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EFFECT OF SAR INDUCERS ON GRAPE SECONDARY METABOLITES

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Chapter 1

Summary

Fungicide application is now the most efficacious method for controlling plant diseases caused by oomycetes and fungi. As legislation is limiting and reducing their use, it is strongly stimulating studies for the identification of additional and environmentally friendly approaches in the control of their associated diseases. 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 used. CHT is a natural and low-cost polymer (from the waste products of the crustacean carapace), obtained 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 compound deeply involved in resistance against pathogens. Two different phytoiatric campaigns were planned, in 2009 and 2010, on two red grapevine (*Vitis vinifera* L.) varieties cultivated in experimental vineyards located at distinct sites: Groppello, an autochthonous cultivar of Lombardia, at Raffa di Puegnago (Brescia), and Merlot at Conegliano Veneto (Treviso). 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; whereas, on Merlot, besides CHT and CHT/Cu, BTH were replaced by 300 g hL^{-1} potassium phosphites. Untreated vines were used as negative control, while plants treated with conventional fungicides (penconazole and methyldinocap) were the positive control. In both 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*. The phytosanitary status of vineyards was assessed weekly on leaves and bunches, by visual inspections, though both sites were not particularly predisposed to severe fungal or other epidemics. The epidemiological evaluations were performed on bunches alone and infection indexes were calculated. All the treatments were effective in controlling fungal infections (downy mildew, *Plasmopara viticola* and powdery mildew, *Erysiphe necator*), in

terms of disease incidence (I %), disease severity (S %) and infection degree (ID %), though these indexes were low in untreated control grapevines, particularly in 2010. 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 melatonin levels [by ultra performance liquid chromatography (UPLC)-MS/MS], total polyphenols (TP, by Folin-Ciocalteau assay) and antiradical activity [by DPPH, 2,2-diphenyl-1-pycryl hydrazyl and ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) assay]. Groppello and Merlot experimental wines were produced, by standard microvinification techniques, in the Centro Vitivinicolo Provinciale of Brescia and Centro di Ricerca per la Viticoltura of Conegliano (TV), respectively, and stored at 4 °C in the dark until analyses. Microvinificates were produced from grapes treated with both elicitors and conventional fungicides (controls), and the following analyses, besides those also conducted for all berry tissues, were carried out: tryptophan, serotonin and melatonin detection (by UPLC-MS/MS); content of *cis* and *trans* resveratrol (by UPLC-MS/MS); levels of mycotoxins (manly ochratoxin A, OTA, by UPLC-MS/MS). In general, berry tissues treated with elicitors and the corresponding experimental wines showed higher levels of melatonin and polyphenols, as well as a higher antiradical activity than samples treated with conventional fungicides. High concentrations of tryptophan were detected in all samples, contrary to serotonin, which was not detected. The most effective elictors were CHT/Cu and CHT. In all wines, the level of OTA was below the allowable threshold of 2 ng/mL. To the best of our knowledge, these results represent the first data on the effects of agrochemicals on the melatonin content of red wine, and the presence of melatonin was reported, for the first time, in berry seeds and flash, after its previously detection in skin. Furthermore, the level of the indolamine in berry tissues varies according to the phenological stage, resulting more abundant in seed at pre-*véraison* and in skin at *véraison*. The good agreement between the data obtained in 2009 and 2010 for Groppello cultivar, and, in 2009, between Groppello and Merlot varieties cultivated in different geographical areas, 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. Finally, the role of melatonin, a powerful antioxidant, in grapevine physiology is still somewhat obscure. It is possible that, at pre-*véraison*, when skin anthocyanic pigmentation is still lacking, this compound may defend berry from damage due to photooxidation or UV radiation, whereas, at *véraison*, the indolamine may protect the germ tissues, particularly rich in storage lipids and membranes and vulnerable to

oxidative damage. In conclusion, the possibility of enhancing the pharmaconutritional potential of grape/wine with phytosanitary treatments should be further tajen into account.

Chapter 2

Introduction

2.1. Induced Resistance

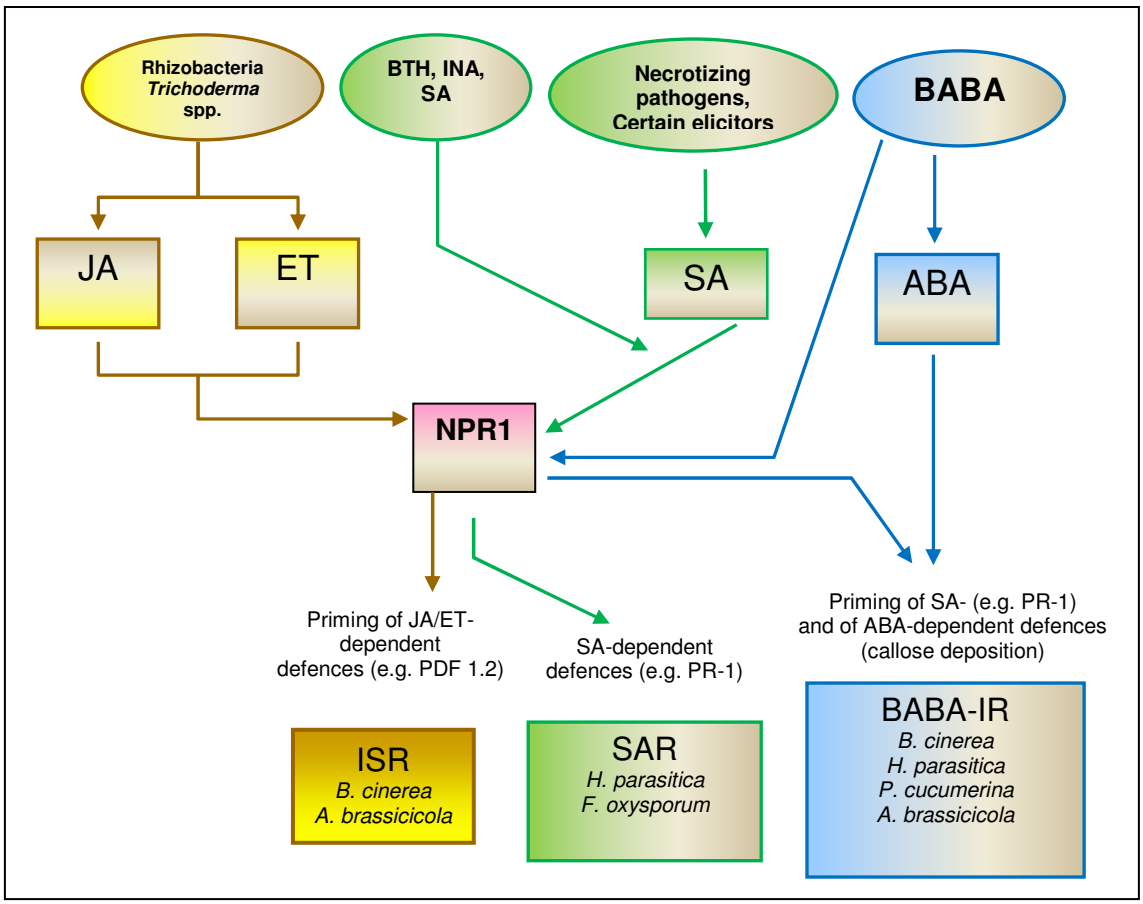
2.1.1. Fungal diseases and induced resistance

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).

2.1.2. 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. Brown 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).

2.1.3. 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).

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

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).

2.1.3.1. 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). 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).

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

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).

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 (Chrikov, 2002; Chen and Xu, 2005; Bautista-Baños *et al*., 2006; Iriti and Faoro, 2009a).

2.1.3.2. 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).

2.1.3.3. Oxidative burst

Immediately downstream of pathogen recognition, early events in plant cell may activate receptor-associated, plasma membrane-bound, heterotrimeric GTP-binding (or simply G) proteins, as noted for a wide variety of animal transmembrane receptors, a family of proteins involved in second messenger cascade. Activation of G proteins may be coupled to ROS generation by the influx of Ca^{2+} from the apoplast due to the opening of calcium channels. The increase of intracellular Ca^{2+} concentration activates a Ca^{2+}-dependent protein kinase that, in turn, stimulates ROS generation. In particular, phosphorylation of a plasma membrane-bound enzyme, a NADPH-dependent oxidase, sharing homology with its mammalian counterpart, stimulates the production of superoxide anion ($^{\cdot}O_2^-$). This radical species is then dismutated to hydrogen peroxide (H_2O_2), by superoxide dismutase (SOD). The role of H_2O_2 is pivotal in plant defence mechanisms, because it is a non-radical, non-charged and membrane permeable species. Therefore, it *i*) contributes to create a hostile environment to the pathogen because of its direct toxicity, *ii*) participates to the oxidative cell wall strengthening and *iv*) acts as a signal molecule (second messenger) for the activation of defence genes. However, the cellular H_2O_2 concentration has to be maintained under a cytotoxic threshold by cell antioxidant defences, because this species can react with transition metals (Cu or Fe), according to Fenton or Haber-Weiss reactions, to form hydroxyl radical ($OH^{\cdot}$), the most reactive and dangerous ROS. Enzymes that regulate the H_2O_2 homeostasis include mainly catalases (CATs), ascorbate peroxidise (APX) and peroxidises (POXs), whereas the main non-enzymatic ROS scavengers are ascorbic acid, glutathione, tocopherols, carotenoids and polyphenols (Mehdy, 1994; Wojtaszek, 1997; Alvarez *et al*., 1998; Apel and Hirt, 2004; Yoshioka *et al*., 2009).

Similarly to animals, nitric oxide (NO) is an important signal molecule in plants too. In mammals, NO is produced by the enzyme NO synthase (NOS) that converts L-citrulline to L-arginine. In plants, there are not homologue genes of animal NOS, though the activity of NOS-like enzymes has been reported in these organisms (Chandok *et al*., 2003, 2004; Guo *et al*., 2003, 2005). Alternatively, plants generate NO from nitrite by nitrate reductase (NR) or via non-enzymatic reduction of apoplastic nitrite (Yamamoto *et al*., 2003; Bethke *et al*., 2004). The physiological role of NO is still not entirely known, though its involvement in stomatal closure, seed germination, fruit ripening, senescence and root organogenesis has been reported. Interestingly, during the pathogen attack, NO may mediate induction of HR and SAR by interacting with H_2O_2 and salicylic acid (SA) (Delledonne *et al*., 2001; Buonaurio *et al*., 2003; Polverari *et al*., 2003; Wendehenne *et al*., 2004; Zaninotto *et al*., 2006).

2.1.3.4. The hypersensitive reaction

Resistant plants often respond with a HR at site of fungal penetration, by localised programmed cell death followed by a wide range of both local and systemic defence reactions, such as lignification, phytoalexin and PR-protein synthesis. This resistance mechanism, which is often associated with both gene-for-gene resistance (effector-triggered immunity) and non-host resistance (microbial-associated molecular-pattern immunity), might involve just a single cell (invisible HR) or extensive and visible tissue areas, to deprive an invading pathogen of an adequate nutrient supply. Additionally, the release of antimicrobial compounds from dying cells and defence responses triggered in cells immediately surrounding infection site contribute to poison and restrict (biotrophic or hemibiotrophic) fungi. Another event at the onset of HR is the generation of molecular signals (SA, ethylene and jasmonic acid) which may alert distal parts of the plant and induce SAR (Glazebrook, 2005; Williams and Dickman, 2008).

PCD in plants shows striking similarities to the hallmarks observed in apoptosis, a typical form of PCD in animals, including chromatin condensation, DNA cleavage (ladders) and activation of caspase (cysteine-aspartic proteases)-like proteases (metacaspases). By contrast, plant cells display unique features lacking in their animal counterparts, such as the presence of a rigid cell wall, chloroplasts and vacuolar proteases (Williams and Dickman, 2008). Depending on pathogen lifestyle, PCD/HR may be either beneficial or detrimental to the host. As previously introduced, in biotrophic pathogen-plant interactions, HR prevents infection, because biotrophs require living cells for growth and colonisation. Conversely, in response to necrotrophic pathogens, which feed on dying or dead tissues, PCD is advantageous to the pathogen and not to the plant (Glazebrook, 2005). This divergence can be explained considering PCD as an essential pathogenicity factor of certain necrotrophic pathogens, which evolved fine strategies to subvert and induce inappropriate PCD in host cells. This is the case of victorin, a host selective toxin of *Cochliobolus victoriae*, the causal agent of victoria blight in oat, and oxalic acid produced by *Sclerotinia sclerotiourum*, a pathogen with an extremely broad host range. In particular, victorin induces PCD in plants with the dominant susceptibility allele *Vb*, whereas oxalic acid is not really a toxin, but functions as a fungal elicitor of PCD (Navarre and Wolpert, 1999; Kim et al., 2008).

2.1.3.5. Cell-wall strengthening

As its basal structure, the primary cell wall is composed of a framework of cellulose microfibrils that are embedded in a matrix of hemicelluloses, pectins and structural proteins. In the epidermis, it constitutes one of the first lines of defence against fungal pathogens, and it typically represents a preformed physical barrier, although the *ex-novo* induction of structural

defences can strengthen the cell wall (table 1). The cell-wall appositions include an array of structures that are involved in the accretion of new cell-wall material. In some pathosystems, the attempted penetration of leaves by phytopathogenic fungi is accompanied by deposition of a plug of material, known as a papilla, directly beneath the penetration site. The epidermal cell wall surrounding the papilla can be modified to form a characteristic disc-shaped zone or halo.

The materials involved in the thickening of host cell wall range from minerals, such as silicon, calcium and sulphur (the fungicidal activity of which is well known), to more or less complex organic polymers, mainly including callose and lignin, polymers of β-1,3-glucose and monolignols, respectively. Moreover, papillae can be impregnated with oxidised phenols, which are directly toxic to pathogens (Hückelhoven, 2007; Hématy *et al*., 2009).

Extensins (hydroxyproline-rich glycoproteins) are the main structural proteins of plant primary cell wall. They have similarities to animal collagen, they form a defined scaffold that sets the spacing of cellulose microfibrils, and they are characterised by post-translationally hydroxylated proline, which makes up about 40% of amino-acid residues. Oxidative cross-linking of hydroxyproline-rich glycoproteins involves the re-arrangement of these pre-existing cell-wall components by peroxidases and hydrogen peroxide (H_2O_2), further improving their resistance to both enzymatic hydrolysis and the physical pressure that can be exerted by pathogens (Merkouropoulos *et al*., 1999; Cannon *et al*., 2008).

Lignin is a polymer that comprises different phenylpropenol units (monolignols) that are connected by covalent linkages. Peroxidases and H_2O_2 are essential for its random polymerisation, which takes place *in muro*, with growing lignin polymer infiltrating the primary cell wall. Lignin is extremely difficult to attack enzymatically, and very few organisms can degrade lignified tissues, as can white rot fungi. Therefore, induced lignification represents an optimal inducible structural barrier for plants, and lignified tissues are also a poor and hostile substrate for pathogen growth and development (Bhuiyan *et al*., 2009).

2.1.3.6. Phytoalexins

Phytoalexins are low-molecular-weight antimicrobial plant secondary metabolites, and they are synthesised *de novo* from essential substrates, including phenylalanine, malonyl-CoA, acetyl-CoA, mevalonic acid and other amino acids (Hammerschmidt, 1999; Iriti and Faoro, 2009b). They have been identified in the majority of plant families, and members of specific plant families usually produce similar types of phytoalexins.

They are synthesised and accumulate locally around infection sites, and although they have never been found in systemically protected tissues before a challenge inoculation, they can rapidly accumulate in induced tissues after a challenge (Van Loon, 2000). Interestingly, they

accumulate both in resistant and susceptible hosts at the same concentrations, though with a different kinetic, thus showing that their efficacy strictly depends on the timing of their synthesis at infection site. The antimicrobial compounds in healthy plant tissues are known as phytoanticipins (VanEtten *et al*., 1994).

Phenylpropanoids, arising from phenylalanine deamination by the enzyme phenylalanine ammonia-lyase (PAL), include several classes of well-studied phytoalexins, for instance isoflavonoids from the Leguminoseae family (Grayer and Harborne, 1994; Dixon and Paiva, 1995). The pathosystem *Phaseolus vulgaris*-*Colletotrichum lindemuthianum* provided a good model for studying the role of phytoalexins in plant resistance against pathogen attack. *C. lindemuthianum*, the causal agent of bean anthracnose, is a hemibiotrophic fungus whose colonization is restricted, in resistant hosts, because of isoflavonoid production, including phaseollin, phaseollidin and kievitone (Bailey *et al*., 1980; Mansfield, 2000). Similar examples include medicarpin and pisatin, two isoflavonoid phytoalexins from alfalfa (*Medicago sativa*) and pea (*Pisum sativum*), respectively (Blount *et al*., 1993, DiCenzo and VanEtten, 2006). However, broad bean (*Vicia faba*) provides a notable exception. Like most other legumes, it produces isoflavonoid phytoalexins, but the main induced antimicrobial compounds are furanoacetylenic wyerone derivatives (Grayer and Harborne, 1994; Mansfield, 2000).

In the Vitaceae family, phytoalexins which have been well characterized constitute a rather restricted group of molecules belonging to the stilbene family, synthesized as a general response to fungal attack. These compounds possess the skeleton based on the *trans*-resveratol (3,5,4′-trihydroxystilbene) structure, including piceids, pterostilbenes and viniferins, that are, respectively glucosides, dimethylated derivatives and oligomers of resveratrol (Jeandet *et al*., 2002). In grapevine (*Vitis vinifera*), activities of chalcone synthase (CHS) and stilbene synthase (STS), enzymes respectively involved in flavonoid and stilbene biosynthesis, are differentially regulated, according to plant developmental stage. During the initial phase of berry ripening (*véraison*), resveratrol accumulation in cells of berry exocarp declines, while anthocyanin synthesis increases, due to competition between two branches of the same pathway. As a consequence, after *véraison*, anthocyanin accumulation confers colour to berry skin, whereas lowering levels of the powerful phytoalexin resveratrol make the grape bunches more susceptible to *Botrytis cinerea* (gray mould) infections (Jeandet *et al*., 1995). The resistance of *Vitis* spp. to *B. cinerea* infection has been shown to correlate with *trans*-resveratrol content (Mlikota Gabler *et al*., 2003). Interestingly, open-field treatment with the plant activator BTH can reverse, to a certain extent, the inverse relationship between resveratrol and anthocyanin content at *véraison*, reducing CHS and STS competition for the same substrate and avoiding metabolic switch from one pathway to the other. Thus, higher levels of resveratrol protect

grapes from gray mould after *véraison*, without interfering with the colouring phase, which is an important qualitative trait (Iriti *et a*l., 2004). Following BABA treatment of grapevines, sporulation of *Plasmopara viticola* was strongly reduced and the accumulation of phytoalexins of the stilbene family increased with time after infection. Induction of *trans*-piceide, *trans*-resveratrol, and more importantly, *trans*-ε-viniferin, *trans*-δ-viniferin and *trans*-pterostilbene was reported for a BABA-primed susceptible grapevine genotype (Slaughter *et al*., 2008).

In the model plant *Arabidopsis thaliana*, camalexin (3-thiazol-2'-yl-indole) represents the main phytoalexin, involved in inducible defence mechanisms against a variety of pathogens, such as the bacterium *Pseudomonas syringae* and the fungus *Alternaria brassicicola*. Camalexin is a N- and S- containing indole phytoalexin synthesized from tryptophan via indole-3-acetaldoxime, a branch point metabolite that also leads to the biosynthesis of glucosinolates, the plant hormone indole acetic acid (IAA) and melatonin (Glawischnig *et al*., 2004).

2.1.3.7. Pathogenesis-related proteins

Some decades ago, it was shown that infection of tobacco plants with tobacco mosaic virus leads to the accumulation of a set of PR proteins (Gianinazzi *et al*., 1970; van Loon and van Kammen, 1970). Acidic extracellular forms of these PR proteins accumulate at the onset of plant resistance, indicating that they have a role as molecular markers for the expression of SAR. PR proteins have a low molecular weight (5-75 kDa), and they are thermostable, highly resistant to proteases, extractable, and stable at low pH (<3). They have a dual cellular localisation, as vacuolar (for basic forms) and apoplastic, the latter being the most important site for their accumulation. First detected in tobacco, PR proteins are now considered to be common-place in the plant kingdom, and have been detected across different genera in all organs of monocotyledonous and dicotyledonous species: leaves, where they are particularly abundant in both mesophyll and epidermis, stems, roots, flowers and seeds. Additionally, exogenous application of SA, or of its functional analogues 2,6-dichloroisonicotinic acid and BTH, can activate PR gene expression and resistance in plants without pathogen inoculation (Edreva, 2005; van Loon *et al*., 2006).

PR proteins are categorised into structurally homologous families. Some of these families have direct antimicrobial activities, whereas, for others, no intrinsic antimicrobial effects have been found yet, suggesting that the latter might have different functions. An important common feature of most antimicrobial PR proteins is their antifungal activity, although some of them also have antibacterial, insecticidal and antiviral properties. Originally, five main groups of PR proteins (PR-1 to PR-5) were characterised in tobacco. Since then, the number of PR protein groups has increased up to PR-17 across many plant species (van Loon and van Strien, 1999;

Sels *et al*., 2008). Todate, the functions of the most abundant family of these PR proteins, PR-1, remain obscure, although direct inhibitory effects against fungal pathogens have been demonstrated. PR-1 inhibits *in-vitro* zoospore germination of *Phytophthora infestans*, as well as *in-vivo* differentiation of infection hyphae of *Uromyces fabae* in leaves of the broad bean (Niderman *et al*., 1995; Rauscher *et al*., 1999). However, the mode of action, the cellular localisation and the molecular target(s) of PR-1 are still not known. PR-2 has β-1,3-glucanase activity, while PR-3, PR-4, PR-8 and PR-11 are chitinases, and PR-5 includes thaumatin-like proteins, such as osmotin. PR-6 is a proteinase inhibitor, and PR-7, PR-9 and PR-10 have endoproteinase, peroxidase and ribonuclease activities, respectively. Membrane-permeabilising properties are characteristic of defensins, thiols and lipid-transfer proteins, which are referred to as PR-12, PR-13 and PR-14, respectively. The germins (oxalate oxidases) and germin-like (oxalate oxidase-like) proteins PR-15 and PR-16, respectively, have multiple enzymatic, structural and receptor functions (van Loon *et al*., 2006; Sels *et al*., 2008). Finally, PR-17 is a secreted protein of tobacco-cultured BY2 cells that has no known function (Okushima *et al*., 2000).

Interestingly, an Arabidopsis *npr1* (nonexpressor of PR genes 1) gene mutant that is also known as non-induced immunity1 (*nim1*) was isolated by a genetic screening in plants that failed to express PR genes after SAR induction with plant activators such as SA, BTH and 2,6-dichloroisonicotinic acid, and avirulent pathogens. Later, NPR1 protein was shown to regulate PR gene expression by a SA-mediated mechanism. Indeed, SA induces nuclear localisation of NPR1, which is essential for induction of PR genes. Once in the nucleus, interaction of NPR1 with TGA transcription factors might be responsible for PR-gene expression. The TGA factors are a family of transcription factors with a basic region/leucine zipper (bZIP) motif that can regulate the induction of PR genes by binding to the *as1-cis* element in the promoters of PR genes (Dong, 2004). There are ten TGA transcription factors in *Arabidopsis*, of which seven (TGA1–TGA7) have been characterised with respect to their interactions with NPR1 (Jakoby *et al*., 2002).

2.1.4. 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, 2003
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*., 2006, 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

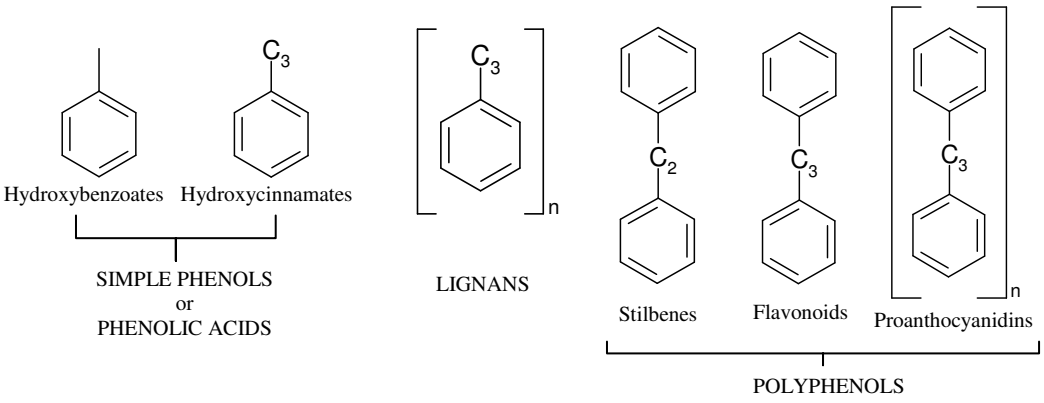
blast disease caused by *Magnaporthe grisea* and it has been widely used in Asian rice production since 1975 (Walters and Fountaine, 2009). Furthermore, BTH has been registered in several countries (as Bion® in Italy) and it is extensively applied to protect a number of crops from fungal diseases (Walters 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.

2.2. Phenylpropanoids

Phenylpropanoids represent one of the most abundant and widely distributed groups of natural compounds originating exclusively from the Plant Kingdom. They consist of metabolites with the basic building unit C_6-C_3, arising from a common precursor (the essential aromatic amino acid phenylalanine) and constituted by an aromatic benzene ring (C_6) and a three-carbon lateral linear chain (C_3, a propane). The main classes include hydroxycinnamates (C_6-C_3), hydroxybenzoates (C_6-C), lignans [$(C_6$-$C_3)_2$] and polyphenols (figure 2). The latter group, in turn, comprises: stilbenes (C_6-C_2-C_6) and flavonoids (C_6-C_3-C_6), compounds with two aromatic rings linked through a two or three-carbon bridge, respectively (figure 2).

Figure 2. Basic moieties of phenylpropanoids. Polyphenols differ from simple phenols and lignans because of a second aromatic ring, whereas proanthocyanidins are oligomeric or polymeric derivatives of flavonoids with polymerization degree ranging from 3 to 11, in grape.



In flavonoids, the three-carbon link forms an oxygenated heterocyclic ring. Flavanols (such as catechin and epicatechin), a subgroup of flavonoids, represent the monomeric units of proanthocyanidins or condensed tannins, therefore considered oligo- or polymeric flavonoid derivatives [$(C_6$-C_3-$C_6)_n$] (figure 2). A number of hydroxyl groups differently distributed on these basic skeletons are further responsible for the polyphenol chemical diversity and biological activity, such as their relevant antioxidant power (Iriti and Faoro 2009c).

2.2.1. The ecological roles of phenylpropanoids

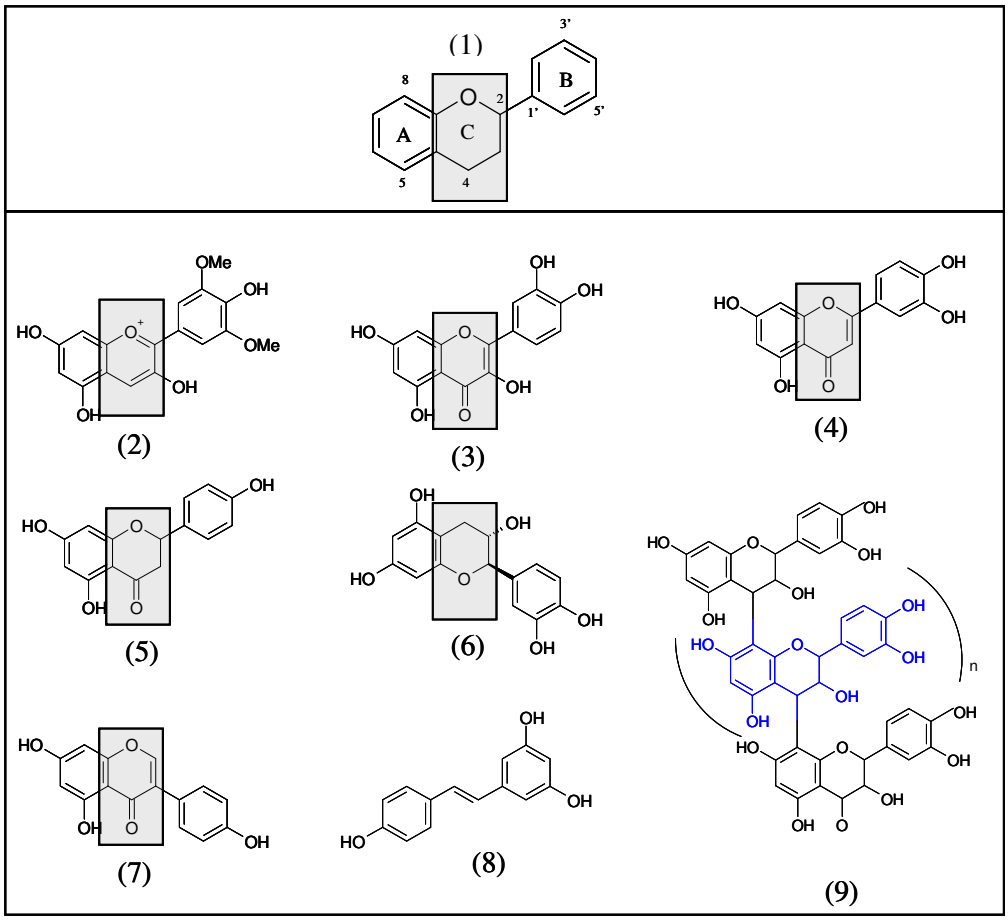
For producing organisms, i.e. plants, phenylpropanoids are secondary metabolites with a plethora of ecological functions (Tahara, 2007). In general, they do not play any role in the plant's primary metabolic requirements, i.e. the synthesis of biomacromolecules (carbohydrates, lipids, proteins, and nucleic acids). Rather, phytochemicals allow interaction of plants with the environment, increasing their overall ability to survive and overcome local challenges (Iriti and Faoro, 2004, 2009b). Polyphenols exert protective functions as antioxidant and UV-absorbing agents. They defend plants against pathogens (fungi, bacteria, virus) and environmental pollutants, and are involved in plant-plant interaction/competition (allelopathy) (Iriti and Faoro, 2000b). These compounds also play a role in herbivore deterrence - mainly against phytophagous insect - acting as agonists or antagonists of neurotransmitter systems or forming structural analogues of (animal) endogenous hormones (Wink, 2000; Miller and Heyland, 2010; Rattan, 2010). Finally, the plant fitness greatly benefits from secondary metabolites which confer colours and scents to flowers and fruits, thus playing important roles in reproduction (attraction for pollinators and seed dispersal) (Harborne, 2001).

2.2.2. Polyphenol structures

This large group of phenylalanine derivatives, are structurally characterized by the presence of at least two aromatic phenolic rings (C_6-OH) (they are, literally, compounds with multiple phenols). In particular, an aromatic ring - formed by the condensation of 3 malonyl Co-A units - is linked to the phenylpropanoid moiety arising from phenylalanine. As previously introduced, these phytochemicals include thousands of metabolites and are grouped into three main classes: flavonoids (C_6–C_3–C_6), stilbenes (C_6–C_2–C_6) and proanthocyanidins (or condensed tannins) [$(C_6$–C_3–$C_6)_n$] (figure 2) (Iriti and Faoro, 2009c).

The flavonoid basic chemical structure is the flavan nucleus, consisting of 15 carbon atoms arranged in three rings (C_6–C_3–C_6): two aromatic rings (A and B) connected by a three-carbon-atom heterocyclic ring, an oxygen-containing pyran ring (C) (figure 3).

Figure 3. The flavan nucleus (**1**) is the basic structure of flavonoids, which include malvidin (**2**) (an anthocyanidin), quercetin (**3**) (a flavonol), luteolin (**4**) (a flavone), naringenin (**5**) (a flavanone), (+)-catechin (**6**) (a flavan-3-ol) and genistein (**7**) (an isoflavonoid) differing in the level of oxidation and saturation of the C ring; other polyphenols comprise resveratrol (**8**) (a stilbene) and proanthocyanidins (**9**) (or condensed tannins), oligo- and polymeric derivatives of flavan-3-ol units.



The main classes of flavonoids (flavanones, flavones, flavonols, flavanols, isoflavonoids, and anthocyanidins) differ in the level of oxidation and saturation of the C ring, while individual compounds within a class vary in the substitution pattern of the A and B rings (Pietta, 2000). Anthocyanidins are the most abundant pigments in plant external tissues. Their conjugated derivatives, anthocyanins, mainly bound to sugars (glycones), hydroxycinnamates or organic acids, are water-soluble pigments conferring blue, dark blue, violet, red and purple hues to flowers, fruits and other organs. Anthocyanins are structurally based on six aglycones/anthocyanidins – malvidin, cyanidin, delphinidin, peonidin, pelargonidin, and petunidin – which differentiate on the basis of number and position of their hydroxyl groups and their degree of methylation (figure 3) (Clifford, 2000). Flavonols mainly include kaempferol, quercetin, and myricetin aglycones, whereas apigenin and luteolin are widely-diffused flavones (figure 3). Flavan-3-ols (or flavanols) provide catechins, the monomeric units for proanthocyanidin biosynthesis (figure 3) (Iwashina, 2000). Flavanones are typical of citrus fruits (genus *Citrus*), such as aglycones hesperetin and naringenin (figure 3) (Peterson *et al*., 2006). Isoflavonoids, also known as phytoestrogens, are important constituents of Fabaceae, including the soy genistein and daidzein (figure 3) (Rusin *et al*., 2010). Molecules belonging to the stilbene family (C_6–C_2–C_6) are almost exclusively present in the genus *Vitis*, and possess the basic chemical structure based on the *trans*-resveratrol skeleton (figure 3). Stilbenes comprise piceids, pterostilbenes, and viniferins that are glucosides, dimethylated derivatives, and oligomers of resveratrol, respectively (Jeandet *et al*., 2002). Proanthocyanidins [$(C_6$–C_3–$C_6)_n$] - also known as condensed tannins or simply tannins - are both oligomeric and polymeric compounds arising from flavanol unit condensation. Common monomers include catechin epimers [(+)-catechin and (-)-epicatechin], whose polymerization degree ranges mainly between 3 and 11 (figure 3) (Santos-Buelga and Scalbert, 2000).

2.2.3. Polyphenols in human diet

The occurrence of polyphenols in plant foods has been widely reported, mainly in a great variety of foodstuffs, including fruit, vegetables, pulses, cereals, olive oil, cocoa/chocolate, and in plant-derived beverages, such as tea, wine and coffee (Pérez-Jiménez *et al*., 2010). As components of (food) plants, polyphenols entered the human diet from earliest stages of human evolution, through the advent of agriculture, approximately 10,000 years ago. This modified human nutritional behaviour and, consequently, the (plant) food-human co-evolution (Iriti and Faoro, 2004). Nowadays, in Western populations, the daily intake of polyphenols has been estimated to be ~ 1000 mg, though fruits and vegetables contain hundreds of bioactive

phytochemicals, apart from polyphenols, that may independently or synergistically promote health benefits (Scalbert and Williamson, 2000; Ovaskainen *et al*., 2008).

In the past few decades, experimental data, mainly recorded from *in vitro* studies on cell cultures or animal models, highlighted the health-promoting effects of polyphenols. Despite the small number of studies on humans, there is compelling epidemiological evidence that consumption of polyphenol-rich plant foods may significantly reduce the risk of some detrimental chronic conditions, such as cardiovascular diseases, certain types of cancer and neurodegenerative disorders (Hertog *et al*., 1993; Scalbert *et al*., 2005; Mink *et al*., 2007). Indeed, epidemiological studies have repeatedly shown an inverse association between the risk of chronic diseases and consumption of polyphenol-rich diets, though there is no relation between the quantity of polyphenols in foods and their bioavailability (Lock *et al*., 2005; He *et al*., 2006; Nöthlings *et al*., 2008). Consequently, the mechanism(s) of action of PPs *in vivo* might be different from the *in vitro* one(s), but the lack of pharmacokinetic and pharmacodynamic investigations on humans does not give a correct and definite understanding of this pivotal aspect. Furthermore, confounding factors may generate a misinterpretation and the above mentioned epidemiological association does not always imply causality: a cause-effect relation still need to be thoroughly described and many further studies are needed before we can prove that these associations do not arise from casualness.

2.2.4. Antioxidant activity of polyphenols

Probably, one of the most investigated biological activity of polyphenols is their antioxidant power, i.e. the capacity to detoxify free radicals, including reactive oxygen and nitrogen species, thus preventing damages to macromolecules (lipids, proteins, DNA) and cell structures. Pathological conditions mechanistically linked to oxidative stress include inflammation, atherosclerosis and carcinogenesis (Mayne, 2003; Pandey and Rizvi, 2010). Therefore, it is not surprising that foods rich in antioxidants, as well as single food components, may play an essential role in the prevention of cardiovascular diseases, cancer, degenerative neurological disorders such as Parkinson's and Alzheimer's diseases, and premature aging, as extensively reported both on cell and animal models (Scalbert *et al.*, 2005; Soobrattee *et al*., 2005).

Flavonoids act as antioxidants by donating electrons and stopping radical chains (Rice-Evans, 2001). This activity is attributed to the phenolic hydroxyls, increasing with the number of OH groups in A and B rings. The structural requirements considered to be essential for effective radical scavenging by flavonoids are the presence of a 3',4'-dihydroxy group (*o*-diphenolic group, a catechol structure) on the B ring, and a double bond between C_2 and C_3 ($\Delta^{2,3}$)

conjugated with a keto function at C_4 of the C ring. Hydroxyl groups on the B ring donate hydrogen and an electron to radical species, stabilizing them and giving rise to a relatively stable flavonoid radical. The C_2–C_3 double bond and the 4-keto group are responsible for electron delocalization from the B ring. Hydroxyl groups in positions 3 and 5, in combination with 4-oxo function and C_2–C_3 double bond, contribute to further enhance the radical scavenging activity (Heim *et al*., 2002; Amic *et al*., 2003; Seyoum *et al*., 2006).

2.2.5 Other biological activities of polyphenols

Polyphenols also possess a plethora of properties more or less correlated to their antioxidant power, such as antimutagenic, anti-inflammatory, antitumoral, antihypertensive, cardio and neuroprotective activities (Pandey and Rizvi, 2010). Some of these have been briefly reported in

Iriti M., Vitalini S. [2011d]. Chemical diversity of grape products, a complex blend of bioactive secondary metabolites. *The Natural Products Journal*, **1**, 71-74.

2.3. Melatonin in plants

Outside the animal kingdom, melatonin was discovered, for the first time, in the photosynthesizing unicellular alga *Lingulodinium polyedrum* (Stein) J. D. Dodge sin. *Gonyaulax polyedra* Stein, belonging to the phylum Dinoflagellata (Balzer and Hardeland, 1991). Since then, the presence of melatonin in food plants and medicinal herbs has been extensively reported. Although there were some preliminary indications (Van Tassel *et al*., 1993; Kolář and Macháková, 1994), the first complete publications reporting melatonin in tracheophytes (vascular or higher plants) were independently provided by two research groups (Dubbels *et al*., 1995; Hattori *et al*., 1995). They found this indoleamine in a number of edible plants and, since then, it has been detected and quantified in roots, shoots, leaves, flowers, fruits and seeds of a considerable variety of spermatophyte species, as attested by numerous publications confirming undoubtedly the presence of this molecule in the Plant Kingdom (Manchester *et al*., 2000; Reiter *et al*., 2001, 2007; Reiter and Tan, 2002; Caniato *et a*l., 2003; Chen *et al*., 2003; Kolář and Macháková, 2005; Murch *et al*., 2009; Paredes *et al*., 2009; Posmyk and Janas, 2009). In flowering plants (angiosperms), the occurrence of melatonin has been described in a number of families belonging to both the mono- and dicotyledons, relevant as food and medicinal plants (Murch *et al*., 1997, 2004; Tettamanti *et al*., 2000; Cole *et al*., 2008; Okazaki and Ezura, 2009; Iriti *et al*., 2010).

The synthesis of melatonin in plants is still not completely known, although some evidence suggests that these organisms are equipped with the enzymatic machinery for its biosynthesis (Murch *et al*., 2000). Similarly, the physiological and pathophysiological role of melatonin *in planta* is still unclear. Because of its structural similarity with plant growth hormones of the auxin family, a hormone-like role has been attributed to melatonin in some plant species, as well as an action in delaying flowering, preventing chlorophyll degradation, protecting against oxidative damage, abiotic stresses, pathogens and environmental pollutants (Lei *et al*., 2004; Afreen *et al*., 2006; Arnao and Hernández-Ruiz, 2006; Iriti and Faoro, 2007; Paredes *et al*., 2009; Posmyk and Janas, 2009).

Among grapevine (*Vitis vinifera* L.) products, melatonin was firstly detected in the berry exocarp (skin) (Iriti et al., 2006) and then in wine (Mercolini et al., 2008). More recently, its presence was confirmed in some different red and white wines (Stege et al., 2010).

2.3.1. Occurrence and analysis of melatonin in food plants

The most common analytical techniques used to detect melatonin in plant foods are both immunological, radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA), and chromatographic, high performance liquid chromatography (HPLC) with electrochemical (HPLC-EC) or fluorimetric (HPLC-F) detection. Additionally, mass-spectrometry (MS) identification represents a powerful tool for an accurate determination of melatonin in plant samples. Apart from analytical techniques used, the chemical complexity of plant tissues and the physicochemical properties of melatonin may influence the analyses, determining, in some cases, opposing results. Furthermore, factors such as characteristics of the place where plants were grown, if agrochemicals or chemical processes were employed in the field, the light regime and circadian behaviour of the plant, the ripening stage and the type of tissue analysed contribute to explain the lack of homogeneity in the data published on the melatonin levels in food plants.

A complete survey on the melatonin content in food plants has been reported in

Iriti M., Vitalini S., Rossoni M., Faoro F. [2010a]. 'Occurrence and analysis of melatonin in food plants', in 'Handbook of Analysis of Active Compounds in Functional Foods', pp. 651-661. Editors Nollet LML and Toldra F, Taylor and Francis Group

2.3.2. Melatonin in traditional Mediterranean foods

As grape products are the main components of the traditional Mediterranean diet which is associated to a reduced risk of chronic degenerative diseases (Trichopoulou *et al*., 2000; Visioli *et al*., 2004; Estruch *et al*., 2006; La Vecchia and Bosetti, 2006), we have suggested that dietary melatonin may contribute to explain these health benefits, mainly attributed to polyphenolic compounds, including resveratrol (Iriti *et al*., 2010). In general, by virtue of their antioxidant power, all these phytochemicals exert protective effects by reducing the oxidative burden due to reactive oxygen species (ROS) involved in etiopathogenesis of cardiovascular diseases, neurological disorders and certain types of cancer (Mayne, 2003).

The role of melatonin in Mediterranean diet has been extensively treated in

Iriti M., Varoni E.M., Vitalini S. [2010b]. Melatonin in traditional Mediterranean diets. *Journal of Pineal Research*, **49**, 101-105.

2.3.3. Melatonin in grape products

As already mentioned, melatonin was first detected in berry skin of Italian and France varieties (Barbera, Croatina, Cabernet Sauvignon, Cabernet Franc, Marzemino, Nebbiolo, Sangiovese and Merlot) grown in north-western Italy, with levels ranging from 0.005 to 0.96 ng/g (Iriti *et al*., 2006). Similar results (from 0.6 to 1.2 ng/g) were reported by Stege and colleagues (2010) for the same tissue of Malbec, Cabernet Sauvignon and Chardonnay varieties cultivated in Argentina. However, much higher melatonin concentrations, between 100 and 150 μg/g, depending on the phenological stage, were measured in the whole berry (i.e. skin, flesh and seeds analyzed together) of Merlot cultivar grown in Canada (Murch *et al*., 2010). Very recently, in berry skin of Malbec variety cultivated in Argentina, melatonin concentration showed similar values during the night (around 10 ng/g), reaching a strong peak at night (159 ng/g) (Boccalandro *et al*., 2011).

As previously mentioned, further studies ascertained the occurrence of melatonin in wine. Firstly, Mercolini and co-workers (2008) detected it at 0.5 and 0.4 ng/mL in Sangiovese red and Trebbiano white wine, respectively. Stege and colleagues (2010) reported melatonin at 0.16, 0.24 and 0.32 ng/mL in Chardonnay, Malbec and Cabernet Sauvignon wines, respectively. More recently, Rodriguez-Naranjo and colleagues (2011a, 2011b) measured higher melatonin concentrations, up to 150 and 400 ng/mL in racked wines. In general, the recent survey of Rayne (2010) defined levels of melatonin near 1 ng/g and 0.5 ng/mL in grapevine berry skin and wine, respectively.

Noticeably, many endogenous and external factors may influence melatonin and polyphenolic levels in grapevine and their products, such as the genetic traits of the cultivar and its geographical origin, the berry tissue/plant organ analyzed, the difference between thin and thick skinned grapes, the phenological stage, day/night fluctuations, pathogen (mainly fungal) infections and phytosanitary treatments, agro-meteorological conditions and environmental stresses, altitude, UV radiation and high light irradiance, the vintage and wine-making procedures (Burkhardt *et a*l., 2001; Hudec *et al*., 2009; Lachman *et al*., 2009; Cohen and Kennedy, 2010; Murch *et al*., 2010; Boccalandro *et al.*, 2011; Rodriguez-Naranjo *et al*., 2011a). In particular, wine-making techniques play a main role in the extraction of phenolics (and possibly of melatonin) from grapes: the maceration time (i.e. skin and seed contact with the must) and the fermentation period improve the extraction and the solubility of phenolic compounds (with an increase in alcoholic content) (Sacchi *et al*., 2005; Pérez-Lamela *et al*., 2007).

2.3.4. Bioavailability of dietary melatonin

In general, an efficient uptake of melatonin from food sources should be expected to influence its circulating levels, basically very low (~200 pg/mL at the maximum night peak and lower than 10 pg/mL during the day) (Bonnefont-Rousselot and Collin, 2010). In animals and humans, the intake of foodstuffs containing melatonin may contribute to increase both the serum levels of this compound and the urinary concentrations of its metabolite 6-sulfatoxymelatonin (Reiter *et al*., 2005; Maldonado *et al.*, 2009; Garrido *et al*., 2010). Finally, the European Food Safety Authority has recently accepted the scientific substantiation of health claims linking melatonin to alleviate subjective feelings of jet leg, the reduction of sleep onset latency and improved sleep quality (EFSA Journal, 2010).

Chapter 3

Aims of PhD Thesis

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,

i) the efficacy of both elicitors was assessed against the most important fungal diseases of grapevine (*Vitis vinifera* L.), downy mildew (*Plasmopara viticola* Berk. et Curtis ex de Bary) and powdery mildew (*Erysiphe necator* Schw.);

ii) the modifications of the grapevine secondary metabolism stimulated by both elicitors were evaluated in terms of increased biosynthesis of polyphenols and melatonin in grape berry (exocarp end endo-mesocarp) and seed tissues;

iii) the content of polyphenols and melatonin was measured in experimental wines (microvinificates), in order to ascertain if the effects of treatments on grapevine secondary metabolites were retained in these products;

iv) the biological activity of grape tissues and microvinificates was assayed relatively to their antiradical capacity;

v) the levels of mycotoxins, especially ochratoxin A, were determined in experimental wines.

Some results obtained from the Ph.D. research project were published or submitted to international journals:

Iriti M., Vitalini S., Di Tommaso G., D'Amico S., Borgo M., Faoro F. [2011a]. New chitosan formulation prevents grapevine powdery mildew infection and improves polyphenol content and free radical scavenging activity of grape and wine. *Australian Journal of Grape and Wine Research*, **17**, 263-269.

Vitalini S., Gardana C., Zanzotto A., Fico G., Faoro F., Simonetti P., Iriti M. [2011b]. From vineyard to glass: agrochemicals enhance the melatonin and total polyphenol contents and antiradical activity of red wines. *Journal of Pineal Research*, **51**, 278-285.

Vitalini S., Gardana C., Zanzotto A., Simonetti P., Faoro F., Fico G., Iriti M. [2011c]. The presence of melatonin in grapevine (*Vitis vinifera* L.) berry tissues. *Journal of Pineal Research*, **51**, 331-337.

Vitalini S., Gardana C., Simonetti P., Fico G., Iriti M. Melatonin, resveratrol and polyphenol content and antiradical activity of traditional Italian grape products. *Food Chemistry* (submitted) (**chapter 4**).

Other results were reported, for the first time, in this thesis document (**chapter 5, 6, 7**).

Chapter 4

Melatonin, resveratrol and polyphenol content and antiradical activity of traditional Italian grape products

Abstract

Grape products are main components of traditional Mediterranean diet. Their regular consumption may exert health benefits mainly attributed to some their bioactive phytochemicals. Though polyphenols represent the paradigm of the health-promoting effects ascribed to grape products, recently, attention has been paid to dietary melatonin, significantly present in Mediterranean foods. Therefore, in this work, we measured the melatonin, resveratrol and total polyphenol (TP) content in some different grape products, namely red and white wines, dessert wines, grape juices and traditional balsamic vinegars of different Italian geographical origin, as well as in experimental wines (microvinificates) produced from grapevines (*Vitis vinifera* L. cv. Groppello) treated with different agrochemicals. Furthermore, in order to explore the biological activity of these commodities, we assessed their antiradical activity by $DPPH^{\cdot}$ (2,2-diphenyl-picrylhydrazyl) and $ABTS^{\cdot+}$ [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] radical-scavenging assay. In general, mass spectrometry analysis revealed that the highest melatonin concentrations were detected in red and dessert wines, whereas the indoleamine was not identified in grape juices. Resveratrol was not found in dessert wines, grape juices and balsamic vinegars, the latter showing a very high TP content (determined by the Folin-Ciocalteau colorimetric assay). The highest DPPH and ABTS radical-scavenging activity was measured in the red wine group. Melatonin and *trans*-resveratrol levels were higher in Groppello wines produced from grapes treated with chitosan (CHT, an elicitor of the plant defence mechanisms) and CHT in combination with copper hydroxide (used in organic agriculture) (CHT/Cu), respectively, than in those obtained from conventional fungicide-treated grapes. To the best of our knowledge, we reported, for the first time, the presence of melatonin in dessert wines and balsamic vinegars.

Keywords: wine, grape juice, balsamic vinegar, polyphenols, resveratrol, melatonin, antioxidant activity, traditional Mediterranean diet

1. Introduction

Outside the Animal Kingdom, melatonin was discovered, for the first time, in the photosynthesizing unicellular alga *Lingulodinium polyedrum* (Stein) J. D. Dodge sin. *Gonyaulax polyedra* Stein, belonging to the phylum Dinoflagellata (Balzer & Hardeland, 1991).

Since then, the presence of melatonin in food plants and medicinal herbs has been extensively reported. Although there were some preliminary indications (Van Tassel, Li, & O'Neill, 1993), the first complete publications reporting melatonin in tracheophytes (vascular or higher plants) were independently provided by two research groups (Dubbels et al., 1995; Hattori et al., 1995). They found this indoleamine in a number of edible plants and, since then, it has been detected and quantified in roots, shoots, leaves, flowers, fruits and seeds of a considerable variety of spermatophyte species, as attested by numerous publications confirming undoubtedly the presence of this molecule in the Plant Kingdom (Kolář & Macháková, 2005). In flowering plants (angiosperms), the occurrence of melatonin has been described in a number of families belonging to both the mono- and dicotyledons, relevant as food and medicinal plants (Paredes, Korkmaz, Manchester, Tan, & Reiter, 2009; Posmyk & Janas, 2009).

The synthesis of melatonin in plants is still not completely known, although some evidences suggest that these organisms are equipped with the enzymatic machinery for its biosynthesis (Murch, Krishna Raj, & Saxena, 2000). Similarly, the physiological and pathophysiological role of melatonin *in planta* is still unclear. Because of its structural similarity with the plant growth hormones of the auxin family, a hormone-like role has been attributed to melatonin in some plant species, as well as an action in delaying flowering, preventing chlorophyll degradation, protecting against oxidative damage, abiotic stresses, pathogens and environmental pollutants (Arnao & Hernández-Ruiz, 2006).

Among grapevine (*Vitis vinifera* L.) products, melatonin was firstly detected in the berry exocarp (skin) (Iriti, Rossoni, & Faoro, 2006) and than in wine (Mercolini et al., 2008). More recently, it was reported in all berry and seed tissues and its presence was confirmed in some different red and white wines (Stege, Sombra, Messina, Martinez, & Silva, 2010; Vitalini et al., 2011a). As grape products are main components of the traditional Mediterranean diet and the latter is associated to a reduced risk of chronic degenerative diseases (Estruch et al., 2006), we have suggested that dietary melatonin may contribute to explain these health benefits, mainly attributed to polyphenolic compounds, including resveratrol (Iriti, Varoni, & Vitalini, 2010). In general, by virtue of their antioxidant power, all these phytochemicals exert protective effects by reducing the oxidative burden due to reactive oxygen species (ROS) and involved in the etiopathogenesis of cardiovascular diseases, neurological disorders and certain types of cancer (Mayne, 2005).

Because, to date, melatonin was detected only in grapevine berry and seed tissues and wines, in this work, we measured the melatonin concentration in some different grape products, namely red and white wines, dessert wines, grape juices and traditional balsamic vinegars of different Italian geographical origin, as well as in experimental wines (microvinificates) produced from

grapevines treated with different agrochemicals. Furthermore, we compared the levels of melatonin with those of resveratrol and total polyphenols and assayed the antiradical activity of these commodities. To the best of our knowledge, we reported, for the first time, the presence of melatonin in dessert wines and balsamic vinegars.

2. Materials and Methods

2.1 Grape products

All the grape products were purchased in a local market. They include: *i*) monovarietal red wines, 2010 vintage (Melag DOC, Alto Adige; Nebbiolo DOC, Piemonte; Terre di Rubinoro DOCG, a 'Chianti Colli Senesi', Toscana; Syrah IGT, Sicilia); *ii*) polyvarietal or blended red wines, 2010 vintage (Placido Rizzotto IGT Centopassi winery, with Nero d'Avola, Syrah, Perricone and other varieties produced in the Belice Corleonese, Sicilia, in the lands seized from 'Cosa Nostra' and dedicated to Peppino Impastato, a young Sicilian that gave up his life in the struggle against mafia; La Segreta IGT, Sicilia, with Nero d'Avola, Merlot and Syrah); *iii*) Chaudelune vin de glace DOC (blanc de Morgex et de la Salle), 2007 vintage (a dessert ice wine produced from grapes that have been frozen when still on the vine), Valle d'Aosta; *iv*) dessert wines, 2009 vintage (Passito and Moscato di Pantelleria, Sicilia); *iiv*) grape juices (Hero® and Vitafit®; *ivv*) traditional balsamic vinegars of Modena IGP.

2.2 Open-field treatments and experimental wines

A phytoiatric campaign was carried out during 2010 in an experimental vineyard of *Vitis vinifera* L. cv. Groppello, an autochthonous red cultivar of Lombardia, located at Raffa di Puegnago (Azienda Agricola San Giovanni, Brescia, Italy) and managed according to the Good Agricultural Practices (http://www.fao.org/prods/gap/). Vine rows ran N-S and planting density was 3 m between rows and 1.25 m between plants. The vineyard was drip-irrigated. Open-field treatments were: (*i*) 0.03% (w/v) chitosan (CHT, 76 kDa molecular weight and 85% deacetylation degree; Sigma-Aldrich, St. Louis, MO, USA), an elicitor of the plant defence mechanisms; (*ii*) 0.03% CHT in combination with 150 g/hL copper hydroxide (Kocide® 3000; Du Pont, Wilmington, DE, USA), the latter employed in organic agriculture (CHT/Cu); (*iii*) 0.3 mM benzothiadiazole [benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester, BTH, trade name Bion®, Syngenta, Basilea, CH], another plant activator. Untreated vines were used as negative control, while plants treated with conventional fungicides (penconazole and dinocap) represented the positive 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. The selected plants were

uniform in size and did not show any nutrient deficiency. Parcels were sprayed every 10 days approximately, according to the meteorological conditions, from the beginning of grape susceptibility to fungal diseases until the complete *véraison* (i.e. approximately from the middle of April to the end of July). To avoid spray drift to neighbouring parcels, treatments were carried out with a spray lance powered by a walking-type motor pump, distributing a volume equivalent to 800-1000 L/ha. The phytosanitary status of vineyards was assessed weekly on leaves and bunches, by visual inspections, though the site was not particularly predisposed to severe fungal or other epidemics.

When grapes reached industrial maturity, they were harvested and transported to the Centro Vitivinicolo Provinciale of Brescia (Italy). Experimental vinifications (microvinification) were conducted in triplicate, by standard techniques, and experimental wines (microvinificates) were stored at 4°C in the dark until analysis.

2.3 Determination of total polyphenol content, DPPH and ABTS assays

All the analyses on samples were carried out in dim light conditions to avoid the photooxidation of bioactive compounds. Total polyphenol (TP) content was measured by the Folin-Ciocalteu colorimetric assay, using gallic acid as standard (Vitalini et al., 2011a). Briefly, an aliquot of the samples was added to 50 μL of Folin-Ciocalteu reagent. The solutions were mixed and allowed to stand for 3 min. Next, 100 μL of a saturated sodium carbonate solution and distilled water to final volume of 2.5 mL were added. After 1 hr of incubation, in the dark, at room temperature, the absorbance was read at 725 nm. Polyphenol quantification was based on a standard curve (0.0-500 mg/L, $y = 0.0375x - 0.0228$, $R^2 = 0.9993$) of gallic acid, and results were expressed as gallic acid mEq.

The 2,2-diphenyl-picrylhydrazyl ($DPPH^{\cdot}$) radical-scavenging activity was performed as previously reported (Vitalini et al., 2011a). In brief, aliquots of each sample, at five different concentrations (from 1 to 100 μm), were added to a 15 μm EtOH solution of $DPPH^{\cdot}$ free radical. After a reaction time of 15 min in the dark, the absorbance at 517 nm was determined by spectrophotometer (Jenway 6310). The IC_{50} was calculated with Prism® 4 (GraphPad Software Inc., La Jolla, CA, USA).

The 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\cdot+}$) radical cation-scavenging capacity was determined according to Vitalini and colleagues (Vitalini et al., 2011a). The $ABTS^{\cdot+}$ radical cation was produced by reacting 7 mM ABTS with 2.45 mM potassium persulfate (final concentration) and maintaining the mixture in the dark at room temperature for, at least, 6 hr before use. The $ABTS^{\cdot+}$ solution was diluted with ethanol to an absorbance of 0.7 (± 0.02) at 734 nm and equilibrated at 30°C. Ten μL of each diluted sample, ethanol (negative

control) and standard solution of the synthetic antioxidant 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox, positive control) were mixed for 30 sec with 1 mL of diluted $ABTS^{\bullet+}$ solution. Their absorbance was read at 734 nm, at room temperature, 50 sec after the initial mixing. The percentage of inhibition was calculated as [($ABS_{control\ 734\ nm}$ - $ABS_{sample\ 734\ nm}$/$ABS_{control\ 734\ nm}$) x 100] and reported as trolox equivalent antioxidant capacity (TEAC, mmol eq trolox/L wine).

2.4 Analysis of melatonin and resveratrol in grape products

All the analyses on samples were carried out in dim light conditions to prevent melatonin and resveratrol alteration.

2.4.1 Reagents and working solutions

Melatonin (*N*-acetyl-5-methoxytryptamine) and *trans*-resveratrol (3,5,4'-trihydroxy-*trans*-stilbene) 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 solutions of melatonin (0.1 mg/mL) and *trans*-resveratrol (1 mg/mL) were prepared in methanol and diluted to give working solutions in the range of 0.1-20 ng/mL and 0.1-10 µg/mL. Stock and working solutions were stored at -80°C and -20 °C, respectively.

2.4.2 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). A 1.7 µm Kinetex C_{18} column (150 x 2 mm; Phenomenex) was used for the separation at a flow-rate of 0.55 mL/min. 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, 0.1% HCOOH in acetonitrile). The capillary voltage was set to 3 kV; the source temperature was 130°C; the desolvating 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 and 5 µL were injected in the UPLC-MS/MS system. Masslynx 4.0 software acquired data with Quan-Optimize option for fragmentation study.

For melatonin determination, the gradient was as follows: 20-50% B in 1.0 min, 50-10% B in 10 sec, and then 80% B for 0.5 min. The fragmentation transition for the multiple reaction monitoring (MRM) was $(m/z)^{+}$ 233→174, with cone voltage (CV) and collision energy (CE) at 16 V and 14 eV, respectively. For resveratrol, analysis the gradient was as follows: 20-60% B in

2.0 min, and then 60% B for 20 sec. the fragmentation transitions for the MRM were $(m/z)^+$ 227→143 (CV 39 V, CE 33 eV)and 227→185 (CV 39 V, CE 18 eV).

2.5 Statistical treatment of data

For each experiment, results are expressed as mean ± standard deviation (SD) of data from three independent extractions each analysed in triplicate. Data were subjected to one-way analysis of variance (ANOVA) and comparison among means was determined according to Fisher's least significant difference (LSD) test. Significant differences were accepted at $p < 0.05$ and represented by different letters.

3. Results

To quantify analytes in the different grape products, the MRM mode was employed, and ion transitions of the standards were recorded by UPLC-MS/MS analysis. For melatonin, the calibration curve was generated with a 233/174 transition area (RT = 1.44 min) for each of the seven melatonin concentrations (from 0.1 to 20 ng/mL), and the LLOD was 0.03 ng/mL. The calibration curve for *trans*-resveratrol (RT = 1.32 min) was linear in the range 0.1-10 μg/mL (n = 6), and the LLOD was 50 ng/mL.

Among the grape products, the highest melatonin content was detected in Melag red wine (0.83 ± 0.02 ng/mL), though the mean concentration of the indoleamine was slightly higher in dessert wines than in red wines (0.26 ± 0.028 vs. 0.25 ± 0.28 ng/mL, difference not statistically significant, $p > 0.05$) (table 1). In the red wine group, a high variability in the metabolite content was found, with a minimum value of 0.06 ± 0.01 ng/mL in Placido Rizzotto wine (table 1). Very low levels of melatonin were measured in traditional balsamic vinegars, with an average of 0.04 ± 0.014 ng/mL, whereas, at the detection limit of 0.03 ng/mL, melatonin was not detected in grape juices (table 1). In red wines, the mean content of *trans*- and *cis*-resveratrol was 0.24 ± 0.09 and 0.87 ± 0.39 mg/L, respectively, with a maximum level of both isomers in Terre di Rubinoro wine (0.38 ± 0.03 and 1.37 ± 0.11 mg/L, respectively) (table 1).

Table 1
Melatonin, resveratrol and total polyphenol content of selected Italian grape products and their corresponding $ABTS^{\cdot+}$ [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] and $DPPH^{\cdot}$ (2,2-diphenyl-picrylhydrazyl) radical-scavenging activity

	Melatonin° **(ng/mL wine)**	**Resveratrol°** **(mg/L wine)**		**Total polyphenols (g/L wine)**	**ABTS (mmol eq trolox/L wine)**	**DPPH IC_{50} (μM) Q = 4.37[#]**
		trans	***Cis***			
Red wines (monovarietal)						
Melag DOC (2010) *Alto Adige*	0.83±0.02a[§]	0.12±0.01a	0.32±0.02a	1.28±0.04a	6.39±0.02a	28.3±1.7a
Nebbiolo DOC (2010) *Piemonte*	0.12±0.01b	0.27±0.02b	0.69±0.06b	2.59±0.18b	12.54±0.22b	13.8±0.9b
Terre di Rubinoro DOCG (2010) *Toscana*	0.11±0.01c	0.38±0.03c	1.37±0.11c	2.23±0.15c	10.62±0.17c	19.6±1.1c
Syrah IGT (2010) *Sicilia*	0.20±0.01d	0.20±0.02d	0.87±0.07d	2.83±0.06d	11.58±0.36d	12.2±0.5d
Red wines (polyvarietal)						
Placido Rizzotto IGT (2010) *Sicilia*	0.06±0.01e	0.33±0.03e	1.29±0.10e	1.83±0.15e	9.48±0.02e	20.1±1.1c
La Segreta IGT (2010) *Sicilia*	0.23±0.02f	0.17±0.01f	0.68±0.05b	2.47±0.10f	11.41±0.17d	19.2±0.9c
White wine						
Chaudelune - vin de glace DOC (2007) *Valle d'Aosta*	0.19±0.01g	ND	ND	0.63±0.04g	0.13±0.02f	84.2±4.6e
Dessert wines						
Passito di Pantelleria (2009) *Sicilia*	0.24±0.02f	ND	ND	0.80±0.17h	1.47±0.01g	66±2.4f
Moscato di Pantelleria (2009) *Sicilia*	0.28±0.03h	ND	ND	0.54±0.03i	0.31±0.01h	75.2±2.6g
Grape juices						
Hero®	ND	ND	ND	2.14±0.02l	4.99±0.03i	92±4.9h
Vitafit®	ND	ND	ND	1.33±0.02m	2.56±0.13l	145±4.4i
Balsamic vinegars						
Modena IGP *(Del Duca)*	0.05±0.01i	ND	ND	5.08±0.18n	6.60±0.17m	25.5±1.3l
Modena IGP *(Cavicchioli)*	0.03±0.01l	ND	ND	5.81±0.40o	7.51±0.10n	16.8±1.1m

°Melatonin and resveratrol were determined by ultraperformance liquid chromatography coupled to mass spectrometry (UPLC-MS/MS), whereas total polyphenols were measured by the Folin-Ciocalteau colorimetric assay
[#]IC_{50} of quercetin used as reference compound
[§]Results are mean ± standard deviation of three independent extractions each analysed in triplicate; different letters indicate means significantly different at $p < 0.05$ (Fisher's least significant difference test)

At the detection limit of 50 ng/mL, resveratrol isomers were not detected in dessert wines, grape juices and balsamic vinegars (table 1). An average of 2.20 ± 0.56 g/L total polyphenols was recorded in red wines, a content statistically different from the TP mean value reported in grape juices (1.73 ± 0.57 g/L, $p < 0.05$) (table 1). The lowest average level of TP was measured in

dessert wines (0.67 ± 0.18 g/L, $p < 0.05$), whereas a very high mean concentration of TP was found in balsamic vinegars (5.44 ± 0.51 g/L, $p < 0.05$) (table 1).

DPPH and ABTS radicals are among the most widely used chromogen compounds to assess the antiradical power of biological samples. The scavenging activity of the different grape products against the DPPH radical, expressed as IC_{50}, is reported in table 1. The mean values of the DPPH radical-scavenging capacity increased in the order: grape juices (118.5 ± 37.47 μM) < dessert wines (70.6 ± 6.5 μM) < balsamic vinegars (21.15 ± 6.15 μM) < red wines (18.86 ± 5.67 μM), with the difference between vinegars and red wines not statistically significant ($p > 0.05$) (table 1). ABTS radical-scavenging assay showed that the TEAC mean values decreased in the order: red wines (10.33 ± 2.18 mmol eq trolox/L) > balsamic vinegars (7.05 ± 0.64 mmol eq trolox/L) > grape juices (3.77 ± 1.71 mmol eq trolox/L) > dessert wines (0.89 ± 0.82 mmol eq trolox/L), with all differences statistically significant ($p < 0.05$) (table 1). In general, the highest DPPH and ABTS radical-scavenging activity was detected in the red wine group. In particular, Syrah and Nebbiolo wines showed the maximum DPPH (IC_{50} = 12.2 ± 0.54 μM) and ABTS (12.54 ± 0.22 mmol eq trolox/L) radical-scavenging capacity, respectively, both the red wines with the highest TP content (2.83 ± 0.06 and 2.59 ± 0.18 g/L, respectively) (table 1).

In Groppello experimental red wines, CHT open field treatments were the most effective in increasing the melatonin content by 38.5% compared with the wine treated with conventional fungicides (0.27 ± 0.01 vs. 0.07 ± 0.009 ng/mL, $p < 0.05$) (table 2). Phytoiatric applications of CHT/Cu and BTH raised by 17% and 24%, respectively, the melatonin levels of the corresponding wines with respect to the conventional wine (0.12 ± 0.01 vs. 0.07 ± 0.009 ng/mL, $p < 0.05$ and 0.17 ± 0.007 vs. 0.07 ± 0.009 ng/mL, $p < 0.05$) (table 2). Moderate variations in the *trans*-resveratrol concentration were recorded in microvinificates. Compared with conventional wine, only CHT/Cu treatments slightly increased the amount of the metabolite in wine (from 0.31 ± 0.01 to 0.33 ± 0.01 mg/L, $p < 0.05$), whereas the wine produced from BTH-treated grapevines showed the lowest *trans*-resveratrol content (0.26 ± 0.001, $p < 0.05$). No difference was observed in the levels of the isomer between CHT and conventional wines. Only slight differences were detected in TP content and antiradical capacity (table 2).

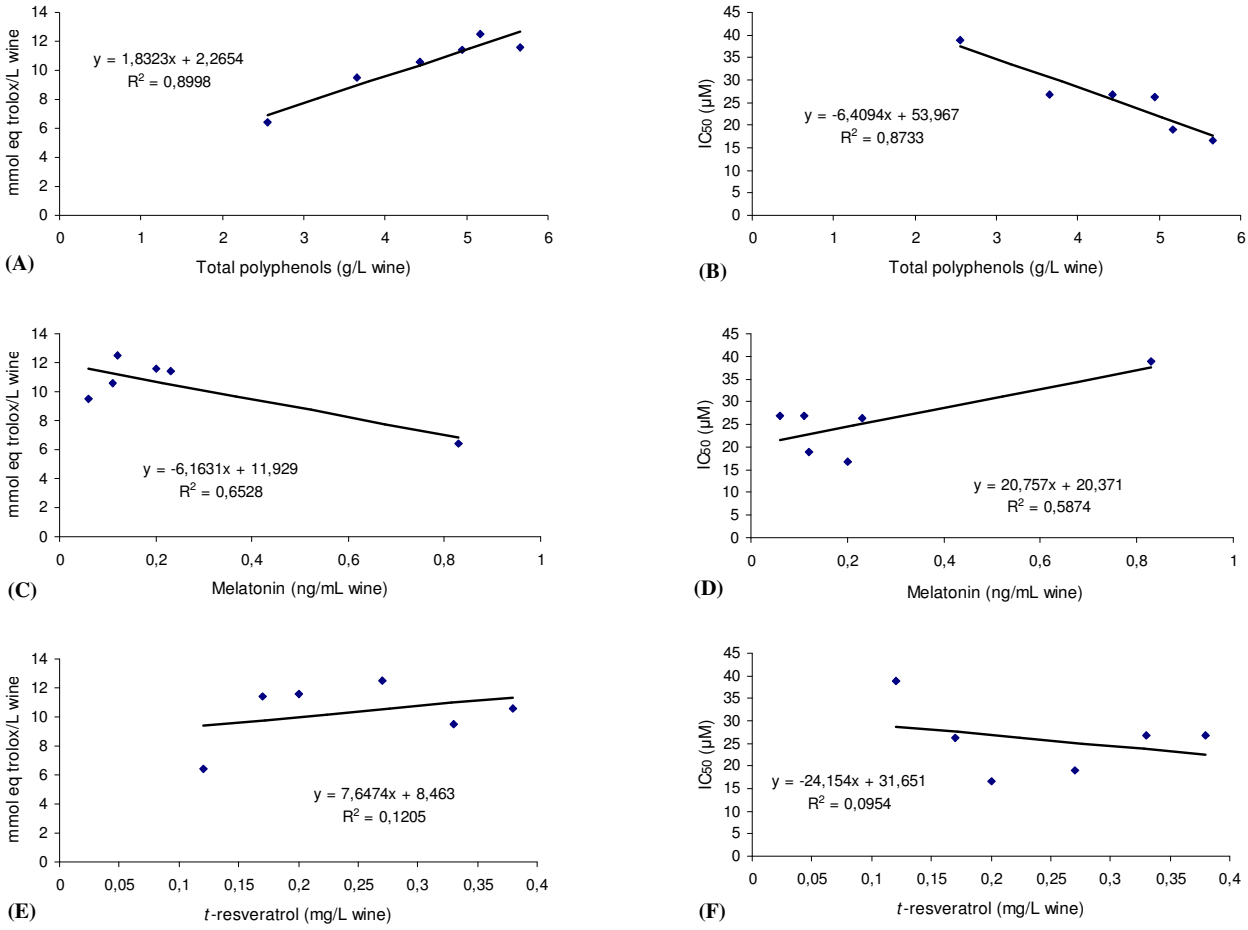
Table 2
Melatonin, resveratrol and total polyphenol content of experimental Groppello red wines (microvinificates) and their corresponding $ABTS^{\bullet+}$ [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] and $DPPH^{\bullet}$ (2,2-diphenyl-picrylhydrazyl) radical-scavenging capacity

	Melatonin° (ng/mL wine)	**Resveratrol°** (mg/L wine)		**Total polyphenols** (g/L wine)	**ABTS** (mmol eq trolox /L wine)	**DPPH** IC_{50} (μM) Q = 4.37[#]
		trans	***Cis***			
Groppello fungicides*	0.07 ±0.01a[§]	0.31 ±0.01a	1.09±0.1a	1.63 ±0.11°	7.85 ±0.03a	15.8 ±1.2a
Groppello CHT	0.27 ±0.01c	0.31 ±0.01a	2.23±0.01c	1.67 ±0.09°	7.36 ±0.05b	17.9 ±1.4b
Groppello CHT/Cu	0.12 ±0.01b	0.33 ±0.01b	1.73±0.07b	1.66 ±0.10°	7.63 ±0.08c	14.9 ±0.9c
Groppello BTH	0.17 ±0.01d	0.26 ±0.01c	1.96±0.02d	1.47 ±0.07b	7.42 ±0.13b	17.4 ±1.2b

°Melatonin and resveratrol were determined by ultraperformance liquid chromatography coupled to mass spectrometry (UPLC-MS/MS), whereas total polyphenols were measured by the Folin-Ciocalteau colorimetric assay
[#]IC_{50} of quercetin used as reference compound
*Groppello wines were obtained by microvinification of grapes from different phytoiatric treatments: conventional fungicides (penconazole and methyldinocap); 0.03% chitosan (CHT); 0.03% CHT in combination with 150 g/hL copper hydroxide (CHT/Cu); 0.3 mM benzothiadiazole (BTH)
[§]Results are mean ± standard deviation of three independent extractions each analysed in triplicate; different letters indicate means significantly different at $p < 0.05$ (Fisher's least significant difference test)

For the red wine group, a correlation analysis based on simple linear regression was performed on the assayed variables at the 95% confidence level (figure 1). TP content was highly correlated with both ABTS (TEAC) and DPPH (IC_{50}) radical-scavenging capacity ($R^2 = 0.8998$ and $R^2 = 0.8733$, respectively) (figure 1A,B). A lower linear correlation coefficient was reported for melatonin and both ABTS and DPPH ($R^2 = 0.6528$ and $R^2 = 0.5874$, respectively) (figure 1C,D), whereas only a very weak correlation was found between *trans*-resveratrol and both radical-scavenging activities ($R^2 = 0.1205$ for TEAC and $R^2 = 0.0954$ for IC_{50}) (figure 1E,F). From these results, it seems that the antiradical power of the assayed red wines may be mostly attributed to their TP content, with melatonin playing a major role in the radical-scavenging capacity of wines compared to *trans*-resveratrol, at least in our experimental conditions.

Figure 1. Correlation analysis based on simple linear regression at the 95% confidence level between: total polyphenol content (g/L wine) determined by the Folin-Ciocalteau colorimetric assay and (A) $ABTS^{\cdot+}$ [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] (mmol eq trolox/L wine) and (B) $DPPH^{\cdot}$ (2,2-diphenyl-picrylhydrazyl) (IC_{50}, μM) radical-scavenging activity; melatonin concentration (ng/mL wine) and (C) $ABTS^{\cdot+}$ and (D) $DPPH^{\cdot}$ scavenging test; *trans*-resveratrol levels (mg/L wine) and (E) $ABTS^{\cdot+}$ and (F) and $DPPH^{\cdot}$ radical-scavenging capacity



4. Discussion

As already mentioned, melatonin was first detected in berry skin of Italian and France varieties (Barbera, Croatina, Cabernet Sauvignon, Cabernet Franc, Marzemino, Nebbiolo, Sangiovese and Merlot) grown in north-western Italy, with levels ranging from 0.005 to 0.96 ng/g (Iriti et al., 2006). Similar results (from 0.6 to 1.2 ng/g) were reported by Stege and colleagues (2010) for the same tissue of Malbec, Cabernet Sauvignon and Chardonnay varieties cultivated in Argentina. However, much higher melatonin concentrations, between 100 and 150 μg/g, depending on the phenological stage, were measured in the whole berry (i.e. skin, flesh and seeds analyzed together) of Merlot cultivar grown in Canada (Murch, Hall, Le, & Saxena,

2010). Very recently, in Merlot berry skin, we found 17.5 and 9.3 ng/g of melatonin at pre-*véraison* and *véraison*, respectively (Vitalini et al., 2011b). In berry skin of Malbec variety cultivated in Argentina, melatonin concentration showed similar values during the night (around 10 ng/g), reaching a strong peak at night (159 ng/g) (Boccalandro, Gonzales, Wunderlin, & Silva, 2011). Finally, the transition from pre-*véraison* to *véraison* raised the melatonin content both in Merlot berry seeds (from 3.5 to 10 ng/g) and flesh (from 0.2 to 3.9 ng/g) (Vitalini et al., 2011b).

As previously introduced, further studies ascertained the occurrence of melatonin in wine. Firstly, Mercolini and co-workers (2008) detected it at 0.5 and 0.4 ng/mL in Sangiovese red and Trebbiano white wine, respectively. Stege and colleagues (2010) reported melatonin at 0.16, 0.24 and 0.32 ng/mL in Chardonnay, Malbec and Cabernet Sauvignon wines, respectively. More recently, our results showed that the levels of melatonin in Groppello and Merlot wines varied between 5.2 and 8.1 ng/mL, depending on agrochemical treatments (Vitalini et al., 2011a), whereas Rodriguez-Naranjo and colleagues (2011a, 2011b) measured higher melatonin concentrations, up to 150 and 400 ng/mL in racked wines. Our results from the phytoiatric campaign are partly in accordance with previous data showing that open-field CHT treatment on Groppello variety raised by 22% the levels of melatonin in wine, whereas CHT/Cu and BTH treatments failed to increase the melatonin content in the corresponding wines (Vitalini et al., 2011a). Conversely, in Merlot cultivar, CHT/Cu was more effective than CHT in improving the melatonin concentration of wine (Vitalini et al., 2011a). Because plant activators include a class of agrochemicals able to trigger the plant defence reaction, mainly stimulating the synthesis of phytoalexins, it would be reasonable to suppose that melatonin may function as a defence metabolite for grapevine, whose synthesis is elicited by treatment with resistance inducers or pathogen attack.

Grapes are probably the most important source of resveratrol in the human diet, found in white, rosé and, mostly, red wines (Stervbo, Vang, & Bonnesen, 2007). It is synthesized mainly in the berry skin in two isomers, *cis*- and *trans*-resveratrol, the latter most widely studied, although the former also possess biological activity (Bertelli et al., 1996). The interest of the scientific community in this phytoalexin has increased over the last years, because of the results of many promising *in vitro*, *ex vivo* and *in vivo* studies indicating the cardioprotective and anticancer potential of this stilbenic polyphenol (Stervbo et al., 2007). In the last decade, an inverse relationship between a moderate, regular red wine consumption at meal and the risk of chronic, degenerative diseases has been reported, with polyphenols considered as the archetype of the observed health benefits (Iriti & Faoro, 2009; Costanzo, Di Castelnuovo, Donati, Iacoviello, & De Gaetano, 2010).

As regards our results, in general, we agree with the recent survey of Rayne (2010), who defined the levels of melatonin near 1 ng/g and 0.5 ng/mL in grapevine berry skin and wine, respectively. However, according to the comprehensive survey of Stervbo and colleagues (2007), red wine contains an average of 1.9 mg/L *trans*-resveratol, a content higher than the average of 0.24 mg/L found in our red wines.

Noticeably, many endogenous and external factors may influence the melatonin and polyphenolic (including resveratrol) levels in grapevine and their products, such as the genetic traits of the cultivar and its geographical origin, the berry tissue/plant organ analyzed, the difference between thin and thick skinned grapes, the phenological stage, day/night fluctuations, pathogen (mainly fungal) infections and phytosanitary treatments, agro-meteorological conditions and environmental stresses, altitude, UV radiations and high light irradiance, the vintage and wine-making procedures (Murch et al., 2010; Boccalandro et al., 2011; Rodriguez-Naranjo et al., 2011a; Vitalini et al., 2011a).

In particular, the wine-making techniques play a main role in the extraction of phenolics from grapes: the maceration time (i.e. the skin and seed contact with the must) and the fermentation period improve the extraction and the solubility of phenolic compounds (with an increase in the alcoholic content) (Sacchi, Bisson, & Adams, 2005). Differently, milder extraction conditions are employed in grape juice production, generally involving a rapid crushing (few seconds), a maceration of 1-3 hours, a pressing of few minutes and then filtration and pasteurization (Gonzáles-Barrio, Vidal-Guevara, Tomás-Barberán, & Espìn, 2009). These different extraction conditions may, at least in part, explain our results reporting differences between wines and juices.

Traditional balsamic vinegar is a typical seasoning produced in Modena and Reggio Emilia (Italy), which has become popular worldwide in recent years. Initially, the must obtained from white or red grapes is subjected to a thermal treatment and is slowly concentrated to produce the cooked must (*vino cotto*), that represents the raw material to produce vinegar. Then, alcoholic fermentation and acetic bioxidation of cooked and concentrated must are carried out in a set of barrels and, after ageing, the product is ready for the consumption (Giudici, Gullo, & Solieri, 2009). In our vinegar samples and experimental conditions, an overestimation of total polyphenols by the methodology used occurred, possibly because the reaction of these compounds with the Folin-Ciocalteau reagent is non-specific and each single phenol shows a different response to this reagent (Naczk & Shahidi, 2004). It is noteworthy that the reported antiradical power of balsamic vinegars may be also due to melanoidins, pigmented polymeric compounds synthesized in the final stage of the Maillard reaction (Verzelloni, Tagliazucchi, & Conte, 2010). Intriguingly, they are rich of phenolic groups and may contribute to 40-50% of

the total antioxidant activity of aged traditional balsamic vinegars (Tagliazucchi, Verzelloni, & Conte, 2010).

5. Conclusions

The discovery of melatonin in edible plants, seeds and medicinal herbs has opened a new field of knowledge in food and nutrition sciences. It is possible that the documented healthy properties of grape/wine are attributed not only to the presence of polyphenolic compounds, but also to melatonin and other hundreds of phytochemicals present in these food matrices. In these terms, it would be of great interest to verify whether the health-promoting effects attributed to certain foods may be, at least in part, due to dietary melatonin, possibly acting synergistically with other bioactive molecules present in food plants. However, at present, the available data regarding the presence of melatonin and its quantity in grape products and other foods are scarce, as well as information on the bioavailability and metabolism of dietary melatonin. Therefore, due to the paucity of data about the melatonin content in foods, it results very difficult to estimate the daily dietary intake of melatonin, and this paper may contribute to provide useful information for food composition tables. In general, an efficient uptake of melatonin from food sources should be expected to influence its circulating levels, basically very low (~200 pg/mL at the maximum night peak and lower than 10 pg/mL during the day) (Bonnefont-Rousselot & Collin, 2010). In animals and humans, the intake of foodstuffs containing melatonin may contribute to increase both the serum levels of this compound and the urinary concentrations of its metabolite 6-sulfatoxymelatonin (Reiter, Manchester, & Tan, 2005; Maldonado, Moreno, & Calvo, 2009; Garrido et al., 2010). Finally, the European Food Safety Authority has recently accepted the scientific substantiation of health claims linking melatonin to the alleviation of subjective feelings of jet leg, the reduction of sleep onset latency and improved sleep quality (EFSA Journal, 2010).

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Chapter 5

Effects of plant activators on the polyphenol content and antiradical activity of grapevine tissues

5.1. Introduction

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) secondary metabolism. In particular, the total polyphenol (TP) content and the antiradical activity were measured in leaf, berry (skin and flesh) and seed tissues at two different phenological stages (pre-*véraison* and *véraison*). Data were collected for two vintages (2009 and 2010) and compared with tissues from conventional fungicide-treated grapevines. For a detailed introduction on the resistance inducers employed in this study, the plant innate immunity and systemic acquired resistance (SAR) the reader should refer to Chapter 5 (Vitalini et al., 2011b).

5.2. Materials and Methods

A detailed description of the phytoiatric campaigns, the scheduling of treatments and their randomization in vineyards has been accurately reported in Chapter 5 (Vitalini et al., 2011b). For the sake of clarity, we only report the list of the analyzed samples (table 1).

Table 1. List of samples analyzed for total polyphenol content and antiradical activity

Samples
Groppello leaves[1] treated with CHT[2], CHT/Cu[3], BTH[4], conventional fungicide (2009, 2010)
Groppello berry skins treated with CHT, CHT/Cu, BTH, conventional fungicide (pre-*véraison* 2009, 2010)
Groppello berry flesh treated with CHT, CHT/Cu, BTH, conventional fungicide (*véraison* 2009, 2010)
Groppello berry seeds treated with CHT, CHT/Cu, BTH, conventional fungicide (pre-*véraison* 2009, 2010)
Groppello berry seeds treated with CHT, CHT/Cu, BTH, conventional fungicide (*véraison* 2009, 2010)

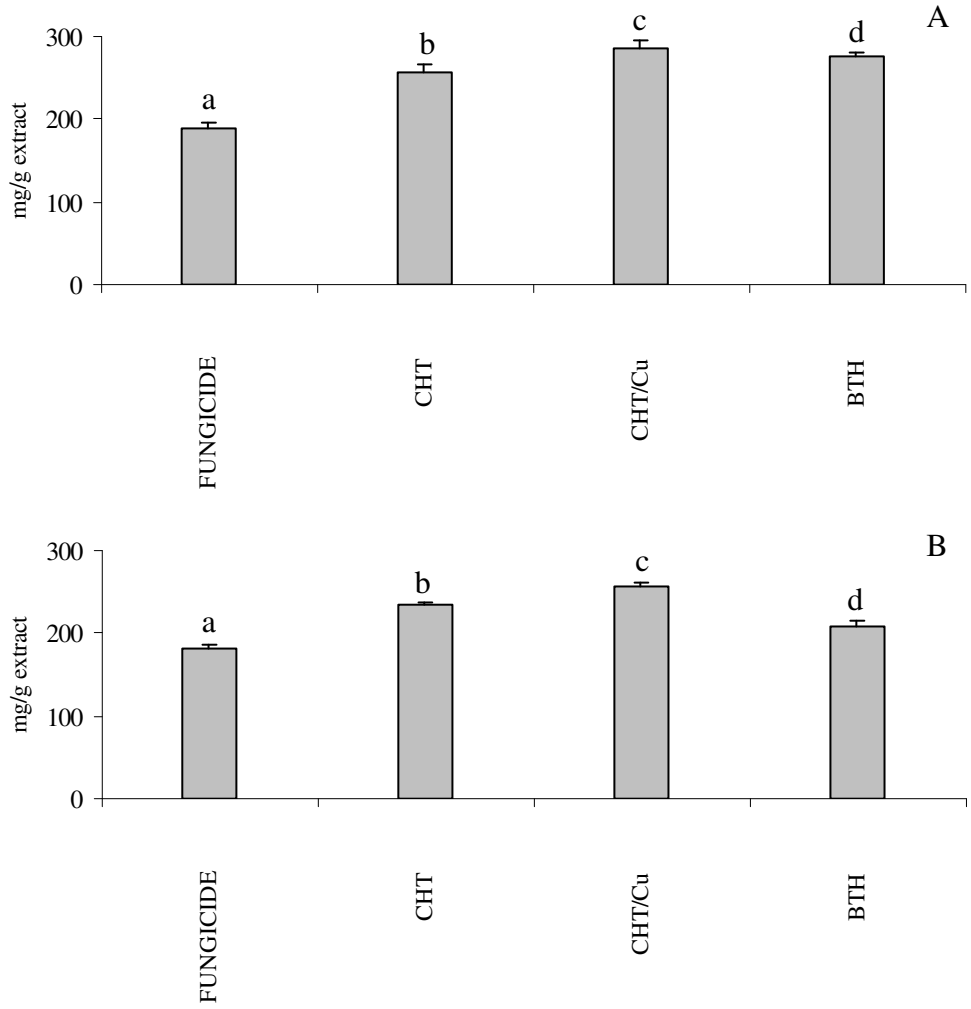
[1]Leaves were collected at pre-*véraison*; [2]CHT, 0.03% chitosan; [3]CHT/Cu, chitosan plus 150 g/hL copper hydroxide; [4]BTH, 0.3 mM benzothiadizole

The TP content of samples was measured by the Folin-Ciocalteau colorimetric assay, and their antiradical activity by DPPH and ABTS radical-scavenging assays. These methodologies, as well as the statistical treatment of data were precisely described in Chapter 6 (Vitalini *et al*., 2011c).

5.3. Results and Discussion

All treatments with elicitors significantly increased the polyphenol content of leaf tissues more than conventional fungicides, both in 2009 and 2010, with CHT/Cu showing the higher efficacy (figure 1A,B).

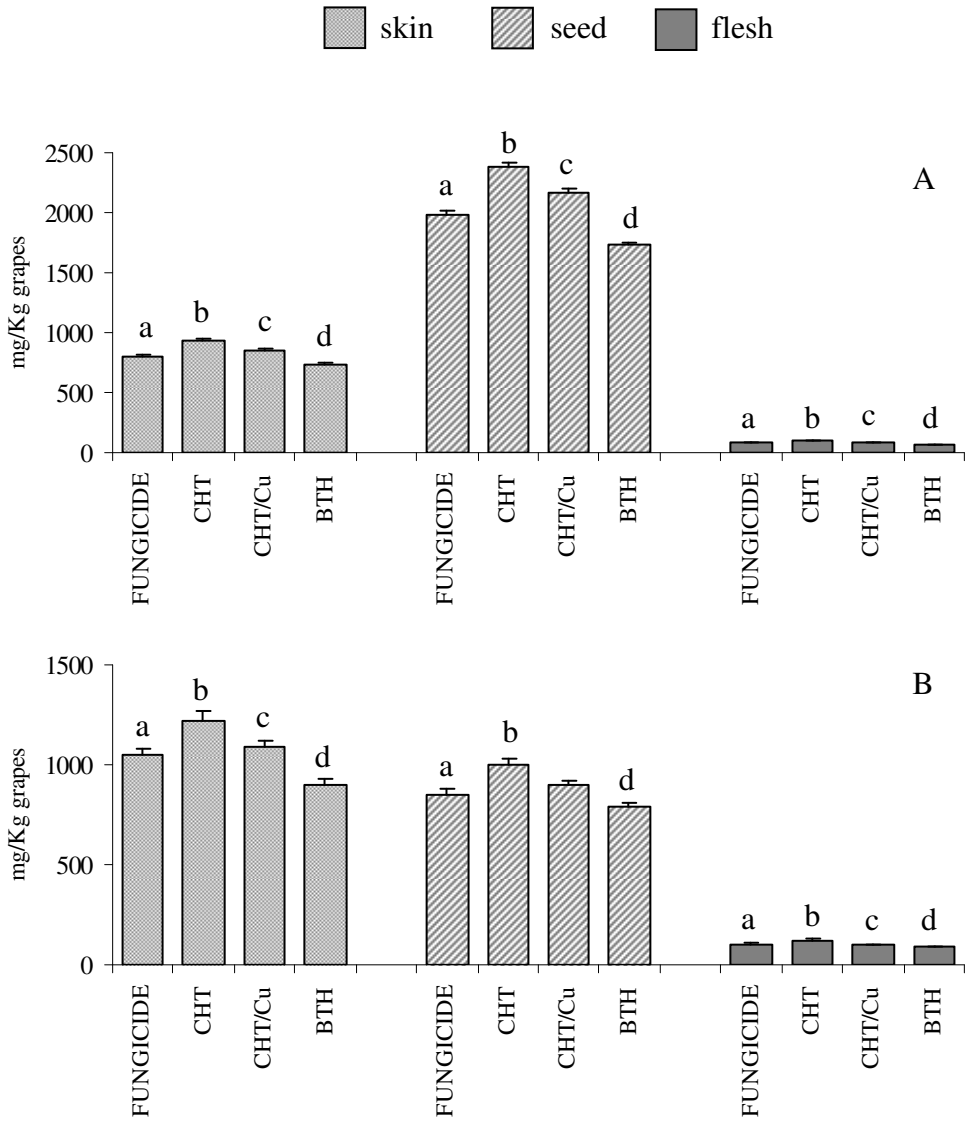
Figure 1. Total polyphenol content (mg/g extract) measured by the Folin-Ciocalteau colorimetric assay in leaf tissues of grapevine (*Vitis vinifera* L. cv. Groppello) collected at pre-*véraison* during the (A) 2009 and (B) 2010 vintage. Results are expressed as mean ± S.D. of data from three determinations recorded for at least two independent extractions. Bars carrying different letters indicate means significantly different at $P < 0.05$ (Tukey's honestly significant difference test).



In berry tissues, in both years, the highest total polyphenol content was detected in seeds, at pre-*véraison* and in skins at *véraison*, with very low levels measured in flesh (figures 2, 3). In berry tissues collected in 2009, CHT/Cu and CHT were the most effective in increasing polyphenols,

mainly in seeds and skins. On the contrary, samples treated with BTH showed lower levels of these metabolites than fungicide (figure 2A,B).

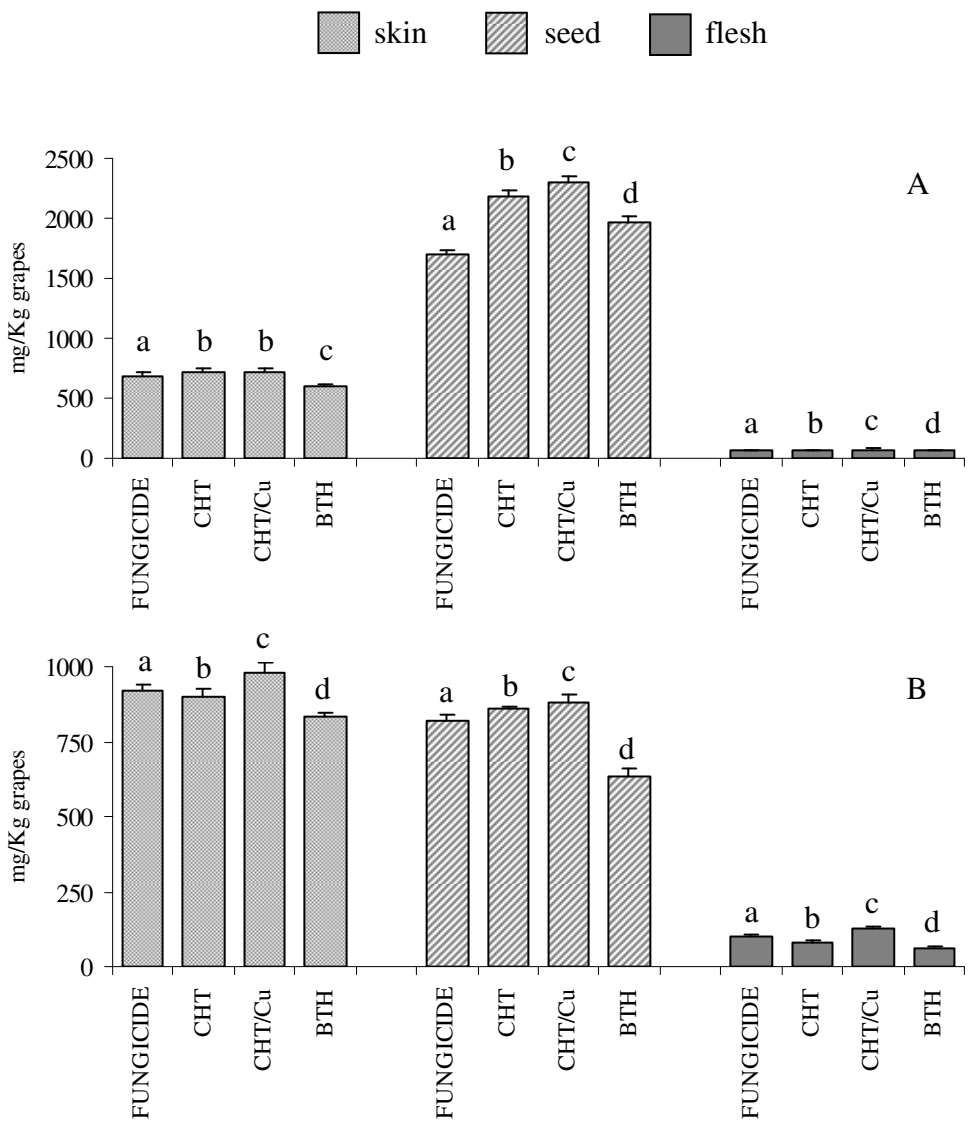
Figure 2. Total polyphenol content (mg/Kg grapes) measured by the Folin-Ciocalteau colorimetric assay in skins, seeds and flesh of grapevine (*Vitis vinifera* L. cv. Groppello) berry sampled at (A) pre-*véraison* and (B) *véraison* during the 2009 vintage. Results are expressed as mean ± S.D. of data from three determinations recorded for at least two independent extractions. Bars carrying different letters indicate means significantly different at $P < 0.05$ (Tukey's honestly significant difference test).



In 2010, at pre-*vèraison*, all the resistance inducers increased the level of polyphenols more than fungicide, in seeds, whereas, in skins, BTH was less effective than fungicide (figure 3A). At *véraison*, in seeds, the highest concentration of polyphenol was detected in samples treated with CHT/Cu and CHT, though, in skins, fungicide was more effective than CHT. In general, at this

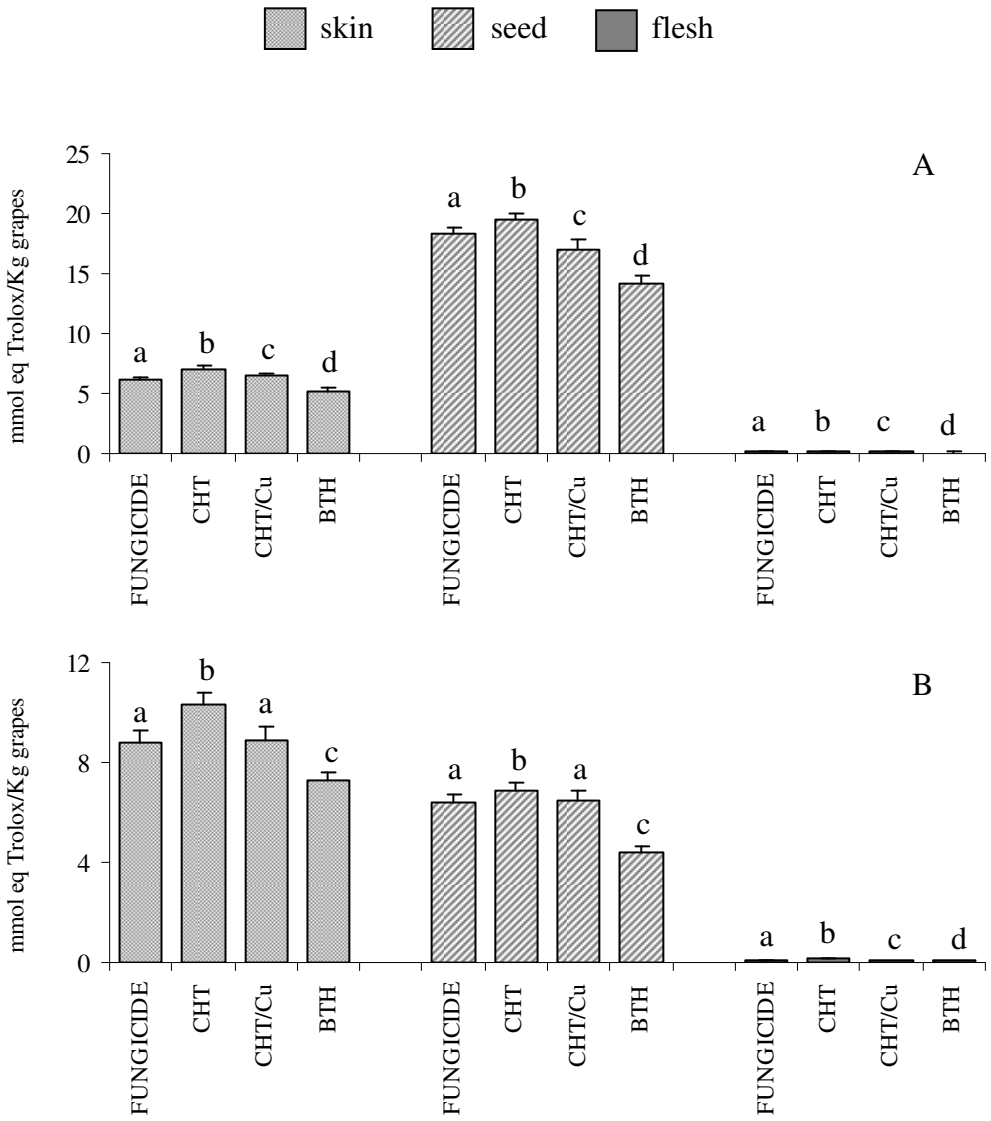
phenological stage, BTH-treated tissues showed the lowest levels of polyphenols in all berry tissue (figure 3B).

Figure 3. Total polyphenol content (mg/kg grapes) measured by the Folin-Ciocalteau colorimetric assay in skins, seeds and flesh of grapevine (*Vitis vinifera* L. cv. Groppello) berry sampled at (A) pre-*véraison* and (B) *véraison* during the 2010 vintage. Results are expressed as mean ± S.D. of data from three determinations recorded for at least two independent extractions. Bars carrying different letters indicate means significantly different at $P < 0.05$ (Tukey's honestly significant difference test).



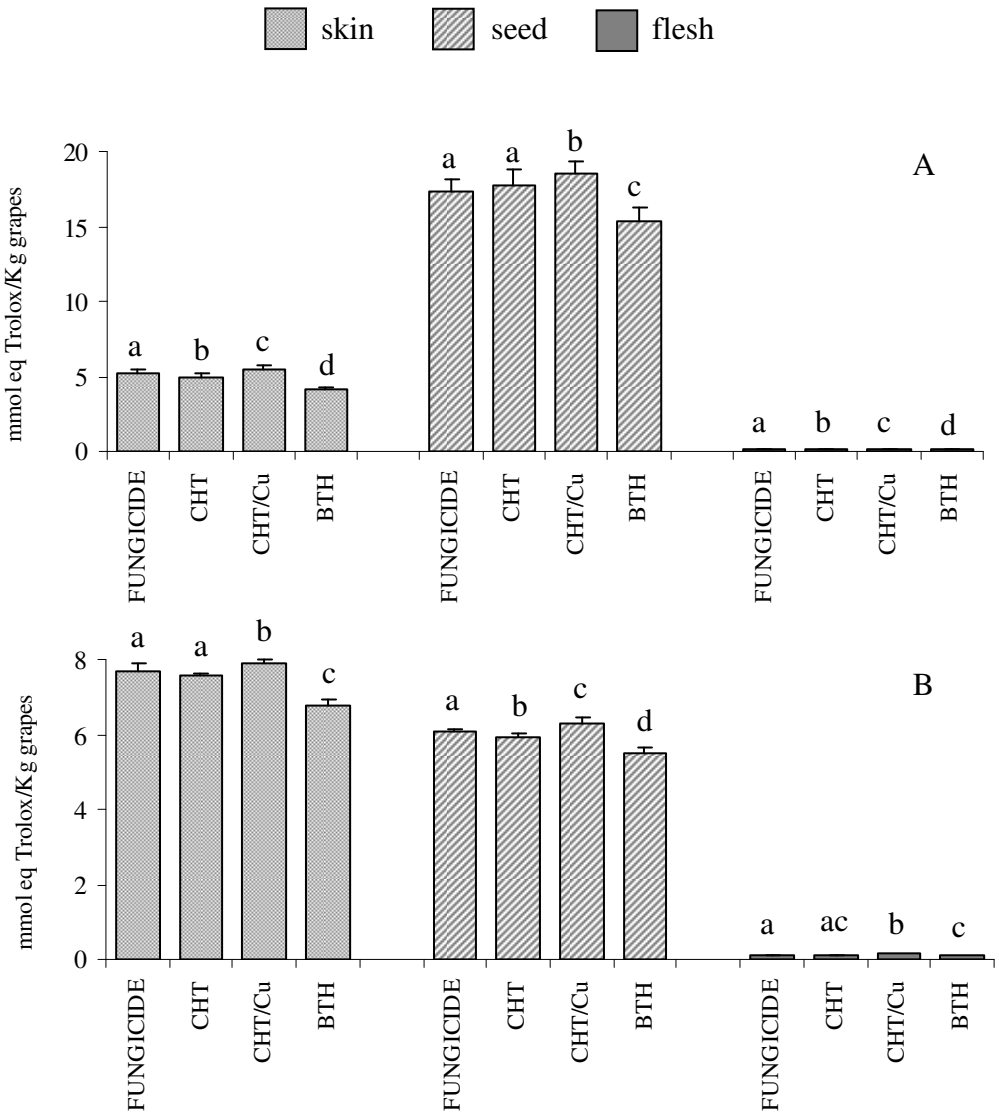
In berry tissues collected in 2009, at pre-*véraison*, the ABTS radical-scavenging capacity decreased in the order: CHT > fungicide > CHT/Cu > BTH, in seeds, whereas, in skins, the order was: CHT > CHT/Cu > fungicide > BTH (figure 4A). At *véraison*, in both tissues, antiradical activity decreased in the order: CHT > CHT/Cu = fungicide > BTH (figure 4B).

Figure 4. ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] radical-scavenging activity (TEAC, mmol eq. Trolox/Kg grapes) measured in skins, seeds and flesh of grapevine (*Vitis vinifera* L. cv. Groppello) berry sampled at (A) pre-*véraison* and (B) *véraison* during the 2009 vintage. Results are expressed as mean ± S.D. of data from three determinations recorded for at least two independent extractions. Bars carrying different letters indicate means significantly different at $P < 0.05$b (Tukey's honestly significant difference test).



In 2010 vintage, at pre-*véraison*, the ABTS radical-scavenging activity decreased in the order: CHT/Cu > CHT = fungicide > BTH and CHT/Cu > fungicide > CHT > BTH, in seeds and skins, respectively (figure 5). At *véraison* the order was: CHT/Cu > fungicide > CHT >BTH and CHT/Cu > CHT = fungicide > BTH, in seeds and skins, respectively (figure 5A,B).

Figure 5. ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] radical-scavenging activity (TEAC, mmol eq. Trolox/Kg grapes) measured in skins, seeds and flesh of grapevine (*Vitis vinifera* L. cv. Groppello) berry sampled at (A) pre-*véraison* and (B) *véraison* during the 2010 vintage. Results are expressed as mean ± S.D. of data from three determinations recorded for at least two independent extractions. Bars carrying different letters indicate means significantly different at P < 0.05b (Tukey's honestly significant difference test).



In 2009, in both phenological stages, the DPPH radical-scavenging capacity increased in the order: BTH < fungicide < CHT/Cu < CHT, in skins and flesh, and BTH < fungicide = CHT/Cu < CHT, in seeds (figure 6A,B). During the 2010 vintage, at pre-*véraison*, the order was: fungicide < BTH < CHT < CHT/Cu, BTH < fungicide = CHT < CHT/Cu and BTH < CHT < fungicide < CHT/Cu, in seeds, skins and flash respectively (figure 7A). At *véraison*, the DPPH radical-scavenging power increase in the order: BTH < CHT < fungicide = CHT/Cu, BTH <

fungicide < CHT < CHT/Cu and BTH < fungicide = CHT < CHT/Cu, in seeds, skins and flash respectively (figure 7B).

Figure 6. DPPH (2,2-diphenyl-picrylhydrazyl) radical-scavenging activity (IC_{50}) measured in skins, seeds and flesh of grapevine (*Vitis vinifera* L. cv. Groppello) berry sampled at (A) pre-*véraison* and (B) *véraison* during the 2009 vintage. Results are expressed as mean ± S.D. of data from three determinations recorded for at least two independent extractions. Bars carrying different letters indicate means significantly different at $P < 0.05$ (Tukey's honestly significant difference test).

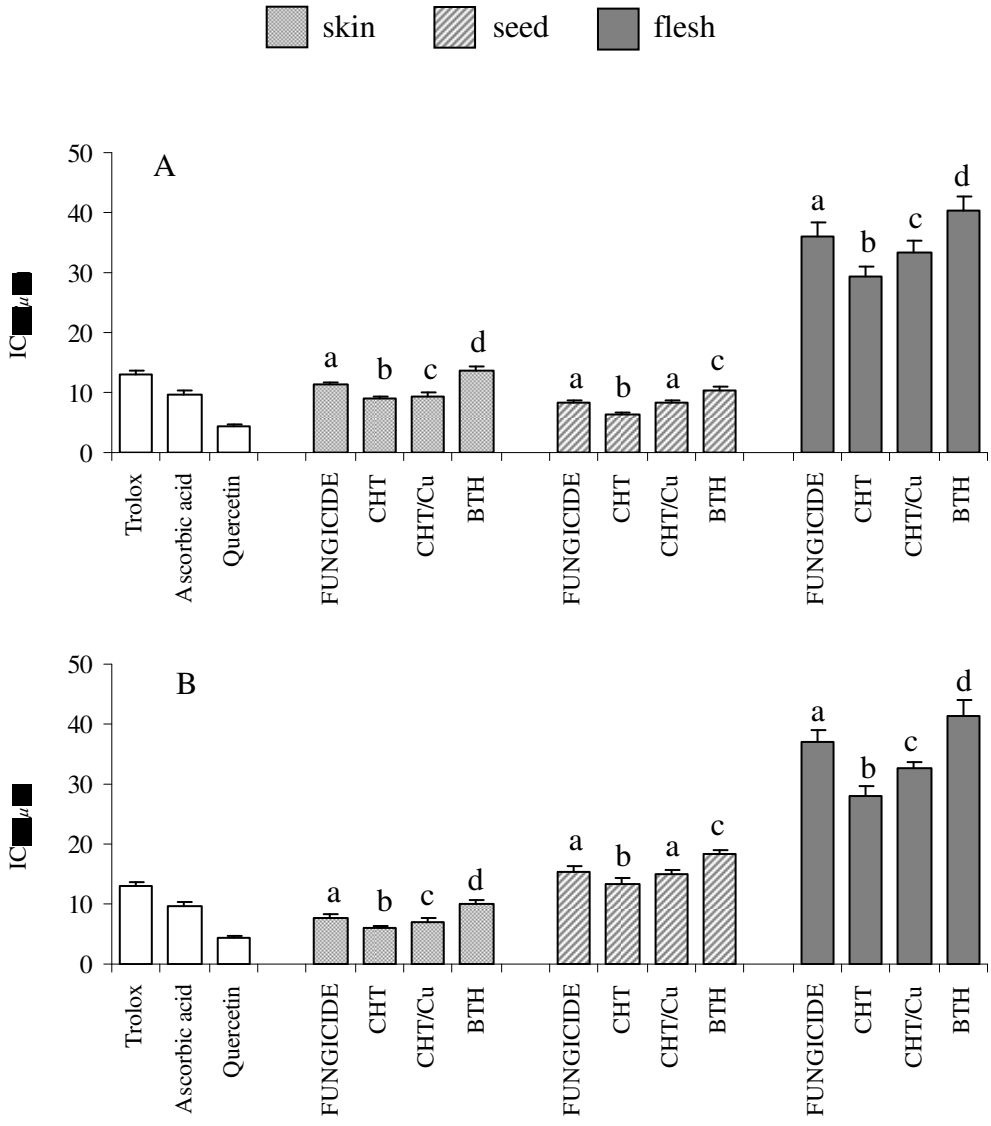
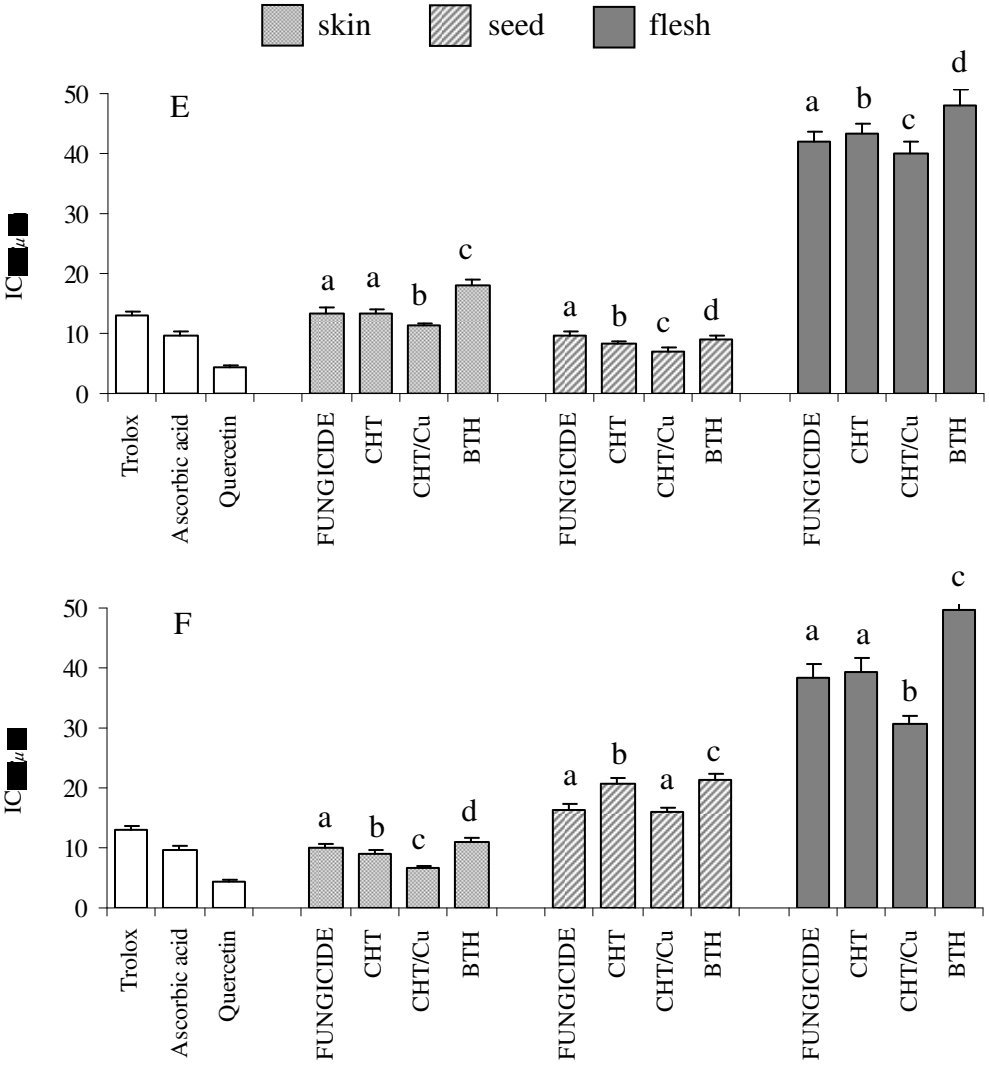


Figure 7. DPPH (2,2-diphenyl-picrylhydrazyl) radical-scavenging activity (IC_{50}) measured in skins, seeds and flesh of grapevine (*Vitis vinifera* L. cv. Groppello) berry sampled at (A) pre-*véraison* and (B) *véraison* during the 2010 vintage. Results are expressed as mean ± S.D. of data from three determinations recorded for at least two independent extractions. Bars carrying different letters indicate means significantly different at $P < 0.05$ (Tukey's honestly significant difference test).



In general, it seems that, in our experimental conditions, elicitors were more effective than conventional fungicides, at least for all the assessed variables, except for BTH in berry tissues, which was the least effective treatment in rising their total polyphenol content and antioxidant activity. Among chitosans, CHT and CHT/Cu were the treatments which elicited the highest responses in the assayed tissues, in 2009 and 2010, respectively, as shown by both polyphenols and ABTS and DPPH tests. According to our opinion, this is the first report in which different resistance inducers have been compared in grapevine, and our results are in agreement with previous data reporting the efficacy of BTH and chitosan in grapevine (Iriti *et al*., 2004, 2005,

2011a). The significance of the observed effects on the production of more healthy and safe wines, as well as the efficacy of treatments on grapevine fungal diseases have been reported in the Chapters 5 (Vitalini *et al*., 2011b), 9 and 10.

Chapter 6

Efficacy of plant activators against grapevine fungal diseases

6.1. Introduction

As previously reported in Chapter 6 (Vitalini *et al*., 2011c) and 8, open field treatments with resistance inducers increased the content of bioactive secondary metabolites and the antiradical activity of grapevine tissues, compared with conventional fungicides. Additionally, these effects were retained in the corresponding experimental wines, in which the levels of mycotoxins remained below the allowable threshold (Chapter 10), thus producing more healthy and safe products. Therefore, in this section, it will be evaluated the potential of elicitors in controlling downy mildew (*Plasmopara viticola*) and powdery mildew (*Erysiphe necator*), the most damaging grapevine fungal diseases.

6.2. Materials and Methods

Two phytoiatric campaigns were planned, in 2009 and 2010, on Groppello, a red grapevine (*Vitis vinifera* L.) variety autochthonous of Lombardia, cultivated in an experimental vineyard located at Raffa di Puegnago (Brescia). Open field treatments 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 both 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 grapevine susceptibility to fungal diseases until the complete *véraison* (i.e., approximately from the middle of April to the end of July). To avoid spray drift to neighbouring parcels, treatments were carried out with a spray lance powered by a walking-type motor pump, distributing a volume equivalent to 800-1000 L ha^{-1}. Symptoms were assessed weekly on leaves and bunches, by visual inspections, though the site were not particularly predisposed to severe fungal or other epidemics, and the following parameters were recorded:

$$\text{Disease Incidence (I\%):} \quad \left(\frac{\text{Symptomatic bunches}}{\text{total bunches}} \right) \times 100$$

Infection Degree (ID%): $\left[\frac{\sum nv}{NV} \right] \times 100$

n = number of bunches in each class
v = class value
N = total amount of assessed bunches
V = the highest class value

Disease Severity (S%): estimated by grouping bunches in 6 classes according to the percentage of infected berries per bunch

6.3. Results and Discussion

The epidemiological evaluations were performed on bunches and infection indexes were calculated in terms of disease incidence (I %), infection degree (ID %) and disease severity (S %). For downy mildew, in 2009 and 2010, I % is reported in figures 1 and 2, respectively, ID % in figures 3 and 4, respectively.

Figure 1. Disease incidence (I %) of downy mildew (*Plasmopara viticola*) on grapevine cv. Groppello in 2009

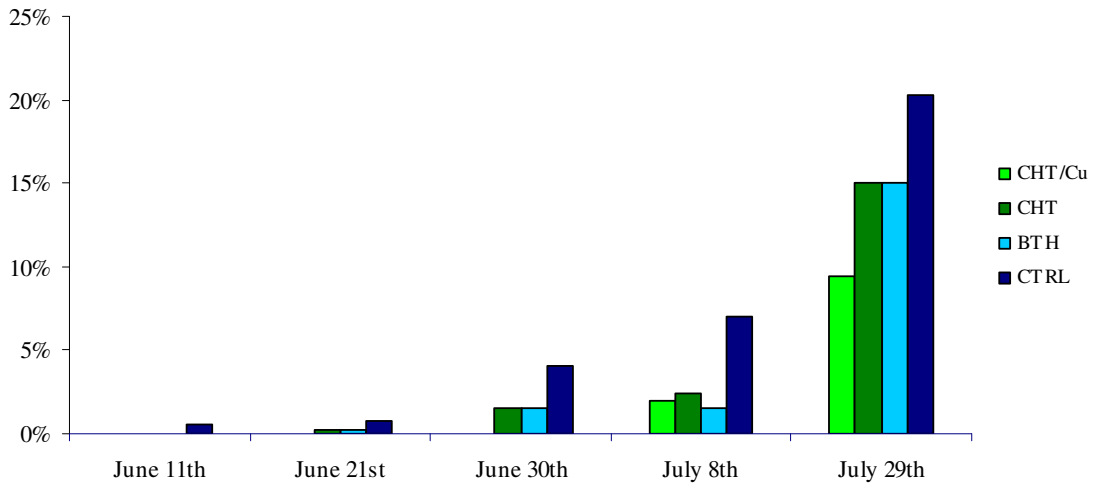


Figure 2. Disease incidence (I %) of downy mildew (*Plasmopara viticola*) on grapevine cv. Groppello in 2010

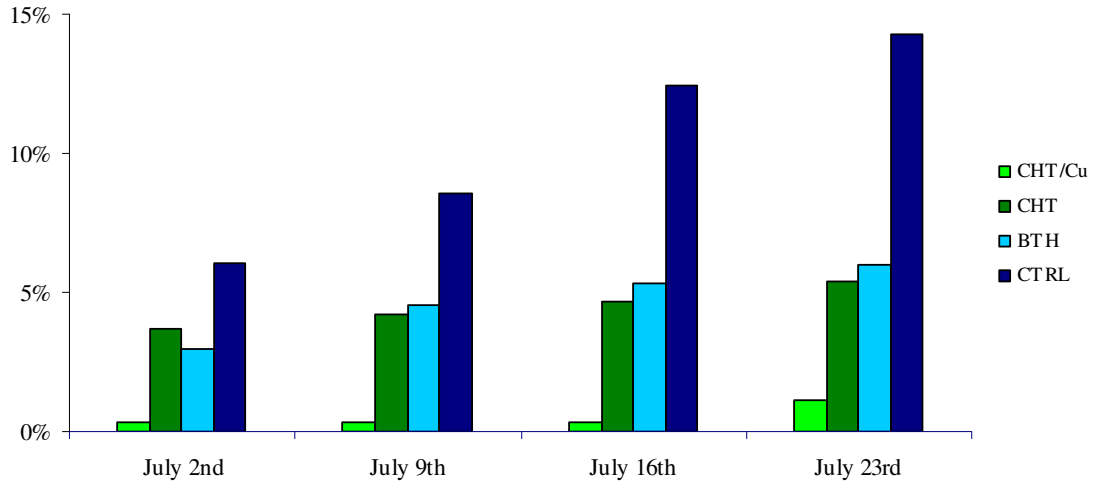


Figure 3. Infection degree (ID %) of downy mildew (*Plasmopara viticola*) on grapevine cv. Groppello in 2009

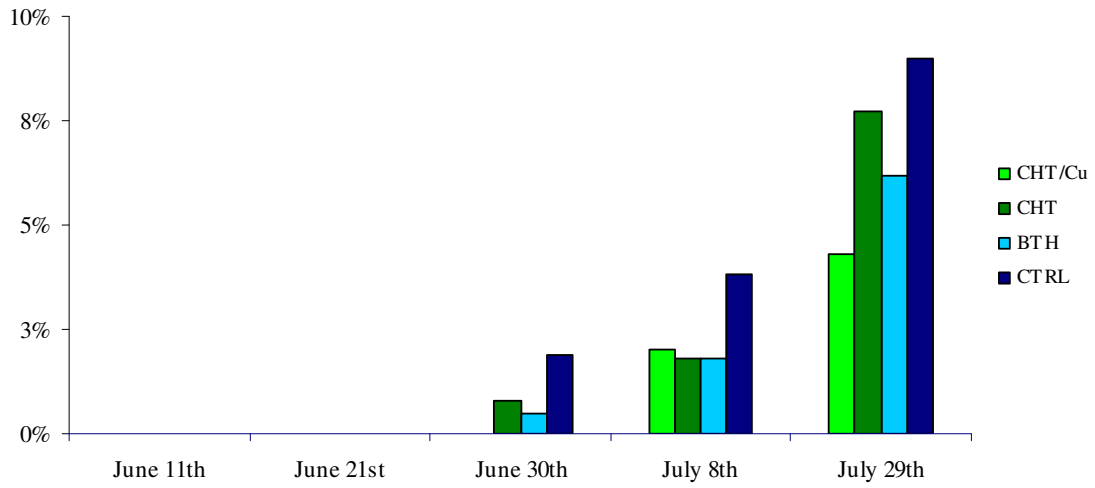
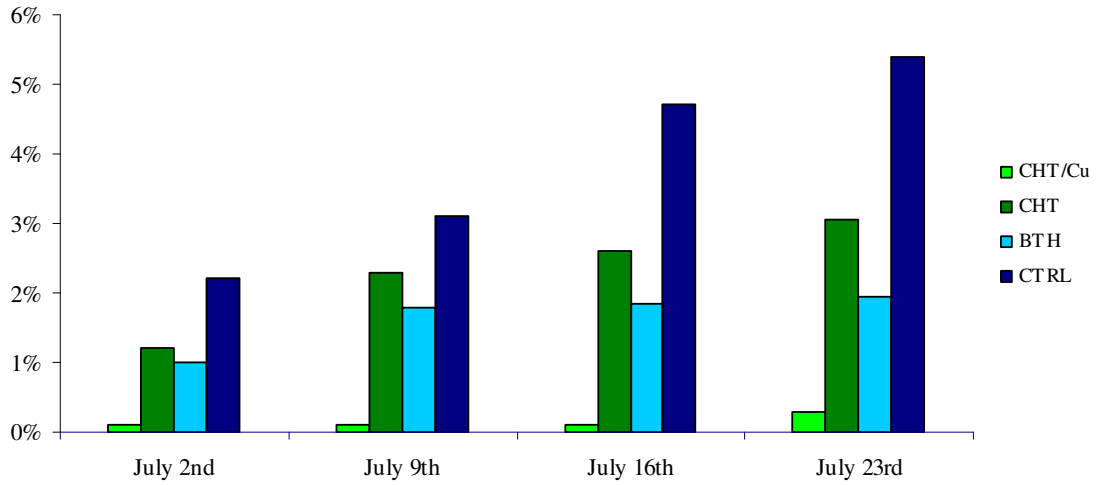


Figure 4. Infection degree (ID %) of downy mildew (*Plasmopara viticola*) on grapevine cv. Groppello in 2010



For powdery mildew, in 2009 and 2010, I % is reported in figures 5 and 6, respectively, ID % in figures 7 and 8, respectively.

Figure 5. Disease incidence (I %) of powdery mildew (*Erysiphe necator*) on grapevine cv. Groppello in 2009

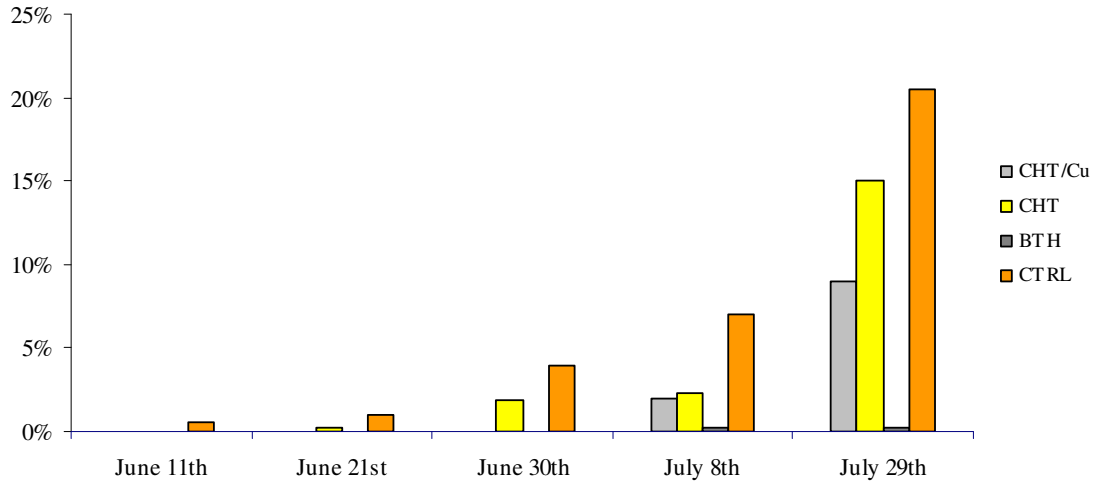


Figure 6. Disease incidence (I %) of powdery mildew (*Erysiphe necator*) on grapevine cv. Groppello in 2010

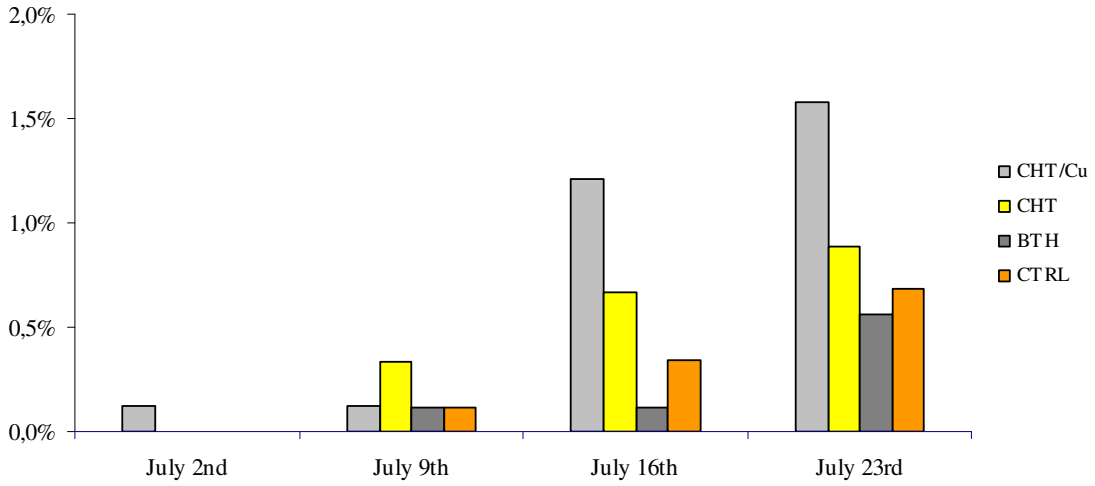


Figure 7. Infection degree (ID %) of powdery mildew (*Erysiphe necator*) on grapevine cv. Groppello in 2009

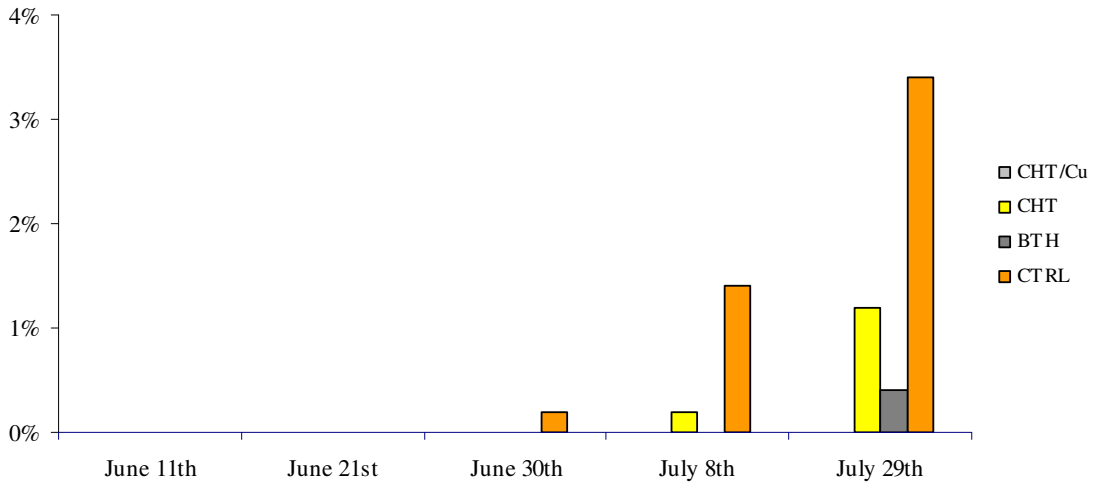
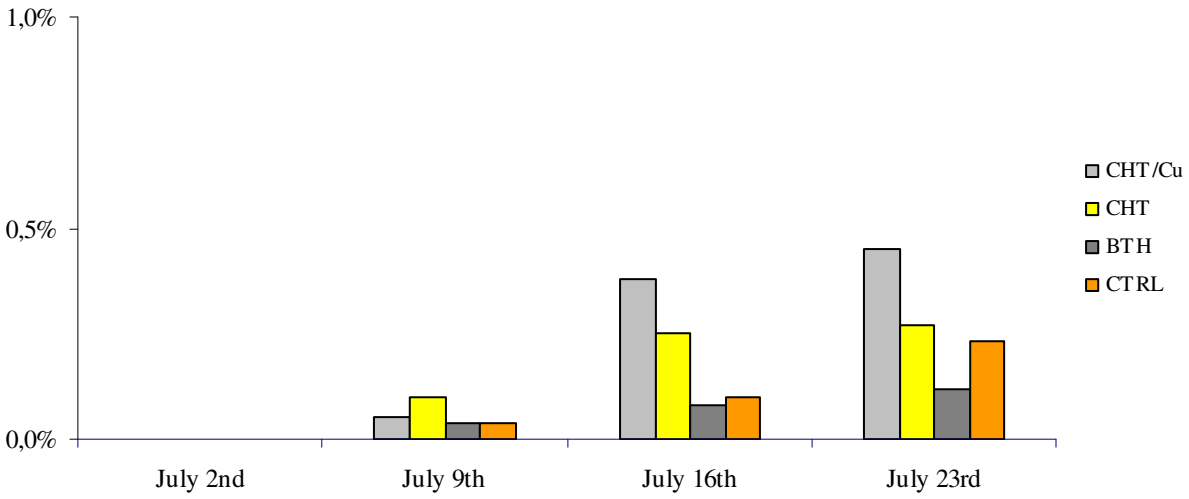


Figure 8. Infection degree (ID %) of powdery mildew (*Erysiphe necator*) on grapevine cv. Groppello in 2010



The S % of downy and powdery mildew in control (CTRL) grapes, in 2009 and 2010, is shown in tales 1 and 2, respectively.

Table 1. Disease severity (S %) of downy and powdery mildew in control (CTRL) grapes in 2009

Downy mildew Powdery mildew

CTRL						
Class	June 30th		July 8th		July 29th	
1 (0-1%)			2			1
2 (1-10%)	9		27	6	55	10
3 (10-25%)		1	4	3	25	9
4 (25-50%)			1		13	5
5 (50-75%)					4	
6 (75-100%)					1	

Table 2. Disease severity (S %) of downy and powdery mildew in control (CTRL) grapes in 2010

Downy mildew Powdery mildew

CTRL								
Class	July 2nd		July 9th		July 16th		July23rd	
1 (0-1%)					4	1	4	1
2 (1-10%)	45		61	1	81	2	98	5
3 (10-25%)	6		9		17		18	
4 (25-50%)	2		5		7		8	
5 (50-75%)								
6 (75-100%)								

The effects of CHT on S %, in 2009 and 2010, is shown in tables 3 and 4, respectively, of CHT/Cu in tables 5 and 6, respectively, of BTH in tables 7 and 8, respectively.

Table 3. Effects of chitosan (CHT) on disease severity (S %) of downy and powdery mildew in 2009

Downy mildew Powdery mildew

	CHT					
Class	**June 30th**		**July 8th**		**July 29th**	
1 (0-1%)						
2 (1-10%)	**3**		**13**	**1**	**53**	**9**
3 (10-25%)			**1**		**17**	**2**
4 (25-50%)					**9**	**1**
5 (50-75%)					**2**	
6 (75-100%)						

Table 4. Effects of chitosan (CHT) on disease severity (S %) of downy and powdery mildew in 2010

Downy mildew Powdery mildew

CHT								
Class	**July 2nd**		**July 9th**		**July 16th**		**July23rd**	
1 (0-1%)						**1**		**2**
2 (1-10%)	**28**		**36**	**3**	**36**	**6**	**42**	**6**
3 (10-25%)	**3**		**7**		**9**		**11**	
4 (25-50%)	**2**		**6**		**8**		**10**	
5 (50-75%)			**1**		**2**		**2**	
6 (75-100%)								

Table 5. Effects of chitosan plus copper (CHT/Cu) on disease severity (S %) of downy and powdery mildew in 2009

Downy mildew Powdery mildew

	CHT/Cu					
Class	**June 30th**		**July 8th**		**July 29th**	
1 (0-1%)						
2 (1-10%)			**9**		**27**	
3 (10-25%)					**7**	
4 (25-50%)					**6**	
5 (50-75%)					**1**	
6 (75-100%)						

Table 6. Effects of chitosan plus copper (CHT/Cu) on disease severity (S %) of downy and powdery mildew in 2010

Downy mildew Powdery mildew

CHT/Cu								
Class	July 2nd		July 9th		July 16th		July23rd	
1 (0-1%)						2		4
2 (1-10%)	3	1	3	1	3	8	9	9
3 (10-25%)								
4 (25-50%)								
5 (50-75%)								
6 (75-100%)								

Table 7. Effects of benzothiadiazole (BTH) on disease severity (S %) of downy and powdery mildew in 2009

Downy mildew Powdery mildew

BTH						
Class	June 30th		July 8th		July 29th	
1 (0-1%)						
2 (1-10%)	2		8		56	2
3 (10-25%)			1		9	
4 (25-50%)					11	
5 (50-75%)					2	
6 (75-100%)					2	

Table 8. Effects of benzothiadiazole (BTH) on disease severity (S %) of downy and powdery mildew in 2010

Downy mildew Powdery mildew

BTH								
Class	July 2nd		July 9th		July 16th		July23rd	
1 (0-1%)	1		1		4		10	5
2 (1-10%)	24		36	1	39	1	39	1
3 (10-25%)	1		3		3		3	
4 (25-50%)			1		1		1	
5 (50-75%)								
6 (75-100%)								

In general, all the treatments were effective in controlling the two fungal infections in terms of I %, ID % and S %, though all these parameters were very low in untreated control grapevines, particularly in 2010. Therefore, it results very difficult to estimate the real performance of the plant activators in open fields. Certainly, as shown in Chapter 4 (Iriti et al., 2011a), chitosan

greatly improved the resistance of grapes against powdery mildew, though in a different variety (Montepulciano d'Abruzzo) cultivated in a diverse geographical area (Chieti, Abruzzo). In any case, the latter results are promising, and a vineyard more predisposed to fungal epidemics is necessary before drafting final conclusions.

Chapter 7

Mycotoxin detection in experimental wines treated with plant activators

7.1. Introduction

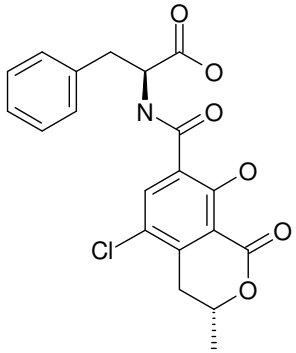
Mycotoxins are fungal secondary metabolites widely distributed in nature, which can be detected in soil and many food plants. They are synthesised in particular growth conditions by some genera of toxigenic fungi (*Aspergillus*, *Penicillum*, *Fusarium*), as a result of field or post-harvest infections, 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 1). 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 1. 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, coffe-, 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 necrotic
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 1). Its IUPAC name is *N*-{[(3*R*)-5-chloro-8-hydroxy-3-methyl-1-oxo-3,4-dihydro-1*H*-2-benzopyran-7-yl]carbonyl}-L-phenylalanine.

Figure 1. 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 effect. 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*., 2006).

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

7.2. Materials and Methods

The description of the phytoiatric campaigns, grapevine cultivars, types of treatment, their scheduling and randomization in vineyards, and microvinificate production has been accurately reported in Chapter 5 (Vitalini et al., 2011a). For the sake of clarity, we only report the list of the agrochemical treatments for each grapevine cultivar and vintage (table 2).

Table 2. Agrochemical treatments for each grapevine cultivar and vintage

Sample
Groppello CTRL[1] 2009
Groppello conventional fungicides 2010
Groppello CHT[2] 2009, 2010
Groppello CHT/Cu[3] 2009, 2010
Groppello BTH[4] 2009, 2010
Merlot CTRL 2009
Merlot conventional fungicides 2009
Merlot CHT 2009
Merlot CHT/Cu 2009

[1]CTRL, control samples; [2]CHT, 0.03% chitosan; [3]CHT/Cu, chitosan plus 150 g/hL copper hydroxide; [4]BTH, 0.3 mM benzothiadizole

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

7.2.2. 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.

7.2.3. 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. 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.

7.3. Results and Discussion

In all the samples (table 2), all mycotoxins were detected at a concentration lower than 6 pg/mL (limit of detection, LOD, 0.15 ng/mL). Figure 2 shows the chromatographic profile a wine extract (sample Groppello CHT 2010), whereas the standards are reported in figure 3. 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 2. 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.

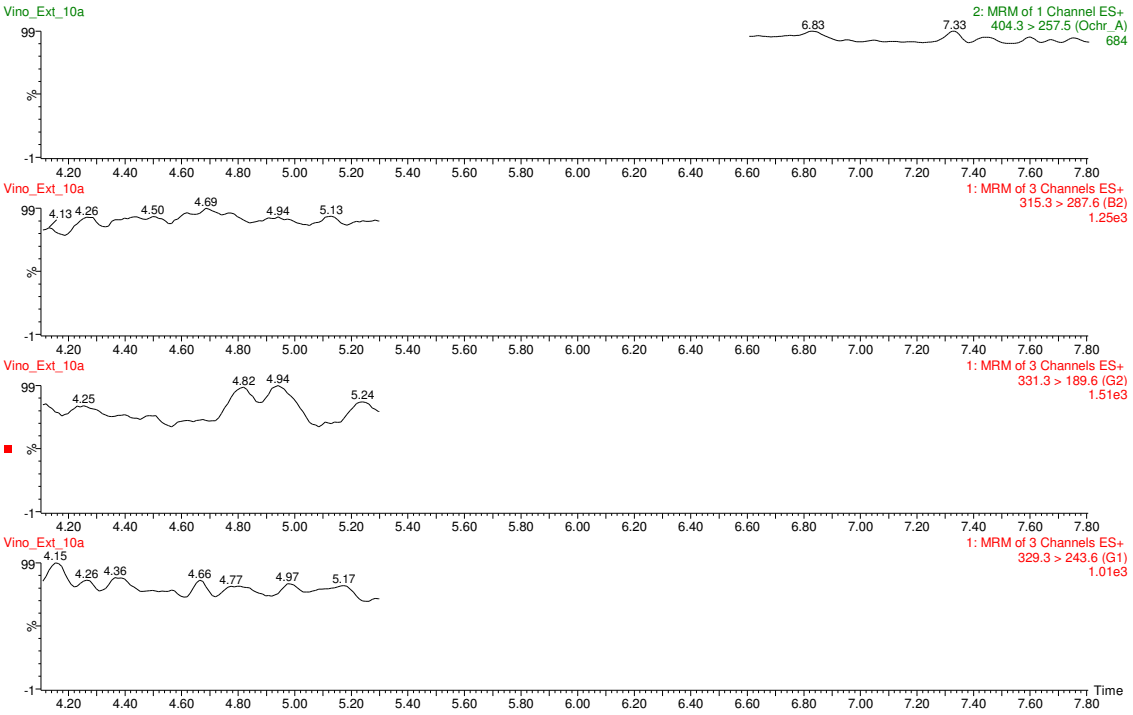
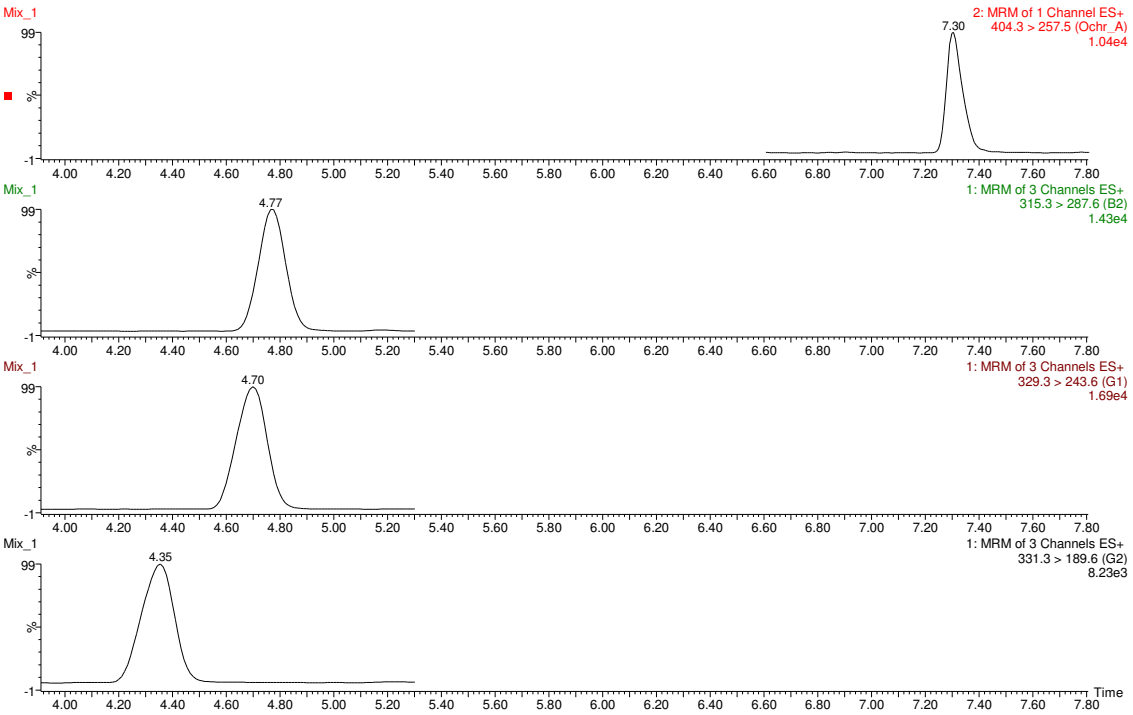


Figure 3. UPLC-MS/MS chromatogram of the mycotoxin standards. The fragmentation transitions were: $(m/z)^+$ 404.3→257.5 for OTA, 315.3→287.6 for AFB2, 329.3→243.6 for AFG1, 331.3→189.6 for AFG2.



Chapter 8

Conclusions

As the results reported, the hypotheses proposed by this PhD project were verified. Of note, some outcomes were highly reproducible, i.e. they presented a similar trend in diverse grapevine cultivars (Montepulciano d'Abruzzo, Groppello, Merlot), in different vintages (2007, 2009, 2010) and geographical areas (Abruzzo, Veneto, Lombardia). In particular, experimental wines obtained from elicitor-treated grapes showed a higher content of bioactive phytochemicals (polyphenols and melatonin) and an enhanced antiradical activity compared with microvinificates produced from grapevines treated with conventional fungicides, irrespective of the plant activator employed (BTH, CHT, CHT + Cu or Kendal Cops®), variety, vintage and geographical area (Montepulciano d'Abruzzo in Abruzzo, 2007; Merlot in Veneto, 2009; Groppello in Lombardia, 2009 and 2010). As regards fungal infections, disease incidence and severity were highly reduced by treatment with Kendal Cops® in Montepulciano d'Abruzzo cultivar, whereas, for other treatments and varieties, it was not possible to draw a similar estimation because of low pathogen pressure registered in 2009 and 2010 in both our sites in Lombardia and Veneto.

The higher antiradical activity measured in wines obtained from grapes treated with resistance inducers may be due to the increased synthesis of plant defence metabolites, relevant for both resistance to pathogen attacks and tolerance to abiotic stressful conditions. Indeed, the antioxidant power of polyphenols and melatonin has been widely described in the Introduction. Therefore, our results convincingly showed that open field treatments with plant activators may improve the health potential of red wine, at least in terms of antiradical capacity.

As previously reported, the discovery of melatonin in edible plants, seeds and medicinal herbs has opened a new field of knowledge in food and nutrition science. In this regard, the occurrence of melatonin in red wine adds a new element in the already complex grape chemistry, probably contributing to explain the plethora of biological activities and healthy properties ascribed to this drink and mainly attributed to polyphenolic compounds. In these terms, it would be of great interest to verify whether health benefits promoted by moderate, regular red wine consumption at meals may be, at least in part, due to melatonin, possibly acting synergistically with the other hundreds of bioactive molecules present in grapes. Similarly, because of the paucity of biokinetic data on dietary melatonin, it would be relevant to evaluate its bioavailability and metabolism in humans.

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.

Certainly, the study of the molecular processes involved in elicitation were beyond the scope of this project, though the mechanism of action of BTH and CHT was extensively investigated in other model pathosystems, as reported in the Introduction. In any case, to gain a more widespread acceptance of induced resistance in crop protection, it is necessary to invest in further basic research. Studies on genome, transcriptome and, especially, proteome and metabolome analyses should provide further information on the complex mechanisms involved in SAR. Otherwise, applied research on SAR expression, through studies on 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 exploitation of SAR. Finally, we need to take into consideration 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.

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