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DOTTORATO DI RICERCA IN
SCIENZE AGRARIE ED AMBIENTALI
CICLO XXXI

COORDINATORE Prof. Giacomo Pietramellara

Effects of *Ascophyllum nodosum* extract on *Vitis vinifera*: Consequences on plant physiology, berry secondary metabolism and drought stress tolerance

Settore Scientifico Disciplinare AGR/03

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Annex IV - Extended abstract published on Acta Italus Hortus

La distribuzione fogliare di un antitraspirante naturale per il miglioramento qualitativo delle produzioni viticole

Acknowledgements

Abstract

Throughout the Mediterranean region, *V. vinifera* is subjected to a wide range of environmental stresses, especially during the summer, when prolonged dry periods are combined with high radiation load and high temperatures.

Furthermore, given the strong influence of the atmospheric factors on this species, seasonal weather pattern can significantly affect grapevine physiology and berry quality under future climate change scenario.

Although grapevines have multiple acclimation strategies to environmental constrains, the evidence for significant climate change in the upcoming decades urges adaptation and mitigation measures to be taken by the whole viticulture and winemaking sectors. Short term adaptation measures can be considered as a first aid protection strategy and should be focused at specific threats, mostly changes in agronomical practices, such as the application of biostimulants to enhance nutrition efficiency, abiotic stress tolerance and quality traits of plants.

Seaweed-based extracts have been recently employed as sustainable tools to improve abiotic stress tolerance and increase grape quality. However, the effect of these extracts on secondary metabolism compounds, that are fundamental for grape and wine quality, is still scarce.

Under this scenario, the objectives of the present PhD project were:

1) to investigate the physiological performances of *V.vinifera* and the biochemical adjustments induced in grape berry skins by *A. nodosum* foliar treatments under field conditions;

2) to evaluate if these treatments affect grapevine water relations and berry phenylpropanoid metabolism, mitigating the effects of a post-*véraison* drought in potted vines.

An enhancement in the biosynthesis of secondary metabolites in berry skins and in leaves in response to these treatments, effective to improve grape quality and help vines to cope with abiotic stresses, was hypothesized.

In order to achieve these goals, two experiments were arranged. The comparison between vines treated with *A. nodosum* extract and non-treated ones was set up in a field experiment carried out in 2016 and 2017 in a commercial vineyard in the Chianti Classico area (Tuscany, Italy), on 18-year-old vines of the red cv. Sangiovese (*V. vinifera*). Furthermore, in order to deeply investigate if *A. nodosum* treatments are effective in help vines to cope with drought stress, a second study was performed in 2017 on potted vines (cv. Pinot noir), subjected to two irrigation regimes (well watered and water stressed) associated with *A. nodosum* foliar treatments, performed with the same protocol as the field experiment. In both experiments, gas exchanges, chlorophyll fluorescence and water potentials on leaves treated with *A. nodosum* extract and non-treated control leaves, were monitored at three phenological stages. In addition, at the same stages, anthocyanins, flavonols and hydroxycinnamic acids were quantified in berry skins.

In general, this PhD thesis provides evidence of *A. nodosum* treatments-induced changes in eco-physiological traits and berry skin metabolism of *V. vinifera*, resulting in different physiological adjustments to counteract environmental stress. Furthermore, in both experiments, the biochemical berry skin analyses revealed that the *A. nodosum* extract likely acted selectively in the phenylpropanoid pathway. In particular *A. nodosum* extract may influence anthocyanin biosynthesis by affecting the activity of enzymes involved in the phenylpropanoid pathway, thus resulting in changes in the content of methoxylated compounds in treated vines.

Moreover, the increases in the phenolic and flavonoid profiles have a significant effect on enhancing multiple stress tolerance as well as improving plant water status and photosynthetic performances. Overall, these results support previous findings on the beneficial effects of *A. nodosum* treatments on plant acclimation to stressful environmental condition. For the first time, this study shows the potential effect of seaweed extracts in promoting higher tolerance to stress in vines. Considering the challenges posed by climate change in the Mediterranean basin, the use of seaweed extracts might represent a sustainable tool to mitigate the negative effects caused by increasing severity of drought events, often associated to heat-waves, in the viticulture sector.

Chapter 1

General introduction

1.1. Socio-economic importance, botanical description and main fruit components of the study species

The genus *Vitis* belongs to the *Vitaceae* family, which comprises of about 10 genera and over ~80 inter-fertile species, that exist almost exclusively in temperate regions of the Northern and Southern Hemispheres (Chen et al., 2007; Wen, 2007; Zecca et al., 2012; Moore and Wen, 2016) (Fig. 2). Among them, *Vitis vinifera* subsp. *vinifera* L., the common grapevine, is the only species of the indigenous genus from Eurasia and is suggested to have first appeared ~65 million years ago. The actual number of vine varieties for the *V. vinifera* species in the world is estimated at 6000 (Lacombe, 2012), generally classified according to the berry final production: wine grapes, table grapes and raisins (Fig. 1).

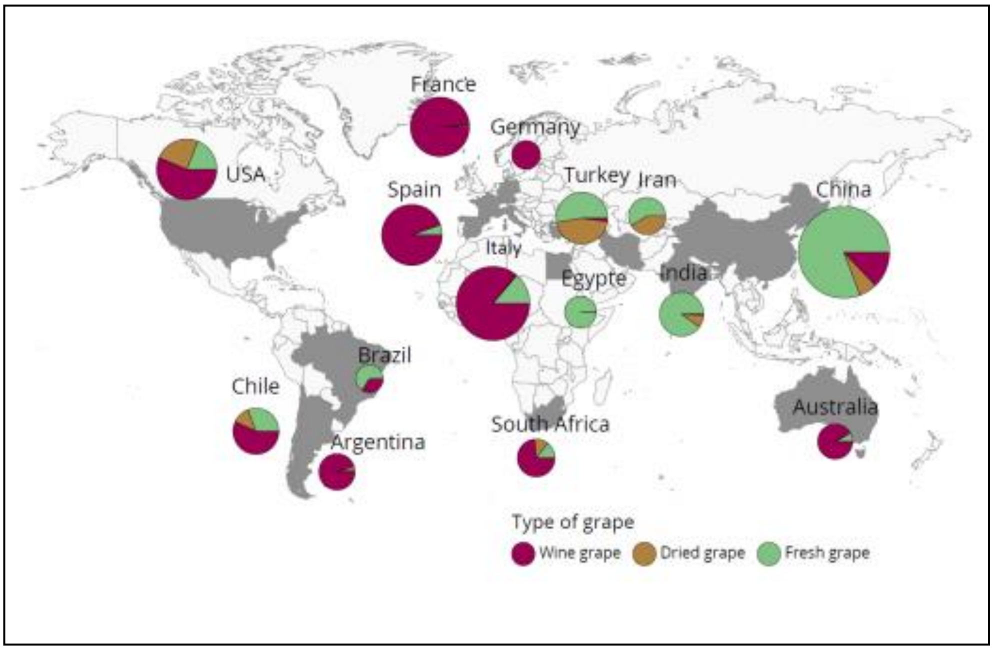


Figure 1. Vine-growing areas and destinations of grape production worldwide. Adapted from Focus OIV 2017 (www.oiv.int).

In the most recent report of the International Organization of Vine and Wine (OIV, 2017), it is estimated that the world's vineyards reached a total area of 7.5 Mha in 2016. Although Europe has lost some of its dominance to Asia, USA, and some southern hemisphere areas (Argentina, Australia, Chile, South Africa), still encompasses the largest vineyard area in the world (38%) and about 39% of total volume of production. The world's top wine-producing countries are France, Italy and Spain representing altogether 33% of the world production. Based on the same report, global wine production stood at 267 MhL in 2016, while wine consumption reached 241MhL, achieving a global turnover of 29 billion of €.

Vitis vinifera subsp. *vinifera* L. is a perennial, woody climbing vine that can be up to 35 m tall, but in cultivation, shoots are usually reduced to 1-3 m, according to training system. Grapevine leaves are circular to circular-ovate, 3–7 lobed, and possess five major veins in a palmate arrangement, with the blade supported by a prominent petiole for optimal light capture. Besides this consistency, all other characters of grapevine leaves vary widely in their hirsuteness, colors, surface contours, size, and dentation, exhibiting striking diversity by cultivars (Galet, 1979; Chitwood et al., 2013). The stomata are usually restricted to the abaxial surface (Moutinho-Pereira, 2009) and their density varies between grapevine cultivars (Gómez-del-Campo et al., 2003; Rogiers et al., 2011). Leaves, as well as current year's shoots and petioles, have non-glandular simple and unicellular trichomes, that generally occur alone on both leaf surfaces (Ma et al., 2016). *V. vinifera* L. produces greenish-white hermaphrodite flowers, which grow together in flower clusters (inflorescences) on the primary shoots and that develop opposite the leaves, typically at the third to six nodes from the base of the shoot (Hellman, 2003). The fruits are oval or oblong berries grouped in clusters, green-yellow to red or purplish-black in color, usually containing 2-3 pyriform seeds enclosed by a lignified endocarp (Chitwood et al., 2013). Fruits are the main reservoir for sugars, organic acids and secondary metabolites, which are differently distributed throughout berry tissues (Fig. 2).

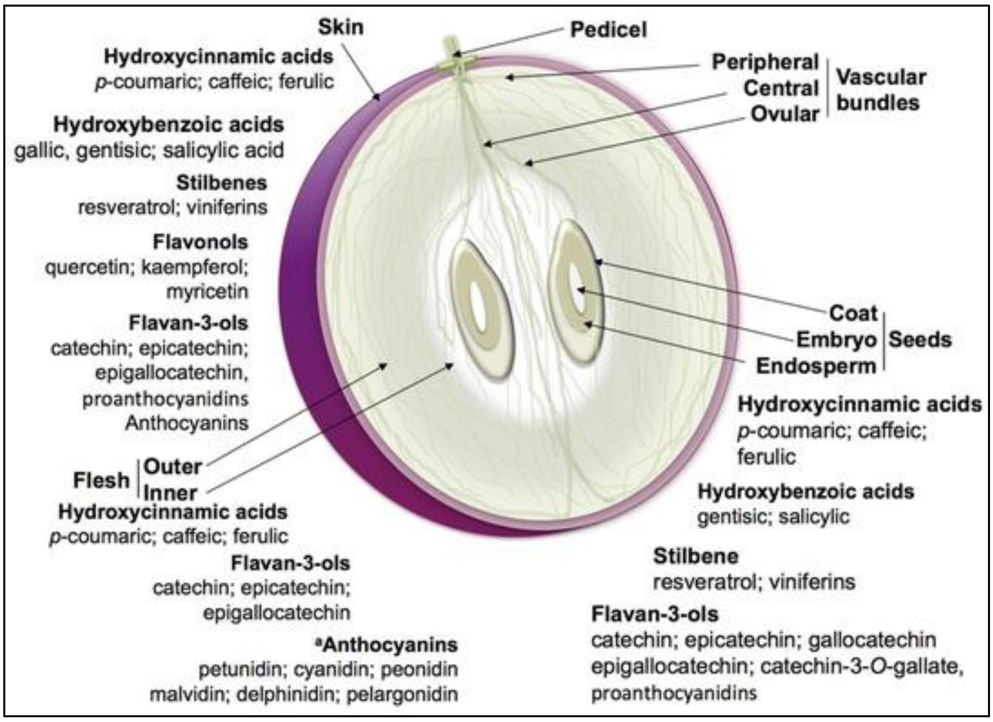


Figure 2. Schematic structure of a ripe grape berry with the distribution pattern of secondary metabolites between tissues. Adapted from Teixeira et al., 2013.

At the end of ripening, high concentrations of glucose and fructose accumulate, while organic acid levels decrease in the berry flesh (Zoccatelli et al., 2013). At the same time, many phenolics such as polyphenols, anthocyanins and tannins accumulate mainly in berry skin (Lund and Bohlmann, 2006). The decisive role of phenolic compounds in red grape is due to their influence on important properties of berries and wines, such as flavor, color and stability against oxidation processes (Waterhouse, 2002; Mattivi et al., 2006; Silva and Queiroz, 2016). Anthocyanins and flavonols are among the most abundant polyphenol subclasses detected in grape berries (Adams, 2006). Each grape cultivar has a unique set of anthocyanins, which include delphinidin-, cyanidin-, petunidin-, peonidin- and malvidin-3-*O*-glucoside, eventually acylated as esters of acetic acid, caffeic acid or p-coumaric acid on the molecule of glucose (Boss et al., 1996; Mattivi et al., 2006; Koyama et al., 2012). The more representative flavonols are glucosides, galactosides and glucuronides of quercetin, myricetin and kaempferol (Makris et al., 2006; Braidot et al. 2008).

1.2. **Climate change impacts on grapevine phenology, eco-physiology, plant water consumption and grape composition**

The last report of the Intergovernmental Panel on Climate Change (IPCC) referred that the average temperature for the European land area for the last decade (2004–2013) is 1.3°C above the pre-industrial level, which makes it the warmest decade of the last millennium.

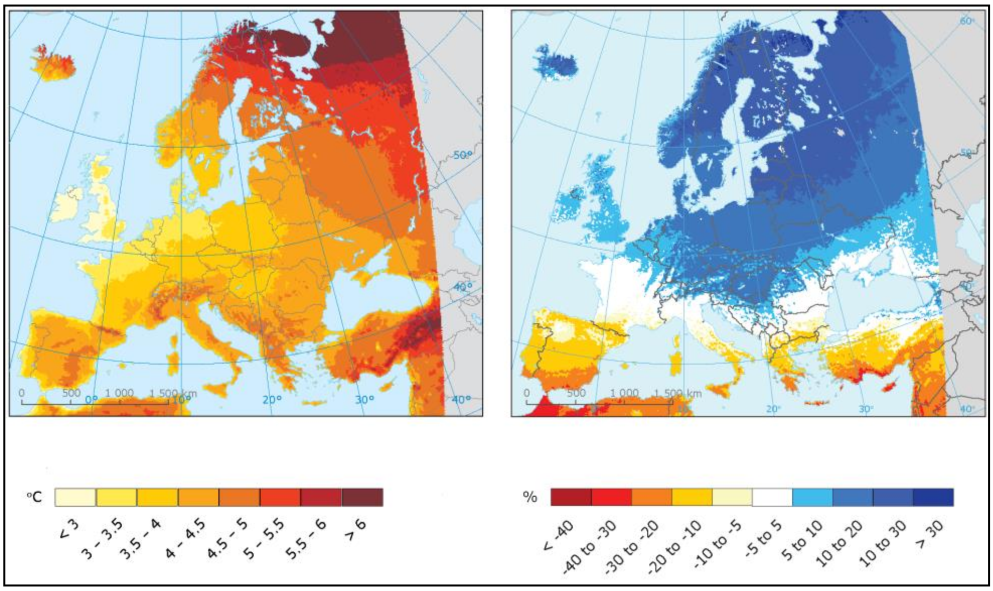


Figure 3. Projected changes for Europe (1971-2000 to 2071-2100) in annual mean temperature (°C) and annual precipitation (%). Adapted from European Environmental Agency (www.eea.europa.eu).

Moreover, annual average land temperature over Europe is projected to continue increasing more than 4.1°C over the rest of this century, with annual precipitation decreasing up to 40% in southern regions and warm extremes becoming more frequent (Fig. 3) (IPCC, 2013).

The impacts of climate change on agriculture are especially evident in the cultivation of grapevines for wine production (viticulture), because weather and climate profoundly influence the production of quality grapes and therefore high-quality wines, with important socio-economic consequences worldwide. High temperatures interacting with high radiation levels and strong atmospheric and soil water deficits, largely affect grapevine metabolism and the biosynthesis of primary and secondary compounds in the berry, which ultimately influence grape quality (Makra et al., 2009; Mosedale et al., 2016) and result in unbalanced wines, with high alcoholic content and low polyphenolic composition (Jones, 2004; Jones et al., 2005; Dalla Marta et al., 2010; Fraga et al., 2012).

Air temperature and solar radiation are considered key factors in the overall growth and productivity of wine grapes (Jones and Alves, 2012).

Grapevines growing under severe heat stress and high radiation load experience a significant decline in productivity and yield attributes, due to stomatal and mesophyll limitations in photosynthesis (P_n) (Moutinho-Pereira et al., 2004), which in turn decrease intrinsic water-use efficiency (iWUE) (Moutinho-Pereira et al., 2004). Extreme heat or heat weaves may also lead to phenological advancement (Chuine et al., 2004; Petrie and Sadras, 2008; Xu et al., 2012), deepening the detrimental effects of climate change by exposing clusters to the higher temperatures that occur earlier in the season (Ramos et al., 2008; Lereboullet et al., 2014).

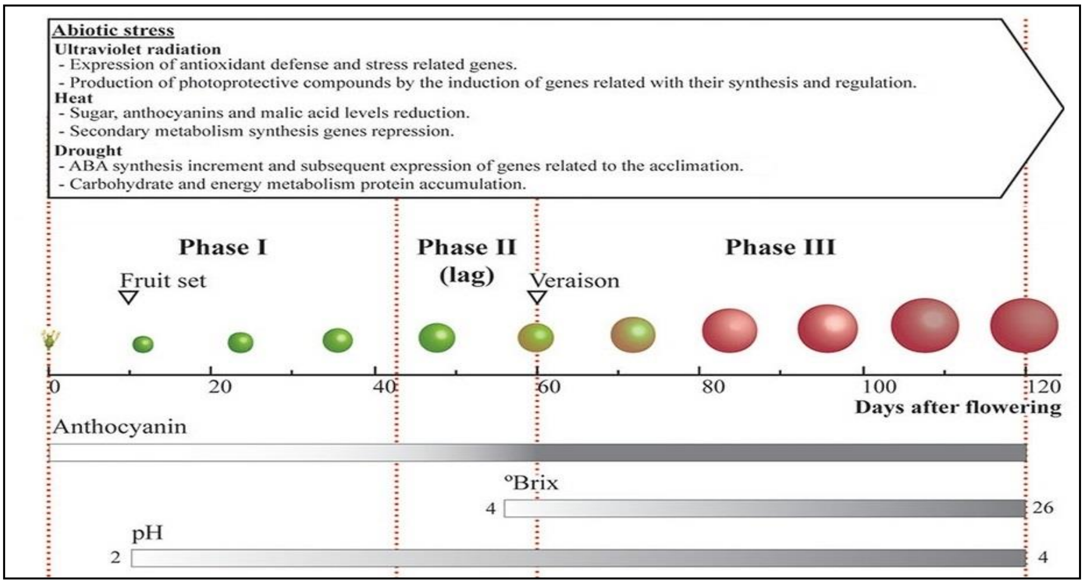


Figure 4. Scheme of the most important changes that berries and seeds undergo during development and the main environmental factors affecting this process. Adapted from Serrano et al., 2017.

In terms of quality, high growing season temperatures combined with high solar radiation promote the accumulation of grape sugars and the breakdown of organic acids, and inhibit the formation of anthocyanin and flavonoid (Haselgrove et al., 2000; Downey et al., 2006; Sadras et al., 2013) (Fig. 4), thus reducing color and the aromatic properties of wines, such as, flavor and aroma components (Jackson and Lombard, 1993; de Orduña, 2010).

Annual precipitation and its seasonality are also key factors influencing viticulture, as water stress can lead to a wide range of effects, yet largely dependent on the stage of development (Cifre et al., 2005, Chaves et al., 2007) (Fig. 5). When soil water stress combines with high atmospheric water demand, water flow into grapevine leaves is insufficient to compensate water losses through transpiration (E), resulting in a depression of leaf water potential (Ψ) (Schultz, 2003; Chaves et al., 2007; Pou et al., 2008; Flexas et al., 2010), that subsequently reduce stomatal conductance (g_s) and photosynthesis (P_n) (Gómez-del-Campo et al., 2004; Lovisolo et al., 2010).

Based on different stomatal behaviors under drought conditions, grapevine cultivars have been classified as isohydric or anisohydric (Schultz, 2003). Some cultivars have greater stomatal control in response to water stress and keep their leaf water potential above the cavitation threshold, that rarely drops below -1.5 MPa. Other cultivars, in turn, exhibit less marked stomatal regulation under drought and substantial depression of Ψ during drought (Griesser et al., 2015; Romero et al., 2017). However, this classification into two categories is controversial and imprecise, and consequently, not always applicable (Chaves et al., 2010; Bota et al., 2016).

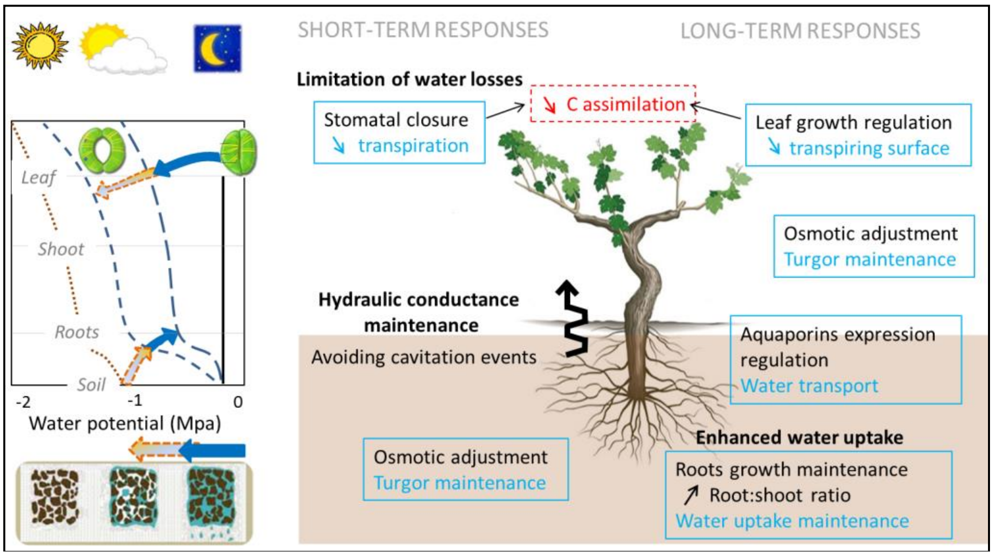


Figure 5. Grapevine physiological responses associated with a drop in plant water potential. The left diagram illustrates the drop of water potential occurring at different intensities depending on the soil water potential and of the evaporative demand (higher from right to left). During the night, water potential equilibrates (vertical line). In the daytime, under high evaporative demand, plant water potentials become

more negative (dashed lines) and further decline in dry soil (dotted line). The right diagram outlines the main physiological adaptations favoring the maintenance of plant water status. Simplified representation adapted from Simonneau et al., 2017.

Moreover, notwithstanding grape quality and anthocyanin profile in berry is greatly dependent on genotype, as differences have been found as a result of vine adaptability to drought and others environmental factors (Guidoni et al., 2002; Downey et al., 2004; Ortega-Regules et al., 2006). Water stress does not imply exclusively negative effects, hence, a moderate dryness can potentially balance vine vegetative and reproductive growth, enhancing berry traits (Bucelli et al., 2004). In particular, it is well known that water deficit from the onset of ripening until full maturation raises the content of anthocyanins in berry skin (Castellarin et al., 2007a; Zarrouk et al., 2012). In addition, both water deficit and radiation load consistently stimulate methoxylation of B-ring-substituted flavonoids (Castellarin et al., 2007b), incrementing the ratio of methoxylated to non-methoxylated anthocyanins, thus enhancing the stability of these compounds (Castellarin and Di Gaspero, 2007; Tarara et al., 2008).

In this context, vineyard management practices with short-term water conservative and sustainable objectives can be considered as a first protection strategy to counteract the effects of climate change and more stressful environment (Costa et al., 2016; Lovisolo et al., 2016). The use of biostimulants, such as seaweed extracts, might represent a tool to enhance plant physiological responses under severe stress conditions to achieve an optimal ripening (Khan et al., 2009; Craigie, 2011).

1.3. **Plant biostimulants: definition and main categories**

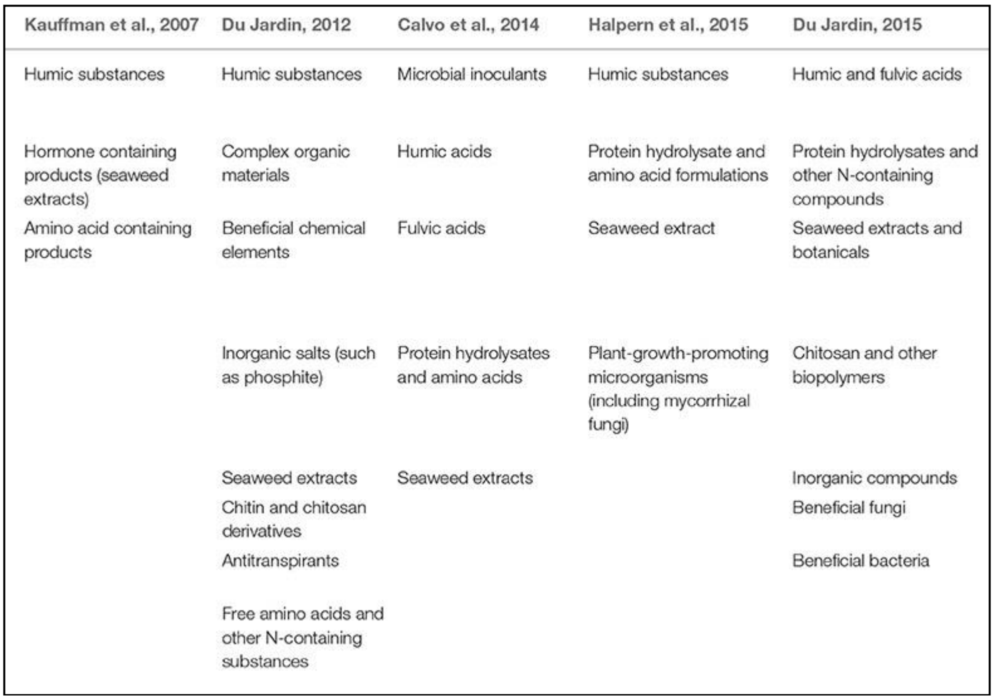
The concept of “biostimulants” as “substances that applied in small quantities promote the growth of plants” was first introduced in 1997 by Zhang and Schmidt of the Virginia Polytechnic Institute and State University (USA).

Ten years later, Kauffman et al. (2007) resumed the definition of biostimulant with some modifications, defining the biostimulants as “materials, other than fertilizers, that promote plant growth when applied in low quantities”. In 2011, the European Biostimulant Industry Council (EBIC), made up of the largest companies in the fertilizers sector, scientific community and stakeholders, was founded with the aims to formulate a rigorous definition of “biostimulant”, through a classification with analytical methods, and to establish this new category of products at legislative level. The first world congress on the use of biostimulants in agriculture took place in Strasbourg (France) in November 2012. More than 700 people from 30 different countries, attempted the congress, witnessing the increasing interest of the industrial sector and academic world.

In 2012, based on the research of du Jardin, a biologist from the University of Liége (Belgium), and the consultations with the European Commission, the EBIC elaborated the first official definition of biostimulants, subsequently amended as follows:

“A plant biostimulant is any substance or microorganism applied to plants with the aim to enhance nutrition efficiency, abiotic stress tolerance and/or crop quality traits, regardless of its nutrients content” (du Jardin, 2015).

In recent years, biostimulants are increasingly been used in agriculture and the sales of these products is estimated to reach a turnover of 2 billion dollars in 2018 (Calvo et al., 2014). Some major categories are widely recognized by scientists, regulators and stakeholders (du Jardin, 2012 ; Halpern et al., 2015), including diverse formulations of substances and other products (Fig. 6), such as seaweed extracts, that are applied to plants or soils to regulate and enhance the crop’s physiological processes.



Kauffman et al., 2007	Du Jardin, 2012	Calvo et al., 2014	Halpern et al., 2015	Du Jardin, 2015
Humic substances	Humic substances	Microbial inoculants	Humic substances	Humic and fulvic acids
Hormone containing products (seaweed extracts)	Complex organic materials	Humic acids	Protein hydrolysate and amino acid formulations	Protein hydrolysates and other N-containing compounds
Amino acid containing products	Beneficial chemical elements	Fulvic acids	Seaweed extract	Seaweed extracts and botanicals
	Inorganic salts (such as phosphite)	Protein hydrolysates and amino acids	Plant-growth-promoting microorganisms (including mycorrhizal fungi)	Chitosan and other biopolymers
	Seaweed extracts	Seaweed extracts		Inorganic compounds
	Chitin and chitosan derivatives			Beneficial fungi
	Antitranspirants			Beneficial bacteria
	Free amino acids and other N-containing substances			

Figure 6. Evolution of biostimulant categories in literature. Adapted from Yakhin et al., 2017.

Seaweeds have been widely used since antiquity in agriculture as soil amendments to improve soil fertility and crop productivity (Newton, 1951; Chapman and Chapman, 1980; Lembi and Waaland, 1988; Metting et al., 1990). Brown seaweeds are the second most abundant group comprising about 2000 species and, among them, *Ascophyllum nodosum* (L.) is the most commonly used in agriculture (Blunden and Gordon, 1986) and most researched (Ugarte et al., 2006). This perennial brown seaweed is recognized as the dominant one in the North Atlantic coastline where water temperatures do not exceed ∼27°C (Keser et al., 2005). *A. nodosum* biostimulant chemical composition and, therefore, also their properties, may vary depending on the season of harvest and the chemical and/or physical methods employed during extraction of the raw material (Craigie, 2011). Generally, the inorganic components of *A. nodosum* extract include nitrogen, phosphorous, potassium, calcium, iron, magnesium, zinc, sodium and sulphur (Rayirath et al., 2009). Besides the mineral components *A. nodosum* extracts also contain varying amounts of organic compounds that include osmolites (e.g. betaines) (Blunden et al., 2009; MacKinnon et al., 2010), vitamins (Battacharyya et al., 2015), bioactive secondary metabolites (e. g. phenolics) (Laetitia et al., 2010; Keyrouz et al., 2011; Balboa et al., 2013). Moreover, one of the major components of commercial extracts of all seaweeds are the polysaccharides, that may account for up to 30–40% of the extract on a dry weight basis (Rayirath et al., 2009) and include alginates, fucoidans, laminarans, lichenan-like glucans and fucose (Khan et al., 2009). Seaweed extracts also contain number of pytohormones including auxins, cytokinins, gibberellins, abscisic acid and brassinosteroids (Stirk et al., 2004).

1.4. **The use of *A. nodosum* as tool to enhance plant tolerance to environmental stresses**

In literature, the traditional application of *A. nodosum* extracts as an organic and mainstream biofertilizer for its properties to promote plant growth, productivity and fruit quality (Chouliaras et al., 2009; Jannin et al., 2013; Hernández-Herrera et al., 2014; Sabir et al., 2014; du Jardin, 2015) is largely documented. *A. nodosum* products have been also recently employed as biostimulants to promote short-term plant acclimation to climate constraints, without adversely impacting on the environment (Craigie, 2011; Sharma et al., 2014; Tanou et al., 2017; Yakhin et al., 2017) (Tab. 1). *A. nodosum* extracts may stimulate numerous metabolic pathways, eliciting plant physiological and biochemical responses to abiotic stress and enhancing plant performance (Khan et al., 2009; Zhang and Ervin, 2008; Rayirath et al., 2009; Parađiković et al., 2011; Nair et al., 2012).

Table 1. Effects of *A. nodosum* extracts on different plant species.

	Treated Species	Treatment effect	Reference
Growth, productivity and fruit quality	Potato	Increased yield	Kuisma, 1989
	Kiwifruit	Increased fruit weight and lenght	Chouliaras et al., 1997
	Citrus	Increased yield	Fornes et al., 2002
	Grapevine	Increased yield	Norrie et al., 2002
	Barley	Increased yield	Steveni et al., 2002
	Grapevine	Increased mineral uptake	Turan and Köse, 2004
	Grapevine	Increased yield	Colapietra and Alexander, 2006
	Grapevine	Enhanced root development and mineral uptake	Mancuso et al., 2006
	Grapevine	Increased yield and berry quality	Norrie and Keathley, 2006
	Apple	Increased yield, fruit size and quality	Basak, 2008
	Grapevine	Enhanced root development and growth	Mugnai et al., 2008
	Olive	Increased yield,oil content and quality	Chouliaras et al., 2009
	Grapevine	Increased yield and berry quality	Kok et al., 2010
	Strawberry	Increased yield and fruit quality	Spinelli et al., 2010
	Pear	Increased yield, fruit diameter and weight	Colavita et al., 2011
	Blueberry	Increased yield and fruit size	Loyola and Munoz, 2011
	Yellow pepper	Increased yield	Parađiković et al., 2011
	Grapevine	Increased growth and mineral uptake	Khan et al., 2012
	Winter rapeseed	Increased yield and nutrient use efficiency	Jannin et al., 2013
	Mango	Increased leaf area and leaf mineral content	Mohamed and El-Sehrawy, 2013
	Grapevine	Increased growth	Popescu and popescu, 2014
	Grapevine	Increased growth, yield, berry quality and leaf nutrient content	Sabir et al., 2014
Environmental stresses and secondary metabolites	Tomato, wheat, barley and maize	Increased chlorophyll content	Whapham et al., 1993
	French bean	Increased chlorophyll and betaines content	Blunden et al., 1996
	Creeping bentgrass	Enhanced antioxidant activity and drought tolerance	Zhang and Schmidt, 2000
	Arabidopsis	Enhanced freezing tolerance	Rayirath et al., 2008; 2009
	Creeping bentgrass	Enhanced heat stress tolerance	Zhang and Ervin, 2008; Zhang et al., 2010
	Citrus	Increased Ψ_w and plant WUE, decreased P_n and g_s	Little and Spann, 2010; Spann and Little, 2011
	Spinach	Enhanced phenolic antioxidant content	Fan et al., 2011
	Grapevine	Enhanced secondary metabolites	Cai et al., 2012
	Pine	Enhanced drought tolerance	MacDonald et al., 2012
	Arabidopsis	Enhanced freezing tolerance	Nair et al., 2012
	Spinach	Enhanced leaf water relations, P_n and g_s	Xu and Leskovar, 2015
	Spiraea and *Pittosporum*	Enhanced phenolic antioxidant content	Elansary et al., 2016
	Arabidopsis	Enhanced drought tolerance	Santaniello et al., 2017
	Grapevine	Enhanced secondary metabolites	Frioni et al., 2018

As an example, *Citrus* spp. plants exposed to treatments with *A. nodosum* commercial extract displayed higher (Spann and Little, 2011) photosynthesis and stomatal conductance than untreated plants under water deficit (Little and Spann, 2010). Other Authors reported that, in greenhouse studies, the treatment of vegetables and turf crops with a commercial extract of *A. nodosum* significantly delayed wilting, decreased water use (i.e. better WUE), increased leaf water content and improved the recovery of drought-wilted plants compared to controls (Little and Neily, 2010; Neily et al., 2008). In addition, applications of seaweed extracts imparted heat tolerance and promoted the performances in creeping bent grass (Ervin et al., 2004; Zhang and Ervin, 2008).

In grapevine, foliar application of seaweed extracts enhances root development (Mancuso et al., 2006; Mugnai et al., 2008), mineral nutrient uptake (Turan and Köse, 2004; Mancuso et al., 2006; Khan et al., 2012; Sabir et al., 2014) and growth (Mugnai et al., 2008; Khan et al., 2012; Popescu and Popescu, 2014; Sabir et al., 2014). There are also several reports on the positive effects of these extracts on yield and grape quality (Norrie et al., 2002; Colapietra and Alexander, 2006; Norrie and Keathley, 2006; Kok et al., 2010; Sabir et al., 2014), but their activity on secondary metabolism is scarcely investigated (Mancuso et al., 2006; Cai et al., 2012; Frioni et al., 2018).

Although the *A. nodosum* extracts mechanisms of action have not been fully elucidated yet (Norrie et al., 2002; Colapietra and Alexander, 2006), recent evidences suggest that the beneficial effects of *A. nodosum* treatments on plant acclimation to stressful condition is mediated by an increase in the concentration of bioactive molecules, including antioxidants in the treated plants (Zhang and Schmidt, 2000; Rayorath et al., 2009). This mechanism involves the activation of antioxidant enzymes and secondary metabolic pathways (Calvo et al., 2014; Santaniello et al., 2017), in particular the biosynthesis of flavonoids (Fan et al., 2011; Cai et al., 2012; Santaniello et al., 2017).

1.5. **Objectives of the thesis**

As stated in the previous paragraphs, *V. vinifera* in the Mediterranean region are subjected to a wide range of environmental stresses, especially during the summer, when low water availability is superimposed on high light and high temperatures.

Furthermore, given the strong influence of the atmospheric factors on this species, seasonal weather pattern can significantly affect grapevine physiology and wine quality under future climate change projections.

Although grapevines have multiple acclimation strategies to environmental constrains, the evidence for significant climate change in the upcoming decades urges adaptation and mitigation measures to be taken by the whole viticulture and winemaking sectors. Short-term adaptation measures can be considered as a first protection strategy and should be focused at specific threats, mostly changes in agronomical practices, such as the application of biostimulants to enhance nutrition efficiency, abiotic stress tolerance and quality traits of plants.

The present PhD project was developed under this scenario. In particular, the objectives of the present work were: 1) to investigate the physiological performances of *V. vinifera* and the biochemical adjustments induced in grape berry skins by *A. nodosum* foliar treatments under field conditions (Chapter 3), and 2) to evaluate if these treatments affect grapevine water relations and berry phenylpropanoid metabolism, mitigating the effects of a post-*véraison* drought in potted vines (Chapter 4).

These issues were approached by choosing two autochthonous European grapevine cultivars, Sangiovese and Pinot noir, distinctive not only for their great socio-economic importance, but specially for their strong phenotypic plasticity, known as the ability of a genotype to produce different phenotypes to face the variability of environmental factors (Nicotra et al., 2010; Palmer et al., 2012; Gratani, 2014).

Sangiovese has an ancient documented history, a high morphological intracultivar heterogeneity and even today it represents the main cultivated variety for Italian red premium wines (Filippetti et al., 2005; Vignani et al., 2002). It is most cultivated in Tuscany (36520 ha; ISTAT, 2010), but it is grown also in France (1564 ha; Ministère de l'Agriculture et de la pêche, 2000), in other European countries (70300 ha; ISTAT, 2010), and also in California (767 ha; USDA, 2008).

The origins of Pinot noir red wine grape cultivar can be traced back to 14th-century in Burgundy, France. Thanks to its reputation to produce high quality wines and its ability to adapt to cooler climates, this variety ranks as the world's fourth most cultivated variety (112000 ha, OIV 2017) and is widely grown in Europe (Germany, Italy, Switzerland, Romania, Hungary, Spain) but also in the vineyards of the New World (United States, New Zealand, Australia, Chile, Argentina, South Africa).

Lastly, this PhD project follows a preliminary study aimed at identifying, by means of a comparative trial, the biostimulant product, its dose and protocol of distribution, more effective in improving the grape quality and the balance between sugar and phenol accumulations in berries. The results of this preliminary study, fundamental for the planning of these PhD experiments, are presented in Chapter 2.

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Chapter 2

Use of natural biostimulants to improve the quality of grapevine production: Firsts results

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L. Salvi, E. Cataldo, S. Secco and G. B. Mattii (2016)

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Use of natural biostimulants to improve the quality of grapevine production: Firsts results

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Abstract

The research was carried out in the 2015 growing season on *Vitis vinifera* cv. Sangiovese to study the effects of biostimulants supplied by foliar feeding, on the vine's eco-physiological and productive characteristics, to improve the quality in a highly vigorous vineyard. The main goal of this study was to separate the technological maturation, in terms of the berry's sugar and acidity accumulations, from the phenolic one (i.e. its anthocyanin and polyphenol contents). Specifically, three biostimulant products were sprayed: two seaweed extracts and one vegetal protein hydrolyzate; an untreated control was used for comparison.

Leaf gas exchanges and chlorophyll content measurements were carried out. From the veraison to harvest, berry samples were collected and total soluble solids, titratable acidity, pH, extractable anthocyanins and polyphenols were analyzed.

In conclusion, all treatments reduced photosynthesis. The balance between technological and phenolic maturities was improved, basically by maintaining the berries' total soluble solids and by increasing their anthocyanin and polyphenol contents.

Keywords: anthocyanins, polyphenols, photosynthesis, *Vitis vinifera*, seaweed extracts, protein hydrolyzate

INTRODUCTION

For years, research in agriculture has focused on improving crop yields, while little attention has been given to product quality. Recently greater importance has been given to environmental protection and production costs reduction, mainly regarding pest treatments as well as foliar nutrition. New formulations, doses and distribution methods have been made available in recent years.

In 2011 the European Biostimulants Industry Council (EBIC) was founded in order to promote this new fertilizers category and to overcome the differences in regulation among member states. EBIC defines the biostimulants as "products containing substances and/or microorganisms whose function, when applied to plants or the rhizosphere, is to stimulate natural processes to enhance/benefit nutrient uptake, nutrient efficiency, tolerance to abiotic stress and crop quality.

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Biostimulants have no direct action against pests, and therefore do not fall within the regulatory framework of pesticides" (European Biostimulants Industry 2012).

Agricultural biostimulants include different formulas and, among these, the most important are seaweed extracts derivatives and hydrolyzed proteins.

The seaweed extracts are obtained from green, red or brown algae, in particular the *Ascophyllum nodosum*, *Ecklonia maxima*, *Laminaria digitata* and *Fucus spp*. types. The method most widely used is that of extraction in cold water at high pressure.

This biostimulant category has different actions of stimulation and/or inhibition of physiological processes, such as effect on germination, vegetative growth and fruit set.

In this case, the biostimulation effects are mainly due to plant hormones, polyphenols, betaines (cytokinin-like action) and polysaccharides (Tagliavini et al., 2006). All these compounds act as elicitors, promoting defense activation responses to stress.

Hydrolyzed proteins (PH) are mostly constituted by free amino acids and peptides, generally obtained by chemical or enzymatic hydrolysis. The protein sources are represented by leather (e.g., collagen), fishing industry and plant biomass residues, each one with its specific mineral and amino acid composition. A recent study reports that treating vine leaves with specific hydrolyzed proteins, activates the enzyme stilbene synthase genes which is responsible for the resveratrol biosynthesis - a defense compound.

Recent research has shown that biostimulants are able to increase plant growth and development. One of the role of biostimulants is to improve nutrients' absorption efficiency (Frankenberger and Arshad, 1995), with fertilization cost and environmental pollution risk reductions. From a nutritional point of view, the biostimulants promote plant growth by changing the radical architecture (Nardi *et al.*, 2006, Paradikovic *et al*., 2011), increasing root development and nutrient uptake (Mugnai *et al.,* 2008, Martin, 2012). It has also been shown that these products increase photosynthetic efficiency, favoring sugar accumulation in fruits (Presutto e Pezzutto, 2006). Moreover, the endogenous gibberellins and cytokinins could lead to an increase in yield, improving economic returns, and the endogenous ABA has the potential to improve total polyphenolic contents (Martin, 2012).

Some authors have demonstrated that biostimulants can make crops less sensitive to stress conditions like extreme temperatures and drought (Subler et al., 1998). In order to maximize their effectiveness, biostimulants should be applied at specific times and in an optimal dose that varies according to the crop and cultivar (Zhang et al., 2003).

Biostimulants are increasingly being used in viticulture: they could be involved in promoting sustainable plant growth, yield and product quality, as well as protecting the crops exposed to environmental stress conditions, such as drought, cold, high temperatures and UV radiation.

In years with suboptimal weather conditions, manipulation of agricultural practices is an essential requirement. Therefore, it is important to improve plant nutrient uptake, by integrating the plant's own metabolic processes. One solution could be the application of vegetal extracts fertilizers, also called biostimulants. In addition, nowadays, consumers prefer well-structured wines with deep color and a pleasant mouthfeel. These kinds of wines can be made from well-ripened grapes with an optimal level of phenolic and sugar maturities. Nevertheless, it is a known fact that the changing climate can modify the ripening process in substantial ways.

For these reasons, the aim of this research was to study the effects of biostimulants’ foliar supply on the vine’s ecophysiological and productive characteristics, to improve quality in a highly vigorous Sangiovese vineyard (*Vitis vinifera*). The main goal was to get act together the technological maturation, in terms of the berry’s sugar accumulation, and the phenolic one, which is linked to the wine’s color and flavor.

MATERIALS AND METHODS

The research was conducted during the 2015 growing season on 25-year-old grapevines (cv. Sangiovese) at the Villa Montepaldi estate, located in San Casciano Val di Pesa (Florence), in the northern part of the Chianti Classico production area (Lat. 43.668 °N, Long. 11.145°E), in Italy. Vines were trained as upwards vertical shoot positioning with spur cordon pruning, with an average of 10 buds per vine and a planting distance of 2.80 m between the rows and 0.90 m between the vines.

The vineyard is positioned on a hill with a medium texture soil (clay: 38,76%; sand: 23,39%; silt: 37.85%), with a slightly alkaline pH (7.81). Cultural practices included shoots topping, grapes thinning and a complete set of treatments against major fungus diseases.

Using the experimental randomized block design with five replication per treatment (15 vines for each treatment of each replication), the comparison between the untreated and four different treatments was set as follows: a vegetal protein hydrolyzate (VPH) was distributed at cluster pre-closure and veraison at a 4 L/ha dose; a seaweed extract (SWE) was applied in two treatments, after fruit set and a week after; another seaweed extract with added minerals (VEM) was distributed in two treatments: 30 and 15 days before harvest, both at 4 L/ha dose.

All products were sprayed on both sides of the trellis with an electric backpack pump. In order to balance the production load, the vines were standardized by canopy hedging, trimming and laterals removal as well as choice of clusters number, according to a regular farming operations. Throughout the season, readings of leaf gas exchange with Ciras 1 (PP Systems) and chlorophyll content using the Spad-502 (Konica Minolta) were carried out. Starting from early September until harvest, 150 berries from each repetition were sampled for analyses of soluble solids (°Brix), titratable acidity (g/L of tartaric acid), pH and berry weight (g). On a comparable sample, analyses of the potential content of anthocyanins and total and extractable polyphenols (mg/L) with the method proposed by Glories were carried out. At harvest, for each treatment fresh weight of yield (kg), the number of clusters/vine and the fresh weight of the bunch (g) were measured.

The data collected were subjected to analysis of variance with SPSS Data Editor. For multiple comparisons between treatments, the Fisher's least significant difference (LSD) was reported and significant differences were assumed for $P < 0.05$.

RESULTS AND DISCUSSIONS

The summer phase of the vegetative season in which this experiment took place featured average temperatures of about 25°C, with scarce rainfall during June and July (from 169 to 212 in Day-of-Year,(DoY)(Figure 1 and Figure 2). The soil's water reserves were replenished by the August rain (from 213 to 243 DoY) and the mild temperatures during harvest favored the complete sugar and phenolic maturities.

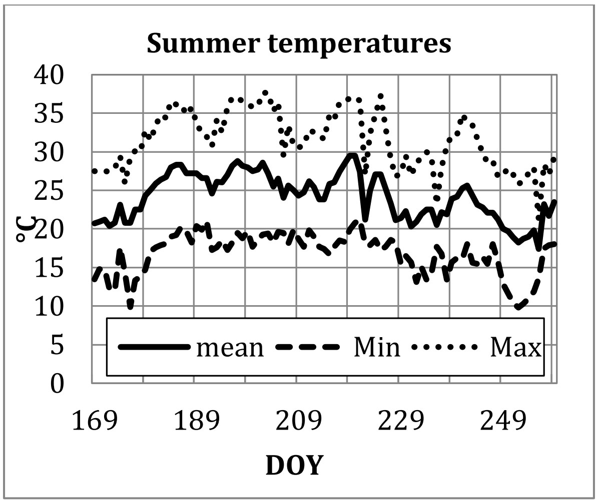


Figure 1. Mean, minimum and maximum summer temperatures in °C. DOY refers to Day-of –Year numbering system.

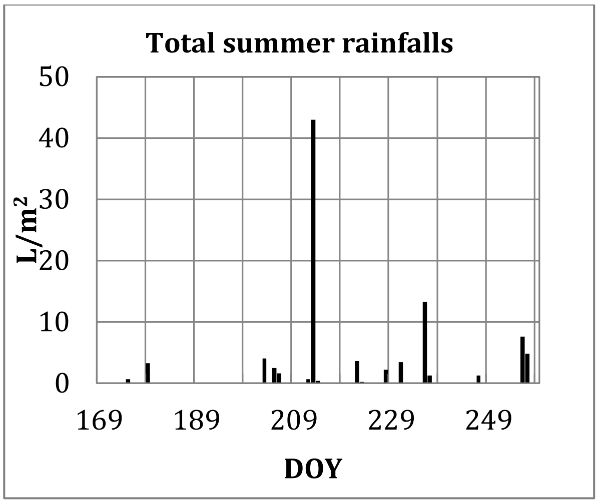


Figure 2. Total summer rainfalls in L/m^2. DOY refers to Day-of –Year numbering system.

Leaf gas exchange

The amount of net photosynthesis (Pn) measured on individual leaves reflects the seasonal trend (Figure 3): the noticeable peaks in Pn are related to rain events (217 and 259 DoY). During the entire season the untreated control showed higher photosynthesis.

Following the second treatment with VPH (215 DoY) a decrease in Pn was detected. This is possibly due to an early leaf ageing. Such decrease was also evident in the stomatal conductance (GS) but it never went below the stress threshold, commonly considered to be around 100 mmol/m²s. It might be useful to apply the treatment in a different period, bringing it forward to the post-closure of the bunch, since the main function of protein hydrolizates (PHs) is related to nitrogen metabolism and assimilation (Ertani et al., 2013; Baglieri et al., 2014; Calvo et al., 2014). The VEM treatment (230 DoY) reduces the photosynthetic activity immediately after the treatment. In the SWE thesis the photosynthetic activity decreases, probably due to the algae extract reducing the vegetative activity of the plant, thus directing the resources towards production and increase of berry and bunch weight (Table 1). Taken collectively, these results suggest that seaweed products elicit abiotic stress tolerance in vines and that the bioactive substances derived from seaweeds lead to a stress tolerance and enhance plant performance. A number of reports suggest that the beneficial anti-stress effects of seaweed extracts may be related to cytokinin activity (Zhang and Ervin, 2004). With reference to transpiration the seasonal trend looks globally rather homogenous among the four theses, seldom showing significant differences (data not shown).

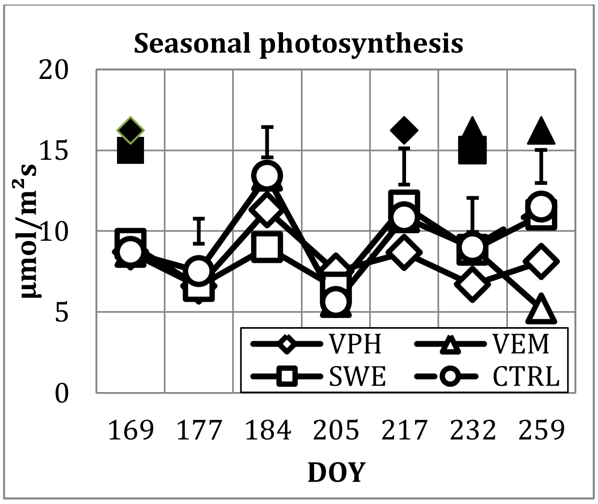


Figure 3. Seasonal leaf photosynthesis in µmol/m²s. Bars represent the least significant difference (P<0.05). Dark symbols represent the day and the type of the treatments.

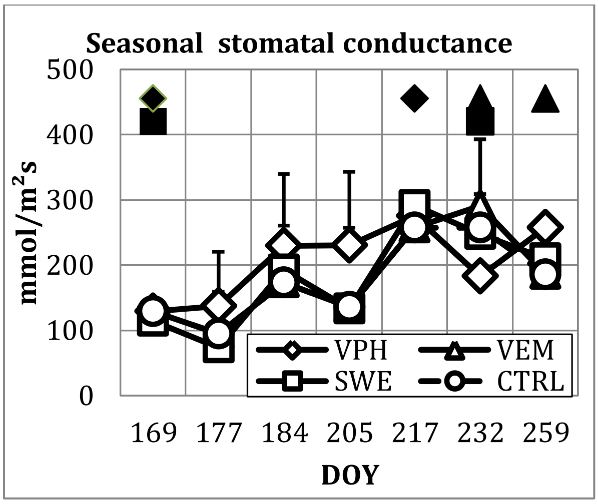


Figure 4. Seasonal leaf stomatal conductance in mmol/m²s. Bars represent the least significant difference (P<0.05). Dark symbols represent the day and the type of the treatments.

GS (Figure 4) has seldom gone below 100 mmol/m²s, the reference threshold in plant water stress evaluation, during the hottest and driest period (June and July, from 169 to 212 DoY). The VPH thesis, during the same period shows higher values, in line with several studies (Kauffman et al., 2007; Apone et al., 2010; Ertani et al., 2013) reporting the positive effects exerted by PHs and amino acids, including proline and betaine, which increase plant defense responses and tolerance to stresses, such as salinity, drought, temperature and oxidative conditions.

The seasonal trend in water use efficiency (WUE in µmol/mmol, photosynthesis/transpiration) appeared to be similar among the treatments and control. Further data on chlorophyll, measured in SPAD Units, was not significantly different (data not shown).

Production and quality parameters

Significant differences were observed in berry weight and bunch weight. Berry weight was highest when treated with protein hydrolyzate (VPH) (Table 1), however the bunch weight was not affected by treatment. The hypothesis is that VPH had an impact on fruit setting by decreasing its ratio, thus affecting the number of berries per bunch. As a consequence the fewer remaining berries grew bigger.

The greatest increase in berry and bunch weight was found when treated with SWE; this is in line with data found in literature. Norrie and Keathley (2006) have reported that *A. nodosum* extracts showed positive effects on the yield of ‘Thompson seedless’ grape (*Vitis vinifera* L.) consistently over a 3-year period. They observed that the *A. nodosum*-treated plants always outperformed (in terms of berry size, berry and bunch weight) the controls maintained under the regular crop management program. This effect is probably due to the seaweed components, such as macro and microelement, amino acids, vitamins, cytokinins, auxins, and abscisic acid (ABA)-like growth substances, that affect cellular metabolism in treated plants leading to enhanced crop yield (Crouch and van Staden 1993a; Reitz and Trumble 1996; Durand et al. 2003; Stirk et al. 2003).

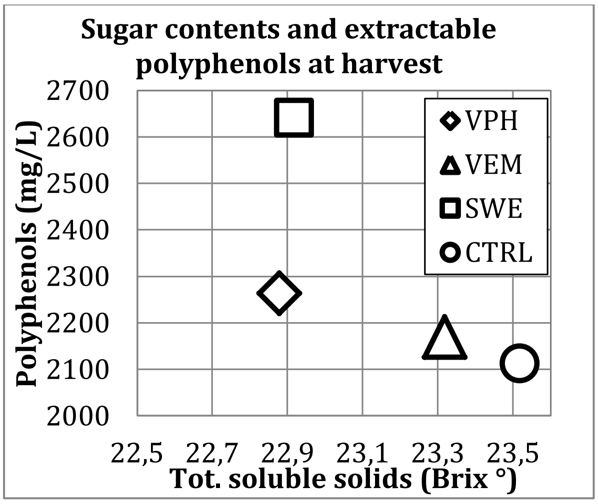
Table 1. Effect of seaweed extract on berry weight, bunch weight and chemical composition

Treatments	Berry weight (g)	Bunch weight (g)	Tot. soluble solids (°Brix)	Extractable anthocyanins (mg/L)	Extractable polyphenols (mg/L)
VPH	3.03b[1]	311a	22.9a	526a	2262a
VEM	2.49a	340ab	23.3a	555b	2169a
SWE	2.53a	384b	22.9a	519a	2639b
CTRL	2.41a	313a	23.5a	402a	2111a

[1] Letters indicate the least significant difference ($P<0.05$).

All treatments have caused a general decrease sugar accumulation, in the berry although there is no significant difference in total soluble solids of any treatment compared to control; no effect also for organic acids and pH (data not shown).

All treatments produced a general increasing trend in the accumulation of extractable polyphenols and anthocyanins in the berry (Table 1).Only the VEM treatment significantly increased the anthocyanin content while only SWE increases polyphenol content.. It can be assumed that the synthesis of anthocyanins is influenced by the nature of the product itself, as well as by the addition of mineral elements such as nitrogen and especially potassium. The thesis treated with SWE shows the highest level of extractable polyphenols. The algae extract has probably played a stress-promoting role that resulted in an increase in biosynthesis of secondary compounds. Correlating the total soluble solids and extractable polyphenols at harvest (Figure 5), the thesis treated with algae extract (SWE) produced the best results, associating a higher quantity of polyphenols with a reasonable sugar level. Such thesis represents a good combination to obtain high quality wines with lower alcohol but higher polyphenolic content. Correlating total soluble solids with extractable anthocyanins at harvest (Figure 6) showed that plants treated with VEM had better levels of extractable anthocyanins with a lower total soluble solids compare to the control. Similar results were observed under protein hydrolyzate treatment and other studies have reported similar findings. For example, Parrado et al. (2007) investigated the effect of the PHs application on the expression of quality parameters such as color and anthocyanin induction in red grapes; PHs gave higher total phenolic and total anthocyanin values compared to untreated plants.



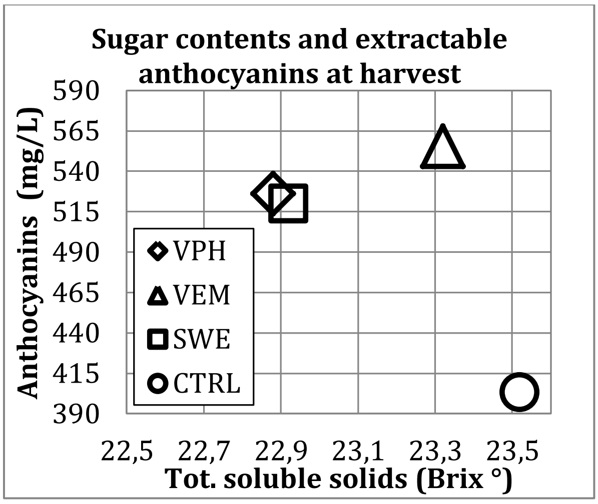


Figure 5. Mean total soluble solids (°Brix) and extractable polyphenols (mg/L) at harvest.

Figure 6. Mean total soluble solids (°Brix) and extractable anthocyanins (mg/L) at harvest.

CONCLUSIONS

To reduce the lag between technological and phenolic maturity due to the interaction between current climatic changes and different canopy management techniques, the foliar application of biostimulant products represents an effective agronomic practice.

Generally it can be argued that by improving nutrient use efficiency and biotic and abiotic stress resistance, such products affect the physiologic activity of plants directing it towards optimal production of secondary metabolites. Moreover, products with biostimulating action delay the accumulation of sugars in the berry, thus resulting in a simple and economical strategy to solve the aforementioned issue of the lag in technological and phenolic maturity at harvest.

SWE appears to contribute in curbing the excess of sugar in the berry at harvest during dry and hot vintages: it delays technological maturity thus promoting production of phenolic compound.

The biostimulant with vegetal extract and mineral elements (VEM) can act as an aid for the production of high quality wines, less alcoholic but well-endowed in terms of color. This product is also helpful in dry and hot vintages to curb excess sugar in the berry at harvest: by delaying technological maturity, it promotes a higher content of extractable anthocyanins.

The benefits of PHs are derived from a better capability to increase plant defense responses and tolerance to stresses as confirmed by the higher levels of stomatal conductance. This has a direct effect in inducing secondary plant metabolites.

The complexity of the products and their mechanisms of action, associated with the variability of the environment, however, make biostimulants, products whose effects are still uncertain, the object of study for many research institutes.

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Chapter 3

Effects of *Ascophyllum nodosum* extract on *Vitis vinifera*: Consequences on plant physiology, grape quality and secondary metabolism

Research paper submitted for publication on Plant Physiology and Biochemistry

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Effects of *Ascophyllum nodosum* Extract on *Vitis vinifera*: Consequences on Plant Physiology, Grape Quality and Secondary Metabolism

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Keywords: *Vitis vinifera*, seaweed-based extract, biostimulants, photosynthesis, stomatal conductance, °Brix, anthocyanins

ABSTRACT

Seaweed-based extracts have been recently employed as sustainable tools to improve abiotic stress tolerance and increase grape quality. However, the effect of these extracts on secondary metabolism compounds, that are fundamental for grape and wine quality, is still scarce. In the present study, the effects of foliar treatments with an *Ascophyllum nodosum* extract on physiological and biochemical parameters of *Vitis vinifera* (cv. Sangiovese) were investigated. We hypothesized an enhancement in the biosynthesis of secondary metabolites in berry skins and in leaves in response to these treatments, effective in improve grape quality and help vines to cope with abiotic stresses. Gas exchanges, chlorophyll fluorescence and midday stem water potential on leaves treated with *A. nodosum* extract and non-treated control leaves, were monitored over two growing seasons at three phenological stages: full *véraison*, mid maturation and full maturation. In addition, anthocyanins, flavonols and hydroxycinnamic acids were quantified both in berry skins and in leaves. The foliar treatments with *A. nodosum* increased photosynthesis and stomatal conductance in treated compared to control plants. Furthermore, extract-treated vines were able to maintain the potential efficiency of photosystem II close to the optimal value even during the hottest periods. No effect of *A. nodosum* extract treatments was observed on stem water potential. *A. nodosum* applications delayed berry ripening, leading to a lower total soluble solids and a higher anthocyanin content in treated berry skins. Interestingly, treatments also affected the content and the partitioning of secondary metabolites in berry skins, as anthocyanins and flavonols contents were higher in treated compared to control plants, while the ratio of methoxylated to non-methoxylated anthocyanins was lower in treated than in control vines. Furthermore, *A. nodosum* extract-treated plants also had higher content of flavonols and hydroxycinnamic acids both in berry skins and in leaves and showed a reduction in the biosynthesis of methoxylated anthocyanins, which are usually accumulated in grapes under environmental constraints. Considering the challenges posed by climate change in the

Mediterranean basin, the use of seaweed extracts might represent a sustainable tool to mitigate the increasing severity of drought, often associated to heat-waves, on the viticulture sector.

1. Introduction

Climate change constitutes a serious challenge for both viticulture and winemaking. These two key economic sectors will have to cope with increasing environmental stresses in the upcoming decades in many regions worldwide. Climate change can strongly affect the development of grapevine and grape quality (Jones and Webb, 2010; Fraga et al., 2012), because high temperatures, combined with high radiation intensities and water deficit, influence vine water status, inhibits photosynthesis, and promote photo-oxidative stress (Bertamini and Nedunchezhian, 2003; Cifre et al., 2005; Hernández et al., 2012; Das and Roychoudhury, 2014; Hossain et al., 2015; Lovisolo et al., 2016). Moreover, environmental consequences related to climate change will accelerate berry ripening processes, that, especially in red varieties, can result in unbalanced wines, with high alcoholic content and low polyphenolic contents (Mosedale et al., 2016).

The decisive role of phenolic compounds in red grape is due to their influence on important properties of berries and wines, such as flavor, color and stability against oxidation processes (Waterhouse, 2002; Mattivi et al., 2006; Silva and Queiroz, 2016). Anthocyanins and flavonols are among the most abundant polyphenol subclasses detected in grape berries (Adams, 2006). The more representative flavonols are glucosides, galactosides and glucuronides of quercetin, myricetin and kaempferol (Makris et al., 2006; Braidot et al. 2008). Each grape cultivar has a unique set of anthocyanins, which include delphinidin-, cyanidin-, petunidin-, peonidin- and malvidin-3-*O*-glucoside, eventually acylated as esters of acetic acid, caffeic acid or p-coumaric acid on the molecule of glucose (Boss et al., 1996; Mattivi et al., 2006; Koyama et al., 2012). It is commonly accepted that anthocyanin profile in grape berry is greatly dependent on genotype, but differences have been found as a result of environmental factors (Guidoni et al., 2002; Downey et al., 2004; Ortega-Regules et al., 2006). In particular, it is well known that water deficit from the onset of ripening until full maturation raises the content of anthocyanins in berry skin (Castellarin et al., 2007a; Zarrouk et al., 2012). In addition, both water deficit and radiation load consistently stimulate methoxylation of B-ring-substituted flavonoids (Castellarin et al., 2007b), incrementing the ratio of methoxylated to non-methoxylated anthocyanins, thus enhancing the stability of these compounds (Castellarin and Di Gaspero, 2007; Tarara et al., 2008). Zarrouk et al. (2016) have recently proposed that increasing anthocyanins stability through methoxylation may constitute an acclimation response to severe environmental constraints, such as the combined effect of drought and heat stress.

It is well known that agronomical practices, such as the use of fertilizers, greatly affect the biosynthesis of flavonoids (Downey et al., 2006; Teixeira et al., 2013). *Ascophyllum nodosum* L. is a brown seaweed, traditionally applied as biofertilizer in agriculture (Chouliaras et al., 2009; Jannin et al., 2013; Hernández-Herrera et al., 2014; Sabir et al., 2014; du Jardin, 2015). Seaweed extract products have been also recently employed as biostimulants to promote short-term plant acclimation to climate constraints, without adversely impacting on the environment (Craigie, 2011; Sharma et al., 2014; Tanou et al., 2017; Yakhin et al., 2017). *A. nodosum* extracts may stimulate numerous metabolic pathways, eliciting plant physiological and biochemical responses to abiotic stress and enhancing plant performance (Khan et al., 2009; Zhang and Ervin, 2008; Rayirath et al., 2009; Paradikovic et al., 2011; Nair et al., 2012). As an example, *Citrus* spp. plants exposed to treatments with *A. nodosum* commercial extract displayed higher drought tolerance (Spann and Little, 2011) by increasing photosynthesis and stomatal conductance (Little and Spann, 2010). Furthermore, recent evidences suggest that the beneficial effects of *A. nodosum* treatments on plant acclimation to stressful condition involve the activation of antioxidant enzymes and secondary metabolic pathways (Calvo et al., 2014; Santaniello et al. 2017), in particular the biosynthesis of flavonoids (Fan et al., 2011; Cai et al., 2012; Santaniello et al. 2017).

In grapevine, foliar application of seaweed extracts enhances root development (Mancuso et al., 2006; Mugnai et al., 2008), mineral nutrient uptake (Turan and Köse, 2004; Mancuso et al., 2006; Khan et al., 2012; Sabir et al., 2014) and growth (Mugnai et al., 2008; Khan et al., 2012; Popescu and Popescu, 2014; Sabir et al., 2014). There are numerous reports also on the positive effects of these extracts on yield and grape quality (Norrie et al., 2002; Colapietra and Alexander, 2006; Norrie and Keathley, 2006; Kok et al., 2010; Sabir et al., 2014), but their activity on secondary metabolism is scarcely investigated (Mancuso et al., 2006; Cai et al., 2012). Thus, we conducted an experiment with Sangiovese variety over two consecutive growing seasons, hypothesizing that treatments with seaweed extracts could promote an enhancement in the biosynthesis of secondary metabolites in grape berry skins and leaves, thus improving grape quality and helping vines to cope with abiotic stresses. The aims of our study were to 1) evaluate how foliar treatments with an *A. nodosum* extract affect physiological performances of *Vitis vinifera* L., and 2) investigate the biochemical adjustments induced in grape berry skins by these treatments under field conditions.

2. Materials and methods

2.1. Grapevine Field Conditions and Experimental Design

Experiments were conducted during the 2016 and 2017 growing-seasons in a commercial vineyard in the Chianti Classico area (Lat. 43.668°N, Long. 11.145°E), Tuscany, Italy, located at an elevation of 250 m a.s.l. facing South-West exposure. The climate is typically Mediterranean, characterized by rainy winters and dry, warm to hot summers. Soil horizons present a clay loam texture with the following average characteristics: clay 38.8%; silt 37.8%; sand 23.4%; organic matter 2.0%; pH (H_2O) 7.8.

The 18-year-old vineyard of the red cv. Sangiovese (*V. vinifera*), clonal selection R 24, grafted on 420 A rootstock, was planted with a spacing of 1.2 m × 3 m (~2778 vines/ha). Vines were trained on a vertical shoot positioning and spur-pruned single cordon system, at 80 cm above ground with a load of 12 buds per vine distributed over 6 spurs. Vines were rainfed-cultivated and grown using standard cultural practices as applied by commercial farmers.

The experimental plots arranged in a randomized complete block design, consisting in four blocks (3 rows each, approximately half a hectare) and one factor (*A. nodosum* extract foliar application). Seaweed extract-treated vines (SWE) were sprayed with 3g of a non-commercial *A. nodosum* extract, diluted in 1 L of water (Santaniello et al., 2017; Frioni et al., 2018). The first application was performed one week after full *véraison* (23 August 2016; 7 August 2017; modified Eichorn and Lorenz (E-L) 36 stage) (Coombe, 1995) and the second one was repeated, on the same vines, after 15 days (6 September 2016; 21 August 2017; E-L 37 stage), according to Salvi et al. (2016). On the same days, untreated control vines (CTRL) were sprayed with the same amount of water.

From the central row of each block, 10 homogeneous vines, for both SWE and CTRL vines, were randomly tagged and used for eco-physiological measurements, yield and biochemical samplings, at three phenological stages: full *véraison* (100% of the berries presented full color change; pre-treatment; 16 August 2016; 31 July 2017; E-L 36 stage), mid maturation (30 August 2016; 14 August 2017; E-L 37 stage), and full maturation (13 September 2016; 28 August 2017; E-L 38 stage).

2.2. Chemical Characterization of the A. nodosum Extract

The *A. nodosum* extract was manufactured using a proprietary process at alkaline pH. Briefly, 25 g of dry *A. nodosum* biomass were treated with 1 N NaOH (100 ml) for 24 h at 150 rpm and 55ºC.

After base treatment, the mixture was centrifuged at 8000 rpm for 10 min and the supernatant was stored at 4°C. Then, the extract was characterized by quantifying proteins, carbohydrates, lipids, ashes, total phenolics, total nitrogen and total organic carbon contents, antioxidant capacity, macro-/micro-nutrients and amino acids. For the analyses of proteins, carbohydrates, lipids and ashes, the *A. nodosum* extract was dried at 80°C for 5 days, and finely ground (< 1mm), with a mortar.

Protein content was determined following Lowry et al. (1951); carbohydrates were determined following Dubois et al. (1956) and lipids following Marsh and Weinstein (1966). Ashes were analyzed following ISTISAN protocols (ISTISAN Report 1996/34).

The total phenolic content in the dried *A. nodosum* extract was determined according to Ganesan et al. (2008), using the Folin Ciocalteu assay. Results were expressed in gallic acid equivalents (mg GAE g^{-1}) of dry powder, using a calibration curve of authentic gallic acid (0 to 500 µg mL^{-1}) (Sigma Aldrich, Italy).

The DPPH (1,1-diphenyl-2-picrylhydrazyl) Radical-Scavenging assay was carried out according to Rajauria et al. (2013). Briefly, the assay was performed in cuvettes with 1:1 (v/v) ratio of 1000 µL of freshly prepared DPPH radical solution (165 mM) and 1000 µL of sample, solubilised in methanol. The reaction mixtures were incubated for 30 min at 25°C in dark condition, and absorbance measured at 517 nm.

Total organic carbon (TOC) was determined with a TOC analyzer (Shimadzu, USA). Macro- and micro-nutrients were analysed by inductively coupled plasma optical emission spectrometry (ICP-OES) using a Perkin Elmer-Optima 2000 DV sequential multi-element instrument (Perkin Elmer, USA). Total nitrogen content was determined by the improved Kjeldahl method for nitrate-containing samples (AOAC, 1990).

Amino acid composition was determined as described by Mustafa et al. (2007). The analysis was performed using a protocol based on the EZ:faast kit (Phenomenex, USA) and the derivatized amino acids were quantified by gas chromatography/mass spectrometry (GC–MS) (Agilent Technologies, USA) using Ala, Asn, Asp, Glu, Gln, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr and Val as external standards and I-norvaline (Sigma Aldrich, Italy) as internal standard.

2.3. Vineyard Microclimate and Eco-physiological Traits

Monthly total precipitations (mm) and mean daily values of maximum and minimum air temperatures (°C) were collected by an automatic meteorological station (Ecotech, Germany) located close to the vineyard. Growing degree days (GDD) or Amerine and Winkler (1944) index was also calculated on a 10°C base temperature. Briefly, GDD expresses the sum of all daily temperatures for the active growth in an area during the vine growing season between the 1st of April and the 31st of October. Net CO_2 assimilation rate (P_n, μmolm^{-2}s^{-1}) and stomatal conductance (g_s, mmolm^{-2}s^{-1}) were measured with a Ciras 3 (PP Systems, USA) on 10 fully expanded leaves (one leaf from each tagged vine) from each block, for both SWE and CTRL vines. Measurements were performed between 10 and 12 a.m., setting the leaf chamber flow at ambient temperature, at ambient CO_2 concentration (400 μmol mol^{-1}) and at saturating photosynthetic photon flux of 1300 μmol m^{-2}s^{-1} during the three phenological stages of full *véraison*, mid maturation and full maturation. Chlorophyll *a* fluorescence transients of dark-adapted leaves were recorded using a saturating flash of actinic light at 3000 μmolm^{-2}s^{-1} for 1s (Handy-PEA®, Hansatech Instruments, UK). Briefly, the maximum quantum yield of photosystem II (PSII) was calculated as $F_v/F_m = (F_m-F_0)/F_m$ where F_v is the variable fluorescence and F_m is the maximal fluorescence of dark-adapted (over a 30-min period) leaves (Maxwell and Johnson, 2000).

Midday stem water potential (Ψ_w, MPa) of dark-adapted leaves (over a 60-min period) was measured by a pressure chamber (PMS Instrument Co, USA) following Scholander et al. (1965). Chlorophyll *a* fluorescence and midday stem water potential were taken on the same leaves used for leaf gas exchanges measurements at the same three phenological stages.

2.4. Berry Composition and Productivity Parameters

A 100-berry sample was collected randomly from the tagged vines of each block, of both SWE and CTRL vines, at full *véraison*, mid maturation and full maturation, taking care to remove berries from all the positions within the cluster. The berry sample was weighed with a digital scale (PCE Italia s.r.l, Italy) and immediately juiced. Soluble solids concentration (°Brix) was measured using a refractometer (ATAGO, USA); must pH was measured using a portable pH meter (Hanna instrument, USA) and titratable acidity (gL^{-1} tartaric acid) was determined on a 10 mL sample by manual glass burette using 0.1 M NaOH to an endpoint of pH 7.0. At full maturation, the tagged vines were harvested, and their production was weighed with a portable electronic scale (Bonso Advanced Technology Ltd., Hong Kong) to obtain yield per vine (kg) and cluster weight (g).

2.5. Phenylpropanoids in Berry Skin and in Leaf

Berry phenylpropanoids content was measured on four replicates, each constituted by a pool of 30 berries (3 berries for each tagged vine), collected from the tagged vines of each block, of both SWE and CTRL vines, at full *véraison*, mid maturation and full maturation. Berry were immediately frozen in liquid nitrogen, then skins were removed and lyophilized (Lio-5P, Cik solution, Germany).

Lyophilized berry skins (0.7g) were ground in a mortar under liquid nitrogen and the obtained powder was extracted with 75% of aqueous ethanol acidified to pH 2 by HCOOH (3×5 mL) and sonicated for 30 min. The supernatant was partitioned with 3×5 mL of *n*-hexane, the extracts were added together and reduced to dryness under vacuum, then, rinsed with MeOH/H_2O (50/50, pH 2). Aliquots of 5 μL were injected into a liquid chromatograph equipped with a quaternary 200Q/410 pump and an LC 200 diode array detector (DAD) (all from Perkin Elmer, USA). Anthocyanins, flavonols and hydroxycinnamic acids were separated in a 250x4.6 mm Agilent Zorbax SB-C18 (5μm) column operating at 30°C, at a flow rate of 0.6 ml min^{-1}, using a gradient solvent system consisting of H_2O (plus 5% HCOOH) (A), MeOH (plus 5% HCOOH) (B), CH_3CN (plus 5% HCOOH (C), during a 25 min run: 0-2 min 90% A, 5% B, 5% C; 2–7 min to 80% A, 10% B, 10% C; 7–12 min to 70% A, 15% B, 15% C; 12–17 min to 60% A, 20% B, 20% C; 17–20 min to 56% A, 22% B, 22% C; 20–24 min to 10% A, 45% B, 45% C; 24–25 min to 90% A, 5% B, 5% C. Quantification of anthocyanins was performed at 530 nm using calibration curves of cyanidin 3-*O*-glucoside, delphinidin 3-*O*-glucoside, petunidin 3-*O*-glucoside, peonidin 3-*O*-glucoside and malvidin 3-*O*-glucoside (Extrasynthese, France). Quantification of flavonols and hydroxycinnamic acids were performed at 330 nm using the calibration curve of quercetin 3-*O*-glucoside, quercetin 3-*O*-galactoside, myricetin 3-*O*-glucoside, *trans*-caftaric acid, *trans*- and *cis*-coutaric acid and kaempferol 3-*O*-glucoside (Extrasynthese, France). The quantification of anthocyanins was used to calculate the proportion of methoxylated/non-methoxylated derivatives and the percentage of methoxylated anthocyanins among 3-glucoside anthocyanins.

Leaf phenylpropanoids content was measured on four replicates, each constituted by a pool of 10 leaves (one leaf each tagged vine) from each block, of both SWE and CTRL vines, collected at full *véraison*, mid maturation and full maturation. The harvested leaves were immediately frozen in liquid nitrogen, stored at -80°C and then lyophilized.

Lyophilized leaf samples (0.3g) were ground in a mortar under liquid nitrogen and the obtained powder was extracted and quantified with the same procedure of phenylpropanoids in berry skin, as reported above. The gradient solvent system consisted of 90% H_2O (plus 1% HCOOH) + 10% CH_3CN/MeOH 25/75 (plus 1% HCOOH) (A), 10% H_2O (plus 1% HCOOH) + 90% CH_3CN/MeOH 25/75 (plus 1% HCOOH) (B), during a 40 min run: 0–3 min 90% A, 10% B; 3–33 min to 50% A, 50% B; 33–38 min to 10% A, 90% B; 38–40 min 90% A, 10% B.

2.6. Statistical Analysis

Year (2016 and 2017), phenological stage (full *véraison*, mid maturation and full maturation) and treatment (SWE and CTRL) were considered as fixed factors. A three-way ANOVA ($P \leq 0.05$) was used to compare SWE and CTRL in different years and phenological stages, and factors interactions.

Then, significant interactions among factors were investigated with one-way ANOVA ($P \leq 0.05$). Mean values were separated by Fisher's least significant difference (LSD) post-hoc test ($P \leq 0.05$). Where appropriate, prior to analysis, the original percentage data were transformed by arcsine function. All statistical analyses were performed using SPSS Statistic 25 (IBM, USA).

3. Results

3.1. Chemical Properties of the A. nodosum Extract

A. nodosum extract was composed by ~39.8% in proteins, ~8.0 % in carbohydrates, ~2.1% in lipids and ~50.1 % in ashes. The DPPH radical-scavenging capacity and total phenolic content were 82.5% ± 3.3 and 74.7 mg GAE g^{-1} of dry weight (DW) ± 5.0, respectively. The *A. nodosum* extract contains mainly organic nitrogen (~4%), organic carbon (~15%), potassium (~103 ppm), phosphorous (~32 ppm), sulfur (~6 ppm) and manganese (~3 ppm) (**Table 1**). The most abundant free amino acids in the *A. nodosum* extract were alanine, phenylalanine, proline and methionine.

Table 1
Main macro/micronutrients and free amino acids quantified in the *A. nodosum* extract.

Macro and Micronutrients	Unit	Amount	Amino Acids	Unit	Amount
Total N	%	4.2	**Alanine**	ppm	12695.8
Organic N	%	4.1	**Phenylalanine**	ppm	7172.3
Total C	%	17	**Proline**	ppm	6561.1
Organic C	%	15.3	**Methionine**	ppm	3796.9
K	ppm	102.6	**Glutamic acid**	ppm	2679.2
P	ppm	31.8	**Alloisoleucine**	ppm	1869.8
Mn	ppm	2.87	**Leucine**	ppm	1489.8
Mg	ppm	0.4	**Tryptophan**	ppm	52.5

3.2. *Vineyard Microclimate and Eco-Physiological Traits*

Vineyard microclimate conditions of 2016 and 2017 are reported in **Figure 1**. Mean air temperature measured from bud break to leaf fall (April-October) was, on average, 1°C higher in 2017 than in 2016. Whereas global radiation was ~15 Wm^{-2} higher in 2017 than in 2016. Annual cumulative precipitations were around 690 mm in 2016 and 492 mm in 2017. Accumulated heat expressed as GDD was lower in 2016 than in 2017 (1987 vs. 2137 GDD, respectively). The hottest and driest period of these two seasons was mid maturation 2017 (August), when minimum and maximum air temperatures were, respectively, ~1° and ~4° higher than in 2016, and precipitations were mostly absent.

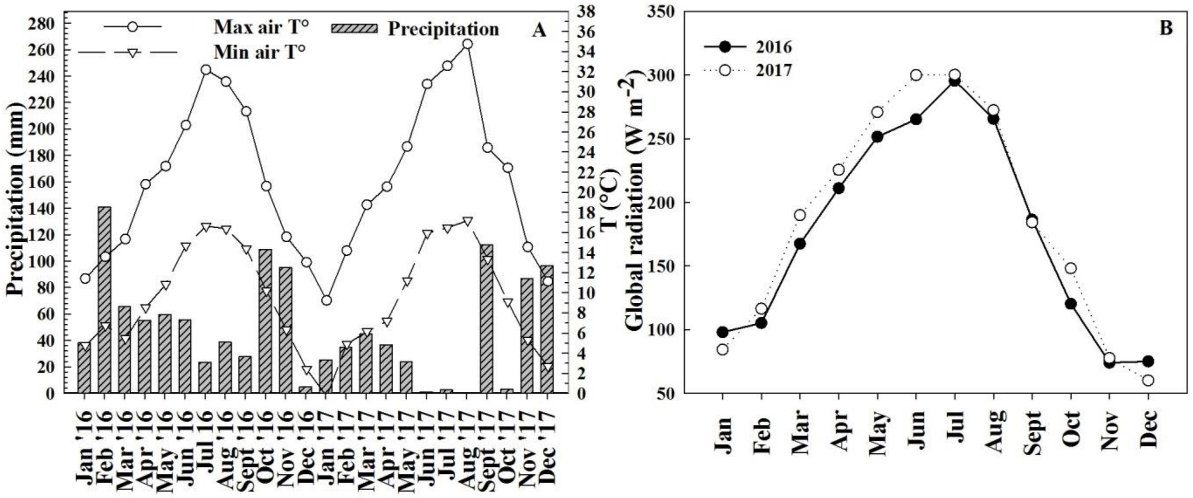

Fig. 1 Vineyard microclimate conditions: monthly total precipitation (mm) and mean daily values of maximum and minimum air temperature (°C) for 2016 and 2017 (A); monthly means of global radiation (W m^{-2}) in 2016 and 2017 (B).

To investigate the role of *A. nodosum* treatments on eco-physiological traits of vines, leaf gas exchanges (P_n and g_s), maximum efficiency of PSII photochemistry (F_v/F_m) and midday stem water potential (Ψ_w) were measured (**Table 2** and **Supplementary Table 1**).

Table 2
Net assimilation rate (P_n), stomatal conductance (g_s), maximum quantum yield of PSII (F_v/F_m) and midday stem water potential (Ψ_w) in leaves of *V. vinifera* treated with *A. nodosum* extract (SWE) and under control conditions (CTRL, untreated plants). Measurements were conducted at the stages of full *véraison* (Full *vérais.*), mid maturation (Mid matur.) and full maturation (Full matur.). Data (mean ± SE, n=10) were subjected to one-way ANOVA. Different letters within the same parameter and row indicate significant differences among treatments (Fisher's LSD test, $P \leq 0.05$).

Year	**Phenology**	**P_n (µmol m^{-2}s^{-1})**		**g_s (mmol m^{-2}s^{-1})**		**F_v/F_m**		**Ψ_w (MPa)**	
		CTRL	SWE	CTRL	SWE	CTRL	SWE	CTRL	SWE
2016	**Full *vérais.***	8.6 ± 1.2 a	10.4 ± 1.2 a	138 ± 20 a	179 ± 23 a	0.78 ± 0.01 a	0.79 ± 0.01 a	-1.11 ± 0.08 a	-1.11 ± 0.10 a
	Mid matur.	12.3 ± 0.7 a	11.9 ± 1.0 a	249 ± 18 a	264 ± 22 a	0.75 ± 0.01 b	0.79 ± 0.01 a	-1.27 ± 0.07 a	-1.15 ± 0.10 a
	Full matur.	6.9 ± 0.5 b	11.0 ± 0.6 a	205 ± 14 b	322 ± 11 a	0.79 ± 0.01 a	0.79 ± 0.01 a	-1.19 ± 0.08 a	-1.12 ± 0.10 a
2017	**Full *vérais.***	15.0 ± 1.5 a	14.0 ± 1.5 a	166 ± 20 a	164 ± 20 a	0.77 ± 0.01 a	0.76 ± 0.01 a	-1.06 ± 0.03 a	-1.01 ± 0.04 a
	Mid matur.	6.6 ± 0.8 b	9.1 ± 0.7 a	46 ± 10 b	87 ± 8 a	0.76 ± 0.01 a	0.78 ± 0.01 a	-1.33 ± 0.02 a	-1.26 ± 0.08 a
	Full matur.	10.5 ± 1.2 b	13.9 ± 1.1 a	164 ± 10 a	179 ± 5 a	0.61 ± 0.08 b	0.77 ± 0.03 a	-1.17 ± 0.04 a	-1.08 ± 0.08 a

The results of the three-way ANOVA show that gas exchanges and F_v/F_m were significantly affected by year, phenological stage and treatment whereas differences in Ψ_w depended mostly on phenological stage and year (**Supplementary Table 1**).

There were no significant differences in all physiological parameters observed at full *véraison* (pre-treatment stage) between SWE and CTRL plants (**Table 2**). In 2016, SWE and CTRL showed similar values of P_n and g_s at mid maturation. By contrast, higher P_n (+59%) and g_s (+57%) values were observed in SWE at full maturation compared to CTRL. The same trend was monitored in 2017, when SWE plants displayed significantly higher P_n and g_s than CTRL plants in the hottest period, at mid maturation (**Table 2**). Moreover, SWE vines were able to maintain significantly higher F_v/F_m than CTRL at mid maturation in 2016 and at full maturation in 2017; Ψ_w was not significantly affected by *A. nodosum* treatments.

3.3. Berry Composition and Productivity Parameters

Year significantly affected ($P \leq 0.05$) berry weight, cluster weight and yield/vine, whereas *A. nodosum* treatments affected significantly only total soluble solids (**Supplementary Table 1**). Berry weight was more than two times higher in 2016 (2.4 g) than in 2017 (1 g), and almost the same difference was also reported for cluster weight (362.3 g in 2016 and 151.5 g in 2017) and yield/vine (2.7 Kg in 2016 and 1.0 Kg in 2017).

The 2016 and 2017 values of total sugar and total acidity contents, pH and berry weight are presented in **Table 3**. Both in 2016 and in 2017, there were no significant differences in all parameters at full *véraison* (pre-treatment stage), whereas significant differences in total soluble solids were observed at full maturation between SWE and CTRL berries. At full maturation SWE presented 1°Brix lower total soluble solids as compared with CTRL. There were no statistical differences between the two treatments in yield/vine and cluster weight (data not shown).

Table 3
Total sugars, titratable acidity, pH and berry weight in berry of *V. vinifera* treated with *A. nodosum* extract (SWE) and under control conditions (CTRL, untreated plants). Measurements were conducted at the stages of full *véraison* (Full *vérais.*), mid maturation (Mid matur.) and full maturation (Full matur.). Data (mean ± SE, n=4) were subjected to one-way ANOVA. Different letters within the same year and phenological stage, indicate significant differences among treatments (Fisher's LSD test, $P \leq 0.05$).

Year	**Phenology**	**Total sugars (°Brix)**		**Titratable acidity (gL^{-1})**		**pH**		**Berry weight (g)**	
		CTRL	SWE	CTRL	SWE	CTRL	SWE	CTRL	SWE
2016	**Full *vérais.***	20.3 ± 0.2 a	20.6 ± 0.2 a	6.7 ± 0.3 a	6.9 ± 0.2 a	3.08 ± 0.04 a	3.03 ± 0.03 a	2.1 ± 0.1 a	2.4 ± 0.2 a
	Mid matur.	23.8 ± 0.1 a	23.6 ± 0.4 a	5.5 ± 0.1 a	5.7 ± 0.1 a	3.16 ± 0.02 a	3.13 ± 0.01 a	2.5 ± 0.1 a	2.7 ± 0.1 a
	Full matur.	23.9 ± 0.1 a	22.9 ± 0.1 b	5.0 ± 0.1 a	5.2 ± 0.1 a	3.25 ± 0.03 a	3.25 ± 0.01 a	2.4 ± 0.1 a	2.5 ± 0.1 a
2017	**Full *vérais.***	18.9 ± 0.2 a	18.6 ± 0.2 a	13.2 ± 0.2 a	13.5 ± 0.2 a	2.80 ± 0.01 a	2.82 ± 0.01 a	0.9 ± 0.0 a	0.9 ± 0.1 a
	Mid matur.	20.7 ± 0.5 a	19.6 ± 0.1 b	10.1 ± 0.2 a	9.7 ± 0.6 a	2.99 ± 0.02 a	3.00 ± 0.04 a	1.2 ± 0.2 a	1.1 ± 0.1 a
	Full matur.	25.3 ± 0.4 a	24.5 ± 0.3 b	7.4 ± 0.3 a	8.2 ± 0.6 a	2.98 ± 0.02 a	3.03 ± 0.04 a	1.0 ± 0.1 a	1.1 ± 0.1 a

3.4. Phenylpropanoids in Berry Skin and in Leaf

Five anthocyanins (i. e. delphinidin-3-*O*-glucoside, cyanidin-3-*O*-glucoside, petunidin-3-*O*-glucoside, peonidin-3-*O*-glucoside and malvidin-3-*O*-glucoside) were identified in the chromatogram of the hydro alcoholic extracts of the berry skins (**Figure 2A**).

Statistical differences related to year and treatment were recorded in all anthocyanin, whereas, on the contrary, their contents were not significantly affected by the phenological stage (**Supplementary Table 2**). Petunidin and peonidin were 80% and 36% lower in 2017 than in 2016, whereas the opposite was observed for delphinidin, cyanidin and malvidin, significantly more abundant in 2017 compared to 2016 (**Supplementary Table 2**).

Figure 2B reports the chromatogram of the hydro alcoholic extracts recorded at 330 nm from SWE and CTRL berry skins. Different derivatives of hydroxycinnamic acids (*trans*-caftaric and *trans*-/*cis*-coutaric acid, ferulic acid derivatives) and flavonols (glucosides, galactosides and glucuronides derivatives of quercetin and kaempferol) were identified (**Figure 2B**).

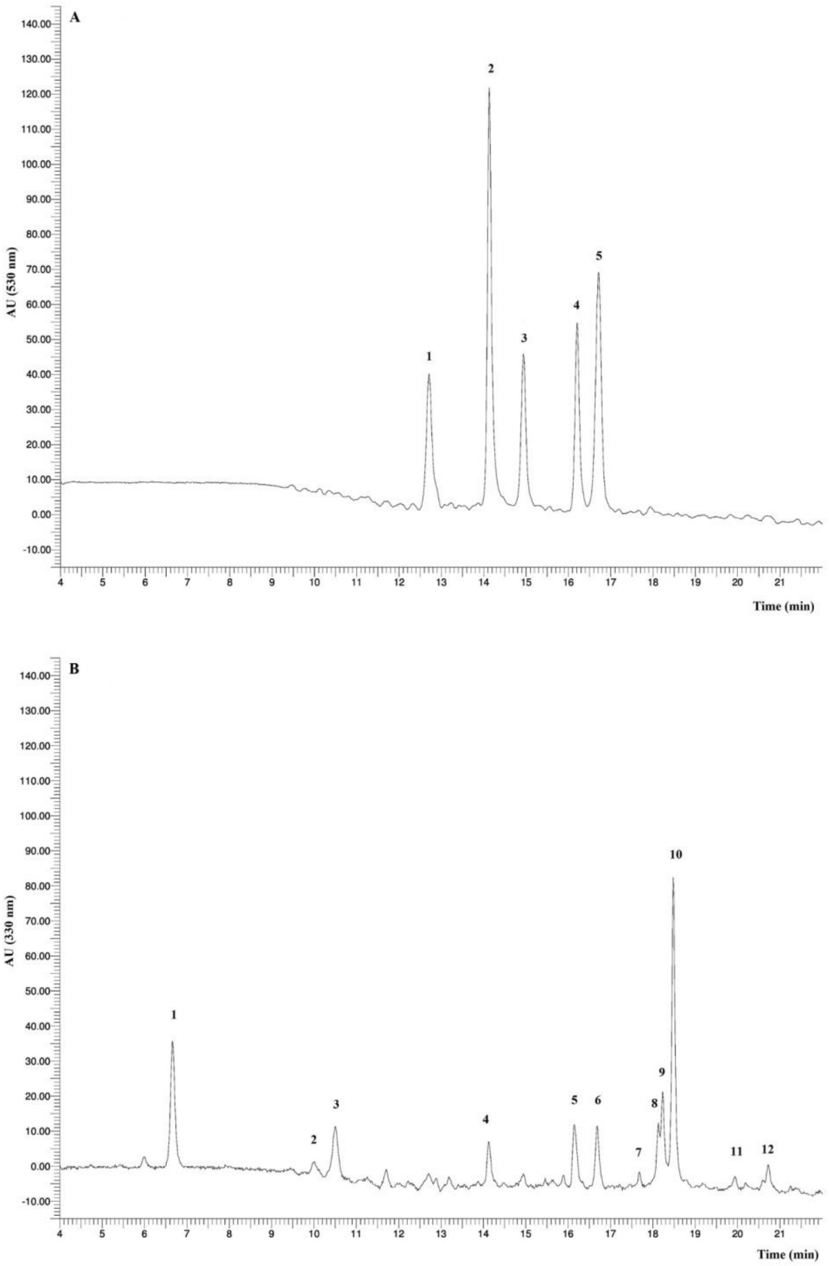

Fig. 2 HPLC-DAD chromatograms of hydroalcholic extract of CTRL berry skins, recorded at 530 nm for anthocyanins (A), and recorded at 330 nm for hydroxycinnamic acid and flavonols (B). Peak numbering in (A): (1) Delphinidin-3-*O*-glucoside; (2) Cyanidin-3-*O*-glucoside; (3) Petunidin-3-*O*-glucoside; (4) Peonidin-3-*O*-glucoside; (5) Malvidin-3-*O*-glucoside. Peak numbering in (B): (1) *trans*-caftaric acid; (2) *cis*-coutaric acid; (3) *trans*-coutaric acid; (4) Ferulic acid; (5), (6) Ferulic acid derivatives; (7) Myricetin-3-*O*-glucoside; (8) Quercetin-3-*O*-galactoside; (9) Quercetin-3-*O*-glucuronide; (10) Quercetin-3-*O*-glucoside; (11) Kaempferol-3-*O*-glucuronide; (12) Kaempferol-3-*O*-glucoside.

Among flavonols, quercetin derivatives were the most represented. It is important to note that myricetin derivatives abundance was strongly and significantly affected by the year (**Supplementary Table 2**), indeed myricetin-3-*O*-glucoside was detected and quantified in 2017 but not in 2016. Anthocyanins (**Figure 3**) were not significantly affected by the *A. nodosum* extract treatments at full *véraison* (pre-treatment stage).

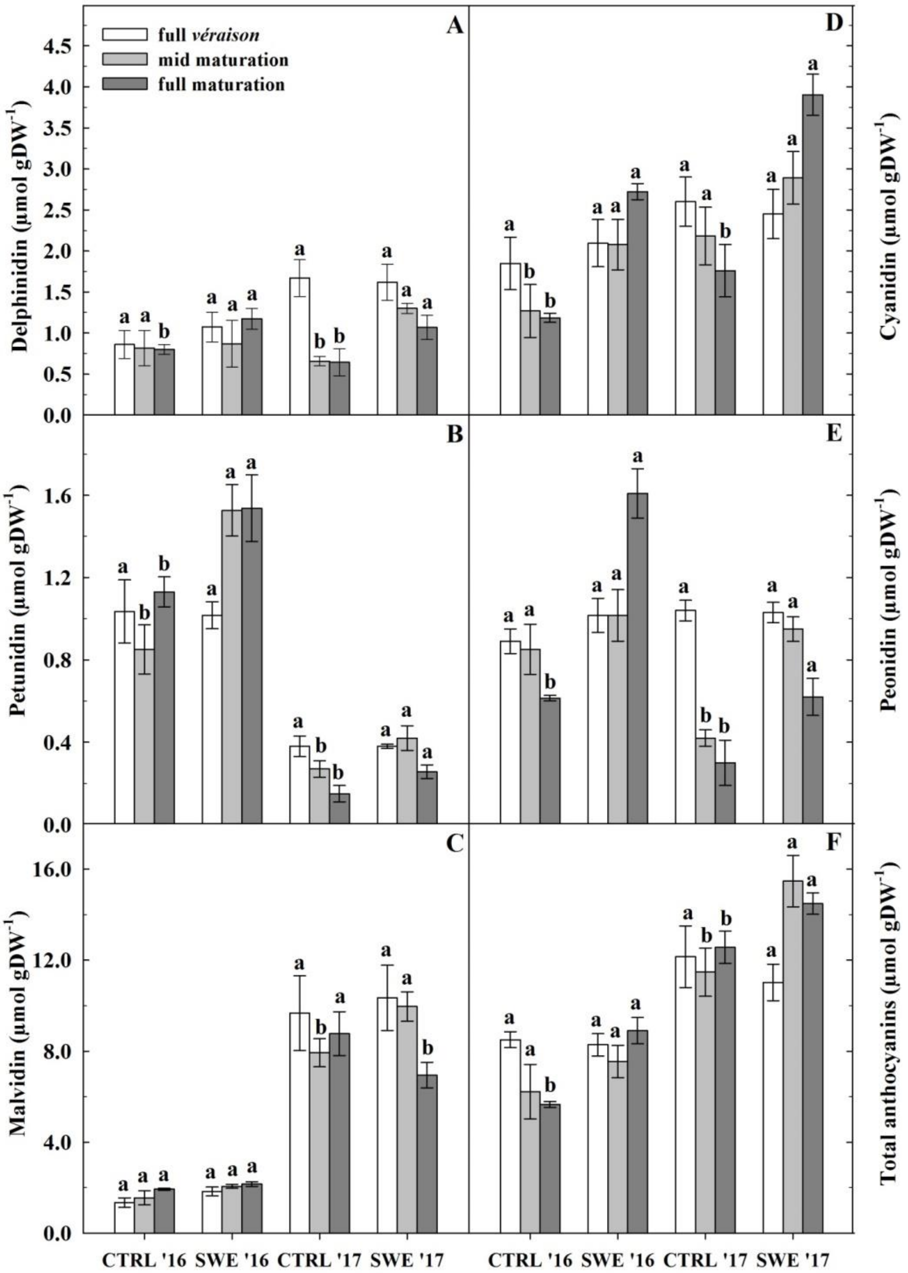

Fig. 3 Delphinidin-3-*O*-glucoside (A), Petunidin-3-*O*-glucoside (B), Malvidin-3-*O*-glucoside (C), Cyanidin-3-*O*-glucoside (D), Peonidin-3-*O*-glucoside (E), and Total anthocyanin content (F) in berry skins (μmol gDW^{-1}) of *V. vinifera* plants treated with *A. nodosum* extract (SWE) and untreated (CTRL) in 2016 and 2017. Analyses were conducted at the stages of full *véraison* (white bars), mid maturation (grey bars) and full maturation (dark grey bars). Data are means ± SE (n=4). Different letters within the same year and phenological stage indicate significant differences between SWE and CTRL, assessed by one-way ANOVA (Fisher's LSD test, $P \leq 0.05$).

In 2016, petunidin (**Figure 3B**) and cyanidin (**Figure 3D**) contents were higher in SWE than CTRL berry skins at mid maturation. Nonetheless, at full maturation, SWE berry skins showed significantly higher total anthocyanin contents than CTRL (**Figure 3F**). In detail, at full maturation, the *A. nodosum* extract treatments increased the abundance of cyanidin (**Figure 3D**) and peonidin (**Figure 3E**) of about 129% and 167% respectively, and of delphinidin (**Figure 3A)** and petunidin (**Figure 3B)** of about 46% and 35%, respectively, compared to control. At the same stage, malvidin contents (**Figure 3C**) remained almost unchanged between SWE and CTRL. In 2017, the contents of all anthocyanins, except malvidin, were higher in SWE than in CTRL, both at mid maturation and at full maturation (**Figure 3**). At full maturation, the *A. nodosum* treatments raised the amounts of cyanidin (117%) (**Figure 3D**), peonidin (107%) (**Figure 3E**), delphinidin (67%) (**Figure 3A**) and petunidin (73%) (**Figure 3B**) in SWE compared to CTRL; on the contrary, malvidin (**Figure 3C**) was 26% higher in CTRL than in SWE.

The methoxylated (peonidin, petunidin and malvidin) to non-methoxylated (cyanidin and delphinidin) anthocyanins ratio (**Figure 4A**), and the percentage of methoxylated anthocyanins on total anthocyanins (**Figure 4B**) were calculated in order to investigate how SWE treatments affected anthocyanin composition.

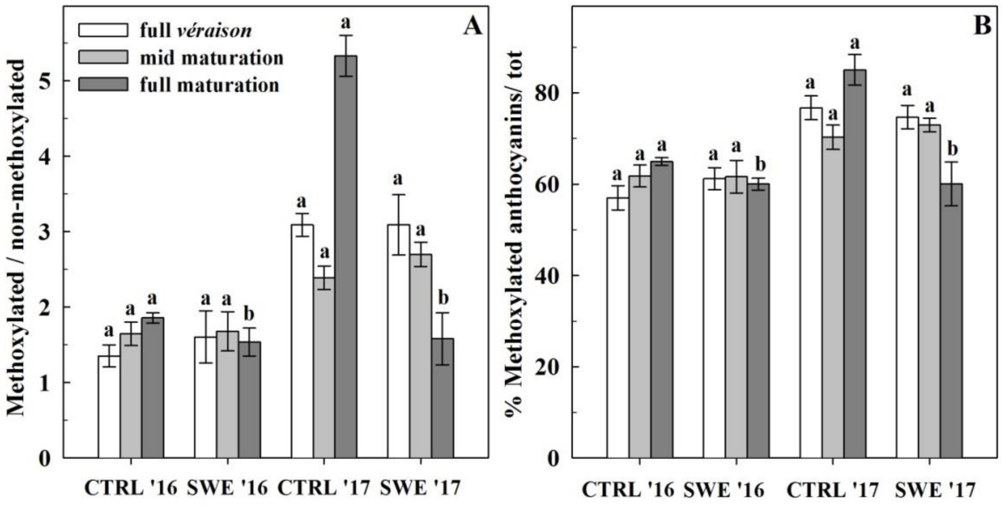

Fig. 4 Ratio of methoxylated to non-methoxylated anthocyanins (A) and percentage of methoxylated anthocyanins on the total amount of anthocyanins (B) in berry skins of *V. vinifera* plants treated with *A. nodosum* extract (SWE) and untreated (CTRL) in 2016 and 2017. Analyses were conducted at the stages of full *véraison* (white bars), mid maturation (grey bars) and full maturation (dark grey bars). Data are means ± SE (n=4). Different letters within the same year and phenological stage, indicate significant differences between SWE and CTRL, assessed by one-way ANOVA (Fisher's LSD test, $P \leq 0.05$).

There was significant year effect ($P \leq 0.05$) on both parameters (**Supplementary Table 2**). Irrespective of the treatment, in 2016, the percentage of methoxylated anthocyanins was about 19% less abundant respect to 2017, resulting in a 54% reduction of the methoxylated to non-methoxylated ratio in 2016 (1.6 μmol g^{-1}DW), than in 2017 (3.5 μmol g^{-1}DW). Both in 2016 and 2017, as a consequence of treatments with *A. nodosum*, the ratio of methoxylated to non-methoxylated anthocyanins was lower in SWE than in CTRL at full maturation (**Figure 4A**).

Similarly, the *A. nodosum* treatments induced a strong reduction in the percentage of methoxylated anthocyanins (**Figure 4B**). In 2016, hydroxycinnamic acids content was higher in CTRL than in SWE berry skins at mid maturation, whereas the opposite was found at full maturation (**Figure 5A**). Quercetin derivatives content (**Figure 5B**) decreased with berry development and was higher in SWE compared to CTRL berry skins at mid maturation and at full maturation. By contrast, kaempferol derivatives (**Figure 5C**) remained almost unchanged among phenological stages and were only marginally affected by the *A. nodosum* treatments. In 2017, the contents of hydroxycinnamic acids and quercetin derivatives increased both in SWE and CTRL berry skins at mid maturation and reached the maximum levels at full maturation (**Figure 5**).

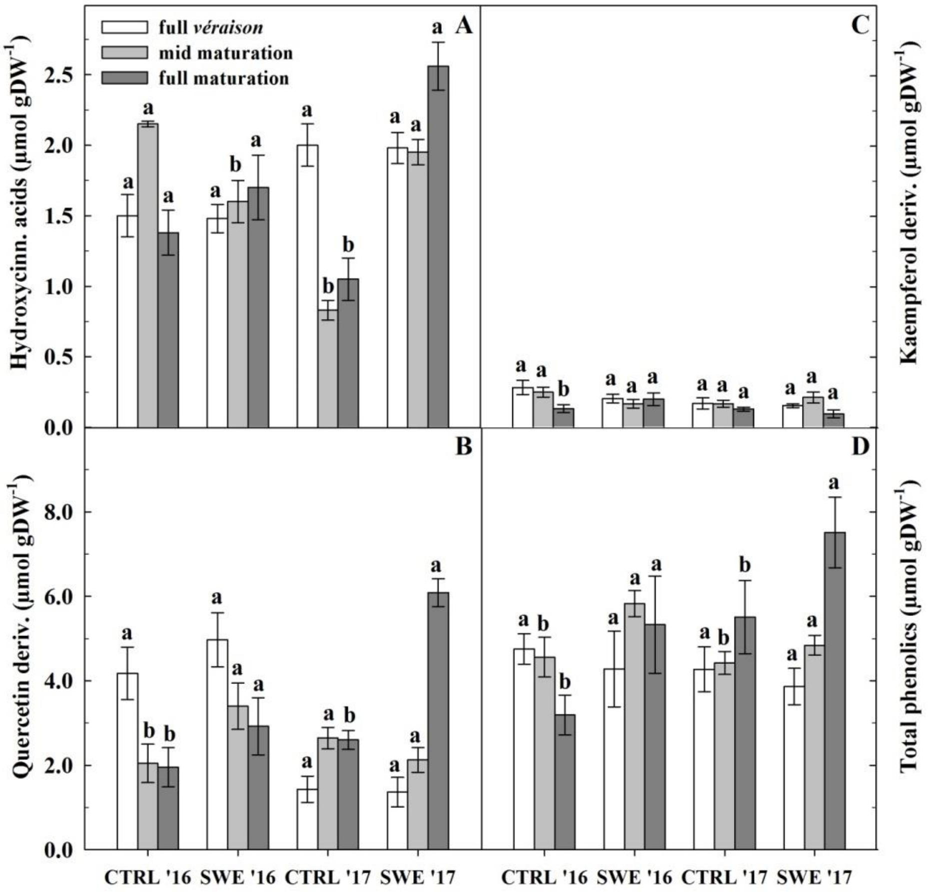

Fig. 5 Hydroxycinnamic acids (A), Quercetin derivatives (B), Kaempferol derivatives (C), and Total phenolic content (D) in berry skins (µmol gDW^{-1}) of *V. vinifera* plants treated with *A. nodosum* extract (SWE) and untreated (CTRL) in 2016 and 2017. Analyses were conducted at the stages of full *véraison* (white bars), mid maturation (grey bars) and full maturation (dark grey bars). Data are means ± SE (n=4). Different letters within the same year and phenological stage, indicate significant differences between SWE and CTRL, assessed by one-way ANOVA (Fisher's LSD test, $P \leq 0.05$).

Hydroxycinnamic acids increased more than twice in SWE compared to CTRL berry skins at mid maturation and at full maturation (**Figure 5A**), and the same trend was observed for quercetin derivatives at full maturation (**Figure 5B**). Myricetin-3-*O*-glucoside was slightly higher in SWE

(3.27 nmol g^{-1}DW) than in CTRL (2.39 nmol g^{-1}DW) (data not shown). The phenolic total contents (**Figure 5D**) were higher in SWE than in CTRL berry skins at mid maturation and at full maturation, in both years.

The same hydroxycinnamic acids and flavonols contained in berry skins were also identified in leaves, with the exception of myricetin derivatives and anthocyanins that were not detected.

Among flavonols, quercetin derivatives represented approximately the 80-85% of the total phenolic content in both years (**Figure 6**). Year significantly affected ($P < 0.05$) quercetin and kaempferol derivatives contents (**Supplementary Table 2**).

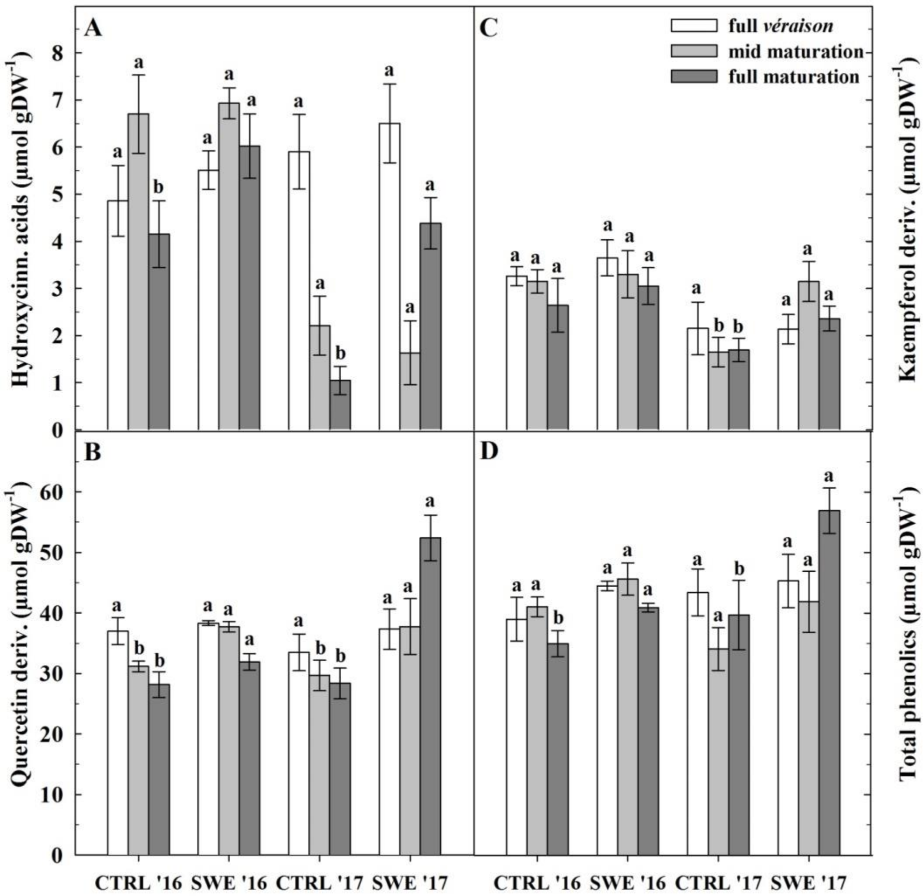

Fig. 6 Hydroxycinnamic acids (A), Quercetin derivatives (B), Kaempferol derivatives (C), and Total phenolic content (D) in leaves (µmol gDW^{-1}) of *V. vinifera* plants treated with *A. nodosum* extract (SWE) and untreated (CTRL) in 2016 and 2017. Analyses were conducted at the stages of full *véraison* (white bars), mid maturation (grey bars) and full maturation (dark grey bars). Data are means ± SE (n=4). Different letters within the same year and phenological stage, indicate significant differences between SWE and CTRL, assessed by one-way ANOVA (Fisher's LSD test, $P \leq 0.05$).

Quercetin derivatives were significantly more abundant in 2017 (37.5 µmol g^{-1}DW) than in 2016 (33.7 µmol g^{-1}DW), while kaempferol derivatives were higher in 2016 (3.2 µmolg^{-1} DW), than in 2017 (2.2 µmol g^{-1}DW). Despite significant differences were not recorded at full *véraison* and mid maturation, *A. nodosum* treatments improved hydroxycinnamic acids accumulation in leaves (**Figure 6A**), in both years at full maturation. Quercetin derivatives (**Figure 6B**) were always higher in SWE than in CTRL, irrespective of the year and phenological stage. In particular, quercetin derivatives were +13% (2016) and +85% (2017) in SWE than in CTRL, at full maturation (**Figure 6B**). As in berry skins, kaempferol derivatives (**Figure 6C**), were not affected by *A. nodosum* in 2016, but significant differences occurred in 2017, with a relevant increase in SWE leaves at mid maturation (+90%) and at full maturation (+40%). Consequently, the total phenolic contents (**Figure 6D**) were higher in SWE than in CTRL at full maturation, both in 2016 (+17%) and in 2017 (+43%).

4. Discussion

Climate change is speeding up the expected frequency and severity of drought periods often associated to heat-waves. The risks deriving from climate change include the impact on primary physiological processes, the anticipation of phenological stages and the uncoupling of technological and phenolic maturity in red grape cultivars, thus inducing a depletion in grape composition at full maturation (Jones and Webb, 2010; Palliotti et al., 2014). In this context, the use of biostimulants, such as seaweed extracts, might represent a sustainable tool to enhance plant physiological responses under severe stress conditions to achieve an optimal ripening (Khan et al., 2009; Craigie, 2011). The positive activity of biostimulants on plant physiology, shown by an increasing number of studies (Khan et al., 2009; Fan et al., 2011; Sharma et al., 2014), is elicited by the presence of “bioactive” compounds, such as small peptides, organic constituents, phenolics. However, the mode of action of bio-stimulatory molecules contained in seaweed extracts remains still largely unknown. To our knowledge, this is the first report analyzing *A. nodosum* effects on eco-physiology and partitioning of secondary metabolites in *V. vinifera*.

4.1. A. nodosum Extract Improved Leaf Gas Exchanges and Protected Photosystem II from Photo-inhibition during Microclimate Limiting Conditions

Stem water potential is commonly used as a main indicator of water stress in grapevine (Lovisolo et al., 2010), ranging from -1.0 to -1.2 MPa and from -1.2 to -1.5 MPa under moderate and severe water stress, respectively (Acevedo-Opazo et al., 2010). However, several studies have clearly shown that in field-grown grapevines g_s is more sensitive to severe water stress, than stem water potential (Dry and Loveys, 1999; Medrano et al., 2002; Flexas and Medrano, 2002; Cifre et al., 2005). In particular, mid-morning g_s is commonly accepted as an integrative parameter reflecting the intensity of water stress in grapevine (Chaves et al., 2010; Tombesi et al., 2015). In our study, we did not record significant differences in Ψ_w between SWE and CTRL vines, in both growing seasons (**Table 2**). By contrast g_s fell in CTRL vines below 50 mmol H_2O m^{-2} s^{-1}, that is considered the threshold indicating the occurrence of severe water stress in grapevine (Medrano et al., 2002; Cifre et al., 2005). *A. nodosum* treatments had a significant effect in reducing stomatal closure and increasing photosynthesis, even under severe water stress (mid maturation 2017, **Table 2**). Diffusional and non-diffusional factors interact to limit down-regulation of the photosynthetic systems (Lawlor and Cornic, 2002; Centritto et al., 2003) to avoid irreversible damage during periods characterized by intense temperature and high radiation load (Palliotti et al., 2009). In 2016 at full maturation and in 2017 at mid maturation, despite lower values of P_n and g_s in CTRL than SWE, untreated plants were able to maintain F_v/F_m values similar to treated vines and close to the optimum (~0.80) (**Table 2**). Hence, at these stages, diffusional limitations were likely the only limitation in photosynthesis (Lawlor and Cornic, 2002; Centritto et al., 2003) in untreated vines. On the contrary, in 2017 at full maturation, CTRL vines showed significantly lower F_v/F_m values than SWE. Therefore, at this phenological stage, initial signs of metabolic limitations to photosynthesis were observed.

The positive effects on plant physiological functions induced by the *A. nodosum* treatments have been previously reported also in *Citrus* spp. (Little and Spann, 2010; Spann and Little, 2011), and other species (Xu and Leskovar, 2015; Elansary et al., 2016). On the basis of the chemical characterization (**Table 1**), we hypothesize that the enhancement of physiological performances promoted by *A. nodosum* treatments may be related to the presence of amino acids and phenolics, which probably confers high DPPH Radical-Scavenging Capacity to the extract. In particular, proline is involved in drought tolerance, offering protection against extreme osmotic stress (Ashraf and Foolad, 2007; Hayat et al., 2012; Sharma et al., 2014), acting both as cytoplasmic osmolyte and as ROS-scavenging compound (Matysik et al., 2002; Cuin and Shabala, 2007).

4.2. A. nodosum Extract Delayed Grape Ripening and Elevated the Balance of Berry Traits

In grapevine, temperature-driven effects on anthocyanin and sugar accumulations have been widely investigated (Bergqvist et al., 2001, Spayd et al., 2002; Mori et al., 2005; Yamane et al. 2006; Tarara et al. 2008; Sadras and Petrie, 2011), because of the pivotal importance of phenolic content for wine quality, especially in regions suitable for premium grape production (e.g. the Chianti Classico production area). However, over the last few decades, grape composition has been altered by global warming, sometimes resulting in poorly balanced wines characterized by an increment of berry total soluble solids uncoupled with anthocyanin accumulation (Jones et al., 2005; De Orduña, 2010; Sadras and Moran, 2012).

This was also observed in our study in 2017, when the 1°C higher temperature respect to 2016, drove the acceleration of sugar accumulation, particularly in CTRL berries (**Table 3**). This effect was less evident in SWE vines, in which we observed a significant lower total soluble solids accompanied with higher total anthocyanin content (**Figure 3**), compared to CTRL plants. This is partially in accordance with previous reports (Sabir et al., 2014; Frioni et al., 2018) and may supports the hypothesis that *A. nodosum* treatments could be involved in delaying ripening, leading to both lower sugar and higher anthocyanin contents in treated berries. However, these positive effects on berry quality were not paralleled by a concomitant increment in yield, probably because *A. nodosum* extract was sprayed in medium-late phenological stages, on berries/clusters fully developed in size (Sabir et al., 2014; Frioni et al., 2018). On the contrary, as previously reported in table grape, early biostimulant applications during berry cell division and enlargement could even induce an improvement in vine productivity and berry weight (Norrie et al., 2002; Norrie and Keathley, 2006; Khan et al., 2012).

4.3. A. nodosum Extract Affected the Content and Partitioning of Anthocyanins in Berry Skin

The total amount of anthocyanins and the relative abundance of single anthocyanins are under genetic control (Ortega-Regules et al., 2006). However, the interaction between genotype and environment plays an important role in determining the berry qualitative traits such as the accumulation of distinctive anthocyanins of a given variety (Tarara et al., 2008). This is particularly evident in some grape cultivars, such as Sangiovese, which shows a broad phenotypic plasticity in response to environmental variables (Dal Santo et al., 2018).

In our study, a large seasonal variation of anthocyanin content in berry skins was observed in two consecutive years. The higher amount of total anthocyanin in 2017 compared to 2016 (**Supplementary Table 2**) may have been driven by higher global radiation and temperatures combined with lower precipitations (Castellarin et al., 2007a; Braidot et al., 2008; Koyama et al., 2012; Romboli et al., 2017) and/or be consistent with a passive accumulation due to water loss during berry dehydration (Moreno et al., 2008). The harsh climatic conditions of 2017 may have also caused a higher ratio of methoxylated to non-methoxylated anthocyanins in berry skins, than in 2016. Furthermore, low water availability, high solar radiations and temperatures promote the conversion of hydroxylated anthocyanins (cyanidin and delphinidin) into their methoxylated derivatives (peonidin, petunidin and malvidin) (Castellarin and Di Gaspero, 2007; Zarrouk et al., 2016). Methoxylation represents a metabolic process that increases anthocyanin stability since the methoxylated derivatives are less sensitive to enzymatic and non-enzymatic oxidation under stressful conditions (Jackman and Smith, 1996; He et al., 2010). A compelling evidence that *A. nodosum* mitigated stressful conditions, particularly in 2017, is the sharp decrease in the ratio of methoxylated to non-methoxylated anthocyanins in SWE than in CTRL (**Figure 4**). In particular, in CTRL berry skins we observed a major flux of the anthocyanin pathway towards more stable malvidin-based derivatives (Azuma et al., 2012), partly compensating the reduction in anthocyanin biosynthesis when P_n resulted depressed by stomatal closure (Zarrouk et al., 2016). By contrast, SWE plants showed higher P_n and accumulated preferentially cyanidin-based anthocyanins in berry skins. We suggest that *A. nodosum* might promote the activity of flavonoid 3′-hydroxylases (F3′H) enzyme, catalyzing the biosynthesis of cyanidin-based anthocyanins, instead of activating the delphinidin parallel branch. This could be due to a direct effect of *A. nodosum* treatments on the activation of specific gene expression (Jayaraman et al., 2011; Santaniello et al., 2017) or to an indirect effect on the improvement of plant physiological responses following environmental constraints. We cannot exclude that this finding may be also related to the treatments protocol, since the first *A. nodosum* application was carried out one week after full *véraison*, when cyanidin and peonidin are the prevalent anthocyanins synthesized in grape berry skins (Downey et al., 2006).

4.4. A. nodosum Extract Promoted the Biosynthesis and Accumulation of Phenolic Compounds in Leaf and Berry Skin

There is substantial literature on the importance of seaweed extracts in triggering the biosynthesis of phenolic compounds in many species, leading to high production of these secondary metabolites with multiple functions (Lakhdar et al., 2010; Kumari et al., 2011; Fan et al., 2011; Krajnc et al., 2012; Lola-Luz et al., 2013, 2014). In grapevine, hydroxycinnamic acids and flavonols constitute two major groups of UV-absorbing phenolic compounds (Kolb et al., 2003; Doshi et al., 2006) and are involved in the stabilization of the flavilium ion of anthocyanins in red wines (Boulton, 2001). In addition, similarly to anthocyanins, their accumulation in berry skins and leaves is strongly affected by environmental conditions of the vineyard (Azuma et al., 2012). In our experiment, non-anthocyanin phenolic compounds in berry skins and leaves were more abundant in 2017 than in 2016 (**Figure 5** and **Figure 6**), probably because of the most stressful conditions registered in 2017 (Castellarin et al., 2007a) and of the dehydration effect mentioned in the previous paragraph.

The phenolic contents in berry skins and leaves were also affected by treatments with *A. nodosum* extract. In fact, at full maturation, SWE berry skins and leaves showed higher contents of hydroxycinnamic acids (**Figures 5A** and **6A**) and quercetin derivatives (**Figures 5B** and **6B**), compared to CTRL, whereas fewer variations were found in kaempferol derivatives (**Figures 5C** and **6C**). The higher content of antioxidant flavonoids, in particular of di-hydroxy B-ring-substituted flavonoids (i.e. quercetin derivatives), may have provided photo-protection to PSII and improved vines performances in SWE leaves, as demonstrated by higher F_v/F_m and P_n values, at full maturation in 2017 (**Table 2**). Moreover, in the berry, the biosynthesis of flavonols is closely related to that of anthocyanins, depending on the same enzymes for both classes of flavonoids (Mattivi et al., 2006): F3′H and F3′5′H (flavonoid 3′-5’-hydroxylases), are involved in the biosynthetic pathway of cyanidin- and delphinidin-based anthocyanins, respectively, as well as of quercetin and myricetin derivatives (Jeong et al., 2006). In our berry samples, quercetin derivatives (**Figure 5B**) were the major flavonols, while traces of myricetin derivatives were only detected in 2017. These results may again suggest that *A. nodosum* treatments is selectively involved in the activation of F3′H enzyme, rather than F3′5′H, thus favoring the accumulation of di-hydroxy B-ring-substituted flavonoids rather that their tri-hydroxy counterparts.

5. Conclusions

Our data indicate that *A. nodosum* treatments during the last stages of berry development may enhance the performances of *V. vinifera* by improving eco-physiological parameters and affecting secondary metabolism, resulting in improved grape quality.

Applications of *A. nodosum* extract had significant effects on phenylpropanoid biosynthesis, both in berry skins and in leaves, and ameliorated leaf gas exchanges, maximum photosystem II efficiency and grape maturity balance. Furthermore, the biochemical analyses revealed that the *A. nodosum* extract likely acted selectively in the phenylpropanoid pathway, influencing anthocyanin partitioning and lowering the biosynthesis of methoxylated compounds. Overall, these results support previous findings on the beneficial effects of *A. nodosum* treatments on plant acclimation to stressful environmental conditions. Furthermore, for the first time in our knowledge, our study shows the potential effect of seaweed extracts in promoting higher tolerance to stress in vines. In order to understand the mechanisms of action of seaweed extracts, it is crucial to focus future research on the identification of seaweed chemical compounds with potential effect on treated plants phenylpropanoid pathway.

Author contributions

The work was conceived and planned by GBM, LS, and CB. LS and EC performed treatments with the *A. nodosum* extract, gas exchange measurements and collected leaf and berry samples. Data processing and analysis were carried out by LS and EC. LS and CB performed the HPLC analysis of phenylpropanoids. The *A. nodosum* extract was chemically characterized by AN. LS, CB and AN drafted the initial manuscript, which was critically revised by all authors.

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Supplementary Table 1

Three-way ANOVA ($P \leq 0.05$) for eco-physiology, chemical features of the berry and productivity parameters. Values are the mean of each parameter, considering treatment (Treat.), phenological stage (Ph. stage) and year as factors, and factors interactions. In the last 6 rows is indicated the significance (Sign.). Other abbreviations: full *véraison* (Full *vérais.*), mid maturation (Mid mat.), full maturation (Full mat.), net assimilation rate (P_n), stomatal conductance (g_s), maximum quantum yield of PSII (F_v/F_m), mid-day stem water potential (Ψ_m), total sugars (Tot. sugars), titratable acidity (TA). For cluster weight and yield/vine parameters, only year and treatment were considered as factors.

Parameter	P_n	g_s	F_v/F_m	Ψ_m	**Total sugars**	**pH**	**TA**	**Berry weight**	**Cluster weight**	**Yield/vine**
Unit	μmolm^{-2}s^{-1}	mmolm^{-2}s^{-1}		MPa	°Brix		gL^{-1}	g	g	Kg
Year										
2016	10.2	228	0.78	-1.16	22.7	3.17	5.7	2.4	362.3	2.7
2017	11.5	144	0.75	-1.10	21.2	2.94	10.4	1.0	151.5	1.0
Ph. stage										
Full *vérais*.	13.0	184	0.77	-1.07	19.5	2.94	10.1	1.5	-	-
Mid mat.	9.9	213	0.77	-1.22	21.9	3.07	7.7	1.9	-	-
Full mat.	9.2	191	0.75	-1.14	24.0	3.16	6.1	1.9	-	-
Treat.										
CTRL	11.2	226	0.78	-1.17	22.3	3.07	7.7	1.7	248.1	1.8
SWE	11.4	181	0.76	-1.11	21.8	3.06	7.9	1.9	265.7	1.9
Sign.										
Year	0.011	0.000	0.001	0.007	0.445	0.330	0.092	0.029	0.000	0.000
Ph. stage	0.028	0.002	0.259	0.000	0.001	0.517	0.185	0.500	-	-
Treat.	0.050	0.002	0.040	0.113	0.208	0.572	0.585	0.492	0.678	0.547
Treat. x Year	0.944	0.071	0.058	0.557	0.499	0.735	0.970	0.920	0.633	0.639
Year x Ph. stage	0.161	0.400	0.051	0.041	0.049	0.081	0.069	0.011	-	-
Treat. x Ph. stage	0.000	0.000	0.028	0.082	0.464	0.315	0.806	0.000	-	-

Supplementary Table 2

Three-way ANOVA ($P<0.05$) for biochemical parameters in berry and leaf. Values are the mean of each parameter (Param.), considering treatment (Treat.), phenological stage (Ph. stage) and year as factors. In the last 3 rows is indicated the significance (Sign.). Other abbreviation: Pre-véraison (Pre-*vér*.), full maturation (Full. mat), delphinidin-3-*O*-glucoside (Delph.), petunidin-3-*O*-glucoside (Petun.), malvidin-3-*O*-glucoside (Malv.), cyanidin-3-*O*-glucoside (Cyan.), peonidin-3-*O*-glucoside (Peon.), total anthocyanin content (Tot. Anth.), methoxylated to non-methoxylated ratio anthocyanins (Methox./non-methox.), percentage of methoxylated anthocyanins among total content (%Methox anth/tot.), hydroxycinnamic acids in berry (Hydr. Acid. Skin), kaempferol derivatives in berry (K. Der. Skin), quercetin derivatives in berry (Q. Der. Skin), Myricetin derivatives in berry (Myr. Der. Skin), total phenolic content in berry (Tot. Phen. Skin), hydroxycinnamic acids in leaf (Hydr. Acid. Leaf), Kaempferol derivatives in leaf (K. Der. Leaf), quercetin derivatives in leaf (Q. Der. Leaf), and total phenolic content in leaf (Tot. Phen. Leaf).

Param.	**Delph.**	**Petun.**	**Malv.**	**Cyan.**	**Peon.**	**Tot. Anth.**	**Methox/ non-methox**	**% Methox anth/tot**	**Hydr. Acid Skin**	**K. Deriv. Skin**	**Q. Deriv. Skin**	**Myr. Deriv. Skin**	**Tot. Phen. Skin**	**Hydr. Acid Leaf**	**K. Deriv. Leaf**	**Q. Deriv. Leaf**	**Tot. Phen. Leaf**
Unit	μmol g^{-1}DW	μmol g^{-1}DW	μmol g^{-1}DW	μmol g^{-1}DW	μmol g^{-1}DW	μmol g^{-1}DW		μmol g^{-1}DW	μmol g^{-1}DW	μmol g^{-1}DW	μmol g^{-1}DW	nmol g^{-1}DW	μmol g^{-1}DW	μmol g^{-1}DW	μmol g^{-1}DW	μmol g^{-1}DW	μmol g^{-1}DW
Year																	
2016	1.0	1.4	2.0	1.8	1.0	7.5	1.6	60.9	1.6	0.6	2.7	0.5	4.7	5.3	3.2	33.7	41.7
2017	1.1	0.3	8.2	2.4	0.6	12.2	3.5	75.3	1.7	0.2	2.7	1.7	4.8	3.4	0.2	2.2	44.1
Ph. stage																	
Pre-vér	1.3	0.9	5.0	2.2	0.8	10.8	3.1	70.0	1.7	0.7	2.2	1.0	4.0	4.7	2.1	18.1	43.4
Vérais.	0.9	0.8	4.9	2.1	0.8	9.9	2.1	66.7	1.6	0.2	2.6	0.6	4.9	4.6	2.0	19.2	41.0
Full mat.	1.0	0.8	4.5	2.0	0.9	9.6	2.5	68.2	1.7	0.1	3.3	1.5	5.4	3.9	1.7	15.6	43.9
Treat.																	
SWE	1.2	1.0	5.2	2.5	1.0	10.7	2.6	67.4	1.7	0.6	3.1	1.2	5.0	4.8	1.9	19.0	46.1
CTRL	0.9	0.7	4.4	1.7	0.7	9.3	2.5	69.1	1.6	0.2	2.3	0.8	4.5	4.0	2.0	16.1	39.2
Sign.																	
Year	0.012	0.000	0.000	0.003	0.021	0.000	0.001	0.000	0.529	0.165	0.718	0.012	0.106	0.125	0.000	0.000	0.547
Ph. stage	0.775	0.571	0.189	0.989	0.686	0.649	0.113	0.505	0.718	0.019	0.712	0.399	0.66	0.688	0.299	0.012	0.531
Treat.	0.031	0.006	0.021	0.007	0.050	0.005	0.281	0.643	0.728	0.091	0.084	0.713	0.571	0.616	0.457	0.100	0.055

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Chapter 4

Eco-physiological traits and phenylpropanoid profiling on potted *Vitis vinifera* L. cv Pinot noir subjected to *Ascophyllum nodosum* treatments under post-*véraison* low water availability

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Eco-physiological traits and phenylpropanoid profiling on potted *Vitis vinifera* L. cv Pinot noir subjected to *Ascophyllum nodosum* treatments under post-*véraison* low water availability

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Keywords: Biostimulants, abiotic stress, water potential, gas exchanges, berry skin metabolites, enzymatic activity, phenylpropanoid pathway

ABSTRACT

In Mediterranean regions, extreme weather conditions characterized by prolonged dry periods and high temperatures during the growing season may alter grapevine physiology and metabolism, thus modifying the quality of wines.

The objective of this study was to investigate the effects of A*scophyllum nodosum* treatments on plant physiology and berry metabolism in *Vitis vinifera* exposed to water stress. The experiment was performed on potted vines (Pinot noir cv.) subjected to two irrigation regimes (well watered, WW, and water stressed, WS) associated with *A. nodosum* extract treatments (SWE and CTRL).

Gas exchanges, chlorophyll fluorescence, relative water content (RWC %), leaf pre-dawn and stem midday water potentials were monitored on SWE and CTRL leaves, both in WW and WS vines at three sampling times. In addition, anthocyanins, flavonols and hydroxycinnamic acids were quantified in berry skins.

The foliar treatments with *A. nodosum* increased photosynthesis and stomatal conductance in SWE compared to CTRL plants, in both irrigation regimes. *A. nodosum* treatments decreased leaf pre-dawn and stem midday water potentials in SWE in comparison with CTRL vines, in water-stressed vines. By contrast, stem water potential did not increase when *A. nodosum* treatments were applied in well-watered vines. No effect of *A. nodosum* extract treatments was observed on the maximum efficiency of photosystem II.

A. nodosum applications did not significantly affect total soluble solids, titratable acidity and productivity parameters. However, treatments raised the content of anthocyanins, flavonols and hydroxycinnamic acids in berries, also affecting the partitioning of these secondary metabolites in berry skins, irrespective on the irrigation regime. The ratio of methoxylated to non-methoxylated anthocyanins and the activity of enzymes involved in the phenylpropanoid pathway were lower in SWE than in CTRL in both WW and WS vines. Consequently, a reduction in the biosynthesis of methoxylated anthocyanins, usually accumulated in grapes under abiotic stresses, was observed.

Our results suggest that foliar application of *A. nodosum* extract may help the acclimation of grapevines to post-*véraison* drought, likely improving plant performances under environmental constrains

1. Introduction

Soil water deficit is the main environmental constraint for viticulture in the Mediterranean basin (Cifre et al., 2005, Chaves et al., 2007). Climate projections for this area predict changes in rainfall and seasonal temperatures patterns in the next decades, with higher frequency of extreme climate events (Schultz and Jones, 2010; Spinoni et al., 2015). This future trends due to global climate change will negatively affect grapevine physiology and grape composition.

Stomatal closure is among the first processes occurring in leaves in response to drought. It is widely reported that gas exchange control by the stomata is the crucial issue for grapevine drought response and water use efficiency (i.e. the ratio of photosynthesis to transpiration; WUE) (Schultz, 2003; Rogiers et al., 2009; Costa et al., 2012; Tomás et al., 2014; Medrano et al., 2015; Bota et al., 2016). Under mild water stress, it is likely that grapevine photosynthesis is depressed almost exclusively by stomatal closure, as indicated by increased WUE. For more prolonged and/or more pronounced water deficits leading to decrease of g_s below 50 mmol H_2O m^{-2} s^{-1}, photosynthetic limitations becomes more dependent on non-stomatal processes, especially decreased mesophyll diffusion conductance to CO_2 and impaired photochemistry (Flexas et al., 2004; Flexas and Medrano, 2002).

Moreover, abiotic stresses such as drought and high temperatures accelerate phenological stages (i.e. budburst, flowering and *véraison*) and berry ripening (Sadras and Petrie, 2011), influencing the primary and secondary metabolism: berry total soluble solids tend to increase (Petrie and Sadras, 2008), leading to high wine alcohol and lower acidity levels; the synthesis of phenolic compounds in berry skin and aromatic ripeness tend to delay (Teixeira et al., 2013), which results in an imbalance between berry sugar accumulation and phenolic ripening (Sadras and Morán, 2012). Anthocyanins and flavonols, the most abundant polyphenol subclasses detected in grape berries (Adams, 2006), are responsible of important properties of berries and wines, such as flavor, color and stability against oxidation processes (Waterhouse, 2002; Mattivi et al., 2006; Silva and Queiroz, 2016). The total amount of anthocyanins and the relative abundance of single anthocyanins are under genetic control (Ortega-Regules et al., 2006). This means that all cultivars express functional genes coding for enzymes that lead to the biosynthesis of di-hydroxylated (peonidin and cyanidin) or tri-hydroxylated (delphinidin, petunidin and malvidin) anthocyanins as well as to the methylation of primary anthocyanins. The interaction between genotype and environmental conditions, such as low water availability, high solar radiations and high temperatures promote the conversion of hydroxylated anthocyanins (cyanidin and delphinidin) into their methoxylated derivatives (peonidin, petunidin and malvidin) (Castellarin and Di Gaspero, 2007; Zarrouk et al., 2016).

Hence, the cultivation of grapevines in a climate change scenario might require a selection of new management practices to maintain grape quality under the future environmental constraints, like the foliar application of biostimulants. The ability of some biostimulant products such as *Ascophyllum nodosum* extracts to enhance the tolerance of crops to drought stress have been reviewed (Craigie, 2011; Sangha et al., 2014). Many studies have reported a broad effectiveness of these kind of extracts in increasing drought stress tolerance of grasses and crops (Richardson et al., 2004; Spann and Little, 2011; Elansary et al., 2016, 2017; Martynenko et al., 2016; Santaniello et al., 2017). Additionally, their application on grapevine resulted in positive effects on yield, berry composition, skin phenolic compounds and plant responses to environmental changes (Norrie et al., 2002; Colapietra and Alexander, 2006; Mancuso et al., 2006; Norrie and Keathley, 2006; Kok et al., 2010; Cai et al., 2012; Sabir et al., 2014; Torres et al., 2016).

Ascophyllum-based algal derivatives may facilitate stress mitigation because of their high concentration of amino acids, that may serve as compatible solutes under osmotic stress (Khan et al., 2009; Di Stasio et al., 2018), as well as bioactive secondary metabolites such as vitamins and their precursors (Berlyn and Russo, 1990; Blunden et al., 1985). Moreover, stress tolerance may be enhanced through high concentrations of phenolic compounds with antioxidant properties that may protect plant tissues against stress-induced reactive oxygen species (ROS) (Laetitia et al., 2010). These peculiar qualities highlight the importance of increasing the knowledge regarding seaweed extracts physiological functions, which are currently unclear.

The aim of this study was to investigate if *A. nodosum* extract treatments were able to positively affect grapevine physiology and berry phenylpropanoid metabolism, mitigating the effects of a post-*véraison* drought in Pinot noir cv. (*Vitis vinifera* L.).

2. Material and methods

2.1. Experimental site and plot layout

Forty 11-year-old vines, *Vitis vinifera* L. cv. Pinot noir clone Entav 115, grafted on 1103 Paulsen rootstock, were grown outdoors at CREA-VIC, Arezzo, Italy (Lat. 43.476°N, Long. 11.824°E; 260 m a.s.l.) in 70 L pots filled by a clay-loam soil. The soil was previously collected from a premium vineyard of the Chianti Classico area (Tuscany), with the following average characteristics: clay 40%; silt 35%; sand 25%, with a volumetric soil water content of ∼34% at field capacity. The vines were trained on vertical shoot positioned trellis, with spur cordon pruning and an average of 10 buds per vine. Every year, at the end of February, each container was fertilized with 40 g of controlled-release fertilizer (Nitrophoska, 12N–12P–17K, Eurochem Agro). To avoid excessive soil over warming, which can negatively affect roots, and maintain a stable temperature, pots were protected by wrapping their lateral surface with a shade net.

At the beginning of the experiment (*véraison*, 13 July 2017; modified Eichorn and Lorenz (E-L) 35 stage), 20 plants were maintained at 90% of maximum water availability as controls (WW, well-watered vines) until harvest (E-L 38 stage), while the other 20 plants were subjected to a water deficit at 40% of maximum water availability (WS, water-stressed) (Palliotti et al., 2014). Until the beginning of treatment, all plants were maintained at field capacity. During water limitation, the surface of the plant containers was covered with aluminum foils to avoid rainfall interference and to minimize soil water evaporation. The water supply per pot was determined monitoring the soil moisture, as volumetric content, by time domain reflectrometry using Trase System 1 (Soil Moisture Equipment Corporation, USA). Readings were taken every day in the early morning with 30 cm long electrodes located in the pots. In each pot, water was supplied at 2 day intervals with drip irrigation emitters.

During the differential irrigation period, ten of the WW vines and ten of the WS ones were treated twice with 3g of a non-commercial *A. nodosum* extract (SWE), diluted in 1 L of water (Salvi et al., 2016; Santaniello et al., 2017; Frioni et al., 2018). The first application was performed approximately 20 days before the expected harvest (27 July 2017; E-L stage 36) and the second one was repeated, on the same vines, after two weeks (10 August 2017; E-L stage 37). On the same days, the other ten vines of WW and WS were sprayed with the same amount of water (CTRL).

Eco-physiological measurements and biochemical samplings, were performed on 10 homogeneous vines (replicates)/treatment at three stages: t_0 (before *A. nodosum* treatments and one week after irrigation regimes differentiation; 20 July 2017; E-L stage 36), t_1 (one week after the first *A. nodosum* treatment; 3 August 2017; E-L stage 37) and t_2 (one week after the second *A. nodosum* treatment; 17 August 2017; E-L stage 38).

2.2. Climate parameters

Daily values of mean, maximum and minimum air temperatures (°C) and global radiation (Wm^{-2}) were collected by an automatic meteorological station (Ecotech, Germany) located close to the experimental site.

2.3. Leaf gas exchange, chlorophyll fluorescence, leaf water potential and content

Leaf gas exchange, net photosynthesis (P_n), stomatal conductance (g_s), transpiration rate (E), were measured on ten fully expanded leaves (one each vines, 10 replicates) per treatment on fully developed and intact leaves using a portable infrared gas analyzer (model Ciras 3, PP Systems, USA). Water use efficiency (WUE) was calculated as the ratio of photosynthesis to transpiration.

Measurements were performed between 10 and 12 a.m., setting the leaf chamber flow at ambient temperature, at ambient CO_2 concentration (400 ppm) and at saturating photosynthetic photon flux of 1300 μmol $m^{-2}s^{-1}$ at t_0, t_1 and t_2. Chlorophyll *a* fluorescence transients of dark-adapted leaves were recorded using a saturating flash of actinic light at 3000 $μmolm^{-2}s^{-1}$ for 1s (Handy-PEA®, Hansatech Instruments, UK). Briefly, the maximum quantum yield of photosystem II (PSII) was calculated as $F_v/F_m = (F_m-F_0)/F_m$ where F_v is the variable fluorescence and F_m is the maximal fluorescence of dark-adapted (over a 30-min period) leaves (Maxwell and Johnson, 2000).

At the same stages, leaf pre-dawn (Ψ_{pd}, MPa) and stem midday (Ψ_m, MPa) water potentials were determined following Scholander et al. (1965) with a pressure chamber (model 600, PMS Instrument Co., USA). Ψ_{pd} was measured between 04.00 and 05.00 hours on ten fully expanded leaves per block and treatment, whereas Ψ_m was measured on ten dark-adapted leaves (over a 60-min period). Other 10 leaves/treatment were taken to calculate Relative Water Content (RWC%, as RWC= ((FM-DM)/(TM-DM))*100 where FM, TM and DM denote fresh, turgid and dry masses respectively).Chlorophyll *a* fluorescence and midday stem water potential were taken on the same leaves used for leaf gas exchanges measurements at the same three phenological stages.

2.4. Berry composition and productivity parameters

A 50-berry sample was collected randomly from each of the 10 vines (10 berry samples per treatment), at t_0, t_1 and t_2, taking care to remove berries from all the positions within the cluster. The berry sample was weighed with a digital scale (PCE Italia s.r.l, Italy) and immediately juiced. Soluble solids concentration (°Brix) was measured using a refractometer (ATAGO, USA); must pH was measured using a portable pH meter (Hanna instrument, USA) and titratable acidity (gL^{-1} tartaric acid) was determined on a 10 mL sample by manual glass burette using 0.1 M NaOH to an endpoint of pH 7.0. At t_2, the vines were harvested and their production was weighed with a portable electronic scale (Bonso Advanced Technology Ltd., Hong Kong) to obtain yield per vine (kg) and cluster weight (g).

2.5. Phenylpropanoids in berry skin

Berry phenylpropanoids content was measured on 5 replicates, each constituted by a pool of 30 berries, collected from 2 of the 10 vines of each treatment, at t_0, t_1 and t_2. Berry were immediately frozen in liquid nitrogen, then skins were removed and lyophilized (Lio-5P, Cik solution, Germany).

Lyophilized berry skins (0.7 g) were ground in a mortar under liquid nitrogen and the obtained powder was extracted with 75% of aqueous ethanol acidified to pH 2 by HCOOH (3×5 mL) and sonicated for 30 min. The supernatant was partitioned with 3×5 mL of *n*-hexane, the extracts were added together and reduced to dryness under vacuum, then, rinsed with MeOH/H_2O (50/50, pH 2). Aliquots of 5 µL were injected into a liquid chromatograph equipped with a quaternary 200Q/410 pump and an LC 200 diode array detector (DAD) (all from Perkin Elmer, USA). Anthocyanins, flavonols and hydroxycinnamic acids were separated in a 250x4.6 mm Agilent Zorbax SB-C18 (5µm) column operating at 30°C, at a flow rate of 0.6 ml min^{-1}, using a gradient solvent system consisting of H_2O (plus 5% HCOOH) (A), MeOH (plus 5% HCOOH) (B), CH_3CN (plus 5% HCOOH (C), during a 25 min run: 0-2 min 90% A, 5% B, 5% C; 2–7 min to 80% A, 10% B, 10% C; 7–12 min to 70% A, 15% B, 15% C; 12–17 min to 60% A, 20% B, 20% C; 17–20 min to 56% A, 22% B, 22% C; 20–24 min to 10% A, 45% B, 45% C; 24–25 min to 90% A, 5% B, 5% C. Quantification of anthocyanins was performed at 530 nm using calibration curves of cyanidin 3-*O*-glucoside, delphinidin 3-*O*-glucoside, petunidin 3-*O*-glucoside, peonidin 3-*O*-glucoside and malvidin 3-*O*-glucoside (Extrasynthese, France). Quantification of flavonols and hydroxycinnamic acids were performed at 330 nm using the calibration curve of quercetin 3-*O*-glucoside, quercetin 3-*O*-galactoside, myricetin 3-*O*-glucoside, *trans*-caftaric acid, *trans*- and *cis*-coutaric acid and kaempferol 3-*O*-glucoside (Extrasynthese, France). The quantification of anthocyanins was used to calculate the proportion of methoxylated/non-methoxylated derivatives and the percentage of methoxylated anthocyanins among 3-glucoside anthocyanins. Moreover, three key enzymatic activities, flavonoid 3',5'-hydroxylase (F3'5'OH, that lead to the formation of delphinidin, petunidin and malvidin from cyanidin and peonidin), 3'-O-methyltransferase (3'-OMT, that convert cyanidin in peonidin), and 5'-O-methyltransferase (5'-OMT, that convert delphinidin in malvidin), linked to known structural genes involved in the synthesis of anthocyanins, were computed (Mattivi et al. 2006), in order to compare the global balance of biosynthesis generating anthocyanins in SWE and CTRL berries, in both irrigation regimes.

2.6. Statistical analysis

We established a two-factorial experiment where two irrigation regimes were combined with *A. nodosum* treatments. Irrigation regimes (WW and WS) and *A. nodosum* treatments (SWE and CTRL) were considered as fixed factors. A three-way ANOVA ($P \leq 0.05$) was used to compare *A. nodosum* treatments effects in different irrigation regimes and sampling times, and factors interactions.

Then, significant interactions among factors were investigated with one-way ANOVA ($P \leq 0.05$). Mean values were separated by Fisher's least significant difference (LSD) post-hoc test ($P \leq 0.05$). All statistical analyses were performed using SPSS Statistic 25 (IBM, USA).

3. Results

3.1. Climate parameters

The 2017 microclimate conditions of the experimental area are reported in **Figure 1**. Average mean, maximum and minimum air temperatures for the *season*, measured from bud break to leaf fall (April-October), were 19.5°, 27.5° and 11.4°C, respectively. The hottest and driest month of this season was August, and the warmest day was the 3rd of this month (t_1), when maximum and minimum air temperatures were, respectively, ~2° and ~6° higher than the monthly average high and low temperatures.

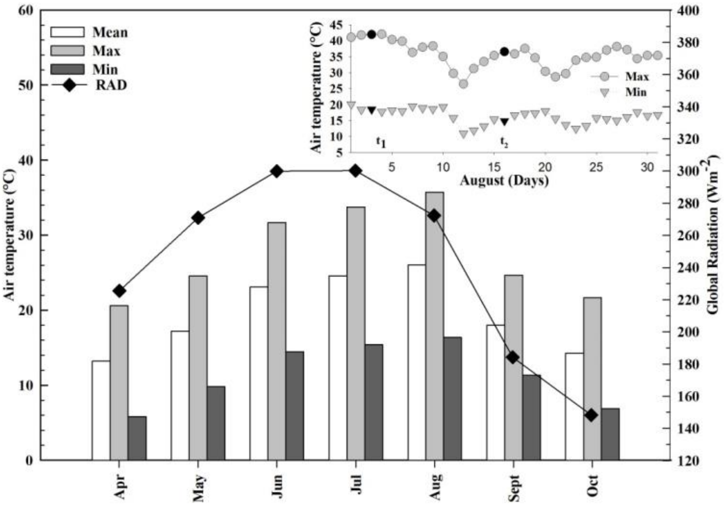

Fig. 1. Experimental site weather parameters. Monthly averages of mean, maximum and minimum air temperature (°C) and global radiation (W m^{-2}) measured from bud break to leaf fall (April-October) (A); in the insert the daily averages of maximum and minimum air temperature (°C) measured in August (B) are shown.

3.2. Leaf gas exchange, chlorophyll fluorescence, leaf water potential and content

To investigate the role of *A. nodosum* treatments on eco-physiological traits of vines, leaf gas exchanges (P_n, g_s, E and WUE), maximum efficiency of PSII photochemistry (F_v/F_m), leaf pre-dawn (Ψ_{pd}) and midday stem water potentials (Ψ_m), RWC% were measured (**Table 1** and **Supplementary Table 1**). The results of the three-way ANOVA show that, in general, gas exchanges, water potentials and RWC% were significantly affected by *A. nodosum* treatments and irrigation regime, whereas no statistical difference was detected in F_v/F_m (**Supplementary Table 1**). Lower values of P_n, g_s, WUE, Ψ_{pd} and Ψ_m were observed in WS than in WW vines. There were no significant differences in all physiological parameters observed at t_0 (pre-treatment stage) between SWE and CTRL plants in both irrigation regimes (**Table 1**). At t_1, similar values of P_n and g_s were observed in SWE than CTRL in both WW and WS vines. At the same stage, WUE was significantly higher in CTRL than in SWE both in WW (+36%) and in WS (+35%) plants. At t_2, in well watered vines, SWE plants displayed significantly higher P_n and g_s than CTRL plants, whereas, WUE was largely unaffected by *A. nodosum* treatments (**Table 1**). Moreover, at the same stage, SWE vines were able to maintain significantly higher P_n, g_s and WUE than CTRL in water stressed plants.

Table 1
Net photosynthesis (P_n), stomatal conductance (g_s), transpiration (*E*) and water use efficiency (*WUE*) in leaves of *V. vinifera* treated with *A. nodosum* extract (SWE) and untreated plants (CTRL), under two irrigation regimes (WW, well watered; WS, water stressed). Measurements were conducted at t_0, t_1 and t_2. Data (mean ± SE, n=10) were subjected to one-way ANOVA. Different letters within the same parameter and row indicate significant differences among treatments (LSD test, $P \leq 0.05$).

		P_n (µmol/m²s)		***gs* (mmol/m²s)**		**F_v/F_m**		***WUE* (mmol/m²s)**	
Irrig. Regime	**Samp. Time**	CTRL	SWE	CTRL	SWE	CTRL	SWE	CTRL	SWE
WW	t_0	13.9±0.7 a	15.0±1.5 a	162.2±19.6 a	146.1±9.3 a	0.77±0.01 a	0.77±0.01a	3.4±0.4 a	3.5±0.4 a
	t_1	6.6±0.6 a	8.6±0.5 a	94.4±3.4 a	92.8±5.4 a	0.76±0.02 a	0.78±0.01 a	4.9±0.2 a	3.7±0.2 b
	t_2	6.6±1.9 b	11.6±0.7 a	86.0±14.7 b	161.5±12.6 a	0.80±0.01 a	0.77±0.03 a	2.6±0.7 a	2.6±0.1 a
WS	t_0	4.8±1.1 a	5.3±2.3 a	92.4±14.8 a	82.2±12.4 a	0.77±0.01 a	0.77±0.01 a	1.8±0.5 a	1.0±0.5 a
	t_1	8.1±0.4 a	7.0±0.6 a	62.3±3.4 a	62.2±6.4 a	0.73±0.03 a	0.76±0.02 a	4.6±0.4 a	3.4±0.1 b
	t_2	1.0±0.2 b	4.3±1.1 a	18.2±5.0 b	49.5±11.4 a	0.80±0.01 a	0.80±0.01 a	1.1±0.4 b	2.1±0.3 a

In WW plants at t_1, Ψ_{pd} and Ψ_m were not significantly affected by *A. nodosum* treatments (**Figs. 2A** and **2B**). On the contrary, under water stress conditions, both at t_1 and t_2, SWE vines displayed higher Ψ_{pd} and Ψ_m values respect to CTRL. In addition, RWC% was higher in SWE than CTRL vines at t_1 only in WS vines (**Fig. 2C**). This was probably due to the highest temperature during the first week of August (**Fig. 1**). At t_2, RWC% values were higher in SWE than CTRL in both water regimes.. F_v/F_m was not significantly affected by *A. nodosum* treatments.

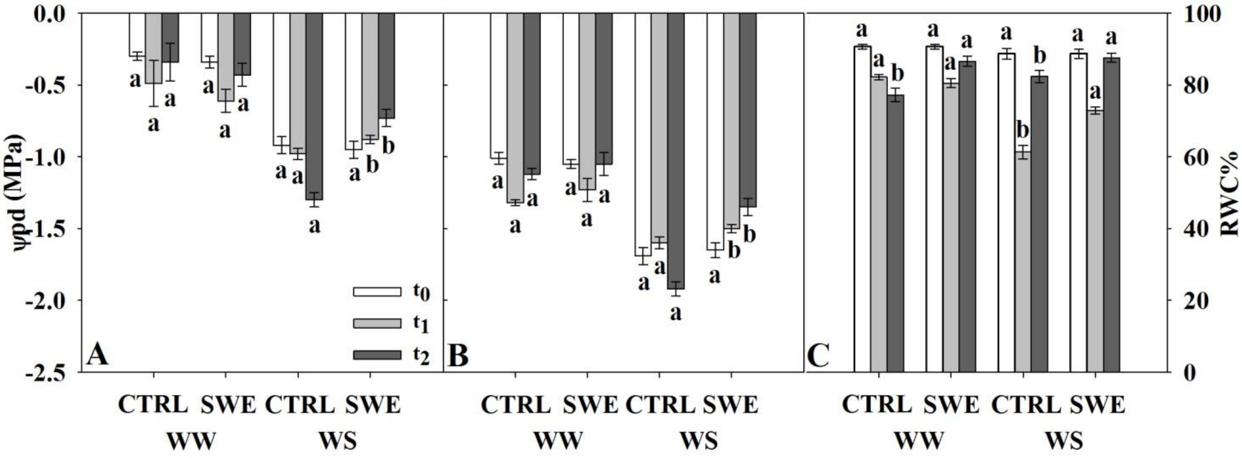

Fig. 2. Leaf pre-dawn (Ψ_{pd}, MPa) (A), midday stem (Ψ_m, MPa) (B) potentials and Relative Water Content (RWC%) (C) in leaves of *V. vinifera* treated with *A. nodosum* extract (SWE) and untreated plants (CTRL), under two irrigation regimes (WW, well watered; WS, water stressed). Measurements were conducted at t_0, t_1 and t_2. Data (mean ± SE, n=10) were subjected to one-way ANOVA. Different letters within the same parameter and row indicate significant differences among treatments (LSD test, $P \leq 0.05$).

3.3. Berry composition and productivity parameters

Irrigation regime significantly affected ($P \leq 0.05$) titratable acidity, pH and berry weight (**Supplementary Table 1**). Titratable acidity, pH and berry weight were ~33%, ~6% and ~29% higher in well watered than in water stressed vines, respectively. Consequently, significant differences were reported in cluster weight and yield/vine. The values of total sugar and titratable acidity contents, pH and berry weight are presented in **Table 2**.

As regards *A. nodosum* treatments effects, there were no significant differences in all parameters between treated and non-treated vines in both water regimes.

Table 2

Total sugars, titratable acidity, pH and berry weight in berry of *V. vinifera* treated with *A. nodosum* extract (SWE) and untreated plants (CTRL), under two irrigation regimes (WW, well watered; WS, water stressed). Measurements were conducted at t_0, t_1 and t_2. Data (mean ± SE, n=10) were subjected to one-way ANOVA. Different letters within the same parameter and row indicate significant differences among treatments (LSD test, $P \leq 0.05$).

		Total sugars (°Brix)		**TA (gL^{-1} tartaric acid)**		**pH**		**Berry weight (g)**	
Irrig. Regime	**Samp. Time**	CTRL	SWE	CTRL	SWE	CTRL	SWE	CTRL	SWE
WW	t_0	16.9±0.2 a	16.4±0.4 a	13.6±1.0 a	13.7±1.0 a	3.09±0.03 a	3.10±0.03 a	1.1±0.1 a	1.1±0.1 a
	t_1	17.8±0.1 a	17.9±0.2 a	10.4±0.6 a	12.4±0.5 a	3.27±0.04 a	3.22±0.07 a	1.1±0.1 a	0.9±0.1 a
	t_2	18.7±0.6 a	18.9±0.5 a	5.4±0.1 a	5.6±0.2 a	3.52±0.03 a	3.47±0.03 a	1.0±0.1 a	1.1±0.1 a
WS	t_0	16.0±0.5 a	16.2±0.5 a	13.1±0.9 a	13.0±0.9 a	3.10±0.04 a	3.09±0.04 a	0.9±0.1 a	0.9±0.1 a
	t_1	18.1±0.1 a	17.2±0.7 a	10.7±1.3 a	12.2±0.5 a	2.99±0.06 a	3.13±0.07 a	1.0±0.1 a	1.0±0.1 a
	t_2	19.1±0.7 a	19.3±0.2 a	5.6±0.3 a	5.9±0.1 a	3.50±0.05 a	3.48±0.01 a	0.9±0.1 a	1.0±0.1 a

3.4. *Phenylpropanoids in berry skin*

Five anthocyanins (i. e. delphinidin-3-*O*-glucoside, cyanidin-3-*O*-glucoside, petunidin-3-*O*-glucoside, peonidin-3-*O*-glucoside and malvidin-3-*O*-glucoside), and different derivatives of hydroxycinnamic acids (*trans*-caftaric and *trans*-/*cis*-coutaric acid, ferulic acid derivatives) and flavonols (glucosides, galactosides and glucuronides derivatives of quercetin and kaempferol) were identified in the chromatograms of the extracts from SWE and CTRL berry skins, recorded at 530 and 330 nm, respectively.

Statistical differences related to irrigation regime were recorded in malvidin, total anthocyanin content, methoxylated to non-methoxylated anthocyanin ratio, kaempferol derivatives and all enzymatic activities computed (**Supplementary Table 2**). Malvidin, total anthocyanin content and methoxylated to non-methoxylated anthocyanin ratio were 43%, 35% and 16% more abundant in WS than in WW. Among flavonols in berry skin, kaempferol derivatives were significantly higher (+93%) in WS compared to WW. F3’5’OH, 3’-OMT and 5’-OMT were similarly enhanced (+24%, +23% and +19%, respectively) by water stress regime (**Supplementary Table 2**).

Anthocyanins (**Fig. 3**) were not significantly affected by the *A. nodosum* extract treatments at t_0. Both in WW and WS vines, delphinidin (**Fig. 3A**), petunidin (**Fig. 3B**), cyanidin (**Fig. 3D**) and peonidin (**Fig. 3E**) contents were higher in SWE than CTRL berry skins at t_1 and t_2. At t_1 SWE berry skins showed also significantly higher malvidin and total anthocyanin contents than CTRL (**Fig. 3F**), irrespective of the irrigation regime; on the contrary, at t_2, malvidin and total anthocyanin contents remained almost unchanged between SWE and CTRL in WW vines and in WS ones.

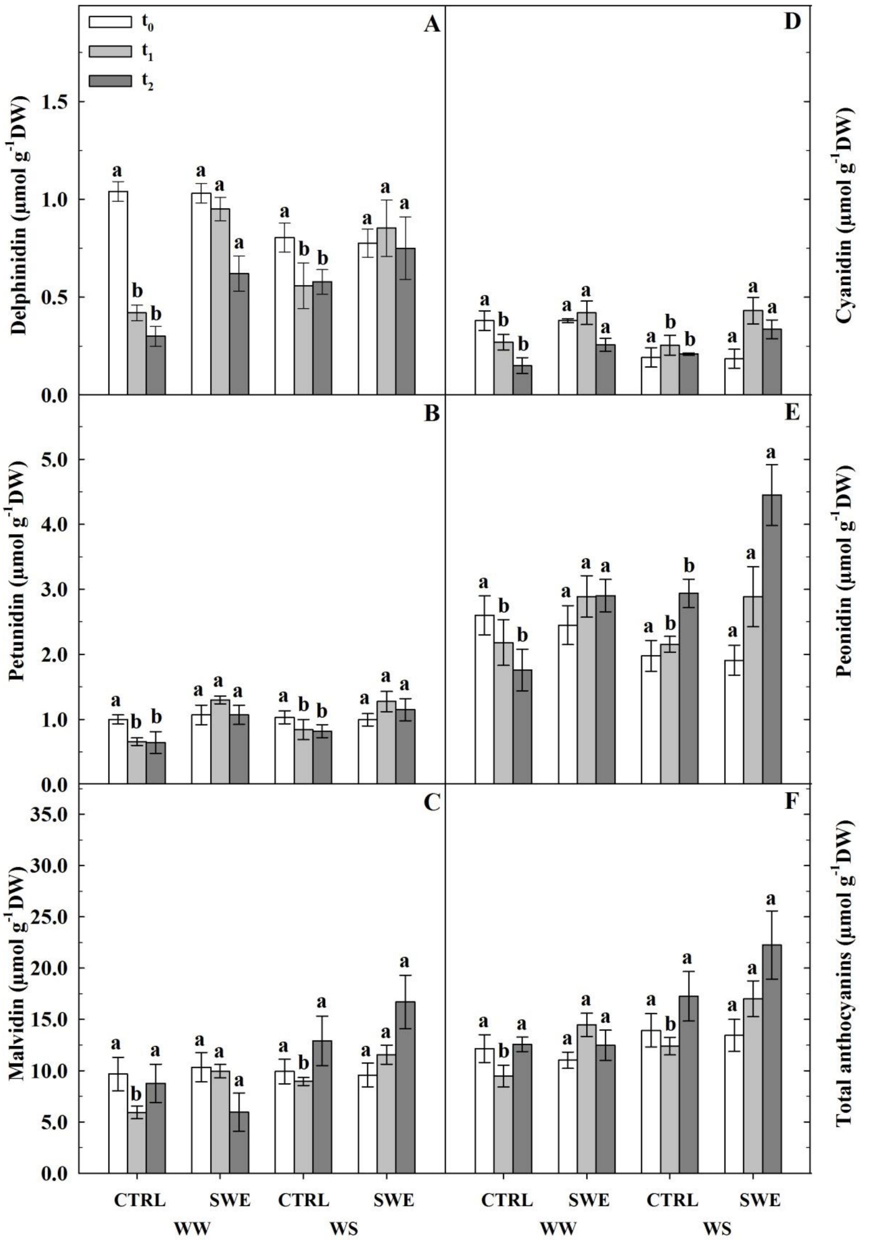

Fig. 3. Delphinidin-3-*O*-glucoside (A), Petunidin-3-*O*-glucoside (B), Malvidin-3-*O*-glucoside (C), Cyanidin-3-*O*-glucoside (D), Peonidin-3-*O*-glucoside (E), and Total anthocyanin content (F) in berry skins (µmol g^{-1}DW) of *V. vinifera* treated with *A. nodosum* extract (SWE) and untreated plants (CTRL) under two irrigation regimes (WW, well watered; WS, water stressed). Analyses were conducted at t$_0$ (white bars), t (grey bars) and t$_2$ (dark grey bars). Data are means ± SE (n=5). Different letters within the same water regime and sampling time indicate significant differences between SWE and CTRL, assessed by one-way ANOVA (Fisher's LSD test, $P \leq 0.05$).

The methoxylated (peonidin, petunidin and malvidin) to non-methoxylated (cyanidin and delphinidin) anthocyanins ratio (**Fig. 4A**), and the percentage of methoxylated anthocyanins on total anthocyanins (**Fig.4B**) were calculated in order to investigate how SWE treatments affected anthocyanin composition. In WW vines, both the methoxylated to non-methoxylated anthocyanins ratio and the percentage of methoxylated anthocyanins on total anthocyanins were higher in CTRL than in SWE at t_1 and t_2 (**Figs. 4A** and **4B**). In WS vines, the methoxylated to non-methoxylated anthocyanins ratio was higher in CTRL than in SWE at t_1 and t_2, but the percentage of methoxylated anthocyanins was largely unaffected by *A. nodosum* treatments (**Figs. 4A** and **4B**).

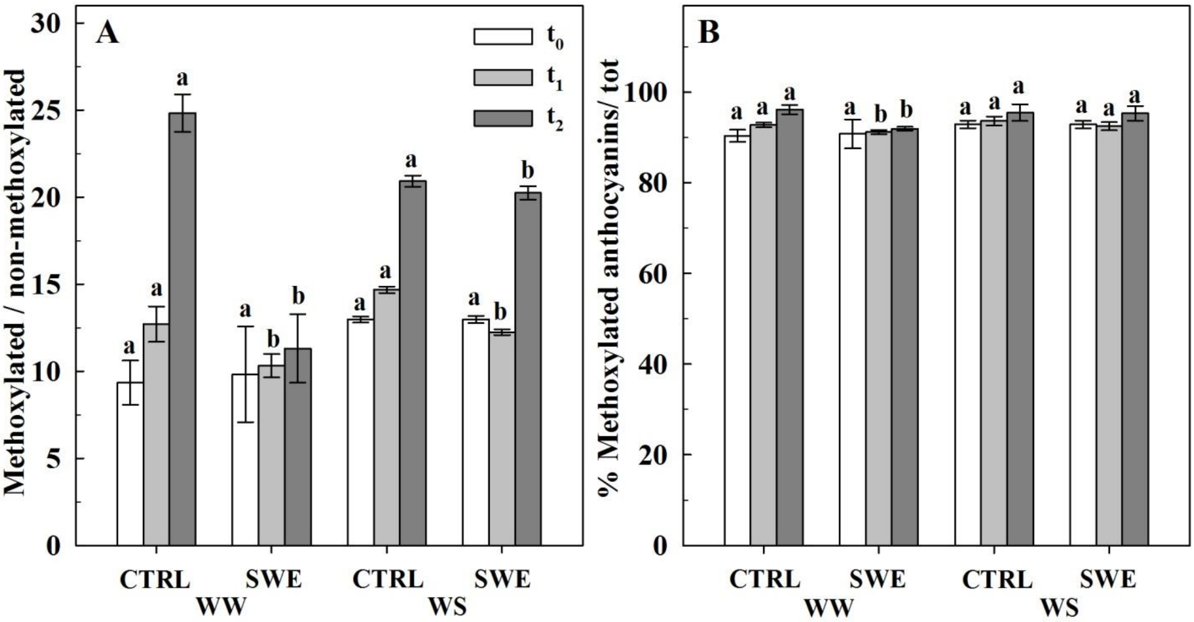

Fig. 4. Ratio of methoxylated to non-methoxylated anthocyanins (A) and percentage of methoxylated anthocyanins on the total amount of anthocyanins (B) in berry skins of *V. vinifera* treated with *A. nodosum* extract (SWE) and untreated plants (CTRL) under two irrigation regimes (WW, well watered; WS, water stressed). Analyses were conducted at t_0 (white bars), t (grey bars) and t_2 (dark grey bars). Data are means ± SE (n=5). Different letters within the same water regime and sampling time indicate significant differences between SWE and CTRL, assessed by one-way ANOVA (Fisher's LSD test, $P \leq 0.05$).

In WW and WS vines, hydroxycinnamic acids (**Fig. 5A**) and total phenolic (**Fig. 5D**) contents were higher in SWE than in CTRL berry skins both at t_1 and t_2. Quercetin derivatives content (**Fig. 5B**), in general, increased with berry development and was more than twice higher in SWE compared to CTRL berry skins at t_1 only in WS vines and both in WW and WS vines at t_2. By contrast, kaempferol derivatives (**Fig. 5C**) remained almost unchanged among sampling times and were only affected by the *A. nodosum* treatments in WS vines, with higher values in SWE than in CTRL at t_2.

The enzymatic activities of F3'5'OH, 3'-OMT and 5'-OMT, computed as ratios of anthocyanin amounts in SWE and CTRL berry skin, in both water regimes, are reported in **Table 3**. In WS vines, the enzymatic activity of F3'5'OH, 3'-OMT and 5'-OMT were generally higher than in WW vines, irrespective of *A. nodosum* treatments (**Supplementary Table 2**).

Both in WW and WS vines, *A. nodosum* treatments did not affect F3'5'OH enzymatic activity at t_1, while decreasing its activity at t_2, leading to lower values in SWE than in CTRL vines.

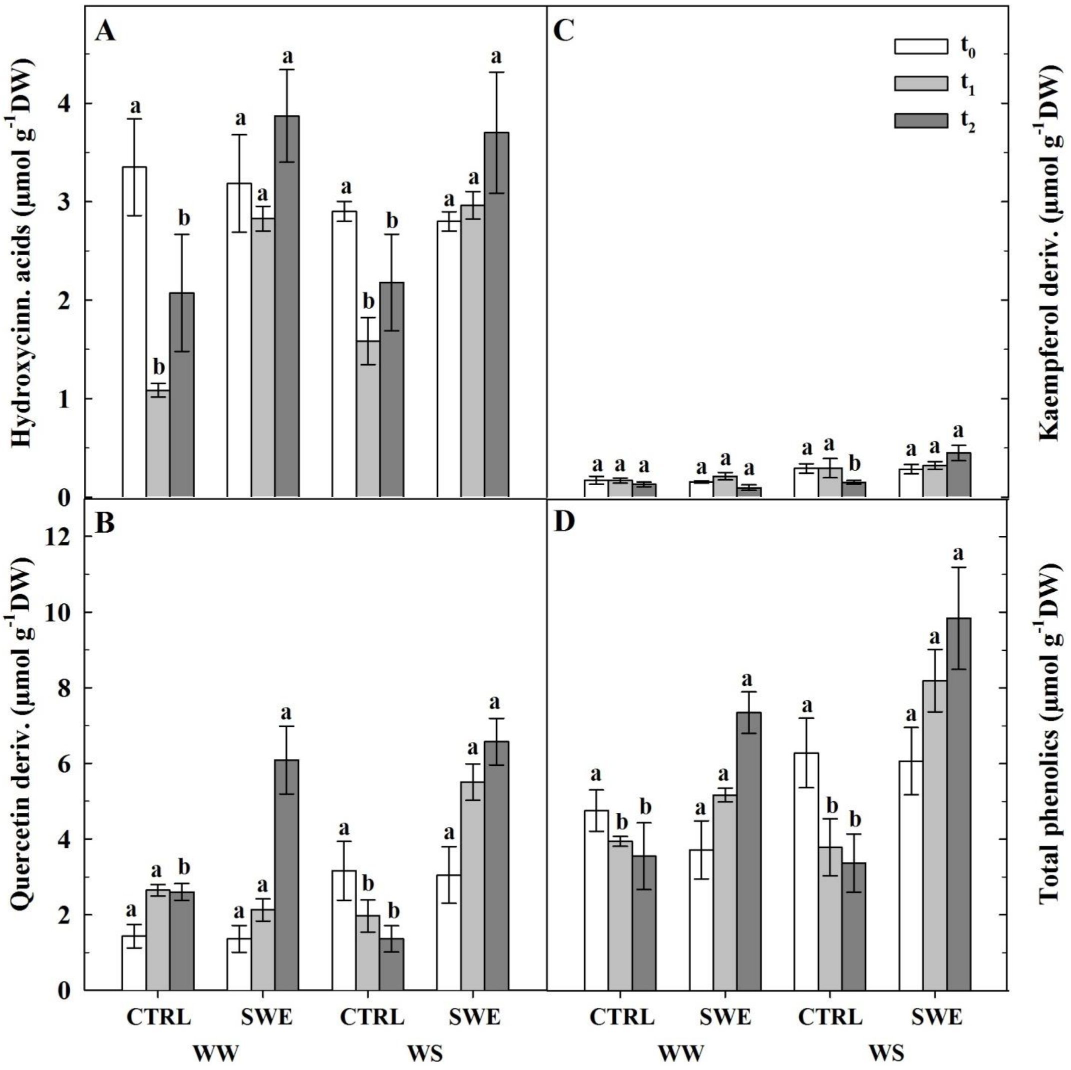

Fig. 5. Hydroxycinnamic acids (A), Quercetin derivatives (B), Kaempferol derivatives (C), and Total phenolic content (D) in berry skins (µmol g^{-1}DW) of *V. vinifera* treated with *A. nodosum* extract (SWE) and untreated plants (CTRL) under two irrigation regimes (WW, well watered; WS, water stressed). Analyses were conducted at t_0 (white bars), t (grey bars) and t_2 (dark grey bars). Data are means ± SE (n=5). Different letters within the same water regime and sampling time indicate significant differences between SWE and CTRL, assessed by one-way ANOVA (Fisher's LSD test, $P \leq 0.05$).

Moreover, SWE showed lower 3'-OMT activity than CTRL only at t_1, in both irrigation regimes. The 5'-OMT enzymatic activity was also affected by *A. nodosum* treatments, that lowered 5'-OMT in WW treated vines at t_1 and t_2, and also in WS vines at t_1 (**Table 3**).

Table 3

F 3’5’OH, 3’-OMT and 5’-OMT enzymatic activities computed as ratios of anthocyanin amounts in berry skin of *V. vinifera* treated with *A. nodosum* extract (SWE) and untreated plants (CTRL), under two irrigation regimes (WW, well watered; WS, water stressed), sampled at t_0, t_1 and t_2. Data (mean ± SE, n=4) were subjected to one-way ANOVA. Different letters within the same parameter and row indicate significant differences among treatments (LSD test, $P \leq 0.05$).

		F 3’5’ activity		**3’-OMT activity**		**5’-OMT activity**	
Irrig. Regime	**Samp. Time**	CTRL	SWE	CTRL	SWE	CTRL	SWE
WW	t_0	3.93±0.05 a	4.40±0.05 a	6.84±0.7 a	6.45±0.7 a	9.30±0.6 a	10.04±0.7 a
	t_1	2.86±0.40 a	3.69±0.56 a	8.08±0.6 a	6.88±1.4 b	14.12±1.4 a	10.48±0.4 b
	t_2	5.09±0.15 a	2.42±0.09 b	11.73±0.9 a	11.31±2.2 a	29.23±3.9 a	9.60±3.2 b
WS	t_0	5.42±0.07 a	5.42±0.07 a	10.30±1.9 a	10.30±1.9 a	12.34±0.4 a	12.20±0.4 a
	t_1	4.29±0.15 a	4.13±0.14 a	8.47±0.3 a	6.69±1.6 b	16.00±1.4 a	13.57±1.1 b
	t_2	4.55±0.06 a	3.89±0.31 b	14.02±0.3 a	13.25±0.9 a	22.36±2.0 a	22.27±4.9 a

4. Discussion

In this study, P_n, g_s, Ψ_{pd}, Ψ_m and RWC% were significantly reduced by drought in WS vines respect to WW ones. These reductions are important physiological responses typically observed in *Vitis vinifera* under moderate stress conditions (e.g. at t_1 in WS vines) and might be explained by changes in plants water status that subsequently affected leaf stomatal conductance, thus reducing the photosynthetic rate (Flexas et al., 2004; Cifre et al., 2005; Lovisolo et al., 2010). When water stress was more severe, in particular at t_2 in WS vines, pot-grown vines responded to water stress with a very strong reduction in WUE and a fall in g_s below 50 mmol H_2O m^{-2} s^{-1}, that is considered the threshold in grapevine under drought conditions (Medrano et al., 2002; Cifre et al., 2005). This indicates that non-stomatal factors were also affected by severe water stress (Flexas et al., 2002, 2004), probably due to an increased mesophyll conductance limitation (data not shown), that impaired the photosynthetic metabolism (Escalona et al., 1999; Galmés et al., 2007; Flexas et al., 2014).

At t_1, the hottest stage, under well-watering conditions, *A. nodosum* extract did not significantly affect P_n, g_s, Ψ_{pd}, Ψ_m and RWC%, whereas WUE was higher in CTRL than in SWE vines, because of lower E in non-treated vines (data not shown). This control of the amount of water lost via transpiration has been identified as an early event in plant response to water deficit to avoid levels of water stress that could compromise their growth and survival (Chaves et al., 2010; Romero et al., 2017). At the same stage, under water stress conditions, there were not significant ameliorating effects in gas exchange parameters induced by the *A. nodosum* treatments, in accordance with previous reports (Spann and Little 2011; Xu and Leskovar, 2015). On the contrary, in WS plants,

Ψ_{pd}, Ψ_m and RWC% were positively affected by *A. nodosum* treatment, with higher water potentials and water content values in SWE than in CTRL plants, as reported also by Di Stasio et al. (2018). At t_2, characterized by lower temperatures compared to t_1 period, there were significant increases in P_n, g_s and RWC% in both WW and WS plants, whereas *A. nodosum* treatments raised Ψ_{pd} and Ψ_m values only in water stressed vines. These results confirm an effect of *A. nodosum* treatments in reducing stomatal closure under drought, hence increasing gas exchange values as previously reported also in *Citrus* spp. (Little and Spann, 2010; Spann and Little, 2011), and other species (Xu and Leskovar, 2015; Elansary et al., 2016). Despite Pinot noir is usually described as an isohydric cv. in post-*véraison* stages (Poni et al., 1993), these physiological responses at t_2 suggest that *A. nodosum* treatments might act switching treated vines towards an anisohydric behavior both under well-water and water-stress conditions, allowing to keep stomata open by increasing leaf water potential (Sade et al., 2012).

The profound changes induced by water deficit on plant physiology during berry ripening have important consequences also on grape maturation and quality. When the water supply is moderately restricted, it improves the quality of red wines by achieving optimal sugar levels, due to competition for carbon assimilates between berry ripening and shoot growth and to a lower sugar concentration in berries because of increased berry size (Van Leeuwen et al., 2009). When the limitation in vine water uptake is severe, the stomatal closure restricts photosynthesis, reducing berry weight, accumulation of sugars and yield (Dry et al., 2001; Medrano et al., 2002; Chaves et al., 2007).

In our study, water availability significantly increased titratable acidity, pH, berry weight and, consequently, cluster weight and yield in WW in comparison with WS vines. However, well-watering conditions did not significantly affect berry sugar accumulation. These results are in contrast with those obtained by other authors who observed either an increase or a decrease (Bravdo et al., 1985; Dokoozlian and Kliewer, 1996; Greer and Weston, 2010) in berry sugars induced by high soil availability.

The evolution of total sugars, titratable acidity and pH were basically unaffected by *A. nodosum* treatments, as reported also by Frioni et al. (2018). In addition, berry weight and vine productivity were not modified by medium-late *A. nodosum* extract applications. On the contrary, as previously reported in table grape, early biostimulant applications during berry cell division and enlargement could even induce an improvement in vine productivity and berry weight (Norrie et al., 2002; Norrie and Keathley, 2006; Khan et al., 2012).

In this study, an increment in malvidin and in total anthocyanin content was found in water stressed vines, suggesting that low water availability drove a major flux of carbon in the anthocyanin pathway, particularly towards more stable malvidin-based derivatives (Azuma et al., 2012). This effect partly compensated the reduction in anthocyanin biosynthesis caused by the reduction in P_n because of stomatal closure (Zarrouk et al., 2016). Moreover, irrespective of the *A. nodosum* treatments, in WS berry skins we observed an increase in the methoxylated to non-methoxylated anthocyanin ratio, confirming that water stress boost anthocyanins stability through methoxylation, as an acclimation response to severe environmental constraints, such as heat stress and high radiation load (Gill and Tuteja, 2010, Rai et al., 2012).

Under both well-watering and water stress conditions, there were increases in the amount of delphinidin, petunidin, cyanidin and peonidin following *A. nodosum* treatments. *A. nodosum* treatment-induced higher level of phenols was described before in other species (Kumari et al., 2011; Fan et al., 2011; Lola-Luz et al., 2014). In addition, the methoxylated to non-methoxylated anthocyanin ratio was lower in SWE than in CTRL vines in both water regimes, and the percentage of methoxylated anthocyanins on total anthocyanins was lower in SWE than CTRL berry skin in WW vines, witnessing a treatment-induced lower investment in the biosynthesis of more stable methoxylated anthocyanins, which are usually accumulated in grapes under environmental constraints (Jackman and Smith, 1996; He et al., 2010). This is also supported by enzymatic activities results, that generally showed an higher enzymatic activity towards the biosynthesis of cyanidin-based derivatives and methoxylated anthocyanins (peonidin and malvidin) than non-methoxylated ones (cyanidin and delphinidin) following *A. nodosum* treatment, as demonstrated by lower F3’5’OH, 3’-OMT and 5’-OMT values in SWE than CTRL in both irrigation regimes. We suggest that *A. nodosum* might affect the activation of specific gene expression (Jayaraman et al., 2011; Santaniello et al., 2017), promoting the activity of flavonoid 3′-hydroxylases (F3′H) enzyme, catalyzing the biosynthesis of cyanidin-based anthocyanins, instead of activating F3′5’OH enzyme and the delphinidin parallel branch. We cannot exclude that this finding may be also related to the protocol of treatments, since the first *A. nodosum* application was carried out approximately at *véraison*, when cyanidin and peonidin are the prevalent anthocyanins synthesized in grape berry skins (Downey et al., 2006). In addition, under both irrigation regimes, we observed an increase in hydroxycinnamic acids and flavonols content in berry skin after *A. nodosum* treatments. In particular, the increment in di-hydroxy B-ring-substituted flavonols (i.e. quercetin derivatives), which are flavonoids with high antioxidant activity, may have improved SWE vines performances, as demonstrated by higher P_n and g_s values at t_2 in both water regimes, and by lower water potentials in water stressed vines. Moreover, in the berry, the biosynthesis of flavonols is closely related to that of anthocyanins, depending on the same enzymes for both classes of flavonoids (Mattivi et al., 2006): F3′H and F3′5′OH, are involved in the biosynthetic pathway of cyanidin- and delphinidin-based anthocyanins, respectively, as well as of quercetin and myricetin derivatives

(Jeong et al., 2006). In our berry samples, quercetin derivatives were the major flavonols, while myricetin derivatives were only detected in traces (data not showed). These results may again suggest that *A. nodosum* treatments is selectively involved in the activation of F3′H enzyme, rather than F3′5′OH, thus favoring the accumulation of di-hydroxy B-ring-substituted flavonoids rather that their tri-hydroxy counterparts.

5. Conclusion

This research provides evidence of *A. nodosum* treatments-induced changes in eco-physiological traits and berry skin metabolism of Pinot noir potted vines, resulting in different abilities to counteract low water availability during post-*véraison* stages.

In summary, in water-stressed vines, *A. nodosum* enhanced water stress tolerance by lowering leaf water potentials, thus allowing the maintenance of optimal water content and reduction in stomatal limitation. Furthermore, in both water regimes, the biochemistry of berry skin was profoundly affected by *A. nodosum* treatments. We suggest a selectivity action of these treatments on lowering the activity of enzymes involved in the the biosynthesis of methoxylated anthocyanins in treated vines. Moreover, the increases in the quercetin derivatives indicate that *A. nodosum* have a significant effect on enhancing *multiple* stress tolerance by increasing the content of antioxidant flavonoids, as well as enhancing plant water status and gas exchange parameters. Overall, these results support previous findings on the beneficial effects of *A. nodosum* treatments on plant acclimation to stressful environmental condition and thus their contribution to the sustainability of agricultural systems.

Contributions

The work was conceived and planned by GBM, LS, and CB. LS and EC performed treatments with the *A. nodosum* extract, gas exchange, water potentials measurements and collected berry samples. Data processing and analysis were carried out by LS and EC. LS and CB performed the HPLC analysis of phenylpropanoids. LS and CB drafted the initial manuscript, which was critically revised by all authors.

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Supplementary Table 1

Three-way ANOVA ($P<0.05$) for eco-physiology, chemical features of the berry and productivity parameters. Values are the mean of each Parameter, considering Treatments, sampling time (Samp. Time) and irrigation regime (Irrig. Regime) as factors. In the last 3 rows is indicated the Significance. Other abbreviations: Net assimilation rate (P_n), stomatal conductance (g_s), transpiration (E), water use efficiency (WUE), maximum quantum yield of PSII (F_v/F_m), predawn water potential (Ψ_{pd}), midday stem water potential (Ψ_m), relative water content (RWC), total acidity (TA). For cluster weight and yield/vine parameters, only Treatments and Irrigation regime were considered as factors.

Parameter	**P_n**	***gs***	**E**	**WUE**	**F_v/F_m**	**Ψ_{pd}**	**Ψ_m**	**RWC**	**Total sugars**	**pH**	**TA**	**Berry weight**	**Cluster weight**	**Yield/vine**
Unit	µmolm^{-2}s^{-1}	mmolm^{-2}s^{-1}	mmolm^{-2}s^{-2}	mmolm^{-2}s^{-3}		MPa	MPa	%	°Brix		gL^{-1}	g	g	g
Treatments														
Treated	8.9152	111.0000	3.2707	2.7552	0.7725	-0.6176	-1.2872	73.5383	17.2526	3.2674	10.4737	0.6873	52.4	312.1
Non-treated	8.1578	95.2000	2.8696	2.8891	0.7693	-0.7547	-1.4238	68.6305	17.4176	3.2235	10.6412	0.6852	46.1	276.7
Samp. Time														
t0	10.3240	130.8000	3.8368	2.5912	0.7683	-0.5747	-1.3083	92.4304	16.4667	3.0933	13.3500	0.9861	-	-
t1	7.5333	65.2381	1.8448	4.1786	0.7582	-0.7973	-1.4174	29.3996	16.9917	3.1525	12.7000	0.9950	-	-
t2	5.1400	74.0000	2.4500	1.9720	0.7924	-0.7382	-1.3583	84.4299	18.5333	3.4942	5.6083	0.9973	-	-
Irrig. Regime														
WW	11.73	133.32	3.73	3.33	0.77	-0.43	-1.10	70.43	17.76	3.32	12.46	0.75	51.72	312.00
WS	5.13	71.00	2.37	2.28	0.77	-0.95	-1.63	71.68	16.66	3.14	9.34	0.58	46.81	289.79
Significance														
Treatments	0.114	0.039	0.019	0.225	0.826	0.001	0.001	0.020	0.337	0.967	0.171	0.971	0.606	0.459
Irrig. Regime	0.000	0.000	0.000	0.000	0.800	0.000	0.000	0.145	0.736	0.034	0.039	0.008	0.031	0.018
Samp. Time	0.000	0.000	0.000	0.000	0.096	0.478	0.000	0.000	0.000	0.000	0.000	0.949	-	-
Treat. x Irrig. Regime	0.109	0.161	0.109	0.002	0.131	0.005	0.003	0.180	0.010	0.188	0.548	0.586	0.694	0.412
Treat. x Samp. Time	0.268	0.170	0.249	0.470	0.161	0.005	0.004	0.440	0.283	0.472	0.345	0.045	-	-
Irrig. Regime x Samp. Time	0.000	0.003	0.001	0.001	0.496	0.000	0.000	0.882	0.001	0.011	0.008	0.113	-	-
Treat. x Irrig. Regime x Samp. Time	0.525	0.548	0.647	0.390	0.530	0.003	0.003	0.698	0.162	0.292	0.599	0.782	-	-

Supplementary Table 2

Three-way ANOVA ($P<0.05$) for biochemical parameters in berry and leaf. Values are the mean of each Parameter, considering Treatments, sampling time (Samp. Time) and irrigation regime (Irrig. Regime) as factors. In the last 3 rows is indicated the Significance. Other abbreviation: Delphinidin-3-*O*-glucoside (Delph.), cyanidin-3-*O*-glucoside (Cyan.), petunidin-3-*O*-glucoside (Petun.), peonidin-3-*O*-glucoside (Peon.), malvidin-3-*O*-glucoside (Malv.), total anthocyanin content (Tot. Anth.), methoxylated to non-methoxylated ratio anthocyanins (Methox./non-Methox.), percentage of methoxylated anthocyanins among total content (% Methox. anth/tot), hydroxycinnamic acids in berry skin (Hydr. Acid. Skin), quercetin derivatives in berry skin (Q. Deriv. Skin), kaempferol derivatives in berry skin (K. Deriv. Skin), total phenolic content in berry skin (Tot. Phen. Skin).

Parameter	Delph.	Cyan.	Petun.	Peon.	Malv.	Tot. Anth.	Methox/non-Methox	% Methox anth/tot	Hydr. Acid Skin	Q. Deriv. Skin	K. Deriv. Skin	Tot. Phen. Skin	F3'5'OH activity	3'OMT activity	5'OMT activity
Unit	μmolg^{-1}DW	μmolg^{-1}DW	μmolg^{-1}DW	μmolg^{-1}DW	μmolg^{-1}DW	μmolg^{-1}DW			μmolg^{-1}DW	μmolg^{-1}DW	μmolg^{-1}DW	μmolg^{-1}DW			
Treatments															
SWE	0.80	0.30	1.01	2.87	11.39	15.94	12.32	92.27	3.06	3.89	0.26	6.92	3.99	9.15	13.05
CTRL	0.61	0.24	0.94	2.17	8.63	12.98	15.91	93.51	2.20	2.13	0.20	4.71	4.36	9.91	17.23
Samp. Time															
t0	0.77	0.23	0.87	2.18	9.63	13.14	9.37	85.50	2.81	2.37	0.22	5.35	4.79	8.47	11.01
t1	0.69	0.33	1.00	2.51	8.94	13.36	7.49	83.91	2.12	2.93	0.25	5.47	3.74	7.53	13.54
t2	0.66	0.24	1.06	2.72	11.55	16.84	12.15	86.28	2.96	3.79	0.22	6.63	3.98	12.58	20.86
Irrig. Regime															
WW	0.64	0.28	0.88	2.36	8.16	12.23	13.06	92.17	2.57	2.68	0.16	5.37	3.73	8.55	13.80
WS	0.77	0.26	1.07	2.60	11.69	16.55	15.18	93.62	2.69	3.31	0.31	6.26	4.62	10.50	16.48
Significance															
Treatments	0.129	0.298	0.463	0.004	0.150	0.001	0.001	0.240	0.006	0.001	0.304	0.001	0.349	0.419	0.002
Irrig. Regime	0.475	0.714	0.073	0.573	0.000	0.005	0.000	0.200	0.248	0.294	0.011	0.181	0.014	0.001	0.034
Samp. Time	0.854	0.064	0.229	0.015	0.001	0.113	0.000	0.321	0.057	0.064	0.818	0.145	0.001	0.064	0.426
Treat. x Irrig. Regime	0.237	0.204	0.025	0.808	0.001	0.272	0.012	0.100	0.866	0.464	0.326	0.602	0.009	0.041	0.027
Treat. x Samp. Time	0.012	0.100	0.000	0.069	0.001	0.270	0.000	0.088	0.029	0.005	0.526	0.01	0.600	0.230	0.757
Irrig. Regime x Samp. Time	0.651	0.564	0.751	0.032	0.010	0.002	0.001	0.748	0.713	0.044	0.888	0.308	0.561	0.042	0.034
Treat. x Irrig. Regime x Samp. Time	0.071	0.620	0.011	0.754	0.002	0.004	0.000	0.028	0.819	0.254	0.370	0.634	0.692	0.966	0.599

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Chapter 5

General conclusion

In the Mediterranean climatic regions, vineyards are subject to periods of high solar radiation, high temperatures and soil water deficit. These conditions may influence vine water status and accelerate berry ripening processes, that sometimes can result in unbalanced wines, with high alcoholic content and low phenolic concentrations. Viticulture provides an example of an agriculture that is highly sensitive to changes in climate conditions, but also of a system on which the capacity for adaptation are subject to socio-economic and cultural determinants.

Adaptation strategies based upon changes in agronomical practices, such as the application of seaweed extracts as biostimulants, can be considered as a first protection tools to enhance nutrition efficiency, abiotic stress tolerance and quality traits of plants.

Despite the increasing number of papers related to the effects of seaweed extract treatments on crop performance under abiotic stress conditions, until now, many works have analyzed the algae biostimulatory potential, mainly focusing separately on gas exchange or phenolic content responses of treated plants. However, in this PhD project, eco-phisiological responses, grape quality factors and antioxidant compounds content in berry and leaves were simultaneously investigated, in order to obtain an integrated view of *A. nodosum* treatment effects on both abiotic stress tolerance and on the regulation of berry ripening.

The first objective of the present thesis was to investigate the physiological performances of *V. vinifera* and the biochemical adjustments induced in grape berry skins by *A. nodosum* foliar treatments under field conditions. To this end, the comparison between vines treated with *A. nodosum* extract and non-treated vines was set up in a field experiment carried out in 2016 and 2017 in a commercial vineyard in the Chianti Classico area (Tuscany, Italy), in which physiological and biochemical parameters were observed at different timescales and related to variation in environmental conditions. The second objective of this thesis was to evaluate if *A. nodosum* treatments affect grapevine water relations and berry phenylpropanoid metabolism, mitigating the effects of a post-*véraison* drought. For this purpose, a second experiment was performed in 2017 on potted vines (cv. Pinot noir), subjected to two irrigation regimes (well watered and water stressed) associated with *A. nodosum* foliar treatments, performed with the same protocol as the field experiment.

The data obtained from both the experiments indicated that *A. nodosum* treatments during the last stages of berry development may enhance the performances of *V. vinifera* by means of physiological and biochemical parameters, as well as enhancing grape quality.

Applications of *A. nodosum* extract ameliorated leaf gas exchanges, maximum photosystem II efficiency and grape maturity balance, and had significant effects on phenylpropanoid biosynthesis, both in berry skins and in leaves. Furthermore, the biochemical analyses revealed that the *A.*

nodosum extract likely acted selectively in the phenylpropanoid pathway, influencing anthocyanin partitioning and lowering the biosynthesis of more stable methoxylated compounds, which are usually accumulated in grapes under environmental constraints. We suggest an higher enzymatic activity towards the biosynthesis of cyanidin-based derivatives and non-methoxylated ones (cyanidin and delphinidin) than methoxylated anthocyanins (peonidin and malvidin) following *A. nodosum* treatment, as demonstrated by lower F3’5’OH, 3’-OMT and 5’-OMT values.

In addition, for the first time in our knowledge, the second experiment shows the potential effect of *A. nodosum* extract in promoting higher tolerance to stress in potted vines, resulting in different abilities to counteract low water availability during post-*véraison* stages. In summary, in water-stressed vines, *A. nodosum* enhanced water stress tolerance by lowering leaf water potentials, which resulted in the maintenance of cell turgor pressure and higher stomatal conductance. Moreover, the changes in the phenolic profile indicate that *A. nodosum* may have a significant effect on enhancing *multiple* stress tolerance by increasing the phenolic content, as well as enhancing plant water status and gas exchange parameters.

Overall, these results support previous findings on the beneficial effects of *A. nodosum* treatments on plant acclimation to stressful environmental conditions, thus contributing to the sustainability of the viticultural system. In order to understand the mechanisms of action of seaweed extracts, it is crucial to focus future research on the identification of seaweed chemical compounds with potential effect on the phenylpropanoid pathway and their mode of action in affecting plant performance.

REGOLAZIONE DEL METABOLISMO SECONDARIO IN *VITIS VINIFERA* ATTRAVERSO UN ESTRATTO DI *A. NODOSUM*: EFFETTI SULLA FISIOLOGIA DELLA VITE E SULLA QUALITÀ DELL'UVA

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Abstract. Heat and drought are major abiotic stresses that impact on many *Vitis vinifera* physiological processes and berry development, especially as global climate change is speeding up the expected frequency and severity of both stress factors. Nevertheless, seaweed extracts might represent a strategy to confer to treated plants the capability to be more prone to counteract short-term periods of severe stresses. In this paper, we report the effects of foliar treatments with an *Ascophyllum nodosum* extract on physiological and biochemical parameters of grapevine. In our study performed in the 2016 season in open field in Tuscany, we assessed differences in leaf gas exchanges, chlorophyll fluorescence and midday stem water potential between treated and untreated vines. In addition, the in-depth analysis of anthocyanins and flavonols in berries presented within the work adds significantly to our knowledge of the influence of seaweed extract on the phenylpropanoid pathway. Moreover, our work witness the treatment-promoting greater tolerance to stress in vines, and support previous studies that hypothesize an influence of treatments with algae extracts on qualitative characteristics in *V.vinifera.*

1 Introduzione

Il clima dell'areale viticolo toscano è caratterizzato da estati con elevate temperature e scarsa piovosità. Queste condizioni influenzano lo stato fisiologico della vite e accelerano i processi di maturazione delle bacche, che a volte possono determinare vini sbilanciati, con alto contenuto alcolico e basse concentrazioni polifenoliche [1].

Negli ultimi anni, i ricercatori hanno studiato il ruolo dei biostimolanti, e tra essi degli estratti di alghe, come possibili promotori dell'acclimatazione delle piante nel breve termine [2]. A questo proposito, gli estratti di alghe sembrano essere responsabili di una maggiore biosintesi dei metaboliti secondari antiossidanti, ad esempio di polifenoli [3], che sono coinvolti sia nella risposta agli stress abiotici, che nella qualità dell'uva.

Nonostante il numero crescente di articoli scientifici sull'effetto dei trattamenti con estratti di alghe sulle prestazioni delle colture in condizioni di stress abiotico, sull'efficienza nell'uso dell'acqua e l'attivazione delle vie biosintetiche di composti antiossidanti [4], molti di questi prodotti non sono stati pienamente sfruttati a causa della mancanza di dati scientifici sulle molecole bioattive presenti nelle alghe e sul loro modo di influenzare le performances delle piante. Fino ad ora, molti ricercatori hanno analizzato il potenziale "biostimolatore" degli estratti di alghe concentrandosi separatamente sugli scambi gassosi o sul contenuto fenolico [5-9]. Con questo progetto ci si propone di esaminare contemporaneamente la risposta eco-fisiologica della pianta, i fattori di qualità dell'uva e i composti antiossidanti negli acini, al fine di ottenere una visione integrata degli effetti del trattamento.

L'obiettivo principale di questo studio è stato quello di valutare l'impatto di un estratto alcalino di *Ascophyllum. nodosum* sull'attività fisiologica, sulla resa e sui parametri qualitativi delle uve (*Vitis vinifera,* cv. Sangiovese), rivolgendo particolare interesse al raggiungimento dell'equilibrio tra maturità tecnologica e fenolica. L'ipotesi di ricerca è che l'applicazione di questi estratti, grazie ai suoi composti bioattivi, potrebbe aumentare sia la tolleranza alla siccità che la qualità dell'uva innescando la biosintesi di metaboliti secondari.

2 Materiali e metodi

La ricerca è stata condotta durante la stagione vegetativa 2016 presso l'azienda agricola Villa Montepaldi, che si trova sulle colline di San Casciano Val di Pesa (FI), nella parte settentrionale della nota zona di produzione del Chianti Classico. Il vigneto nel quale è stata effettuata la sperimentazione è stato impiantato nel 1998 con viti di Sangiovese (clone R 24) innestate su 420A, esposto a Sud-Ovest, con sesto d'impianto di 2,8 x 0,9 e forma d'allevamento a controspalliera, potata a cordone speronato. Il suolo è di medio impasto, con tessitura franco-argillosa (argilla 38.8%; sabbia 23.4%; limo 37.8%), con pH leggermente alcalino (7.8). Avvalendosi del disegno sperimentale a blocchi randomizzati con 5 ripetizioni per tesi di 3 filari ciascuna, è stato impostato

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il confronto tra il non trattato (CTRL) e il trattatoa con un estratto di *A. nodosum* (SWE), distribuito per via fogliare con dose di 4 l/ha, ad inizio invaiatura e due settimane dopo. Negli stessi giorni dei trattamenti con *A. nodosum*, le viti non trattate sono state spruzzate con acqua. Dal filare centrale di ogni ripetizione, sono state selezionate e cartellinate 15 viti omogenee/tesi, usate per i rilievi eco-fisiologici, produttivi e per i campionamenti di acini, in tre fasi fenologiche: pre-invaiatura (pre-trattamento), piena invaiatura (una settimana dopo il primo trattamento) e maturazione (una settimana dopo il secondo trattamento). Ad ogni rilievo, campionando 15 foglie adulte e sane poste nella fascia mediana della parete dalle piante cartellinate di ogni tesi, sono stati effettuati rilievi di fotosintesi (P_n, μmol m^{-2} s^{-1}) e conduttanza stomatica (g_s, mmol m^{-2} s^{-1}) con un analizzatore di gas all'infrarosso (Ciras 3, PP Systems, USA), di efficienza massima del fotosistema II (Fv/Fm) con un fluorimetro portatile (Handy-PEA®, Hansatech Instruments, UK), di potenziale idrico di tralcio (Ψ_m, Mpa) con una camera a pressione (PMS Instrument Co, USA). Inoltre, nelle stesse tre fasi fenologiche, sono stati effettuati campionamenti di 150 acini da ogni ripetizione/tesi per la determinazione degli zuccheri solubili (g/L), dell'acidità titolabile (g/L di acido tartarico), del pH e del peso dell'acino (g). Su un sottocampione analogo di 30 acini sono state effettuate le analisi relative alla composizione di antociani e polifenoli nelle bucce (μmol g di sostanza secca^{-1}), utilizzando un HPLC-DAD (Perkin Elmer, USA).

Alla raccolta, sulle 10 piante cartellinate per ogni ripetizione di ogni tesi sono stati determinati il peso fresco della produzione (Kg), il numero di grappoli/pianta e il peso fresco del grappolo (g).

I dati raccolti sono stati sottoposti all'analisi della varianza ad una via con SPSS Data Editor (IBM, USA). Le differenze statisticamente significative sono state assunte per P<0.05.

3 Risultati e discussioni

3.1 Effetto dell'estratto di *A. nodosum* sui parametri eco-fisiologici

In **Tab. 1** sono riassunti i valori medi stagionali dei parametri eco-fisiologici misurati durante la stagione. SWE mostra valori medi di P_n, *gs* e Fv/Fm significativamente maggiori di CTRL. Al contrario, nel nostro studio, non abbiamo registrato differenze significative in Ψ_m tra SWE e CTRL. I trattamenti con *A. nodosum* hanno avuto un effetto significativo nel ridurre la chiusura stomatica e aumentare la fotosintesi. In più, mentre le piante non trattate hanno mostrato segni di moderata foto-inibizione, le viti trattate sono state in grado di mantenere i valori Fv/Fm più vicini all'optimum indicato per la vite (~ 0,80) [10-11]. Gli effetti positivi sulle funzioni fisiologiche delle piante indotte dai trattamenti con *A. nodosum* sono stati precedentemente riportati anche in spinacio, Spiraea e Pittosporum [9; 12]. Ipotizziamo che il miglioramento delle prestazioni fisiologiche promosse dai trattamenti con *A. nodosum* possa essere correlato alla presenza di aminoacidi e composti fenolici all'interno dell'estratto, che, con probabilità, hanno conferito un'elevata capacità antiossidante all'estratto stesso.

Tab 1. Valori medi stagionali dei parametri eco-fisiologici.

Parametro	SWE	CTRL
P_n (μmol m^{-2} s^{-1})	11.1±0.8 a	9.3±0.6 b
***gs* (mmol m^{-2} s^{-1})**	245±16.5 a	197±16 b
Fv/Fm	0.79±0.01 a	0.76±0.01 b
Ψ_m (Mpa)	-1.13±0.1 a	-1.23±0.07 a

3.2 Effetto dell'estratto di *A. nodosum* sulle caratteristiche qualitative e produttive in *Vitis vinifera*

I valori a vendemmia degli zuccheri totali e dei parametri produttivi sono presentati nella **Tab. 2**. Non sono state registrate differenze statistiche tra i due trattamenti nell'acidità totale e nel pH (dati non mostrati) e nei parametri produttivi. Al contrario, differenze significative nel tenore di zucchero totale sono state osservate a vendemmia tra acini SWE e CTRL. A piena maturazione, SWE presentava un contenuto di zuccheri totali inferiore di circa 10 g/L rispetto a CTRL.

Tab 2. Zuccheri solubili e parametri produttivi a vendemmia.

Parametro	SWE	CTRL
Zuccheri (g/L)	229±1 b	239±1 a
Peso di 150 acini (g)	375±15 a	360±15 a
Peso di 10 grappoli (Kg)	3.8±0.1 a	3.5±0.3 a
Resa di 10 piante (Kg)	27±2 a	26±3 a

Per quanto riguarda la composizione delle bucce, sono stati quantificati i maggiori contenuti di antociani sia metossilati (peonidina, petunidina, malvidina) che non metossilati (delfinidina e cianidina) (**Fig. 1** e **Fig. 2**), a piena invaiatura e a maturazione. Il rapporto tra i derivati della quercetina e del kampferolo è anche esso maggiore nelle bucce SWE rispetto a CTRL, in tutte le fasi fenologiche post trattamento (**Fig. 3**).

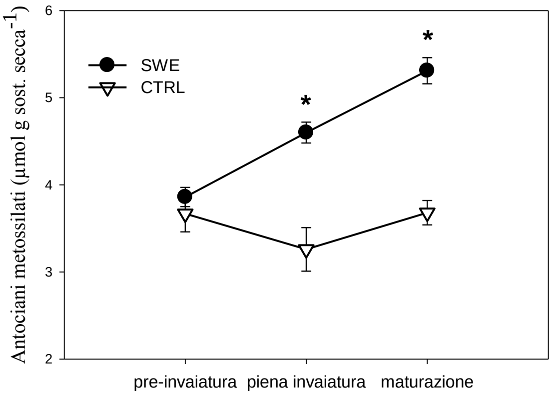

Fig 1. Valori medi (± SE, n=5) di antociani metossilati nelle bucce, nei tre punti di rilievo. * indica significatività (P≤ 0.05)

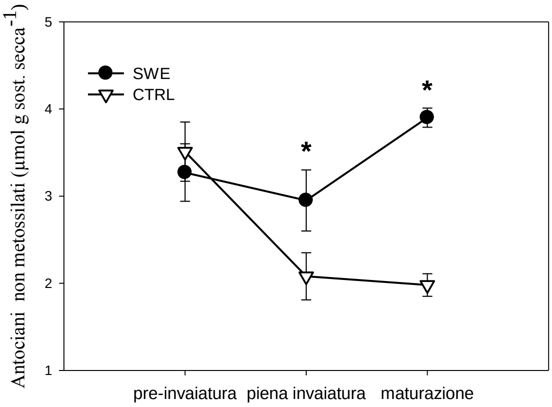

Fig 2. Valori medi (± SE, n=5) di antociani non metossilati nelle bucce, nei tre punti di rilievo. * indica significatività (P≤ 0.05)

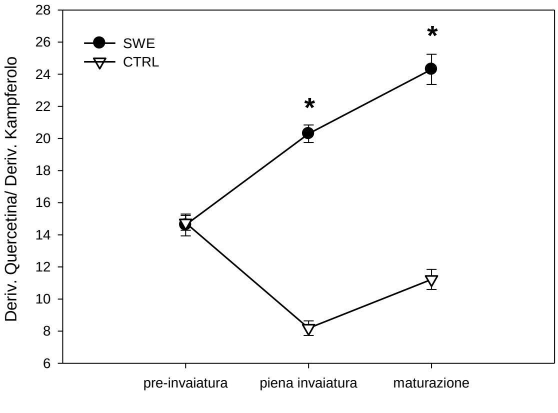

Fig 3. Valori medi (± SE, n=5) del rapporto tra derivati della quercetina e derivati del kampferolo nelle bucce, nei tre punti di rilievo. * indica significatività (P≤ 0.05)

Analizzando collettivamente questi risultati, possiamo ipotizzare che le elevate temperature stagionali abbiano guidato l'accelerazione dell'accumulo di zucchero in CTRL. Questo effetto è meno evidente in SWE, in cui abbiamo osservato un contenuto zuccherino significativamente inferiore accompagnato da un più elevato contenuto di antociani rispetto alle piante CTRL. Ciò è in accordo con studi precedenti [8;13] e sostiene fortemente l'ipotesi che i trattamenti con *A. nodosum* possano essere coinvolti nel ritardare la maturazione, bilanciando la maturazione tecnologica con quella fenolica. Tuttavia, questi effetti positivi sulla qualità dell'uva non sono stati accompagnati da un concomitante incremento della resa, probabilmente perché l'estratto di *A. nodosum* è stato distribuito in stagione avanzata su acini completamente sviluppati in dimensioni. Al contrario, come riportato in precedenza nell'uva da tavola, applicazioni di biostimolanti durante la divisione e distensione delle cellule dell'acino potrebbero persino indurre un miglioramento della produttività della vite e del peso delle bacche [14-16].

4 Conclusioni

Nell'ottica di ridurre i fenomeni di sfasamento tra maturità tecnologica e fenolica ascrivibili alle interazioni tra i cambiamenti climatici in atto ed alle mutate tecniche di gestione della chioma, la distribuzione fogliare di prodotti biostimolanti può rappresentare una valida pratica agronomica.

I nostri dati indicano che i trattamenti con *A. nodosum* durante le ultime fasi dello sviluppo delle bacche possono potenziare le prestazioni di *V. vinifera* migliorando i parametri eco-fisiologici e influenzando il metabolismo secondario, con conseguente miglioramento della qualità dell'uva.

In dettaglio, i trattamenti con l'estratto di *A. nodosum* hanno aumentato la fotosintesi, riducendo la chiusura stomatica, massimizzato l'efficienza del fotosistema II e bilanciato la maturazione dell'uva. Questo prodotto risulta essere un valido aiuto in annate siccitose e calde, permettendo di ovviare al problema di un eccesso di zucchero nell'acino a vendemmia, riuscendo a ritardare la maturità tecnologica della bacca e apportando un corredo polifenolico più alto.

Al fine di comprendere i meccanismi di azione degli estratti di alghe marine, è fondamentale concentrare la ricerca futura sull'identificazione dei composti chimici delle alghe con un potenziale effetto sulla via dei fenilpropanoidi delle piante trattate.

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S034

Effects of vegetal protein hydrolysate on ripening and berry quality in viticulture

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Introduction

The phenomenon of global warming is creating major climatic changes marked by unforeseeable with damaging consequences for agriculture. The consequences on the physiology of vines are very evident, as demonstrated by the effects of water shortage (rainfall reduction in the last 100 years has been 5% with greater concentrations in some periods), phase shift phenomena (anticipation of harvest times, but especially for the accumulation processes that coincide with high temperatures), the oxidative effects on photosynthetic activity, the altered synthesis of secondary compounds (polyphenols and aromas) which are the basis of wine quality.

Viticulture is assisted in the phasing out of the technological maturation with respect to the phenolic and aromatic ones: the grapes arrive in the cellar too hot, with high sugar gradients which are affected by the high alcohol content and the possible problems of fermentation stoppages.

The aim of the research is to balance the grape technological and phenolic maturity by the use of biostimulant products, making the two maturations balanced. The research deals with the effects of three vegetal protein hydrolysates supplied by foliar application on vine ecophysiological, productive and qualitative berry characteristics, to improve yield and quality in case of abiotic stress conditions.

Materials and methods

The used products are three protein hydrolyzed of vegetable origin obtained by enzymatic hydrolysis, rich in easy-absorbing and utilizing L-amino acids. Particularly present are glutamic and aspartic acid (64% of free amino acids) the basis from which all other amino acids are obtained for transaminase. They contain natural complexes that favor the absorption of microelements.

- A Formulation: concentrated Suspension. Dosage 4 l/ha - 2 treatments: cluster pre-closure and veraison
- B Formulation: concentrated Suspension. Dosage 4 l/ha - 2 treatments: cluster pre-closure and veraison
- C Formulation: hydrodispersible granules. Dosage 100 gr/ha - 3 treatments: cluster pre-closure, veraison and 20 days before harvest

The experimental research was carried out on *Vitis vinifera* Sangiovese grapevines in central Italy. Vines are trained as spur-pruned upward vertical shoot positioning. Clorophyll content and leaf gas exchanges with Ciras 1 (PP Systems) were measured. Berries from each replication were sampled for analyses of soluble solids (Brix), berry weight (g), anthocyanin and polyphenol contents (mg/L), with Glories method.

The data collected were subjected to analysis of variance with SPSS Data Editor, and the Fisher's least significant difference (LSD) were assumed for $P<0.05$.

Results

Thesis	Dose /Ha	° Brix	Acidity (g/L tartaric ac.)	pH	Berry weight (g)	Cluster weight (g)	Extractable anthocyanins (mg/l)	Extractable polyphenols (mg/l)
A	4 L/Ha	23,0	5,4	3,21	2,5	566	599	1988
B	4 L/Ha	22,6	5,0	3,20	2,6	469	585	1729
C	100 g/Ha	23,2	5,0	3,20	2,5	475	615	1983
CTRL		24	5,0	3,26	2,4	297	572	1815
LSD		0,91	0,282	0,06		154,3	25,7	131,5

Table 1: Grapes quality summary. LSD indicates statistical differences between the treatments ($P<0.05$) - All treatments reduced sugar content with statistic differences versus control, and, at the same time, increased anthocyanins and polyphenols. Moreover we can observe an increase in cluster weight in all treatments

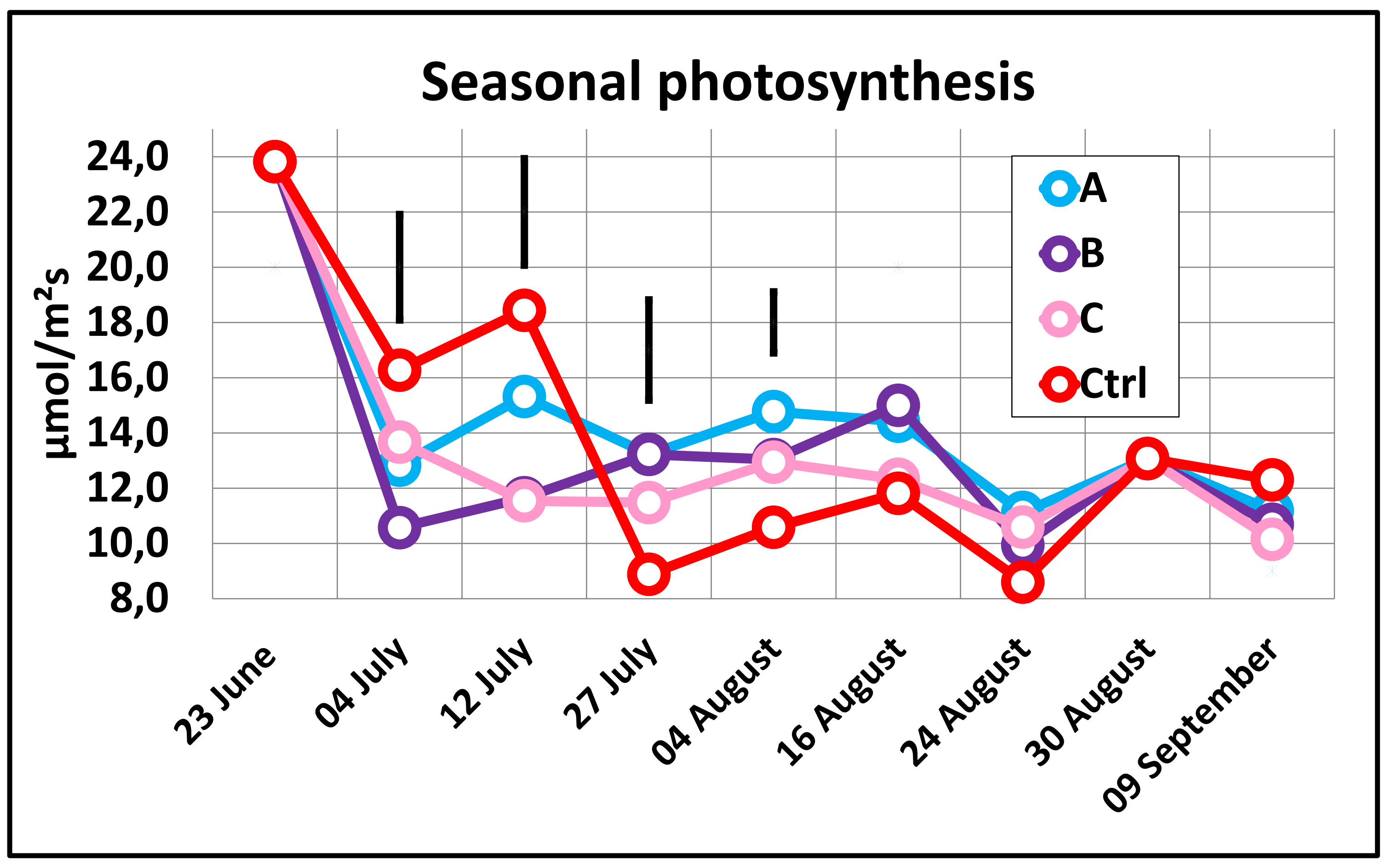

Figure 1: Seasonal performance photosynthesis. Bar on the line indicates statistical differences between the treatments ($P<0.05$). In the hottest time (end of July-end of August) all treatments increased net photosynthesis, and, in general, leaf gas exchange

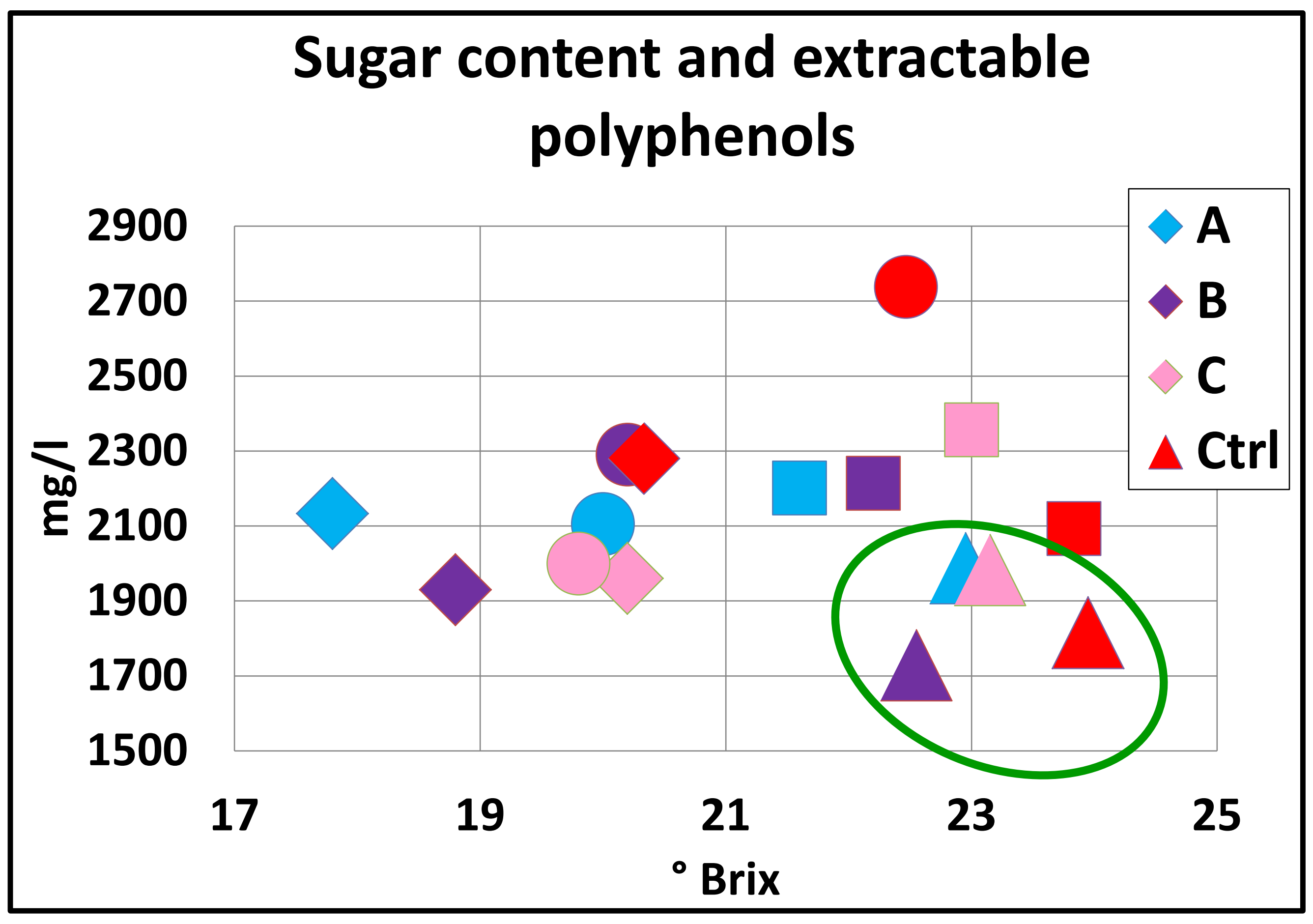

Figure 2

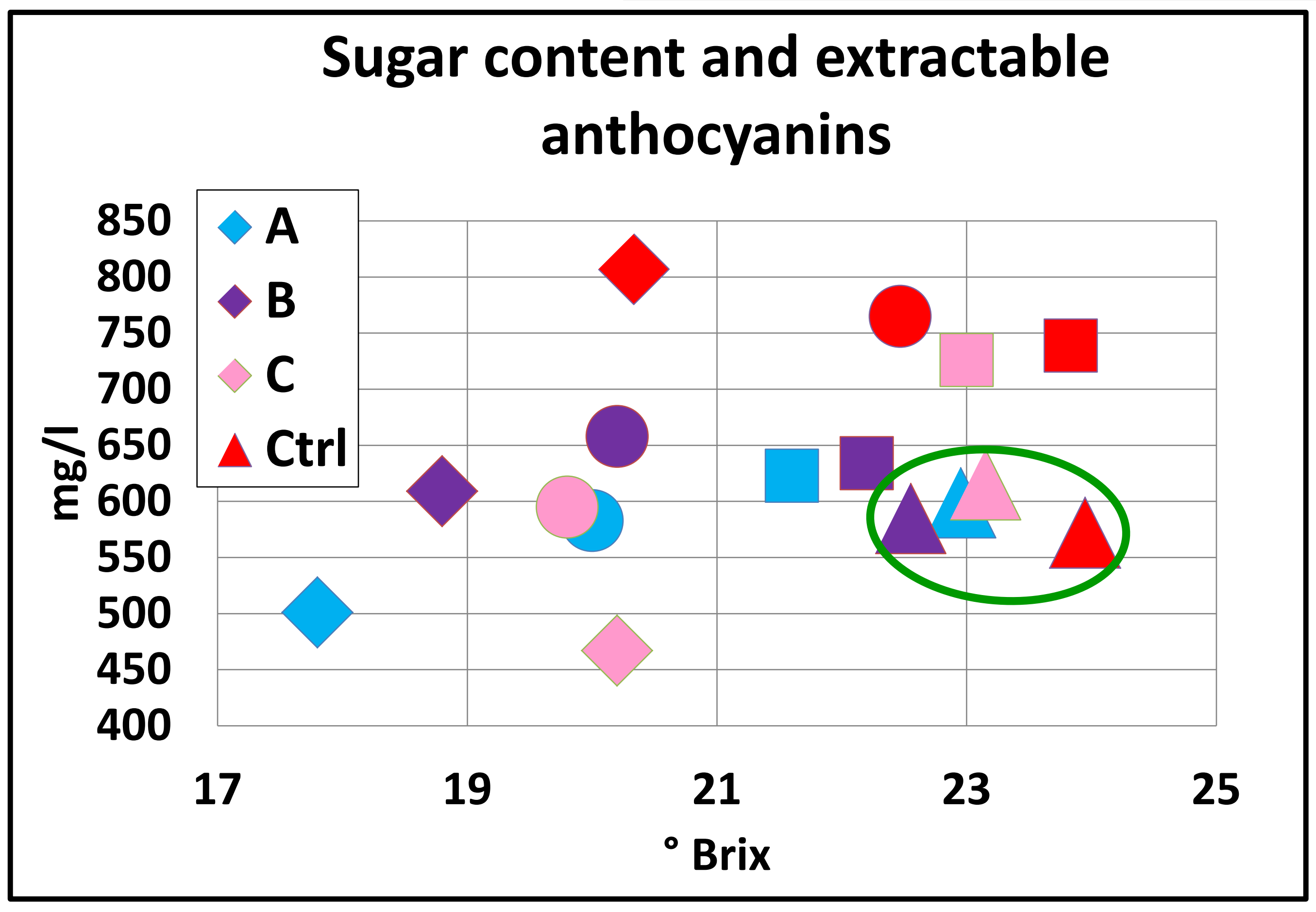

Figure 3

Figure 2: Seasonal correlation between sugar content and extractable polyphenols. Circled values represent vintage data.

Figure 3: Seasonal correlation between sugar content and extractable anthocyanins. Circled values represent vintage data.

Conclusions

The sprayed products improved the amount of production, while maintaining high qualitative standards, even in the presence of abiotic stress. They acted on plant physiology, stimulating the full expression of the potential of cultivation. The product improveed the photosynthetic efficiency of the vines during the warmest and drought period; they also promoted a balanced early maturation, in terms of sugar content and color (anthocyanin accumulation). In general, it can be assumed that these products, improving the efficiency of nutrient utilization and resistance to biotic and abiotic stress, by directing physiological activity towards maximizing the accumulation of secondary plant metabolic substances. The acting products biostimulating also delayed the accumulation of berry sugars, representing a simple and economic strategy to the problem of the mismatch between technological and phenolic maturity at harvest.

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Acta Italus Hortus 19: 39-40

Uso di biostimolanti naturali per il miglioramento qualitativo delle produzioni viticole

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Use of natural bio-stimulants to improve the grapevine production quality

Abstract. The research, carried out in the 2014 and 2015 growing seasons on Sangiovese vines, studied the effects of bio-stimulants, supplied by foliar feeding, on the vine's eco-physiological and productive characteristics, to improve the quality in a cv. Sangiovese vineyard. In fact, the main goal of this study was to separate the technological maturation, in terms of the berry's sugar and acidity accumulations, from the phenolic one (i.e. its anthocyanin and polyphenol contents). Specifically, three bio-stimulant products were sprayed: two seaweed extracts and one protein hydrolyzate; an untreated control was used for comparison. In conclusion, all treatments reduced photosynthesis; moreover, the balance between technological and phenolic maturities was improved, basically by maintaining the berries' sugar content level and by increasing their anthocyanin and polyphenol contents.

Introduzione

L'European bio-stimulants industry council (EBIC), fondato nel 2011, definisce i biostimolanti come "prodotti che contengono sostanze e/o microrganismi la cui funzione è quella di stimolare i naturali processi fisiologici in modo da incrementare l'assorbimento dei nutrienti e la loro efficienza, la tolleranza agli stress e la qualità del prodotto".

Da un punto di vista nutrizionale, le ricerche più recenti hanno dimostrato che i biostimolanti sono in grado di modificare l'architettura radicale delle piante (Paradikovic et al., 2011), incrementando lo sviluppo delle radici e migliorando l'efficienza di assorbimento (Mugnai et al., 2008; Martin, 2012).

Peraltro, le gibberelline, le citochinine e l'acido abscissico endogeni contenuti in alcune categorie di biostimolanti, ad esempio negli estratti di alghe, possono indurre un incremento nelle rese e nel contenuto di polifenoli totali dei frutti (Martin, 2012). Alla luce del cambiamento climatico in atto, il miglioramento delle pratiche colturali è più che mai una necessità e tutti questi aspetti suggeriscono il possibile contributo positivo dei biostimolanti nel ridurre le dosi di fertilizzanti da distribuire, pur mantenendo/innalzando la qualità del prodotto, riducendo i costi di produzione e limitando i rischi di inquinamento ambientale.

L'obiettivo di questo studio è stato quello di valutare l'impatto dei biostimolanti sull'attività fisiologica, sulla resa e sui parametri di qualità delle uve di un clone molto produttivo di Sangiovese (Vitis vinifera), rivolgendo particolare interesse al raggiungimento dell'equilibrio tra maturità tecnologica e fenolica.

Materiali e metodi

La ricerca è stata condotta presso l'azienda agricola Villa Montepaldi, a San Casciano Val di Pesa (FI), durante le stagioni vegetative 2014 e 2015, su viti di Sangiovese allevate a controspalliera. Avvalendosi del disegno sperimentale a blocchi randomizzati con 5 ripetizioni per tesi, è stato impostato il confronto tra il non trattato e 3 diversi biostimolanti: un idrolizzato proteico e due estratti di alghe (estratto alghe 1 e estratto di alghe 2). Durante entrambe le stagioni sono stati effettuati rilievi di scambi gassosi fogliari con il Ciras 1 (PPSystems) e di contenuto di clorofilla mediante lo strumento Spad-502 (Konica Minolta).

A partire dai primi di settembre fino alla vendemmia sono stati effettuati prelievi di 150 acini da ogni ripetizione per la determinazione di zuccheri solubili (°Brix), dell'acidità titolabile (g/L di acido tartarico), del pH e del peso dell'acino (g). Su un campione analogo sono state effettuate le analisi relative al contenuto potenziale di antociani e polifenoli totali ed estraibili (mg/L) con il metodo proposto da Glories.

Alla raccolta per ogni tesi sono stati determinati il peso fresco della produzione (kg), il numero di grappoli/pianta e il peso fresco del grappolo (g).

I dati raccolti sono stati sottoposti all'analisi della varianza con SPSS Data Editor. Per i confronti multi-

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pli tra i trattamenti è stata riportata la differenza minima significativa (DMS) di Fisher e le differenze statisticamente significative sono state assunte per P<0.05.

Risultati e discussione

Le due stagioni vegetative in cui si è svolta la sperimentazione si sono caratterizzate per andamenti meteorologici quasi opposti: nel 2014 la temperatura media estiva non ha mai superato i 25°C, tra giugno e luglio sono precipitati circa 150 mm d'acqua, un sesto della piovosità annuale della zona, determinando il ritardo della maturazione e l'insorgenza di muffe sui grappoli che hanno costretto a vendemmiare le uve prima della loro completa maturazione. Nel 2015 le temperature medie del periodo estivo si sono attestate sui 25-30°C, accompagnate da scarsi eventi piovosi nei mesi di giugno e luglio. Le riserve idriche del terreno si sono reintegrate con le piogge di agosto e le temperature miti in vendemmia hanno favorito la completa maturazione zuccherina e fenolica. Nonostante queste notevoli differenze meteorologiche è stato possibile individuare una tendenza negli effetti dei prodotti distribuiti analoga per entrambi gli anni.

Per quanto riguarda gli scambi gassosi su foglia singola, si registrano valori tendenzialmente più bassi per le tesi trattate con i biostimolanti rispetto al controllo. Questo è più evidente per fotosintesi netta (fig.1), mentre i valori di traspirazione si allineano maggiormente. La conduttanza stomatica in entrambe le stagioni non è mai scesa al di sotto delle 100 mmol/m2s , soglia che viene presa come riferimento nella valutazione dello stress idrico della pianta.

Sia nel 2014, che nel 2015 non si evidenziano differenze significative nei parametri della maturazione tecnologica delle uve, mentre le sostanze coloranti ed il corredo polifenolico risultano superiori nelle uve delle tesi trattate con i biostimolanti (fig.2).

Conclusioni

Nell'ottica di ridurre i fenomeni di sfasamento tra maturità tecnologica e fenolica ascrivibili alle interazioni tra i cambiamenti climatici in atto ed alle mutate tecniche di gestione della chioma, la distribuzione fogliare di prodotti biostimolanti può rappresentare una valida pratica agronomica.

In generale, si può ipotizzare che questi prodotti, migliorando l'efficienza di utilizzo dei nutrienti e la resistenza agli stress biotici ed abiotici, deprimano l'attività fisiologica della pianta indirizzandola verso la massimizzazione dell'accumulo di sostanze del metabolismo secondario.

Tuttavia, la complessità dei prodotti e dei loro meccanismi di azione, associata alla variabilità dell'ambiente e delle condizioni climatiche, fanno si che queste sostanze, dagli effetti ancora incerti, siano oggetto di studio di molti enti di ricerca.

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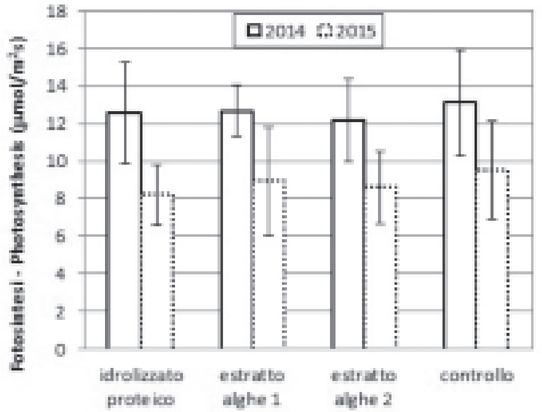

Fig. 1 - Valori medi di fotosintesi netta delle stagioni 2014 e 2015 (µmol/m^2s). Le barre indicano la deviazione standard.

Fig. 1 - Mean of seasonal net photosynthesis values for 2014 and 2015 (µmol/m^2s). Histograms are means ± sd.

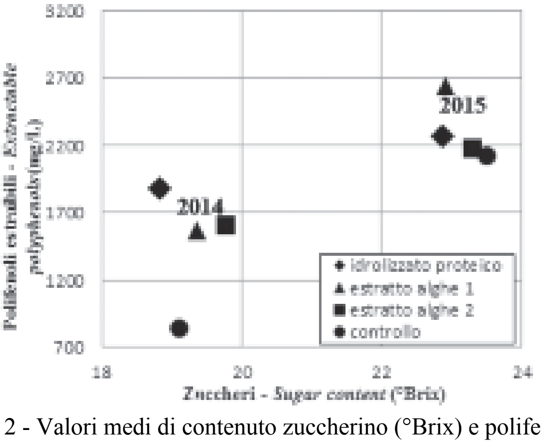

Fig. 2 - Valori medi di contenuto zuccherino (°Brix) e polifenoli estraibili (mg/L) delle uve a vendemmia (stagioni 2014 e 2015).

Fig. 2 - Mean of sugar (°Brix) and polyphenol (mg/L) contents at harvest for 2014 and 2015.

Acta Italus Hortus 19: 47-48

La distribuzione fogliare di un antitraspirante naturale per il miglioramento qualitativo delle produzioni viticole

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Leaf distribution of a natural anti-traspirant for the qualitative improvement of wine production

Abstract. In recent decades the increasing trend in global temperatures has affected the accumulation of sugars in the berries and then the alcohol degree in wines. The purpose of this study was to evaluate the effect of a natural anti-traspirant on the physiology and quality of the Sangiovese grapes , with particular focus towards the balance between technological and phenolic maturity. The use of vegetal anti-traspirant allows to limit transpiration by increasing the resistance to the passage of water vapor, with a consequent increase in the leaf water potential and improvement of the water status of the vine. Applications around veraison delay the maturation leading to a decrease of the sugar content, and an increase of the polyphenolic substances. The results show that the application reduced transpiration, stomatal conductance and photosynthesis by carrying out more the film-forming effect in the warmer months and driest vintage (2015). Overall, the treatment decreased the sugar content and raised the polyphenolic anthocyanins and improved the balance between technological and phenolic maturity, compared to untreated control.

Key words: grape quality, leaf gas exchange, Sangiovese, spur cordon

Introduzione

L'uso di filmogeni antitraspiranti permette di limitare la traspirazione aumentando la resistenza al passaggio del vapore acqueo, determinando un aumento del potenziale idrico fogliare. Gli antitraspiranti minimizzano la perdita d'acqua dalle foglie riducendo la conduttanza stomatica, migliorando lo stato idrico della pianta e riducendo l'avvizzimento e l'abscissione fogliare. (Marallo, 2015).

Data l'efficienza nell'uso di antitraspiranti nella regolazione della fotosintesi, indipendentemente dalla data di applicazione, è ipotizzabile che applicazioni tardive in viticoltura (attorno all'invaiatura) potrebbero essere prese in considerazione per ritardare la maturazione in zone calde dove, sotto la pressione del riscaldamento globale, l'accumulo di zuccheri è troppo veloce, l'acidità è troppo bassa e i sapori sono atipici. Questo potrebbe garantire, oltre ad una diminuzione del tenore zuccherino, anche un aumento delle sostanze polifenoliche, più lente ad accumularsi rispetto agli zuccheri (Davenport *et al.,* 1972).

Recentemente, negli areali viticoli collinari caratterizzati da scarsa disponibilità idrica e estati siccitose, sono stati riscontrati fenomeni di sfasamento tra maturità tecnologica e fenolica ascrivibili alle interazioni tra i cambiamenti climatici e alle tecniche di gestione della chioma. Ciò comporta un'accelerazione dell'accumulo di solidi solubili nelle bacche che raggiungono gradazioni zuccherine corrispondenti a livelli ottimali di alcool potenziale, non associati ad un'opportuna maturità fenolica.

L'obiettivo di questo studio è stato quello di valutare l'effetto dell'antitraspirante sul metabolismo, sull'assorbimento dei nutrienti e sui parametri quanti-qualitativi di uve di Sangiovese. In una prima fase, è stato misurato l'effetto dell'antitraspirante vegetale sui parametri fisiologici delle viti. Successivamente è stato valutato l'impatto sulla resa e sui parametri della maturità tecnologica e fenolica delle uve.

Materiali e metodi

La ricerca è stata condotta presso l'azienda agricola Villa Montepaldi, sulle colline di San Casciano Val di Pesa (FI), durante la stagione vegetativa 2015. Il vigneto nel quale è stata effettuata la sperimentazione è stato impiantato nel 1998 con Sangiovese come cultivar, esposto a Sud-Est, con sesto d'impianto di 2,80 x 0,90 e forma d'allevamento a controspalliera, potata a cordone speronato.

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Il prodotto utilizzato è un antitraspirante vegetale con aggiunta di sali minerali ancora non in commercio. Per ottenere le 2 tesi (1 tesi trattata e una tesi controllo), nel campo sperimentale sono stati effettuati trattamenti col metodo a blocchi randomizzati, ottenendo 5 ripetizioni per tesi. Sono stati irrorati 6,25 ml di prodotto a ripetizione pari a 2,5 l/ha con pompa a zaino elettrica ElettroEASY.

I due trattamenti sono stati effettuati in prechiusura grappolo (18 giugno 2015) e all'invaiatura (3 agosto 2015). Per rilevare gli scambi gassosi su foglia singola è stato usato l’analizzatore di gas all’infrarosso, CIRAS 1 (PP Systems Herts, UK). Per determinare il livello di clorofilla è stato utilizzato SPAD-502. Le analisi tecnologiche hanno determinato il grado zuccherino con rifrattometro ottico, il pH e l’acidità totale del mosto; mentre le analisi fenoliche determinano il potenziale totale ed estraibile di antociani e polifenoli in mg/L col il metodo Yves-Glories.

I dati raccolti sono stati sottoposti all’analisi della varianza con SPSS Data Editor.

Risultati e discussione

Il trattamento antitraspirante si rivela molto efficace nel periodo più secco, abbassando significativamente fotosintesi e traspirazione rispetto al controllo.

Nella conduttanza stomatica (gs) non ci sono state grosse divergenze tra trattato e controllo e non si è mai avuto un vero stress nell’arco della stagione (i valori di gs non sono mai scesi sotto 100 mmol/m²s).

Nell’efficienza nell’utilizzo dell’acqua WUE la tesi con antitraspirante mantiene per tutta la stagione i migliori rapporti di pn su traspirazione e su gs.

Per le analisi tecnologiche si nota una tendenza nel maggior accumulo zuccherino nella tesi controllo; da qui si evince che il trattamento ha esercitato un rallentamento nell’accumulo zuccherino nella bacca.

Il controllo raggiunge prima un alto °Brix: in data 23 settembre il °Brix del controllo è 23,5 rispetto alla tesi con trattamento 22,2 °Brix (tab. 1).

Questa strategia è stata efficace nel ridurre il ritmo di accumulo di zuccheri nella bacca segnando un -1,3 °Brix al momento del raccolto e abbassando il tenore alcolico nei vini ottenuti.

Nelle misure di pH e acidità non si riscontrano differenze significative fra il trattato e il riferimento.

Sulle analisi fenoliche: la sintesi dei polifenoli nella tesi trattata con l’antitraspirante è fin dall’invaiatura più alta rispetto al controllo: il trattamento con antitraspirante (552 mg/L) apporta un contenuto significativamente maggiore a vendemmia degli antociani estraibili rispetto al controllo (402,85 mg/L).

Mettendo in relazione il contenuto zuccherino dell’acino con il contenuto in polifenoli ed antociani estraibili a vendemmia si identifica con chiarezza che l’antitraspirante ha il miglior equilibrio tra maturazione tecnologica e fenolica.

Infine i parametri produttivi (produzione/pianta e peso del grappolo) sono molto simili fra loro, senza differenze statisticamente significative tra le tesi.

Conclusioni

Nell’ottica di ridurre i fenomeni di sfasamento tra maturità tecnologica e fenolica ascrivibili alle interazioni tra i cambiamenti climatici in atto ed alle mutate tecniche di gestione della chioma, la distribuzione fogliare di un prodotto antitraspirante può rappresentare una valida pratica agronomica. In generale, si può ipotizzare che il prodotto, migliorando l’efficienza degli scambi gassosi nella pianta, deprime l’attività fisiologica della pianta indirizzandola verso la massimizzazione dell’accumulo di sostanze del metabolismo secondario. L’emulsione antitraspirante ritarda inoltre l’accumulo di zuccheri nell’acino, rappresentando così una strategia semplice ed economica alla problematica dello sfasamento tra maturità tecnologica e fenolica alla vendemmia. I parametri qualitativi hanno infatti messo in evidenza un ritardo nella maturazione delle bacche provenienti dalle viti trattate con antitraspirante rispetto al controllo.

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Tab. 1 - Tabella riassuntiva dei principali parametri misurati.
Tab. 1 - Summary table of the main measured parameters.

Tesi	°Brix	pH	Ac. tartarico (g/L)	Antociani tot. (mg/L)	Antociani estraibili (mg/L)	Polifenoli tot (mg/L)	Polifenoli estraibili (mg/L)
Antitraspirante	22,2	3,3	5	961	552	2.488	2.329
Controllo	23,5	3,4	4,7	773	403	2.225	2.112

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