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Effects of SAR Inducers on Quality and Safety of the Grape Products

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PREFACE

0.1 Summary

Fungicide application is now the most efficacious method for controlling plant diseases caused by fungi and oomycetes. As legislation is limiting and reducing their use, studies for the identification of additional and environmentally friendly approaches in the control of their associated diseases are strongly stimulating. Among these, systemic acquired resistance (SAR) offers the prospect of long-lasting, broad-spectrum disease control through activation of the resistance defence machinery of the plant itself. Plant activators are products employed in crop protection able to elicit SAR. Therefore, they may trigger the plant own defence response against pathogen attacks, mainly stimulating mechanisms such as the biosynthesis of phytoalexins, plant secondary metabolites with a broad spectrum biological activity.

In this study, two plant defence inducers, benzothiadiazole (BTH) and chitosan (CHT), deserving particular attention because of their efficacy and low toxicity, have been tested. CHT is a natural and low-cost polymer obtained from the waste products of the crustacean carapace, by chitin deacetylation. Its effectiveness is higher when molecular weight is between 10 and 100 kD and the deacetylation degree range is from 80 to 90 percent. BTH [benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester] is a synthetic compound and a functional analogue of salicylic acid, a plant hormone-like metabolite deeply involved in resistance against pathogens. The phytoiatric campaigns were planned, in 2009 and 2010, on a red grapevine (*Vitis vinifera* L.) variety cultivated in an experimental vineyard: Groppello, an autochthonous cultivar of Lombardia, located at Raffa di Puegnago (Brescia, Italy). Open field treatments carried out on Groppello grapevines were: *i*) 0.03% (w/v) CHT (76 kDa molecular weight and 85% deacetylation degree), *ii*) 0.03% CHT in combination with 150 g hL^{-1} copper hydroxide (CHT/Cu) and *iii*) 0.3 mM BTH. Untreated vines were used as negative control, while plants treated with conventional fungicides (penconazole and methyldinocap) were the positive control. In field surveys, the trial was set up as a complete randomized block design in 4 replications, with 10 vines (a parcel) per treatment in each block. Plants were sprayed approximately every 10 days, according to the meteorological conditions, from the beginning of grape susceptibility to fungal diseases until the complete *véraison*. Sampling was scheduled at two phenological phases: pre-*véraison* and 100% *véraison*; bunches were randomly collected from plants during the morning and stored at -20 °C until analyses. Berry tissues were separated into skin, flesh and seeds, powdered and

extracted. Then, extracts were tested to evaluate their phytosterol levels by gas chromatography (GC). Groppello experimental wines were produced, by standard microvinification techniques, in the Centro Vitivinicolo Provinciale of Brescia and stored at 4 °C in the dark until analyses. Microvinificates were produced from grapes treated with both elicitors and conventional fungicides (control), and the following analyses were carried out: phytosterol detection (by GC); content of volatile compounds (by GC/MS); levels of mycotoxins (manly ochratoxin A, OTA, by ultra performance liquid chromatography (UPLC)-MS/MS).

To the best of our knowledge, these results represent the first records on the effects of agrochemicals on the phytosterol content of grape berry and seed tissues, at different phenological stages, and experimental wines. In general, *β*-sitosterol is the main component in the berry tissues, with the highest levels in the flesh, followed by skin and seeds respectively. Moreover the content of *β*-sitosterol and campesterol decreased during ripening. Stigmasterol and campesterol were present in very low quantities in both growth stages. With regard to the effects of inducers on *β*-sitosterol content, the most effective treatments in seeds were CHT and BTH in pre-*véraison* and in *véraison*, respectively, in 2009, only CHT in the two phenological stages in 2010. In fleshes and skins, the most effective treatment on *β*-sitosterol was CHT/Cu at *véraison*, in 2009, and at pre-*véraison*, in the 2010. In fleshes, at *véraison* 2010, the highest levels of *β*-sitosterol were recorded in BTH treated sample.

In general, the data on experimental wines indicated that the treatments with SAR inducers increased the plant sterols. In fact, *β*-sitosterol, stigmasterol and campesterol were more abundant in microvinificates obtained from grapes treated with CHT, CHT/Cu and BTH compared to those from conventional fungicide-treated grapes.

Aroma is an important factor in quality control and quality assurance of foods, but, in wine, this factor is possibly the most important. In 2009 the highest concentrations of acetals and alcohols were detected in CHT/Cu and CHT microvinificates, respectively. BTH treatment raised the levels of all esters in wines: acetates, butanoates, propionates and aliphatic esters. Elicitors had no effects on aldehydes and ketones, whereas CHT/Cu was the most effective treatment in increasing the terpene linalool. The analyses on experimental wines from vintage 2010 are still in progress.

In all wines, the level of OTA was below the allowable threshold of 2 ng/mL.

The good agreement between the data obtained in 2009 and 2010 for Groppello cultivar suggests that, in general, plant activators may improve some qualitative/healthy treats ascribed to red wine, though their efficacy in

controlling grapevine fungal diseases should be better ascertained. In conclusion, the possibility of enhancing the pharmaconutritional potential of grapes/wine with phytosanitary treatments would deserve particular attention, even if it should be further investigated.

STATE OF THE ART

1.1 Induced resistance

1.1.1 Fungal diseases and induced resistance

More than 10,000 species of oomycetes and fungi can cause diseases in plants (here referred to as 'fungal diseases'), with the resultant severe reduction in the quantity and quality of plant products (Agrios, 2005). To control these diseases and to prevent crop losses, regulatory, cultural, biological, physical and chemical control methods are used. Although fungicide application is, in general, the most effective and widely used method, legislation is now limiting and reducing this method. "Non-chemical methods to be preferred" and "reduction of pesticide use to necessary levels" are two of the eight general principles for Integrated Pest Management that have recently been detailed by the European Commission. Therefore, there is a clear and urgent need for additional approaches to control fungal diseases. Among these, induced resistance (IR) is particularly promising, a phenomenon in which, once appropriately stimulated, a plant has enhanced resistance upon inoculation challenge with the relevant pathogen. IR can be both localised and systemic, and it can be induced by limited pathogen infection, avirulent pathogens, beneficial non-pathogenic bacteria and fungi, and certain chemicals (Walters and Fountaine, 2009). IR was first documented in begonia plants by Beauverie (1901), which obtained protection against *Botrytis cinerea* through soil application of an attenuated strain of the same fungus. In the early 1900's, several studies were carried out on IR to fungi in plants, some of which were also in Italy, and these were well documented in the review of Arnaudi (1930). However, only since the mid-1980's there has been increasing interest in IR as a new and environmentally safe means of control of fungal diseases, which was mainly due to the studies of Joseph Kuć and colleagues (Walters et al., 2007).

1.1.2 Induced resistance and the signalling pathways involved

At least three types of systemic IR are known, which have been shown to be effective against both biotrophic and necrotrophic oomycetes and fungi: systemic acquired resistance (SAR), induced systemic resistance (ISR) and β-aminobutyric-acid-induced resistance (BABA-IR) (Walters et al., 2007; Pieterse et al., 2009). The signalling pathways controlling these types of IR and the defence responses associated with them are well characterised in the model plant *Arabidopsis thaliana* (Van der Ent et al., 2008; Pieterse et al., 2009), as schematised in figure 1.

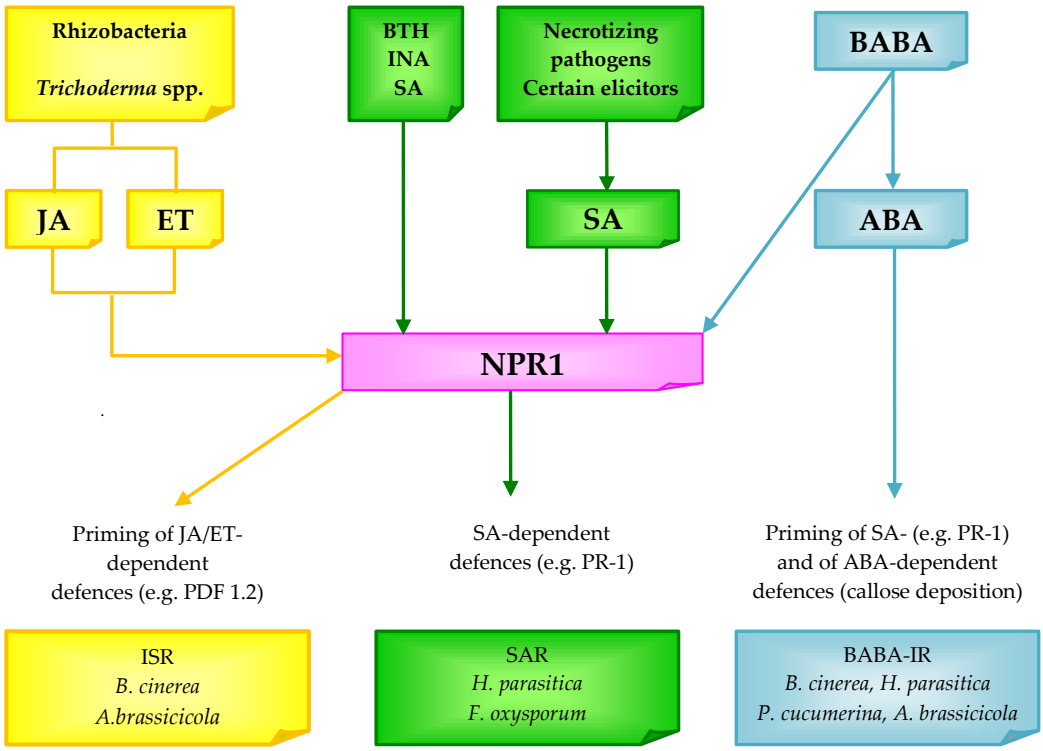

Figure 1. Model of the signal transduction network in *Arabidopsis thaliana* that controls induced systemic resistance (ISR), systemic acquired resistance (SAR), and β-aminobutyric-acid-induced resistance (BABA-IR). ABA, abscisic acid; BTH, benzothiadiazole; BABA, β-aminobutyric acid; ET, ethylene; INA, 2,6-dichloroisonicotinic acid; JA, jasmonic acid; NPR1, non-expressor of PR-genes 1; PDF1.2, plant defensin; SA, salicylic acid. Yellow lines, ISR pathway; green lines, SAR pathway; blue lines, BABA-IR pathway (adapted by Buonaurio et al., 2009).

SAR can be induced by necrotising pathogens and by treatment with various agents, such benzothiadiazole (BTH), a photostable functional analogue of salicylic acid (SA) that is associated with the accumulation of SA and pathogenesis-related (PR) proteins, and is dependent on the regulatory protein NPR1 (nonexpressor of PR genes 1) (Durrant and Dong, 2004). ISR, which can be induced by beneficial rhizobacteria (Van Loon et al., 1998) and *Trichoderma* spp. colonising the roots (Van der Ent et al., 2009), does not require SA, can occur without the production of PR proteins, and is dependent on ethylene and jasmonic acid signalling as well as NPR1 (Pieterse et al., 1998). BABA-IR involves both SA-dependent and abscisic-acid-dependent defence mechanisms; the importance of these defences can vary according to the nature

of the challenging pathogen. Indeed, BABA-IR against *B. cinerea* resembles SAR, in that it requires endogenous accumulation of SA and functional NPR1 (Zimmerli et al., 2000, 2001), while the abscisic-acid-dependent pathway, which is associated with callose apposition, is necessary for the development of resistance against *Hyaloperonospora parasitica* and *Plectosphaerella cucumerina* (Zimmerli et al., 2000; Ton and Mauch-Mani, 2004).
Although there are several exceptions, it can generally be stated that fungi with a biotrophic lifestyle are more sensitive to SA-mediated induced defences (as SAR), whereas necrotrophic fungi are controlled more through jasmonic acid/ ethylene-mediated defences (as ISR) (Thomma et al., 2001; Glazebrook, 2005). Furthermore, both biotrophic and necrotrophic fungi and oomycetes are contrasted in BABA-protected plants (Walters and Fountaine, 2009). Of note, there is also cross-talk between the above-mentioned phytohormone signalling pathways, which is believed to provide the plant with powerful regulatory potential (Spoel and Dong, 2008; Pieterse et al., 2009). As illustrated in figure 1, the interactions between these pathways can be either (mutually) antagonistic or synergistic, thus providing either negative or positive functional outcomes. It is believed that this cross-talk helps the plant to minimise energy costs and to create a flexible signalling network that allows it to finely tune its defence response towards an invader (Van der Ent et al., 2008; Pieterse et al., 2009). IR appears to be associated with direct activation of these defences and/or the ability to 'recall' a previous infection, root colonisation or chemical treatment. This latter phenomenon is known as priming, and it results in plants that respond more rapidly and effectively when exposed to the subsequent pathogen attack (Conrath et al., 2006; Goellner and Conrath, 2008).

1.1.3 Mechanisms of induced resistance to fungal pathogens

Disease is a rare outcome in the spectrum of plant-microbe interactions and plants have (co)evolved a complex set of defence mechanisms to hinder pathogen challenging and, in most cases, prevent infection. The battery of defence reactions includes physical and chemical barriers, both preformed (or constitutive or passive) and inducible (or active), depending on whether they are pre-existing features of the plant or are switched on after challenge (table 1). When a pathogen is able to overcome these defences, disease ceases to be the exception (Keen, 1999; Iriti and Faoro, 2003a).Three main explanations support this rule: *i*) plant is not a substrate for microbial growth and does not support the lifestyle of the invading pathogen; *ii*) constitutive barriers prevent colonization of plant by pathogen; *iii*) plant recognizes pathogen by its innate

immune system, then activates inducible defences (Nürnberger and Lipka, 2005).

Table 1. Plant defence mechanisms

	Structural	**Chemical**
Constitutive (passive, preformed)	Anatomical barriers (trichomes, cuticle, cell wall)	Preformed inhibitors (phytoanticipins: glucosides, saponins, alkaloids) and antifungal proteins (lectins)
Inducible (active)	Cell wall strengthening (callose, lignin and suberin appositions; oxidative extensin cross-linking	Oxidative burst, hypersensitive response, phytoalexins, pathogenesis-related proteins

Plant innate immunity. The host ability to respond to an infection is determined by genetic traits of both the plant itself and the pathogen. Some resistance mechanisms are specific for plant cultivars and certain pathogen strains. In these cases, plant resistance (R) genes, encoding for receptors, recognize pathogen-derived molecules (specific elicitors) resulting from the expression of avirulence (*avr*) genes (table 2). This gene-for-gene relation, also known as host resistance, triggers inducible barriers, i.e. a cascade of events leading to SAR (Ellis et al., 2007). In addition, another type of resistance is activated through recognition, by plant receptors, of general (race-nonspecific) elicitors, microbe-associated molecular patterns (MAMPs) including mainly lipopolysaccharides, peptidoglycans, flagellin, fungal cell wall fragments, lipid derivatives (sterols and fatty acids), proteins, double stranded RNA and methylated DNA (table 2).

Table 2. Plant innate immunity

Type of resistance	**Elicitors**
Host (specific) resistance	Specific elicitors, encoded by the *avr* genes of certain pathogen strains (gene-for-gene)
Non-host (basal) resistance	General exogenous (race-nonspecific MAMPs[1]) and endogenous (plant-derived oligogalacturonides) elicitors

[1]MAMPs: microbe-associated molecular patterns

This non-host or basal resistance can also be induced by endogenous, plant-derived, general elicitors, such as oligogalacturonides, released from the plant cell wall by fungal hydrolytic enzymes (table 2) (de Wit, 2007; Boller and He, 2009; Galletti et al., 2009). Among MAMPs, chitosan (CHT), is a deacetylated chitin derivative which is worthy of special attention because of its use in chemical-induced resistance and efficacy against plant diseases (Iriti and Faoro, 2009a). Like a general elicitor, CHT is able to prime an aspecific, long-lasting and systemic acquired immunity (SAR) by binding to a specific receptor in the plant cell (Chirkov, 2002; Chen and Xu, 2005; Bautista-Baños et al., 2006; Iriti and Faoro, 2009a).In any case, the spectrum of defence reactions elicited by both types of resistance, that collectively represent the plant innate immune system, is rather similar (Iriti and Faoro, 2007). Immunity may be expressed locally, in the infected cells, or in uninfected distal tissues, probably because of one or more endogenous systemically translocated (or volatile) signals that activate defence mechanisms in plant organs distal from the initial site of infection (Kumar and Klessig, 2008).

Recognition of a pathogen by plant cell entails physical interaction of a stimulus (elicitor) with a receptor. According to the receptor/ligand model, the constitutively expressed R genes encode proteins that possess domains characteristic of typical receptors responsible for the innate immunity in mammals and *Drosophila*. These proteins, also known as pattern-recognition receptors (PRR), can be grouped into different classes according to certain common structural motifs. Many R proteins contain a leucine-rich repeat (LRR) domain involved in recognition specificity (Zipfel, 2008; Padmanabhan et al., 2009).

Plant defence responses. As previously introduced, receptor function entails signal perception, and recognition at the site of infection activates the effectors of IR. Many biochemical, cytological and molecular changes are associated with IR in plants that are systemically protected against pathogens, which are probably directly and/or indirectly responsible for the resistance phenomenon. The defence machinery in plants that show IR to fungal pathogens should be very similar to mechanisms used by plants that show gene-for-gene or non-host resistance based on the development of structural and/or chemical barriers after the chemical induction or challenge inoculation (Iriti and Faoro, 2007).

One of the earlier cell responses upon infection includes a rapid burst of reactive oxygen species (ROS), in some cases leading to a hypersensitive response (HR) and the programmed death of cells at the site of attempted pathogen penetration (table 1) (Alvarez et al., 1997). The following activation of allosteric enzymes initiates callose apposition via a calcium-dependent callose synthase, oxidative extensin (hydroxyproline-rich glycoproteins) cross-linking and, finally, lignin deposition, processes responsible for the cell wall strengthening (table 1). A second line of barriers results from transcriptionally activated defence genes, encoding for enzymes that catalyse the synthesis of phytoalexins, defence metabolites arising from secondary metabolic pathways, and pathogenesis-related proteins (PRs) (table 1) (Keen, 1999).

Benzothiadiazole. In a screen of various benzothiadiazole derivatives, benzo-1,2,3-thiadiazole-7-carbothioic acid S-methyl ester (BTH, acibenzolar-*S*-methyl) emerges as a strong inducer of SAR in numerous plant-pathogen combinations, with much lower phytotoxicity than either SA or INA (Friedrich et al., 1996; Schurter et al., 1987).

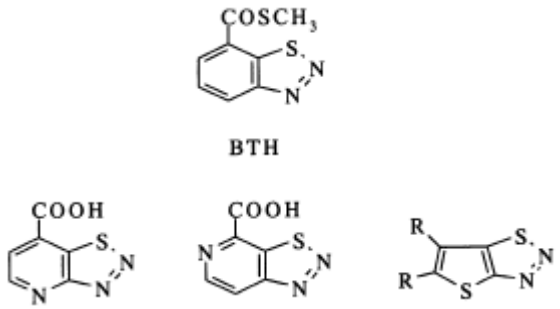

Figure 2. Structure of benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester and analogues.

Like SA, BTH inactivats catalase, ascorbate peroxidase, and a mitochondrial NADH : ubiquinone oxidoreductase (van der Merwe and Dubery, 2006; Wendehenne et al., 1998). Treatment of barley (*Hordeum vulgare* L.) with BTH does not immediately induce ROS production, but conditions the plants for a faster and stronger response upon infection with the powdery mildew fungus *Blumeria graminis* (Faoro et al., 2008). This potentiated, or "primed" (Conrath et al., 2006) response includes a more intense HR-associated oxidative burst and more extensive formation of cell wall appositions (papillae), coupled with greater accumulation of phenolic compounds at sites of attempted fungal

penetration (Faoro et al., 2008). The activity of BTH varies between different pathosystems though, as BTH-induced resistance of bean (*Phaeseolus vulgaris*) to the rust fungus *Uromyces appendiculatus* involves an oxidative burst but no HR-related cell death (Iriti and Faoro, 2003). With regards to SA signaling, BTH acts downstream of SA accumulation (Friedrich et al., 1996) and may contribute to the establishment of SAR through an interaction with SABP2, a methyl salicylate esterase that is critical for the perception of defence-inducing signals in systemic tissues (Du and Klessig, 1997; Forouhar et al., 2005; Park et al., 2007).

Chitosan. Chitosan (CHT), deacetylated chitin, is a natural biodegradable compound derived from crustaceous shells such as crabs and shrimps (Sandford, 1989). Chitosan is a low acetyl form of chitin mainly composed of glucosamine, 2-amino-2-deoxy-β-D-glucose (Freepons, 1991). The positive charge of chitosan confers to this polymer numerous and unique physiological and biological properties with great potential in a wide range of industries such as cosmetology (lotions, facial and body creams) (Lang and Clausen, 1989), food (coating, preservative, antioxidant, antimicrobial) (Benjakul et al., 2000), biothecnology (chelator, emulsifier, flocculent) (Sandford, 1989) pharmacology and medicine (fibers, fabrics, drugs, artificial organs) (Liu et al., 2001) and agriculture (soil modifier, films, fungicide, elicitor) (Ren et al., 2001). The elicitor activity of chitosan was first demonstrated in a screen of fungal cell wall components that were assayed for their ability to induce phytoalexin accumulation in pea pods and induce resistance to the fungal pathogen *Fusarium solani* (Hadwiger, 1979). Chitosan induced resistance is associated with an increased content of polyphenolic phytoalexins in treated plant tissues, because of the stimulation of phenylpropanoid pathway, the biosynthetic route leading to polyphenol synthesis. Elicitation of this metabolic pathway by CHT has been reported in grape and other plants and correlated with the increase in both activity and transcript levels of phenylalanine ammonia lyase and chalcone synthase, key enzymes of the phenylpropanoid route (Nandeeshkumar P, et al., 2008; Chen H, et al., 2009).These effects are also related to improved quality and increased antioxidant power of foodstuffs derived from CHT-treated crops (Iriti M et al., 2011, Cho MH et al., 2008). Chitin and chitosan are known to associate with plasma membranes (Baureithel et al., 1994), and a chitin-binding protein (CE-BiP) was identified in rice (Kaku et al., 2006). A number of studies have shown the induction of local and systemic acquired resistance against viral infections in chitosan-treated plants, with efficacy depending more on plant species than on type of virus.

1.1.4 Induced resistance to control fungal plant diseases and its practical application

Induction of plant defences is an innovative and fascinating approach for the control of fungal diseases, an aspect that has stimulated many studies to find and develop novel resistance inducers and to determine their efficacies under controlled and/or open-field conditions. The protective effects of SAR have been evaluated in many plant diseases, such as those caused by *Oomycota*, *Zygomycota*, *Ascomycota* and *Basidiomycota*, in both monocotyledonous and dicotyledonous plants. Some examples of SAR efficacy in the control of fungal plant diseases are given in table 3, with the most used inducers being BTH, chitosan and methyl jasmonate.

Table 3. Examples of induced resistance for the control of fungal plant diseases in the field

Disease	Causal agent	Crop	Inducer	Reference
Gray mold	*Botrytis cinerea*	Grapes	Chitosan	Romanazzi et al.,2002
Downy mildew	*Plasmopara viticola*	Grapes	Chitosan	Dagostin et al., 2006
Late blight	*Phytophthora infestans*	Tomato	BTH, Phosetyl-Al, Kendal® and Pom-PK®	Veronesi et al., 2009
Fusarium wilt	*Fusarium oxysporum* f. sp. *melonis*	Melon	Methyl jasmonate	Buzi et al., 2004b
Gummy stem blight	*Didymella bryoniae*	Melon	BTH, methyl jasmonate	Buzi et al., 2004a
White mold	*Sclerotinia sclerotiorum*	Melon	BTH, methyl jasmonate	Buzi et al., 2004a
Rust	*Puccinia recondita*	Wheat	BTH	Vallad and Goodman, 2004
	Uromyces appendiculatus	Bean	BTH	Iriti and Faoro, 2003b
Powdery mildew	*Blumeria graminis* f. sp. *tritici*	Wheat	BTH	Stadnik and Buchenauer, 1999
	Blumeria graminis f. sp. *hordei*	Barley	BTH, Chitosan	Faoro et al., 2008
	Erysiphe necator	Grape	Chitosan	Iriti et al., 2008 Vitalini et al., 2009
	Podosphaera xanthii	Melon	Chitosan	Iriti et al., 2008
Septoria leaf blotch	*Septoria tritici*	Wheat	BTH	Vallad and Goodman, 2004

BTH is effective against downy mildews, *Phytophthora* late blight, *Fusarium* wilt, rusts, powdery mildews, gray mold, septoriosis, and others (Iriti and Faoro, 2003b; Buzi et al., 2004a, 2004b; Vallad and Goodman, 2004; Faoro et al., 2008; Veronesi et al., 2009). Chitosan applications control the main grapevine

diseases, such as gray mold, downy mildew and powdery mildew (Romanazzi et al., 2002; Dagostin et al., 2006; Iriti et al., 2008; Vitalini et al., 2009).
The efficacy of SAR has also been evaluated in the control of postharvest decay of fruit (e.g. stone fruit, strawberry, sweet cherry), in which fungi are mainly involved. For many fruits, the use of synthetic fungicides is not allowed after the harvest, while for others, such as citrus fruits, there are few active ingredients allowed, as over a short time the fungus, e.g. *Penicillium digitatum*, can develop resistant strains (Kinay et al., 2007). For this reason, it is very difficult to protect these fruits, and particularly for organic growers, where pre-harvest treatments against postharvest decay using organic fungicides are forbidden. In this context, many studies have been carried out with the aim of controlling postharvest diseases, such as blue mold, brown rot, gray mold, green mold and *Rhizopus* rot (table 4).

Table 4. Examples of treatments with resistance inducers for the control of postharvest fungal diseases of fresh fruit and vegetables in storage

Disease	Causal agent	Crop	Inducer	References
Blue mold	*Penicillium expansum*	Apple	Quercetin,umbelliferone	Sanzani et al., 2008
			trans-2-hexenal	Neri et al., 2006
			BTH, BABA, methyl jasmonate	Quaglia et al., 2009
		Grapes	Chitosan	Romanazzi et al., 2009
		Pear	*trans*-2-hexenal	Neri et al., 2006
		Peach	BTH	Liu et al., 2005
Brown rot	*Monilinia laxa*, *M. fructigena*, *M. fructicola*	Sweet cherry	Chitosan	Romanazzi et al., 2003
Gray mold	*Botrytis cinerea*	Apple	BTH, BABA, methyl jasmonate	Quaglia et al., 2009
		Grapes	Chitosan	Romanazzi et al., 2002, 2006, 2009
		Strawberry	Chitosan	El Ghaouth et al., 1992; Zhang and Quantick, 1998; Reddy et al., 2000; Romanazzi et al., 2000
			Chitosan, BTH, Fitocalcio®,Algition®	Santini et al., 2009
		Sweet cherry	Chitosan	Romanazzi et al., 2003
		Grapes	BTH	Iriti et al., 2004, 2005
		Tomato	BTH	Iriti et al., 2007
Green mold	*Penicillium digitatum*	Grapefruit	BABA	Porat et al., 2003
Rhizopus rot	*Rhizopus stolonifer*	Strawberry	Chitosan	Romanazzi et al., 2000
			Chitosan, BTH, Fitocalcio®,Algition®	Santini et al., 2009

In trials that have been carried out after harvesting, where the aim was to protect these high value commodities, an effectiveness that is even lower than that achieved in field treatments can still have a relevant economic impact. Moreover, the postharvest environment is more stable with respect to the field, so better control of diseases can be achieved. All of these compounds can increase host resistance, although some of them also have direct inhibitory effects on growth of pathogen, coupling their antimicrobial and eliciting activities. The results with chitosan are typical: reduction of growth and induction of growth malformations of decay-causing fungi, such as *B. cinerea* and *Rhizopus stolonifer* (El Ghaouth et al., 1992), as well as increasing host resistance and eliciting fruit defences. As an example, chitosan treatment has been shown to increase chitinase, β-1,3 glucanase and phenylalanine ammonia-lyase activities in strawberry (El Ghaouth et al., 1992; Zhang and Quantick, 1998; Romanazzi et al., 2000) and to reduce H_2O_2 production (Santini et al., 2009). Although several elicitors of SAR are reported to be effective in controlling many fungal plant diseases (Schreiber and Desveaux, 2008), only some of them have been applied to crop protection in practice, especially those where their effectiveness is durable and high, and comparable with that provided by treatment with synthetic fungicides. For example, the plant activator probenazole (3-allyloxy-1,2-benziothiazole-1,1-oxide) is very effective against rice blast disease caused by *Magnaporthe grisea* and it has been widely used in Asian rice production since 1975 (Walters and Fountaine, 2009). Furthermore, BTH has been registered in several countries (as Bion® in Italy) and it is extensively applied to protect a number of crops from fungal diseases (Walters and Fountaine, 2009). For more than a decade, Stadnik and Buchenauer (1999) have reported success in field experiments with single applications of BTH in the control of wheat powdery mildew, although there was no improvement in the control of this disease with additional applications of the plant activator, as compared to single applications, and no improvements in yield were associated with the use of BTH, compared to untreated controls. Similar results on crop yield were reported by Iriti and Faoro (2003c) in the pathosystem *Phaseolus vulgaris*/*Uromyces appendiculatus*. Recently, there has been increasing interest in Italy for the exploitation of SAR (Bugiani, 2006), which has also been stimulated by reductions in available fungicides, with agrochemical companies decreasing their residue thresholds to levels lower than those prescribed by law. However, there are some problems that slow the exploitation of SAR for crop protection in practice: *i*) in open fields, the effectiveness of some inducers may be variable; *ii*) consumers, many farmers and crop protectionists ask for agricultural products with very

high performances; and *iv*) a number of inducers are not registered as plant protection products, but as biostimulants of plant defences, and their compositions are not known (e.g. Kendal®, Pom-PK®). In addition, the efficacy of SAR in the field is variable, as it can be influenced by environment, crop genotype and nutritional status, and the extent to which plants have already been induced (Reglinski et al., 2007; Walters and Fountaine, 2009). Unfortunately, our understanding of the impact of these factors on the expression of SAR is poorly developed.

1.2 Isoprenoids

Isoprenoids, also named terpenoids, are the chemically and functionally most diversified class of low molecular mass lipids in plants, both primary and secondary metabolites (Iriti and Faoro, 2009). The common denominator of all these compounds is their biosynthetic precursor, isoprene. The term "isoprenoid" includes all substances that derive from one or more isoprene units. Among the important isoprenoids are compounds such as electron carriers (quinones), membrane constituents (sterols), vitamins (A, D, E and K), plant hormones (side chain of cytokinins, abscisic acid, gibberellins and brassinosteroids), photosynthetic pigments (chlorophyll, phytol and carotenoids) and essential oils (Sacchettini and Poulter, 1997).

1.2.1 Isoprenoid pathway

Acetyl coenzyme A (CoA) represents the precursor for the isoprenoid biosynthesis (Fig. 3). Firstly, two molecules of acetyl CoA react to give acetoacetyl CoA and, then, with a further acetyl CoA to produce β-hydroxy-β-methylglutaryl-coenzime A (HMG-CoA). In plants, the same enzyme, HMG-CoA synthase, catalyses both reaction. The conversion of HMG-CoA into mevalonate, via HMG-CoA reductase, is the rate limiting enzyme of this pathway (Chappel, 1995). Mevalonate kinase and mevalonate phosphate kinase phosphorylate, respectively, mevalonate and, then, mevalonate 5-phosphate, yielding mevalonate 5-diphosphate. Further, mevalonate diphosphate decarboxylation, via mevalonate diphosphate decarboxylase, produces isopentenyl diphosphate (IPP), the five-carbon building block for the formation of isoprenoid chains. The enzyme IPP:dimethylallyl-PP isomerase converts IPP into dimethylallyl diphosphate (DMAPP), the acceptor for successive transfers of isopentenyl residues (Bach, 1987; Chappell, 1995).
Hemiterpenes (C_5), such as isoprene, originate from dimethylallyl-PP, upon the release of diphosphate. Differently, dimethylallyl-PP can condense with IPP, to form geranyl-PP via geranyl-PP synthase. In the same way, further chain elongation is attained by head to tail condensation of geranyl-PP to IPP, to produce farnesyl-PP via farnesyl-PP synthase. Analogously, geranylgeranyl-PP synthase catalyses the head to tail condensation of farnesyl-PP to IPP, thus yielding geranylgeranyl-PP (Poulter and Rilling, 1978).

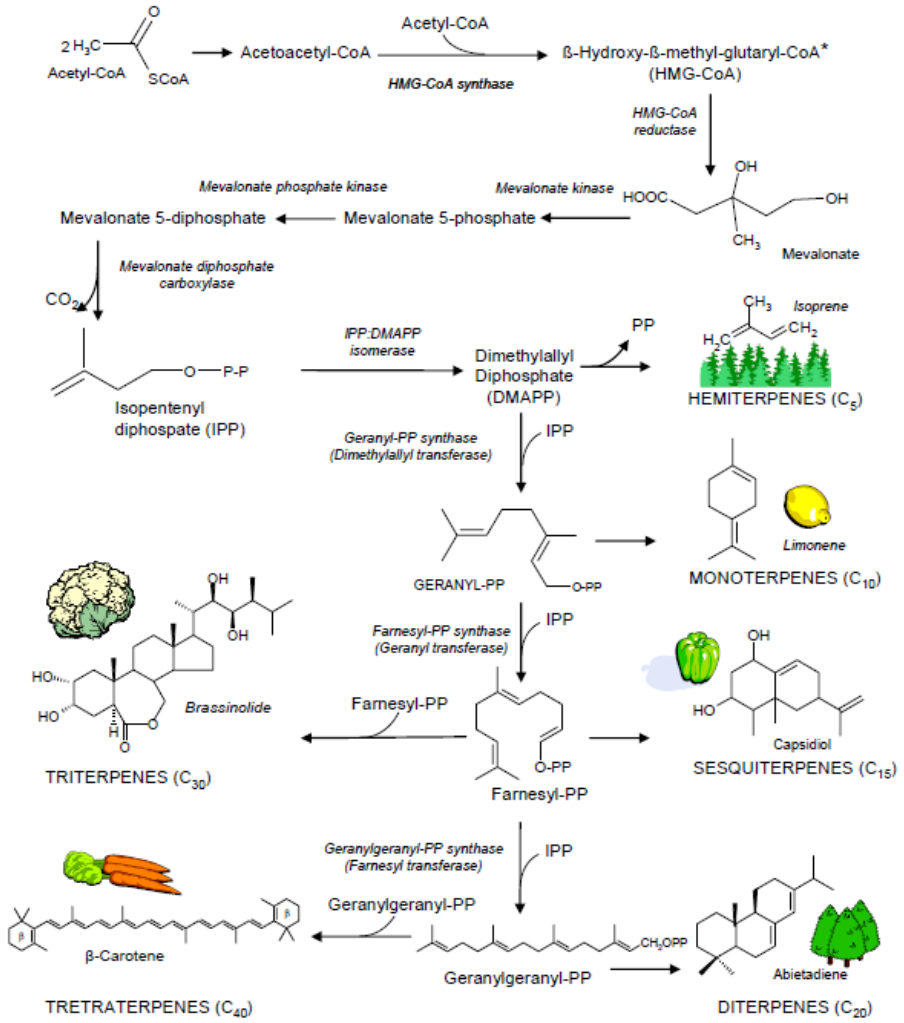

Figure 3. Isoprenoid pathway from acetyl-CoA.

Geranyl-PP is the precursor for the formation of monoterpenes (C_{10}) or essential oils, including highly volatile open chain and cyclic compounds, such as menthol, limonene, geraniol, linalool and pinene. They are active in plant-microbe, plant-pronubi, plant-phytophagous and plant-plant interactions, due to their attractiveness and repulsiveness (Croteau, 1987).

Farnesyl-PP is the precursor for the synthesis of open chain and cyclic sesquiterpenes (C_{15}), the largest group of isoprenoids, including essential oils and antibiotic compounds (phytoalexins) (Cane, 1990). Diterpenes (C_{20}) derive from geranylgeranyl-PP, consisting of phytoalexins, plant hormones, the

phytol side chain of chlorophylls, tocopherols and phylloquinone (Dogbo and Camara, 1987).

Furthermore, triterpenes (C_{30}) are synthesized from two molecules of farnesyl-PP (C_{15}), by a reductive head to head condensation. Squalene triterpene is the precursor for sterols, important membrane constituents, via squalene synthase (Abe et al. 1993). Analogously, head to head condensation of two molecule of geranylgeranyl-PP (C_{20}) leads to tetratepenes (C_{40}), such as carotenoids (carotene, lycopene) and xanthophylls (lutein, zeaxanthin, violaxanthin). Besides, isoprenoids are involved in protein prenylation, that is the synthesis of variously lengthened isoprenoid chains, anchoring proteins in membranes, such as G proteins, ubiquinone, plastoquinone and cytochrome-*a*. Finally, natural rubber is a polyterpen, composed of over 1000 isoprene units and deriving from polymerisation of geranylgeranyl-PP units (Wendt and Schulz, 1998).

1.3 Sterols

Sterols are a class of the great family of isoprenoids. The conversion of FPP to squalene constitutes the starting point of the biosynthesis of sterols. Found in all eukaryotic organisms, they are membrane components which regulate the fluidity and the permeability of phospholipid bilayers. In addition, sterols participate in the control of membrane-associated metabolic processes, which require the action of a few specific sterols. In general, however, the sterols are precursors of a wide range of compounds that are involved in cellular processes in animals, fungi and higher plants. In fact, they play an important role in cellular and developmental processes in plants as precursors of the brassinosteroids. Lastly, in plants, act as substrates for a wide variety of secondary metabolites such as cardenolides, glycoalkaloids and saponins.

1.3.1 Structure, biosynthesis and cellular localization of sterols

Sterol was the name originally proposed to describe a 3β-monohydroxy compound based upon the perhydro-1,2-cyclopentanophenanthrene ring system, with methyl substitution at C10 and C13 and a side chain with 8–10 carbon atoms (Fig. 4). Structural variations arise from different substitutions in the side chain and number and position of double bonds in the tetracyclic skeleton.

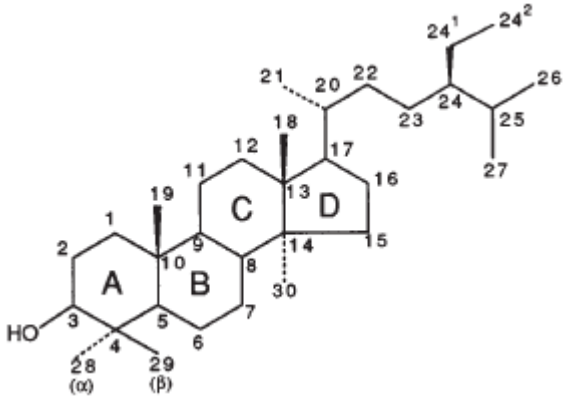

Figure 4. Basic structure of a sterol.

According to the different double bond position in the sterol nucleus, phytosterols may also be categorized as *Δ*5- and *Δ*7 sterols. They also may be categorized in three subclasses based on the number of methyl group on C-4: 4,4′ –dimethylsterols, 4-methylsterols and 4-desmethylsterols.

Plant sterols have been reported to include over 250 different sterols in various plants. The most common representatives are *β*-sitosterol, stigmasterol and campesterol (4-desmethylsterols). Chemical structures of these sterols are similar to cholesterol, differing in the side chain. For instance, sitosterol and stigmasterol have an ethyl group at C-24, and campesterol a methyl group in the same position (Fig. 5).

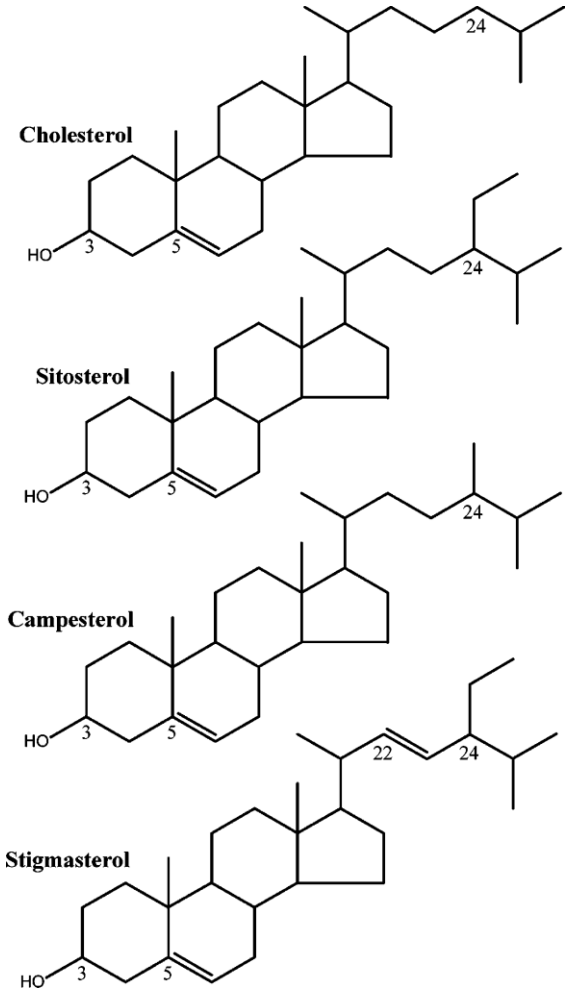

Figure 5. Cholesterol and common Δ^5-phytosterols.

A recent convention among nutritionists divided phytosterols into the two categories of "sterols" indicating a double bond at position 5, and "stanols," indicating 5α-reduction of that double bond (Katan, 2002). These classes are reflected in the common names of the compounds. For example, sitosterol is structurally identical to sitostanol except for the double bond at position 5, and campesterol bears the same relationship to campestanol.

In most higher plants, sterols with a free 3β-hydroxyl group, also called free sterols, are the major end products. However, sterols also occur as conjugates in which the 3-hydroxyl group is either esterified (by a long-chain fatty acid to give steryl esters) or β-linked (to the 1-position of a monosaccharide, usually

glucose) to form steryl glucosides or, when the 6-position of the sugar is esterified by a fatty acyl chain, acylated steryl glucosides (Akihisa, 1991) (Fig. 6).

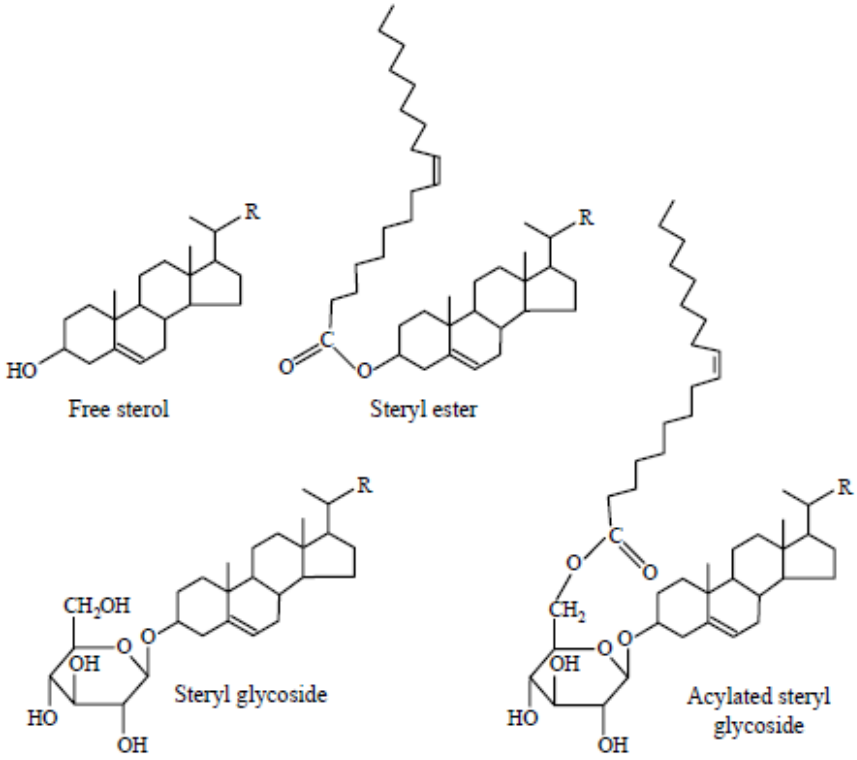

Figure 6. Examples of basic structures of free sterol and steryl conjugates. R (side chain) varies among different sterols.

In plants the sterol pathway (Fig. 7) consists of a sequence of more than 30 enzyme-catalysed reactions, all of which are localized in the cell membranes (Benveniste, 1986). The enzymatic steps in plants with a photosynthetic apparatus convert squalene oxide into cycloartenol, whereas non-photosynthetic fungi convert squalene oxide into lanosterol and finally ergosterol (Hartmann, 1997). Both lanosterol and cycloartenol can be converted into Δ^5-24-alkyl sterol end-products. Plants are thought to transform sterols by a series of interacting pathways. The major post-squalene biosynthetic pathway is regulated by critical rate-limiting steps such as the methylation of cycloartenol into cycloeucalenol. The 9β, 19-cyclopropane ring of cycloeucalenol is opened by cyclopropyl-sterol isomerase to form obtusifol.

Further downstream of the pathway, 24-methylene lophenol is transformed into 24-ethylidene lophenol by a sterol-methyltransferase which confers to the plant kingdom the capability to produce 24-ethyl sterols as the predominant molecular species. A minute amount of sterols, predominantly campesterol and its epimer 22(23)-dihydrobrassicasterol, form the pool of brassinosteroid precursors (Yokota, 1997).

Most of the higher plant sterols are found as free sterols (β-OH at C3) which serve as membrane components. In fact, the side chain extends into the hydrophobic core and interacts with fatty acyl chains of phospholipids and proteins with the free 3-hydroxyl facing the aqueous phase. Sterols are present in low amounts in ER, tonoplast and mitochondrial membranes. Also, a relatively small proportion of sterol molecules is present in the envelope of chloroplasts, but they are absent from thylakoid membranes. It appears that there is no specific association of an individual sterol molecule with a given membrane compartment, and that all the membranes contain the same sterols in similar proportions. All plant sterols are able to regulate membrane fluidity, though with different efficiencies. Sitosterol and campesterol are the most efficient.

A low-abundance class of sterols are esters linked to fatty acids. The roles of steryl esters also include storage and transport; this is supported by their presence in soluble forms, such as in lipoprotein complexes.

An involvement of these esters in the structure or function of the pollen coat has been suggested (Murphy, 2001). The esters could serve to facilitate germination of the pollen tube on the stigmas of plants from the dry stigma-type as is the case for triglycerides (Wolters, 1998) and as an adhesive component of the pollen coat for insect-mediated pollination.

A second low-abundance class of sterols constitutes sterol glucosides and sterol acyl glucosides (Mudd, 1980; Warnecke et al., 1997). These conjugated molecules comprise mainly Δ^5-sterols and are synthesized and located in the plasma membrane (Hartmann, 1987); variations in their relative concentrations together with other lipids have been related to the adaptability of plants to low temperatures (Palta, 1993). Free and conjugated forms of sterols are distributed in most of the plant cell types. The regulation of the proportion of these two forms is, thus far, unknown but it is most probably involved in a fine tuning of the concentration of free sterols in the plasma membrane (Ullmann 1993).

Figure 7. Sterol biosynthetic pathway of higher plants. SMT1, cycloartenol-C24-methyltransferase; SMT2, 24-methylene lophenol-C24-methyltransferase; FACKEL, sterol-C14-reductase; STE1/DWF7/BUL1, Δ^7-sterol-C5(6)-desaturase; DWF5, $\Delta^{5,7}$-sterol-Δ7-reductase; DIM, $\Delta^{24(25)}$-sterol reductase

1.3.2 Phytosterols in grapes and wines

The results of different study illustrate that phytosterols are mainly localized in the cuticular wax (Higgins and Peng, 1976) and berry skins (Le Fur et al. 1994) of grapes. Quantitative analysis of the lipid composition of grapes shows that the lipid fraction of pulp represents only 0.10% (fresh weight), whereas grape skin contains up to 0.32% (Higgins and Peng, 1976). Some works have focused on the evolution of fatty acids in several anatomical parts of grape berries during ripening (Roufet et al. 1987; Miele et al. 1993). The main lipid fraction evidenced in grape skins was phospholipids. The neutral lipid fraction represented around 40% of lipid content in grape skins. The major lipid components were polyunsaturated fatty acids, especially oleic, linoleic and linolenic acids. Studies on the evolution of phytosterols in grape skins and pulp confirmed β-sitosterol to be the main phytosterol (85–90%), while campesterol and stigmasterol amount to ca. 5% each (Dagna et al. 1982; Le Fur et al. 1994). Grape maturation induced a similar trend in β-sitosterol, campesterol and stigmasterol, i.e. a loss of these phytosterols in grape skins (Le Fur et al. 1994).

1.3.3 Assimilation of grape phytosterols by *Saccharomyces cerevisiae*

During alcoholic fermentation, yeast cells are progressively exposed to a very stressful environment due to the strong decrease of external pH and to the rising accumulation of ethanol in the external medium (Salmon, 1996). The main effects are to render the cellular plasma membrane leaky to protons, and to partially inhibit the activity of the ATPase-proton pump. Yeast cells are therefore obliged to consume more energy (in the form of ATP) to maintain pH homeostasis, until the equilibrium between passive entrance of protons and their expulsion by the ATPase-proton pump activity can not be sustained, leading to cell death.

Plasma membrane is mainly constituted by lipidic bilayer formed by a fluid arrangement of different classes of phospholipids, creating a non polar environment surrounding the yeast cell. This very fluid architecture is stabilized by smaller rigid molecules, sterols, mainly in the membrane regions where transporters are located. Sterols play major roles in the building and maintenance of yeast membranes: they mainly regulate membrane fluidity and permeability, ethanol resistance and H^+-ATPase activity; and they also regulate aerobic cell metabolism, cell cycle and exogenous sterol uptake (Daum, 1998).

In enological conditions, yeast cells are only able to synthesize sterols in the presence of trace amounts of oxygen (Rosenfeld et al, 2003). In this condition, the main sterol of yeast plasma membrane is ergosterol. In complete anaerobiosis, yeasts can not synthesize ergosterol but can incorporate a wide variety of exogenous sterols. For example, during wine fermentation, yeast growth occurs by assimilating grape phytosterols.
Grape phytosterols in strongly anaerobic conditions are easily incorporated by yeast cells for promoting yeast growth and the initiation of fermentative activity, but they rapidly perturb the yeast membrane properties by being the predominant sterols. However, since squalene and lanosterol are detected in biomass, phytosterol assimilation in anaerobic conditions does not exert any negative regulation on squalene synthesis (Fornairon-Bonnefond et al. 2002; M'Baya et al. 1989) nor the first steps in the ergosterol biosynthesis pathways. All grape phytosterols are incorporated in yeast cells in a very similar way. However, their final behavior within biomass differs: stigmasterol is directly incorporated in the plasma membrane in its free form, whereas β-sitosterol appears mainly as steryl esters within lipid particles. Steryl esters represent the major constituents of yeast lipid particles (up to 50%) (Leber et al. 1994) and are utilized for membrane formation under conditions of lipid depletion (Daum and Paltauf 1980).

1.3.4 Phytosterol in human nutrition

Phytosterol metabolism.
Phytosterols occur naturally in plants either esterified with fatty acids in the cell membranes or in free form within the cells (Beck et al., 2007). After ingestion, phytosterols, like cholesterol and other lipids, are emulsified by bile salts secreted into the small intestine to form micelles for digestion. After micelle formation, the esterified phytosterols are hydrolyzed to free phytosterols probably by cholesterol esterase and pancreatic lipase enzymes (Normen et al., 2006). Free phytosterols are then absorbed into enterocytes by ATP-binding cassette transporters that are encoded by ABC G5 and G8 genes, which are also involved in cholesterol absorption (Igel et al., 2003). In the enterocytes, these compounds become esterified to fatty acids by acyl-CoA cholesterol acyltransferases, and combined with cholesterol, triacylglycerols and apolipoproteins to form chylomicrons (Gylling et al., 2006). The chylomicrons are secreted into the lymph and then transferred to the bloodstream, where they are transformed to chylomicron remnants after the uptake of triacylglycerols by cells and transported to the liver. In the liver, the

phytosterols may either be used for synthesis of bile salts (Hamada et al., 2007) or be incorporated into very low density lipoproteins and be secreted into the blood, from where they are converted to low-density lipoproteins and presented to cells for uptake (Sanders et al., 2000; Gylling et al., 2006; Hamada et al., 2007). In the tissues, phytosterols are incorporated into the cell membranes (Awad et al., 2004) and have been found to be highly concentrated in the lungs, adrenal cortex, intestinal epithelia and ovaries (Sanders et al., 2000). Phytosterols that are either not taken up by cells or secreted back into the blood by cells are transported to the liver, from where they are excreted into the bile (Sanders et al., 2000).

Phytosterols as cardioprotective dietary components.

Cardiovascular diseases (CVD) are the leading cause of morbidity and mortality worldwide. It is well established that lifestyle, particularly the diet, plays an important role in the prevention and treatment of CVD (Krauss, 2000). A major target for dietary interventions is reducing the increased serum low-density lipoprotein cholesterol (LDL-C) concentration (Lauer, 2001). Foods enriched with fatty acid esters of plant sterols or stanols, i.e. plant sterol or stanol esters, are well known for their serum LDL-C lowering effect (Demonty, 2009), which is not transient, as shown in an 85-week intervention study (de Jong, 2008). The effectiveness of these compounds is further supported by the fact that they are nowadays incorporated into national and international guidelines such as the National Cholesterol Education Program guidelines. These guidelines encourage a daily incorporation of 2 g plant sterols or stanols into a healthy diet low in saturated fatty acids to reduce CVD risk for subjects with elevated LDL-C concentrations. In this case, addition of plant sterols and stanols can lower serum LDL-C concentrations by up to 10% (Lauer, 2001).

Phytosterols are plant-derived sterols that are structurally similar and functionally analogous to cholesterol in vertebrate animals. When incorporated as functional food ingredients, plant sterols and stanols are frequently esterified with a fatty acid ester to increase the solubility in the food matrix (Devaraj, 2006). The rate of absorption of cholesterol and plant sterols/stanols is very different. About 40–60% of cholesterol is absorbed, whereas plant sterols/stanols are absorbed for 15% or less, depending on the specific isoform (Ling, 1995; Demonty, 2009; Mensink, 2010).

Although the LDL-C lowering effect of food enriched with plant sterol and stanol esters is sustained and widely accepted, the discussion whether the type of food (food matrix) influences its efficacy is still ongoing (Abumweiss, 2008). Moreover the type of food carrier used and the frequency of intake seem to be

important as well (Demonty, 2009). Furthermore, Abumweis et al. (2001) concluded that the time of intake is also crucial, since consumption before or with breakfast only failed to reduce serum LDL-C, while the expected serum LDL-C lowering effect was observed when plant sterols were consumed together with a main meal being either lunch or dinner. To better understand all discrepancies between the individual studies, understanding the effects of plant sterol/stanol esters on cholesterol metabolism is essential.

The first studies, mentioning a role for plant sterols in the regulation of serum cholesterol concentration were published by Peterson et al. in 1951. Even in those early studies, the hypocholesterolemic effect of plant sterols was confirmed in patients (Pollak, 1953) However, the underlying mechanism was completely unknown, but was thought to be related to effects on intestinal cholesterol absorption.

During sixties, it became more or less generally accepted that plant sterols and stanols competed with dietary cholesterol for incorporation into mixed micelles, which is a crucial step for cholesterol absorption (Borgstrom, 1967). If plant sterols/stanols replace micellar cholesterol, less cholesterol will be taken up into the enterocyte. After uptake, cholesterol is normally esterified by intestinal acylcoenzyme A cholesterol acyltransferase 2 (ACAT-2). The so-formed cholesteryl esters are incorporated into chylomicrons and secreted into the lymph. In contrast, plant sterols/stanols are poor substrates for ACAT-2 and remain in their free form inside the enterocyte.

Although displacement of cholesterol from mixed micelles in the intestinal lumen seemed to be an important mechanism of plant sterol- and stanol-induced inhibition of intestinal cholesterol absorption, several other mechanisms involving actively regulated processes have been suggested.

More recently, Plosch et al. (2006) and Davis et al. (2004) have described the crucial role of different sterol transporters such as ATP-binding cassette G5 and G8 transporter (ABC G5/ABC G8) and Nieman-pick C1 like 1 protein (NPC1L1) and their regulatory mechanisms. It is questioned whether plant sterols and stanols interact with intracellular cholesterol sensors such as liver X receptor (LXR), leading to an increased expression of ABC G5/ABC G8 and ABCA1. The latter transports sterols to a nascent HDL particle, whereas ABC G5/ABC G8 promotes the efflux of sterols back into the intestinal lumen, resulting in decreased cholesterol absorption. At the same time, possible regulation of NPC1L1 by plant sterols/stanols is proposed. Recently, transintestinal cholesterol excretion (TICE) has been suggested as a possible target for the plant sterol/stanol mediated cholesterol lowering effect. Stimulation of TICE increases fecal neutral sterol loss. However, further

research is needed to explore the effects of the plant sterols/stanols on the intestinal cholesterol absorption into more detail. For example, the transporters responsible for basolateral and apical cholesterol secretion need to be identified. It is also debated whether TICE alone or possibly together with other mechanisms described explain the full cholesterol lowering effect of plant sterols and stanols.

Phytosterols as anticancer dietary components.

In recent years, a great deal of interest has been given to the role of phytosterols in the protection from some common cancers, such as lung (Mendilaharsu, 1998), stomach (De Stefani, 2000), ovary (McCann, 2003) and estrogen-dependent human breast cancers (Ju, 2004).

Phytosterols seem to act through multiple mechanisms of action, including inhibition of carcinogen production, cancer-cell growth, angiogenesis, invasion and metastasis, and through the promotion of apoptosis (programmed cell death) of cancerous cells. Phytosterols consumption may also increase the activity of antioxidant enzymes and thereby reduce oxidative stress.

Reactive oxygen species produced by oxidatively stressed cells can damage DNA, resulting in carcinogenesis. Vivancos and Moreno (2005) reported that *β*-sitosterol increased the activities of antioxidant enzymes, superoxide dismutase and glutathione peroxidase in cultured macrophage cells with oxidative stress induced by phorbol 12-myristate 13-acetate, indicating that phytosterols can protect cells from damage by reactive oxygen species.

Studies on the effect of phytosterols on cell growth and multiplication have shown a negative relationship between phytosterols and cancer development and progression at various concentrations ranging from 8 to 32 mM concentrations (Awad et al., 2007; Moon et al., 2007; Park et al., 2007).

Angiogenesis plays a vital role in cancer cell growth and multiplication as these cells require nutrients for growth (Prescott, 2000), whereas metastasis is the major cause of death due to cancer (Awad et al., 2001a). Awad and colleagues reported reduced invasiveness and adhesiveness of breast cancer cells in vitro due to *β*-sitosterol treatment by 78 and 15%, respectively, compared with the control. This group also reported reduced metastasis of murine cancer cells of the lungs and lymph nodes by 62 and 33%, respectively, after inoculating the mice with prostrate cancer cells and feeding them with diets containing 2% phytosterols. Awad et al. (2001b) have also reported that phytosterol treatment of breast cancer cells in vitro resulted in reduced invasiveness of the cells by reducing their adhesiveness.

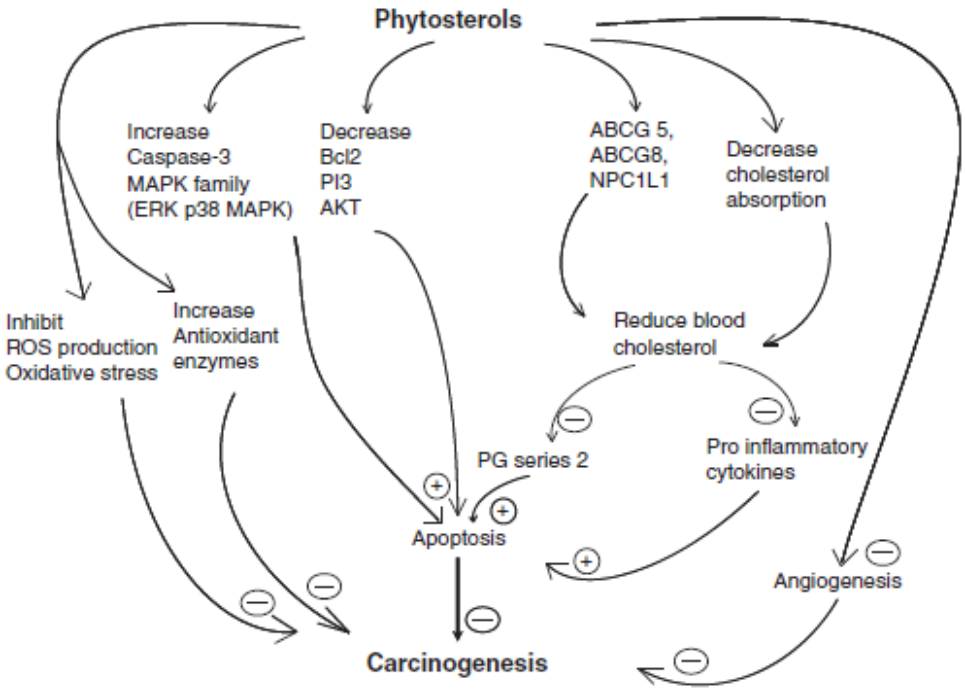

Figure 8. Proposed mechanisms of action of phytosterols on carcinogenesis (Woyengo et al., 2009).

Phytosterols seem to inhibit the development of various cancers mainly by promoting apoptosis of cancer cells through the activation of caspases. The increased activity of these enzymes could be attributed to the incorporation of phytosterols into cancer cell membranes. Phospholipids have been reported to interact more strongly with cholesterol than with phytosterols, resulting in changes in their membrane structure and function. These changes increase the activities of proteins involved in extra- and intracellular signal-transduction pathways that activate caspase enzymes. Phytosterols could also inhibit cancer development by lowering blood cholesterol level. They could reduce the incorporation of cholesterol in the lipid rafts of cancer cells and hence promote the apoptosis of cancer cells by reduction in anti-apoptotic signal transduction.
In summary, mounting evidence supports a role for phytosterols in protecting against cancer development. Hence, phytosterols could be incorporated in diet not only to lower the cardiovascular disease risk, but also to potentially prevent cancer development.

1.4 Wine aroma

The volatile fraction of wine plays a prominent role in its organoleptic characteristic. It determines its aroma, which is the most important parameters influencing wine quality and consumer acceptance (Boulton et al., 1995, Câmara et al., 2006). The volatile compounds are perceived by the olfactory receptors in the nose which trigger an odorous sensation. Therefore, flavour is the sensation perceived by the brain when the olfactory epithelium is reached by a fraction of molecules, which were vaporized in the glass – orthonasal route – or put in contact with the mouth – retronasal route. Consequently, the intensity of the olfactory sensation is not simply dependent on the concentration of the volatile compound in the liquid phase, but also depends on its volatility, its vapour pressure and its perception threshold.
The olfactory perception threshold can be defined as the minor stimulus which is able to promote an olfactory sensation in at least 50 % of a jury of a sensory panel. If the tasters are able to identify the odour, a recognition threshold is specified. On the other hand, if the volatile compound is already present in the tasting solution, a difference threshold could be defined as the minor addition of the substance susceptible to promote a change in the sensory stimulus (Vilanova and Oliveira,).

1.4.1 Classification and origin of volatile compounds

The wine aroma is the result of a long biological, biochemical and technological sequence. It is a complex balance of more than 1000 volatile compounds, but less than 10% may contribute to the flavour. These are small molecules (from 30 g/ mol to 300 g/ mol) having different physicochemical properties regarding concentration (ranging from several mg L^{-1} to less than a few ng L^{-1}) polarity, volatility and odour impact (Perestrelo et al., 2008, Polášková et al., 2008). Furthermore, each volatile compound presents its own olfactory perception threshold which is, in turn, influenced by the other constituents of the wine (Table 5). Some compounds, present in trace amounts, can play an important role in the aroma, whereas other compounds, present at very high concentrations, are odourless or participate only in a minor way.
Wine aroma complexity is due to the diversity of the mechanisms occurring during the formation of the compounds involved (Ribéreau-Gayon, 2006):

- grape metabolism, influenced by the variety, soil, climate, agronomic, sanitary conditions and degree of ripeness (varietal aroma)

- ❖ biochemical and pre-fermentation phenomena (oxidation and hydrolysis), triggered during berry crushing and maceration (pre-fermentative aroma)

- ❖ metabolisms of the microorganisms (yeasts and bacteria) carrying out alcoholic and malo-lactic fermentations, (fermentative aroma)

- ❖ chemical reactions occurring during wine development and its bottle conservation generating the aging "bouquet" (post-fermentative aroma)

Table 5. Perception thresholds (μg L^{-1}) and odour descriptors of relevant wine volatile compounds

Family/Compound	Perception Threshold [a]		Descriptor [b]	References
Monoterpenes				
Z-rose oxide	0.2	hydro-alcoholic solution; retronasal	green, floreal	[a]Guth, 1997; [b]Ong & Acree, 1999
Nerol oxide	100	water	fragrant	[ab]Simpson, 1979
Linalool	25.2	model wine solution; orthonasal	lemon	[a]Ferreira et al., 2000; [b]Escudero et al.,2004
HO-trienol	110	water	linden	[a]Simpson, 1979; [b]Ribéreau-Gayon et al., 2000
α-terpinol	250	model wine solution; orthonasal	pine	[a]Ferreira et al., 2000; [b]Meilgaard, 1975
Nerol	400	water	lime, roses	[b]Meilgaard, 1975
Geraniol	36	model wine solution; orthonasal	rose-like, citrus-like	[a]Escudero et al., 2004; [b]Czerny et al., 2008
Wine lactone	0.01	hydro-alcoholic solution; retronasal	sweet, coconut	[a]Guth, 1997; [b]Guth, 1996
Methoxipyrazines				
3-isobutyl-2-methoxypyrazine	0.0039	water; orthonasal	bell pepper-like	[a,b] Czerny et al., 2008
3-isopropyl-2-methoxypyrazine	0.0062	water; orthonasal	earthy, pea-like	[a,b] Czerny et al., 2008

Family/Compound	Perception Threshold [a]		Descriptor [b]	References
C_{13}-norisoprenoids				
β-damascenone	0.05	hydro-alcoholic solution; retronasal	sweet, apple	[a]Guth, 1997; [a]Escudero et al., 2004;
β-ionone	0.09	model wine solution; orthonasal	flowery, violet-like	[a] Ferreira et al., 2000; [b] Czerny et al., 2008
Thiols				
4-mercapto-4-methyl-2-pentanone	0.0008	model wine solution; orthonasal	box-tree, broom	[a,b] Tominaga et al 1998a
4-mercapto-4-methyl-2-pentanol	0.0055	model wine solution; orthonasal	citrus zest	[a,b] Tominaga et al., 1998a
3-mercaptohexyl acetate	0.0042	model wine solution; orthonasal	box tree, passion fruit	[a] Tominaga et al., 1996; [b] Tominaga et al., 1998a
3-mercapto-1-hexanol	0.06	model wine solution; orthonasal	passion fruit, grapefruit	[a,b] Tominaga et al., 1998a
C_6-compounds				
Z-3-hexen-1-ol	400	hydro-alcoholic solution; retronasal	lettuce like	[a] Guth, 1997; [b] Czerny et al., 2008
Alcohols				
2-methyl-1-butanol	1200	water; orthonasal	alchol, solvent	[a] Czerny et al., 2008; [b] Meilgaard, 1975
3-methyl-1-butanol	30000	hydro-alcoholic solution;retronasal	alchol, solvent	[a] Guth, 1997; [b] Meilgaard, 1975
2-phenylethanol	14000	model wine solution; orthonasal	roses, perfumed	[a] Ferreira et al., 2000; [b] Escudero et al., 2004
Esters				
Ethyl butyrate	20	hydro-alcoholic solution; retronasal	fruity	[a] Guth, 1997; [b] Czerny et al., 2008
Ethyl hexanoate	14	model wine solution; orthonasal	fruity, apple	[a] Ferreira et al., 2000; [b] Meilgaard, 1975
Ethyl octanoate	5	model wine solution; orthonasal	fruity, fresh	[a] Ferreira et al., 2000; [b] Escudero et al., 2004
Ethyl decanoate	200	model wine solution;orthonasal	fruity, fatty acid	[a] Ferreira et al., 2000; [b] Meilgaard, 1975

Family/Compound	Perception Threshold [a]		Descriptor [b]	References
Ethyl 2-methylbutyrate	18	model wine solution; orthonasal	fruity	[a] Ferreira et al., 2000; [b] Czerny et al., 2008
Ethyl 3-methylbutyrate	3	model wine solution; orthonasal	fruity, blueberry-like	[a] Ferreira et al., 2000; [b] Czerny et al., 2008
3-methylbutyl acetate	30	hydro-alcoholic solution; retronasal	banana	[a] Guth, 1997; [b] Meilgaard, 1975
2-phenylethyl acetate	250	hydro-alcoholic solution; retronasal	flowery	[a] Guth, 1997; [b] Escudero et al., 2004
Fatty acids				
3-methylbutyric acid	33.4	model wine solution; orthonasal	fatty acid, rancid	[a] Ferreira et al., 2000; [b] Escudero et al., 2004
2-methylbutyric acid	3300	hydro-alcoholic solution; retronasal	sweaty, cheesy	[a] Guth, 1997; [b] Czerny et al., 2008
Hexanoic acid	420	model wine solution; orthonasal	sweaty, cheesy	[a] Ferreira et al., 2000; [b] Meilgaard, 1975
Octanoic acid	500	model wine solution; orthonasal	fatty, unpleasant	[a] Ferreira et al., 2000; [b] Escudero et al., 2004
Phenols				
4-ethylguaiacol	33	model wine solution; orthonasal	smoky, gammon-like	[a] Ferreira et al., 2000; [b] Czerny et al., 2008
4-vinylguaiacol	1100	model wine solution; orthonasal	clove-like, smoky	[a] Ferreira et al., 2000; [b] Czerny et al., 2008
Sulphur Compounds				
Dimethyl sulphide	10	hydro-alcoholic solution; retronasal	asparagus-like, putrid	[a] Guth, 1997; [b] Czerny et al., 2008
3-(methylthio)-1-propanol	500	hydro-alcoholic solution; retronasal	cooked potato like	[a] Guth, 1997; [b] Czerny et al., 2008
Carbonyl Compounds				
Acetaldehyde	10000	hydro-alcoholic solution; orthonasal	fresh, green	[a] Moreno et al., 2005; [b] Czerny et al., 2008

Family/Compound	Perception Threshold [a]		Descriptor [b]	References
3-hydroxy-2-butanone	30000	hydro-alcoholic solution; orthonasal	fruity, moldy, woody	[a] Moreno et al., 2005; [b] Meilgaard, 1975
2,3-butanedione	100	hydro-alcoholic solution; retronasal	buttery	[a] Guth, 1997; [b] Czerny et al., 2008

1.4.2 Varietal compounds

The wine molecules related to grape variety are the monoterpenols, the methoxypyrazines, the C_{13}-norisoprenoids, the volatile thiols, the volatile phenols and the dimethyl sulphide. A few aroma compounds have been directly linked to specific varietal flavors and aromas (Polášková, 2008) (Table 6).

Table 6. Constituens contributing to varietal aromas of selected wines

Compounds	Variety	References
Linalool	Muscat	Fenoll et al., 2009
Geraniol	Muscat	Fenoll et al., 2009
Nerol	Muscat	Fenoll et al., 2009
3-Isobutyl-2-methoxypyrazines	Cabernet	Allen et al., 1991
4-mercapto-4-methyl-2-pentanone	Sauvignon	Tominaga et al 1998a
3-Mercapto-1-hexanol	Sauvignon	Tominaga et al 1998a
Dimethyl sulphide	Syrah	Guth, 1997

These molecules, excepting the methoxypyrazines, occur in grapes in the form of non-volatile precursors like unsaturated fatty acids, glycosides, carotenoids, cysteine S-conjugates and phenolic acids, which can generate flavour compounds during or after the technological sequence of winemaking.

Monoterpenes. Monoterpenes, major components of essential oils, are C10 representatives of isoprenoids, arising from geranyl pyrophosphate following the head-to-tail condensation of two molecules of isopentenyl pyrophosphate (Fig. 9) (Iriti-Faoro, 2006). Primary wine aroma is largely due to the presence of isoprenoid monoterpens in grape, namely linalool, HO-trienol, α-terpineol, nerol and geraniol, and two monoterpenic oxides, rose oxide and nerol oxide (Ribéreau-Gayon et al., 2000).

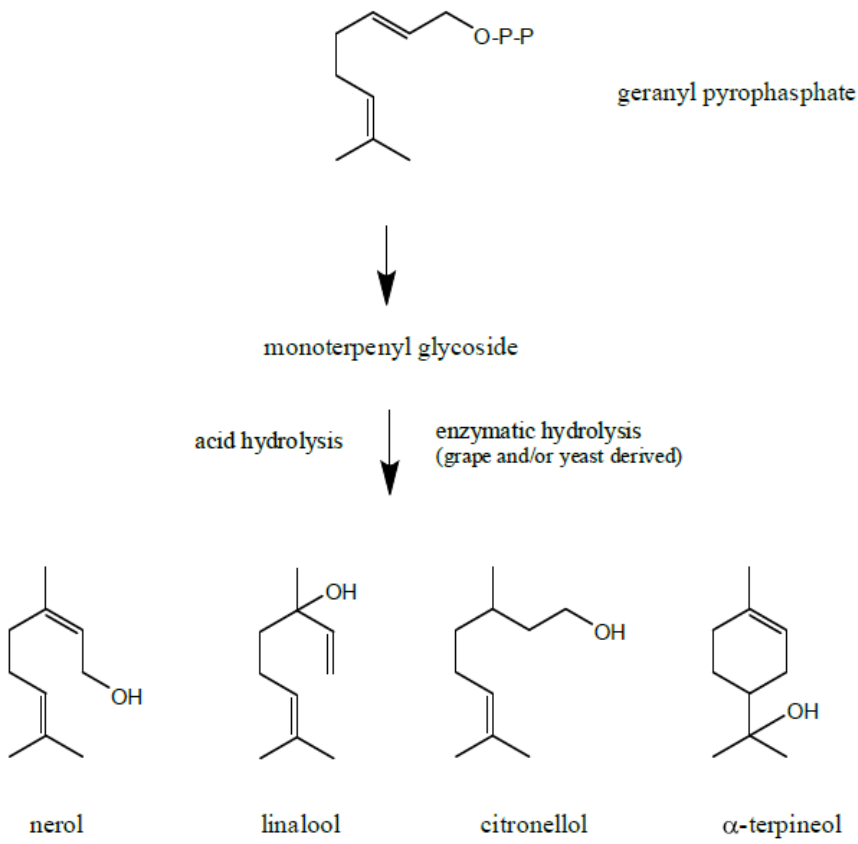

Figure 9. Biosynthetic pathway of monoterpenes in grapes

They exist in grapes both in a free state and in the form of glycosidic precursors, which can be hydrolyzed by the acidic conditions present in wines or by means of grape or yeast derived enzymes (Francis et al., 1992). These compounds, located mainly in grape skin, have low perception thresholds, in the range of μg L^{-1}, and may contribute to the floral notes of wines (Table 1). The terpene content varies considerably from cultivar to cultivar, and these compounds are particularly abundant in aromatic grape varieties such as Muscat, Riesling and Gewürztraminer (Park and Noble, 1993)

Methoxypyrazines. Methoxypyrazines are nitrogenated heterocycles produced by the metabolism of amino-acids. This family contains several compounds, and the most important contributor appears to be 3-isobutyl-2-methoxypyrazine, which is very pronounced in Sauvignon Blanc grapes and wines (Allen et al, 1991, Allen and Lacey, 1993). Other methoxypyrazines, 3-

isopropyl-2-methoxypyrazine and 3-*sec*-butyl-2-methoxypyrazine are present at lower concentrations. Methoxypyrazines are highly odoriferous showing low perception thresholds in water (of the order of 1 ng L^{-1}) and are responsible for the earthy and vegetable notes – green pepper and asparagus – of wines (Czerny et al., 2008). These volatile compounds exist in a free state mainly in grapes skin and their content decrease after *véraison* until harvest (Lacey et al., 1991). According to several studies, the levels of methoxypyrazines in grapes and wines are influenced mainly by climatic conditions during the ripening stage; especially hot climates are not suitable for the production of typical Sauvignon Blanc wines because high temperatures and light radiation levels adversely affect the formation and preservation of methoxypyrazines (Marais et al., 1999).

C_{13}-norisoprenoids. The carotenoids, terpenes with 40 carbon atoms, also play a role in varietal aroma In fact, oxidation of carotenoids produces volatile fragments, C_{13}-norisoprenoids. These are strongly odoriferous compounds, such as *β*-ionone (aroma of viola), *β*-damascenone (aroma of exotic fruits), *β*-damascone (aroma of rose), and β-ionone (aroma of flowers and fruits) (Baumes et al., 2002).

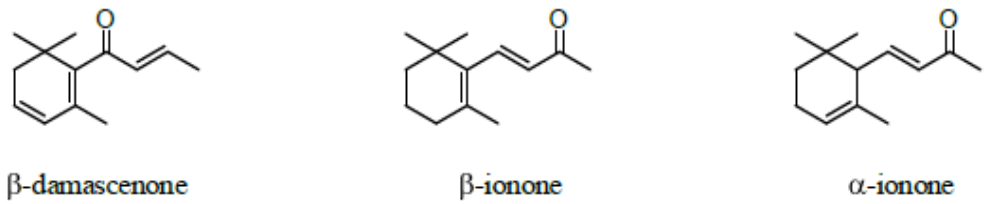

Figure 10. Structures of the main C^{13} norisoprenoids identified in wines

Norisoprenoids are present in the grape berries and leaves in the form of non-volatile precursors, namely carotenoids (such as *β*-carotene, lutein, neoxanthin and violaxanthin) and glucosides (Ribéreau-Gayon et al., 2006). These precursors are released during crushing by hydrolysis under acidic conditions or by fungal enzymes. Norisoprenoids can be divided in megastigmanes and non-megastigmanes. C_{13}-norisoprenoids belong to the megastigmane group. The megastigmanes have complex aromas: *β*-ionone and *β*-damascenone, with their floral and fruit notes, have low concentration thresholds (Baumes et al., 2002). Moreover *β*-damascenone is thought to be important in the aroma of some wines, such as Chardonnay and Riesling. It must be noted, that solar exposition favours the synthesis of carotenoids in grapes before *véraison* and its

degradation to C_{13}-norisoprenoids after this phonological stage (Razungles et al., 1993).

Volatile thiols. The contribution of the varietal thiols to wine aroma has been known. Tominaga in several works (1996, 1998a, 1998b, 2000, 2000b), has identified four volatile thiols: 4-mercapto-4-methyl-2-pentanone (4MMP), 3-mercapto-1-hexanol (3MH), 4-mercapto-4-methyl-2-pentanol (4MMPOH) and the 3-mercaptohexyl acetate (3MHA) (Fig. 11). They were first found in Sauvignon Blanc (Tominaga et al., 1996), and then in many other wine varieties such as Gewürztraminer, Riesling, Pinot Blanc, Semillon, Cabernet-Sauvignon, Merlot. Vegetal notes –box tree and broom– as well as fruit notes –passion fruit, grapefruit– are associated to these compounds; additionally, perception thresholds are extremely low, of few ng L^{-1}.

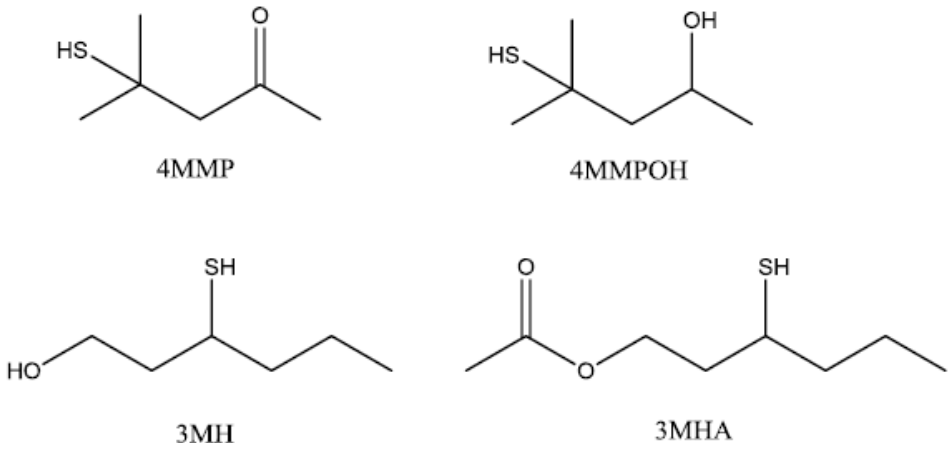

Figure 11. Structures of the main volatile thiols identified in wines

These aroma compounds are not present in the must but appear in wines during alcoholic fermentation. Under yeast action, some varietal thiols can be generated by release from *S*-cysteine conjugate precursors present in the must.

Volatile phenols. Some phenolic acids like caffeic acid, *p*-coumaric acid and ferulic acid can act as precursors of volatile phenols, which could contribute positively to wine aroma, when they are present at low concentrations; associated descriptors are smoky, clove-like and leather (Table 1). Yeasts can conduct the decarboxylation of phenolic acids to volatile phenols, as well as esterase activities present in enzymatic preparations used in winemaking. During wine storage and ageing, volatile phenols may be further transformed.

Dimethyl sulphides. They belong to the family of thioesters. Thioesters are organosulfur compounds characterized by the presence of one or more sulfur atoms bonded between two carbon atoms. The occurrence of these compounds has been described in several publications, but mainly in red wines (de Mora et al., 1987; Park et al., 1994). They can impart very bad odours in wine and have low perception thresholds. Nevertheless, most of these compounds can be easily eliminated by simple aeration (Mestres et al., 2002).

1.4.3 Pre-fermentative compounds

Pre-fermentative compounds are formed during harvesting, transport, crushing and pressing, as well as during eventual must heating or grape maceration. This group comprises C_6-aldehydes and C_6-alcohols; the main structures found in wines are hexanal, cis-3- hexenal, trans-2-hexenal and the corresponding alcohols (Moio et al., 2004). These compounds, with vegetal and freshly cut grass aromas, are formed in the must before fermentation, by the sequential action of grape lipases, lipoxygenases, and alcohol dehydrogenases on the lipidic precursors of linoleic and linolenic acids. During fermentation, aldehydes are reduced by yeasts to alcohols, principally hexanol, which is not very odorant, and hexenol, which is more odorant but found in minor amount.

1.4.4 Fermentative compounds

Fermentation microorganisms are the major contributors to wine aroma, especially yeasts are responsible for synthesis of aroma active primary and secondary metabolites, the biotransformation of grape must constituents into flavor-active compounds, and the production of enzymes that can transform odorless compounds present in grapes into aroma active compounds (Fleet 2003, Styger et al. 2011). These fermentative compounds are alcohols, fatty acids, esters, carbonyl compounds, sulphur compounds and some volatile phenols (Figure 12).

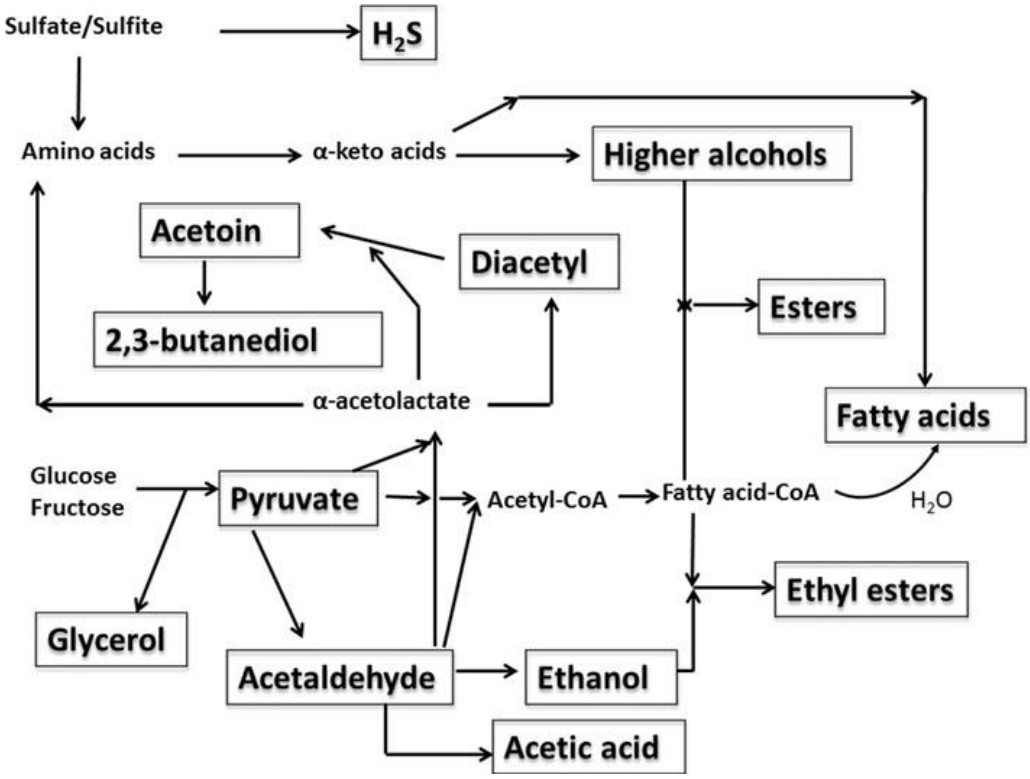

Figure 12. Some of the major classes of aroma compounds (shown in blocks) produced by yeasts during alcoholic fermentation (adapted from Lambrechts and Pretorius 2000).

Higher alcohols They are alcohols with more than two carbons They are practically absent in grapes and musts, though they are found in wine at concentrations typically between 150 and 550 mg L^{-1} (Ribéreau-Gayon et al., 2000).They have positive impact on wine aroma at levels below 300 mg L^{-1}, imparting fruity characteristics, and a negative impact at concentrations exceeding 400 mg L^{-1} that result in strong, pungent aromas (Sweigers et al. 2005). The main higher alcohols present in wine include, 2- and 3-methyl butanol, 2-methyl propanol and propanol. Most of the higher alcohols present in the wine occurs as by-products of yeast fermentation. Higher alcohols are formed either from metabolism of the sugars (anabolic pathway), via pyruvate, having the keto acids as intermediates, or from grape amino acids through the Erhlich mechanism (catabolic pathway), where amino acids undergo successively a deamination, a decarboxylation and a reduction (Ribéreau-Gayon et al., 2006) (Fig. 13).

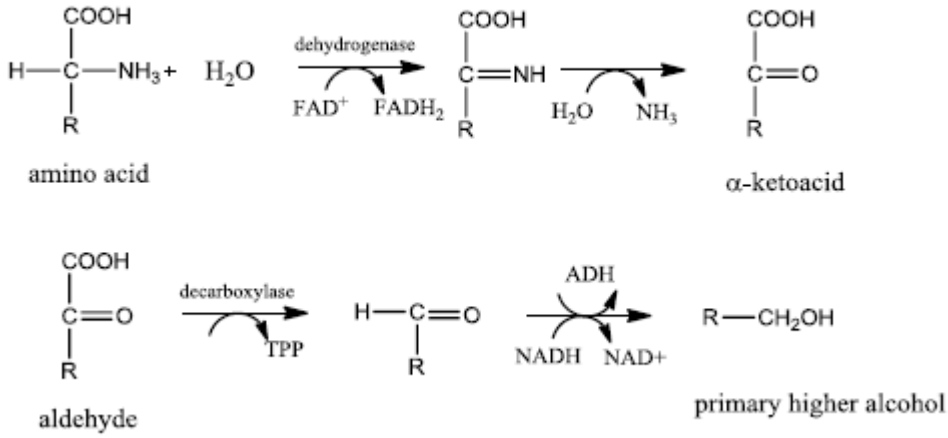

Figure 13. Biosynthesis of higher alcohols, according to Ehrlich (Ribéreau-Gayon et al., 2006)

Fatty Acids. Volatile fatty acids are carboxylic acids with long unbranched aliphatic chains which can be saturated or unsaturated. Fatty acids present in wine may derive from the anabolism of lipids, resulting in compounds with even number of carbon atoms, by oxidative decarboxylation of α-keto acids or by the oxidation of aldehydes (Fig. 13). During fermentation, yeasts produce small amounts of hexanoic, octanoic and decanoic acids. These fatty acids possess cheesy, goat-like odours and usually occur at levels below the perception threshold in wines, although their occurrence at or just below the threshold level can contribute to the overall complexity of wine aroma. Fatty acids with short chain lengths (between C8 and C14) are toxic to yeasts and exhibit strong antimicrobial activity and this effect is intensified if these fatty acids are unsaturated (Bardi et al., 1998).

Esters. Most esters found in alcoholic beverages are secondary metabolites produced by *Saccharomyces cerevisiae* during fermentation (Engan, 1974). They represent the secondary metabolites with the greatest impact on wine aroma and their presence contributes to the overall fruity aroma of the wine. Esters are formed as a result of the reaction between the carboxyl group of an organic acid and the hydroxyl group of an alcohol or phenol, and water molecules are eliminated (Ribéreau-Gayon et al., 2000).
Esters can be divided into straight-chain (aliphatic) and cyclic (phenolic) esters. Esters which are based on acetic acid and higher alcohols, such as isoamyl and isobutyl alcohols, are classified as low molecular weight esters and are termed

as fruity esters because of their pleasant aromas. Considering the constituents of wine (alcohols and acids), esters are formed easily and over 160 have been identified in wine, although most in trace amounts with low volatility and moderate odour intensities.

These esters play a significant role in the generation of the bouquet of young white wines, the "fermentation bouquet". Isoamyl acetate (3-methylbutyl acetate) presents a banana-like flavour. The high molecular weight fatty acid ethyl esters (ethyl caprate) have brandy, fruity and grape-like aromas. Ethyl octanoate has an apple-like aroma (Rojas et al., 2001). Their concentrations are typically below 10 mg L^{-1}, but this value is approximately 10 times their perception threshold. Esters are produced in grapes only to a negligible extent. The concentration found in wines is mainly due to their production by yeasts during alcoholic fermentation. Ester formation in wine is influenced by many factors, such as yeast strain chosen, fermentation temperature, nitrogen level in must, oxygen availability, sulfur dioxide concentration, juice clarification and grape variety.

Aliphatic esters make up the largest ester group in wine, with the monocarboxylic acid esters being the most aromatic. This group of esters have esterification based on ethanol and various saturated fatty acids, acetic acid and higher alcohols. They are divided into monocarboxylic acid esters (containing a single carboxyl group in the parent acid), di- or tricarboxylic acid esters (containing two or three carboxyl groups), and hydroxyl and oxo-acid esters (containing a hydroxyl or ketone group in the parent acid). Among the monocarboxylic acid esters, the most important saturated fatty acids are hexanoic, octanoic and decanoic acids. This group of esters contributes pleasant, fruit-like aromas.

Fatty acid ethyl esters (ethyl hexanoate and ethyl octanoate) are produced by yeast activity during alcoholic fermentation (Ribéreau-Gayon et al., 2000). They are synthesised by ethanolysis of the acyl-S-CoA that is formed during fatty acid synthesis or by degradation processes and reach a maximum concentration during fermentation. Reaction with malonyl-S-CoA produces a new acyl-S-CoA with two additional carbon atoms (Ribéreau-Gayon et al., 2000). The ethyl acetates of fatty acids have very pleasant wax and honey odours which contribute to the aromatic finesse of white wines. They are present at total concentrations of a few mg L^{-1} (Ribéreau-Gayon et al., 2000). Formation of these esters is promoted during slow and difficult fermentations.

Acetate esters are produced by yeasts from the reaction of acetyl-CoA with a higher alcohol. The formation of these compounds is favoured by low temperatures and anaerobic fermentations. This results in hydrolysis back to

their component alcohols and acetic acid. These esters lend intense, unusual odours such as banana, pear drops, and apple to wines. Furthermore, some of these compounds have been described as odour molecules very active in wine and have been found to be present in all wines, forming the base of wine aroma (Ferreira, 2010). These compounds are ethyl butyrate, ethyl hexanoate, ethyl octanoate, ethyl decanoate, isobutyl acetate, isoamyl acetate, β-phenylethyl acetate, ethyl isobutyrate and ethyl isovaleratedy. For clarity purpose, the family of esters will be separated into three groups, namely ester acetates, ester of fatty acids and esters of other acids.

Sulphur compounds. In addition to producing compounds with a positive impact on wine aroma, yeasts also produce several compounds that are considered to negatively impact wine aroma. This includes the sulfur containing compounds: mainly thiols, sulfides, polysulfides, and thioesters.
Based on their volatility, they can be classified in two categories. The light sulfur compounds have a boiling point below 90°C, are highly volatile and generally have low perception thresholds. They can impart very bad odours in wine, such as rotten eggs, cabbage, onion, and garlic. Nevertheless, most of these compounds can be easily eliminated by simple aeration (Etiévant, 1991). The heavy sulfur compounds have high boiling points (above 90 °C) and react with other compounds present in the wine to produce off-flavour complex, detrimental to the wines.
Light sulphur molecules present at the end of alcoholic fermentation apart from sulphur dioxide are methyl mercaptans (e.g. methanethiol), ethyl mercaptans (e.g. ethanethiol) and respective thioacetates, sulphides (e.g. hydrogen sulphide, carbonyl sulphide) and disulphides (e.g. carbon disulphide). Main heavy sulphur compounds are secondary products of amino acids metabolism (cysteine, methionine and homomethionine). The most common cited are 2-mercaptoethanol, 2-methylthioethanol, 3-(methylthio)-1-propanol (methionol), methionyl acetate, methional and 3-methylthiopropanoic acid (Ribéreau-Gayon et al., 2006). These compounds may have several origins. They can be generated by yeast metabolism, during alcoholic fermentation, or by lactic acid bacteria during malolactic fermentation (Moreira et al., 2010; Vallet et al., 2008). In fact yeast need organic sulfur compounds such as cysteine, methionine, S-adenosyl methionine, and glutathione for growth and have to biosynthesize them using inorganic forms of sulfur if they are not present (Sweigers et al. 2005).Treatment in the vineyard (pesticides, fungicides) can be other sources of these off-odours in wine

(Mestres et al., 2000). On the contrary, they can be eliminated by copper sulfate fining or through the use of micro-oxygenation (Cano-López et al., 2006).

1.4.5 Post-fermentative compounds

The wine aroma and flavor are generated through an immensely complex interaction of various classes of volatile compounds and various environmental and biological factors. During ageing the wine acquires aromatic complexity as a result of important modifications consequence of different phenomena, such as: esterification/hydrolysis reaction, redox reactions, spontaneous clarification, CO_2 elimination, slow and continuous diffusion of oxygen through wood pores and transfer of tannins and aromatic substances from wood into wine (Camara et al., 2005). Generally, aging of wines leads to a loss of the characteristic aromas linked to the grape varietal and fermentation, and to the appearance of new aromas distinctive of older wines or atypical aromas associated with wine deterioration (Hernanz et al., 2009). In particular the contents of ethyl esters of branched-chain fatty acids vary during ageing (Diaz-Maroto et al., 2005). Moreover the ageing of wine on the lees (mainly residue yeast cells) was found to reduce the concentrations of volatile compounds imparting a fruity aroma and increasing long-chain alcohols and volatile fatty acids (Perez-Seradilla et al., 2008). Interestingly, it has also been shown that the lees can remove some of the unpleasant wine volatile phenols due to its biosorbent qualities (Chassagne et al., 2005).
In order to form the lees, the yeast cells must first undergo the process of autolysis. Autolysis is a slow, complex process and can be induced by a variety of factors such as temperature and the activation of lytic cell enzymes. To a large extent this process also seems strain-dependent (Martınez-Rodrıguez and Polo, 2000). Autolysis is of enological importance because, as the cells lyse, they release nitrogen, amino acids, peptides, proteins and mannoproteins into the wine (Martınez-Rodrıguez et al., 2001) These latter are cell wall-associated proteins liberated into the wine that play a role in protection against haze formation, as well as color stabilization (Comuzzo et al., 2006). Lipids are also released during autolysis and liberated fatty acids can give rise to volatile components such as esters, aldehydes, and ketones—thus impacting on the aroma and flavor of the wine (Pueyo et al., 2000).
Additionally, the structural characteristics of the wood, i.e., the grain, porosity, and permeability, and its chemical composition, including polyphenols, tannins, and volatile compounds, can influence the complex biochemical processes that take place during the oxidative ageing of wine in barrels,

changing the composition of the wine and adding to its stability. The simple extraction of aromatic compounds (volatiles and polyphenols) and tannins from wood can add a richness and complexity to the aroma and taste of wines (Jarauta et al., 2005).

1.4.6 Yeast influence on volatile composition of wines

The fermentation process in winemaking can occur either naturally, with the indigenous yeast of the grape surface, or by inoculating the must with selected starters. The use of locally selected yeast strains (usually belonging to the species *Saccharomyces cerevisiae*), with strain-specific metabolic characteristics can positively affect the final quality of the wine (Romano et al., 2003). Several studies have clearly shown the effects of indigenous and inoculated yeast populations on the wine volatile composition Vilanova and Sieiro (2006a) showed that Albariño wines produced by spontaneous fermentation had higher contents in higher alcohols, ethyl esters, acetates and were more aromatic than those obtained with the selected yeast strain. Another study (Vilanova and Masneuf-Pomarède, 2005a, 2005b) was performed with *S. cerevisiae* yeast strains (ASln1, ASln2 and ASln20) isolated from musts obtained from Albariño grapes harvested in the Rías Baixas region (Spain). ASln20 produced the highest amounts of alcohol. Marked differences in the volatile composition of the fermented musts, determined by gas chromatography (GC) were dependent upon the yeast strain used. *S. cerevisiae* ASln1 and ASln20 produced the greatest quantities of esters, (ethyl butyrate, ethyl hexanoate, ethyl octanoate and ethyl decanoate) which can give to wine a fruity aroma. These results suggest that the production level of these compounds is characteristic of the individual yeast strains, which highlights the importance of characterizing yeast strains for industrial use.

1.4.7 *Terroir* effect on grape and wine volatile composition

Terroir has been acknowledged as an important factor in grape and wine quality, particularly in European viticulture. The *terroir* concept was born in the Europe Appellations of Origin (AO) and was used for many purposes: to guarantee the authenticity of the products against frauds, to justify an economical advantage linked to a specific property, to synthesize an historical local experience, to strengthen the defence of a community of growers facing economical competition and to explain the characteristics of the wines. Today

terroir concept can be defined as an interactive ecosystem, including climate, soil and the vine (van Leeuwen et al., 2004).
The Atlantic Ocean (Winegrowing Region of the European Communities) and the relief markedly influence the climate. The produced wine is unique and its specific characteristics are mainly due to the climate and soil. Several studies have been conducted to know the grape and wine volatile composition in basis to the *terroir* in Atlantic viticulture (Oliveira et al., 2000; Vilanova et al., 2007b; Zamuz & Vilanova, 2006a, 2006b).
Oliveira and colleagues investigated the influence of the climate and the soil on the volatile compounds of Vinho Verde grapes from two autochthonous varieties, Alvarinho and Loureiro, in two subregions for each one of them (Alvarinho in Monção and Lima; Loureiro in Lima and Cávado). The results showed that the global characteristics of the grapes from the two varieties depend on the harvest factor (climate) rather than on the sub-region where the vine is planted. The characteristics of the soil have an even lesser influence on the variableness of the samples, although the Alvarinho variety picked in the Lima sub-region seemed to have different characteristics.
Albariño must and wine from NW Spain were studied in three different areas from Rías Baixas AO (Vilanova et al., 2007b; Zamuz and Vilanova, 2006a). The Rías Baixas was originally made up of three subzones, Val do Salnés, O Rosal and Condado do Tea. The Atlantic climate, with wet winters and sea mists, varies between the subzones. The coolest is Val do Salnés, and the hottest is southerly Condado do Tea and O Rosal, with occasional temperatures over 35°C and colder winters. Differences of climate and geography make the wines from the different origins individual in their own right. Non-terpenic compounds were the most abundant compounds in the free aroma fraction of the Albariño musts from the three geographic areas; of these, 2-phenylethanol (rose aroma) was the most important. The Albariño must from O Rosal should be the most aromatic since it had significantly higher volatile compounds content, with the bound compounds making up the largest group, quantitatively. The Albariño must from Val do Salnés had the lowest concentrations of volatile compounds and should therefore be the least aromatic; this could be related to its lower maturation index.
Respecting wines, Albariño from O Rosal was characterized by a high content of higher alcohols, while wines from Val do Salnés show the highest concentrations of free terpenes, acetates and ethyl esters. Wines of Condado do Tea show the highest concentrations of C13-norisoprenids, principally due to α-ionone (Zamuz and Vilanova, 2006b). The results obtained in the study on Albariño wines showed that significant differences have been found among

different geographic areas of Rías Baixas AO (northwestern Spain) in terms of the concentrations of most aromatic compounds.

Another comparative study was conducted on Albariño wine produced from musts from northern and southern Galicia (NW Spain) (Vilanova et al., 2007b). The influence of *terroir* on varietal and fermentative volatile compounds was studied. Data obtained from GC showed that differences were present in wine volatiles. The Albariño wines from northern Galicia showed the highest total concentration of volatiles analyzed, dominated by higher contents in total free terpenes and acetates. Total higher alcohols and ethyl esters characterised the Albariño wine from the south. Among the terpenes found, geraniol was markedly abundant in the north, while nerol and linalool were most abundant in the south. Among the alcohols, 2-phenylethanol and benzyl alcohol showed the highest concentrations in the south and in the north, respectively. Albariño wines from the south were more heterogenic than those from the north. Differentiation of these wines was possible. This behavior could be due to the predominance of *terroir* over the varietal character of the wines.

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AIMS

2.1 Aims of the study

The main aims of this PhD thesis were to evaluate the performance of two plant activators, benzothiodiazole (BTH) and chitosan (CHT), in vineyard, and to compare their effects with those induced by treatments with conventional agrochemicals. In particular,

- ✓ the modifications of the grapevine secondary metabolism stimulated by both elicitors were evaluated on the phytosterols amount in different grape berry (exocarp end endo-mesocarp) and seed tissues, at different phenological stages, and in experimental wines treated with SAR (systemic acquired resistance) inducers;

- ✓ the content of volatile compounds was measured in experimental wines (microvinificates);

- ✓ the levels of mycotoxins, especially ochratoxin A, were determined in experimental wines.

RESULTS AND DISCUSSION

3.1 Effects of plant activators on the phytosterol content in grapevine tissues and in experimental wines

In this chapter, the effects of plant activators chitosan (CHT), chitosan plus copper (CHT/Cu) and benzothiadiazole (BTH) were assessed on grapevine (*Vitis vinifera* L, cv. Groppello) lipid metabolism. In particular, the phytosterol (*β*-sitosterol, stigmasterol and campesterol) content was measured in berry (skin and flesh) and seed tissues at two different phenological stages (pre-*véraison* and *véraison*), and experimental wines (microvinificates). Data were collected for two vintages (2009 and 2010) and compared with products from conventional fungicide-treated grapevines.

3.2 Materials and Methods

3.2.1 Phytoiatric campaign

Open field treatments with plant activators (Table 1) were performed in 2009 and 2010 on an autochthonous cultivar of Lombardia, Groppello, cultivated in an experimental vineyard located at Raffa di Puegnago (Azienda Agricola San Giovanni, Brescia, Italy). The treatments were: (i) 0.03% (w/v) chitosan (CHT, 76 kDa molecular weight and 85% deacetylation degree; Sigma-Aldrich, St. Louis, MO, USA), (ii) 0.03% CHT in combination with 150 g/hL copper hydroxide (Kocide - 3000; Du Pont, Wilmington, DE, USA) (CHT/ Cu) and (iii) 0.3 mM benzothiadiazole [benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester, BTH, trade name Bion®, Syngenta, Basilea, CH]. Untreated grapevines were used as negative control, while plants treated with conventional fungicides (penconazole and methyldinocap) were used as the positive control (Table 1).

Table 1 Open field treatments applied every 10 days on Groppello parcels

Treatment
CHT (0.03% chitosan)
CHT/Cu (0.03% chitosan + 150 g hL^{-1} copper hydroxide)
BTH (0.3 mM benzothiadiazole)
Fungicides (penconazole + methyldinocap, positive control)
No treatment (negative control)

The trial was set up as a complete randomized block design in four replications, with ten vines (a parcel) per treatment in each block. Plants were sprayed every 10 days approximately with a spray lance, according to the meteorological conditions, from the beginning of grape susceptibility to fungal diseases until the complete *véraison* (i.e. approximately from the half of April to the end of July). Sampling was scheduled at two phenological phases: pre*véraison* ('pea-size' stage, before the end of the berry's growth cycle) and 100% *véraison* (stage when berry turn color and soften). Bunches were randomly collected from plants during the morning and stored at -20°C. Different tissues (seeds, fleshes and skins) of frozen berries, randomly selected from bunches, were carefully separated with a chilled scalpel and were lyophilised (Leybold-Heraeus GT2) for 20 h. After lyophilisation, the homogeneous sample of each tissue was pulverized with a chilled mortar and pestle. The powders were kept at -20°C and under shelter from the light until analyses. Experimental Groppello wines (microvinificates) were produced, by standard microvinification techniques, in the Centro Vitivinicolo Provinciale di Brescia (Italy) and stored at 4 °C in the dark until analysis.

3.2.2 Sterol extraction from seed and berry tissues

In all plant tissues, phytosterols occur in the form of free sterols, steryl esters, steryl glycosides and acylated steryl glycosides (Moreau et al., 2002, Phillips, 2002). Therefore, the optimal sample preparation procedure for total sterol determination should include sterols from all possible conjugates. Acid hydrolysis prior to alkaline hydrolysis has been used to release sterols in their free and conjugated forms, including glycosidic sterols (Toivo, 2001). Cholesterol was added to each sample (1 g), as internal standard (0.5 mL of a stock solution of cholesterol in ethanol, 1 mg mL^{-1}), together with 2 mL of ethanol and 10 mL of 6 N HCl. The suspension was stirred at 80 °C for 60 min. After cooling, 10 mL of ethanol was added, and the mixture was stirred for a further 5 min. Finally, 50 mL of hexane/diethyl ether (1:1 v/v) was added and, after 10 min of stirring, 35 mL of the organic layer was removed and evaporated under vacuum by a Büchi Rotavapor R-114 (temperature not greater than 40 °C). The residue was treated with ethanolic pyrogallol (10 mL of a solution 2% w/v) and KOH (6 mL of a solution 25% w/v). After 30 min at 80 °C, the solution was cooled, and 20 mL of water and 40 mL of cyclohexane were added therein. The mixture was centrifuged at 6500 rpm for 5 min at 4 °C. Part of the organic layer (30 mL) was removed, and the solvent was evaporated by rotavapor. Dry samples were stored at -20 °C.

3.2.3 Sterol extraction from experimental wines

The extraction process consisted in transferring 50 mL of experimental wine to a separatory funnel and in adding 0.5 mL of a solution of 1 mg mL^{-1} cholesterol in ethanol as internal standards. Sterols were extracted by shaking, with three successive 50 mL portions of $CHCl_3$, in a separatory funnel. In order to break down the water- chloroform emulsion, when it occurred, the mixtures were centrifuged for 5 min at 5000 rpm to completely separate the chloroformic and aqueous phases. The organic phase was transferred to a rotary evaporator flask at 30°C for concentrating to about 1 mL and, after the addition of an opportune amount of anhydrous Na_2SO_4, it was transferred into a screw-capped tube washing with approximately 1 mL of $CHCl_3$. The extracts were dried under a nitrogen flux (Cocito and Delfini, 1994).

3.2.4 Solid phase extraction of phytosterols

Sep-pak Vac (1 g) silica cartridge for SPE were purchased from Waters (Milford, USA). The SPE cartridge was conditioned with 15mL of hexane. Each sample extract was dissolved in 500 μL hexane-2-propanol (98:2, v/v) mixture and applied on the cartridge equilibrated with 15 mL of the same mixture. The sterol fraction was eluted with hexane-2-propanol mixtures of increasing polarity: 5 mL of 98:2 (v/v), 3,5 mL of 96:4 (v/v). The eluates were collected, joined together and dried under vacuum by a rotavapor.

3.2.5 Gas chromatographic (GC) analysis of phytosterols

Dry samples obtained were analyzed by GC to determine the content of sterols. Standards of the principal phytosterols (campesterol, stigmasterol, and *β*-sitosterol) and cholesterol were transformed into the corresponding trimethylsilyl ethers, according to the following general procedure: 1 mg of a single sterol and 0.5 mL of a mixture of bis(trimethylsilyl) trifluoroacetamide (BSTFA, Aldrich) and dry pyridine (1:1 v/v) were left for 2 h at 60°C, the reaction mixture was directly analysed by GC utilizing a Dani 1000 instrument equipped with a fused silica capillary column WCOT-CP-Sil-5 CB (Chrompack, 25 m x 0.32 mm ID, film 0.11 μm) and a FID detector (hydrogen 0.9, air 1.0 and nitrogen 1.0 bar) The running conditions were: carrier helium (0.9 bar); injector 300°C, detector 320°C, oven 180°C (4 min) then to 300°C at 5°C min^{-1}; injection volume 2 μL. Detector signal output was monitored by a computer and all chromatograms and data were generated and processed by

Dani Data Station version 1.7 software. The structures of the sterols were confirmed by injection of the same samples in a Dani 3800 gas chromatograph equipped with the same capillary column and connected to a VG 7070 EQ mass spectrometer. The obtained spectra were compared with literature data. The extracts were treated and analyzed with the same procedure previously described for the standards.

3.3 Results and discussion

3.3.1 Microvinificates

Standard analyses on Groppello experimental wines produced from grapes treated with elicitors or conventional fungicides in 2009 and 2010 are reported in table 2.

Table 2. Standard analysis on microvinificates obtained from red grapes (*Vitis vinifera* L. cv. Groppello) treated with different agrochemicals.

	Ethanol[A] (%, v/v)	Reducing sugars[A] (g L^{-1})	Total titratable acidity[A] (g tartaric acid L^{-1})	Total volatile acidity[A] (g acetic acid L^{-1})	pH[A]	Malic acid[A] (g L^{-1})	Total extract[A] (g L^{-1})	Total polyphenols[B] (mg L^{-1})
Vintage 2009								
Control	10.8	0.9	6.3	0.46	3.48	2.6	23.1	1907 ± 7
CHT	11.1	1.0	6.3	0.50	3.54	2.5	24.9	2473 ± 16
CHT/Cu	10.7	1.6	5.3	0.62	3.62	2.7	23.4	2038 ± 15
BTH	9.7	1.8	5.2	0.59	3.64	1.5	20.7	1549 ± 40
Conventional fungicides	11.3	1.6	6.4	0.53	3.48	2.4	21.1	1491 ± 23
Vintage 2010								
Control	11.9	< 2	6.4	0.42	3.46	3.08	26.8	1591 ± 29
CHT	10.7	< 2	6.6	0.46	3.54	3.57	27.4	1673 ± 9
CHT/Cu	11.9	< 2	6.3	0.42	3.51	3.10	26.5	1669 ± 10
BTH	11.4	< 2	6.5	0.47	3.50	3.45	26.1	1472 ± 7
Conventional fungicides	12.4	< 2	6.6	0.38	3.39	2.83	25.5	1635 ± 11

[A]these parameters were determined by the Fourier Transform Infrared-Spectroscopy (FTIR); [B]total polyphenols were measured by the Folin-Ciocalteu colorimetric assay; results are means ± standard deviation of three independent extractions each analysed in triplicate.

3.3.2 Phytosterols

Phytosterols are members of the triterpene family of isoprenoids. Phytosterols have been receiving particular attention because of their capability to lower serum cholesterol levels in human, resulting in significant reduction in the risk of cardiovascular disease (Pattel and Thompson, 2006). Furthermore, they are also regarded as a kind of natural products with anti-inflammatory (Bouic, 2002), anti-bacterial (Zhao et al., 2005), anti-oxidant (van Rensburg et al.,2002,) and anti-cancer properties (Awad et al., 2000). It is known there are more than 200 phytosterols in nature (Laakso, 2005), but the more abundant are *β*-sitosterol, stigmasterol and campesterol. The plant sterol content may vary depending on many factors, such as genetic background, growing conditions, tissue maturity and postharvest changes (Piironen et al., 2003). A detailed analysis of the sterol content in grapevine products (berries and wine) was reported, for the first time, in this work. The table 3 shows the phytosterol amounts in all berry (flesh and skin) and seed tissues from Groppello cultivar, at two different phenological stages, pre-*véraison* and *véraison*, and in two years (vintage 2009 and 2010). In general, *β*-sitosterol was the main component in the berry tissues, with the highest levels in the flesh, followed by skin and seeds, respectively (Fig. 1). According to a previous work (Bauman, 1977), the sterol content decreased during ripening, with values less than half. Our results also showed the same trend in fleshes (Fig. 1). About seeds, it is similar only for *β*-sitosterol and campesterol (Fig. 1-2-3). Stigmasterol and campesterol were present in very low quantities in both growth stages (Fig.2-3). With regards to the effects of plant resistance inducers on *β*-sitosterol content, the most effective treatments in seeds were CHT and BTH in pre-*véraison* and in *véraison*, respectively, in 2009, only CHT in the two phenological stages in 2010. In fleshes and skins, the most powerful treatment was CHT/Cu at *véraison*, in 2009, and at pre-*véraison*, in the 2010. In fleshes, at *véraison* 2010, the highest levels of *β*-sitosterol were recorded in BTH-treated samples (Tab. 3).
We observed the same trend in seeds for stigmasterol (Tab.3), while in skins the most efficient treatments were CHT at *véraison* 2009/2010 and BTH at pre-*véraison* 2009. In flesh, CHT and CHT/Cu were effective respectively at *véraison* 2009 and in both phenological stages in 2010 (Tab. 3). Moreover, in 2009, CHT was the best treatment both in seeds and in fleshes for campesterol. Finally, at pre-*véraison* 2010, CHT/Cu and CHT increased the campesterol content: the first in seeds and in fleshes, and the last in skins. At *véraison,* in the vintage 2010, all tissues treated with the plant activators (CHT/Cu, BTH and CHT) had the highest level of campesterol (Tab. 3).

The figure 4 shows the β-sitosterol content found in Groppello experimental wines vintage 2009 and 2010 produced from grapes treated with plant resistance inducers compared to microvinificates obtained from untreated sample and conventional fungicide-treated grapes. We detected and identified all the main plant sterol, i.e. campesterol, stigmasterol and β-sitosterol, but only the latter was quantified because the most predominat in all wines.
In general, the data on experimental wines indicated that the treatments with SAR inducers increased the content of plant sterols. In fact, β-sitosterol was more abundant in microvinificates obtained from grapes treated with CHT, CHT/Cu and BTH compared to those from conventional fungicide-treated grapes (Fig. 4).

Table 3. Phytosterol contents in grape seeds, fleshes and skins of Groppello vintage 2009 and 2010 (mg/100 g dry weight). Results are mean ± standard error of three independent extractions each analysed in triplicate.

	β-Sitosterol		**Stigmasterol**		**Campesterol**	
			Seeds			
Vintage 2009	pre-*vèraison*	*vèraison*	pre-*vèraison*	*vèraison*	pre-*vèraison*	*vèraison*
CTRL	100.30±3.03	72.43±0.39	7.48±0.80	8.25±0.11	10.80±0.37	6.37±0.05
CHT	**112.25±3.07**	70.58±2.01	**9.52±0.42**	8.88±0.38	**12.20±0.42**	**6.62±0.14**
CHT/Cu	92.96±1.97	64.62±1.43	8.21±0.16	8.89±0.32	10.67±0.23	5.72±0.17
BTH	86.36±0.67	**72.43±0.38**	8.12±0.38	**9.27±0.38**	10.37±0.24	6.20±0.11
Fungicides	86.03±0.94	62.53±0.20	8.29±0.14	7.06±0.17	10.19±0.24	4.62±0.14
			Fleshes			
CTRL	289.00±8.20	53.30±2.15	10.14±0.76	2.65±0.56	16.05±0.79	2.79±0.75
CHT	262.97±4.45	52.91±0.28	8.19±0.14	**2.68±0.06**	15.04±0.21	**3.80±0.64**
CHT/Cu	262.24±8.55	**60.25±3.74**	8.37±0.27	2.50±0.22	14.9±0.27	2.69±0.20
BTH	282.59±6.30	59.38±2.02	8.70±0.44	2.31±0.42	14.84±0.28	2.87±0.19
Fungicides	282.63±2.99	46.79±1.12	8.87±0.42	1.89±0.38	15.23±0.41	2.29±0.56
			Skins			
CTRL	170.61±2.38	73.67±2.23	10.95±0.44	2.77±0.18	11.05±0.46	4.05±0.27
CHT	179.32±3.74	75.45±1.18	10.86±0.64	**3.18±0.19**	10.81±0.11	3.91±0.09
CHT/Cu	172.59±2.62	**79.76±3.98**	10.72±0.41	3.04±0.20	10.81±1.30	3.85±0.14
BTH	169.99±2.91	77.38±1.48	**11.52±0.37**	3.019±0.19	10.45±0.07	3.74±0.11
Fungicides	183.34±4.90	71.55±1.37	10.55±0.21	2.14±0.13	10.10±0.49	3.80±0.33

Sample	**β-Sitosterol**		**Stigmasterol**		**Campesterol**	
			Seeds			
Vintage 2010	pre-*vèraison*	*vèraison*	pre-*vèraison*	*vèraison*	pre-*vèraison*	*vèraison*
CTRL	120.91±1.18	77.47±2.06	10.55±0.29	9.93±0.23	13.77±0.23	7.52±0.08
CHT	**140.91±3.21**	**81.72±1.40**	**12.40±0.31**	**11.58±0.17**	**15.82±0.25**	7.50±0.10
CHT/Cu	107.53±4.12	79.25±2.21	9.94±0.74	10.49±0.11	12.04±0.34	**9.13±1.08**
BTH	108.07±2.88	70.20±2.63	9.20±0.33	9.41±0.30	11.27±0.37	6.42±0.15
Fungicides	116.23±2.16	66.88±0.28	9.59±0.29	8.50±0.03	13.09±0.15	5.49±0.04
			Fleshes			
CTRL	285.65±4.32	64.53±0.28	10.06±0.31	2.34±0.22	15.41±0.33	3.28±0.06
CHT	283.96±6.86	67.75±1.31	10.11±0.37	2.05±0.04	15.22±0.55	3.38±0.07
CHT/Cu	**311.65±3.60**	69.12±0.76	**12.19±0.52**	**2.35±0.15**	**17.94±0.32**	2.73±0.10
BTH	251.28±0.80	**69.47±0.32**	9.68±0.49	2.21±0.09	14.07±0.33	**3.54±0.14**
Fungicides	264.35±0.20	56.65±1.94	10.03±0.46	1.67±0.10	13.39±0.44	2.69±0.08
			Skins			
CTRL	244.63±6.80	97.99±2.94	16.33±0.30	4.63±0.23	12.11±0.28	5.49±0.43
CHT	212.84±3.08	**106.18±2.26**	14.27±0.70	**5.89±0.43**	12.05±0.29	**5.53±0.40**
CHT/Cu	**252.76±1.33**	92.97±2.65	16.21±0.22	3.81±0.06	**13.93±0.28**	4.70±0.13
BTH	214.06±6.06	95.14±4.48	14.60±0.59	4.78±0.50	11.76±0.92	4.86±0.25
Fungicides	231.53±10.25	96.16±0.72	16.30±0.59	3.58±0.04	12.54±0.41	5.15±0.24

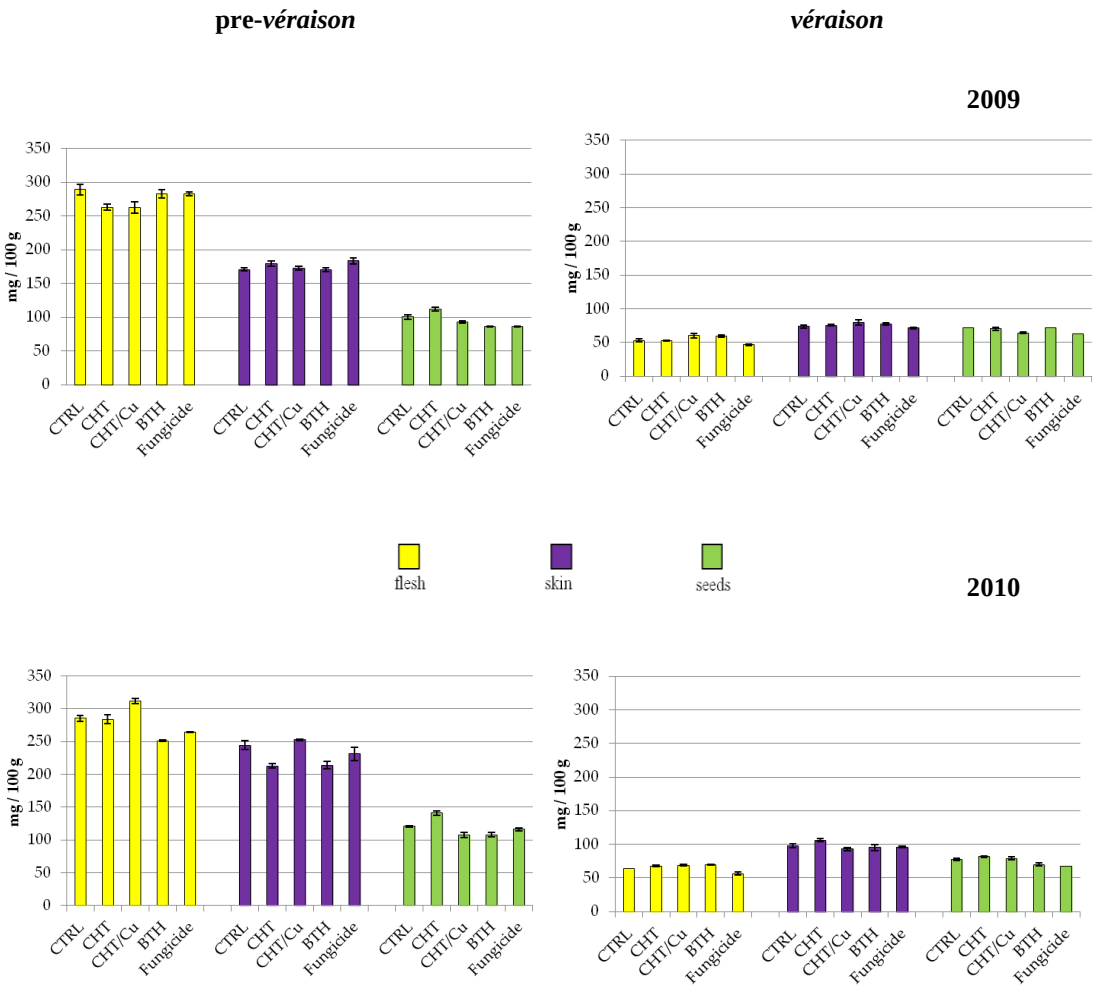

Figure 1. *β*-sitosterol contents in grape fleshes, skins and seeds of Groppello vintage 2009 and 2010 (mg/100 g dry weight) and in two phenological stage, pre-*véraison* and *véraison*. Results are mean ± standard error of three independent extractions each analysed in triplicate.

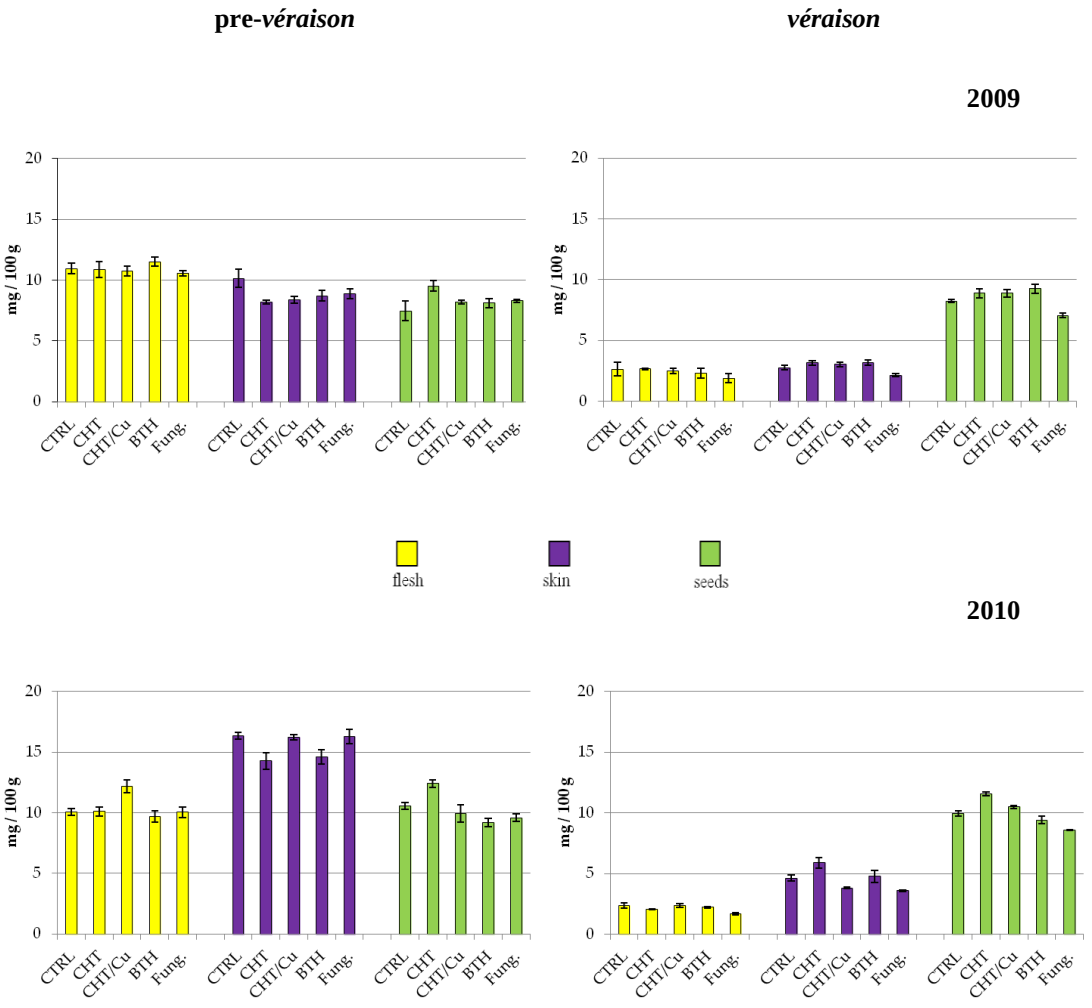

Figure 2. Stigmasterol contents in grape fleshes, skins and seeds of Groppello vintage 2009 and 2010 (mg/100 g dry weight) and in two phenological stage, pre-*véraison* and *véraison*. Results are mean ± standard error of three independent extractions each analysed in triplicate.

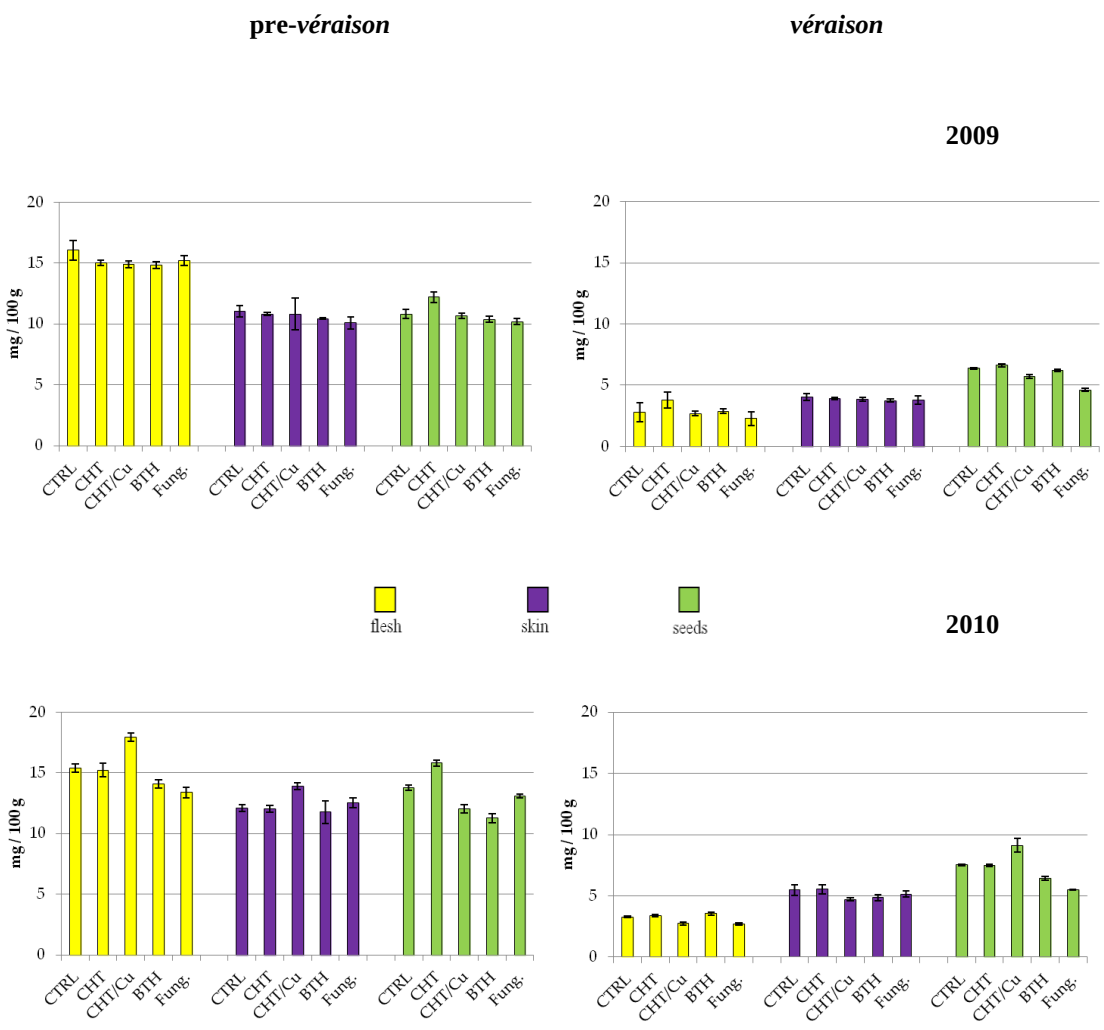

Figure 3. Campesterol contents in grape fleshes, skins and seeds of Groppello vintage 2009 and 2010 (mg/100 g dry weight) and in two phenological stage, pre-*véraison* and *véraison*. Results are mean ± standard error of three independent extractions each analysed in triplicate.

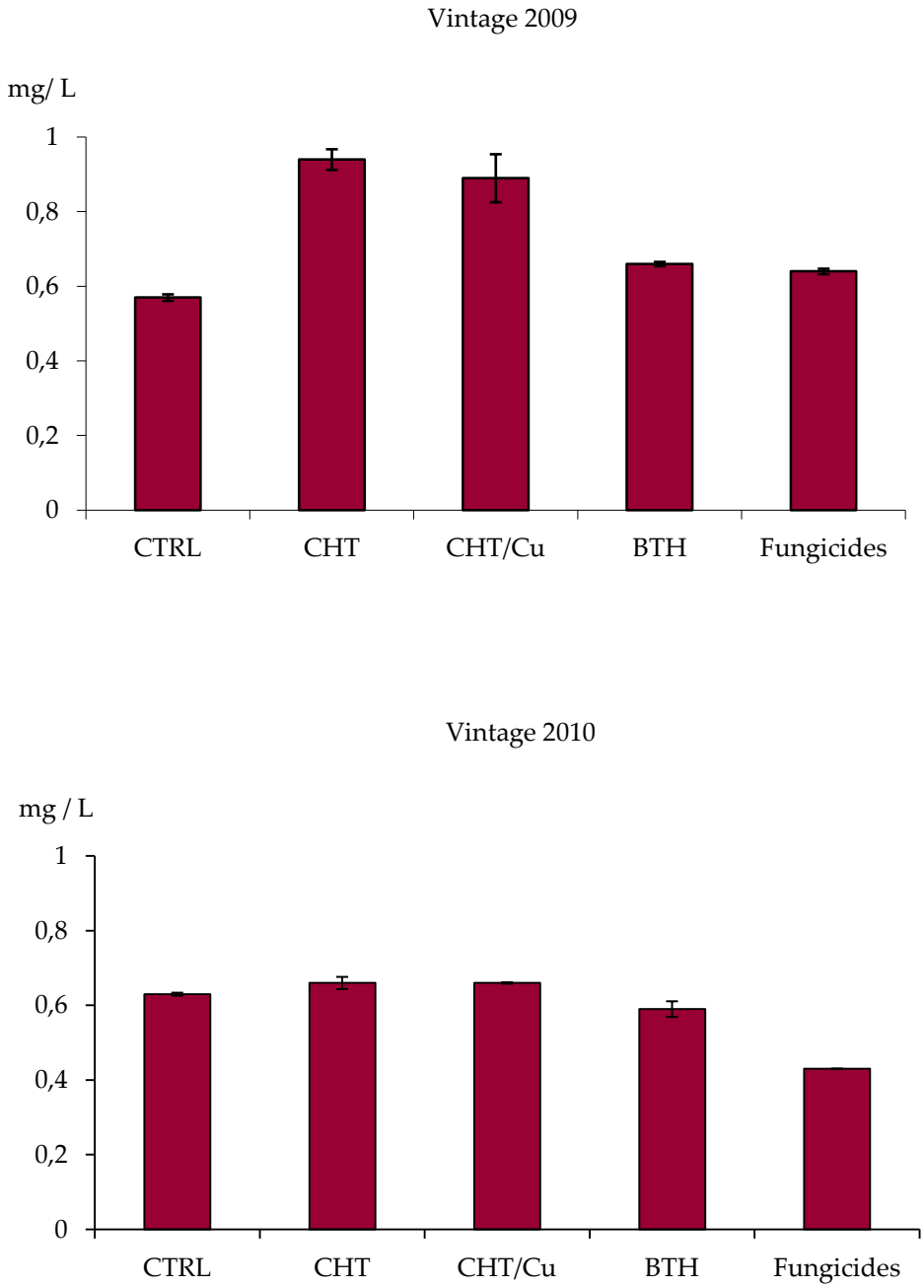

Figure 4. *β*-sitosterol contents in experimental wines of Groppello vintage 2009 and 2010 (mgl/L). Results are mean ± standard error of three independent extractions each analysed in triplicate.

3.4 Conclusion

As previously showed, our results convincingly demonstrated that open field treatments with plant activators may enhance the phytosterol content of berry tissues, and, more interestingly, this effect seems to be retained in the experimental wines. In these terms, plant resistance inducers may improve the healthy potential of red wine, by virtue of the biological activities of plant sterols. Therefore, the occurrence of phytosterol in wine, reported for the first time, may add a new element in the already complex grape chemistry, probably contributing to explain the plethora of healthy properties ascribed to this beverage and mainly attributed to polyphenolic compounds. It would be of great interest to verify whether the health benefits promoted by the moderate, regular red wine consumption at main meals may be, at least in part, due to phytosterols, possibly acting synergistically with the other hundreds of bioactive metabolites present in grapes. Finally, pharmacokinetic data are necessary to evaluate their bioavailability and metabolism in humans.

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3.6 Effects of plant activators on aroma compounds of Groppello wine

Volatile aroma compounds are important factors that affect grape flavour and quality and also contribute to the organoleptic character of wine. In this study, we report, for the first time, the effects of plant activators on red wine volatile aromatic compounds.

3.7 Materials and methods

3.7.1 Experimental

Aliquot of 30 mL were transferred in a 50 mL glass container and placed in a thermostatic bath kept a 25°C to prevent alteration of the volatiles. Gas extraction was performed by passing 0.8 L of pure He at a rate of 100 mL min^{-1} into the glass container. The gas inlet was located at 0.5 cm from the bottom of the extraction vessel. The outlet of the extraction system was connected to a sampling two-stage trap consisting of a glass tube filled with 0.120 g of of Tenax TA® (Restek Corp., USA), 20/35 mesh. The adsorbent tubes measured 160 mm long, have an external diameter of 6 mm and an internal diameter of 3 mm. Before sample collection, the traps were cleaned by passing a stream of ultrapure dry N_2 at a flow rate of 100 mL min^{-1} and under heating up to 250°C for 3 h. After sampling, sample was passed through the tube to eliminate any moisture and excess of ethanol. In order to verify possible environmental contamination, blank analyses of headspace of the empty glass container were carried out following the same procedure. To assess the presence of carry-over effects, the adsorbent trap was also desorbed before and after each entire sampling procedure. Cartridges were stored in a refrigerator at 4°C until analysis.

3.7.2 CG-MS Analysis

Volatile organic compounds (VOC) retained on the adsorption traps were thermally desorbed at 250°C and released compounds focused in a fused silica liner kept at -150°C. The concentrated sample was then injected into the chromatographic column by raising the temperature of the liner up to 230°C in 1 min. The thermal desorption, cryo-focusing and injection steps were carried out using a Thermal Desorption Cold Trap Injector (Chrompack, Middleburg, The Netherlands) connected to a 5890 gas chromatograph (Hewlett Packard,

Palo Alto, CA, USA) and using a 5970 quadrupole mass spectrometer (Hewlett Packard) as detection system. VOC, transferred to the capillary column (60 m x 0.25 mm I.D.; 0.25 μm film of polymethylsiloxane; HP1, Hewlett Packard) by heating the fused silica liner to 230°C, were separated maintaining the oven temperature at 40°C for 10 min and programming to 220°C at 5°C min^{-1}. The ionization of the samples was achieved at 70 eV under the full-scan (SCAN) mode. The mass range studied was from 30 to 350 m/z. Terpene compounds were recorded in the selective ion monitoring (SIM) mode. The identity of the compounds of interest was achieved by comparison of their retention time and mass spectra with those provided by authentic standards and by the Wiley 275 library. Quantification of VOC and terpenes was performed using multilevel calibration curves, calculating the response factors for each compound and using d_{14}-cymene as internal standard (ISTD). Authentic standard compounds were supplied by Aldrich Chemical Co. (USA). For quantification of monoterpenes the comparison of the response for the m/z 93 ion was generally used, while m/z 130 for the internal standard. Known aliquots of d14-cymene were added to the sampling traps just before starting collection, allowing an overall normalization of the analytical system (sampling and MS response). For compounds which commercial standards were not available, the response factors of compounds with similar chemical structure were used. The concentration of each compound is calculated after subtracting the average amount found in the headspace of empty glass container at the same sampling time and is expressed in relation to mL of wine at sampling time.

3.8 Results and discussion

Aroma is an important factor in quality control and quality assurance of foods, but, in wine, this trait is possibly the most important. It is produced by a complex balance of more than 800 volatile compounds in different ranges of concentrations, and with different volatilities and polarities. These compounds have differentiated origins, from grapes (varietal aroma), from alcoholic fermentation under anaerobic conditions (fermentative aroma) and the *bouquet*, which results from the transformation of the aroma during ageing.

Acetals are tipically produced during aging and distillation. They are formed when an aldehyde reacts with the hydroxyl groups of two alchohols. Acetals, mainly 1,1-diethoxy-ethane, usually contribute fruity or green aroma characteristics. The highest concentrations of acetals were detected in CHT/Cu microvinificates (Tab. 4).

Higher alcohols are one of the quantitatively most important fractions. They are released as secondary products of the metabolism of yeasts and are responsible for the fermentative aroma of wines. In Groppello wines, this group was mainly composed of 2-methyl-1-propanol and 3-methyl-1-butanol (the alcohol detected at the highest relative concentration). The main amounts of alcohols were detected in the wine from CHT-treated grapes (Tab.4).
Most esters found in alcoholic beverages are secondary metabolites produced by *Saccharomyces cerevisiae* during fermentation. Among the esters, total acetates were the most abundant. They result from the reaction of acetyl-CoA with higher alcohols. BTH treatment rised the levels of all esters in wines: acetates, butanoates, propionates and aliphatic esters (Tab. 4). Elicitors had no effects on aldehydes and ketones.
Terpenes are considered to be related to the grapevine variety and are important for the expression of varietal characteristics in wine. Terpenes have low olfactory thresholds and are generally associated with floral aromas. In this study limonene, linalool and α-terpinolene were detected, but their relative concentrations were not very high. CHT/Cu was the most effective treatment in increasing the terpene linalool (Tab. 4). The analyses on experimental wines from vintage 2010 are still in progress.

Table 4. Volatile compounds detected in experimental wines[A] obtained from red grapes (*Vitis vinifera* L. cv. Groppello) treated with different agrochemicals during the phytoiatric campaigns planned in 2009.

Class	Compound	Concentration (μg L^{-1})			
		Conventional fungicides	BTH	CHT	CHT/Cu
Acetals	1-methoxy-ethoxy-ethane	ND	0.044 ± 0.024a[G]	0.011 ± 0.011b	0.034 ± 0.034a
	1,1-diethoxy-ethane	2.227 ± 0.527a	8.121 ± 0.974b	9.008 ± 0.215c	12.176 ± 0.533d
	1-ethoxy-1-pentoxyethane	0.471 ± 0.175a	1.718 ± 0.275b	2.033 ± 0.476bc	2.501 ± 1.061c
	1,1-di(1-methyl-butoxy)-ethane	0.241 ± 0.087a	0.659 ± 0.164b	0.935 ± 0.251c	1.116 ± 0.671c
Total acetals		2.939 ± 0.791a	10.541 ± 0.559b	11.986 ± 0.951c	15.828 ± 1.163d
Alcohols	2-methyl-1-propanol (isobutyl alcohol)	6.359 ± 0.151a	4.597 ± 0.082b	4.917 ± 0.035c	3.554 ± 0.636d

		1-butanol	0.097 ± 0.013a	0.072 ± 0.015b	0.647 ± 0.127c	0.078 ± 0.002b
		3-methyl-1-butanol (isoamyl alcohol)	43.479 ± 2.056a	46.602 ± 1.461b	48.938 ± 2.946c	33.189 ± 6.053d
		2-tetralol (1,2,3,4-tetrahydronaphthalen-1-ol)	0.044 ± 0.044a	0.087 ± 0.045b	0.032 ± 0.027a	ND
		1-octanol	ND	ND	ND	0.003 ± 0.003
Total alcohols			49.981 ± 2.266a	51.359 ± 1.482a	54.501 ± 2.845b	36.825 ± 6.689c
Aldehydes		3-methyl butanal	0.091 ± 0.021a	0.168 ± 0.019b	0.129 ± 0.008c	0.182 ± 0.033b
		2-methyl butanal	0.029 ± 0.001a	0.033 ± 0.001b	0.017 ± 0.007c	0.028 ± 0.001d
		benzaldehyde	ND	ND	ND	ND
		4-methyl benzaldehyde (*p*-tolualdehyde)	0.232 ± 0.061	ND	ND	ND
Total aldehydes			0.353 ± 0.084a	0.201 ± 0.019b	0.146 ± 0.015c	0.211 ± 0.032b
Esters	Acetates	ethyl acetate	32.709 ± 1.065a	44.709 ± 1.754b	19.805 ± 5.097c	20.931 ± 6.094c
		ethanethioic acid S-methyl ester (S-methyl thioacetate)	0.149 ± 0.028a	0.220 ± 0.051b	0.086 ± 0.064c	0.044 ± 0.044c
		propyl acetate	0.634 ± 0.044a	0.710 ± 0.126a	0.182 ± 0.072b	0.201 ± 0.057b
		3-methyl butyl acetate (iso-amylacetate)	6.979 ± 0.636a	8.827 ± 0.864bc	7.767 ± 1.986ac	3.569 ± 1.965d
		2-methylbutylacetate	1.486 ± 0.603a	1.818 ± 0.341ab	2.051 ± 0.486bc	1.528 ± 1.149ac
		hexyl acetate	0.051 ± 0.011a	0.082 ± 0.011b	0.066 ± 0.031ab	0.026 ± 0.011c
	Total acetates		42.007 ± 2.298a	56.368 ± 2.792b	29.958 ± 6.763c	26.301 ± 9.232c
	Butyrates	ethyl butyrate	1.127 ± 0.158a	1.256 ± 0.201a	0.761 ± 0.136b	0.466 ± 0.243c

		ethyl 2-methyl butyrate	0.457 ± 0.058a	0.263 ± 0.038b	0.194 ± 0.078c	0.155 ± 0.061c
		ethyl-3-methyl butyrate (ethyl isovalerate)	0.465 ± 0.053a	0.306 ± 0.043b	0.274 ± 0.108b	0.128 ± 0.063c
		diethyl butyrate (diethyl succinate)	0.119 ± 0.001a	0.374 ± 0.015b	0.299 ± 0.012c	0.206 ± 0.052d
	Total butyrates		2.169 ± 0.047a	2.201 ± 0.271a	0.1529 ± 0.335b	0.955 ± 0.316c
	Propionates	ethyl propionate	2.638 ± 0.423a	2.515 ± 0.136a	1.382 ± 0.362b	0.907 ± 0.286c
		ethyl lactate (lactic acid ethyl ester, ethyl 2-hydroxypropionate)	1.455 ± 0.011a	7.467 ± 0.137b	4.987 ± 0.247c	3.319 ± 1.026d
	Total propionates		4.093 ± 0.434a	9.982 ± 0.001b	6.369 ± 0.609c	4.227 ± 0.740a
	Alkans	ethyl hexanoate (ethyl caproate)	1.672 ± 0.258a	2.761 ± 0.326b	1.957 ± 0.654a	1.223 ± 0.123 c
		ethyl 3-hexenoate	0.029 ± 0.007a	0.048 ± 0.001b	0.042 ± 0.012b	0.011 ± 0.011c
		ethyl heptanoate	0.019 ± 0.002a	0.027 ± 0.001b	0.36 ± 0.017b	0.024 ± 0.002c
		methyl octanoate	0.005 ± 0.002a	0.008 ± 0.002b	0.005 ± 0.001a	0.002 ± 0.002c
		methyl salicylate (2-hydroxybenzoic acid methyl ester)	ND	ND	ND	ND
		ethyl octanoate	0.847 ± 0.092a	1.229 ± 0.134b	0.828 ± 0.351ac	0.648 ± 0.089c
		ethyl nonanoate	0.001 ± 0.001a	ND	ND	0.002 ± 0.002a
		ethyl decanoate	0.018 ± 0.001a	0.187 ± 0.160b	0.027 ± 0.018a	0.011 ± 0.005c
	Total alkans		2.593 ± 0.354a	4.262 ± 0.624b	2.897 ± 1.055a	1.921 ± 0.231c
Total esters			50.864 ± 3.040a	72.813 ± 3.689b	40.754 ± 8.764c	33.404 ± 9.038 c
Ketones		2-pentanone	0.160 ± 0.009a	0.149 ± 0.023ac	0.161 ± 0.024a	0.136 ± 0.016bc
		2,3-pentanedione	0.495 ±	ND	ND	0.083 ±

		0.078a			0.083b
	4-methyl 2-heptanone	0.034 ± 0.034acd	0.089 ± 0.009b	0.056 ± 0.012c	0.038 ± 0.005d
	beta-damascenone	0.008 ± 0.001a	0.012 ± 0.003b	0.023 ± 0.004c	0.008 ± 0.001 a
Total ketones		0.697 ± 0.053a	0.251 ± 0.029be	0.241 ± 0.041ce	0.266 ± 0.061de
Terpenes	limonene	ND	ND	ND	0.009 ± 0.001
	linalool	0.008 ± 0.001a	0.009 ± 0.003a	0.008 ± 0.003a	0.007 ± 0.004a
	alpha-terpinolene	0.026 ± 0.004a	0.018 ± 0.001b	0.002 ± 0.002c	0.004 ± 0.004c
Total terpenes		0.034 ± 0.004a	0.027 ± 0.002b	0.011 ± 0.001c	0.021 ± 0.009d
Miscellaneous	1,3-di-tert-butyl benzene	0.031 ± 0.001a	0.035 ± 0.013b	0.018 ± 0.013c	0.019 ± 0.003c
Total		104.897± 6.238a	135.22± 4.613b	107.65± 12.632a	86.574 ± 16.803c

ND: not detectable; results are mean ± standard error of three independent extractions each analysed in triplicate, and different letters within the same row indicate means significantly different at $p < 0.05$ (Fisher's least significant difference test).

3.9 Conclusions

In general, open field treatments with plant activators increased the levels of volatile aroma compounds in microvinificates. Certainly, the study of the molecular processes involved in elicitation are beyond the scope of this study, though the mechanism of action of BTH and CHT was extensively investigated in other plant models, as reported in the Introduction. In any case, to gain a more widespread acceptance of induced resistance in crop protection, it will be necessary to invest further in basic research. Studies on genome, transcriptome and, especially, proteome and metabolome analyses should provide further information on the complex mechanisms involved in SAR and responsible for the elicitation of volatile aroma compounds. Otherwise, applied research on the SAR expression, through studies on the effects of environmental factors and agronomic practices should contribute to explain the variable effectiveness of SAR in the field, which is the main limitation in the commercial sxploitation of SAR.

3.10 Mycotoxin detection in experimental wines treated with plant activators

Mycotoxins are fungal secondary metabolites widely distributed in nature, which can be detected in soil and many food plants. They are synthesised in particular growth conditions by some genera of toxigenic fungi (*Aspergillus*, *Penicillum*, *Fusarium*), as a result of field or post-harvest infections, and many important plant and animal foods are susceptible of contamination (flours, pasta, wine, fruit juices, coffee, milk, yoghurt and cheese). The most important groups of mycotoxins include aflatoxins, ochratoxins, and *Fusarium* toxins, the latter including fumonisins, trichothecenes and zealarenone, highly toxic because of their carcinogenicity, nephrotoxicity and hepatoxicity (table 5). They can be introduced in the consumers organism by contaminated foodstuffs, and intoxication caused by mycotoxins are defined mycotoxicoses (Steyn, 1995; Hussein and Brasel, 2001).

Table 5. Producing fungi, occurrence in food and toxic effects of the main mycotoxins

Group	Compounds	Toxin-producing fungi	Occurrence	Toxic effects
Aflatoxins	B_1, B_2, G_1, G_2, M_1, M_2	*Aspergillus flavus, A. parasiticus, A. nomius*	oily seeds, corn, cereals, soya, spices, milk, milk products	carcinogen, hepatotoxic, immunosupressive
Ochratoxins	A, B, C	*Penicillium verrucosum Aspergillus* species	cereals, coffee, cacao- and soya bean, grapes, wine	carcinogen, teratogen, nephrotoxic, immunosupressive, neurotoxic
Patulin		*Aspergillus* and *Penicillium* species	apple, apple juice, other fruits,vegetables	enzyme inhibition, genotoxic, oedema inducing
Fusarium toxins: Trichothecenes	T-2 and HT-2 toxin, deoxynivalenol	*Fusarium* species	cereals (wheat, barley, ryes, oats, rice), cereal products	protein synthesis inhibition, hemato- and immunotoxic necrotizing
Zearalenone	F-2 toxin	*Fusarium* species	cereals (corn, wheat, barley, rice)	oestrogenic effect, fertility disturbances, infertility, damaging of spermatogenesis

Fumonisins	B_1, B_2, B_3	*Fusarium moniliforme*, other *Fusarium* species	corn and corn-based foodstuffs	nephro- and hepatotoxic, pulmonary oedema, encephalomalacy, oesophagus and liver carcinoma

The main mycotoxin of concern in grape is ochratoxin A (OTA), produced by the black *Aspergillus* spp. (*Aspergillus* section *Nigri*). In particular, it is produced primarily when *A. carbonarius* infects berries before harvest, whereas the relatively few toxigenic strains of the related species, *A. niger*, may also contribute to contamination, as the latter is by far the most common species of *Aspergillus* present on grapes. Toxigenic isolates of *A. ochraceus* have only occasionally been isolated from grapes (Varga and Kozakiewicz, 2006; Hocking et al., 2007). OTA structure consists of a dihydrocoumarin moiety linked to a molecule of L-β-phenylalanine via an amide bond (figure 5). Its IUPAC name is *N*-{[(3*R*)-5-chloro-8-hydroxy-3-methyl-1-oxo-3,4-dihydro-1*H*-2benzopyran -7-yl]carbonyl}-L-phenylalanine.

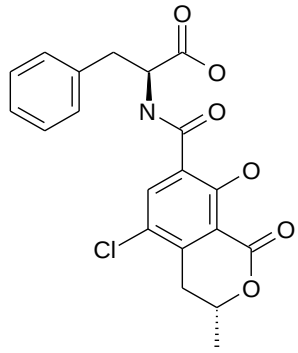

Figure 5. Chemical structure of ochratoxin A (OTA)

The aflatoxigenic species, *Aspergillus flavus* and *A. parasiticus*, have also occasionally been isolated from grapes. Moreover, *Trichothecium roseum* growing on grapes affected by grey rot (*Botrytis cinerea*) is the likely source of mycotoxins such as trichothecin. However, mycotoxins such as these have seldom been detected in wine and other grape products, and are currently of little concern for the grape and wine industries (Varga and Kozakiewicz, 2006; Hocking et al., 2007).

OTA is slowly though relatively well absorbed from the gastrointestinal tract and it is accumulating in the highest concentration in the kidneys, in less quantity in the liver, muscle and fat of animal and human organisms. Therefore, this mycotoxin is nephrotoxic in mammals, causing tubulo-nephrosis and kidney fibrosis. The proximal tubule is the primary site of its cytotoxic and carcinogen effects. Furthermore, it can penetrate the placenta thus resulting embryotoxic and teratogen (EFSA, 2006).
Many environmental and ecological factors may affect both the incidence of *Aspergillus* spp. on grapes and OTA biosynthesis, such as temperature, water activity, susceptibility of grape varieties to infection and toxin production, mechanical damages to berries. In particular, minimising damage to bunches is pivotal in reducing the risk of OTA contamination, which can be achieved through a careful vineyard management, including the control of fungal diseases (e.g. bunch rot caused by *B. cinerea*) by agrochemicals and the use of grape varieties with resistance to rain damage (Varga and Kozakiewicz, 2006; Hocking et al., 2007). Of note, much OTA is removed during vinification at the solid-liquid separation stages. During pressing to separate the wine from skins, 50-80% of the total OTA content originally present in the crushed grapes remains bounded to the discarded skins and seeds (Varga and Kozakiewicz, 2006; Hocking et al., 2007).
The European Community has established the limit of 2 μg/kg (ppb, 2 ng/mL) as the maximum allowable concentration of ochratoxin A in wine, must and grape juice [COMMISSION REGULATION (EC) No 1881/2006]. Starting from April 2006, it will be forbidden to market batches that will not satisfy. A tolerable weekly intake (TWI) of up to 120 ng/kg b.w. was also established (EFSA, 2006).
As previously introduced, because the proper use of agrochemicals is part of a careful vineyard management, in addition to other viticultural practices, we evaluated the level of OTA, aflatoxin B2 (AFB2), G1 (AFG1) and G2 (AFG2) in experimental wines produced with grapes treated with both elicitors and conventional fungicides.

3.11 Materials and Methods

3.11.1. Sample preparation

An aliquot (1 mL) of each wine was mixed with a double volume of ethyl acetate, vortexed for 1 min and allowed to stand till the partition of the two layers. The organic portion (200 *m*L) was dried under nitrogen gas and then

resuspended in 250 *m*L of ethyl acetate. The mixture was dried under nitrogen and, after the addition of methanol (50 *m*L), centrifuged at 12,000 g for 1 min. The supernatant was subjected to analysis.

3.11.2. Chemicals

The standards of OTA, AFB2, AFG1 and AFG2 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol, acetonitrile and formic acid were from Merck (Darmstadt, Germany). Water was obtained from a MilliQ apparatus (Millipore, Milford, MA, USA). The stock solution of each toxin was prepared in methanol and diluted to give standard solutions in the range of 0.25-10 ng/mL. Stock and working solutions were stored at -80°C and -20°C, respectively.

3.11.3. UPLC-MS/MS conditions

The chromatographic system consisted of an UPLC (ultra-performance liquid chromatograph) mod. Acquity (Waters, Milford, MA, USA) coupled to a triple quadrupole mass spectrometer mod. Quattromicro (Waters), equipped with an electrospray ionization (ESI) source and operating in the positive ion mode. A 1.7 μm Kinetex C_{18} column (150 x 2 mm; Phenomenex) was used for the separation at a flow-rate of 0.550 mL min^{-1}. The column was maintained at 60°C and the separation was performed by means of a linear gradient elution (eluent A, 0.1% HCOOH; eluent B, acetonitrile). The capillary voltage was set to 3.50 kV; the source temperature was 130°C; the desolvation temperature was 350°C; and argon was used at 2.5 x 10^{-3} mbar to improve fragmentation in the collision cell. The dwell time was 0.2 sec for AFB2, AFG1 and AFG2, and 0.4 for OTA. Five μL were injected in the UPLC-MS/MS system. Masslynx 4.0 software acquired data with Quan-Optimize option for fragmentation study. The elution gradient was as follows: 90% A and 10% B in 1.0 min, 30% A and 70% B in 10 min, and then 90% A and 10% B for 0.5 min. Multiple reaction monitoring (MRM) was performed using the following precursor-to- fragment transitions: $(m/z)^+$ 404.3→257.5, with cone voltage (CV) and collision energy (CE) at 25 V and 20 eV, respectively, for OTA; 315.3→287.6 (CV 35 V, CE 25 eV) for AFB2; 329.3→243.6 (CV 35 V, CE 25 eV) for AFG1; 331.3→189.6 (CV 40 V, CE 35 eV) for AFG2.

3.12. Results and Discussion

In all the samples, all the tested mycotoxins were detected at a concentration lower than 6 pg/mL (limit of detection, LOD, 0.15 ng/mL). Figure 6 shows the chromatographic profile of a wine extract (sample Groppello CHT 2010), whereas the standards are reported in figure 7. Therefore, all microvinificates contained levels of OTA below the limit of 2 ng/mL previously introduced.
In general, it seems that, all treatments in open field prevent the infection of grapes with toxigenic fungi, or reduce their synthesis of mycotoxins.
To the best of our knowledge, the efficacy of plant activators in reducing the mycotoxin contamination of foodstuffs has been previously investigated only in wheat and barley. In both cereals, CHT treatment significantly reduced the Fusarium head blight disease caused by *Fusarium culmorum*, as well as the contamination of grains with the trichothecene mycotoxin deoxynivalenol, under both glasshouse and field conditions (Khan and Doohan, 2009).

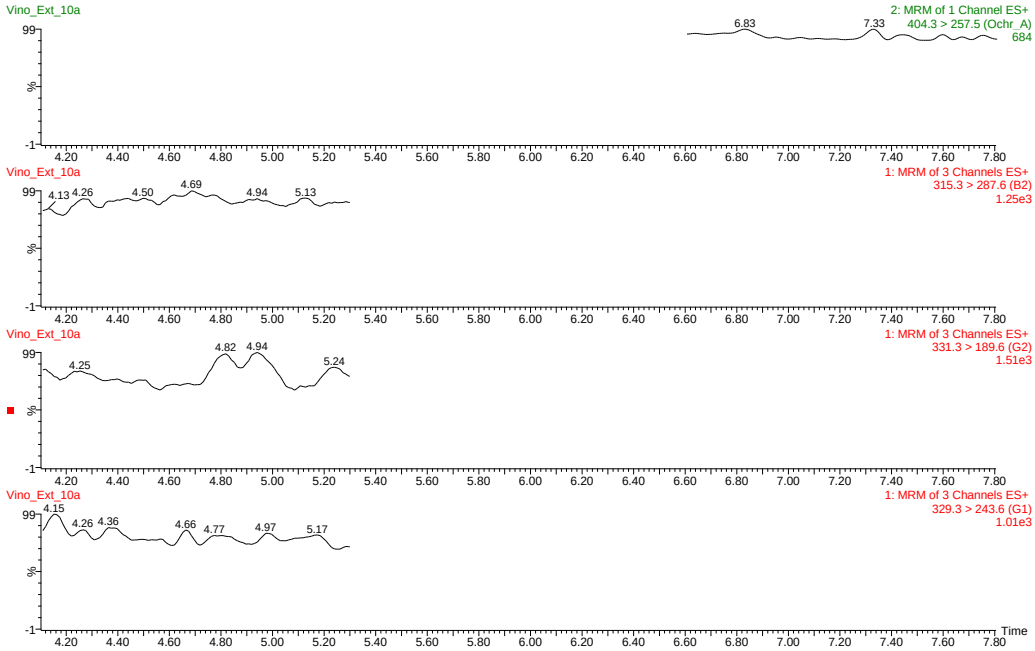

Figure 6. Typical UPLC-MS/MS chromatogram of a wine extract (sample Groppello CHT 2010). The fragmentation transitions were: $(m/z)^+$ 404.3→257.5 for OTA, 315.3→287.6 for AFB2, 329.3→243.6 for AFG1, 331.3→189.6 for AFG2.

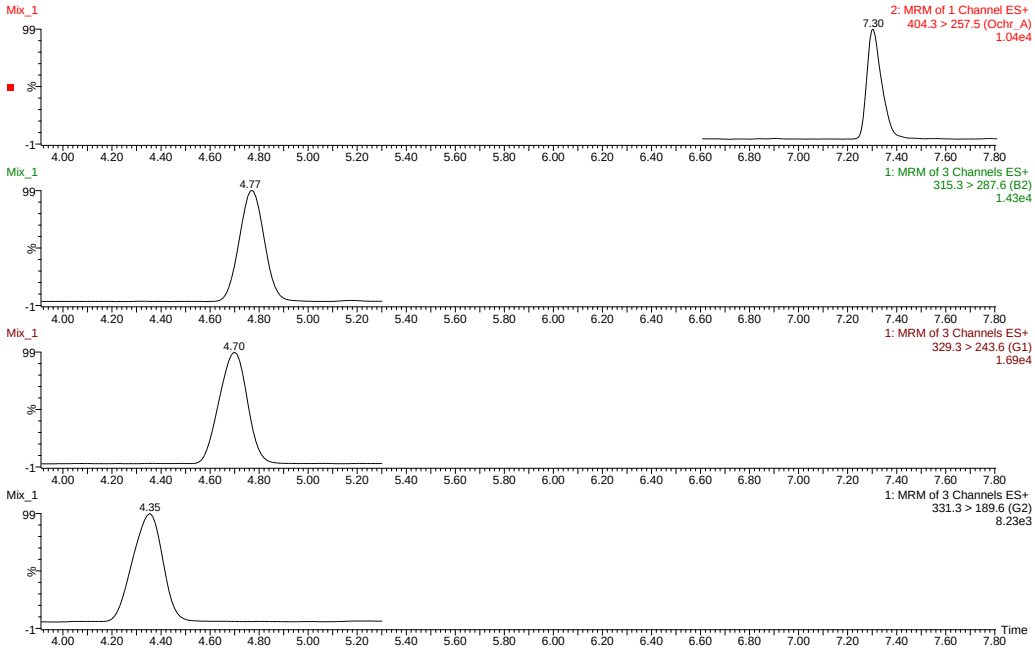

Figure 7. UPLC-MS/MS chromatogram of the mycotoxin standards. The fragmentation transitions were: $(m/z)^+$ 404.3→257.5 for OTA, 315.3→287.6 for AFB2, 329.3→243.6 for AFG1, 331.3→189.6 for AFG2.

3.13. Conclusions

Interestingly, treatments with elicitors did not favour mycotoxin (particularly ochratoxin A) contamination of wines, compared with conventional fungicides, thus suggesting a long lasting efficacy of resistance inducers, able to restrict the colonization of grapes by toxigenic fungal strains both in vineyard and postharvest. Finally, we need to take into account that, from 2014, Integrated Pest Management will be mandatory in all the European Union, and, therefore, it is necessary (or crucial) to consider the use of resistance inducers in crop protection.

3.14. References

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