3-Sulfanylhexanol Precursor Biogenesis in Grapevine Cells: The Stimulating Effect of *Botrytis cinerea*

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ABSTRACT: Volatile thiols, compounds that contribute strongly to the varietal aroma, are present in much higher concentrations in sweet wines than in dry wines. This positive effect, due to the presence of *Botrytis cinerea* on the berries, in fact results from a strong enrichment of cysteine *S*-conjugate precursors in botrytized berries. In the present study, a convenient model was investigated to reproduce and therefore study this phenomenon. A *Vitis vinifera* cell culture was used as a simple model, and we focused on *S*-3-(hexan-1-ol)-L-cysteine (P-3SH), the cysteinylated precursor of 3-sulfanylhexanol. We demonstrated that grapevine cells were able to produce P-3SH and that the presence of *B. cinerea* considerably increased the precursor level (up to 1000-fold). This positive result was determined to be due to metabolites secreted by the fungus. These molecules were temperature sensitive, unstable over time, and their production was activated in the presence of grapevine cells. Moreover, part of the pathway leading to P-3SH was deciphered: it was directly derived from the cleavage of *S*-3-(hexan-1-ol)-L-glutathione, which itself was generated after a conjugation of glutathione on (*E*)-2-hexenal.

KEYWORDS: *Vitis vinifera*, noble rot, cysteinylated conjugates, glutathionylated conjugates, thiol precursor, (*E*)-2-hexenal, glutathione

■ INTRODUCTION

One of the most famous types of French dessert wine, Sauternes, is produced from *Vitis vinifera* L. cv. Sauvignon blanc and Semillon grapes. Specific climatic conditions and, above all, the presence of *Botrytis cinerea* in its noble-rot form, allow a powerful over-ripening process and thus the production of these great wines. Particular aroma ranges are obtained in this way, with typical sweet nuances that evoke honey, caramel, and crystallized fruit, as well as citrus aromas, such as orange peel or grapefruit, together with walnut or spicy-curry overtones.[1−4] Several volatile thiols are known to contribute to the varietal aroma of wines made from the Sauvignon blanc and Semillon varieties.[5−7] The presence of *Botrytis cinerea* in grapes induces a strong modification of the volatile thiol content.[1−4] The principal polyfunctional thiol contributors of the Sauternes aroma range are 3-sulfanylhexanol (3SH), 3-sulfanylpentan-1-ol (3SP), 3-sulfanylheptan-1-ol (3SHp), 2-methyl-3-sulfanylbutan-1-ol (2M3SB), and 4-methyl-4-sulfanylpentan-2-one (4MSP), which smell of grapefruit and passion fruit, citrus and sulfur, raw onion, and boxwood, respectively. 3SH and 4MSP can be detected in wines made from healthy grapes, whereas other thiols are present in only very trace concentrations. On the contrary, wines obtained from botrytized grapes contain volatile thiols in significant amounts. 3SH, 3SP, and 3SHp concentrations in botrytized wines are particularly affected by the development of *B. cinerea* on grapes (more than 30-fold for 3-SH).

These polyfunctional thiols are not present in grape juice and are released from their corresponding cysteine *S*-conjugates during alcoholic fermentation.[8−11] As a result, 3SH, 3SP, 3SHp, and 2M3SB are present in grape juice in a cysteinylated conjugate precursor form: *S*-3-(hexan-1-ol)-L-cysteine (P-3SH), *S*-3-(pentan-1-ol)-L-cysteine (P-3SP), *S*-3-(heptan-1-ol)-L-cysteine (P-3SHp), and *S*-3-(2-methylbutan-1-ol)-L-cysteine (P-2M3SB), respectively.[11,12] The positive effect of *B. cinerea* on the terminal volatile thiol concentration in sweet wines is in fact due to an enhancement of the cysteine *S*-conjugate; volatile thiol concentrations in Sauvignon blanc and Semillon must are considerably higher when *B. cinerea* had developed on the grapes. Thibon et al. showed that, within a one week period, the P-3SH levels increased approximately 100-fold between the healthy and first botrytized stages.[13] These authors suggested that *B. cinerea* stimulates the grapevine metabolic pathway implicated in P-3SH formation, thus explaining the observed increase.

The origin of the cysteinylated precursor in grapevines has not been completely determined. The presence of *S*-3-(hexan-1-ol)-glutathione (P-GSH) in white grape juice may indicate that P-GSH is a pro-precursor of P-3SH.[14−17] Glutathione *S*-conjugates are often involved in the detoxification systems of living organisms. First, toxic compounds are conjugated with glutathione by glutathione *S*-transferase[18,19] and two enzymes, γ-glutamyltranspeptidase (to eliminate glutamic acid) and carboxypeptidase (to eliminate glycine), are used to split these compounds to form a cysteine *S*-conjugate.[20,21] In plants, the detoxification pathways are activated in response to a variety of stresses, including senescence, as well as abiotic (oxidation, injury, etc.) and biotic (pathogens) factors. *Botrytis cinerea* development on grapes may stimulate this pathway as it has been demonstrated that the lipoxygenase VvLOXC and -O transcripts are rapidly accumulated in berries infected by *B. cinerea*.[22] The lipoxygenase-hydroperoxide lyase enzyme pathway

releases (E)-2-hexenal[23−25] and other reactive aldehydes.[19] (E)-2-Hexenal induces glutathione S-transferases,[19,26] thus increasing the formation of S-glutathione conjugates such as the P-GSH pro-precursor. Therefore, the cleavage of P-GSH by detoxification enzymes may be responsible for the rapid and large increase in P-3SH levels in botrytized grapes.[27]

The exact pathway leading to the production of P-3SH is still unclear due to the complex nature of the composition of grapes and the involvement of different actors (B. cinerea and V. vinifera). As far as we know, our experiment is the first to use a simple grape model to clarify our understanding of the role of B. cinerea in P-3SH formation. This model is likely to be valuable in future studies as it allows the impact of a single parameter to be tested. Moreover, it is relevant because experiments can continue year-round under controlled conditions. Vitis vinifera cell suspension cultures were inoculated with B. cinerea in order to assess the role of the fungus on P-3SH formation, confirm the role of P-GSH with respect to the aroma pro-precursor, and validate our model. We first verified the ability of plant cells to produce P-3SH alone and in the presence of B. cinerea. Then, the specificity of the inductor treatment was studied, and a preliminary investigation of which fungal elements act as a stimulator was carried out. Finally, we show that the synthesis of the precursor P-3SH is produced by the plant, and we emphasize the intermediate compounds involved in this biosynthesis.

■ MATERIALS AND METHODS

Reagents. (E)-2-Hexenal (95%), methyl jasmonate, L-glutathione reduced (98%), casein hydrolysate, 1-naphtalene-acetic acid, benzyl-aminopurin, sodium borohydride (98%), cesium carbonate (99%), diethyl ether (99.8%), and DOWEX 50WX8 were purchased from Sigma-Aldrich (France). N-Methyl-N-(tert-butyldimethylsilyl)-trifluoroacetamide with 1% t-butyl-dimethychlorosilane (MTBSTFA/t-BDMCS) was obtained from Perbio Science (Brebières, France). Chelating Sepharose Fast Flow was purchased from GE Healthcare (Buckinghamshire, England).

Plant Materials. Grapevine cells of Vitis vinifera L. cv. Cabernet Sauvignon (CS6) were cultured in maintenance medium, described previously as B5 medium.[28] This medium was buffered to pH 5.8 and contained 20 g/L sucrose, 250 mg/L casein hydrolysate, 0.5 mg/L 1-naphtalene-acetic acid, and 0.12 mg/L benzylaminopurine. The cultures were grown under continuous fluorescent light (5000 lx) at 24 °C on an orbital shaker at 100 rpm. Suspension cultures were subcultured weekly in 50 mL of maintenance medium by inoculating the cells at a 1/5 (v/v) ratio in fresh medium. For the experiments (treatments as well as control), 7-day-old cell suspensions were inoculated into an induction medium with the same composition as the maintenance medium but containing 40 g/L sucrose at a 1/5 (v/v) ratio.[28] The cell treatments were carried out after 7 days of culture in induction medium. Two days post-treatment, the grapevine cells were harvested by vacuum filtration on a 30 μm membrane, and the filtrate (extracellular medium) was kept. The total cells (around 10 g per flask) were rapidly frozen in liquid nitrogen and ground in a mortar, and the resulting powder was extracted twice with 3 mL of ethanol (70%). The filtrates of ethanolic extracts were centrifuged at 8000g for 15 min at 4 °C, and the supernatants were stored at −20 °C until analysis.

Fungus Materials. Two Botrytis cinerea strains were used: C77-4 (Faculté d'Oenologie collection; Bordeaux, France), isolated from Château d'Yquem grapes in 1977 and UMRSV01M103 (M1 strain, INRA-UMR Santé Végétale collection, Bordeaux, France), and collected from the Medoc appellation Cabernet Sauvignon in 2001. Other vine fungi (INRA-UMR Santé Végétale collection, Bordeaux, France), Guignardia

bidwellii (GUI-BID-BA1) and Cladosporium spp. (00BR5302), were collected from the Barsac appellation and Château d'Arche (Sauternes), respectively. Pure isolates of fungi were grown in Petri dishes containing 30 mL of malt agar medium.

Treatments on Cell Suspension Cultures. Seven-day-old cell cultures were treated according to the different modalities. A S-3-(hexan-1-ol)-glutathione (P-GSH) or (E)-2- hexenal and glutathione (GSH) mix was added at a final concentration of 10 and 300 μM, respectively. Elicitor treatments consisted of adding either methyl jasmonate (MeJA, 25 μM final concentration), a fungal mycelium plug of B. cinerea or a sterile filtrate of either the B. cinerea culture or V. vinifera/B. cinerea coculture. A control method, carried out in similar conditions, was used for each treatment. Water, ethanol (the methyl jasmonate solvent), or induction medium was added to the control cell, depending on the experiments tested.
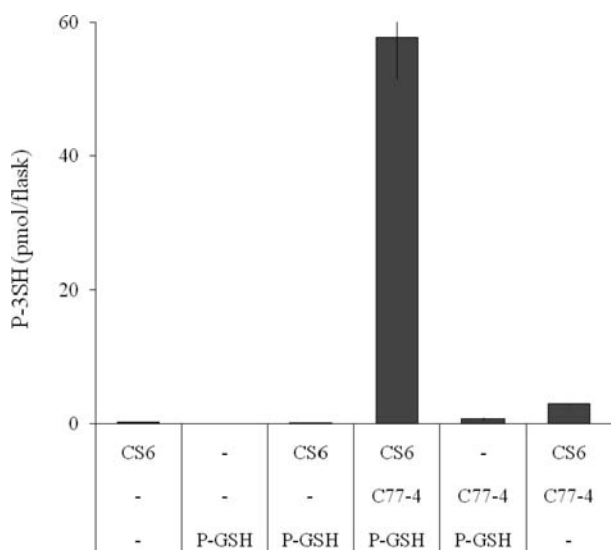
S-3-(Hexan-1-ol)-glutathione Synthesis. S-3-(Hexan-1-ol)-glutathione was synthesized as follows: (E)-2-Hexenal (20 mmol) was added to a stirred solution of glutathione (18 mmol) and cesium carbonate (9 mmol) in distilled water. The mixture was stirred at room temperature overnight. The mixture was extracted with diethyl ether (2 × 30 mL) to eliminate excess (E)-2-hexenal, and S-3-(hexan-1-al)-glutathione was reduced in S-3-(hexan-1-ol)-glutathione with sodium borohydride (4 mmol). After 4 h, the aqueous layer was adjusted to pH 2−3 with 1% citric acid in water. The crude product was concentrated and purified by ion exchange chromatography (DOWEX 50WX8). The fraction eluted with 20% NH_4OH was acidified to pH 2 with 10% HCl. Lyophilization of the aqueous solution gave a white powder compound with approximately 90% purity (contamination with glutathione). The purity was confirmed by liquid chromatography coupled to mass spectrometry (electrospray ionization). An ion-trap mass spectrometer (LCQ Advantage, Thermo Electron, San Jose, CA, USA), equipped with a standard electrospray source, was used for the MS and MS/MS analyses. Spectra were recorded in the positive mode.

MS, m/z (%) 430 (55) $[M + Na]^+$, 408 (100) $[M + H]^+$, 390 (9), 349 (45), 305 (19), 293 (11), 279 (6).

MS^2 m/z 408, m/z (%) 279 (100), 262 (52), 162 (13).

Precursor Extraction and Its Quantification in the Cell and Culture Filtrates. The method for purifying P-3SH from must was based on the protocol described by Thibon et al.[12] as follows: the pH of the Chelating Sepharose column, containing immobilized copper, was adjusted to 10 by percolation with 3 mL of sodium carbonate buffer (Na_2CO_3, 50 mM, pH 10). For the analysis of the cell culture filtrate, 100 μL of the sample was added in 400 μL of ultrapure water containing 200 pg of S-3-(nonan-1-ol)-cysteine as an internal standard. The mix was adjusted to pH 10 by using a sodium carbonate buffer (Na_2CO_3, 1 M, pH 10) and was directly loaded onto the column, which was then washed with 2 mL of potassium phosphate buffer (50 mM, pH 7) and eluted by percolation with 3 mL of hydrochloric acid solution (25 mM). The eluate containing S-cysteine conjugates was evaporated to dryness and dispersed in 500 μL of ethanol. The soluble fraction was again evaporated dry under vacuum in a 2 mL vial. For the P-3SH analysis of the cell extract, 2 mL of ethanolic extract, previously obtained, was supplemented with 200 pg of S-3-(nonan-1-ol)-cysteine as an internal standard, evaporated to dryness at 70 °C to eliminate the ethanol. The dry residue was dispersed in 1 mL of water, adjusted to pH 10, and loaded onto the column using the same procedure as described above.

The vial containing the dry residue, purified as described previously, was capped under a nitrogen stream. For derivatization, MTBSTFA/t-BDMCS (20 μL), and anhydrous pyridine (40 μL) were introduced with a syringe. The mixture was heated to 70 °C for 15 min. A 2-μL derivatized sample was analyzed on a Trace GC gas chromatograph (Thermo Electron Corporation, Milan, Italy) interfaced with a Polaris Q mass spectrometer, equipped with an ion trap analyzer (Thermo Electron). GC-MS/MS analysis conditions were as described by Thibon

Figure 1. *S*-3-(Hexan-1-ol)-cysteine (P-3SH) production in the culture medium of *Vitis vinifera* cell suspensions (CS6 strain) from *S*-3-(hexan-1-ol)-glutathione (P-GSH) and in the presence of *B. cinerea* C77-4 strain. Seven-day-old cultures were either supplemented with 10 μM of P-GSH or not supplemented, and/or they were inoculated with the *B. cinerea*. The data are expressed in pmol per flask (50 mL) at 48 h after treatment and show the means and standard deviations of the duplicate assays ($n = 2$).

Table 1. P-3SH Production in Grapevine Cultures from P-GSH in the Presence of *B. cinerea*: Quantification in Cells and Extracellular Medium[a]

				P-3SH (pmol/flask)		conversion
	V. vinifera	substrate	*B. cinerea*	supernatant	cell	rate (%)
1	CS6			0.2 ± 0.01	2.0 ± 0.3	0.4
2	CS6	P-GSH		0.1 ± 0.03	2.4 ± 0.2	0.5
3	CS6	P-GSH	C77-4	$57.6^* \pm 6.1$	$9.2^* \pm 1.1$	13.3^*
4	CS6		C77-4	$5^* \pm 0.09$	0.6 ± 0.1	1.12^*

[a] P-GSH (10 μM) and/or *B. cinerea* (C77-4 strain) were added to grapevine cells ($n = 2$). The P-3SH levels were quantified in the culture medium (supernatant) and in the cells (cell), 48 h post-treatment, and expressed in pmol per flask (50 mL). The conversion rates of P-GSH in P-3SH are calculated and expressed as a percentage (%). *, statistically different from the negative control (line 1), ANOVA $P = 0.01$.

et al.[11], selecting the following ions: $m/z\ 506 \rightarrow m/z\ 478$ for *t*-BDMS-P-3SH, and $m/z\ 548 \rightarrow m/z\ 520$ for *t*-BDMS derivative of *S*-3-(nonan-1-ol)-cysteine.

■ RESULTS

P-3SH Can Be Produced by Grapevine Cell Cultures. Plant cell suspension cultures have been often used to understand plant defense responses, in particular to decipher the signalization pathway against pathogens.[29−32] In this work, we used cell suspension cultures of *Vitis vinifera* L. cv. Cabernet Sauvignon (CS6). This CS6 strain has already been used in our research institute as a cell culture model allowing, for example, grapevine defense studies[31] and the monitoring of the impact of abscisic acid on anthocyanin biosynthesis.[33] Using this reliable CS6 strain, we can now examine the role of *S*-3-(hexan-1-ol)-glutathione (P-GSH) in *S*-3-(hexan-1-ol)-cysteine (P-3SH) biogenesis. Cell cultures of *Vitis vinifera* (CS6) were cultured for 2 days either in the presence or not of P-GSH, and then P-3SH production was assayed (Figure 1). Grapevine cells, alone, only produced trace levels of P-3SH in the culture medium as well as in the presence of P-GSH (approximately 0.2 pmol/flask). Cells cultured for 2 days with *B. cinerea* (C77-4 strain) and in the presence of P-GSH produced large amounts of P-3SH in the culture medium. Indeed, the P-3SH levels were 250-fold higher (60 pmol/flask) in these conditions compared to those in the cell cultures with P-GSH but without the fungus. The conversion rate of P-GSH to P-3SH by the coculture of grapevine cells and *B. cinerea* was determined to be at 12%.

Then, it was important to confirm that this enrichment of the P-3SH compound, measured only in the culture medium and obtained when the plant cells were cultured with *B. cinerea*, was due to a real stimulation of P-3SH synthesis and not to a potential release of P-3SH in the extracellular media due to the action of

the cell wall degrading enzyme of *B. cinerea*. Thus, the same treatment was repeated, and the P-3SH levels were quantified in both the culture medium and cells (Table 1). As we already noted, CS6 cells alone are able to synthesize P-3SH but only in trace amounts. This compound was primarily kept in the cells (99%). P-GSH addition in grapevine cells triggered a similar production (quantities and localization of P-3SH) as in the control cells. On the contrary, when cells were supplemented with P-GSH and challenged with *B. cinerea*, a high P-3SH production was observed, mainly in the culture medium (86%). With this last experiment, we can conclude that *B. cinerea* was an essential activator for P-3SH production and that P-3SH measurement in the culture medium was an accurate reflection of the total production by cells. Moreover, *B. cinerea* alone is not capable of producing P-3SH, and plant cells by themselves only produce small quantities of this compound. However, in the presence of both *B. cinerea* and grapevine cells, a large amount of P-3SH was released, indicating that the plant cells are the source of P-3SH production and that this production is activated by the presence of *B. cinerea*. This is correlated to what occurred in the grapes. Indeed, the main effect of grape botrytization on Sauvignon blanc and Semillon must is to enhance its aromatic potential; the *S*-3-(hexan-1-ol)-cysteine (P-3SH) concentrations were considerably higher when *B. cinerea* had developed on the grapes. A 100-fold increase was observed only one week after the grapes were infected by *B. cinerea*.[13] Thus, the fungus might stimulate P-3SH formation and increase its release in the medium. Therefore, *B. cinerea* could reveal the grapevine metabolic pathway implicated in P-3SH formation and may help determine the origin of volatile thiol precursors. To conclude, we validated the in vitro model which is a good reflection of grape responses in vineyard conditions. Moreover, we confirm that like some cysteine *S*-conjugates, P-3SH was produced by the breakdown of the corresponding glutathione *S*-conjugate (P-GSH).
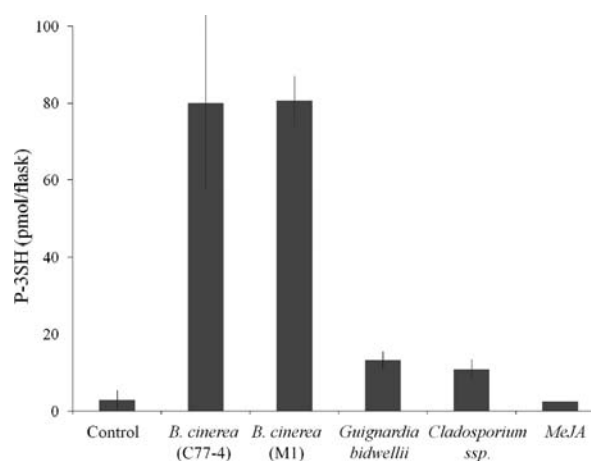
Kinetics of P-3SH Production. P-3SH production was monitored for one week after P-GSH addition to grapevine cells and fungal treatment (Figure 2). In the meantime, 1-mL aliquots were withdrawn from the flasks. The cysteinylated precursor started to be produced in the culture medium 18 h post-treatment; it peaked at 2 days and then gradually decreased. In natural conditions, a similar process was observed during the botrytization of the grapes.[13] In fact, a considerable and rapid increase (approximately 100-fold) in the P-3SH concentrations was observed as *B. cinerea* developed on the grapes, when the
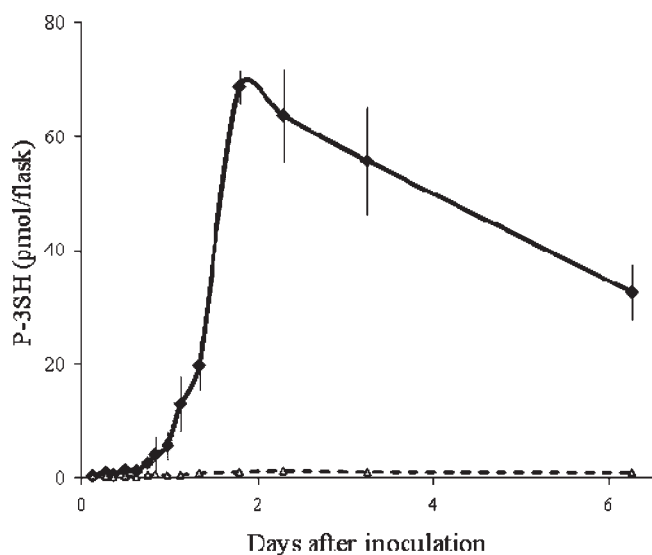
Figure 2. Time-course of P-3SH production in the culture medium of *V. vinifera* cells from P-GSH after inoculation with *B. cinerea* (C77-4 strain, black diamond, ■) or not (negative control, white triangle, △). The data are expressed in pmol per flask and show the means and standard deviations of the duplicate assays ($n = 2$).

Figure 3. Study of *B. cinerea* specificity on the induction of P-3SH production by *V. vinifera* cells from P-GSH after 48 h. Two strains of *B. cinerea* (C77-4, the positive reference, and M1), two other grapevine pathogen fungi *Guignardia bidwellii* (GUI-BID-BA1 strain), and either *Cladosporium* spp. (00BR5302 strain) or methyl jasmonate (MeJA, 25 μM), a general elicitor of grapevine defense, were added to CS6 cells in the presence of P-GSH. P-3SH production was expressed in pmol per flask ($n = 2$).

berries were entirely botrytized but slightly desiccated. On the contrary, a decrease in P-3SH quantities was observed in later stages of botrytization. This decrease, due to the permanent presence of fungus on the grapes or in grapevine cells, could be explained by a possible use of cysteinylated compounds such as nitrogen or sulfur sources for *B. cinerea* and by the fact that the plant cells were unable to continue P-3SH production. These results first confirm that our choice to study P-3SH production 2 days post-treatment was correct, and second, they once again validate our use of grapevine cells as a convenient model to study the biogenesis pathways of volatile thiol precursors in grapes.

Specificity of *B. cinerea* As an Enhancer of Cysteinylated Precursor Biogenesis. The specificity of *B. cinerea*, as a P-3SH production inductor, was verified in conditions similar to those previously described. Two strains of *B. cinerea*, two other pathogen fungi, and one elicitor of plant defense (methyl jasmonate (MeJA)) were tested. The *B. cinerea* C77-4 strain, collected in grapes infected by noble rot and used for the previous assays in this study, was used as a positive control. The *B. cinerea* M1 strain was isolated from grapes that were infected by another strain associated with gray rot. We studied Cladosporium rot (*Cladosporium* spp.), which is associated with a common disease in black cultivars, particularly in Cabernet Sauvignon and *Guignardia bidwellii*, the causing agent of grape Black Rot disease. For the experiment assays, 10 mM P-GSH and either mycelium plugs or MeJA (25 μM) were added to 7-day-old grapevine cell cultures. The P-3SH levels were determined in the culture medium 2 days after treatment (Figure 3). Treatment with MeJA did not trigger a larger synthesis of P-3SH. Otherwise, *Guignardia bidwellii* and *Cladosporium* spp. induced a slight production of P-3SH (around 4-fold higher than the control). On the contrary, the two *B. cinerea* strains showed a similar high enhancer precursor production rate. These results confirm the essential role and, above all, the specificity of the *B. cinerea* species, irrespective of which strain was used to induce the grapevine pathways implicated in P-3SH formation.

Origins of Cysteinylated and Glutathionylated Precursors in Grape Must. As shown above, the results obtained from P-GSH demonstrated that this glutathionylated form was an upstream cysteinylated precursor of volatile thiols. In plants, glutathione S-conjugates are usually involved in the detoxification systems of living organisms. Toxic compounds present in cells are conjugated with glutathione by glutathione S-transferase. When a plant is attacked by a pathogen, they often produce (*E*)-2-hexenal and other reactive aldehydes in response. These compounds, toxic to the pathogen, might be toxic to the plant cell itself at a critical level.[23−25] They can be conjugated with glutathione by the plant in order to decrease this toxicity and then are broken down to form cysteine S-conjugates. In order to determine whether the glutathionylated precursor in plant cells was synthesized by following this pathway, the cell cultures were supplemented with (*E*)-2-hexenal and/or glutathione (300 μM each) instead of P-GSH. The P-3SH assays are presented in Table 2. The addition of (*E*)-2-hexenal and glutathione to grapevine cells allowed a slightly more significant P-3SH formation (88 pmol/flask, with a total conversion rate approaching 0.6%) in relation to the control method (2.5 pmol/flask, 0.02%, line 1). When *B. cinerea* (strain C77-4) was added with (*E*)-2-hexenal and glutathione to the plant cells, a considerable increase in the P-3SH synthesis was observed (2925 pmol/flask, 19.5%, line 5). However, fungal inoculation in plant cells, in the presence of glutathione but without (*E*)-2-hexenal, resulted in a P-3SH formation (line 3) similar to that of the control method (line 1). On the contrary, plant cells supplemented with the fungus and (*E*)-2-hexenal but without glutathione produced large quantities of P-3SH, close to 1300 pmol/flask (about 8.7%, line 4), i.e., 2-fold less than that in the same condition with glutathione (line 5). Therefore, a pool of glutathione from plant cells can be mobilized to synthesize P-3SH in the presence of *B. cinerea* and (*E*)-2-hexenal. However, (*E*)-2-hexenal has to be added to the plant cells in order to obtain a high production of P-3SH: plant cells cannot naturally produce this compound in these conditions. In any case, these results seem to validate our initial hypothesis. It appears that P-3SH was produced in grapes by the

Table 2. P-3SH Production in Grapevine Cells (CS6) from Glutathione (GSH) and (*E*)-2-Hexenal in the Presence of *B. cinerea*[a]

	V. vinifera	substrates	*B. cinerea*	P-3SH (pmol/flask)		conversion rate (%)
				total	(supernatant/cell)	
1	CS6			2.5 ± 0.1	(0.3/2.2)	0.02
2	CS6	GSH	(*E*)-2-hexenal	88* ± 57	(5.2/83)	0.6*
3	CS6	GSH	C77-4	3.7 ± 1.3	(3.7/0)	0.025
4	CS6		(*E*)-2-hexenal C77-4	1320* ± 154	(1010/310)	8.8*
5	CS6	GSH	(*E*)-2-hexenal C77-4	2925* ± 315	(2311/614)	19.5*

[a] GSH, (*E*)-2-hexenal (300 μM each) and/or *B. cinerea* were added to *V. vinifera* cell cultures (n = 2). The P-3SH levels were monitored in the extracellular medium (supernatant) and in the cells (cell), 48 h post-treatment, and expressed in pmol per flask. The conversion rates of P-GSH in P-3SH are calculated and expressed as a percentage (%). *, statistically different from the negative control (line 1), ANOVA P = 0.01.

Table 3. P-3SH Production in *V. vinifera* Cell Suspensions (CS6) Treated with Filtrates of a Grapevine/*B. cinerea* Coculture, 48 h Post-Treatment[a]

	treatment	added volume (mL)	P-3SH			conversion rate (%)
			mean (pmol/flask)	SD	relative amount (%)	
1	*B. cinerea*		2130	224	100	14.2
2	F$_{coculture}$	10	2173	469	102	14.5
3	F$_{coculture}$	5	2343	341	110	15.6
4	F$_{coculture}$	1	254*	107	12*	1.7*
5	AF$_{coculture}$	10	150*	22	7*	1*
6	H$_2$O	10	19*	2	1*	0.1*

[a] Either (*E*)-2-hexenal and/or glutathione (300 μM each), *B. cinerea* (positive reference, line 1) and/or different volumes of an autoclaved (AF) or 0.22 μm membrane sterilized (F) filtrate were added to the grapevine cells. This filtrate consisted in the supernatant of a 2-day-old coculture of CS6 grapevine cells in the presence of *B. cinerea* C77-4. P-3SH was quantified in the culture medium, and data are expressed in pmol per flask (50 mL). The means and standard deviations (SD) of the duplicate assays, the relative amount (as a percentage) of P-3SH production of the positive reference (*B. cinerea*-line 1), and the conversion rate of (*E*)-2-hexenal and glutathione in P-3SH (n = 2) are calculated. *, statistically different from the reference (*B. cinerea*, line 1), ANOVA P = 0.01.

intermediate of the detoxification pathways. This was amplified by *B. cinerea* infection. Moreover, we estimated the conversion rates of (*E*)-2-hexenal and P-GSH in P-3SH and obtained a rate of 20% and 12%, respectively. Therefore, (*E*)-2-hexenal is a more valuable and useful compound, compared to P-GSH, in terms of helping the plant in P-3SH production. This result may be explained by the fact that reactive aldehydes including (*E*)-2-hexenal induce glutathione *S*-transferase.[19]

Preliminary Investigation of *B. cinerea* Metabolites Involved in P-3SH Induction by *V. vinifera* Cell Cultures. In *V. vinifera* suspension cells, we have shown that P-3SH formation was initiated from P-GSH or from (*E*)-2-hexenal and glutathione addition in the presence of mycelium plugs of *B. cinerea*. This P-3SH production may be induced by the infection of plant cells by either *B. cinerea* or compounds acting as elicitors, released in the coculture medium. To further specify this point, a sterile filtrate of a 2-day-old coculture of grapevine cells (CS6) and *B. cinerea* (C77-4) was initially used as an inductor in order to determine if fungal presence was necessary. The coculture was filtrated and sterilized by two processes. First, the supernatant was filtrated on a 0.22 μm membrane in order to eliminate all fungus mycelium and spores (filtrate F$_{coculture}$). For the second assay, the same filtrate was autoclaved for 15 min at 110 °C (autoclaved filtrate: AF$_{coculture}$). Different quantities of the sterilized coculture filtrate were added to the CS6 cell cultures in the presence of (*E*)-2-hexenal and glutathione (300 μM each). The P-3SH content was quantified 2 days after supplementation, as previously described (Table 3). *Botrytis cinerea* (C77-4 strain),

Table 4. Effect of Two Types of Filtrate on P-3SH Production in Grape Cells in Relation to the Age of the Pre-Culture (Five States)[a]

age of preculture (days)	P-3SH (pmol/flask)	
	F$_{B.\ cinerea}$	F$_{coculture}$
0	5 ± 5.7	10 ± 0.4
1	3 ± 0.3	1218 ± 74
2	2 ± 0.7	1897 ± 137
3	244 ± 102	1895 ± 254
7	2081 ± 312	1020 ± 166

[a] A pre-culture of *B. cinerea* was done either in the presence of plant cells (F$_{coculture}$) or not (F$_{B.\ cinerea}$). Their filtrates were withdrawn at different times and sterilized on the membrane. They were tested on grapevine cells supplemented with (*E*)-2-hexenal and glutathione (300 μM each). P-3SH production was quantified post 2-days of treatments, expressed in pmol per flask (50 mL). The means and standard deviations of the duplicate assays are provided.

used as a positive reference, induced a synthesis of P-3SH by plant cells of approximately 2100 pmol/flask (relative amount, 100%, line 1). A similar P-3SH production was observed in the modality with either 5 or 10 mL of the filtrate sterilized on the membrane (F$_{coculture}$) with a relative amount of 110% and 102%, respectively (Table 3, line 3 and 4). The induction of P-3SH formation by the coculture filtrate F$_{coculture}$ appeared to be dependent on the volume added. Indeed, the 1-mL aliquot only
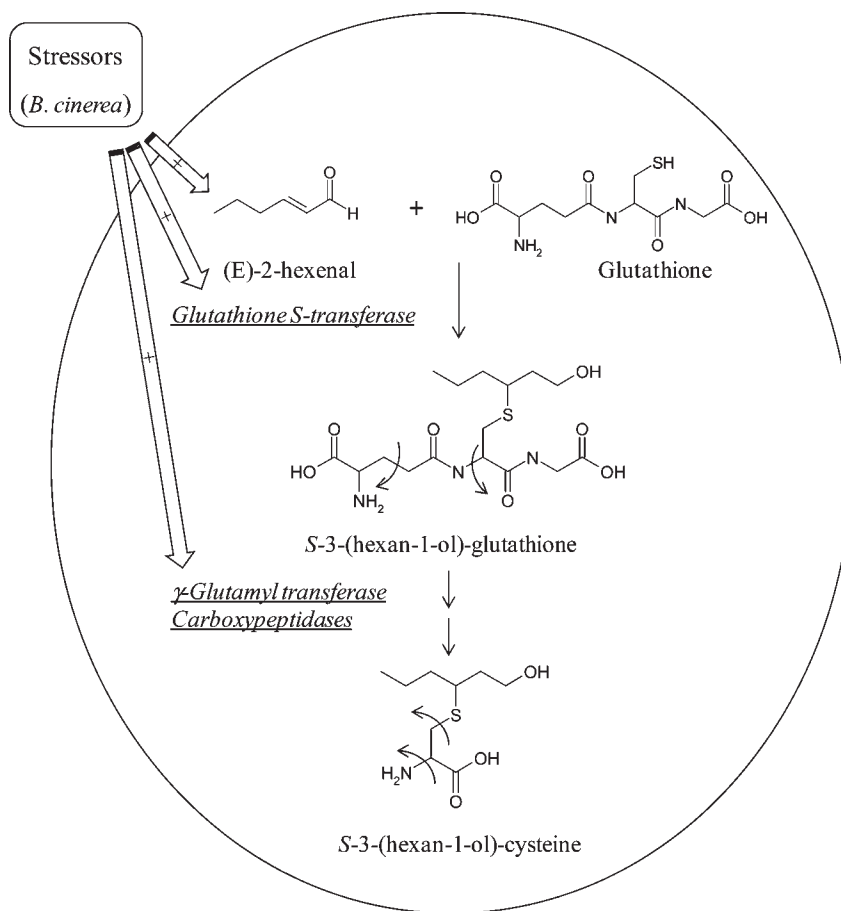
Figure 4. Hypothetical S-3-(hexan-1-ol)-glutathione (P-GSH) and S-3-(hexan-1-ol)-cysteine (P-3SH) formation pathway in grapevines, induced by abiotic or biotic (*B. cinerea*) stressors. Stressors increased glutathione S-transferase and γ-glutamyl transferase enzyme activities in grape berries.[27]

induced 12% of the P-3SH release compared to the positive reference *B. cinerea* (Table 3, line 1 vs line 4). Moreover, the positive effect of F$_{coculture}$ was in part degraded by the thermal treatment at 110 °C (only 7% compared to the reference, line 1—2 vs line 5).

In a second step, we sought to clarify the origin of these stimulating compounds, i.e. to determine whether or not they were produced by the fungus or by the plant in response to the fungus, such as in stress signals. In order to do this, a preculture was done: *B. cinerea* was cultured in the plant medium either with (F$_{coculture}$) or without (F$_{B.\ cinerea}$) grapevine cells. The cultures were stopped at several time points by sterile filtration. The filtrate activity was verified on CS6 fresh plant culture by adding 10 mL of F$_{coculture}$ or F$_{B.\ cinerea}$ in the presence of (*E*)-2-hexenal and glutathione, as previously described. The P-3SH assays are presented in Table 4. As noted in the previous experiment, F$_{coculture}$ rapidly acquired a cell inductive activity with a maximum at 2 days (allowing a P-3SH production close to 2000 pmol by flask). *Botrytis cinerea*, cultivated alone in the plant medium, was equally able to release active compounds in the culture medium, but it synthesizes these inducing molecules more slowly. In fact, F$_{B.\ cinerea}$ was as active as the F$_{coculture}$ or *B. cinerea* mycelium plug in the production of P-3SH but only if it was cultivated for 7 days in the plant medium (allowing a P-3SH production of 2081 pmol per flask). Nevertheless, active compounds were present in both filtrates, F$_{B.\ cinerea}$ and F$_{coculture}$. Thus, we have shown that they were produced and released by *B.*

cinerea, and moreover, their synthesis was stimulated when the fungus was in the presence of plant cells in the culture medium.

Therefore, P-3SH induction was due to *B. cinerea* secreted metabolites and not to the simple infection of *B. cinerea* in plant cells. In addition, the filtrate of the *B. cinerea* culture or its coculture lost its inducing capacity after 2 weeks of storage at either 4 °C or −18 °C (data not shown). Therefore, besides being unstable to heat, the stimulating metabolites released by *B. cinerea* are also unstable over time. The purification of *B. cinerea* active metabolites will be undertaken in future studies to target the fungus effect.

In conclusion, the use of a simple grape cell model allowed the role of *B. cinerea* in P-3SH formation to be clarified. We demonstrated that the addition of *B. cinerea* to grapevine cells considerably increased precursor production, as observed in the vineyard. This model has allowed us to determine the origin of P-3SH in grapes. It derived from the cleavage of S-3-(hexan-1-ol)-L-glutathione, which was itself generated after a conjugation of glutathione on (*E*)-2-hexenal (Figure 4).

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■ ABBREVIATIONS USED

3SH, 3-sulfanylhexan-1-ol; 3SP, 3-sulfanylpentan-1-ol; 3SHp, 3-sulfanylheptan-1-ol; 2M3SB, 2-methyl-3-sulfanylbutan-1-ol; 4MSP, 4-methyl-4-sulfanylpentan-2-one; P-3SH, S-3-(hexan-1-ol)-L-cysteine; P-3SP, S-3-(pentan-1-ol)-L-cysteine; P-3SHp, S-3-(heptan-1-ol)-L-cysteine; P-2M3SB, S-3-(2-methylbutan-1-ol)-L-cysteine; P-GSH, S-3-(hexan-1-ol)-L-glutathione; MeJA, methyl jasmonate; GSH, glutathione; ANOVA, analysis of variance.

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