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Review

# Use of Elicitors and Beneficial Bacteria to Induce and Prime the Stilbene Phytoalexin Response: Applications to Grapevine Disease Resistance

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**Abstract:** Phytoalexins are naturally occurring molecules with antimicrobial activity deriving from the secondary metabolism of plants; they are synthesized in response to physical agents or stresses and phytopathogenic agents (fungi, bacteria and viruses), as well as numerous chemical compounds and biological control agents. Among these, grapevine phytoalexins, which belong to the chemical group of stilbenes, exhibit biocidal activity against a large number and variety of plant pathogens. It is important to investigate whether induction of stilbene phytoalexin production can serve to protect this plant against its pathogens. Quite a few chemical compounds, derivatives of phytohormones bio-elicitors as well as biocontrol agents, have been used to induce the synthesis of stilbene phytoalexins with the aim of conferring protection to grapevine against its main diseases (gray mold, downy mildew, powdery mildew and esca). This article reviews the experiments that have been carried out in this direction during the last 30 years and shows that the observed protective effects towards pathogens are generally linked to induction and priming of the grapevine phytoalexin response, confirming the interest in using, in a more general way, stimulation of the production of phytoalexins in plants as a basis for crop protection.

**Keywords:** stilbenes; phytoalexins; resveratrol; elicitors; plant defenses; chemicals; biological control agents



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## 1. Introduction

According to the fundamental observation made by Müller and Börger [1] 80 years ago, infection with an incompatible strain of *Phytophthora infestans*, capable of initiating hypersensitivity reactions in potato tubers (*Solanum tuberosum*), significantly prevented the effect of a subsequent infection with another strain (compatible) of *P. infestans*. This inhibition phenomenon was linked to the synthesis by the plant of a “chemical principle”, making the tissues resistant to infection, which these two researchers named “phytoalexin”. Phytoalexins are produced in a certain number of plant families, mainly (but not exhaustively) Leguminosae, Fabaceae, Solanaceae, Malvaceae, Poaceae, Brassicaceae (Cruciferae) and Vitaceae [2,3]. Phytoalexins are compounds with an antimicrobial activity generally ranging between 10 and 100 µM, with a molecular mass over 1500 Da, for example, high molecular-ordered stilbene oligomers such as pauciflorol D having a mass of 1587.624 Da [4–6]. Their production essentially corresponds to a de novo synthesis in response to various biotic and

abiotic stresses. Their study has given rise over the last 30 years to many works on the elucidation of their biosynthetic pathways and the regulation of their syntheses, finding interesting applications in engineering of these pathways in microbes and plants [2,7–9]. Phytoalexins exhibit a very broad spectrum of biological activity against a number of living organisms, from viruses, bacteria, fungi and plants to animals that also take part in allelochemical processes among plants. Namely, the antimicrobial function of phytoalexins and the role they play in the defense mechanisms of plants have been a central topic of study among the plant pathologist community. Of prime concern for plant pathologists is to find out if the stimulation of the synthesis of phytoalexins by different means (chemical substances, biological elicitors, beneficial microorganisms) can be of interest in terms of crop protection [10].

In the phytoalexin world, stilbene phytoalexins have received special attention, without doubt linked to their proven role in human health as anti-cancer, antioxidant and cardio- and neuroprotective agents. They are not strictly limited to the Vitaceae, where they were first identified in 1976 following the pioneering works of Langcake and Pryce [11–13]. Here, we unveil the different ways of inducing the synthesis of these compounds in grapevine during experiments carried out on detached leaves or potted grapevine, including also describing some experiments conducted in the vineyard. The challenge is to verify whether the stimulation of stilbene phytoalexin production can contribute to protecting grapevine from pathogens' attacks with possible applications in the vineyard. More generally, extending the results described in this review may also open the use of stimulating phytoalexin synthesis as a basis for crop protection.

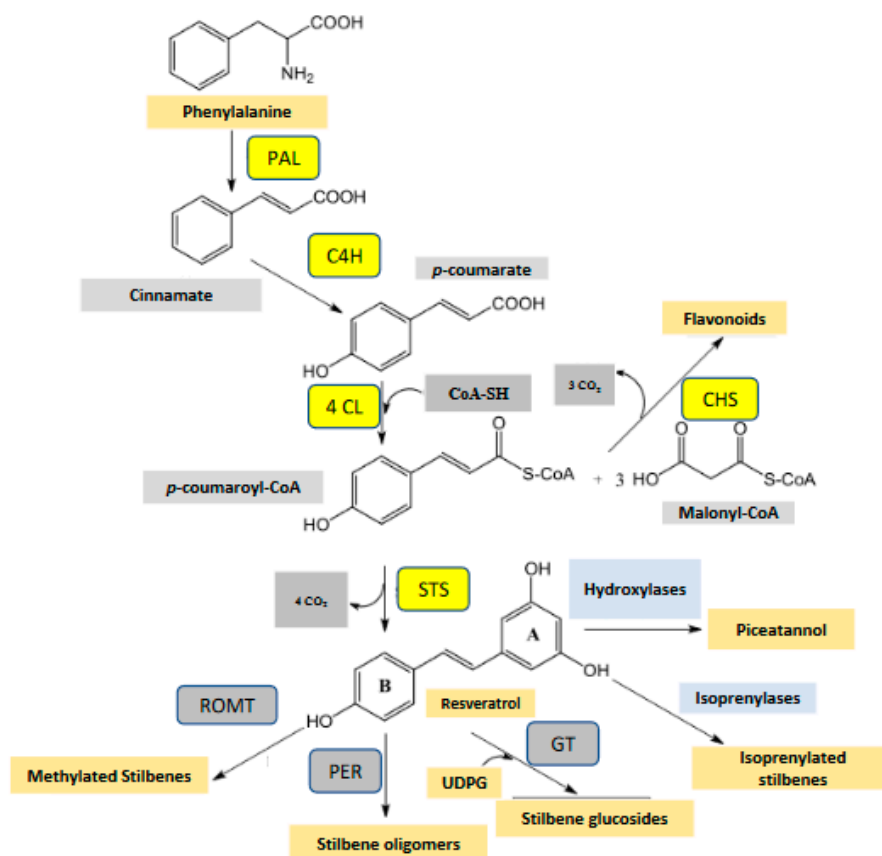
## 2. Chemistry and Antifungal Activity of Some Stilbenes of Grapevine: A Brief Overview

A recent and comprehensive study on these aspects has recently been published [5]. Stilbenes belong to a restricted chemical group compared to the large family of flavonoids [14]. But it is in grapevine that this class of compounds presents a spectacular diversity [5,15]. In fact, 48 different coding genes have been counted in the stilbene synthase family in grapevine, of which 33 are potentially active [16,17].

Building of the resveratrol core begins with phenylalanine [5,18] undergoing oxidative deamination catalyzed by phenylalanine ammonia lyase (PAL), leading to trans-cinnamic acid (Figure 1). The latter is hydroxylated to para-coumaric acid (trans isomer) by a specific cytochrome P450 hydroxylase, C4H, with para-coumaric acid being then converted into its coenzyme A thioester, para-coumaroyl-CoA, by binding with a coenzyme A molecule via a cinnamoyl-CoA ligase (4CL). This is a dichotomous system that is unique in the plant kingdom and comprises two enzymes (chalcone synthase (CHS) and stilbene synthase (STS)) using the same substrates (p-coumaroyl-CoA and three malonyl-CoA units (formed from glycolysis)) that affords flavonoids, on the one hand, and stilbenes, on the other (Figure 1). Resveratrol undergoes intense metabolism involving methylation, hydroxylation, glycosylation and, especially, peroxidation reactions, leading to the formation of stilbene oligomers [6].

Certain methylated, glycosylated and hydroxylated stilbene derivatives, as well as numerous oligomers, have been identified in grapevine [5,15]. Despite that, few enzymes catalyzing the transformation of resveratrol into its derivatives have been characterized both at the genomic and functional level. Piceatannol, a hydroxylated derivative of resveratrol, was characterized for the first time in grapevine [19] (not pictured). Though its structural relationship with resveratrol suggests that it could be synthesized directly by hydroxylation of the latter by a flavonoid-3-hydroxylase-like enzyme (F3H) as in flavonoid biosynthesis, no experimental evidence of the genesis of piceatannol from resveratrol has been reported to date. Pterostilbene, which is a dimethylated stilbene identified in 1979 by Langcake's group [20], is undoubtedly the best-known resveratrol derivative (Figure 2). The direct biosynthesis of pterostilbene from resveratrol is catalyzed by a resveratrol-O-methyl transferase (ROMT), which was cloned from mildew-infected grape leaves and function-

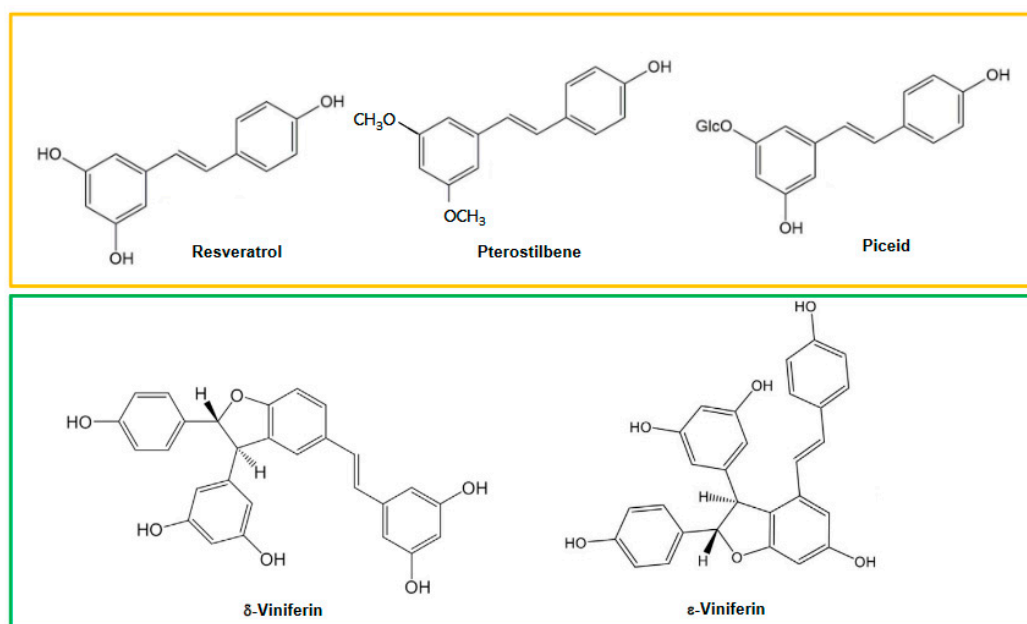
ally characterized. This enzyme ensured the methylation of resveratrol into pterostilbene in vitro and in vivo [21]. Isorhapontigenin (not pictured) is a monomethylated stilbene likely resulting from the direct methylation of piceatannol. Piceid, the 3-*O*-resveratrol glucoside, plays an important role in the metabolism of resveratrol where it is considered a storage form (Figure 2). Glycosylation reactions are generally catalyzed by glycosyltransferases using uridine diphospho-glucose (UDPG) as a glucose donor. Although there are 240 putative coding genes for glycosyltransferases (GTs) in grapevine [22], only two genes (*VLRSGt* and *VvUGT72B27*) encoding GTs which are active on resveratrol, leading to piceid synthesis, have been identified in grapevine [23,24].



**Figure 1.** Biosynthetic pathway from phenylalanine to resveratrol and subsequent metabolism. Abbreviations: PAL, phenylalanine ammonia lyase; C4H, cinnamate-4-hydroxylase; 4CL, cinnamoyl-CoA ligase; STS, stilbene synthase; CHS, chalcone synthase; ROMT, resveratrol-*O*-methyltransferase; PER, peroxidases; GT; glycosyltransferases; UDPG, uridine diphospho-glucose. Note that isoprenylated stilbenes are not present in grapevine.

Stephenson and his group have brilliantly demonstrated that resveratrol constitutes the building block of all oligomers whose levels of condensation can reach seven resveratrol units in the case of pauciflorol D. Oligomer formation initially proceeds by the oxidation of resveratrol into several radicals, which then condense with each other according to defined coupling modes [4]. Oxidation of resveratrol is linked to the action of various peroxidases, some of which have previously been described in grapevine, namely, the peroxidases A1 and B2 located in the apoplasmic compartment and the vacuolar peroxidase B5 [25–27]. Horseradish peroxidase (HRP) was employed in Langcake’s group’s pioneering works reporting, in that case, the formation of  $\delta$ -viniferin [28]. It is unknown, however, whether peroxidases are able to orientate the polymerization of resveratrol radicals towards specific oligomers; the use of these enzymes, such as HRPs, in vitro generally affords complex mixtures of stilbene oligomers [4,5,29]. Current hypotheses favor the involvement

of chemical reactions between the radicals formed (Friedel–Crafts reactions, oxa-Michael additions) to explain these oligomerization processes [4].



**Figure 2.** Structures of the five main stilbenes evoked in this study. Stilbene monomers, resveratrol; pterostilbene and piceid (3-O-resveratrol-β-D-glucoside). Resveratrol dimers, δ-viniferin and ε-viniferin, present in the Vitaceae. Glc = β-D-glucosyl moiety.

Many grapevine pathogenic microorganisms can elicit both the synthesis and the accumulation of stilbene phytoalexins. Following the pioneering work of Langcake's group, other researchers have described the synthesis of resveratrol and its derivatives in response to *B. cinerea* [30,31], *Plasmopara viticola* [32,33], *Aspergillus carbonarius*, an ochratoxin A-producing fungus [34] or the Esca complex of diseases responsible for the leaf-stripe disease symptoms in grapevine [35,36]—this list obviously not being exhaustive. Conversely, the produced stilbenes exerted a biocidal activity against these pathogens.

The antifungal activity of stilbenes has recently been described elsewhere [5]. Due to their low water-solubility, the antifungal activity of stilbenes has initially been greatly underestimated. Langcake and Pryce [11], by solubilizing resveratrol in water, reported concentrations > 870 μM for obtaining 50% inhibition of the spore germination in *B. cinerea* (ED<sub>50</sub>), for example, though lower values of 438 μM were reported for the inhibition of the development of the mycelium of this fungus when minute quantities of an organic solvent (acetone) were added to the medium (and even less (88 μM) for *Fusarium oxysporum*) [11]. Data regarding inhibition of the spore germination of *B. cinerea* have since been revisited by incorporating a minimum amount of ethanol into the spore incubation medium to ensure resveratrol solubility. Under these conditions, ED<sub>50</sub> values of 390 μM were noted [37]. Fairly low inhibition values have been reported concerning the reduction of zoospore motility of the oomycete *Plasmopara viticola*, responsible for grapevine downy mildew, with ED<sub>50</sub> of 192 μM and 500 μM [38,39]. Methylated stilbenes such as pterostilbene and isorhapontigenin, respectively derived from resveratrol and piceatannol, exhibited higher biocidal activities, on the order of 2.5 to 10 times compared to the hydroxylated compounds, depending on the type of pathogen. Several studies have reported ED<sub>50</sub> for pterostilbene around 70 μM for the inhibition of the germination of *B. cinerea* spores [28,37,40] and 39–390 μM for reduction in the fungal growth of Esca disease-associated fungi [41]. The biocidal activity of pterostilbene was more accentuated regarding the decreased *P. viticola* zoospore motility, with ED<sub>50</sub> of 9 μM [13] and 14.6 μM [38]. As confirmation of the greater activity of methylated stilbenes compared to their non-methylated counterparts, isorhapontigenin has been reported to be 2.5 times more inhibitory to the development

of downy mildew (ED<sub>50</sub> of 116 µM) than the highly hydroxylated piceatannol (ED<sub>50</sub> of 254 µM) [42].

Surprisingly, dimeric stilbenes such as  $\epsilon$ -viniferin showed quite high biocidal activity, with ED<sub>50</sub> of 220 and 230 µM for the respective inhibition of spore germination and mycelial development in *B. cinerea* [13], and even more strong activity regarding sporulation decrease in *P. viticola* (ED<sub>50</sub> of 12.7 and 70 µM) [38,43]. Finally, several highly molecular-ordered stilbenes—the trimer miyabenol C and three tetramers, namely, hopeaphenol, vitisin A and vitisin B—exhibited remarkable inhibitory activities on the development of downy mildew with very low ED<sub>50</sub> values: 18 µM for hopeaphenol; 40 µM for miyabenol C and, respectively, 20 and 12 µM for the vitisins A and B [43]. The relationship between the chemical structure and the antifungal activity of stilbenes has been particularly discussed in the case of monomers [39,44–46]. It appears that the presence of methoxy groups or electron-withdrawing groups on 4'-hydroxystilbenes in positions 3, 3–4 and 3–5 increases their antifungal activity and their ability to form charge complexes with membrane proteins [39,44,45]. Furthermore, the presence of methoxy groups on the stilbene ring enhances the hydrophobic and lipophilic characters of the corresponding compounds. Thus, pterostilbene or pterostilbene analogues containing a furan moiety exhibited log*P*—this parameter representing the hydrophobicity and the lipophilic character of a given compound—of, respectively, 3.54 and >4 [45,46], whereas resveratrol had a log*P* of only 3.09 [46]. The lipophilic nature of a phytoalexin depends on its ability to penetrate cell membranes, as discussed in Jeandet et al. [5]. The strong antifungal activity of certain oligomeric stilbenes could also be explained by their highly hydrophobic properties and their greater capacity to cross cell membrane systems [6].

The fact that the synthesis of phytoalexins is induced by a large number of pathogenic fungi, their production being possibly associated with an increase in the resistance of plants to infection, has led researchers to wonder about ways to stimulate the production of these compounds with different types of elicitors or by the use of microorganisms through numerous assays carried out in potted plants or in the vineyard [47,48]. The crucial question of transferring the data acquired in the field has been more rarely addressed [49,50]. These questions are developed in the following sections.

### 3. Induction of Stilbene Phytoalexin Synthesis by Chemicals and Possible Applications in the Vineyard

#### 3.1. Organic and Inorganic Metallic Salts

Among the chemicals which can induce phytoalexin production, numerous metallic salts have been described as having the ability to trigger the synthesis of these compounds with more or less application in the vineyard (Table 1). Fairly old studies have reported the use of heavy metallic salts such as, among the most active, silver (AgCl), mercury (HgCl<sub>2</sub>) and copper (CuCl<sub>2</sub>) salts for the induction of pisatin synthesis in pea (*Pisum sativum*) [51]. Also, in pea, copper chloride at a concentration of 5 mM was found to induce the activity of a methyltransferase, catalyzing the methylation of (+)-6 $\alpha$ -hydroxymaackiain into (+)-pisatin [52].

Copper chloride and other metallic salts such as copper sulfate (CuSO<sub>4</sub>), which is widely used in viticulture, have been recognized as inducers of the synthesis of several stilbene phytoalexins and flavonoid glucosides in *Veratrum grandiflorum* [53] and the synthesis of resveratrol,  $\epsilon$ -viniferin and piceid in grapevine plantlets in vitro [54]. Organic or inorganic aluminum-based metallic salts have also been employed in grapevine with potential applications for the protection of this plant in the field.

The specific mechanisms of activation of the grapevine defenses by aluminum, particularly implementation of the phytoalexin system, have been deciphered very recently. Adrian et al. [55] reported a phenomenon of hyper-elicitation of the resveratrol synthesis in detached grapevine shoots treated with aluminum chloride (AlCl<sub>3</sub>) at a concentration of 7 mM in *V. rupestris*. In a similar way to what was described for pisatin biosynthesis in pea, methylation of resveratrol to its dimethylated derivative, pterostilbene, was observed

on grapevine leaf discs by induction of a resveratrol-*O*-methyltransferase in the presence of 1% AlCl<sub>3</sub> [21]. Among the organic aluminum salts, fosetyl-Al has been the subject of numerous studies in plants with applications in the vineyard.

Fosetyl<sup>®</sup>-Al (Aluminum Tris-*O*-ethylphosphonate) is a well-known fungicide (Aliette, Bayer Crop Science) which has shown a fair efficacy against downy mildews and, particularly, grapevine downy mildew (*P. viticola*) [56–59], while also being applied for the treatment of Esca [60]. A relationship between the protective action of fosetyl-Al and a stimulation of the defense mechanisms in tobacco [61,62] and, more specifically, of the phytoalexin system in tobacco [63], citrus [64] and grapevine has been reported [65,66]. These latest works, carried out on detached grapevine leaves or on leaf discs, have reported that it was difficult to distinguish which part could be attributed to the stimulation of phytoalexin synthesis following infection by sporangial suspensions of *P. viticola* and which part was due solely to the application of the fungicide. For Raynal et al. [65], fosetyl-Al alone did not induce phytoalexin synthesis in grapevine (resveratrol and cis- $\epsilon$ -viniferin), whereas these compounds saw their concentration increase in the tissues when this fungicide was applied simultaneously with—or 24 h after—infection (priming effect) (Table 1). For Dercks and Creasy [67], the sole application of fosetyl-Al induced resveratrol production but at modest levels (4.7  $\mu$ g/g fresh weight). Post-infectious applications of fosetyl-Al, that is, 22 h after inoculation with *P. viticola*, led to a significant rise in resveratrol amounts (19.1  $\mu$ g/g fresh weight in the susceptible variety, Riesling; 69  $\mu$ g/g fresh weight in the mid-resistant species, *V. rupestris* and 107.38  $\mu$ g/g fresh weight in the resistant variety, Castor). On the other hand, the 2 to 4 times increase in the content of resveratrol compared to the control during the pre-infectious application of fosetyl-Al (24 h before inoculation) was particularly interesting since it suggested that, prior to application of the fungicide, it displayed a priming effect on the synthesis of resveratrol and implementation of the phytoalexin response [66]. Fosetyl-Al efficacy towards the sporulation of *P. viticola* depended on the date of application of the treatment, the dose used and, above all, the grapevine variety. The doses of fosetyl-Al required to totally suppress sporulation at the pre-infectious stage varied from 200  $\mu$ g/mL for *V. rupestris* (mid-tolerant) and the cv Castor (resistant) to 400  $\mu$ g/mL for the cv Riesling (susceptible). At the post-infectious stage, the sensitive variety was no longer protected by fosetyl-Al, regardless of the dose; *V. rupestris* was protected only with the highest dose, while the resistant variety (Castor) remained protected by both treatment doses.

To appreciate the capacity of aluminum to trigger stilbene phytoalexin production in grapevine, tests were carried out for 90 s using a fungicide called Synermix (Table 1). This contained aluminum chloride in combination with a seaweed extract. Previously, trials conducted in compliance with regional treatment programs against gray mold in the vineyard of Bordeaux, Champagne and the Val de Loire over an eight-year period revealed an average efficacy of this fungicide of 33.6% against *B. cinerea* [67,68].

**Table 1.** Induction of stilbene phytoalexin synthesis by chemicals associated with resistance of grapevine to diseases.

Chemicals	Plant Material	Biological Inputs	References
Fosetyl-Al	Potted grapevine (cv Carignan)	Protection against <i>P. viticola</i> infection. No effect of fosetyl-Al alone on stilbene accumulation, but active when applied at the time of infection or shortly after.	[65]
Fosetyl-Al	cv Riesling (susceptible), <i>V. rupestris</i> (mid-tolerant) and cv Castor (resistant) leaf discs	Total suppression of <i>P. viticola</i> sporulation for pre-infectious applications. No protection for the susceptible cultivar during post-infectious applications. Induction of stilbene production and priming effect.	[66]

Table 1. Cont.

Chemicals	Plant Material	Biological Inputs	References
Synermix (aluminum chloride in combination with a seaweed extract)	Eight-year experiments in a vineyard ( <i>V. vinifera</i> )	Efficacy of 33.6% towards <i>B. cinerea</i> infection vs. Iprodione (42.2%). Efficacy of 70.7% for the combination Synermix/Iprodione. High AlCl <sub>3</sub> -induced resveratrol production.	[55,68]
Copper sulfate	In vitro plantlets and potted grapevine (cv Chardonnay)	Protection against <i>B. cinerea</i> infection (>60%) but only 38% against <i>P. viticola</i> , associated with an induction of chitinase and β-1,3-glucanases along with stilbene production.	[54]
Fosetyl-Al	Nine-year experiments in a vineyard (cvs Albana and Sangiovese)	Reduction of the foliar symptoms of Esca decorrelated with stilbene production.	[60]

At the same time, application of a fungicide known for the treatment of gray mold, Iprodione, demonstrated an effectiveness close to 42.2%. Interestingly, use of the two fungicide products in combination showed an efficacy in a vineyard of 70.7%, that is, a synergistic effect of +9.1% compared to the calculated theoretical efficacy of the combination of the two fungicides according to Colby's formula [69], where TE is the theoretical efficacy of the combination of the two fungicides and  $\alpha$  and  $\beta$  are, respectively, the efficacy of fungicides A and B measured in the fields. The synergy is deduced by subtracting the efficacy of the fungicide combination in the fields from the theoretical efficacy of the fungicide combination:

$$TE = \alpha + \beta - \left[ \frac{\alpha \times \beta}{100} \right] \quad (1)$$

In an attempt to explain the effectiveness of Synermix in the control of gray mold in the vineyard, the hypothesis of a stimulation by this fungicide of the natural defenses in grapevine (phytoalexin system) has been put forward. On the basis of previous works carried out with metallic salts (salts of copper and mercury), the inducing effect of aluminum chloride, one of the components of Synermix, was evaluated on the synthesis of phytoalexins in this plant. A study published in 1996 clearly demonstrated a hyper-elicitation of resveratrol production, resulting in quantities on the order of a few hundred micrograms per gram of fresh weight on grapevine shoots (*V. rupestris*) dipped in tubes containing solutions of AlCl<sub>3</sub>·6H<sub>2</sub>O ranging from 7 to 90 mM with 15 h of incubation [55]. In contrast, no eliciting activity was observed on resveratrol synthesis using the seaweed extract. Synermix was marketed as an adjuvant for fungicidal products and not as a fungicide but was withdrawn from the market.

Wang et al. [70] recently elucidated the mechanisms by which the aluminum cation Al<sup>3+</sup>, which is present both in organic and metallic salts, triggered phytoalexin production in grapevine cell cultures. Aluminum initiated intracellular actin remodeling (Al-actin bundling) through the activation of NADPH oxidases located at the plasma membrane level, the membrane-associated NADPH oxidases known as Reactive Burst Oxidase Homologs (RBOHs). These proteins are responsible for the oxidative burst that produces reactive oxygen species, which play a crucial role in plant development and growth as well as in plant responses to stresses [71,72]. An induction of two subfamilies of genes encoding resveratrol synthase (*STS47* and *STS27*), as well as the gene coding for the transcription factor *MyB14* involved in the stilbene synthase promoter, was observed through aluminum action, a phenomenon which was RBOH-actin-dependent. This resulted in resveratrol synthesis. Interestingly, similar observations were reported in planta in two cultivars of *V. vinifera* and *V. sylvestris*, demonstrating that these mechanisms are validated from the cell



to the whole plant. The fact that RBOHs mediate the remodeling of actin in response to aluminum, involving the activation of several genes' coding for the synthesis of stilbene phytoalexins in grapevine, would make this enzyme a privileged target to prime host defenses upstream from the infection process. The authors, therefore, suggested looking for ways other than aluminum, whose toxicity is proven, to stimulate RBOH proteins as alternative approaches [70].

With copper sulfate ( $\text{CuSO}_4$ ) applied at a concentration of 50  $\mu\text{g}/\text{mL}$  to in vitro grapevine plantlets or potted plants (cv Chardonnay), a protective effect of more than 60% in the extension of *B. cinerea* necrosis and only 38% in the case of downy mildew development was observed [54]. This protection was associated with an induction of chitinase and  $\beta$ -1,3-glucanase activities in the plant as well as a significant rise in stilbene synthesis in the leaves (resveratrol: 20  $\mu\text{g}/\text{g}$  FW;  $\epsilon$ -viniferin: 45  $\mu\text{g}/\text{g}$  FW and piceid: 25  $\mu\text{g}/\text{g}$  FW). These experiments showed that pre-treatment with non-toxic concentrations of  $\text{CuSO}_4$  led to increased protection against gray mold and, to a lesser extent, against downy mildew, correlated with a stimulation of the plant defense mechanisms. This effect can be potentiated by co-treatment with chitosan (see Section 3.3).

Application of fosetyl-Al for the control of the main pathogens responsible for Esca in grapevine showed a protective activity of this treatment against the incidence of the disease foliar symptoms during experiments carried out in a vineyard from 1999 to 2007 on cvs Albana and Sangiovese [60]. However, the protective effect of fosetyl-Al appeared to be decorrelated from the production of stilbenes (resveratrol and  $\epsilon$ -viniferin), evidenced by the application of fosetyl-Al alone not inducing their synthesis.

### 3.2. Phytohormone Derivatives with a Stimulating Effect on Grapevine Natural Defenses

A certain number of phytohormones along with their derivatives (salicylic acid, SA; jasmonic acid, JA, and its methylated derivative methyljasmonate, MeJA; ethylene; etc.) display an inducing effect on plant systemic acquired resistance (SAR) including active responses such as accumulation of pathogenesis-related proteins, phytoalexins or plant cell wall strengthening. Several substances (benzothiadiazole, methyljasmonate and ethephon) have been used as resistance inducers in grapevine against biotrophic, hemi-trophic or necrotrophic pathogens. These operate through the activation of different phytohormone signaling networks mediated by salicylic acid, jasmonic acid and/or ethylene, although several pathways can be activated simultaneously [73,74]. Some of these molecules (benzothiadiazole, methyljasmonate, alone or in combination) have been used in particular with the aim of modifying polyphenol metabolism in grapes, increasing stilbene biosynthesis through induction of the genes encoding enzymes of the phenylpropanoid/stilbene pathway [75,76].

Benzothiadiazole (S-methyl benzo[1,2,3]thiadiazole-7-carbothioate, Bion, Syngenta) (BTH) is a salicylic acid (SA) mimic which provides plants with protection against pathogens without possessing a direct antifungal activity, instead acting on the induction of systemic acquired resistance (SAR) by SA-dependent pathways [77,78]. In the study conducted by Iriti et al. [79], BTH (0.3 mM) was applied in three different treatments within one week to detached grape bunches (*V. vinifera* cv Merlot) at veraison time (Table 2).

Subsequent infections of the bunches by *B. cinerea* carried out at the fourth week after treatment with BTH clearly showed a protective effect of the treatment regarding contamination of the grape clusters by gray mold, since 87% of them only exhibited between 0 and 10% infection, while 92% of the untreated ones showed a contamination rate of between 50 and 100%. The authors then attempted to explain the observed protective effect of BTH by an increase, particularly, in the synthesis of resveratrol in the berries. A 40% increase in the resveratrol content of the berries treated with BTH compared to the control (0.546 mg/kg FW versus 0.390 mg/kg FW) was observed, but these very low levels in absolute values were, in this case, not high enough to support the hypothesis of a stimulation of the phytoalexin system by BTH [79]. Another work has reported the protective effect of BTH used at a dose of 0.05% on the grapevine *V. vinifera* cv Grüner Veltiner, a variety susceptible to

downy mildew (*P. viticola*), against the infection by this pathogen, resulting in a reduction in the incidence of the disease to only 15.8% in greenhouses [80]. The priming effect of BTH was linked to the activation of various genes encoding PR proteins, peroxidase, PAL (phenylpropanoid pathway) and STS (resveratrol synthesis) (Table 2).

Application of BTH (1.9 mM) to the leaves of grapevine plants of *V. vinifera* cv Cabernet Sauvignon was carried out prior to inoculation with different isolates of downy mildew (*P. viticola*) and powdery mildew (*Erysiphe necator*) [81]. BTH exhibited a growth inhibition for all the tested isolates ranging from 60 to 98% (*P. viticola*) and from 65 to 75% (*E. necator*), thus conferring grapevine a high protection. In addition to an overexpression of a number of genes encoding PR proteins, lipoxygenase (LOX-9) and glutathione-S-transferase (GST) following pretreatment with BTH at different times following inoculation by the two isolates of each of the two pathogens, an overexpression of the *STS* gene was observed, as well as a significant accumulation of piceid (up to 60 µg/g dry weight (DW)), whereas the levels of resveratrol remained very low (2 to 8 µg/g DW). Quite surprisingly, taking account of the fact that pterostilbene is generally only a minor component, in quantitative terms, of grapevine phytoalexin response [82,83], the latter accumulated significantly in the leaves under BTH treatment (whether contaminated or not), reaching, for example, 16 to 21 µg/g DW in plants treated with BTH and inoculated with *E. necator*. The high fungistatic and fungitoxic activities of this compound compared to its non-methylated derivative, resveratrol, partly explained the protective effect exerted by BTH against subsequent contaminations by *P. viticola* and *E. necator* [81]. These experiments confirmed that the protection provided by BTH treatment involved an activation of SAR (Table 2).

Very interesting experiments were conducted in the vineyard over two consecutive years (2014 and 2015), comparing two protection treatments, one with a classic fungicide, pyrimethanil, and the other with the chemical elicitor BTH, on grapevine cv Sémillon, which is very susceptible to gray mold (*B. cinerea*) [84]. In vitro activity tests showed that pyrimethanil had an  $IC_{50}$  51 times lower than BTH (67.5 mg/L vs. 3450 mg/L). In the vineyard, the differences observed regarding the protective effects of these two compounds were considerably reduced: 35% reduction in disease incidence for pyrimethanil and 20% for BTH in 2014, 29% and 25%, respectively, in 2015, demonstrating the relative efficacy of BTH treatments. Establishing a correlation between the reported protection activities with an overexpression of the grapevine defense genes was even trickier. The genes coding several PR proteins were up-regulated as well as those encoding flavonoid biosynthesis, *VvCHS* and *VvF3H*, resulting in a 42% increase in the total polyphenol content 48 h after the treatments' application; an up-regulation of the genes encoding stilbene biosynthetic pathways, *VvSTS* and *VvROMT*, was delayed seven days after the treatment. Overexpression of these two genes was not accompanied by a significant production of stilbenes (piceid and pterostilbene), making it difficult to correlate the protective activity of BTH in the vineyard and activation of the phytoalexin biosynthesis [84] (Table 2).

The eliciting effect of methyljasmonate, a derivative of jasmonic acid, a phytohormone implicated in the regulation of plant defense responses, also possessing regulatory functions in plant growth and development, has been the subject of numerous studies on grapevine cell cultures [5,85]. We limit ourselves here to works carried out on grapevine plants. *V. vinifera* cv Cabernet Sauvignon grapevine plants placed in a confined atmosphere and subjected to methyljasmonate (MeJA) vapors at a concentration of 400 nmol/L saw, 15 and 30 days after veraison, their piceid concentration increasing in the leaves for the two phenological stages (1200 and 990 nmol/g DW), but without any effect on resveratrol concentration, which remained at very low levels (21 and 74 nmol/DW) [86]. In grape berries, stilbenes were almost undetectable or in very low amounts in every the phenological stage. Interestingly, MeJA showed a potentiating effect on the production of resveratrol in the leaves in response to UV stress (1632 and 2658 nmol/g DW), but without any significant effect on the berry resveratrol content. Treatments with airborne MeJA could find application in the vineyard for protecting leaves against the attacks of pathogenic agents at the last stages of ripening but did not produce notable effects on that in grape

berries. This type of treatment found, on the other hand, application for the protection of flowers and young berries whose stilbene phytoalexin production was very active [87]. Vezzuli et al. [88] reported similar experiments by spraying MeJA at different phenological stages on potted grapevine of the Barbera variety (fruit set, veraison and maturity, or by combining these treatments over the entire ripening period). The activating effect of MeJA on stilbene production (resveratrol +  $\epsilon$ -viniferin) was mainly observed in ripe berries, but the levels of phytoalexins attained were not high enough ( $< \mu\text{g/g DW}$ ) to inhibit further pathogen development.

Pre-treatment of foliar cuttings (cv Cabernet Sauvignon) or in the vineyard (cv Merlot) with MeJA used at doses of 5 mM (in a single application for potted plants or in several applications in the vineyard every 7–10 days, from bloom to veraison) conferred a 75 and 73% reduction, respectively, in the symptom incidence of powdery mildew (*E. necator*), while treatment with fosetyl-Al provided with an 83% protection [89]. However, no protective effect against the development of downy mildew (14%) was reported. A rise in the resistance to powdery mildew was correlated with a pre-infectious increase in the transcript levels of the class 4 acid chitinase (25 times), as well as in PAL and STS (44 and 11 times, respectively). Activation of the PAL and STS genes was accompanied by an increase in the stilbene content of the leaves (244 nmol/g DW for piceid; 176 nmol/g DW for resveratrol; 10 nmol/g DW for pterostilbene; 80 and 8 nmol/g DW for the dimers  $\epsilon$ - and  $\delta$ -viniferin, respectively). These data showed a correlation between pre-treatment with MeJA, stimulation of grapevine defense mechanisms including phytoalexin response and protection against powdery mildew in the vineyard [89].

Three different elicitors [90] were tested as regard to the protection of potted grapevines (cv Cabernet Sauvignon) against infection by downy mildew (*P. viticola*), MeJA, BTH and phosphonates (PHOS), a constituent of fosetyl-Al [91]. Pretreatment with the three elicitors at the respective doses of 2 g/L, 1.5 g/L and 1.09 g/L applied six days before inoculation provided a protective effect of 98.5, 97 and 85.8%, respectively towards infection by downy mildew. At the gene level, BTH induced the overexpression of many PR proteins unlike leaves treated with MeJA and PHOS. The three treatments caused an increase in the concentration of flavanols, which are antifungal compounds (MeJA > BTH > PHOS), as well as a significant induction of stilbene biosynthesis (piceid up to 8  $\mu\text{g/mg DW}$ ;  $\epsilon$ -viniferin: 9  $\mu\text{g/mg DW}$ ) and, to a lesser extent, of resveratrol and pterostilbene ( $< 1 \mu\text{g/mg DW}$ ) for MeJA. This strong stilbene accumulation appeared, however, to be decorrelated from the expression of the *VvPAL* and *VvSTS* genes (Table 2).

Ethylene is a gaseous phytohormone that plays a crucial role in plant development and growth, plant stress responses, flowering and maturation, as well as senescence and germination [92]. Ethephon (2-chloroethylphosphonic acid) is an ethylene-releasing compound. Used at a dose of 0.5 g/L (Sierra, Bayer, CropScience), it showed a protective effect against *E. necator* both on leaf discs and grapevine cuttings of Cabernet Sauvignon, with disease control rates ranging from 64 to 70% [93]. In addition to providing protection against powdery mildew, there was a significant induction in the expression levels of PR proteins, as well as those of PAL and STS genes by factors of 10 and 67, respectively, associated with an increase in the synthesis of piceid (312 nmol/g DW), resveratrol (133 nmol/g DW) and  $\epsilon$ -viniferin (58 nmol/g DW), as well as  $\delta$ -viniferin and pterostilbene (12 and 10 nmol/g DW). While MeJA or ethephon applied individually to foliar cuttings (cv Cabernet Sauvignon) at the respective doses of 5 nM and 6.94 nM reduced powdery mildew (*E. necator*) colonization by 60%, fosetyl-Al showed an 85% effectiveness under the same conditions; however, the MeJA/ethephon combination only reduced the incidence of the disease by 30% [94]. The effect of the combination of the two chemicals on the stimulation of defense genes (*GLU*,  $\beta$ -1,3-glucanase, *PAL1* and *STS*) and the production of stilbenes was difficult to interpret since gene expression analysis was carried out on cell suspensions, a model very different from that used for the protection tests. The MeJA/ethephon co-treatment, although not conferring a similar protection level compared to each of the treatments, led to an overexpression of the *PAL1*, *STS* and *GLU* genes (by, respectively, 120,

125 and 4500 times), as well as an increase in the contents of resveratrol (10.8 nmol/mg DW) and piceid (18.3 nmol/mg DW). The antagonistic effect observed during the application of the two products in combination at the plant protection level could be due to their different actions on signaling pathways and is probably linked to suppression of the MeJA-induced expression of some *PR proteins* and *LOX* genes by ethephon [94].

**Table 2.** Induction of grapevine disease resistance linked with stilbene phytoalexin synthesis by phytohormone derivatives.

Compounds	Plant Material	Biological Inputs	References
Benzothiadiazole	Detached grape bunches (cv Merlot)	Significant decrease in <i>B. cinerea</i> incidence of the grape clusters (87% of them exhibited between 0 and 10% infection). Low stilbene accumulation.	[79]
Benzothiadiazole	Potted grapevine (cv Grüner Veltiner)	Reduction in downy mildew disease incidence to 15.8%. Strong activation of <i>PAL</i> and <i>STS</i> genes as well as peroxidases and PR-proteins.	[80]
Benzothiadiazole	Leaves of grapevine plants (cv Cabernet Sauvignon)	Growth inhibition in <i>P. viticola</i> (60–98%) and <i>E. necator</i> (65–75%). Overexpression of the <i>STS</i> gene with high piceid accumulation and significant accumulation of pterostilbene. Overexpression of several genes coding for PR proteins, LOX and GST.	[81]
Benzothiadiazole vs. Pyrimethanil	Two-year experiments in a vineyard (cv Semillon)	Decreases in gray mold incidence by 35 and 20% for pyrimethanil and benzothiadiazole, respectively, in 2014; –29 and –25% in 2015. Up-regulation of <i>VvSTS</i> and <i>VvROMT</i> genes with no correlated accumulation of stilbenes.	[84]
Methyljasmonate	Plant cuttings (one application; multiple applications in a vineyard from floraison to veraison) (cv Cabernet Sauvignon)	Decreases in powdery mildew incidence of 75 and 73% in plant cuttings or in their vineyard, but not for downy mildew. Up-regulation of genes' coding for PR proteins as well as <i>PAL</i> and <i>STS</i> , along with increase in stilbene accumulation.	[89]
Methyljasmonate (MeJA), Benzothiadiazole (BTH), Phosphonates (PHOS)	Plant cuttings (cv Cabernet Sauvignon)	Respectively, 98.5, 97.3 and 85.8% protection towards downy mildew infection with MeJA, BTH and PHOS. High induction of stilbene production, decorrelated from <i>VvPAL</i> and <i>VvSTS</i> expression. High overexpression of PR proteins with BTH, lower with MeJA and PHOS.	[90]
Ethephon	Plant cuttings and leaf discs (cv Cabernet Sauvignon)	Increases in protection (64–70%) against powdery mildew. Up-regulation of defense genes ( <i>PAL</i> and <i>STS</i> ) along with strong stilbene accumulation (piceid, resveratrol and dimers).	[93]

Table 2. Cont.

Compounds	Plant Material	Biological Inputs	References
Methyljasmonate/ Ethephon	Plant cuttings and cell suspensions (cv Cabernet Sauvignon)	Decreases of 60% of the colonization by downy mildew by methyljasmonate or ethephon alone (only 30% when applied in combination; 85% with fosetyl-Al). Increases in <i>PAL1</i> and <i>STS</i> along with increase in piceid and resveratrol production in cell suspensions. Inhibition of some PR proteins and <i>LOX</i> gene-induced expression by ethephon.	[94]

### 3.3. Bio-Elicitors

This section treats bio-elicitors such as, among others, laminarin, chitosan, fungal sterols, bacterial rhamnolipids,  $\beta$ -amino-butyric acid and tagatose, which are all able to induce stilbene synthesis [95,96] (Table 3).

Among polysaccharides, laminarin, a  $\beta$ -1,3-glucan extracted from a brown alga, *Laminaria digitata*, has shown protective effects against *B. cinerea* and *P. viticola* by reducing the symptoms of these two diseases by, respectively, 55% and 75% on leaves of grapevine in vitro plantlets of the Chardonnay and Gamay varieties, both susceptible to gray mold and downy mildew [97]. Although carried out on a different plant model, parallel experiments realized with cell suspensions showed an induction of responsive defense genes (preceded by an oxidative burst), particularly the genes coding PR proteins as well as a strong overexpression (20 times higher than that of the controls) of the *PAL* and *STS1* genes with a concomitant accumulation of resveratrol and  $\epsilon$ -viniferin (Table 3).

Chitosan is also a polysaccharide composed of D-glucosamine and of N-acetyl-D-glucosamine units, partially deacetylated and obtained from the chitin of crabs, shrimps and other crustaceans, known for its bio-stimulant and elicitor properties [98]. The protective properties of chitosan alone or in combination with copper sulfate have been studied towards gray mold and downy mildew as well as its action on the stimulation of grapevine defense mechanisms [54,99]. Pretreatment of grapevine plantlets (cv Chardonnay) with chitosan at a dose of 150 mg/L resulted in the total suppression of *B. cinerea* lesions on leaves, demonstrating the protection effect of chitosan regarding gray mold [99]. Interestingly, this protective effect was observed after removal of the elicitor, by washing the leaves, and before inoculation by *B. cinerea*, seeming to indicate that the action of this elicitor was not only linked to its direct antifungal activity but to the activation of some plant defense mechanisms (overexpression of chitinase, PAL and LOX activities), even if parallel works seemed to question the direct antifungal activity of chitosan [54]. Similarly, pretreatment with chitosan, with a molecular mass of 1500 Da and partially deacetylated (20%), at a dose of 200  $\mu$ g/mL, induced 60 to 71% protection towards, respectively, the infection of grapevine in vitro plantlets or potted plants by gray mold and downy mildew [54]. This protective effect was associated with an induction of plant chitinase and  $\beta$ -1,3-glucanase activities and an increase in stilbene contents (resveratrol: 18  $\mu$ g/g FW;  $\epsilon$ -viniferin: 50  $\mu$ g/g FW and piceid: 40  $\mu$ g/g FW). The protection rate increased to 85% in the case of downy mildew upon combination of chitosan (200  $\mu$ g/mL) and copper sulfate (50  $\mu$ g/mL), with potentiation of the phytoalexin response [54] (Table 3).

Ergosterol is a major sterol found in fungi that plays a role in the fluidity and permeability of cell membranes. Pretreatment with ergosterol administered at a concentration of 200  $\mu$ M on grapevine plants (cv Ugni Blanc) conferred a protective effect of 75% against infection by *B. cinerea* [100]. Conversely, only 55% protection was achieved in the case of pretreatment with BTH. The resistance provided by ergosterol was accompanied by an induction, mediated by the mobilization of the trans-acting elements of the transcription factor WRKY, of stilbene synthase gene (*VST1*) expression and, incidentally, by the over-

expression of the *VvLTP1* gene coding for the lipid transfer protein 1. Overexpression of the *VST1* gene was correlated with low resveratrol synthesis in the plants treated with ergosterol (227 ng/g FW) (Table 3).

$\beta$ -amino-butyric acid (BABA) is a non-protein amino acid known to induce resistance against oomycetes in plants [101,102]. Pretreatment with aqueous solutions of BABA (1 mM) prior to infection by downy mildew (*P. viticola*) in two cultivars (plant cuttings), one susceptible (Chasselas), the other resistant (Solaris), induced a strong reduction in sporulation and growth of *P. viticola* hyphae [103]. The priming effect exerted by BABA resulted in an increase in the overall amounts of stilbenes (resveratrol, piceid and  $\epsilon$ -viniferin). Priming activity of BABA on the expression of *PAL* and *C4H* was mainly observed in the downy mildew-resistant variety, Solaris, whereas *STS* was up-regulated in both the BABA-primed cultivars (Table 3).

Works have reported that rhamnolipids can potentially be used for the protection of plants against bacterial and fungal diseases, protecting, for example, oilseed rape and tomato from *B. cinerea* attacks [104,105]. In grapevine (*V. vinifera*), pretreatment with a mixture of rhamnolipids (RLs) extracted from *Pseudomonas aeruginosa* at concentrations of 0.1 and 1 mg/L showed a protective effect on in vitro whole plantlets against infection by *B. cinerea* [106]. RL activity resulted in a strong increase in the transcript accumulation of genes encoding various PR proteins and LOX, as well as *PAL* and *STS* genes. Because of their lipo-solubility, RLs exerted a direct antifungal action and were able to potentiate the action of other bio-elicitors such as chitosan.

The role of sugars in the interactions between plants and microbes and in the resistance of the former to numerous pathogens, reported as sweet immunity, is well established [107,108]. In this context, rare sugars have been used to stimulate the defenses of plants and, thus, increase their resistance to phytopathogenic agents [109]. Among these, D-tagatose has shown protective activity, especially against members of the class of oomycetes [110,111]. Root or aerial pretreatments of grapevine in vitro plantlets (cv Chardonnay) by IFP48, an 80% D-tagatose-based product currently under registration on the European market and used at a dose of 5 g/L, showed an anti-oomycete activity, reducing sporangia density of *P. viticola* by 35 to 50% [112,113], but exhibited no effect, on the other hand, on the control of gray mold (*B. cinerea*) [113]. The protective effect towards *P. viticola* was correlated with an overexpression of genes coding for PR proteins, *LOX9* as well as *PAL* and *STS* genes (with a respective induction of 10 and 8 times) with only a low accumulation of stilbenes (3–5  $\mu$ g/g FW for resveratrol and a few micrograms per gram of FW for viniferins  $\epsilon$  and  $\delta$ ). Overexpression of these responsive defense genes in grapevine explained, at least in part, the protection conferred by IFP48, as overexpression levels of those genes remained high after inoculation with downy mildew. The protection mechanisms of IFP48 resulted in a modulation of the expression of some responsive defense genes controlled by the salicylic acid pathway (*PR1* and *PR2* genes) and that of jasmonic acid (*ERF1*, Ethylene Response Factor 1 and *PR3c* genes) in the absence of pathogen infection.

**Table 3.** Use of bio-elicitors for grapevine protection and stimulation of the phytoalexin response.

Elicitor	Plant Material	Biological Inputs	References
Laminarin	Grapevine in vitro plantlets (cv Chardonnay and Gamay)	Offered 55 to 75% protection against, respectively, gray mold and downy mildew. Overexpression of genes coding PR proteins as well as <i>PAL</i> and <i>STS1</i> genes with concomitant accumulation of phytoalexins (cell suspensions).	[97]
Chitosan partially deacetylated	Grapevine in vitro plantlets (cv Chardonnay)	Total suppression of <i>B. cinerea</i> necrosis on leaves. Overexpression of chitinases, <i>PAL</i> and <i>LOX</i> activities. Direct antifungal activity. No mention of stilbene production.	[99]

Table 3. Cont.

Elicitor	Plant Material	Biological Inputs	References
Chitosan partially deacetylated (20%)	Grapevine in vitro plantlets and potted plants (cv Chardonnay)	Gave 60 to 70% protection against respectively gray mold and downy mildew, linked with an increase in chitinase, $\beta$ -1,3-glucanase activities and augmentation of stilbene content (resveratrol, piceid and $\epsilon$ -viniferin).	[54]
Chitosan + copper sulfate	Potted plants (cv Chardonnay)	Gave 83% protection against downy mildew. associated with a potentiation of the phytoalexin response.	[54]
Ergosterol	Grapevine plants (cv Ugni Blanc)	Gave 75% protection against <i>B. cinerea</i> contamination (55% with benzothiadiazole). Overexpression of the WRKY transcription factor, <i>VvST1</i> and <i>VvLTP1</i> with correlated resveratrol production.	[100]
$\beta$ -amino-butyric acid	Plant cuttings of cvs Chasselas and Solaris	Strong inhibition of <i>P. viticola</i> sporulation and hyphal growth linked with a priming effect on stilbene accumulation correlated with overexpression of <i>PAL</i> and <i>C4H</i> genes in Solaris and <i>STS</i> in both cultivars.	[103]
Bacterial rhamnolipids	Grapevine in vitro plantlets	Protection of leaves against <i>B. cinerea</i> infection. Overexpression of <i>PR proteins</i> , <i>LOX</i> , <i>PAL</i> and <i>STS</i> genes.	[106]
D-Tagatose (IFP48)	Grapevine in vitro plantlets (cv Chardonnay)	Reduction by 35% of sporangia density of <i>P. viticola</i> , associated with a direct anti-oomycete activity. Overexpression of genes encoding PR proteins, <i>LOX9</i> , <i>PAL</i> and <i>STS</i> along with low stilbene production (resveratrol, piceid and dimers).	[112]
D-Tagatose (IFP48)	Grapevine in vitro plantlets (cv Chardonnay)	Reduction by 50% of sporangia density of <i>P. viticola</i> but no effect towards <i>B. cinerea</i> . Overexpression of <i>PR1</i> and <i>PR2</i> genes (SA pathway), <i>ERF1</i> and <i>PR3c</i> genes (JA pathway). Low stilbene production (resveratrol, piceid and dimers).	[113]

#### 4. Control of Grapevine Diseases by Beneficial Organisms Involving Stimulation of Phytoalexin Synthesis

The biological control of plant diseases can be defined as partial or total, direct or indirect inhibition of the growth and development of a pathogenic agent responsible for a given disease by another living organism (or groups of organisms), considered as a beneficial organism, called biological control agent (BCA) [114–119]. The fungus *Trichoderma harzanium*, strain T39, has been reported, for example, as a biocontrol agent for downy mildew in grapevine showing an 86% reduction in disease symptoms on potted grapevine, cv Pinot Noir [120]. Among the differentially induced defense responsive genes analyzed, *PAL* and *STS* genes were found to be up-regulated without mention regarding stilbene phytoalexin production. There are works describing induction of phytoalexin biosynthesis by BCAs, for example, to name just a few, the accumulation of scoparone and scopoletin in the control of *Penicillium digitatum*, *P. italicum* and *B. cinerea* by the yeast *Rhodotorula glutinis* in orange fruit [121], the synthesis of dianthranilide-type phytoalexins during the biocontrol of fusarium wilt by a *Pseudomonas* strain in carnation [122] or camalexin priming in the induced resistance of Arabidopsis by beneficial bacteria in the control of *B. cinerea* and *P. syringae* [123]. To our knowledge, the first study reporting the biocontrol of a

grapevine disease, gray mold, by a soil bacterium causing elicitation of the biosynthesis of the phytoalexin resveratrol dates back to 1998 [124]. Co-inoculation of in vitro grapevine plantlets of *V. vinifera* (susceptible) and *V. rupestris* (mid-tolerant) with *B. cinerea* and the uncharacterized soil bacterium B-781 led to a complete suppression of the disease symptoms. The biocontrol of *B. cinerea* could have been linked to an increase in the production of resveratrol in *V. vinifera* (6 µg/g FW) and in greater amounts in *V. rupestris* (13 µg/g FW). A potentiating effect of the fungus/bacterium co-inoculation was observed on resveratrol production (78 µg/g FW) in *V. vinifera* and to a lesser extent in *V. rupestris* (31 µg/g FW) [124] (Table 4).

Two rhizobacteria of the genus *Pseudomonas*, *Pseudomonas fluorescens* CHA0 and *Pseudomonas aeruginosa* 7NSK2 induced resistance in vitro in leaves of grapevine plantlets of the Chardonnay variety (susceptible) to *B. cinerea* [125], the protection conferred towards gray mold infection being greater than 20% with *P. fluorescens* CHA0 and about 35% with *P. aeruginosa* 7NSK2 (Table 4). In both cases, induction of the systemic resistance to *B. cinerea* could be correlated with a priming effect on stilbene phytoalexin accumulation in the leaves, this effect being initially relatively weak at the pre-infection stage (before *B. cinerea* inoculation), but significant compared to the control (10–25 µg/g FW for resveratrol and 15 to 30 µg/g FW for ε-viniferin). At 3 days post-inoculation with *B. cinerea*, these concentrations reached very high values (800 µg/g FW for resveratrol and 250 µg/g FW for ε-viniferin with *P. fluorescens* CHA0; >700 µg/g FW for resveratrol and 180 µg/g FW for ε-viniferin with *P. aeruginosa* 7NSK2). Phytoalexin production was preceded by an early oxidative burst. Very interestingly, two mutants of the 7NSK2 strain, the mutant KMPCH, deficient in pyochelin (Pch) and pyoverdine (Pvd), two bacterial compounds that act as inducers of resistance, and the mutant KMPCH-567, deficient in Pch, Pvd and SA, showed differential induction profiles in the resistance to *B. cinerea*. The KMPCH mutant (Pch- and Pvd-negative) showed a protective effect of 35% against *B. cinerea*, that is, an effect comparable to that of the parental strain 7NSK2, the KMPCH-567 mutant (Pch-, Pvd- and SA-negative) inducing little or no protection against gray mold infection (<10%). The protective activity linked to the KMPCH mutant was found to be correlated with a potentiation of the production of resveratrol in the leaves, the KMPCH-567 mutant only inducing weak resveratrol amounts at two days post-inoculation. These data, therefore, reinforced the role played by phytoalexins in the bacteria-induced disease resistance to *B. cinerea* [125].

Several bacteria isolated in the Champagne vineyard, *Bacillus subtilis*, *Pantoea agglomerens*, *Acinetobacter lwoffii* and *Pseudomonas fluorescens*, induced a protective effect against *B. cinerea* on in vitro grapevine plantlets (cv Chardonnay), *B. subtilis* showing a 35% reduction in gray mold symptoms, with *P. fluorescens* showing the highest protection rate (70%) and *P. agglomerens* and *A. lwoffii* resulting in a 60% reduction in leaf symptoms [126]. All the bacteria triggered an early oxidative burst preceding an induction of the biosynthesis of the phytoalexins resveratrol and ε-viniferin on the order of 10 to 20 µg/g FW. A priming effect on phytoalexin accumulation was observed with *P. fluorescens* and *A. lwoffii*, but not in the case of *P. agglomerens* and *B. subtilis*, where pretreatment with these bacteria had no effect on subsequent *B. cinerea*-induced phytoalexin production. Later studies focused specifically on the mechanisms of action of the bacterium *P. fluorescens* PTA-CT2 on grapevine [127,128]. Induction of the systemic resistance in grapevine against *B. cinerea* resulted in a 60% reduction in the symptoms caused by this fungus on in vitro grapevine plantlets cv Chardonnay, through the differential expression of defensive response genes at the root level (organ colonized by the bacterium) and at the leaf level (lack of colonization). Genes of the phenylpropanoid pathway and resveratrol synthesis (*PAL* and *STS*) as well as those of the anthocyanin biosynthetic route (*CHS*, chalcone synthase, *CHI*, chalcone isomerase, *ANS*, anthocyanidin synthase) displayed a much higher overexpression level in the leaves compared to the roots [127]. The PTA-CT2 bacterium was able to prime phytoalexin synthesis (resveratrol, piceid and ε-viniferin) in response to *B. cinerea* inoculation, the amounts of stilbenes accumulated in the leaves, in response to *P. fluorescens* (3 days after



the beginning of the infection), being higher in the leaves (piceid: 20 µg/g FW; resveratrol: 4 µg/g FW; ε-viniferin 10 µg/g FW) than in the roots (piceid: ~0 µg/g FW; resveratrol: 1 µg/g FW ε-viniferin 5 µg/g FW). Stilbene accumulation was fully consistent with the up-regulation of *PAL* and *STS* genes: high in the leaves and low in the roots. Other defense genes, particularly those encoding PR proteins, showed a differential response depending on the organs analyzed (Table 4).

Although the effectiveness of the PTA-CT2 strain of the bacterium *P. fluorescens* has already been demonstrated for the biocontrol of gray mold (*B. cinerea*) [125–127,129–131], that of this bacterium against *P. viticola* (downy mildew) had not been the subject of study until the work of Lakkis et al. [128]. The effectiveness of the PTA-CT2 strain was evaluated on two-year-old potted grapevines of the varieties Pinot Noir (susceptible) and Solaris (tolerant) by soil drenching with the bacterial solution. Application of the bacterium led to a reduction in the growth development of *P. viticola* of 80% in Pinot Noir and only 55% in Solaris; this reduction was 73–80% for Pinot Noir and 43% for Solaris following infection by *B. cinerea* (Table 4). In the absence of any infection, PTA-CT2 did not induce changes in the basal defenses of the plant, but induced changes at the hormonal level and an improvement in the photosynthetic capacities for the two varieties. In contrast, PTA-CT2 primed defensive pathways including *PAL* and *STS* gene overexpression, which was correlated with increased phytoalexin levels in both varieties. The two varieties showed quite similar phytoalexin accumulation profiles after PTA-CT2/*P. viticola* or PTA-CT2/*B. cinerea* co-inoculation—resveratrol: 15 µg/g FW, ε-viniferin: 6–8 µg/g FW and δ-viniferin: 12–17 µg/g FW and resveratrol: 40 µg/g FW, ε-viniferin: 12–25 µg/g FW and δ-viniferin: 5–10 µg/g FW, respectively. These results clearly demonstrated that the effectiveness of the biocontrol exerted by *P. fluorescens* was mediated by up-regulation of the genes involved in the stilbene biosynthetic pathway, resulting in phytoalexin response priming, and reinforced the role played by stilbenes in grapevine/plant pathogens interactions.

There are a few experiments describing the use of beneficial bacteria as biocontrol agents of fungal diseases in the vineyard and linking a possible protective effect of these bacteria to the stimulation of grapevine defense systems [129–131]. Tests carried out in the vineyard for four consecutive years from 2002 to 2005 regarding the biological control of *B. cinerea* by grapevine-associated bacteria reported interesting results towards the reduction on grapevine leaves and berries of the *B. cinerea* symptoms (from 50 to 75%) by application of bacteria via the soil drenching method or foliar spray [129,130]. This resistance to *B. cinerea* was correlated with a stimulation of β-1,3-glucanase and chitinase activities in grape leaves and berries, but no analysis of phytoalexins was described.

The only experiment describing use of endophytic and rhizospheric bacteria for the biocontrol of gray mold in vineyard conditions and linking this protective effect to the induced production of phytoalexins both in the leaves and the berries was that conducted by Aziz et al. [131] (Table 4). Three living bacteria isolated from grapevine in the Champagne vineyard, one rhizospheric, *B. subtilis* (PTA-271), the other two being endophytic bacteria obtained from tissues of healthy grapevine plants, *P. fluorescens* (PTA-CT2) and *P. agglomerens* (PTA-AF2), previously studied on grapevine in vitro plantlets [126], were brought to grapevine plants (cv Chardonnay) in a vineyard by drenching the soil, either individually or in the form of binary mixtures, in two treatments in June and July. On grapevine leaves, it was interesting to note that it was the combination PTA-AF2 + PTA-271 which triggered the highest level of systemic resistance (80 to 90% reduction in symptoms towards *B. cinerea*), 75 days after the first bacterial application, which also displayed the highest level of total phytoalexins (35 to 40 µg/g FW) as well as the highest values for resveratrol (25 µg/g FW) and ε-viniferin (7 µg/g FW) confirming the good correlation between protection towards contamination by *B. cinerea* and accumulation rates of antifungal phytoalexins. The results were more difficult to interpret regarding bacteria alone, since PTA-271, which provided the best protection level on leaves (75% reduction in *B. cinerea* symptoms), was not the one which induced the highest phytoalexin response compared to the PTA-CT2 + PTA-AF2 mixture or PTA-CT2 alone.

**Table 4.** Control of grapevine diseases by beneficial organisms involving stimulation of phytoalexin synthesis.

Biocontrol Agent	Plant Material	Biological Inputs	References
Uncharacterized soil bacterium B-781	In vitro grapevine plantlets of <i>V. vinifera</i> (susceptible) and <i>V. rupestris</i> (mid-tolerant)	Complete suppression of gray mold symptoms with increase in resveratrol accumulation.	[124]
<i>Pseudomonas fluorescens</i> CHA0 and <i>Pseudomonas aeruginosa</i> 7NSK2	In vitro grapevine plantlets cv Chardonnay	Protection against gray mold > 20% with <i>P. fluorescens</i> CHA0 and about 35% with <i>P. aeruginosa</i> 7NSK2. At 3 days post-inoculation with <i>B. cinerea</i> , stilbene concentrations reached very high values, on the order of several hundred µg/g FW.	[125]
<i>Bacillus subtilis</i> , <i>Pantoea agglomerens</i> , <i>Acinetobacter lwoffii</i> and <i>Pseudomonas fluorescens</i>	In vitro grapevine plantlets cv Chardonnay	A 35% reduction in gray mold symptoms with <i>B. subtilis</i> , 70% with <i>P. fluorescens</i> and 60% with <i>P. agglomerens</i> and <i>A. lwoffii</i> . Priming effect on phytoalexin accumulation with <i>P. fluorescens</i> and <i>A. lwoffii</i> but not with <i>P. agglomerens</i> and <i>B. subtilis</i> .	[126]
<i>Trichoderma harzanium</i> strain T39	Potted grapevine, cv Pinot Noir	A 86% reduction in disease symptoms towards downy mildew; induction of <i>PAL</i> and <i>STS</i> genes, but no mention of stilbene production.	[120]
<i>P. fluorescens</i> PTA-CT2	In vitro grapevine plantlets cv Chardonnay	A 60% reduction in gray mold symptoms. Differential expression of <i>PAL</i> and <i>STS</i> genes (higher in leaves than in roots), correlating with stilbene accumulation in the two organs.	[127]
<i>P. fluorescens</i> PTA-CT2	Two-year-old potted grapevins of the varieties Pinot Noir (susceptible) and Solaris (tolerant)	Reduction in the growth development of <i>P. viticola</i> of 80% in Pinot Noir and only 55% in Solaris, 73–80% for Pinot Noir and 43% for Solaris towards <i>B. cinerea</i> . No induced changes in the basal defenses of the plant with PTA-CT2 alone. PTA-CT2 primed defensive pathways including <i>PAL</i> and <i>STS</i> gene overexpression, which was correlated with increased phytoalexin levels in both varieties.	[128]
<i>B. subtilis</i> (PTA-271), <i>P. fluorescens</i> (PTA-CT2) and <i>P. agglomerens</i> (PTA-AF2), alone or as binary mixtures	One-year experiment on grapevine plants (cv Chardonnay) in a vineyard, including leaves and berries	An 80 to 90% reduction in symptoms towards <i>B. cinerea</i> with PTA-AF2 + PTA-271 on leaves. A 93% reduction in <i>B. cinerea</i> symptoms with PTA-CT2 + PTA-AF2 on berries, well-correlated with phytoalexin accumulation.	[131]

*B. cinerea* is a particularly redoubtable pathogen that damages the harvested grape quality because of its late attacks on berries and due to the fact that the amounts of inducible phytoalexins during stress at this stage of maturity are low [87,132]. Specific attention must, therefore, be paid to the level of protection of the grape clusters towards this fungus. The effectiveness of bacteria alone in terms of reduction of the *B. cinerea* symptoms on berries was very good, varying from 78% (PTA-CT2) to 87% (PTA-271), which correlated well with the accumulation of total phytoalexins (15 µg/g FW for PTA-271 and around 40 µg/g FW for PTA-CT2) along with a significant production of the antifungal dimer, ε-viniferin (>12 µg/g FW), 75 days after application of the first bacterial treatment. The PTA-CT2 + PTA-AF2 combination displayed the highest level of protection on grape berries (93% reduction of the *B. cinerea* symptoms) correlating with a significant accumulation of total phytoalexins (>20 µg/g FW). It was interesting to note that, in almost all cases,

accumulation levels of phytoalexins in grape berries remained high, even 91 and 99 days after application of the first bacterial treatment, i.e., at dates close to maturity, stages where they are most vulnerable to gray mold attacks [131].

### 5. Limitations to the Transfer of the Eliciting Treatments for Field Applications

Most of the of treatments used to stimulate the phytoalexin response in grapevine (basal levels and priming) have been shown to provide effective protection against different pathogens, achieving a 93% reduction in *B. cinerea* symptoms on grape berries in the vineyard through the use of bacteria [131]. Limits as to the effectiveness of these treatments have nevertheless appeared. While fosetyl-Al is able to confer some level of protection to plants of *V. rupestris* (mid-tolerant) or cv Castor (resistant), this compound, even at the highest doses, proved unable to protect Riesling, a susceptible *V. vinifera* variety, towards downy mildew for post-infectious applications [66]. Some elicitors exhibited some protective effects against a given pathogen but there may be little or no protection towards another pathogen [54,89,113]. In the case of biocontrol agents, a variable level of effectiveness can be obtained, depending on the type of bacteria used [125,126] or, for the same bacterium, according to the grapevine variety treated [128].

Bio-elicitors like ergosterol and rhamnolipids (RLs) do not seem transferable for use in the vineyard due to their cost. The high cost of RLs, for example, is mainly linked to the fermentation process of the microorganisms and the subsequent purification steps of the RLs produced [133]. Other compounds such as D-tagatose have not yet been the subject of studies in the vineyard, notwithstanding the fact that this compound, tested under the IFP48 formulation, is in the process of being registered on the European market [112,113]. Metallic cation-based fungicide products (fosetyl-Al, Synermix), which have shown protective effects against mildew or towards gray mold, can cause an accumulation of aluminum, the toxicity of which has been proven. Even copper sulfate, which confers a satisfactory protective effect against *B. cinerea* and whose practice is ancestral in the form of the *bouillie bordelaise*, is currently subject to restrictions as to its possible use in the vineyard.

Although not mentioned in the presented studies, certain phytohormone derivatives could have negative effects on the hormonal balance of grapevine. For example, application of salicylic acid to grape bunches of the Shiraz variety in the vineyard resulted in a delay of two to four weeks in the maturation process of fruits through a possible inhibition of abscisic acid production, whose synthesis peak marks the beginning of veraison [134]. One can wonder if some treatments, namely, using BTH, which mimics the action of salicylic acid, could not modify the hormonal balance of the plant.

Finally, the use of bacterial biocontrol agents can be hampered by the difficulty in controlling the bacterial inoculum and the problem of selecting the most effective bacterial strains or mixtures in terms of disease protection [131].

### 6. Conclusions

Most of the treatments described in this study (chemicals, phytohormone-derivatives, bio-elicitors, biocontrol agents) led to variable protective effects against various pathogens, which number among the phytopathogenic agents responsible for grapevine major diseases, this protection being most often correlated with overexpression of genes of the phenylpropanoid pathway (*PAL* and *C4H*) and of resveratrol synthesis (*STS*) alongside an up-regulation of the expression of other responsive defense genes (*LOX*, *GST*, *PER*, *ERF1* and genes encoding PR proteins). Induction of *PAL* and *STS* genes was generally accompanied by a notable increase in the stilbene content (resveratrol, piceid, pterostilbene and dimers), which may explain the protection observed towards pathogens. However, in some cases, the level of accumulation of these compounds regarding the doses required for them to exert their antifungal activity remained insufficient to support the protective effects observed [79,88,112,113] and, sometimes, a decorrelation was described between *PAL* and *STS* genes' up-regulation and stilbene production [84,90,112,113]. It has been suggested

that this decorrelation could be linked to the time of sampling and the production kinetics of certain stilbenes, some of which exhibiting a biphasic synthesis profile [90,135]

Even if some data were difficult to interpret, there were compelling facts to support the role of stilbene phytoalexin inducers in controlling various pathogens thereby providing grapevine with relative protection against diseases. Although phytoalexins represent only one component of the plant response to stress, it appeared that their production and the overexpression levels of the genes involved in their biosynthesis (*PAL*, *C4H*, *STS*), were often correlated with those of other responsive defense genes (*PR proteins*, *LOX*, *GST*, *PER*, etc.).

The different elicitation methods used (elicitors and biocontrol agents) were able to act on the stimulation of the basal levels of phytoalexin biosynthesis (treatment at the pre-infectious stage) [54,55,81,86,89,90,93,100,124], by a priming effect (synergy of the effect linked to the pre-treatment combined with the phytoalexin response following infection by the pathogen) [65,66,103,124–128] or both the stimulation and priming [84,124,125]. Levels of phytoalexin accumulation in the plant treated with the elicitor and co-infected with the pathogen was then greater than that in the plant only infected with the pathogen.

The tests carried out in the vineyard face the versatility of experimental conditions (climate, disease pressure, way of applying the treatments and frequency of applications). Because of these constraints, few trials have been carried out in the vineyard [68,84,89,131]. Experiments conducted with Synermix ( $\text{AlCl}_3$  + seaweed extract) obtained good results in terms of grapevine protection towards *Botrytis cinerea* [66,67]. Experiments including applications of MeJA in the vineyard conferred a 73% reduction in the symptom incidence of powdery mildew [89]. This protective effect was correlated with a large increase in pre-infectious accumulation of various stilbene phytoalexins (resveratrol, piceid and dimers).

Input of endophytic or rhizospheric bacteria, alone or as a mixture, to grapevine plants grown in the vineyard by drenching the soil in two treatments showed very promising results in terms of protection of this plant against infection by gray mold, with reduction rates of the disease symptoms reaching 90% in the leaves and 93% in grape berries [131]. This protection effect was accompanied by a significant increase in the phytoalexin basal levels. Most importantly, phytoalexin amounts in the grape berries remained high, even 91 and 99 days after application of the first bacterial treatment, i.e., at stages close to fruit maturity when the fruits are particularly susceptible to *B. cinerea* attacks.

What questions remain to be answered to achieve effective phytoalexin stimulation under field conditions?

Metallic salts possess a significant activity on the stimulation of stilbene synthesis, particularly, aluminum chloride and copper sulfate, which are associated with a certain level of protection of grapevine against gray mold [54,67,68] and, to a lesser extent, against downy mildew [54]. The aluminum contained in  $\text{AlCl}_3$  was a very effective inducer of phytoalexin synthesis, leading to the accumulation of huge amounts of resveratrol in the leaves (>500  $\mu\text{g/g}$  FW) [55]. The mechanisms by which this metal cation triggers resveratrol hyperproduction include an overexpression of two *STS* gene sub-families and the transcription factor *MYB14*, which controls the *STS* gene promoter. Initiation of the overexpression of these genes, which depends on Al-induced remodeling of intracellular actin, involves RBOH proteins [70]. Compounds capable of mimicking the action of aluminum on the RBOH-actin complex could be sought, representing an interesting path for further technological development.

Another prospective way would be to improve the efficaciousness of the elicitors by increasing their penetration into the plant inner tissues of the plant by the use of nanoencapsulation methods. Such an approach has already been considered recently with MeJA [136,137]. Encapsulation of MeJA on amorphous calcium phosphate (ACP)-nanoparticles and their application to the Tempranillo grapevine variety in the vineyard showed a stimulatory effect on the accumulation in the grape clusters of polyphenols (flavanols and flavonols) as well as stilbenes [137]. Similar experiments showed a 10 times greater effect of ACP-nanoparticles doped with MeJA compared to MeJA alone on wine stilbene concentrations and, therefore, on the upstream increase in the grape berry stilbene

content [136]. Application of elicitors carried by nanoparticles could not only increase the penetration of these elicitors into the plant, prolonging retention and release, but also the persistence of their activity within the tissues.

The use of beneficial bacteria for disease control in the vineyard will require improvement of the density of the bacterial inoculum as well as of the level of the colonization efficiency of the host by the bacteria. It is likely that the treatment of plant roots in the vineyard by the soil drenching method will remain the most effective one for the application of bacteria. The use of bacterial mixtures should also be the subject of more in-depth studies, the observed relative inefficiency of certain combinations possibly being the result of competition phenomena between bacteria from different bacterial populations in the field or being the consequence of negative interactions between the signaling pathways mediated by these bacteria. In the experiments carried out by Aziz et al. [131], it was indeed noticed that the highest protective effect towards *B. cinerea* in the vineyard (leaves and berries) was obtained with the mixture of the two bacteria having individually the weakest protection activity.

In sum, all the works presented and discussed in this review showed that the protective effect observed towards phytopathogenic agents by the application of different elicitors was correlated with an increase in the biosynthetic basal levels of stilbene phytoalexins as well as phytoalexin priming in grapevine, making it possible to validate the concept of using phytoalexin induction as a means for crop protection.

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