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Effects of a biostimulant derived from the brown seaweed *Ascophyllum nodosum* on ripening dynamics and fruit quality of grapevines

Tommaso Frioni[a,b,c,*], Paolo Sabbatini[b], Sergio Tombesi[c], Jeffrey Norrie[d], Stefano Poni[c], Matteo Gatti[c], Alberto Palliotti[a]

[a] Dipartimento di Scienze Agrarie, Alimentari e Ambientali, Università degli studi di Perugia, 06128 Perugia, Italy
[b] Department of Horticulture, Michigan State University, 48824-11325 East Lansing, MI, USA
[c] Dipartimento di Scienze delle Produzioni Vegetali Sostenibili, Università Cattolica del Sacro Cuore, 29122 Piacenza, Italy
[d] Acadian Seaplants Limited, B3 B 1X8 Dartmouth, NS, Canada

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ABSTRACT

Most modern and traditional grape-growing regions are facing challenging times due to the unpredictability of weather conditions and warming trends. Innovative and sustainable tools such as seaweed-based biostimulants may play a key-role in the development of environment-friendly viticultural strategies to improve yields, biotic/abiotic stress tolerance and fruit and wine quality. A sprayable *Ascophyllum nodosum* extract was tested on grapevines cv. Sangiovese grown under Mediterranean conditions (central Italy) and on grapevines cv. Pinot Noir and Cabernet Franc within a cool-climate viticulture region (Michigan, USA). The product was sprayed on the canopies at label doses (1.5 kg/ha) five times during the season, starting two weeks before veraison. The seaweed extract did not affect leaf gas exchanges, yield or cluster and berry size, but hastened veraison, improved anthocyanins accumulation in all cultivars and increased phenolic content particularly in Sangiovese. Therefore, medium-late application of the seaweed extract can be a simple way to favour chromatic and chemical proprieties of grapes and wines. This is the first report of positive effects of *Ascophyllum nodosum* extracts on the quality of cultivated wine grapes. The adoption of the technique can be particularly suitable to cool-climate viticulture, especially as it pertains to short growing seasons and genotypes with a limited phenolic profile.

1. Introduction

Efforts to improve agricultural sustainability are being encouraged worldwide. Sustainable production includes ensuring yield with particular attention to food safety and conservation of rural ecosystems (Pretty, 2008). Biostimulants, natural fertilizers and plant defense activators/elicitors, are tools gaining consideration within modern crop management (Colla and Rouphael 2015). Seaweed extracts are natural compounds described by Du Jardin (2015) as one of the main groups of biostimulants. Concentrates obtained by different marine plants have been studied for their positive effects in different agricultural systems (Battacharyya et al., 2015; Khan et al., 2009). The brown seaweed *Ascophyllum nodosum* (L.) Le Jol. is one of the more interesting seaweed species given its widespread application and potential in agriculture (Khan et al., 2009). *Ascophyllum nodosum* (AN) extracts have been reported to promote growth and yield in many crops and to increase quality. They can trigger specific metabolic pathways in treated plants and provide organic compounds having diverse effects in plant metabolism (Battacharyya et al., 2015; Khan et al., 2009). Foliar applications of AN extracts have been reported to increase crop tolerance towards pathogens (Battacharyya et al., 2015; Khan et al., 2009) and to affect plant hormone biosynthesis (Wally et al., 2013). Moreover, experiments on model plants such as *Arabidopsis thaliana* (L.) suggested that AN extracts can modulate genetic signalling related to secondary metabolism and phenolic biosynthesis (Goñi et al., 2016). Molecular and genomic studies are supported by several researches that reported an increased content of anthocyanins, phenolics, flavonoids and anti-oxidant compounds in response to AN extract treatments (Fan et al., 2011, 2013; Lola-Luz et al., 2013, 2014a,b; Ochmian et al., 2008; Roussos et al., 2009).

In premium red wine grape production, phenolic content is of pivotal importance for wine quality and economic return. This remains a challenge for growers given wide seasonal climatic variability due to climate change (Jones et al., 2005; Schultze et al., 2016b).

Abbreviations: AN, *Ascophyllum nodosum* (L.) Le Jol; T min, Minimum temperature; T max, Maximum temperature; T avg, Average temperature; GDD, Growing Degree Days; TSS, Total soluble solids; TA, Titratable acidity; DOY, Day of the year; DAFB, Days after full bloom

* Corresponding author. Present address: Dipartimento di Scienze delle Produzioni Vegetali Sostenibili, Università Cattolica del Sacro Cuore, 29122 Piacenza, Italy.
E-mail address: tommaso.frioni@unicatt.it (T. Frioni).

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Furthermore, the extensive range of climatic areas where thousands of grapevine cultivars are spread imposes different challenges to growers. In warmer climate regions, advanced phenological stages and the uncoupling of technological and phenolic maturity in red grape cultivars depletes grape composition at harvest (Jones et al., 2005; Palliotti et al., 2014). On the other hand, in cool climate regions winter and spring minimum temperatures, short growing season and high disease pressure challenge vineyard productivity and grape quality (Jones et al., 2005; Schultze et al., 2016a). Such variability causes different problems and can influence the effectiveness of cultural techniques (Frioni et al., 2017). Although several experiments report on positive effects of AN extracts applications in grapevines (Khan et al., 2012; Kok et al., 2010; Norrie et al., 2002; Norrie and Keathley 2006; Sabir et al., 2014), a comprehensive evaluation of the potentiality of AN-based products on *Vitis vinifera* under different climatic conditions is lacking.

The aim of this work was to evaluate canopy applications of a AN extract on grapevines grown in two different viticultural areas: central Italy, a typical Mediterranean environment, and Michigan, a cool-cold viticulture region (Schultze et al., 2016b). More specifically, this study examined the effect of AN extract on phenolic maturity and tested its replicability under different climatic conditions and on different genotypes. Taking into account the promotion of anthocyanins and polyphenols reported on other crops and considering the challenge that climatic conditions represent in different environments for achieving an optimal ripening, our general hypothesis was that AN extracts can be a useful tool to improve grape quality for red wine production.

2. Materials and methods

2.1. Experiment 1: site, plant material and experimental design

The first experiment (Exp. 1) was conducted in 2013 in central Italy (Deruta, Umbria, 42° 96′ 15″ N, 12° 40′ 78″ E, 405 m asl, loamy soil type, south exposition, north-south row orientation) on 48 fifteen-years-old vines of *Vitis vinifera* L. cv. Sangiovese (clone VCR30) grafted on 420A. Vines were planted at 1.00 × 2.50 m between vines and rows, respectively, and trained with vertical shoot positioned trellis system (VSP), spur-pruned during winter to ~10 buds per vine. Cordons were trained 0.9 m aboveground and three pairs of catch wires were forming canopy walls of 1.2 m above the cordons. The plot was organized using a Randomized Complete Block Design (RCBD), consisting of four blocks of 12 vines each and one factor (AN extract foliar application), with 16 vines per treatment. Two weeks after full bloom all vines were adjusted to a crop load of about 13 clusters per vine. Shoot trimming was performed when the shoot tips reached a length of ~30 cm higher than the top wire and standard pest management practices were applied, according to local standards. Three weeks after the pea-size stage (as described by Coombe, 1995), vines were assigned to the following treatments: 16 vines, four per block, were assigned to the first treatment, consisting of multiple applications of the AN extract Acadian Marine Plant Extract Powder (Acadian Seaplants Limited, Dartmouth, NS, Canada) at label rates, 1.5 kg/ha (SWE1); another set of 16 vines were assigned to SWE2, consisting of multiple applications of the same AN extract at 3.0 kg/ha; the remaining 16 vines were assigned to CONTROL, consisting in application of water. A surfactant was added to all treatments as suggested on the product's label. Treatments were repeated on the same vines four times before harvest, at application intervals of ten to twenty days. Treatment dates were 22 Jul 2013 (56 Days After Full Bloom – DAFB), 5 Aug 2013 (70 DAFB), 23 Aug 2013 (88 DAFB), 2 Sep 2013 (98 DAFB), 15 Sep 2013 (105 DAFB).

2.2. Experiment 2: site, plant material and experimental design

A second experiment (Exp. 2) was carried out in 2014 on 64 five-years-old vines of *Vitis vinifera* L., divided into two plots. The first plot was composed of grapevines cv. Pinot Noir (clone 114 grafted on 101-14 MGt) while the second one of grapevines cv. Cabernet Franc (clone 332 grafted on 101-14 MGt). Plots were situated in a commercial vineyard in Benton Harbor, MI, USA (42° 13′ 30″ N, 86° 37′ 36″ W). Soils were spinks sandy loam (USDA, 1957) and the vineyard had a barely perceptible slope, with south exposition and a north-south row orientation. Each plot consisted of 32 vines of the same cultivar, planted with a spacing of 1.50 × 3.00 m for Pinot Noir and 1.80 × 3.00 m for Cabernet Franc between vines and rows, respectively. Both cultivars were trained with a vertical shoot positioned trellis system (VSP), cane-pruned during winter to about 30 nodes per vine for Pinot Noir and 50 nodes per vine for Cabernet Franc. Multiple trunks were retained to ensure survival during low winter temperatures and re-trained after severe damage from extreme freezing temperatures recorded during winter 2012/2013. The two sections were organized with a Randomized Complete Block Design (RCBD), consisting of four blocks of eight vines each and one factor (AN extract application), with 16 vines per treatment. Two weeks after full bloom all vines were adjusted to a crop load of about 45 clusters per vine in Pinot Noir and 100 clusters per vine in Cabernet Franc. Shoot trimming was performed when the shoot tips reached a length of ~30 cm higher than the top wire. Standard commercial disease management was applied based on experience and weather conditions.

Phenological stages were identified as described by Coombe (1995). Grape veraison was considered when 50% of the berries presented full color change. For Pinot Noir, vines were assigned to the two treatments three weeks after vines reached the pea-size stage. For the late-ripening Cabernet Franc, vines were assigned to the two treatments four weeks after the pea-size stage. Half of the vines of each cultivar were assigned to the AN extract application (SWE) and the remaining 16 vines to the untreated control (CONTROL). On the same day the first application was performed. At application, SWE vines were treated with a full canopy spray at 1.5 kg/ha of the AN extract (Acadian Marine Plant Extract Powder, Acadian Seaplants Limited, Dartmouth, NS, Canada), diluted in water, including the addition of an adjuvant, as suggested in the product's label. CONTROL vines were sprayed only with water and the adjuvant. Treatments were repeated on the same vines four times before harvest, at ten to twenty day intervals. Treatment dates for Pinot Noir were 30 Jul 2014 (44 DAFB – Days After Full Bloom), 6 Aug 2014 (51 DAFB), 16 Aug 2014 (61 DAFB), 26 Aug (71 DAFB), 7 Sep 2014 (83 DAFB) and for Cabernet Franc 16 Aug 2014 (57 DAFB), 30 Aug (71 DAFB), 7 Sep 2014 (80 DAFB), 20 Sep 2014 (93 DAFB), 11 Oct 2014 (114 DAFB).

2.3. Weather data

Environmental conditions during both experiments were data-logged by two automated weather stations located nearby the vineyards. Daily maximum (T max), average (T avg) and minimum temperature (T min) and precipitation from 1 Apr to 31 Oct of 2013 (Exp. 1) and 2014 (Exp. 2) were collected. Cumulative growing-degree-days (GDD) (Baskerville and Emin 1969) were then calculated. Same data were obtained also for the same period of the ten previous years, to calculate the ten-year running average.

2.4. Gas exchanges parameters, leaf composition and canopy architecture

Throughout Exp. 2, two weeks after full bloom shoots were counted and three representative shoots were identified, tagged and numbered. Leaf net photosynthesis (P_n), stomatal conductance (g_s) and transpiration rate (E) were measured on 22 Aug 2014 for Pinot Noir (T max = 29.4, T min = 21.2, T avg = 25.3) and on 3 Sep 2014 for Cabernet Franc (T max = 28.7, T min = 15.2, T avg = 22.0), which corresponded to about one week after veraison. Gas exchange parameters were measured between 1200 h and 1300 h on the third leaf of first tagged shoot of each vine, using a CIRAS-2 portable photosynthesis machine (PP Systems Version 2.02; Amesbury, MA, USA). Readings

were taken on a leaf surface of 2.5 cm^2 at light saturation and ambient relative humidity.

On the same leaves chlorophyll fluorescence was measured between 1300hr and 1400hr with a lightweight portable fluorimeter (Handy-PEA, Hansatech Institute Ltd, Norfolk, UK). Lightweight leaf clips were left closed on the leaf surface for at least 20 min to allow for dark adaptation. Opening the plate exposed the dark-adapted leaf tissue to an actinic light flash (wavelength of 650 nm, intensity > 3000 µmol/m^2/s) at which time the instrument provided the F_v/F_m ratio (F_m = fluorescence maximum over the induction curve; F_v = difference between F_m and F_o, where F_o is the ground fluorescence), an indicator of PSII maximum efficiency (Strasser and Srivastava 1995).

Finally, using the same leaves, relative chlorophyll concentrations were estimated between 1400hr and 1500hr using a SPAD-502 chlorophyll meter (Minolta, Tokyo, Japan). The leaves were then sampled and stored at −80 °C. The frozen samples were then used to determine leaf soluble solids and starch content. Leaves were lyophilized and ground to powder; 0.01 g of powder was placed in 15 ml tubes and mixed into a solution of 80% ethanol and placed in a warm bath at 80 °C for 1 h. After 10 min of centrifugation at 10000 rpm, 10 µl of supernatant was sampled and used for the determination of alcohol soluble sugars by the Anthrone method (Loewus 1952). For starch determination, pellet material was then washed with sodium acetate buffer and then added with 0.5 ml of sodium acetate buffer. Tubes were placed in warm bath with temperature set at 80 °C for 1 h. One milliliter of solution of amyloglucosidase and α-amylase in 0.05 M sodium acetate buffer was added as described by Chow and Landhäusser (2004) and bath temperature was set at 50 °C. Sugar content was measured on the supernatant by the anthrone method as previously described Absorbance was read with a UV–vis spectrophotometer (Model UV-1800, Shimadzu Corporation, Kyoto, Japan) at values of 620 nm.

Two days before hedging (executed in mid-August for both genotypes, with Pinot Noir already subjected to three sprays and Cabernet Franc to only one), shoot length of all tagged shoots was assessed. At harvest, all tagged shoots were sampled and total leaf weight and leaf area was measured, using a AAM-7 leaf area meter (Hayashi-Denko Co., Tokyo, Japan). The total leaf area per vine was then estimated based on the shoot count. Leaves from each shoot were then oven-dried at 95 °C to constant weight and dry matter content was measured.

2.5. Harvest data, cluster morphology and bunch rot incidence\severity

Grapevines were harvested once soluble solids reached ~22 Brix for Sangiovese, ~22 Brix for Pinot Noir and ~20 Brix for Cabernet Franc.

For both experiments, at harvest, yield per vine was measured recording also the total number of clusters per vine. For Exp. 2, clusters from tagged shoots were sampled, placed in a cooler and brought to the lab where cluster weight, number of berries per cluster and berry weight were measured. Bunch rot incidence and severity were measured counting the number of clusters showing symptoms (incidence) of fruit rot, independently by the disease aetiology, and recording the percentage of affected berries (severity). Finally, thirty berries from each cluster were used to measure equatorial diameter and then frozen at −80 °C. After several days berries were weighed before and after skin separation to determine skin/pulp ratio. Skins and pulps were then oven-dried at 95 °C to constant weight, to calculate skin dry matter content. Equatorial diameter was used to calculate average berry surface and volume.

2.6. Grape chemical composition

For both experiments, from veraison to harvest three groups of 100 berries per treatment per block were periodically sampled from untagged shoots (totally 12 bags per treatment at each sampling date). From each bag 76 berries were used to count the number of fully coloured berries and then were crushed. The juice was filtered to obtain the must on which total soluble solids, pH and titratable acidity were determined. Soluble solids (Brix) were measured with a digital refractometer (ATA-3810 PAL-1 Pulse Inc., Van Nuys, CA, USA). A 370 Thermo Orion pH meter (Thermo Fisher Scientific Inc., Logan, UT, USA) was used to measure pH. Titratable acidity (TA) was measured with a Multi-T 2.2 digital titrator (Laboratory Synergy Inc., Goshen, NY, USA) with each sample consisting of 10 ml clear juice diluted with distilled water to 100 ml and titrated with 0.1 M sodium hydroxide (NaOH) to a pH of 8.2 using an equation to yield the titratable acidity (g/l), according to Iland et al. (2004).

The remaining berries (24 berries × 12 bags per treatment, repeated for all the sampling dates) were frozen and after several days total skin anthocyanins and phenolics were determined, according to Ough and Amerine (1980) and Slinkard and Singleton (1977), respectively. From each berry, a 10 mm diameter disks of the grape skin were separated from the pulp. Disks were taken from the external, middle portion of well-exposed berries. Two skin disks (1.5 cm^2) were macerated in 50 ml methanol containing 0.1% HCl (v/v) at pH 1 and maintained at ~25 °C for 24 h in the dark with periodic shaking. Total anthocyanin content was determined by reading absorbance at 520 nm at pH 1 using an extinction coefficient (molar absorbance value) of 28.000 and molecular weight of 529 (typical of malvidin-3-glucoside). Total soluble phenolics were then assayed from a 0.2 ml sample, to which was added 1.8 ml distilled water, followed by 10 ml 10% aqueous Folin-Ciocalteau reagent (Sigma) and 8 ml 7.5% (w/v) aqueous Na_2CO_3. The mixture was maintained at 24 °C and after 2 h the absorbance was measured at 750 nm and compared to a gallic acid standard curve. Absorbance was measured with a UV–vis spectrophotometer (Model UV-1800, Shimadzu Corporation, Kyoto, Japan). Skin anthocyanins and phenolics were expressed as mg/cm^2 of malvidin-3-glucoside equivalent and gallic acid equivalent, respectively; average berry surface was used to report the data as a concentration (mg/g), so to calculate anthocyanins/Brix ratio.

2.7. Statistical analysis

Results were tested for normality and homogeneity of variance was calculated separately for the two experiments. For Exp. 2 data collected for Pinot Noir and Cabernet Franc was analysed separately. Data was subjected to a one-way analysis of variance (ANOVA) using the PROC MIXED in SAS (version 9.1.3; SAS Institute, Cary, NC, USA). Means were then separated using the Student-Newman-Keuls (SNK) test for Exp. 1 (three treatments: CONTROL, SWE1, SWE2) and using the Student's *t*-test for Exp. 2 (two treatments: CONTROL, SWE).

3. Results

3.1. Weather evolution

During 2013 an amount of 2034 GDD were accumulated from 1 Apr to 31 Oct in the site where exp. 1 was conducted (Fig. 1a), a value which is similar to the ten-year average (2026 GDD). In detail, May, June and July were slightly cooler than the average for the region. On the other hand July, August and September were slightly warmer than usual (+28, +27 and +15 GDD, respectively). The highest T max of the season (39° C) was recorded on 5 Aug 2013. Rains (Fig. 1b) were concentrated in the months of May (153 mm, + 52 mm than the ten-year average) and October (122 mm), meanwhile June, July and August were drier than usual (−24 mm, −29 mm and −30 mm than the ten-year average, respectively). Totally, between 1 Apr and 31 Oct 2013 440 mm of rain fell on the site of exp. 1, a value which is similar to the average of the ten previous years, but rains were not uniformly distributed along the summer. Overall, in central Italy weather evolution in 2013 was in line with the usual trend for the region.

In 2014 April, May and June followed a trend which is typical for south-western Michigan, with a T avg rising to values of about 20 °C

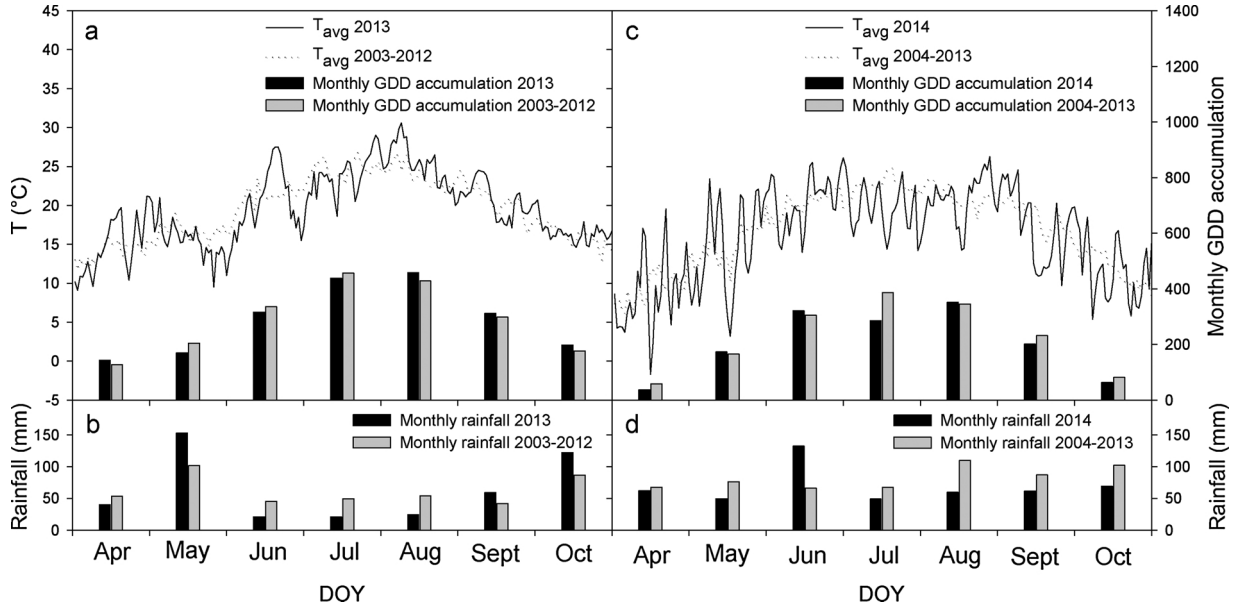

Fig 1. Weather evolution in central Italy in 2013 (panels a and b) and in south-western Michigan in 2014 (panels c and d), in comparison with the average for the ten previous seasons. T avg = daily average temperature, GDD = growing-degree-days, DOY = day of the year.

(Fig. 1c). July and August 2014 were cooler than the average for the same period, with no consistent T max over 30 °C and with some unusual negative peaks of T min (15 Aug 2014: T max = 22.7 °C, T min = 6.3 °C). September 2014 begun with cool air stationing in the region, then in the second part of the month warmer temperatures were alternating to periods with lower temperatures than the average. October 2014 was no different than the expected, with T max rarely rising above 15 °C and T min dropping in the coldest nights close to freezing temperatures (19 Oct 2014: T max = 12.4 °C, T min = 0.4 °C). At the end of the growing seasons a total of 1431 GDD were accumulated from 1 Apr to 31 Oct 2014, 143 GDD less than the average of the ten previous years. Rainfall recorded in 2014 for the same period was 579 mm (Fig. 1d), with an even distribution between the seven months examined, and a main concentration in June (192 mm), especially in the second half of the month (22 Jun 2014: 46 mm; 30 Jun 2014: 45 mm).

3.2. Exp. 1 – productivity and ripening dynamics

In 2013, Sangiovese vines achieved technological maturity on 30 Sep (126 DAFB). Vine productivity was not affected by AN extract: SWE1 and SWE2 yielded 3.1 and 3.3 kg/vine, respectively, and yield per vine was not significantly different than the CONTROL (Table 1). Similarly, cluster weight and number of clusters per vine was not impacted by AN extract. At harvest total soluble solids, pH and TA in grapes from SWE1 or SWE2 was not different from CONTROL.

During the period between veraison and harvest, soluble solids in the juice did not change with the AN treatments (Fig. 2a). Skin total anthocyanins (Fig. 2b) were higher in SWE1 (+25% than CONTROL) and SWE2 (+18% than CONTROL) beginning with the first sampling at 93 DAFB. After that, corresponding with the active accumulation of anthocyanins, no further significant differences were observed. However, once biosynthesis slowed down SWE1 and SWE2 again were found to have a higher anthocyanin content than CONTROL (SWE1 +0.12 mg/cm^2 and SWE2 +0.07 mg/cm^2 at 113 DAFB). At harvest (Table 1), anthocyanin content remained higher in SWE1 (+0.09 mg/cm^2) and SWE2 (+0.06 mg/cm^2) than CONTROL. Skin total phenolics (Fig. 2c) showed a similar pattern. At the first sampling no differences were found between treatments. Between 100 and 107 DAFB, phenolic compounds in skins were actively accumulating without significant effects due to the AN extract. After the final AN extract applications and once the biosynthesis rate began to decrease, SWE1 and SWE2 phenolics content was found to be higher than CONTROL. At 113 DAFB, SWE1 had +33% more phenolics than CONTROL. At harvest (Table 1) differences remained significant, with SWE1 having +3.1 mg/cm^2 and SWE2 having +2 mg/cm^2, when compared to CONTROL.

3.3. Exp. 2 – phenology, canopy architecture and leaf characteristics

Bud-break in grapevines cv. Pinot Noir occurred on 8 May 2014. Full bloom was recorded on 16 Jun 2014 and pea-size stage was reached on 9 Jul 2014 (Table 2). Up until the pea-size stage, no difference was found between CONTROL and SWE, with vines not having been subjected to AN extract treatment. On 11 Aug 2014 (939 GDD accumulated) Pinot Noir SWE vines were found in the stage of veraison (50% of coloured berries). At that moment Pinot Noir vines already received two AN extract applications. CONTROL vines showed color on 50% of the berries only on 16 Aug 2014. This amounted to five days and 45 GDD later. Harvest, fixed as the moment when grapes reached 22 Brix, occurred on the same day (30 Sep 2014) for both CONTROL and SWE vines cv. Pinot Noir.

Table 1
Productivity and fruit composition of grapevines cv. Sangiovese grown in central Italy in 2013 and subjected to multiple canopy applications of a *Ascophyllum nodosum* extract at 1.5 kg/ha (SWE1) and at 3 kg/ha (SWE2), in comparison with untreated vines (CONTROL).

	Yield kg/vine	Clusters per vine n°	Cluster weight g	Soluble solids Brix	pH	Titratable acidity g/L	Skin total anthocyanins mg/cm^2	Skin total phenolics mg/cm^2
CONTROL	3.2	12	267	22.4	3.21	5.5	0.277 b[1]	0.753 b
SWE1	3.3	12	274	21.6	3.16	5.8	0.374 a	1.063 a
SWE2	3.1	11	281	22.1	3.16	5.6	0.344 a	0.951 a

[1] Different letters indicate significant difference per $P < 0.05$ (SNK test). Absence of letters means that significant differences between treatments were not found.

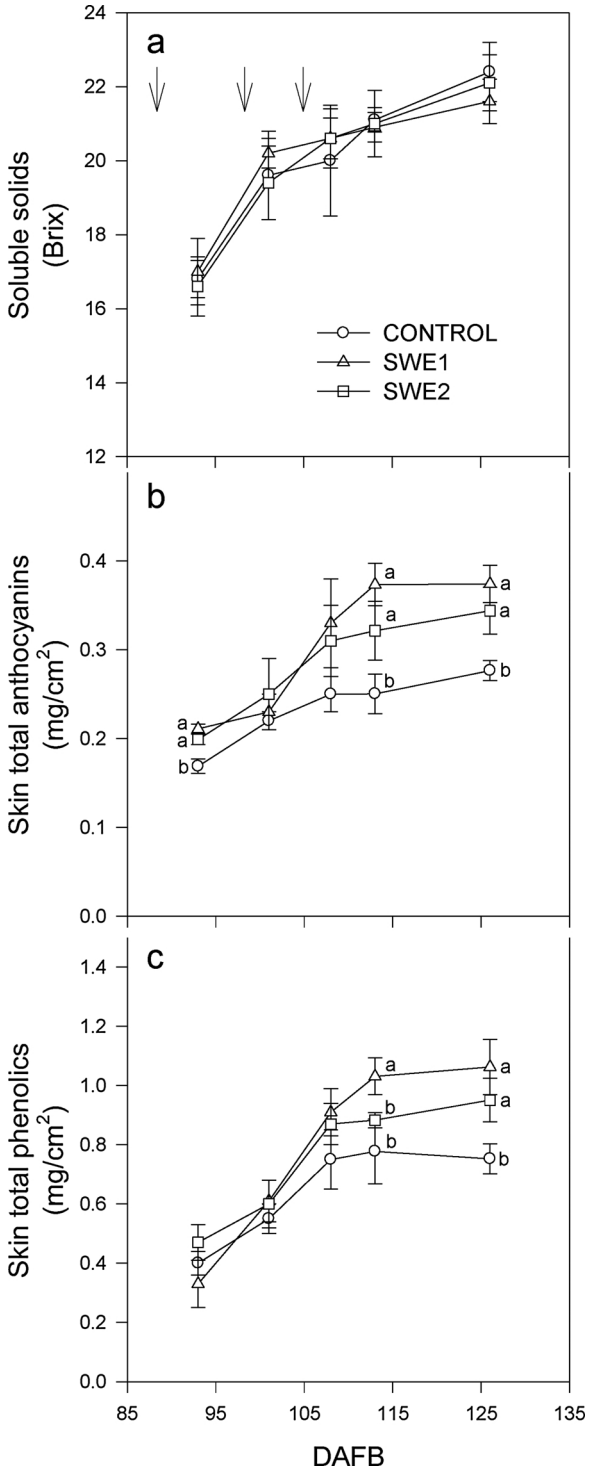

Fig. 2. Evolution of soluble solids (panel a), skin total anthocyanins (panel b) and skin total phenolics (panel c) during ripening for grapevines cv. Sangiovese grown in central Italy in 2013 and subjected to multiple canopy applications of a *Ascophyllum nodosum* extract at 1.5 kg/ha (SWE1) and at 3 kg/ha (SWE2), in comparison with untreated vines (CONTROL). Arrows represent treatment applications during the considered period. If coinciding in the same day, treatments were executed subsequently to the samplings. Vertical bars represent standard errors (n = 12). Different letters indicate significant difference per $P < 0.05$ (SNK test). Absence of letters means that significant differences between treatments were not found. DAFB = days after full bloom.

Cabernet Franc showed bud-break on 14 May 2014, full bloom on 20 Jun 2014 and pea-size stage on 17 Jul 2014. Veraison on Cabernet Franc occurred on the same day (30 Aug 2014, 1153 GDD accumulated), with no effect from AN extract application. Grapes were harvested at 20 Brix on 28 Oct 2014 for both SWE and CONTROL.

The AN extract sprays had no effect on shoot length either in Pinot Noir or Cabernet Franc (Table 3). Similarly no difference was found in both genotypes for vine leaf area or leaf weight. However, a higher dry matter content was found in leaves from SWE vines (+2% Pinot Noir, +2% Cabernet Franc). Moreover, leaves from both genotypes treated with AN extract resulted in a significantly higher soluble sugar content (+30% in Pinot Noir vines and +22% in Cabernet Franc) while no statistical difference was found in starch content.

Despite different leaf morphology and composition, leaf physiological functionality was not altered by AN extract (Table 4). SWE vines for both cultivars had comparable leaf P_n, g_s and E relative to CONTROL vines. Similarly, no difference was found in photosystems' efficiency (f_v/f_m) and SPAD values, either in Pinot Noir or Cabernet Franc between SWE and CONTROL.

3.4. Exp. 2 – vine productivity, cluster morphology and grape sanity

Pinot Noir CONTROL vines produced 2.6 kg of grapes per vine (Table 5). The AN extract application did not improve vine productivity (2.4 kg/vine). The AN extract did not change either number of clusters per vine or cluster weight at harvest (60.1 g–60.8 g). No effects were found on cluster morphology, with berry size not differing between treatments. Interestingly, the skin to pulp ratio was not changed by AN extract sprays, but SWE had a significantly higher skin dry matter content (+8%), compared to CONTROL.

For Cabernet Franc, no difference was found in vine productivity (7.4 kg in CONTROL vines vs 7.5 kg in SWE vines), cluster number or morphology and berry size. As in Pinot Noir vines, skin to pulp ratio was similar between SWE and CONTROL berries, with skins from SWE having a higher dry matter content (+6%).

Pinot Noir CONTROL vines had 46% of clusters presenting bunch rot symptoms, with SWE vines showing similar values. Affected clusters had an average of 20–25% rotten berries. In Cabernet Franc the bunch rot incidence was appreciably lower. Only 1–2% of clusters had symptoms, with no statistical difference between treatments.

3.5. Exp. 2 – ripening dynamics

In Pinot Noir, vines subjected to AN extract applications had significantly higher total soluble solids immediately after veraison (Fig. 3a). At 61 days after full bloom (DAFB), SWE reached 10.2 Brix (+2.2 Brix than CONTROL). Soluble solids were higher in SWE at 71 DAFB (+18%) and at 76 DAFB (+6%). Later in the season no significant differences were found in sugars content between SWE and CONTROL. No consistent differences in pH or TA were found during ripening (Fig. 3b and c), even if pH was significantly higher on SWE vines at 71 DAFB (+2%). At harvest, AN extract had no significant effect on soluble solids, must pH and titratable acidity (Table 6)

In Cabernet Franc ripening followed a typical trend with final soluble solid above 20 Brix with TA below 6 g/l. (Table 6). AN extract did not have significant effect on soluble solids, must pH or TA in Cabernet Franc either during ripening or at harvest (Fig. 3d–f).

3.6. Exp. 2 – skin total anthocyanins and phenolics

SWE grapevines cv. Pinot Noir reached veraison five days earlier than CONTROL vines (Fig. 4a). At 56 DAFB SWE vines had 40 ± 17% (mean ± s.e.) of berries presenting full colouration, meanwhile CONTROL vines had 22 ± 13%. CONTROL vines were considered at veraison at 61 DAFB when grapes had 51 ± 9% of full coloured berries and grapes from SWE vines had already reached 76 ± 12%. For subsequent samplings, the percent of coloured berries rose quickly to 100% and no significant differences were found.

Total anthocyanins content in the skins of Pinot Noir grapes (Fig. 4b) was no different between treatments at 61 DAFB. However, at

Table 2
Date and growing-degree-days (GDD) accumulated for each stage of development in 2014 for grapevines cv. Pinot Noir and Cabernet Franc grown in south-western Michigan and subjected to multiple canopy applications of a *Ascophyllum nodosum* extract (SWE) at 1.5 kg/ha, in comparison with untreated vines (CONTROL).

	Bud-break[a]		Full bloom[a]		Pea-size[a]		Veraison[a]		Harvest	
	Date	GDD[b]	Date	GDD[b]	Date	GDD[b]	Date	GDD[b]	Date	GDD[b]
Pinot Noir										
CONTROL	8 May	61	16 Jun	352	9 Jul	618	16 Aug	984	30 Sep	1368
SWE	8 May	61	16 Jun	352	9 Jul	618	11 Aug	939	30 Sep	1368
Cabernet Franc										
CONTROL	14 May	102	20 Jun	403	17 Jul	687	30 Aug	1153	28 Oct	1431
SWE	14 May	102	20 Jun	403	17 Jul	687	30 Aug	1153	28 Oct	1431

[a] Phenological stages identified as described by Coombe (1995).
[b] GDD calculated from 1 Apr 2012–31 Oct 2012 with base temperature of 10 °C (Baskerville and Emin, 1969).

Table 3
Vegetative parameters, vine architecture and characteristics of leaves in grapevines cv. Pinot Noir and Cabernet Franc subjected to multiple canopy applications of a *Ascophyllum nodosum* extract (SWE) at 1.5 kg/ha, in comparison with untreated vines (CONTROL).

	Shoot length[a]	Leaf area[b]	Leaf specific weight[b]	Leaf dry matter[b]	Leaf-area-to-yield ratio	Leaf soluble sugars[b]	Leaf starches[b]
	cm	m^2/vine	mg/cm^2	%		mg/g DW	mg/g DW
Pinot Noir							
CONTROL	78	3.3	27	29.6	1.27	81	24
SWE	82	3.1	29	30.2	1.30	105	16
	ns[c]	ns	ns	*	ns	*	ns
Cabernet Franc							
CONTROL	105	6.8	33	28.5	0.92	111	32
SWE	116	6.2	32	29.2	0.83	135	24
	ns	ns	ns	*	ns	*	ns

[a] Measurement executed before hedging.
[b] Measurements and samplings executed at harvest.
[c] * and ns mean respectively significance and not per $P < 0.05$ (Student's *t*-test).

Table 4
Net photosynthesis (P_n), stomatal conductance (g_s), transpiration (E), photosystems efficiency (f_v/f_m) and SPAD index of medium leaves of grapevines cv. Pinot Noir and Cabernet Franc subjected to multiple canopy applications of a *Ascophyllum nodosum* extract (SWE), in comparison with untreated vines (CONTROL).

	P_n[a]	g_s[a]	E[a]	f_v/f_m[a]	SPAD value[a]
	µmol CO_2/m^2/s	mmol/m^2/s	mmol H_2O/m^2/s		
Pinot Noir					
CONTROL	14.7	285	6.4	0.743	21
SWE	14.5	269	6.7	0.759	24
	ns[b]	ns	ns	ns	ns
Cabernet Franc					
CONTROL	13.9	288	7.2	0.797	25
SWE	14.4	291	7.0	0.788	28
	ns	ns	ns	ns	ns

[a] Measurements performed on 23 Aug 2014 for Pinot Noir and on 3 Sep 2014 for Cabernet Franc, 4–7 days after that respective CONTROL vines resulted in veraison (Coombe, 1995).
[b] * and ns mean respectively significance and not per $P < 0.05$ (Student's *t*-test).

71 DAFB Total anthocyanins were significantly higher in SWE (+0.07 mg/cm^2). At 76 DAFB and 84 DAFB again no difference between treatment was found, but, after the last AN extract application, anthocyanins content remained higher until harvest (+0.03 mg/cm^2 at 91 DAFB and +0.05 mg/cm^2 at 99 DAFB).

Skin total phenolics (Fig. 4c) followed a similar trend. Values were not different at 61 DAFB, but at 71 DAFB SWE had a phenolic content significantly higher (+0.26 mg/cm^2) than the CONTROL. Phenolics on skins of grapes from SWE vines were higher at 76 DAFB (+0.13 mg/cm^2), then at 84 DAFB no significant difference was found. After subsequent AN extract application, at 91 DAFB, phenolics were again significantly higher on SWE berries' skins (+0.06 mg/cm^2). During final ripening, SWE and CONTROL grapes showed no significant differences in content of skin phenolics. At harvest (Table 6), Pinot Noir grapes from SWE vines had skin total anthocyanins content of 0.29 mg/cm^2, significantly higher (+0.03 mg mg/cm^2) than the CONTROL, and skin phenolic content of 0.71 mg/cm^2. Even though anthocyanins content at harvest was higher in SWE, the ratio between anthocyanins concentration and soluble sugars (anthocyanins to Brix ratio) did not result in significant differences between treatments.

In Cabernet Franc colouring of berries demonstrated a similar evolution between treatments (Fig. 4d). Veraison occurred at 71 DAFB when SWE vines had 52 ± 8% of coloured berries and on CONTROL vines 43 ± 14%. At 94 DAFB, berry colouring was almost complete (88% for CONTROL, 92% for SWE), and was complete at 115 DAFB, with no statistical differences between treatments.

Anthocyanins concentration on Cabernet franc berries' skins (Fig. 4e) was similar between SWE and CONTROL at the beginning of ripening. After late applications of the AN extract, SWE had significantly higher content at 102 DAFB (+0.04 mg/cm^2) and at 107 DAFB (+0.03 mg/cm^2). At 115 DAFB the difference between treatments was not significant, but after the last application of AN, anthocyanins in SWE increased and at harvest SWE had higher content than CONTROL (+0.03 mg/cm^2).

Differently from anthocyanins evolution, Cabernet Franc skins phenolics content (Fig. 4f) showed no differences between treatments during ripening. The concentration rose from 0.25 mg/cm^2 at 71 DAFB until 0.84 mg/cm^2, but SWE and CONTROL were not statistically significantly different each other.

At harvest, fruit had a skin total anthocyanins concentration of 0.43 mg/cm^2, significantly higher than CONTROL, and phenolics content was similar between treatments. As in Pinot Noir, Cabernet Franc showed no difference due to the AN extract in the relationships between anthocyanins and sugars.

4. Discussion

The AN extract was tested on three of the most relevant grapevine cultivars for the production of premium red wines. Environmental and edaphic conditions in two experiments were consistently different and representative of the typical conditions in warm- and cool- climate producing regions. The first experiment was carried out in a hilly vineyard in a warm viticultural area in central Italy while the second experiment was carried out in a flat vineyard in a cool-cold climate viticultural area in Michigan, USA.

AN applications had minor effects on vine physiological performances as related to carbon assimilation and vegetative growth. Under non-limiting conditions (during the previous weeks, rainfall was well

Table 5
Harvest data, cluster morphology, berry composition and bunch rot occurrence in grapevines cv. Pinot Noir and Cabernet Franc subjected to multiple canopy applications of a *Ascophyllum nodosum* extract (SWE) at 1.5 kg/ha, in comparison with untreated vines (CONTROL).

	Yield[a] kg/vine	Clusters per vine[a] n°	Cluster weight[b] g	Berries per cluster[b] n°	Berry weight[b] g	Skin/pulp ratio[b]	Berry skin dry matter[b] %	Bunch rot incidence[b] %	Bunch rot severity[b] %
Pinot Noir									
CONTROL	2.58	46	60.1	48	1.25	0.277	30.2	46	25
SWE	2.38	42	60.8	46	1.33	0.283	32.6	52	20
	ns[c]	ns	ns	ns	ns	ns	*	ns	ns
Cabernet Franc									
CONTROL	7.37	101	105.4	74	1.43	0.299	27.3	2	5
SWE	7.46	107	108.6	73	1.49	0.290	28.9	1	5
	ns[c]	ns	ns	ns	ns	ns	*	ns	ns

[a] Measured/counted in field on each vine at harvest.
[b] Measured/counted in the lab on model tagged shoots.
[c] * and ns mean respectively significance and not per $P < 0.05$ (Student's *t*-test).

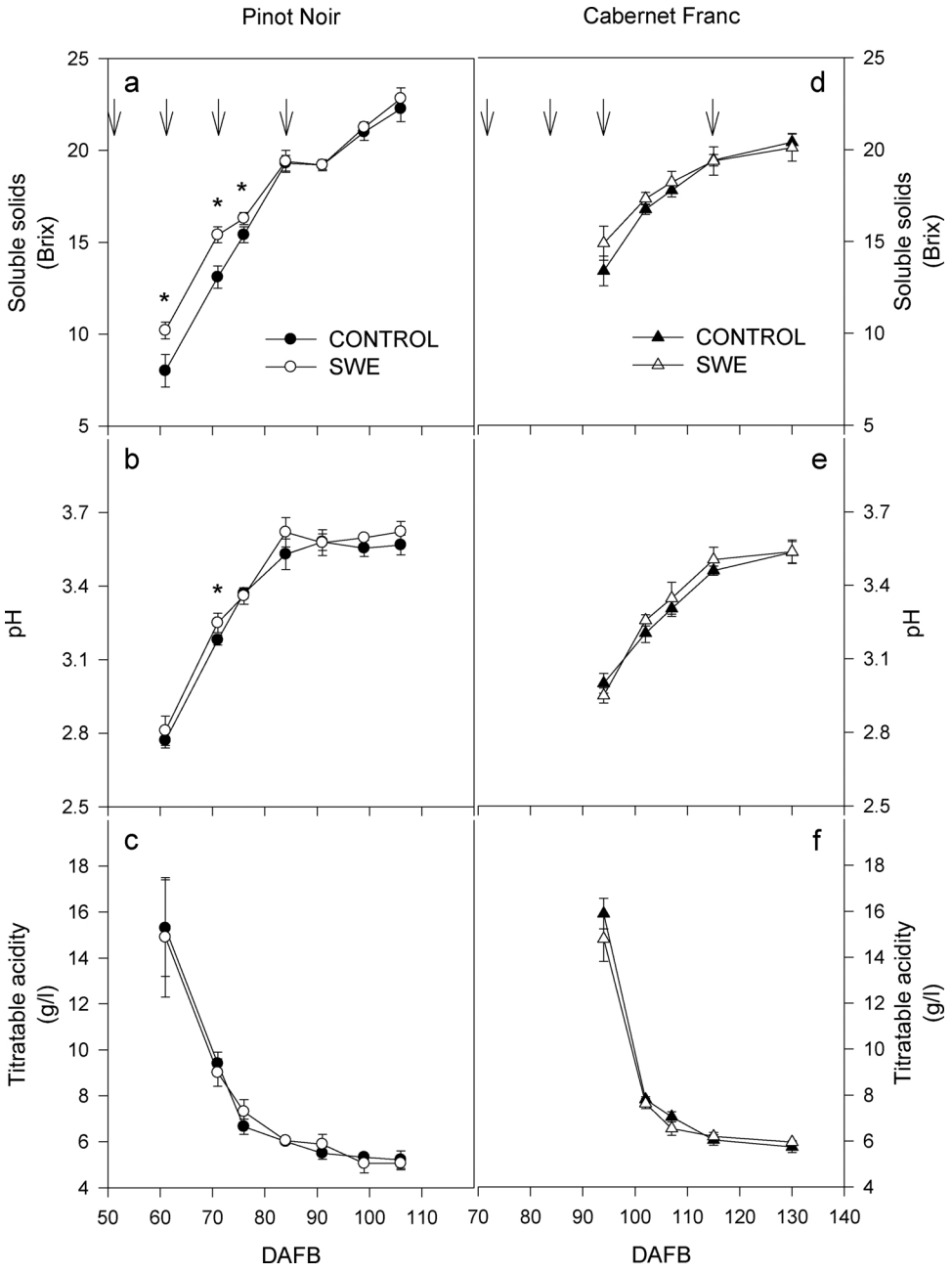

Fig. 3. Evolution of soluble solids (panels a and d), pH (panels b and e) and titratable acidity (panels c and f) during ripening for grapevines cv. Pinot Noir (panels a–c) and cv. Cabernet Franc (panels d–f) subjected to multiple canopy applications of a *Ascophyllum nodosum* extract (SWE), in comparison with untreated vines (CONTROL). Arrows represent treatment applications during the considered period. If coinciding in the same day, treatments were executed subsequently to the samplings. Vertical bars represent standard errors (n = 12), points with asterisk are different per $P < 0.05$ (Student's *t*-test). DAFB = days after full bloom.

Table 6
Quality of fruit at harvest in grapevines cv. Pinot Noir and Cabernet Franc subjected to multiple canopy applications of a *Ascophyllum nodosum* extract (SWE), in comparison with untreated vines (CONTROL).

	Soluble solids Brix	pH	Titratable acidity g/L	Skin total anthocyanins mg/cm^2	Skin total phenolics mg/cm^2	Anthocyanins to Brix ratio
Pinot Noir						
CONTROL	22.3	3.67	5.06	0.265	0.690	0.068
SWE	22.8	3.72	5.22	0.292	0.714	0.067
	ns[a]	ns	ns	*	ns	ns
Cabernet Franc						
CONTROL	20.1	3.63	5.51	0.402	0.698	0.107
SWE	20.4	3.64	5.65	0.434	0.709	0.102
	ns[a]	ns	ns	*	ns	ns

[a] * and ns mean respectively significance and not per $P < 0.05$ (Student's *t*-test).

distributed and temperatures rarely rose above 30 °C) AN extract treatment had no effects on leaf gas exchanges, in accordance with previous reports (Spann and Little 2011; Xu and Leskovar, 2015). However these results do not allow for the exclusion of AN extract treatment effects under abiotic stress conditions.

No differences were found between SWE and CONTROL vines at early phenological stages (Table 2). In Pinot Noir SWE vines, veraison occurred a few days earlier than in Cabernet Franc, probably due to the

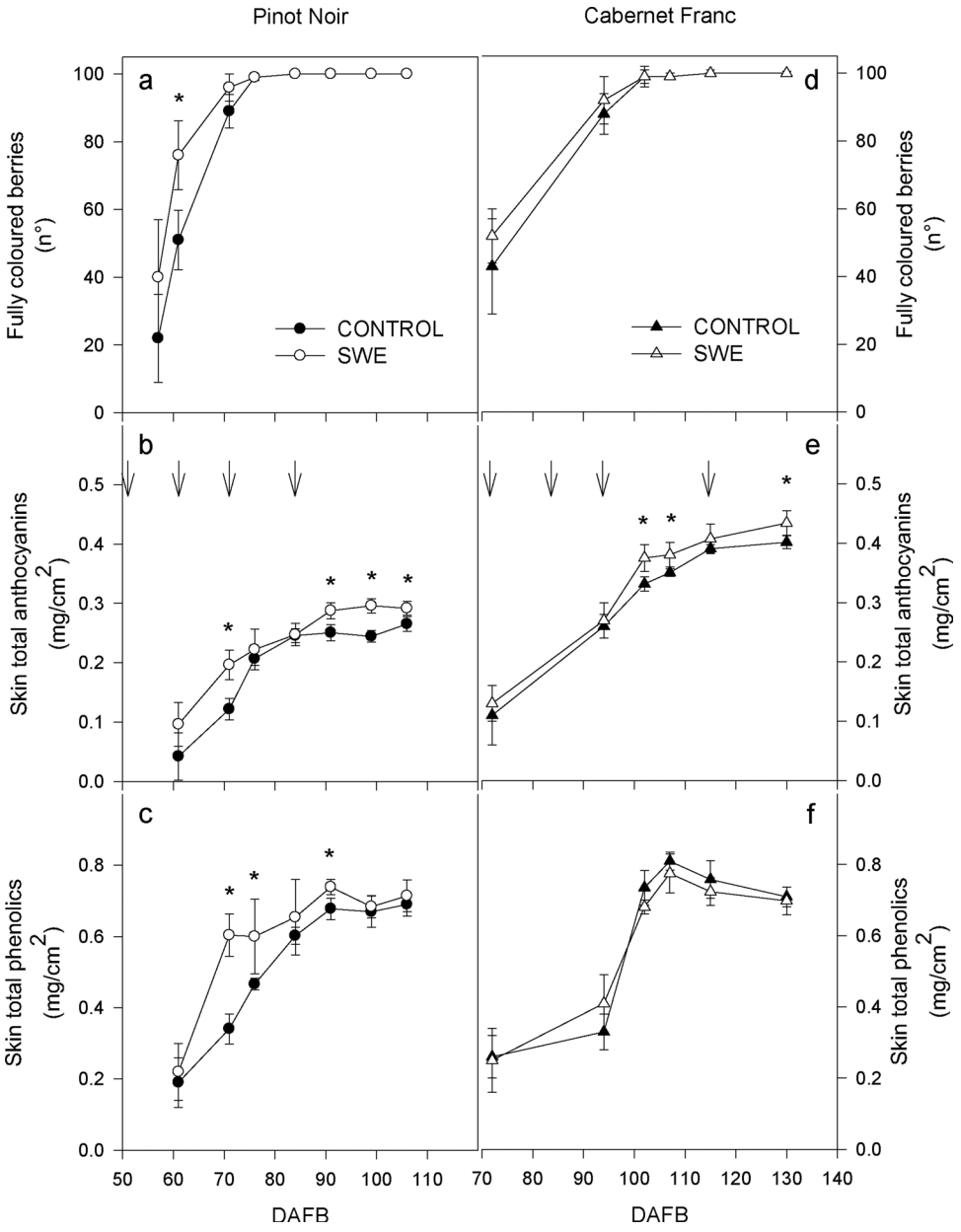

Fig. 4. Evolution of veraison (panels a and d), skin total anthocyanins (panels b and e) and skin total phenolics (panels c and f) during ripening for grapevines cv. Pinot Noir (panels a–c) and cv. Cabernet Franc (panels d–f) subjected to multiple canopy applications of a *Ascophyllum nodosum* extract (SWE), in comparison with untreated vines (CONTROL). Arrows represent treatment applications during the considered period. If coinciding in the same day, treatments were executed subsequently to the samplings. Vertical bars represent standard errors (n = 12), points with asterisk are different per $P < 0.05$ (Student's *t*-test). DAFB = days after full bloom.

earlier application of SWE (Fig. 4). These results are in accordance with the advancement of phenological stages found in different species in response to AN extract treatments (Crouch and Van Staden, 1992; Sabir et al., 2014). Evolution of pH and acidity were basically unaffected by the AN extract while TSS evolution was slightly positively affected in Pinot Noir during the first part of the ripening process.

At harvest, no difference was found on vine productivity in either experiment. Cluster morphology was not modified by medium-late AN extract applications. These data partially differ from previous reports for table grape but, unlike our experiment, authors applied the biostimulant earlier in the season, when inflorescences and fruits were actively developing and growing (Khan et al., 2012; Norrie et al., 2002; Norrie and Keathley 2006).

The incidence and severity of bunch rot symptoms were not affected by AN treatment. Although AN extracts have been proposed as systemic resistance inducers toward several pathogens in different crops (Battacharyya et al., 2015; Khan et al., 2009), the cool season, without high temperature peaks during ripening and with constant rainfall, set up favourable conditions for pathogens during Exp. 2. Under these conditions, with reasonable disease pressure, AN extract had no effect on bunch rot incidence.

In our experiments, the major effect of AN extract was the significant increase in anthocyanins biosynthesis in grape skin. The chromatic potential of treated grapes was boosted in both environments and on all the cultivars evaluated, without any significant effect of the concentration used for sprays. AN extract did not change the ratio between skin and pulp of the berry, excluding any effect on phenol concentration. Overall, the biostimulant boosted anthocyanins and phenolics close to veraison and then later in the season, maintaining active accumulation before harvest. This can be partially related to the fact that AN extract slightly anticipated veraison and triggered anthocyanins and phenolics biosynthesis in grape skins, with some peculiarities due to the ripening progression; in Sangiovese and Pinot Noir, the biostimulant was particularly effective at early stage of ripening. Probably, the anticipated veraison found in Pinot Noir can be a consequence of promotion of the phenylpropanoid metabolism induced by the higher sugars content, generating a pool of secondary metabolites including anthocyanin and flavonoids (Vogt 2010; Dai et al., 2011). Taking into account anthocyanins and phenolics, AN extract was quantitatively more effective in Sangiovese and Pinot Noir than in Cabernet Franc, in comparison with control. This may be due to the genetically higher anthocyanins and phenolic profile of Cabernet Franc in comparison with other tested cultivars (Robinson et al., 2013).

The results point out for the first time a positive effect of AN extract on phenols and anthocyanins in grapevine cultivars for red wine production on a wide range of climatic conditions. Furthermore, our experiments demonstrate an impact on the phenol accumulation distributed over the ripening process and particularly significant at the early stages of ripening and right before harvest. These findings are consistent with observation on other horticultural crops that reported an increase of anthocyanins and phenolic content (Ochmian et al., 2008; Lola-Luz et al., 2013, 2014a,b; Roussos et al., 2009; Fan et al., 2011, 2013). The authors attributed the effects to the AN extract's ability to modulate plant endogenous growth regulators (mostly cytokinins and abscisic acid) metabolism and catabolism (Lola-Luz et al., 2013, 2014a,b; Fan et al., 2011, 2013; Wally et al., 2013). The response to AN extract treatments observed in Sangiovese, Pinot Noir and Cabernet Franc can be theoretically due to a modulation of grapevine endogenous phytohormones. Zeatin and abscisic acid are known to be affected by AN extract applications (Wally et al., 2013; Goñi et al., 2016) and to be involved in the induction of veraison, berry ripening and anthocyanins biosynthesis (Deikman and Hammer 1995; Wheeler et al., 2009).

The positive effects of AN extract in red wine grapes are pivotal, being the phenolic maturity a main component of fruit quality. These results suggest that AN extracts could be useful to growers under different and variable environmental conditions to achieve an optimal fruit maturity for the production of premium red wines.

5. Conclusions

Ascophyllum nodosum extracts are natural products that can be useful in different viticultural regions and situations. Medium-late seaweed extract applications significantly increased anthocyanins content in three of the most important grape cultivars for red wine production under warm and cool climate conditions.

This is the first report about positive effects of a biostimulant obtained by the brown seaweed *Ascophyllum nodosum* in ripening dynamics and fruit quality at harvest in wine grape. Medium-late multiple applications of the seaweed extract can be a useful tool to improve quality of grapes for the production of premium red wines. The technique can be particularly suitable if chromatic proprieties of the product are pivotal or for wines that are supposed to age. Further studies are needed to clarify mechanisms of action involved and metabolites dynamics after the seaweed extract's application.

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