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Effects of Elicitors, Viticultural Factors, and Enological Practices on Resveratrol and Stilbenes in Grapevine and Wine

Luigi Bavaresco[1,*], Fulvio Mattivi[2], Mirko De Rosso[1] and Riccardo Flamini[1]

[1]*CRA-Centro di Ricerca per la Viticoltura, Conegliano (TV), Italy;* [2]*IASMA Research and Innovation Centre, Fondazione Edmund Mach, San Michele all'Adige (TN), Italy*

Abstract: The ability of grapevine to activate defense mechanisms against some pathogens has been shown to be linked to the synthesis of stilbenes by the plant (inducible viniferins). Metabolized viniferins may also be produced or modified by extracellular enzymes released by the pathogen in an attempt to eliminate undesirable toxic compounds. Due to the important properties of resveratrol, there is increasing interest in producing foods with higher contents of this compound and higher nutritional value. The production of high resveratrol-containing grapes and wines relies on quality-oriented viticulture (suitable terroirs, sustainable cultural practices) and wine-making technologies which avoid degradation of the compound. The technique of skin extraction and enzymatic hydrolysis of glucoside forms in wine-making plays an important role in resveratrol wine concentration. Other factors affecting its final concentration and balance in wine are conditions for promoting *trans-cis* isomerization of aglycones, the type of yeast used, and the presence of lactic bacteria with β-glucosidase activity. In general, the enological practices commonly used to stabilize wine after fermentation do not significantly affect resveratrol concentrations, which show considerable stability.

Keywords: Phytoalexins, resveratrol, stilbenes, viniferins, grapes, wine, health.

1. CHEMISTRY OF VINE STILBENES

1.1. Inducible *vs* Metabolized Viniferins

It is convenient for the understanding of the biosynthesis and role of grape viniferins to distinguish between "inducible" and "metabolized" viniferins [1].

The mechanisms of formation of grape viniferins in *Plasmopara viticola* infected leaves have been discussed in a recent paper [1]. The enzymes involved in the formation of viniferins are expressed both in pathogens and plants. Evidence of the ability of grapevine to directly synthesize viniferins comes from their constitutive presence in large amounts in some parts of the plant, as a diversity of stilbenes [2,3] and stilbenoid oligomers [4] which are present at gram per kilogram levels in vine roots; ε-viniferin has also been reported to be a constitutive stilbene of grapevine clusters stems [5]. Moreover, synthesis of 3 dimers (two δ-viniferin glucosides and pallidol) has been proved in *V. vinifera* cv Gamay Freaux var. Tenturier cell cultures [6].

"Inducible" viniferins can arise from the oligomerisation of *trans*-resveratrol in grape tissues as an active defence strategy by the plant. They are hardly detectable in healthy leaves, and a number of reports proved the induction of substantial accumulation of these compounds in infected leaves.

"Metabolised" viniferins could be produced or modified by extracellular enzymes released from the pathogen in an attempt to eliminate undesirable toxic compounds. The pathogenesis of *Botrytis cinerea* is essentially linked to excretion of lytic enzymes such as polyphenoloxidases or laccases. One enzyme, stilbene oxidase, can detoxify grape stilbenic phytoalexins, destroying grapevine defence mechanisms and allowing the fungus to grow [7]. Convincing evidence of such a process was provided by Cichewicz *et al.* [8], demonstrating that incubation of *B. cinerea* with resveratrol resulted in the production of six oxidized resveratrol dimers (A-C restrytisols, δ-viniferin, leachinol F and pallidol), and by Sbaghi *et al.* [9], who observed that the pathogenicity of different strains of *B. cinerea* was directly correlated to their capacity to degrade the grape viniferins.

In the case of grapevine leaves infected with *P. viticola*, the importance of oxidative dimerisation of resveratrol in comparison to the extent of its glycosylation in the defense reaction was elucidated by Pezet *et al.* [10]. Resistant varieties produced a higher concentration of ε- and δ-viniferin. Resveratrol and piceid have little or no toxicity for *P. viticola*, whereas δ-viniferin is highly toxic and can be considered an important marker for the grapevine resistance to *P. viticola*. In susceptible cultivars, either resveratrol is glycosylated, or its concentration is very low and consequently viniferin concentrations are also too low [10].

1.2. Mechanisms of Formation of Viniferins

Takaya *et al.* studied the mechanism of viniferin formation in depth, analysing the reaction produced using HPLC equipped with a CD detector using a chiral column [11]. They showed that several inorganic reagents are capable of producing a mixture of (±)-ε-viniferins. On the other hand, *in-vitro* experiments with guaiacol peroxidases

*Address correspondence to this author at the CRA-Centro di Ricerca per la Viticoltura, Viale XXVIII Aprile 26, 31015 Conegliano (TV), Italy;
Tel: +39-0438-456725; Fax: +39-0438-64779;
E-mail: luigi.bavaresco@entecra.it

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belonging to Class III (GPx) (EC 1.11.1.7) from soybean, a fungus (*Arthromyces ramosus*), and horseradish did not lead to ε-viniferin, although other viniferins such as (±)-pallidol, (±)-δ-viniferin, (±)-leachianol F and (±)-quadrangularin B and C, were obtained. In other words, in terms of molecular structure and chirality, this mechanism does not reproduce the chemical route to main known grape viniferins. This experiment showed, in agreement with previous results of Li and Ferreira [12] that the main products obtained via these peroxidases proceed via different intermediates as compared to those required for ε-viniferin production.

Peroxidases catalyse the oxidation of *trans*-resveratrol in the presence of H_2O_2, giving the corresponding resveratrol radical and H_2O. In the absence of ascorbic acid, as in the case of grapevine leaves [13], resveratrol radicals react with one another, giving rise to the corresponding polymers. The formation of ε-viniferin-like compounds (Fig. **1**) formally requires reaction between a (sterically hindered) oxidized radical intermediate in position 2 of resveratrol (A), with a second intermediate (less hindered) with the radical in the α position, on the stilbenic double bond (B). Formation of the whole class of viniferins observed in literature also requires the involvement of these two intermediates and of a third intermediate (less hindered) with the radical in position 3' of *trans*-resveratrol (C) [11,12]. The diversity of coupling modes leads to the structural complexity of the oligomers, which increases the difficulty of their regiocontrolled synthesis [14].

Other extracellular, laccase-like enzymes (EC 1.10.3.2) secreted by fungi are also capable of metabolising *trans*-resveratrol [15,16]. Stilbene oxidase isoenzymes from *B. cinerea* have been isolated and characterised [7,17]. It was suggested that they mainly produce δ-viniferin [18]. The formation of δ-viniferin requires the involvement of two resveratrol radicals (B+C, Fig. 1) [11].

The diversity of "inducible" grape oligomers is thought to derive from the effect on resveratrol of plant peroxidase isoenzymes localized both in the cell wall, vacuole and apoplast [19]. It has been suggested that these isoenzymes are involved in both the constitutive and inducible defense of grapevine against fungi [20]. *In vitro* experiments with suspension cultured cells suggested that elicitation induced a modified pattern for the extracellular peroxidases which are involved in the formation of grape viniferins [21,22]. Interestingly, in the genetic part of a recent study by Malacarne *et al.* [23], the induction of two grapevine peroxidase genes in a resistant offspring of the cross, has been observed 24 hours after *P. viticola* infection. It will be of great importance to test if these peroxidases are indeed capable of catalyzing the production of specific classes of viniferins starting form resveratrol as substrate.

Some as yet unknown auxiliary or dirigent proteins are also required to capture free radical intermediates (A, B and C, Fig. **1**), providing a structure for the production of stereoselective radical coupling, yielding an optically active product.

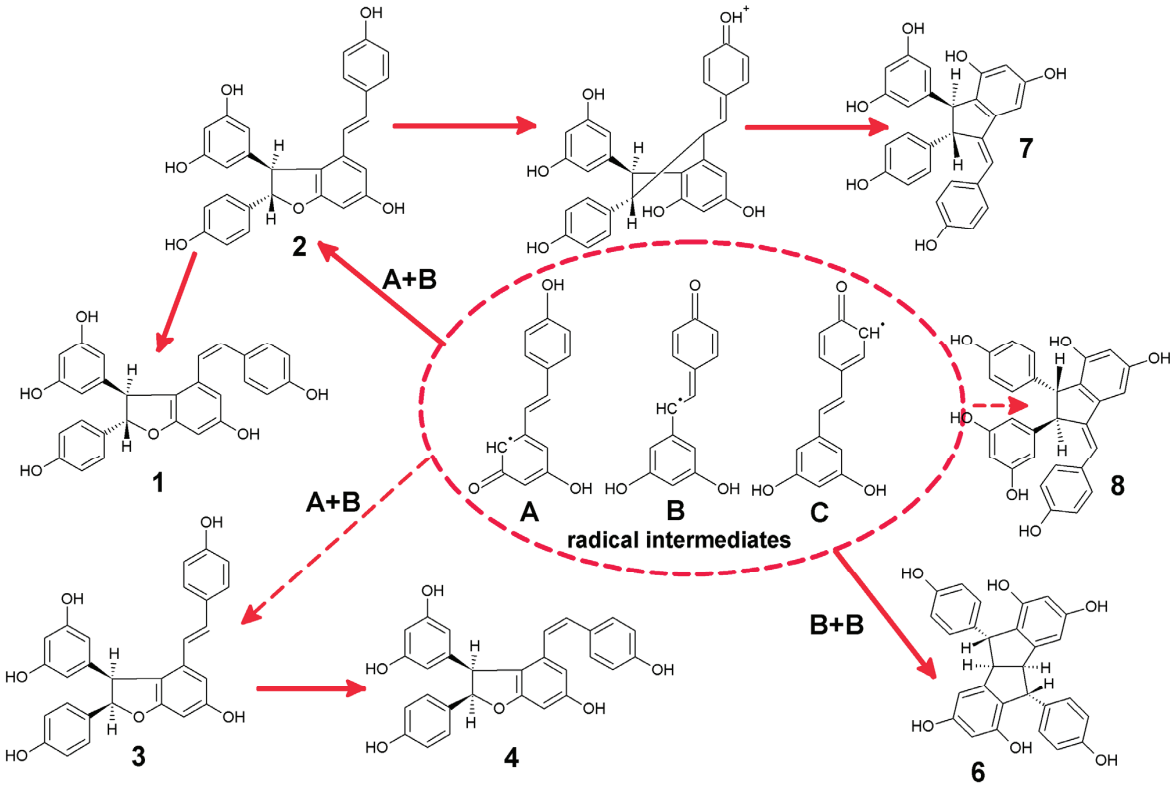

Fig. (1). Roadmap of formation of resveratrol dimers, reporting the resveratrol radicals or the intermediate involved in their formation, according to the literature [11,12,24,25]. All the dimers found in this study formally require an A+B or B+B reaction scheme, while the B+C path, leading to δ-viniferin, was not observed. Legend: structures of stilbenoid dimers in grapevine leaves: Z-ε-viniferin (1), E-ε-viniferin (2), E-ω-viniferin (3), Z-ω-viniferin (4), pallidol (6), E-ampelopsin D (7) and E-quadrangularin A (8).

The sequential mechanism, so far not elucidated, is expected to progress from the initial synthesis of resveratrol, towards the formation of dimers (Fig. **1**) and then higher oligomers (Figs. **2** and **3**). Growth through the subsequent addition of one resveratrol unit to an existing dimer should leave one part of the initial structure unchanged. The formation of a tetramer may derive either from sequential addition of another resveratrol unit to a trimer or from direct condensation of two dimers. Again the resulting tetramer should retain part of the configuration of the parent structures.

Characterisation of the whole class of viniferins present in infected vine leaves [1] provided some indirect evidence of their possible relationships, which may be useful for understanding the possible biogenetic pathways according to which viniferins are biosynthesized. Figs. **1-3** show some speculative ideas regarding these pathways, which are compatible with the structural information obtained in this study, and derived from comparison with the literature and with the assumption that the stereochemistry of precursors should be partially retained in higher oligomers. The availability of a routinary HPLC method for their accurate quantification in infected grape leaves [26] should allow the researchers to design novel experiments in order to further explore these hypotheses.

The pattern of viniferins found in infected leaves lacked some of the constitutive viniferins to date elucidated in healthy grape tissues, such as ampelopsin A and hopeaphenol [4], r-viniferin [3], r-2-viniferin [2], gnetin H [3], *trans*-miyabenol C [27], *trans*-amurensin B, amurensin G and ampelopsin F [28]. While the presence of many optically active viniferins, rather than racemates, in the partially resistant genotypes characterized by Mattivi and coworkers [1] suggested a high level of control of the biosynthesis, supporting the theory of "inducible" viniferins, it was not possible to rule out the possible presence of metabolites driven by fungal laccases and peroxidases. It was not observed in this study the presence of δ-viniferin or any other stilbenoid formed through the involvement of a (C) resveratrol radical (Fig. **1**).

The difference in the pattern of viniferins in these recent results [1] and those obtained in previous studies [10,29-32], could be due to differences in the grape varieties or methods used. As it was reported in a complementary parts of the same study [23,26], substantial diversity in patterns was observed between different high stilbenoid producer genotypes, supporting the hypothesis that the plant, rather than the pathogen, is responsible for the complex pattern of viniferins observed in *P. viticola* infected leaves. In perspective, to assess whether the outcome of this study holds in general, it will be very interesting to investigate other resistant varieties challenged with different pathogens, and analyze the viniferins accumulation profile.

2. PHARMACOLOGICAL AND ANTIOXIDANT PROPERTIES OF RESVERATROL

A French epidemiological study (1979-80) showed that, in France, despite the high consumption of foods rich in saturated fatty acids, the incidence of mortality from cardiovascular diseases was lower than in other comparable countries. This phenomenon, known as the "French paradox", has been linked to the consumption of red wine [33]. In 1990, consumption of red wine, but not of white wine or ethanol, was found to induce platelet hypoaggregation [34]. Two years later, resveratrol was identified in wine [35].

In the last 15 years, this compound has become an important qualitative parameter of wine, because of its various beneficial effects on human health, as shown by a number of *in vitro* and *in vivo* studies (although clinical efficacy of stilbenes has been not still fully demonstrated): anticancer activity, inhibition of platelet aggregation and cardioprotection, antioxidant and anti-inflammatory activity [36-41].

2.1. Absorption and Metabolism

The most significant data on the absorption and metabolism of resveratrol have been provided by *in vivo* studies on rats: after oral administration of single doses of 13-26 g daily for two weeks, resveratrol quickly enters the bloodstream, and is found in significant concentrations in several organs [42,43]. Concentrations in heart, liver and kidney tissues were found to be similar to or higher than in plasma (1 ng/mL serum, 1 ng/g tissue); in rat plasma treated with *trans*-resveratrol, a concentration of resveratrol of 0.18 mg/L was observed about 15 minutes after intake [44].

In vitro studies show that resveratrol in liver and duodenum undergoes sulfation and glucuronidation, reactions which may limit its bioavailability and favor its elimination from the organism. Conversely, it has also been demonstrated that quercetin, another polyphenol present in wine, is a potent inhibitor of these reactions [45]. *In vivo* and *in vitro* studies have shown neither the presence of resveratrol, nor of its oxidative and hydrolysis products in human microsomes after 1 h incubation. However, large amounts of *trans*-resveratrol-3-O-glucuronide and *trans*-resveratrol-3-sulfate were found in rat urine, mouse serum, and after incubation with both human and rat hepatocytes. In both models, only trace amounts of *cis*-resveratrol were found, indicating that isomerization is not an important mechanism for elimination of the compound [46,47].

In lymphoblast microsomes expressing human CYP1B1 enzyme belonging to the cytochrome P450 family, transformation of resveratrol to piceatannol, another *V. vinifera* stilbene characterized by important anticancer activity, has been observed [48]. An immunohistochemical study showed that this enzyme is over-expressed in tumors of several organs. Its functional role is not well-known, but it shows aromatic hydroxylation activity and catalyzes the conversion reaction of estradiol to 4-hydroxyestradiol. As estradiol (an estrogen) and resveratrol (a phytoestrogen) have similar molecular structures, it has been suggested that resveratrol is a substrate of CYP1B1 after the addition of a hydroxyl group in position 4, like estradiol.

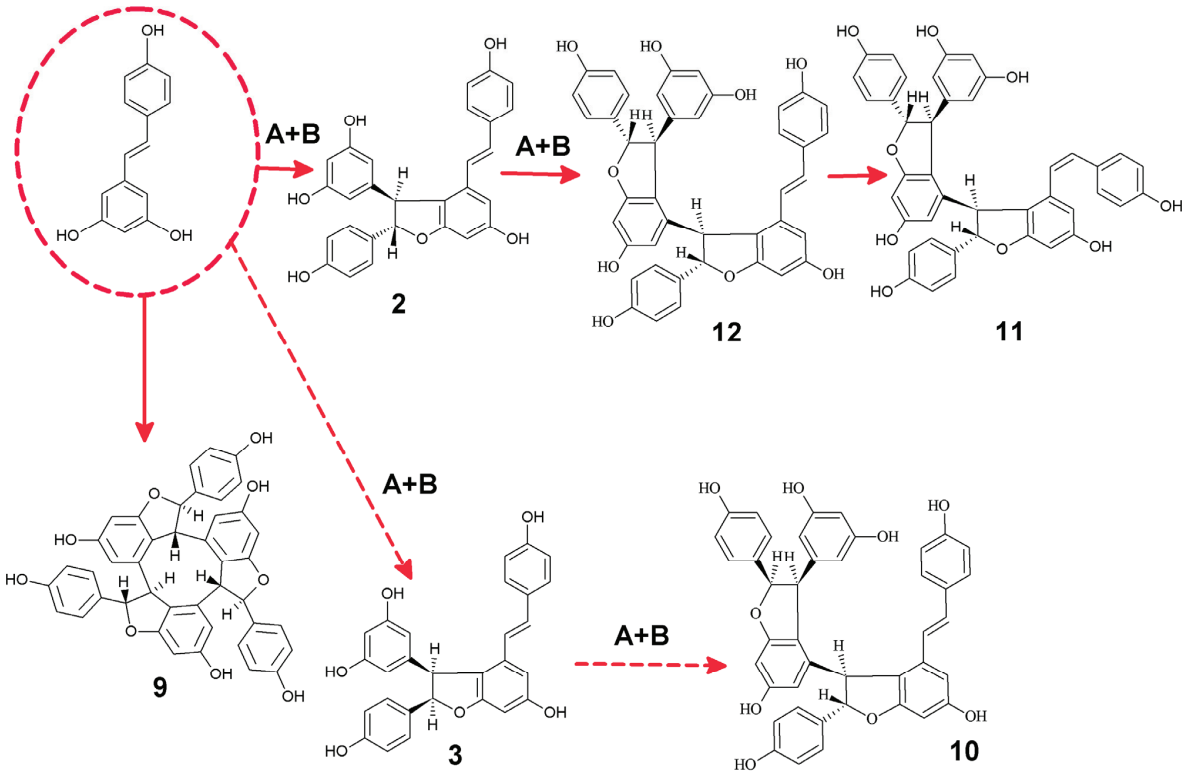

Fig. (2). Roadmap of formation of resveratrol trimers, suggesting the resveratrol radicals formally involved in their formation. Legend: structures of stilbenoid trimers in grapevine leaves: α-viniferin (9), E-*cis*-miyabenol C (10), E-miyabenol C (12), and Z-miyabenol C (11).

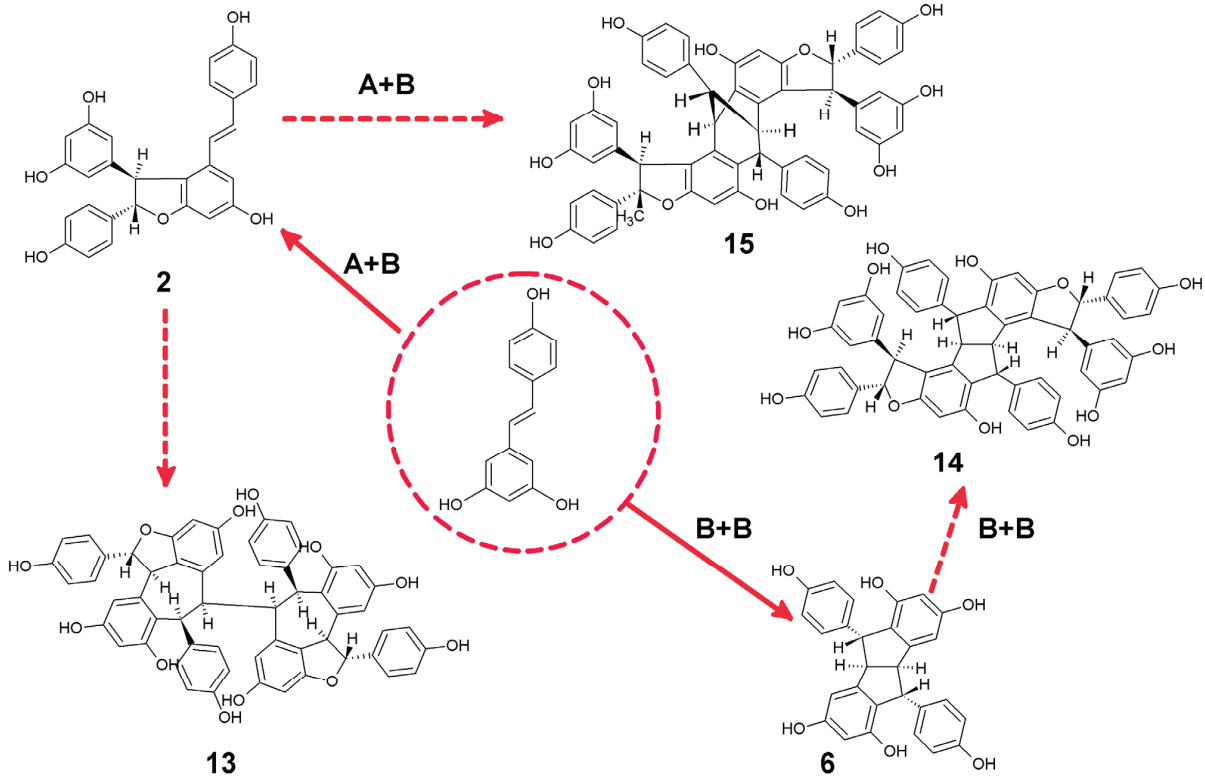

Fig. (3). Roadmap of formation resveratrol tetramers, suggesting the resveratrol radical formally involved in their formation. Legend: structures of stilbenoid tetramers in grapevine leaves: isohopeaphenol (13), ampelopsin H (14), vaticanol C isomer (15), and hopeaphenol (16).

2.2. Proprieties of Resveratrol

2.2.1. Antioxidant

Antioxidant activity is typical of polyphenols. Their activity as peroxyl radical scavengers and in the formation of complexes with metals (Cu, Fe, etc.) has been clearly shown in *in vitro* studies. The ability of polyphenols to cross the intestinal wall of mammals confers biological and antioxidant properties on these compounds.

The first studies on the antioxidant properties of *trans*-resveratrol demonstrated that, when added to human low density lipoprotein (LDL), it reduces oxidation catalyzed by copper [38]. The activity of resveratrol is about two times lower than that of quercetin and epicatechin, two other grape polyphenols. These results indicated that regular intake of these substances present in wine (especially red) may have beneficial effects on human health, e.g., protection against atherogenesis.

Measuring the formation of degradation products of polyunsaturated fatty acids (PUFA) during oxidation of pig LDL revealed the ability of resveratrol to inhibit peroxidation, like flavonoids, either by chelation (particularly of Cu) or as a radical scavenger [49]. The importance of Cu chelation in the protective activity of resveratrol against oxidation of PUFA was confirmed in a later study: resveratrol was more effective than flavonols in Cu-promoted oxidation and, vice versa, oxidation in the presence of free radicals [39].

Another study tested the ability of some anthocyanins (peonidin and malvidin 3-O-glucoside), (+)-catechin, (-)-epicatechin, and stilbenes (*cis*- and *trans*-resveratrol, *cis*- and *trans*-piceid, piceatannol and astringin) to prevent lipid peroxidation induced by Fe in microsomes and by Cu in LDL. *Trans*-resveratrol showed activity about six times higher than the *cis* isomer, but piceatannol was more active than resveratrol [50].

2.2.2. Anti-Inflammatory and Vasoprotective

The anti-inflammatory and vasoprotective proprieties of resveratrol are due to its ability to inhibit platelet aggregation and cyclooxygenase, and to modulate lipid and lipoprotein metabolism. Resveratrol can inhibit the formation of molecules involved in inflammation, such as the products of lipoxygenase and thromboxane B2 (TXB2), in rat peritoneal polymorphonuclear leukocytes [51].

At a concentration of 3.6 g/L, *trans*-resveratrol reduced the aggregation induced by collagen by 50% in platelet-enriched human blood. Activity in human platelet aggregation and in the synthesis of three eicosanoids deriving from arachidonic acid [TXB2, hydroxyeicosatetraenic acid (HHT), hydroxyheptadecatrienic acid (12-HETE)] was similar to that of quercetin, inducing dose-dependent inhibition in both thrombin-induced and ADP-induced platelet aggregation. It was inferred that intake of *trans*-reveratrol, in combination with other antioxidant polyphenol compounds, contributes to low levels of platelet aggregation, resulting in a lower risk of blood clot formation [40,41].

Resveratrol also inhibits cyclooxygenase (with the lowest effective dose of 15 μM), the key enzyme in the formation of the inflammatory response [36]. Cyclooxygenase is responsible for converting arachidonic acid to prostaglandins which, in addition to being mediators of inflammation, are promoters of tumor growth and angiogenesis in processed tissues, and suppress immune surveillance [52-54].

Resveratrol has been shown to prevent the increase of leukotriene B4 and prostaglandin E2 levels in cells by inhibiting 5-lipoxygenase and prostaglandin-H-synthase (cyclooxygenase and peroxidase functions). It also blocks lipid peroxidation of cell membranes and inhibits apoptosis induced by H_2O_2 in human erythroleukemia cells (K562) probably by inhibition of enzymes involved in the arachidonic acid metabolic pathway [55,56].

After the beneficial effects of resveratrol on rat lipid metabolism had been observed [57,58], some studies on a human hepatoma cell line, HepG2, were developed [59]. A significant decrease in the intracellular concentration of apoprotein B (constituents of lipoproteins) in response to increased *trans*-resveratrol in the culture medium (up to 50 μM) was observed. Resveratrol also induced decreased secretion of cholesteryl esters and triglycerides, suggesting a fall in the production of very low density lipoproteins (VLDL) and consequently of LDL, with positive effects due to the atherogenic proprieties of LDL [59,60].

The anti-inflammatory activity of resveratrol on fibroblasts was studied by analysing its action on the nuclear transcription factor (NF_kB), a protein related to the inflammatory response. Resveratrol inhibited I_KB-kinase (a protein responsible for the activation of NF_kB), and expression of mRNA for the monocyte chemotactic factor (responsible for regulating gene coding for NF_kB): as a consequence, it is a potent inhibitor of NF_kB [61].

2.2.3. Antimutagenic, Antiproliferative, and Anticancer

In the presence of Cu ions, resveratrol promotes DNA damage by acting as a reducing agent [62] but, when glutathione or ascorbate are present, it shows antioxidant and DNA protective action. In the ascorbate system, resveratrol acts as a radical scavenger, but in the glutathione system, it prevents the formation of hydroxyl radicals by inhibiting the formation of glutathione homodimers (GSSG) [63].

Resveratrol added to DNA plasmids in the presence of Cu ions does not protect against H_2O_2 damage, but rather increases it [64].

Treatment of *Salmonella typhimurium* strains (Ames test) with *Yucca schidigera* extract containing resveratrol showed suppression of the SOS response induced by 3-amino-1,4-dimethyl-5H-pyridate[4.3-b]indole(Trp-P-1) and also had a weak inhibitory effect on the SOS response induced by *N*-methyl-*N*'-nitro-*N*-nitroso guanidine (MNNG), reducing the effects of these mutagenic agents [65]. A study of the antimutagenic activity of resveratrol and other stilbene compounds on various kinds of tumor cells treated with MNNG demonstrated the cytotoxic effect of stilbenes: in particular, *trans*-resveratrol showed a strong dose-dependent

cytotoxicity against human liver hepatoma (HepG2) and human colon cancer cells (HT-29) [66]. In a preparation consisting of 0.5 mL of buffer solution at pH 7.4 and 500 micrograms of DNA from calf thymus cells, in the presence of H_2O_2 0.5 mM in order to create an oxidizing environment and Cr(III) 500 mM, a concentration of 0.10 mM of resveratrol reduced DNA oxidative damage by 50% [67].

The activity of resveratrol with respect to inhibition of DNA damage induced by H_2O_2 has also been studied in hamster ovary cells [68]. Treatment with resveratrol was shown to induce a slight increase in endogenous oxidation and also chromosomal aberrations (*double strand breaks*), but not damage to primary DNA (*single strand breaks*). Simultaneous treatment with resveratrol and H_2O_2 showed a slight protective effect, whereas pre-treatment with resveratrol promoted an increase in cellular oxidation. It protected from chromosomal aberrations only in the case of simultaneous treatment with the reagents.

Treatment of normal mouse fibroblasts with resveratrol, at concentrations of 30 and 90 μM for 24 hours, and 100-200 μM of H_2O_2 for 15-45 minutes, showed decreased intracellular oxidation (dichlorofluorescein test). The activity on cells treated with condensed cigarette smoke also showed a decrease in the production of reactive oxygen species (ROS) and in *single strand breaks* (Comet test) [69].

In vivo studies on the protective activity of resveratrol against oxidative damage showed significant decreases in the amount of basic nitrogen oxides in the urine of resveratrol-treated rats [70].

Resveratrol showed dose-dependent antiproliferative properties against proliferation of calf aortic smooth muscle cells induced by mitogens such as serum, endothelin and platelet-derived growth factor (PDGF) [71,72].

Human endothelial cells taken from umbilical vein cells showed dose-dependent inhibitory effects, with inhibition of vascular endothelial growth factor (VEGF) (45.5% with resveratrol 10 micromol/L, 52.6% with resveratrol 100 micromol/L) [73].

The dose-dependent antiproliferative activity of resveratrol was also observed in bovine pulmonary artery endothelial cells after treatment for 72 hours at concentrations of 50 and 100 μM. However, in this case, the accumulation of cells in the S and G_2 phases of the cell cycle was observed. This blockage was accompanied by an increase in the expression of proteins p53 (tumor suppressor) and p21 (a cyclin-dependent kinase inhibitor) [74]. Similar effects were reported against malignant cells [75-77].

Another study showed the anti-proliferative activity of resveratrol on human epidermoid carcinoma (A431) cells. Cell lines treated with resveratrol at concentrations of 1-50 μM for 24 h showed a cell cycle block in the G_1 phase and apoptotic induction. In the treated cells, increased expression of WAF1/p21, decrease of expression of cyclin D1-D2-E, and decrease of CDK2 kinase-cdk4-cdk6 were observed. The induction of WAF/p21 caused by resveratrol inhibits the assembly of the cyclin-kinase complex, stopping the cycle in G_1/S. This block is irreversible and leads to apoptosis of cancer cells [78]. The dose- and time-dependent antiproliferative activity of resveratrol on human breast epithelium tumor cells has also been reported [79].

Another important propriety of resveratrol is that it can inhibit some key enzymes of the cell cycle, such as DNA polymerase α and δ, and ribonucleotide reductase. Studies on the ability of resveratrol to inhibit the replication of mammalian DNA polymerase have been performed with purified enzymes [80]. *In vitro* resveratrol showed significant inhibition of ribonucleotide reductase, responsible for DNA synthesis in mammalian cells [81]. This was explained by the ability of this phytoalexin to eliminate the radical tyrosyl radical cation, essential for deoxyribonucleotide synthesis, contained in the minor subunit of the enzyme.

The chemical structures of resveratrol, estradiol and the synthetic estrogen diethylstilbestrol show similarities, as all these compounds contain phenol ring A. Competition between resveratrol and estradiol in estrogen-receptor binding has been studied in estrogen receptor-positive and estrogen receptor-negative breast adenocarcinoma cells. Resveratrol was observed to have estrogenic activity and exhibits variable degrees of estrogen receptor agonism in several systems [82].

Other studies have shown the direct effect of resveratrol in inhibiting angiogenesis in animals. Resveratrol blocked the growth of bovine capillary endothelial cells previously stimulated with fibroblast growth factor (FGF-2) with dose-dependent activity. One of the targets of resveratrol appeared to be an enzyme important in proliferative signal transduction, MAP-kinase, phosphorylation of which is inhibited. The inhibitory effect of resveratrol on the growth and migration of pig endothelial cells in the presence of growth factors FGF-2 and VEGF has also been observed [83]. In developing chick embryos, resveratrol inhibited the vascularization of the chorioallantoic membrane, with a dose-dependent effect. Similar results were obtained in mice treated with beverages containing resveratrol in concentrations comparable to three glasses of red wine. The anti-angiogenic action of resveratrol on pathological and physiological processes (e.g. wound healing) has also been highlighted [83].

In general, the chemopreventive activity of other *V. vinifera* stilbenes (e.g. piceatannol) is often similar to or higher than that of resveratrol, but has different dose-dependent effects [84].

3. PHYTOALEXINS AND ELICITORS

Plant resistance against various biotic stress factors involves a large number of constitutive and inducible (active) defense mechanisms. Typical constitutive defenses are structural barriers (waxes, cutin, suberin, lignin, phenols, cellulose, callose and cell-wall proteins) which are often rapidly reinforced upon stress [85]. Active defense mechanisms mainly involve the oxidative burst, rapid, localized cell death (hypersensitive response), accumulation of phytoalexins, and synthesis of pathogenesis-related (PR) proteins [85,86]. Stilbenes intervene in both constitutive (conferring resistance to pathogens, nematodes, insects and herbivores) and active defense responses [87].

Stilbenes are low molecular weight phenolic compounds present in the woody or fleshy tissues of some plant families such as *Pinaceae*, *Mirtaceae*, *Fagaceae*, *Liliaceae*, *Moraceae*, *Papilionaceae*, and *Vitaceae*. They play a role in resistence to decay of woody tissues, and in fleshy ones have specific functions of defence. They are classified within a family of antibiotic substances called phytoalexins, which are produced by the plant in response to pathogen attack. They are synthesized and accumulated into lipid vesicles in the cytoplasm, further secreted nearby infected sites in order to limit pathogen proliferation [88]. As a consequence, phytoalexins play a direct role in the mechanisms of resistance and tolerance of plants to diseases and abiotic stresses, but are not essential for the normal life of the plant [89].

The process by which the plant is stimulated to produce secondary metabolites is called "elicitation", indicating an external stressful stimulus applied to the crop [90].

In grapevine, the synthesis of stilbenes is induced in response to biotic and abiotic stresses and may occur in various plant tissues: berries, leaves, and cluster stems. It may be induced by biotic elicitors such as *Botrytis cinerea*, *Plasmopara viticola*, *Tricoderma viride*, *Erysiphe necator, Rhizopus stolonifer*, *Bacillus* spp., *Aspergillus carbonarius*, *Aspergillus japonicus* and *Laminarina* spp., or abiotic ones such as UV irradiation, aluminum chloride, fosetyl-Al, ozone, sucrose, dimethyl–β-cyclodextrin, methyl-jasmonate, benzothiadiazole, chitosan oligomers, salicylic acid, anoxic treatments, ABA, BABA and emodin [91-94].

The fungitoxic activity of phytoalexins is partly due to the destabilization of the pathogen cells by delocalizing membrane protein electrons. In susceptible plants, phytoalexins can be detoxified and metabolized by pathogens [89].

The occurrence of stilbenes in *Vitaceae* has been known since 1976. After *Botrytis cinerea* infection, the leaves of various grapevine species irradiated at 366 nm show a bright blue color on the marginal zone of infection; this fluorescence is attributed to *trans*-resveratrol. Oligomeric structures of resveratrol, called viniferins, have also been found [95,96].

The study of stilbene contents in leaves of various aged plants shows that resveratrol is produced in smaller quantities than other stilbenes, and production increases with infection because, although stilbene does not exert high fungitoxic action, it is the precursor of viniferins (Figs. **1-3**) [97]. This is because resveratrol is considered to be the most important stilbene in grape and the precursor of this class of compounds. However, other stilbenes such as piceatannol - a phytoalexin with antifungal properties - also occur in grape. Stilbene was isolated for the first time from the stem of Vouacapoua and legumes in both free and glucoside forms, and was detected for the first time in Cabernet Sauvignon grapes by Bavaresco *et al.* [98].

Table **1** lists resveratrol, piceid and piceatannol contents in berries of *V. vinifera* cv. Barbera in relation to berry growth stage, fungus species (*Aspergillus*), berry status, and incubation temperature. Relevant increases in stilbenes (resveratrol in particular), promoted by all fungus species with respect to controls, have been observed [99].

4. VITICULTURAL FACTORS AND VINE STILBENES

Besides biotic and abiotic stresses, other factors may affect stilbene contents in grapevines, such as variety, rootstock [100,101], meteorological conditions [100,102], soil [103] and cultural practices [104-107].

4.1. Grape Variety and Rootstock

Variety plays an important role in stilbene concentrations in grape and plant tissues, as clearly shown in Fig. **4** (*trans*-resveratrol, *cis*-piceid and *trans*-piceid levels in 78 red and white grape varieties) and Fig. **5** (*trans*-resveratrol in the

Table 1. Effect of Growth Stage, Fungus Species, Berry Status, and Incubation Temperature on the main Phytoalexins Content in Grape [99]

		Symptoms (%) [c]	Ochratoxin A (µg/kg FW)	*trans* -resveratrol (µg/g FW)	*trans* -piceid (µg/g FW)	Piceatannol (µg/g FW)
growth stage [a]	at veraison	32 a	4.80	2.24	0.53	0.00 a
	during ripening	68 b	2.06	1.86	0.96	0.18 b
fungus species [b]	*A. carbonarius* (I)	73 a	9.30 a	2.45 b	0.95	0.05 b
	A. carbonarius (II)	76 a	8.65 a	1.68 b	0.56	0.05 b
	A. japonicus	63 b	0.06 b	4.26 a	0.93	0.09 b
	A. ochraceus	57 b	2.31 b	1.97 b	0.90	0.26 a
	A. fumigatus	32 c	0.02 b	1.40 bc	0.84	0.08 b
	control	0 d	0.00 b	0.49 c	0.27	0.06 b
berry status [a]	intact	43 b	2.16 b	1.50 a	0.73	0.09
	punctured	65 a	4.69 a	2.59 b	0.75	0.09
incubation temp [a]	25 °C	45 b	3.00	2.05	0.68	0.09
	30 °C	55 a	3.85	2.05	0.81	0.10

[a,b] Each value is the man of 84 (a) or 24 (b) data. [c] Values in each column without the same letters are significantly different ($P<0.05$).

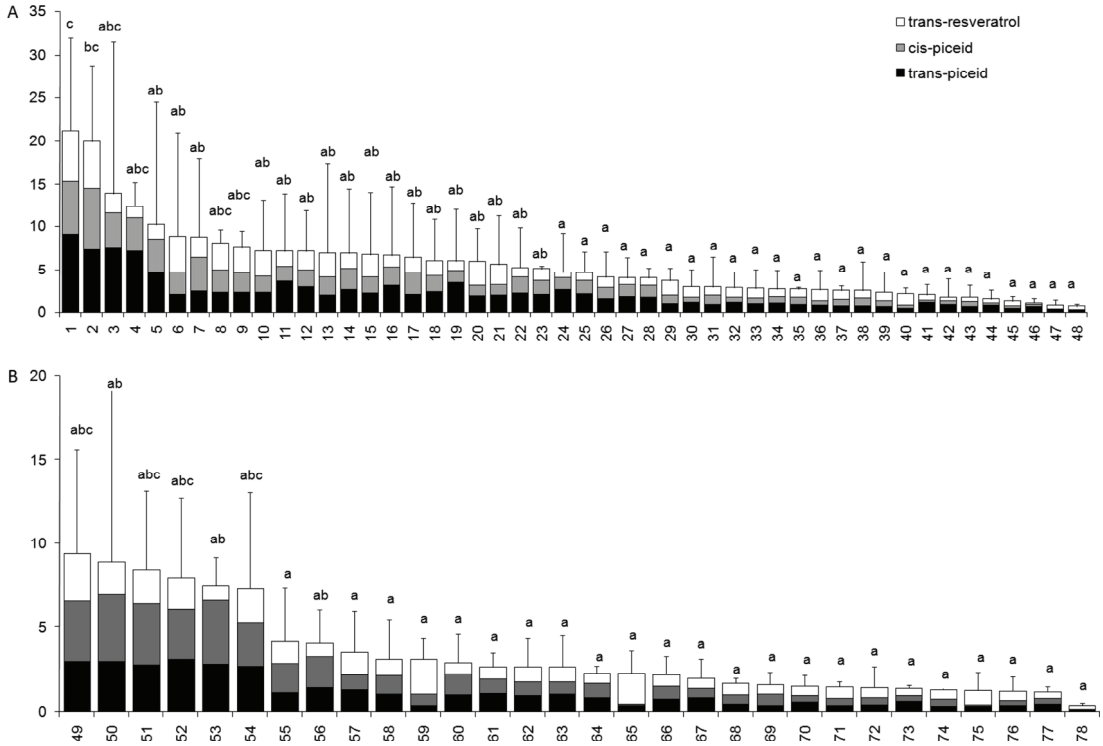

Fig. (4). Influence of the variety on stilbene concentration in grape [101]. Stilbene content of *V. vinifera* red (A) and white or pink (B) grapes harvested during vintages 2000, 2001, and 2004. The height of the bars refers to the average of the total resveratrol content as a sum of *trans*- and *cis*-piceid and *trans*-resveratrol of 3 years expressed as mg/kg of fresh weight of total grape berry. The standard deviation is also reported. The letters indicate groups of means within which there are not statistically significant differences (p value ≤ 0.05), according to Tukey's honestly significant difference (HSD) procedure applied to within-year normalized data. The numbers correspond to the variety names as follows: Pinot Noir (1), Pinot Tete de Negre (2), Tarrango (3), Franconia (4), Alicante Bouquette (5), Carmenere (6), Schioppettino (7), Ancellotta (8), Gaglioppo (9), Nero (10), Bovale Sardo (11), Lambrusco Salamino (12), Schiava Lombarda (13), Merlot (14), Pinotage (15), Dolcetto (16), Lambrusco Oliva (17), Negro Amaro (18), Tempranillo (19), Primitivo (20), Frappato (21), Zweigelt (22), Montepulciano (23), Cesanese (24), Uva di Troia (25), Cigliegiolo (26), Sangiovese (27), Pavana (28), Sagrantino (29), Primitivo di Gioia (30), Enantio (31), Cannonau (32), Croatina (33), Raboso del Piave (34), Casetta (35), Moscato Rosa (36), Grignolino (37), Marzemino (38), Tannat (39), Aleatico (40), Cabernet Sauvignon (41), Nera dei Baisi (42), Aglianico (43), Cabernet Franc (44), Schiava Grossa (45), Nebbiolo (46), Teroldego (47), Rebo (48), Marsanne (49), Pinot Gris (50), Rousanne (51), Italia (52), Malvasia Puntinata (53), Inzolia (54), Xinomavro (55), Garganega (56), Muscat Rouge de Madere (57), Madeleine Angevine (58), Sauvignon Blanc (59), Peverella (60), Malvasia di Candia Aromatica (61), Verdicchio (62), Viogner (63), Grechetto (64), Riesling (65), Verduzzo Friuliano (66), Kozma Palne Muskotali (67), Prosecco (68), Petit Manseng (69), Fiano (70), Chardonnay (71), Ribolla Gialla (72), Nosiola (73), Verduzzo Trevigiano (74), Gewuerztraminer (75), Pignoletto (76), Ortrugo (77), and Greco di Tufo (78). (with permission of American Chemical Society)

rachis of 10 different varieties). Pinot noir and Marsanne have the highest levels of total stilbenes in ripe berries within red and white varieties, respectively.

According to the data in Fig. **5**, Barbera has the highest concentration of *trans*-resveratrol and Marzemino the lowest. Small pieces of cluster stems, which may drop into the fermenting must after grape mashing and destemming, can therefore contribute to resveratrol concentration in the wine.

Rootstock may also affect stilbenes in the plant, K5BB inducing a higher level of resveratrol than SO4 or 1103P (Table **2**).

4.2. Soil and Vineyard Elevation

Soil type may also play an important role in the concentration of grape stilbenes (Fig. **6**) [103]. Fig. **6** shows the results on grapes from vines growing on calcareous soils, which have higher stilbene concentrations that those from vines grown on neutral soils.

Table **3** shows the influence of vineyard altitude on *trans*-resveratrol and *trans*-piceid concentrations in grapes at harvest. Altitudes of up to 320 m a.s.l. showed increased stilbene concentrations, although a decrease at the highest altitude was observed [104].

4.3. Cultural Practices

Stilbenes in grapevine tissues may also be affected by cultural practices. Figs. **7** and **8** show the effect of nitrogen (N) supply on the accumulation of resveratrol in vine leaves and grapes. In general, there is a negative effect of increasing N rates on resveratrol concentrations, possibly contributing to explain because grapevines fertilized at high N rates are more susceptible to diseases.

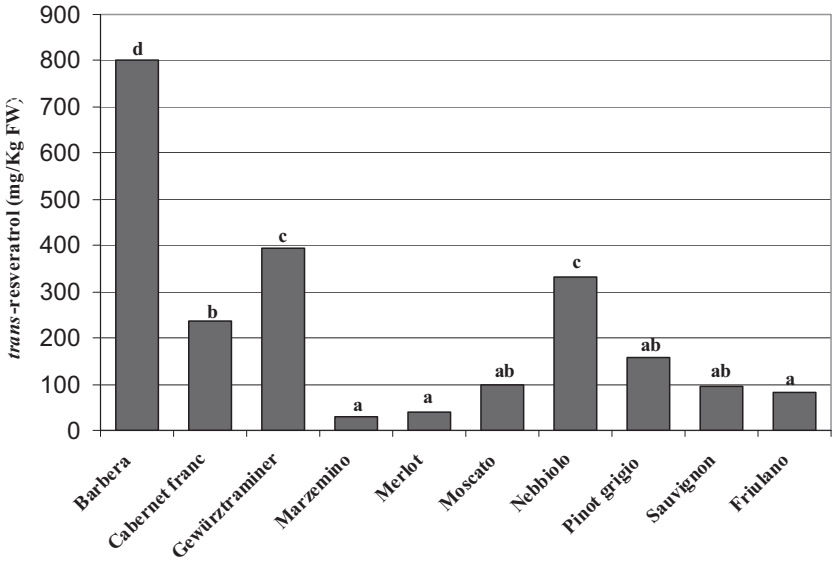

Fig. (5). Concentration of *trans*-resveratrol in rachis of 10 varieties at harvest. The data with the same letter are not significantly different for $p<0.05$ (Tukey test) [5,108].

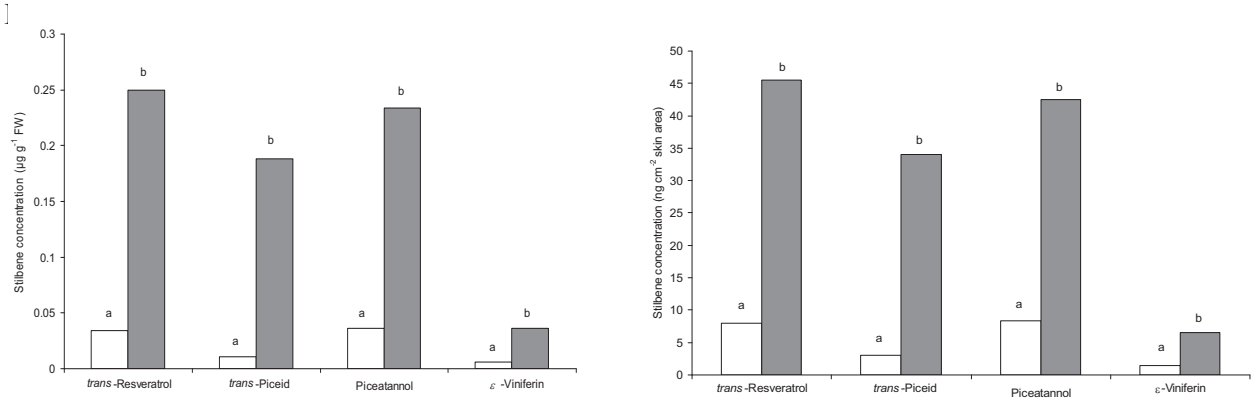

Fig. (6). Effect of the soil on the stilbene concentration of Merlot grape berries. Bars: white, non calcareous soil; grey, calcareous soil [103].

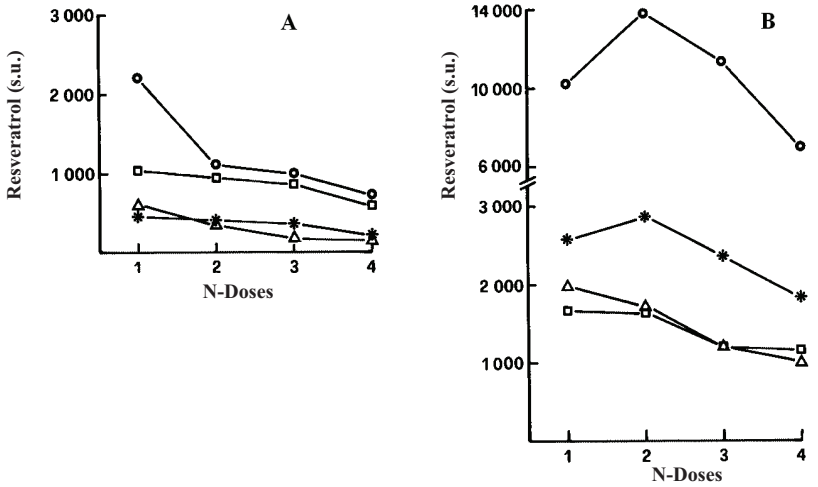

Fig. (7). Effect of nitrogen supply to the vineyard on leaf resveratrol content of four vine varieties [110]. (**A**) first sampling date; (**B**) third sampling date; s.u.: scan units; Δ Riesling; * Kerner (Trollinger × Riesling); □ Merzling (French Hybrid Seyve-Villard 5-276 × [Riesling × Ruländer]); ○ Orion (Optima × Seyve-Villard 12-375).

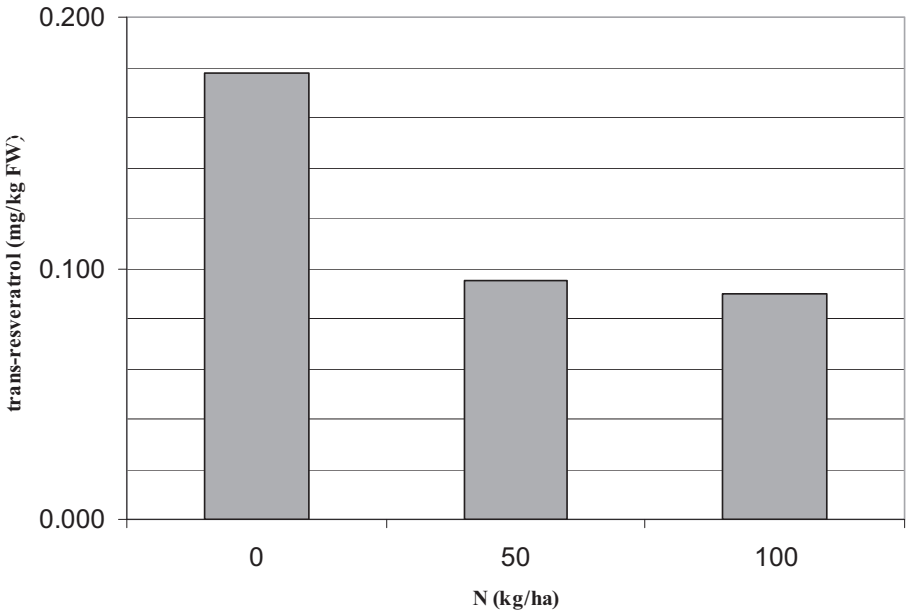

Fig. (8). *trans*-resveratrol content in ripe berries (*cv*. Barbera) at the harvest depending on nitrogen supply (mean data 2000-2002) [111].

Table 2. Effect of Rootstock on *trans*-resveratrol Content in Pinot Blanc Leaves. s.u.: Scan Units [109].

	Kober 5BB	SO4	1103 P	LSD 0.05
trans-resveratrol (s.u.)	3390	2020	1840	1050

Table 3. Effect of Vineyard Elevation above the Sea Level on the Stilbene Concetrations in Grape (mean data of Barbera, Croatina, and Malvasia di Candia Aromatica Ripe Grapes, Harvested in 2000-2002) [100].

Elevation	*trans*-resveratrol (mg/kg FW)	*trans*-piceid (mg/kg FW)
150 m a.s.1.	0.012 a	0.065 a
240 m a.s.1.	0.059 b	0.086 a
320 m a.s.1.	0.087 b	0.186 b
420m a.s.1.	0.070 b	0.074 a

The effect of removing leaves at *véraison* in several grapevine varieties was investigated in a field trial [105]. This method gave different results (in terms of stilbene concentrations in grapes at harvest) depending on genotype and meteorological conditions in the four years studied.

The concentration of stilbenes in wines can be also influenced by crop load and irrigation. Fig. **9** shows that high grape production and irrigation induces lower synthesis of stilbenes in the wine.

4.4. Genomic and Transcriptomic Advances

Recent studies on the heterozygous Pinot noir (clone ENTAV 115) genome have elucidated that at least 21 stilbene synthase (StSy) copies (Fig. **10**) can be predicted [112]. In the near-homozygous PN40024, 43 StSy copies were predicted [113]. Most StSy copies are clustered in chromosome 16 and, among the several peroxidase genes predicted, some may participate in the formation of viniferins. A resveratrol glucosyltransferase, putatively involved in piceid synthesis [114], is present as a single gene on chromosome 3.

Several studies have been carried out on the effects of stress conditions on StSy gene expression, both abiotic [115-117] and biotic [118,119]. Recent genome-wide transcriptome analyses on the expression modulation of StSy genes at constitutive level, in several grape varieties [101], induced by various elicitors [120-123] have been reported.

Due to the biological properties of resveratrol, there is great interest in StSy gene transfer experiments to other plants, with the aim of increasing their resistance to disease. Studies on transferring StSy genes have shown that the introduction of a single gene is sufficient to synthesize resveratrol in heterologous plant species. This is because StSy uses precursor molecules already present throughout the plant kingdom as substrates: for example, increased resistance to *B. cinerea* and *O. tuckeri* has been observed in wheat [124]. Stable transformation in a homologous system has been described, such as increased tolerance of 41B rootstock against *B. cinerea* [125] and transient transformation in grape leaves, performed before infection with *P. viticola,* affected the development of the infection [126]. Conversely, experiments in which plants transformed

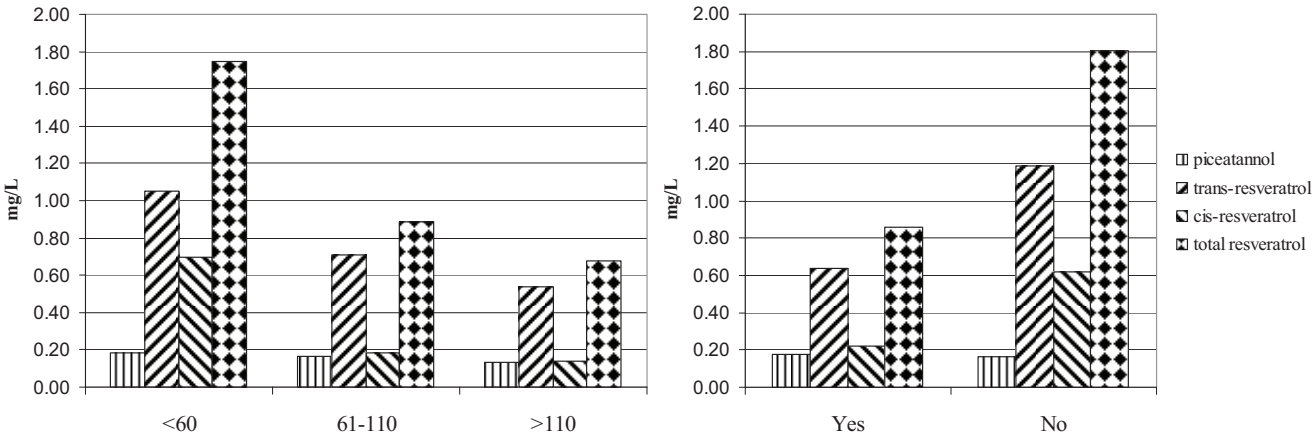

Fig. (9). Stilbene concentration in Sicilian red wines depending on grape production (q/ha) (left) and irrigation (right) [107].

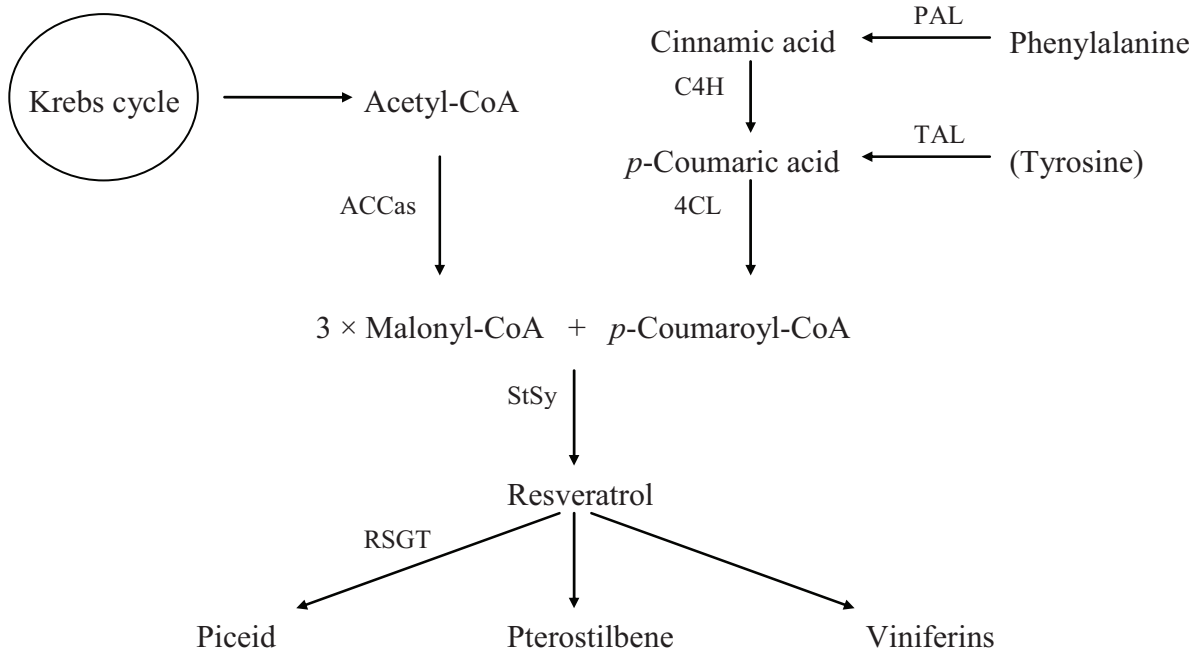

Fig. (10). Stilbene biosynthesis in grapevine. ACCase, acetyl CoA carboxylase; PAL, phenylalanine ammonia lyase; C4H, cinnamate-4-hydroxylase; TAL, tyrosine ammonia liase; 4CL, 4-coumarate-CoA ligase; StSy, stilbene synthase; RSGT, resveratrol glucosyltransferase.

with StSy genes did not show increased resistance to pathogens have also been reported [127]. At present, no marker-assisted-selection (MAS) data for disease resistance based on stilbene synthesis are available. Fingerprinting based on StSy genes has been used to characterize Barbera clones [128] and Chardonnay, Pinot Noir, Sangiovese and Touriga Nacional varieties [129,130].

5. WINE RESVERATROL AND OENOLOGICAL PRACTICES

In light of the data presented in the previous section, it is evident that the composition of grape is the key factor limiting the concentration and pattern of resveratrols in wine. It is also very important a complete maturity of the berry, since it was demonstrated that the amount of resveratrols in the berry rises with the progress of the ripening [101].

5.1. Extraction from Grape

Red wines contain resveratrols in a considerable higher concentration than white wines. This is the obvious consequence of the fact that resveratrols are synthesized in the solid parts of the berry and that white wines are produced with no or limited maceration with the pomaces. The content of resveratrols in red wines is mainly influenced by the duration of the skin contact. At the beginning of the fermentation, *cis*-resveratrol is absent in juice since this compound is usually absent in grape [101]. Both *trans*-resveratrol and their glucosides (*trans*- and *cis*-piceid) are

easily transferred from the grape to the juice already during the early phases of skin contact. Their concentration in wine increases quickly up to the maximal production of ethanol, and a prolonged skin contact can complete the extraction from the solid parts of grape. The two isomers of piceid, being more polar, are extracted from the grape skins before the extraction of the relevant aglycons [106]. Their concentration decreases in the juice during the fermentation process, due to hydrolysis with release of the free resveratrols. The extraction from grape skin of *trans*-resveratrol is delayed since it requires the presence of ethanol to increase its solubility and mobility.

The final balance is the consequence of three concomitant factors, according to Fig. **11**, i.e. direct extraction from the grape skins, enzymatic hydrolysis of the glucosides which leads to the formation of aglycons, and *trans-cis* isomerisation [106].

Therefore, the formation of the aglycons is mainly due to the presence of both a direct extraction of free compounds from the grape skins and the hydrolysis of glucosides. The contemporaneous reaction of *trans-cis* isomerization can also play a role, especially with regard to the aglycons, which are less stable than the relevant glucosides.

5.2. Role of the Yeast

Both grape tissues and yeasts have β-glucosidase activities [131-134]. The exogenous β-glucosidase of yeast cell membrane usually reaches its utmost activity during a short time (2-3 days) around the exponential growth of yeast population; after that, it undergoes a rapid decrease because of its weak stability at acidic pH [135,136]. The choice of the yeast can therefore influence the final concentration and balance of resveratrol in wine [137]. Also some lactic bacteria were shown to possess β-glucosidase activity [138]. More studies are required to verify if these activities can affect the final balance between the bound and free forms of resveratrols in wine.

5.3. Enological Practices which affect the Concentration of Resveratrol in Wine

The low levels of fining agents necessary for the stabilization of red wines do not significantly reduce the concentration of *trans*-resveratrol in wines [139]. Resveratrols are relatively stable compounds, which can remain stable for years in wines, provided that they are properly stored (i.e. avoiding exposure to excess heat) and with the presence of exogenous antioxidants (i.e. sulphur dioxide) at the levels which are normally considered adequate to prevent the alterations of wines [140]. The study of the stability of both red and white wines added with exogenous *trans*-resveratrol for the production of functional beverages confirmed the stability of this compound in wine [141]. This assumption, however, is not always true for all winemaking processes. Sherry wines in particular, have been shown to be subject of great losses of resveratrols, due to the peculiar winemaking process and ageing, and in particular to oxidative phenomena and a combination with acetaldehyde and "flor" biofilm growth [142].

More interesting is the application of specific post-harvest techniques, which are able to modulate the resveratrols in grape just before the processing. The irradiation of grape with ultraviolet-C light could be particularly favorable for the production of raisin wines, as in the example of Amarone della Valpolicella. It has been demonstrated that the biosynthesis of resveratrol in grape can be induced during 2-3 months after the harvest, and resulted in the production of experimental wines greatly enriched in resveratrols [143]. Similar techniques are under evaluation and were confirmed to be a feasible way to produce high quality red wines

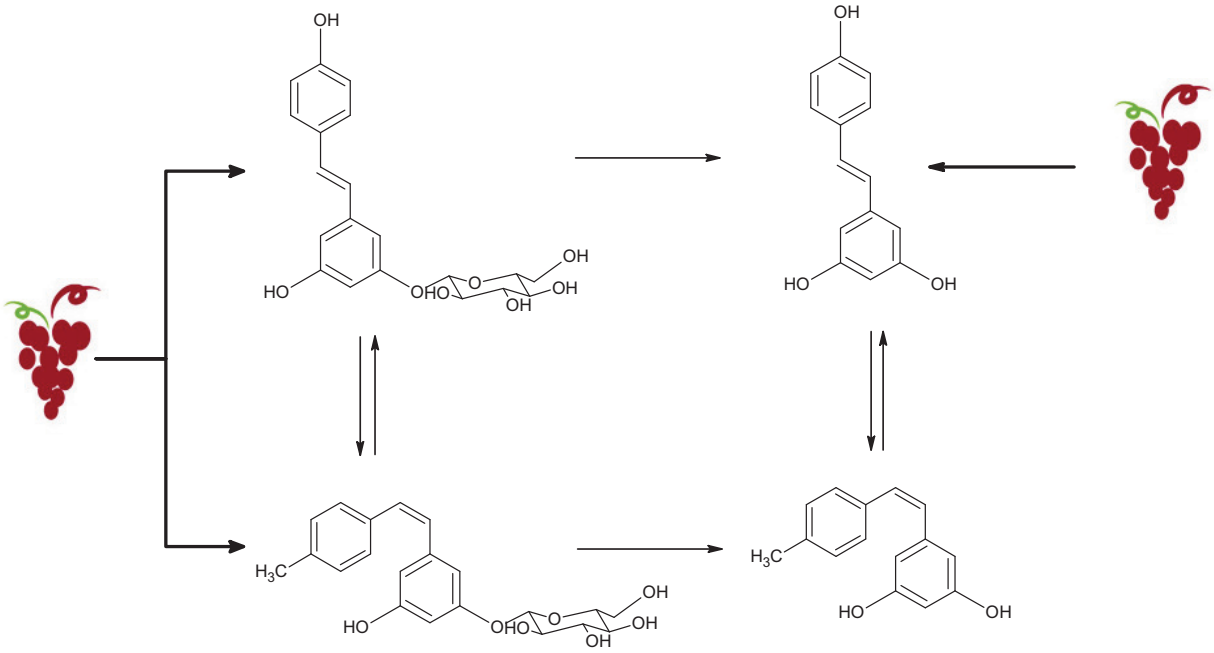

Fig. (11). Factors influencing resveratrol concentration during red wine vinification (thick arrow, direct extraction from solid parts of grape; single arrow, hydrolysis yield free aglycons; double arrow, *cis-trans* isomerization).

enriched in resveratrol and piceatannol [144]. On the other hand, the same technique was not successful for the production of white wines enriched in resveratrols, since the extended contact with the pomace required for this process was reported to compromise the overall quality of the enriched wines [145].

6. CONCLUSION

In the next few years, genetic research should focus on stilbene synthesis and plant-environment interactions, to enhance grapevine resistance toward diseases and to improve the beneficial proprieties of grape-based products. In addition, due to the increased demand for stilbene compounds for nutraceutical, cosmetic and pharmaceutical uses, their production from sustainable sources is desirable, and the use of biotechnology through recombinant microorganisms and plant cell suspensions has been reported to be particularly promising [146]. SAR – systemic acquired resistance – inducers are of particular interest in the production of more healthy grape products.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflicts of interest.

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