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## **NMR spectroscopy and chemometrics-based analysis of grapevine**

Ali, K.

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## **CHAPTER 2**

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### **Metabolic constituents of grapevine and grape-derived products**

Kashif Ali, Federica Maltese, Young Hae Choi, &  
Robert Verpoorte

Natural Products Laboratory, Institute of Biology, Leiden University,  
The Netherlands

## Abstract

The numerous uses of the grapevine fruit, especially for wine and beverages, have made it one of the most important plants worldwide. The phytochemistry of grapevine is rich in a wide range of compounds. Many of them are renowned for their numerous medicinal uses. The production of grapevine metabolites is highly conditioned by many factors like environment or pathogen attack. Some grapevine phytoalexins have gained a great deal of attention due to their antimicrobial activities, being also involved in the induction of resistance in grapevine against those pathogens. Meanwhile grapevine biotechnology is still evolving and biotechnologists are making huge efforts to produce grapevine cultivars of desired characteristics. In this chapter, important metabolites from grapevine and grape derived products like wine will be reviewed with their health promoting effects and their role against certain stress factors in grapevine physiology.

## Introduction

Grapevine (*Vitis* spp.) is globally one of the most important fruit species due to the numerous uses of its fruit in the production of wine, grape juice, and other foods. It has a connection with an ancient historical development of human culture. In the earliest writings and archives associated to all sorts of agricultural and religious activities, grapevine and its products were given a significant place (Thomas et al. 1993). The oldest records of the use of grapevine products by humans dated back to 3500 - 2900 B.C. (Bowers et al. 1999). Among the *Vitis* species, *Vitis vinifera* is currently one of the most cultivated because of its use in wine production (Lodhi and Reisch 1995).

The grapevine has been easily cultivated and across the world gives rise up to eight million hectares of vineyards (Vivier and Pretorius 2000). Up to now *V. vinifera* has reached all continents but is successfully cultivated only in temperate climate regions with sufficient rain, warm and dry summers and relatively mild winters. The Vitaceae family consists of almost one thousand species, grouped in seventeen genera. Grapevines are classified in the *Vitis* genus and it is generally accepted that the most cultivated *V. vinifera* comprises up to 5000 true cultivars, used in wine, table (fresh fruit) and dried grape manufacturing around the world. Improvements to these cultivars initially relied largely on random selections of natural mutations which resulted in

enhanced cultivation or improvement in some aspects of fruits and/or wine quality. This was later followed by more directed clonal selection schemes (Bowers et al. 1999).

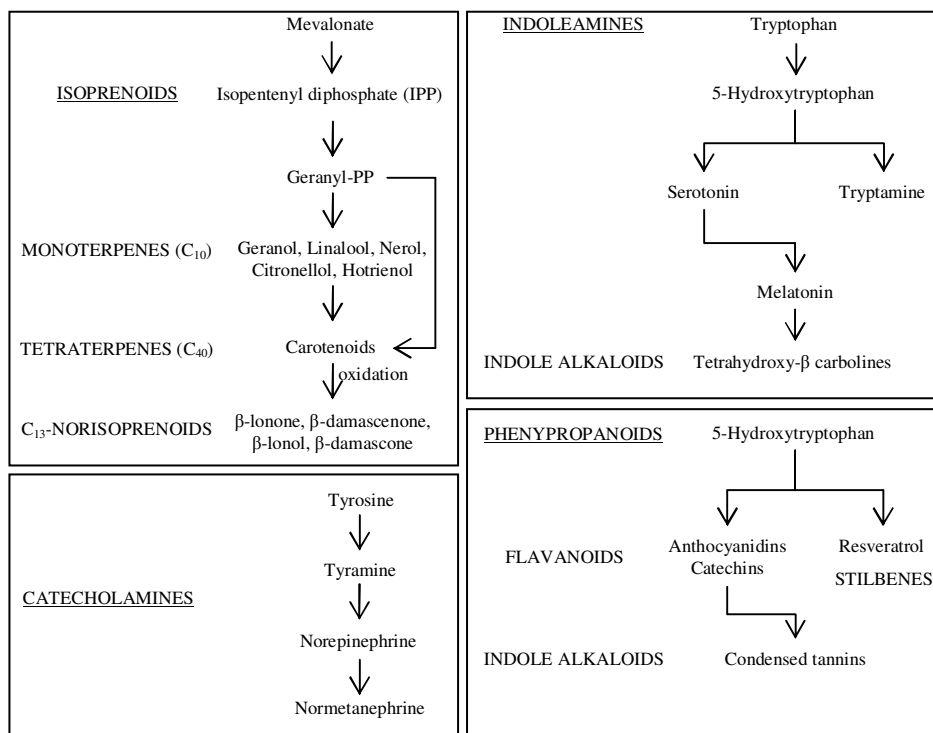
The quality of grapevine, as with most plants, mainly depends on its metabolites. The production of these metabolites is especially sensitive to external conditions. In particular, the chemical diversity of grapevine is mostly affected by secondary metabolites. These secondary metabolites consist of a wide array of species-specific chemicals and belong to different phytochemical groups many of which have proved to be of great value to the pharmaceutical, agrochemical, food and fragrance industries (Zhang et al. 2005). In general, secondary metabolites are known to play key physiological functions in plants including their adaptation to the environment (Lewinsohn et al. 2001), acquired resistance to pests and diseases, pollinator attractant capacity, and the building of symbiotic relations with microorganisms (Harborne 2001). They are also often very crucial in the determination of the quality of food attributes (color, taste, and aroma), and colors and pigments of ornamental plants (Dixon 2001; Iriti and Faoro 2006).

This review attempts to present detailed features of grapevine chemistry including primary and secondary metabolites and the biological activities associated to these chemical constituents. A detailed account of grapevine diseases and their resistance to them is also presented, followed by a brief discussion on grape biotechnology and its prospects.

### **Overview on phytochemistry of grapes and wine**

Plant metabolism is mainly divided into two types i.e. primary and secondary metabolism. The primary and secondary metabolites are known to perform completely different tasks in plants. A “primary metabolite” is directly involved in normal growth, development, and reproduction of plant species while a “secondary metabolite” is not directly involved in those processes, but usually has important ecological functions like defenses against predators, parasites and diseases, for interspecies competition, and to facilitate the reproductive processes (coloring agents, attractive smells, etc). Many of these primary and secondary metabolites from plants are famous for their beneficial effects on human health.

The phytochemical composition of grapevine includes a great variety of known bioactive compounds. Among other beneficial effects, the active compounds in grapes have been proved to have powerful antioxidant properties (Yilmaz and Toledo 2004). Antioxidants found in grapes can neutralize free radicals and may reduce or even help prevent some of the damage caused by them. Resveratrol (a key component in grapevine phytochemistry) has gained much popularity as an antioxidant supplement (Sovak 2001). Figure 1 shows the biosynthetic pathways of important phytochemicals from grapevines.



**Figure 1.** Schematic diagram of the syntheses of important grapevine metabolites.

The chemical composition of grapes and wine has been intensely studied in the recent decades and the number of compounds identified increased exponentially as different analytical techniques such as gas chromatography (GC), high performance liquid chromatography (HPLC) and high performance thin layer chromatography (HPTLC)

coupled with mass spectrometry (MS) and nuclear magnetic resonance (NMR) has been developed (Brun et al. 1986).

The study of the chemical composition of wine has so far allowed the identification of more than 500 compounds of which 160 compounds were esters. The concentration of these compounds ranges from  $10^{-1}$  to  $10^{-6}$  mg/L. Even at these concentrations many of the individual compounds are likely to play a very important role in the human taste perception. The carbonyls, phenols, lactones, terpenes, acetals, hydrocarbons, sulfur, and nitrogen compounds, although present in very low concentrations, contribute to the fragrance of wine. However, the taste and mouth-feel sensations are primarily due to compounds like water, ethanol, organic acids, amino acids, sugars, and glycerol.

## **Primary metabolites in grapes and wine**

### ***Sugars and amino acids***

Glucose and fructose are the principal grape sugars while sucrose and other sugars are rarely detected in *V. vinifera* grapes. Cultivars other than *V. vinifera* may contain up to a 10% of sucrose. Amount of sugars in *V. vinifera* can be affected by cultivar type, maturity and health of the berries (Crippen and Morrison 1986). High sugar concentrations can also result in the increase of the volatility of aromatic compounds. Generally, sweetness is detected at levels higher than 1% (w/v) of overall sugars, and this is influenced by other constituents such as acids and tannins.

The amino acids contribute to wine aroma, taste, and appearance (Hernandez-Orte et al. 2003). Carrying out studies of partial least-squares regression models, Hernandez-Orte et al. (2002) found that amino acid composition accounts for a high proportion of the variance in the volatile composition. Moreover, the composition of the amino acids in the wine has an influence on aromas during the maturing process (Escudero et al. 2000). In the wineries, obtaining young wines with fruity aromas is now gaining much interest. In order to improve the kinetics of wine fermentation, activators are usually added, mainly ammonium salts, also auxiliaries to avoid stuck and sluggish fermentations (Bisson 1999; Blateyron and Sablayrolles 2001). Daily-based research is carried out into new additives and therefore it is interesting to know how the addition of nitrogen sources affects amino acid uptake by the yeast and how the addition affects the synthesis of aroma compounds. The activators are metabolized by the yeasts and modify

the consumption of amino acids and ammonium in the juice thus causing a variation in the wine aroma. As occurs with esters, the pool of intracellular nitrogen regulates the formation of higher alcohols (large 1986).

### ***Biogenic amines***

Biogenic amines (BAs) in wines are produced from the free amino acids by microbial decarboxylation. They can be originated from the different wine making processes or from the grape berries and have unpleasant effects at high concentrations and caused a health risk to sensitive individuals (Gardini et al. 2005). Studies on BA showed that yeasts not appear to be responsible for the production of BAs in commercial red wines (Marcobal et al. 2006). The wine contamination with BAs mostly occurred during natural or spontaneous malolactic fermentation by lactic acid bacteria present in low population in healthy grapes (Herbert et al. 2005; Landete et al. 2005). Although there are no legal limits for BAs but some countries, like Austria and Switzerland, have their own suggestions. The export of wines to those countries could be affected by the higher levels of BAs, making BAs a potential economic threat.

### ***Polysaccharides***

In comparison with other macromolecular fractions, wine polysaccharides have been less studied. Some of their properties were determined (Riou et al. 2002) along with the characterization of their structures (Vidal et al. 2000). Polysaccharides are generally low in final wine products, being partially water soluble and extracted into the juice during crushing and pressing. The effects of polysaccharides in wines are probably direct i.e. ‘mellowness’ or indirect by modulating astringency (Pellerin et al. 1996).

### ***Alcohols***

According to Fraile et al. (2000), the formation of different alcohols takes place at the end of the fermentation, when most of the amino acids have been consumed, whereas, according to Rapp and Versini (1991), this synthesis occurs at the same time as ethanol production. Ethanol is the most abundant and important alcohol in wine. Ethanol concentration in wine generally ranges from 10-13 mL/100 mL, depending mainly on sugar content and lesser on temperature and yeast strain. Ethanol is crucial to the

stability, aging, and sensory properties of wine. It also influences the type and amount of aromatic compounds produced by affecting the metabolic activity of yeast. The dissolving action of ethanol is also probably involved in the reduction of the evaporation of aromatic compounds during fermentation (Williams and Rosser 1981). Moreover, it is also involved in the production of acetals by reacting with the aldehydes. Methanol is also present in wines in fairly large amounts (0.1-0.2 gm/L) (Jackson 2000). In wines methanol is formed by the degradation of pectic substances, present in crushed grapes, by enzyme action (Ribereau-Gayon et al. 2000). The methanol content in wines is dependent on number of factors like the variety and condition, maceration treatment, fermentation temperature, and pectolytic enzyme treatment. The *in vivo* oxidation of methanol produces formaldehyde and formic acid which are toxic to the central nervous system. Due to this methanol content is one of the critical control point parameters which should be controlled during the alcoholic fermentation stage to produce wine safe for human consumption (Kourtis and Arvanitoyannis 2001).

Other potentially significant higher alcohols in wine are n-propanol, 2-methyl-1-propanol, 2-methyl-1-butanol, and 3-methyl-1-butanol (Rapp and Mandery 1986). The higher alcohols are produced as a by-product of yeast fermentation and their concentration is markedly influenced by vinification practices such as temperature, presence of oxygen, suspended solids and yeast strain (Sponholz 1988). Higher alcohols may produced by deamination of the amino acids and grape-derived aldehydes, and by the denitrification of amino acids (Chen 1978).

### ***Organic acids***

In grape juice and wine, organic acids composition is very important as it highly influences the organoleptic properties, microbiologic control and a parameter of critical control in wine stabilization. The processes like alcoholic fermentation, malolactic fermentation, and oxidation of ethanol are involved in the production of these compounds or they may directly come from grapes. Tartaric and malic acids are the major organic acids in grape juice while it also contains succinic and citric acid in low amounts. Wine contains both volatile and fixed acids. Volatile acids, principally acetic acid, can be readily removed by reverse osmosis. The content of fixed acids such as tartaric, malic, and citric acid (from grapes) along with lactic, succinic, oxalic, fumaric,



and citric acid (from fermentation process) influences the pH of wine (Singleton 1982). Also small amounts of different acids like galacturonic, citramalic, pyruvic acid, and ketoglutaric acid etc. are also there (Castellari et al. 2000).

## **Secondary metabolites in grapes and wine**

### ***Phenolics***

Phenolics composed a large group of complex metabolites particularly contributing to the characteristics of grapes and red wines. Phenolic are known to contribute to pigmentation of different organs along with their role against different biotic and abiotic stresses. A large range of structures has been shown by phenolic compounds, hence properties as well. Different properties of wine like appearance, taste, mouth-feel, fragrance, and antimicrobial activity can be affected by phenolics and related compound (Kennedy 2008). Grapevine phenolics may arise from the fruit (skins and seeds) and vine stems, or may be products of yeast metabolism. Figure 2 shows structures of some important phenolic compounds. The concentration of phenolics in wine increases during fermentation but begins to fall as they bind and precipitate with proteins and yeast hulls. During fining and maturation, the phenolics concentration continues to decrease, suffering a dramatic fall during the aging stage. The three primary phenol groups that occur in grapes and wine are simple phenolics, flavonoids, and stilbenoids. The schematic biosyntheses of these three groups are shown in Figure 3.

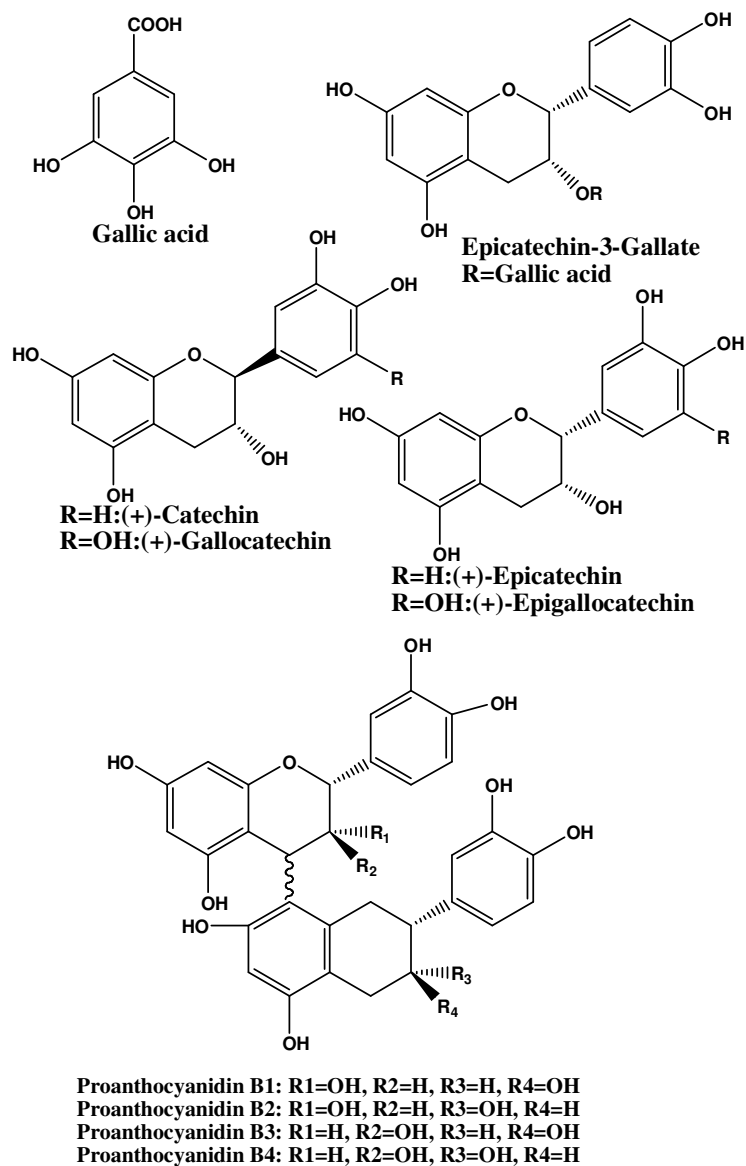
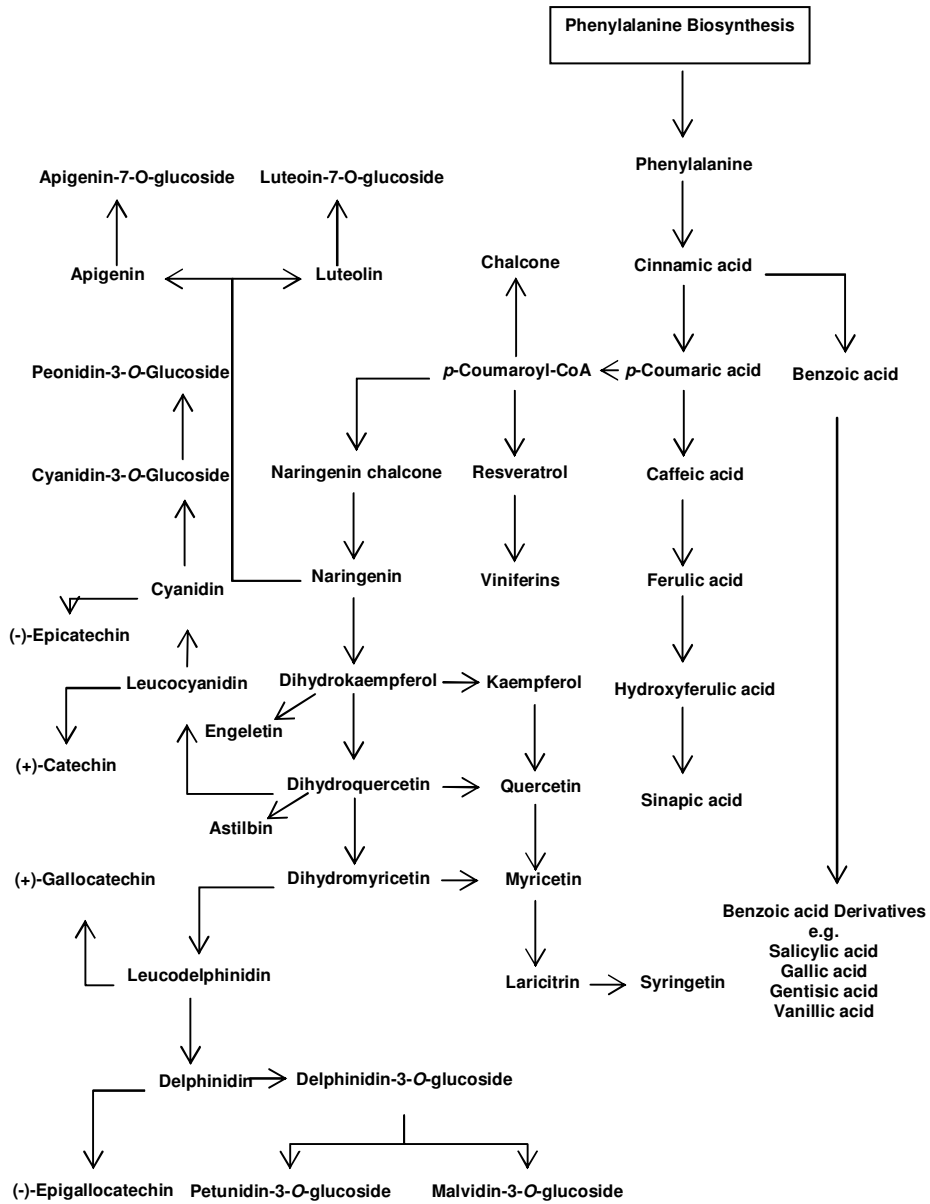


Figure 2. Structures of major polyphenols identified in grapes.



**Figure 3.** Biosynthetic pathway from phenylalanine to different classes of phenolics found in grapevine and wine.

### **Simple phenolics and phenylpropanoids**

Simple phenolics of grape origin are initially synthesized from phenylalanine (Hrazdina et al. 1984), whereas those of yeast origin are derived from acetic acid (Packter 1980). Simple phenolics are derivatives of hydroxycinnamic acids (HCA) and hydroxybenzoic acids (HBA). Primarily, they are stored in cell vacuoles of grape cells and can easily be released by crushing. Common hydroxycinnamic acids are *p*-coumaric acid, caffeic acid, sinapic acid, and ferulic acid. In grapes and wine, esterified form of HCA with tartaric acid is more common than free HCA. The caftaric acid (caffeic acid conjugated with tartaric acid) is predominant (up to 50%) of the total HCA. Since the concentration of HCA depend on factors like grape variety, growing conditions, climate etc., different studies shows different concentrations. Compounds like *p*-coutaric acid (*p*-coumaric acid conjugated with tartaric acid) and fertaric acid (ferulic acid conjugated with tartaric acid) are found in the concentrations of 55 mg/L and 16 mg/L, respectively (Ricardo-Da-Silva et al. 1993). Derivatives of HCA like ethyl esters of caffeic and coumaric acid along with ethyl esters and diethyl esters of caftaric acid were also reported (Baderschneider and Winterhalter 2001). Evidence of glucosides like 4-*O*-glucosides of ferulic and coumaric acids (both *cis*- and *trans*- forms), glucosides of caffeic, coumaric, ferulic, and sinapic acids have been shown by some studies (Cooper and Marshall 2001; Monagas et al. 2005; Baderschneider and Winterhalter 2001). Previous reports of NMR characterization showed *cis*- isomers of coumaric and coutaric acids in Chardonnay grape pomace and Riesling wine (Baderschneider and Winterhalter 2001). The hydroxycinnamic esters are more concentrated (2 to 100 fold) in grape skin than in pulp. Differences in the total amount and proportion (Cheynier et al. 1986) have been reported based on the varietal differences. In addition, HCAs are also important constituents of acylated anthocyanins. The relation of acetylated and coumaroylated anthocyanins can be used in the authenticity control of red wine.

When compared with HCA, grapes and wines have lower levels of hydroxybenzoic acids (HBAs) and their derivatives. The most common HBAs are gallic acid, gentisic acid, protocatechuic acid, and *p*-hydroxybenzoic acid, which are mainly found in their free form (Pozo-Bayon et al. 2003; Vanhoenacker et al. 2001). Hydroxybenzoic acids are mostly represented by gallic acid, which is found free as well as acyl substituent of flavan-3-ols. Two forms of gallic acid, i.e. 3-*O*- $\beta$ -glucopyranoside and 4-*O*- $\beta$ -

glucopyranoside, have been reported in grape (Lu and Foo 1999). In wine the level of gallic acid has been shown by many studies (Pozo-Bayon et al. 2003; Pena-Neira et al. 2000; Sladkovsky et al. 2004) that gallic acid is ranging from 0.3 mg/L to 4.8 mg/L. Other benzoic acids such as protocatechuic, vanillic, and syringic acids are reported in Riesling wine from Germany (Baderschneider and Winterhalter 2001). Several derivatives of HBA were also identified including the ethyl esters of vanillic and *p*-hydroxybenzoic acid, methyl esters of vanillic and protocatechuic acid, ethyl esters of protocatechuic acid, the glucose ester of vanillic acid, from German Riesling wine (Baderschneider and Winterhalter 2001).

### **Flavonoids**

Flavonoids are found primarily in the skins and grape seeds. Flavonoids can be further divided into several sub-groups like flavones, flavonols, dihydroflavanols, flavanols, and anthocyanidins, with the same skeleton but different oxidation state of their central pyran ring. The most common flavonoids in wine are flavonols, catechins (flavan-3-ols), and anthocyanins. Flavonoids are synthesized from the combination of the shikimic and polyketide pathway. Polymerization of polyhydroxy flavan-3-ol units, (+)-catechin and (-)-epicatechin, and their gallate esters produces oligomers and polymers called proanthocyanidins (often referred to as procyanidins). Even though procyanidins occur primarily as dimers in grapes (Kennedy 2008), they tend to be polymerized and predominate in wine as condensed tannins (mid-size procyanidin polymers containing three to five subunits). In some cultivars, structural differences exist among skin, stem, and seed procyanidins. Also, there are considerable differences in types and concentrations between cultivars (Boselli et al. 2004). In grape seeds and skin, about twenty procyanidin dimers and trimers have been identified (Fulcrand et al. 1999). Seeds contained procyanidin of catechins, epicatechin, and epicatechin gallate units while grape skin and wine showed procyanidins mostly based on epicatechin and epigallocatechin units (De Pascual-Teresa et al. 2000). The total amount of procyanidins is reported to vary from 1.7 to 4.4 g/kg of berries in skin, 1.1 to 6.4 g/kg in seeds, and 0.2 to 1 g/kg in pulp (Mane et al. 2007).

Anthocyanins, which are responsible for the red or pink color in grape berries, belong to the flavonoid family. They are glycosylated derivatives and their parallel aglycones are

known as anthocyanidins. In *V. vinifera*, 3-monoglucosides are the major anthocyanins where glucose can be acylated by acetic, *p*-coumaric, and caffeic acid (Alcalde-Eon et al. 2006; Vidal et al. 2004). Some additional anthocyanins, like 3-caffeoylglucosides of peonidin, cyanidin and delphinidin, have also been reported in grapes using HPLC-MS (Vidal et al. 2004). The anthocyanin profiling can be used for the chemotaxonomic classification as the total amounts (from 500 mg/kg to 3 g/kg of berries) and proportions of the various anthocyanins are characteristic of each variety (Mattivi et al. 2006). Grape anthocyanin composition depends not only on the maturity but also on different vine growing parameters like soil and climate together with practices such as pruning, fertilization, or watering (Gonzalez-Neves et al. 2002). The accumulation of anthocyanins starts at the veraison stage with occasional decrease towards the end of the ripe stage, especially in hot climates (Fournand et al. 2006).

In wine, apart from the anthocyanins, another class of compounds has been evolved known as pyranoanthocyanins. They are basically derived from anthocyanins having an additional pyran ring attached to position 4 and 5 of anthocyanin. These pyranoanthocyanins, e.g. vitisin A and B, varied in concentration at the different stages of wine (Asenstorfer et al. 2003). Pyranoanthocyanins are found more resistant against color loss due to sulfites and pH variation (Hakansson et al. 2003). The contribution of the pyranoanthocyanins in the color of red wine has been estimated by number of studies but resulted with different conclusions (Alcalde-Eon et al. 2006; Schwarz et al. 2003; Boido et al. 2006) which might be due to the wine type and the way to calculate the pigments. A particular type of pyranoanthocyanin derivatives are vinylpyranoanthocyanin-flavanol pigments, also known as Portisins (Mateus et al. 2004) as they are isolated from the Port wine. These pigments have increased stability towards sulfites and pH-induced color loss (Oliveira et al. 2006). The formation of this new class of pigment involves pyranoanthocyanins and vinyl-flavanols, representing an evolution in the wine pigments having pyranoanthocyanins as a main precursor.

Flavanols are also reported in grape berries in the form of 3-*O*-glycoside of quercetin, myricetin, kaempferol, isorhamnetin, laricitrin, and syringetin, both in red and white cultivars (Mattivi et al. 2006; Talcott and Lee 2002). Mostly 3-*O*-glucoside and 3-*O*-glucuronide of flavanols are present on grapes but other mono and diglycosides are also reported present in lower amounts (Downey et al. 2003). Both grape and wine have also

been reported for the presence of dihydroflavonols like astilbin and engeletin (Guebailia et al. 2006).

### ***Stilbenoids***

Stilbenoids (1,2-diarylethenes) are another class of phenolic compounds (non flavonoids) belonging to the phenolic group and attracted great interest because of their potential health valuable effects (Guebailia et al. 2006). Stilbenes are essentially located in grape skin (Creasy and Coffee 1988; Roggero and Garcia-Parilla 1995) but also reported in grape seeds (Sun et al. 2006) and grape stem (Bavaresco et al. 1997). Grapes and red wine are two of the major dietary sources of stilbenes but their concentration is relatively low as compared to other phenolic compounds (Pace-Asiak et al. 1995). Grape seeds contain low amounts of stilbenes (Pezet and Cuenat 1996), but a substantial amount of these compounds may be found in grape stems (Sun et al. 2003). However, grape stems contribute little to these compounds in wine due to their poor diffusion property (Sun et al. 2006).

Resveratrol (a monomeric stilbene) is the major stilbene of grapes. It is synthesized from phenylalanine and can exist in two isomeric forms. In wines, both isomeric forms were detected (Sun et al. 2003) due to photochemical isomerization of *trans*- form into *cis*- form during wine making (Jeandet et al. 1995; Roggero and Garcia-Parilla 1995). Stilbenes can also occur in oligomeric and polymeric forms, known as viniferins. They are induced by oxidative polymerization of monomer resveratrol through the activity of a peroxidase (Jean-Denis et al. 2006). Many viniferins have been reported so far in grapes including  $\epsilon$ -viniferin and  $\delta$ -viniferin, dimeric resveratrol (Pezet et al. 2003; Vitrac et al. 2005) and  $\alpha$ -viniferin, trimeric resveratrol (Pryce and Langcake 1977). Stilbenoids such as ampelopsin-A (dimeric resveratrol) and hopeaphenol (dimeric ampelopsin A) have also been identified (Jeandet et al. 2000; Guebailia et al. 2006).

The stilbene contents in wines varied considerably due to several factors like climate, grape variety, fungal infection (Perrone et al. 2007), UV light, metal ions (Pussa et al. 2006) and enological methods (Gambutì et al. 2004). It has been shown that red wine is usually more concentrated in stilbenes than rose and white wine as this depends on the skin contact of the must and the high phenolic content of grape cultivars during fermentation (Perrone et al. 2007). Many reports have been published targeted towards

the quantification of stilbenes in different wines (Vitrac et al. 2005; Guebaila et al. 2006; Naugler et al. 2007).

### **Importance of grape phenolics to human health**

The medicinal and nutritional value of grapes has been proclaimed for thousands of years. Several ancient Greek philosophers praised the healing power of grapes usually in the form of wine (Sun and Spranger 2005). Grape leaves have been used to stop bleeding, inflammation, pain, and diarrhea. Unripe grapes used to treat sore throats and dried grapes (raisins) to cure constipation and thirst. Round, ripe, and sweet grapes have been used to treat a range of health problems including cancer, cholera, smallpox, nausea, eye infections, skin, kidney and liver diseases (Shi et al. 2003). Grape berries are prescribed as laxatives and as a diuretic in case of gastrointestinal and circulatory disturbances, or for the treatment of liver or kidney diseases (Stervbo et al. 2007). Grape phenolics have been proved to possess several health promoting properties playing an important role in the inhibition of carcinogenesis, mutagenesis, and cardiovascular diseases. These activities have been associated to their *in vivo* and *in vitro* antioxidative activities. Flavonoids in grape seeds have also been reported to exhibit activities against peptic ulcer (Saito et al. 1998) and several dermal disorders (Bomser et al. 1999).

#### ***Antioxidant activity***

Phenolics including flavonoids and related polyphenols from wine and grapes have generated remarkable interest based on several reports (both *in vivo* and *in vitro*) of their antioxidant and free radical scavenging properties. A clinical report has shown that procyanidin oligomers from grape seeds are 20 and 50 times more potent antioxidant than vitamin C and E, respectively (Uchida 1980). Silva et al. (1991) found that monomeric and polymeric grape seed proanthocyanidins have superoxide and hydroxyl radicals scavenging activity. They also reported that certain polyphenols, for example, epicatechin-3-*O*-gallate, procyanidins B2 and B2-3-*O*-gallate, had higher superoxide radical trapping activity at pH 9 than at pH 7.5.

Kanner et al. (1994) found that red wine phenolics inhibit myoglobin, cytochrome C, and iron ascorbate catalyzed lipid peroxidation. They also compared the antioxidative capacity of wine phenolics and  $\alpha$ -tocopherol and concluded that wine phenolics were



twice more potent antioxidants than that of  $\alpha$ -tocopherol. It was shown that gallic acid, resveratrol, and tannic acid exerted same inhibition on lipid peroxidation which was greater than D-L- $\alpha$ -tocopherol. Cuendet et al. (2000) proved that *trans*-piceide, a glucoside derivative of resveratrol, could effectively scavenge free radicals.

An *in vivo* study regarding the antioxidant effect showed that grape seed proanthocyanidins exhibited a superior protective effect than vitamin E, vitamin C, vitamin E plus C, and  $\beta$ -carotene against lipid peroxidation and DNA fragmentation in mice (Bagchi et al. 1998). Resveratrol was also reported to inhibit platelet aggregation and LDL oxidation, protecting liver from lipid peroxidation in rats, apart from its anticarcinogenic activities (Teissedre et al. 1996). Another *in vitro* study showed that grape seed procyanidins might reduce the oxidation of polyunsaturated fatty acids in mouse liver microsomes (Bouhamidi et al. 1998). Tedesco et al. (2000) found antioxidant activity of nonalcoholic constituents of red wine.

#### ***Anti-cardiovascular diseases activity***

Coronary heart disease (CHD), a serious health condition affecting a significant population of the developed countries, is usually associated to high cholesterol levels in blood. In particular, high levels of low density lipoproteins (LDL) in plasma may play a role in the initiation of atherosclerotic plaque. Grape, wine, and grape seed extracts inhibit the oxidation of LDL and platelet aggregation and preventing CHD have been shown by several *in vitro* and clinical studies (Frankel et al. 1993; 1995; Gryglewsk et al. 1987; Mangiapane et al. 1992). Polyphenols (mainly catechins and procyanidins) of grape seeds play a major role in the observed inhibition of LDL oxidation due to their antioxidant activity and platelet aggregation inhibition (Manthey et al. 2002).

The consumption of purple grape juice offered a good protection against the oxidation of LDL cholesterol, as shown by an *in vivo* study with dogs, monkeys, and humans, from which it could be inferred that the flavonoids of purple grape juice and red wine may inhibit the initiation of atherosclerosis (Folts 2002). Another study shows that moderate red wine consumption for four weeks is associated with desirable changes in HDL-C (High Density Lipoprotein-Cholesterol) and fibrinogen compared with water with or without red grape extract (Hansen et al. 2005). A study done by Sato et al. (1999) showed that animals fed with GSP (Grape Seed Phenolics) had a lower myocardial

infarction rate due to scavenging of free radicals produced during ischemia and reperfusion. Sato et al. (2001) also showed that in rats, fed with 50-100 mg/kg of GSP, the number of apoptotic cells could be reduced along with the lessening in free radicals production.

An *in vitro* study of Kaur et al. (2007) on peripheral blood mononuclear cells showed that resveratrol derivatives are 20-10 folds more efficient in inhibiting the tissue factor induction which is responsible for blood coagulation and thrombosis. The same group previously reported the same effect of these derivatives on the vascular (Pendurthi et al. 1999) and endothelial cells (Pendurthi et al. 2002). Some studies were also carried out to determine whether the alcohol in wine is responsible for the reduced levels of LDL in blood of moderate wine drinkers. Frankel et al. (1993) diluted red wine till 10  $\mu\text{mol/L}$  total phenolics (1000 times dilution) in order to evaluate *in vitro* effect of wine phenolics on human LDL oxidation inhibition. Diluted wine and quercetin (10  $\mu\text{mol/L}$ ) evenly repressed the human LDL oxidation, proving that this inhibition was from constituents other than alcohols. Interestingly, the inhibition was considerably higher than that induced by  $\alpha$ -tocopherol. Another study indicated that several red grape polyphenols inhibit cardiac voltage-gated sodium channel and that this effect may contribute to the documented cardioprotective efficacy of red grape products (Wallace et al. 2006).

#### ***Anti-inflammation and anti-ulcer activity***

Apart from the other described activities, grape seed polyphenols (procyanidin) are also capable of inhibiting some enzymes which catalyze the release of many substances promoting inflammation, such as, histamine, serine protease, prostaglandin, and leukotrienes (Amella et al. 1985; Middleton and Drzewieki 1983; Pearce et al. 1984). Procyanidins was also proved to be able to inhibit the activation of hyaluronidase, a proteoglycan splitting enzyme that attacks various tissues during inflammation. This activity was associated to the antihistaminic effect and to their ability to strengthen cell membranes of basophils and mast cells, which contain the allergens, thus preventing the hypersensitivity to pollens and food allergens (Hansen 1995).

In rats, grape seed extracts containing high or low flavonol contents showed anti-ulcer activity (Saito et al. 1998). This protective effect was higher in high flavonol containing

extracts than in those with a low content. The authors also found that (+)-catechin, procyanidin B3 and dimeric and trimeric procyanidins did not show any defensive activity against ulcer while tetramers, pentamers, and hexamers of these were active. The authors postulated that the activity shown by longer oligomeric procyanidins might be due to their ability to bind to the proteins present on the stomach surface.

#### ***Anticancer and antimutagenic activities***

A variety of procyanidins from grapes and their seeds have been shown to prevent the growth of cancer cells. The anti-mutagenic activity of procyanidins from *V. vinifera* on *Saccharomyces cerevisiae* was examined by Liviero and Puglisi (1994). They found that procyanidins from grapes at 0.5 mg/ml induced a 65% decrease of the mitochondrial spontaneous mutability in *Saccharomyces* strain S288C. At the same concentration of procyanidins from grapes, 92 % reduction rate was observed in the mutation rate for the nuclear genetic determinant canavanine sensitivity. Another report (Tyagi et al. 2003) also shows that grape seed extract has a possible role in antiproliferation and apoptosis of human prostate carcinoma.

Nakagawa et al. (2001) reported the effect of resveratrol on the inhibition of the growth of breast cancer cells. Suppression in the growth of these cells by resveratrol was reported to occur through apoptosis. Another anti-carcinogenic study showed that resveratrol was capable to inhibit tumor initiation, promotion and progression and also explaining how it intervened in each stage (Jang et al. 1997). At first stage, resveratrol acted as an antioxidant and drug metabolizing enzymes of phase II were induced. At the second step, it mediated anti-inflammatory effects and the enzymes like cyclooxygenase and hydroperoxidase were inhibited. Finally human leukemia cell differentiation was induced by resveratrol (antiproliferation activity) along with the inhibition of the development of preneoplastic lesions in carcinogen treated mouse mammary glands.

Bomser et al. (2000) noted that ornithine decarboxylase (ODC), a rate-limiting enzyme in polyamine biosynthesis, could have an essential role in diverse biological processes including cell proliferation and differentiation. High levels of ODC are associated with increased risk for cancer. Grape seed polyphenols extract containing mainly oligomeric and polymeric proanthocyanidins was shown to inhibit epidermal ODC activity in mice. Previously, Bomser et al. (1999) had found that grape seed extract containing mainly

oligomeric and polymeric proanthocyanidins showed anti-tumor activity in mouse skin epidermis. A methylated derivative of resveratrol is also reported for the human colon cancer cell lines inhibition, about 100 folds more active than resveratrol (Schneider et al. 2003).

The p53 gene suppresses tumor formation and induces apoptosis. Modulation of p53 expression by red wine and its polyphenolic contents was studied on three human breast cancer cell lines and one colon cancer line by Soleas et al. (2001), which showed that these polyphenols, including quercetin, catechin, *trans*-resveratrol, and caffeic acid did not affect p53 gene expression in two of the breast cancer lines. Resveratrol, on the other hand, did decrease the expression of this gene in breast cancer cells of a wild type (MCF-7), while catechin and caffeic acid increased its expression but in a concentration independent manner. Caffeic acid and resveratrol reduced the expression of p53 in colon cancer cell lines, but this reduction again was not dose-responsive. Soleas et al. (2001) concluded that anticarcinogenic properties of wine should not be attributed to the modulation of p53 gene expression by these wine polyphenolic constituents. An *in vitro* study showed that methylated derivative of resveratrol induces apoptosis in human lymphoblastoid cell independently of their p53 status (Schneider et al. 2004).

Basly et al. (2000) studied the estrogenic/antiestrogenic effects of resveratrol isomers on the *in vitro* human breast cancer cells, finding that the *cis* form was less effective than *trans* in both cell lines. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) and FeCl<sub>3</sub> reduction assays showed that these isomers could be both antioxidant and prooxidant, depending on their concentrations.

#### ***Other biological activities***

The effect of flavonoids on intestinal microflora has also been investigated. Tebib et al. (1996) reported that monomeric proanthocyanidins of grape seeds did not affect the activities of fecal bacterial enzymes of rats. However, polymeric proanthocyanidins showed 'a beneficial colonic protective effect' by reducing colonic enzymatic activity of  $\beta$ -glucosidase,  $\beta$ -glucuronidase, mucinase, and nitroreductase due to a dilution effect. Polymeric tannin supplementation was shown to stimulate fermentative activities without increasing the activity of harmful enzymes on animal models. Chung et al. (1998) reported that tannic acid inhibited the growth of intestinal bacteria such as

*Clostridium perfringens*, *Enterobacter cloacae*, *Escherichia coli* and *Salmonella typhimurium* while showing no inhibitory effect on *Bifidobacterium infantis* or lactic acid bacteria *Lactobacillus acidophilus*.

Khanna et al. (2001) showed that grape seed proanthocyanidin extract containing 5 mg/g *trans*-resveratrol induced the expression of vascular endothelial growth factor (VEGF) in keratinocytes. Therefore, GSP extract containing resveratrol can be used to treat dermal wounds and other dermal disorders. Polyphenols in grape seed extract have been shown to reduce food intake in animal models. A study on humans showed that grape seed reduced energy intake without further effects on satiety, mood or tolerance (Vogels et al. 2004). Flechtner-Mors et al. (2004) also showed that moderate consumption of white wine is effective in weight loss in overweight and obese subjects.

### **Major diseases and defence strategies**

As a crop, grapes are susceptible to many diseases. Downy mildew, powdery mildew, grey mold, anthracnose, black rot, and crown gall are the major grape diseases around the world. All of these-with the exception of crown gall, are caused by fungi that attack the berries, reducing yield and quality. The degree of susceptibility varies depending on the variety, but damage can be harsh if no pest management is carried out.

The most threatening diseases to the grapevines are powdery and downy mildew caused by two different fungi, *Uncinula necator* and *Plasmopara viticola*, respectively. Downy mildew, caused by *P. viticola* (oomycete fungi), is the disease which affects both leaves and fruit (Madden et al. 2000). Powdery mildew is another widespread and destructive disease of grapevines worldwide. The damage caused by the pathogen, *U. necator*, has been noticed from the studies of the disease on cultivars of the European grape species (David et al. 2001).

*Botrytis cinerea*, the causal agent of grey mould or botrytis bunch rot in grapes, is responsible for considerable economic damage in vineyards worldwide. During the last 50 years, management of *B. cinerea* has relied heavily upon the use of synthetic chemicals (Rosslenbroich and Stuebler, 2000). Grapevines are also attacked by viruses such as the Nepovirus and GFLV (Grapevine Fan Leaf Virus). GFLV is responsible for an important and widespread degeneration of grapevine, causing a substantial loss in

grape yield (up to 80%), low fruit quality, and a progressive decline of infected vines that can eventually lead to plant mortality (Martelli 1993; Martelli and Savino 1990).

### ***Pest and disease control***

In nearly all parts of the world, pest and disease control in grapevines is generally achieved by widespread application of fungicides and pesticides, which results in high costs and a negative environmental impact. Scientists have been working hard to obtain grapevine varieties with an increased resistance against pathogens (Peixe et al. 2004). A great effort has been made to obtain improved grapevines through controlled cross-breeding between American and Asian cultivars and the resulting plants have been selected and their resistance evaluated (This et al. 2006). While this method presents unquestionable advantages, it is a slow process which also contributes in the reduction of genetic variability and loss of important quality of wine. Therefore, the use of biotechnological methods for this purpose is gaining much attention now.

### ***Resistance in grapes***

Plants like other organisms have developed different mechanisms to respond to different stresses such as unfavorable environmental conditions, nutrition deficiency and pathogen attack (Downie et al. 2004; Matsumoto et al. 2004). Plant defense system (resistance) against pathogens comprises a number of mechanisms, the most significant of which is the biosynthesis of secondary metabolites. A certain group of compounds have been found to be involved or associated with plant defense mechanisms. These compounds, known as phytoalexins, are low molecular weight, antimicrobial secondary metabolites (Harborne 1999; Kuc 1995; Purkayashita 1995).

In grapevine, the most frequently observed and best characterized defense reactions to fungal infection are the accumulation of phytoalexins as well as Pathogen Related (PR) proteins (Derckel et al. 1999). Figure 4 shows some of the phytoalexins isolated from grapevine. Phytoalexins from the Vitaceae in general, have been the subject of numerous studies as these compounds are thought to have implications in both phytopathology and human health. Upon infection, grapevines rely upon preformed and inducible resistance mechanisms for defense (Gabler et al. 2003; Keller et al. 2003). The cuticle layer and cell wall physically resist the penetration of hyphae while tannins and

phenolics inhibit fungal enzymes involved in pathogenesis (Goetz et al. 1999; Sarig et al. 1998). Early inducible responses include the deposition of new cell wall material, the release of reactive oxygen species (ROS) and hypersensitive cell death (HR) at the infection site (Hammerschmidt and Smith-Becker 1999). It has been demonstrated that various biotic and abiotic agents can activate defense mechanism in grape berries and leaves enhancing the resistance against diseases (Table 1).

### ***Phytoalexins from grapes***

Stilbenes are generally biologically active compounds that also have antifungal activities against various pathogens such as *Cladosporium cucumerinum*, *Pyricularia oryzae* (Langcake and Pyrcce 1976), *Plasmopara viticola* (Dercks and Creasy 1989; Langcake, 1981), and *Sphaeropsis sapinea* (Celimene et al. 2001). Resveratrol is the major phytoalexin produced in grapevines along with its derivatives such as piceide, pterostilbene and  $\epsilon$ -,  $\alpha$ -,  $\beta$ -, and  $\delta$ -viniferin (Jeandet et al. 2002; Pezet et al. 2004).

Resveratrol was found to accumulate in the abaxial surface of leaves and in skin of berries in the range of 40-400  $\mu\text{g/g}$  fresh weight (Adrian et al. 2000; Jeandet et al. 1991). A study on grape plantlets proved the existence of a positive correlation between resveratrol synthesis in leaves (induced by UV) and field resistance (Sbaghi et al. 1995). Regarding fungal infection resistance, an *in vitro* study showed that at the concentration of 90  $\mu\text{g/mL}$ , resveratrol was able to inhibit conidial germination by 50% while reduction in mycelial growth was observed in the range of 60 to 140  $\mu\text{g/mL}$  (Adrian et al. 1997).

Pterostilbene is the dimethylated derivative of resveratrol. A study showed that it is 5-fold more toxic than resveratrol, completely inhibiting conidial germination at concentrations ranging from 52 to 60  $\mu\text{g/mL}$  ( $\text{ED}_{50}$ ) and 18 to 20  $\mu\text{g/ml}$  (Adrian et al. 1997; Langcake 1981). The methylation of the hydroxyphenyl groups in phenolics could potentially resulted in an enhanced biocidal activity as these two different stilbenes indicated (Pezet and Pont 1990). The oxidative dimers of resveratrol have formed a new class of compounds known as viniferins. A study by Pezet et al. (2004) showed that  $\delta$ -viniferin exhibited toxicity similar to that of pterostilbene against *P. viticola*.

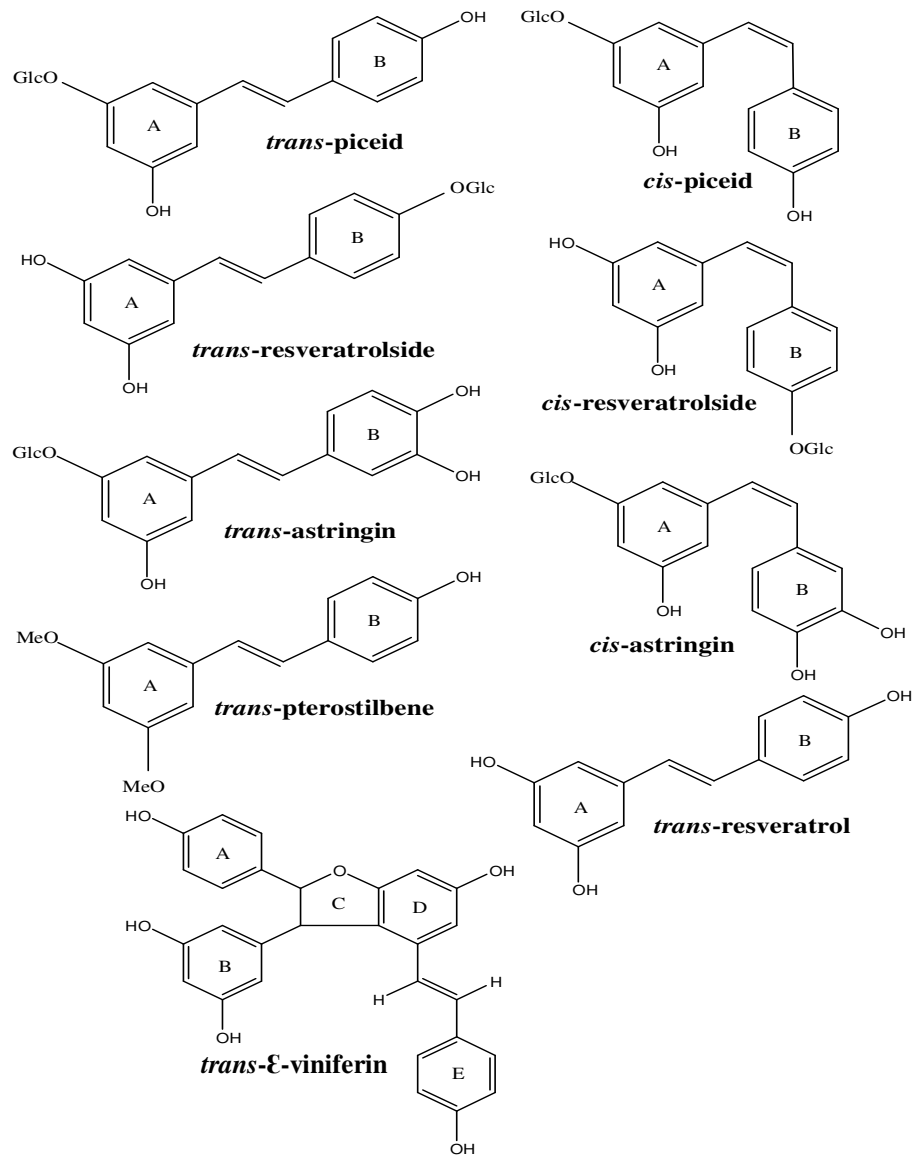


Figure 4. Chemical structures of stilbene phytoalexins. Glc: glucosyl (C<sub>6</sub>H<sub>11</sub>O<sub>5</sub>).



**Table 1.** Examples of inducing agents with demonstrated cellular and biological activity on grape tissues (Partially adapted from Elmer and Reglinski 2006).

Inducing Agent	Reported Activity	Reference
Methyl jasmonate	Accretion of phytoalexins and defense-related proteins along with the induction of the oxidative burst	Repka et al. 2004; Belhadj et al. 2006; Vezzulli et al. 2007
Aluminium chloride	Accumulation of phytoalexins in grapevine leaves	Adrian et al. 1996 ; Borie et al. 2004
Laminarin ( $\beta$ -1,3-glucan from <i>Laminaria digitata</i> )	Induction of defense-related genes and oxidative burst. Enhancement in resistance against <i>B. cinerea</i> was also observed	Aziz et al. 2003
Benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester	High levels of resveratrol and anthocyanins with the induced resistance against <i>B. cinerea</i> with the accumulation of chitinase class III transcript	Owen et al. 2002; Iriti et al. 2004
Oligogalacturonide	H <sub>2</sub> O <sub>2</sub> production, induction of defense-related genes in cell suspensions, and elevation of resistance to <i>B. cinerea</i> in detached leaves	Poinssot et al. 2003; Aziz et al. 2004
Chitosan	Phenylalanine ammonia-lyase activity induction and elevation of resistance to <i>B. cinerea</i>	Romanazzi et al. 2002; Patricia et al. 2006
5-Chlorosalicylic acid	Enhanced production of phenolics, and field efficacy against <i>B. cinerea</i>	Duxbury et al. 2004; Reglinski et al. 2005
Chitogel	Reduced <i>B. cinerea</i> infection on plantlet leaves	Ait-Barka et al. 2004

The above reports constitute clear evidence of the substantial antifungal activity of stilbenes, which in turn suggests their participation in the resistance against fungal grapevine diseases, thus supporting their proposal as indicators of resistance. Pool et al. (1981) studied the relationship between phytoalexins production potential and resistance to diseases in grapevine, concluding that there was a positive correlation in both the speed and the intensity of resveratrol synthesis and the resistance of grapevine varieties to fungal diseases caused by *B. cinerea* or *P. viticola*. Unfortunately, the production of resveratrol in field-growing grapevines has proved to be very sensitive to a wide range of environmental factors, thus limiting its use as a marker for disease resistance (Barlass et al. 1987).

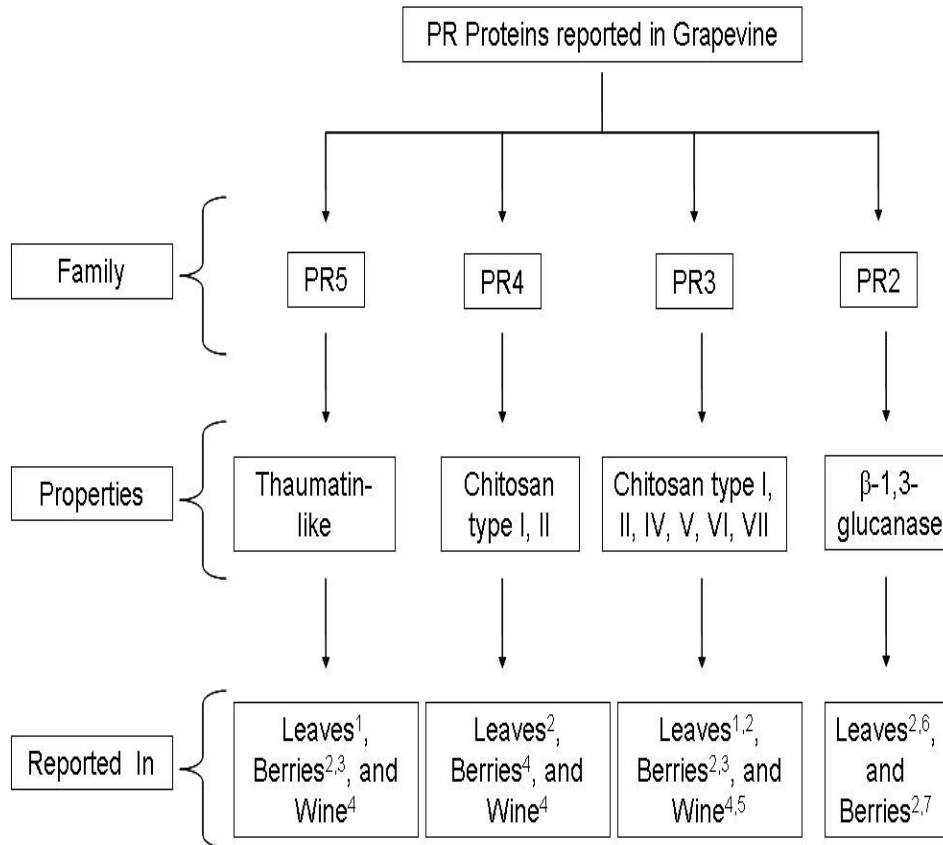
### ***Pathogenesis-related proteins from grapes***

Pathogenesis-related (PR) proteins are typically acidic, of low molecular mass and highly resistant to proteolytic degradation. They accumulate in berries and leaves of grapevines (Figure 5) in response to pathogen attack and are thought to contribute in resistance (Giannakis et al. 1998). The type of tissue, developmental stage, and type of infecting pathogen were observed to vary the pattern of PR-protein expression (Busam et al. 1997; Derckel et al. 1999; Robert et al. 2002). In grape berries, PR-protein levels are also produced constitutively as a normal part of the ripening process (Robinson et al. 1997; Tattersall et al. 1997) and this has been a prophylactic measure against environmental stress and pathogen attack (Davies and Robinson 2000). Chitinase and thaumatin-like proteins represent the predominant PR-proteins in grape berries. These two protein families account for half of the soluble protein in ripe grapes (Waters et al. 1998). Bezier et al. (2002) showed that grapevine leaves and berries expressed polygalacturonase inhibitor proteins (PGIPs) in response to infection by *B. cinerea*. PGIPs are thought to contribute to disease resistance by inhibiting the degradation of the plant cell wall by fungal polygalacturonases.

PR proteins can also be induced in leaves and berries as part of a defensive response to the classical PR protein gene-inducers (wounding, chemical elicitors, pathogen attack, or abiotic stress) by the expression of specific PR genes (Jacobs et al. 1999; Robinson and Davis 2000). These processes modulate the levels and proportions of the PR proteins in grapes, apparently depending on the factors like cultivar, region, climate, and agricultural practices (Monteiro et al. 2003).

### **Biotechnology of grapevine**

Grapevine biotechnology is one of the most promising developments but on the other hand increasingly faced with many contradictions. As with plant biotechnology in general, several technical factors dictate the speed of the current progress in this field. These factors include the easiness with which the relevant plant species can be operated in tissue culture, be transformed with foreign DNA and be regenerated into new plantlets.



**Figure 5.** Pathogen Related (PR) proteins in Grapevine. 1- Busam et al. 1997; 2- Ferreira et al. 2001; 3- Hayasaki et al. 2001; 4- Viver and Pretorius 2002; 5- Colova-Tsolova 2000; 6- Renault et al. 1996; 7- Kraeva et al. 1998.

*Vitis vinifera*, being a woody perennial, initially proved to be reluctant to these manipulations. Very few of the early attempts, to transform grapevine, were successful. The establishment of the genetic transformation protocols is mainly limited by the development of *in vitro* regeneration system for different grapevine cultivars. Now, several groups have produced transgenic grapevines that are presently at varying stages of evaluation. Still there is no universal protocol to transform or genetically engineer all grapevine cultivars. Significant progress has been made, however, for the establishment of this technology over the world and the success rate should increase with the coming years (Kikkert et al. 2001).

**Table 2.** Targets for the genetic improvement of grapevine cultivars.

Desirable properties	Examples of current potential target genes	Focus area	References
Pathogen tolerance	Virus coat proteins, Glucanase- and chitinase- encoding genes, Anti-microbial peptides	Pathogen-derived resistance strategies, Defence and defence signaling against fungal and bacterial pathogens	Yamamoto et al. 2000; Vidal et al. 2006; Mauro et al. 1995
Resistance to abiotic stress	Tonoplast integral proteins, Carotenoid biosynthetic genes, antifreez genes	Carotenoid biosynthesis and control, polyamines and their role in stress, Aquaporins	Tesvetkov et al. 2000
Improvement in quality factors	UDP-glucose:flavanoid 3-O-glucosyltransferase, Silencing of polyphenol oxidase, sucrose transporters, Baranase gene	Phloem loading and unloading, isolation of seed-specific promoters, anthocyanin biosynthesis and control, Oxidation reactions	Fillion et al. 1999; Kobayashi et al. 2001; Kobayashi et al. 2002; Boss et al. 1996

Genetic engineering is commended as an amazing achievement in the grapevine industries. This technology and its supporting disciplines lead to the establishment of stress tolerant and disease resistant varieties of *Vitis vinifera*, with increased productivity, efficiency, sustainability and environmental friendliness, especially regarding improved pest and disease control, water use efficiency and grape quality. The implementation and successful commercialization of genetically improved grapevine varieties will only materialize if an array of hurdles posed by scientific, legal and regulatory issues, intellectual property and patenting, (Kikkert et al. 2001), political, economic, and not least, the negative public perception of genetically modified products (Pretorius, 2000) can be solved. Grapevine biotechnology is currently practiced in all major viticultural research centers worldwide and is focused on several issues (Table 2).

### Conclusion and perspectives

Economically, grapevine is one of the most important crop in the world and has a deeply rooted significance in human culture. The functional ingredients of grapevine include several metabolites which have been reported to exhibit different activities including antioxidant, anti-cancer, and prevention of cardiovascular disease and the treatment of several skin disorders. Resistance of plants to infection by phytopathogenic microorganisms is the result of multiple defense reactions comprising both constitutive

and inducible barriers. In grapevine, the best characterized defense mechanisms are the accumulation of phytoalexins and the synthesis of PR-proteins. Particular attention has been given to stilbene phytoalexins (e.g. resveratrol) produced by Vitaceae. Despite the enormous amount of research carried out on grapevine phytochemistry, there is still a lot to be done in this field. The current advances achieved in analytical platforms and methodologies can and in fact will, really facilitate the isolation of new phytochemicals from grapevine with higher medicinal potential or novel bioactivities. The identification and characterization of different disease-resistant grapevine cultivars, at the gene, protein or metabolite level, is also a focal point of the grapevine research today. By the integration of different 'omics' technologies, it will be possible to better understand the total network of plant responses to various external factors including pathogen attack. In this way, a systems biology approach will be developed to explore and exploit the possible synergism between genes, proteins and metabolites.