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Metabolic and transcriptional changes associated with the use of *Ascophyllum nodosum* extracts as tools to improve the quality of wine grapes (*Vitis vinifera* cv. Sangiovese) and their tolerance to biotic stress

Tommaso Frioni,[a,b*] Sergio Tombesi,[a] Mara Quaglia,[b] Ornella Calderini,[c] Chiaraluce Moretti,[b] Stefano Poni,[a] Matteo Gatti,[a] Alessandro Moncalvo,[a] Paolo Sabbatini,[d] Julian Garcìa Berrìos[e] and Alberto Palliotti[b]

ABSTRACT

BACKGROUND: Recent studies report that *Ascophyllum nodosum* extracts, once applied on the canopy of different crops, deliver positive effects, increasing yield, inducing tolerance to biotic stress, and improving the quality of products. However, the mechanisms of action are still unclear. In this research, vines subjected to multiple foliar applications of an *A. nodosum* extract (ANE) at label doses were compared with untreated vines (NTV) in accordance with a comparative approach. The investigation coupled a field experiment with a second trial conducted under semi-controlled conditions, to clarify the mechanisms of action involved.

RESULTS: The biostimulant did not affect soluble solids or the acidity of grapes; instead, it improved their anthocyanin and phenolic concentrations and the respective profiles. At the time of harvest, anthocyanin, and phenolic concentration were increased by 10.4% and 14.5%, respectively, when compared to the NTV. These effects correlated with a specific modulation of genes involved in the flavonoid metabolic pathways. Moreover, grapes from ANE vines witnessed a significant reduction in the spreading of gray mold when they were either assessed in field conditions or *in vitro*, compared to the grapes of NTV vines. This was related to a significant upregulation of the defense-related genes of the plant.

CONCLUSIONS: Overall, the results showed that *A. nodosum* extracts can be valuable tools in viticulture considering the emergence of challenging environmental conditions; hence, the regulation of specific metabolic pathways is the mechanism of action that leads to an increased tolerance of biotic stress and of changes in the content of grape metabolites.

Supporting information may be found in the online version of this article.

Keywords: seaweed extract; grapevine; flavonoids pathway; *botrytis cinerea*; induced resistance; grape composition

INTRODUCTION

Climate change is affecting modern and traditional viticulture regions worldwide. Several profitable wine districts are facing the consequences of the unpredictability of extreme weather conditions and warming trends.[1,2] This scenario may lead to a drastic impairment of yields and may become detrimental to fruit and wine quality.[1–3] One of the most relevant components of fruit quality for the production of red wines is phenolic maturity.[3] It consists in the achievement of the desired concentration and profile of chromatic and phenolic substances in fruits.[3] Erratic weather conditions during ripening may uncouple phenolic maturity and technological maturity, leading to an asynchronous achievement of the optimal sugar and phenolic content.[2,3] Simultaneously, the change of environmental conditions may also increase the pressure of pathogens.[1] One of the main biotic stresses that affect grapevine

* *Correspondence to: T Frioni, Università Cattolica del Sacro Cuore, Via Emilia Parmense 84 – 29122 Piacenza, Italy. E-mail: tommaso.frioni@unicatt.it*

a *Department of Sustainable Crop Production (DI.PRO.VE.S.), Università Cattolica del Sacro Cuore, Piacenza, Italy*

b *Department of Agricultural, Food and Environmental Sciences (DSA3), Università degli Studi di Perugia, Perugia, Italy*

c *Institute of Biosciences and Bioresources (IBBR), National Research Council of Italy (CNR), Perugia, Italy*

d *Department of Horticulture, Michigan State University, East Lansing, MI, USA*

e *Departamento de Produccion Vexetal, Universidad de Santiago de Compostela, Escola Politecnica Superior, Lugo, Spain*

yield and fruit quality, especially in humid vintages and in susceptible genotypes, is bunch rot. *Botrytis cinerea* Pers. has been reported to be the main cause of bunch rot, which also affects wine quality.[4] Pest and disease protection of the vineyard is economically and environmentally expensive and, if not correctly implemented, may reduce economic returns and/or the quality of the environment. For these reasons, viticulture is seeking new alternative solutions to increase its sustainability.

Biostimulants are gaining interest among specialists for the positive effects they have on different crops.[5–8] Among them, the extracts obtained by the brown seaweed *Ascophyllum nodosum* (L.) Le Jolis (AN) are those most investigated for their potential application in agriculture.[7–9] Recent studies recognize that most of the effects of seaweed extracts and other biostimulants on crops rely on the modulation of specific metabolisms of target plants and not on the simple contribution of nutrients and chemical compounds, as previously hypothesized.[7–12] AN extracts modulate the hormonal plant signaling and enhance plant growth.[7–9,11,12] Moreover, AN extracts activate the systemic defense of the plants, leading to improved tolerance to pathogens.[13,14] The protective effects of *A. nodosum* extracts against *B. cinerea* and other plant pathogens have been reported for different host plants, such as carrot[10] and cucumber.[13,14] However, no report has been found that analyzes the effects of *A. nodosum* extracts on grapevines in relation to those of *B. cinerea* bunch rot.

Only a few published research papers have described the effects of AN extracts on grapevines, and these were mainly on table grapes, evidencing positive effects on productivity.[15,16] Recently, considering the increased phenolic content found in the treated tissues of other cultivated species,[17–19] foliar applications of AN extracts in viticulture have been proposed with the goal of improving the phenolic maturity of red grapes.[20–22] However, the mechanisms of action leading to higher anthocyanin and phenolic compound content are actually unknown and only hypothesized.

The aim of this paper was to evaluate the effects of foliar applications of an *A. nodosum* extract on the biosynthetic pathways of phenolic compounds of grapevines and on tolerance to biotic stress. Results observed in the field have been integrated with a trial conducted under semi-controlled conditions, with the aim of clarifying the mechanisms of action. The physiological and metabolic results were coupled with the analysis of the expression of specific genes involved in the pathways involved.

MATERIALS AND METHODS

Experiment 1

Site, plant material and treatments

A first experiment (Experiment 1) was performed in 2014 and 2015 in Deruta (PG), central Italy (42° 96′ 15″ N, 12° 40′ 78″ E, 250 m above sea level (a.s.l.), south exposition, north–south row orientation, loamy soil). The vineyard consisted of 32 × 15-year-old vines of *Vitis vinifera* L. (cv. Sangiovese clone VCR30, grafted on 420A rootstock), planted with spacing of 1.00 × 2.50 m (density of 4000 vines ha^{-1}) and trained to a vertical shoot positioned (VSP), spur-pruning trellis system. Further details of the vineyard's features and management are given in Frioni *et al.* (2018).[21]

The experimental layout consisted of 32 vines organized in a randomized complete block design (RCBD) with four blocks consisting of eight vines each and one factor (AN extract foliar application), 16 vines per treatment.

Treatments were assigned in 2014, 3 weeks after the pea-size stage (as described by Coombe).[23] Half of the experimental vines were assigned to applications of the *A. nodosum* extract (Acadian® Marine Plant Extract Powder, Acadian Seaplants Limited, Dartmouth, NS, Canada) at labeled rates of 1.5 kg ha^{-1} (ANE). The product is a commercial *A. nodosum* extract, mainly composed of amino-acids, alginates, organic acids, and macro- and micro-nutrients in different concentrations. A full description of the product composition is reported in Hurtado *et al.*[24] The remaining vines were regarded as a control (NTV). The NTV vines were sprayed only with water at the same time and volume as the other treatment, following the approach of Faurie *et al.*[25] and Jayaraman *et al.*[14] A surfactant was added to all treatments, following the manufacturer instructions. Sprays were applied at 09:00 h, and this action was repeated on the same vines six times between the pea-size phenological stage[23] and harvest; this was done at intervals of 7–15 days. ANE solution was prepared by diluting 1.5 g of product per L of water (equal to 1.5 kg ha^{-1}), applying 0.25 L of solution per vine (1000 L/4000 vines). Details of the spraying schedule for Experiment 1 are given in Table S1 in the supporting iinformation.

Ripening kinetics, harvest parameters and fruit composition. Three groups of 100 berries per treatment were sampled periodically from veraison to harvest (DOY 209, 220, 232, 240, 251, 259, 267 in 2014 and DOY 218, 224, 232, 239, 246 in 2015, with intervals of 6 to 12 days) to describe the ripening progression as total soluble solids (TSS), pH, titratable acidity, and total concentration of anthocyanins and phenolics in skins. The harvest date was set at the time when grapes from NTV reached a TSS concentration of ∼22 Brix or a skin total anthocyanin concentration of 0.3 mg cm^{-2}. At the time of harvest, all the vines under experiment were manually harvested; the vine yield was measured, and the number of bunches per vine was recorded. The incidence of bunch rot (percentage of the total number of bunches showing symptoms) on each vine was assessed. Three representative bunches per vine were sampled and brought to the lab. The bunch and the rachis weight were recorded, the number of berries per bunch was counted, and the average weight of berries was calculated. The severity of bunch rot was calculated as the percentage of berries showing symptoms with regard to the total number of berries counted in each bunch. A sample of 90 healthy berries per vine (30 from each sampled bunch) was collected from each vine to analyze the fruit chemistry – total soluble solids (TSS), titratable acidity (TA), must pH, and total skin anthocyanins and phenolics. Another group of ten berries from each sampled bunch was used to determine the skin : pulp ratio by weighing the berry, carefully peeling the skin from each of them and weighing the skin separately. Berries pertaining to samples that were collected to analyze fruit chemistry were crushed to obtain a must. The TSS concentration was analyzed with a temperature-compensating RX 5000 refractometer (Atago-Co Ltd.), and the pH was assessed using a digital PHM82 pH meter (radiometer). A Titrex Universal Potentiometric Titrator (Steroglass S.R.L.) was used to measure the TA by titrating with 0.1 N NaOH until an end-point of pH 8.2. The concentration of the titrating agent needed to obtain a pH of 7 was assumed as TA value and expressed in g L^{-1} as tartaric acid equivalent.

Skin total anthocyanin and phenolic concentration in the berry skins was determined following the procedures reported by Frioni *et al.*[21] and the methods described in Iland *et al.*[26]

Experiment 2

Site, plant material, and treatments

A second experiment (Experiment 2) was conducted in 2015 in an outdoor space of the Department of Agricultural, Food and Environmental Sciences, University of Perugia, in the urban area of the city of Perugia, central Italy (43° 10′ 30″ N 12° 39′ 45″ E, 405 m a.s.l). Twenty × 60 L pots were filled with loamy soil having a field capacity of 30.2% {(volume water/volume soil) × 100} and a wilting point of 16.7%. Each pot contained a 5-year-old vine cv. Sangiovese (clone VCR30, grafted on 1103 Paulsen). Vines were spur-pruned in winter with three spurs carrying two buds each. During growth, developing shoots were directed upright using suitable stakes. Pots were maintained at field capacity throughout the experiment by an automated water-supply system providing water to each pot for 1 min three times per day (08:00 h, 13:00 h and 18:00 h, 6 L per vine per day). Pest control was carried out based on expertise and as per local standards.

During the season, half of the vines were subjected to multiple foliar applications (ANE) of Acadian Marine Plant Extract Powder (Acadian Seaplants Limited). The product was sprayed at the rate of 3 g plant^{-1}, which had already been tested for grapevines and other species;[21,22] the formulate was adequately diluted in water, and a surfactant was added. The other ten vines were assigned to the control treatment (NTV) at the same dates and sprayed only with water, according to Faurie *et al.*[25] and Jayaraman *et al.*[14] The full-canopy sprays were performed at 08:00 h; this was repeated five times during the season. Details of the spraying schedule for Experiment 2 are given in Table S2 in supporting information.

Harvest parameters, fruit composition and total phenolics in the skin

The harvest date was set at the time when grapes reached a TSS concentration of ∼22 Brix. During harvest, all vines except three were manually harvested, the weight of the grapes was measured, and the number of bunches per vine was recorded. Three representative bunches were sampled from each vine and then brought to the laboratory. The average weight of bunch and berry was determined as described for Experiment 1. A sample of 90 berries (30 berries from each sampled bunch) was collected from each vine to analyze TSS, pH, TA and total anthocyanin and phenolic concentrations in the skins with the same methods as those adopted for Experiment 1.[21,26]

Metabolic analysis and flavonoids pathway genes

Based on the results of a recent study on the effects of an *A. nodosum* extract on the phenolic maturity of grapevines, three different stages were identified during ripening.[21] The first stage corresponded to the achievement of full veraison (i.e., all berries showing complete pigmentation) and a TSS concentration of ∼16 Brix (stage FV, DOY 222). The second stage corresponded to a TSS concentration of ∼19 Brix (stage MR, DOY 226). The third stage coincided with the technological maturity (stage TM, DOY 240), which was set when the grapes achieved a TSS concentration of ∼22 Brix.

At each stage, a ten-berry sample was collected at 10:00 h from each vine by carefully cutting each berry with scissors at the pedicel in order to avoid any damage or juice loss. Berries were always manipulated with gloves, immediately frozen in liquid nitrogen, and then stored at −80°C for subsequent gene expression analyses on skins by quantitative real-time polymerase chain reaction (RT-PCR).

For RNA extraction, the skin of half of each frozen berry was removed by using liquid nitrogen during the procedure. Approximately 100 mg of skin was isolated for each sample, and the rest of the berry was immediately stored at −80 °C to be processed for high-performance liquid chromatography (HPLC) analysis (see next paragraph). The total RNA was extracted with the Spectrum Plant Total RNA Kit (Sigma-Aldrich, St. Louis, MO, USA) following the manufacturer's protocol for fruits. The RNA was treated with DNase I (Ambion, Thermo Fisher Scientific, Waltham, MA, USA), and 250 ng of the total RNA was reverse transcribed using oligo(dT)18 primer and SuperScript III Reverse Transcriptase (Life Technologies, Carlsbad, CA, USA), in accordance with the manufacturer's instructions. The Q-PCR mix was as follows: 3 µL of cDNA (1:100 dilution), 5 µL of Power SYBR™ Green PCR Master Mix (Thermo Fisher Scientific), 1.5 µL of each primer (both forward and reverse at a concentration of 2.5 µµM), and 1.5 µL of molecular biology grade water for a total volume of 12.5 µL. The following PCR program was used on an ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA): 50 °C for 2 min and 95 °C for 10 s; 40 cycles of 95 °C for 15 s and 60 °C for 1 min, with a final cycle of 95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s and 60 °C for 15 s respectively. After each assay, a dissociation kinetic analysis was performed to verify the specificity of the amplification products. Three biological and three technical replicates were used.

Relative amounts of each transcript were calculated using the 2-ΔΔCT method.[27] The housekeeping gene ubiquitin conjugating factor (UbiCF) was used as an endogenous reference locus to normalize the expression levels of the target genes.[27] The primers to be used for Quantitative reverse transcription polymerase chain reaction(RT-qPCR) analysis for genes involved in the anthocyanin pathway were retrieved according to Castellarin *et al.*[28] and the literature available therein (namely UFGT = flavonoid 3-*O*-glucosyltransferase; OMT2 = flavonoid *O*-methyl transferase 2; LDOX = leucocyanidin dioxygenase; GST = glutathione S-transferase; F3′H = flavonoid 3′ hydroxylase; F3′5′H = flavonoid 3′5′ hydroxylase; DFR = dihydroflavonol reductase).

HPLC analysis

The remaining portion of the skin, from the same berries used for the above RT-PCR, was used to determine the concentration of phenolic compounds, including the assessment of the anthocyanin profile. After the separation of skin, the flesh of the berries in the same sample was mixed and crushed to obtain a must. This juice was used to determine the TSS, pH and TA for each sample to better describe and characterize stages FV, MR and TM. The methods adopted were the same as previously detailed.

All solvents used for analytic determinations were of the HPLC grade. The water of Milli-Q quality, acetonitrile, and methanol were obtained from VWR (Radnor, PA, USA). The concentration of the following compounds was accordingly measured: *t*-resveratrol, *t*-piceid, myricetin (Myr), myricetin 3-*O*-glucoside (Myr 3-*O*-glc), quercetin 3-*O*-glucuronide (Quer 3-*O*-glu), quercetin 3-*O*-glucoside (Quer 3-*O*-glc), kaempferol 3-*O*-glucoside (Kmp 3-*O*-glc), delphinidin 3-*O*-glucoside (Dp 3-*O*-glc), cyanidin 3-*O*-glucoside (Cy 3-*O*-glc), petunidin 3-*O*-glucoside (Pt 3-*O*-glc), peonidin 3-*O*-glucoside (Pn 3-*O*glc), malvidin 3-*O*-glucoside (Mv 3-*O*-glc), (+)-catechin, and (−)-epicatechin. Commercial standards from Extrasynthese (Genay, France) were used. L-(+)-tartaric acid, L-(−)-malic acid, citric acid, (+)-catechin, and (−)-epicatechin standards were purchased from Sigma-Aldrich (St Louis, MO, USA).

The grape samples were carefully manually peeled, and the resulting skins and seeds were immediately frozen and lyophilized.

Phenolic compounds were extracted from grape skins, following the reports of Downey and Rochfort.[29] In brief, 0.1 g of lyophilized grape skin was extracted in 1.0 mL of 50% (v/v) methanol in water for 15 min with sonication. The extracts were centrifuged (5 min at 10000×*g* at 4 °C), filtered through a 0.22 µm polypropylene syringe for HPLC analysis and transferred to HPLC auto-sampler vials.

A chromatographic method was developed using an Agilent 1260 Infinity Quaternary LC (Agilent Technology, Santa Clara, CA, USA), which consisted of a G1311B/C quaternary pump with an inline degassing unit, a G1329B autosampler, a G1330B thermostat, a G1316B thermostated column compartment, a G4212B diode array detector fitted with a 10 mm path, and a 1 µL Max-Light cartridge flow cell. The instrument was controlled using the Agilent Chemstation software version A.01.05.

Separation was achieved on a reverse-phase C18 Synergi Hydro-RP 80A, 250 × 4.6 mm, 4 µm (Phenomenex manufacturers, Torrance, CA, USA), according to Nicoletti *et al.*[30] The solvent used comprised 5% (v/v) formic acid (solvent A) and acetonitrile (solvent B). The flow rate was 0.5 mL min^{-1}, with a linear gradient profile consisting of solvent A with the following proportions (v/v) of solvent B: 0–10 min, 2–10% B; 10–25 min, 10–12% B; 25–35 min, 12–30% B; 35–43 min, 30% B; 43–48 min, 30–40% B; 48–52 min, 40–50% B; 52–55 min, 50–60% B; 55–58 min, 60–98% B; 58–63 min, 98% B; 63–66 min, 98–2% B; 66–72 min, 98% B. The column temperature was maintained at 40 ± 0.1 °C and 5 µL of the sample extract was injected. The elution was monitored at 200–700 nm, and the detection was done through UV–visual absorption with diode-array detector (DAD) scanning between 280, 320, 370, and 520 nm. Phenolic compounds were identified using authentic standards and by comparing the retention times. Quantification was based on peak areas and performed by external calibration with standards (Figure S1 in the supporting information). All anthocyanins were thereby expressed as malvidin 3-*O*-glucoside.

Organic acid concentrations at harvest were detected by analyzing the juice obtained from the collected samples. The must, diluted four times by water, was filtered through a 0.22 µm polypropylene syringe for HPLC analysis and transferred to the HPLC auto-sampler vials. For this analysis, an Allure Organic Acid Column, 300 × 4.6 mm, 5 µm (Restek, Bellefonte, PA, USA) was used. Separation was performed in isocratic conditions using water; the pH was adjusted at 2.5 using ortho-phosphoric acid, and the flow rate was 0.8 mL min^{-1}. The column temperature was maintained at 30 ± 0.1 °C, and 15 µL of sample was injected. The elution was monitored at 200–700 nm, and detection was done by UV–visual absorption with DAD at 210 nm. The organic acids were identified using authentic standards, and quantification was based on peak areas, performed by external calibration with standards.

Induction of defense-related gene transcripts accumulation

The induction of defense-related gene expression and the effectiveness of the induced resistance against the agent of gray mold were evaluated with regard to the three non-harvested vines per treatment.

For RNA extraction and real-time PCR analysis, 15 berries per vine were sampled carefully by cutting and handling with scissors and gloves; each berry was cut at the pedicel in order to avoid any damage or loss of juice. Dates of sampling were August 14, 2015 (DOY 226) and August 15, 2015 (DOY 227), at 0 h post last treatment (hpt) and 24 hpt; ANE vines were subjected to a whole canopy *A. nodosum* extract spray on August 14, 2015, as previously described. Samples were immediately frozen in liquid nitrogen and then stored at −80°C. RNA extraction was performed using Spectrum™ Plant Total RNA Kit (Sigma-Aldrich) on grape skin separated from the flesh as reported above, following the manufacturer's instructions for fruit sample. RNA concentration was measured with NanoDrop (Thermo Fisher Scientific). cDNA was synthetized using 250 ng of total RNA per sample as well as the SuperScript II RNAse H+ reverse transcriptase (Invitrogen). A real-time polymerase chain reaction was carried out according to the protocol of Santi *et al.*[31] in a CFX96 Real-Time PCR Detection System (Bio-Rad®, Hercules, CA, USA) using EvaGreen® dye (Bio-Rad ®, Hercules, CA, USA) and primers for *V. vinifera* – pathogenesis-related protein 1 (VvPR1),[32] callose synthase 2 (VvCaS2),[31] lipoxygenase (VvLOX), actin (VvActin) and Elongation factor 1-Cabernet sauvignon (VvEF1-Cs)[33] (Table S3 in supporting information). VvPR1 is an acidic protein that is dependent on the salicylic acid (SA) defense pathway,[32] VvLOX is an enzyme involved in the synthesis of jasmonic acid (JA) and thus in the JA defense pathway,[33] while VvCas2 is an enzyme whose upregulation can lead to a reinforcement of plant cell walls by apposition of the β-1,3-glucan callose (induction of callose apposition is a morphological host defense response common to different resistance pathways).[31] VvActin and VvEF1-Cs were used as reference genes.[33] Three technical and three biological replicates were performed for each gene. Analysis of defense-related gene expression was performed by CFX Manager Software (Bio-Rad®, Hercules, CA, USA) and the relative expression of the genes (RE) was calculated using the 2-ΔΔCT method as reported above.[27]

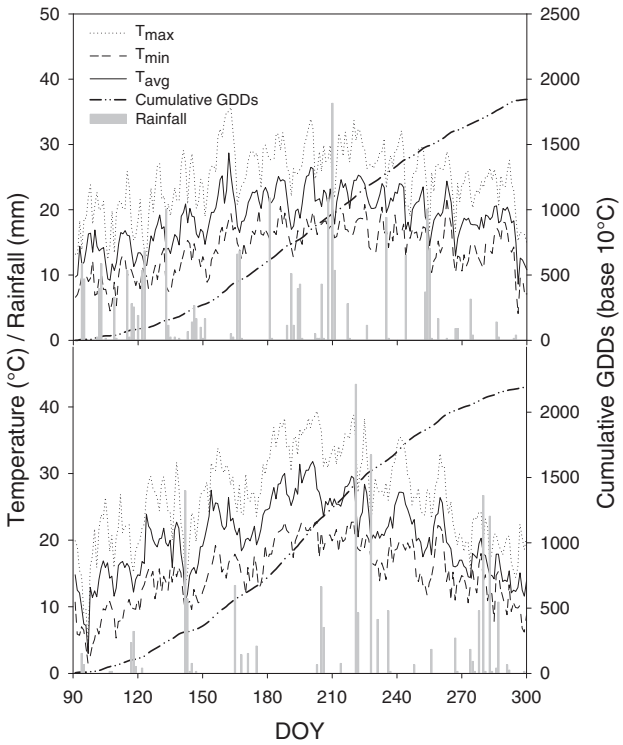

Figure 1. Weather evolution in the site of Experiment 1 (Deruta, PG, central Italy) in 2014 (upper panel) and in 2015 (lower panel) from April 1 to October 31. T_{max} = daily maximum temperature; T_{min} = daily minimum temperature; T_{avg} = daily average temperature; GDDs = growing degree days (calculated after Winkler);[39] DOY = day of year.

In vitro *gray mold assay*

An *in vitro* assay was also performed to test the protection conferred by the treatment with ANE against *B. cinerea*. Healthy

Table 1. Harvest parameters and berry morphology in 2014 and 2015 for field-grown grapevines cv. Sangiovese subjected to multiple canopy applications of an *A. nodosum* extract (ANE), in comparison with untreated vines (NTV)

	Yield kg vine^{-1}	Bunches per vine n	Bunch weight g	Berries per bunch n	Berry weight g	Skin/pulp ratio g/g	Bunch rot incidence %	Bunch rot severity %
NTV	2.99	14	213	113	1.89	0.156	20	12
ANE	3.15	15	208	116	1.80	0.163	13	8
2014	2.21	14	158	101	1.56	0.171	31	18
2015	3.90	15	259	136	1.92	0.149	2	4
T	ns[a]	ns	ns	ns	ns	ns	*	*
Y	***	ns	***	**	***	**	***	***
T × Y	ns	ns	ns	ns	ns	ns	***	***

[a] *, **, *** and ns indicate significant differences at $P \leq 0.05$, 0.01, and 0.001 or not significant, respectively.
T, Treatment; Y, Year.

Table 2. Basic fruit chemistry and phenolic maturity in 2014 and 2015 for field-grown grapevines cv. Sangiovese subjected to multiple canopy applications of an *A. nodosum* extract (ANE), compared with untreated vines (NTV)

	TSS Brix	pH	Titratable acidity g L^{-1}	Total anthocyanins mg cm^{-2}	Total phenolics mg cm^{-2}
NTV	20.4	3.27	6.20	0.336	0.812
ANE	20.3	3.32	6.31	0.371	0.930
2014	18.3	3.38	7.10	0.322	0.689
2015	22.5	3.23	5.38	0.385	1.053
T	ns[a]	ns	ns	**	*
Y	***	*	***	***	***
T x Y	ns	ns	ns	ns	ns

[a] *, **, *** and ns indicate significant differences at $P \leq 0.05$, 0.01, and 0.001 or not significant, respectively.
T, Treatment; Y, Year.

berries, which were uniform in size, were sampled by cutting at the pedicel on October 8, 2015 (DOY 261) at 1000 h (2 hpt), when they were more susceptible to *B. cinerea* infection,[34] from those three vines per treatment not harvested. Prior to their use, the surface of the detached berries was sterilized with 70% ethanol (v/v), rinsed in tap water, and wounded by sterile lancets, following the method advocated by Martinez-Romero *et al.*[35] Inoculation was performed with a suspension containing 1×10^5 conidia mL^{-1} of the PVUPG 240 isolate of the pathogen, which was prepared as described by Moretti *et al.*[36] and sprayed with a hand atomizer. Inoculated ANE and NTV berries were incubated in growth chambers (one chamber per vine, each containing 32 berries) at 21 ± 2 °C, 100% relative humidity (RH) and photoperiod of 12 h at 240 µmol m^{-2} s^{-1}. At 72 h post-inoculation (hpi), the incidence of infected berries was counted and the severity of symptoms on each berry was assessed visually using the five-point scale of Marois *et al.*[37] The data obtained were used to calculate the percentage disease index (PDI) using to the formula of McKinney.[38]

Weather conditions

Data regarding weather, during both experiments, was obtained by an automated weather station located near the vineyard (Experiment 1) and the outdoor area (Experiment 2). It recorded the daily minimum temperature (T_{min}), the daily maximum temperature (T_{max}), the daily average temperature (T_{avg}), and the daily rainfall. Thereafter, growing degree days and the Winkler index were calculated, as described by Winkler.[39]

Statistical analysis

The experiment 1 data were analyzed by a two-way ANOVA (treatment × year) using SigmaStat 3.5 (Systat Software, San Jose, CA, USA). When significant interactions between treatment and year were found, the results for the two experimental factors were shown separately in a figure included in the supporting information. The means between different treatments, Regarding ripening kinetics, the means of different treatments were analyzed distinctly for the 2 years involved and then separated by the Student's t-test .

Experiment 2 data were instead analyzed by a one-way ANOVA using the same software and the mean values were separated by Student's t-test. Regression analysis was performed using Sigma Plot 11.0 (Systat Software, San Jose, CA, USA). Data regarding defense-related gene expression were analyzed using a two-way (time × treatment) analysis of variance (ANOVA) and Duncan's-multiple comparison test ($P \leq 0.01$). Thereafter, a combined one-way (treatment) ANOVA and Duncan's-Multiple Comparison test ($P \leq 0.01$) were performed by combining the data mentioned above with the data derived from the *in vitro* assessment of infection, which was expressed in terms of the PDI.

RESULTS

Experiment 1

Weather evolution

The years 2014 and 2015 had distinctive weather patterns, which were experienced in the vineyard where Experiment 1 was carried

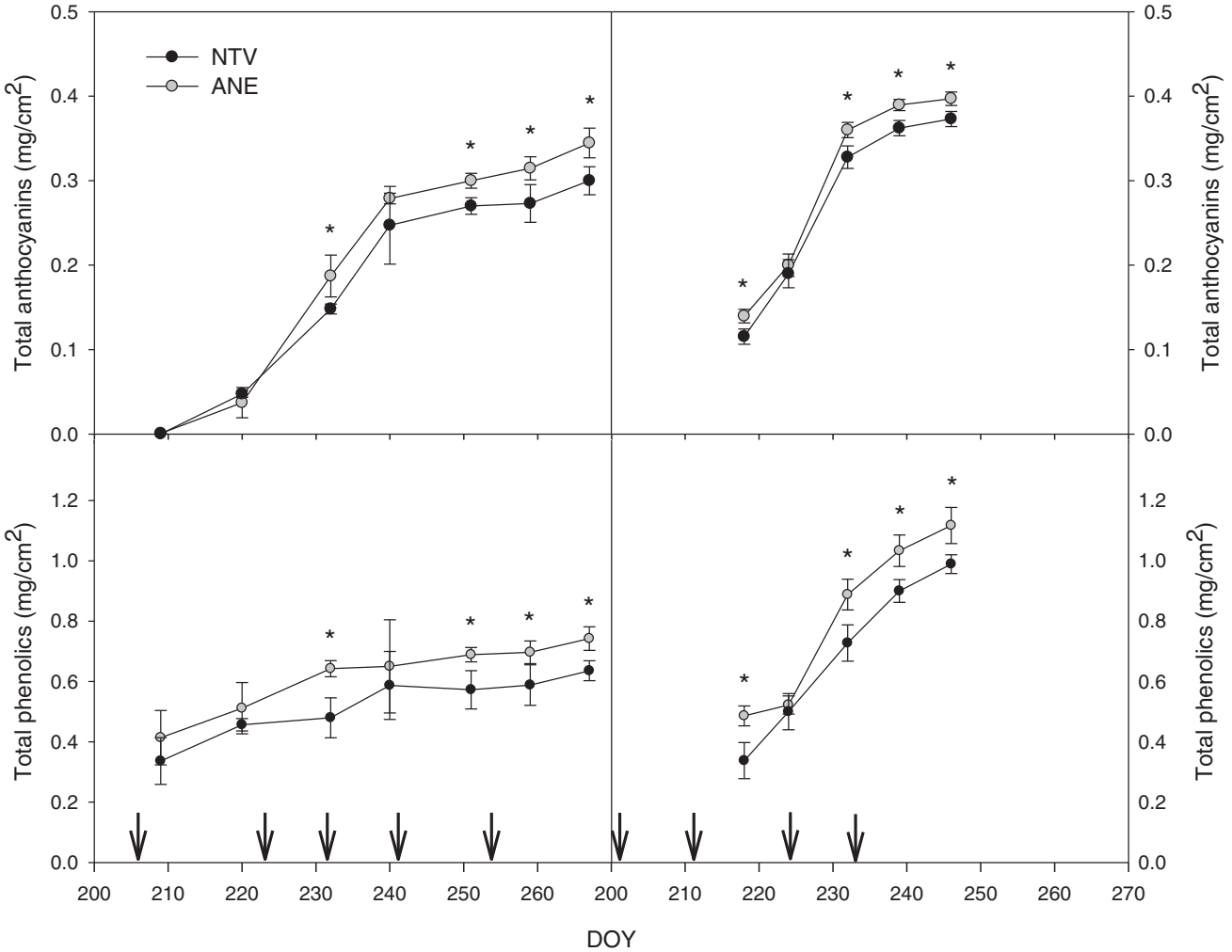

Figure 2. Evolution of skin total anthocyanins (upper panels) and total phenolics (lower panels) during ripening in 2014 (left panels) and 2015 (right panels) for field-grown grapevines cv. Sangiovese subjected to multiple canopy applications of an *A. nodosum* extract (ANE), in comparison with untreated vines (NTV). Each point is the mean of four values ± SE. Arrows represent dates of treatment application. Asterisks mean significant difference where $P \leq 0.05$ (Student's t-test).

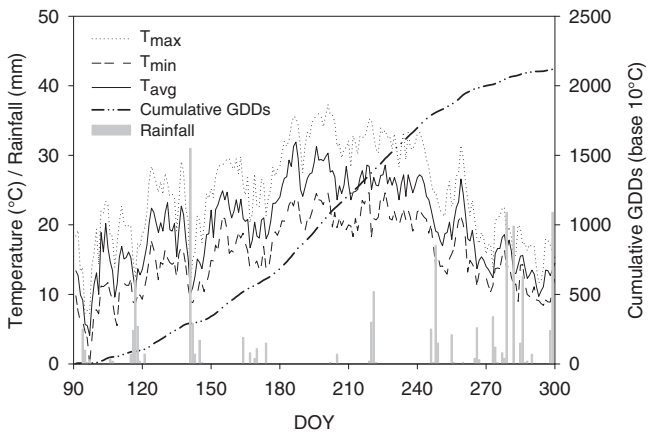

Figure 3. Weather evolution in the site of Experiment 2 (Perugia, PG, central Italy) from April 1 to Oct 31, 2015. Tmax = daily maximum temperature; Tmin = daily minimum temperature; Tavg = daily average temperature; GDDs = growing degree days (calculated after Winkler);[39] DOY = day of year.

out (Fig. 1). In the first year of the experiment, a relevant amount of rain (403 mm) was recorded between March 1 and October 31, with a total heat accumulation of 1848 GDDs, whereas in the same period in 2015, the GDD accumulation was much higher (2210 GDDs) and a lower amount rainfall (323 mm) was recorded. Considering the evolution of the two seasons, in 2014, T_{max} rarely rose above 33 °C (only in 5 days out of the entire summer), with rain being uniformly distributed throughout July, August, and September. On the other hand, in 2015, a dry period of intense hot air blew in the region from July onwards (550 GDDs were accumulated, with T_{max} peaking at 39.4 °C and T_{avg} ranging between 26 and 32 °C for the whole month). Rainstorms in mid-August (104 mm) were followed by another dry period comprising high temperatures, between late August and mid-September (334 GDDs were accumulated from August 25, 2015, and September 19, 2015).

Yield, bunch morphology, fruit sanity, and basic chemistry at harvest

The vines undergoing experiment reached harvest parameters on September 25, 2014 (DOY 268) and on September 3, 2015 (DOY 246).

No differences were found due to the application of seaweed extract on vine yield and bunch morphology that were, instead,

Table 3. Description of the three stages adopted for skin transcriptomic and metabolic analysis of berries sampled on potted grapevines cv. Sangiovese subjected to multiple canopy applications of an *A. nodosum* extract (ANE), in comparison with untreated vines (NTV)

	Stage FV[a]			Stage MR[a]			Stage TM[a]		
	TSS[b] Brix	pH	TA[b] g L^{-1}	TSS[b] Brix	pH	TA[b] g L^{-1}	TSS[b] Brix	pH	TA[b] g L^{-1}
NTV	15.7	2.83	13.7	18.9	3.07	9.8	21.5	3.31	6.0
ANE	15.9	2.75	12.9	18.8	3.11	9.6	21.7	3.33	5.8
	ns[c]	ns	ns	ns	ns	ns	ns	ns	ns

[a] FV, full veraison August 10, 2015 (DOY 222); MR, mid-ripening August 14, 2015 (DOY 226); TM, technological maturity August 28, 2015 (DOY 240).
[b] TSS, total soluble solids; TA, titratable acidity.
[c] * and ns mean respectively significance and not significant where $P < 0.05$ (Student's *t*-test).

significantly affected by the season (Table 1). Despite a similar number of bunches per vine being produced between the 2 years, in 2014, yield was lower (−43%) due to a reduction not only in the size of berries (−19%) but also the number of berries per vine (−26%). In 2014, berries had a higher skin : pulp ratio. The seaweed extract significantly reduced the incidence of *Botrytis* bunch rot and severity (−7% of rot symptomatic bunches, −4% of berries with symptoms) in comparison with NTV. Moreover, the spread of bunch disease was very different between the two seasons, with 31% of bunches presenting mold and an average of 18 rotten berries out of 100 in 2014, and only 2% of hit bunches and 4% of rotten berries in the subsequent year. As highlighted by the significant interaction between treatment and year, the seaweed extract delivered different results with respect to the two vintages that underwent field-trial.

The detail of interaction reported in Figure S2 in supporting information shows that the biostimulant's effects were relevant in 2014 but not in 2015.

At the time of harvest, September 25, 2014 (DOY 268) and September 3, 2015 (DOY 246), no difference was found in relation to basic fruit chemistry due to the application of the seaweed extract (Table 2). Again, the seasonal weather pattern had a primary role on fruit composition. In 2014, the grapes never achieved the threshold of 22 Brix, and at harvest, they had a TSS concentration 4.2 Brix lower than the subsequent year, with a higher pH and TA (+1.72 g L^{-1}).

Skin total anthocyanins and phenolics

The AN extract increased total anthocyanin (+10%) and phenolic (+15%) concentration in the skin consistently in the two seasons. The effects were found to be significant throughout the entire ripening course and to be similar between different vintages (Fig. 2). In 2014, treatments around veraison produced an increase in total anthocyanins and phenolics, which was already significant at DOY 232. Later (DOY 240), NTV reduced the differences to ANE, but subsequent seaweed extract applications delivered an increase of anthocyanins and phenolics concentrations that resulted significant until harvest. In 2015, applications around veraison led to an increase in concentrations from the first fruit sampling (DOY 217). Differences between treatments were found to be not significant later during the season (DOY 224), which was similar to what was observed in the previous year, but after new biostimulant applications the total anthocyanins and phenolics were observed to be higher in ANE and the differences were maintained until harvest. In 2015, biosynthesis of anthocyanins and phenolics in the skins was faster, and at harvest, the total concentrations were significantly higher than in the previous season.

Experiment 2

Weather evolution

In the outdoor area where Experiment 2 was performed, 2129 GDDs were accumulated from April 1, 2015 to October 31, 2015 (Fig. 3). In the same period, a total rainfall of 240 mm was recorded, with precipitation particularly concentrated during spring (31 mm on DOY 142) and in October (totally 90 mm). During the summer, a long hot period occurred between DOY 175 and 218, when the recorded T_{avg} was between 23.1 and 31.9 °C – just interrupted by 1.6 mm of rainfall between DOY 202 and DOY 205 – without a significant decrease in temperature. The warmest day of the year was July 20 (DOY 201), when the T_{min} was 24.6 °C and T_{max} was 37.3 °C. After 17 mm of rainfall between DOY 219 and DOY 222, a new dry period was recorded from DOY 223 until DOY 245, with a T_{avg} of ∼26°C and no rain.

Regulation of flavonoid biosynthetic pathway and evolution of skins' phenolic profile

During Experiment 2, no difference was found in the TSS concentration of grapes and acidity of NTV and ANE grapes at the three evaluation stages (Table 3). At the FV stage, grapes had a must of ∼15.8 Brix, a pH of ∼2.79 and a TA of ∼ 13.3 g L^{-1}. At the MR stage, the TSS concentration of grapes was ∼18.9 Brix, with a pH of 3.09 and a TA of 9.7 g L^{-1}. At the last stage of evaluation, which coincided with the technological maturity of grapes, the sugars comprised between 21.5 and 21.7 Brix, with a pH of ∼3.32 and a TA of ∼5.9 g L^{-1} independent of the treatment.

Coinciding with the FV stage, no difference between treatments was found in the relative expression (RE) of the seven tested genes (Fig. 4(a)). At that moment, independent of the treatment, OMT2 and F3′5′H were the most expressed transcripts. At the MR stage (Fig. 4(b)) most of the evaluated genes were exhibiting higher levels of transcripts on ANE grapes (UFGT +35%, LDOX +52%, GST +63%, F3′H + 68%, F3′5′H + 79% and DFR + 98%, as compared with NTV grapes). Only for OMT2 was no difference found between treatments. Independent of the treatment, all of the transcripts were quantitatively less expressed than the previous stage except DFR, which was the most expressed gene at the MR stage, and F3′H, which exhibited a difference between treatments (NTV -24% and ANE +50%, when compared to the transcripts found at the FV stage). When the berries achieved technological maturity (Fig. 4(c)), RE of UFGT, OMT2, LDOX, GST and F3′H was higher in NTV grapes; meanwhile, no difference was found between F3′5′H and DFR. UFGT, OMT2, LDOX and F3′5′H in NTV grapes had a higher RE than the previous stage and ANE grapes had a lower expression than in the previous stage. At

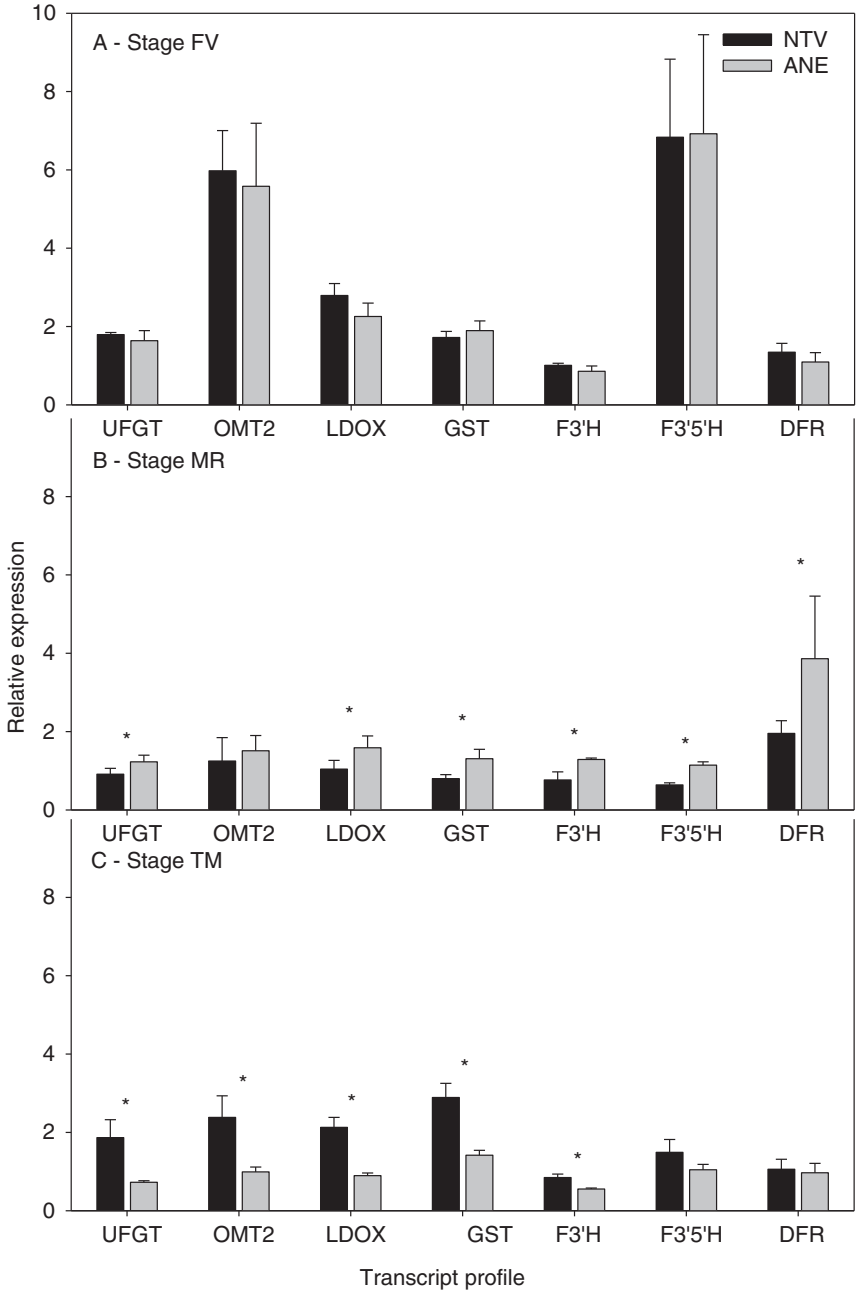

Figure 4. Relative expression of seven genes of the flavonoid pathway in skins of berries sampled on potted grapevines cv. Sangiovese subjected to multiple canopy applications of an *A. nodosum* extract (ANE), in comparison with untreated vines (NTV). Vertical bars represent the average of three replicates ± standard error. Asterisks mean significant difference where $P \leq 0.05$ (Student's t-test). FV = full veraison August 10, 2015 (DOY 222); MR = mid-ripening August 14, 2015 (DOY 226); TM = technological maturity, August 28, 2015 (DOY 240). UFGT = flavonoid 3-*O*-glucosyltransferase; OMT2 = flavonoid *O*-methyl transferase 2; LDOX = leucocyanidin dioxygenase; GST = glutathione *S*-transferase; F3'H = flavonoid 3′ hydroxylase; F3′5′H = flavonoid 3′5′ hydroxylase; DFR = dihydroflavonol reductase.

this stage, DFR had a significantly lower RE than the MR stage, independent of the treatment.

At the FV stage, the skins of ANE had higher concentrations of Dp 3-*O*-glc (+5%), Pt 3-O-glc (+24%) and Mv 3-*O*-glc (+45%) than the skins of NTV (Fig. 5(a)). Instead, the concentration of Cy 3-*O*-glc and Pn 3-*O*-glc was not different, as observed between treatments. Consequently, at this stage the proportion of tri-substituted anthocyanins was higher on the skins of ANE, as well as the total concentration of 3-*O*-glc anthocyanins (+0.75 mg g^{-1}). At the MR stage, only the concentration of Cy 3-*O*-glc was significantly higher (+8%) on ANE grapes (Fig. 5(b)). Again, the total concentration of 3-*O*-glc anthocyanins was higher in ANE than NTV (+0.84 mg g^{-1}). Independent of the treatment in comparison with the previous stage, the abundance of all the different compounds increased, by values included between 0.2 and 0.06 mg g^{-1}, except for Pt 3-*O*-glc, whose values remained similar to the ones detected at the FV stage. Once technological maturity was achieved (Fig. 5(C)), Dp 3-*O*-glc, Cy 3-*O*-glc, and Pt 3-*O*-glc were again higher in skins of ANE (+22%, +24%, and + 12%, respectively, as compared to NTV), while no differences were found in the abundance of Pn 3-*O*-glc and Mv 3-*O*-glc. Between treatments. The total concentration of 3-*O*-glc anthocyanins at Stage TR were significantly higher in ANE

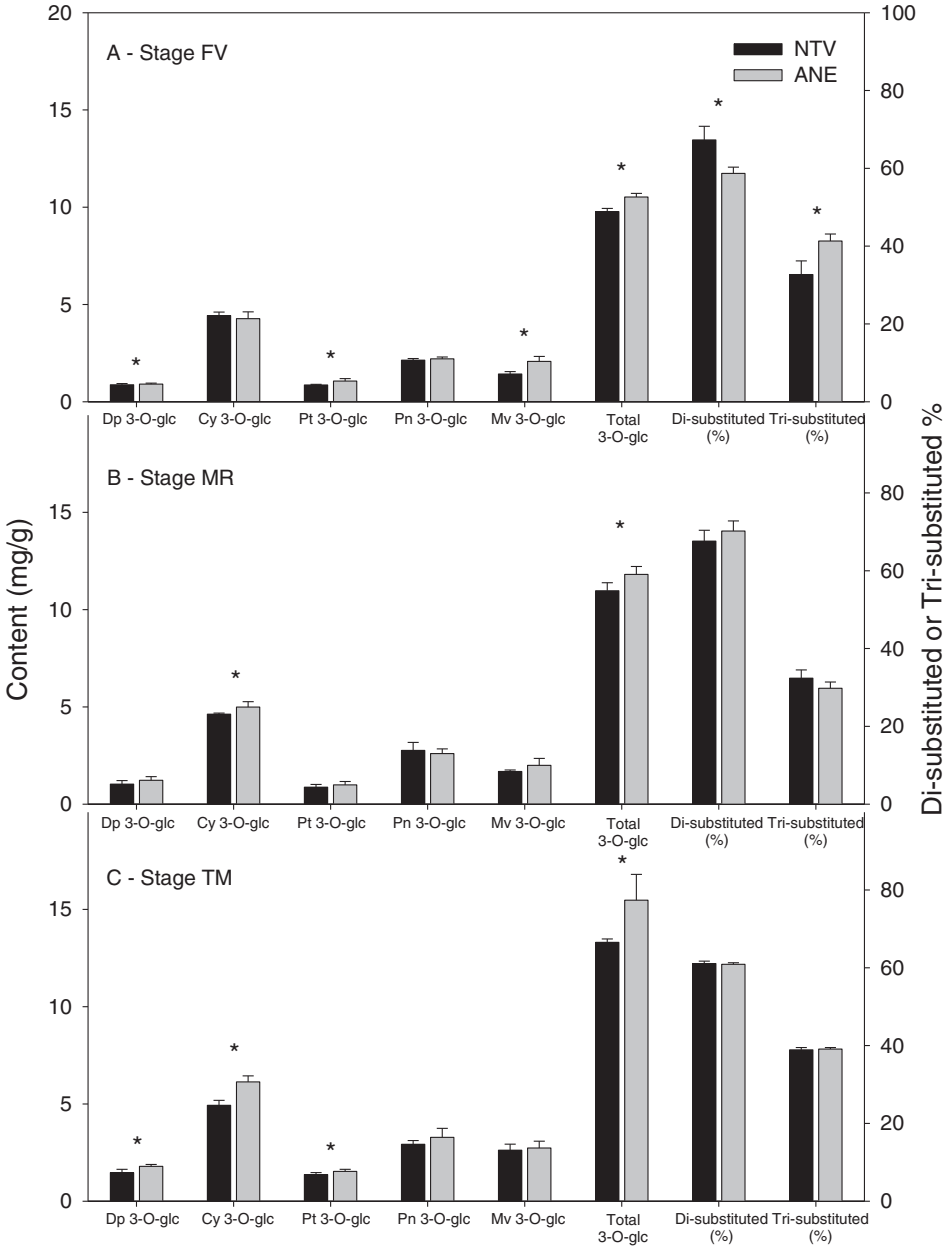

Figure 5. Skin anthocyanin profile of berries sampled on potted grapevines cv. Sangiovese subjected to multiple canopy applications of an *A. nodosum* extract (ANE), in comparison with untreated vines (NTV). Vertical bars represent the average of three replicates ± standard error. Asterisks mean significant difference where $P \leq 0.05$ (Student's t-test). FV = full veraison, August 10, 2015 (DOY 222); MR = mid-ripening August 14, 2015 (DOY 226); TM = technological maturity, August 28, 2015 (DOY 240). Data expressed as mg of Mv 3-*O*-glc equivalent per g of dry weight. Dp 3-*O*-glc = delphinidin 3-*O*-glucoside, Cy 3-*O*-glc = cyanidin 3-*O*-glucoside, Pt 3-*O*-glc = petunidin 3-*O*-glucoside, Pn 3-*O*-glc = peonidin 3-*O*-glucoside, Mv 3-*O*-glc = malvidin 3-*O*-glucoside.

(+2.17 mg g^{-1}), without any change of the proportions between di-substituted and tri-substituted compounds.

Regression between TSS and skin 3-*O*-glc anthocyanidins concentration across the three ripening stages confirmed that the effects of the seaweed extract were different in relation to the different compounds and to the ripening progression (Fig. 6). All the correlations were described by the exponential growth function. Confidence (R^2) of the correlation between Dp 3-*O*-glc and TSS was 0.95 for NTV and 0.99 for ANE (Fig. 6(a)). Considering Cy 3-*O*-glc, the R^2 of the relations was 0.74 for NTV and 0.95 for ANE (Fig. 6(b)). Regression analysis between Pt 3-*O*-glc and TSS revealed an R^2 of 0.89 for NTV and 0.79 for ANE (Fig. 6(c)). The correlation between Pn 3-*O*-glc and sugars had a confidence (R^2) of 0.84 for NTV and 0.81 for ANE (Fig. 6(d)). Finally, relations between Mv 3-*O*-glc and TSS had an R^2 of 0.94 for NTV and 0.68 for ANE (Fig. 6(e)).

Yield, bunch morphology and fruit quality at harvest

Potted grapevines satisfied the harvest criteria (TSS ~22 Brix) on August 28, 2015 (DOY 240). At harvest, the ANE and NTV vines were carrying an average of 1.11–1.21 kg $vine^{-1}$ of fruit with no differences being induced by the treatments (Table S4). The average bunch weight and bunch morphology was not affected by the seaweed extract – neither was the basic fruit chemistry. The concentration of the different organic acids was

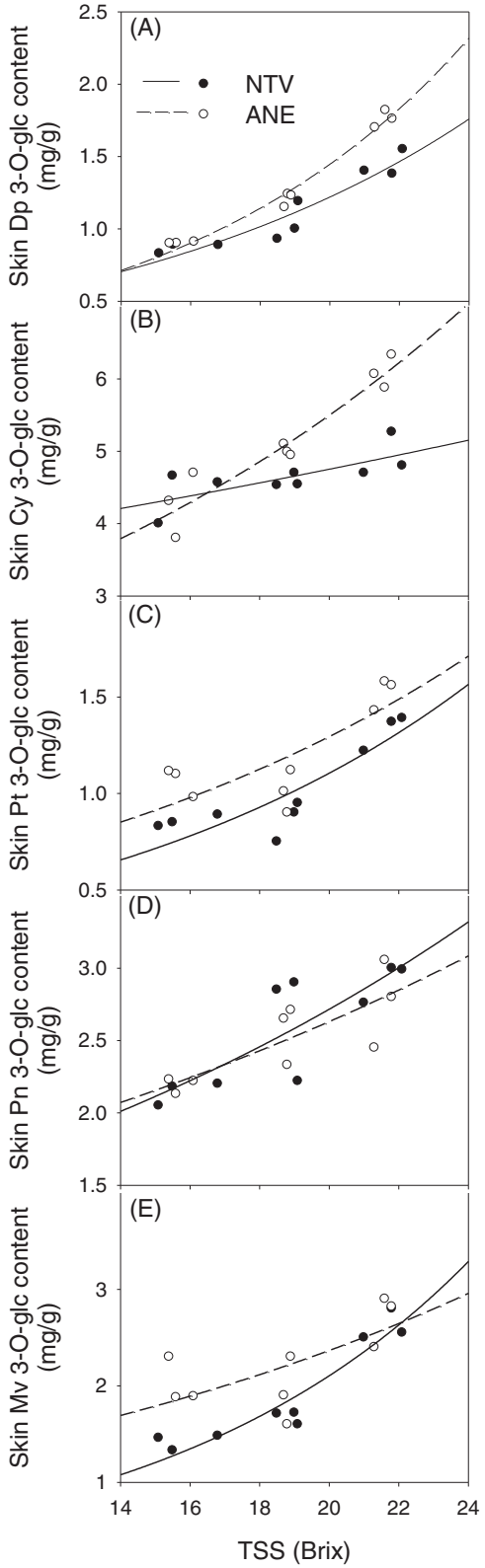

Figure 6. Correlation between total soluble solids (TSS) and the content of different anthocyanidins in skins of berries sampled on potted grapevines cv. Sangiovese subjected to multiple canopy applications of an *A. nodosum* extract (ANE), in comparison with untreated vines (NTV). Data expressed as mg of Mv 3-*O*-glc equivalent per g of dry weight. Dp 3-*O*-glc = delphinidin 3-*O*-glucoside (panel A), Cy 3-*O*-glc = cyanidin 3-*O*-glucoside (panel B), Pt 3-*O*-glc = petunidin 3-*O*-glucoside (panel C), Pn 3-*O*-glc = peonidin 3-*O*-glucoside (panel D), Mv 3-*O*-glc = malvidin (panel E).

similar in grapes belonging to ANE as well as NTV. At the time of harvest, Dp 3-*O*-glc, Cy 3-*O*-glc, and Pt 3-*O*-glc were higher in ANE (see previous paragraph), whereas no difference in the proportion between di-substituted and tri-substituted anthocyanins and in the concentration of acylated or coumarated was found after the treatment (Table 4). The skins of ANE grapes, when compared with skins of NTV grapes, had +5% of their total anthocyanin concentration expressed on the skin surface basis and + 16% of total 3-*O*-glc anthocyanins expressed on the skin mass basis.

The seaweed extract application led to a change in the phenolic profile of grapes at harvest (Table 5). Skins of ANE grapes had a significantly lower amount of (+)-catechin and (−)-epicatechin (−14% and − 15% respectively). In addition, the concentrations of Kmp 3-*O*-glc, Quer 3-*O*-glc, and Quer 3-*O*-glu were significantly lower in ANE (−3.6 μg g^{-1}, −222 mg g^{-1}, and −194 mg g^{-1} than NTV respectively); meanwhile the abundance of Myr and Myr 3-*O*-glc increased with the application of the seaweed extract (+65.83 μg g^{-1} and + 20.11 μg g^{-1} more than NTV respectively). Instead, the foliar sprays did not affect the t-resveratrol and t-piceid concentration (not shown). With regard to Experiment 2, the total concentration of phenolic compounds at the time of harvest was observed to be significantly higher in skins of ANE grapes (~25%).

Defense-related gene expression and reduction of B. cinerea *infections*

At 24 hpt, in berries that were harvested from ANE treated plants, the RE of the defense-related genes VvPR1 and VvCas2 was significantly higher in comparison with NTV (Fig. 7). At the same time, no difference between treatments was found in the RE of the *VvLOX* (Fig. 7) and no differences were detected between treatments at 0 hpt in the RE for the three defense-related genes (Fig. 7).

At 72 hpi, the PDI was significantly different and lower in ripe berries harvested from the ANE-treated plant and artificially inoculated with *B. cinerea*, in comparison with those of berries harvested from NTV (Fig. 8).

DISCUSSION

The lack of information about the physiological response of grapevines to the application of seaweed extracts and about the mechanisms of action impairs a full evaluation of the technique profitability. In this research, we evaluated the effects of an *A. nodosum* extract in field conditions (Experiment 1). We then investigated the mechanisms of action in a semi-controlled environment, integrating transcriptomic, metabolic, and physiological results (Experiment 2).

The effects of the seaweed extract were consistent over field and semi-controlled conditions. Productivity and cluster morphology were not affected by medium-late application of the seaweed extract, in accordance with previous findings.[20–22] Under field conditions, the *A. nodosum* extract improved anthocyanin and phenolic concentration in the skins, without significantly affecting the basic fruit chemistry. The evaluation of ripening evolution revealed that the effects were particularly evident around veraison and over the last part of ripening, in agreement with Frioni *et al.*[21] and Salvi *et al.*[22] Although the two vintages were notably different, with 2014 having low heat accumulation and high pathogen pressure, resulting in a slow ripening despite a lower vine yield, the effects of the *A. nodosum* extract on phenolic concentration were consistent between seasons.

Table 4. Berry skin anthocyanin profile at harvest for potted grapevines cv. Sangiovese subjected to multiple canopy applications of an *A. nodosum* extract (ANE), in comparison with untreated vines (NTV)

	Di-substituted[a] %	Tri-substituted[a] %	Acylated[a] mg g^{-1}	Coumarated[a] mg g^{-1}	Total anthocyanins[b] mg cm^{-2}	Total 3-*O*-glc anthocyanins[a] mg g^{-1}
NTV	61.1	38.9	Trace	0.07	0.322	13.31
ANE	60.9	39.1	Trace	0.07	0.348	15.48
	ns[c]	ns		ns	*	*

[a] Determined by HPLC, data expressed as mg of Mv 3-*O*-glc equivalent on g of dry weight.
[b] Determined after Iland.[26]
[c] * and ns mean respectively significance and not significant where $P < 0.05$ (Student's *t*-test).

Table 5. Berry skin phenolic profile at harvest for potted grapevines cv. Sangiovese subjected to multiple canopy applications of an *A. nodosum* extract (ANE), in comparison with untreated vines (NTV)

	(+)-catechin µg g^{-1}	(−)-epicatechin µg g^{-1}	Kmp 3-*O*-glc [a] µg g^{-1}	Myr 3-O-glc [a] µg g^{-1}	Quer 3-*O*-glc [a] µg g^{-1}	Quer 3-*O*-glu [a] µg g^{-1}	Myr[a] µg g^{-1}	Total phenolics[b] mg cm^{-2}
NTV	44.2	89.4	13.4	149.2	1258	1996	167.6	0.89
ANE	38.1	75.9	9.8	169.3	1036	1802	233.4	1.11
	*c	*	*	*	*	*	*	*

[a] Determined by HPLC, data expressed as µg on g of dry weight., Kmp 3-*O*-glc = kaempferol 3-*O*-glucoside, Myr = myricetin, Myr 3-*O*-glc = myricetin 3-*O*-glucoside, Quer 3-*O*-glc = quercetin 3-*O*-glucoside, Quer 3-*O*-glu = Quercetin 3-*O*-glucoronide.
[b] Determined after Iland.[26]
[c] * and ns mean respectively significance and not significant where $P < 0.05$ (Student's *t*-test).

Results from the pot trial (Table 4, Table 5) were coherent with the field trial. Moreover, Experiment 2 demonstrated, in agreement with Salvi *et al.*,[22] that the functional effects of the class of anthocyanins under evaluation might be different and that functional changes in each anthocyanidin, during the ripening stage, may be different. Mv 3-*O*-glc concentration was affected, especially when the TSS comprised between 14 and 20 Brix (Fig. 6(e)), whereas Dp 3-*O*-glc and Cy 3-*O*-glc concentration was particularly enhanced over 20 Brix (Fig. 6(a), Fig. 6(b)). ANE grapes had higher Pt 3-*O*-glc independent of the TSS concentration (Fig. 6(C)); on the other hand, Pn 3-*O*-glc seemed to be unaffected by the treatment all throughout ripening (Fig. 6(D)). Salvi *et al.*[22] proposed that *A. nodosum* extracts can have different effects on single anthocyanidines but, as far as we know, this was the first time that the differential effect of a biostimulant on a single anthocyanidin compound was described in relation to the TSS concentration in grapes.

In Experiment 2, after veraison (FV stage) – corresponding to a TSS concentration of ∼15.8 Brix – the relative expression (RE) of the seven genes under analysis was similar between ANE and NTV. The concentration of all the tri-substituted anthocyanins and the total concentration of the 3-*O*-G chromatic compounds was significantly higher in ANE. The analysis of the MR stage revealed a higher relative expression of all the analyzed transcripts (except for OMT2) in ANE. At the stage of technological ripening (the TM stage) – corresponding to 14 days after the last application of *A. nodosum* extract – a higher 3-*O*-G anthocyanin concentration in ANE grapes coincided with a higher RE of all the analyzed genes, except for F3′5′H and DFR, in the skins of NTV berries. Considering that ANE grapes already had a considerable number of 3-*O*-G anthocyanins (16% higher than NTV), it is likely that the RE of anthocyanin-related genes was lower because tissues were closer to the genotype's total natural accumulation of chromatic compounds, whereas NTV, at the TM stage, was still actively accumulating anthocyanins to reach the varietal maximum abundance. This explanation is compatible with the reported decrease in the RE of UFGT, LDOX, and GST activity in the last stages of ripening.[40] Overall, *A. nodosum* extracts were capable of inducing a genetically related flavonoid biosynthesis after veraison, especially in the hours that followed the application. Instead, at full veraison, or after several days from the treatment, the RE of the flavonoid pathway was unaffected.

Considering the ripening progression, the biostimulant's applications at veraison were particularly effective on tri-substituted anthocyanins. Foliar sprays at the mid-ripening stage (MR stage) could modulate biosynthesis of anthocyanins and phenolics, and increase their concentration until harvest, anticipating the achievement of the final concentration of different metabolites. This confirms and explains previous findings of various authors who increased anthocyanins, flavonoids and phenolic compounds in tissues of different plants subjected to applications of *A. nodosum* extract.[17–22,41–43]

Interestingly, the seaweed extract very much changed the phenolic profile of berries' skins at harvest. The ANE grapes had lower (+)-catechin, (−)-epicatechin, Kmp 3-*O*-glc, Quer 3-*O*-glc, and Quer 3-*O*-glu concentration, and higher Myr 3-*O*-glc and Myr concentration, if compared to NTV. The seaweed extract therefore stimulated the tri-substituted flavonol biosynthesis. This can be related to the higher levels of transcripts found for F3′5′H at the MR stage and the lower levels of transcripts for F3′H found at the TM stage. The two enzymes, indeed, competitively control the biosynthesis of tri-substituted and di-substituted flavonoids.[44,45] Quer 3-*O*-glc, (+)-catechin and (−)-epicatechin can be related, from a sensorial point of view, to sensations of bitterness and astringency and their abundance can be detrimental to certain styles of wine.[46] On the other hand, flavonols can be essential for the co-pigmentation of anthocyanins and the stability of color and of wine in general.[47] The reduction of Quer 3-*O*-glc, Quer 3-*O*-glu,

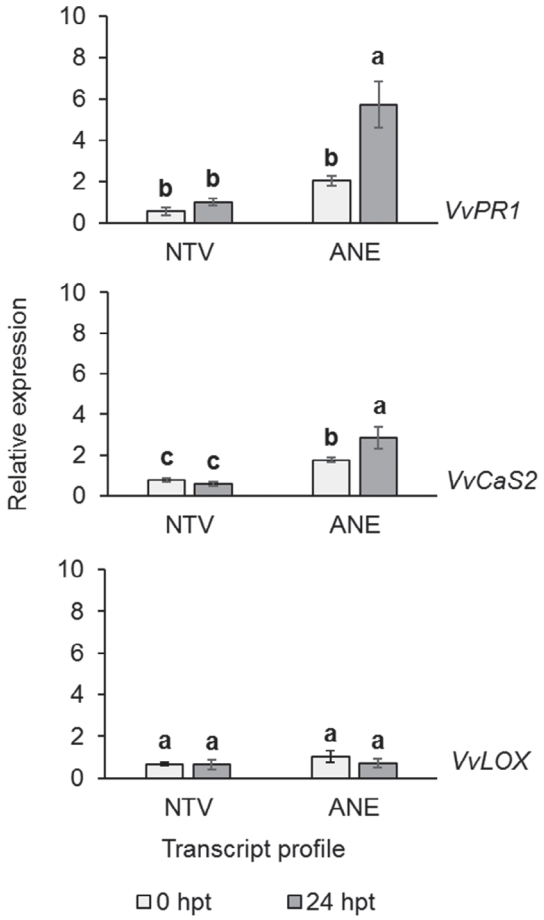

Figure 7. Relative expression of defense-related genes in skins of grape berries sampled on potted vines, cv. Sangiovese, subjected to multiple foliar applications of Acadian Marine Plant Extract Powder (ANE) or on water sprayed control vines (NTV) at 0 h and 24 h post-treatment (hpt) on August 14, 2015 (DOY 226). Each column represents the mean ± standard error of three technical and three biological replicates. Different letters indicate significant difference (Duncan's test, $P \leq 0.01$). VvPR1 = *V. vinifera* Pathogenesis related protein 1; VvCaS2 = *V. vinifera* Callose Synthase 2; VvLOX = *V. vinifera* Lipoxygenase.

(+)-catechin and (−)-epicatechin, coupled with an increased concentration of Myr, Myr 3-*O*-glc, total anthocyanins and phenolics, found on ripe grapes of ANE, can be considered a relevant change of fruit quality for the production of distinctive styles of red wines. Although an increase of flavonol derivatives and total phenolics in grape skins had already been observed,[20–22] the effects of the seaweed extract on the different class of phenolic compounds was never investigated before.

The genetic induction of flavonoid biosynthesis by *A. nodosum* extracts was never demonstrated and this is the first time that the RE of genes that are involved in the flavonoid pathway was analyzed together with determination of metabolites in plant tissues subjected to the seaweed extract. The positive modulation of the pathway is of special interest in relation to red grapes, as anthocyanin and phenolic profiles are essential components enhancing the quality of grapes required for premium red wine.

Consistent with findings reported for other plant species,[10,13,14] our work suggested, for the first time as far as we are aware, the protective effect of treatment – with *A. nodosum* extracts – against *B. cinerea* bunch rot on grapes. As the berries were thoroughly disinfected and rinsed before artificial inoculation, we can exclude the notion that the protection observed here was connected to the direct antimicrobial effect of *A. nodosum* extracts.[48] An indirect effect of the treatment, which could be linked to the induction of host defenses, has thus been supposed. Similar to what had been observed on carrot[10] and cucumber,[13,14] vines treated with *A. nodosum* extracts resulted in increased levels of transcripts of defense-related genes. In particular, and in accordance with Jayaraj *et al.*[10] and Jayaraman *et al.*,[14] berries harvested at 24 hpt from ANE treated vines had higher levels of the transcripts of the VvPR-1, which could be used as a marker of the SA defense pathway.[32] At the same time, the level of the transcript of VvCaS2, an enzyme involved in the synthesis of the β-1,3-glucan callose,[31] was higher in the ANE berries. These data are comparable with the induction of β-1,3 glucanase observed at 24 hpt with *A. nodosum* extract in cucumber leaves.[14] Unlike the reports by Jayaraj *et al.*[10] and Jayaraman *et al.*[14] about lipoxygenases, the level of VvLOX transcripts did not increase in berries sampled from ANE vines. Thus, *A. nodosum* extracts modulated the induction of defense-related genes in grapevines and reduced *B. cinerea* infections. Moreover, a combined effect of the systemic induction of defenses and higher phenolic concentration in skins should not be excluded. Polyphenols are known to be antimicrobial compounds involved in plant resistance to pathogens.[14]

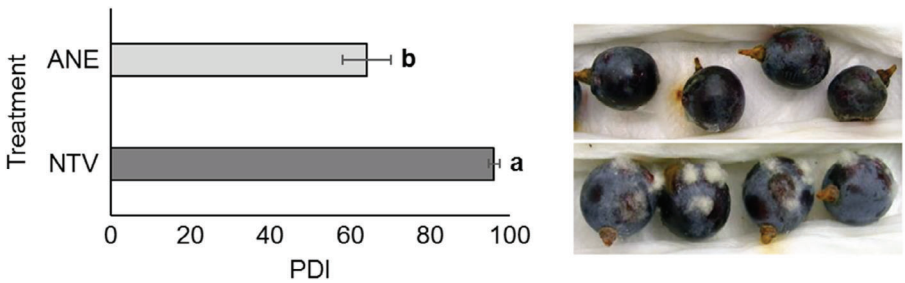

Figure 8. Percentage disease index (PDI) calculated according to McKinney's[34] formula at 72 h post-inoculation (hpi) with *B. cinerea* on ripe berries sampled on October 8, 2015 (DOY 261) from potted vines cv. Sangiovese subjected to multiple foliar applications of Acadian Marine Plant Extract Powder (ANE) or on water sprayed control vines (NTV). Each column represents the mean ± standard error of four replicated trials. Different letters indicate significant difference (Duncan's test, $P \leq 0.01$). Representative berries are shown at the side of each column.

CONCLUSIONS

A. nodosum-based biostimulants reduce the impact of a key-pathogen of grapevine, *B. cinerea*, and at the same time can improve the chromatic and phenolic profiles of wines. Considering the need for alternative solutions for pathogen control in viticulture, the biostimulant can become a useful option through which to develop sustainable and integrated pest-management strategies, with the opportunity to improve wine quality while reducing the use of chemical pesticides. The present work points out, for the first time, that the modulation of genes relating to flavonoid pathways and the upregulation of some defense-related genes are the mechanisms of action that lead to the observed effects in grapevine that were discussed above. The increase in anthocyanin and phenolic concentration found in the skins of grapes sprayed with *A. nodosum* can be essential for the production of premium red wines and may improve the stability of wines in general. Considering the efforts to increase sustainability of viticulture, the use of the seaweed extracts can be a valuable choice in developing new strategies to achieve different pivotal tasks in the production of high-quality wines, with lower environmental ramifications.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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