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STUDY OF THE CONTENT OF STILBENES ON GRAPE CANES WASTE. USE OF FOOD BY-PRODUCTS AS NATURAL PESTICIDES ON GRAPEVINE

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STUDIO DEL CONTENUTO DI STILBENI NEI TRALCI DI VITE. UTILIZZO DI SOTTOPRODOTTI ALIMENTARI COME ANTIPARASSITARI NATURALI SU VITE

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“Faça o que for necessário para ser feliz. Mas não se esqueça que a felicidade é um sentimento simples, você pode encontrá-la e deixá-la ir embora por não perceber sua simplicidade”.

Mario Quintana

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SUMMARY

Grapes (*genus Vitis*) are an important fruit crop from economic and cultural point of view in many countries of Europe, Asia and America. Currently, countries as France, Spain and Italy produce on average 7% of the fresh grape and 90% of the wine grape. At the same time, chemical pesticides are a ubiquitous element in agriculture and are among the most widely used chemicals in the world. In addition, grape canes represent a large source of waste derived from the viticulture industry, with an estimated volume of 1−3 t/ha/year. Likewise, the seafood processing industry produces a large quantity of by-products and discards (heads, tails, skins, scales, viscera, backbones, and shells). Yet, these food wastes may often contain several usable substances of high value including grape cane extract and chitosan that may have important environment, agriculture and health benefits. Considering this important topic, this work proposes the use of by-products from grape cane waste (stilbene extract) and the fishing industry (chitosan), as substitutes of pesticides on grapevine against *Botrytis cinerea*. In the present study, the effect of different processes of storage and different pruning time on the stilbene accumulation on Pinot noir canes was investigated. Considering the effect of different processes of storage and different pruning time on the stilbene accumulation on Pinot noir canes, stilbene extract could be easily obtained collecting canes on the pruning time in December and storing them for 12 weeks at room temperature to reach the highest accumulation of stilbenes. Moreover, we analyzed the effect of the stilbene accumulation on grape canes of seven autochthonous grape varieties from Veneto region. The cultivars Verdiso and Incrocio Manzoni 13.0.25 showed the highest accumulation of stilbenes when harvested in October and stored for twelve weeks at room temperature, highlighting the importance of the cultivar on stilbene accumulation. Chitosan is a highly investigated biopolymer with well-known antimicrobial properties, that are largely influenced by the molecular weight, the degree of acetylation as well as the derivatization and preparation methods used. The biological activity of a commercial chitosan soluble in acid solution, obtained from shrimp shell waste, with a molecular weight of 173 kDa and a degree of acetylation of 17% was investigate. Exogenous application of stilbene extract or chitosan demonstrated to protect grapevine against the necrotrophic fungus *Botrytis cinerea*. Stilbene extract (SE) demonstrated to induce satisfactory protection toward *Botrytis cinerea* on grapevine plants, thus being a promising natural fungicide. SE possessed a direct antifungal activity inferred by the inhibitory effect

of the mycelium growth observed on nutrient agar medium and through the reduction of the necrotic lesions caused by *B. cinerea* on grapevine leaves. Furthermore, it was verified that the acquired protection derived also from an induction of some grapevine defense mechanisms. After its perception, SE induced specific defense events, such as the activation of MAPKs and a higher expression of a gene encoding a glutathione-S-transferase (*GST1)* and some PR genes, but negatively regulated new stilbene production. This result suggests the activation of an immune-ready state on SE-treated plants. Besides, thanks to direct fungistatic activity and filmogenic properties chitosan (173/17) conferred a good level of protection for grapevine leaves against *B. cinerea.* Moreover, it induced grapevine defense response with some delay. From three days from the treatment there was an induction of the JA/ET-mediated response and a repression of the SA-mediated signaling, and a transient accumulation of *trans*-resveratrol. The finding demonstrated that these food byproducts derived from the practice of viticulture and from the fishing industry, respectively, could be an alternative for the development of novel natural fungicides.

RESEARCH BACKGROUND AND JUSTIFICATION

The 2017 total world area under vines, corresponding to the total surface area planted with vines, including that not yet in production or not yet harvested, is estimated to be almost reaching 7.6 mha (OIV, 2018). Because of the economic importance and the beneficial effects on human health, grape is one of the fruit crops most widely grown in many areas of the world and widely consumed in different forms as fresh, raisins, wine, vinegar, molasses, grape juice, etc., being that 80% of the cultivated grape is utilized for wine-making (Zhu *et al.*, 2015).

Chemical pesticides are a ubiquitous element in agriculture and are among the most widely used chemicals in the world. In addition, they are designed to have a biological effect (i.e., to be toxic to at least one organism) and they are released into the environment deliberately and in large quantities (Muir *et al.*, 2004). Besides the growing society demand of sustainability policies for decrease the use of pesticides in agriculture, there is also a large preoccupation with the generation of food and agricultural by-products, such as chitosan by-products from the fishing industry (crustaceans) and grape cane waste.

Chitosan is the name used for low acetyl substituted forms of chitin (deacetylated), which possesses the property to induce plant defences against pathogens (resistance inducer). Also, depending on the deacetylation degree and molecular weight, chitosan has sticky properties, so that it could help even other molecules to adhere to the leaves increasing their effectiveness. Chitin is the second most abundant natural biopolymer in the world after cellulose and is the major structural component of the exoskeleton of invertebrates and cell walls of fungi (Shahidi *et al.*,1999).

Moreover, several recent works highlight the valorisation of by-products of the wine industry, such as grape cane waste, which are still not extensively recovered for alternative uses, but contain high quantity of stilbenes, including *trans*-piceatannol, *trans*-resveratrol and *trans*-viniferin (Rayne *et al.*, 2008; Vergara, *et al.*, 2012; Lambert *et al.*, 2013; Gorena *et al.*, 2014; Houillé *et al.*, 2015; Ewald *et al.*, 2017; Billet *et al.*, 2018). These molecules are produced by plants in response to infections, and it has been showed to possess antifungal properties.

Nevertheless, it will be necessary to evaluate existing techniques and innovative approaches for efficient use of the by-products of fishing industry (chitin and chitosan) and grape cane waste, as a substitute for pesticides, thereby contributing to the creation of sustainable value chains in the farming.

INTRODUCTION

Global production of grape

Grapes (*genus Vitis*) represent one of the most consumed fruits and this fact may be related to intrinsic sensory factors (taste, freshness, aroma) as well as commercial availability, reasonable price, and more recently because of their health properties demonstrated by a large number of *in vitro*, *in vivo*, and clinical/epidemiological studies (Chou *et al.*, 2001; Jung *et al.*, 2006; Lima *et al.*, 2014; Oliboni *et al.* 2007; Toaldo *et al.*, 2015; Vilas Boas *et al.*, 2014; Vinson *et al.*, 2001).

Grape is extensively cultivated and consumed as raw and processed material such as raisin, juice, jelly, jam, vinegar and wine. Grape is a nutritious crop that contains minerals, dietary fiber, and various compounds such as vitamin C, vitamin B6 and antioxidants (Jayaprakasha *et al.*,2001). According to the Food and Agriculture Organization (FAO) statistics, 75,866 square kilometers of the world are dedicated to the grape cultivation that indicates the great importance of this strategic product. Global grape production has been estimated in 2016 to be 39% in Europe, 34% in Asia and 18% in America. Currently, countries as France, Spain and Italy produce on average 7% of the fresh grape and 90% of the wine grape (OIV, 2017). Although viticulture has spread around the globe, most of the world's grapes are still produced in Mediterranean Europe. About 40% of the world's vineyard hectarage occurs in Europe, of which 33% is located in Spain, Italy, and France. Although global vineyard hectarage has declined over the past 10 years, coverage has increased in countries such as China, United States, Australia and New Zealand. Grape usage varies considerably from region to region, and country to country. For example, most grapes grown in Germany are used in wine production, whereas most Turkish grapes are used for raisin production or as a fresh fruit crop (OIV, 2017).

Pathogenic fungi and chemical pesticides or fungicides

During cultivation grapes face frequent attack from a large variety of pathogenic agents. Pesticide number, type and quantities applied on grapes vary significantly every year, depending on climate conditions, enemies' growth, wrong application from the producer etc. Many pesticide residues are often detected in grapes and wine, depending on the quantity of their use in the field, the way, the number of applications and the time from application to harvest (Doulia *et al.*, 2016). Serious problems have arisen by the use of pesticides for both the environment and human health. However, the crop protection with

chemical methods is still a normal practice in viticulture for wine making, due to the specificities of the cultivation of the vine and its diseases and pests.

Botrytis cinerea is a pathogenic fungus causing gray mold, which affects several fruits and plants all over the world. Once a product is attacked by *B. cinerea*, it cannot be recovered, with great economic losses (Williamson *et al.*, 2007). One of the most important problems regarding *B. cinerea* is that it infects the plants during the blooming, but it is not noticeable until the ripening (Timperio *et al.*, 2012). Commonly, chemicals are used to fight this disease, for instance azoles (Stehmann and De Waard, 1996), anilinopyrimidines, phenylpyrroles or hydroxyanilides (Rosslenbroich and Stuebler, 2000). However, resistant strains are developed quickly by the fungus (Elad *et al.*, 1995; Pappas, 1997).

Many key fungicides have been withdrawn from the market because of concerns about their safety in the food supply and in the environment. Sulfur dioxide fumigation effectively controls gray mold, but it can cause bleaching injuries on the berries and browning of the rachis (Nelson and Richardson, 1967), and its residues can be harmful to people allergic to sulfites (Karabulut *et al.*, 2003). In addition, sulfur dioxide is not allowed for use in organically certified grapes (Mlikota *et al.*, 2001). Therefore, there is a need for developing alternative strategies to control gray mold on grapes that are safe, effective, economical, and compatible with commercial handling practices (Karabulut *et al.*, 2005).

Fortunately, there is a tendency towards replacing the use of toxic substances by natural, environmentally friendly products (Ali *et al.*, 2015). There has been an interest with respect to better use of chitosan and the use of stilbene against development of gray mold on grapevine *in vitro* and *in vivo* (Ait Barka *et al.*, 2004; Aziz, *et al.*, 2006; Trotel-Aziz *et al.*, 2006; Reglisnki *et al.*, 2010, Schnee *et al.*, 2013; Richard *et al.*, 2016; Gabaston *et al.*, 2017).

Chitin Preparation and Characterization

Chitin is a fairly ubiquitous compound produced by many organisms: fungi and algae cell walls, insects (exoskeleton), mollusks (endoskeleton of cephalopods) and crustaceans (shell). Annually, it has been estimated that on the order of $10^{10} – 10^{11}$ tons are produced by living organisms (Revathi *et al.*, 2012). However, commercially chitin is mainly recovered from marine sources, i.e., the crustaceans processing industries. In fact, more than 10,000 tons could be available every year from shellfish waste (Merzendorfer, 2011), which would provide sufficient raw material if the appropriate commercial procedures for value-added processes will be developed.

Modern food production generates a large quantity of by-products most of which are still underutilized. Yet, these food wastes may often contain several usable substances of high value including some that may have important health benefits. Generation of waste during the processing of food is unavoidable and disposal can be one of the major problems for those industries and for society. Especially if not done properly it can have negative impacts on the environment (i.e., pollution), create risks to human health, and a loss of income to the waste generator. For example, the seafood processing industry produces a large quantity of by-products and discards (heads, tails, skins, scales, viscera, backbones, and shells). These residual materials may be an excellent source of proteins, lipids, pigments, and small molecules. In addition, the shell materials may be a source of chitinous materials (Hamed *et al.*, 2016).

Chitin and chitosan are co-polymers of *N*-acetyl-*D*-glucosamine and *D*-glucosamine, where the ratio of each monomer in the polymer chain defines its physical, chemical and biological properties, and whether it is characterized as chitin or chitosan. The *N*-acetyl-*D*-glucosamine and *D*-glucosamine residues in chitin and chitosan are linked together via β-1,4-glycosidic linkages similarly to cellulose.

Chitin is distinguished from chitosan by the higher proportion of *N*-acetyl-*D*-glucosamine over *D*-glucosamine in the polymer chain, with typically more than 95% *N*-acetyl-*D*-glucosamine and less than 5% *D*-glucosamine being found in chitin derived from crab shells, shrimp shells and squid pens (Rinaudo, 2006; Sagheer *et al.*, 2009). Taking into account the arrangement of the chitin polymer in its native form, two major types of chitin can be observed: the α-chitin found in shrimp shells and the β-chitin found in squid pens (Rinaudo, 2006). α-Chitin isomorph is by far the most abundant; it occurs in fungal and yeast cell walls, in krill, lobster and crab tendons and in shrimp shells, as well as in insect cuticle (Younes and Rinaudo, 2015). The rarer β-chitin is found in association with proteins in squid pens (Rudall and Kenchington, 1973; Rudall, 1969) and in the tubes synthesized by pogonophoran and vestimetiferan worms (Blackwell *et al.*, 1965; Gaill, 1992). Although chitin can be found in various sources in nature, it is normally produced from shrimp or crab shells by deproteinization and demineralization (Figure 1) (Rinaudo, 2006; Younes and Rinaudo, 2015).

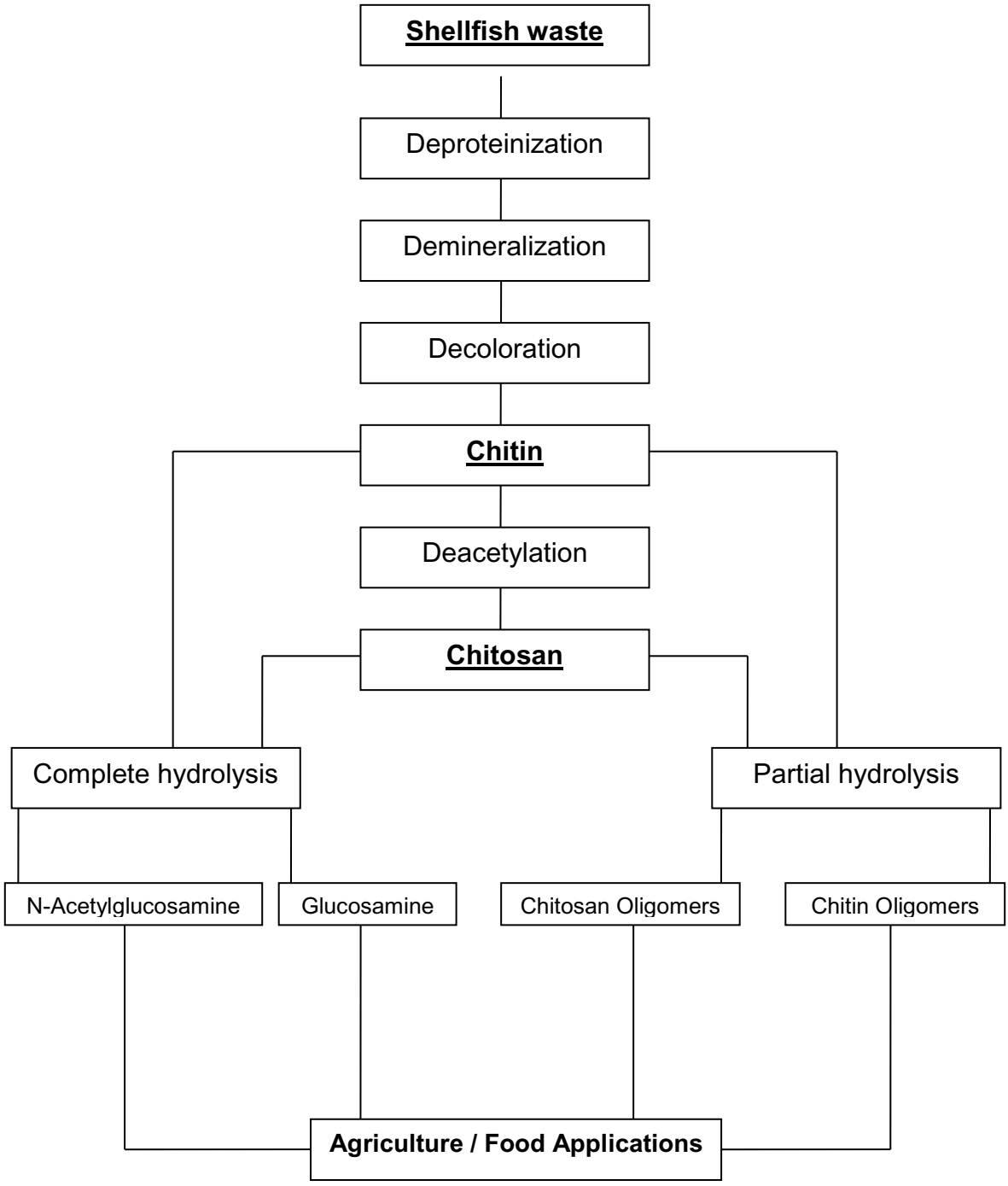

Figure 1. Simplified flow chart for preparation of chitin, chitosan, their oligomers and monomers from shellfish waste. Adapted from (Shahidi *et al.*, 1999).

The deproteinization consists in the disruption of chemical bonds between chitin and proteins. The complete removal of protein is especially important for biomedical applications, as a percentage of the human population is allergic to shellfish, the primary culprit being the protein component (Younes and Rinaudo, 2015).

A wide range of chemicals have been tested as deproteinization reagents including NaOH, Na_2CO_3, $NaHCO_3$, KOH, K_2CO_3, $Ca(OH)_2$, Na_2SO_3, $NaHSO_3$, $CaHSO_3$, Na_3PO_4 and Na_2S. Reactions conditions vary considerably in each study. NaOH is the preferential reagent and it is applied at concentration ranging from 0.125 to 5.0 M, at varying temperature (up to 160°C) and treatment duration (from few minutes up to few days). In addition to deproteinization, the use of NaOH invariably results in partial deacetylation of chitin and hydrolysis of the biopolymer lowering its molecular weight (Younes and Rinaudo, 2015).

Demineralization consists in the removal of minerals, primarily calcium carbonate. Demineralization is generally performed by acid treatment using HCl, HNO_3, H_2SO_4, CH_3COOH and HCOOH (No and Hur, 1998; Percot *et al.*, 2003). Among these acids, the preferential reagent is dilute hydrochloric acid. Demineralization is easily achieved because it involves the decomposition of calcium carbonate into the water-soluble calcium salts with the release of carbon dioxide.

Demineralization treatments are often empirical and vary with the mineralization degree of each shell, extraction time, temperature, particle size, acid concentration and solute/solvent ratio. The latter depends on the acid concentration, since it needs two molecules of HCl to convert one molecule of calcium carbonate into calcium chloride. In order to have a complete reaction, acid intake should be equal to the stoichiometric amount of minerals, or even greater (Johnson and Peniston, 1982; Shahidi and Synowiecki, 1991). Since, it is difficult to remove all minerals (due to the heterogeneity of the solid), larger volume or more concentrated acid solution is used. Demineralization can be followed by acidimetric titration: the evolution of pH towards neutrality corresponds to acid consumption while the persistence of acidity in the medium indicates the end of the reaction (Tolaimate, *et al.*, 2003). In addition, a decolorization step is often added in order to remove pigments and obtain a colorless pure chitin (Younes and Rinaudo, 2015). Acetone or an organic solvent mixture are used to remove the pigments such as carotenoids (Kaur and Dhillon, 2013; Abdulkarim *et al.*, 2013; Benhabiles *et al.*, 2012; Mohammed *et al.*, 2013).

Degree of Deacetylation

Chitosan is produced commercially by deacetylation of chitin. In the process of deacetylation, acetyl groups from the molecular chain of chitin are removed to form amino groups (Younes and Rinaudo, 2015). The degree of deacetylation (DDA), which determines the content of free amino groups in polysaccharides, can be employed to differentiate between chitin and chitosan. It is very well known that the degree of deacetylation is one of

the most important chemical characteristics, which could influence the performance of chitosan in many applications (Tolaimate *et al.*, 2003). Overall, the larger is the DDA, the higher is the solubility in acidic conditions. Generally, chitosan with higher DDA, which has a higher positive charge, would be expected to have stronger antibacterial activity (Taskin *et al.*, 2014).

The *N*-deacetylation of chitin is either performed heterogeneously (Chang *et al.*, 1997) or homogeneously (Sannan *et al.*, 1976). Commonly, in the heterogeneous method, chitin is treated with a hot concentrated solution of NaOH during few hours, and chitosan is produced as an insoluble residue with deacetylation up to about 85%–99%. According to the homogeneous method, chitin is prepared by dispersion in concentrated NaOH (30g NaOH/ 45g H_2O /3g Chitin) at 25°C for 3 h or more, followed by dissolution in crushed ice around 0°C. This method results in a soluble chitosan with an average degree of acetylation of about 48%–55% (Kurita *et al.*, 1977). This process produces deacetylation with acetyl groups uniformly distributed along the chains, for example chitosan with DDA = 10% after 580 h at 25°C (Sannan *et al.*, 1976).

Aiba (1991) showed that deacetylation reaction performed under heterogeneous conditions gives an irregular distribution of *N*-acetyl-D-glucosamine and *D*-glucosamine residues with some block wise acetyl group distribution along polymeric chains. Thus, solubility and degree of aggregation of chitosan can vary in aqueous solutions leading to changes in their average characteristics. For instance, physicochemical properties of such chitosan's may differ from those of randomly acetylated chitosan's obtained under homogeneous conditions.

Furthermore, variations in chitosan preparation may also result in changes of: DDA, distribution of acetyl groups along the chains, molecular weight and viscosity in solution (Berger *et al.*, 2005; Rong and Hwa, 1996). In fact, many parameters in the deacetylation reaction can impact the characteristics of the final chitosan (Li *et al.*, 1992). For instance, Rege and Block (1999) investigated the effect of temperature, processing time and mechanical shear on chitosan characteristics, and found that temperature and processing time have a significant effect on DDA and molecular weight.

Tolaimate *et al.* (2000) reported that chitosan DDA is greatly affected by temperature and repetition of alkaline steps. Tsaih and Chen (2003) also examined the effect of time reaction and temperature. All these studies were performed using a classical one-variable-at-a-time experimentation. These reports indicate that molecular weight and DDA of chitosan are mainly affected by NaOH concentration, reaction time, temperature and

repetition of alkaline steps. Additional factors such as reaction reagent, atmosphere, particle size, chitin and solvent ratio, and source of raw material were also tested in other studies (No and Meyers, 1995; Chang *et al.*, 1997; Tolaimate *et al.* 2000; Sannan *et al.*, 1977).

Molecular Weight and Solubility of Chitosan

The physical properties of chitosan in solution depend strongly on DDA and on the acetyl group distribution along the chains. Block-wise distribution of acetyl groups, caused by heterogeneous deacetylation performed on solid state chitin causes chain association even in dilute solutions and formation of aggregates as well as difficulties in molecular weight determination (Philippova *et al.*, 2001; 2012).

The first step in chitosan characterization is the determination of their molecular weights (after dissolution), then DDA and eventually the distribution of acetyl group along the chain by Nuclear magnetic resonance (NMR). At present, NMR seems to be the most convenient technique to get the correct DDA for soluble samples. The molecular weight absolute values also may be obtained from steric exclusion chromatography equipped with viscometer and light scattering detector on line allowing to determine the Mark-Houwink parameters without fractionation and to obtain the relation between the radius of gyration and the molecular weight (Brugnerotto *et al.*, 2001). Besides, determinations performed with glass capillary viscometer are also well widespread in characterization of molecular weight and solubility of chitosan (Mao *et al.*, 2004). Additionally, different solvents based on acetic acid have been proposed, for instance: 0.3 M acetic acid added with sodium acetate (up to 0.1 or 0.2 M) in aqueous solution. Chitosan is also soluble in acetic acid or hydrochloric acid at pH lower than 6.

The chitosan's can be distinguished by their molecular weight (MW): high molecular weight (HMW) chitosan, low molecular weight (LMW) chitosan, and oligochitosan (short chain chitosan) (Kulikov *et al.*, 2014). HMW chitosan cannot pass through the microbial membrane and hence stack on the cell surface, which blocks nutrient transport into the microbial cell membrane, resulting in cell lysis (Li *et al.*, 2010; 2010; Tokura *et al.*, 1997; Cuero *et al.*,1991). On the other hand, dissociated chitosan molecules in solution, with lower molecular weight (< 5000 kDa), could bind with DNA and inhibit synthesis of mRNA through penetration toward the nuclei of the microorganisms (Kulikov *et al.*, 2015; Chien *et al.*, 2015; Kong *et al.*, 2008; Sudarshan *et al.*, 1992).

Activity of chitosan in agriculture

Chitin and chitosan from the fishing industry by-products have been proved to be nontoxic, biodegradable and biocompatible, and have shown to induce a wide spectrum of defensive reaction in plants (Shibuya and Minami, 2001; Henryk *et al.*, 1991). Chitosan, as one of the most important elicitors, has been proved to elicit plant defense response to a broad spectrum of phytopathogens including plant fungi (Chirkov, 2002; Terry and Joyce, 2004). Four main approaches have been used for chitin and chitosan application in agriculture:

- ✓ Protection of plants from pests and diseases before and after harvest;
- ✓ Enhancing of antagonist microorganisms' action;
- ✓ Enhancing of beneficial symbiotic plant microorganism interactions;
- ✓ Regulating plant growth and development.

The biological activity of chitosan is related to the size of the oligomer and the degree of deacetylation (Kauss *et al.*, 1989; Hadwiger *et al.*, 1994).

The *in vitro* activity of low molecular weight chitosan against *Botrytis cinerea* on excised Chardonnay leaves was reported by Trotel-Aziz *et al.* (2006) and by Aziz *et al.* (2006). In the first study, treatments of the leaves induced the activity of lipoxygenase, phenylalanine ammonia-lyase (PAL) and chitinase. The best protection was achieved at the dose of 75–100 mg/L. The second study showed that a 48-h incubation with chitosan (degree of deacetylation of 80%) at 200 µg/ml, induces a high phytoalexin production. Results obtained by Reglinski *et al.* (2010) supported the work of Trotel-Aziz *et al.* (2006) confirming that chitosan act in a concentration-dependent manner, but also reporting a direct antifungal activity. Besides, a commercial chitosan-based product, named Chitogel®, enhanced the development of Chardonnay plantlets and inhibited the growth of *B. cinerea* (Ait Barka *et al.*, 2004). The application of a chitosan solution to vines showed both preventive and curative control of infection by *B. cinerea* (Amborabé *et al.*, 2004).

Alternative methods to control postharvest grey mold on table grapes have been reviewed by Romanazzi *et al.* (2012), and further research by Feliziani *et al.* (2013) described the effects of preharvest chitosan treatments using different commercial formulations of chitosan on the quality and postharvest decay of table grapes.

Grape Canes

Grape canes represent a large source of waste derived from the viticulture industry, with an estimated volume of 1−5 tons/hectare/year depending upon plantation density, climate, and vigor of the grape variety. Currently, no or limited valorization of grape canes exists because they are usually burnt in the field or composted (Devesa-Rey *et al.*, 2011).

For the best quality grapes, some cultural practices including irrigation, fertilization and plant protection should be performed in vineyards, annually. Pruning, one of the cultural operations carried out in the vineyards, has important implications for vine function as it influences the form and size of the vine, the balance between vegetative and fruit growth in the vine, the quantity and quality of grape production. Balanced pruning is the concept of equating the nodes retained at pruning with the production capacity, the aim being to maintain a balance between vegetative growth and fruit production (Tassie and Freeman, 2001). An average vine before pruning may have 25 canes and 750 buds Winkler *et al.* (1997), and it is important to practice removal of some of them from the vine for the best quality grapes. Reynolds *et al.*, (1995) found that cane pruning weight varied from 0.56 kg/vine to 2.01 kg/vine depending on the trellis systems and the years.

Most of the solid waste material, such as grape cane waste, pomace, seeds and stems generated from the viticulture and winemaking processes are usually composted or burned every year. Among these by-products, grape canes can be considered as an unexploited source of stilbenes and other phenolics, as proposed by several authors (Karacabey and Mazza, 2008; Çetin *et al.*, 2011).

Agricultural wastes, largely ignored and unevaluated economically, can be the source of high-value phytochemicals and value-added industrial products (Das and Singh, 2004).

Stilbenoids on grape canes

Stilbenoids are members of the non-flavonoid phenolic compound family and are produced by plant secondary metabolism. Plants possess an innate immune system that prevents their infection by most of microorganisms such as oomycetes and fungi (Nürnberger *et al.*, 2004). This self-defense potential includes the production of the secondary metabolites phytoalexins, antimicrobial compounds synthesized and accumulated in response to biotic and/ or abiotic stress (Kuc, 1995; Jeandet *et al.*, 2013).

Stilbenoids in *Vitis* genus accumulate in branches or canes, axillary buds and roots, whereas leaves, which are more exposed to environmental stress, have a low concentration of these compounds. Wang *et al.* (2010) found the highest concentration of resveratrol in

branches, specifically in phloem tissue, and the lowest concentration in leaves; however, the concentration of the enzyme stilbene synthase (STS) was highest in the leaves (almost twice that in the stem). These findings suggest a differential response of the plant to stress. The stilbenoid induction capacity is increased in leaves to provide enhanced protection, given their higher exposure to the environment and resulting susceptibility to attack by pests and diseases.

In grapevines, stilbenes are synthesized via the phenylalanine/polymalonate pathway. Grapevine stilbenes include a number of substances, such as resveratrol (*trans* – and – *cis* isomers, 3,5,4'-trihydroxystilbene), resveratrol glucoside piceid (*trans* – and – *cis*-resveratrol-3-O-β-D-glucopyranoside) and resveratroloside (resveratrol-4'-O-β-D-glucopyranoside), viniferins, pterostilbene (*trans* -3,5 dimethoxy-4'-hydroxistilbene), piceatannol or astringinin (piceatannol-3-O-β-D-glucopyranoside), pallidol (*trans*-resveratrol dimmer) and others resveratrol trimers and tetramers (Bavaresco *et al.*, 2009).

The key compound resveratrol is formed by condensation of one molecule of *p*-coumaroyl-CoA and three molecules of malonyl-CoA by stilbene synthase. Subsequent glycosylation, methoxylation or dimerization reactions provides a spectrum of resveratrol derivatives (Chong *et al.*, 2009). Such modifications are essential for the biological activity of the so- formed compounds (Pont and Pezet, 1990; Regev-Shoshani *et al.*, 2003). For instance, antimicrobial activities of resveratrol and its dimer viniferin have been reported for downy mildew (Pezet *et al.*, 2004), powdery mildew (Schnee *et al.*, 2008) and gray mold (Adrian and Jeandet, 2012). Piceatannol, a resveratrol analogue, is induced by fungal elicitors (Yang *et al.*, 2010) and under ultraviolet (UV) irradiations (Ku *et al.*, 2005).

A body of reports has demonstrated that resveratrol was associated with reduced coronary heart disease mortality and atherosclerosis (Wu *et al.*, 2008), inhibited low density lipoprotein oxidation (Manna *et al.*, 2000), and carcinogenesis (Jang *et al.*, 1997; Signorelli and Ghidoni, 2005). In comparison, the antimicrobial properties of stilbenes have been less investigated. Due to the dual significances in both plant protection and human health, the content and distribution of the resveratrol in grape and wine had attracted more and more attentions over the past several years, particularly regarding the by-products for the grape and wine production.

In grapevines, stilbenes are constitutively accumulated at high concentrations in the heartwood where they act as phytoanticipins and can prevent the development of wood decay (Hart and Shrimpton, 1979; Hart, 1981). In other tissues, they are accumulated in response to various microorganisms including the following pathogens: *Plasmopara viticola,*

Erysiphe necator, Botrytis cinerea, Phaeomoniella chlamydospora, Fusarium solani, Cladosporium cucumerinum, Pyricularia oryzae, Aspergilli, Rhizopus stolonifer (Chong *et al.*, 2009; Jeandet *et al.*, 2010; Adrian *et al.*, 2013). Whereas resveratrol generally shows a moderate antimicrobial activity, it is the precursor of more active derivatives such as pterostilbene and viniferins (Chong *et al.*, 2009; Jeandet *et al.*, 2010; Adrian, *et al.*, 2013).

Recently, interest in the bioproduction and chemical synthesis of stilbenes has emerged to identify highly active molecules that could be used for medical applications and/or plant disease control (Mazue *et al.*, 2010; Jeandet *et al.*, 2013).

Different levels of stilbenoids have been reported in the cane of *Vitis vinifera*. In particular, *trans*-resveratrol a level of 4250 mg/kg DW (dry weight) wes reported in a Pinot noir cultivar from Canada (Karacabey and Mazza, 2008), similar to levels reported by Vergara *et al.* (2012) for the same cultivar in South Chile (up to 5590 mg/kg DW *trans*-resveratrol), and to levels of up to 6533 mg/kg DW reported in Gewürztraminer canes. Lower levels of *trans*-resveratrol were reported in China by Zhang *et al.* (2011), who found concentrations between 570 and 1751 mg/kg FW (fresh weight) in different *Vitis* species. In hybrid cultivars from Estonia, no differences in the concentrations of resveratrol and viniferin were found in canes collected in winter, spring and summer; the range for resveratrol was 100 – 4700 mg/kg and that for viniferin was lower, 100 – 1500 mg/kg (Aaviksaar *et al.*,2003). Püssa, *et al.* (2006) reported between 1100 and 3200 mg/kg DW of resveratrol and between 700 and 1700 mg/kg DW viniferin for the same hybrid varieties. The differences in the concentration ranges of stilbenoids can be attributed in part to differences in extraction and quantification methodology (Karacabey and Mazza, 2008; Pawlus *et al.*, 2013). However environmental factors mediated by elicitors may affect stilbenoid biosynthesis.

Despite recent advances in the evaluation of stilbenoid composition of grape canes (Karacabey and Mazza, 2008; Pawlus *et al.*, 2013), varying results have been reported. Several factors can explain this variability. First, the variety and provenance of the grape can greatly influence stilbene composition (Lambert *et al.*, 2013; Vergara *et al.*, 2012; Zhang *et al.*, 2011). The time elapsed between sample collection and analysis is another important factor. Vergara *et al.* (2012) reported that the stilbenoid concentration in Pinot noir canes increased by 35% after storage for over 2 months, whereas stilbenoid levels decreased in Cabernet Sauvignon canes. Houillé *et al.* (2015) evaluated the changes in the resveratrol concentration in eight varieties of grape canes after a 6-week storage period at 20ºC; and reported that the *trans*-resveratrol concentration in Pinot noir canes had the highest

accumulation of *trans*-resveratrol from 250 mg/kg DW (at 0 week) to 4725 mg/kg DW (at 6 week).

Lambert *et al.* (2013) evaluated the *trans*-resveratrol, *trans*-viniferin and *trans*-piceatannol concentration in sixteen varieties of grape canes dried at 40°C for 15 days. The highest concentrations of *trans*-resveratrol were obtained with Pinot noir 1526 mg/kg DW and Merlot 1181 mg/kg DW, while the lowest one was in Chardonnay 190 mg/kg DW. The highest concentrations of *trans*-viniferin was obtained with Pinot noir 3737 mg/kg DW and Sauvignon Blanc 2697 mg/kg DW, while the lowest one was in Carignan 967 mg/kg DW. The highest concentrations of *trans*-piceatannol was obtained with Pinot noir 1710 mg/kg DW and Chenin 1227 mg/kg DW, while the lowest one was in Cinsault 298 mg/kg DW.

Based on these results, future studies should elucidate the mechanisms and processes that induce the increase in stilbenoid levels in grape canes during storage after pruning. Whether stilbenoid concentration increases due to biosynthesis in pruned canes or due to their liberation from other biomolecules to which they were bound in the fresh cane, is a question that remains to be answered (Gorena *et al.*, 2014). Very recently the first experiments on this topic revealed a transient expression of *phenylalanine ammonia-lyase* (*PAL*) and *stilbene synthase* (*STS*) genes after the wounding, and these processes seems to be triggered involving *jasmonate signaling* (Billet *et al.*, 2018).

Few studies were found about the use of stilbene crude extract from grapes canes against *Botrytis cinerea.* Only the authors Schnee *et al.* (2013) evaluated the biological activity *in vitro* of stilbene extract against gray mold development.

GENERAL OBJECTIVE

This work proposes the use of by-products from grape cane waste and the fishing industry (chitosan), as substitutes of pesticides on grapevine against *Botrytis cinerea* on grapevine leaves (*in vitro*) and whole-plants (*in vivo*).

SPECIFIC OBJECTIVES

a) According to research of literature (Rayne *et al.*, 2008; Vergara *et al.*, 2012; Lambert et al, 2013; Gorena *et al.*, 2014; Houillé *et al.*, 2015) the *Vitis vinifera* L. cv. Pinot noir was selected to study the influence of several processes, such as storage times and health status on the content of stilbenes in grape canes assays;

b) Following the conditions established in the previous study, the content of stilbenes was studied on grape canes of seven Veneto region varieties, such as, white varieties Bianchetta, Glera VCR Sel. Lungo, Incrocio Manzoni 6.0.1.3, Verdiso, and red varieties Incrocio Manzoni 13.0.25, Marzemino and Raboso, with the objective to found those with the highest yield in stilbenes;

c) The best combination of harvest time and storage times of Pinot noir variety it was chosen to prepare a crude stilbene extract for the treatment on grapevine. At the same time the commercial chitosan was characterized for degree of deacetylation, molecular weight and viscosity. The biological activities of stilbene extract (SE) and chitosan (Chit) were evaluated on grapevine (*in vitro*) and (*in vivo*) against the grape pathogen *Botrytis cinerea*;

d) The influence of SE-treatment or Chit-treatment on grapevine plants was further evaluated on the expression of some defense and stress-related genes, such as, genes involved in the *hormone-mediated signaling*, *salicylic acid-mediated signaling*, *jasmonic acid-mediated signaling*, gene involved in *redox status*, and on *transcription factor*, genes coding for *PR-protein* and genes involved in *phenylpropanoid pathway*.

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CHAPTER I

Influence of pruning time and health status on stilbenoid levels in Pinot noir grape canes

Influence of pruning time and health status on stilbenoid levels in Pinot noir grape canes

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ABSTRACT

Grapevine canes represent a large source of waste derived from the grape cultivation. In the present study, the effect of different processes of storage and different pruning time on the stilbene accumulation on Pinot noir canes was analyzed. Besides, it was investigated if the activation of defense mechanisms accompanying leafroll symptoms expressions could affect the stilbenoid accumulation in canes harvested at the pruning time. The maximum accumulation of *trans*-resveratrol and *trans*-piceatannol was obtained in canes harvested in October and submitted to drying at 40 ºC. Even in grape canes harvested in October, November and December and stored for different times at room temperature (20 ± 2 °C) it was evident a marked increase of *trans*-resveratrol and *trans*-piceatannol which reached a maximum around eight weeks of storage. In addition, it was found a significant higher accumulation of *trans*-resveratrol and *trans*-piceatannol in canes harvested from symptomatic plants compared to those harvested from asymptomatic plants for all the pruning times.

Keywords: Stilbenoids, Grape canes, Accumulation, Pruning time, Health status

INTRODUCTION

Stilbenoids are a small family of plant secondary metabolites derived from the phenylpropanoid pathway, and they act as plant phytoalexins displaying different bioactivities making them compounds of high current interest (Fernández-Mar *et al.*, 2012).

In the Vitaceae, stilbenoids accumulate in response to various biotic and abiotic stresses such as the attack of pathogen *Erysiphe necator* (Schnee *et al.*, 2008), *Plasmopara viticola* (Alonso-Villaverde *et al.*, 2011), *Botrytis cinerea* (Gruau *et al.*, 2015) and UV-C irradiation (Yin *et al.*, 2016). They can also be induced in response to plant hormones, such as ethylene and jasmonates (D'Onofrio *et al.*, 2009; Jiang *et al.*, 2015). The accumulation of resveratrol in different grapevine organs induced by several external stimuli has been extensively studied, while the contribution of bacterial endophytes and viral infections was scarcely considered (Hart and Shrimpton, 1979; Hart, 1981; Chong *et al.*, 2009; Jeandet *et al.*, 2010; Adrian *et al.*, 2013)

During grape development, stilbenoids are accumulated in the whole plant and especially in woody tissues (Wang *et al.*, 2010). Later, during autumn-winter pruning, large amounts of stilbenoids can be retrieved from canes, making these by-products a valuable source of bioactive and antioxidant compounds (Houillé *et al.*, 2015; Gorena *et al.*, 2014; Lambert *et al.*, 2013; Vergara *et al.*, 2011).

Stilbenoids are valuable natural products due to their potential health benefit effects. A body of reports has demonstrated that resveratrol was associated with reduced coronary heart disease mortality and atherosclerosis (Wu *et al.*, 2008), inhibited low density lipoprotein oxidation (Manna *et al.*, 2000), and carcinogenesis (Jang *et al.*, 1997; Signorelli and Ghidoni, 2005). In comparison, the antimicrobial properties of stilbenes have been less investigated. Due to the dual significances in both plant protection and human health, the content and distribution of the resveratrol in grape and wine had attracted more and more attentions over the past several years, particularly the by-products for the grape production and wine.

Grape cane extracts have been shown to prevent cardiovascular, metabolic, and inflammatory risk, such as atherosclerosis (Romain *et al.*, 2012) as well as antioxidant activities (Müller *et al.*, 2009). Since stilbenoids are found in only small amounts in human diet, food supplements based on grape by-products represent an interesting source of dietary stilbenoids. Indeed, there is still a need for alternative sources of resveratrol, as can be seen by the recent permission of resveratrol as a novel food ingredient in the European Union (Commission Implementing Decision (EU) 2016/1190).

The main objective of the present work was the establishment of procedure that allowed the greater content of stilbenoids on grape cane waste, considering also the contribution of a systemic viral infection.

MATERIALS AND METHODS

Plant materials

Grape canes waste of *Vitis vinifera* L. cultivar Pinot noir was obtained from vineyards at the Oenological School of Conegliano I.S.I.S.S (Istituto Statale di Istruzione Secondaria Superiore "G.B. Cerletti"), Conegliano, Province of Treviso, Italy (latitude 45° 87' 69" N and longitude 12° 28' 53" E and an average height of 63 M).

Field experiment and growth conditions

A field experiment was conducted during autumn-winter 2015−2016 on grape canes randomly harvested on plants of a conventional vineyard over the October, November, December 2015 and January 2016, before pruning. The canes were cut into 10 cm long sections and submitted to different processes of storage, such as freezing at – 20 ºC, freeze-drying, drying at 40 ºC until a constant weight, and finally storing for four, eight and twelve weeks in well-aerated conditions in the dark, at room temperature. As control, a sample was immediately extracted after each pruning.

In addition, during autumn–winter 2016−2017, a second sampling was performed on the same vineyard, collecting separately canes from plants showing severe symptoms of grapevine leafroll disease (red coloration and downwardly-rolled margins of leaves) and asymptomatic plants. Serological analyses (ELISA), carried out with commercial kits (BIOREBA AG and Agritest) confirmed the presence of *Grapevine leafroll-associated virus 3* (GLRaV-3) in symptomatic plants, while other viral infections (GLRaV-1, -2, *Grapevine Virus A* and *B*, *Grapevine fanleaf virus* and *Arabis mosaic virus*) were excluded. The harvest was performed in October, November and December 2016, before pruning. The canes were cut into 10 cm long sections and stored for zero (used as control), three, six, nine and twelve weeks in the dark at room temperature.

Stilbenoid extraction

The stilbenoid extraction was performed according to the procedure described by Rayne *et al.* (2008) with some modifications. Briefly, the grape canes were ground with a coffee grinder (Imetec, Azzano San Paolo, BG, Italy). Three-stage extraction was performed

by continuous stirring at room temperature using an 8:1 (v/w) 80% ethanol:sample ratio over a 60-min period for each extraction. During the first extraction, 250 µL of t-OH-stilbene 200 µg/mL in ethanol were added ad internal standard. The extracts were vacuum filtered at 1.6 µm on glass microfibre filter (GF/A, Whatman) and combined and the solvent removed by rotary evaporation (Büchi model R-114, Flawil, Switzerland), then stored at −20 °C for quantification. All analyses were performed in triplicate.

HPLC analysis

The analysis of stilbenoids were performed according to the procedure described by Vincenzi *et al.* (2013) with some modifications. Stilbenes were separated on a C18 Lichrospher column (4 mm x 250 mm, 5 µm, Agilent Technologies, Milano, Italy) at 40 °C, using an HPLC system (Waters Corporation, Milford, MA, USA) equipped with a Dual Band UV detector Waters 2487 (Waters Corporation, Milford, MA, USA). The mobile phase gradient will be 0.5% v/v formic acid in deionized water (solvent A) and 2% v/v formic acid in methanol (solvent B). The gradient program will be 0 to 10% (solvent B) in 3 min, followed by 10 to 30% (solvent B) in 5 min, 30 to 44% (solvent B) in 35 min, 44 to 55% (solvent B) in 2 min, 55 to 75% (solvent B) in 15 min and 75 to 100% (solvent B) in 1 min. After washing for 2 min with solvent B, the column was re-equilibrated with solvent A. The flow rate will be 1.0 mL/min and injection volume 20 µL. Detection was performed at 306 nm for trans-isomers for *trans*-resveratrol, *trans*-ε-viniferin and *trans*-piceatannol. The concentration of individual stilbenes was quantified on the basis of peak areas using calibration curves of commercially available standards of *trans*-resveratrol, *trans*-ε-viniferin and *trans*-piceatannol, and correcting the value for the internal standard recovery. All the stilbene standards were obtained from Extrasynthese (Genay Cedex, France). Data were analysed by the Waters BreezeTM Chromatography Software (Version 3.30). The limits of detection (LOD) and quantification (LOQ) were performed according to the procedure described by (Shrivastava and Gupta 2011).

Statistical analysis

The results were evaluated by one-way analysis of variance (ANOVA), and the mean values were analyzed by Tukey's test using the software STATISTICA® 12.0 (StatSoft Inc, Tolson, USA).

RESULTS AND DISCUSSION

Effect of the different processes of storage on stilbenoid levels in Pinot noir grape canes waste

Grape canes can be recovered after the pruning process (generally performed in autumn−winter). It has already been demonstrated that, after the cutting, the grape canes accumulate large quantities of stilbenoid (Karakabey and Mazza, 2008; Houillé *et al.*, 2015), however the effect of the pruning time on the stilbene accumulation rate has never taken in account. Considering the stilbenoid accumulation in cut canes depends on activation of related genes followed by active synthesis of resveratrol and its derivatives, the physiological state of the starting material (i.e. full active or dormant, healthy or infected) could have an important effect on newly activated stilbene metabolism. In this study the samples were harvested at different times during the pruning period (autumn-winter 2015−2016), and a control sample for each sampling time was obtained by immediate extraction. Other samples were submitted to different processes of storage, such as freezing at –20 ºC, freeze-drying or drying at 40 ºC until a constant weight to ensure the stability of the biological material. At the same time, the grape canes were stored for four, eight and twelve weeks at room temperature (RT), in order to simulate the normal situation of canes left on the ground after pruning. The canes were stored in an airy place in the dark to avoid the development of molds.

The average water loss for the different processes were approximately 3% for frozen canes, 30% for the oven-dried, 43% for the freeze-dried, and 33, 45 and 46% for canes after four, eight and twelve weeks of storage at RT, respectively.

The stilbenoid content in grape canes of Pinot noir submitted to different processes of storage are presented in the Tables 1-2-3.

First of all, it was possible to see that the basal level of *trans*-piceatannol and *trans*-resveratrol in fresh canes is quite low (less than 100 mg/kg DW of canes) whereas the dimer *trans*-viniferin is already present at concentrations 10-20 times higher. These results confirm the literature data (Gorena *et al.*, 2014; Houillé *et al.*, 2015; Billet *et al.*, 2018), only Vergara and colleagues (2012) found very high content of *trans*-resveratrol (between 2500 and 3500 mg/kg) already at time zero.

Regarding the pruning time, the differences in stilbene content, though significant, are small and fluctuating, indicating that, at least over the period we considered and in absence of stresses, the stilbene metabolism in canes is inactive. Gorena *et al.* (2014)

found the same results on Pinot noir canes collected at different times from grape harvest to leaf senescence, with only a small increase over an 8 months period.

The frozen samples showed in general no significant differences in the stilbenoid concentration ($p > 0.05$) from the control extracted immediately. In some cases, the frozen samples demonstrated lower stilbene content than control. The use of the conventional freezing process form ice crystals inside the cell, facilitating the rupture of the cell walls and increasing the chance of degradation of the phenolic compounds. Gorena *et al.* (2014) evaluated the effect of storage at −20 °C during four months on cane of Pinot noir immediately ground after pruning. The authors observed that the stilbenoid levels remained constant from time zero until the fourth month of storage. Also, Houillé on Cabernet Franc variety (2015) demonstrated that when canes were stored at − 20 °C, *trans*-resveratrol was not induced. Zhang *et al.* (2011) found high level of *trans*-resveratrol in grape canes frozen in liquid nitrogen, however they did not specify the time elapsed between the cutting and the freezing of samples, which is very important for the stilbene accumulation in grape canes.

The freeze-dried samples showed a behavior similar to the frozen ones, indicating that only little modifications in stilbene content can be achieved by this method.

The drying process at 40 °C showed a significant increase ($p < 0.05$) of stilbenes in samples harvested at all pruning times, however the highest increase was observed in canes harvested in October, where *trans*-piceatannol and *trans*-resveratrol increase of 26.8 and 27.5 times, respectively. The increase of these two stilbenoids fall down to about five-fold in canes harvested in November and become even less pronounced in those harvested in December and January. The increase of *trans*-ε-viniferin in canes dried at 40 °C, though significant at all harvest times, was less than doubled, probably due to the high initial content of this stilbene in fresh canes. A temperature of 40 °C appears able to increase the kinetic rate of stilbene synthesis without a denaturation effect on the enzymes involved in the stilbene metabolism. Other researchers found an increase in stilbenoid content in grape canes submitted to 40 °C (Lambert *et al.*, 2013) or 45 °C 48h (Rayne *et al.*, 2008), whereas Houillé and colleagues (2015) found that a temperature of 65 °C is able to inhibit the stilbenoid synthesis. The latter authors tried also a storage at 15, 20 and 28 °C and found a faster increase of *trans*-resveratrol in canes with increasing temperature, however at 28 °C the maximum amount of *trans*-resveratrol reached was 2/3 than that obtained at 15 and 20 °C, indicating a possible negative effect of temperature during a long-term exposure. The behavior of canes harvested at different times is very interesting and seems to indicate a more “ready” stilbene metabolism in canes harvested in October, while the synthesis of

stilbenes is progressively less rapid with the evolution of canes toward winter dormancy. The same experiment was repeated also in 2016, giving similar results (44, 19.7 and 2 times increase of *trans*-resveratrol in canes harvested in October, November and December, respectively). This behavior could suggest the presence of high level of enzymes involved in resveratrol synthesis in fresh canes, with their natural decrease during senescence. Lambert *et al.* (2013) found high level of stilbenoid accumulation (1710 ± 224 mg/kg, 1526 ± 293 mg/kg and 3737 ± 421 mg/kg DW, for *trans*-piceatannol, *trans*-resveratrol and *trans*-viniferin, respectively) after a treatment at 40 °C on Pinot noir grape canes harvested in January. In this case, however, the treatment lasted for 15 days, probably allowing the time for the new synthesis of enzymes because of the stress induced by the cutting. On the contrary, the same results were not noticed by Rayne *et al.* (2008) which found up to 3.32 mg/kg of *trans*-resveratrol in Pinot noir grape canes harvested in March after a treatment at 45 °C for only 48h.

In grape canes stored for four, eight and twelve weeks at RT (20 ± 2 °C) it was evident the marked increase of *trans*-resveratrol and *trans*-piceatannol reaching a maximum around eight weeks of storage as already reported by many authors. Vergara *et al.* (2012) reported that the stilbenoids concentration in Pinot noir canes increased by 35% after storage at room temperature for 2 months. Houillé *et al.* (2015) evaluated the changes in the *trans*-resveratrol concentration in eight varieties of grape canes after a ten weeks storage period at 20 ºC and reported that the *trans*-resveratrol concentration in Pinot noir canes had the highest accumulation after six weeks (from 250 mg/kg DW to 4725 mg/kg DW). In this case, it is important to consider also the length of the cane cuts submitted to storage. In fact, as reported by Billet *et al.* (2018), the stilbenoid accumulation is much more rapid in cuttings of 0.5 cm compared to those of 10 cm. In our samples, the increase of stilbenoids was constant over the eight weeks period, confirming the results of Houillè *et al.* (2015) for Pinot noir cuttings of the same length (10 cm). Comparing the canes harvested at different pruning times some difference can be observed. In fact, contrarily to what observed for 40 °C treatment, in this case the stilbene synthesis is faster and a higher content of *trans*-piceatannol and *trans*-resveratrol is reached when increasing the pruning time from October to December. When the canes were recovered in January, however, a drop in the accumulation rate and in the total amount of stilbenes was observed, except for *trans*-ε-viniferin, whose concentration continued to increase.

Considering this increase is due to a *de novo* synthesis of the stilbene synthase enzyme after the injury, as demonstrated by Billet *et al.* (2018), we can hypothesize that the

presence of higher quantity of enzyme molecules in grape canes harvested in October, can have an inhibitory effect, so being responsible for a less intense stilbene synthase induction.

Influence of grapevine leafroll symptoms on the stilbenoid concentrations in Pinot noir grape cane waste

Grapevine leafroll disease is one of the most important grapevine viral diseases affecting grapevines worldwide. Disease symptoms are presumably linked to indirect effects of viral-induced interference with the network of host metabolic pathways. The virus induces changes in transcript profiles in a variety of biological function, such as photosynthesis, modulation of transcription factors, translation, transport, and secondary metabolism, in symptomatic leaves (Espinoza *et al.*, 2007; Gutha *et al.*, 2010). Enhanced expression of defense- and stress-related genes suggests activation of host responses concomitant with symptom development (Naidu *et al.*, 2015). Among the secondary metabolites, plant phenols constitute one of the most common and widespread group of defensive compounds and for the grapevine, resveratrol is the most important stilbene phytoalexine. Accumulation of *trans*-resveratrol was reported in leaves collected from GLRaV-3-infected plants showing evident foliar rolling and alterations in leaf color (Repetto *et al.*, 2012). In the present work, it was investigated if activation of defense mechanisms accompanying leafroll symptoms expressions could lead to a larger increase in the stilbenoid content (i.e. resveratrol and its derivatives) on canes harvested from symptomatic grapevines at the pruning time.

The grape canes harvested from symptomatic and asymptomatic plants in three different times (October, November and December autumn−winter 2016−2017) were evaluated with respect to three stilbenoids compounds (*trans*-resveratrol, *trans*-piceatannol, and *trans*-ε-viniferin), in four storage times at room temperature (from three to 12 weeks), dried at oven and control (Supplementary tables 1-3). Analyses performed immediately after pruning confirmed the presence of low levels of *trans*-resveratrol and *trans*-piceatannol and higher quantity of *trans*-ε-viniferin, as reported for samples harvested in 2015-2016. Interestingly, the amount of *trans*-resveratrol and *trans*-piceatannol showed an increase of eight and four times, respectively, in canes coming from symptomatic plants harvested in December compared to those harvested in October (from 15.56 ± 1.6 mg/kg to 124.14 ± 0.8 mg/kg for *trans*-resveratrol; from 7.23 ± 0.3 to 28.35 ± 1.7 for *trans*-piceatannol). Moreover, differences in the stilbenoids content in canes harvested from symptomatic and asymptomatic grapevines were evaluated after different storage processes. The drying process at 40 °C, according to the above-reported data, showed a significant increase ($p <$

0.05) of stilbenoids in samples harvested in October from both symptomatic and asymptomatic plants, with the only exception of *trans*-ε-viniferin in the symptomatic grapevines, whose content remain constant. The storage of canes led to an increase of stilbene content with a trend resembling that reported for samples harvested in 2015-2016. A deeper analysis revealed a significant higher amount of *trans*-resveratrol and *trans*-piceatannol in canes harvested from symptomatic plants compared to those harvested from asymptomatic plants for all the pruning times (Figure 1-2). On the opposite, the *trans*-ε-viniferin content was mainly higher in canes coming from asymptomatic plants, with a significant difference in particular for the pruning performed in October (Figure 3).

In compatible plant-virus interactions, pathogens spread through all plant tissues without inducing a resistance response, generating global cellular stress and development defects (Whitham *et al.*, 2006). Although unable to stop viral replication and systemic infection, susceptible hosts are not passive against viruses. The plant response involves changes in the expression of defense and stress-associated genes (Whitham *et al.*, 2006). GLRaV-3 infection is systemic in vine, being generally localized in the vascular plant tissue (phoem). After leaves falling, during the dormancy period, virus particles persist and replicate in the vascular system of the grapevine, and they could alter the expression profile of defense and stress-associated genes. The increased level of *trans*-resveratrol and *trans*-piceatannol observed in canes harvested from symptomatic plants during winter suggests an induction in transcription of genes involved in the phenylpropanoid pathway, responsible for stilbene biosynthesis, caused by GLRaV-3 infection. Contrariwise, lower quantity of *trans*-ε-viniferin was generally detected in canes harvested from symptomatic plants compared to those coming from asymptomatic grapevines. Viniferins, oligomers originated from oxidative dimerization of resveratrol units by means of peroxidase enzymes, accumulate in grapevine leaves upon fungal infection or UV irradiation and they showed high toxicity against *Plasmopara viticola* (Jeandet *et al.*, 2002; Pezet *et al.*, 2004). On resistant grapevine cultivars, resveratrol synthesized upon *P. viticola* infection was rapidly oxidized into toxic viniferins, while in susceptible grapevine it was glycosylated into a non-toxic piceide (Pezet *et al.*, 2004). In the present paper, the lower level of *trans*-ε-viniferin in the virus-infected canes, could be related to the establishment of a compatible interaction between the virus and the plant, without any resistance response, thus assuring the viral replication and propagation.

CONCLUSION

Collectively, the data reported in the present study confirm that the biosynthetic enzyme activities and, particularly, those involved in the stilbene pathway persist during grape cane storage. The grape canes by-products can be a rich source of stilbenoids, but many factors should be to considerated to obtain the higher yield of these compounds. In particular, the pruning time, which has never taken in account before, can be an important factor affecting the canes response. In addition, the presence of viruses can be a factor able to increase the stilbenoid accumulation in grape canes during storage.

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TABLES

Table 1. *trans*-resveratrol content in grape canes Pinot noir harvested before pruning in the autumn-winter 2015−2016

Processes	*trans*-resveratrol (mg/kg DW)			
	October	November	December	January
Control	65.84 ± 5.08^{dC}	80.99 ± 0.16^{eB}	45.62 ± 0.92^{eD}	92.31 ± 0.46^{fA}
Freezing	59.45 ± 1.63^{dC}	57.07 ± 0.74^{eC}	89.73 ± 0.90^{eB}	115.81 ± 4.56^{efA}
Freeze-drying	110.47 ± 4.74^{dB}	88.61 ± 3.78^{eC}	143.43 ± 1.78^{dA}	155.14 ± 1.24^{deA}
Drying 40ºC	1809.80 ± 21.43^{aA}	377.84 ± 2.72^{dB}	183.94 ± 1.40^{dC}	180.35 ± 1.87^{dC}
4 weeks stored	640.92 ± 4.46^{cD}	1228.81 ± 1.52^{bB}	1516.14 ± 1.31^{cA}	1173.47 ± 12.27^{cC}
8 weeks stored	1431.25 ± 28.58^{bC}	1562.10 ± 4.86^{aB}	1780.04 ± 15.82^{bA}	1430.62 ± 2.10^{bC}
12 weeks stored	673.32 ± 23.80^{cC}	626.06 ± 29.71^{cC}	2850.21 ± 25.09^{aA}	2268.14 ± 27.21^{aB}

Means ± SD (standard deviation) followed by the same lowercase letter in the column and uppercase in the line not represent significant difference $p < 0.05$ by Tukey's test.

Table 2. *trans*-piceatannol content in grape canes Pinot noir harvested before pruning in the autumn-winter 2015−2016

Processes	*trans*-piceatannol (mg/kg DW)			
	October	November	December	January
Control	31.51 ± 0.69^{eC}	34.07 ± 0.66^{eB}	26.32 ± 0.98^{eD}	38.82 ± 0.36^{eA}
Freezing	15.38 ± 0.37^{eD}	22.46 ± 0.17^{eC}	32.83 ± 0.28^{eA}	29.23 ± 0.08^{fB}
Freeze-drying	24.11 ± 1.29^{eA}	18.27 ± 1.25^{eB}	26.45 ± 0.20^{eA}	23.99 ± 0.85^{fA}
Drying 40ºC	846.42 ± 0.49^{aA}	187.06 ± 0.23^{dB}	48.80 ± 0.02^{dC}	47.09 ± 1.59^{dC}
4 weeks stored	324.92 ± 4.02^{dD}	528.22 ± 1.14^{bB}	708.61 ± 9.22^{bA}	410.89 ± 3.85^{cC}
8 weeks stored	557.64 ± 3.77^{bB}	684.84 ± 11.82^{aA}	536.54 ± 0.77^{cC}	499.94 ± 0.86^{bD}
12 weeks stored	450.26 ± 3.66^{cB}	232.41 ± 3.15^{cC}	804.81 ± 1.52^{aA}	574.75 ± 0.21^{aB}

Means ± SD (standard deviation) followed by the same lowercase letter in the column and uppercase in the line not represent significant difference $p < 0.05$ by Tukey's test.

Table 3. *trans*-ε-viniferin content in grape canes Pinot noir harvested before pruning in the autumn-winter 2015−2016

Processes	*trans*-ε-viniferin (mg/kg DW)			
	October	November	December	January
Control	1511.69 ± 9.23^{cdA}	1260.28 ± 11.71^{cB}	1449.30 ± 51.23^{cdA}	1161.68 ± 8.66^{eB}
Freezing	1018.75 ± 14.69^{eC}	995.81 ± 9.35^{cC}	1326.41 ± 22.65^{dA}	1231.30 ± 13.39^{eB}
Freeze-drying	1905.76 ± 154.79^{abA}	1188.85 ± 13.14^{cB}	1490.78 ± 0.44^{cB}	1424.59 ± 10.40^{dB}
Drying 40ºC	2097.28 ± 17.18^{aA}	1194.56 ± 14.31^{dC}	1885.11 ± 7.29^{bB}	2029.77 ± 28.95^{bA}
4 weeks stored	1761.13 ± 24.78^{bcA}	1122.79 ± 20.67^{cdC}	1446.61 ± 32.83^{cdB}	1446.83 ± 10.30^{dB}
8 weeks stored	1626.68 ± 12.16^{bcC}	1760.21 ± 3.22^{bB}	1778.24 ± 1.42^{bB}	1951.85 ± 23.87^{cA}
12 weeks stored	1329.69 ± 14.60^{deC}	2029.70 ± 11.70^{aB}	2424.15 ± 72.47^{aAB}	2616.91 ± 17.78^{aA}

Means ± SD (standard deviation) followed by the same lowercase letter in the column and uppercase in the line not represent significant difference $p < 0.05$ by Tukey's test.

FIGURES

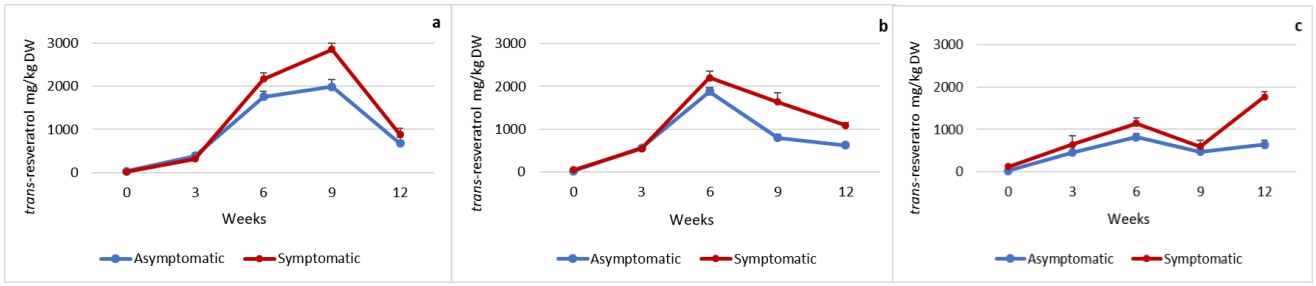

Figure 1. Content of *trans*-resveratrol on pinot noir canes asymptomatic and symptomatic harvested before pruning in (a) October, (b) November and (c) December on autumn-winter 2016-2017.

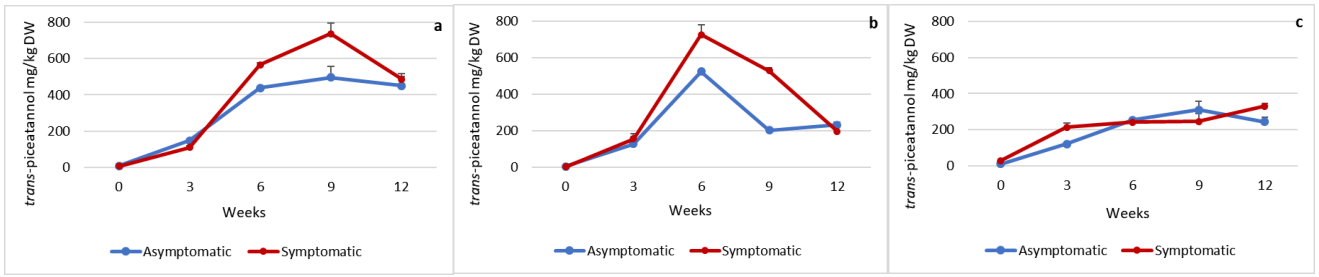

Figure 2. Content of *trans*-piceatannol on pinot noir canes asymptomatic and symptomatic harvested before pruning in (a) October, (b) November and (c) December on autumn-winter 2016-2017.

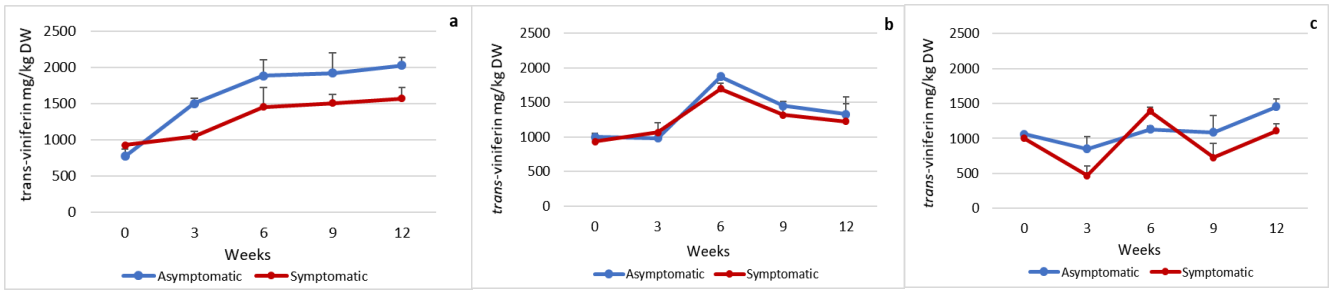

Figure 3. Content of *trans*-ε-viniferin on pinot noir canes asymptomatic and symptomatic harvested before pruning in (a) October, (b) November and (c) December on autumn-winter 2016-2017.

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SUPPLEMENTARY TABLES 1-3

Table 1. *trans*-resveratrol content in Pinot noir canes (A) asymptomatic and (S) symptomatic harvested before pruning in the autumn−winter 2016−2017

Processes	*trans*-resveratrol (mg/kg DW)		
	October	November	December
Control A	30.09 ± 7.5^{f}	19.29 ± 0.6^{f}	28.45 ± 3.2^{e}
Control S	15.56 ± 1.6^{f}	46.18 ± 3.8^{f}	124.14 ± 0.8^{de}
Drying 40ºC A	1326.11 ± 34^{cd}	374.15 ± 37^{ef}	57.28 ± 2.5^{e}
Driyng 40ºC S	1045.79 ± 177^{d}	192.76 ± 16^{f}	64.31 ± 4.6^{e}
3 weeks stored A	388.22 ± 6.0^{ef}	561.74 ± 22de	455.55 ± 9.2^{cde}
3 weeks stored S	320.20 ± 24^{ef}	547.84 ± 58de	646.08 ± 296^{c}
6 weeks stored A	1760.56 ± 115^{bc}	1877.19 ± 94^{ab}	796.58 ± 91^{bc}
6 weeks stored S	2165.71 ± 140^{ab}	2198.50 ± 161^{a}	1141.99 ± 130^{b}
9 weeks stored A	1989.94 ± 162^{bc}	801.21 ± 62^{cd}	477.85 ± 77^{cde}
9 weeks stored S	2851.83 ± 437^{a}	1632.56 ± 221^{b}	594.55 ± 281^{cd}
12 weeks stored A	673.32 ± 238de	626.06 ± 29de	641.06 ± 95^{c}
12 weeks stored S	885.70 ± 346de	1082.47 ± 73^{c}	1716.99 ± 222^{a}

Means ± SD (standard deviation) of three replicates (n = 3). Different letters on the same column represent significant difference $p < 0.05$ by Tukey's test.

Table 2. *trans*-piceatannol content in Pinot noir canes (A) asymptomatic and (S) symptomatic harvested before pruning in the autumn−winter 2016−2017

Processes	*trans*-piceatannol (mg/kg DW)		
	October	November	December
Control A	11.68 ± 11^{d}	4.98 ± 3.7^{e}	10.17 ± 7.7^{e}
Control S	7.23 ± 0.3^{d}	3.78 ± 4.7^{e}	28.35 ± 1.7^{e}
Drying 40ºC A	403.76 ± 19^{bc}	Nd	13.03 ± 0.7^{e}
Driyng 40ºC S	466.86 ± 41^{b}	1.94 ± 1.5^{e}	7.98 ± 8.6^{e}
3 weeks stored A	148.30 ± 6.1^{cd}	129.55 ± 39^{d}	121.06 ± 7.7^{d}
3 weeks stored S	107.13 ± 9.6^{d}	157.12 ± 27^{cd}	214.53 ± 22^{c}
6 weeks stored A	437.28 ± 5.9^{b}	524.28 ± 6.3^{b}	254.98 ± 1.1^{bc}
6 weeks stored S	565.66 ± 10^{ab}	727.20 ± 54^{a}	242.57 ± 8.3^{bc}
9 weeks stored A	496.71 ± 59^{ab}	203.68 ± 9.4^{cd}	309.43 ± 49^{ab}
9 weeks stored S	737.44 ± 238^{a}	524.29 ± 18^{b}	246.61 ± 42^{bc}
12 weeks stored A	450.25 ± 66^{b}	232.41 ± 15^{c}	243.88 ± 24^{bc}
12 weeks stored S	485.89 ± 14^{ab}	197.60 ± 36^{cd}	326.49 ± 15^{a}

Means ± SD (standard deviation) of three replicates (n = 3). Different letters on the same column represent significant difference $p < 0.05$ by Tukey's test. Nd = not detected ($Piceatannol_{LOQ}$ = 0.06 mg/kg)

Table 3. *trans*-viniferin content in Pinot noir canes (A) asymptomatic and (S) symptomatic harvested before pruning in the autumn−winter 2016−2017

Processes	*trans*-ε-viniferin (mg/kg DW)		
	October	November	December
Control A	774.60 ± 97^{e}	1001.19 ± 31^{d}	1060.33 ± 2.7^{bcd}
Control S	930.21 ± 0.97^{de}	907.31 ± 113^{d}	1001.75 ± 52^{cd}
Drying 40ºC A	1720.61 ± 125^{ab}	1122.89 ± 10^{cd}	794.11 ± 83^{cde}
Driyng 40ºC S	983.15 ± 72de	1046.19 ± 74^{cd}	929.71 ± 5.3^{cd}
3 weeks stored A	1503.54 ± 73^{abc}	982.63 ± 86^{d}	891.91 ± 169^{cde}
3 weeks stored S	1028.02 ± 74^{cde}	1066.42 ± 136^{cd}	465.56 ± 140^{e}
6 weeks stored A	1887.85 ± 223^{ab}	1872.74 ± 31^{a}	1131.67 ± 44^{abc}
6 weeks stored S	1454.45 ± 276^{ab}	1695.20 ± 83^{ab}	1404.44 ± 55^{ab}
9 weeks stored A	1925.13 ± 274^{ab}	1451.85 ± 56^{abc}	1086.01 ± 237^{abcd}
9 weeks stored S	1507.26 ± 126^{abc}	1289.59 ± 126^{bcd}	729.07 ± 203de
12 weeks stored A	2029.70 ± 111^{a}	1329.69 ± 146^{bcd}	1429.78 ± 109^{a}
12 weeks stored S	1574.86 ± 153^{ab}	1226.45 ± 350^{cd}	1108.93 ± 104^{abcd}

Means ± SD (standard deviation) of three replicates (n = 3). Different letters on the same column represent significant difference $p < 0.05$ by Tukey's test.

CHAPTER II

Grapevine canes waste from Veneto region as a new source of stilbenoids content

Grapevine canes waste from Veneto region as a new source of stilbenoids content

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ABSTRACT

On the one hand there is an enormous amount of underutilized side-streams rich in bioactives during wine production, while on the other hand there is a growing demand for promising phytochemicals for e.g. food or cosmetic industries. In the present we analyzed the stilbene accumulation in grape canes of seven autochthonous grape varieties from Veneto region, Italy. In addition, we investigated the effect of pruning time on the stilbenes accumulation rate during the storage. Taking into account the effect of the pruning time on the cultivars harvested in October, November and December and maintained for twelve weeks at room temperature, cultivar Verdiso and Incrocio Manzoni 13.0.25 showed the highest increase of *trans*-resveratrol, *trans*-piceatannol, and *trans*-ε-viniferin when harvested in October, highlighting the importance of the cultivar and effect of pruning time in the accumulation of stilbene in grape canes.

Keywords: Resveratrol, Viniferin, Piceatannol, Grape canes, Autochthonous varieties

INTRODUCTION

The winemaking industry is responsible for large part of grape waste as pomace, grape canes, seeds and stems. Some waste as pomace and seeds are valued from food industry being popular as source of antioxidant polyphenols or for grape seed oil production. At the same time, there is still a need for alternative sources of resveratrol, as can be seen by the recent permission of resveratrol as a novel food ingredient in the European Union (Commission Implementing Decision (EU) 2016/1190).

Stilbenoids accumulate in different parts of grapevine, however, Wang *et al.* (2010) found the highest concentration of *trans*-resveratrol in grape canes. Grape canes waste is generated during winter annual pruning and represent a large source of waste derived from the viticulture industry, with an estimated volume of 1 to 3 t/h year depending upon plantation density, climate, and vigor of the grape variety. Currently, emission protection regulations mostly prohibit the burning of grape canes, which was the traditional way of disposal of these woody residues (Ewald *et al.*, 2017).

Grape canes can be considered as an unexploited source of stilbenoids, as proposed by several authors (Vergara *et al.*, 2012; Lambert *et al.*, 2013; Gorena *et al.*, 2014; Houillé *et al.*, 2015; Guerrero *et al.*, 2016; Ewald *et al.*, 2017). Different content of stilbenoids have been found in grape canes of *Vitis vinifera* stored at 40 to 45 °C or at room temperature (20 ± 3 °C). Vergara *et al.* (2012) compared the stilbenoid content in canes of several grape varieties cultivated in different regions and in two different years in Chile, finding *trans*-resveratrol in the range 446 to 6533 mg/kg DW, with the highest content found in Gewurztraminer variety. Lower values of *trans*-resveratrol were reported by Lambert *et al.* (2013) comparing grape canes harvested from 16 different varieties in France. These authors found the lowest content of *trans*-resveratrol in Chardonnay (190 mg/kg DW) and highest content in Pinot noir cultivar (1526 mg/kg DW) while the *trans*-piceatannol and *trans*-ε-viniferin content were significant highest in all cultivars when compared with (Vergara *et al.*, 2012). Guerrero *et al.* (2016) described that most abundant stilbenoid was *trans*-viniferin in all cultivars, which reached the highest concentration in Gewürztraminer cultivar. While Ewald *et al.* (2017) found the higher levels of *trans*-resveratrol and *trans*-viniferin in Pinot blanc and Sauvignon blanc harvested in Germany (3199 – 3329 mg/kg DW, respectively). Zhang *et al.* (2011) studied the content of resveratrol in grape canes of many different grape varieties, including local varieties, cultivated in the seven major Chinese grape producing regions finding high variability.

Beside the genetic determinants, several other factors could explain these different results, such as the climate, the solvent used for the extraction of stilbenoids (Rayne *et al.*, 2008), the temperature and time of grape canes storage (Houillé *et al.*, 2015). In addition, other factors, such as the pruning time, could affect the stilbene accumulation rate in canes. This factor has never taken in account before, in fact in several articles this data is not reported at all, and, when present, show to be highly variable, with pruning times varying from 1 to 4 months after the grape harvest.

Up to date there are no data available concerning stilbenoid content in grape cane waste of Italian grape varieties. In the present paper the stilbene accumulation in grape canes of seven autochthonous grape varieties from Veneto region, one of the most important wine producing regions in Italy, has been studied. In addition, the effect of pruning time on the stilbenes accumulation rate during the storage was taken in account.

MATERIALS AND METHODS

Plant materials

Grape canes of *Vitis vinifera* L. from Veneto region white varieties, such as Bianchetta, Glera VCR Sel. Lungo, Incrocio Manzoni 6.0.1.3, Verdiso; and red varieties Incrocio Manzoni 13.0.25, Marzemino Biotipo 13, Raboso and, as reference, international varieties as Sauvignon blanc INRA 316 and Pinot noir, were collected randomly from plants from a conventional vineyard at Oenological School of Conegliano, Province of Treviso, Italy (I.S.I.S.S – Istituto Statale di Istruzione Secondaria Superiore “G.B. Cerletti”, Italy) (latitude 45° 87’ 69” N and longitude 12° 28’ 53” E and an average height of 63 M). The canes were collected monthly in October, November and December (autumn−winter 2016−2017). The canes were cut into 10−20 cm long sections and stored for three, six, nine and twelve weeks in well-aerated conditions in the dark, at room temperature. For control, a sample was immediately extracted after each pruning sampling point.

Stilbenoid extraction

The stilbenoid extraction was performed according to the procedure described by Rayne *et al.* (2008) with some modifications. Briefly, the grape canes were ground with a coffee grinder (Imetec, Azzano San Paolo, BG, Italy). Three-stage extraction was performed by continuous stirring at room temperature using an 8:1 (v/w) 80% ethanol:sample ratio over a 60-min period for each extraction. During the first extraction, 250 µL of t-OH-stilbene 200 µg/mL in ethanol were added ad internal standard. The extracts were vacuum filtered at 1.6

μm on glass microfibre filter (GF/A, Whatman) and combined and the solvent removed by rotary evaporation (Büchi model R-114, Flawil, Switzerland), then stored at −20 °C for quantification. All analyses were performed in triplicate.

HPLC analysis

The analysis of stilbenoids were performed according to the procedure described by Vincenzi *et al.* (2013) with some modifications. Stilbenes were separated on a C18 Lichrospher column (4 mm x 250 mm, 5 μm, Agilent Technologies, Milano, Italy) at 40 °C, using an HPLC system (Waters Corporation, Milford, MA, USA) equipped with a Dual Band UV detector Waters 2487 (Waters Corporation, Milford, MA, USA). The mobile phase gradient will be 0.5% v/v formic acid in deionized water (solvent A) and 2% v/v formic acid in methanol (solvent B). The gradient program will be 0 to 10% (solvent B) in 3 min, followed by 10 to 30% (solvent B) in 5 min, 30 to 44% (solvent B) in 35 min, 44 to 55% (solvent B) in 2 min, 55 to 75% (solvent B) in 15 min and 75 to 100% (solvent B) in 1 min. After washing for 2 min with solvent B, the column was re-equilibrated with solvent A. The flow rate will be 1.0 mL/min and injection volume 20 μL. Detection was performed at 306 nm for trans-isomers for *trans*-resveratrol, *trans*-ε-viniferin and *trans*-piceatannol. The concentration of individual stilbenes was quantified on the basis of peak areas using calibration curves of commercially available standards of *trans*-resveratrol, *trans*-ε-viniferin and *trans*-piceatannol, and correcting the value for the internal standard recovery. All the stilbene standards were obtained from Extrasynthese (Genay Cedex, France). Data were analysed by the Waters BreezeTM Chromatography Software (Version 3.30). The limits of detection (LOD) and quantification (LOQ) were performed according to the procedure described by (Shrivastava and Gupta 2011).

Statistical analysis

The results were evaluated by one-way analysis of variance (ANOVA), and the mean values were analyzed by Tukey's test using the software STATISTICA® 12.0 (StatSoft Inc, Tolson, USA).

RESULTS AND DISCUSSION

Considering that stilbenoid accumulation in cut canes depends on activation of related genes followed by active synthesis of resveratrol and its derivatives, as already reported by Houillé *et al.* (2015) and Billet *et al.* (2018), it is expected that different grape varieties respond in different way after the injury for both total amount of stilbenoid produced and rate of their accumulation. Also, the climate and other environmental factors can affect the way the canes respond during the storage period, for this reason the canes of the seven varieties (more two international varieties as reference) taken in consideration in this study were collected in the same year from plants grown in the same vineyard. The samples were harvested at different times on the autumn-winter 2016−2017 (October, November and December) during the pruning period. At the same time, the grape canes were stored for three, six, nine and twelve weeks at room temperature (RT), in order to simulate the normal situation of canes left on the ground after pruning. The canes were stored in an airy place in the dark to avoid the development of molds. The control sample for each sampling time was obtained by immediate extraction.

As a first observation, the basal level of *trans*-piceatannol and *trans*-resveratrol in fresh canes was quite low (less than 40 mg/kg DW) whereas the dimer *trans*-ε-viniferin was already present at concentrations 10 to 30 times higher (Supplementary tables 1-15). These results confirm the literature data (Gorena *et al.*, 2014; Houillé *et al.*, 2015; Billet *et al.*, 2018), only Vergara and colleagues (2012) found very high content of *trans*-resveratrol (between 2500 and 3500 mg/kg) already at time zero.

Collectively, the canes pruned in October showed a gradual increase of stilbenes during all the storage period, whereas the canes collected in November showed a notable peak of accumulation of stilbene content after 6 weeks of storage for almost all the varieties (Figure 1, 2). Houillé *et al.* (2015) found the same results, i. e. a peak of accumulation of *trans*-resveratrol and *trans*-piceatannol after 6 weeks of storage, on eight cultivars (collected in December) and stored for 2, 4, 6, 8 and 10 weeks. On the other hand, the pruning carried out in December demonstrated a different behavior in the accumulation of stilbenes (Figure 3). For many varieties, in particular for Verdiso and Incrocio Manzoni 13.0.25 (IM 13.0.25) the peak of stilbene accumulation was retarded to 9 weeks of storage. This behavior of canes harvested at different times seems to confirm the results obtained in the previous Chapter on Pinot noir, that is a slower stilbenoid response in canes harvested in October, probably due to the high quantity of stilbene synthase enzymes still present in the woody tissue, which under regulate the induction of the related genes after the injury. The synthesis

of stilbenoids is instead more rapid with the evolution of canes toward winter dormancy until November, then the accumulation rate slow down in canes harvested in December.

Taking into account the effect of the pruning time on the single samples, among the cultivars harvested in October and maintained for twelve weeks at RT, cultivar Verdiso, IM 13.0.25 and Marzemino showed the highest increase of *trans*-resveratrol, *trans*-piceatannol, and *trans*-ε-viniferin (Figure 1). Among the white varieties, Verdiso is one of the last to be harvested. Similarly, IM 13.0.25 and Marzemino are, among red varieties, those with the later harvest. It is not clear if this common behavior could be related to the similar accumulation rate of stilbenes in pruned canes. Regarding resveratrol, even though after 9 weeks Pinot noir was the cultivar with the highest content, confirming the high stilbene metabolism of this variety, after 12 weeks IM 13.0.25 increased again reaching the higher resveratrol content among all the varieties taken in consideration. Even for piceatannol, IM 13.0.25 presented concentration almost always higher than Pinot noir.

Following the pruning time, the canes harvested in November demonstrated a significant increase ($p < 0.05$) for all stilbene compounds when maintained for twelve weeks at RT. Again the cultivar IM 13.0.25 presented a constant increase of *trans*-resveratrol content (2016 ± 365 mg/kg DW) with values comparable with those found in October. In this group, a high increase of the content of *trans*-piceatannol (633 ± 64 mg/kg DW) and *trans*-ε-viniferin (2193 ± 213 mg/kg DW) was detected even on the cultivar Bianchetta (Figure 2). Compared to Pinot noir, which reached after 6 weeks the maximum content of piceatannol and resveratrol among all varieties, Bianchetta and IM 13.0.25 were able to reach the same or higher quantities of both compounds after a storage of 12 weeks.

In grape canes harvested in December Pinot noir showed a very small accumulation of resveratrol and piceatannol, while again Verdiso and IM 13.0.25 were able to reach a high content of both compounds (Figure 3).

Our findings confirm the results previously found by Guerrero *et al.* (2016) which highlight the importance of the cultivar in the accumulation of stilbene in grape canes after pruning. In addition, we demonstrated how the pruning time affect both the quantity and the accumulation rate of stilbenes in pruned canes.

The information on the maximum stilbene content recoverable from canes of different grapevine cultivars could be interesting for grape producers in order to obtain cane extracts with high stilbenes concentration from their own grape canes waste. These extracts can be the base for the purification of stilbenes to be used in the food or cosmetic industries, with a big economic income considering the value of food-grad resveratrol is about 2000-3000

US$/kg (Zhang *et al.*, 2011). However, the crude cane extract could be also reused by grape growers in the same vineyard in an idea of circular economy. In fact stilbenes have shown antifungal activity against different fungi. Until now, the antifungal activity *in vitro* of the crude cane extracts from Pinot noir, Gamaret and Divico cultivars was reported by Schnee *et al.* (2013).

CONCLUSION

The information on the maximum stilbene content recoverable from canes of different grapevine cultivars could be interesting for grape producers, but many factors have to be taken in to account to obtain the higher yield of these compounds. In summary, the data reported in the present study confirm the importance of the cultivar in the accumulation of stilbene in grape canes after pruning. In addition, we demonstrated how the pruning time affect both the quantity and the accumulation rate of stilbenes in pruned canes.

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FIGURES

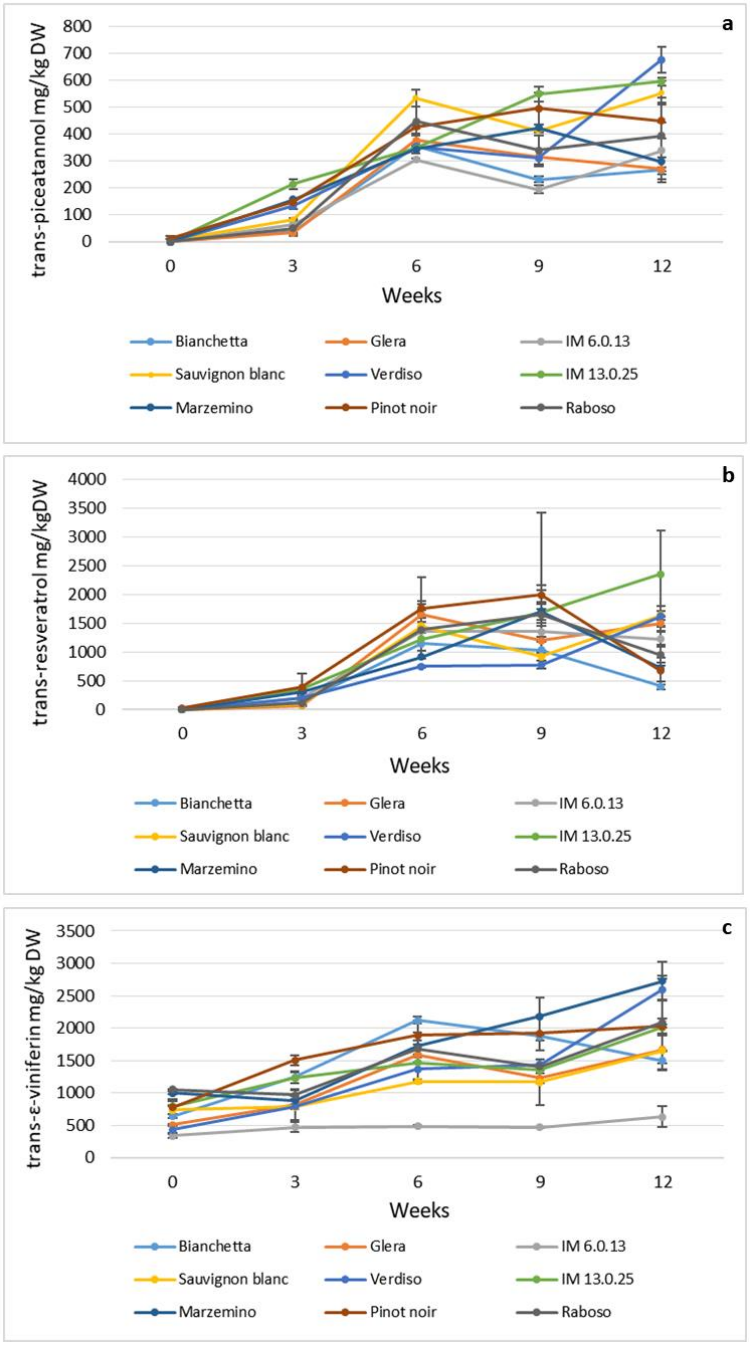

Figure 1. Stilbenoid concentrations in grape canes harvested in October (autumn−winter 2016−2017) from white varieties, Bianchetta, Glera VCR Sel. Lungo, Incrocio Manzoni 6.0.1.3, Verdiso, and red varieties Incrocio Manzoni 13.0.25, Marzemino Biotipo 13, Raboso, Sauvignon blanc INRA 316, Pinot noir and stored at room temperature for 3, 6, 9, and 12 weeks.

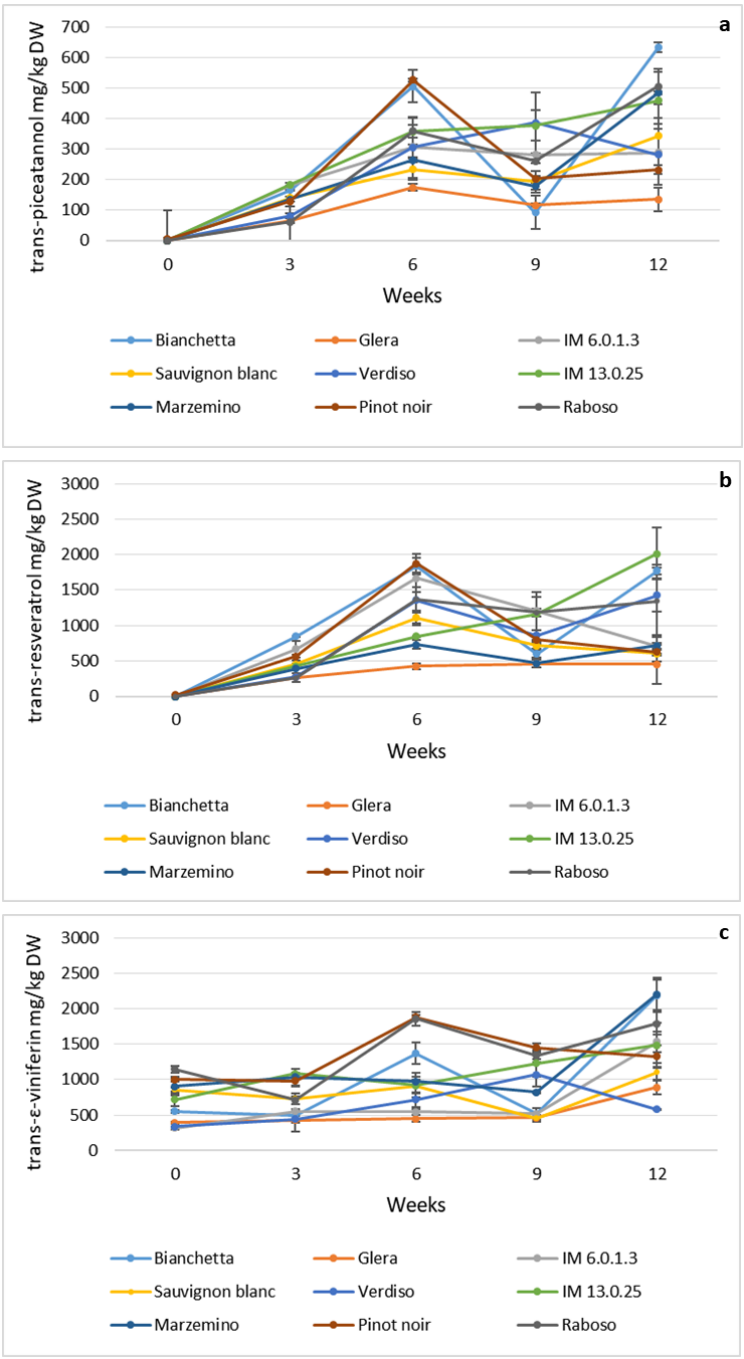

Figure 2. Stilbenoid concentrations in grape canes harvested in November (autumn−winter 2016−2017) from white varieties, Bianchetta, Glera VCR Sel. Lungo, Incrocio Manzoni 6.0.1.3, Verdiso, and red varieties Incrocio Manzoni 13.0.25, Marzemino Biotipo 13, Raboso, Sauvignon blanc INRA 316, Pinot noir and stored at room temperature for 3, 6, 9, and 12 weeks.

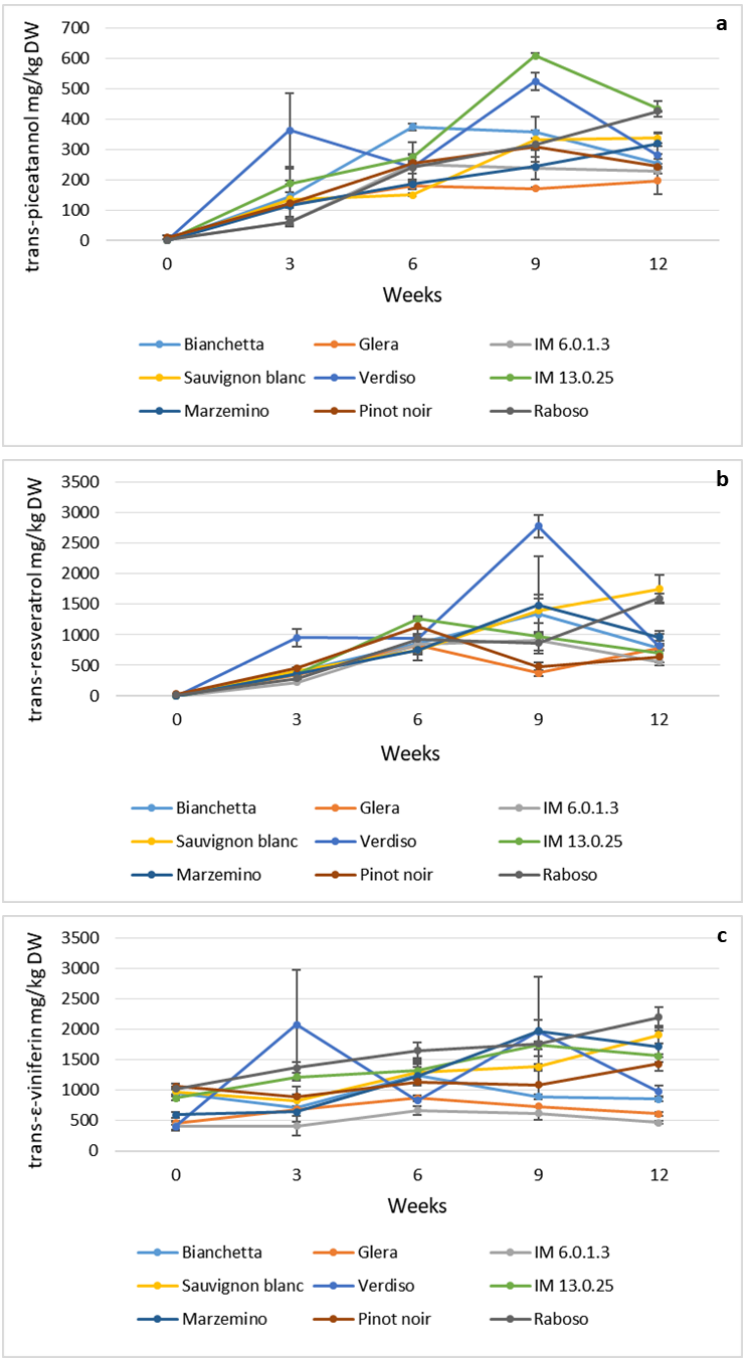

Figure 3. Stilbenoid concentrations in grape canes harvested in December (autumn−winter 2016−2017) from white varieties, Bianchetta, Glera VCR Sel. Lungo, Incrocio Manzoni 6.0.1.3, Verdiso, and red varieties Incrocio Manzoni 13.0.25, Marzemino Biotipo 13, Raboso, Sauvignon blanc INRA 316, Pinot noir and stored at room temperature for 3, 6, 9, and 12 weeks.

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SUPPLEMENTARY TABLES1-15

Tables 1-2-3. *trans*-piceatannol, *trans*-resveratrol and *trans*-ε-viniferin content on grape canes harvested in October, November and December (autumn−winter 2016−2017) and submitted at immediate extraction (control)

	Control		
	trans-piceatannol (mg/kg DW)		
Canes	October	November	December
Bianchetta	Nd	Nd	Nd
Glera	Nd	Nd	Nd
I.M 6.0.13	Nd	Nd	2.66 ± 0.1^{b}
Sauvignon blanc	3.56 ± 7.2^{a}	Nd	Nd
Verdiso	Nd	Nd	Nd
I.M 13.0.25	Nd	Nd	Nd
Marzemino	Nd	Nd	Nd
Pinot noir	11.68 ± 11^{aA}	4.98 ± 3.7^{aA}	10.17 ± 7.8^{aA}
Raboso	Nd	Nd	2.43 ± 2.7^{b}

Means ± SD followed by the same lowercase letter on the column and uppercase on the line for the not represent significant difference $p < 0.05$ by Tukey's test.
Nd = not detected ($Piceatannol_{LOQ}$ = 0.06 mg/kg), ($Resveratrol_{LOQ}$ = 0.32 mg/kg)

	Control		
	trans-resveratrol (mg/kg DW)		
Canes	October	November	December
Bianchetta	Nd	Nd	Nd
Glera	Nd	Nd	Nd
I.M 6.0.13	Nd	Nd	Nd
Sauvignon blanc	Nd	Nd	Nd
Verdiso	Nd	Nd	Nd
I.M 13.0.25	Nd	Nd	Nd
Marzemino	Nd	Nd	Nd
Pinot noir	30.09 ± 7.5^{aA}	19.29 ± 0.6^{aA}	28.45 ± 3.2^{aA}
Raboso	Nd	Nd	11.15 ± 0.6^{b}

Means ± SD followed by the same lowercase letter on the column and uppercase on the line for the not represent significant difference $p < 0.05$ by Tukey's test.
Nd = not detected ($Piceatannol_{LOQ}$ = 0.06 mg/kg), ($Resveratrol_{LOQ}$ = 0.32 mg/kg)

	Control		
	trans-ε-viniferin (mg/kg DW)		
Canes	October	November	December
Bianchetta	642 ± 34^{cdB}	556.59 ± 21^{deB}	959.39 ± 44^{abA}
Glera	504.53 ± 20^{deA}	395.56 ± 16^{efB}	456.18 ± 31^{cdAB}
I.M 6.0.13	338.27 ± 29^{eA}	321.18 ± 10^{fA}	413.33 ± 64^{dA}
Sauvignon blanc	739.30 ± 71^{cA}	856.72 ± 30^{bcA}	972 ± 50^{abA}
Verdiso	434.93 ± 49^{deA}	341.58 ± 48^{fA}	404.28 ± 77^{dA}
I.M 13.0.25	795.41 ± 101^{bcA}	716.76 ± 94^{cdA}	865.46 ± 33^{bA}
Marzemino	1000.19 ± 20^{abA}	907.61 ± 133^{bcAB}	595.35 ± 48^{cB}
Pinot noir	774.60 ± 97^{cB}	999.43 ± 31^{aA}	1060.33 ± 2.8^{aA}
Raboso	1057.16 ± 1.24^{aA}	1144.78 ± 46^{aA}	1018.43 ± 83^{abA}

Means ± SD followed by the same lowercase letter on the column and uppercase on the line for the not represent significant difference $p < 0.05$ by Tukey's test.

Tables 4-5-6. *trans*-piceatannol, *trans*-resveratrol and *trans*-ε-viniferin content on grape canes harvested in October, November and December (autumn−winter 2016−2017) and stored for 3 weeks at room temperature

	Stored for 3 weeks at room temperature		
	trans-piceatannol (mg/kg DW)		
Canes	October	November	December
Bianchetta	37.40 ± 17^{bA}	166.30 ± 22^{aA}	145.79 ± 12^{abA}
Glera	33.85 ± 0.1^{dB}	65.66 ± 6.7^{cdA}	119.02 ± 66^{bA}
I.M 6.0.13	64.62 ± 14^{cdB}	182.35 ± 23^{aA}	62.17 ± 16^{bB}
Sauvignon blanc	83.40 ± 3.7^{cB}	140.13 ± 27^{abA}	135.89 ± 63^{bB}
Verdiso	133.40 ± 3.3^{bB}	81.24 ± 1.2^{bcdB}	362.24 ± 121^{aA}
I.M 13.0.25	215.15 ± 19^{aA}	184.86 ± 6.7^{aA}	187.16 ± 50^{abA}
Marzemino	158.31 ± 11^{bA}	135.75 ± 8.0^{abAB}	114.41 ± 6.8^{bB}
Pinot noir	148.30 ± 6.1^{bA}	129.55 ± 39^{abcA}	122.89 ± 7.7^{bA}
Raboso	50.54 ± 2.7^{cdA}	61.54 ± 0.4^{dA}	62.15 ± 9.7^{bA}

Means ± SD followed by the same lowercase letter on the column and uppercase on the line for the not represent significant difference $p < 0.05$ by Tukey's test.

	Stored for 3 weeks at room temperature		
	trans-resveratrol (mg/kg DW)		
Canes	October	November	December
Bianchetta	140.63 ± 23^{cdC}	848.98 ± 6.1^{aA}	395.55 ± 40^{aB}
Glera	76.31 ± 6.1^{dB}	262.51 ± 65^{dA}	338.54 ± 62^{aA}
I.M 6.0.13	206.64 ± 63^{cB}	669.18 ± 118^{abA}	223.16 ± 28^{aB}
Sauvignon blanc	100.32 ± 1.7^{dB}	453.07 ± 57^{cdA}	414.87 ± 40^{aA}
Verdiso	204.48 ± 20^{cA}	275.21 ± 3.7^{dA}	951.44 ± 551^{aA}
I.M 13.0.25	354.18 ± 9.6^{abA}	427.25 ± 40^{cdA}	355.96 ± 82^{aA}
Marzemino	309.96 ± 11^{bA}	389.17 ± 67^{cdA}	370.68 ± 94^{aA}
Pinot noir	388.22 ± 6.0^{aB}	561.74 ± 22^{bcA}	455.55 ± 9.2^{aB}
Raboso	114.95 ± 7.1^{dB}	257.84 ± 8.1^{dA}	282.34 ± 32^{aA}

Means ± SD followed by the same lowercase letter on the column and uppercase on the line for the not represent significant difference $p < 0.05$ by Tukey's test.

	Stored for 3 weeks at room temperature		
	trans-ε-viniferin (mg/kg DW)		
Canes	October	November	December
Bianchetta	1241.68 ± 93^{abA}	491.68 ± 10^{dB}	705.66 ± 6,7^{abB}
Glera	815.61 ± 438^{bcA}	430.34 ± 158^{dA}	687.58 ± 23^{abA}
I.M 6.0.13	467.59 ± 78^{cA}	552.02 ± 10^{dA}	409.99 ± 261^{bA}
Sauvignon blanc	784.31 ± 35^{bcA}	733.12 ± 77^{bcdA}	831.66 ± 97^{abA}
Verdiso	795.15 ± 22^{bcA}	442.54 ± 46^{dB}	2077.63 ± 905^{aA}
I.M 13.0.25	1228.70 ± 86^{abA}	1083.44 ± 10^{aA}	1216.74 ± 69^{abA}
Marzemino	883.50 ± 63^{bcA}	1038.17 ± 119^{abA}	643.95 ± 171^{abA}
Pinot noir	1503.54 ± 73^{aA}	982.63 ± 86^{abcB}	891.91 ± 169^{abB}
Raboso	968.48 ± 71^{abcB}	709.45 ± 57^{cdC}	1371.01 ± 89^{abA}

Means ± SD followed by the same lowercase letter on the column and uppercase on the line for the not represent significant difference $p < 0.05$ by Tukey's test.

Tables 7-8-9. *trans*-piceatannol, *trans*-resveratrol and *trans*-ε-viniferin content on grape canes harvested in October, November and December (autumn−winter 2016−2017) and stored for 6 weeks at room temperature

	Stored for 6 weeks at room temperature		
	trans-piceatannol (mg/kg DW)		
Canes	October	November	December
Bianchetta	357.40 ± 2^{bcdB}	506.68 ± 57^{abA}	373.68 ± 11^{aAB}
Glera	378.49 ± 23^{bcdA}	175.33 ± 12.50^{eB}	180.07 ± 12^{cdB}
I.M 6.0.13	305.62 ± 4.6^{dA}	308.94 ± 63^{cdA}	250.43 ± 72^{bcdA}
Sauvignon blanc	533.60 ± 31^{aA}	234.12 ± 35^{deB}	150.59 ± 5.5^{dB}
Verdiso	354.22 ± 11^{bcdA}	306.75 ± 54^{cdAB}	241.34 ± 21^{bcdB}
I.M 13.0.25	348.59 ± 18^{cdA}	358.65 ± 44^{bcA}	273.61 ± 12^{bB}
Marzemino	344.50 ± 12^{cdA}	265.44 ± 8.6^{deB}	185.13 ± 13^{cdC}
Pinot noir	427.28 ± 5.9^{bcB}	526.88 ± 6.3^{aA}	254.98 ± 1.2^{bcC}
Raboso	448.63 ± 53^{aA}	360.57 ± 21^{cdAB}	240.71 ± 40^{bcdB}

Means ± SD followed by the same lowercase letter on the column and uppercase on the line for the not represent significant difference $p < 0.05$ by Tukey's test.

	Stored for 6 weeks at room temperature		
	trans-resveratrol (mg/kg DW)		
Canes	October	November	December
Bianchetta	1146.40 ± 9.1^{cdB}	1839.47 ± 115^{aA}	856.88 ± 125^{aB}
Glera	1658.53 ± 71^{abA}	422.96 ± 105^{eB}	835.75 ± 38^{aC}
I.M 6.0.13	1359.29 ± 12^{bcB}	1669.72 ± 71^{abA}	847.30 ± 195^{aC}
Sauvignon blanc	1453.43 ± 74^{abcA}	1105.62 ± 4.3^{cdAB}	752.20 ± 72^{aB}
Verdiso	748.94 ± 12^{eB}	1356.99 ± 65^{bcA}	942.05 ± 362^{aA}
I.M 13.0.25	1221.47 ± 202^{cA}	847.87 ± 188^{dA}	1260.84 ± 3.2^{aA}
Marzemino	916.55 ± 33^{deA}	728.29 ± 62^{deB}	751.90 ± 64^{aB}
Pinot noir	1760.56 ± 115^{aA}	1877.19 ± 94^{aA}	1141.99 ± 130^{aB}
Raboso	1388.78 ± 56^{bcA}	1370.58 ± 35^{bcA}	926.85 ± 164^{aB}

Means ± SD followed by the same lowercase letter on the column and uppercase on the line for the not represent significant difference $p < 0.05$ by Tukey's test.

	Stored for 6 weeks at room temperature		
	trans-ε-viniferin (mg/kg DW)		
Canes	October	November	December
Bianchetta	2120.63 ± 57^{aA}	1366.34 ± 152^{bB}	1251.30 ± 180^{abcB}
Glera	1588.47 ± 7.4^{abcA}	452.23 ± 47^{dB}	872.40 ± 47^{bcdC}
I.M 6.0.13	486.56 ± 16^{dB}	543.11 ± 40^{dAB}	666.57 ± 71^{dA}
Sauvignon blanc	1177.29 ± 30^{cA}	904.77 ± 94^{cA}	1295.70 ± 195^{abA}
Verdiso	1371.76 ± 176^{bcA}	719.46 ± 102^{cdB}	827.15 ± 6.5^{cdB}
I.M 13.0.25	1469 ± 461^{bcA}	922.82 ± 172^{cA}	1318.11 ± 150^{abA}
Marzemino	1718.01 ± 26^{abA}	977.31 ± 58^{cC}	1229.16 ± 144^{abcB}
Pinot noir	1887.85 ± 223^{abA}	1878.03 ± 31^{aA}	1131.67 ± 44^{bcdB}
Raboso	1685 ± 120^{abcA}	1855.83 ± 103^{aA}	1653.85 ± 135^{aA}

Means ± SD followed by the same lowercase letter on the column and uppercase on the line for the not represent significant difference $p < 0.05$ by Tukey's test.

Tables 10-11-12. *trans*-piceatannol, *trans*-resveratrol and *trans*-ε-viniferin content on grape canes harvested in October, November and December (autumn−winter 2016−2017) and stored for 9 weeks at room temperature

	Stored for 9 weeks at room temperature		
	trans-piceatannol (mg/kg DW)		
Canes	October	November	December
Bianchetta	232.48 ± 12^{deAB}	91.97 ± 44^{dB}	356.79 ± 50^{bA}
Glera	316.62 ± 27^{cdA}	116.77 ± 4.6^{cdB}	170.51 ± 3.3^{dB}
I.M 6.0.13	193.83 ± 15^{eA}	282.21 ± 62^{abA}	238.21 ± 37^{cdA}
Sauvignon blanc	411.55 ± 0.6^{bcA}	193.29 ± 36^{bcdB}	332.50 ± 31^{bcA}
Verdiso	312.14 ± 1.2^{cdeB}	386.76 ± 55^{aAB}	523.37 ± 30^{aA}
I.M 13.0.25	550.77 ± 28^{aA}	377.28 ± 50^{aB}	607.63 ± 8.2^{aA}
Marzemino	423.81 ± 31^{bcA}	179.07 ± 12^{bcdB}	243.50 ± 3.8^{cdB}
Pinot noir	496.71 ± 59^{abA}	203.68 ± 9.4^{bcdB}	309.43 ± 49^{bcAB}
Raboso	340.22 ± 55^{cdA}	260.99 ± 6.9^{abcA}	315.86 ± 19^{bcA}

Means ± SD followed by the same lowercase letter on the column and uppercase on the line for the not represent significant difference $p < 0.05$ by Tukey's test.

	Stored for 9 weeks at room temperature		
	trans-resveratrol (mg/kg DW)		
Canes	October	November	December
Bianchetta	1032.79 ± 6.4^{cdeA}	598.59 ± 56^{bB}	1338.52 ± 318^{bA}
Glera	1196.41 ± 13^{cdA}	462.70 ± 56^{bB}	374.38 ± 44^{bB}
I.M 6.0.13	1358.25 ± 93^{bcA}	1204.21 ± 272^{aA}	912.11 ± 216^{bA}
Sauvignon blanc	923.19 ± 74^{deAB}	716.56 ± 45^{abB}	1396.09 ± 201^{bA}
Verdiso	782.20 ± 68^{eB}	863.19 ± 63^{abB}	2781.19 ± 181^{aA}
I.M 13.0.25	1686.03 ± 178^{abA}	1166.95 ± 237^{aA}	982.74 ± 63^{bA}
Marzemino	1709.43 ± 30^{abA}	464.18 ± 2.9^{bB}	1485.72 ± 795^{bA}
Pinot noir	1989.94 ± 162^{aA}	801.21 ± 62^{abB}	477.85 ± 77^{bC}
Raboso	1649.46 ± 97^{abA}	1183.34 ± 53^{aB}	864.59 ± 118^{bB}

Means ± SD followed by the same lowercase letter on the column and uppercase on the line for the not represent significant difference $p < 0.05$ by Tukey's test.

	Stored for 9 weeks at room temperature		
	trans-ε-viniferin (mg/kg DW)		
Canes	October	November	December
Bianchetta	1834.18 ± 62^{abA}	519.18 ± 81^{deC}	891.65 ± 42^{abB}
Glera	1231.26 ± 77^{cA}	460.38 ± 26^{eB}	729.96 ± 10^{bC}
I.M 6.0.13	473.38 ± 4.3^{dA}	519.54 ± 30^{cdA}	620.48 ± 112^{bA}
Sauvignon blanc	1168.32 ± 352^{cAB}	454.87 ± 53^{eB}	1382.57 ± 41^{abA}
Verdiso	1436.63 ± 89^{bcB}	1074.03 ± 172^{bcC}	1968.68 ± 178^{aA}
I.M 13.0.25	1350.99 ± 15^{bcB}	1229.90 ± 186^{abAB}	1735.67 ± 60^{abA}
Marzemino	2181.91 ± 289^{aA}	822.39 ± 10^{cdB}	1972.10 ± 895^{aA}
Pinot noir	1925.13 ± 274^{abA}	1451.85 ± 56^{aA}	1086.01 ± 237^{abA}
Raboso	1408.91 ± 26^{bcA}	1339.68 ± 44^{abA}	1763.53 ± 200^{aA}

Means ± SD followed by the same lowercase letter on the column and uppercase on the line for the not represent significant difference $p < 0.05$ by Tukey's test.

Tables 13-14-15. *trans*-piceatannol, *trans*-resveratrol and *trans*-ε-viniferin content on grape canes harvested in October, November and December (autumn−winter 2016−2017) and stored for 12 weeks at room temperature

	Stored for 12 weeks at room temperature		
	trans-piceatannol (mg/kg DW)		
Canes	October	November	December
Bianchetta	267.58 ± 47^{cB}	633.91 ± 64^{aA}	253.92 ± 15^{bcdB}
Glera	271.04 ± 7.7^{cA}	136 ± 38^{fA}	197.02 ± 45^{dA}
I.M 6.0.13	338.29 ± 106^{bcA}	289.36 ± 30^{cdefA}	229.25 ± 8.6^{cdA}
Sauvignon blanc	553.85 ± 45^{abcA}	344.22 ± 60^{bcdeB}	337.56 ± 18^{abB}
Verdiso	678.59 ± 78^{aA}	282.55 ± 16^{defB}	280.77 ± 29^{bcdB}
I.M 13.0.25	596.19 ± 15^{abA}	460.63 ± 92^{abcdA}	434.23 ± 26^{aA}
Marzemino	298.95 ± 48^{cB}	485.30 ± 5.2^{abcA}	319.68 ± 34^{bcB}
Pinot noir	450.25 ± 66^{abcA}	232.41 ± 15^{efB}	243.88 ± 24^{cdB}
Raboso	394.86 ± 142^{abcA}	505.95 ± 58^{abA}	424.89 ± 1.8^{aA}

Means ± SD followed by the same lowercase letter on the column and uppercase on the line for the not represent significant difference $p < 0.05$ by Tukey's test.

	Stored for 12 weeks at room temperature		
	trans-resveratrol (mg/kg DW)		
Canes	October	November	December
Bianchetta	409.88 ± 66^{cC}	1769.46 ± 50^{aA}	776.63 ± 236^{bB}
Glera	1498.69 ± 134^{abcA}	452.99 ± 283^{bB}	786.64 ± 29^{bAB}
I.M 6.0.13	1222.43 ± 135^{abcA}	709.21 ± 46^{bB}	557.09 ± 93^{bB}
Sauvignon blanc	1634.88 ± 79^{abA}	603.99 ± 116^{bB}	1752.10 ± 226^{aA}
Verdiso	1624.75 ± 172^{abA}	1426.98 ± 234^{abAB}	821.66 ± 86^{bB}
I.M 13.0.25	2353.27 ± 746^{aA}	2016.16 ± 365^{aA}	699.22 ± 203^{bB}
Marzemino	719.96 ± 233^{bcA}	713.73 ± 144^{bA}	960.83 ± 108^{bA}
Pinot noir	673.32 ± 238^{bcA}	626.06 ± 29^{bA}	641.06 ± 95^{bA}
Raboso	946.53 ± 186^{bcA}	1343.89 ± 512^{abA}	1601.40 ± 80^{aA}

Means ± SD followed by the same lowercase letter on the column and uppercase on the line for the not represent significant difference $p < 0.05$ by Tukey's test.

	Stored for 12 weeks at room temperature		
	trans-ε-viniferin (mg/kg DW)		
Canes	October	November	December
Bianchetta	1498.70 ± 157^{bcB}	2193.46 ± 213^{abA}	853.27 ± 41^{deC}
Glera	1668.79 ± 218^{abcA}	893.02 ± 103^{deB}	609 ± 28^{deB}
I.M 6.0.13	632.74 ± 157^{cB}	1531.79 ± 141^{abcdA}	464.50 ± 26^{eB}
Sauvignon blanc	1649.34 ± 14^{abcA}	1107.93 ± 122^{cdeB}	1909.42 ± 145^{aA}
Verdiso	2597.18 ± 167^{abA}	577.64 ± 8.5^{eC}	976.41 ± 99^{cdB}
I.M 13.0.25	2015.47 ± 35^{abA}	1483.30 ± 320^{bcdB}	1561.67 ± 25^{bB}
Marzemino	2731.26 ± 285^{aA}	2203.79 ± 227^{aAB}	1714.50 ± 255^{bB}
Pinot noir	2029.70 ± 111^{abA}	1329.69 ± 146^{cdB}	1429.78 ± 109^{bcB}
Raboso	2086.93 ± 728^{abA}	1792.81 ± 162^{abcA}	2198.13 ± 170^{aA}

Means ± SD followed by the same lowercase letter on the column and uppercase on the line for the not represent significant difference $p < 0.05$ by Tukey's test.

CHAPTER III

Stilbene extract from grape canes reduces *Botrytis cinerea* infection in grapevine by inducing some defense mechanisms and negatively regulating new stilbene production

Stilbene extract from grape canes reduces *Botrytis cinerea* infection in grapevine by inducing some defense mechanisms and negatively regulating new stilbene production

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ABSTRACT

Crude extract of *Vitis vinifera* canes (SE) represent a source of stilbene compounds with well knows antifungal properties. Exogenous application of SE demonstrated to induce satisfactory protection toward *Botrytis cinerea* on grapevine plants, thus being a promising natural fungicide. SE possessed a direct antifungal activity inferred by the inhibitory effect of the mycelium growth observed on nutrient agar medium and through the reduction of the necrotic lesions caused by *B. cinerea* on grapevine leaves. Furthermore, it was verified that the acquired protection derived also from an induction of some grapevine defense mechanisms. After its perception, SE induced specific defense events, such as the activation of MAPKs and a higher expression of a gene encoding a glutathione-S-transferase (*GST1)* and some PR genes, but negatively regulated new stilbene production. This result suggests the activation of an immune-ready state on SE-treated plants.

Keywords: Cane extract, Resveratrol, Stilbenes, Phytoalexins, *Botrytis cinerea*, Grapevine, Defense response

INTRODUCTION

Grapevine (*Vitis vinifera*) is a major fruit crop worldwide. During cultivation grapes face frequent attacks from a large variety of pathogenic agents, thus requiring an intensive use of phytochemicals to limit pathogen infections. Many pesticide residues are often detected in grapes and wine, depending on the quantity of their use in the field, the way, the number of applications and the time from application to harvest (Doulia *et al.*, 2016). Serious problems have arisen using pesticides for both the environment and human health. However, the crop protection with chemical methods is still a normal practice in viticulture for wine making, due to the specificities of the cultivation of the vine and its diseases and pests. Therefore, there is a need for developing alternative strategies to control fungal disease of grapes that are safe, effective, economical, and compatible with commercial handling practices (Karabulut *et al.*, 2005). Fortunately, there is a tendency towards replacing the use of toxic substances by natural, environmentally friendly products (Ali *et al.*, 2015).

Grape canes represent a large source of waste derived from the viticulture industry, with an estimated volume of 1−5 tons hectare year depending upon plantation density, climate, and vigor of the grape variety. Most of the solid waste material, such as grape cane waste, generated from the viticultural and winemaking processes are usually composted or burned every year. Grape canes constitutively accumulate high concentrations of stilbenes, a family of phenolic secondary metabolites, including resveratrol and its derivates, involved in plant responses to various biotic and abiotic stresses (Houillé *et al.*, 2015; Chong *et al.*, 2009). Cane extracts of various polarity as well as a series of pure compounds contained in the grape cane extracts, above all resveratrol and viniferin, exhibited significant antifungal activities against the major fungal pathogens affecting grapevines, *Plasmopara viticola, Erysiphe necator* and *Botrytis cinerea* (Schnee *et al.*, 2013; Pezet *et al.*, 2004; Schnee *et al.*, 2008; Adrian and Jeandet, 2012). However, the antimicrobial activity of cane extracts was mainly assayed toward in vitro tests, measuring spore germination and sporulation (Schnee *et al.*, 2013; Gabaston *et al.*, 2017). The potential of cane extract and that of stilbene compounds to be used as fungicides by exogenous application on plants has only been reported in few studies. Resveratrol exogenously applied to apples inhibited the penetration of *Venturia inaequalis*, the causal agent of apple scab (Schulze *et al.*, 2005). Moreover, external application of resveratrol improved the postharvest quality of fruits (Montero *et al.*, 2003; Gonzalez Urena *et al.*, 2003). Recently, it was demonstrated that treatments of grapevine plants with stilbene extract, obtained from grape canes, were able

to offer a considerable protection against *P. viticola* infection (Richard *et al.*, 2016). The potential of grapevine cane extract and that of the main stilbene compounds, to induce elicitation on grapevine, with the activation of plant defense reactions, was scarcely investigated. Elicitors are molecules that trigger a cascade of signaling events that enables the activation of defense genes, the expression of which leads to defense reactions including phytoalexin production, pathogenesis related (PR) proteins synthesis, cell-wall strengthening, and in some cases the hypersensitive response (Thakur and Sohal, 2013).

In the present work it was explored whether exogenous application of stilbene extract (SE), obtained from grape canes, was able to induce protection toward *B. cinerea* on grapevine plants. Furthermore, it was verified if the acquired protection was derived from a direct antifungal activity of the added stilbenes or if there was an induction of grapevine defense mechanisms.

MATERIALS AND METHODS

SE extraction and characterization by HPLC analysis

Grape canes were obtained from plants of *Vitis vinifera* cv. Pinot noir cultivated on vineyards of the Oenological School of Conegliano, in the North-East Italy.

The stilbene extraction was performed according to the procedure described by Rayne *et al.* (2008) with some modifications. Briefly, the grape canes were ground with a coffee grinder (Imetec, Azzano San Paolo, BG, Italy). Three-stage extraction was performed by continuous stirring at room temperature using an 8:1 (v/w) 80% ethanol:sample ratio over a 60-min period for each extraction. During the first extraction, 250 µL of t-OH-stilbene 200 µg/mL in ethanol were added ad internal standard. The extracts were vacuum filtered at 1.6 µm on glass microfibre filter (GF/A, Whatman) and combined and the solvent removed by rotary evaporation (Büchi model R-114, Flawil, Switzerland), then stored at − 20 ºC for quantification. All analyses were performed in triplicate.

The analysis of stilbenes was performed according to the procedure described by Vincenzi *et al.* (2013) with some modifications. Stilbenes were separated on a C18 Lichrospher column (4 mm x 250 mm, 5 µm, Agilent Technologies, Milano, Italy) at 40 °C, using an HPLC system (Waters Corporation, Milford, MA, USA) equipped with a Dual Band UV detector Waters 2487 (Waters Corporation, Milford, MA, USA). The mobile phase gradient will be 0.5% v/v formic acid in deionized water (solvent A) and 2% v/v formic acid in methanol (solvent B). The gradient program will be 0 to 10% (solvent B) in 3 min, followed by 10 to 30% (solvent B) in 5 min, 30 to 44% (solvent B) in 35 min, 44 to 55% (solvent B) in

2 min, 55 to 75% (solvent B) in 15 min and 75 to 100% (solvent B) in 1 min. After washing for 2 min with solvent B, the column was re-equilibrated with solvent A. The flow rate will be 1.0 mL/min and injection volume 20 µL. Detection was performed at 306 nm for *trans*-isomers of resveratrol, trans-ε-viniferin and piceatannol. The concentration of individual stilbenes was quantified on the basis of peak areas using calibration curves of commercially available standards of *trans*-resveratrol, trans-ε-viniferin and *trans*-piceatannol, and correcting the value for the internal standard recovery. All the stilbene standards were obtained from Extrasynthese (Genay Cedex, France). Data were analyzed by the Waters BreezeTM Chromatography Software (Version 3.30). The limits of detection (LOD) and quantification (LOQ) were performed according to the procedure described by Shrivastava and Gupta (2011).

Grapevine material

The plantlets of grapevine *Vitis vinifera* L. cv. Merlot Ampelos TEA 20, susceptible to *B. cinerea*, were placed in individual pots (20 cm × 30 cm) containing a mixture of blond peat and perlite (3:2, v/v). They were grown in a glasshouse at a temperature of 24 and 18 ºC (day and night, respectively) with a photoperiod of 12 h of light and at a relative humidity (RH) of 70 ± 10 % until they developed twenty leaves. Plants were sub-irrigated every two days with water.

Grapevine (*Vitis vinifera* L. cv. Gamay) cell suspensions were cultivated in Nitsch-Nitsch medium as previously described by Krzyzaniak *et al.* (2018). Seven-day-old cultures were diluted twice in Nitsch-Nitsch medium 24 h prior to use for experiments. For ROS and MAPK analyses, cells were washed twice with the equilibration buffer M10 (10 mM MES, 175 mM mannitol, 0.5 mM K_2SO_4, 0.5 mM $CaCl_2$; pH 5.3), then re-suspended at 0.1 g fresh weight of cells (FWC) per mL in M10 and equilibrated for 2 h under light (25°C, 125 rpm), before treatments. For phytoalexins analyses, cells were adjusted at 0.1 g FWC/ mL in Nitsch-Nitsch medium before treatments.

Biological assays

The *Botrytis cinerea* was isolated from *Brassica oleracea* (kindly provided by Simone Ferrari, Sapienza University of Rome, Italy) and cultured as described by Ferrari *et al.* (2003). The fungi were grown and maintained on malt extract agar (Amresco, Solon, Ohio, USA) in 90 mm Petri dishes at 21 °C under a photoperiod of 12 h light. Conidia were harvested from 15- to 20-day-old cultures and collected by rubbing the plates with a glass

rod with 10 mL of sterile distilled water, filtered on sterile gauze to remove mycelia and conidia were pelleted by centrifugation at 5,000 × g (5 min) and resuspended in 5 mL of sterile distilled water at a final concentration of 1 × 10^6 conidia/mL, using a Malassez counting chamber.

In vitro antifungal assays were carried out adding SE 30 µM (based on the resveratrol concentration) in 90 mm Petri dishes containing potato dextrose agar (PDA) and the same conditions were used for the controls with water or ethanol solution 50%. *B. cinerea* mycelium was inoculated on the center of plate and maintained at 21 °C under a photoperiod of 12 h light. The mycelial development was observed and measured until full plate coverage.

Assays to evaluate the presence of antifungal effects of stilbene extract were firstly carried out on detached leaves incubated in Petri dishes (*in vitro* assays) and then on the whole grapevines (*in vivo* assays).

For *in vitro* assays grapevine leaves excised from plants (12 to 16-week-old) were washed with water, surface-sterilized by immersion in 3% sodium hypochlorite for 4 min, and then rinsed twice in distilled water for 1 min (Danti *et al.*, 2002). Then, the leaves were placed in Petri dishes, the adaxial side facing wet adsorbent paper (Whatman). Treatments were performed by spraying leaves with a solution of stilbene extract as a final resveratrol concentration of 440µM (100 µg/mL), and *B. cinerea* infection were achieved after 24 hours. Depending on size of the leaves, one to four lesions were applied to each leaf, and the fresh wounds were covered with 5 µL drops of the conidial suspension of 1 × 10^6 conidia/mL in 39 g/mL potato dextrose broth (Carlo Erba, Rodano, Milan, Italy). The plates were incubated at 24 ºC with a 12 h photoperiod. Humidity was maintained by covering the plates with a transparent plastic lid.

For whole plant infections (*in vivo* assay), twenty leaves per plants were inoculated after 24 hours from treatments, as described above, with 10 µL drops of the conidial suspension (1 ×10^6 conidia/mL). The plants were covered with transparent plastic bag and incubated at 24 ºC with a 12 h photoperiod.

Disease development was measured as average diameter of lesions formed from one to ten days post infection (dpi).

To assess plant protection level induced by SE-treatment, the biological assays were repeated with the same procedures but with lower *B. cinerea* inoculum (2 µL drops of the conidial suspension of 1 × 10^5 conidia/mL) to obtain a slower evolution of fungal development.

H_2O_2 production measurement

In grapevine cell suspensions, H_2O_2 production was detected using the chemiluminescence of luminol, as described in Dubreuil-Maurizi *et al.* (2010).

Immunodetection of phosphorylated MAPKs

Twenty µg of protein per sample were solubilised in Laemmli buffer (Laemmli, 1970), submitted to 12% SDS-PAGE before Western blotting. After transfer, the nitrocellulose membrane (Hybond ECL, Amersham Biosciences, Munchen, Germany) was pre-incubated first during 1 h at room temperature with TBST buffer (10 mM Tris-HCl, 150 mM NaCl, 0.05% Tween-20, pH 7.5) and 2% BSA, then incubated for 1h with an anti-phospho Thr202/Tyr204 peptide of human ERK1/2 mouse antibody, (Cell Signaling, Danvers, MA, USA), 1/20000 diluted in TBST buffer. After 3 washes with TBST buffer, probing and detection were performed by an ECL detection kit (Perkin Elmer, Little Chalfont, UK).

Phytoalexin quantification on cell suspensions

Treatments of cells were performed by adding of 87 µL of SE or ethanol solution 50% to 20 mL of cell suspension to obtain the final concentration of 100 µL/mL. As controls, 200 µL of oligogalacturonide (OG), provided by Elicityl OligoTech (Crolles, France), or 200 µL of ultrapure water were added to 20 mL of cell suspension. A volume of 1.5 mL of cell suspension was collected at 0, 1, 3, 6, 9 and 24 hours. Cells were separated from the medium by filtration under vacuum through GF/A filters (Whatman), and they were frozen at – 80 ºC. Three biological replicates were performed.

Analysis of stilbenes in cell suspensions was performed as previously described (Krzyzaniak *et al.* 2018). The ground frozen samples were resuspended in 1.0 mL ethanol 80% (HPLC grade) and extracted at 4 °C. Phytoalexins were analyzed by RP-HPLC using a Beckman System Gold chromatography system equipped with a diode array detector Model 168 and a Beckman 507 sample injector equipped with a 20 µL sample loop. Phenolics were separated on a Kinetex C18 column (4.6 x 100 mm, 2.6 µm, Phenomenex) using a ow rate of 1 mL/min, and a mixture of solvent A (1.5 ml/L phosphoric acid in MilliQ water) and solvent B (100% acetonitrile) as mobile phase. Phytoalexins were eluted with a linear gradient from 0 to 40% solvent B. Retention times were 11.37 min for *trans*-piceid, 11.65 min for *trans*-piceatannol, 13.58 min for *trans*-resveratrol, 17.62 min for *trans*-ε-viniferin and 19.32 min for *trans*-Δ-viniferin. Quantification was performed with standard calibration curves using peak areas at 310 nm.

Gene expression studies: sampling, RNA extraction and quantitative RT-qPCR

The expression of selected defense-related genes was investigated on leaves collected from grapevine plants. The experimental design included four conditions: treated and infected leaves (SE+Bc), treated and uninfected leaves (SE), untreated and infected leaves (positive control) and untreated and uninfected leaves (negative control). Leaves were collected before the treatment and one-day post treatment which correspond to inoculation day. Moreover, three samplings were carried out after *B. cinerea* inoculation (2, 5 and 8 dpi). Three independent biological replicates were collected from each experimental condition and each sampling time point, by excising 10 mm disks from four different leaves. After the collection, the leaves were immediately frozen in liquid nitrogen and stored at – 80 ºC.

The frozen samples (100 mg) were homogenized in liquid nitrogen and total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen) with a protocol described by (MacKenzie *et al.*, 1997). One mg of RNA was treated with 1 U of RNase-free DNase I (Invitrogen) for 45 min at 37 ºC and the reaction was stopped with 1 µl of 25 mM EDTA. After denaturation at 95 ºC for 5 min, RNA was reverse transcribed at 42 ºC for 50 min with Moloney Murine Leukemia Virus reverse transcriptase (Invitrogen) and DNA random primers (Roche Diagnostic) (Bertazzon *et al.*, 2012).

Real-time PCR assays were carried out on a Bio-Rad thermal cycler (model CFX 96) in 96-well plates using the 2× Platinum SYBR Green qPCR Supermix UDG (Invitrogen). The PCR reactions were performed at least in duplicate, in a total volume of 10 µL, including 0.3 mM of each primer and 1 µL of cDNA. The thermal protocol included a decontamination step of 3 min at 50 ºC to allow for optimal UDG (Uracyl DNA Glycosylase) enzymatic activity, followed by a step of 3 min at 95 ºC in order to activate the Platinum Taq polymerase, to deactivate the UDG and to denature the DNA sample. Subsequently, 50 cycles of a two-step protocol, consisting of 5 s of denaturation at 95 ºC followed by 30 s of annealing/extension at 60 ºC, were performed. Identical thermal cycling conditions were used for all the targets.

For the selection of reference genes, a set of five *V. vinifera* candidate reference genes (*actin, cytochrome oxidase, pyruvate decarboxylase, glyceraldehyde-3-phosphate dehydrogenase* and *26S rRNA*) was tested in the experimental conditions (Supplementary table 1). The qbasePLUS software (Biogazelle) was used identifying *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) and *cytochrome oxidase* (*COX*) as the two most stably-expressed genes.

The expression of five categories of defense and stress-related genes were monitored. Several genes involved in the hormone-mediated signaling were considered: *Non expressor of PR1* (*NPR1*) and *Enhanced disease susceptibility 1* (*EDS1*) important activators of the salicylic acid signaling; genes encoding JAZ1.1, 9-lipoxygenase (LOX9) and 13-lipoxygenase (LOX13) involved in the jasmonic acid-mediated signaling; *1-aminocyclopropane-1-carboxylate oxidase 1-like* (*ACO*) involved in ethylene biosynthesis. Moreover, *glutathione-S-transferase* (*GST1*), a gene involved in redox status, and the transcription factor WRKY1 were analyzed. The expression level of five genes coding for PR-protein was evaluated: *PR1*, *PR5* (*Thaumatin-like*/*Osmotin*), *PR6* (*Proteinase inhibitor*), *PR10* (*Ribonuclease-like*) and *PR14* (*Lipid transfer protein*). Finally, two genes were selected from the phenylpropanoid pathway: *PAL (phenylalanine ammonia-lyase)* and *STS1* (*stilbenes synthase 1*), both responsible for phytoalexin synthesis. The gene specific primers were selected from the literature (Supplementary table 1).

Statistical analysis

The results were evaluated by one-way analysis of variance (ANOVA), and the mean values were analyzed by Tukey's test using the software STATISTICA® 12.0 (StatSoft Inc, Tolson, USA).

RESULTS AND DISCUSSION

SE induced protection of grapevine leaves against *Botrytis cinerea*

SE obtained from grape canes, whose content was 4.52 mg/mL of *trans*-piceatannol, 23.01 mg/mL of *trans*-resveratrol and 5.23 mg/mL of *trans*-ε-viniferin, was used for treatments of grapevine plants. The antifungal effect of SE on grapevine was investigated by its application before or after *B. cinerea* inoculation. Initially, the spreading of necrotic lesions was tested on grapevine leaves that were treated with SE 24 h before inoculation with *B. cinerea*, both *in vitro* (detached leaves incubated in Petri dishes) and *in vivo* (on plants). A first trial was performed on leaves at high doses of SE (0.3 mg/mL of resveratrol) and symptoms of phytotoxicity were never observed after treatment. Generally, for *in vivo* assays the evolution of *B. cinerea* infection was slower when compared to those observed on detached leaves, albeit a similar protection induced by SE treatment was detected in both trials. A significant reduction of lesion diameters was observed already after two days post *B. cinerea* inoculation in treated leaves in comparison to untreated ones (Figure 1). The higher protection induced by treatment with SE, was reached at 10 dpi with reductions of

lesion diameter, respect to the positive control, of 50% and 48% for *in vitro* and *in vivo* assays, respectively. Moreover, the curative activity of SE, i.e. the capacity to reduce the infection when the fungus has already attacked the host, was investigated by treating detached leaves or whole grapevines 24 h after *B. cinerea* inoculation. In both experiments the treatment with SE showed to be ineffective, without any significative reduction of lesion diameter (Figure 2).

For our knowledge this is the first report on the use of cane extract as preventive treatment on grapevine leaves against *B. cinerea* infection. Interestingly, only one treatment with a concentration of SE much lower than that used for previously reported antifungal assay against *B. cinerea* (5 mg/mL from Schnee *et al.*, 2013), prompted a satisfactory protection against grey mold attacks in controlled conditions.

SE showed direct antifungal effect against *Botrytis cinerea*

The direct antifungal activity of SE against *B. cinerea* was screened by monitoring the mycelium growth on nutrient agar medium. The development of *B. cinerea* mycelium started at 2 dpi on the controls and at 7dpi after the SE treatment. In addition, the SE treatment slowed down the growth of *B. cinerea* mycelium, which covered the plate after 26 dpi, while on the controls, the mycelium covered the plate in only 9 dpi (Figure 3). A further assay was performed adding different concentrations of SE into the PDA medium (from 0 to 200 µM, based on the resveratrol concentration, four replicates for each concentration). The IC_{50} (considered as the concentration able to halve the mycelium growth with respect to the control) after 96 h of incubation was 150 µM, corresponding to about 35 µg/mL of resveratrol.

The antifungal effect of different purified stilbenes was previously described against *B. cinerea* infection in agar Petri dishes (Adrian *et al.*,1997; Adrian *et al.*,1998; Schnee *et al.*, 2013). Among them, Schnee *et al.* (2013) also demonstrated fungitoxic activity of Pinot noir canes extract against *Plasmopara viticola, Erysiphe necator* and *B. cinerea.* Adrian and colleagues (1997) found an effect on the hyphal mean length of *B. cinerea* only with concentrations higher or equal to 60 µg/mL of resveratrol. However, it must take in account that, in addition to resveratrol, the SE used for the inhibition assays in the present work contained also viniferin and piceatannol, which could have a synergistic effect against the fungal development.

Early signaling events induced by SE in grapevine cell suspensions

To determine whether SE perception by grapevine triggers some defense responses, the early signaling events induced by SE in *V. vinifera* cell suspensions were firstly characterized.

One of the most peculiar events in the early stages upon elicitor application is H_2O_2 formation that has been reported to exert various effects on plant defense response, including the reinforcement of the cell wall, hypersensitive response, activation of defense genes, and induction of defensive compounds (Torres, 2010). Timely production of H_2O_2 monitored after treatment with SE showed that SE did not induce any significative oxidative burst (data not shown).

Mitogen-activated protein kinase (MAPK) cascades are implicated in signaling defense responses of plants as major components downstream of receptors or sensors that transduce extracellular stimuli into intracellular responses (Zhang *et al.*, 2001). A rapid activation of MAPKs has been described for different plant system in response to elicitors (Gomez-Gomez and Boller, 2002; Lebrun-Garcia *et al.*, 1998, Romeis *et al.*, 1999; Zhang *et al.*, 1998). The time course of activation of MAPKs was analyzed in control (ethanol) and SE-treated grapevine cells. From 5 to 45 min, SE induced rapid and transient phosphorylation of a MAPK with relative molecular masses of 49 kDa, which was not observed in control cells. Moreover, a transient activation of another MAPK with relative molecular masses of 45 kDa was detected after 15 min (Figure 4).

Production of ROS and activation of MAPKs are two of the earliest responses in plants under biotic or abiotic stresses. In the present paper, it was shown that SE treatment on grapevine cells was able to activate MAPKs cascade without the induction of oxidative burst.

However, the H_2O_2 burst was found not to require MAPK activation in several studies and it was also reported that fungal-induced MAPK activation is not dependent on the H_2O_2 burst, suggesting that H_2O_2 itself acts as a stress, rather than as a signal for other stresses to activate MAPKs (Romeis *et al.*, 1999; Ligterink *et al.*, 1997; Zhang *et al.*, 2001).

Phytoalexin production in SE-treated grapevine cells

Grapevine cell cultures respond to pathogens and elicitors by synthesizing and accumulating stilbenoid phytoalexins, mainly resveratrol and its dimer viniferin. The release of resveratrol in the culture medium after SE-treatment was firstly monitored and compared with that observed after treatment with OG, well known elicitor of grapevine defense, and

that accumulated after ethanol treatment, solvent in SE (Figure 5a). Resveratrol accumulated in higher amount in the medium of SE-treated than in that of OG-treated cells. However, very high values were detected immediately after the addition of SE (510 ± 45 µg/g, followed by a progressively decreasing of the resveratrol amount until it almost disappeared after 24 h (16.69 ± 14.25 µg/g). By contrast accumulation of resveratrol in the medium of OG-treated cells was transient and it peaked among 6 and 9 h after treatment (62.38 ± 24 and 63.79 ± 28.46 µg/g at 6 and 9 h, respectively). Surprisingly piceatannol was only detectable in the medium of SE-treated cells immediately after the treatment (86.22 µg/g) and completely disappeared after 1 h, whereas ε-viniferin concentration progressively decreased until 24 h (from 52.02 µg/g at To, to 3.29 µg/g after 24 h). We therefore failed to detect any stilbene accumulation in the medium of SE-treated cells, which could have been indicative of an induction of stilbene biosynthesis triggered by SE.

Accumulation of stilbenes was also monitored inside the cells (Figure 5b). Resveratrol, ε-viniferin and piceatannol were detected in SE-treated cell from 1 h after treatment with levels that slowly increased to reach a maximum of approximately 17.88 ± 9.0, 13.07 ± 6.01 and 9.09 ± 5.28 µg/g at 9 h. After 24 h from the treatment only ε-viniferin could be detected in higher level (8.60 ± 5.13 µg/g), while the amounts of resveratrol and piceatannol dramatically decreased. OG-treatment induced stilbene production with different time courses, type of molecules and levels of accumulation. Piceid and Δ-viniferin, in addition to resveratrol and ε-viniferin, were induced in OG-treated cell, without any trace of piceatannol. As for SE-treated cells, the higher accumulation of resveratrol was observed after 9 h from the treatment (3.0 ± 0.88 µg/g), but, unlike SE-treated cells, the levels of all the detected stilbenes remained at higher level at 24 h (1.94 ± 0.23, 1.18 ± 0.52, 1.80 ± 0.75, 0.62 ± 0.04 µg/g for piceid, resveratrol, ε-viniferin and Δ-viniferin, respectively).

Several experiments in grapevine cell cultures have shown that cells synthesize and accumulate resveratrol in the extracellular space, in response to several elicitors (Calderon *et al.*, 1993; Belhadj *et al.*, 2008; Tassoni *et al.*, 2005; Ferri *et al.*, 2009, Zamboni *et al.*, 2006; Almagro *et al.*, 2015). By contrast, piceids and ε-viniferin were generally accumulate after elicitor-treatment in higher concentration inside the cells (Martinez-Marquez *et al.*, 2017; Adrian *et al.*, 2012). In the present work, after SE-treatment, only resveratrol, ε-viniferin and piceatannol, which were the stilbene molecules contained in SE, were detected in the culture medium with a proportion between the three molecules reassembling that calculated for the SE used for the treatment. Furthermore, the same molecules were detected inside the cell from 1 h after the treatment. Unlike OG-treatment, neither piceid nor

Δ-viniferin were detected in SE-treated cells. Moreover, the time course of resveratrol, e-viniferin and piceatannol accumulation after SE-treatment showed that there was a general decreasing until 24 h, except for e-viniferin whose level remain relatively higher inside the cells. Several reports have shown that after treatment with different elicitors of the grapevine defense response the level of stilbenes increased after the treatment, with a maximum pick within 24 h followed by a decreasing (Aziz *et al.*, 2003; Belhdj *et al.*, 2008; Saigne-Soulard *et al.*, 2015). Collectively, these data lead to exclude the activation of the stilbene biosynthesis induced by SE treatment. However, the three stilbenes, added with the treatment, were clearly detectable in the cell fraction, indicating a transport of these molecules from the extracellular medium to the cells, although the detected levels represented only a very small fraction of the initial amount added to the medium with the treatment. After SE-treatment it was difficult to know whether stilbenes detected by HPLC were really inside the cells or simply linked to the cell-wall. Indeed, it was reported that resveratrol or derivates can be linked to the cell wall by a peroxidase-mediated cross-linking with cell-wall constituents, thus participating to the cell wall strengthening (Calderon *et al.*, 1990; Adrian *et al.*, 2012). The permanence of ε-viniferin inside SE-treated cells after 24 h suggested the possible oxidation of a portion of resveratrol. Resveratrol does not possess a high antimicrobial activity (Adrian *et al.*, 1997) but it is the precursor of more active derivates, as viniferin (Pezet *et al.*, 2004). Therefore, these data suggest that SE-treatment did not induce any new stilbene production, but that the added stilbene molecules were partially transported inside grapevine cells, remaining in part as active antifungal compounds (viniferin), or being cross-linked to the cell wall therefore contributing to its reinforcement.

Defense-related gene expression in SE-treated grapevines

Plant response to several biotic and abiotic stimuli, included elicitors, involves the modulation of the expression of many defense-related genes. In the present work, the expression profile of typical grapevine defense marker genes was followed on SE-treated grapevine plants. Moreover, it was followed the transcriptional response of defense-related genes in pretreated leaves after *B. cinerea* challenge.

The expression of genes involved in the metabolic pathways leading to the production of antimicrobial molecules, such as PR proteins and stilbene phytoalexins, was investigated. It was observed that the treatment with SE caused the modulation of genes coding for PR proteins. In detail, *PR5*, a thaumatine-like, and *PR6*, a serine protease inhibitor, were markedly expressed at 1 dpt, contrariwise *PR1* resulted downregulated at the same time

point (Figure 6). From 3 dpt the expression of the investigated *PR* genes decreased and after 9 dpt most of them were downregulated compared with controls. Contrarywise, the expression of *PAL* and *STS*, the main genes involved in the biosynthesis of phenylpropanoid, didn't show any significant alteration after SE treatment**.** The transcriptional regulation of a gene encoding glutathione S-transferase GST1 was also monitored. Interestingly this gene was clearly upregulated after SE treatment, showing a transient mRNA accumulation at 1 dpt. For most of the time point, the expression of genes involved in signaling pathways mediated by hormones (*NPR1, EDS1, JAZ1.1, LOX9, LOX 13* and *ACO*) and that of a gene coding for WRKY1 transcription factor did not show any consistent difference between untreated and SE-treated leaves (Figure 6).

To further investigate the mechanisms induced by SE treatment on grapevine, the transcript level of the above reported set of defense marker genes was investigated after *B. cinerea* infection. The analysis was performed over the first 8 dpi, when *B. cinerea* displayed a comparable development in control an SE-treated leaves. Most of the genes involved in the production of phytoalexins and PR proteins showed a lower expression in the SE-treated leaves compared with untreated grapevines (Figure 7a, 7b). The higher expression of *PAL* and *STS*, observed after *B. cinerea* infection on untreated plants, was confirmed for all the timing point analyzed (Figure 8a, 8b). Moreover, a marked upregulation on untreated plants, in comparison to SE-treated plants, was observed for *PR6* at 2 dpi. A strong increase in *GST1* expression, evident on untreated plants, was observed in the early days after *B. cinerea* infection, after which the expression returned at the same level of the control plants (Figure 8c).

Collectively, data reported in the present work showed that SE-treatment triggers slight induction of some *PR* genes, though the late beginning of sampling probably led to losing information about the signaling pathways activated after the treatment. The observed upregulation of some defense-related genes, above all *GST1*, could explain the protection acquired by grapevine leaves treated by stilbenes extract against *B. cinerea* attacks. GSTs participate in the detoxification processes by conjugating glutathione to electrophilic compounds and, in addition, they act as non-enzymatic carrier proteins enabling intracellular shuttling of endogenous compounds (Conn *et al.*, 2008). It was showed that GSTs have a role in the transport and accumulation of phenylpropanoid compounds into the vacuole (Tavares *et al.*, 2013). Gruau and collaborators (2015) reported a high upregulation of *GST1* associated with an enhanced phytoalexin accumulation during the local and systemic immune response triggered by *Pseudomonas fluorescens* PTA-CT1 against *B. cinerea* in

grapevine. The upregulation of *GST1* observed in *A. thaliana*, that was associated to the enhanced phytoalexin camalexine accumulation and to wound-induced resistance against *B. cinerea,* seemed to be necessary to prevent damages related to phytoalexin toxicity at high concentration (Chassot *et al.*, 2008). In the present work, the increased level of *GST1* expression did not correlate with any accumulations of endogenous resveratrol but it can be hypothesized that this is triggered by the increasing of exogenous stilbenes provided by the treatment. Recently, it was reported that some particular GST isoforms, classified as multidrug resistance-associated proteins (MRP), were involved in the resveratrol transport out of the grapevine cells (Martinez-Marquez *et al.*, 2017).

Despite SE-treatment upregulated only few defense-related genes, most of the monitored genes were downregulated after challenge with *B. cinerea*. The transcription of *PAL*, *STS1* and *GST1*, that was clearly enhanced after *B. cinerea* attacks, was downregulated in SE-treated plants.

CONCLUSION

Collectively, it could be summarizing that SE treatment was perceived by cells and leaves, induced some defense events, such as activation of MAPKs and higher expression of some PR genes and *GST1*, but negatively regulated new stilbene production. This result suggests the activation of an immune-ready state on SE-treated plants. In plant defense, the physiological process by which a plant prepares to more quickly or aggressively respond to future biotic or abiotic stress was known as priming. The “primed” state has been related to increased, more efficient activation of the defense response and enhanced resistance to challenging stress (Conrath, 2009). It was reported that this increasing alertness could correlate with no or minimal gene induction, and that it results from the improved perception and/or amplification of defense response- inducing signals, rather than from the direct activation of these defense responses (Slaughter *et al.*, 2012).

To our knowledge, this is the first study that investigated at transcriptional and metabolic levels defense mechanisms induced by treatment with stilbenes on grapevine.

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FIGURES

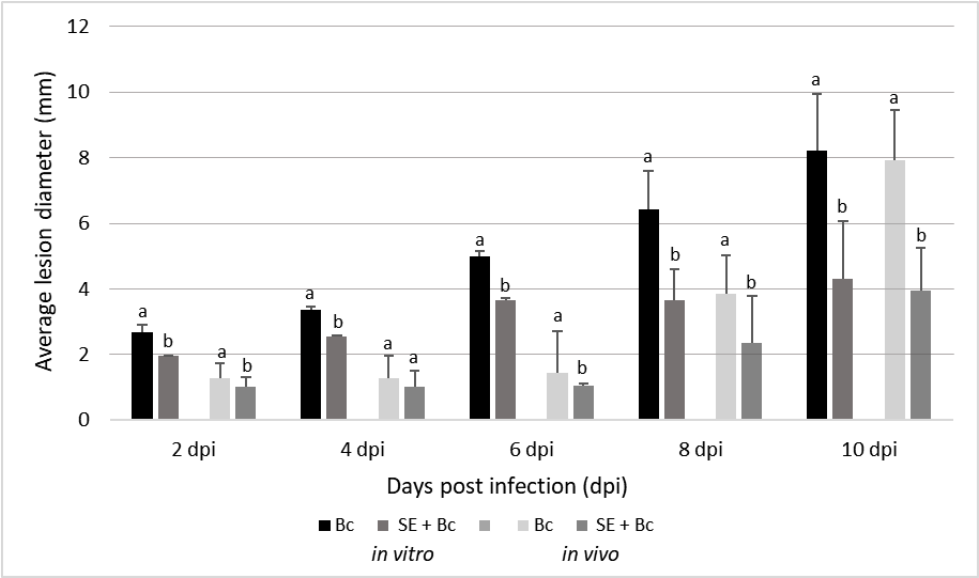

Figure 1. Results of preventive SE-application. Average lesion diameter of *Botrytis cinerea* infection measured on untreated leaves (Bc) and on leaves treated with SE 24 h before *B. cinerea* inoculation (SE + Bc) at different days post infection (dpi) observed in the *in vitro* and *in vivo* assays. Different letters present significant difference $p < 0.05$ by Tukey's test.

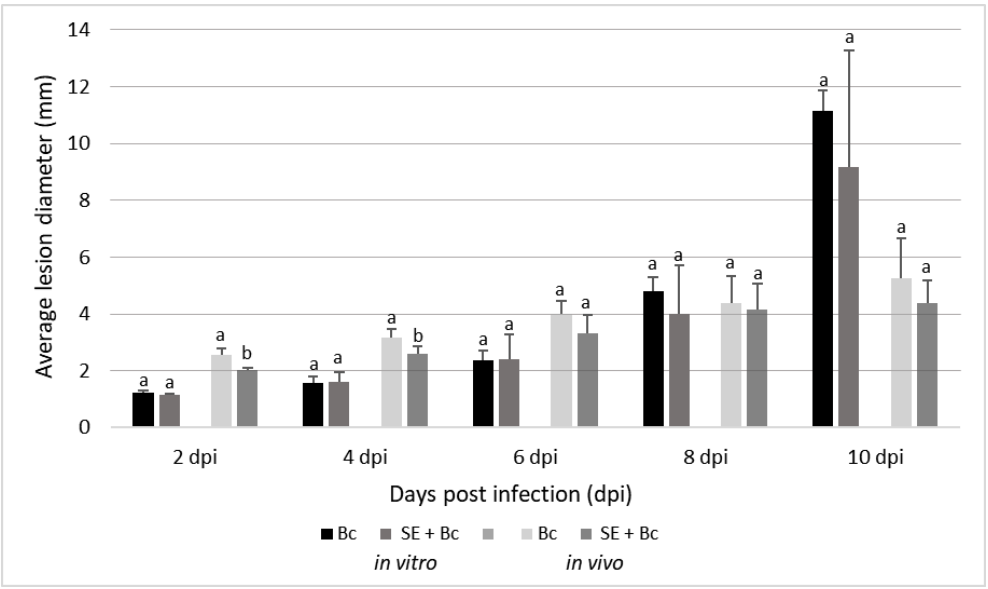

Figure 2. Results of curative SE-application. Average lesion diameter of *Botrytis cinerea* infection measured on untreated leaves (Bc) and on leaves treated with SE 24 h after *B. cinerea* inoculation (SE + Bc) at different days post infection (dpi) observed in the *in vitro* and *in vivo* assays. Different letters present significant difference $p < 0.05$ by Tukey's test.

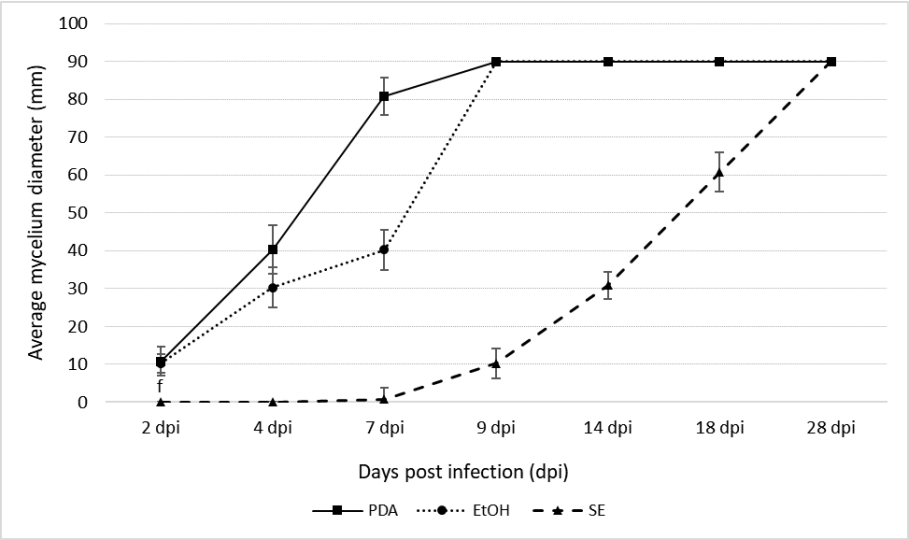

Figure 3. Average mycelium diameter (mm) of *Botrytis cinerea* infection on PDA medium (PDA), on PDA medium added with ethanol (EtOH) and on PDA medium added with SE (SE) measured from two days post inoculation. Results are expressed as means ± standard deviation.

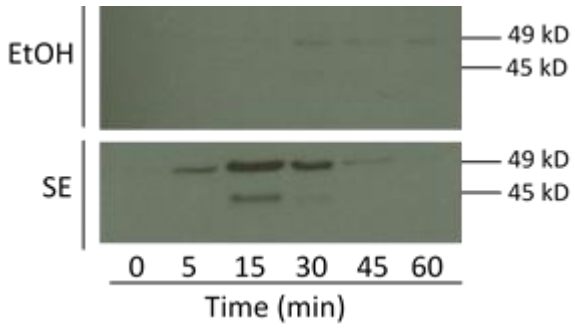

Figure 4. Activation kinetics of two mitogen-activated protein kinases (MAPKs) detected by Western blot with a human phosphorylated extracellular regulated protein kinase 1/2 (α-pERK1/2), monitored on SE-treated cells (SE) and ethanol-treated cells as a control (EtOH).

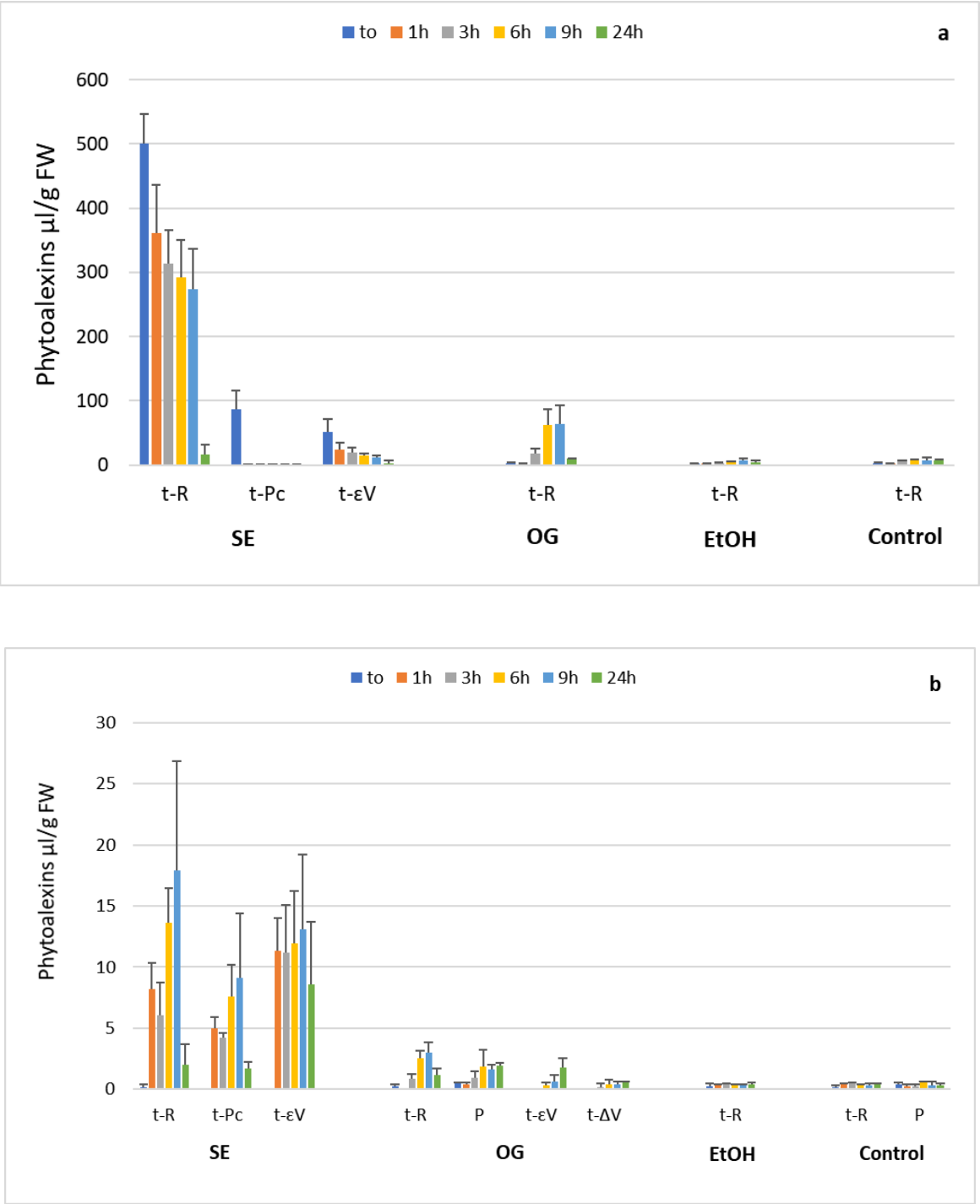

Figure 5. Stilbenoid accumulation by the grapevine cell culture in the presence of SE, oligogalacturonide (OG), ethanol (EtOH) and in the control. **a**) Extracellular accumulation; **b**) Intracellular accumulation. Levels of *trans*-resveratrol (t-R), *trans*-piceatannol (t-Pc), ε-viniferin (t-εV), Δ-viniferin (tΔ-V) and piceid (P) are indicated. Results are expressed as means ± standard deviation.

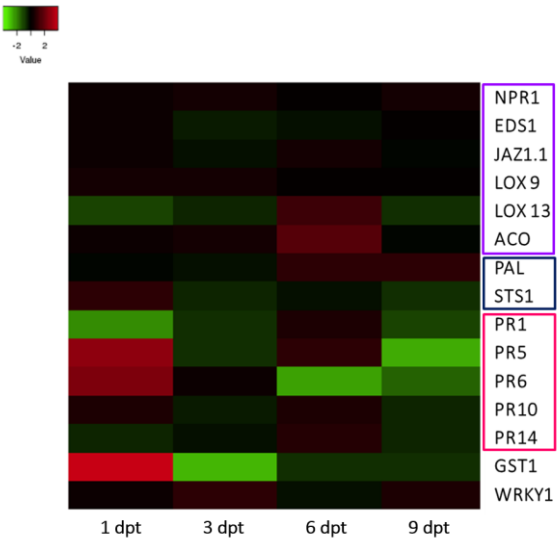

Figure 6. Transcript levels of defense-related genes in leaves induced by treatment with grapevine plants. Each column represents the time in days post treatment and each row represents one gene. A tree color scale was used to show fold induction of each gene (log transformed). The fold induction values were normalized to the reference genes *GAPDH* and *COX* and to untreated leaves as the control samples.

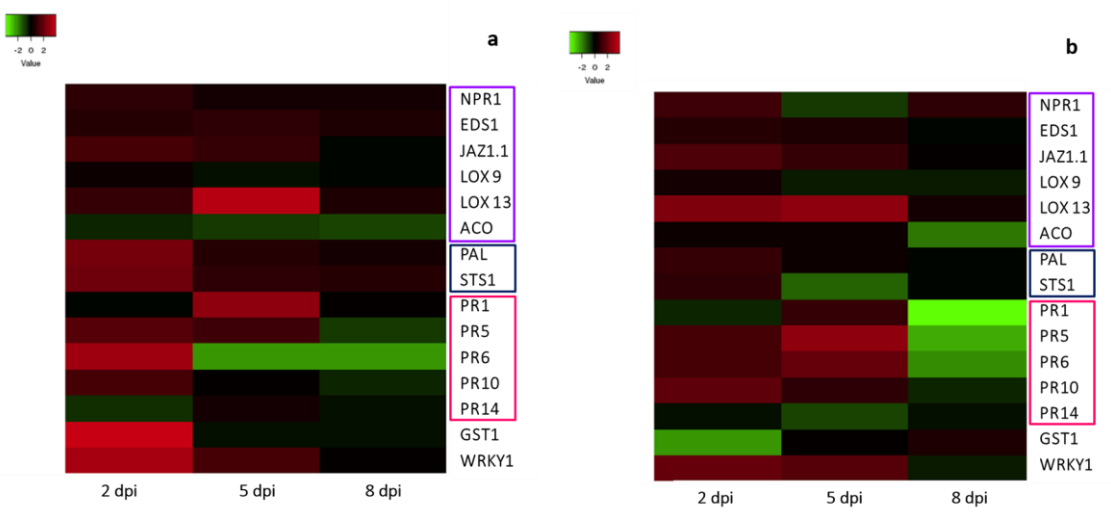

Figure 7. Transcript levels of defense-related genes induced by *Botrytis cinerea* infection on untreated plants **(a)** and on plants pretreated with SE **(b)**. The fold induction values were normalized to the reference genes *GAPDH* and *COX* and to untreated leaves as the control samples.

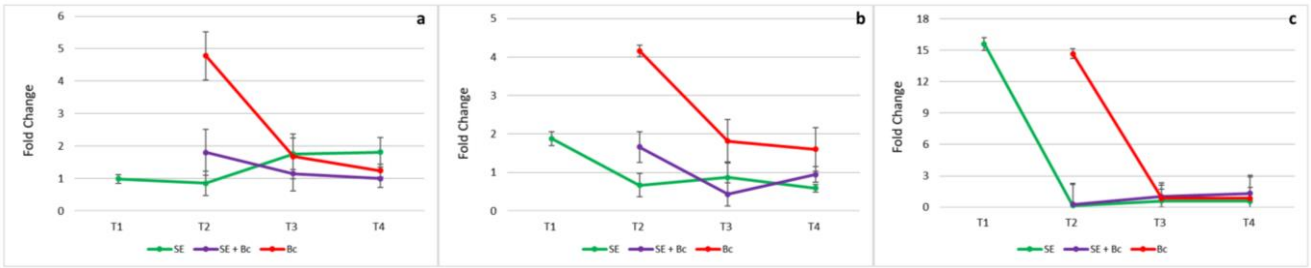

Figure 8. Transcriptomic profiles of *PAL* **(a)**, *STS1* **(b)** and *GST1* **(c)** observed after SE treatment and induced by *Botrytis cinerea* infection on untreated and SE treated plants for four timing point (T1: 1 dpt, T2: 3 dpt and 2 dpi, T3: 6 dpt and 5 dpi, T4: 9 dpt and 8 dpi).

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SUPLEMENTARY TABLE 1

Table 1. Primer pairs used for quantitative RT-qPCR analysis

Pathway	Target	Forward primer	Reverse primer	Reference
Salicylic acid	EDS1	ATATCGCCGCATAGTTGA	CGTAGGTTCCTGTGTCTT	Liu *et al.*, 2016
	NPR1	GGCGGTTTTGGGGTATTTGT	AGAGCACCTCCACCATGAAA	Shangguan *et al.*, 2017
Jasmonic acid	JAZ1.1	CCTCCACAGGTTCTTGGAG	GGTTTGTGAGGACCTGCAGG	Marchive *et al.*, 2013
	LOX-9	GACAAGAAGGACGAGCCTTG	CATAAGGGTACTGCCCGAAA	Dufour *et al.*, 2016
	LOX-13	ACTGCCCCGACCTTCTTC	AGCCAACCCTAACATTCCTG	Pilati *et al.*, 2014
Ethylene	ACO	TCTTGGTTTGGAGAAGGGCT	CCCTAACAAGCTCAGGTCGA	Shangguan *et al.* 2017
Phenylpropanoid metabolism	PAL	AGGGGCATTCGAGGAGGAGTTG	ACCAGTCAGCAGCCCTGTTCC	Repetto *et al.*, 2012
	STS1	ATCGAAGATCACCCACCTTG	CTTAGCGGTTCGAAGGACAG	Dufour *et al.*, 2016
PR protein	PR1	CCCAGAACTCTCCACAGGAC	GCAGCTACAGTGTCGTTCCA	Dufour *et al.*, 2016
	PR5	GGAGGCAATGGTTTCCACCTTGGG	ACTTGGACGGGACCATAGAGGTTAG	Dufour *et al.*, 2016
	PR6	TGGGAAGCAGGCTTGGCCTGA	ACCTGGCTCTCACCGAAGGG	Dufour *et al.*, 2016
	PR10	GCTCAAAGTGGTGGCTTCTC	CTCTACATCGCCCTTGGTGT	Dufour *et al.*,2016
	PR14	ACAGTTGATCGCCAGGCCGC	GCCCGGAAGCCCACTTGCAA	Dufour *et al.*, 2016
Redox status	GST1	GGGATCTCAAAGGCAAAACA	AAAAGGGCTTGCGGAGTAAT	Dufour *et al.*, 2016
Transcription factor	WRKY1	GAAGCCCACCGAGAACTTTGAAC	ATATGGGTGTCCACCACTCTTTCC	Repetto *et al.*, 2012
Reference genes	ACT	ATGTGCCTGCCATGTATGTTGCC	AGCTGCTCTTTGCAGTTTCCAGC	Bézier *et al.*, 2002
	COX	CGTCGCATTCCAGATTATCCA	CAACTACGGATATATAAGAGCCAAAACTG	Bertazzon *et al.*, 2012
	PDC	GCTTGCGTCGTCACCTTCAC	TGCCGTAGTCGTTGGAGTTGG	Bertazzon *et al.*, 2012
	GAPDH	AATGAAGGACTGGAGAGGTGGAAG	CCGACACATCAACAGTAGGAACAC	Bertazzon *et al.*, 2012
	26S rRNA	TCCCACTGTCCCTGTCTACTATCC	TGGTATTTCACTTTCGCCGTTTCC	Bertazzon *et al.*, 2012

CHAPTER IV

Chitosan with filmogenic properties induce delayed grapevine defense mechanisms and protect grapevine against *Botrytis cinerea*

Chitosan with filmogenic properties induce delayed grapevine defense mechanisms and protect grapevine against *Botrytis cinerea*

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ABSTRACT

Chitosan is a highly investigated biopolymer with well-known antimicrobial properties, that are largely influenced by the molecular weight, the degree of acetylation (DA) as well as the derivatization and preparation methods used. In the present paper it was investigated the biological activity of a commercial chitosan soluble in acid solution, obtained from shrimp shell waste, with a molecular weight of 173 kDa and a degree of acetylation of 17%. Thanks to direct fungistatic activity and filmogenic properties chitosan (173/17) conferred a good level of protection for grapevine leaves against *B. cinerea.* Moreover, it induced grapevine defense response with some delay. From three days from the treatment there was an induction of the JA/ET-mediated response and a repression of the SA-mediated signaling, and a transient accumulation of *trans*-resveratrol.

Keywords: Chitosan, Elicitor, *Botrytis cinerea*, Grapevine, Defense response, Gene expression

INTRODUCTION

Grapevine (*Vitis vinifera* L.), one of the most economically important fruit species grown around the world, is susceptible to many fungal pathogens, such as *Botrytis cinerea* which induces gray mold disease at various developmental stages and on different plant organs (Mohamed *et al.*, 2007). Generally, the disease is controlled through spraying of phytochemicals. On the other hand, due to negative impact of phytochemicals on environment, quality of food and beverage products and on health human, there is a demand of society for alternatives based on natural methods of disease control.

Several alternatives to use of fungicides on grapevine has been reported in literature against *Botrytis cinerea*, such as seaweed (Jeandet *et al.*, 2000), laminarin (Aziz *et al.*, 2003), oligogalacturonides (Aziz *et al.*, 2004; De Miccolis Angelini *et al.*, 2009), plant extract of *Reynoutria sachalinensis* (Elmer and Reglinski 2006), rhamnolipids (Varnier *et al.*, 2009), *Saccharomyces* (Pujos *et al.*, 2014), and chitosan (Ait Barka *et al.*, 2004, Aziz *et al.*, 2006, Trotel-Aziz *et al.*, 2006, Reglinski *et al.*, 2010).

Chitosan is a natural non-toxic polymer of β-1,4-linked glucosamine obtained by deacetylation of chitin which is an important waste product from shrimps and crabs (crustaceans shell), mollusks (endoskeleton of cephalopods), fungi and algae cell walls and insects (exoskeleton). However, commercially chitosan is mainly recovered from marine sources, i.e., the crustaceans processing industries. In fact, more than 10,000 tons could be available every year from shellfish waste (Merzendorfer, 2011).

Its biological activity is related to the size of the polymer and to the degree of deacetylation (Kauss *et al.*, 1989; Hadwiger *et al.*, 1994). The *in vitro* activity of formulations containing putative low molecular weight chitosan (LMW), but without any specification on the exact molecular weight, as Armour-Zen® and Chitogel®, has been reported on grapevine plantlets and detached leaves against *B. cinerea* (Ait Barka *et al.*, 2004; Reglinski *et al.*, 2010). In detail, Aziz *et al.* (2006) and Trotel-Aziz *et al.* (2006) used chitosan with MW 1.5-10 kDa and 5 kDa, respectively, and both demonstrated an elevation of defense-related enzyme activity and induced resistance to infection by *B. cinerea*. They showed that chitosan elicits a variety of defense reactions in plants such as the stimulation of phenylalanine ammonia lyase (PAL), peroxidase, and lipoxygenase activities, as well as the accumulation of phytoalexins and PR proteins. Comparing samples of chitosan with different MW, Trotel-Aziz *et al.* (2006) showed also that the induction activity decreased with the polymer dimension, moving from 1.5 to 10 kDa. Regarding the direct antifungal effect of chitosan, these authors found contrasting results on *B. cinerea*, with only small inhibitory

effect until 300 mg/L in one case (Aziz et al, 2006) and a maximum inhibitory effect at 150 mg/L in the other case (Trotel-Aziz *et al.*, 2006). The deacetylation degree was similar in the two experiments, so this parameter could not be responsible for such a difference.

Few data are available on the use of chitosan with higher MW. In this case, it has to take in account that the increase of the polymer dimension affects also its solubility: chitosan with molecular weight below 20 kDa are still water-soluble (Qin *et al.*, 2002) while higher MW polymers need diluted acids to be dissolved, giving viscous solutions. This aspect can constitute a difficulty when formulating commercial preparations to be applied on plants but, on the other hand, the film-forming ability of viscous chitosan solutions could increase the protection effect against fungi by creating a physical barrier, as suggested by Ait Barka *et al.* (2004). Reddy *et al.* (2000) using a HCl-soluble chitosan found a protective effect against *B. cinerea* in strawberry, hypothesizing a direct fungistatic effect and suggesting (but without demonstrating) even an effect on the induction of defense response. Hernández-Muñoz *et al.* (2006) demonstrated the ability of HCl-soluble chitosan to inhibit the *in vitro* growth of *B. cinerea*, finding an IC_{50} of 1.77 g/L.

In the present study, a commercial chitosan soluble in acid solution, obtained from shrimp shell waste, was chemically characterized, and its antifungal activity against *B. cinerea* infections was investigated by conducting *in vitro* assays (conidia germination and mobility; mycelium growth). Furthermore, the ability of chitosan in protecting grapevine leaves from *B. cinerea* was demonstrated, and it was explored whether this protection is related to the activation of several defense mechanisms.

MATERIALS AND METHODS

Chemical characterization of chitosan

The commercial chitosan from shrimp shell was purchased from Qingdao Yunzhou Biochemistry Co., Ltd (Jimo, Qingdao, China).

The degree of deacetylation was determined according to the procedure described by Tolaimate *et al.* (2000) with some modifications. The chitosan (0.2 g) was dissolved in 20 mL of 0.1 M HCl solution and 25 mL of deionized water. After 30 min. of continuous stirring, a second portion of 25 mL of deionized water was added and stirring continued for other 30 min. When chitosan was completely dissolved, solution was titrated with NaOH 0.1 N solution using an automatic titrator (Hanna Instruments, model HI 901, Woonsocket, RI, USA) and a curve with two inflexion points were obtained. The difference of the volumes of

these two points correspond to the acid consumed for the salification of amine groups that was used to determine the degree of acetylation of the chitosan.

The intrinsic viscosity of chitosan was determinated according to the methodology of Mao *et al.* (2004). The chitosan (0.050 g) was dissolved in 100 mL of 2% HAc/0.2M NaAc, and the viscosity was measured using an Ubbelohde glass capillary viscometer, with a viscosity range 2.000 to 10.000 cSt (Fungilab, ASTM size 4, Sant Feliu del Llobregat, Barcelona) in a constant-temperature water bath at 25 ± 0.01 ºC in triplicate. The capillary diameter used was 0.63 mm. Solution concentrations were adjusted based on the viscosity of the samples and the flow through time was kept in the range of 100-150 s. Five different concentrations were tested, and the calculation of intrinsic viscosity was obtained by common intercept of both Huggins and Kraemer plots.

Botritys cinerea **culture**

The *B. cinerea* was isolated from *Brassica oleracea* (kind gift of Simone Ferrari, Sapienza University of Rome, Italy) and cultured as described by Ferrari *et al.* (2003). The fungi were grown and maintained on malt extract agar (Amresco, Solon, Ohio, USA) in 90 mm Petri dishes at 21 °C under a photoperiod of 12 h light. Conidia were harvested from 15- to 20-day-old cultures and collected by rubbing the plates with a glass rod with 10 mL of sterile distilled water, filtered on sterile gauze to remove mycelia and conidia were pelleted by centrifugation at 5,000 × g (5 min) and resuspended in 5 mL of sterile distilled water at a final concentration of 1×10^6 conidia/mL, using a Malassez counting chamber.

Biological assays

The antifungal properties of chitosan were investigated both on nutrient agar medium and grapevines (detached leaves and whole plants).

Antifungal assays were carried out in 90 mm Petri dishes adding 1 mg/mL chitosan on potato dextrose agar (PDA) medium. The same conditions were used for the controls with water and 0.5% acetic acid solution. *B. cinerea* mycelium was inoculated on the center of plate and maintained at 21 °C under a photoperiod of 12 h light. The mycelial development was observed and measured until full plate coverage.

For assays performed on grapevine leaves (*in vitro*), leaves were excised from plants (12-16-week-old) and washed with water, surface-sterilized by immersion in 3% sodium hypochlorite for 4 min, and then rinsed twice in distilled water for 1 min (Danti *et al.*, 2002). Then, the leaves were placed in Petri dishes, the adaxial side facing wet adsorbent paper

(Whatman). Treatments were performed by spraying leaves with a solution of 1% chitosan and after 24, 72 and 120 h leaves were infected with *B. cinerea*. Depending on the size of the leaves, one to four lesions were applied to each leaf, and the fresh wounds were covered with 5 µL drops of the conidial suspension of 1 × 10^6 conidia/mL in potato dextrose broth (PDB) (Carlo Erba, Rodano, Milan, Italy). The plates were incubated at 24 ºC with a 12 h photoperiod. Humidity was maintained by covering the plates with a transparent plastic lid.

For whole plants assays (*in vivo*), 24 h after treatments with 1 mg/ml chitosan, twenty leaves per plants were inoculated with 10 µL drops of the conidial suspension in PDB (1 ×10^6 conidia/mL). The plants were covered with transparent plastic bag and incubated at 24 ºC with a 12 h photoperiod.

Fungal development was measured as average diameter of lesions formed from one to ten days post infection (dpi) for both experiments.

Gene expression studies: sampling, RNA extraction and quantitative RT-qPCR

To investigate whether chitosan treatment induced the activation of defense mechanisms, the expression of selected defense-related genes was investigated on leaves collected from uninfected plants during *in vivo* assay. Leaves were collected from 1 to 9 days after treatment (dpt). Untreated leaves were collected as controls. Three independent biological replicates were collected from each experimental condition and each sampling time point, by excising 10 mm disks from four different leaves. After the collection, the leaves were immediately frozen in liquid nitrogen and stored at – 80 ºC.

Frozen samples (100 mg) were homogenized in liquid nitrogen and total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen) with a protocol described by (MacKenzie *et al.*,1997). One µg of RNA was treated with 1 U of RNase-free DNase I (Invitrogen) for 45 min at 37 ºC and the reaction was stopped with 1 µl of 25 mM EDTA. After denaturation at 95 ºC for 5 min, RNA was reverse transcribed at 42 ºC for 50 min with Moloney Murine Leukemia Virus reverse transcriptase (Invitrogen) and DNA random primers (Roche Diagnostic) (Bertazzon *et al.*, 2012).

Real-time PCR assays were carried out on a Bio-Rad thermal cycler (model CFX 96) in 96-well plates using the 2× Platinum SYBR Green qPCR Supermix UDG (Invitrogen). The PCR reactions were performed at least in duplicate, in a total volume of 10 µL, including 0.3 mM of each primer and 1 µL of cDNA. The thermal protocol included a decontamination step of 3 min at 50 ºC to allow for optimal UDG (Uracyl DNA Glycosylase) enzymatic activity, followed by a step of 3 min at 95 ºC in order to activate the Platinum Taq polymerase, to

deactivate the UDG and to denature the DNA sample. Subsequently, 50 cycles of a two-step protocol, consisting of 5 s of denaturation at 95 ºC followed by 30 s of annealing/extension at 60 ºC, were performed. Identical thermal cycling conditions were used for all the targets.

For the selection of reference genes, a set of five *V. vinifera* candidate reference genes (*actin, cytochrome oxidase, pyruvate decarboxylase, glyceraldehyde-3-phosphate dehydrogenase* and *26S rRNA*) was tested in the experimental conditions (Supplementary table 1). The qbasePLUS software (Biogazelle) was used identifying *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) and *cytochrome oxidase* (*COX*) as the two most stably-expressed genes.

The expression of five categories of defense and stress-related genes were monitored. Several genes involved in the hormone-mediated signaling were considered: *Non expressor of PR1* (*NPR1*) and *Enhanced disease susceptibility 1* (*EDS1*) important activators of the salicylic acid signaling; genes encoding JAZ1.1, 9-lipoxygenase (*LOX9*) and 13-lipoxygenase (*LOX13*) involved in the jasmonic acid -mediated signaling; *1-aminocyclopropane-1-carboxylate oxidase 1-like* (*ACO*) involved in ethylene biosynthesis. Moreover, *glutathione-S-transferase* (*GST1*), a gene involved in redox status, and the transcription factor WRKY1 were analyzed. The expression level of five genes coding for PR-protein was evaluated: *PR1*, *PR5* (*Thaumatin-like*/*Osmotin*), *PR6* (*Proteinase inhibitor*), *PR10* (*Ribonuclease-like*) and *PR14* (*Lipid transfer protein*). Finally, two genes were s*elected from the phenylpropanoid pathway: PAL (phenylalanine ammonia-lyase)* and *STS1* (*stilbenes synthase 1*), both responsible for phytoalexin synthesis. The gene specific primers were selected from the literature (Supplementary table 1).

Phytoalexin extraction and characterization by HPLC analysis

To investigate whether chitosan treatment induced production of phytoalexins, the level of stilbene compounds was investigated on the same leaves collected for transcriptomic assays.

The stilbenoid extraction was performed according to the procedures described Repetto *et al.* (2012) with some modifications. An amount of 200 g of frozen leaves were ground in a mortar with liquid nitrogen, then extracted using 10 mL of methanol with 100 µL of trans-4-idrossistilbene (internal standard). The extract was filter vacuum and evaporated under vacuum at 35 ºC and the residue dissolved in methanol and water (1:1 v/v). The

samples were filtered with a 0.2 mm membrane and stored at −20 °C for quantification by HPLC.

The analysis of stilbenes was performed according to the procedure described by Vincenzi *et al.* (2013) with some modifications. Stilbenes were separated on a C18 Lichrospher column (4 mm x 250 mm, 5 µm, Agilent Technologies, Milano, Italy) at 40 °C, using an HPLC system (Waters Corporation, Milford, MA, USA) equipped with a Dual Band UV detector Waters 2487 (Waters Corporation, Milford, MA, USA). The mobile phase gradient will be 0.5% v/v formic acid in deionized water (solvent A) and 2% v/v formic acid in methanol (solvent B). The gradient program will be 0 to 10% (solvent B) in 3 min, followed by 10 to 30% (solvent B) in 5 min, 30 to 44% (solvent B) in 35 min, 44 to 55% (solvent B) in 2 min, 55 to 75% (solvent B) in 15 min and 75 to 100% (solvent B) in 1 min. After washing for 2 min with solvent B, the column was re-equilibrated with solvent A. The flow rate will be 1.0 mL/min and injection volume 20 µL. Detection was performed at 306 nm for trans-isomers of resveratrol, trans-ε-viniferin and piceatannol. The concentration of individual stilbenes was quantified on the basis of peak areas using calibration curves of commercially available standards of *trans*-resveratrol, *trans*-ε-viniferin and *trans*-piceatannol, and correcting the value for the internal standard recovery. All the stilbene standards were obtained from Extrasynthese (Genay Cedex, France). Data were analyzed by the Waters BreezeTM Chromatography Software (Version 3.30). The limits of detection (LOD) and quantification (LOQ) were performed according to the procedure described by Shrivastava and Gupta (2011).

Statistical analysis

The results were evaluated by one-way analysis of variance (ANOVA), and the mean values were analyzed by Tukey's test using the software STATISTICA® 12.0 (StatSoft Inc, Tolson, USA).

RESULTS

Chemical characterization of chitosan

The degree of deacetylation of chitosan was 82.75 %, whereas viscosity [*n*] and molecular weight were 0.31 and 173 kDa, respectively.

Direct antifungal properties of chitosan against *Botrytis cinerea*

Antifungal assays on nutrient agar medium revealed that treatment with 1 mg/mL chitosan allowed a total blockage of *B. cinerea* mycelium. Contrariwise, on the controls the mycelium growth started at 2 dpi and reached the coverage of the plates at 5 and 10 dpi on plates with PDA and PDA added with acetic acid, respectively. Another assay was performed adding different concentrations of chitosan into the PDA medium (from 0 to 0.3 mg/mL, four replicates for each concentration). The IC_{50} after 120h of incubation was 0.3 mg/mL, explaining the complete inhibition of mold growth in the previous experiment.

Chitosan treatment reduced *B. cinerea* infection on grapevine

A preliminary test was performed to exclude any phytotoxic effects caused by chitosan-treatment on grapevine leaves. The highest dose of chitosan application (1 mg/mL) did not showed phytotoxic effects (data not shown).

Different assays were performed inoculating *B. cinerea* on grapevine detached leaves 24, 72 and 120 h after chitosan treatment. The highest protection levels were reached for treatments made 24 and 72 h before *B. cinerea* inoculation. In both assays, the highest percentage of disease reduction was observed at 10 dpi, with a reduction of necrotic lesion diameter of 41% and 69% for treatments made 24 and 72 h before *B. cinerea* inoculation, respectively (Figure 1).

The protection level induced on grapevine detached leaves by chitosan-treatment, carried out 24 h before *B. cinerea* inoculation, was compared to that obtained on whole plants. The evolution of *B. cinerea* infection was slower on assays performed on detached leaves compared to assays with plants, albeit a similar protection induced by chitosan-treatment was detected in both trials (Figure 2). For both assays, treated leaves showed a significative reduction of the average lesion diameter from two days after *B. cinerea* inoculation, in comparison to untreated leaves. As for detached leaves, the highest protection induced by treatment on plants was reached at 10 dpi with reductions of lesion diameter respect to the positive control of 43%.

Chitosan modulated the expression of some grapevine defense-related genes

The expression level of a set of defense-marker genes was evaluated to investigate if the protection induced by chitosan-application derived from the elicitation of grapevine defense responses.

Activation of defense response in plants is mediated by an interconnected network of signal transduction pathways depending mainly by hormones, such as salycilic acid (SA), jasmonic acid (JA) and ethylene (Et). Modulation of the expression level of six genes involved in hormone-mediated signaling was investigated at different times after chitosan-treatment of grapevine plants (Figure 3). Generally, few changes in gene expression were observed for all the investigated genes at one day after treatment. The more interesting results emerged from 3 to 6 dpt when it was observed the downregulation of *NPR1* and *EDS1*, two marker-genes of SA-mediated signaling, and the upregulation of *JAZ1.1*, *LOX9* and *LOX13*, three genes involved in the JA-mediated defense response, beside that of *ACO*, a key-gene of Et-mediated response. Later, at 9 dpt differences in genes expression level between treated and untreated leaves became less evident. Moreover, transcription of *GST1*, a gene involved in the regulation of the redox status, was transiently upregulated one day after treatment, but it was strongly downregulated from 3 dpt. Contrariwise, the expression level of *WRKY1* was slightly enhanced until 9 dpt.

The expression pattern of five *PR* genes, that are reliable defense markers in grapevine, was investigated on plants treated with chitosan (Figure 4). The expression of most *PR* genes was downregulated after chitosan treatment, except for *PR5*, encoding a thaumatin-like protein, whose transcription was substantially higher at 1 dpt and remained high thereafter, up to decrease at 9 dpt.

After one day from the treatment, any modulation of *PAL* and *STS* expression, two key genes of the phenylpropanoid pathway, was induced by chitosan (Figure 5). Successively, the transcription of both genes was slight downregulated at 3 dpt and then, from six days, there was an increasing in the transcription of *PAL*, while the mRNA level of *STS1* continued to decrease until 9 dpt.

Chitosan induced transient accumulation of phytoalexins

The accumulation of some stilbene compounds following chitosan-treatment was investigated on the same leaves collected for transcriptomic studies. Among three searched stilbenes, it was possible to quantify only *trans*-resveratrol, while both *trans*-piceatannol and *trans*-ε-viniferin were undetectable. Significant differences, between treated and untreated plants, was observed only at 3 dpt, with a higher accumulation of *trans*-resveratrol detected on treated plants (Figure 6).

DISCUSSION

Chitosan is a highly investigated biopolymer with well-known antimicrobial properties. This antimicrobial effect is largely influenced by the molecular weight, the degree of acetylation (DA) as well as the derivatization and preparation methods used. On grapevine, chitosans with a minimum chain length (1.5 kDa) and with DA lower that 20% were identified as the best inducers of plant defense (Aziz *et al.*, 2006). The characterization of chitosan used in this work revealed that it had a MW of 173 kDa and a DA of 17%. According to the classification reported from some review, a chitosan with polymer dimension of 173 kDa should be classified as low MW chitosan (LMW) (Verlee *et al.*, 2017). However, this MW, which affect both solubility and viscosity, is higher than that of chitosans used in the majority of the reported antifungal trials *in vitro* and *in vivo*. The degree of acetylation (17%) was similar to that which showed the best antifungal activity in previous experiments (Aziz *et al.*, 2006). In fact, it has been suggested that the presence of at least a small percentage of acetylated groups (i. e. chitin portion) could be better recognized by the receptors on the plasma membrane.

Chitosan used in the present work showed a direct fungistatic effect against *B. cinerea* that confirmed data reported by other authors. Ait Barka *et al.* (2004) found a 44% mycelium reduction when incorporating in the PDA medium 1% of Chitogel (a commercial preparation of chitosan, in this case no information about chitosan molecular weight and concentration were reported). Reglinski *et al.* (2010) found an IC_{50} of 0.175 mg/mL adding a commercial preparation of chitosan (Armour Zen) in the liquid medium. Even in this case the molecular weight of chitosan was not reported. Only Aziz-Aziz *et al.* (2006) excluded a direct effect of chitosan on *B. cinerea* observing only slight growth inhibition with chitosan concentration up to 0.3 mg/mL. However, the chitosan used in those experiments exhibited very low molecular weight (1.5 kDa) in comparison with the chitosan used in the present experiment (173 kDa).

Assays performed on grapevine leaves and plants revealed that chitosan used in the present work conferred a good level of protection against *B. cinerea*. The protective effect of chitosan was reported by many authors on different plant species. Ait Barka *et al.* (2004) reported the efficacy against *B. cinerea* of foliar application of Chitogel on V*itis vinifera*. Aziz-Aziz *et al.* (2006) showed a 50% reduction of *B. cinerea* lesion diameter after application of 0.05 mg/mL of low molecular weight chitosan. Reglinski *et al.* (2010) observed a significant effect on *B. cinerea* development only after application of 5 mg/mL of chitosan. All these authors started the infection 24-48 h after the chitosan application, because it is well known

that chitosan is a resistance inducer and need a delay in order to show its effect. It has been shown that chitosan, mainly at LMW, elicits on grapevine a variety of defense reactions, such as transient increasing of LOX, PAL and chitinase activities (Trotel-Aziz *et al.*, 2006). However, the grapevine response to chitosan was never investigated at the transcriptomic level so far. In the present work, after treatment with chitosan many genes involved in hormone-signaling pathways resulted slightly modulated (Figure 3). Interestingly, the higher modulation observed after six days from the treatment revealed an induction of the JA/ET-mediated response and a repression of the SA-mediated signaling. In particular, *LOX-13*, a gene encoding a lipoxygenase that catalyze the initial step of jasmonate formation in plants, and *ACO*, a gene involved in the synthesis of a precursor of ET, were significative upregulated. JA, SA and ET are central players in mediating responses to pathogens and wounds. SA is usually associated with response to biotrophic pathogens whereas JA/ET are most often thought to function in response to wounding and to necrotrophic pathogens (Glazebrook *et al.*, 2005). Several reports demonstrated that application of chitosan to many plant species, including rice, led to a rapid increase in the JA content through the activation of the octadecanoic pathway (Doares *et al.*, 1995; Rakwal *et al.*, 2002). Enhanced expression of LOX genes was reported by many authors on grapevine in response to elicitors, such as laminarin, and it agrees with the increased lipoxygenase activity reported on grapevine leaves after treatment with LMW chitosan (Aziz *et al.*, 2003; Trotel-Aziz *et al.*, 2006). The main significative event observed one day after chitosan treatment was a higher expression of *GST1*, followed by a marked downregulation of the same gene. *GST1* encodes for an enzyme which takes part in the detoxification of elicitor-generated oxidants and the increasing of its expression was reported in response to the oxidative burst (Levine *et al.*, 1994; Mauch and Dudler 1993; Van Acker *et al.*, 2000).

Despite some transcriptomic modulation of several genes involved in signaling pathways induced by chitosan, the only significant event observed at downstream level was the upregulation of a *PR5* gene until six days after treatment (Figure 4). On the opposite, the transcription of the other PR genes was generally downregulated or otherwise it was at the same levels on treated and untreated leaves. Unexpectedly, the expression of two genes involved in the biosynthesis of stilbenic phytoalexins showed few modulations after chitosan treatment, in spite of the finding of a transient accumulation of *trans*-resveratrol in treated plants at 3 dpt (Figure 6). Aziz and collaborators (2006) reported a pick of *trans*-resveratrol accumulation in grapevine leaves at 48 h after treatment with chitosan with dependency on

DA and MW. The highest phytoalexin accumulation was triggered with chitosan with a DA of 2 to 20% and MW from 1.5 to 3 kDa.

Data reported in the present work highlight that addition of chitosan (173/17) activated the grapevine response with some delay (highest modulation of signaling genes at 6 dpt and higher accumulation of *trans*-resveratrol at 3 dpt). Therefore, it seems that a partial degradation of chitosan polymers, caused by the hydrolytic activity of plant chitinases, could be needed to induce a substantial grapevine defense response. However, it was observed a significant reduction on the spreading of necrotic lesions caused by *B. cinerea* already from one day after chitosan treatment. Therefore, it is likely that chitosan applied on grapevine leaves acts also as a direct inhibitor of *B. cinerea* development. Moreover, as previously suggested by Ait Barka *et al.* (2004), beside the induction of defense mechanisms, chitosan, thanks to its filmogenic property, may also act as a physical barrier to fungal attack. It has to take in consideration, however, that filmogenic properties of chitosan are strictly dependent on its molecular weight, and the low molecular weight chitosan generally used for foliar application lose this property.

Concluding, data presented in this paper show that the good level of protection for grapevine leaves against *B. cinerea*, conferred by chitosan (173/17) could be the result of three properties of chitosan. Indeed, chitosan can act on grapevine leaves as a physical barrier to fungal attack and directly by affecting fungal growth, mainly during the first time after treatment, and successively, as an inducer of grapevine defense reactions.

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FIGURES

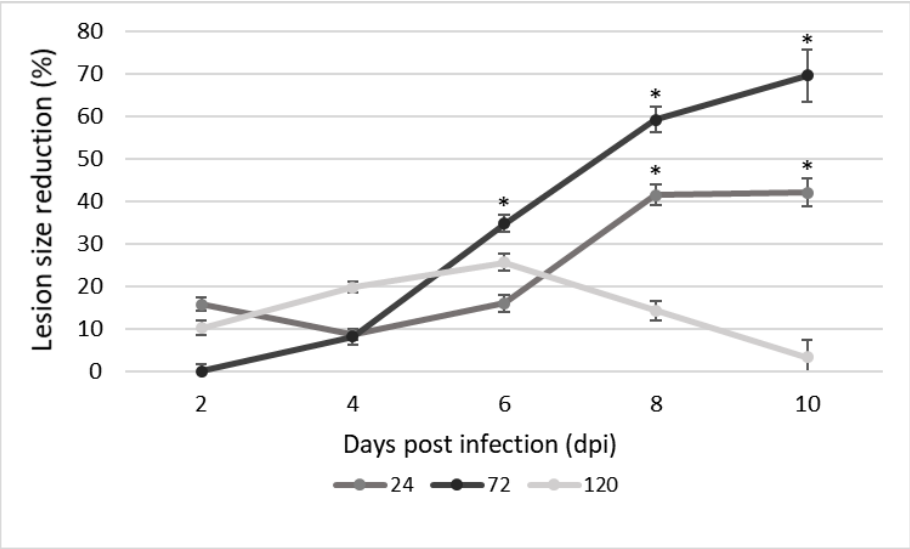

Figure 1. Results of preventive application of chitosan (Chit). Average lesion diameter of *Botrytis cinerea* infection measured on leaves treated with chitosan 24, 72 and 120 h before *B. cinerea* inoculation at different days post infection (dpi) observed in the *in vitro* and *in vivo* assays. The samples with (*) present significant difference $p < 0.05$ by Tukey's test.

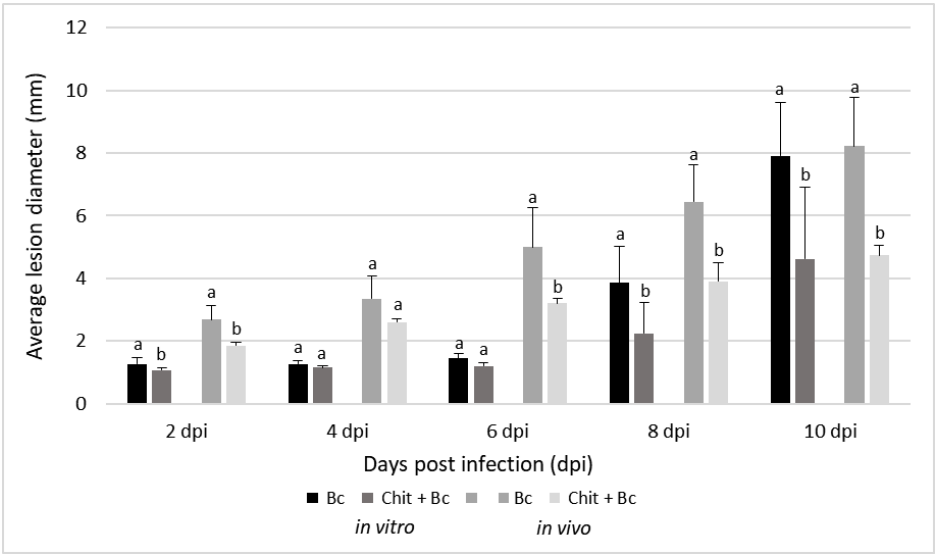

Figure 2. Results of preventive application of chitosan (Chit). Average lesion diameter of *Botrytis cinerea* infection measured on untreated leaves (Bc) and on leaves treated with chitosan 24 h before *B. cinerea* inoculation (Chit + Bc) at different days post infection (dpi) observed in the *in vitro* and *in vivo* assays. Different letters present significant difference $p < 0.05$ by Tukey's test.

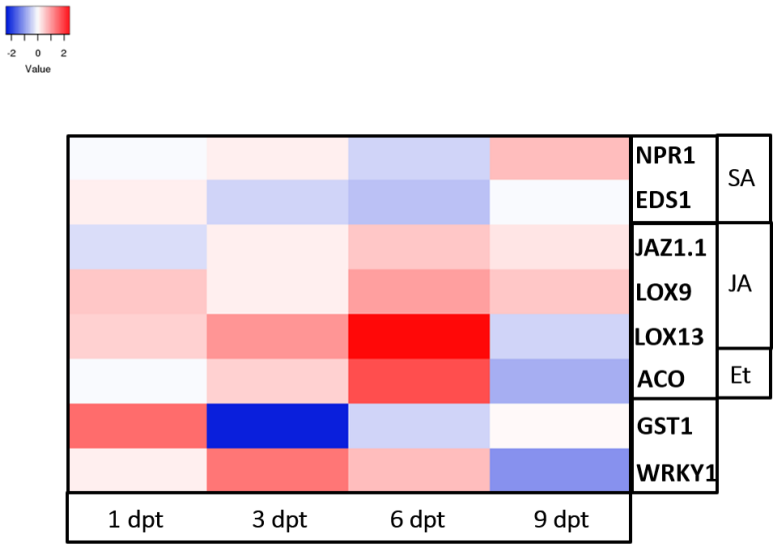

Figure 3. Transcript levels of genes involved in different signaling pathways on grapevine induced by chitosan treatment Each column represents the time in days post treatment and each row represents one gene. A tree color scale was used to show fold induction of each gene (log transformed). The fold induction values were normalized to the reference genes *GAPDH* and *COX* and to untreated leaves as the control samples.

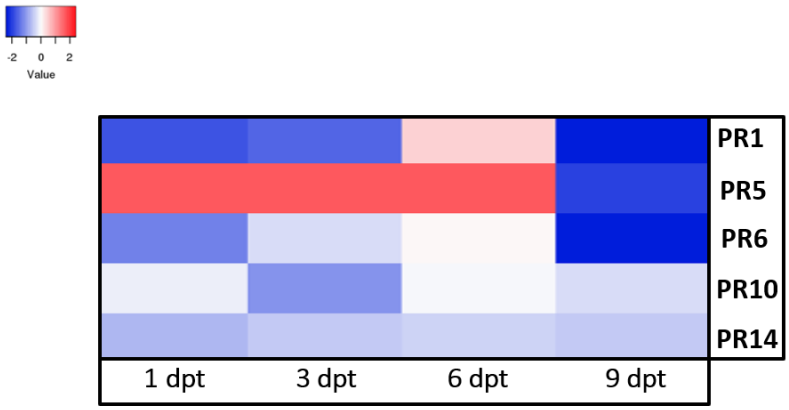

Figure 4. Transcript levels of some PR genes on grapevine after chitosan treatment. Each column represents the time in days post treatment and each row represents one gene. A tree color scale was used to show fold induction of each gene (log transformed). The fold induction values were normalized to the reference genes *GAPDH* and *COX* and to untreated leaves as the control samples.

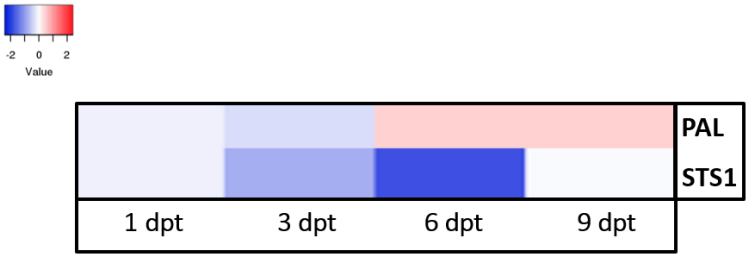

Figure 5. Transcript levels of marker genes of the phenylpropanoid pathway on grapevine after chitosan treatment. Each column represents the time in days post treatment and each row represents one gene. A tree color scale was used to show fold induction of each gene (log transformed). The fold induction values were normalized to the reference genes *GAPDH* and *COX* and to untreated leaves as the control samples.

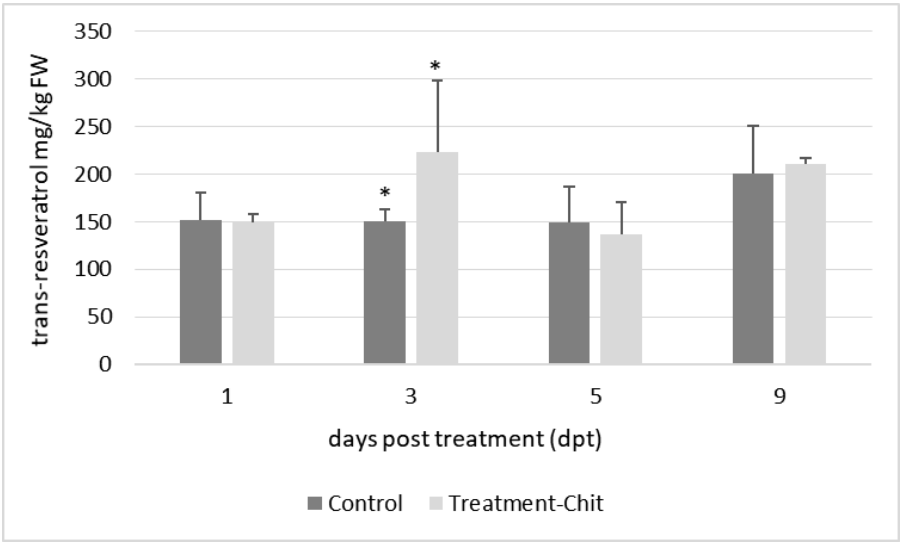

Figure 6. Content of *trans*-resveratrol measured on grapevine leaves after treatment with chitosan (Treatment-Chit) and on untreated plant (Control). The samples with (*) present significant difference $p < 0.05$ by Tukey's test.

SUPLEMENTARY TABLE 1

Table 1. Primer pairs used for quantitative RT-qPCR analysis

Pathway	Target	Forward primer	Reverse primer	Reference
Salicylic acid	EDS1	ATATCGCCGCATAGTTGA	CGTAGGTTCCTGTGTCTT	Liu *et al.*, 2016
	NPR1	GGCGGTTTTGGGGTATTTGT	AGAGCACCTCCACCATGAAA	Shangguan *et al.*, 2017
Jasmonic acid	JAZ1.1	CCTCCACAGGTTCTTGGAG	GGTTTGTGAGGACCTGCAGG	Marchive *et al.*, 2013
	LOX-9	GACAAGAAGGACGAGCCTTG	CATAAGGGTACTGCCCGAAA	Dufour *et al.*, 2016
	LOX-13	ACTGCCCCGACCTTCTTC	AGCCAACCCTAACATTCCTG	Pilati *et al.*, 2014
Ethylene	ACO	TCTTGGTTTGGAGAAGGGCT	CCCTAACAAGCTCAGGTCGA	Shangguan *et al.* 2017
Phenylpropanoid metabolism	PAL	AGGGGCATTCGAGGAGGAGTTG	ACCAGTCAGCAGCCCTGTTCC	Repetto *et al.*, 2012
	STS1	ATCGAAGATCACCCACCTTG	CTTAGCGGTTCGAAGGACAG	Dufour *et al.*, 2016
PR protein	PR1	CCCAGAACTCTCCACAGGAC	GCAGCTACAGTGTCGTTCCA	Dufour *et al.*, 2016
	PR5	GGAGGCAATGGTTTCCACCTTGGG	ACTTGGACGGGACCATAGAGGTTAG	Dufour *et al.*, 2016
	PR6	TGGGAAGCAGGCTTGGCCTGA	ACCTGGCTCTCACCGAAGGG	Dufour *et al.*, 2016
	PR10	GCTCAAAGTGGTGGCTTCTC	CTCTACATCGCCCTTGGTGT	Dufour *et al.*,2016
	PR14	ACAGTTGATCGCCAGGCCGC	GCCCGGAAGCCCACTTGCAA	Dufour *et al.*, 2016
Redox status	GST1	GGGATCTCAAAGGCAAAACA	AAAAGGGCTTGCGGAGTAAT	Dufour *et al.*, 2016
Transcription factor	WRKY1	GAAGCCCACCGAGAACTTTGAAC	ATATGGGTGTCCACCACTCTTTCC	Repetto *et al.*, 2012
Reference genes	ACT	ATGTGCCTGCCATGTATGTTGCC	AGCTGCTCTTTGCAGTTTCCAGC	Bézier *et al.*, 2002
	COX	CGTCGCATTCCAGATTATCCA	CAACTACGGATATATAAGAGCCAAAACTG	Bertazzon *et al.*, 2012
	PDC	GCTTGCGTCGTCACCTTCAC	TGCCGTAGTCGTTGGAGTTGG	Bertazzon *et al.*, 2012
	GAPDH	AATGAAGGACTGGAGAGGTGGAAG	CCGACACATCAACAGTAGGAACAC	Bertazzon *et al.*, 2012
	26S rRNA	TCCCACTGTCCCTGTCTACTATCC	TGGTATTTCACTTTCGCCGTTTCC	Bertazzon *et al.*, 2012

CONCLUSION AND PERSPECTIVES

Collectively, data obtained during PhD work verified that two food byproducts, cane extract and chitosan, derived from the practice of viticulture and from the fishing industry, respectively, could be effective candidates for the development of novel natural fungicides.

Cane extract could be easily obtained collecting canes after pruning on December and storing them for 12 weeks at room temperature to reach the highest accumulation of stilbenes. Moreover, it was showed that canes harvested from some varieties extensively cultivated in the Veneto region could be good source of stilbenes.

Commercial chitosan, inexpensive and easy to find, demonstrated to have direct fungitoxic effects, filmogenic properties and elicitor activity on grapevine plants.

Exogenous application of cane extract or chitosan demonstrated to protect grapevine against the necrotrophic fungus *Botrytis cinerea*. However, the antifungal properties of cane extract and chitosan were investigated only experimentally with controlled environmental condition. It will be interesting to investigate if they will able to maintain the same level of protection also under field conditions. Moreover, it will be useful to investigate the activity of cane extract and chitosan against other grapevine pathogens.