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Research Article

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Posted Date: November 12th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-11468022/v1

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Additional Declarations: No competing interests reported.

Stage-Dependent Effects of Abscisic Acid and Seaweed Extract on Cold Hardiness and Protective Metabolism in Grapevine (*Vitis vinifera* L. *cv*)

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Abstract

Late winter and early spring frost events have become an increasing threat to grapevine production under changing climatic conditions, highlighting the need for practical strategies to improve bud freezing tolerance during dormancy. Although exogenous abscisic acid (ABA) and seaweed (SW) extracts have been reported to enhance plant tolerance to abiotic stresses, their comparative effectiveness throughout different dormancy stages and among grapevine cultivars remains insufficiently understood. This study evaluated the effects of foliar ABA and SW applications on bud cold hardiness and associated biochemical responses in three *Vitis vinifera* cultivars over two consecutive growing seasons. Field-grown vines were treated with ABA or SW, and bud freezing tolerance was determined by low-temperature exotherm (LTE) analysis during cold acclimation, mid-winter dormancy, and deacclimation. To investigate the physiological basis of treatment responses, proline, reducing sugars (RS), and malondialdehyde (MDA) contents were quantified, and multivariate relationships among variables were examined using principal component analysis (PCA). Both ABA and SW significantly improved bud freezing tolerance, although the magnitude of the response depended on cultivar, dormancy stage, and seasonal conditions. The strongest improvements were generally observed during the deacclimation period, when ABA increased freezing tolerance by up to 2.72 °C and SW by up to 3.96 °C. The treatments also induced distinct biochemical responses. ABA consistently promoted proline accumulation in responsive cultivars, whereas both treatments reduced lipid peroxidation in specific cultivar–stage combinations but were ineffective or even increased oxidative stress in others, particularly in the cold-sensitive cultivar Red Globe. PCA further demonstrated clear associations between freezing tolerance and biochemical traits and confirmed that treatment responses were strongly genotype and stage dependent.The results provide physiological evidence supporting the use of these treatments as practical tools for improving winter resilience in grapevine while emphasizing that their effectiveness is highly dependent on genotype and dormancy stage.

Keywords: Viticulture, Low Temperature Exotherm, Proline, Reducing Sugar, MDA, PCA

Introduction

The development and maintenance of cold hardiness are critical determinants of winter survival and productivity in grapevine (*Vitis vinifera* L.). During dormancy, grapevine buds gradually acquire freezing tolerance through cold acclimation, a process regulated by environmental cues such as decreasing temperature and photoperiod [1, 2]. However, ongoing climate change has increased the frequency of erratic winter warming events followed by sudden frost episodes, disrupting the natural acclimation–deacclimation cycle and substantially increasing the risk of bud injury and yield loss in viticultural regions worldwide.

To reduce freezing damage, considerable attention has been directed toward exogenous compounds capable of enhancing plant stress tolerance. Among these, abscisic acid (ABA) has been extensively investigated because of its central role in regulating dormancy, cold acclimation, osmotic adjustment, and antioxidant defense [3]. Likewise, seaweed (SW) extracts have attracted increasing interest as natural biostimulants owing to their diverse bioactive constituents, including phytohormones, polysaccharides, betaines, and mineral nutrients, which collectively improve plant resilience to abiotic stresses [4, 5].

Although both ABA and seaweed extracts have individually been reported to improve tolerance to drought, salinity, and low temperatures, their comparative effects on grapevine freezing tolerance throughout the successive stages of dormancy remain poorly understood. In particular, little information is available regarding whether their physiological effectiveness changes during acclimation, mid-winter dormancy, and deacclimation, and whether these responses differ among commercially important grapevine cultivars. Furthermore, the relationships between treatment-induced improvements in freezing tolerance and associated biochemical adjustments have not been comprehensively evaluated under field conditions. ABA functions as a major signaling hormone coordinating grapevine responses to environmental stress. Previous studies demonstrated that exogenous ABA enhances freezing tolerance [6, 7] by promoting osmotic adjustment, reducing membrane damage [8], stimulating antioxidant systems, and regulating cold-responsive gene expression [9–11]. These physiological responses are frequently accompanied by the accumulation of compatible solutes, particularly soluble sugars and proline, which stabilize cellular structures during freezing stress [10]. Recent transcriptomic studies have further shown that ABA regulates extensive stress-responsive metabolic pathways in grapevine, emphasizing its central role in cold adaptation [12, 13].

Seaweed extracts represent an environmentally friendly alternative for improving stress tolerance in perennial crops. Extracts derived from brown algae such as Ascophyllum nodosum contain numerous biologically active compounds capable of enhancing plant metabolism, antioxidant capacity, and membrane stability [14–16]. In grapevine, seaweed-based biostimulants have been associated with improved vegetative growth, photosynthetic performance, and stomatal conductance [17–19]. Nevertheless, compared with drought or salinity, their potential role in enhancing bud freezing tolerance has received considerably less attention, and direct comparisons with ABA under field conditions remain scarce.

Cold hardiness is a highly dynamic trait that differs among cultivars and changes continuously throughout dormancy [20]. Consequently, evaluating freezing tolerance at a single developmental stage provides only limited insight into treatment efficacy. Integrating physiological measurements with biochemical indicators across the entire dormancy period offers a more comprehensive understanding of how exogenous applications influence cold adaptation under naturally fluctuating environmental conditions.

Therefore, the present study compared the effects of foliar ABA and seaweed extract applications on freezing tolerance in three *Vitis vinifera* cultivars over two consecutive seasons. Bud cold hardiness was assessed using low-temperature exotherm (LTE) analysis during acclimation, mid-winter, and deacclimation, while proline, reducing sugars, and malondialdehyde were quantified to characterize treatment-induced physiological responses. Principal component analysis was further employed to integrate physiological and biochemical traits and identify the major factors associated with improved freezing tolerance. The findings provide new insights into the stage-dependent and cultivar-specific effectiveness of ABA and seaweed extracts and their potential application as practical tools for improving winter resilience in grapevine.

Materials and Methods

Study area and plant material

Dormant grapevine canes were collected from privately owned commercial vineyards located in Pamukova, Sakarya, Türkiye. The cultivars investigated were ‘Trakya İlkeren’ (40°28′22.9″N, 30°06′12.5″E), ‘Michele Palieri’ (40°28′23.6″N, 30°06′39.7″E), and ‘Red Globe’ (40°28′14.2″N, 30°07′18.2″E). All plant material was collected with the explicit permission of the vineyard owner. The collection and experimental procedures were conducted in accordance with relevant institutional, national, and international guidelines and legislation. Cultivar identities were confirmed by Dr. Gülhan Gülbasar Kandilli. The collected plant material was maintained in cold storage facilities at the Atatürk Horticultural Central Research Institute, Yalova, Türkiye, as part of research project TAGEM/BBAD/A/21/A1/P6/2477.

The study area is characterized by fertile alluvial soils originating from deposits of the Sakarya River [21]. Based on meteorological records long-term (1959-2020) mean annual maximum and minimum temperatures of 19.8 °C and 8.8 °C, respectively, and an annual precipitation of 561.2 mm [22]. During the experimental seasons (2019–2021), January was the coldest month; the lowest recorded daily minimum temperature was −9.9 °C on 20 January 2021. August was the warmest month, with a maximum temperature reaching 31.7 °C. The highest mean daily relative humidity was observed in December (93.9%) during the study period.

The plant material consisted of grafted grapevine cultivars grown under standard commercial practices: 'Trakya İlkeren' (TI) grafted onto Kober 5BB rootstock, 'Michele Palieri' (MP) grafted onto 1103 Paulsen, and 'Red Globe' (RG) grafted onto 140 Ruggeri. Vine spacing was 3.0 m × 2.0 m for TI and MP, whereas RG vines were planted at 3.0 m × 1.5 m. Vines were 5–8 years old and trained on a high-wire cordon system positioned approximately 4.0–5.5 feet above ground level. Vines were pruned at the end of March each year using the double Guyot training system, retaining 24 buds per vine. Air temperature data were monitored using a HOBO U12–013 data logger.

Cane samples were collected from each cultivar at three time points per season: 19 December 2019, 23 January 2020, and 12 March 2020 (Year I), and 20 December 2020, 21 January 2021, and 12 March 2021 (Year II). Approximately 80 one-year-old canes of uniform thickness were randomly sampled from vines per cultivar, placed in plastic bags, and transported to the laboratory. For each cane, the first four buds starting from the basal position were excised and used for subsequent analyses. Prior to differential thermal analysis, samples were stored at 4 °C.

ABA and Seaweed Treatments

Abscisic acid (ABA) was applied to four vines of each cultivar at a concentration of 400 mg L^{-1} active ingredient, using a commercial S-ABA formulation (Protone™, Valent BioSciences, Libertyville, IL, USA). Unlike standard commercial practices focused solely on cluster coloration, the treatment in this study was applied to the entire green canopy at the veraison stage [23].

Seaweed extract was applied to four vines of each cultivar at a concentration of 3 ml L^{-1} using a commercial liquid formulation (Seasol®, Seasol International Pty Ltd, Bayswater, Victoria, Australia). The application was performed at two distinct phenological stages: preveraison and postharvest.

Vines in the control group received no treatment.

Low-temperature exotherm (LTE) determination

Bud cold hardiness was assessed using differential thermal analysis (DTA) performed with a programmable freezing chamber (Tenney Junior Environmental Test Chamber) located at Atatürk University. Dormant buds were carefully excised from canes using a sharp razor blade, while retaining approximately 1–2 mm of woody tissue at the bud base [24]. Silicone grease was applied to the cut surface to minimize dehydration effects.

For DTA measurements, eight to nine buds were positioned cut-side down onto a thermoelectric module (TEM). One TEM located in the middle of each tray was left empty and served as a reference control for signal normalization across modules [20]. Trays were tightly sealed and placed into the programmable freezer. The chamber temperature was initially stabilized at 4 °C and then lowered at a constant rate of 4 °C h^{-1} until reaching a final temperature of –35.2 °C.

A data acquisition system recorded voltage outputs generated during exotherm events associated with tissue supercooling. Voltage signals were automatically exported to Microsoft Excel, and the low-temperature exotherm (LTE) was defined as the temperature corresponding to the peak exotherm signal for each bud. LT50 values were calculated as the mean of LTE peak temperatures, as previously described [24].

Cane diameters measurements

Shoot and cane diameters were measured using a high-precision digital caliper. For each cane, four separate measurements were taken across the first five internodes (starting from the base). A total of six canes were sampled for each combination of cultivar and treatment per period. Overall, 54 canes were evaluated during each measurement period to ensure statistical robustness.

Bud sampling for biochemical analyses

Compound bud samples obtained from the cane collections described above were used for biochemical assays. Excised buds were immediately frozen in liquid nitrogen and stored at –80 °C until analysis. Frozen samples were ground into a fine powder using a pre-chilled mortar and pestle under liquid nitrogen. All biochemical measurements were conducted using three biological replicates per treatment.Malondialdehyde (MDA) quantification

Lipid peroxidation was estimated by measuring malondialdehyde (MDA) content according to Heath and Packer (1968) [25]. Briefly, 0.1 g of frozen bud powder was homogenized in 2 mL of 5% trichloroacetic acid (TCA). Homogenates were centrifuged at 12,000 × g at 4 °C for 30 min. The resulting supernatant (1 mL) was transferred

into glass tubes and mixed with 1 mL of 0.6% thiobarbituric acid (TBA). Samples were incubated at 90 °C for 30 min and then centrifuged again at 12,000 × g for 15 min.
Absorbance of the final supernatant was measured at 532, 600 and 450 nm using a spectrophotometer, with the reaction mixture without tissue used as blank. MDA concentration was calculated as:
MDA (nmol g^{-1} FW) = [(6.45 × (A532 − A600)) − (0.56 × A450)] / FW [26].

Reducing sugars (RS) determination
Reducing sugars (RS) were determined following the method described by [27]. Briefly, 0.2 g of ground bud tissue was mixed with 0.2 mL of 15% potassium ferrocyanide and 0.2 mL of 30% zinc sulfate, and the total volume was adjusted to 10 mL with distilled water. The mixture was filtered, and 0.5 mL of the filtrate was combined with 6.0 mL of dinitrophenol reagent and 1.5 mL of distilled water.

Samples were heated in a boiling water bath (100 °C) for 6 min, cooled under running water for 3 min, and absorbance was recorded at 600 nm using a spectrophotometer (Hitachi U-2900, Japan). A dinitrophenol–water mixture without sample extract served as the negative control.

Proline determination
Free proline was determined according to Bates et al. (1973) [28]. Bud samples were ground in liquid nitrogen and homogenized in 5 mL of 3% (w/v) sulfosalicylic acid. After filtration through Whatman No. 2 paper, the filtrate was reacted with acid-ninhydrin and glacial acetic acid at 100°C for 1 hour. The reaction was terminated in an ice bath, followed by extraction with 4 mL of toluene. The absorbance of the toluene phase was measured at 520 nm. Proline concentrations were calculated via a standard curve and expressed as μmol g^{-1} FW. Analyses were performed across three periods (autumn, winter, and spring) concurrently with DTA tests.

Statistical analysis
All statistical analyses were performed using JMP statistical software (version 7.0; SAS Institute Inc., Cary, NC, USA) (SAS, 2003). Mean comparisons were conducted using the LSMeans Student's t-test at a significance level of $p \leq 0.05$. Pearson's correlation coefficients were calculated to assess relationships between LTE50 values and the measured physiological parameters. Dot plot visualizations were generated using Python (Matplotlib) and exported at high resolution.

Results

Climatic conditions during the experimental seasons
Daily mean air temperatures from September to April differed between the two experimental seasons (Figure 1). In both years, temperatures gradually declined from autumn to winter and increased again from March onward. Compared with Year 1 (2019–2020), Year 2 (2020–2021) exhibited greater temperature fluctuations and lower minimum temperatures, particularly during January and February.
Daily mean temperature data recorded between September and April highlighted distinct climatic patterns for both experimental years (Figure 1). While Year 1 exhibited a more stabilized decline in temperature, Year 2 was characterized by more pronounced fluctuations and lower minimum mean temperatures, particularly during January and February. In both years, a sharp increase in mean temperatures was observed starting from March, marking the onset of the deacclimation phase for the grapevines.

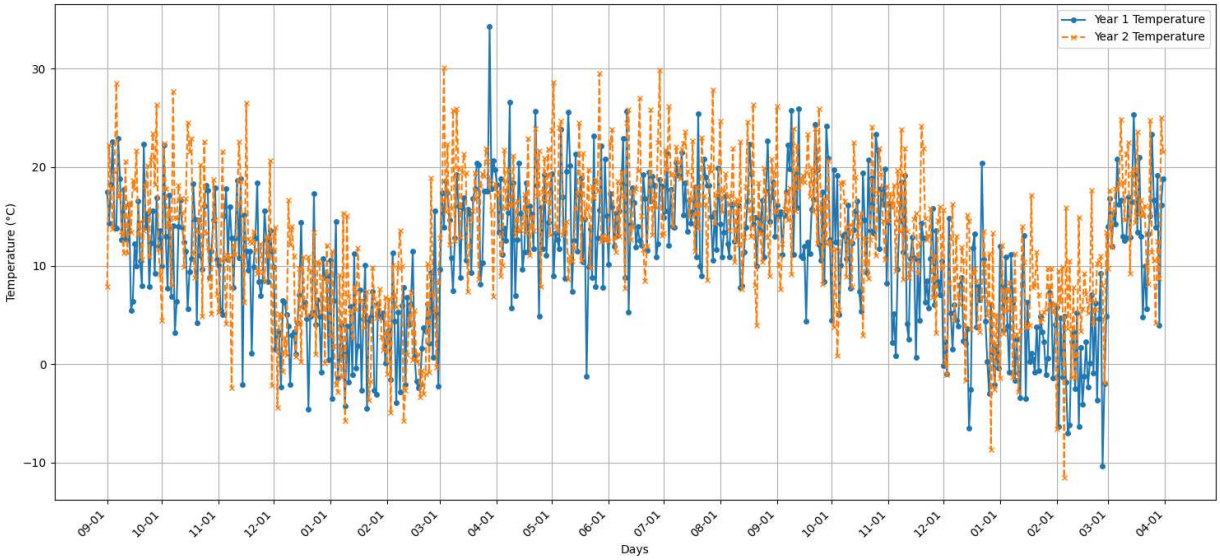


Figure 1. Daily mean temperature fluctuations during the two consecutive research seasons (Year 1: September 2019 – April 2020; Year 2: September 2020 – April 2021).

Effect of Treatments on Low Temperature Exotherms

Foliar applications of ABA and seaweed (SW) extract affected bud freezing tolerance throughout dormancy, although the magnitude of the response varied among cultivars, sampling periods, and years (Figure 2). During the first sampling period of 2020, ABA improved freezing tolerance by 0.40–1.76 °C depending on the cultivar. In the same period, SW increased freezing tolerance by 1.23 °C in the RG cultivar (Figure 2a). During the corresponding period in 2021, ABA enhanced freezing tolerance by 1.10 °C in MP (Figure 2b).

During the second sampling period of 2020, ABA significantly improved freezing tolerance by 1.65 °C in MP and 0.82 °C in RG. Seaweed application increased freezing tolerance by 1.07 °C in MP and 0.78 °C in TI. No significant treatment effects were detected during the corresponding period in 2021.

The largest treatment responses were observed during the third sampling period. In 2020, ABA enhanced freezing tolerance by 2.72 °C in MP and 1.81 °C in RG. Seaweed treatment produced comparable or greater improvements, increasing freezing tolerance by 3.96 °C in MP and 1.00 °C in RG. During the same period in 2021, ABA increased freezing tolerance by 0.53 °C in TI and 0.84 °C in RG, whereas SW improved freezing tolerance by 1.01 °C in MP and 0.59 °C in RG.

Overall, both ABA and SW treatments improved bud freezing tolerance across the dormancy period, although treatment responses differed among cultivars and between growing seasons.

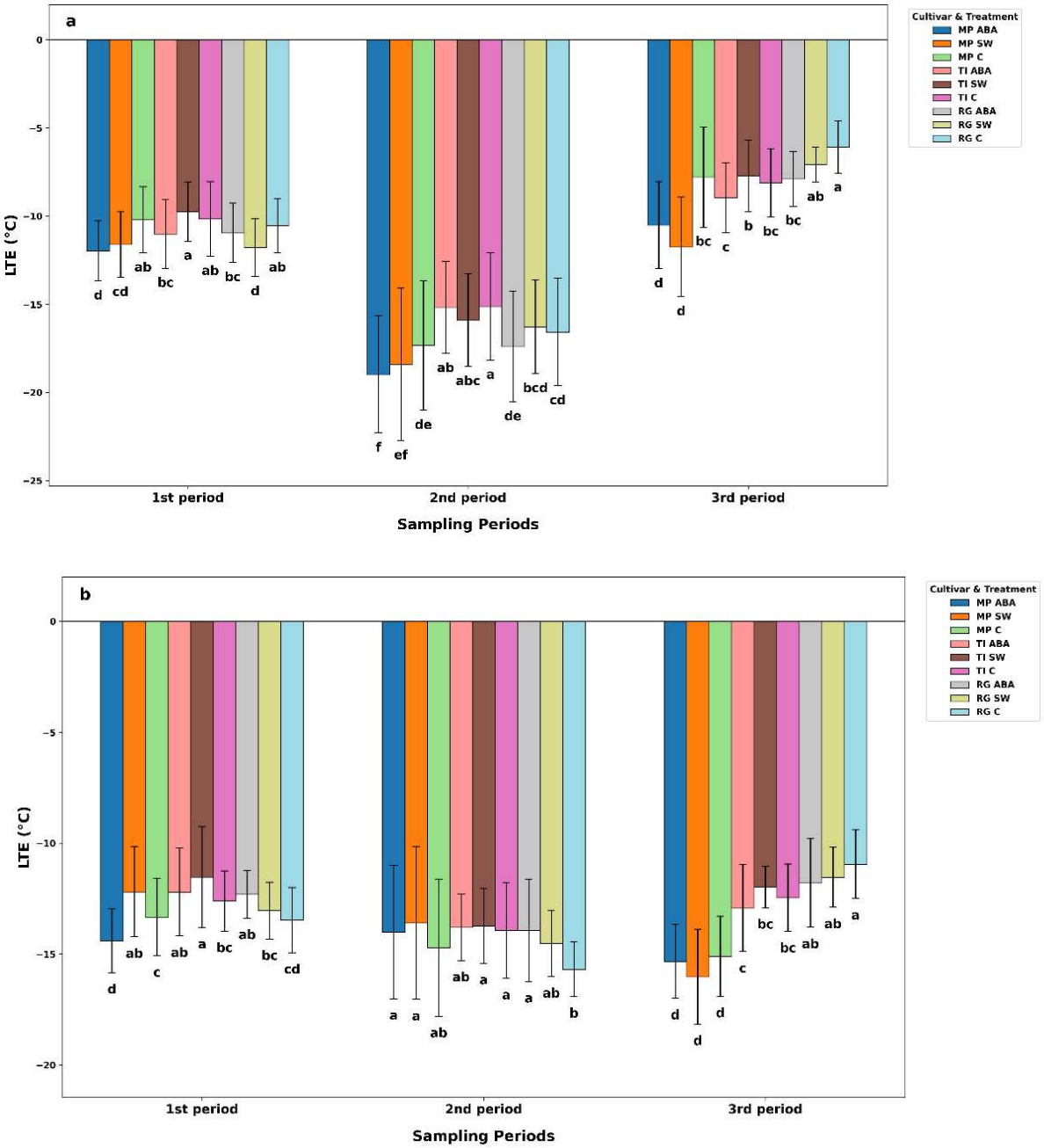


Figure 2. Comparative analysis of low-temperature exotherm (LTE) values during two consecutive research seasons: (a) Year 1 (2019–2020) and (b) Year 2 (2020–2021). Bars represent the mean values of three grapevine cultivars under ABA and SW treatments across three sampling periods. Different letters within each period indicate statistically significant differences according to LS Means Student test ($p < 0.001$). Error bars denote the standard deviation (SD). (MP: Michele Palieri, TI: Trakya Ilkeren, RG: Red Globe; ABA: Abscisic acid, SW: Seaweed extract, C: Control)

Effect of treatments on cane diameter

Cane diameter was measured to determine whether foliar ABA and SW applications affected vegetative growth during the experimental period (Table 1). Across all cultivars, cane diameter ranged from 6.8 to 11.8 mm in 2020 and from 7.9 to 14.7 mm in 2021.Minor differences among treatments were detected within individual sampling periods; however, no consistent treatment effect was observed across cultivars or years. In MP, SW-treated vines exhibited slightly greater cane diameters than the control during the second sampling period of both years, whereas treatment-related differences in TI and RG were small and inconsistent. Overall, cane diameter remained comparable among ABA-, SW-, and control-treated vines throughout the study.Linear regression analysis showed no significant relationship between cane diameter and bud freezing tolerance (LTE) in any sampling period (Figure 3; Supplementary Figure a-f). Correlation coefficients were consistently low, indicating that variation in cane diameter was not associated with variation in LTE values.

Table 1 Effects of foliar ABA and seaweed (SW) applications on cane diameter (mm) in three grapevine cultivars during two consecutive seasons.

Cult/Treatments	2020			2021		
	1st period	2nd period	3rd period	1st period	2nd period	3rd period
MP ABA	7.66±0.92abc	9.75±0.74b	11.08±1.03a	8.87±1.54de	11.19±1.89ab	9.79±1.34
MP SW	7.9±1.16ab	11.37±0.99a	10.23±0.68b	10.47±1.14a	11.65±1.39a	9.3±1.59
MP C	8.13±0.96a	8.78±1.34de	11.15±1.04a	10.13±1.02ab	11.83±1.4a	14.78±1.55
TI ABA	7.76±0.96ab	9.36±0.98bc	9.13±0.81cd	9.7±0.08a	10.22±10.22c	7.99±1.08
TI SW	6.8±0.9d	9.79±1.28b	9.47±1.18c	8.69±1.44e	9.35±1.02d	9.27±1.22
TI C	8.3±0.59a	9.0±1.19cd	8.34±1.29e	9.69±1.48bc	10.28±1.17c	9.18±0.95
RG ABA	7.16±0.64cd	8.27±0.67ef	8.47±0.55de	9.36±0.81cd	11.29±1.43a	11.36±1.44
RG SW	7.39±0.67bc	7.95±0.77f	8.05±1.34e	9.37±1.25cd	11.82±1.25a	9.44±1.55
RG C	7.38±1.59bc	8.25±0.91ef	8.63±1.1de	10.46±1.08a	10.45±1.72bc	10.65±1.06

Values represent the means ± standard deviation (SD). Mean separation within columns by LS Means Student's multiple comparison test at the $p < 0.05$ level.

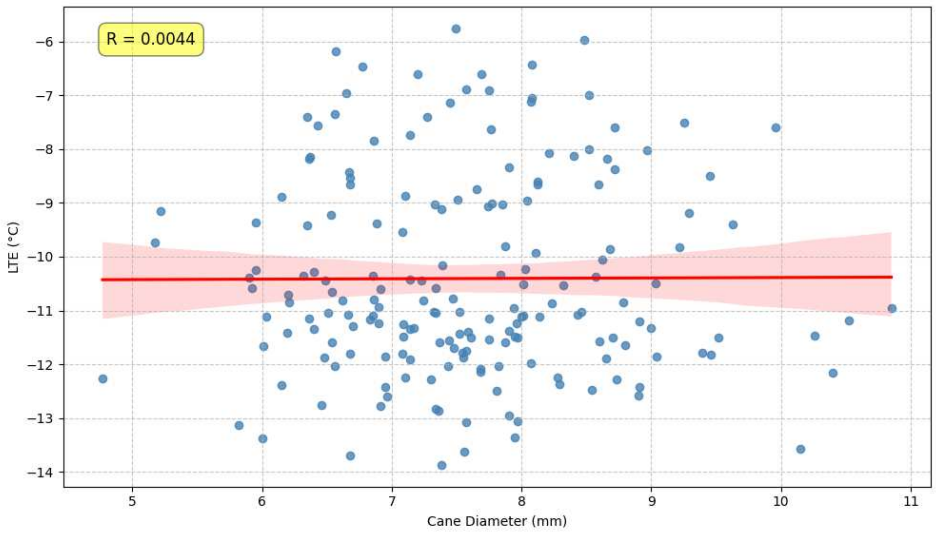


Figure 3. Representative relationship between cane diameter and bud freezing tolerance (LTE) during the first sampling period of the 2019–2020 season. The regression analysis showed no significant association (R = 0.0044; n = 54). Similar relationships were observed in all other sampling periods (Supplementary Figure a-f).

Effect of treatments on bud biochemicals

Foliar ABA and SW applications altered bud biochemical characteristics, although responses varied among cultivars, sampling periods, and years (Table 2).

2020 season

During the first sampling period, neither ABA nor SW significantly affected reducing sugar (RS) concentrations in any cultivar. In contrast, both treatments reduced malondialdehyde (MDA) levels in TI, decreasing MDA from 6.73 nmol g^{-1} FW in the control to 3.33 and 2.21 nmol g^{-1} FW following ABA and SW applications, respectively. During the second sampling period, ABA and SW increased proline accumulation in MP, with the highest value recorded in ABA-treated vines (18.48 µmol g^{-1} FW). RS concentrations remained similar among treatments, whereas MDA levels in TI decreased to 1.78 and 1.53 nmol g^{-1} FW following ABA and SW applications, respectively.

During the third sampling period, proline concentrations were not significantly affected by either treatment. However, both ABA and SW reduced MDA concentrations in MP to 0.76 and 0.66 nmol g^{-1} FW, respectively, compared with the control.

2021 season

During the first sampling period, SW increased proline concentrations in MP and TI. In MP, ABA and SW also increased RS concentrations compared with the control. In TI, SW increased RS concentration, whereas MDA was lower than in the control. In contrast, SW-treated RG vines exhibited higher MDA concentrations than untreated vines.

During the second sampling period, MP showed the strongest biochemical response to both treatments. Proline increased to 6.34 and 5.30 μmol g^{-1} FW following ABA and SW applications, respectively, while RS concentrations also increased relative to the control. In TI, neither treatment substantially affected proline accumulation, and RS concentrations remained highest in the control. In RG, SW increased proline concentration, whereas RS values were comparable among treatments.

During the third sampling period, treatment responses differed among cultivars. In MP, ABA and SW increased RS concentrations relative to the control. In RG, both treatments increased proline concentrations. MDA concentrations increased in TI and RG following both applications, whereas only minor differences were observed among treatments in MP.

Overall, biochemical responses to ABA and SW were dependent on cultivar, dormancy stage, and growing season. Proline accumulation and lipid peroxidation exhibited the greatest treatment-dependent variation, whereas reducing sugar concentrations generally showed smaller responses across treatments.

Table 2. Effects of foliar ABA and SW applications on proline (Pro, µmol g^{-1}), reducing sugars (RS, mg g^{-1}), and malondialdehyde (MDA, nmol g^{-1}) levels in buds of three grapevine cultivars

Year	2020									2021								
Cult/ Treat	1st period			2nd period			3rd period			1st period			2nd period			3rd period		
	Pro	RS	MDA	Pro	RS	MDA	Pro	RS	MDA	Pro	RS	MDA	Pro	RS	MDA	Pro	RS	MDA
MP ABA	ND	3.54±0.06d	1.67±0.63c	18.48±0.21c	5.82±0.08c	6.79±0.46a	9.51±0.06d	1.91±0.20ab	2.7±0.40c	0.98±0.12f	2.44±0.11c	4.62±0.11c	6.34±0.29a	4.92±0.06b	4.9±0.37e	6.89±0.06c	1.85±0.20b	6.88±0.40b
MP SW	ND	4.19±0.33c	3.25±0.13bc	17.62±0.24d	5.91±0.02c	2.79±0.58bc	10.69±0.48c	1.77±0.09b	2.6±0.11cd	1.45±0.0c	2.09±0.05d	5.17±0.06b	5.3±0.08b	4.51±0.05c	5.92±0.09b	3.28±0.48f	2.02±0.09a	6.1±0.11c
MP C	ND	3.75±0.21d	3.49±0.24bc	12.25±0.17e	6.21±0.25b	1.78±0.52cd	9.51±0.14d	2.08±0.02a	3.36±0.35b	1.03±0.33ef	1.53±0.02e	3.85±0.19d	3.39±0.06d	3.33±0.07e	4.89±0.29de	7.71±0.14b	0.7±0.02c	6.61±0.35bc
TI ABA	ND	4.45±0.13bc	3.33±0.58bc	10.18±0.18f	6.73±0.12a	1.78±0.65cd	6.18±0.2f	0.46±0.13d	3.63±0.29b	1.66±0.06b	2.37±0.06c	4.34±0.13c	0.7±0.27h	4.16±0.12d	6.59±0.61a	7.51±0.02b	0.22±0.01e	8.89±0.08a
TI SW	ND	4.63±0.0ab	2.21±0.36bc	18.28±0.23cd	6.47±0.23ab	1.53±0.21cde	2.98±0.38g	1.24±0.25c	2.37±0.87cd	1.82±0.05a	3.33±0.06a	5.19±0.18b	0.26±0.04i	4.48±0.13c	5.59±0.08c	5.27±0.35e	0.21±0.06e	8.37±0.15a
TI C	ND	4.82±0.1a	6.73±0.36a	18.62±0.72c	6.63±0.15a	3.54±0.42b	8.04±0.73e	1.08±0.12c	3.72±0.53b	1.36±0.35cd	2.85±0.02b	5.22±0.23b	1.22±0.04g	5.39±0.17a	5.21±0.30d	8.12±0.18a	0.58±0.17d	3.8±0.16d
RG ABA	ND	3.53±0.04d	3.23±0.39bc	21.02±0.33a	5.24±0.16d	0.96±0.06de	10.9±0.13c	0.27±0.0e	5.58±0.01a	1.08±0.02e	1.28±0.11fg	4.31±0.16c	2.35±0.25f	1.01±0.09g	4.21±0.08f	6.26±0.04d	ND	6.5±0.27bc
RG SW	ND	3.74±0.03d	4.31±1.01b	19.48±0.3b	5.0±0.11e	0.49±0.41de	13.04±0.09b	0.34±0.05de	2.11±0.36de	1.32±0.06d	1.2±0.06g	5.8±0.32a	4.01±0.06c	1.01±0.03g	3.82±0.08g	5.51±0.06e	ND	6.05±0.23c
RG C	ND	3.52±0.13d	2.53±0.08bc	6.08±0.13g	5.28±0.05d	0.2±0.53e	14.98±0.15a	ND	1.77±0.01e	1.89±0.02a	1.36±0.01f	3.67±0.26d	2.91±0.16e	1.8±0.031f	5.66±0.14bc	2.19±0.21g	ND	3.99±0.22d

Data are expressed as Mean ± SD (n=3). ND: Not Detected (below the limit of detection). MP: Michele Palieri; TI: Trakya İlkeren; RG: Red Globe; ABA: Abscisic Acid; SW: Seaweed; Pro: Proline; RS: Reducing Sugar; MDA: Malondialdehyde. Significance $p < 0.001$)

Principal component analysis of physiological and biochemical traits

Principal component analysis (PCA) was performed to examine the relationships among bud freezing tolerance (LTE), proline, reducing sugars (RS), and malondialdehyde (MDA) across sampling periods (Figures 4–6). The first two principal components explained 84.2%, 75.1%, and 78.7% of the total variation during the first, second, and third sampling periods, respectively.

First sampling period

During the first sampling period, PC1 and PC2 accounted for 61.8% and 22.4% of the total variation, respectively (Figure 4). LTE and RS were positively associated along PC1, whereas proline was positioned in the opposite direction. Samples from 2020 and 2021 formed distinct clusters. ABA-treated samples were distributed closer to the proline vector, while many 2021 samples were located nearer the MDA vector.

Second sampling period

During the second sampling period, PC1 explained 50.8% and PC2 explained 24.3% of the total variation (Figure 5). Proline and RS remained positively associated, whereas MDA was positioned in the opposite direction along PC1. LTE was primarily aligned with PC2. Sample distribution differed among treatments, with ABA- and SW-treated vines showing broader dispersion than the control.

Third sampling period

During the third sampling period, PC1 and PC2 explained 50.2% and 28.5% of the total variation, respectively (Figure 6). LTE and proline were similarly oriented, whereas RS showed a stronger association with PC2. Samples from 2021 were generally positioned closer to the MDA vector than those from 2020. ABA- and SW-treated samples occupied regions distinct from the control group.

Overall, PCA demonstrated clear differences in sample distribution among growing seasons, cultivars, and treatments, reflecting temporal variation in physiological and biochemical responses during dormancy.

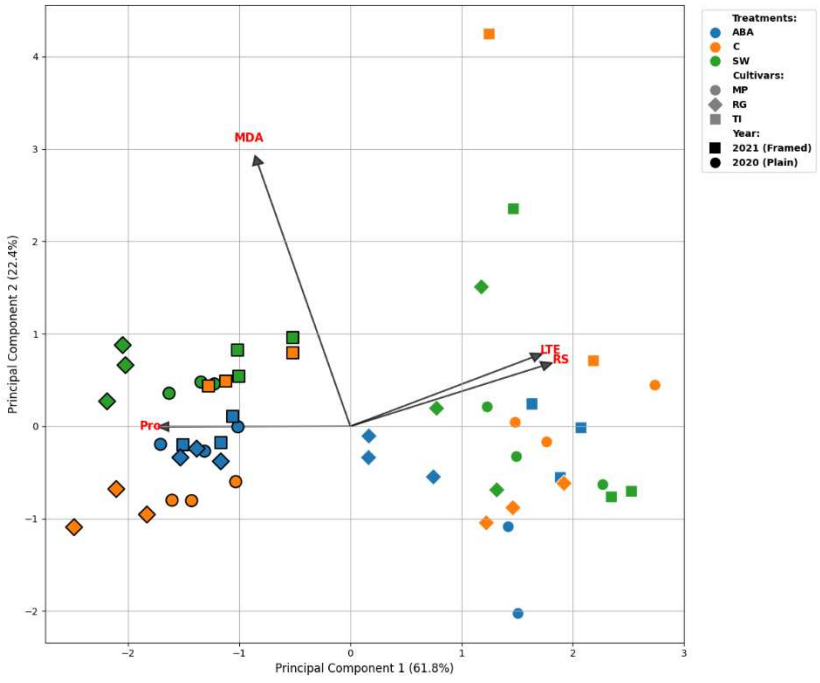


Figure 4: 1st periods of two years Principal Component Analysis (PCA) biplot illustrating the relationships between bud cold hardiness (LTE) and biochemical markers (Pro (μmol g^{-1}) , RS (mg g^{-1}) , MDA (nmol g^{-1}), cultivars (MP, RG, TI), and foliar treatments (Control, ABA, and SW). The vectors represent the loading of each physiological variable, while symbols denote individual samples, highlighting the distinct metabolic shifts and the potential modulating effects of foliar applications on vine resilience during the onset of cold acclimation.

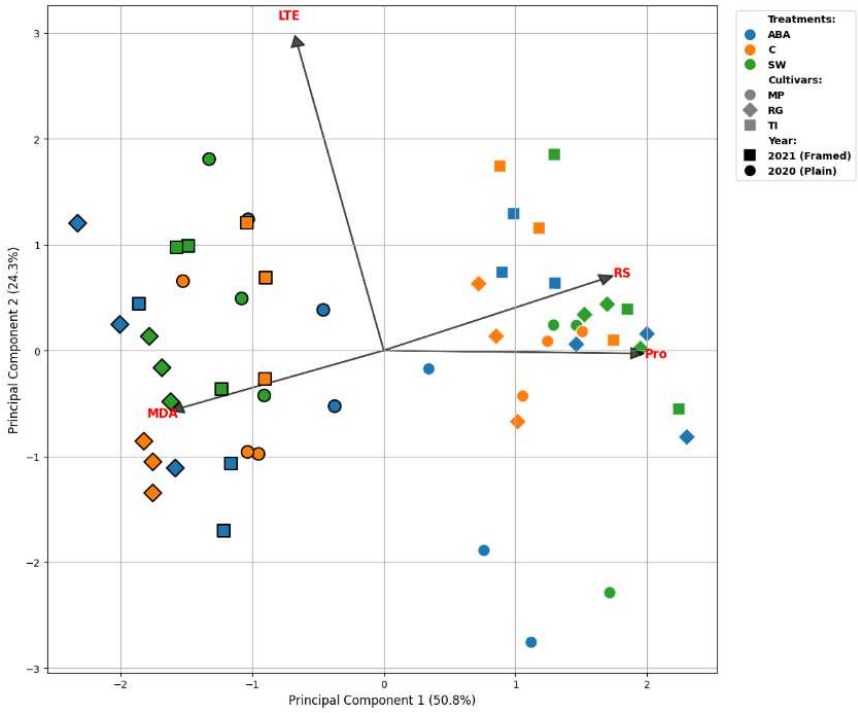


Figure 5: 2nd periods of two years Principal Component Analysis (PCA) biplot illustrating the relationships between bud cold hardiness (LTE) and biochemical markers (Pro (µmol g^{-1}) , RS (mg g^{-1}) , MDA (nmol g^{-1}), cultivars (MP, RG, TI), and foliar treatments (Control, ABA, and SW).

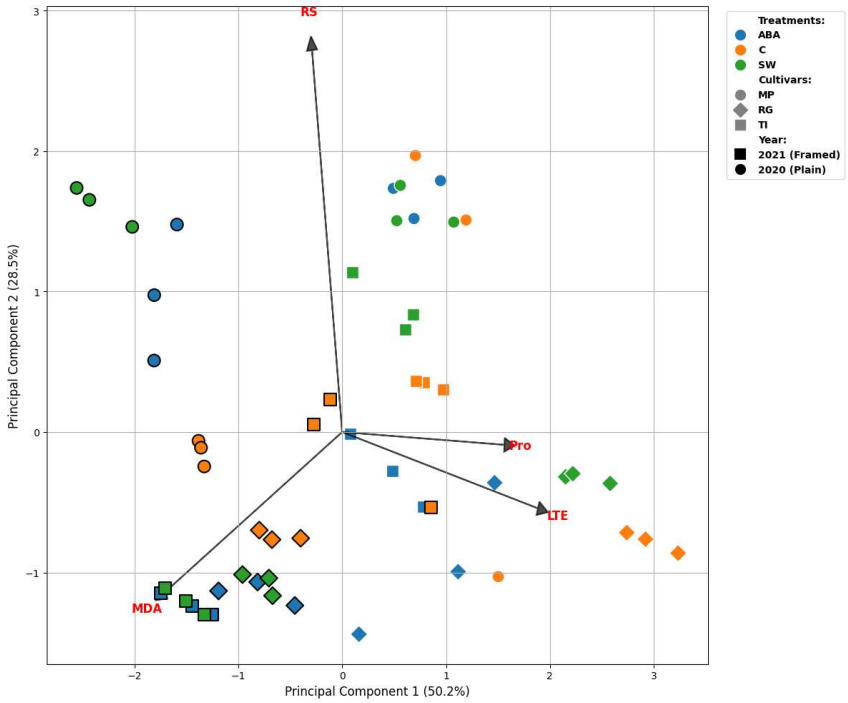


Figure 6: 3rd periods of two years Principal Component Analysis (PCA) biplot illustrating the relationships between bud cold hardiness (LTE) and biochemical markers (Pro (µmol g^{-1}) , RS (mg g^{-1}) , MDA (nmol g^{-1}), cultivars (MP, RG, TI), and foliar treatments (Control, ABA, and SW).

Discussion

Overall effects of ABA and seaweed extract on grapevine freezing tolerance

The present study demonstrates that foliar applications of abscisic acid (ABA) and seaweed (SW) extract improve bud freezing tolerance in grapevine throughout dormancy, although the magnitude of the response depends strongly on cultivar, dormancy stage, and seasonal climatic conditions. Unlike many previous studies that evaluated cold hardiness at a single developmental stage, the present work followed the dynamic progression of dormancy from cold acclimation to deacclimation over two consecutive seasons. This experimental approach revealed that the effectiveness of exogenous treatments is not constant but changes throughout dormancy, highlighting the importance of developmental stage when evaluating strategies for improving grapevine winter survival.

The improvement in freezing tolerance observed following ABA application agrees with previous reports describing ABA as a central regulator of cold acclimation and dormancy in grapevine [9, 29, 30]. In the present study, ABA consistently reduced LTE values, with the greatest improvements occurring during the deacclimation period. This finding suggests that exogenous ABA remains physiologically effective even after maximum winter hardiness has been reached and may contribute to maintaining bud cold tolerance during periods of fluctuating spring temperatures. Such a response is particularly relevant under current climatic conditions, where premature warming events frequently accelerate deacclimation and increase the risk of subsequent frost injury.

Seaweed extract also enhanced freezing tolerance, although its effects were generally more variable than those of ABA. This variability is not unexpected because commercial seaweed formulations contain complex mixtures of bioactive compounds whose physiological effects depend on species composition, extraction methods, environmental conditions, and plant developmental stage. Nevertheless, the improvements observed in the present study support previous reports demonstrating that brown seaweed extracts, particularly those derived from *Ascophyllum nodosum*, enhance tolerance to abiotic stresses through multiple physiological pathways [5, 14, 31]. Although direct evidence for freezing tolerance in grapevine remains limited, the present results indicate that seaweed extracts can contribute to improved bud cold hardiness under field conditions.

One of the most important findings of this study is that treatment efficacy was strongly influenced by environmental conditions. During the 2019–2020 season, both ABA and SW substantially improved freezing tolerance, whereas treatment responses were generally smaller during the colder 2020–2021 season. This difference likely reflects the stronger natural cold acclimation induced by lower winter temperatures in the second year. Under such conditions, endogenous cold acclimation mechanisms may have already approached their physiological maximum, leaving less opportunity for additional improvement through exogenous applications. Similar interactions between environmental conditions and ABA-mediated cold acclimation have been reported previously [29, 30], indicating that treatment efficacy depends not only on the applied compound but also on the intensity of naturally induced cold responses.

The present findings further demonstrate that freezing tolerance cannot be explained solely by treatment effects but results from interactions among environmental conditions, genotype, and dormancy stage. Such interactions are frequently overlooked in studies evaluating cold hardiness under controlled environments or during a single sampling period. By examining multiple cultivars across three stages of dormancy over two consecutive years, the present study demonstrates that the physiological benefits of foliar ABA and seaweed applications are highly dynamic. Consequently, recommendations for the practical use of these compounds should consider both seasonal climatic conditions and cultivar-specific responses rather than assuming uniform treatment efficacy.

Seasonal and cultivar-dependent responses to ABA and seaweed applications

A major outcome of the present study is that the effectiveness of both ABA and seaweed applications was strongly dependent on dormancy stage, cultivar, and seasonal climatic conditions. Rather than producing a uniform increase in freezing tolerance throughout the dormant period, both treatments displayed dynamic responses that varied among sampling periods and between years. This finding emphasizes that exogenous applications should be considered as modulators of the natural acclimation process rather than universal enhancers of cold hardiness.

The contrasting responses observed between the two experimental seasons illustrate the importance of environmental conditions in determining treatment efficacy. Although ABA and SW substantially improved freezing tolerance during the 2019–2020 season, their effects were generally less pronounced during 2020–2021. Meteorological data indicated that the second season experienced lower winter temperatures and greater temperature fluctuations, conditions that likely promoted stronger natural cold acclimation. Under these circumstances, grapevine buds may have approached their maximum genetically determined freezing tolerance, thereby reducing the additional benefit provided by exogenous applications. Similar interactions between environmental cues and ABA-regulated cold acclimation have been reported previously, demonstrating that endogenous acclimation processes largely determine the extent to which external treatments can further improve freezing tolerance. [32–34]

Another important observation was the marked variation among cultivars. Michele Palieri generally exhibited the greatest improvement following ABA treatment, particularly during the second and third sampling periods, whereas Red Globe showed a more variable response and Trakya İlkeren displayed intermediate sensitivity. These cultivar-specific responses are consistent with previous studies reporting considerable genetic variation in grapevine cold acclimation capacity and dormancy behaviour [20, 32]. Such variation probably reflects differences in endogenous ABA metabolism, cold-responsive signalling pathways, membrane stability, and osmotic adjustment capacity among cultivars.

The particularly strong response observed during the deacclimation period deserves special attention. In viticulture, deacclimation represents one of the most vulnerable phases of the annual cycle because warming temperatures accelerate the loss of freezing tolerance while the risk of late spring frost remains high. The substantial reduction in LTE values following ABA application during this period suggests that exogenous ABA may delay or moderate the decline in cold hardiness as buds transition toward renewed growth. Although the present study did not directly evaluate hormonal regulation during deacclimation, the physiological responses observed are consistent with previous reports showing that ABA contributes to the maintenance of dormancy [29, 30] and suppression of premature bud development under fluctuating spring temperatures.

Seaweed extract showed a response pattern comparable to ABA in several cultivar–stage combinations but with greater variability. Unlike ABA, whose physiological role in cold acclimation has been extensively characterised, seaweed extracts contain multiple biologically active compounds that influence several metabolic pathways simultaneously. Consequently, their effectiveness is expected to depend more strongly on environmental conditions and genotype. Nevertheless, the present results demonstrate that seaweed extract can substantially enhance freezing tolerance in specific cultivar–stage combinations, indicating that biostimulant-based approaches deserve greater attention as complementary tools for improving winter resilience in perennial fruit crops.

Collectively, these findings demonstrate that treatment timing is as important as treatment selection. The physiological benefits of ABA and seaweed applications cannot be generalized across all cultivars or throughout the entire dormant season. Instead, successful implementation requires consideration of genotype, seasonal temperature patterns, and the physiological stage of dormancy. This dynamic perspective provides a more realistic framework for developing management strategies aimed at mitigating frost damage under increasingly variable climatic conditions.

Physiological mechanisms underlying improved freezing tolerance

The enhanced freezing tolerance observed following ABA and seaweed applications was accompanied by coordinated changes in proline accumulation, reducing sugar content, and membrane lipid peroxidation, indicating that improved cold hardiness resulted from integrated physiological adjustments rather than from a single metabolic response. Although the magnitude of these responses differed among cultivars and sampling periods, the overall pattern suggests that osmotic regulation and maintenance of membrane integrity were the primary physiological processes associated with enhanced freezing tolerance.

Among the measured biochemical parameters, proline exhibited the most consistent treatment-dependent response. Increased proline accumulation following ABA application was particularly evident in Michele Palieri during the second and third sampling periods and, to a lesser extent, after seaweed application. Proline is widely recognized as an important compatible solute that contributes to osmotic adjustment, stabilization of proteins and cellular membranes, and scavenging of reactive oxygen species during abiotic stress [35]. Consequently, increased proline accumulation may have contributed to maintaining cellular homeostasis under freezing conditions. However, the present results also indicate that proline accumulation alone cannot fully explain differences in freezing tolerance, since treatment responses varied among cultivars and developmental stages. This observation agrees with previous studies suggesting that proline functions as one component of a broader cold-acclimation network rather than serving as an independent predictor of freezing tolerance. [36].

Reducing sugars displayed a more variable response than proline. Significant increases were observed only in specific cultivar–treatment combinations, particularly during deacclimation. Soluble sugars are known to contribute to freezing tolerance by lowering cellular osmotic potential, protecting membrane phospholipids, and stabilizing proteins during extracellular ice formation. [37, 38]. They also provide readily available carbon sources that support recovery following freezing stress. The inconsistent changes observed in the present study therefore suggest that sugar metabolism is regulated not only by exogenous treatments but also by genotype and environmental conditions. Rather than acting independently, soluble sugars likely function together with other osmoprotective compounds to maintain cellular integrity throughout dormancy.

Changes in malondialdehyde (MDA) provided complementary evidence for treatment-induced protection against freezing injury. MDA is widely used as an indicator of membrane lipid peroxidation and oxidative damage under

abiotic stress [39]. In several cultivar–stage combinations, ABA and seaweed applications reduced MDA concentrations relative to untreated vines, indicating improved membrane stability during cold exposure. Nevertheless, reductions in MDA were not observed consistently across all cultivars or sampling periods, demonstrating that oxidative stress responses remained strongly genotype-dependent. Similar cultivar-specific variation has been reported previously in grapevine, where cold-tolerant genotypes generally maintain lower lipid peroxidation than cold-sensitive cultivars during winter stress [40, 41].

When considered together, the biochemical responses observed in the present study indicate that neither proline accumulation, reducing sugar concentration, nor MDA alone adequately explains the improvements in freezing tolerance. Instead, the physiological benefits of ABA and seaweed applications appear to arise from the coordinated regulation of osmotic adjustment and membrane protection. This integrated response is consistent with current models of plant cold acclimation, in which multiple protective mechanisms operate simultaneously to preserve cellular function during freezing stress. Consequently, the improvement in LTE values observed after foliar applications is most likely the outcome of coordinated physiological regulation rather than the modification of a single biochemical pathway.

Integration of physiological and biochemical responses

Principal component analysis provided an integrated view of the relationships among freezing tolerance and the measured biochemical traits throughout dormancy. Rather than identifying individual treatment effects alone, PCA demonstrated that the physiological responses induced by ABA and seaweed applications reflected coordinated changes involving multiple biochemical variables. This multivariate approach therefore complements the univariate analyses by illustrating how freezing tolerance is associated with broader metabolic adjustment.

One of the most consistent patterns revealed by PCA was the clear separation between the two experimental seasons. Samples collected during 2021 were generally associated with higher MDA values, whereas many 2020 samples were more closely related to proline accumulation and improved LTE values. This seasonal separation supports the conclusion that environmental conditions substantially influenced the physiological status of grapevine buds and modified the effectiveness of exogenous treatments. Consequently, the multivariate analysis reinforces the view that treatment responses cannot be interpreted independently of climatic conditions.

PCA also demonstrated distinct cultivar-dependent response patterns. Although all cultivars responded to ABA and seaweed applications, the magnitude and direction of biochemical adjustment differed among genotypes. Michele Palieri generally clustered closer to variables associated with improved freezing tolerance, whereas Red Globe frequently showed stronger associations with oxidative stress indicators. These observations agree with the physiological data presented in the Results section and further support the conclusion that genetic background is a major determinant of treatment responsiveness [42].

Another important outcome of the multivariate analysis was the consistent separation of treated vines from untreated controls. Although some overlap occurred among sampling periods, ABA- and seaweed-treated samples generally occupied regions of the PCA space distinct from the control group, indicating that both treatments modified the overall physiological state of dormant buds rather than influencing only individual biochemical parameters. This observation suggests that improvements in freezing tolerance resulted from coordinated metabolic regulation rather than isolated changes in proline, reducing sugars, or membrane stability alone.

Taken together, the PCA results strengthen the overall interpretation of this study by demonstrating that improvements in freezing tolerance are associated with integrated physiological responses involving osmotic adjustment, oxidative stress regulation, and genotype-dependent metabolic plasticity. The multivariate approach therefore provides additional evidence that ABA and seaweed applications influence grapevine cold hardiness through coordinated physiological regulation rather than through a single dominant mechanism.

Conclusion:

This study demonstrates that foliar applications of abscisic acid (ABA) and seaweed (SW) extract significantly improve bud freezing tolerance in grapevine during dormancy. However, the present findings clearly show that the effectiveness of these treatments is not a fixed characteristic but is strongly governed by the interaction among cultivar, dormancy stage, and seasonal climatic conditions. Improvements in freezing tolerance were consistently associated with coordinated physiological adjustments involving proline accumulation, changes in reducing sugar content, and reduced membrane lipid peroxidation, indicating that enhanced cold hardiness results from integrated metabolic regulation rather than from modification of a single biochemical trait.

The principal contribution of this study lies in demonstrating that the physiological benefits of exogenous ABA and seaweed applications are highly dynamic throughout the dormancy cycle. By integrating physiological, biochemical, and multivariate analyses across three grapevine cultivars, three dormancy stages, and two consecutive growing seasons, this work provides field-based evidence that treatment efficacy is determined by complex interactions between genotype, developmental stage, and environmental conditions. These findings move

beyond the conventional view that ABA or seaweed extracts simply enhance cold tolerance and instead demonstrate that successful cold acclimation depends on context-dependent physiological regulation.
From a viticultural perspective, both ABA and seaweed extract represent promising management tools for mitigating freezing injury, particularly during deacclimation, when grapevine buds become increasingly susceptible to late spring frosts. Nevertheless, the present results indicate that effective implementation should rely on cultivar-specific management strategies that account for seasonal temperature patterns rather than uniform application programmes. Such an adaptive approach is likely to become increasingly important as climate change continues to intensify temperature instability during winter and early spring.
Future research should focus on elucidating the molecular mechanisms underlying the genotype-dependent responses identified in this study. Integrating transcriptomic, metabolomic, hormonal, and long-term field analyses will improve our understanding of how ABA- and seaweed-mediated signalling networks coordinate cold acclimation and deacclimation in grapevine. Ultimately, such knowledge will support the development of scientifically informed, environmentally sustainable, and cultivar-specific strategies for improving winter resilience and reducing frost-related yield losses in perennial fruit crops.

Acknowledgements
The authors gratefully acknowledge Metin Cömert for his invaluable collaboration and for generously providing the experimental vineyard where this research was conducted.

Ethics approval and consent to participate:
This study involved only cultivated grapevine plant material and did not involve human participants or animals. Details regarding the origin and identification of the plant material are provided in the Materials and Methods section.

Consent for publication:
Not applicable.

Availability of data and materials:
The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests:
The authors declare no conflict of interest..

Clinical trial number: Not applicable

Funding:
This study was supported by the Republic of Türkiye Ministry of Agriculture and Forestry General Directorate of Agricultural Research and Policies (Project No: TAGEM/BBAD/A/21/A1/P6/2477).

Author Contributions:
Conceptualization, G.G.K, and G.S.; methodology, G.G.K, and G.S.; software, G.G.K.; validation, G.G.K, and G.S; formal analysis, G.G.K.; investigation, G.G.K, and G.S.; resources, G.G.K, and G.S.; data curation, G.G.K.; writing original draft preparation, G.G.K, and G.S.; writing, review and editing, G.G.K, and G.S.; visualization, G.G.K.; supervision, G.S.; project administration, G.G.K, and G.S.; All authors have read and agreed to the published version of the manuscript.

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