cultivar, climate, soil conditions, growth stage, irrigation, and viral infection, have been related to their accumulation in grape (*18*−*22*).

Anthocyanins are strong antioxidants. Their double bond conjugate system allows electron delocalization, resulting in very stable structures and powerful antioxidant activity. Furthermore, the degree and position of hydroxylation and methoxylation, in the B ring, modulate their stability and reactivity (*23*, *24*).

Pharmacological properties of anthocyanins include anti-inflammatory, anti-oedema, antimutagenic, antitumor, vasodilatation, and free radicals scavenging activity. Thus, they are utilized as pharmaconutrients in capillary fragility, cardioprotection, and chemoprevention.

Plant resistance activators are a class of either natural or synthetic compounds that stimulate active plant defense mechanisms (reviewed in *2*). Among these, one of the most interesting is benzothiadiazole (BTH, Bion) (*25*), which induces a broad spectrum, long lasting, and systemic immunity (SAR, systemic acquired resistance) against different pathogens of numerous species, such as wheat, arabidopsis, tobacco, and bean (reviewed in *26*). Furthermore, BTH and its acid derivative are completely translocated and degraded in plant tissues not incurring in persistence and residues (*27*, *28*).

BTH is a functional analogue of the plant endogenous hormone-like compound salicylic acid (SA), that is required for the induction of defense genes leading to SAR establishment. These genes encode for pathogenesis related (PR) proteins and key enzymes of secondary metabolic pathways, such as the above-mentioned PAL (*29*−*31*).

We have previously demonstrated that BTH efficacy in bean depends on the dose employed in treatments and on the induction time (*26*). We have also shown that PAL induction by BTH leads to lignin biosynthesis, and possibly, phytoalexins production, thus making plants resistant to bean rust caused by *Uromyces appendiculatus* (*32*).

On this basis, we wondered if BTH treatment of grapevine could enhance the synthesis of phytoalexin *trans*-resveratrol and

other secondary metabolites, such as anthocyanins, all of them playing an important role both as nutraceuticals and phytoalexins. To verify this hypothesis, grapevine plants have been treated with BTH at pre-harvest, and the content of the above metabolites has been analyzed in post-harvest berries. An improved HPLC method has been set up to detect simultaneously stilbenes and anthocyanins. Furthermore, being that these phenylpropanoids are involved in plant defense mechanisms, we have tested the resistance of berries from treated plants to post-harvest *B. cinerea* infection.

MATERIALS AND METHODS

Plant Materials and Treatments. Plant (*Vitis vinifera*, cv. Merlot) treatments were carried out in an experimental vineyard at Conegliano Veneto (Treviso, Italy). Plants were sprayed on the first, fourth, and seventh day of the last week of August 2003, at the end of vèraison, with a water suspension of BTH (Benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester) (trade name Bion, Syngenta, CH) at the concentration of 0.3 mM, prepared from a wettable formulation containing 50% (w/w) active ingredient. This concentration has been selected on the basis of the results obtained in a previous study on bean plants (*26*). Control plants were sprayed with a water suspension of wettable powder alone. Mature clusters were collected from treated and untreated plants at harvest, that is 4 weeks after BTH treatments, for chemical analysis and pathogen inoculation.

Standards. Malvidin-3-monoglucoside and *trans*-resveratrol were purchased from Sigma Chemical Co (St. Louis, MO). *Cis*-resveratrol was obtained by isomerization of *trans*-resveratrol with 1 day light exposure. Identification and quantification of anthocyanins and stilbenes peaks were obtained by calibration curve of standards.

Sample Preparation for Stilbene Analysis. Stibene analysis was performed on frozen grapes, after removing pulp and seeds. Frozen berries ($n = 30$), randomly selected from each sample, were peeled without thawing, and the obtained skins were weighed. To standardize the process, 5 g of skin samples was used for each polyphenol extraction. All extraction steps were done in the dark, to prevent *trans*-resveratrol isomerization and oxidation.

Samples were homogenized in liquid nitrogen with a chilled mortar and pestle, and 30 mL of 95% methanol was added to the obtained powder. After 15 min of sonication (Braunsonic 1510 Sonicator) and a further 20 min of mixing in a rotary shaker, samples were centrifuged at 10000*g* for 30 min at 4 °C. Afterward, samples were extracted once again, with 25 mL of methanol, under the same conditions as above. Extracts were mixed together and concentrated by evaporation in a rotary evaporator at 40 °C. Residues were reconstituted with 4 mL of absolute methanol and stored at −20 °C.

Sample Preparation for Anthocyanin Analysis. Anthocyanin extraction was performed from skins of 20 frozen berries, randomly selected from each sample, by adding 100 mL of 95% methanol. Samples were kept in the dark at 4 °C overnight, then extraction mixture was filtered and skins were extracted again with 50 mL of methanol for 2 h. Extracts were dried in a rotary evaporator at 40 °C, and residues were redissolved in 10 mL of methanol/perchloric acid (0.3%) in water (23:73). All extracts were stored in foil-wrapped glass vials at −20 °C.

High-Performance Liquid Chromatography (HPLC) Analysis of Stilbenes (Conventional Method). A Shimadzu LC-10ADvp, SIL-10ADvp HPLC equipment, with an SPD-10Avp, RF-10Axl detectors, was used. HPLC pumps, autosampler, and detectors were controlled via Class vp 3.4 software. A modified version of Mattivi method (*33*, *34*) was used to resolve the phenolic content of grape extracts. The analytical column was a Luna RP C18 (4.6 × 250 mm, particle size = 5 μm), provided of guard column obtained from Chemtek Analytica. The column temperature was maintained at 25 °C. Gradient elution profile was as follows: 0−5 min, 90% solvent B (phosphate buffer 0.02 M KH_2PO_4/H_3PO_4 pH = 3), 10% solvent A (acetonitrile); 5−8 min, 80% B, 20% A; 8−13 min, 80% B, 20% A; 13−25 min, 60% B, 40% A; 25−27 min, 90% B, 10% A; 27−34 min, 90% B, 10% A. Flow-rate was 1 mL/min. A volume of 5 μL of each sample solution was injected, after centrifugation at 10000*g* for 30 min, and subsequent filtration through a PTFE 0.45-μm membrane syringe filter. Fluorimetric detections were recorded for 34 min at $\lambda_{ex} = 330$ nm and $\lambda_{em} = 374$ nm; wavelengths of 285 and 310 nm were used for absorbance detector in two separated channels.

HPLC Analysis of Anthocyanins and Stilbenes (Simultaneous Method). This method has been set up with the aim of simultaneously detecting stilbenes and anthocyanins. HPLC equipment was the same as above. Chromatographic separation was performed with a Purospher LiChroCART RP-18 HPLC column (4.6 × 250 mm, particle size = 5 μm), provided of precolumn LiChroCART (4 × 4 mm) (Merk, VWR international). Chromatographic analyses were made with a mobile phase linear gradient of absolute methanol, solvent A and 0.3% perchloric acid in water, solvent B at a flow-rate of 0.45 mL/min (*35*). Gradient elution profile was as follows: 0 min, 27% B, 73% A; 1−32 min, 43% B, 57% A; 32−45min, 68.5% B, 31.5% A; 45−47min, 100% B; 3 min constant 100% B. Samples were centrifuged at 10000*g* for 30 min and then filtered using a PTFE 0.45-μm membrane syringae filter (Corning, Inc.). Injection volume of each sample was 10 μL. Fluorimetric detections were recorded for 60 min at $\lambda_{ex} = 330$ nm and $\lambda_{em} = 374$ nm for stilbene quantification. A wavelength of 520 nm was used for the absorbance detector in a separated channel.

Fungal Inoculum. *B. cinerea* was cultured in Petri dishes containing 20 mL potato dextrose agar (PDA) and incubated at 20 °C, high humidity, and 12-h light photoperiod, for 2 weeks. Conidial suspension was prepared by flooding the cultured plates with 2 mL of sterile solution (0.01% Tween 20) and gently scraping the agar surface with a sterile platinum loop, to dislodge the conidia. The suspension was filtered through a double layer of gauze, to separate the conidia from mycelium fragments. The conidia concentration was measured and adjusted to $5 \times 10^4 \cdot mL^{-1}$, using a Bürker chamber (*36*).

Berry Inoculation and Disease Evaluation. Both treated and untreated clusters were washed in running water for 2 h, surface sterilizated with 2% NaOCl for 5 min, and rinsed twice in sterile water. Berry skin was pricked, at constant depth, with a sterile needle close to the pedicel, and a drop of inoculum (20 μL) was placed upon it. Control berries were inoculated similarly with 20 μL of Tween solution without conidia. After inoculation, clusters were placed in humidity saturated air, at 20 °C and 12/12 h photoperiod, until symptom appearance.

Disease severity was estimated on 50 treated and 50 control clusters, by grouping them in six classes according to the percentage of infected berries per cluster: 0% (class 1), 1−10% (class 2), 10−25% (class 3), 25−50% (class 4), 50−75% (class 5), and 75−100% (class 6).

Presentation of Results. Mean and standard deviation of values from biological assays were analyzed by using MSTAT-C software (Michigan State University). Infection experiments, extraction of metabolites, and HPLC analysis were repeated at least three times unless otherwise stated.

RESULTS

HPLC Analysis of Anthocyanin and Resveratrol Content. To validate our hypothesis on BTH elicitation of stilbene synthesis in *V. vinifera*, the HPLC conventional method of Mattivi (*33*) was applied with resveratrol standard applications (**Figure 2**).

Four different extractions were made on BTH and control samples and tested by reversed-phase gradient HPLC. *Trans*- and *cis*-resveratrol peaks were resolved at 285 and 310 nm, respectively, and identified according to elution order and retention times.

BTH treatment enhanced both trans- and cis-isomer accumulation in berry skin, as shown in BTH-control overlaid chromatograms (**Figure 3**). However, the increment of the trans-isomer was significantly higher than that of the cis one and was estimated to be of about 40%, with respect to the control (**Table 1**). The same chromatograms showed that BTH treatment had brought about a striking increase of three specific peaks

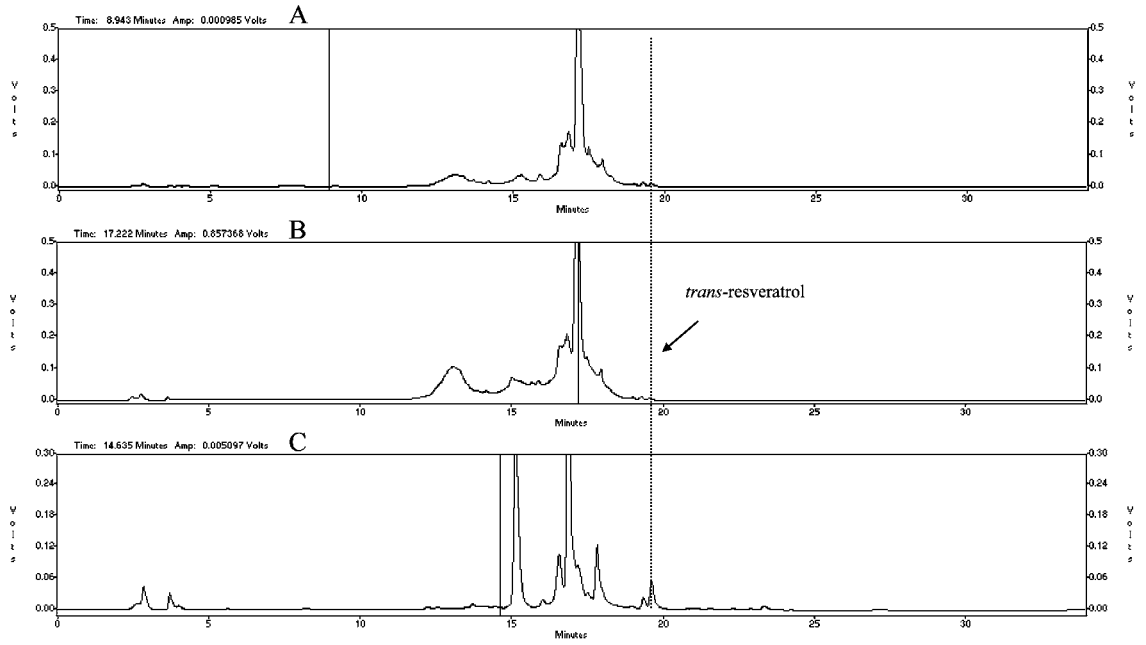

Figure 2. Chromatograms of a skin extract from Merlot grape (*conventional method*), recorded at 310 nm (**A**), 285 nm (**B**), and with fluorimetric detection (**C**).

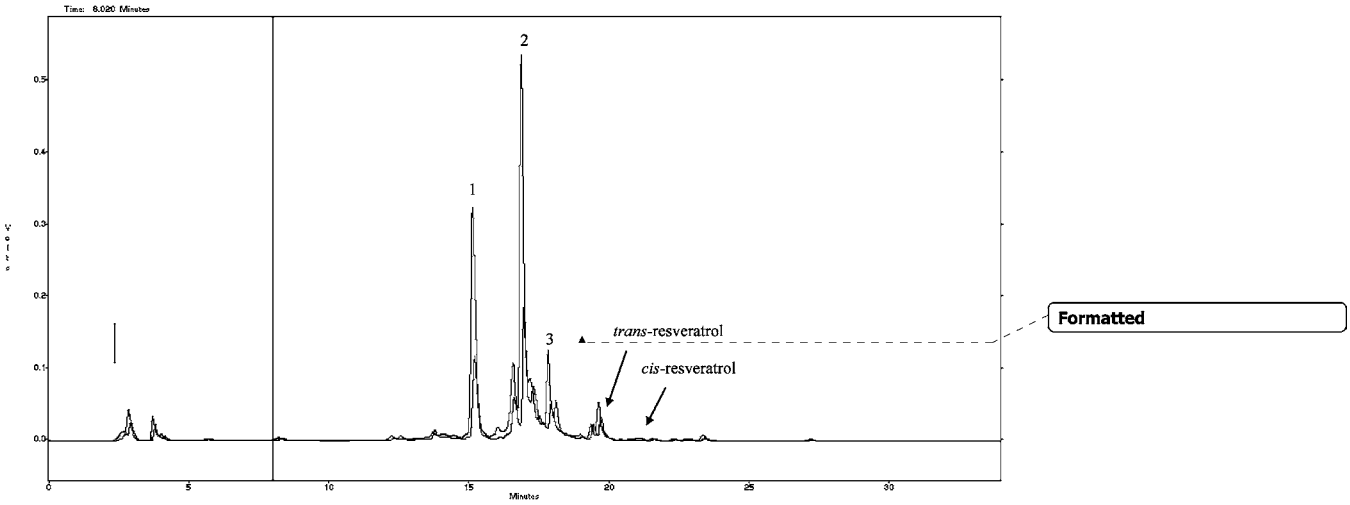

Figure 3. Chromatographic overlaid separation of stilbenes detected by fluorescence (*conventional method*) of extracts of BTH-treated (upper profile) and control (lower profile) samples.

Table 1. *Trans*- and *cis*-Resveratrol Content in Berry Skins from BTH and Control Plants, as Estimated from the Chromatograms in **Figure 3**

treatments	*trans*-resveratrol (mg/kg grapes)	*cis*-resveratrol (mg/kg grapes)
BTH	0.546	0.148
control	0.390	0.115

(**Figure 3**; peaks **1**, **2**, and **3**). We expected these peaks were due to anthocyanins because of the great content of these compounds in grapevine (*13*).

Anthocyanins were, in fact, detected with absorbance at 520 nm, after extraction from BTH-treated and control berry skin tissues. Different concentrations of malvidin 3-glucoside were used to make a calibration curve and optimize the method (*35*). A total of 15 different anthocyanins derivatives were identified, as shown in BTH-control overlaid chromatograms (**Figure 4**). Three main groups, in both grape extracts, could be clearly distinguished: five monoglucosides of the anthocyanidins delphinidin, cyanidin, petunidin, peonidin and malvidin (anthocyanins **1**−**5**), as well as the corresponding acetylated (**6**−**10**) and *p*-coumaroyl (**11**−**15**) derivatives. Elution of extracts of untreated samples yielded the three most abundant anthocyanins, which were identified by comparing their retention times and elution order as malvidin 3-glucoside (**5**), malvidin 3-(6-*O*-acetyl) glucoside (**10**), and malvidin 3-(6-*O*-coumaroyl) glucoside (**15**). Regarding the other anthocyanins, the glucosides of delphinidin (**1**), petunidin (**3**), and peonidin (**4**) were present in minor amounts, while cyanidin 3-glucoside (**2**) was noticed in traces. The whole anthocyanin content, particularly malvidine conjugates, increased strongly after BTH treatment (more than 100%), as further confirmed by the HPLC comparative analysis between 20 mL of extract from control plants and 10 mL of extract from BTH treated ones, whose chromatograms clearly showed the above-mentioned quantitative difference (not shown).

Finally, we set up a novel and simple method for the simultaneous detection of stilbenes and anthocyanins in grape that differs noticeably from the one previously proposed by Ali

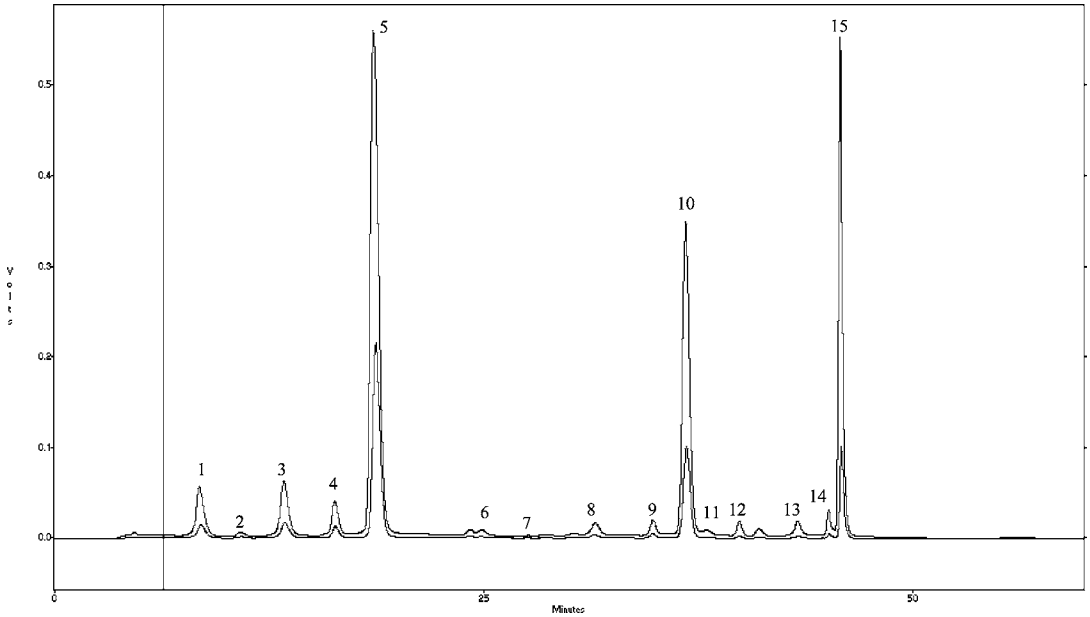

Figure 4. Chromatographic separation ($\lambda = 520$ nm) of anthocyanins (*simultaneous method*): chromatographic overlaid obtained with 10-μL injections of BTH (upper profile) and control (lower profile) samples. Peaks **1**–**5**: 3-glucosides of Delphinidin, Cyanidin, Petunidin, Peonidin, and Malvidin, respectively, Peaks **6**–**10**: 6-*O*-acetylated derivatives of glucosides 1–5; peaks **11**–**15**: 6-*O*-*p*-coumaroyl derivatives of glucosides 1–5 (see **Figure 1** for molecular structures).

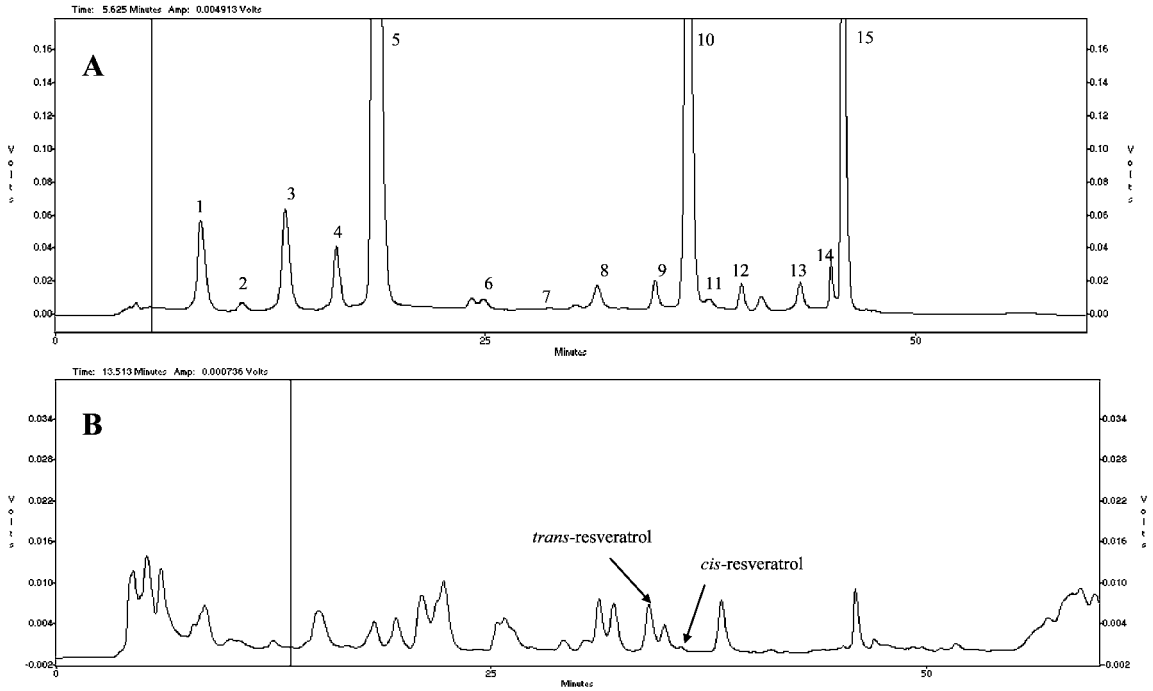

Figure 5. Absorbance and fluorescence of chromatographed (*simultaneous method*) skin extracts from Merlot grapes. (**A**) Chromatographic separation ($\lambda = 520$ nm) of anthocyanins: delphinidin 3-monoglucoside (**1**), cyanidin 3-monoglucoside (**2**), petunidin 3-monoglucoside (**3**), peonidin 3-monoglucoside (**4**), malvidin 3-monoglucoside (**5**), acetylated forms (**6**–**10**), and *p*-coumaroylated forms (**11**–**15**). (**B**) Stilbenes detected by fluorescence, with excitation and emission at 330 and 374 nm, respectively.

and Strommer (*37*). A detection with an RF-10Axl fluorimetric detector instrument, at $\lambda_{ex} = 330$ nm and $\lambda_{em} = 374$ nm, was performed at the same time as one with a SPD-Avp UV detector (wavelength 520 nm), following anthocyanin detection (**Figure 5**). Quantification of stilbenes in control and treated Merlot variety, performed following this method, again showed an increase of about 40% in the *trans*-resveratrol content of berry skin from BTH treated plants, confirming data obtained with the conventional method (**Table 1**). Furthermore, with the simultaneous method, it was possible to identify malvidins as the main anthocyanins enhanced by BTH treatment, and accumulated in part as their acetylated and *p*-coumaroylated forms of the malvidin-3-glucoside.

BTH-Induced Resistance in Berries. Berries were inoculated with fungal spore suspension at harvest, that is two weeks after the end of véraison. This specific developmental stage coincided with the fourth week after the last of BTH multiple treatments, which, accordingly, had been performed two weeks before the end of véraison. Pale grayish mycelial growth soon became apparent 3 or 4 days after inoculation, nearby the

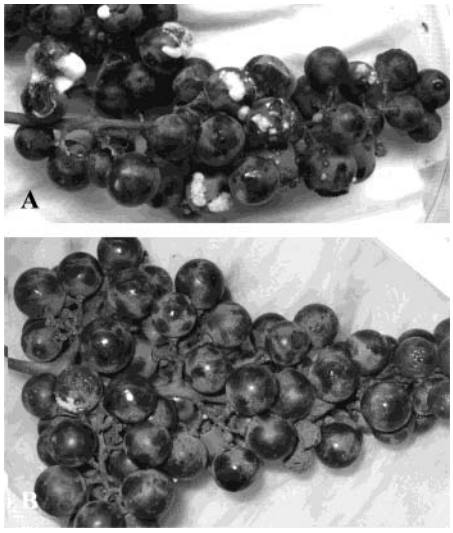

Figure 6. Grape clusters from control (**A**) and BTH-treated (**B**) plants, inoculated at postharvest with *Botrytis cinerea*; gray mould symptoms are more severe in the control (**A**) than in BTH-treated sample.

Table 2. Distribution of Grape Clusters in Different Severity Classes, Depending on the Percentage of Infected Berries Per Cluster[a]

	class severity					
treatments	0% (class 1)	1–10% (class 2)	10–25% (class 3)	25–50% (class 4)	50–75% (class 5)	75–100% (class 6)
control	0% ± 0	0% ± 0	0% ± 0	8% ± 3	73% ± 12	19% ± 7
BTH	31% ± 5	56% ± 8	23% ± 2	0% ± 0	0% ± 0	0% ± 0

[a] *Botrytis cinerea* was inoculated at harvest, 4 weeks after BTH treatment.

pricking point, particularly in the control clusters (**Figure 6**). Afterward, hyphal development continued rapidly for another week, at the end of which (10 days after inoculation) disease severity was assessed **(Table 2**).

In control clusters, class 5 was the most recurrent (50−75% of infected berries within the cluster), which included 73% of inoculated clusters, followed by class 6 (75−100% of infected berries) with a 19% incidence of clusters, and class 4 (25−50% of infected berries) with 8% of symptomatic clusters. None of the clusters could be attributed to classes 1−3.

Clusters from BTH-treated plants showed a significantly reduced incidence of gray mould infection. In fact, 56% of diseased ones belonged to class 2 (1−10% of infected berries), while the other representative classes were 1 (0% of infected berries) and 3 (10−25% of infected berries), with an incidence of 31 and 23% respectively. None of the clusters from treated grapevines showed a percentage of infected berries exceeding 25%.

DISCUSSION

The aim of this study was to enhance resveratrol content in grape, meanwhile improving its resistance to *B. cinerea* by means of BTH treatments. According to the results, the two goals appear to have been achieved, at least in the case of the examined cv. Merlot, which is highly susceptible to gray mould. However a third, very interesting result, consequent to treatments, was the great increase of anthocyanins content in berries, particularly malvidin 3-monoglucoside and its acetyl and coumaryl esters. In addition to their credits for beneficial effects on human health, anthocyanins are pigments responsible for wine color and stability. Therefore, many studies have been carried out to improve their content in grape. Most of these studies have been done by elicitation with different compounds of cell suspension cultures, succeeding in increasing both anthocyanins and stilbenes syntheses, as summarized in **Table 3**. However, as far as we know, only one elicitation has been done directly on grape berries, by exogenous treatment with ethephon (ethylene) at véraison, demonstrating that this hormone stimulates long-term expression of genes related to anthocyanin biosynthesis, including the limiting and final step of UDP glucose-flavonoid 3-*O*-glucosyl transferase (UFGT, **Figure 1**) (*41*). Interestingly, we have previously shown that BTH induces a sort of "ethylene priming" in bean plants, possibly as a consequence of a cross talk between ethylene and BTH transduction pathways (*52*). Thus, it is possible that BTH treatments on grapes stimulates a similar mechanism.

Table 3. Defense Responses Induced in *Vitis vinifera* Cell Cultures and Plants, after Treatment with Different Elicitors[a]

elicitors	effects on cell cultures	effects on plants	refs
methyl jasmonate	stilbenes, anthocyanins		38
	PAL, CHI, PR proteins		39
jasmonic acid	anthocyanins		40
ethylene		CHS, F3H, UFGT transcripts and anthocyanins (berries)	41
		PR proteins (leaves)	42
salicylic acid	PR proteins, CCoAOMT and STS transcripts		43–44
	PAL, CHI, PR proteins		39
		PR proteins (leaves)	45–47
		PR proteins (berries)	48
2,6-dichloroiso-nicotinic acid	PR proteins, CCoAOMT and STS transcripts		43–44
benzothiadiazole	PR proteins		43–44
laminarin	stilbenes, PR proteins, PAL, and STS transcripts	PR proteins (leaves)	49
chitosan	PAL, CHI, PR proteins		39
sugars	anthocyanins, stilbenes		50–51

[a] PAL, phenylalanine ammonia-lyase; CHI, chalcone isomerase; CHS, chalcone synthase; CCoAMOT, caffeoyl-coenzime A 3-O-methyltrasferase; F3H, flavanone-3-hydroxylase; PR, pathogenesis related proteins; STS, stilbene synthase; UFGT, 3-*O*-glucosyl transferase.

The great increase in anthocyanins, observed in this study, deserves further interest because, among them, malvidin-3-(6-*O*-*p*-coumaroyl) glucoside showed the highest content. Coumarate derivatives are relevant in winemaking, as they may undergo intramolecular copigmentation, increasing light absorption, and in turn, red wine color (*53*).

As regards stilbenes and anthocyanins HPLC separation, the simultaneous method we set up proved to be more simple and less-time-consuming that the conventional one. Furthermore, it allows the simultaneous analysis of the major classes of stilbenes and anthocyanins from grape skin with a better separation of well identifiable peaks than the method recently proposed by Ali and Strammer (*37*). In any case, the results obtained for the controls are in good agreement with those reported by others on the same grapevine cultivar and with different separation methods (*62*)

The finding that BTH treatment greatly increased anthocyanins and stilbenes content in the present study could also be the consequence of a combined effect of BTH induction of PAL (*52*), the key enzyme of phenylpropanoid pathway, and the timing of treatments, concentrated in a week during véraison,

that is the critical period during which the developmental changes of polyphenols assume an important role (*54*). In this regard, it must be noted that during ripening, stilbene accumulation in skin cells of berries declines, while anthocyanin biosynthesis increases, possibly due to a competition between the two branches of phenylpropanoid pathway (**Figure 1**), with a differential regulation of the key enzymes CHS and STS. Besides, anthocyanin enhanced synthesis coincides with sugars accumulation, both in flesh and skin (*55*, *56*). Thus, it seems that BTH reverses, to a certain degree, the inverse relationship between resveratrol and anthocyanin metabolic pathways, reducing CHS and STS competition for substrate binding and raising both anthocyanin and resveratrol synthesis. Hence, in BTH treated berries, anthocyanin accumulation appears not to impair resveratrol production at ripening. In other words, the usual metabolic switch between the two metabolic branches of the same pathway seems to be avoided (*57*).

The apparent lack of this competition could explain the significant resistance to gray mould we have observed at postharvest. In fact, the high phytoalexin stilbene level present in grape before véraison is considered to be one of the main factors explaining the often reported resistance to *B. cinerea*, in contrast to the low stilbene concentration and high susceptibility observed at ripening (*58*−*60*).

Besides phenylpropanoids, other important compounds are involved in the induction of SAR to *B. cinerea*, particularly pathogenesis-related proteins (PRs) (*61*) that are, more generally, considered a marker of SAR (*2*). PRs induction, namely type I and III acid chitinases, following BTH treatment, have been reported in grapevine cell culture (*43*, *44*). Though many other elicitors induce PRs synthesis in in vitro systems, few of them have been applied directly to plants (**Table 3**). Nevertheless, elicitation of PRs in grapevine leaves and/or berries have been demonstrated with ethephon (*42*), laminarin (*49*), and salicylic acid (*45*−*48*). Particularly, laminarin, from brown alga *Laminaria digitata*, and chitosan, from crustacean shell, elicit grapevine defense against *B. cinerea* and *Plasmopara viticola*. (*49*, *62*). Thus, it is admittedly possible that also the BTH-induced SAR be in part due to PRs accumulation in the berries.

In conclusion, BTH treatments performed at véraison not only increase significantly in grape berries the content of two important pharmaconutrients, such as *trans*-resveratol and anthocyanins, but also induce SAR against the detrimental *B. cinerea* infection. Being that SAR is long lasting and effective against a broad spectrum of plant pathogens, its induction with BTH could represent an interesting strategy to protect the grapevine throughout the whole vegetative season, as an alternative, or complement to fungicide treatments. This strategy would greatly reduce the environmental impact of crop treatments, by virtue of the very low toxicological risk associated with BTH (*63*), which is also rapidly degraded in plant tissues and lacks any antibiotic activity (*27*, *28*). Moreover, SAR establishment, due to its multigenic trait, should not incur the risk of selecting agrochemical resistant pathogen strains or overcoming single transgene resistance (*2*).

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