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CICLO XXXVII

Grapevine biochemical defense mechanisms under biotic stress: a focus on phenolic compounds accumulation and pathogen resistance.

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Abstract

Climate change is profoundly reshaping viticultural landscapes by altering temperature regimes, precipitation patterns, and increasing the frequency of extreme weather events. These changes affect grapevine phenology, accelerate ripening, reduce fruit quality, and increase susceptibility to pathogens, particularly fungi, posing significant challenges to sustainable viticulture. While agronomic practices such as canopy management, rootstock selection, and the predictive models offer partial solutions, the physiological and biochemical responses of the grapevine, especially the production of secondary metabolites, are critical for long-term adaptation. Among secondary metabolites, phenolic compounds—particularly stilbenes—play a central role in inducible defense mechanisms. Stilbenes are synthesized in response to biotic stress, such as infections by *Plasmopara viticola*, *Botrytis cinerea*, and wood-colonizing fungi like *Phaeomoniella chlamydospora*, often accumulating in infected or adjacent tissues as part of a complex and localized response. These compounds, including resveratrol, ε-viniferin, and their derivatives, exhibit antimicrobial properties through mechanisms such as membrane disruption and inhibition of microbial respiration. Stilbene biosynthesis is influenced by environmental factors and closely associated with cultivar-specific resistance, as reflected in distinct gene expression profiles across different genotypes. In addition to their role in defense, phenolic compounds contribute to fruit quality and offer potential human health benefits, making their study relevant from both agricultural and nutraceutical perspectives. Understanding the regulation and function of phenolic compound pathways offers promising insights for developing disease-resistant cultivars and integrated management strategies under climate stress scenarios. This thesis highlights the intersection of climate-driven stress, plant defense responses, and the metabolic plasticity of *Vitis vinifera*, emphasizing stilbenes as pivotal compounds in the grapevine adaptive and protective system.

Abstract

Il cambiamento climatico sta profondamente rimodellando i paesaggi vitivinicoli, modificando i regimi termici, i pattern di precipitazione e aumentando la frequenza degli eventi meteorologici estremi. Tali alterazioni influenzano la fenologia della vite, accelerano i processi di maturazione, riducono la qualità delle uve e aumentano la suscettibilità della pianta ai patogeni, in particolare ai funghi, rappresentando sfide rilevanti per una produzione sostenibile. Sebbene pratiche agronomiche come la gestione della chioma, la selezione dei portinnesti e l'impiego di modelli previsionali offrano soluzioni parziali, le risposte fisiologiche e biochimiche della pianta, in particolare la produzione di metaboliti secondari, risultano fondamentali per l'adattamento a lungo termine. Tra i metaboliti secondari, i composti fenolici—e in particolare gli stilbeni—rivestono un ruolo centrale nei meccanismi di difesa inducibili. Gli stilbeni sono sintetizzati in risposta a stress biotici, come le infezioni da *Plasmopara viticola*, *Botrytis cinerea* e funghi lignicoli come *Phaeomoniella chlamydospora*, accumulandosi frequentemente nei tessuti infetti o adiacenti come parte di una risposta complessa e localizzata. Questi composti, tra cui resveratrolo, ε-viniferina e i loro derivati, esercitano attività antimicrobica attraverso meccanismi quali la disgregazione delle membrane cellulari e l'inibizione della respirazione microbica. La biosintesi degli stilbeni è influenzata da fattori ambientali ed è strettamente correlata alla resistenza specifica del genotipo, come evidenziato da distinti profili di espressione genica osservati tra diverse cultivar. Oltre al loro ruolo nella difesa, i composti fenolici contribuiscono alla qualità delle uve e possiedono potenziali benefici per la salute umana, rendendone lo studio rilevante sia dal punto di vista agronomico che nutraceutico. La comprensione della regolazione e della funzione delle vie metaboliche dei composti fenolici offre prospettive promettenti per lo sviluppo di cultivar resistenti alle malattie e l'implementazione di strategie gestionali integrate in scenari di stress climatico. La presente tesi pone l'attenzione sull'intersezione tra stress indotto dal clima, risposte difensive della pianta e plasticità metabolica di *Vitis vinifera*, enfatizzando il ruolo degli stilbeni come composti chiave nel sistema adattativo e protettivo della vite.

1. Introduction

Grapevine (*Vitis vinifera* L.) is the most cultivated perennial crop worldwide and the major Vitis species used for wine production, playing a pivotal role due to its significant cultural, economic, and ecological importance. First domesticated in the Middle East and the Caucasus Mountains, grapevine cultivation gradually spread to many regions, globally. Today, the leading grape-growing and wine-producing countries are primarily located within the so-called "vine-growing belts", which extend between the 30th and 50th parallels in the Northern Hemisphere and between the 30th and 40th parallels in the Southern Hemisphere. Vineyards cover approximately 7.3 million hectares (OIV, 2023), with more than half of this area concentrated in Europe, particularly in Italy, France and Spain. These regions benefit from a favorable habitat with hot summers and mild winters, which supports optimal vine and grape growth. However, despite ideal climate conditions, global warming is increasingly threatening traditional viticultural regions. Higher temperatures, reduced precipitation regimes and unpredictable weather events are dramatically changing the climate of most cultivation areas (Wolkovich *et al.,* 2018). Thus, conventional vine-growing regions are progressively shifting towards warmer climate profiles, while cooler areas at higher latitudes or altitudes have become increasingly suitable for viticulture (Hannah *et al.,* 2013). Rising temperatures are accelerating the vine phenological cycle, leading to earlier grape ripening (Mozell *et al.,* 2014; Aschenfelter *et al.,* 2016), which affects negatively berry chemical and organoleptic properties (Malheiro *et al.,* 2013; Fraga *et al.,* 2017), typically resulting in higher total soluble solid content, increasing the wine alcoholic concentration, and in a decrease in total acidity. Therefore, in historic vine- and wine-producing regions, where viticulture and winemaking are closely tied to cultural heritage and local economies, it is increasingly urgent to develop new strategies to help grapevines adapt to changing environmental conditions. Possible adaptations to improve grapevine yield and quality may involve changes in plant material, such as i) the use of varieties and clones with different thermal requirements and higher resistance to summer stress (Fraga *et al.,* 2013); ii) improving rootstock selection to enhance vine yield, slow down berry ripening and improve drought tolerance (Morando, 2001; Patono *et al.,* 2022); iii) implement training systems that promote vine growth under a diffused light regime (Palliotti *et al.,* 2011); iv) apply minimal pruning techniques to help delay veraison and harvest (Palliotti *et al.,* 2014), and v) the development of climate-based predictive models (Hannah *et al.,* 2013).

Another significant consequence of climate change is the heightened vulnerability of grapevines to pests, pathogens and diseases, particularly under unexpected conditions of extreme heat and drought during the summer (Corredor-Moreno and Saunders, 2020), strongly influencing the expression and severity of disease symptoms (Boyer, 1995). A notable rise in fungal diseases has been reported

(Gullino *et al.*, 2018), likely linked to the impact of abiotic stress on plant physiology and biology. In grapevines with latent infections, the presence of pathogenic agents does not necessarily lead to the immediate appearance of symptoms; rather, symptom expression often occurs following exposure to abiotic stress. Drought events and high temperatures, in particular, play a critical role in modulating both the timing and severity of disease development (Di Marco and Osti, 2008; Lecomte *et al.*, 2012). Abiotic stress disrupts the balance between plant growth and defense mechanisms, weakening the plant's ability to trigger effective responses and, ultimately, influencing its tolerance or susceptibility to infections (Huang *et al.*, 2008; Amtmann *et al.*, 2008; Leakey *et al.*, 2009; Kontunen-Soppela *et al.*, 2010; Mittler and Blumwald, 2010). As a result, this increased vulnerability to fungal diseases necessitates a greater reliance on preventive and curative chemical treatments throughout the grapevine phenological cycle. In viticulture, copper-based products are among the most widely used active ingredients to prevent major fungal diseases such as downy mildew (*Plasmopara viticola*). However, the intensive use of copper in vineyards has led to an increase in its concentration in soil, negatively affecting microbial biodiversity and the overall ecosystem balance. In response to this situation, the European Union has restricted the use of copper-based products, limiting their application to a maximum of 4 kg/ha per year (De la Fuente *et al.*, 2021). This limitation poses a significant challenge, particularly for organic viticulture (Berkelmann-Löhnertz *et al.*, 2012), where viable alternatives to copper-based fungicides are lacking. Consequently, there is a growing demand for more sustainable and healthier practices to reduce the number of treatments required to control fungal diseases. This has stimulated research toward new strategies for effective disease management and plant protection. Among the most promising solutions, integrated pest management is particularly notable, standing out for the coordinated use of natural and chemical products with biocontrol agents, such as Trichoderma spp. (Reis *et al.*, 2022).

However, even if innovative agronomic and cultural practices can enhance grapevine tolerance to climate change-induced stresses, a deeper understanding of the biological mechanisms governing the grapevine metabolic responses is equally crucial. Despite the development of numerous strategies aimed at mitigating the impact of biotic and abiotic stresses, these approaches often fail to examine the intrinsic biological complexity of species. In particular, the grapevine genetic variability and phenotypic plasticity can significantly modulate responses to environmental pressures, potentially buffering or amplifying the effects of external stimuli. This becomes particularly important when considering the accumulation of secondary metabolites involved in the grapevine defense system. Previously, research focused on the composition and concentration of berry secondary metabolites, mainly assessing their impact on fruit and wine quality, as well as their potential benefits for human well-being (Iriti *et al.*, 2010). However, beyond their well-known role in berry ripening, secondary

metabolites are now recognized as central players in plant defense. Gouot and collaborators (2019) investigated the effect of rising temperatures on flavonoid metabolism in grape, while Rienth and colleagues (2021a) examined how abiotic stresses — including elevated temperatures, drought, excessive UV-B radiation and increased CO_2 concentrations — affected both the concentration and distribution of these compounds in berries. Nevertheless, some diseases — including powdery and downy mildews, wood diseases, viral and bacterial infections — are known to spread in the leaves, stems, wood, and roots, as well. Thus, research has increasingly broadened to encompass a higher range of secondary metabolites in vegetative organs, highlighting their pivotal role in plant defense against biotic stresses. Secondary metabolites act as natural fungicides, enhancing resistance against a broad array of pathogens, including fungi, oomycetes, bacteria, phytoplasmas and viruses. This protective role is extensively attributed to their antioxidant capacity, which enables them to scavenge free radicals (Ramalhosa *et al.*, 2013; Wei *et al.*, 2016).

The following sections provide an overview of the secondary metabolites identified in grapevine organs, highlighting their role in tolerance mechanisms against biotic stresses.

2. Secondary metabolites: focus on phenolic compounds

Secondary metabolites include a diverse group of phenylalanine-derived compounds with a multitude of functions in biology and ecophysiology of plants (Taylor and Grotewold, 2005). Among secondary metabolites, phenolic compounds are particularly abundant and display a wide variability of molecular structures. They possess potent bioactivity and their accumulation at key points of the phenological phases can constitute important adaptation strategies, often conferring properties that are useful to plant survival. Overall, phenolics are present in various tissues and organs where they serve as pigments, antioxidants, signaling molecules, structural components and act as direct UV protectants (Braidot *et al.*, 2008; Rienth *et al.*, 2019; Santos-Sánchez *et al.*, 2019). Several reproductive processes are mediated by different phenolics acting to attract pollinators (Mol *et al.*, 1998), contributing to pollen structure and function (Coe *et al.*, 1981; Mo *et al.*, 1992; Taylor and Jorgensen, 1992), and facilitating seed dispersal (Debeaujon *et al.*, 2000; Lepiniec *et al.*, 2006). Moreover, they are often determinant in the interactions with other organisms, including allelopathic agents in plant–plant competition (Bais *et al.*, 2004), acting as insect and herbivore deterrents (McAllister *et al.*, 2005), mediating signaling processes with symbiotic nitrogen-fixing bacteria (Wasson *et al.*, 2006) and contributing to defense mechanisms against pathogens (Treutter, 2005). Beyond their ecological and physiological roles in plant adaptation and survival, phenolic compounds are also of considerable value to humans. They are employed in producing a wide range of natural products - including dyes, fibers, adhesives, oils, waxes, flavorings, fragrances - and their structural

diversity and bioactivity make them valuable candidates for developing new drugs, antimicrobials, insecticides, and herbicides. Moreover, despite phenolic compounds are not classified as essential nutrients like vitamins in human diet, research indicates that their consistent intake over time may influence metabolic processes in ways that reduce the risk of chronic degenerative diseases, such as cardiovascular disorders and metabolic syndromes (Fraga, 2010).

All phenolic compounds derive from the same precursors and share common genes, involving a complex network of routes based principally on the shikimate and phenylpropanoid pathways (**Fig. 1**). Their biosynthesis begins with glucose, which triggers the formation of erythrose-4-phosphate and phosphoenolpyruvate. Subsequently, they are converted into amino acids, l-tryptophan, l-tyrosine, and l-phenylalanine (Schmid and Amrhein, 1995), with the latter two being the main precursors of the phenylpropanoids. It is estimated that under normal growth conditions without stress, more than 20% of the total fixed carbon during photosynthesis flows through the shikimate pathway (Tohge *et al.,* 2013). The phenylpropanoid pathway begins with the aromatic amino acid l-phenylalanine, derived from the shikimate pathway, and leads to the formation of cinnamate, the precursor of all flavonoid and non-flavonoid phenolic compounds. Phenylpropanoid derivatives typically have a ring backbone with hydroxyl groups or other substitutions. Flavonoids, with a C6-C3-C6 carbon framework, are classified based on the degree of oxidation and the substitution pattern on the central heterocyclic ring. Non-flavonoids, on the other hand, have a C6-C2-C6 backbone (stilbenes) or a simpler C6 structure (cinnamic and benzoic acid derivatives; Braidot *et al.,* 2008; Rienth *et al.,* 2021a). Each enzyme families catalyze specific steps of the biosynthetic pathway. Phenylalanine ammonia-lyase initiates the pathway by converting L-phenylalanine into cinnamate, which is subsequently hydroxylated and activated by cinnamate-4-hydroxylase and 4-coumarate-CoA ligase, to form p-coumaroyl-CoA. This intermediate, along with malonyl-CoA, serves as a substrate for chalcone synthase and stilbene synthase, which catalyze the initial committed steps in the biosynthesis of flavonoids and stilbenes, respectively (Rienth *et al.,* 2021a). Overall, phenolics can undergo various modifications, including hydroxylation, methylation, or O-glycosylation of hydroxyl groups, as well as C-glycosylation directly to the carbon atom of the flavonoid skeleton (Stobiecki and Kachlicki, 2006). These biochemical variations generate a wide range of compounds with varying complexity. The main classes of flavonoidic phenolic compounds include anthocyanins, flavonols, flavan-3-ols, and proanthocyanidins, these last also referred to as condensed tannins (Schwinn *et al.,* 2004; Kitamura *et al.,* 2006). In addition to these, hydroxycinnamic and hydroxybenzoic acids, along with stilbene compounds, represent the most prevalent group of non-flavonoidic phenolics in grapevine (Ferrandino *et al.,* 2012; Rienth *et al.,* 2021a).

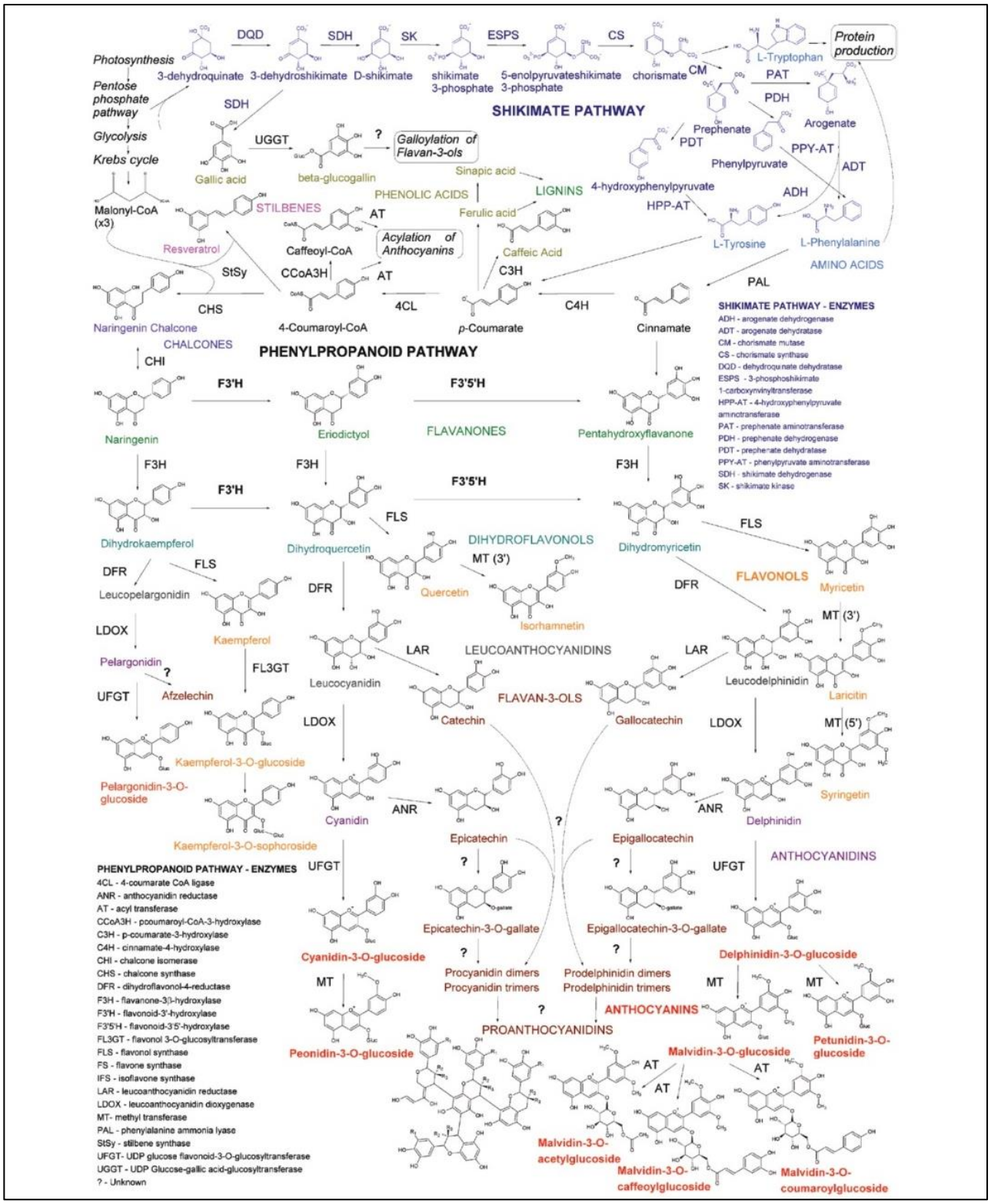


Figure 1. Detailed shikimate and phenylpropanoid pathways in grapevine. Examples of glycosylation and acylation are only shown for kaempferol and malvidin (Gouot *et al.*, 2018).

3. Flavonoid phenolic compounds

Flavonoids are considered the most relevant quality-determining compounds in berries and wines as they contribute to color, flavor, and astringency. First, flavonoids were thought not to contribute to plant development and survival, but they are now acknowledged as important molecules in protecting vines against abiotic and biotic stress (Pourcel *et al.,* 2007; Castellarin *et al.,* 2012; Di Ferdinando *et al.,* 2012; Petrussa *et al.,* 2013). In grapevine, they are present as constitutive compounds and/or as induced substances in the lignified organs, leaves, stems and berries. The first step of flavonoid biosynthesis is catalyzed by chalcone synthase, which, starting from 4-coumaroyl-CoA and 3-malonyl-CoA, leads to the formation of naringenin chalcone. Naringenin chalcone, through the action of chalcone isomerase, is converted into flavanone, and then into dihydroflavonols by flavonoid-3-hydroxylase. At this point, the biosynthetic pathway diverges into two main branches, catalyzed by two distinct flavonoid hydroxylases: flavonoid-3′-hydroxylase and flavonoid-3′,5′-hydroxylase, which lead to the production of di-hydroxylated and tri-hydroxylated flavonoids, respectively (**Fig. 2**; Azuma, 2018; Gouot *et al.,* 2019). They have a C6–C3–C6 structure, consisting of two hydroxylated benzene rings linked by a three-carbon chain. Based on the oxidation state of the C3 ring, these compounds are classified into three main groups: flavonols, flavan-3-ols (which include both simple flavan-3-ols and their polymeric forms, proanthocyanidins), and anthocyanins (Castellarin *et al.,* 2012).

Despite a comprehensive understanding of the grapevine flavonoid biosynthetic pathway, only a limited number of studies have explored the cellular localization, transport across endomembrane and subsequent storage of flavonoids (Braidot *et al.,* 2008). The biosynthesis of flavonoids occurs in the cytoplasm, but the majority of these compounds are subsequently transported and compartmentalized, primarily within the cell wall and vacuoles. For instance, microscopic analyses revealed that anthocyanins accumulate within the vacuoles of the outer hypodermal layers of the berry skin, except in *teinturier* varieties, where anthocyanins are also present in the pulp cells (Cheynier, 2006). In contrast to anthocyanins, flavanols and tannins are localized not only in the vacuoles but also in the cell walls. Once synthesized in the cytosol, flavonoids need an efficient transport system to reach their final storage locations. The transport of flavonoids, particularly anthocyanins, into the vacuole is thought to be primarily driven by the establishment of a proton gradient between the cytosol and the vacuole (or the cell wall), generated by the activity of H+-ATPases and, in the tonoplast, H+-PPases. When they reach the vacuole, the acidic pH of the vacuolar environment, together with flavonoid acylation, is crucial for inducing specific conformational changes, which facilitate the effective sequestration and stabilization of the metabolites within the vacuole (Kitamura, 2006). Based on prior findings in *Arabidopsis* and other species, two distinct transport mechanisms have

been proposed in grapevine to account for the movement of flavonoids from the endoplasmic reticulum to the vacuole, as well as their redistribution from storage compartments to other cellular targets, such as the cell wall, where they perform physiological roles (Zhao *et al.*, 2015). These two pathways, known as membrane vesicle trafficking and membrane transporter-mediated trafficking, may operate simultaneously or be active in different tissues or phenological stages. This observation supports earlier interpretations of flavonoid transport as a complex multifactorial process that relies on various mechanisms and the involvement of multiple enzymes (Petrussa *et al.*, 2013).

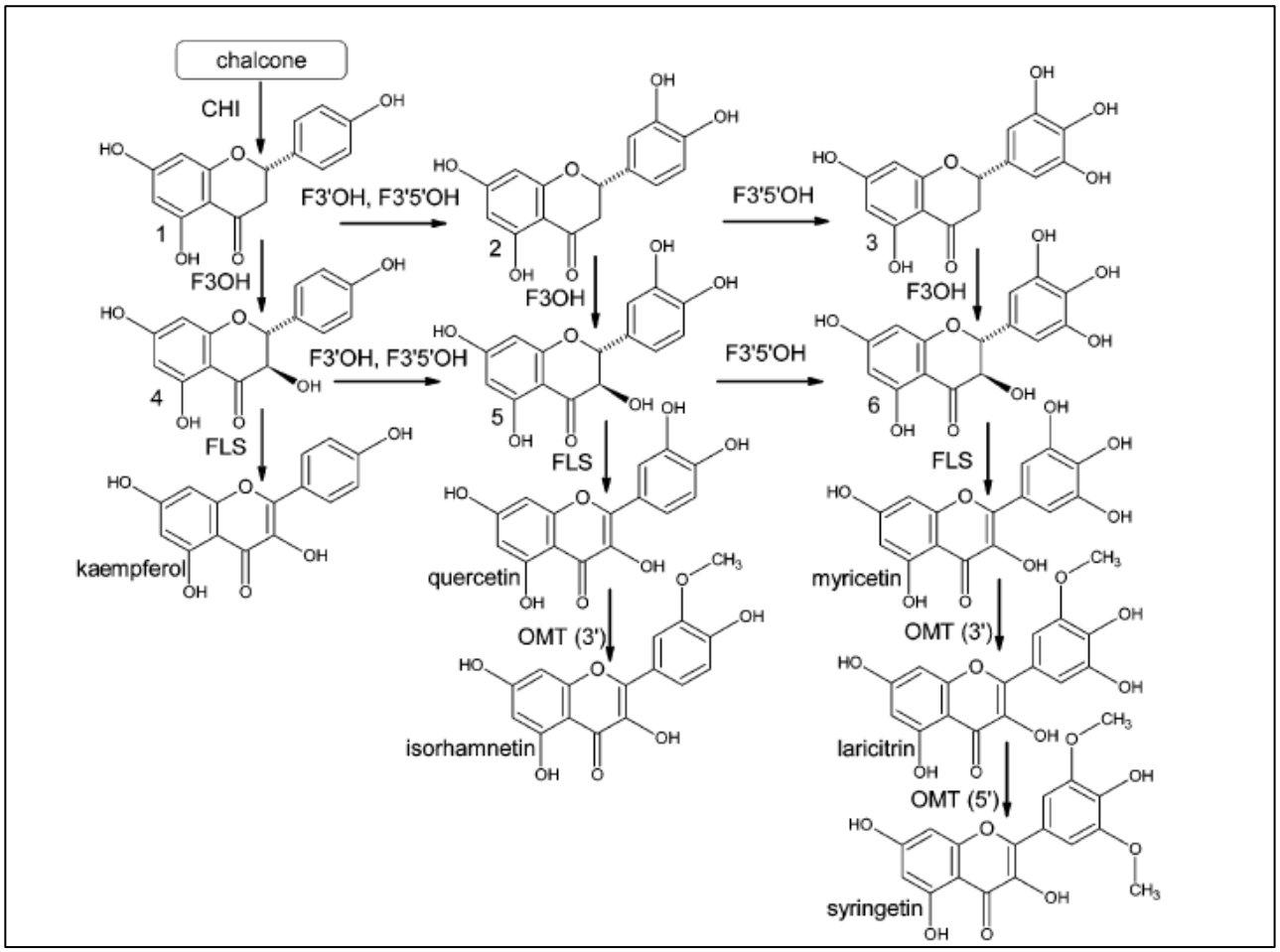


Figure 2. General pattern for flavonol biosynthesis. Precursors: 1, naringenin; 2, eriodictyol; 3, 3',4',5,5',7-pentahydroxyflavanone; 4, dihydrokaempferol; 5, dihydroquercetin; and 6, dihydromyricetin. Enzymes: CHI, chalcone isomerase; F3OH, flavanone 3-hydroxylase; FLS, flavonol synthase; F3'OH, flavonoid 3'-hydroxylase; F3'5'OH, flavonoid 3',5'-hydroxylase; and OMT, methyltransferases (Mattivi *et al.*, 2006).

3.1 Flavonols

Flavonols are an important class of polyphenols in grapevines, primarily localized in the leaves and in the skins of both white and red grapes. Their synthesis begins in the flower buttons and the highest concentrations peak a few weeks after veraison. The concentration stabilizes subsequently during early fruit development and decreases as the grape berries increase in size (Downey *et al.,* 2003). They are considered to act as UV- and photo-protectors because they absorb strongly at both UV-A and UV-B wavelengths, and have a powerful antioxidant activity (Montoro *et al.,* 2005; Flamini *et al.,* 2013). Thus, vineyard practices that promote sunlight penetration can significantly increase flavonol levels in grapevine seasonal organs (berries, stems and leaves). Flavonols contribute to stabilizing the color of red wines, particularly through co-pigmentation processes of anthocyanins. Lastly, they are considered bioactive compounds of possible importance for human health and nutrition (Boulton, 2001; Flamini *et al.,* 2013). Like all phenolic compounds, flavonols are products of the phenylpropanoid pathway, specifically synthesized by the activation of flavonol synthases, and regulated by a light-induced transcription factor (Downey *et al.,* 2004; Czemmel *et al.,* 2009), through a side branch of anthocyanin biosynthesis (Downey *et al.,* 2003). They are primarily found as 3-glycosides, including glucosides, glucuronides, and galactosides. The most common flavonols identified are quercetin (3-O-glucoside and 3-O-glucuronide) and myricetin (3-O-glucoside), although other compounds such as kaempferol, isorhamnetin, laricitrin, and syringetin, also in glucoside forms, have been detected (**Fig. 3A**). Flavonoid-3′-hydroxylase and flavonoid-3′,5′-hydroxylase enzymes catalyze the hydroxylation of the B ring of naringenin, resulting in the formation of 3-hydroxy (via pentahydroxy-flavone) and 3,5-hydroxy (via eriodictyol) flavonols (Mattivi *et al.,* 2006). Pentahydroxy-flavone leads to the formation of dihydromyricetin, while eriodictyol leads to dihydroquercetin. These intermediate compounds, dihydromyricetin and dihydroquercetin, are key precursors in the biosynthesis of myricetin and quercetin, respectively. Additionally, the biosynthetic pathways involving dihydroquercetin and dihydromyricetin contribute to the production of the corresponding 3'-hydroxy and 3',5'-hydroxy anthocyanins (Bogs *et al.,* 2006). The total content and composition of flavonols vary significantly among genotypes and are influenced by genetic and environmental factors. White and rose (light-red) cultivars predominantly produce mono- and di-hydroxylated B-ring flavonols such as kaempferol, quercetin, and isorhamnetin, while red varieties also accumulate tri-hydroxylated forms like myricetin, laricitrin and syringetin (Mattivi *et al.,* 2006; Castillo-Muñoz *et al.,* 2010). For this reason, flavonol profiles can serve as useful chemo-taxonomic markers due to their cultivar-specific patterns.

3.2 Flavan-3-ols

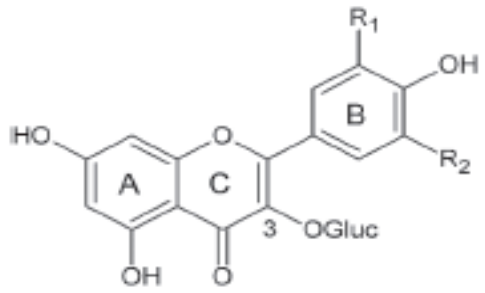
Flavan-3-ols are localized in vacuoles and in the cell wall of berries, seeds, leaves and stems. Studies demonstrated that flavan-3-ols exist in distinct forms: as free compounds within vacuoles and as bound forms associated either with the tonoplast (protein-linked) or with the cell wall (polysaccharide-linked; Amrani-Joutei *et al.*, 1994). Flavan-3-ols are synthesized from flowering to the lag phase through the action of leucoanthocyanidin reductases or anthocyanidin reductase. In grapevine, the main flavan-3-ols are catechin, epicatechin, epicatechingallate, gallocatechin and epigallocatechin which are the building blocks for proanthocyanidins or condensed tannins (**Fig. 3C**; Mattivi *et al.*, 2009). Catechin and epicatechin are the most abundant flavan-3-ols, while gallocatechin and epigallocatechin are present in smaller amounts. Epicatechin and epigallocatechin can be acylated with gallic acid, forming galloylated esters such as epicatechin gallate and epigallocatechin gallate. Catechin, epicatechin and epicatechingallate are dihydroxylated, derived from leucocyanidin and cyanidin, and are classified as procyanidins, whereas gallocatechin, epigallocatechin, and epigallocatechin gallate are trihydroxylated derivatives of delphinidin. Small quantities of flavan-3-ols can be found as free monomers in skin, seeds, pulp, or polymerized as proanthocyanidins in skin and seeds (Cohen and Kennedy, 2012a). Proanthocyanidins are composed of flavan-3-ol subunits interconnected via C4–C8 and C4–C6 interflavan-bonds. Flavan-3-ols, comprising monomers and condensed polymers, are the main class of flavonoids in seeds (Braidot *et al.*, 2008). From *véraison*, seed colour changes from green to brown, probably due to proanthocyanidins oxidation or complexation with other compounds (Hanlin *et al.*, 2010). Overall, the relationship between temperature and flavan-3-ol accumulation appears complex, as results across studies are inconsistent. While Cohen and Kennedy (2012a) observed an increase in the synthesis of flavan-3-ol monomers under elevated temperature conditions, in Sangiovese berries, Pastore and collaborators (2017) reported no significant effect of heat stress on flavan-3-ol levels. Similarly, Gouot *et al.* (2019) found no change in flavan-3-ol concentrations under high temperature treatments. In addition, this relationship also remains ambiguous when considering polymerized flavan-3-ols, as different studies have reported variable effects. On one hand, some studies reported no effect on skin proanthocyanidins when high temperature treatments were applied at the whole-vine level (Mori et al., 2007). On the other hand, other studies reported an effect of high temperature on proanthocyanidins, displaying a decrease in both in seeds and in skins (Bonada and Sadras, 2015).

3.3 Anthocyanins

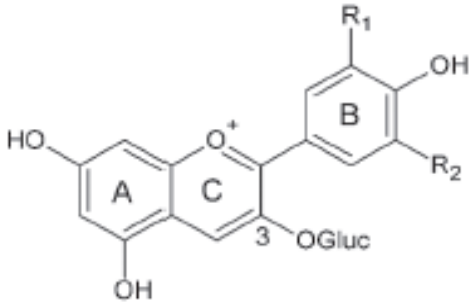
Anthocyanins represent the main pigments contributing to the coloration of red grapes. In *Vitis vinifera*, their biosynthesis occurs in the epidermal and subepidermal cells of the berry skin, beginning

at *véraison* and they are predominantly stored in the vacuoles. However, a limited number of red grape cultivars - named "*Teinturier*" - exhibit anthocyanin in the berry pulp (Castellarin *et al.,* 2011; Falginella *et al.,* 2012). Anthocyanins serve biological roles, including protection against UV radiation and excessive solar exposure, defense against pathogens and oxidative stress, as well as scavenging free radicals. Additionally, they contribute to seed dispersal by attracting animals and participate in the modulation of various signaling pathways (He *et al.,* 2010). In grapevine, these compounds are primarily found as monoglucosides of methoxylated and/or hydroxylated anthocyanidins. The diversity in anthocyanins arises from the different substituents on the B-ring. Specifically, cyanidin-3-O-glucoside and peonidin-3-O-glucoside have two hydroxyl groups (3'-hydroxylated), while delphinidin-3-O-glucoside, petunidin-3-O-glucoside, and malvidin-3-O-glucoside are characterized by three hydroxyl groups (3',5'-hydroxylated; **Fig. 3B**). The flavonoid 3'-hydroxylase and flavonoid 3',5'-hydroxylase enzymes are crucial for the formation of the 3'-hydroxylated and 3',5'-hydroxylated forms, respectively. The anthocyanin monoglucosides can also undergo acylation by various acids, including acetic, caffeic and p-coumaric acids. Each anthocyanin has a particular hue, ranging from red to blue, with delphinidin derivatives associated with blueness and cyanidin derivatives with reddishness. Methylation may enhance color stability, as anthocyanin-O-methylation reduces the chemical reactivity of hydroxyl groups (Sarni *et al.,* 1995). The composition of anthocyanins, including the relative abundance of each compound and the ratio between di-hydroxylated and tri-hydroxylated forms, as well as the proportion of acylated versus non-acylated derivatives, varies considerably among different grapevine cultivars. This variability is influenced by genetic factors, environmental conditions, and vineyard management practices (Monagas *et al.,* 2003; Ferrandino *et al.,* 2010; Theodorou *et al.,* 2019).

A



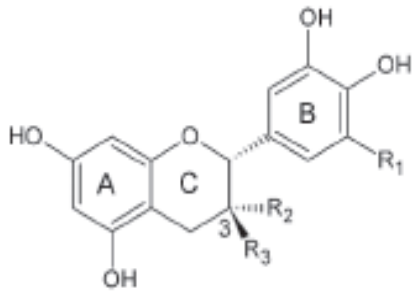
B



Flavonols	R_1	R_2
Quercetin	OH	H
Myricetin	OH	OH
Kaempferol	H	H
Laricitrin	OCH_3	OH
Isorhamnetin	OCH_3	H
Syringetin	OCH_3	OCH_3

Anthocyanins	R_1	R_2
Pelargonidin	H	H
Cyanidin	OH	H
Peonidin	OCH_3	H
Delphinidin	OH	OH
Petunidin	OCH_3	OH
Malvidin	OCH_3	OCH_3

C



Flavan-3-ols	R_1	R_2	R_3
Catechin	H	H	OH
Epicatechin	H	OH	H
Gallocatechin	OH	H	OH
Epigallocatechin	OH	OH	H
Epicatechin-gallate	H	gallate	H
Epigallocatechin-gallate	OH	gallate	H

Figure 3. Structure of flavonols (A), anthocyanins (B) and flavan-3-ols (C).

4.Non-flavonoid phenolic compounds

Non-flavonoid phenolic compounds accumulate in different grapevine tissues. They consist of simple C6 backbone and C6-C2-C6 backbone compounds, such as hydroxybenzoic and hydroxycinnamic acids (C6 backbone) and stilbenes, respectively (Braidot *et al.*, 2008; Rienth *et al.*, 2021a).

4.1 Cinnamic and benzenic acids and their derivatives

Among the simple C6 backbone compounds, the hydroxycinnamic acids -primarily p-coumaric, caffeic and ferulic acids - and their esterified with tartaric acid derivatives (coutaric, caftaric, and fertaric acids) are the most abundant (Ferrandino *et al.*, 2010) in grapevine tissues. Their biosynthesis occurs during the early stages of berry development, up to the lag phase, and is catalyzed by caffeic acid 3-O-methyltransferase and caffeoyl-CoA 3-O-methyltransferase. In grapevine, the prevalent hydroxycinnamic acids in the skins are caftaric acid, p-coumaroyltartaric acid, and feruloyltartaric acid, with the trans isomers generally more abundant than their *cis* counterparts; caftaric acid is the main form accumulated in grapevine pulps (Adams, 2006). Although hydroxycinnamic acids accumulate mainly in the berry pulp, they are distributed across all berry tissues (Cadot *et al.*, 2006; Braidot *et al.*, 2008), and can also form conjugates with anthocyanins (Cadot *et al.*, 2006; Castellarin *et al.*, 2012). In addition to hydroxycinnamates, grapevine contains the minor concentration of hydroxybenzoic acids, including gentisic acid, salicylic acid, gallic acid and p-hydroxybenzoic acid (Vanhoenacker *et al.*, 2001; Ali *et al.*, 2010). Among them, gallic acid is the most abundant, occurring both in free form and, particularly in the seeds, as an acyl group linked to flavan-3-ol structures (Adams, 2006). These compounds play key roles in must and wine browning reactions, serve as precursors to volatile phenols and exhibit antimicrobial and antioxidant properties (Falchi *et al.*, 2006).

4.2 Stilbene compounds

Another class of non-flavonoid phenolic compounds produced in the *Vitaceae* family is that of stilbenes. Despite the structural diversity of polyphenols, stilbenes form a relatively narrow group of compounds, all sharing a common backbone derived from resveratrol. The major role ascribed to stilbenes in several plant families, including *Vitaceae*, is to act as phytoalexins (Langcake *et al.*, 1977), which are defensive substances of low molecular weight produced in response to infection. Indeed, stilbenes play important defensive roles, functioning as antimicrobial, deterrent, or repellent agents against pathogens such as fungi, bacteria, and nematodes, as well as herbivores (Jeandet *et al.*, 2002; Chong *et al.*, 2009). In addition, recently, resveratrol and its derivatives have attracted

considerable attention for their potential health-promoting properties (Anekonda *et al.,* 2006), including anti-cancer, antioxidant, anti-inflammatory and cardioprotective properties (Bavaresco *et al.,* 2012). Stilbenes are synthesized through the phenylalanine pathway, with stilbene synthase catalyzing the final step by condensing one molecule of p-coumaroyl-CoA with three malonyl-CoA units in a single reaction (**Fig. 4**). Stilbene synthase is closely related to chalcone synthase, the key enzyme involved in flavonoid ring formation. Both enzymes share the same substrates and catalyze similar condensation reactions, but the resulting ring closures differ, leading to the formation of distinct products: chalcones and stilbenes (Jeandet *et al.,* 2010). Grapevine stilbenes include *cis*- and *trans*-resveratrol, piceatannol, *cis*- and *trans*-piceid, astringin, pallidol and viniferins. Resveratrol, the simplest and most studied stilbene, exists in

both *cis* and *trans* isomeric forms (Kiselev *et al.,* 2017) and serves as the precursor to several derivatives, including piceid, astringin, piceatannol, and pterostilbene (which exhibits enhanced antifungal activity compared to the non-methylated forms; Chong *et al.,* 2009). Viniferins, oligomeric oxidation products of resveratrol catalyzed by 4-hydroxystilbene peroxidases, include several structurally diverse forms: α-viniferin (cyclic trimer), β-viniferin (cyclic tetramer), γ-viniferin (highly polymerized form), δ-viniferin (isomeric dehydrodimer), and ε-viniferin (cyclic dehydrodimer). Among these, ε-viniferin, is the most abundant in grapevine berries (Castellarin *et al.,* 2012). Generally, stilbenes can be accumulated in free, glycosylated and methylated forms. Glycosylation enhances hydrophilicity and chemical stability, allowing safer storage and facilitating intracellular transport. Glycosylated stilbenes are typically stored in the vacuoles, where they are protected from enzymatic degradation and oxidative stress. Methylation may also occur, increasing lipophilicity and biological activity, particularly antifungal potency. Additionally, the biosynthesis of oligomeric forms, such as viniferins, is catalyzed by peroxidases in the vacuole and cell wall. These forms can be integrated into cell wall structures, chemically and physically contributing to pathogen defense (Sak *et al.,* 2021). Transport mechanisms for stilbenes involve ATP-binding cassette (ABC) transporters. In *Vitis vinifera*, the transporter VvABCB15 has been identified as a key exporter of resveratrol to the apoplast, especially under stress conditions such as UV exposure or pathogen attack. This apoplastic localization supports its function as a phytoalexin, directly targeting invading microbes (Martínez-Márquez *et al.,* 2025).

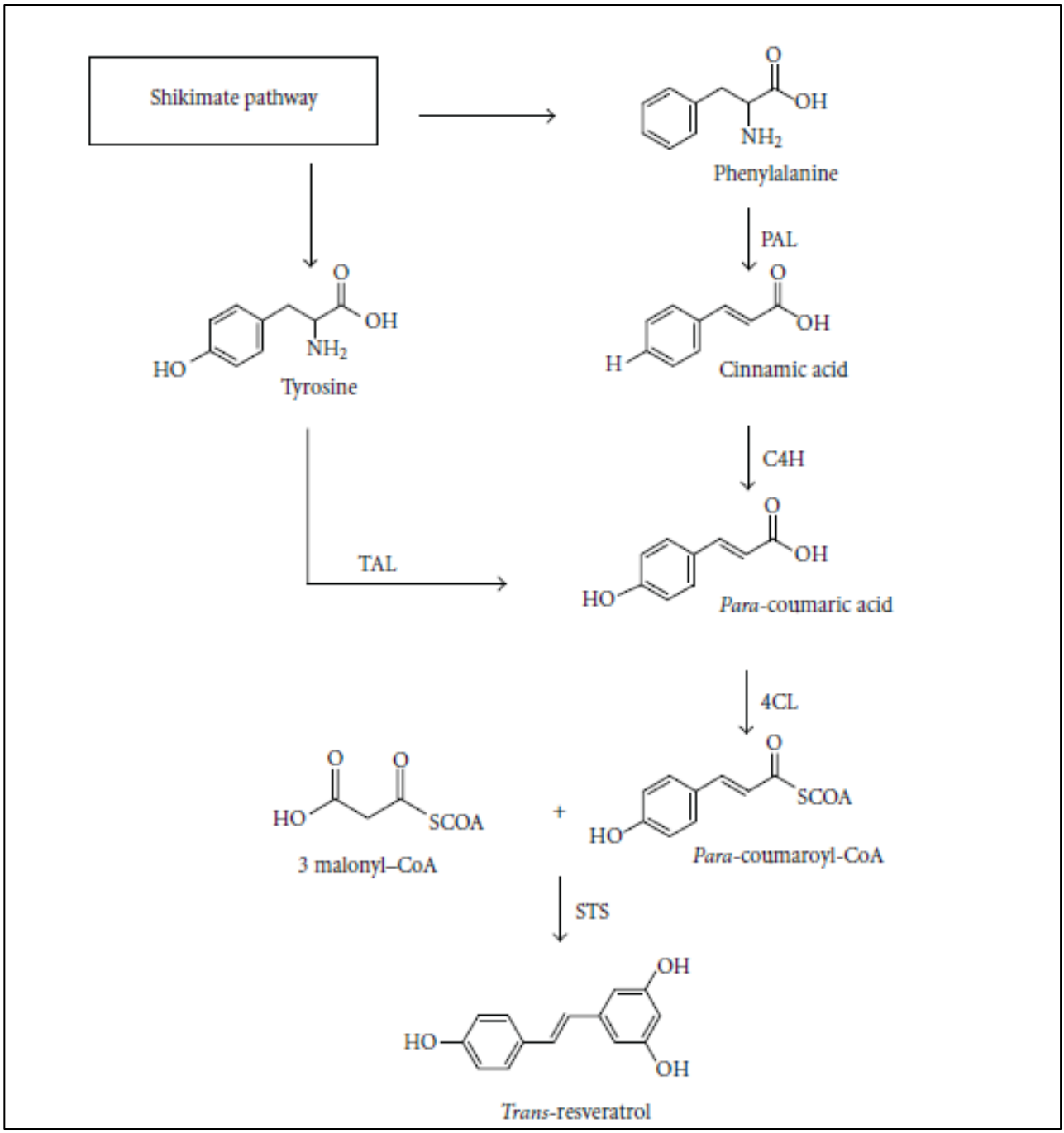


Figure 4. Biosynthesis of resveratrol via the phenylalanine/polymalonate pathway. PAL, phenylalanine ammonia lyase; TAL, tyrosine ammonia lyase; C4H, cinnamate-4-hydroxylase; 4CL, para-coumaric acid: coenzyme A ligase; STS, stilbene synthase. (Jeandet *et al.,* 2012).

4.2.1 Monomeric stilbenes

Resveratrol is the main biologically active stilbene compound, and consequently, it has been the primary focus of many studies. It exists in two isomeric forms, *cis* and *trans*, which differ in both their chemical and biological activities (**Fig. 5**). The *trans*-isomer is more stable, although

interconversion between the two forms can occur under exposure to heat or ultraviolet light. Besides resveratrol, other simple stilbenes such as *trans*-pterostilbene and piceatannol have been identified in *Vitis vinifera* for longtime (Langcake *et al.*, 1979). Various glucoside derivatives of resveratrol have been described, including piceid (β-glucoside of resveratrol) and astringin (the 3-O-β-glucoside of piceatannol), which are found in both *cis* and *trans* configurations (Waffo-Teguo *et al.*, 1998). Resveratrol exhibits antifungal activity (Jeandet *et al.*, 2002; Adrian and Jeandet, 2006). In wood tissues, it is constitutively produced and acts as a phytoanticipin (VanEtten *et al.*, 1994), whereas in leaves and berries, it mainly acts as a phytoalexins, synthesized in response to biotic and abiotic stresses (Langcake and Pryce, 1976). Furthermore, genetic engineering approaches have introduced the stilbene synthase gene into various plants - including tobacco, tomato and poplar - to enable *trans*-resveratrol production and study its potential in promoting plant health and disease resistance (Giorcelli *et al.*, 2004; Halls and Yu, 2008; Delaunois *et al.*, 2009).

Monomers	R_1	R_2	R_3	R_4
Trans-resveratrol	OH	OH	H	OH
Cis-resveratrol	OH	OH	H	OH

Figure 5. Molecular structures of trans- and cis-resveratrol.

4.2.2 Oligomeric stilbenes

In addition to monomeric stilbenes, several oligomeric forms have been isolated from *Vitis vinifera*, including dimers, trimers and tetramers, which are products of oxidative condensation of resveratrol units. Stilbene dimers are divided into two main groups. The first group contains oxygen heterocyclic ring bearing an aromatic ring; examples include ε-viniferin and δ-viniferin, which may be either

glycosylated or non-glycosylated (Langcake *et al.*, 1977; Baderschneider *et al.*, 2000; Waffo-Teguo *et al.*, 2001). The second group consists of dimers without an oxygen heterocyclic ring, such as pallidol (Baderschneider *et al.*, 2000). A resveratrol trimer, tentatively identified as α-viniferin, has been detected in grapevines infected with downy mildew (Jean-Denis *et al.*, 2006). Lastly, tetramers can be classified into three structural groups: the first group contains a bicyclic undecane ring system, the second group features a bicyclic decane ring system and the third group possesses a benzofuran scaffold (Waffo-Teguo *et al.*, 2008).

5. Phenolic Compounds in grapevine vegetative organs

Although considerable studies have focused on phenolic compounds in grapevine, the limited- or non-edible vegetative organs - such as leaves and canes - have historically received far less attention. This limited exploration is largely due to the traditional focus on fruit-bearing tissues, given their commercial relevance to the wine and food industries. However, vegetative organs have increasingly been recognized as valuable and underexploited sources of bioactive molecules with promising health-related properties. In fact, *V. vinifera* leaves have a long history of use in traditional medicine, attributed to their wide range of biological activities, including hepatoprotective, spasmolytic, hypoglycemic, antibacterial, antifungal, anti-inflammatory, antiviral, and antioxidant effects (Katalinic *et al.*, 2009; *Xia et al.*, 2010). As a result, in recent years, there has been a growing interest in the isolation, identification and quantification of phenolic compounds from grapevine vegetative organs to characterize their complex chemical profiles and explore their potential applications in the nutraceutical and pharmaceutical industry and in organic agriculture as natural fungicides. A mapping of the distribution of phenolic compounds in grapevine reveals that both composition and concentration greatly vary depending on the analyzed organ. From an anatomical perspective, flavonoid compounds are primarily localized in the leaves, with lower concentrations found in canes; contrary, non-flavonoids compounds are primarily localized in the canes, with lower concentrations found in leaves (Goufo et al., 2020). Stilbenes appear to be constitutively accumulated in woody parts, roots, canes and stems, where they are thought to play a role in protecting against wood decay (Gabaston *et al.*, 2017). This constitutive accumulation likely explains the higher and more consistent levels of stilbenes in these tissues, compared to leaves where stilbene synthesis is primarily induced in response to pest and disease attacks (Wei *et al.*, 2016; Gabaston *et al.*, 2017).

- **Leaves:** The chemical composition of *Vitis vinifera* leaves is remarkably complex and diverse, a factor that underlies their broad spectrum of biological activities. Phytochemical studies have revealed the presence of numerous classes of bioactive compounds, including organic acids, phenolic acids, flavonoids, tannins, procyanidins, anthocyanins, carotenoids, terpenes,

lipids, enzymes, vitamins, and both reducing and non-reducing sugars (Felicio *et al.,* 2001; Xia *et al.*, 2010; Doshi *et al.,* 2006). Among flavonoid polyphenols, flavonols are particularly abundant, with quercetin-3-O-glucuronide, quercetin-3-O-glucoside and kaempferol-3-O-glucoside identified as the major compounds (Kedrina- Okutan *et al.,* 2018; Goufo *et al.,* 2020). These flavonols are constitutively present in leaves, although their accumulation is modulated by environmental factors, notably exposure to ultraviolet light (Morales *et al.,* 2011). It has been reported that in the leaf blade the concentration of phenolic compounds is higher than in the petiole (Kedrina-Okutan *et al.,* 2018). In response to environmental stresses such as nutrient deficiency, drought, low temperatures and pathogenic infections, leaves may accumulate anthocyanins, contributing not only to stress resistance but also to changes in leaf pigmentation (Margaria *et al.,* 2014; Del Frari *et al.,* 2025). Flavonol concentration is followed by that of hydroxycinnamic acids, hydroxybenzoic acids and flavan-3-ols (Goufo *et al.,* 2020). Conversely, stilbenes are present in much lower concentrations compared to flavonoids. The most prevalent stilbenes detected in grapevine leaves are *trans*-resveratrol, piceid and piceatannol. However, these compounds are often undetectable in healthy leaves under normal conditions and are typically synthesized in response to biotic stress. For instance, in a study of two Serbian grapevine varieties, total stilbene content was significantly higher in leaf extracts from infected plants compared to healthy ones, highlighting their role as inducible defense compounds (Andelkovic *et al.*, 2015). In addition, the spatial distribution of phenolic compounds in grapevine leaves, analyzed by matrix-assisted laser desorption/ionization, revealed a specific localization of *trans*-resveratrol, pterostilbene and viniferins around the veins of healthy leaves (Becker *et al.,* 2014).

- **Canes:** Grapevine canes, the lignified one-year old shoots pruned annually from the plant, have emerged as exceptionally rich sources of stilbenes, often containing higher concentrations than leaves (Besrukow *et al.,* 2022; Ingrà *et al.,* 2024). Stilbenes in canes, although present constitutively, are principally synthesized in response to external stresses such as pathogen attack, mechanical damage, or UV exposure, functioning predominantly as phytoalexins (Jeandet *et al.*, 2010). The major stilbenes identified in canes include *trans*-resveratrol, ε-viniferin, piceatannol and piceid (Besrukow *et al.,* 2022; Ingrà *et al.,* 2024). Notably, ε-viniferin is found in particularly high concentrations, contributing to the cane's antioxidant and antimicrobial properties. The authors concluded that the degree of stilbene oligomerization increases from aerial organs towards the root system. Moreover, a manual dissection of the cortex, pith, and conducting tissues of grape canes indicated that stilbene monomers predominate in the conducting tissues, whereas oligomers are more abundant in

the cortex and pith (Houillé *et al.,* 2015). There are few studies describing the presence of flavonols and anthocyanins in grapevine canes (Goufo et al., 2020), whereas canes are rich in a range of other flavonoid compounds such as catechin, procyanidin B2, epicatechin, ellagic acid, and gallic acid (Cebrián-Tarancón *et al.,* 2019), which further enhance their potential value for pharmaceutical and nutraceutical applications.

6. Factors influencing the biosynthesis and concentration of phenolics in vegetative organs

The concentration of phenolic compounds in grapevine vegetative organs shows a considerable degree of variability. This wide variation arises from the combined effect of several factors. Among the most influential, as extensively documented in the literature, are the grapevine genotype (cultivar and rootstocks), climatic conditions, vineyard management practices and exposure to biotic and abiotic stresses (Harb *et al.*, 2015; Wallis *et al.*, 2013; Kedrina-Okutan *et al.*, 2018; Besrukow *et al.*, 2022).

6.1 Grapevine cultivar and rootstocks

Grapevine cultivars are not genetically homogeneous, with varying levels of intra-varietal diversity. They are typically propagated through vegetative methods, meaning a group of vines derived from the same mother plant, producing a clonal population. Initially, the primary aim of clonal selection was to develop virus-free populations from a single healthy vine. Over time, the selection process has evolved to include additional viticultural factors. Early criteria included grapevine yield and sugar concentration, but as the understanding of grapevine chemistry grew, more advanced factors, including the concentrations of phenolic compounds, were also considered. Research on clonal diversity within grapevine cultivars reveals variations in numerous characteristics. For instance, clones of *V. vinifera* cv Pinot Noir cultivated in California and Champagne regions showed considerable differences in yield, sugar content and acidity levels (Anderson *et al.*, 2008). Moreover, each clone is often grafted on different rootstocks, further influencing their growth and characteristics (Harb *et al.*, 2015). These factors help explain why grapevine polyphenol content can vary so widely. When comparing the phenolic profiles of canes from various cultivars, it was found that Pinot noir and Cabernet Sauvignon exhibited higher concentrations of *trans*-resveratrol and *trans*-ε-viniferin compared to Chardonnay, Syrah, Merlot and Sauvignon Blanc (Lambert *et al.*, 2013). However, the accumulation of resveratrol in Pinot Noir and Cabernet Sauvignon depends on the specific clones and rootstocks being examined (Leeuwen *et al.*, 2013; Lambert *et al.*, 2013). Besrukow and colleagues (2022), showed that Pinot Noir exhibited higher average stilbene concentrations than Riesling, in

alignment with the observations reported by Lambert and co-workers (2013). Interestingly, in Pinot Noir, the variation in stilbene composition was predominantly attributable to differences in profile distribution rather than to the overall quantity. Instead, a difference attributable to the selection of different rootstocks is that of Pinot Blanc grafted onto Kober 5B, S04, and 1103P, where the accumulation of resveratrol in the leaves varied considerably (Bavaresco *et al.,* 2001). Several studies have shown that the stems and leaves of red grapevine cultivars contain higher levels of proanthocyanidins, flavonols, hydroxycinnamic acids, anthocyanins and stilbenes compared to white cultivars (Püssa *et al.,* 2006; Dias *et al.,* 2015). However, the biosynthesis of phenolic compounds is also strongly influenced by the developmental stage of the plant. In particular, very young and very old leaves tend to produce lower amounts of phenolic compounds (Schneider *et al.,* 2008; Schoedl *et al.,* 2012), and the accumulation is strongly dependent on the leaf position on the shoot and the sampling time. For example, in the study by Bouderias and collaborators (2020), quercetin 3-O-glucuronide displayed the highest levels in young leaves and its concentration slightly decreased during the season.

6.2 Climate and geographical location

The seasonal climatic variability of the region along with geographical location - particularly its latitude and elevation - are well-known factors that influence the grapevine tissue phenolic composition. Among environmental variables, light, temperature and water availability play central roles in regulating phenolic biosynthesis. Varying levels of light exposure revealed that shading reduces the flavonoid content in leaves, confirming the hypothesis that these compounds contribute to UV protection (Piñeiro *et al.,* 2013; Kocsis *et al.,* 2015). Leaves fully exposed to sunlight showed higher accumulations of quercetin-3-O-glucoside, kaempferol-3-O-glucoside and quercetin-3-O-galactoside compared to partially shaded leaves (Schoedl *et al.,* 2011). Moreover, phenolic biosynthesis appears highly sensitive to daily temperature fluctuations, although the impact varies depending on the timing. Both excessively low and high temperatures have been shown to suppress flavonoid production (Bavaresco *et al.,* 2012). Prolonged drought conditions, as well as episodes of heavy rainfall, can have a significant impact on grapevine physiology (Harb *et al.,* 2015; Martín *et al.,* 2019). Water stress has been shown to upregulate the expression of genes involved in the anthocyanin biosynthetic pathway (Cui *et al.,* 2017) leading also to increased levels of various polyphenols in the leaves, particularly *cis*-resveratrol-3-O-glucoside, kaempferol-3-O-glucoside and quercetin-3-O-glucoside (Griesser *et al.,* 2015). Conversely, excessive irrigation has been associated with a reduction in proanthocyanidin levels (Bavaresco *et al.,* 2012). Notably, clear differences in the phenolic profiles of leaves and stems have been documented among grapevine cultivars originating

from different geographical regions (Schneider *et al.*, 2008; Gorena *et al.*, 2014; Pantelic *et al.*, 2017; Martín *et al.*, 2019; Kedrina-Okutan *et al.*, 2019).

In our recent study, we investigated the total accumulation and profiles of stilbenes in Cabernet Sauvignon and Syrah canes, grown in two distinct geographical areas, Italy and Portugal. Differences in temperature and precipitation between these two areas affected grapevine growth, phenology and stress response, influencing the accumulation of stilbenes. Major quantitative differences were found between the two cultivation areas: in both cultivars, the average stilbene concentration, was higher in canes collected in Italy with respect to that detected in canes from Portugal. The inhibitory impact of high temperatures combined with scarce rainfall provides a plausible explanation for the lower stilbene levels observed in Portugal (Ingrà *et al.*, 2024). Lastly, with regard to further spatial vineyard differences, stilbene levels were tendentially lower in the center plot of a vine row compared to the outsets, and this effect was particularly distinctive for Chardonnay, Cabernet Sauvignon and Merlot (Besrukow *et al.*, 2022). It can be hypothesized that the "inner vines" experienced reduced levels of stress, potentially benefiting from the shielding effect of the surrounding "outer vines." These outer rows may have acted as a buffer, mitigating exposure to various stress factors such as fungal pathogens, insect pressure, UV radiation and other environmental challenges. Supporting this hypothesis, previous studies have shown that vines located in more inward vineyard positions may benefit from a degree of protection, for example, against grapevine leafroll disease (Cooper *et al.*, 2018).

6.3 Agronomic practices

Many cultural practices affect the production and accumulation of phenolic compounds in grapevine organs. For instance, pruning greatly influences the levels of stilbenes in the canes, leaves and stems (Gorena *et al.*, 2014). Besrukow and colleagues (2022) conducted a three-year study to assess the influence of pruning time on stilbene accumulation, collecting samples at different pruning periods. Across all vintages, the highest total stilbene concentrations were consistently observed in canes pruned in December. Similarly, De Bona and co-workers (2020) reported the highest stilbene levels in canes harvested in October, November, and December, with December showing the greatest accumulation. Besrukow and colleagues (2022) also included a later pruning point in February. While previous literature generally indicates a progressive increase in stilbene levels from October to December, their study provided additional insight into the effects of even later pruning.

In our recent study, we investigated the effect of varying pruning times on the stilbene accumulation in grapevine canes by extending the pruning period to the phenological stages of the sixth extended leaf and visible inflorescence. This approach aimed to broaden the evaluation window for assessing

the seasonal accumulation patterns. The results revealed contrasting trends depending on the cultivation area. In particular, while samples from Italy showed a progressive decline in stilbene levels over the course of the growing season, those from Portugal exhibited a slight, though not statistically significant, increase in stilbene concentration during the same period. Thus, our findings suggest that, in addition to pruning time, environmental conditions significantly influence stilbene concentrations (Ingrà *et al.,* 2024).

Generally, practices promoting plant vigor, such as fertilizer application, have been shown to impact the biosynthesis of phenolic compounds in grapevines. For instance, higher nitrogen doses applied to one-year-old potted vines reduced *trans*-resveratrol accumulation in the leaves. In contrast, increasing potassium doses led to higher levels of *trans*-resveratrol (Bavaresco *et al.,* 2001). Additionally, iron deficiency has been found to stimulate anthocyanin production in the apical leaves of grapevines (Caramanico *et al.,* 2017). The agrochemicals, such as plant hormones or chitosan, aimed at promoting vegetative growth and improving grape quality, influence phenolic biosynthesis (Singh *et al.,* 2019). It has been observed that the use of abscisic acid, auxin and ethylene generally increases flavonoid accumulation, whereas the application of gibberellic acid and ethylene receptor inhibitors tends to have the opposite effect, reducing flavonoid concentrations (Braidot *et al.,* 2008). The indirect consequences of applying fertilizers and hormones could lead to lower polyphenol levels as they promote the growth of thick foliage, which may limit the sunlight exposure of certain plant organs, limiting their phenolic biosynthesis.

Grapevines are vulnerable to various pests and diseases, which are typically managed through chemical and biological treatments. These treatments can introduce additional variability when studying phenolic accumulation. Grapevine plants mycorrhized with *Rhizophagus irregularis* showed a significant increase in stilbene production, accompanied by an up-regulation of genes involved in the leaf stilbene biosynthesis pathway (Bruisson *et al.,* 2016). Treatment with *Trichoderma* species has been shown to induce the expression of key genes like PAL and STS (Leal *et al.,* 2021). However, in a study by Chervin and co-workers (2022), the use of another *Trichoderma*-based product resulted in a mild plant metabolomic response. Some studies have shown that copper treatments can significantly upregulate STS expression in grapevine leaves. For instance, copper application to the canopy of two-year-old Summer Black vines resulted in a marked increase in STS activity. Similarly, spraying $CuSO_4$ on Chardonnay leaves plantlets in *in vitro* conditions elicited a response. Furthermore, the use of a copper-based product, LC2017, has been demonstrated to activate PAL and STS in the wood of Cabernet Sauvignon cuttings, indicating its role as an elicitor. However, in our study in field conditions, after applying pruning wound protection products, such as Cuprocol (a copper-oxychloride based contact fungicide) and Esquive (a *Trichoderma*-based biocontrol agent) we

did not observe any significant changes in the total concentration and profile of stilbene in treated plants compared to the control ones (Ingrà *et al.*, 2024).

6.4 Biotic stress

High variability in grapevine phenolic levels is due to biotic stress. Studies have shown that the plant infection status significantly impacts the phenolic profiles, often more than other environmental or agricultural factors. Following pathogen attacks and insect infestations, all vegetative organs of the grapevine undergo modifications in both the composition and concentration of polyphenolic compounds (Wallis *et al.*, 2013). However, the relationship between phenolic accumulation and disease susceptibility can be contradictory in the literature. The expression of flavonoid pathway genes and the accumulation of phenolic compounds were detected in both healthy and diseased leaves, confirming that the pathway is active under both conditions (Cui *et al.*, 2017). Polyphenols that are present before a pathogen infection are known as preformed ('phytoanticipins'), and are part of a passive resistance mechanism (Gindro *et al.*, 2012; Gabaston *et al.*, 2017). In contrast, active resistance involves the synthesis, degradation or transformation of these compounds into phytoalexins in response to pathogen attacks (Bavaresco *et al.*, 2001). Phytoalexin biosynthesis involves three main metabolic pathways: the acetate- mevalonate, acetate-malonate and shikimate pathways. When necessary, some precursors from these metabolic routes are redirected towards phytoalexin production, leading to enhanced enzyme activity in the biosynthetic pathways of vital compounds, along with the appearance of enzymes involved in the steps closely associated with phytoalexin biosynthesis. Pathogens can metabolize and detoxify phytoalexins, resulting in increased plant susceptibility. Additionally, pathogens may suppress phytoalexin accumulation by secreting phytotoxins or other metabolites.

An induction in the synthesis of stilbene compounds in tissues has been reported in response to the main grapevine pathogens, namely, *Botrytis cinerea* (Bruisson *et al.*, 2016), *Plasmopara viticola* (Mattivi *et al.*, 2011; VanLeeuwen *et al.*, 2013; Gabaston *et al.*, 2017), *Erysiphe necator* (Schnee *et al.*, 2008), fungi associated with grapevine trunk diseases (Lima *et al.*, 2017; Amalfitano *et al.*, 2011; Goufo *et al.*, 2019), *Rhizopus stolonifera* of berry rot (Bavaresco *et al.*, 2001), grapevine leafroll-associated virus 3 of grapevine leafroll diseases (Cui *et al.*, 2017), *Xylella fastidiosa* (Wallis *et al.*, 2013), and *Aspergillus carbonarius* of sour rot (Bavaresco *et al.*, 2009). Specifically, in grapevine canes, studies demonstrated an increase in stilbene concentration, namely E-resveratrol and E−ε-viniferin within the affected discolored wood (Amalfitano *et al.*, 2011), as well as in xylem vessels and adjacent tissues, following inoculation with pathogens such as *Phaeoacremonium minimum* and *Phaeomoniella chlamydospora* (Del Rio *et al.*, 2001; Martin *et al.*, 2009). Regarding flavescence dorée phytoplasma infection, it was demonstrated a strong activation of anthocyanin and

proanthocyanidin biosynthesis and accumulation in infected leaves respect to healthy ones (Margaria *et al.,* 2014). The variability in grapevine phenolic profiles due to biotic stress highlights the complex interplay between plant defense mechanisms and pathogen attack. It is clear that both preformed and *de novo* synthesized polyphenols play crucial roles in grapevine resistance. The induction of phenolic compounds, particularly in response to major grapevine pathogens, underscores the importance of these compounds in plant defense.

7. Focus on the relationship between stilbene and biotic stress

Already in 1962, Hillis and Hasegawa demonstrated that stilbenes are a class of secondary metabolites produced in plants in response to pathogenic infection or environmental *stimuli* (Dixon *et al.,* 1990). These external factors can inhibit flavonoid biosynthesis, redirecting metabolic pathways toward stilbene production. Plant defense system against stresses is generally categorized into constitutive and inducible mechanisms. Constitutive defense refers to structural features that act as physical barriers, limiting damage from herbivores and/or the spread of microorganisms. In contrast, inducible defense mechanisms are activated when the plant perceives a stress signal, triggering a cascade of physiological and biochemical responses that comprise constitutive/preformed and inducible chemical barriers. Inducible chemical barriers involve the synthesis of pathogenesis-related proteins (PR) and the accumulation of phytoalexins. The *de novo* synthesis of phytoalexins, including stilbenes, plays a crucial role in limiting the spread of pathogens and mitigating damage caused by external *stimuli* (Tyunin *et al.,* 2017). These inducible mechanisms can be triggered by biotic stress - including pathogenic microbes and pests - or by abiotic factors such as elevated temperature, drought, ultraviolet radiation and exposure to chemicals like surfactants, heavy metals, salts or antibiotics (Bruno *et al.,* 2007). In response to biotic stress conditions, genes involved in the stilbene biosynthetic pathway exhibit differential expression patterns, resulting in variable antimicrobial activity and a complex defense mechanism (Gao *et al.,* 2024). In particular, the induction of stilbene synthesis in response to biotic stress is well documented in the genus *Vitis*, especially following infection by fungal pathogens such as powdery mildew, downy mildew, and gray mold (Zhu *et al.,* 2004; Frobel *et al.,* 2019). In viticulture, downy mildew, caused by the biotrophic oomycete *Plasmopara viticola*, is among the most damaging diseases, with severe impacts on grape quality and significant yield losses. In infected plants, the synthesis of stilbenes is activated and the accumulation levels of these compounds in affected tissues allow for the differentiation between resistant and susceptible grapevine cultivars. Maul and collaborators (2014) investigated defense-related metabolic pathways in various grapevine varieties infected with *P. viticola*, identifying resistance loci (Rpv, Resistance

to *Plasmopara viticola*) associated with the plant defensive response. In a subsequent study, Rossarolla and coworkers (2021) inoculated *P. viticola* onto the leaves of cv Bronner, presenting a very strong activation of the stilbene pathway. This genotype exhibited a pronounced activation of the stilbene biosynthetic pathway, with marked upregulation of STS, GT, and ROMT genes at 1- and 3-day post-inoculation. The disease resistance mechanism mediated by stilbenes may also operate through the inhibition of microbial systems. In *Vitis vinifera*, pterostilbene has been shown to inhibit the respiration of *Botrytis cinerea* conidia, with its toxicity likely related to its ability to permeate lipophilic membranes. Ultrastructural changes in *B. cinerea* following pterostilbene treatment included disorganization of the endoplasmic reticulum and disappearance of ribosomes, indicating a rapid cellular response. In contrast, the lower antifungal activity of resveratrol compared to pterostilbene may be attributed to its more hydrophilic nature, which limits its diffusion into hydrophobic biofilm membranes (Gao *et al.,* 2024). Notably, chemical modifications of resveratrol - such as methylation, glycosylation, isomerization and oligomerization - have been shown to enhance its antimicrobial activity compared to the unmodified molecule (Chong, *et al.,* 2009).

Moreover, the grapevine can be affected by trunk diseases, a widespread and destructive grapevine disease that affects grape yield and quality. The diseases are generally associated with the development of diverse wood pathogens among which the ascomycetes *Phaeomoniella chlamydospora* and *Phaeoacremonium minimun* and the basidiomycete *Fomitiporia mediterranea* are most commonly cited (Goufo et al., 2019). Physical and biochemical responses are activated when the grapevine is under stress caused by wood-colonizing fungi. Among the earliest is the formation of tyloses, which help isolate the infected area and limit pathogen spread (Fontaine *et al.,* 2016). However, this process is often accompanied by the localized accumulation of polyphenols, particularly stilbenes. Stilbenes comprise a diverse range of compounds (Pawlus *et al.,* 2016; Riviere *et al.,* 2012) and their biosynthesis and accumulation can vary depending on several factors. These include the *Vitis* species and cultivar, cultivation and environmental conditions, vineyard age, rootstock, water availability, soil composition and the type of fungal diseases (Vergara *et al.,* 2012; Lambert *et al.,* 2013). Moreover, grapevine varieties exhibit different levels of susceptibility (Larignon *et al.,* 2009; Lorrain *et al.,* 2012) associated with their capacity to accumulate specific stilbenes (Guan *et al*., 2016). *Trans*-resveratrol has been shown to inhibit the growth of *Phaeomoniella chlamydospora* and *Phaeoacremonium angustius*, suggesting that its presence may contribute to disease resistance mechanisms in grapevines (Santos *et al.,* 2006a). Amalfitano and collaborators (2000) reported that both resveratrol and ε-viniferin levels were significantly higher in discolored (brown-red) wood compared to healthy wood from the same vine, indicating localized phenolic accumulation in response to fungal colonization. Overall, *trans*-resveratrol and its oligomers

constitute the main phenolic compounds identified in grapevine woody tissues, with ε-viniferin, resveratrol dimers, and tetramers being among the most abundant (Martin *et al.,* 2009). Among all cultivars analyzed by Martin and co-workers (2009), ε-viniferin was consistently the most prevalent phenolic compound, followed by *trans*-resveratrol, whose concentration appeared to be strongly influenced by the presence of fungal pathogens in the wood tissue. Interestingly, the accumulation of phenolic compounds, both in total content and as individual stilbenes, was often confined to colonized tissues. Two distinct accumulation patterns were frequently observed: in some cases, the fungal-induced increase in phenolic content was also accompanied by a significant rise in adjacent non-colonized tissues. In other cases, when phenolic levels showed minimal variation in colonized tissues, a reduction was occasionally detected in non-colonized tissues (Martin *et al.,* 2009). Other studies demonstrated an increase in stilbene concentration within the affected discolored wood (Agrelli *et al.,* 2009) as well as in xylem vessels and adjacent tissues, following inoculation with pathogens such *as Phaeoacremonium minimum* and *Phaeomoniella chlamydospora* (Del Rio *et al.,* 2001). Lima and collaborators (2012) demonstrated that elicitation of *Vitis vinifera* cell cultures with an extract of *Phaeomoniella chlamydospora*, as well as with methyl jasmonate, triggered significant alterations in the phenolic profile of the cultures. Notably, these treatments induced the *de novo* biosynthesis of three viniferin-type stilbenes, specifically identified as ε-viniferin-diglucoside, ε-viniferin-glucoside, and a dimeric polymer composed of two ε-viniferin units. An increase in phenolic compounds was observed in esca-symptomatic leaves of *Vitis vinifera* cv Alvarinho, a Vinho Verde variety known for its susceptibility to esca. This finding indicates that, despite their vulnerability, susceptible cultivars are capable of activating defense responses against the disease. However, the timing and intensity of this response may be insufficient to effectively contain or overcome the progression of the infection (Lima *et al.,* 2012).

In summary, stilbenes constitute a fundamental component of the inducible defense system in *Vitis vinifera*. Their biosynthesis is tightly regulated and context-dependent, involving complex signaling pathways that lead to the production of structurally diverse and biologically active compounds with antimicrobial properties. Despite variability in the intensity and effectiveness of this response across cultivars and environmental conditions, the localized and systemic accumulation of stilbenes underscores their pivotal role in limiting pathogen colonization and disease progression. Further elucidation of the regulatory networks and biosynthetic fluxes governing stilbene metabolism may provide critical insights for the development of disease-resistant grapevine genotypes and the implementation of integrated disease management strategies.

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Aim of the thesis

Grapevine trunk diseases (GTDs) are a group of fungal diseases that affect the perennial organs of *Vitis vinifera* L., significantly impacting and compromising vineyard productivity and lifespan worldwide. These diseases are caused by a wide range of wood-colonizing fungi belonging to various genera and species. Once established, fungi infect the vascular system, causing internal necrosis, wood discoloration, cankers, and white rot. The most prominent GTDs include *Botryosphaeria dieback*, *Eutypa dieback*, and the esca complex (Mondello *et al.,* 2018). In recent years, their incidence has increased markedly, driven, in part, by environmental stresses such as extreme drought and water deficiency, partly linked to climate change. Among GTDs, the esca complex is considered one of the most destructive. Affected vines may show symptoms in woody tissues and above-ground parts, particularly the foliage. Based on the nature of wood damage and the fungal species involved, affected vines are classified into two categories: grapevine leaf stripe disease (GLSD) and esca proper (Surico *et al.,* 2009; Mondello *et al.,* 2018). Wood symptoms - such as brown streaks, necrosis and white rot interfere with the vine's capacity to transport water and nutrients, reducing vigor, yield, grape quality and, sometimes, leading to premature vine death (Fontaine *et al.,* 2016).

One of the most debated and least understood aspects of the esca complex involves the stripe leaf symptoms, known as "tiger stripes," which appear inconsistently and irregularly from year to year (Del Frari *et al.,* 2019). Interestingly, they are not always present in vines with wood infections and appear to be influenced by a complex interplay of biotic, abiotic and management/agronomic factors (Del Frari *et al.,* 2021). This observation supports the hypothesis that the origin of the foliar symptoms is more complex than the mere presence of fungal pathogens. Indeed, multiple attempts to reproduce foliar symptoms through artificial inoculation with esca-associated fungi isolated from symptomatic vines have largely failed (Del Frari *et al.,* 2021). Despite years of research, the exact mechanisms behind the development of leaf symptoms remain poorly understood, posing a challenge to the progression of effective and sustainable disease management strategies.

Moreover, the spread of GTDs is closely linked to specific vineyard practices that facilitate pathogen entry, particularly winter pruning, which produces wounds highly susceptible to infection (Mondello *et al.,* 2018). Additionally, grafting processes and nursery propagation are recognized as critical stages in the spread of these pathogens. Other critical stages include disease management, because of the limited availability of effective control measures and the restriction of certain plant protection product use in viticulture (Gramaje *et al.,* 2018). Today, best practices are focused on prevention, employing targeted viticultural techniques such as delayed pruning, double pruning, and the application of pruning-wound protection products that act as physical barriers to infection (Mondello *et al.,* 2018; Gramaje *et al.,* 2018; Del Frari *et al.,* 2023). In light of these challenges, we designed two

complementary studies to investigate key aspects of grapevine defense and symptom expression associated with GTDs.

1)**The first study** focused on the grapevine's intrinsic defense mechanisms, particularly its biochemical responses, including the localized accumulation of stilbenes. E-resveratrol and E-ε-viniferin are key stilbene compounds known to accumulate near areas of fungal colonization, where they contribute to the inhibition of pathogen growth. However, the grapevine's ability to produce and accumulate these defensive compounds may be influenced by the use of plant protection products, potentially weakening its natural defense mechanisms. Although the mode of action of these products and their interactions with microbiota have been widely studied (Larignon *et al.,* 2008; Bruez *et al.,* 2021), their specific impact on stilbene accumulation in woody tissues remains largely unexplored. To address this gap, our study investigated stilbene accumulation in the pruning wounds of one-year-old shoots following the application of various protective treatments. The research was conducted on two internationally cultivated grapevine cultivars, Cabernet Sauvignon and Syrah, grown under contrasting environmental conditions, in Italy and Portugal. The objective was to gain deeper insight into the dynamics of stilbene accumulation in grapevine wood and to clarify the role in the plant defense response against GTD-associated pathogens.

2)**The second study** investigated a relatively unexplored aspect of the esca complex: the presence of three distinct phenotypic variants among symptomatic leaves. While these phenotypes share certain features, such as interveinal necrosis and green tissue adjacent to the main veins, they differ notably in terms of onset, progression and pigmentation patterns. Although physiological, anatomical, genetic and metabolomic changes associated with striped leaves have been extensively studied (Magnin-Robert *et al.,* 2017; Calzarano *et al.,* 2021; Weiller *et al.,* 2024; Bortolami *et al.,* 2023), the role of the leaf-associated microbiome has received limited attention. Notably, esca-related fungal pathogens are rarely detected directly in leaf tissues; nevertheless, the potential influence of the foliar endophytic mycobiome on grapevine physiology and leaf biochemistry remains poorly understood. Emerging evidence suggests a possible relationship between the foliar fungal community and the accumulation of anthocyanins, secondary pigments involved in plant defense against abiotic and biotic stressors, and commonly associated with senescence. Endophytic fungi have also been shown to modulate both metabolic pathways and anthocyanin profiles in several crop species, including grapevine (Chen *et al.,* 2020). Building on these insights, the aim of this study was threefold: (1) to characterize the endophytic fungal community of grapevine leaves through DNA metabarcoding using next-generation sequencing technologies; (2) to track shifts in the microbial community structure throughout symptom development; and (3) to compare fungal composition, anthocyanin content and pigment profiles across different symptomatic phenotypes and asymptomatic leaves.

3) **In a preliminary study**, we aimed to investigate the seasonal dynamics of polyphenolic profiles in *Vitis vinifera* L. leaves, focusing on the effects of leaf ageing and variability due to the position on the shoot. While numerous studies have addressed knowledge on polyphenol accumulation in grape berries and the influence of agronomic practices on bunch microclimate and phenolic content, comparatively little is known about the fluctuation of these compounds in leaves throughout the growing season. Given the key role of phenolic compounds in mitigating oxidative stress during senescence and their potential protective functions against environmental stress, this work explores the qualitative and quantitative variation in specific phenolic classes, namely flavonols, low molecular weight flavan-3-ols, and hydroxycinnamic acid esters, in both mature and senescing basal leaves. By comparing mature and basal senescing leaves, the study seeks to clarify how polyphenol profiles are modulated by leaf developmental stage and insertion nodes on the shoot, contributing to a better understanding of the physiological role of foliar polyphenols in grapevine ecophysiology and their possible interactions with ageing-related processes.

4) **During my research stay at the University of Castilla-La Mancha** (Albacete, ES), I focused on the analysis of volatile compounds extracted from vine shoots, exploring their potential valorisation within a circular economy framework. The intensifying climate crisis has brought sustainability to the forefront of agri-food systems, emphasizing the need to adopt circular models that minimize waste and promote resource reutilization. In this context, viticulture offers significant opportunities for innovation, particularly through the exploitation of pruning residues. Among these, vine shoots—generated annually by winter pruning—represent one of the most abundant yet underutilized sources of lignocellulosic biomass. While pruning is essential for controlling vine vigor and ensuring fruit quality, pruning residues are traditionally treated as waste, often burned or left to decompose, practices that contribute to greenhouse gas emissions, the release of polycyclic aromatic compounds, and the proliferation of soilborne pathogens. In response to these challenges, recent studies have explored the potential of vine shoots beyond energy recovery, with a growing interest in their application as oenological coadjuvants during wine ageing. Vine shoots have shown promising results in enhancing wine complexity through the release of volatile and phenolic compounds, offering a sustainable alternative to traditional oak chips. Notably, they are rich in *trans*-resveratrol and free of ellagitannins—compounds typical of oak wood—contributing to distinctive aromatic profiles and potentially enhancing the terroir expression of wines. They also contain amounts of volatile organic compounds, which can further contribute to wine aroma complexity. Beyond oenology, vine shoots represent a valuable source of bioactive compounds, including stilbenes, phenolic acids, and flavanols, with antioxidant, antimicrobial, and anti-ageing properties. Given the innovative nature and compositional uniqueness of vine shoots, the study conducted at the University of Castilla La

Mancha (Albacete, Catedra de Quimica Agricola) investigated the qualitative and quantitative profile of volatile organic compounds in untreated vine shoots, alongside a broader phytochemical characterization. The goal is to assess their suitability for diverse industrial applications, including food, pharmaceutical, and cosmetic sectors, thereby supporting the sustainable transformation of viticultural by-products into high-value resources. This work aligns with the objectives of the 2030 Agenda and contributes to the green transition in agriculture through environmentally responsible practices. Overall, among the volatile organic compounds identified, vanillin emerged as the most abundant, indicating its central role in defining the aromatic profile. Within the terpene fraction, linalool, trans-linalool oxide, and nerol were the predominant constituents, with floral and citrus notes. Geranyl acetone was the principal compound detected among the norisoprenoids, underscoring its importance in shaping the overall volatile composition. However, although preliminary analytical steps have been undertaken, the results will not be presented in this work, as the complexity of the target molecules requires extended data processing and interpretation.

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Effects of Growing Areas, Pruning Wound Protection Products, and Phenological Stage on the Stilbene Composition of Grapevine (*Vitis vinifera* L.) Canes

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ABSTRACT: Applying plant protection products (PPP) on grapevine pruning wounds is a viticultural practice used to mitigate the spread of grapevine tuck disease, which is posing serious economic losses in the vine-wine industry. However, the impact of PPP on woody tissues remains unclear. Our study, conducted in two European vineyards, investigated the effects of Cuprocol, Tessior, Esquive, and Bentogran on stilbenes, in canes of Cabernet sauvignon and Syrah, at three phenological stages. Main stilbenes, quantified by HPLC-UV-DAD (1260 Agilent Infinity System) and identified by HPLC-ESI/MS (Thermo Scientific LCQ FLEET system), included E-resveratrol, E−ε-viniferin, E-piceatannol, and E-polydatin. Canes exhibited varying proportions of individual stilbenes, reflecting differences based on climatic conditions and phenological phases, rather than on the application of specific PPP. Vines grown in cool-climate conditions exhibited higher levels of E-resveratrol, whereas vines from the Mediterranean climate area exhibited higher levels of E−ε-viniferin. We also observed divergences in the accumulation trend of wood stilbenes throughout the season in canes collected in the two different growing areas.

KEYWORDS: *grapevine trunk diseases, wood, Cuprocol, Tessior, Esquive, Bentogran, resveratrol, ε-viniferin, climatic conditions, HPLC-UV/DAD, HPLC-ESI/MS*

INTRODUCTION

Grapevine trunk diseases (GTDs) comprise a group of fungal diseases involving several wood-colonizing fungi from diverse genera and species. These fungi infest perennial organs, instigating vascular infections that result in wood discoloration, necrotic lesions, and white rot. Infected vines occasionally exhibit foliar symptoms that, altering leaf photosynthetic capability, cause yield reduction and berry quality deterioration. While extensive research has examined various aspects of GTDs and their causal agents, the factors influencing the appearance and progression of leaf symptoms remain elusive.[1] The three primary GTDs (*Botryosphaeria* dieback, *Eutypa* dieback, and the esca complex−are detrimental diseases, with a serious economic impact on the vine-wine industry, worldwide. Their rising incidence and severity are exacerbated by stressful environmental conditions, often due to climate change issues, mainly severe water stress. The spread of GTDs is primarily attributed to cultural practices, particularly winter pruning.[1] Winter pruning, an essential practice in viticulture, inflicts numerous wounds in differently aged wood depending on the adopted pruning method. Pruning cuts serve as major entry points for GTD pathogens. Additionally, GTD fungi can invade vines during the grafting and nursery processes. The limited availability of effective control strategies and the current regulatory restrictions have added complexity to managing GTDs. In Europe, sodium arsenate was used to control their spread until its prohibition in 2003, because of its adverse impact on human health and environment,[1] leaving no effective treatments. The use of other chemicals, including Fosetyl-Al[2,3] and triazoles[4] as alternatives to sodium arsenate was proposed but their effectiveness is contingent upon factors such as the mode of application, the specific location (nursery or vineyard), and the targeted pathogens [for review, see ref 5]. Currently, an efficient approach to mitigate infection threats is to focus on preventing field infections, by choosing reasoned cultural practices such as delayed pruning,[6] double pruning,[7] and the application of wound protection products.[8] Delaying pruning and/or double pruning reduce exposure to rainfall, constricting the time frame of wound vulnerability to infection. Double pruning is suitable for spur-pruned vineyards, providing a preliminary pruning phase, followed by conventional pruning. Pruning wound protection product distribution has gained widespread popularity[1,5,9] despite this approach being time-consuming and costly. Mastics and pastes, alone or supplemented with fungicides, serve as protectors by creating a physical barrier that hinders the entry of fungi. When external factors compromise this physical barrier, fungicides may still actively limit pathogens, ensuring wound protection.[5] In vineyards, copper-based fungicides are extensively used to

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Table 1. Seasonal Weather Conditions at the DISAFA (Italy, Continental Climate Conditions) and Almotivo (Portugal, Mediterranean Climatic Conditions) Vineyards from January to May[a]

year	month	MTMIN	MTMAX	MTMED	nFD	nRD	TR
			DISAFA Vineyard				
2022	January	−2.4	10.4	3	27	0	2.6
	February	0.5	13.8	6.9	13	1	4.6
	March	2.8	14.3	8.6	6	3	9.4
	April	7.5	19.7	13.4	0	4	22.8
	May	14.4	26.3	19.9	0	8	82
			Almotivo Vineyard				
2022	January	7.7	14.9	11.0	0	0	1
	February	9.1	17.4	12.9	0	0	0.5
	March	10.4	16.2	13.0	0	12	51.6
	April	11.1	18.5	14.4	0	1	4.1
	May	14.4	25.7	19.3	0	0	0

[a]MTMIN: monthly average of minimum temperatures (°C); MTMAX: monthly average of maximum temperatures (°C); MTMED: monthly average of daily average temperatures (°C); nFD: number of frost days, with minimum average temperatures below 0; nRD number of rainy days; TR: total rainfall (mm).

control fungal and bacterial diseases,[2,10] also thanks to their ability to elicit several genes associated with plant defense mechanisms.[11] However, copper accumulation adversely affects the ecosystem biodiversity due to its phytotoxic effects and accumulation in soil and water, highlighting the need for a reevaluation of its use in plant protection strategies. These aspects led the EU to introduce stringent limitations on copper distribution in vineyards (28 kg/ha in 7 years). Another promising approach is the utilization of biological control agents, notably *Trichoderma* species, or the application of natural products.[12,13] In viticulture, two commercial solutions based on *Trichoderma* are used to control GTDs: Vintec (Belchim Crop Protection) and Esquive (Agrauxine by Lesaffre). However, despite several promising solutions for pruning wood protection, managing GTD using a single approach appears to be challenging. The evolutionary adaptation has enabled grapevines to trigger defense mechanisms in response to stress. In reaction to pathogen infection and wood colonization, grapevine deploys physical and biochemical barriers. Initially, the production of tyloses serves to compartmentalize the spread of fungi.[14] Subsequently, the induction of polyphenol-rich areas, particularly of stilbenes, inhibits the pathogen growth.[15] It seems well-established that no grapevine species or varieties are inherently resistant to trunk diseases. However, grapevine varieties exhibit different levels of susceptibility,[16,17] associated with their capacity to accumulate specific stilbenes.[18] Stilbenes comprise a diverse range of compounds,[19,20] and their biosynthesis and accumulation can vary depending on several factors. These include the *Vitis* species and cultivar, cultivation and environmental conditions, vineyard age, rootstock, water availability, soil composition, and the type of fungal diseases.[21−23] Studies demonstrated an increase in stilbene concentration within the affected discolored wood,[24,25] as well as in xylem vessels and adjacent tissues, following inoculation with pathogens such as *Phaeoacremonium minimum* and *Phaeomoniella chlamydospora*.[15,26] Specifically, E-resveratrol and E−ε-viniferin accumulate close to the colonized tissues, suggesting their role in inhibiting wood colonization.[27,28] However, plant protection products (PPPs) may influence the capacity of treated plants to accumulate wood stilbenes, and this interference can be decisive in countering pathogen attacks. While extensive studies investigated the mode of action of PPPs against GTD pathogens,[29,30] their behavior within the plant after application,[29,30] and endophyte microbial ecology,[9] research on the effects of PPPs on stilbene accumulation remains limited.[31,32] This knowledge gap underscores the need for a comprehensive understanding of how PPPs influence the stilbene dynamics in grapevine wood. The objective of this research was to investigate the accumulation of stilbenes in one-year-old wood pruning wounds after applying various pruning wound protection products. The study involved two internationally recognized grapevine cultivars, Cabernet Sauvignon and Syrah, grown in Italy and Portugal, to evaluate the interactions with environmental conditions. By examining the relationship between PPP applications and the grapevine response, the study aimed to elucidate the stilbene accumulation dynamics in grapevine wood and its potential role in defending against pathogens.

MATERIALS AND METHODS

Reagents and Standards. E-resveratrol, E-piceatannol, E−ε-viniferin, and E-polydatin were purchased from Extrasynthèse (Genay, France). HPLC-grade acetonitrile was provided by Sigma-Aldrich (Milan, Italy) and Honeywell (Seelze, Germany). Methanol used for extraction was supplied by Sigma-Aldrich (Milan, Italy) and PanReac (Barcelona, Spain). Formic acid was supplied by Sigma-Aldrich (Milan, Italy).

Vineyard Description and Seasonal Weather Conditions. During winter 2021/2022, one-year-old canes of Cabernet Sauvignon and Syrah vines were sampled in the collection vineyards of the University of Turin (DISAFA vineyard; 45 30530 0 N, 7350320 0 E) in Grugliasco (TO), Italy, and of the Instituto Superior de Agronomia (Almotivo vineyard; 38 42032.70 0 N, 911011.50 0 W) in Lisbon, Portugal. The DISAFA vineyard, planted in 2008, has a density of 4400 vines/ha and is situated 293 m above sea level in a flat area. Vines are vertical-shoot-positioned and trained to the Guyot pruning system. The soil composition is sandy; further details can be found in.[33] Integrated agricultural management practices are applied. Cabernet sauvignon is grafted onto 779P (*Vitis berlandieri* × *Vitis rupestris*), and Syrah onto SO4 (*Vitis berlandieri* × *Vitis riparia*). The Almotivo vineyard, established in 1998, has a vine density of 3333 plants/ha and employs the Cordon Royat bilateral training system with spur pruning. The soil is categorized as vertisol. Integrated agricultural practices are applied. Cabernet Sauvignon and Syrah are grafted on 140 RU rootstock (*Vitis berlandieri* × *Vitis rupestris*). The seasonal weather conditions are listed in Table 1.

Experimental Setup. In the autumn of 2021, externally asymptomatic canes were carefully selected and labeled. In January

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Table 2. Protection Products Applied to the Pruning Wounds of One-Year Old Canes of Cabernet Sauvignon and Syrah Vines Grown in Continental (DISAFA, Italy) and Mediterranean (Almotivo, Portugal) Climatic Conditions and Relative Tested Concentrations

trade name	manufacturer	active ingredients or biological control agent (BCA)	tested concentration
Cuprocol	Syngenta (Basel, Switzerland)	copper oxychloride	36.5 g L^{-1}
Esquive	Agrauxine (Marcq-en-Barœul, France)	*Trichoderma atroviride* I-1237 (1×10^{8} CFU g^{-1})	100.0 g L^{-1}
Tessior	BASF (Ludwigshafen, Germany)	boscalid 10 g L^{-1} + Pyraclostrobin 5 g L^{-1}	10.0 g L^{-1} boscalid + 5.0 g L^{-1} pyraclostrobin
Bentogran	AEB Bioq. Port. (Singapore)	sodium bentonite	80% (v/v) sodium bentonite in water

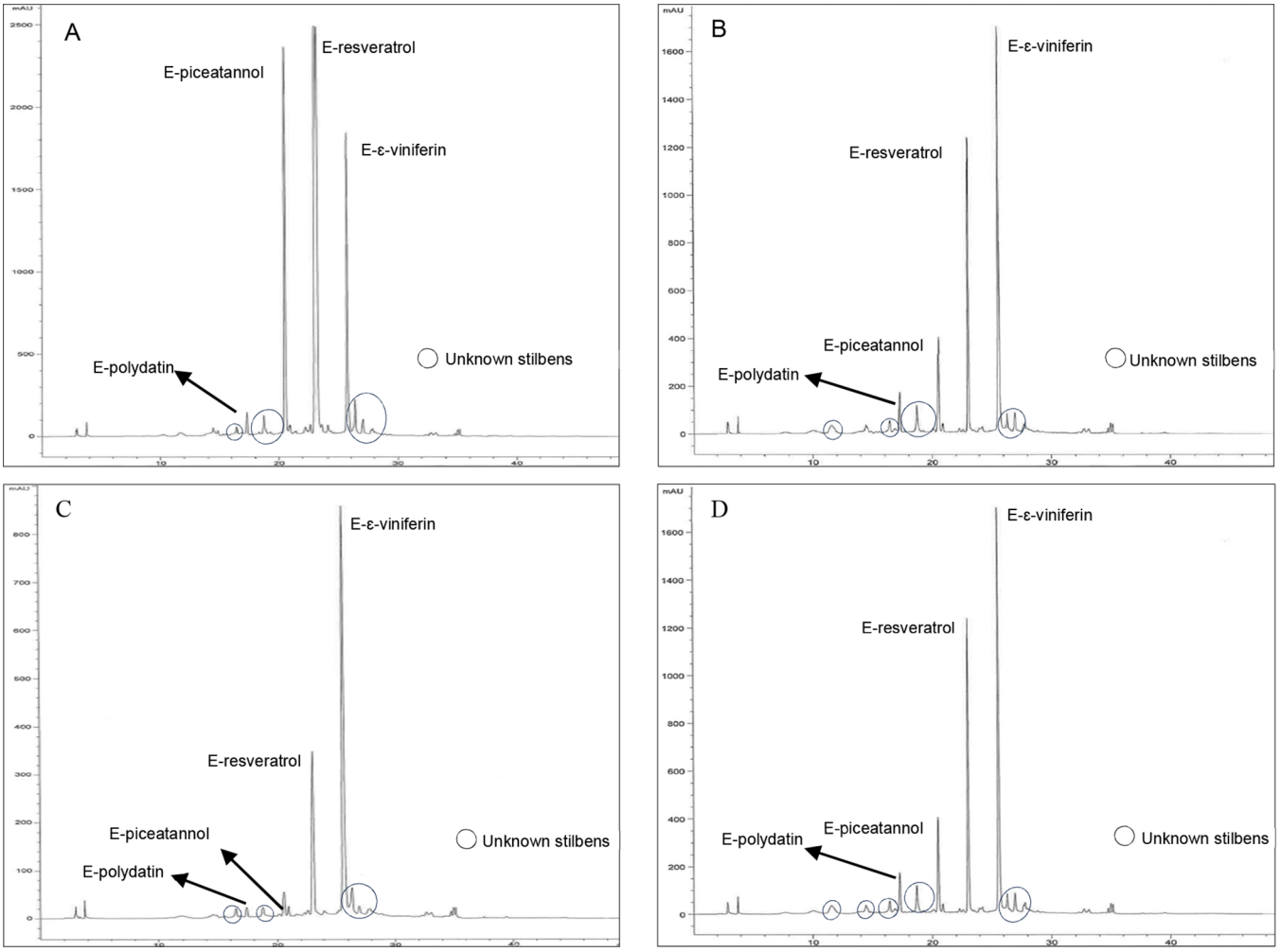

Figure 1. HPLC-UV/DAD chromatograms (1260 Agilent Infinity System), acquired at 320 nm, of one-year-old Cabernet sauvignon wood extracts. A and B: chromatographic separation of the wood extracts collected in continental climate conditions (Italy), at the phenological stage of dormant bud (A) and at the phenological stage of sixth extended leaf and visible inflorescence (B). C and D: chromatographic separation of the wood extracts collected in Mediterranean climate conditions (Portugal), at the phenological stage of dormant bud (C) and at the phenological stage of sixth extended leaf and visible inflorescence (D).

2022, when vines were dormant, we performed manual prepruning by shortening the one-year-old canes 2−3 cm above the fourth node (approximately 20 cm above the fruiting cane in Guyot-pruned vines, DISAFA vineyard) and 20 cm above the spur in Cordon Royat pruned vines (Almotivo vineyard). A three-day monitoring period was implemented to ensure minimal or no bleeding from the pruning wounds before applying the treatments. The pruning wound protection products used were: Cuprocol, a copper-oxychloride-based contact fungicide; Tessior, a liquid polymer containing two systemic fungicides, boscalid and pyraclostrobin; Esquive, a *Trichoderma*-based biocontrol agent; Bentogran, a sodium bentonite inert clay acting as a physical barrier, largely used in cellars (Table 2). Cuprocol, Esquive, Tessior, and water (as control) treatments were applied with a micropipette (25 μL) on the surface of the wound and covered with Parafilm. Bentogran was applied with a brush. Each treatment was applied to 3 canes on 4 different vines (each vine represented a biological replicate), obtaining 12 treated canes per treatment. Vines were treated with a unique pruning wound protection product to avoid potential interference among the treatments. Four canes per treatment were sampled at three different time points, corresponding to three phenological phases: dormant bud, second extended leaf, and sixth extended leaf with visible inflorescence.[9] Globally, 120 canes were sampled in each vineyard (4 canes per 5 treatments per 2 varieties per 3 sampling times). In Italy, samplings were carried out in March (dormant bud), April (second extended leaf), and May (sixth extended leaf and visible inflorescence). In Portugal, due to different climatic conditions, samplings were conducted approximately one month earlier compared

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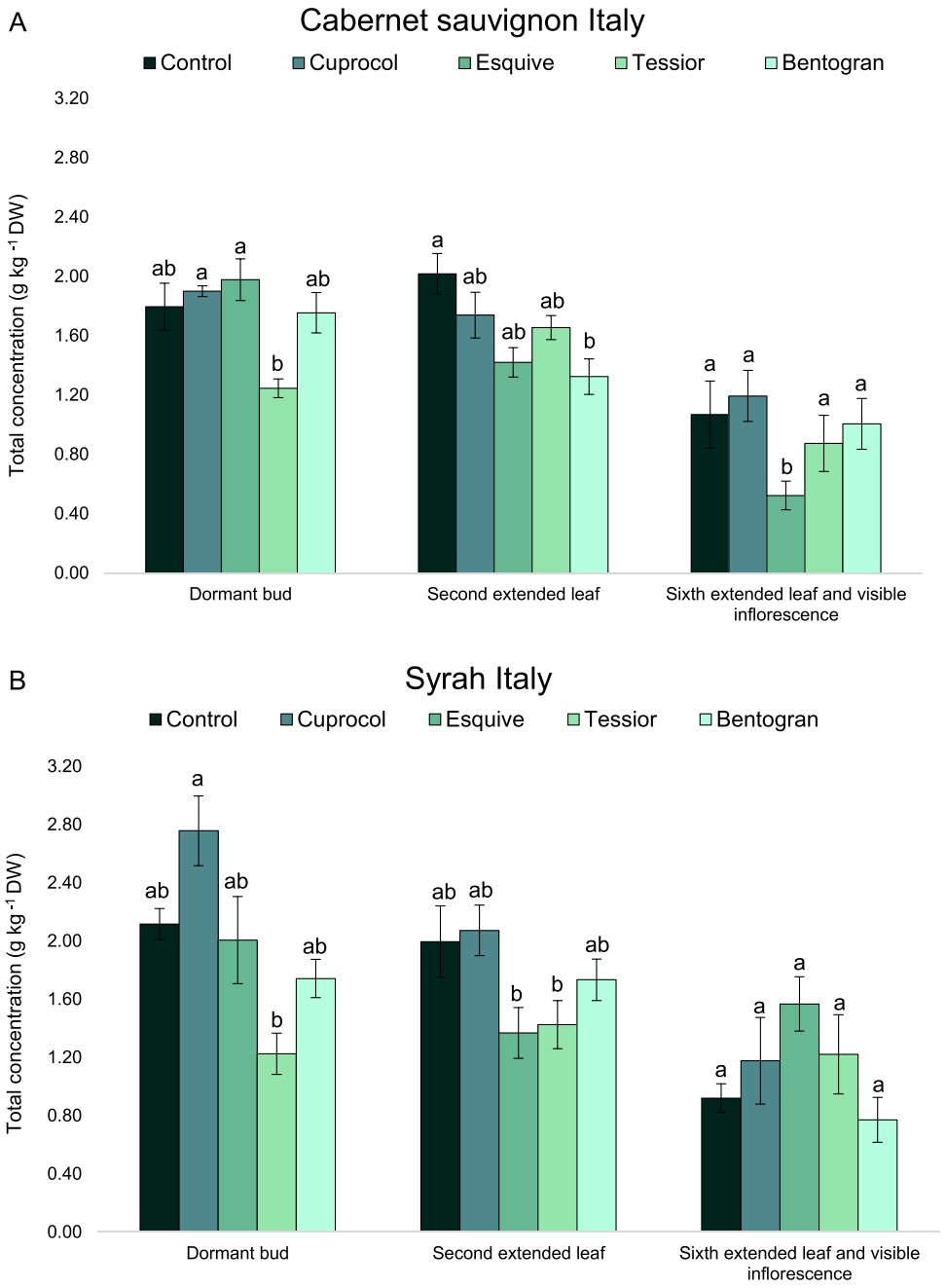

Figure 2. Total stilbene concentrations in one-year-old canes of Cabernet sauvignon (A) and Syrah (B), sampled at the DISAFA vineyard (Italy) at three phenological phases. Average values of four replicates ± standard errors; statistical differences among treatments within the same phenological phase were assessed by the posthoc Bonferroni test at $P \leq 0.05$.

to Italy, with the first sampling at the end of February, the second at the end of March, and the third at the end of April.

Sample Extraction. Collected canes were immediately stored at −80 °C for 1 week. After, they were freeze-dried. To avoid unrealistic results due to the possible effect of the activation of polyphenol oxidases, we discarded the upper 5 mm of the cane and we grounded samples to dust in a mortar, with the aid of liquid nitrogen.[9] Two hundred milligrams of each sample were extracted in 1 mL of methanol: water (8:2 v/v) acidified with 0.1% (v/v) acetic acid in an ultrasonic bath, for 1 h. The extracts were then centrifuged at 4000 g/min for 10 min. The supernatants were filtered (0.2 μm pore size) and stored in vials.[34]

Quantitative and Qualitative Analysis by HPLC−UV/DAD and HPLC-ESI/MS. Wood extracts were analyzed by high-performance liquid chromatography with photodiode array detection (HPLC−UV/DAD) using a 1260 Agilent Infinity System equipped with a reverse-phase HPLC column Licrosphere 100 RP-18 (5 μm particle size) packed with LiChroCART 250-4 (25 × 0.4 cm ID) HPLC-Cartridge (Merck KGaA, Germany) with a guard column (LiChroCART 4−4). The mobile phase consisted of water added with 0.1% (v/v) formic acid (solvent A) and acetonitrile (solvent B), according to a previously published method.[35] Briefly, the flow rate was kept constant at 0.8 mL min^{-1} and the separation was performed at 25 °C under gradient elution conditions, starting with 5% B, increasing linearly to 100% B in 35 min, and keeping 100% B for 5 min. At the end, a reconditioning of the column was carried out eluting the column with 100% B to 5% B in 10 min, followed by a re-equilibration phase under isocratic conditions. Chromatograms were acquired at 320 nm and the injection volume was 20 μL. The system was fully operated by Agilent Technologies (2001−2013) Chem-

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Table 3. (A) Evolution of the Total Stilbene Concentrations (g kg^{-1} Dry Weight) in Cabernet Sauvignon and Syrah One-Year-old Canes, Treated with Different Plant Protection Products, and Sampled at the DISAFA Vineyard (Italy); (B) Significance of the Cultivar Effect, within Each Phenological Phase and Regardless of the Treatment[a]

A) phenological phases				
Cabernet sauvignon				
treatments	dormant bud	second extended leaf	sixth extended leaf and visible inflorescence	level of significance
control	1.80 ± 0.16 ab	2.02 ± 0.14 a	1.07 ± 0.23 b	*
Cuprocol	1.90 ± 0.04	1.74 ± 0.15	1.19 ± 0.17	ns
Esquive	1.98 ± 0.14 a	1.42 ± 0.10 b	0.52 ± 0.10 c	***
Tessior	1.25 ± 0.06 ab	1.66 ± 0.08 a	0.88 ± 0.19 b	*
Bentogran	1.76 ± 0.14 a	1.33 ± 0.12 ab	1.01 ± 0.17 b	*
Syrah				
control	2.12 ± 0.11 a	2.00 ± 0.24 a	0.92 ± 0.10 b	**
Cuprocol	2.76 ± 0.24 a	2.07 ± 0.17 ab	1.18 ± 0.30 b	*
Esquive	2.01 ± 0.30	1.37 ± 0.17	1.57 ± 0.19	ns
Tessior	1.22 ± 0.14	1.43 ± 0.17	1.22 ± 0.27	ns
Bentogran	1.74 ± 0.13 a	1.73 ± 0.14 a	0.77 ± 0.15 b	**

B			
cultivars	dormant bud	second extended leaf	sixth extended leaf and visible inflorescence
Cabernet sauvignon vs Syrah	ns	ns	ns

[a]Averages of four replicates ± standard errors. Per each treatment, different letters (Bonferroni test) represent significant differences among phenological phases (* = $P \leq 0.05$; ** $P = \leq 0.01$: *** $P = 0.001$; ns= no significant).

Station (version 01.05 35; instrument 1). Targeted stilbenes were quantified by the external standard method through calibration curves via HPLC−UV/DAD analysis. The between-lab reliability of the HPLC-UV method was evaluated using an additional HPLC apparatus (instrument 2, see Supplementary Table 1). Superimposable UV chromatographic profiles were obtained in terms of both relative retention times and the ratio between peak areas of the main analytes. Wood extracts were also analyzed by HPLC-ESI/MS, applying the experimental conditions described above (column and elution mode, injection volume 10 μL), using a Thermo Scientific LCQ FLEET system (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a quaternary pump and a Surveyor autosampler connected to an LCQ Fleet ion trap spectrometer through an ESI source, operating under negative (ESI−) ion conditions. The ion spray and capillary voltage were set at 5 kV and −24 V in negative ion mode, respectively. The capillary temperature was 300 °C. The acquisition was performed in Full Scan mode (mass range 50−1000 Da). The Thermo Fisher Xcalibur MS Software Version 2.2 (Thermo Scientific, San Jose, CA, USA) was used for data acquisition and processing. Target stilbenes (E-resveratrol, E-piceatannol, E−ε-viniferin, and E-polydatin) were identified by comparing retention times, UV spectra, and MS patterns of fragmentation of authentic reference standards to those of the compounds detected in wood extracts. The total stilbene concentration was calculated by summing individual stilbenes to those categorized as "unknown" and attributed to stilbenes (Figure 1), based on the similarity of their spectra with those of the UV-spectra of standard E−ε-viniferin and E-resveratrol, particularly in the range of wavelength between 250 and 350 nm (maximum absorption at about 306 and 308 nm). Data were expressed in grams kg^{-1} of dry weight (DW).

Statistical Analysis. All data were analyzed by the RStudio Build 372 software program version for Windows. Analysis of variance was performed by one-way ANOVA and followed by the Bonferroni posthoc test at $P \leq 0.05$. All measurements were performed in quadruplicate, and results were expressed as means ± standard errors.

All normalized data were submitted to a Principal Component Analysis (PCA), using Past 4.03.[36]

RESULTS

DISAFA Vineyard, Italy. *Treatment-Related Stilbene Concentrations and Seasonal Evolution.* In Cabernet sauvignon, at the phenological stage of dormant bud, canes treated with Esquive (1.98 g kg^{-1}), Cuprocol (1.90 g kg^{-1}), Tessior (1.25 g kg^{-1}) and Bentogran (1.76 g kg^{-1}) did not exhibit any significant differences in stilbene accumulation compared to control samples (1.80 g kg^{-1}). Tessior treatment significantly lowered the stilbene concentration with respect to Cuprocol and Esquive treatments ($P \leq 0.05$; Figure 2A). At the phenological stage of the second extended leaf, Bentogran-treated samples (1.33 g kg^{-1}) displayed a lower stilbene concentration compared to controls (2.02 g kg^{-1}; $P \leq 0.05$). Cuprocol, Esquive, and Tessior (1.74, 1.42, and 1.66 g kg^{-1}, respectively) did not show any differences in stilbene total accumulation compared to controls (Figure 2A). At the phenological stage of six extended leaves and inflorescence appearance, Esquive (0.52 g kg^{-1}) significantly reduced cane stilbene concentration compared to control samples (1.07 g kg^{-1}; $P \leq 0.05$) whereas Cuprocol (1.19 g kg^{-1}), Tessior (0.88 g kg^{-1}) and Bentogran (1.01 g kg^{-1}) treatments did not show any significant differences compared to controls (Figure 2A).

In Syrah, at the dormant bud stage, Cuprocol induced the highest stilbene concentration (2.76 g kg^{-1}) although the amount was not statistically different from that observed in controls (2.12 g kg^{-1}; Figure 2B). Esquive (2.01 g kg^{-1}) and Bentogran (1.74 g kg^{-1}) treatments showed stilbene accumulation similar to that of control samples. Syrah exhibited the lowest concentration under the Tessior treatment (1.22 g kg^{-1}), significant if compared to Cuprocol ($P \leq 0.01$; Figure 2B). At the phenological stage of the second extended leaf, statistically significant differences were observed in Esquive (1.37 g kg^{-1}) and Tessior (1.43 g kg^{-1}) samples compared to controls (2.00 g kg^{-1}) and to Cuprocol-treated samples (2.07 g kg^{-1}; significant for $P \leq 0.05$; Figure 2B). When the sixth leaf was extended and the inflorescence became visible, no significant differences were found between the control and treated canes (Figure 2B).

Through the season, Cabernet sauvignon control canes and Tessior-treated canes showed a significant decrease ($P \leq 0.05$) in the total stilbene concentration (−47%), from the second

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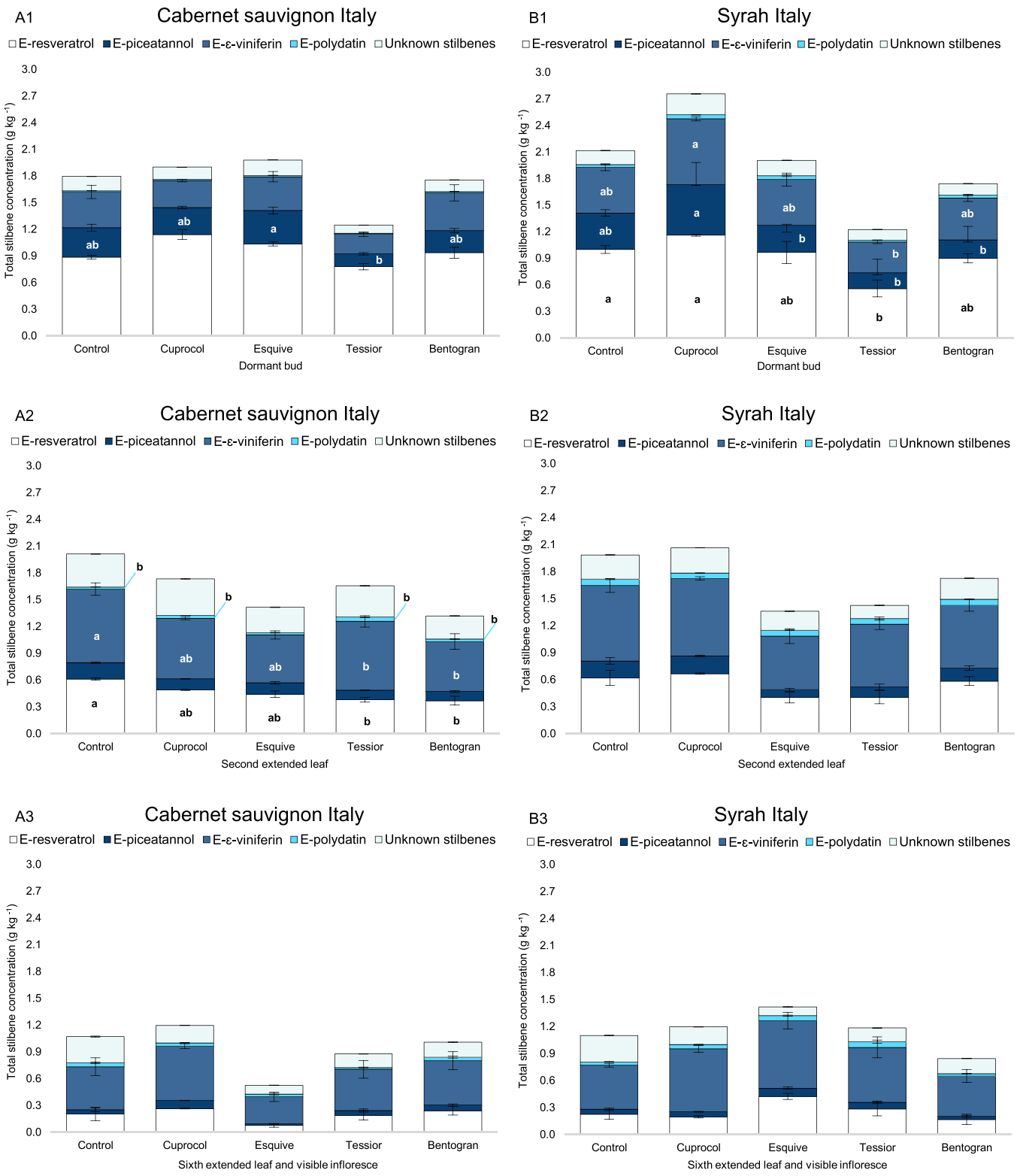

Figure 3. Stilbene profiles of one-year-old canes of Cabernet sauvignon (A1−A2−A3) and Syrah (B1−B2−B3), sampled at the DISAFA vineyard at three phenological phases. Average values of four replicates ± standard errors; per each individual stilbenes, different letters (Bonferroni test at $P \leq 0.05$) represent significant differences among treatments.

phenological phase to the third; similarly, Bentogran samples reduced the total stilbene concentration by 43% (significant between the first and the third sampling time for $P \leq 0.05$; Table 3A). Esquive treatment gradually decreased the total stilbene concentration during the three phenological phases, reaching a reduction of 74% ($P \leq 0.001$; Table 3A). No significant differences were detected in Cuprocol-treated canes over the sampling period. In Syrah, the evolution of stilbene concentrations during the season showed a gradual decrease in control samples (−57%; $P \leq 0.01$), in Cuprocol (−57%; $P \leq$

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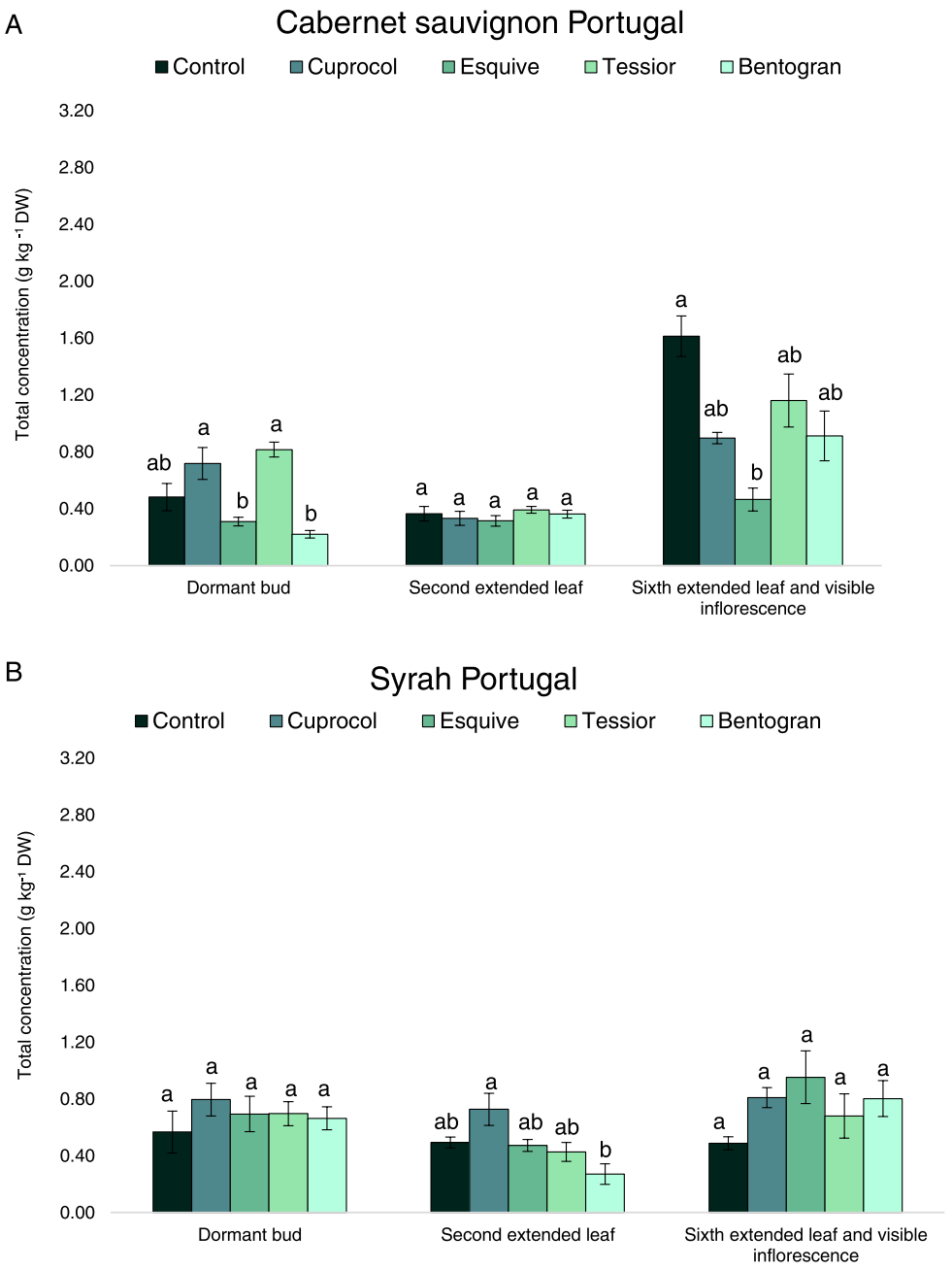

Figure 4. Total stilbene concentrations in one-year-old canes of Cabernet sauvignon (A) and Syrah (B), sampled at the Almotivo vineyard (Portugal) at three phenological phases. Average values of four replicates ± standard errors; statistical differences among treatments, within the same phenological phase, were assessed by the posthoc Bonferroni test at $P \leq 0.05$.

0.05), and in Bentogran (−56%; $P \leq 0.01$) samples, whereas no significant differences were detected in Esquive and Tessior-treated canes over the sampling period (Table 3A).

Taking the average values of the stilbene concentrations of samples collected in Italy per each cultivar, regardless of the applied treatment, we did not find any cultivar-related influence at each phenological phase (Table 3B).

Stilbene Profiles. At the dormant bud phase, E-resveratrol was the predominant molecule in Cabernet sauvignon canes, with an average concentration of 1.00 g kg^{-1}, without significant differences among the treatments (Figure 3A1). At the second phenological phase, its concentration decreased on average up to 0.46 g kg^{-1}. Control samples (0.61 g kg^{-1}) displayed significantly higher concentration ($P \leq 0.01$) compared to Tessior (0.38 g kg^{-1}) and Bentogran-treated canes (0.37 g kg^{-1}; Figure 3A2 and Supplementary Table 2). At the third sampling time, the E-resveratrol average concentration was 0.19 g kg^{-1} and no major significant differences were detected among treatments (Figure 3A3). E−ε-viniferin average concentrations were 0.40, 0.67, and 0.47 g kg^{-1} at the first, second, and third phenological phases, respectively. During the season, no statistically significant variations were detected between treated and control samples (Figure 3A1−A3). At the dormant bud phase, the E-piceatannol concentration was 0.28 g kg^{-1}. Control samples did not show any significant difference compared to treated canes, however, E-piceatannol concentration was significantly higher ($P \leq 0.05$) in Esquive-treated canes (0.38 g kg^{-1}) with

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respect to Tessior-treated canes (0.15 g kg^{-1}; Figure 3A1 and Supplementary Table 2). At the second extended leaf stage, the E-piceatannol average concentration was 0.13 g kg^{-1}. Control samples (0.18 g kg^{-1}) exhibited a significantly higher concentration ($P \leq 0.01$) compared to Tessior-treated canes (0.11 g kg^{-1}) and Bentogran-treated canes (0.10 g kg^{-1}; Figure 3A2 and Supporting Information Table 2). At the third phenological phase, the mean concentration of E-piceatannol was 0.06 g kg^{-1}, without significant differences among the treatments (Figure 3A3). E-polydatin was always found in trace amounts, regardless of the treatment (Figure 3A1−A3). However, at the second extended leaf stage, Tessior-treated canes showed significantly higher concentrations of E-polydatin ($P \leq 0.01$) compared to the other treatments and controls (Figure 3A2 and Supporting Information Table 2). The concentration of unknown stilbenes was on average 0.14, 0.33, and 0.18 g kg^{-1} at the first, second, and third phenological phases, respectively. During the season, no statistically significant variations were detected between treated and control samples (Figure 3A1−A3).

Also in Syrah, at the dormant bud phase, E-resveratrol was the predominant stilbene, with an average concentration of 0.90 g kg^{-1}. Control samples (1.00 g kg^{-1}) and Cuprocol-treated canes (1.16 g kg^{-1}) displayed a significantly higher concentration ($P \leq 0.01$) compared to Tessior-treated canes (0.56 g kg^{-1}; Figure 3B1 and Supplementary Table 3). In the second and third phenological phases, the average concentrations of E-resveratrol were 0.56 and 0.26 g kg^{-1}, respectively. No significant differences among treatments were detected at these two phenological phases (Figure 3B2,B3). At the dormant bud phase, E−ε-viniferin was the second most abundant molecule, with an average concentration of 0.52 g kg^{-1} (Figure 3B1). Control samples did not exhibit any significant differences compared to treated canes; however, Cuprocol induced higher E−ε-viniferin concentration compared to Tessior treatment ($P \leq 0.05$; Figure 3B1 and Supporting Information Table 3). At the stages of the second extended leaf and sixth extended leaf with visible inflorescence, E−ε-viniferin became the main molecule, exhibiting average concentrations of 0.70 and 0.60 g kg^{-1}, respectively, with no major differences between control and treated samples (Figure 3B2,B3). At the dormant-bud phase, E-piceatannol concentration was on average 0.34 g kg^{-1}. Control samples did not show any significant difference compared to treated canes; however, Cuprocol induced higher E-piceatannol concentration compared to Esquive, Tessior and Bentogran-treated samples ($P \leq 0.001$; Figure 3B1 and Supporting Information Table 3). At the second extended leaf, the average concentrations were 0.15 and 0.06 g kg^{-1} at the sixth extended leaf and visible inflorescence stage. No significant differences were found among treatments (Figure 3B2,B3). E-polydatin was present in trace amounts during the three phenological phases, and no differences between treated and control samples were ever detected (Figure 3B1−B3). Unknown stilbenes showed an average concentration of 0.16, 0.23, and 0.17 g kg^{-1} at the first, second, and third phenological phase, respectively. No significant differences were found between control and treated canes (Figure 3B1−B3).

Almotivo Vineyard, Portugal. *Treatment-Related Stilbene Concentrations and Seasonal Evolution.* In Cabernet sauvignon, at the dormant bud phase, control samples (0.48 g kg^{-1}) exhibited an intermediate stilbene concentration with respect to Cuprocol (0.72 g kg^{-1}) and Tessior (0.82 g kg^{-1}) treated samples on one hand and Bentogran (0.22 g kg^{-1}) and Esquive (0.31 g kg^{-1}) on the other (Figure 4A). No significant differences were found between control and treated canes. However, differences were detected (significant for $P \leq 0.001$) between Tessior and Cuprocol (higher concentration) and Esquive and Bentogran (lower concentrations; Figure 4A). At the second sampling time, regardless of the treatment, the total stilbene concentration was averagely 0.35 g kg^{-1}, and no major differences were found among treated and control samples (Figure 4A). At the stage of the sixth extended leaf with visible inflorescences, Esquive significantly reduced the stilbene total concentration compared to control samples ($P \leq 0.01$; Figure 4A). In Syrah at the first sampling time, control canes displayed a concentration of 0.57 g kg^{-1}, which was not significantly different compared to the concentration of treated canes (average 0.71 g kg^{-1}; Figure 4B). During the second sampling time, Cuprocol (0.73 g kg^{-1}) showed significant differences ($P \leq 0.05$) compared to Bentogran-treated samples (0.27 g kg^{-1}) and no significant differences compared to controls (Figure 4B). At the stage of sixth extended leaf and visible inflorescence, the stilbene concentration in controls was 0.49 g kg^{-1}, and no major differences were found between treated and control canes (Figure 4B).

The Cabernet sauvignon total stilbene concentrations exhibited significant differences over the sampling period with the exception of Esquive-treated canes (Table 4A). Control samples showed a seasonal increase of stilbene concentrations of +30% (significant for $P \leq 0.001$, from the second extended leaf to the sixth extended leaf and visible

Table 4. Evolution of the Total Stilbene Concentrations (g kg^{-1} Dry Weight) in Cabernet Sauvignon and Syrah One-Year-old Canes, Treated with Different Plant Protection Products, and Sampled at the Almotivo Vineyard (Portugal); (B) Significance of the Cultivar Effect, within Each Phenological Phase and Regardless of the Treatment[a]

A) phenological phases			
Cabernet sauvignon			
treatments	dormant bud	second extended leaf	sixth extended leaf and visible inflorescence
control	0.48 ± 0.10 b	0.36 ± 0.05 b	1.61 ± 0.14 a
Cuprocol	0.72 ± 0.11 a	0.33 ± 0.05 b	0.90 ± 0.04 a
Esquive	0.31 ± 0.03	0.31 ± 0.04	0.46 ± 0.08
Tessior	0.82 ± 0.05 ab	0.39 ± 0.02 b	1.16 ± 0.19 a
Bentogran	0.22 ± 0.03 b	0.36 ± 0.03 b	0.91 ± 0.18 a
Syrah			
control	0.57 ± 0.15	0.49 ± 0.04	0.49 ± 0.05
Cuprocol	0.80 ± 0.12	0.73 ± 0.11	0.81 ± 0.07
Esquive	0.69 ± 0.12	0.47 ± 0.04	0.95 ± 0.19
Tessior	0.70 ± 0.08	0.43 ± 0.07	0.68 ± 0.16
Bentogran	0.66 ± 0.08 ab	0.27 ± 0.07 b	0.80 ± 0.13 a
B			
cultivar	dormant bud	second extended leaf	sixth extended leaf and visible inflorescence
Cabernet sauvignon vs Syrah	*	***	**

[a]Averages of four replicates ± standard errors. Per each treatment, different letters (Bonferroni test) represent significant differences among phenological phases (* = $P \leq 0.05$; ** $P = \leq 0.01$: *** $P = 0.001$; ns = no significant).

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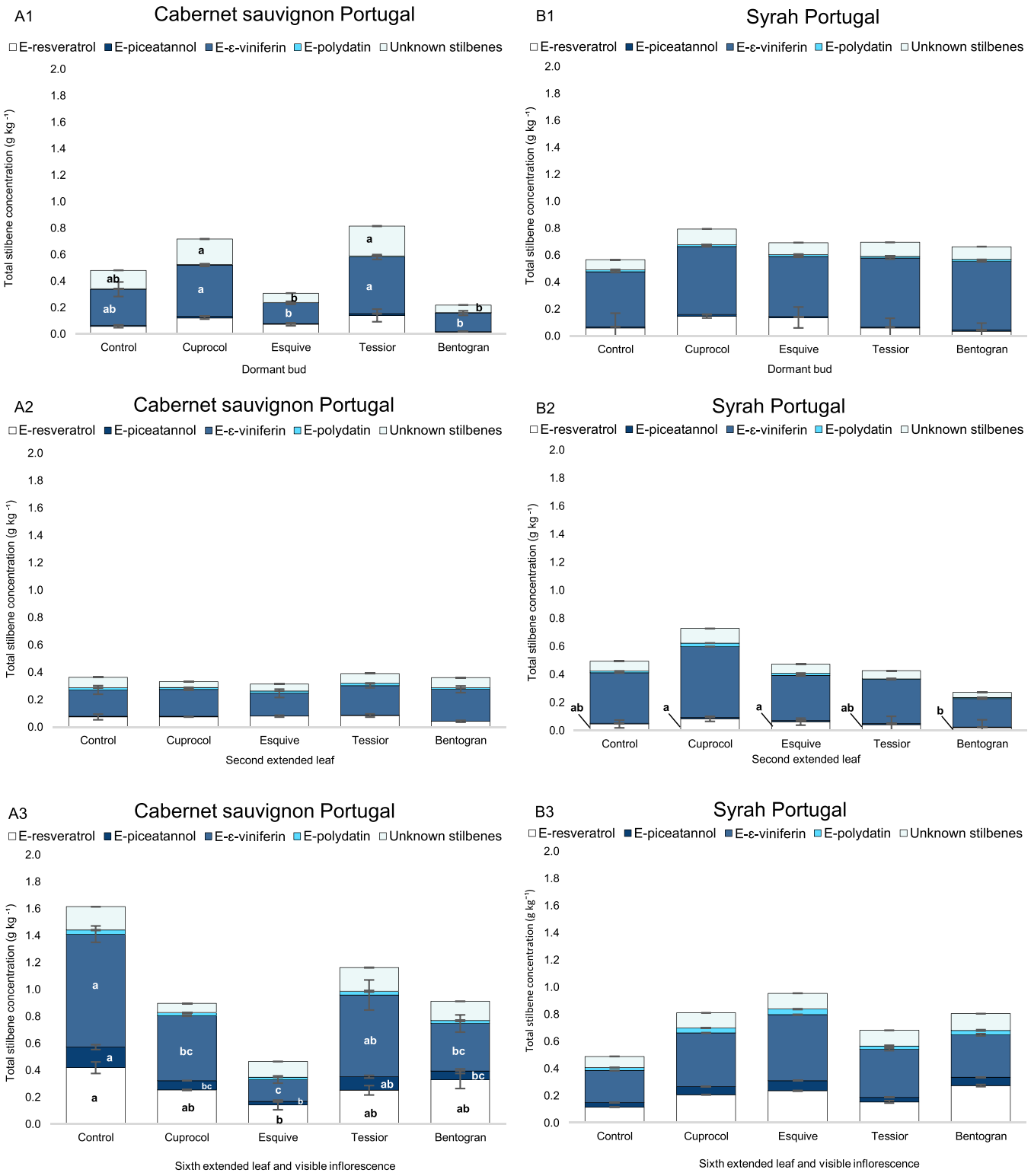

Figure 5. Stilbene profiles of one-year-old canes of Cabernet sauvignon (A1−A2−A3) and Syrah (B1−B2−B3), sampled at the Almotivo vineyard at three phenological phases. Average values of four replicates ± standard errors; per each individual stilbenes, different letters (Bonferroni test at $P \leq 0.05$) represent significant differences among treatments.

inflorescence; Table 4A). Cuprocol and Tessior-treated sample stilbene concentration increase was significant for $P \leq 0.01$ (+37 and +34%, respectively, from the second extended leaf to the sixth extended leaf; Table 4A). In Bentogran-treated samples, the stilbene concentration significantly increased ($P \leq 0.01$; +24% from the second extended leaf to the sixth extended leaf and visible inflorescence; Table 4A). In Syrah, the seasonal evolution of total stilbenes exhibited no major differences throughout the three phenological phases, with the exception of Bentogran-treated samples where concentrations increased by +34% ($P \leq 0.05$, from the second phenological phases to the third; Table 4A).

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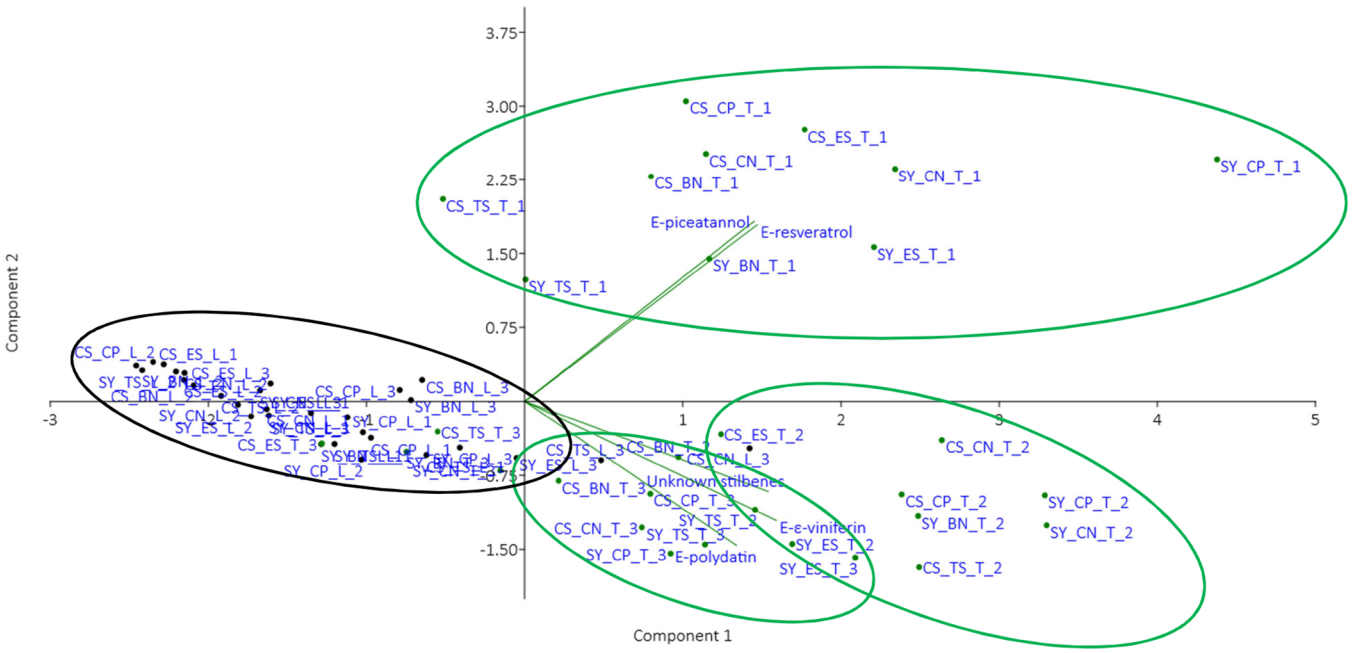

Figure 6. Distribution of the observations on the two first principal components (Component 1 and Component 2) and the 5 variables. CS Cabernet sauvignon, SY Syrah. CN control, CP Cuprocol, ES Esquive, TS Tessior, BN Bentogran. 1 dormant bud, 2 second extended leaf, 3 sixth extended leaf and visible inflorescence.

Comparing the average stilbene total concentrations of the two cultivars, regardless the treatment, differences emerged at the dormant bud stage ($P \leq 0.05$) and at the stage of six extended leaf and visible inflorescence ($P \leq 0.01$. Table 4B).

Stilbene Profiles. In Cabernet sauvignon, at the dormant bud and at the second extended leaf phases, E-resveratrol concentration was on average 0.08 and 0.07 g kg^{-1}, respectively, regardless of the treatment. During these phenological phases, control samples did not exhibit any significant differences compared to treated canes (Figure 5A1,A2). In the third phenological phase, Esquive treatment lowered the concentration of E-resveratrol compared to controls ($P \leq 0.05$; Figure 5A3 and Supporting Information Table 4). At the dormant bud phase, E−ε-viniferin was the predominant molecule with an average concentration of 0.30 g kg^{-1}, regardless of the applied treatment. No significant differences between the control and treated samples were found during this phase (Figure 5A1). However, the E−ε-viniferin concentration between Cuprocol and Tessior (higher concentration) and Esquive and Bentogran (lower concentration) was significant ($P \leq 0.001$; Figure 5A1 and Supporting Information Table 4). At the second phenological phase, the concentration of E−ε-viniferin was on average 0.20 g kg^{-1}, with no differences between treated and control samples (Figure 5A2). At the third phenological phase, the E−ε-viniferin in control samples reached 0.84 g kg^{-1} and it was significantly higher compared to Cuprocol, Esquive, and Bentogran but not when compared to Tessior ($P \leq 0.001$; Figure 5A3 and Supplementary Table 4). During the first and the second phenological phases, E-piceatannol, regardless of the applied treatment, was found in traces (Figure 5A1,A2). At the third phenological phase, E-piceatannol reached a concentration of 0.15 g kg^{-1} in control samples, significantly higher compared to Cuprocol, Esquive, and Bentogran ($P \leq 0.001$; Figure 5A3 and Supporting Information Table 4). E-polydatin was found in trace amounts over the three phenological phases (Figure 5A1−A3), with no significant differences between control and treated samples. At the dormant bud phase, the concentration of unknown stilbenes was similar between controls and treated canes. Cuprocol and Tessior reached higher concentrations compared to Esquive and Bentogran ($P \leq 0.001$; Figure 5A1 and Supplementary Table 4). At the second and third phenological phases, the unknown stilbene concentrations were 0.06 g kg^{-1} and 0.14 g kg^{-1}, respectively, with no statistical differences among treatments (Figure 5A2,A3).

In Syrah, at the dormant bud, the E-resveratrol average concentration was 0.09 g kg^{-1}, without significant differences between control and treated samples (Figure 5B1). At the second extended leaf phase, the E-resveratrol average concentration was quite stable. However, we found significant differences ($P \leq 0.01$) at the second sampling time between Cuprocol and Esquive (higher concentration) and Bentogran (lower concentration), without significant differences with respect to control samples (Figure 5B2 and Supporting Information Table 5). In the third phenological phase, the E-resveratrol average concentration increased, but no significant differences were found among samples (Figure 5B3). In Syrah, like in Cabernet sauvignon, at the dormant bud phase, E−ε-viniferin was the predominant molecule with an average concentration of 0.48 g kg^{-1}, and no major differences among treatments were observed (Figure 5B1). At the second and third sampling times, the concentration decreased to 0.34 and 0.36 g kg^{-1}, respectively, with no significant differences among treatments (Figure 5B2,B3). During the three phenological phases, E-piceatannol, E-polydatin, and the sum of unknown stilbenes accumulated in trace amounts, with no significant differences between control and treated canes (Figure 5B1−B3).

Comparison between the Two Geographical Areas. As marked quantitative and qualitative differences emerged in the comparison between the two growing areas, normalized

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concentration data were used to perform a PCA that showed a net separation of the two geographical areas on the first principal component (PRIN1) which justified 60.6% of the total variance (Figure 6). Variables associated with PRIN1 were E−ε-viniferin, unknown stilbenes, and E-resveratrol; E-piceatannol was associated with the second principal component (PRIN2), justifying 25.4% of the total variance. Based on this variable association, the two geographical areas were clearly separated: samples collected at DISAFA (Italy) were characterized by higher concentrations of E−ε-viniferin, unknown stilbenes, and E-resveratrol at all sampling times, with respect to Almotivo (Portugal). Within the DISAFA area, the first sampling time was clearly separated from the other two samplings based on the high concentrations of E-resveratrol and E-piceatannol (cases were distributed in the positive quadrant of the Cartesian plane). Oppositely, the second and the third samplings were characterized by high amounts of E-polydatin and E−ε-viniferin, and they were distributed in the fourth quadrant of the Cartesian plane. Within the Almotivo area, the association of variables did not allow separating sampling times and cases, with all distributed in the third quadrant. No cultivar discrimination emerged in any cultivation area.

DISCUSSION

In this study, we investigated the total accumulation and profiles of stilbenes in one-year-old canes of Cabernet sauvignon and Syrah, at three different phenological stages, after the application of pruning wound protection products, Cuprocol, Tessior, Esquive, and Bentogran, a mineral product used as a cellar additive. Stilbenes play a crucial role in grapevine defense mechanisms, and understanding their dynamics in separate vineyards, at different phenological stages and in response to PPP distribution, is essential for improving disease management and sustainable viticulture, particularly considering the high reliance of viticulture on fungicides. The susceptibility of grapevines to various pathogens can necessitate up to 20 treatments per year. However, with increasing restrictions, there is an urgent need for more sustainable crop protection methods including organic-based solutions. To ensure a comprehensive evaluation of the interactions between PPPs and wood stilbene accumulation, we conducted our study in two distinct viticultural districts: Piedmont (Turin, Italy) and Lisbon (Portugal). By considering their contrasting climates and geographical features, we aimed to explore how these factors might have influenced grapevine responses to treatments. Lisbon experiences a Mediterranean climate with mild, wet winters and hot, dry summers, while Turin has a continental climate with colder winters and temperate summers (Table 1). The variations in temperature and precipitation between these two areas affect grapevine growth, phenology, and exposure to stressors, potentially influencing the accumulation of stilbenes. The results obtained from the two locations revealed important variations in stilbene accumulation patterns and profiles (Figures 3 and 5). We observed an advancement of phenological phases in Lisbon, occurring approximately one month earlier compared to Turin. Moreover, the seasonal trend in stilbene accumulation showed a striking contrast with a decrease observed in Turin and an increase observed in Lisbon. Major quantitative differences were found between the two cultivation areas (Figures 2, 4, and 6): in both cultivars, the average stilbene concentration, regardless of the treatment, was higher in canes collected in the continental climate (Turin) with respect to that detected in canes from the Mediterranean-type climate (Portugal).

As previously hypothesized,[37] variations in stilbene biosynthesis and concentration suggest the influence of climate and environmental conditions: Pinot noir cultivated in Canada exhibited an average *trans*-resveratrol concentration ranging from 1.3 to 4.1 g kg^{-1} DW;[38] in Chile, in the same cultivar, concentrations up to 3.7 g kg^{-1} DW were found.[21] Additionally, the concentration of *trans*-resveratrol found in Pinot noir cultivated in France was 1.5 g kg^{-1} DW,[39] and in Germany, it ranged between 1.0 and 2.3 g kg^{-1} of DW.[40] An extensive transcriptome analysis of Sangiovese berry skins during ripening[41] highlighted that high temperatures (averages of 26 °C) had an inhibitory effect on stilbene biosynthesis, whereas lower temperatures (averages of 21 °C) induced the expression of several members of the stilbene synthase (STS) and phenylalanine ammonia-lyase (PAL) multigene families. Also Rienth et al., 2014[42] reported an inhibitory impact of high temperature on the grapevine stilbene biosynthetic pathway and Besrukow et al., 2022[40] found that warm and dry conditions could potentially lead to lower stilbene amounts in grapevine canes. Turin and Lisbon displayed notable differences in their average temperatures. In Turin, medium winter temperatures were lower with respect to Lisbon, with January, February, and March average temperatures of 3.0, 6.9, and 8.6 °C, respectively, in Turin versus 11.0, 12.9, and 13.0 °C, in Lisbon. Furthermore, there were notable differences in terms of rainfall between the two locations. In Turin, the rainy days were distributed from January to May, resulting in a total of 121.4 mm and 16 days of rain. Lisbon experienced rainy days exclusively in March (12 days), with a recorded rainfall of 51.6 mm. The contrasting climatic conditions between Turin and Lisbon likely played a pivotal role in shaping the accumulation of wood stilbenes in the respective vineyards. The inhibitory impact of high temperatures combined with scarce rainfall provides a plausible explanation for the lower stilbene levels observed in Lisbon. Beyond climatic factors, the endophytic microbiome represents an additional element that could influence the stilbene accumulation and composition. For example, some endophytes within grapevine leaves can modify their host metabolome.[43] Within wood, certain pathogens trigger the synthesis of stilbenes,[15] while others efficiently metabolize them.[44] Additionally, various fungal endophytes isolated from grapevines are capable of producing resveratrol independently,[45] while others can biodegrade it.[46] In our recent work,[9] we observed major differences in the microbial composition of canes between the vines grown in Turin and those cultivated in Lisbon, and the extent to which certain dominant taxa contribute to stilbene abundance will be evaluated in an upcoming study.

We also observed a divergence in the accumulation trend of wood stilbenes throughout the season in the two vineyards. In the continental-type climate, (Turin) concentrations showed a gradual decline as the phenological stages advanced in both cultivars (always significant, except in Cabernet sauvignon Cuprocol-treated canes and in Syrah Esquive and Tessior-treated canes). In contrast, in Mediterranean climatic conditions (Lisbon), the Cabernet sauvignon stilbene concentration showed an increasing trend from the phenological stage of the second extended leaf onward, excluding Esquive-treated samples, whereas in Syrah the concentration showed a stable trend over the sampling times. Besrukow and collaborators

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(2022)[40] reported that time of sampling and pruning time, influence stilbene total amounts. They observed the highest concentrations in samples harvested in full winter (December) in Pinot Noir, which aligns with the findings reported by De Bona et al., (2020).[47] In addition,[40] also highlighted a hibernal decrease in stilbene concentration from December to February, which is in line with the decreasing trend observed in samples collected in Turin. The increasing trend observed in Cabernet Sauvignon from the Lisbon vineyard, which contrasts with the overall stable trend of Syrah, remains difficult to explain. A plausible explanation for this increase could be attributed to a significant rainfall event in March that created favorable climatic conditions for disease development. In response to this potential threat, grapevines likely activated their defense mechanisms, leading to the accumulation of stilbenes. Despite also Syrah vines being exposed to the same rainfall event, no stilbene accumulation increase was found. This could suggest the existence of another influential factor beyond environmental conditions, the genotype. Cabernet Sauvignon has been recognized as a highly susceptible grapevine cultivar to wood diseases, whereas Syrah displays a lower susceptibility. We can thus hypothesize that Cabernet sauvignon has evolved a more sensitive and rapid response system in wood tissues to react to stress conditions, especially those conducive to fungal inoculation. This heightened responsiveness might explain its higher level of stilbenes. Acknowledging the potential impact of different sampling/pruning times on stilbene concentration, we based our sampling on the vine phenological stage rather than on Julian Day. This method ensured that we collected samples at specific growth stages, trying to guarantee the acquisition of homogeneous and representative samples from the two growing areas. Consequently, we minimized the influence of the sample collection time on the observed differences. By adopting this strategy, any variations in stilbene concentration between Turin and Lisbon were more likely attributable to the contrasting environmental conditions rather than to the timing of sample collection.

In both vineyards, following the application of the pruning wound protection products, we did not observe any distinct responses in stilbene total concentration with respect to control samples. In the continental climatic conditions, we found a reduced concentration in Bentogran-treated samples (second sampling) and Esquive-treated samples (third sampling) in Cabernet sauvignon and after the application of Tessior and Esquive in Syrah (second sampling for both). In Mediterranean climate conditions, Esquive reduced total stilbene concentration in Cabernet sauvignon (at the third sampling). Thus, from our study, it appears that there is no uniform response dynamic following the application of treatments, except a general capability of Esquive to reduce the concentration of stilbenes. In the literature, some studies evaluated the role of protection products on the accumulation of phenolic compounds. They demonstrated a significant up-regulation of STS in the leaves of a grapevine hybrid (Summer black) after the distribution of copper on the canopy of two-year-old vines[48] and an eliciting effect when $CuSO_4$ was sprayed on the leaves of Chardonnay plantlets in *in vitro* conditions.[49] Other authors[11] described the role of a copper-based product (LC2017) as an elicitor, demonstrating its capacity to activate PAL and STS, in the wood of three-node cuttings of Cabernet sauvignon. Copper treatment commonly leads to the rapid accumulation of reactive oxygen species (ROS) within plant cells, subsequently triggering oxidative stress and interfering with plant vital processes, including photosynthetic activity and electron transport and affecting the functionality of chloroplast membranes.[11] Further investigations into the assessment of chemicals employed as protectants against GTDs, revealed that vines treated with copper might develop necrotic lesions when infected with GTD pathogens and that symptoms may manifest even in copper-treated but noninoculated vines,[50] implying a potential phytotoxic effect. In our study, conducted in "in field" conditions and on mature vines, Cuprocol did not lead to a visible stimulatory effect on stilbene accumulation with respect to control samples nor in Cabernet sauvignon or in Syrah, in both continental and Mediterranean climates.

Several studies deal with the evaluation of the efficacy of *Trichoderma* species on vine pruning wound protection, as reviewed in a previous study,[1] showing encouraging results. *Trichoderma* species have been described as plant growth stimulators and defense stimulators. Treatment with *Trichoderma* species induced the expression of PAL and STS.[51] but in another study,[31] the use of another *Trichoderma*-based product, resulted in a mild plant metabolomic response that was sufficient to differentiate between treated and untreated samples, but it was not enough to conclude that the treatment had a significant stimulating effect on the plant defense activation. In our study, Esquive treatment led to a reduction in stilbene concentration in Cabernet sauvignon in both climatic conditions, at the stage of the sixth extended leaf and visible inflorescence, whereas in the former phenological stages, Esquive occasionally induced a higher stilbene accumulation. However, as stated in[31] the metabolic response was mild and not definite enough to assert that it effectively and definitively elicits a defensive response in the vines.

To the best of our knowledge, there are no studies on the possible elicitor effects of Tessior treatment on grapevine defense response. Some studies focused on the effectiveness of Tessior application on pruning wounds demonstrating its capability to reduce GTD infections.[52] The findings of our study do not provide a clear interpretation. In fact, in the continental climate area, both cultivars exhibited a lower concentration of stilbenes after Tessior treatment (in some sampling stages) compared to the control samples. In the Mediterranean climate conditions, no differences in stilbene concentration in Cabernet sauvignon and Syrah Tessior-treated samples compared to the controls were detected. Further investigations are necessary to fully comprehend the mechanisms of action of Tessior and its possible impact on grapevine stilbene metabolism.

Finally, we used Bentogran, an enological cellar product, for the clarification of wines. The mechanisms of action of sodium bentonite in enology rely on its ability to absorb unstable proteins present in must and wine. It acts as a clearing agent, binding unstable proteins, and forming insoluble complexes that precipitate and settle at the bottom, allowing the removal of impurities. We were aware that this product has no direct fungicidal effect, but our purpose was to investigate the possible reuse of this waste product from the cellar, to reduce waste, and to increase recycling in the vine-wine sector. Considering that sodium bentonite solidifies when rehydrated, we hypothesized to propose it as a physical protection for pruning wounds. In the continental climate area, in both cultivars, the results were satisfactory. Even though its application did not lead to a significantly higher accumulation of stilbenes with respect to control samples, it often induced a

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higher accumulation, compared to the other treatments. For example, both in Cabernet sauvignon and in Syrah, its application resulted in a higher accumulation compared to the Tessior treatment. Further research on this product would be potentially interesting to gain a comprehensive understanding of its effects on stilbene metabolism. Moreover, it would be interesting to investigate the effects it could have on "plant pathogenesis-related proteins", given its ability to bind proteins. This information would provide valuable insights into its potential role in enhancing plant defense mechanisms and its broader implications in viticulture and vine health.

Qualitative differences in the stilbene profiles emerged between the canes collected in continental climate and Mediterranean conditions. In continental climate conditions, E-resveratrol was the predominant molecule in both cultivars, and E−ε-viniferin became the predominant molecule with the advance of the season. In the Mediterranean climate, E−ε-viniferin was the main molecule, in both cultivars, during all three phenological phases. STS represents crucial enzymes facilitating the condensation of three malonyl-CoA units and one cinnamic-acid-derived CoA-ester (p-coumaroyl-CoA). This process results in the initial production of E-resveratrol (3,5,4′-trihydroxystilbene); oligomerization and glycosylation reactions of E-resveratrol happen later in the biosynthetic pathway, resulting in the formation of E−ε-viniferin through the dimerization process.[53] A decrease of *trans*-resveratrol with a simultaneous increase in E−ε-viniferin was already observed,[40] confirming a negative correlation between the contemporaneous presence of monomers and oligomers. Moreover Chong et al., (2009)[54] highlighted a positive correlation between resveratrol and other monomeric stilbenes, such as piceatannol. In our study, we confirmed the findings from these two previous studies. In fact, in both continental and Mediterranean climates, the E-resveratrol concentration was positively correlated with the concentration of E-piceatannol and negatively correlated with that of E−ε-viniferin. However, the pattern of individual stilbene accumulation differed in continental climates compared with the Mediterranean conditions for both cultivars. Drier and warmer conditions were associated with a reduced abundance of oligomeric stilbenes[40] whereas cold and wet conditions induced a lower abundance of monomeric stilbenes. In our studies, the concentration of E−ε-viniferin, in both localities, increased following a rainy period, creating favorable conditions for fungi development. In the vineyard grown in continental conditions, 100 mm of rain was recorded between April and May (corresponding to the phenological phase of the second extended leaf and the sixth extended leaf with visible inflorescence). In the Mediterranean climate vineyard, in March (dormant bud phase), a precipitation event allowed the accumulation of 52 mm of rain. Thus, we could hypothesize that the increased concentration of E−ε-viniferin, possessing a higher antifungal efficacy than E-resveratrol, is directly related to the rate of environmental humidity, which creates favorable conditions for pathogen development and increases the need for vines to produce phytoalexins, to face pathogens.

We observed minimal differences in the concentrations of individual stilbenes between the control and treated canes, with few exceptions. Indeed, we did not detect a consistent pattern of the accumulation of individual stilbenes following treatment application. Furthermore, the PCA analysis revealed sample clustering based on the accumulation of individual molecules, particularly in cultivars grown in continental climates, which appeared to be more influenced by the phenological phase rather than by the treatment. Conversely, in cultivars cultivated in Mediterranean climates, there was no discernible differentiation in the accumulation of individual stilbenes, regardless either the phenological phase or the applied treatment. Therefore, we can conclude by highlighting the role of the seasonal evolution of wood stilbenes and the incidence of the cultivation area on both total concentrations and profiles, rather than the possible effect of fungicide treatments.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.4c00583.

Table 1: additional HPLC apparatus used to evaluate the between-lab reliability of the HPLC-UV method utilized in the experiment; Table 2: concentrations of individual stilbenes of Cabernet sauvignon canes collected at the DISAFA vineyard; Table 3: concentrations of individual stilbenes of Syrah canes collected at the DISAFA vineyard; Table 4: concentrations of individual stilbenes of Cabernet sauvignon canes collected at the Almotivo vineyard; Table 5: concentrations of individual stilbenes of Syrah canes collected at the Almotivo vineyard (PDF)

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Author Contributions

C.I., G.D.F., R.B.F. and A.F. conceived the study. C.I. and G.D.F. performed the field experiment. C.I. prepared the extracts and performed the HPLC-UV/DAD analysis, with the help of M.F. and M.P., under the supervision of A.F. E.T. performed the HPLC-UV and HPLC-ESI/MS analysis under the supervision of D.R. D.R. and S.C. evaluated the between-lab reliability of the analytical method. C.I. and A.F. analyzed data and wrote the draft. C.I., G.D.F., and A.F. edited the final manuscript. All authors read and agreed to the published version of the manuscript.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

GTDs	Grapevine trunk diseases
PPPs	Plant protection products
DW	Dry weight
PAL	Phenylalanine ammonia-lyase
STS	Stilbene synthase

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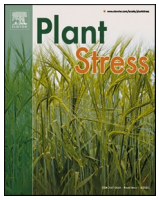

Fungal community dynamics and anthocyanin profiling of grapevine leaves in a vineyard affected by esca☆

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ABSTRACT

Leaves in grapevines affected by esca, a grapevine trunk disease, may occasionally manifest a symptom known as the 'esca leaf stripe symptom.' Most frequently, this symptom appears as scorching in the interveinal tissue, with red pigmentation observed between scorched and healthy tissue. However, purple pigmentation or an absence of pigmentation is occasionally reported. The synthesis and accumulation of anthocyanins may drive these different symptom phenotypes. Recent evidence also implicates fungal endophytes in host manipulation, potentially influencing grapevine metabolic profiles, including anthocyanins. In this study, working on cultivars Cabernet Sauvignon and Touriga Nacional, we used DNA metabarcoding (i) to explore the microbial dynamics of endophytes during symptom progression in esca-affected leaves, and (ii) to reveal the fungal diversity for different symptom phenotypes, along with their qualitative and quantitative anthocyanins composition.

The endophytic mycobiome profiling revealed a large fungal richness (260 taxa), and a beta diversity influenced by cultivar ($P < 0.01$) and vintage ($P = 0.001$). We observed significant differences in beta diversity between leaves affected by chlorotic spots and asymptomatic ones ($P < 0.05$), revealing major shifts in fungal community composition during early stages of esca symptom progression. Comparing asymptomatic leaves and different symptom phenotypes, we detected cultivar and vintage-dependent alterations in alpha and beta diversity, as well as in individual taxa abundance (e.g. *Botrytis caroliniana* over-represented in red leaves). Total anthocyanin accumulation was influenced by cultivar ($P \leq 0.0001$) but not by vintage. In Touriga Nacional, and to a lesser extent in Cabernet Sauvignon, purple leaves accumulated significantly lower amounts of trihydroxylated anthocyanins and acyl-derivatives, when compared to red leaves.

Fungal communities significantly alter in composition during esca symptom progression, and for different symptom phenotypes, suggesting a strong correlation between microbial structure and the physiological and biochemical processes that occur in leaves.

1. Introduction

Charming to the eye, yet a sign of doom, the esca leaf stripe symptom is one of the least understood aspects of the esca complex of diseases (Del Frari et al., 2022; Del Frari et al., 2021; Lecomte et al., 2012) (Fig. 1). Symptomatic grapevines (*Vitis vinifera* L.) are currently included in the

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syndromes grapevine leaf stripe disease (GLSD) and esca proper, depending on wood symptomatology and fungal pathogens found in perennial organs (Surico, 2009; Mondello et al., 2018). Typical wood symptoms found in grapevines manifesting the esca leaf stripe symptom, also known as 'tiger stripes', are brown wood streaking, cankers, and, most frequently, white rot (Del Frari et al., 2021; Surico, 2009; Mugnai et al., 1999; Gramaje et al., 2018), which lead to losses in plant vigor, yield quantity and quality, and premature plant death (Fontaine et al., 2016). Interestingly, not all wood-symptomatic vines manifest tiger stripes. In GLSD and esca proper-affected vines, the leaf stripe symptom is known to appear discontinuously, from year to year; its onset may occur over an extended period of time, between late spring and mid-summer; it may affect the whole canopy or only portions of it, *e.g.* single shoots, multiple shoots on the same spur or an entire arm (Lecomte et al., 2012; Del Frari et al., 2019; Serra et al., 2018). Moreover, numerous variables influence symptoms onset and severity, such as biotic and abiotic factors, and agricultural practices (Del Frari et al., 2021). Overall, this suggests that wood symptoms and their causal agents are not the sole responsible for tiger stripes, but they are only one piece of the puzzle. This conclusion is further supported by the overwhelming lack of success in past attempts of reproducing symptoms by artificial inoculations with esca-associated fungi, with only a few exceptions (e.g. Del Frari et al., 2021), and by the controversy that still surrounds the identity of the leaf stripe symptom triggering factor(s) (Del Frari et al., 2022; Del Frari et al., 2021). The microbiological aspect of perennial organs in grapevines manifesting tiger stripes and the available control strategies to mitigate symptoms incidence and severity have been recently reviewed in Del Frari et al. (2022), Del Frari et al. (2021).

A yet unexplored aspect of symptomatic leaves concerns three symptom phenotypes that may manifest in GLSD or esca-affected grapevines (Fig. 1). The similarities among them are the interveinal necrotic lesions and the green lamina portions in proximity of the main veins, while the differences concern symptom onset, progression and the lamina pigmentation between necrotic lesions and green tissue.

(1) Esca tiger stripes is the most frequently observed symptom phenotype in vineyards, and the first described in the scientific literature (Mugnai et al., 1999; Viala, 1926). Phenotypic alterations in leaves start with the appearance of scattered chlorotic spots that may expand and coalesce (Fig. 1, C). Subsequently, necrotic lesions appear in correspondence of some chlorotic spots and portions of the lamina surrounding chlorotic tissue acquire pigmentation (Fig. 1, II, D), typically described as tonalities of red (Lecomte et al., 2012; Surico, 2009; Mugnai et al., 1999; Serra et al., 2018). Leaf margins may or may not turn necrotic and interveinal lesions may coalesce or remain of constant size throughout the growing season (Lecomte et al., 2012).

(2) In apoplectic leaves, portions of interveinal tissue undergo a rapid phase of discoloration, turning pale green to gray, they necrotize (Fig. 1, III), and wilt. After necrotizing, affected shoots typically lose all leaves in a matter of days (Lecomte et al., 2012; Spagnolo et al., 2012; Magnin-robert et al., 2017). Alterations in the pigmentation of the lamina between necrotic lesions and green tissue -in proximity of the main veins- are minimal or absent, although a thin violet strip is occasionally observed bordering the necrotic tissue.

(3) In 2001, Larignon and colleagues brought attention over the black dead arm (BDA) syndrome, a putative grapevine wood disease caused by *Botryosphaeriaceae* fungi, characterized by esca leaf stripe-like phenotype (Larignon et al., 2001; Larignon and Dubos, 2001). In this third symptom phenotype, symptoms manifest earlier in the season, when compared to esca tiger stripes, and they appear as wine-red to purple areas on the margins and interveinal areas of the lamina. Subsequently, necrotic lesions appear in correspondence of some pigmented areas and may coalesce similarly to esca tiger stripe. Necrotic lesions remain surrounded by varying proportions of wine-red to purple tissues, which divide them from green tissues, localized in proximity of the main veins (Fig. 1, I) (Lecomte et al., 2012; Larignon et al., 2001; Úrbez-Torres, 2011; Kuntzmann et al., 2010). In addition, researchers detected metabolic, cytological and histological differences between BDA and esca leaves (Valtaud et al., 2009; Valtaud et al., 2011), or associated canes (Fleurat-Lessard et al., 2013). However, over the following years, multiple authors challenged the view that the BDA syndrome is distinct from esca, bringing forward three main issues: (i) the BDA phenotype may occasionally transition to esca phenotype during the growing

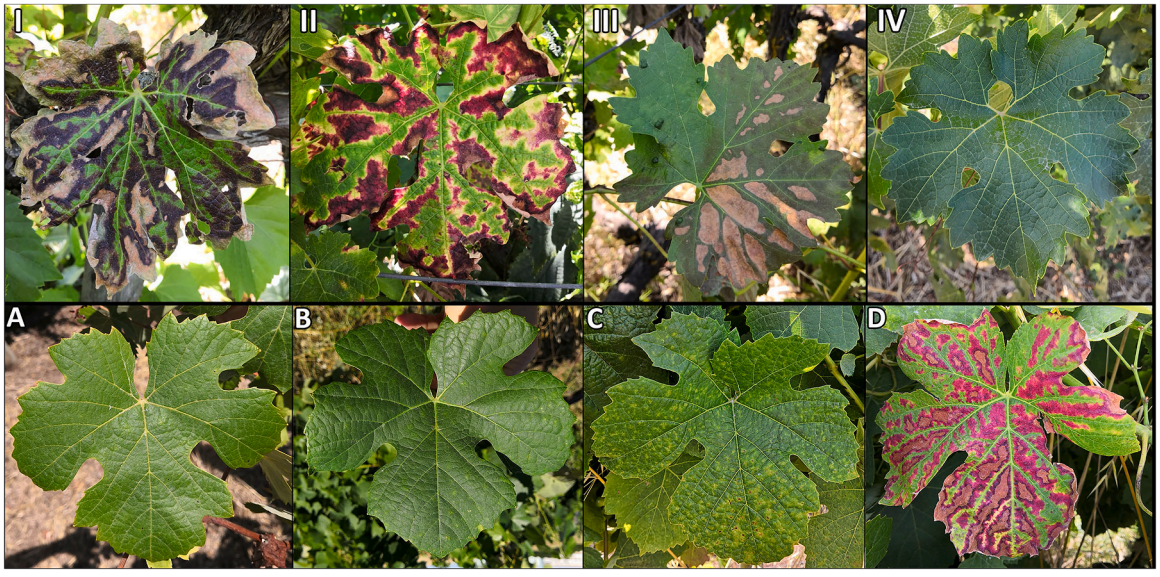

Fig. 1. Grapevine leaves (cv. Cabernet Sauvignon) manifesting different symptom phenotypes (I, II, and III), and asymptomatic leaves (phenotype IV; Asy). Phenotype I is identified as BDA (black dead arm, *sensu* Larignon (Larignon et al., 2009)), phenotype II as Esca / esca tiger stripes, and phenotype III as apoplectic leaves (Apox). Grapevine leaves (cv. Touriga Nacional) in asymptomatic shoots, sampled between the fifth and seventh internode (A; YAsy), and two internodes below (B; Asy); in symptomatic shoots, sampled between the fifth and seventh internode, affected by scattered chlorotic spots (C; Chl), and two internodes below, Esca leaves (D).

season; (ii) in red varieties, esca tiger stripes may exhibit a broad range of coloring patterns, and it is possible – albeit infrequent – to find both wine-red to purple and tonalities of red pigmentation on the same leaf or on different leaves on the same shoot; (iii) the BDA phenotype has not been entirely reproduced by artificial inoculation with putative BDA-associated fungi (Lecomte et al., 2012; Úrbez-Torres, 2011; Kuntzmann et al., 2010; Lecomte et al., 2006; Surico et al., 2006). For these reasons, the BDA phenotype is currently included in the cluster of symptoms manifested in esca-affected grapevines, therefore not a separate syndrome, yet, experimental evidence explaining the observed differences in pigmentation, symptom onset and progression is still missing.

A significant body of research has been carried out to assess anatomical, physiological, gene expression, and metabolomic alterations occurring in tiger striped leaves (Magnin-robert et al., 2017; Valtaud et al., 2011; Lima et al., 2010; Goufo and Cortez, 2020; Goufo and Singh, 2021; Magnin-Robert et al., 2011; Calzarano et al., 2016; Calzarano et al., 2021; Weiller et al., 2024; Bortolami et al., 2023). However, the microbiological aspect has been largely neglected. While it is known that esca-associated pathogens are not found in symptomatic leaves (Mugnai et al., 1999), the role of the endophytic mycobiome and its interaction with grapevine leaf physiological and biochemical processes remains to be evaluated. In a recent paper, Chen *et al.* (2020) identified multiple fungal taxa over- or under-represented in differently colored grapevine leaves, suggesting a potential correlation between microbiota and total anthocyanins (Chen et al., 2020). Anthocyanins are secondary metabolites responsible for berry pigmentation (Castellarin et al., 2006; Castellarin and Di Gaspero, 2007); they can also accumulate in vegetative organs, mainly leaves, as a line of defense against environmental stress, such as exposure to high light radiation (Tattini et al., 2005; Tattini et al., 2014), to protect chloroplasts and to reduce the incidence of photo-oxidative stress (Lev-Yadun and Gould, 2008; Feild et al., 2001). In leaves, anthocyanins are accumulated in the cytosol and stored in the vacuole of epidermal and/or mesophyll cells (Kedrina-okutan et al., 2018; Flamini et al., 2013; Hatier and Gould, 2008), but their localization may change during leaf ontogenesis (Merzlyak et al., 2008). In addition, anthocyanins are involved in grapevine-pathogen interactions: they are absent in adult and healthy leaf blades (Kedrina-okutan et al., 2018), whereas they may accumulate in large amounts after pathogen attack, like in the case of *flavescence dorèe* and *bois noir* phytoplasmas, and grapevine leafroll-associated virus (Margaria et al., 2014; Negro et al., 2020; Gutha et al., 2010). Moreover, anthocyanins accumulate at specific developmental stages and/or during physiological changes, such as leaf senescence (Hoch et al., 2001; Matus et al., 2017). In fact, a popular hypothesis is that anthocyanins are the end products of leaf senescence because of the overflow of carbohydrates during the recovery of photosynthetic products (Feild et al., 2001).

Fungal endophytes are known to manipulate their host (Collinge et al., 2022; Dupont et al., 2015; Giauque et al., 2019), and recent evidence revealed endophyte-dependent alteration in metabolic (Pan et al., 2020) and anthocyanin (Yu et al., 2020) profiles in grapevine leaves and other crops (Ważny et al., 2021; de Vries et al., 2018). In an attempt of clarifying the involvement of grapevine leaf fungal endophytes and anthocyanins in the three symptom phenotypes described above, we set three main objectives. (1) – To characterize the endophytic mycobiome of grapevine leaves using DNA metabarcoding (next-generation sequencing; NGS); (2) – To assess the microbial dynamics during the progression of esca tiger stripes, from scattered chlorotic spots to fully symptomatic leaves. (3) – To explore fungal community composition, anthocyanin concentration and profile, and the relationship between the two, when comparing different symptom phenotypes and asymptomatic leaves.

2. Materials and methods

2.1. The vineyard, field sampling, and samples processing

2.1.1. The vineyard

Field sampling took place in the experimental vineyard (Almotivo) of the Instituto Superior de Agronomia, University of Lisbon, Lisbon, Portugal (38°42′32.7″N, 9°11′11.5″W). The vineyard has a density of 3333 plants/ha, the soil is classified as vertisol, it is managed under conventional agricultural practices and rainfed. The selected cultivars were Cabernet Sauvignon, known for its high susceptibility to grapevine trunk diseases (Gastou et al., 2024; Csótó et al., 2023) and Touriga Nacional, a less susceptible cultivar, both grafted on 140 Ruggeri rootstock (*Vitis berlandieri* × *Vitis rupestris*), trained as bilateral cordon Royat and spur-pruned. Grapevines were planted in 1998, and they were 22 and 23 years-old at the time of sampling. The vineyard has a history of esca, with leaf-symptomatic grapevines accounting for ≤1 % of the total plants in all recorded years (from 2015 to 2022). An in-depth microbiological analysis of fungal endophytic communities detected in cv. Cabernet Sauvignon can be found in Del Frari et al. (2019) and Del Frari et al. (2023). Symptoms correlated to other grapevine trunk diseases were also detected (Vaz et al., 2020).

2.1.2. Field sampling

Sampling took place on June 23rd, 2020, and on June 17th, 2021, at the phenological stage of 'pea-sized' berries, at the beginning of the seasonal manifestation of symptoms. Plant protection products (Pergado F, Syngenta, 5 % Mandipropamid + 40 % Folpet; Indar 5EW, Dow, 4.95 % Fenbuconazole) were sprayed three (2020) or two (2021) weeks before sampling. Due to symptom discontinuity, individual grapevines sampled in 2021 were not the same sampled on the previous year.

To address Objective 2, we examined cvs Cabernet Sauvignon and Touriga Nacional, in 2020. We established four categories, based on symptomatology and leaf age (Fig. 1, A - D). On asymptomatic shoots, we sampled (A) – 'Young asymptomatic leaves' (YAsy), between the fifth and seventh internode, and (B) – 'Asymptomatic leaves' (Asy), two internodes below YAsy leaves. On symptomatic shoots, we sampled (C) – leaves affected by 'scattered chlorotic spots' (Chl), between the fifth and seventh internode, and (D) – 'Esca leaves' (Esca), characterized by interveinal necrosis followed by chlorotic, red and green tissues, two internodes below Chl leaves. The term 'Esca' (with a capital E) is used in the text specifically for the leaf symptoms just described, as opposed to 'esca', which refers to the disease complex. Asymptomatic shoots were monitored throughout the growing season to confirm that leaves remained asymptomatic.

To address Objective 3, we examined cvs Cabernet Sauvignon and Touriga Nacional, both in 2020 and 2021. Symptomatic and asymptomatic leaves were collected between the third and fifth internode and, at the time of sampling, approximately 10–25 % of the surface area of the symptomatic leaf blade presented necrotic lesions. We established four categories based on leaf symptom phenotypes (Fig. 1, I - IV). (I) – 'BDA leaves' (BDA), *sensu* Larignon (Larignon et al., 2009), characterized by interveinal necrosis followed by dark purple and green tissues; (II) – 'Esca leaves', as described above (Objective 2, category D); (III) – 'Apoplectic leaves' (Apox), characterized by interveinal necrosis followed by green tissue; (IV) – 'Asy leaves', as described above (Objective 2, category B). Overall, from the day of sampling, Esca and BDA leaves persisted on the shoots for at least two weeks, while Apox leaves withered and fell within one week. Apox leaves were sampled exclusively in 2020, as this symptom phenotype was not detected in 2021. In Esca leaves (symptom phenotype II), chlorotic tissue was less evident/absent in cv. Touriga Nacional, while always present in cv. Cabernet Sauvignon (Fig. 1, II, D). At the time of sampling, there were no visible symptoms of a leaf pathogen infection (e.g. downy or powdery mildews) or mineral deficiency.

For each of the above-mentioned categories (Objectives 2 and 3), we

collected between 8 and 12 biological replicates, each of them corresponding to leaves sampled from a single shoot, on a single vine. While in the field, sampled leaves were stored in ice, in sterile plastic bags, and then moved to a −80 °C freezer until sample processing.

2.2. Sample processing, DNA extraction, library preparation and sequencing

Leaves were deprived of necrotized tissue with the use of sterile scissors and they were surface sterilized to remove epiphytes, following a procedure similar to that described in Fan et al. (2020). Briefly, for each category, all biological replicates underwent double rinsing in sterile distilled water followed by immersion in a solution of sodium hypochlorite 3 % (v/v) for 5 min, followed by a triple rinse in sterile distilled water. Aliquots of the water from the last rinse were plated on potato dextrose agar and incubated at 25 °C, in the dark, for two weeks to confirm the absence of microbial growth.

After sample processing, five surface sterilized leaves per each category*cultivar*year were ground to dust using sterile mortars and pestles with the aid of liquid nitrogen. Aliquots of 0.15 g from each ground biological replicate were used for DNA extraction. This step was performed by combining the method described in Cenis (1992) and the ZymoBIOMICS™ DNA Miniprep Kit. Briefly, in lysis tubes, we added leaf sample and extraction buffer as in Cenis (1992), we then processed the samples in a bead beater fitted on a vortex for 10 min followed by heating at 65 °C for 10 min. The DNA extraction protocol was then followed as per (Cenis, 1992) up until the DNA precipitation step, which was followed by DNA binding, washing, and elution steps as described in ZymoBIOMICS™ manufacturer instructions. Two negative controls of the DNA extraction procedure were added.

The amplicon chosen in this study targets the internal transcribed spacer region 1 (ITS1), and the primer set selected was ITS1F2 – ITS2 with overhang recommended by Illumina. The full sequence of the primers, including Illumina overhangs, is the following: ITS1F2 (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-GAACCWGCGGARGGATCA-3′) and ITS2 (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-GCTGCGTTCTTCATCGATGC-3′) (Gaylarde et al., 2017). For building libraries, we used a double-step PCR approach as reported by Feld et al. (2015). Each first-step PCR reaction contained 12.5 μL of Supreme NZYTaq II 2x Green Master Mix™ (NZYtech™), 0.5 μL of forward and reverse primers from a 10 μM stock, 1.5 μL of sterile water, and 5 μL of template. Each reaction was pre-incubated at 95 °C for 2 min, followed by 39 cycles of 95 °C for 15 s, 55 °C for 15 s, 72 °C for 40 s; a further extension was performed at 72 °C for 10 min. Second PCR-step for barcoding, fragment-purification by using MagBio beads, and Qubit quantification was performed as reported in Gobbi et al. (2019). Final pooling was performed at 10 ng/sample.

DNA Sequencing was performed using an in-house Illumina® MiSeq instrument and 2 × 250 paired-end reads with V2 Chemistry.

2.3. Bioinformatics

Data produced by sequencing have been demultiplexed using our Illumina® Miseq platform. Demultiplexed reads were analyzed using QIIME2 v 2022.2 (Caporaso et al., 2010) with an analogous pipeline described in Gobbi et al. (2019). To summarize the data processing, raw reads were clipped to 12 bp on the 5′ end removing the primers. The reads were then denoised using DADA2 (Callahan et al., 2017) discarding singletons. In order to reduce the effect of low-abundant amplicon sequence variants (ASVs), on downstream statistical analysis, any feature appearing <25 times in the complete dataset was filtered out. Taxonomical assignment was performed at 99 % identity through the plugin QIIME feature classifier (Bokulich et al., 2018) using BLAST and the UNITE (Koljalg et al., 2013) v9 database for ITS. If taxonomy was not assigned with reasonable precision, further attempts were performed on the dominant features, using BLAST against the NCBI database to confirm or refine the result.

The raw data of this study is available in the European Nucleotide Archive (ENA accession number PRJEB80528).

2.4. Sample processing and quantitative analyses of anthocyanins by HPLC-DAD

Leaves deprived of necrotic tissue were freeze-dried. Anthocyanins were extracted from lyophilized leaves (0.5 mg) in 25 mL of pH 3.9 hydroalcoholic buffer (40 % v/v ethanol, 2 g/L Na2S2O5, 5 g/L tartaric acid, 22 mL/L 1 N NaOH) and left to macerate for a week at −20 °C. The samples were centrifuged for 10 min at 4000 rpm. The supernatant was separated and kept in the dark, the pellet was resuspended in the same buffer, and then centrifuged again. The two extracts were mixed and brought to a final volume of 50 mL. Extracts were stored at −20 °C until further analysis.

Leaf extracts were retained on a Sep-Pak C18 silica-based bonded phase cartridge (Waters Corp., WAT051910, Milford, MA) eluted with methanol. The extracts were evaporated to dryness in a rotary evaporator (Laborota 4000, Heidolph Instruments GmbH & Co. KG, Schwabach, Germany), and then were resuspended with solvent B for HPLC and filtered through 0.20 μm membrane filter GHP Acrodisc (PALL Italia, Buccinasco, Milano, Italy).

Samples were analyzed by high-performance liquid chromatography (HPLC) with an Agilent 1260 (Agilent, Waldbronn, Germany), equipped with a diode array detector (DAD) detector (G1316A) with a reverse-phase column Purosphere STAR RP-18 end-capped (5 μm) packed into LiChroCART 250-4 HPLC—Cartridge (25 cm × 0.4 cm ID; Merck KGaA, Germany) with a guard column LiChroCART 4-4 of the same packing material. DAD detector was set at 520 nm. Solvent A was 10 % (v/v) formic acid and solvent B was water/methanol/formic acid (40:50:10, v/v/v). The chromatographic separation was carried out at a flow rate of 1 mL/min with a gradient from 28 % to 72 % of B in 63 min, with the following gradient of solvent A: from 72 to 55 % in 15 min; to 30 % in 20 min; to 10 %, in 10 min; to 1 % in 10 min; to 72 % in 5 min and holding 72 % for 3 min. Results were expressed as mg of malvidin 3-O-glucoside chloride equivalent per kg of leaves dry weight.

The profile of anthocyanins was identified based on their UV–VIS spectra and retention times by comparison with standards. Malvidin-, peonidin-, petunidin-3-O-glucoside chloride, myrtillin chloride (delphinidin-3-O-glucoside chloride) and kuromanin chloride (cyanidin-3-O-glucoside chloride) were purchased from Extrasynthèses (Lyon, Genay Cedex, France). Acylated anthocyanin, not available as pure standard molecules, were tentatively identified on the basis of previous studies conducted on the leaf anthocyanin extracts of V. *vinifera* cultivars by HPLC-ESI-MS/MS (Kedrina-okutan et al., 2018) and based on the current laboratory identification of acyl-derived anthocyanin in grapes. Since anthocyanins acyl-derivatives were tentatively identified, we did not consider them separately but we summed the individual peak areas and calculated their concentration and their relative percentage incidence over total anthocyanins. A tentative identification is found in supplementary materials (Table S1), where average retention times of individual peaks in the described chromatographic conditions are reported.

2.5. Statistical analyses and data visualization

2.5.1. Data analysis for NGS

The frequency table and its taxonomy were combined, converted to biom format in QIIME (Caporaso et al., 2010), then merged with a table of metadata into an S4 object and analyzed in R (version 3.6.3). The 'phyloseq' (version 1.30.0) (McMurdie and Holmes, 2013) and 'biomformat' (version 1.14.0) (McMurdie and Paulson, 2016) packages were used to create the primary data object. The 'vegan' (version 2.5.5) (Oksanen et al., 2013), 'mvabund' (version 4.1.3) (Wang et al., 2017), 'metacoder' (version 0.3.4) (Foster et al., 2017), 'taxa' (version 0.3.4)

(Foster et al., 2018), 'tidyverse' (version 1.3.0) (Wickham et al., 2019), 'microbiome' (version 1.8.0) (Lahti and Sudarshan), ggplot2, (version 3.3.2) (Valero-Mora, 2015), 'pairwiseAdonis' (version 0.4.1) (Arbizu, 2020), 'gridExtra' (version 2.3), and directlabels (version 2024.1.21) packages were used for data manipulation, visualization, and statistical analysis. The R code used for these analyses is publicly available at https://github.com/Marieag/LeaSyBiome/blob/main/LeaSyBiome_Study1.R.

The alpha diversity was measured using the Simpson's (D) and inverse Simpson's (1/D) diversity indexes and tested with one-way ANOVA with a post hoc Bonferroni correction to determine significant differences between cultivars (Cabernet Sauvignon, Touriga Nacional), year (2020 and 2021), and between leaf symptoms (BDA, Esca, Apox and Asymptomatic leaves).

We analyzed the β-dispersion to measure between-sample variances in abundance, computing the distances of group members to the group centroid. The resulting ordination was plotted using a Bray-Curtis distance matrix. To assess overall inter-group variance (beta diversity) for each year and cultivar, we also performed permutational multivariate analysis of variance (PERMANOVA) with 999 permutations, using the "vegan" package. Post hoc pairwise tests were performed to evaluate the differences between categories using the "pairwiseAdonis" wrapper, applying FDR to correct for multiple comparisons.

Relative abundance for each taxon was calculated and visualized using barplots.

Additionally, we generated heat trees to visualize effect size of the relative abundance of fungal taxa at different taxonomic levels using the 'MetacodeR' package, which calculates the log2 fold change (LfC) in genus and family abundance. A Wilcoxon Rank Sum test was applied to test differences between the same species in different categories, and the resulting p-values were corrected for multiple comparisons using FDR, as implemented in MetacodeR. We focused our analysis on taxa present at RA>0.1 %, and P-value threshold was set to 0.05.

2.5.2. Data analysis for anthocyanins

All data were analyzed by RStudio Build 372 software program version for Windows. Analysis of variance was performed by two-way ANOVA, normalizing data with $\log_{10}$, and followed by Bonferroni post-hoc test at $P \leq 0.05$, $P \leq 0.01$, $P \leq 0.001$, and $P \leq 0.0001$. All measurements were performed in triplicates, and results were expressed as means ± standard errors.

2.5.3. Data visualization for NGS and anthocyanins

Finally, we correlated anthocyanin levels with microbial presence by overlaying anthocyanin level contours on top of samples and fungal taxa, based on a Bray-Curtis dissimilarity matrix.

The ordisurf function was utilized to visualize potential relationships between anthocyanin levels and fungal species presence in grapevine leaves (McCaig et al., 2024; Yang et al., 2022). This fits a smooth surface to environmental data over a pre-existing ordination plot, using a generalized additive model (GAM) to create contour lines representing the variation of the environmental variable—in this case, total anthocyanin levels—across the ordination space.

3. Results

3.1. Sequencing dataset description

A total of 96 samples were sequenced, 90 representing samples included in the study and the remaining 6 resulting from the DNA sequencing of negative controls for the DNA extraction ($n = 3$) and PCR ($n = 3$). After denoising the raw reads removing singletons and low abundant features, the sequencing dataset used for the analysis consist in 90 samples and contains a total of 2.638.250 denoised reads. These reads are represented by 1424 unique features. All samples included in this study were retained while the negative controls were discarded due to insufficient number of reads.

3.2. Endophytic mycobiome overview – objective 1

When looking at the total dataset (objective 1), the microbial composition of endophytic fungi in grapevine leaves, in terms of taxa assigned to the different features, corresponds to 260 taxa. Among them, 56 were assigned to genus level (21.5 %), and 174 to species level (67 %). Ten taxa are detected at a relative abundance (RA) greater than 1 %, corresponding to 85 % of the total dataset RA, 21 taxa at a RA between 1 and 0.1 %, and 229 are considered rare taxa (RA<0.1). Among non-rare taxa (i.e. RA>0.1 %), those identified to genus and/or species level are listed in Table 1.

Ascomycetes are dominant (54.9 %), followed by oomycetes (29.1 %) and basidiomycetes (6.9 %). The most abundant families are *Peronosporaceae* (29.1 %), *Cladosporiaceae* (18.1 %), *Pleosporaceae* (13.9 %), *Aureobasidiaceae* (12.5 %), and *Debaryomycetaceae* (6.6 %). Among the RA>1 % taxa, the most abundant are *Plamopara viticola, Cladosporium delicatulum, Aureobasidium* sp., *Debaryomyces* sp. and *Stemphylium* sp., the former being the only known pathogen affecting grapevine leaves. Three taxa are reported for the first time in grapevine, namely *Botrytis caroliniana, Pyrenophora chaetomioides and Daldinia raimundi* (Table 1). GTD-associated fungi were not detected among non-rare taxa. However, small abundances (RA<0.01 %) of *Diplodia, Phaeomoniella chlamydospora, Fomitiporia* and *Neofusicoccum* are present in the dataset.

In adult leaves of different categories, i.e. three symptom phenotypes and asymptomatic (dataset for objective 3), all non-rare taxa are found both in Cabernet Sauvignon and in Touriga Nacional. Taxa *Plasmopara*

Table 1
Taxonomic classification and relative abundances (RA) of non-rare taxa (RA>0.1 %) in the total dataset, identified at genus or species level.

Phylum (RA, %)	Family (RA, %)	Genus/Species	RA (%)
Oomycetes (29.1)	*Peronosporaceae* (29.1)	*Plasmopara viticola*	29.1
	Cladosporiaceae (18.1)	*Cladosporium delicatulum*	17.6
		Cladosporium ramotenellum	0.5
	Aureobasidiaceae (12.5)	*Aureobasidium* sp.	9.9
		Aureobasidium pullulans	2.6
		Stemphylium sp.	7.5
		Alternaria sp.	3.7
	Pleosporaceae (13.9)	*Alternaria infectoria*	1.9
		Alternaria metachromatica	0.5
		Paradendryphiella arenariae	0.2
		Pyrenophora chaetomioides †	0.1
	Debaryomycetaceae (6.6)	*Debaryomyces* sp.	6.6
	Sclerotiniaceae (1.2)	*Botrytis caroliniana* †	1.2
Ascomycetes (54.9)	*Mycosphaerellaceae* (0.8)	*Mycosphaerella tassiana*	0.8
	Didymellaceae (0.9)	*Epicoccum nigrum*	0.8
		Didymella sp.	0.1
	Didymosphaeriaceae (0.3)	*Pseudopithomyces chartarum*	0.3
	Phaeosphaeriaceae (0.2)	*Sclerostagonospora lathyri*	0.2
	Xylariaceae (0.2)	*Daldinia raimundi*†	0.2
	Periconiaceae (0.1)	*Periconia byssoides*	0.1
	Saccharomycetaceae (0.1)	*Hanseniaspora guilliermondii*	0.1
	Sporidiobolaceae (5.1)	*Sporobolomyces roseus*	4.9
		Sporobolomyces oryzicola	0.2
Basidiomycetes (6.9)	*Filobasidiaceae* (1.6)	*Filobasidium* sp.	0.9
		Filobasidium chernovii	0.7
	Bulleribasidiaceae (0.2)	*Vishniacozyma* sp.	0.2

† first report in grapevine.

viticola, Botrytis caroliniana and *Alternaria infectoria* are over-represented in Cabernet Sauvignon (2-fold difference or more), while *Aureobasidium* sp., *Epicoccum nigrum* and *Filobasidium chernovii* are more abundant in Touriga Nacional. When examining vintages, all non-rare taxa are found both in 2020 and 2021, exception for taxon *Aureobasidium pullulans*, detected exclusively in year 2020. Vintage-wise, numerous taxa are differentially abundant (at least 3-fold difference), some of them being *P. viticola* and *F. chernovii,* over-represented in 2020, and *Debaryomyces* sp., *Cladosporium ramotenellum* and *E. nigrum*, more abundant in 2021.

3.3. Mycobiome and Esca symptoms progression in leaves – objective 2

3.3.1. Alpha and beta diversity

The alpha diversity, measured using Simpson and InvSimpson indices, does not vary between leaf age groups, although a strong trend suggests differences when comparing cultivars ($P = 0.051$). In Cabernet Sauvignon, the Simpson index varies significantly when comparing young asymptomatic leaves (YAsy) and Esca leaves ($P = 0.012$), while strong trends are observed when comparing asymptomatic leaves (Asy) and Esca, and YAsy and leaves affected by chlorotic spots (Chl, $P = 0.06$). Concerning the InvSimpson index, trends are observed when comparing Asy and YAsy leaves with Esca (Fig. 2). In Touriga Nacional, neither significant differences nor trends are observed for both examined indices.

The beta dispersion varies between leaf age groups ($P = 0.001$). Significant variation is evident when comparing the four leaf categories, both in Cabernet Sauvignon ($P = 0.014$) and Touriga Nacional ($P = 0.002$). In Cabernet Sauvignon, the clustering pattern reveals that Esca and Chl leaves diverge from YAsy leaves ($P = 0.051$), and a strong trend of divergence separates Esca and Asy leaves ($P = 0.056$) (Fig. 2). A similar pattern is observed in Touriga Nacional, where significant differences are found when comparing YAsy leaves with all other leaf categories ($P \leq 0.047$), and Chl with Asy leaves ($P = 0.042$). In both cultivars, no differences in clustering are observed when comparing Esca and Chl leaves ($P > 0.05$).

3.3.2. Taxa abundance

The qualitative and quantitative microbial composition varies when comparing cultivars and leaf categories (Figs. 3 and 4).

When comparing young asymptomatic leaves (YAsy) and leaves affected by chlorotic spots (Chl), both in Cabernet Sauvignon and Touriga Nacional, *Sporobolomyces roseus, Filobasidium* sp., *Aureobasidium* sp. and *Epicoccum nigrum* are more abundant in Chl leaves, while no taxa are over-represented in YAsy leaves.

When comparing Chl and Esca leaves, in both cultivars, only *E. nigrum* and, to a lesser extent, *Alternaria infectoria* are more abundant in the former, while only *Debaryomyces* sp. is moderately over-represented in the latter.

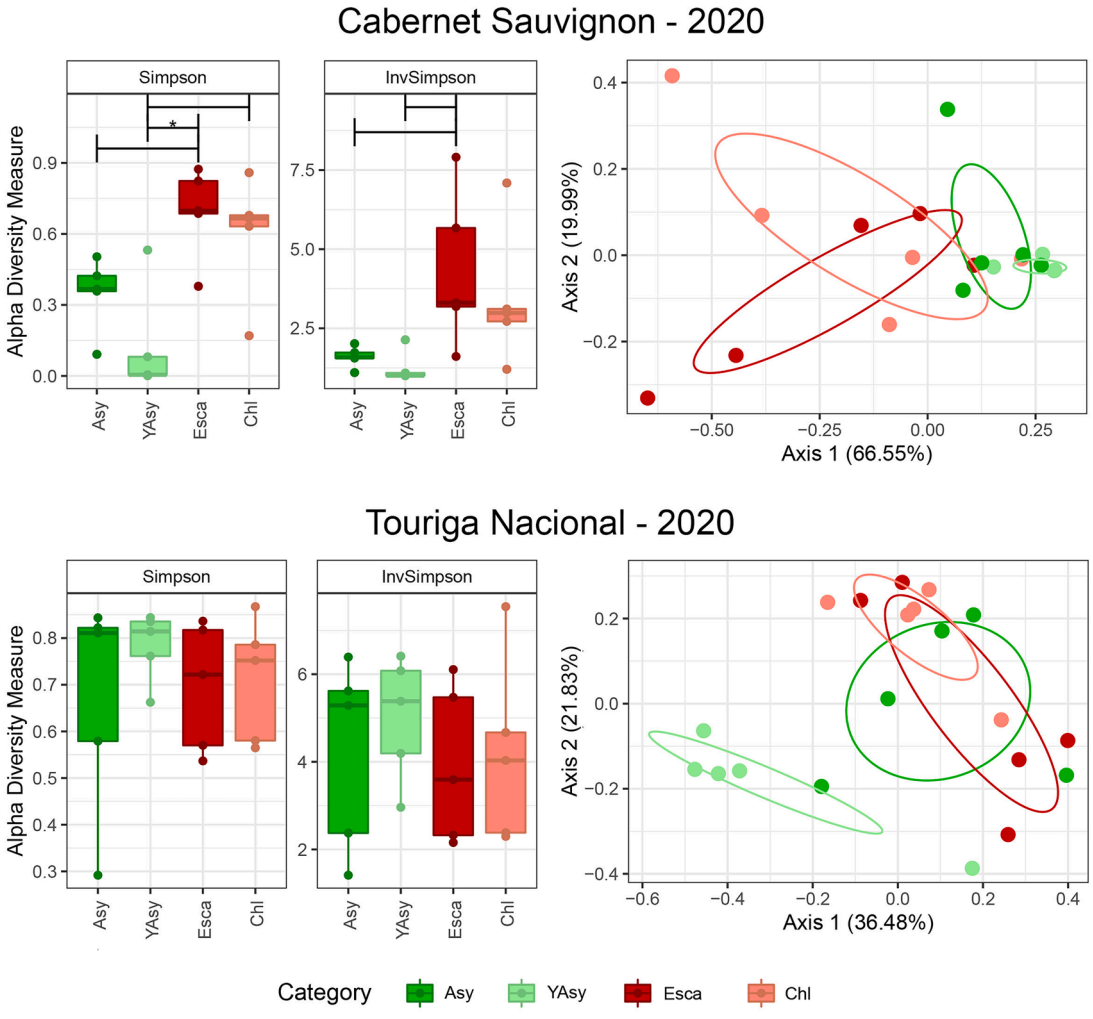

Fig. 2. Box plots of alpha diversity indices (Simpson and InvSimpson) and PCoA plots of beta dispersion based on the Bray–Curtis dissimilarity of the fungal communities in different leaf categories (Asy, YAsy, Esca, Chl) in grapevine cvs. Cabernet Sauvignon and Touriga Nacional, in 2020. The horizontal brackets marked with an asterisk (*) indicate statistical differences, according to a one-way ANOVA with a post hoc Bonferroni correction, while brackets without asterisks indicate trends ($0.10 < P < 0.05$). Ellipses illustrate the multivariate normal distribution of samples within the same leaf category.

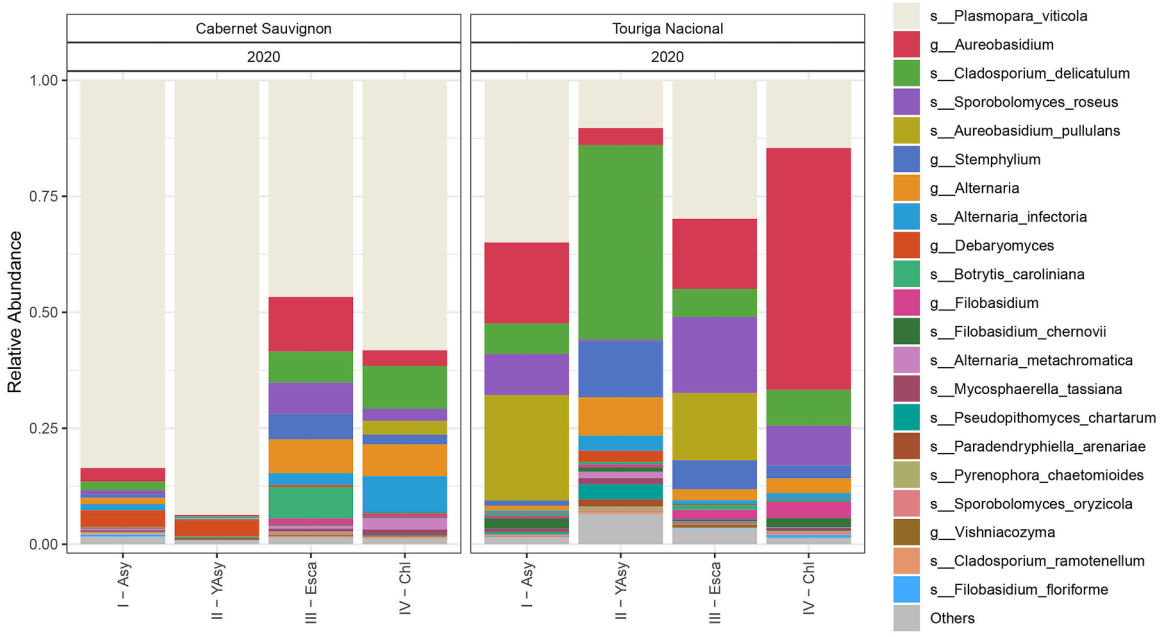

Fig. 3. Bar plots of the relative abundance of the 21 most abundant taxa identified at the genus (g_) or species (s_) level found in different leaf categories (Asy, YAsy, Esca, Chl), in grapevine cvs. Cabernet Sauvignon and Touriga Nacional, in 2020. 'Others' are taxa not included among the 21 most abundant.

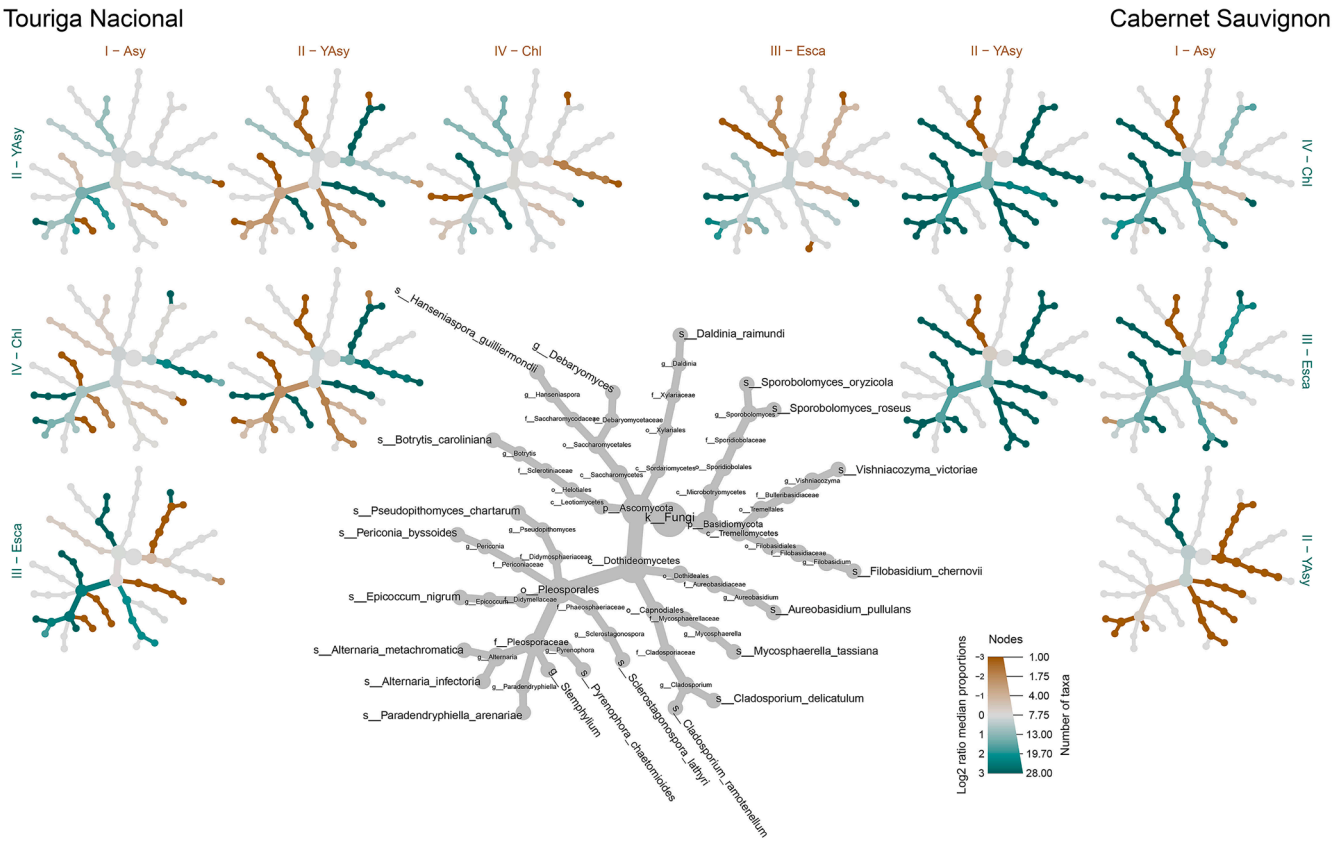

Fig. 4. Differential heat tree matrices depicting changes in fungal taxa abundance between leaf categories (Esca, Chl, Asy, YAsy) in grapevine cvs. Cabernet Sauvignon and Touring Nacional, in 2020, represented in the dataset, and in the grey cladogram, at a relative abundance (RA) > 0.1 %. The smaller cladograms show pairwise comparisons between each category, with the color illustrating the $\log_2$ fold change: a green node indicates a lower abundance of the taxon in the category on the abscissa than in the category on the ordinate. A brown node indicates the opposite.

3.4. Mycobiome and anthocyanins for different symptom phenotypes – objective 3

3.4.1. Mycobiome in different symptom phenotypes

3.4.1.1. Alpha diversity. The alpha diversity of the mycobiome does not vary when comparing cultivars, vintages, and symptom phenotypes ($P > 0.05$).

However, when looking at year/cultivar combinations, the Simpson and Inverted Simpson (InvSimpson) indices vary significantly among symptoms phenotypes (Fig. 5). In cv. Cabernet Sauvignon (2020), the Simpson index differs significantly when comparing Asy and Esca leaves ($P = 0.03$), and a trend is observed for the InvSimpson index ($P = 0.078$). However, in the same year, neither statistical differences nor trends are observed for cv. Touriga Nacional.

In Cabernet Sauvignon (2021), both Simpson and InvSimpson indices show trends of divergence when comparing BDA and Esca leaves ($P = 0.088$, $P = 0.096$). In the same year, in Touriga Nacional, both the Simpson and InvSimpson indices vary significantly when comparing BDA and Asy leaves ($P = 0.02$, $P = 0.019$) (Fig. 5).

3.4.1.2. Beta diversity. The beta diversity of the microbial composition varies significantly when comparing cultivars ($P = 0.006$) and vintages ($P = 0.001$), but not symptom phenotypes ($P > 0.05$).

When looking at year/cultivar combinations, the Bray–Curtis dissimilarity, represented in PCoA plots of the beta dispersion, varies significantly when comparing symptom phenotypes in Cabernet Sauvignon (2020, $P = 0.041$; 2021, $P = 0.001$), and Touriga Nacional (2021, $P = 0.007$), while a trend is found in Touriga Nacional (2020, $P = 0.08$) (Fig. 6).

In Cabernet Sauvignon (2020), the clustering pattern reveals that both Apox and Esca leaves have a trend of divergence from Asy leaves; in 2021, this trend is significant (Esca vs Asy; $P = 0.017$), along with a difference between Esca and BDA leaves ($P = 0.017$). In Touriga Nacional (2021), the post-hoc test show strong trends of divergence when comparing Esca and Asy leaves, as well as BDA and Asy leaves ($P = 0.085$). No differences in clustering are observed when comparing Asy and BDA leaves, in both cultivars and vintages.

3.4.1.3. Taxa abundance. The bar plots in Fig. 7 offer a visual representation of the 21 most abundant taxa detected in leaves of different symptom phenotypes and asymptomatic, in both cultivars and vintages.

When comparing symptom categories, in different years and cultivars, numerous taxa vary in abundance, as shown in Fig. 8. To find recurring patterns in taxa over- or under-representation when comparing symptom categories, we focus on those taxa that are similarly differently abundant either in the same cultivar in both years, or in the same year in both cultivars.

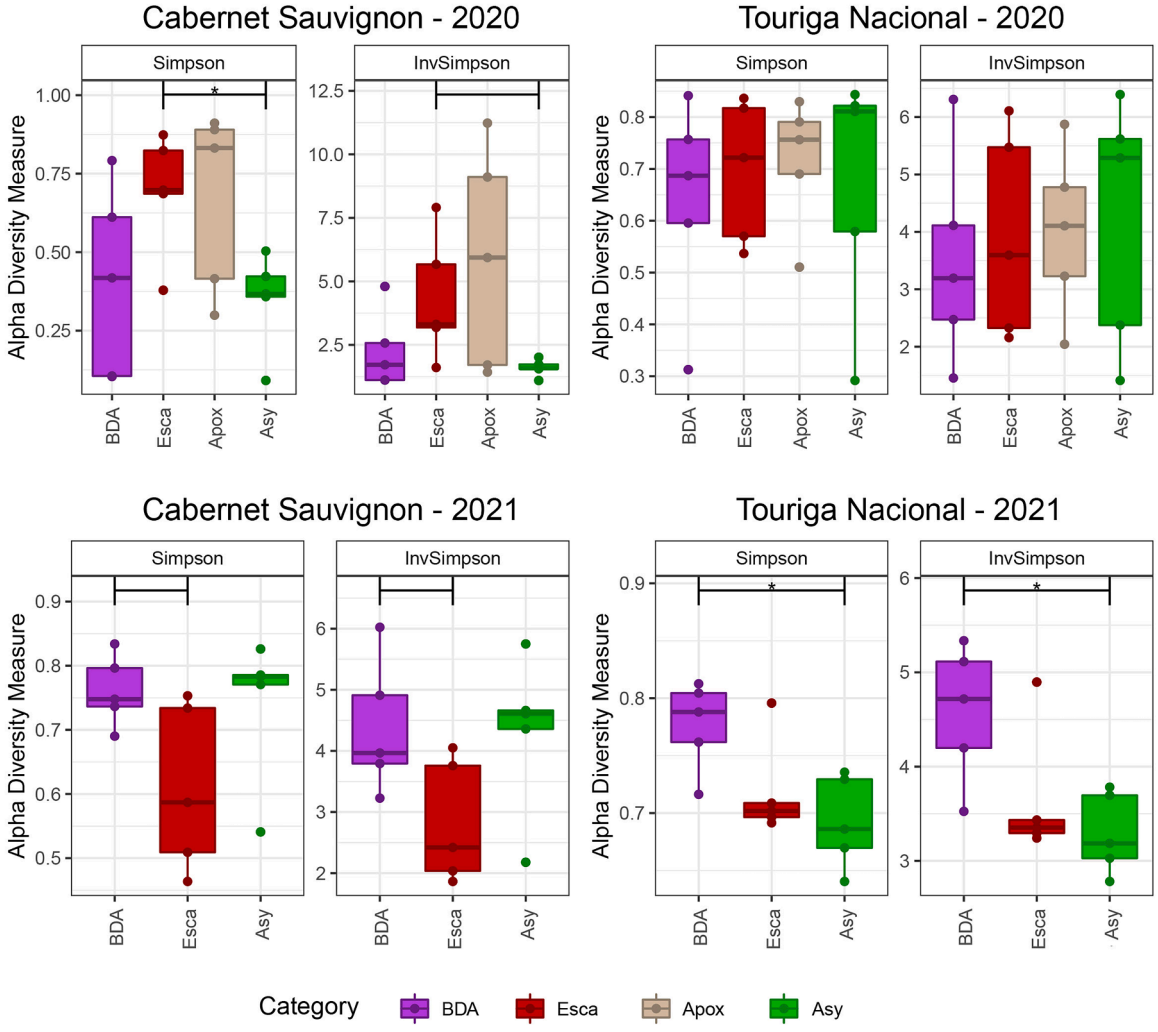

Fig. 5. Box plots of alpha diversity indices (Simpson and InvSimpson) showing the richness of the fungal communities in grapevine leaves under different symptom phenotypes (black dead arm [BDA], Esca, apoplectic [Apox]) and asymptomatic (Asy), in grapevine cvs. Cabernet Sauvignon and Touring Nacional, in 2020 and 2021. The horizontal brackets marked with an asterisk (*) indicate statistical differences, while brackets without asterisks indicate trends ($0.10 < P < 0.05$).

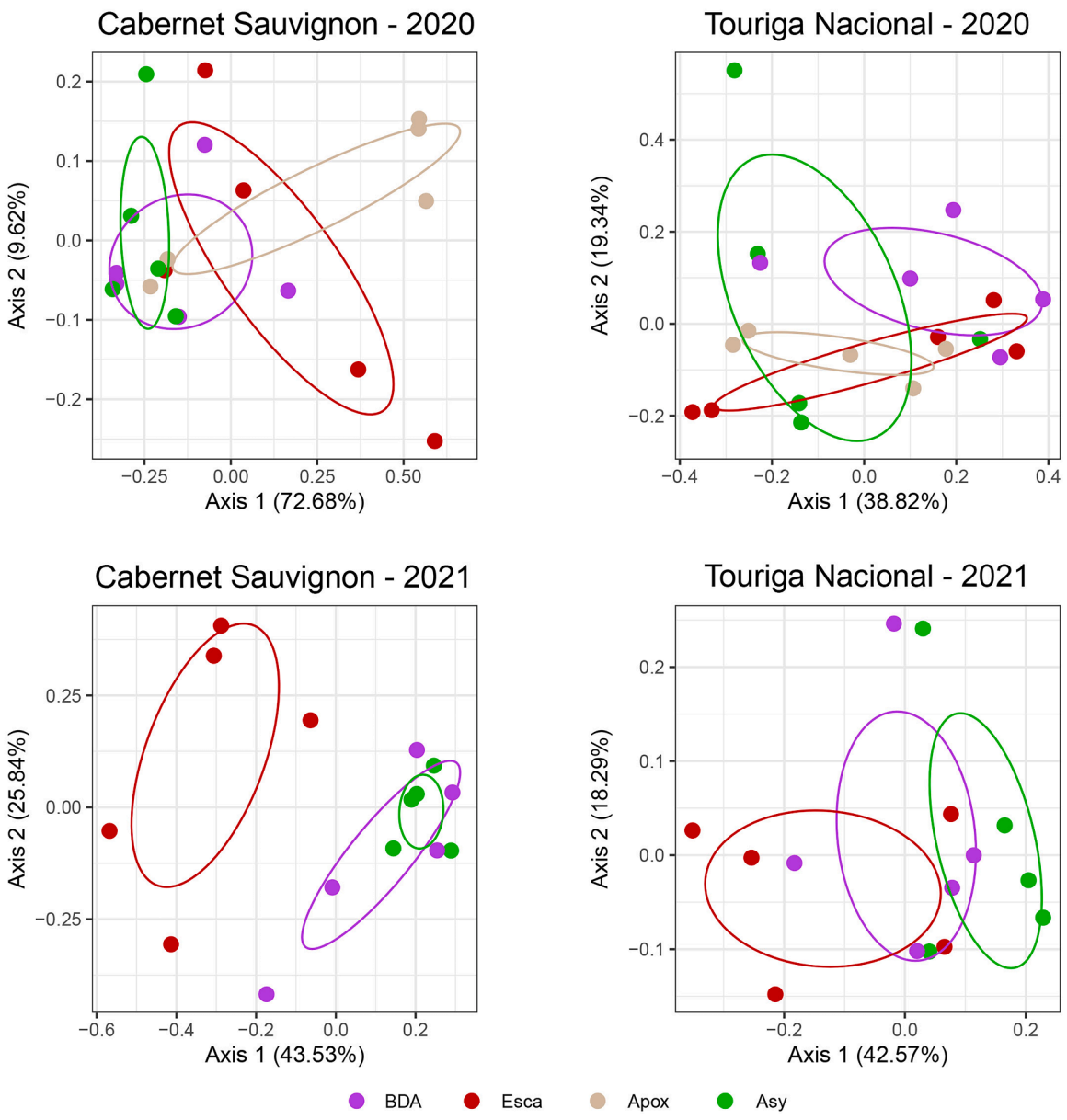

Fig. 6. PCoA plots of beta dispersion based on the Bray–Curtis dissimilarity of the fungal communities in grapevine leaves under different symptom phenotypes (black dead arm [BDA], Esca, apoplectic [Apox]) and asymptomatic (Asy), in grapevine cvs. Cabernet Sauvignon and Touring Nacional, in 2020 and 2021. Ellipses illustrate the multivariate normal distribution of samples within the same symptom category.

Esca* vs *Asy. In both vintages, *Botrytis caroliniana, Stemphylium* sp. and *Debaryomyces* sp. are consistently over-represented in Esca leaves, the first two taxa in Cabernet Sauvignon, the third in Touriga Nacional. *Stemphylium* sp. and *Daldinia raimundi* are two taxa over-represented in Esca leaves, in both cultivars, the former exclusively in 2020, the latter exclusively in 2021. On the other hand, *Epicoccum nigrum* and *Pseudopithomyces chartarum* are over-represented in Asy leaves, the former in both cultivars, only in 2021, the latter in both vintages, only in Touriga Nacional.

BDA* vs *Asy. No taxa are similarly over- or under-represented when comparing cultivars, in 2020, or vintages, in Cabernet Sauvignon. In both vintages, *Debaryomyces* sp. is over-represented in BDA leaves, while *P. chartarum* is more abundant in Asy leaves, exclusively in Touriga Nacional. In both cultivars, *Filobasidium* sp. and *D. raimundi* are over-represented in BDA leaves, while *E. nigrum* is more abundant in Asy leaves, only in 2021.

Esca* vs *BDA. In both vintages, *B. caroliniana, Stemphylium* sp., *Alternaria metachromatica* and *P. chartarum* are consistently over-represented in Esca leaves, the former two taxa in Cabernet Sauvignon, the latter two in Touriga Nacional. *P. chartarum, B. caroliniana and Paradendryphiella arenariae* are taxa over-represented in Esca leaves, in both cultivars, the former exclusively in 2020, the latter two exclusively in 2021. *Debaryomyces* sp., *Filobasidium* sp. and *Mycosphaerella tassiana* are over-represented in BDA leaves, either in both vintages, in a single cultivar, or in both cultivars, in a single year.

Apox* vs *Esca, BDA, Asy. When compared to all other categories, taxa over-represented in Apox leaves are *Filobasidium chernovii, Periconia byssoides* and *Vishniacozyma* sp., in Cabernet Sauvignon, and *Sporobolomyces oryzicola* and *E. nigrum*, in Touriga Nacional.

No taxon is exclusively present or consistently over- or under-represented in a specific symptom phenotype, in both vintages and cultivars.

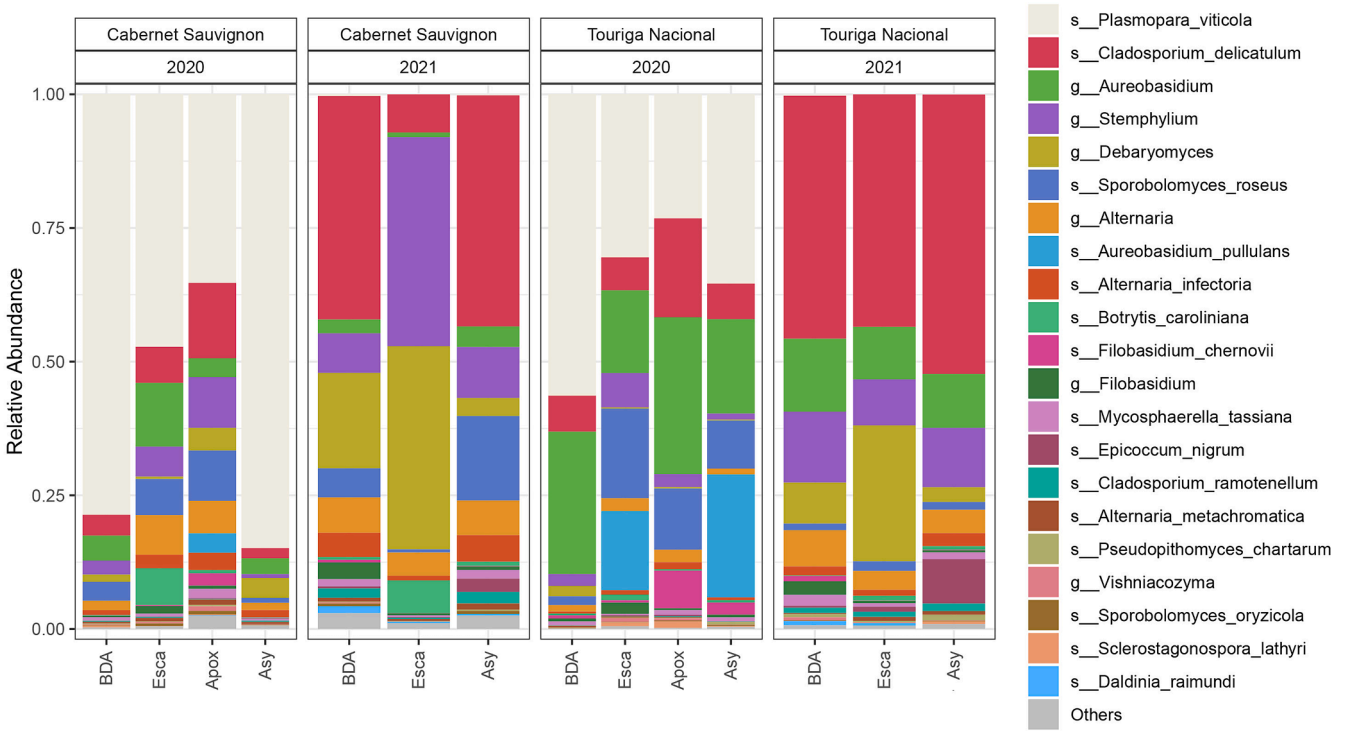

Fig. 7. Bar plots of the relative abundance of the 21 most abundant taxa identified at the genus (g_) or species (s_) level found in different symptom phenotypes (black dead arm [BDA], Esca, apoplectic [Apox]) and asymptomatic leaves (Asy), in grapevine cvs. Cabernet Sauvignon and Touriga Nacional, in 2020 and 2021. 'Others' are taxa not included among the 21 most abundant.

3.4.2. *Anthocyanins under different symptoms categories*

3.4.2.1. Total anthocyanins. In both vintages and cultivars, anthocyanins accumulated in Esca and BDA symptomatic leaves, while they were not detected in asymptomatic ones (Table 2). Regardless of symptom phenotype and vintage, total leaf anthocyanin concentration was always higher in Cabernet Sauvignon (452.2 - 724.4 mg kg^{-1}), when compared to Touriga Nacional (47.6 - 213.2 mg kg^{-1}). In Cabernet Sauvignon, the total anthocyanin concentration did not significantly differ when comparing Esca and BDA leaves, both in 2020 and 2021. Conversely, in Touriga Nacional, Esca leaves reached higher concentrations of total anthocyanins in both years, when compared to BDA (Table 2). In 2020, the total anthocyanin concentration depended on the factor 'cultivar' ($P \leq 0.0001$), and on the interaction 'cultivar*symptom phenotype' ($P \leq 0.001$), but not on 'symptom phenotype'. Instead, in 2021, significant differences were ascribable to 'cultivar' ($P \leq 0.0001$), to 'symptom phenotype' ($P \leq 0.001$), and to the interaction 'cultivar*symptom phenotype' ($P \leq 0.01$; Table 2).

3.4.2.2. Anthocyanin profile. The anthocyanin profile of Esca and BDA leaves highlights differences between the two cultivars and symptom phenotypes. The di-hydroxylated forms always displayed higher concentrations when compared to tri-hydroxylated and acyl-derivative anthocyanins, in both cultivars, symptom phenotypes, and vintages, with a prevalence of peonidin 3-*O*-glucoside, followed by cyanidin 3-*O*-glucoside, in most cases. This is especially evident in Touriga Nacional with BDA phenotype, where di-hydroxylated forms reached nearly 100 % of the total anthocyanin amount (Fig. 9). In 2020, significant differences in the accumulation of di-hydroxylated forms were ascribable to cultivar ($P \leq 0.0001$), symptom phenotype ($P \leq 0.01$), and the interaction cultivar*symptom phenotype ($P \leq 0.01$; Table 2). Instead, in 2021, the accumulation of di-hydroxylated anthocyanins was similar in Esca and BDA leaves within the same cultivar, but it differed significantly for cultivar ($P \leq 0.0001$), symptom phenotype ($P \leq 0.01$), and not for the interaction cultivar*symptom phenotype (Table 2). The concentration of tri-hydroxylated forms and acyl-derivatives was always higher in Esca leaves, regardless cultivar and vintage (Table 2). In 2020, tri-hydroxylated anthocyanins showed significant differences depending on symptom phenotype ($P \leq 0.0001$), and on the interaction cultivar*symptom phenotype ($P \leq 0.01$), but not on cultivar (Table 2), whereas in 2021, significant differences were also ascribable to cultivar ($P \leq 0.0001$; Table 2). The profile of tri-hydroxylated anthocyanins, regardless cultivar and symptom phenotype, showed malvidin 3-*O*-glucoside as the prevalent form, followed by petunidin and delphinidin 3-*O*-glucosides (Fig. 9). In 2020, acyl-derivatives showed highly significant differences depending on cultivar, symptom phenotype, and interaction cultivar*symptom phenotype ($P \leq 0.0001$); in 2021, highly significant differences were associated to cultivar ($P \leq 0.0001$) and symptom phenotype ($P \leq 0.01$), but not to the interaction cultivar*symptom phenotype.

3.4.3. *Correlation mycobiome - anthocyanins*

We analysed the relationship among fungal communities, single taxa and anthocyanins, both total and classes of different anthocyanin forms (di-hydroxylated, tri-hydroxylated, and acyl derivatives), using ordisurf plots (Fig. 10).

In 2021, the total anthocyanin levels significantly ($P = 0.017$) mapped onto the NMDS plot, revealing fungal species associated with varying anthocyanin concentrations. The value of anthocyanins at the location of each taxon-associated number, on each individual plot, indicates the anthocyanin concentration with which that taxon is associated, suggesting significant correlations. For example, *B. caroliniana, Stemphylium* and *Hanseniaspora guilliermondii* are more abundant in regions with high total anthocyanin concentrations, while *Filobasidium, Cladosporium ramotenellum* and *Aureobasidium* are associated with low concentrations. When examining classes of different anthocyanin forms (di-hydroxylated fit, $P = 0.038$; tri-hydroxylated fit, $P = 0.0085$; acyl derivates fit, $P = 9.34\ e^{-05}$), *B. caroliniana* and *Stemphylium* remain associated with the highest concentrations across all three classes, while

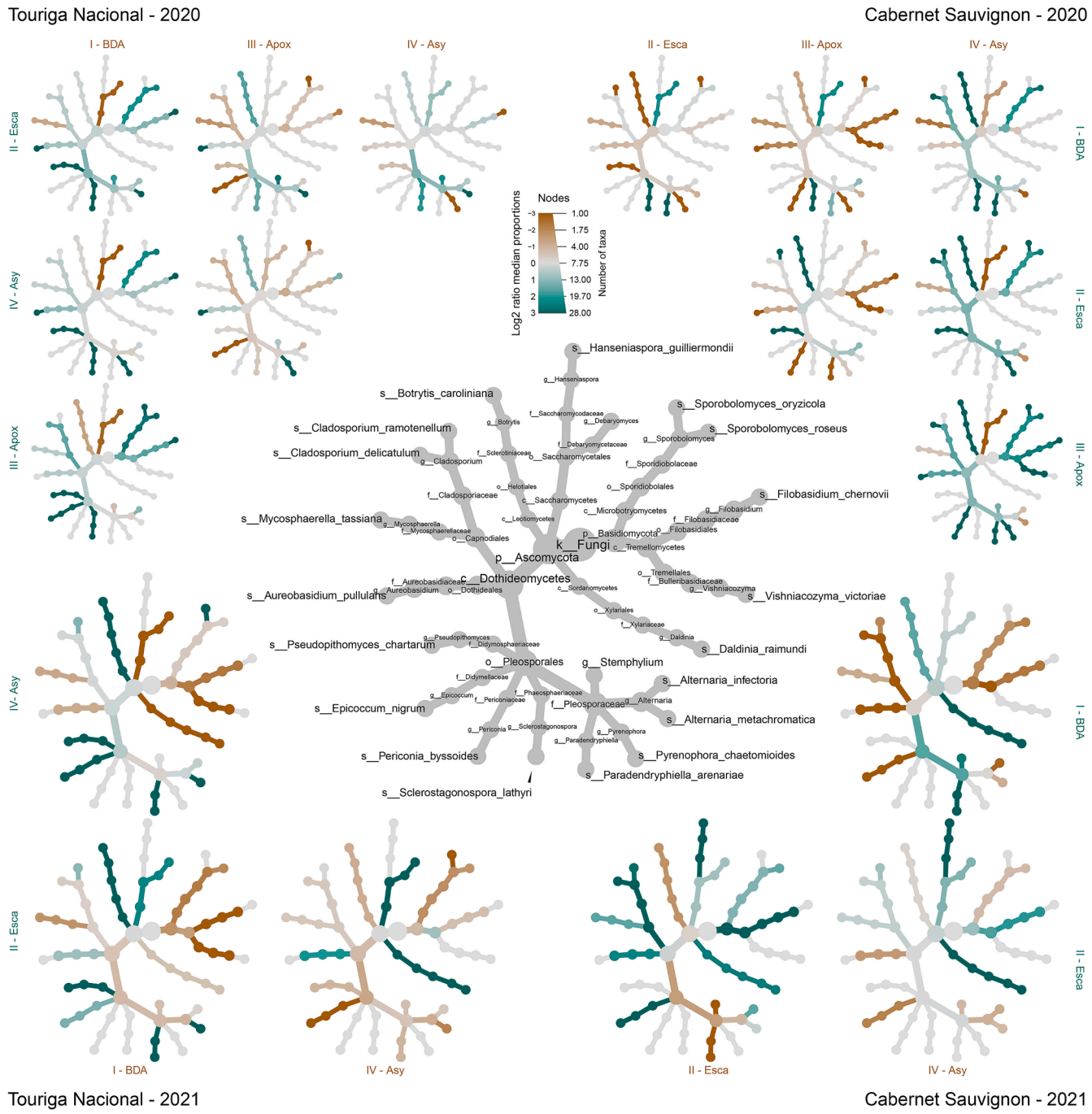

Fig. 8. Differential heat tree matrices depicting changes in fungal taxa abundance between leaf symptom phenotypes (black dead arm [BDA], Esca, apoplectic [Apox]) or asymptomatic (Asy), in grapevine cvs. Cabernet Sauvignon and Touring Nacional, in 2020 and 2021, represented in the dataset,and in the grey cladogram, at a relative abundance (RA) > 0.1 %. The smaller cladograms show pairwise comparisons between each symptom category, with the color illustrating the $\log_2$ fold change: a green node indicates a lower abundance of the taxon in the symptom category on the abscissa than in the symptom category on the ordinate. A brown node indicates the opposite.

H. guilliermondii is linked to high concentrations of di-hydroxylated anthocyanins and acyl derivatives, but only intermediate levels of tri-hydroxylated anthocyanins.

In Cabernet Sauvignon, the contour plots indicate higher anthocyanin concentrations in Esca leaves, with a clear separation of microbial species when compared to BDA leaves. In Touriga Nacional, samples are clustered on lower anthocyanin concentrations, and differences between Esca and BDA phenotypes are less clear.

In 2020, the dataset fit was found significant only for tri-hydroxylated anthocyanin ($P = 0.024$; Figure S1) and it is not further discussed.

4. Discussion

4.1. Endophytic mycobiome using NGS – objective 1

In recent years, numerous studies investigated the grapevine leaf microbiome, either employing whole leaves (i.e. endophytes and epiphytes) (Knapp et al., 2021; Liu and Howell, 2020; Pinto et al., 2014; Molnár et al., 2022; Kernaghan et al., 2017), or focusing on the leaf

Table 2
Total anthocyanin concentration (mg/kg dry weight) in Cabernet Sauvignon and Touriga Nacional leaves with different symptom phenotypes (Esca, black dead arm [BDA]) or asymptomatic (Asy), in 2020 and in 2021. Within each cultivar, mean values of total anthocyanins and class of different anthocyanin forms followed by different letters are significantly different for $P \leq 0.05$, according to a two-way ANOVA with a post hoc Bonferroni correction. Significant differences among cultivars, symptom phenotypes and their interactions were tested for $P \leq 0.01$ (*), $P \leq 0.001$ (**) and $P \leq 0.0001$ (***); ns = *not significant.*

2020	Symptom phenotype	Di-hydroxylated (mg/kg)	Tri-hydroxylated (mg/kg)	Acyl-derivatives (mg/kg)	Total anthocyanins (mg/kg)
Cabernet Sauvignon					
	Esca	214.2 ± 26.5 b	68.9 ± 6.7 a	169.1 ± 17.4 a	452.2 ± 34.7 a
	BDA	549.0 ± 85.4 a	18.0 ± 2.3 ab	94.0 ± 12.3 ab	661.0 ± 83.6 a
	Asy	0.0	0.0	0.0	0.0
Touriga Nacional					
	Esca	92.2 ± 6.8 c	78.9 ± 1.6 a	42.1 ± 12.2 b	213.2 ± 16.4 b
	BDA	96.8 ± 10.8 c	4.1 ± 3.3 b	0.02 ± 0.01 c	100.9 ± 11.1 c
	Asy	0.0	0.0	0.0	0.0
Cultivar		***	ns	***	***
Symptom phenotype		*	***	***	ns
Cultivar*Symptom phenotype		*	*	***	**

2021	Symptom phenotype	Di-hydroxylated (mg/kg)	Tri-hydroxylated (mg/kg)	Acyl-derivatives (mg/kg)	Total Anthocyanins (mg/kg)
Cabernet Sauvignon					
	Esca	483.7 ± 15.0 a	83.9 ± 9.7 a	156.8 ± 7.3 a	724.4 ± 4.9 a
	BDA	422.0 ± 88.1 a	36.7 ± 6.5 b	91.1 ± 15.0 a	549.8 ± 109.3 a
	Asy	0.0	0.0	0.0	0.0
Touriga Nacional					
	Esca	85.0 ± 3.7 b	39.9 ± 1.8 b	12.2 ± 5.3 b	137.1 ± 6.9 b
	BDA	47.6 ± 9.5 b	0.01 ± 0.01 c	0.01 ± 0.01 c	47.6 ± 9.5 c
	Asy	0.0	0.0	0.0	0.0
Cultivar		***	***	***	***
Symptom phenotype		*	***	*	**
Cultivar*Symptom phenotype		Ns	***	ns	*

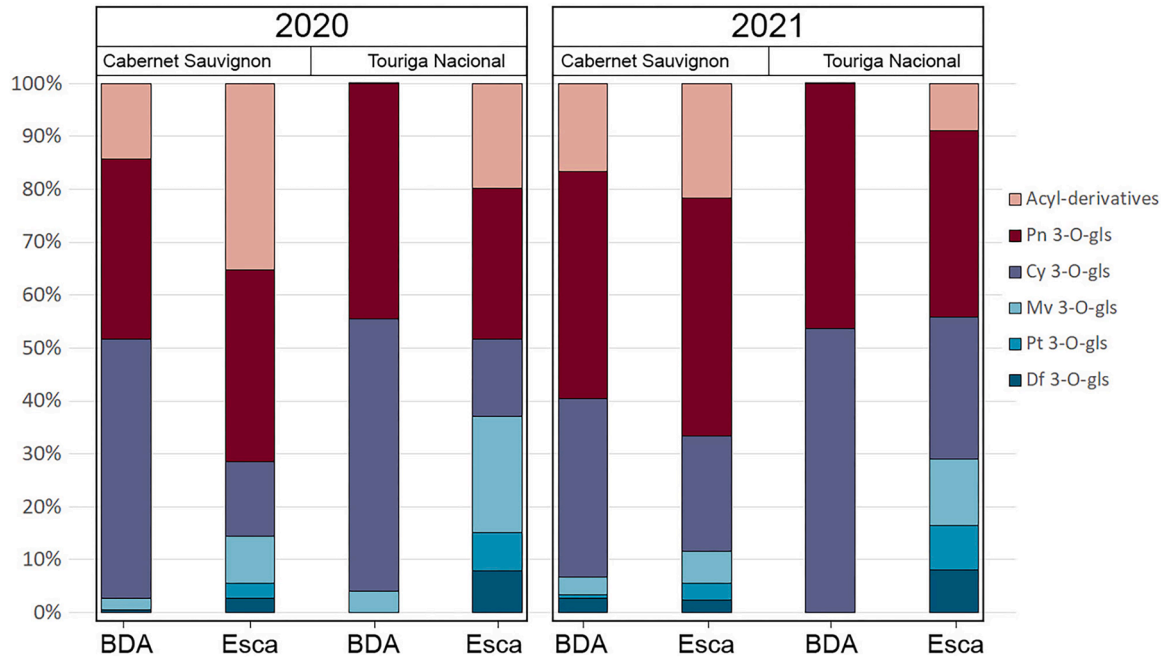

Fig. 9. Anthocyanin profile (%) of Cabernet Sauvignon (CS) and Touriga Nacional (TN) leaves under Esca or black dead arm (BDA) phenotypes, in 2020 and 2021. Mv 3-*O*-gls = malvidin 3-O-glucoside; Pn 3-*O*-gls = peonidin 3-*O*-glucoside; Pt 3-*O*-gls = petunidin 3-*O*-glucoside; Cy 3-*O*-gls = cyanidin 3-*O*-glucoside; Df 3-*O*-gls= delphinidin 3-*O*-glucoside and sum of acylated forms (acyl-derivatives).

phyllosphere (Singh et al., 2018a; Singh et al., 2018b; Gobbi et al., 2020; Papadopoulou et al., 2022). To our knowledge, only three studies looked exclusively into the *Vitis* spp. leaf endophytic mycobiome (Fan et al., 2020; Aleynova et al., 2022; Aleynova et al., 2023), using NGS techniques.

When compared to the fungal communities found in whole leaves or phyllosphere, leaf endophytes are believed to contribute to a smaller extent to the total species richness (Behrens and Fischer, 2022). Our results are in line with those obtained in a previous study (Fan et al., 2020), where the authors detected 150 fungal genera in V. *vinifera* and

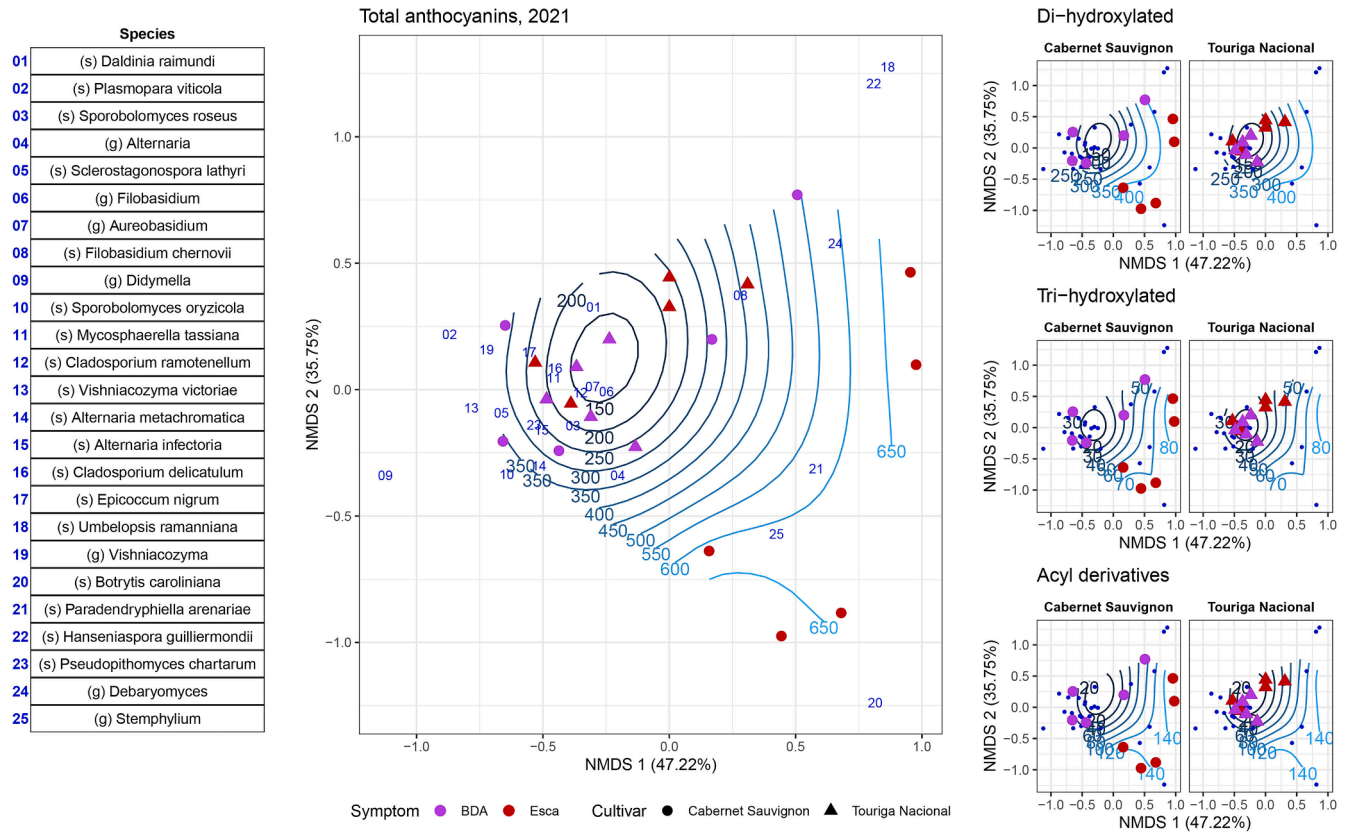

Fig. 10. Non-metric multidimensional scaling plots representing microbial and anthocyanin data for year 2021. Microbiome samples are plotted as large dots (circles and triangles) while single fungal taxa (RA>0.1 %) are represented as blue numbers in the "Total Anthocyanins" plot and corresponding species list (leftmost panel). Contours indicate anthocyanin concentration gradients. Three sets of plots display di-hydroxylated, tri-hydroxylated, and acyl derivative anthocyanin profiles, separated by grapevine cultivar. In these plots, species are represented by small blue dots, and contours show variations in specific anthocyanin levels.

V. *amurensis* leaves (162 genera in the present study), but considerably greater than what was reported a couple of years before by Aleynova and colleagues, in V. *amurensis* (64 genera) (Aleynova et al., 2022), and later in other *Vitis* spp. hybrids (62 to 87 genera) (Aleynova et al., 2023). Excluding *Plasmopara viticola*, whose presence in 2020 was due to improper timing in the application of plant protection products, brought about by Covid-related restrictions, numerous abundant taxa found in our study, such as *Cladosporium* spp., *Alternaria* spp., *Aureobasidium, Mycosphaerella* and *Filobasidium*, have been previously reported in the grapevine endosphere (Aleynova et al., 2022; Aleynova et al., 2023) and in other studies that analyzed whole leaves or phyllosphere (Knapp et al., 2021; Liu and Howell, 2020; Singh et al., 2018b; Gobbi et al., 2020; Papadopoulou et al., 2022; Barroso-Bergadà et al., 2023). Surprisingly, only few abundant taxa (RA>1 %), e.g. *Cladosporium*, are shared between our study and that of Fan and colleagues (Fan et al., 2020), in *Vitis* spp., suggesting large genotype- or environmental-driven variability in microbial composition.

Interestingly, *P. viticola* was detected in high abundances (in 2020) despite leaves being free of visible lesions produced by this oomycete. A similar observation was recently reported (Aleynova et al., 2023), highlighting a lag period between pathogen infection, tissue colonization and symptoms manifestation. GTD-associated fungi were not expected in leaves (Mugnai et al., 1999), and our data confirms that they do not constitute a sizable component of the endophytic mycobiome. However, the detection of small abundances (RA<0.01 %) of *Diplodia, Phaeomoniella chlamydospora, Fomitiporia* and *Neofusicoccum* suggests that their genetic material may find its way to leaves.

When looking at whole leaves or leaf phyllosphere, cultivar-dependent differences in mycobiome composition, either in terms of community indices or individual taxa abundance, are reported in multiple studies (Molnár et al., 2022; Singh et al., 2018a; Singh et al., 2018B; Papadopoulou et al., 2022). Concerning leaf endophytes, (Fan et al., 2020; Aleynova et al., 2023) detected significant differences when comparing *Vitis* species or hybrids, however, in literature, there are no reports over the cultivar effect on the mycobiome composition of V. *vinifera*, using culture-independent approaches. In this study, we reveal, for the first time in the leaf endosphere, differences in taxa composition, abundance and beta diversity between Cabernet Sauvignon and Touriga Nacional.

Vintage is a strong predictor of fungal community diversity at the phyllosphere level (Knapp et al., 2021; Papadopoulou et al., 2022). This is suggested to be due to multiple factors, one of the most mentioned being meteorological conditions. In our study, vintage is a highly significant factor in predicting community diversity; however, the infection by *P. viticola* (year 2020) seems the main driver of observed differences (Barroso-Bergadà et al., 2023).

4.2. *Mycobiome during symptoms progression in Esca leaves – objective 2*

To explore microbial dynamics during esca symptom progression, from the scattered chlorotic spots stage (Chl) to fully symptomatic leaves (Esca; Fig. 1A-D), we compared the mycobiome profile of symptomatic and asymptomatic leaves of different ages, in Cabernet Sauvignon and Touriga Nacional, in 2020. We focus the following discussion on two key observations.

First, the mycobiome beta diversity of Chl leaves differs from that of asymptomatic leaves of the same age (YAsy), along with several taxa over- or under-represented, in both cultivars (Fig. 4). This suggests that the physiological/biochemical events that lead to the appearance of chlorotic spots alter the abundance of fungal endophytes in leaves,

favoring certain taxa (e.g. *Aureobasidium* sp., *E. nigrum*) over others (*Debaryomyces* sp.). Second, the mycobiome profile of Chl and Esca leaves does not differ for any measured index, and only few taxa are over- or under-represented (e.g. *B. caroliniana, E. nigrum*). These observations suggest that the mycobiome composition is mostly shaped during the early stages of symptom development, and only a small number of taxa are influenced by subsequent events, such as the appearance of pigmentation typical of Esca leaves (Fig. 4, Table 2). We find reasonable to hypothesize that community alterations may occur even before the appearance of chlorotic spots, along with physiological alterations that are known to precede symptoms expression (Magnin-robert et al., 2017; Valtaud et al., 2011; Goufo and Cortez, 2020; Magnin-Robert et al., 2011; Calamai et al., 2014; Christen et al., 2007; Goufo and Cortez, 2023). From this perspective, fungal communities may be passive spectators that adapt to environmental circumstances, or they may be actively involved in the events that lead to (or prevent) symptoms onset. For example, certain fungi may negatively affect plant physiology or gene expression, as suggested by the activation of plant defenses in pre-symptomatic leaves (Magnin-Robert et al., 2011), or they may positively affect leaves -that remain asymptomatic- by detoxifying putative leaf symptoms-inducing molecules (Del Frari et al., 2022; Giauque et al., 2019). The fungi over-represented in Chl leaves, when compared to YAsy leaves (Fig. 4), i.e. *S. roseus, E. nigrum, Aureobasidium* sp. and *Filobasidium* sp., are not known grapevine pathogens, despite *A. pullulans* abundance in grapevine wood being recently associated to increased severity of Esca tiger stripes (Karácsony et al., 2023). These, and other, fungi may even alter their lifestyle in response to leaf senescence-like processes, that are reported during early symptoms development (Bortolami et al., 2023). On the other hand, *Debaryomyces*, a genus involved in the biological control of several plant pathogens (Hernandez-Montiel et al., 2018; Medina-Córdova et al., 2018; Droby et al., Aug. 1989), and whose role in the grapevine endosphere remains unknown, is abundant in YAsy leaves but absent in Chl leaves, in both cultivars (Fig. 4). This genus may be especially sensitive to the plant physiological changes that precede symptoms onset, and its temporary absence may -hypothetically- deprive the plant of a beneficial taxon useful to maintain community homeostasis or involved in detoxification.

4.3. Symptoms phenotypes: mycobiome and anthocyanins – objective 3

In this study, we present the first microbiological evaluation of grapevine leaves affected by interveinal necrosis and manifesting different symptom phenotypes, as well as their qualitative and quantitative anthocyanin composition. In previous studies, other authors demonstrated that endophytes can manipulate their host, leading to alterations in their metabolite profile, including that of anthocyanins (Yu et al., 2020), therefore, we aimed at understanding whether there is a correlation between leaf mycobiome and anthocyanins.

There are numerous factors that contribute to shaping microbial composition and anthocyanin amounts and profiles, complicating the establishment of a direct correlation. Two well-known factors are grapevine genotype and vintage (Papadopoulou et al., 2022; Aleynova et al., 2023); a third is sampling time, and a fourth is the inherent phenotypic variability observed in each individual leaf. Sampling timing is a critical factor for three main reasons. (i) A pathogen attack, such as the one observed in 2020 for *P. viticola*, may rapidly alter microbial and metabolic profiles (Barroso-Bergadà et al., 2023; Nascimento et al., 2019), affecting genotypes differently, depending on their susceptibility to this oomycete. (ii) The application of plant protection products, which typically precedes symptom onset, likely alters leaf microbial composition (Gobbi et al., 2020; Rantsiou et al., 2020; Sumby et al., 2021), leading to an over- or under-representation of certain taxa in the sequence of events preceding symptom development. (iii) The anthocyanin profile may vary rapidly depending on the symptom 'stage,' as suggested in a preliminary study (Larignon et al., 2004). Regarding phenotypic variability, we based our sampling on the percentage of necrotic tissue, *i.e.*, 10–25 % of the total leaf area; however, there is also variability in the area of pigmented leaf blade, when comparing individual leaves. Some exhibit approximately a 50:50 ratio of pigmented-green tissue (Fig. 1, I), while others have less pigmented area, *e.g.*, 20:80. This may influence both anthocyanin measurements and fungal abundance, as certain fungi may preferentially colonize pigmented tissue. Future studies should keep in consideration these factors to refine our understanding of specific aspects of esca leaf stripe symptom.

Despite these challenges, our results reveal major differences in microbial composition and anthocyanin profiles between symptomatic and asymptomatic leaves, as well as among different symptom phenotypes.

4.3.1. Mycobiome

The symptom phenotypes under examination and asymptomatic leaves often differ in terms of community indices and individual taxa abundance, though this is cultivar- and/or vintage-dependent. When examining community indices, significant differences or strong trends are observed when comparing Esca and Asy leaves in Cabernet Sauvignon. This highlights the major alterations occurring in the fungal community profile during Esca symptom development, as described earlier. Trends are also observed in Touriga Nacional, although not across both examined years (Figs. 5 and 6). *Botrytis caroliniana* and *Stemphylium* sp. are often over-represented (or exclusively present) in Esca leaves, particularly in Cabernet Sauvignon, where an over-representation of *Botrytis* sp. in red-colored leaves was previously reported (Chen et al., 2020), supporting our findings. It remains to be determined whether *B. caroliniana* colonizes exclusively red-pigmented tissue or the entire leaf (both pigmented and green tissues) and to evaluate whether this taxon engages in host manipulation or finds a preferential niche in leaves undergoing physiological alterations that lead to the development of red pigmentation.

On the other hand, neither BDA nor Apox leaves differ from Asy leaves in any community index across both vintages and cultivars, with only one exception (Fig. 5). In this case, the sequence of events leading to these symptom phenotypes did not significantly affect fungal communities, and individual taxa are only moderately affected (Fig. 8). Notably, none of the taxa over-represented in purple leaves in (Chen et al., 2020) are similarly over-represented in BDA leaves in our study.

Examining community indices, Esca and BDA leaves differ significantly in one cultivar/vintage combination (beta diversity in Cabernet Sauvignon – 2021), although multiple trends are observed. Among the several taxa over- or under-represented in each symptom phenotype (Fig. 8), *B. caroliniana* is significantly more abundant (or exclusively present) in Esca leaves, further emphasizing this fungus involvement in this symptom phenotype.

Overall, this suggests that, among the 'Esca – BDA – Asy' leaves, BDA leaves occupy an intermediate position, as their endophytic fungal communities differ moderately from both Esca and Asy leaves (Fig. 6).

It is difficult to identify a clear pattern that suggests a strong involvement of microbial structures or specific taxa in the Apox symptom phenotype, since the evaluation was conducted for only one year. It is possible that the rapid sequence of events leading to symptom onset and eventual leaf fall occurs so quickly that fungal endophytes have a very limited time to respond (Magnin-robert et al., 2016).

4.3.2. Anthocyanins

In this study, in both cultivars, Esca and BDA leaves accumulated significant amounts of anthocyanins, while no anthocyanins were detected in Asy leaves. The absence -or the presence of trace amounts- of anthocyanins in healthy and non-senescing leaves has been previously demonstrated in V. *vinifera* (Kedrina-okutan et al., 2018; Negro et al., 2020; Gutha et al., 2010; Guidoni et al., 1997).

The total anthocyanin accumulation in Cabernet Sauvignon leaves was higher than in Touriga Nacional, in both vintages, regardless of the symptom phenotype. This confirms that the ability to accumulate

anthocyanins in vegetative organs (beyond berries) is a cultivar-specific trait. Touriga Nacional accumulated more anthocyanins in Esca leaves compared to BDA leaves, highlighting that, in this cultivar, anthocyanin accumulation also depends on the interaction cultivar*symptom phenotype (Table 2).

Unlike berries, few studies have explored the anthocyanin profile of grapevine leaves. It is known that grapevine leaves mainly accumulate cyanidin-based anthocyanins and, to a lesser extent, tri-hydroxylated forms, as the expression of flavonoid 3′5′ hydroxylase in grapevine leaves is limited (Kobayashi et al., 2009). For example, Pinot Noir leaves accumulate di-hydroxylated anthocyanins (Matus et al., 2017), and cyanidin 3-*O*-glucoside is the prevalent form in Sangiovese leaves (Negro et al., 2020). Similarly, in Yan73 leaves, over 40 % of total anthocyanins were peonidin-derivatives, followed by cyanidin-derivatives (20 %) (Xie et al., 2020; Guan et al., 2012). In the present study, di-hydroxylated anthocyanins made up 69 % of the total concentration in Cabernet Sauvignon and 75 % in Touriga Nacional, consistent with findings in other varieties (Fig. 9). Unlike leaves, tri-hydroxylation in berries is generally prevalent relative to di-hydroxylation (Ferrandino et al., 2012), and acylation, besides being genotype–dependent, is largely a response to stress conditions, for example high temperatures and limited water availability. Besides, in berries, acylation of free anthocyanins happens later relative to the formation of the free forms, increasing thermostability.

Both Cabernet Sauvignon and Touriga Nacional primarily accumulated di-hydroxylated anthocyanins, with the highest levels observed in Touriga Nacional BDA phenotype (2021), where they accounted for 100 % of the total anthocyanin concentration. Since methylation occurs after the formation of the simplest non-methylated di-hydroxylated anthocyanin (i.e. cyanidin 3-*O*-glucoside), the BDA phenotype may represent an early stage of symptom development, which could either progress to the Esca phenotype or remain as BDA (Lecomte et al., 2012). This transition may trigger the methylation of the initial non-methylated anthocyanins. This is likely associated with BDA pigmentation (Fig. 1, I), wine-red to dark purple, with an average cyanidin 3-O-glucoside incidence of 50 % of the total amount. If the leaves alter to Esca pigmentation, dominated by forms with higher levels of methylation (peonidin 3-*O*-glucoside and the tri-hydroxylated malvidin 3-*O*-glucoside), the color shifts to tonalities of red (Fig. 1, II). This is especially relevant in Touriga Nacional, where tri-hydroxylated anthocyanins and acyl-derivatives accumulated in Esca leaves but were absent or only present in trace amounts in BDA leaves. In Cabernet Sauvignon, tri-hydroxylated anthocyanins and acyl-derivatives were detected in both symptom phenotypes, however, always in lower amounts in BDA leaves.

The higher level of acylation found in the combination grapevine*Esca leaves, regardless the cultivar, could be considered as a specific response of grapevine leaf tissue to esca, in line with what happens in berries, where acylation happens later relative to the formation of the simplest non-acylated free forms. Thus, through the analysis of the anthocyanin profile of symptomatic leaves, we can support the hypothesis of the transition from BDA infection, resulting in the prevalent accumulation of the simplest cyanidin 3-*O*-glucoside, to the esca syndrome. This development, particularly in Cabernet Sauvignon leaves, resulted in a high total anthocyanin concentration, a high incidence of tri-hydroxylated and acylated anthocyanins (average of the two seasons 41.5 % over total amounts), respect to BDA-affected leaves where their average percentage incidence stopped at 20 % (Fig. 9).

4.3.3. *Mycobiome and anthocyanins*

When comparing the Esca and BDA phenotypes, the differences observed both in mycobiome and anthocyanin profiles suggests a strong correlation between the two (Fig. 10).

Our result show that there is no single taxon exclusively present or significantly over-represented only in Esca or only in BDA leaves, across cultivars and vintages. This is not in support of the hypothesis that fungi are responsible for manipulating their host toward the accumulation of anthocyanins (Collinge et al., 2022; Pan et al., 2020; Yu et al., 2020), leading to either symptom phenotype, even though the role of *B. caroliniana* should be further explored. Instead, we hypothesize that physiological and biochemical events that occur in leaves lead to alterations in fungal endophytes abundance. In this scenario, taxa under- or over-represented in specific symptom phenotypes suggest different tolerance to such events. These include alterations in hormonal and lipidic profiles (Goufo and Cortez, 2020), phytoalexins accumulation (Calzarano et al., 2016), the synthesis of anthocyanins precursors, such as p-coumaric acid and quercetin, molecules with known antifungal activity (Hu et al., 2023; Nguyen and Bhattacharya, 2022), and the expression of defense-related genes (*e.g.* chitinases and other PR-proteins) (Valtaud et al., 2009; Magnin-Robert et al., 2011).

The microbial structure and lack of anthocyanin accumulation in Asy leaves are altered when examining BDA leaves. Here, we observe mild changes in microbial composition and the exclusive accumulation of di-hydroxylated anthocyanins in Touriga Nacional, with moderate amounts of tri-hydroxylated anthocyanins and acyl derivatives in Cabernet Sauvignon (Figs. 6 and 9). This indicates a BDA-specific triggering factor, occurring earlier in the season, leading to minor or no cytological and histological changes in affected leaves and canes (Valtaud et al., 2011; Fleurat-Lessard et al., 2013). In contrast, Esca leaves show more pronounced microbial alterations, accompanied by a greater accumulation of tri-hydroxylated anthocyanins and acyl derivatives in both cultivars, both associated with stronger stress responses (Figs. 6 and 9). This suggests an Esca-specific triggering factor, occurring slightly later in the season, leading to major cytological and histological changes in affected tissues (Valtaud et al., 2011; Fleurat-Lessard et al., 2013).

Alternatively, a single triggering factor could regulate the expression of either BDA or Esca phenotypes in a concentration-dependent manner, with low concentrations leading to BDA and high concentrations resulting in Esca.

Overall, the evidence and insights presented in this study support the view that the BDA and Esca leaf phenotypes are distinct. We can speculate that intermediate phenotypes, or the transition from BDA to Esca (and never *vice versa*), may result from the simultaneous or sequential action of the Esca and BDA triggering factors. This hypothesis does not deal with the origin of the triggering factors, therefore, Larignon's original view that the BDA phenotype has different etiological agents from Esca remains to be verified (Larignon et al., 2009). The present study, however, highlights the need to identify the triggering factor(s) to move a step forward.

On this matter, it has been demonstrated that during esca progression in woody tissues, besides the reduction of the sapwood functionality (Gómez et al., 2016), there may be impairments also in the sap flow of the leaves. This leads to a vascular occlusion and cavitation phenomena (Bortolami et al., 2023; Bortolami et al., 2019) which could affect the leaf xylemic vessels and, probably, bring about disruptions of phloem transport, involving, to some extent, the regular translocation of nutrients, especially sugars. The impairment in sugar transport out of the leaf can trigger sugar accumulation, and consequently, anthocyanin accumulation. It is well and longtime known (Pirie and Mullins, 1976) that carbohydrate accumulation induces the up-regulation of the phenylpropanoid pathway, notably increasing the anthocyanin accumulation (Weiss, 2000; Teng et al., 2005; Solfanelli et al., 2006; Murakami et al., 2008; Peng et al., 2008). However, sugar increase is not the only driver of anthocyanin accumulation; in fact, the berry anthocyanin accumulation involves a synergic effect between sugar accumulation and abscisic acid (Pirie and Mullins, 1976), a key-mediator of stress (Ferrandino and Lovisolo, 2014; Cao et al., 2011), and a senescence-related hormone (Pirie and Mullins, 1976; Gao et al., 2016; Zhao et al., 2016). It was previously demonstrated that carbohydrate metabolism is altered in Esca leaves, with an accumulation of fructose (Valtaud et al., 2011). Additionally, there is an increased abundance of

senescence-related hormones, such as abscisic acid, jasmonic acid, and salicylic acid, when compared to asymptomatic leaves (Goufo and Cortez, 2020). The accumulation of anthocyanins, along with the presence of senescence-related hormones and vascular occlusions, shares several similarities with leaf senescence processes, as previously suggested by (Bortolami et al., 2023).

5. Conclusion

This study provides new insights into the interplay between fungal communities and anthocyanin profiles in grapevine leaves affected by esca and related symptom phenotypes. Using DNA metabarcoding, we observed that Esca symptom progression correlates with shifts in fungal diversity, suggesting early biochemical events that precede the appearance red pigmentation. In addition, our findings highlight significant differences in fungal composition and anthocyanin profiles across symptom phenotypes, with *Botrytis caroliniana* being especially associated with red pigmentation. While di-hydroxylated anthocyanins dominated in BDA leaves, tri-hydroxylated anthocyanins accumulated more in Esca leaves, suggesting a potential progression between these phenotypes driven by biochemical and microbial changes. No single fungal taxon was uniquely linked to a specific symptom phenotype, indicating that microbial shifts likely respond to, rather than trigger, host physiological and biochemical alterations. Anthocyanins emerged as both stress-response metabolites and potential indicators of disease progression.

Future studies should investigate the molecular interactions between fungal communities and host metabolism to better understand the mechanisms driving these changes. Additionally, exploring the genetic and environmental factors influencing symptom variability could aid in developing strategies to mitigate trunk diseases impact and improve vineyard sustainability.

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CRediT authorship contribution statement

Giovanni Del Frari: Writing – review & editing, Writing – original draft, Visualization, Investigation, Funding acquisition, Conceptualization. **Chiara Ingrà:** Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Marie Rønne Aggerbeck:** Writing – review & editing, Writing – original draft, Visualization, Formal analysis, Data curation. **Alex Gobbi:** Investigation, Data curation. **Teresa Nascimento:** Investigation. **Ana Cabral:** Investigation. **Helena Oliveira:** Writing – review & editing, Funding acquisition. **Lars Hestbjerg Hansen:** Writing – review & editing, Funding acquisition. **Alessandra Ferrandino:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition. **Ricardo Boavida Ferreira:** Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

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Data availability

Sequencing data is publicly available - see manuscript

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Season evolution of polyphenols during leaf aging in cv Nebbiolo.

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Abstract. The different organs of the grapevine show quantitative and qualitative differences in phenolic compounds. High concentrations of flavonols, depending on variety and environmental conditions, characterize leaf blades and veins, where they cover the role of protectors of the tissues from light. Knowledge of seasonal fluctuations of leaf phenols is limited: the purpose of our project was to study the seasonal evolution of concentrations and profiles of flavonols, flavan-3-ols, and hydroxycinnamic acid esters (HCA) in blades and veins of aging leaves opposite to the cluster, collected in young and old vineyards, in cv Nebbiolo. The concentration of flavonoid polyphenols decreased during the season and showed re-arrangements of the profiles. HCA concentration was stable during the season but the caftaric acid percentage incidence over total amounts slightly decreased (-5%). Comparing leaves collected from old and young plants for flavonols and HCA, no major differences were found, regardless of the leaf compartment analyzed. Instead, flavan-3-ol concentration was higher in the leaves of older plants. We noticed a clear response in terms of leaf polyphenolic accumulation following the adoption of canopy management practices such as defoliation; the detected increase was dependent both on the age of plants (high response in old vines) and on the leaf compartment (high response in blades).

1. INTRODUCTION

Grapevine (*Vitis vinifera* L.) phenolic compound profiles and accumulation are affected by genotype, environmental conditions, and cultural practices and vary among plant organs, tissues, and phenological stages. They are synthesized from the amino acid phenylalanine through the phenylpropanoid pathway [1]. Phenolic compounds have peculiar properties and play important roles in grapevine biology and ecology. One of their most important properties in response to a wide range of stress is their antioxidant activity, which leads to inhibiting the generation of reactive oxygen species (ROS) or reducing their amounts. Polyphenols take part in the grapevine adaptation mechanisms to the environment; they protect vegetative and reproductive organs from ultraviolet radiation and they are implicated in the resistance against pathogens. It is longtime known and well-detailed that grapevine berry quality, particularly that of wine-grape varieties, is largely dependent on polyphenol accumulation [2, 3, 4]. Profiles of polyphenols in berries, such as the ratio of tri-hydroxylated and di-hydroxylated anthocyanins and caftaric and coutaric acid, allow classifying *Vitis vinifera* varieties and clones [2, 5]. Agronomic and cultural practices influence phenolic compound biosynthesis; in fact, practices that favor exposing bunches to light, such as leaf and/or lateral shoot removal, influence polyphenol accumulation in berries, influencing grape health and quality [6, 4].

Grapevine leaves contain a wide range of phenolic compounds. In a study conducted by Kedrina-Okutan and co-workers, it was shown that concentrations and profiles of main polyphenols in the leaves of *Vitis vinifera* varieties [7] and *Vitis* species [8] varied according to different leaf compartments (blades and veins). To date, much less is known about polyphenol fluctuation during leaf aging. Leaves are the main carbon absorption source in plants, having an important role in CO_2 fixation and biomass production during the season. In addition to the changes that occur in photosynthetic activity due to environmental factors, photosynthesis is influenced by leaf senescence. The chlorophyll content, Rubisco activity, and photosynthetic electron transport are strongly associated with leaf aging [9]. Leaf senescence is linked with increased content of ROS [10]; therefore, compounds related to oxidative stress, such as phenolic compounds, could play important roles during this process. The final step of the grapevine leaf annual cycle is also delineated by differences in color, and this is caused by the degradation of chlorophyll. In addition, some genotypes also synthesize anthocyanins, which are responsible for the red color appearance. These compounds can protect photosynthetic apparatus, shielding leaves from potential damage due to light [11]. Some studies consider the effects due to leaf aging on the photosynthetic apparatus and secondary metabolites [12, 13], but few studies relate leaf aging throughout the season, according to insertion level, to phenolic compound fluctuation. The present research explores the polyphenolic concentration and profiles of Nebbiolo grapevine mature and basal aging leaves during the season, to individuate changes in the phenylpropanoid concentration, particularly that of flavonols, low molecular weight flavan-3-ols and hydroxycinnamic acid esters (HCA).

2. MATERIALS AND METHODS

2.1 Plant Material

Nebbiolo healthy leaves (no visible signs of any biotic or abiotic alteration were evident) opposite to the cluster and alternative sampled in sun-exposed and shaded conditions were sampled in four vineyards located in the municipality of Grinzane Cavour (CN), within the geographical mention Raviole, located between 230 m and 260 m asl, West/South-West exposed and about 300 meters apart. We examined two young vineyards (5 and 6 years old) and two old vineyards (more than 30 years old): within the different vineyard ages, one vineyard was managed following the integrated protocol for plant protection, and the other was organically managed. Vines were vertical shoot positioned and Guyot pruned. The vineyard was organized into randomized blocks of 12 vines each. Since leaf age decreases towards the shoot tip, analyzing changes in the profile and accumulation of phenolic compounds of the leaf opposite to the cluster (i.e., the first leaves formed after bud-break) allows evaluating the polyphenolic evolution during leaf aging. Leaves opposite to the clusters were sampled at eight different time points: 1) 9th of June (160 day of the year, DOY 160, 7 days after flowering, beginning of berry set), 2) 22th of June (DOY 173, cluster closure), 3) 15th of July (DOY 196, 35 days after berry set), 4) 28th of July (DOY 209, 14 days before veraison), 5) 11th of August (DOY 223, veraison, 50%), 6) 9th of September (DOY 252, 29 days after veraison), 7) 28th September (DOY 271, 48 days after veraison) and 8) 6th of October (DOY 279, harvest) in 2021.

2.2 Sample Extraction

We worked on fresh leaves, immerging blades, and veins separately in a hydroalcoholic buffer at pH 3.9 (40% ethanol, 2 g/L of $Na_2S_2O_5$, 5 g/L of tartaric acid, 22 mL/L of 1 N NaOH) and preparing extracts accordingly to Kedrina-Okutan *et al.,* 2018).

2.3 Analyses of Individual Phenolic Compounds

2.3.1 Sample Preparation

Leaf extracts were diluted with 1 M phosphoric acid (1.1-fold) and filtered (0.20 μm) into dark the vials (Di Stefano and Cravero, 1992).

2.3.2 Quantitative Analyses of Phenolic Compounds by HPLC-DAD

Individual phenolic compounds of leaf extracts were separated by a reverse-phase column Licrosphere 100 RP-18 (5 μm particle size) packed with LiChroCART 250-4 (25 × 0.4 cm ID) HPLC-Cartridge (Merck KGaA, Germany) with a guard column (LiChroCART 4-4); the column was thermostated at 25 °C. Solvent A was phosphoric acid 10^{-3} M and solvent B was pure methanol. Chromatograms were acquired at 280, 320, and 360 nm simultaneously, and the run time was 50 min. Compounds were identified based on spectrum correspondence with authentic standards and quantified by the external standard method through calibration curves. Data were expressed in mg/kg of fresh weight (FW) and per unit of leaf surface area.

2.4 Agronomic measurements

During the vegetative season, we measured the canopy development, to be able to express data per foliar surface unit and to evaluate influences attributable to different leaf densities tied to the agronomic practices applied. Four leaf contact surveys were carried out during the season, at DOY 160 (7 days after flowering, beginning of berry set), at DOY 196 (35 days after berry set), at DOY 223 veraison, 50%), and at DOY 252 (29 days after veraison), according to the Point Quadrat method (Smart, 1985). Thirty contacts per replicate were taken in the canopy area between the hedge wire and the first couple of wires. The canopy density (d) was obtained through the following ratio: SAr/LA, where: 1) $SA_{reviewed*}$ = (2 x H x S) where H = average wall height; S = wall thickness; *in the original formula no reference is made to wall thickness; 2) LA = (H x N - b) where H = average wall height; N = the total number of foliar contacts obtained through the Point Quadrat method / n° of wall insertions; b = percentage of canopy holes.

2.5 Statistical Analysis

All data were analyzed by RStudio Build 372 software program version for Windows. Analysis of variance was performed by two-way ANOVA and followed by Bonferroni posthoc test at $P \leq 0.05$. All measurements were performed in triplicates, and results were expressed as means ± standard errors.

3. RESULTS

We analyzed polyphenol accumulation in the blades and veins of younger and older plants of cv Nebbiolo during the vegetative season, expressing data based on fresh weight.

3.1 Flavonols

Flavonol glycosides were quantitatively the most abundant phenolic compounds in the leaves of young and old plants. The total flavonol glycosides content (Fig. 1) in blades of young plants ranged from 4387.06 to 3232.36 mg/kg and from 2023.67 to 1249.87 mg/kg in veins. In old plants, the total concentration in blades varied from 4471.66 to 2887.30 mg/kg and in veins from 1773.88 to 1300.52 mg/kg. The highest total concentration was measured at DOY 160 (14 days before veraison) in leaf blades, and the content of flavonols tended to decrease during the season regardless of plant age. Oppositely, in the veins of young plants, the highest concentration of

total flavonols was assessed at DOY 209 (14 days before veraison) but the concentration was generally stable regardless of the age of the plants. In blades and veins, the profile was similar: the main compound was quercetin 3-O-glucuronide which accounted for up to 70% of the total concentration followed by quercetin 3-O-glucoside.

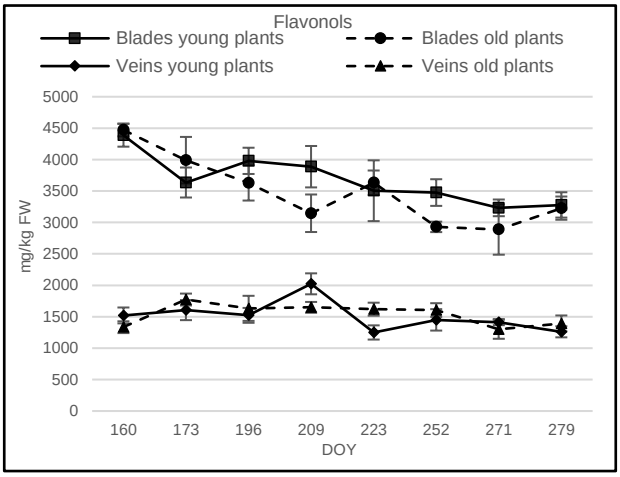

Fig. 1. Total flavonol evolution during the season of Nebbiolo young and old plants in leaf and blades (n=3 ± s.e).

3.2 Hydroxycinnamic acid esters

Hydroxycinnamic acid esters (HCA; Fig. 2) were the most abundant non-flavonoid phenolic compounds in grapevine leaves. Amounts ranged from 2725.66 to 1308.67 mg/kg in blades and from 1342.48 to 738.58 mg/kg in veins in leaves picked in young plants. The total concentration in blades of older plants ranged from 3044.14 to 1562.62 mg/kg and in veins from 1474.22 to 812.45 mg/kg. Generally, a decreasing trend was assessed during the season, particularly in blades in which the highest concentration was measured at DOY 160 (7 days after flowering, beginning of berry set), regardless of the age of the plant. The prevalent HCA was *trans*-caftaric acid (82-87%) followed by *trans*-coutaric acid.

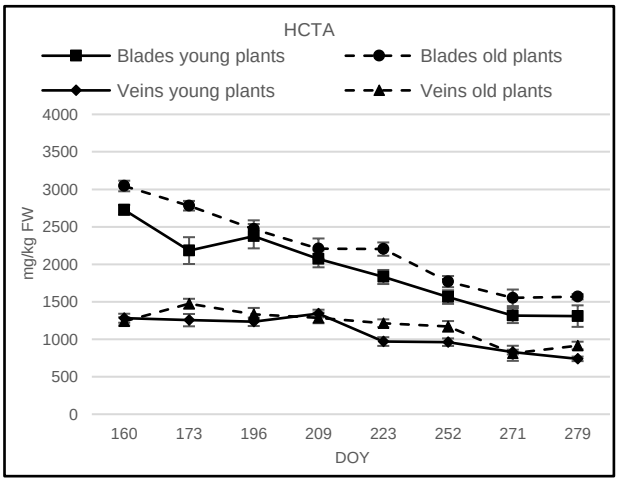

Fig. 2. Total HCA evolution during the season of Nebbiolo young and old plants in leaf and blades (n=3 ± s.e).

3.3 Flavan-3-ols

Different from the flavonols and HCA, the concentration of flavan-3-ols was similar in the two leaf compartments of the leaves collected from young plants, with a stable or slightly increasing trend. In old plants, the trend was similar in the two leaf compartments with high concentrations, especially in the blades, compared with young plants (Fig. 3).

In young vines, the leaf blade concentrations of flavan-3-ols ranged from 741.39 to 560.84 mg/kg and from 957.17 to 414.32 mg/kg in the leaf vein. In older plants, the amounts in blades ranged from 1489.50 mg/kg to 1072.58 mg/kg of FW, and in veins from 1031.81 mg/kg to 515.74 mg/kg of FW. The (+)-catechin was found to be the main compound in the leaf, accounting for 35% of the total concentration in leaf blades followed by (-)-epicatechin with 10%, whereas in veins (+)-catechin accounted for 40%, followed by (-)-epicatechin, accounting for about 7%.

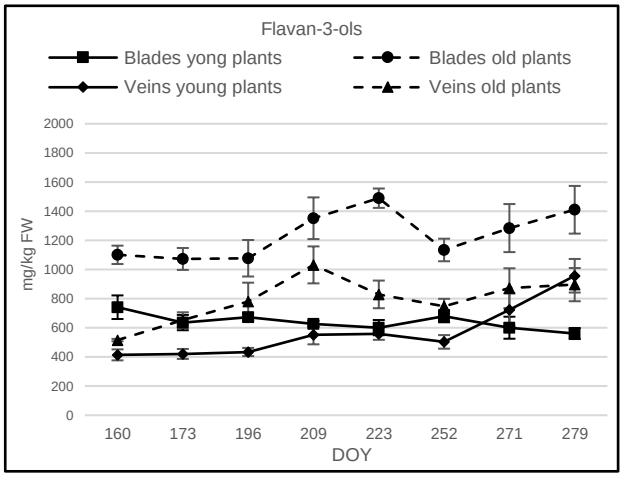

Fig. 3. Total flavan-3-ols evolution during the season of Nebbiolo young and old plants in leaf and blades (n=3 ± s.e).

3.4 Phenolic compounds and canopy density

Measurements of the leaf wall during the season allowed relating the concentrations of the three classes of phenolic compounds analyzed to the canopy development. Studying the ratio between the concentration of polyphenols (mg/kg fresh weight) and the canopy density, measured between the first wire and the first couple of wires (the area where sampling was carried out), differences between young and old plants emerged. The graphs show that close to veraison (DOY 223), the concentration of the three classes of polyphenols increases, and the trend is the same for all molecules, regardless of the leaf compartment analyzed and the age of the plants. As regards flavonols (Fig. 4A), the highest concentration was found in blades rather than in veins and the trend was consistent regardless of the age of the plants. HCTA (Fig. 4B) showed similar concentrations between blades and veins except for blades of old plants. Finally, for flavan-3-ols (Fig. 4C), higher concentrations were found in old plants in both tissues compared to young plants.

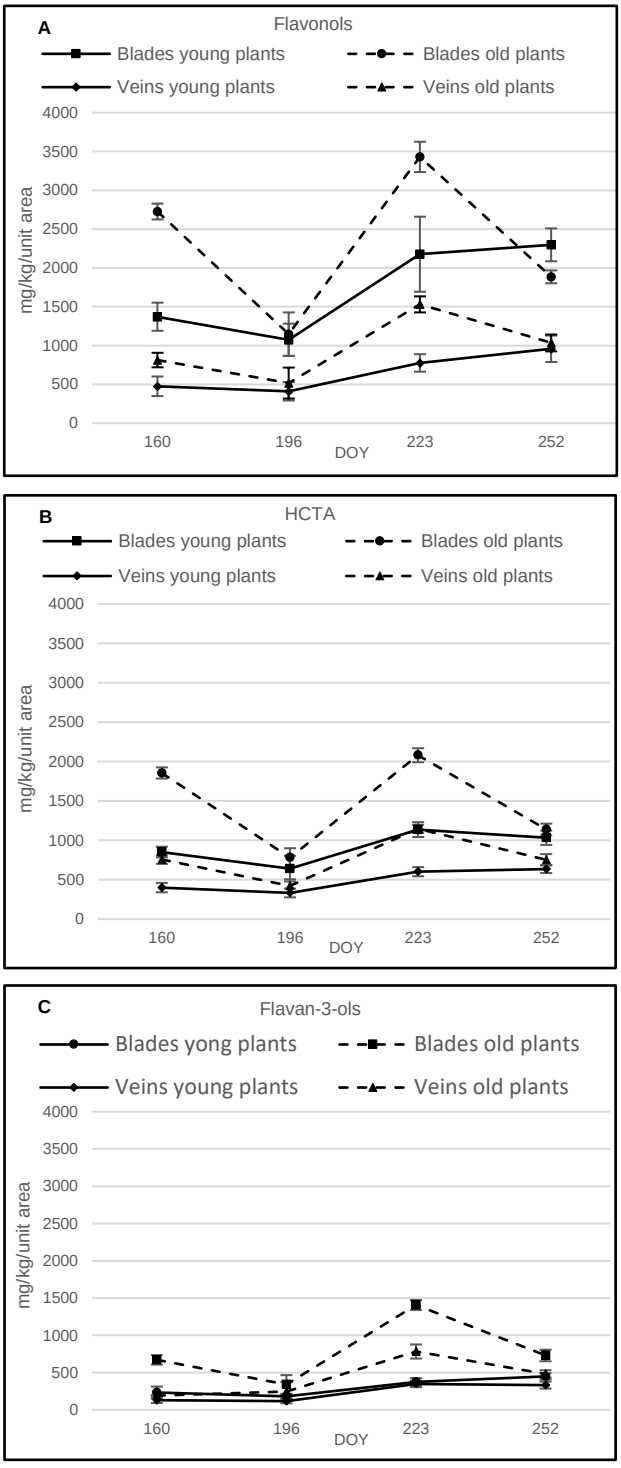

Fig. 4. The ratio between the concentration of (A) flavonols, (B) HCA and (C) flavan-3-ols and the canopy density of Nebbiolo young and old plants in leaf and blades (n=3 ± s.e).

4. DISCUSSION

We examined the accumulation and the profile of flavonols, HCA, and flavan-3-ols in Nebbiolo leaves collected in young (5 and 6 years old) and old (more than 30 years old) vineyards. Our sampling model ensured the investigation of aging leaves during the season as we always sampled the leaf opposite to the cluster. Initially, we aimed to assess whether the different vineyard management, as to the grapevine protection against pathogens, had repercussions on the accumulation of leaf polyphenols. In fact, within the same plant age, one of the two vineyards was organically conducted and the other was managed accordingly to the integrated pest management protocol. As no statistically significant differences were found comparing the total polyphenol concentrations of the leaves collected in the vineyards managed accordingly to the two different pest-management regimes (data not shown), we exclusively took into account the age of the plants and we evaluated the polyphenol seasonal evolution of aging leaves collected from young and old vines.

The accumulation trend of total flavonols in blades and veins did not differ among plants of different ages. Flavonols play key roles in the physiological processes of plants; these compounds, mainly located in epidermal cells, are involved in the photoprotection of tissues and excess energy dissipation, and they are also strongly correlated with diseases [14] as in the case of infection by *Plasmopara viticola* [15]. These compounds have also been indicated as scavengers of the abscisic acid-dependent reactive oxygen species [16]. Our results showed that during the season there is a slight decreasing trend in flavonol concentration, in line with previous results, obtained in another season and leaves collected in a different location [7]. In that study, it emerged that during the season in leaves randomly collected from the 4th to the 7th node on the shoot of old Nebbiolo vines, the quercetin glycoside type incidence on total concentrations changed. The percentage incidence of quercetin 3-O-glucuronide decreased during the season (-20%), whereas the glucoside form incidence increased (+30 %). Results of the present study obtained analyzing fully expanded and aging leaves, show similar behavior (a progressive seasonal decrease of the glucuronide percentage incidence, -8% and a slight increase of the glucoside (+6%) but to a minor extent.

Also, the study by Bouderias and collaborators [17], showed how the position of the leaf on the shoot and the sampling time affected the accumulation and profile of flavonols. Quercetin 3-O-glucuronide displayed the highest levels in young leaves and its concentration slightly decreased during the season, in line with our results. According to Kőrösi and collaborators [18], photocatalytic oxidative stress induces a decrease in the concentration of quercetin 3-O-glucuronide in the leaf, thus emphasizing the importance of this compound during plant defense processes. However, considering that flavonols are precursors to the biosynthesis of other molecules, we can speculate that during the season their total accumulation decreases in favor of the accumulation of other compounds (mainly anthocyanins, not measured in the present study), following the beginning of the leaf senescence processes.

Hydroxycinnamic acid esters are the most abundant group of non-flavonoid phenols in grapevine. HCA are accumulated as constitutive molecules in grapevine organs [19] and they protect tissues from visible light but not from UV light [20]. Leaf hydroxycinnamates showed a decreasing trend during the season, particularly in blades, regardless of plant age. *Trans*-caftaric acid was found to be the main non-flavonoid compound in leaves

followed by *trans*-p-coutaric; during the aging *trans*-caftaric acid undergoes a slight decrease in concentration, equal to 5%, both in blades and in veins of young and old plants. Conversely, the concentration of *trans*-p-coutaric acid remained unchanged during the season, both in blades and in veins (7 and 9%, respectively). These compounds have been indicated as possible molecules with anti-fungal activity in *Vitis vinifera* and in *Arabidopsis* [21, 22]. Considering that the leaves of this study were healthy the amounts we detected can be considered constitutive and not stress-induced amounts. To date, there are no studies in the literature that highlight the fluctuations of these compounds during leaf aging.

Some flavan-3-ols, like epicatechin, can act by inhibiting pro-oxidant enzymes, such as NADPH oxidases and lipoxygenases [23]. To date, little is known about the flavan-3-ols fluctuation in grapevine leaves; present results show that the concentration remains quite stable or slightly increases throughout the leaf aging. Unlike the other compounds, whose concentration is markedly higher in blades than in veins, the concentration of flavan-3-ols was similar in the two leaf compartments, especially in young plants. In old plants, the concentration of flavan-3-ols was higher than in young plants; therefore, one could speculate that high concentrations of leaf monomeric flavan-3-ols are a peculiar trait of old vines. (+)-catechin was found to be the main flavan-3-ol in the leaf, accounting for 30% of the total concentration followed by (-)-epicatechin (10%) in blades whereas in leaf veins (+)-catechin reached 40% and (-)-epicatechin 7%. Moreover, the percentage of (+)-catechin displayed a decreasing trend during the season: in young plants, the percentage of decrease in leaf blades and veins was 13%. In old plants, this behavior was more marked in veins, in which there was a drastic seasonal decrease of (+)-catechin, which reached 44%. On the contrary, in leaf blades of old plants, between DOY 160 and 279, the percentage of (+)-catechin decreased by 24%. Further studies are necessary to understand if this profile variation could be ascribed to the leaf tissue aging and/or to the plant age. As high concentrations of constitutive proanthocyanidins (polymeric flavan 3-ols) can be related to the low susceptibility to certain diseases, such as flavescence dorée [24], we can speculate that a high quantity of monomeric proanthocyanidins could represent an important element of tolerance to stressors.

Grapevine canopy management is an important factor to define the composition of grapes and wines. Leaves and/or lateral shoots (= defoliation) can be removed in the cluster area, between fruit set and ripening, to reduce the canopy density, improve intra-canopy air circulation, and influence bunch microclimate. Considering that defoliation affects berry phenolic composition, as well as detailed in numerous papers, we evaluated if this agronomic practice could also affect the leaf phenolic composition. UV-B radiation is one of the strongest influences on the biosynthesis of phenolic compounds. For all the compounds that we analyzed, close to berry veraison and after the leaf removal (which viticulturists practiced approximately during the second half of July, DOY 196-206), there was an increase in concentration in both blades and veins, and this increase was more pronounced in old plants. This could suggest that the remaining leaves after defoliation are more exposed to sunlight, the ROS concentration increases and the plant responds by accumulating antioxidants.

In conclusion, we found a seasonal variation in the concentrations of the main leaf polyphenols; this variation, related to the aging of the leaf, was more marked in the blades respect to the veins. Moreover, we noticed a clear response in terms of leaf polyphenolic accumulation following the adoption of canopy management practices such as leaf/lateral shoot removal, which was performed around mid-July. This increase seems to be dependent both on the age of plants (high response in old vines) and on leaf compartments (high response in blades). Other investigations are underway to clarify what happens to leaf polyphenols following defoliation. In addition, given the recognized importance of certain secondary metabolites in mediating tolerance/resistance responses to pathogens, further studies in this field appear to be of interest to provide knowledge on the innate and/or inducible self-defense abilities of the grapevine.

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8. Conclusion and future perspective

This study explored two complementary and still partially unexplored aspects of grapevine (*Vitis vinifera* L.) response to plant protection products and grapevine trunk diseases, with particular focus on the esca complex—one of the most severe and widespread diseases affecting vineyards worldwide. Through a multidisciplinary approach, we investigated the grapevine intrinsic defence mechanisms, with particular emphasis on stilbene accumulation in response to pruning wound protection treatments, as well as the dynamics of the foliar endophytic fungal community and its possible relationship with the appearance and variability of esca-associated leaf symptoms. The decision to investigate these two specific aspects of grapevine response, with particular focus on the esca complex, stems from the need to address two major challenges associated with this pathology: on the one hand, the difficulty of diagnosing the presence of pathogenic fungi inside the plant at an early stage; on the other hand, the availability and actual effectiveness of management or curative practices capable of activating or supporting the vine natural defense mechanisms. The fungal pathogens responsible for esca colonize exclusively woody tissues, making early detection extremely challenging without invasive interventions, such as the removal of trunk sections. Moreover, foliar symptoms—typically expressed as leaf reddening—are not caused by the physical presence of the pathogens in leaf tissues. For this reason, we aimed to assess whether such pigmentation might result from a metabolic imbalance in the plant, particularly the accumulation of anthocyanins as part of a systemic stress or defense response to the infection. In parallel, we focused on the analysis of stilbene compounds in woody tissues to evaluate the local defense mechanisms activated in response to stress conditions, a process well-documented in the literature. However, our study aimed to further investigate whether the application of pruning wound protection products could modulate this innate response—either enhancing or suppressing it—and how such effects might vary depending on environmental conditions or grapevine cultivar. In parallel, we focused on the analysis of stilbene compounds in woody tissues to evaluate the local defense mechanisms activated in response to stress conditions, a process well-documented in the literature. However, our study aimed to further investigate whether the application of pruning wound protection products could modulate this innate response—either enhancing or suppressing it—and how such effects might vary depending on environmental conditions or grapevine cultivar. Investigating both the indirect and systemic signals of stress (such as foliar alterations) and the local metabolic responses (such as stilbene accumulation in the wood) allows for a broader and more integrated understanding of the pathology and of the possible strategies to contain it sustainably.

In the first segment of the research, the analysis of stilbene accumulation in one-year-old canes allowed us to investigate the role of these phenolic compounds, especially E-resveratrol and E-ε-

viniferin, piceatannol and piceid, in the grapevine response to plant protection products. However, in both vineyards under investigation, applying pruning wound protection products did not result in any distinct differences in total stilbene concentration and profile compared to untreated control samples. However, a particularly relevant finding was the marked differences between continental and Mediterranean climates. Environmental factors emerged as a key driver in modulating both the quantitative accumulation of wood stilbenes and the qualitative composition of the stilbene profile. In the continental climate, stilbene concentrations showed a gradual decline with the progression of phenological stages in both cultivars, with a few specific exceptions. Conversely, under Mediterranean conditions, the two genotypes exhibited contrasting accumulation trends over the season, highlighting a genotype-specific response to the environment: Cabernet Sauvignon displayed an increasing accumulation trend, while Syrah maintained a stable pattern, emphasizing their divergent responses. These results suggest that, at least within the specific context of our study, no uniform or predictable response dynamic to the treatments was observed. Instead, the outcomes were influenced by multiple factors, mainly environmental conditions, cultivar and plant phenological stage. In particular, our findings highlight that climate conditions play a crucial role in shaping grapevine defence mechanisms, affecting both the total stilbene accumulation and the specificity of the metabolic profile. This underlines the importance of carefully considering the environmental background when developing and evaluating plant protection strategies, advocating for a targeted and adaptable agronomic approach that accounts for both varietal traits and the climatic characteristics of different viticultural areas. In this perspective, it is essential to explore the relationship between cultural practices and the activation of grapevine natural defences to optimize increasingly effective and sustainable integrated management strategies.

The second part of this study investigated the symptomatic variability associated with Esca in grapevine leaves, identifying three distinct phenotypes and providing, for the first time, an in-depth characterization of the leaf endophytic fungal community using DNA metabarcoding techniques. The fungal microbiome may play a role in modulating the grapevine metabolic response, particularly by influencing the accumulation of anthocyanins, pigments involved in plant defence mechanisms. However, our analyses revealed a correlation between symptom progression and shifts in fungal composition, suggesting the occurrence of early biochemical events that precede the appearance of red pigmentation in leaves. Notably, *Botrytis caroliniana* was associated with pigmentation-related symptoms, while differences in anthocyanin profiles among phenotypes pointed to a symptomatic progression shaped by both microbial and biochemical dynamics. No single fungal taxon was found to be exclusively associated with a specific symptomatic phenotype, supporting the hypothesis that changes in microbial communities are more likely a consequence of the host physiological stress

responses than a direct cause of symptom expression. In this context, anthocyanins emerged not only as key metabolites in the grapevine stress response but also as potential indicators of disease progression. These findings offer new insights into esca-associated symptoms, suggesting that their expression results from a complex interaction among the host plant, its microbiota, and environmental factors, rather than solely the presence of wood-inhabiting pathogens.

Regarding the seasonal evolution and tissue-specific distribution of flavonols, hydroxycinnamic acids, and flavan-3-ols in leaves of young and old grapevines, the results highlighted that leaf ageing significantly influences the polyphenolic profile, with a progressive decline in several compounds. This decrease, particularly marked for flavonols, can be attributed both to natural leaf senescence, characterized by reduced photosynthetic capacity and selective metabolite remobilization, and to a possible metabolic conversion into other compounds, such as anthocyanins, which were not analyzed in the present study. Anthocyanins tend to accumulate in senescent leaves, where they serve a photoprotective role by filtering excess light and mitigating oxidative damage, while facilitating nitrogen recycling through the protection of tissues during chlorophyll degradation. In contrast, other compounds, such as flavan-3-ols, showed an increasing trend, suggesting a potential adaptive function in response to aging. Furthermore, a summer defoliation event carried out around véraison led to an increase in polyphenol concentrations in the remaining leaves, likely as a response to increased light exposure and the associated rise in reactive oxygen species production. These findings underline the role of foliar polyphenols as reliable indicators of the plant physiological state and its tolerance potential to environmental and biotic stresses, offering a promising perspective for the sustainable agronomic management of vineyards.

A deeper understanding of the grapevine defence mechanisms in response to trunk diseases and protection/preventive treatments requires the integration of physiological and metabolic studies with molecular-level analyses. In this context, transcriptomic and proteomic approaches represent powerful tools to unravel the complex signalling networks that regulate the grapevine defence response. Transcriptomic analysis enables the identification of genes expressed in response to pathogen infection and the application of plant protection products, while also providing insight into the temporal dynamics of gene regulation. This approach may reveal the activation of signalling pathways related to biotic stress perception, production of reactive oxygen species (ROS), and hormone-mediated defence mechanisms, such as those involving salicylic acid, ethylene, and jasmonic acid, well-known regulators of resistance against necrotrophic pathogens. In parallel, proteomics allows for the identification of proteins expressed and modified at the post-translational level, offering a functional perspective closer to the grapevine actual physiological state. In particular, this technique can clarify which enzymes are directly involved in the biosynthesis of phenolic

compounds, such as stilbenes and anthocyanins, which are closely linked to grapevine defence. Stilbenes, including resveratrol and viniferin, function as antimicrobial phytoalexins, while anthocyanins may contribute to mitigating oxidative stress. Understanding how the biosynthesis of these metabolites is regulated at both the transcript and protein levels could provide critical insights into the mechanisms of resistance and the onset and variability of foliar symptoms observed in the field. This knowledge would support the development of more tailored management strategies that account for genotype-specific responses, phenological stages, and local environmental conditions. Moreover, the integration of omics data—metabolomics, microbiomes, transcriptomics, and proteomics—into predictive modelling frameworks offers one of the most promising avenues for sustainable management of the grapevine. Combined analysis of environmental (climate, soil, humidity, exposure), agronomic (cultural practices, pruning methods, vineyard age), and biological data can facilitate the development of high-resolution predictive models capable of more accurately assessing the symptom emergence and disease progression. Metabolomic profiles provide direct insight into the physiological status of the plant and the activation of defence pathways. Concurrently, microbiome analyses can shed light on the resilience or susceptibility of grapevines based on microbial dynamics associated with specific stress conditions or symptom phenotypes. The integration of these multidimensional datasets using machine learning algorithms or advanced statistical modelling can enable the creation of decision-support systems capable of identifying site- and cultivar-specific risk factors. These systems would guide timely and precise agronomic interventions. Ultimately, the implementation of data-driven, adaptive management strategies represents a concrete opportunity to enhance phytosanitary efficiency, reduce unnecessary chemical inputs, and increase the overall sustainability of viticultural systems.

9. List of Publications

1. Del Frari, G., Aggerbeck, M. R., Gobbi, A., Ingrà, C., Volpi, L., Nascimento, T., Ferrandino, A., Hestbjerg Hansen, L., Ferreira, R. B. (2023) Pruning wound protection products induce alterations in the wood mycobiome profile of grapevines. *Journal of Fungi*, 9(4), 488.

2. Tumminelli, E., Cavalloro, V., Ingrà, C., Ferrandino, A., Porta, A., Marrubini, G., Martino, E., Rossi, D., Collina, S. (2025). Vineyard pruning-wood waste valorisation: sustainable extraction of bioactive compounds. *Frontiers in Chemistry*, 13, 1597833.