The effects of glycine betaine application on frost tolerance in three table grape cultivars

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Abstract

Cryoprotectants like glycine betaine (GB), have colligative properties that both delay the intracellular ice crystals formation and reduce the potential for osmotic damage during the freezing and thawing processes. Here the study, in which three different grape cultivars of *Vitis vinifera* L. were used, the effects of GB application three days before sampling on frost tolerance were tried to be determined. Samples were taken at three different stages of dormancy (transition to acclimation, hardening and deacclimation) and low temperature exotherm for 50% (LTE_{50}) degrees were determined by testing the winter buds with differential thermal analysis (DTA). In addition, two different biochemical analyses, which were stated in previous studies to be important in frost tolerance, were performed to determine the physiological effects of GB. The effect of GB treatment varied according to cultivars, years and dormancy stages but the greatest effect was an increase of 3.07°C frost tolerance in the third dormancy stage, during 2020 for the 'Michele Palieri' cultivar. The LTE_{50} data showed that the GB application enhanced frost tolerance during two stages during the dormant season (1st stage 1.72°C and 3rd stage 1°C) in 2021 for the same cultivar. Also, the GB application provided 1.75°C greater frost tolerance in the spring of 2020 for the 'Red Globe' cultivar. Our results showed that there was no relationship between GB treatment and biochemical content (proline and MDA).

Keywords: GB, *Vitis vinifera* L., dormancy, application, DTA

INTRODUCTION

Glycine betaine (GB) is dipolar at physiological pH, but electrically neutral in nature. GB accumulates at an osmotically significant level in many halophytic plants and cyanobacteria (Rhodes and Hanson, 1993). Many plants accumulate low molecular weight compounds with cryoprotectant activity in response to low temperatures (Guy, 1990; Rajashekar, 2000). Therefore, GB has an osmoprotectant function and stabilizes membranes during freezing (Karabudak et al., 2014; Coughlan and Heber, 1982; Zhao et al., 1992). It has been suggested that compatible solutes such as GB protect macromolecules such as nucleic acids, proteins and lipids, even at low levels, and also act as reservoirs of carbon and nitrogen sources (Umezawa et al., 2006). In addition, it has been reported that GB and glycine N-trimethyl derivative compatible solutes are the most efficient osmolytes to reduce biotic stress in plants (Waheed et al., 2021).

It has been suggested that GB acts not only as an osmoprotectant, but also as a membrane stabilizer that contributes to the stabilization and integrity of cellular membranes when plants are exposed to low temperature conditions (Park et al., 2006). However, the mode of action of this protective mechanism on membranes remains unclear, and the expression of GB-induced, cold-regulated genes has been suggested to be more associated with genes responsible for reducing the generation of ROS under cold stress conditions (Chen et al., 2000). Even in small amounts, GB has a protective effect in cases of short-term exposure to cold. However, during prolonged periods of cold stress, higher GB accumulation is required to facilitate desirable effects such as osmotic adjustment and clearance of ROS (Gibon et al.,

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1997; Demiral and Türkan, 2004).

During the cold acclimation treatment to *Arabidopsis thaliana*, the GB level in the leaves increased sharply as the plant gained frost tolerance (Xing and Rajashekar, 2001). In other studies, it has been determined that the treatment of GB in many plants provides a significant increase in frost tolerance (Zhao et al., 1992; Allard et al., 1998; Rajashekar et al., 1999; Somersalo et al., 1996; Karabudak et al., 2014). The GB biosynthetic genes from plants or bacteria have been cloned and used to genetically regulate GB biosynthesis in a series of species which lead, in most cases, to and the resultant GB accumulation in most cases improve tolerance to low temperatures (Hayashi et al., 1997; Holmström et al., 2000).

The osmotic role of GB in grapevine and its effect on stress tolerance have not been investigated yet. There is ample evidence that GB plays an important role in stress tolerance (Mickelbart et al., 2006). Foliar GB applications for the accumulation of low GB concentrations in plants is a promising approach to reduce the adverse effects imposed by various abiotic factors (Chen and Murata, 2008). GB foliar application was a recommended and functional method in cases where low solutes provided tolerance in plants against stress conditions (Bhatti et al., 2013).

MATERIALS AND METHODS

Material

The material of the study consisted of three commercial *V. vinifera* grape cultivars in a producer's vineyard in Sakarya, Pamukova. Eight-year-old 'Trakya İlkeren' (Tİ) grafted on Kober 5BB rootstock, 6-year-old 'Michele Palieri' (MP) grafted on 1103 Paulsen rootstock, and 4-year-old 'Red Globe' (RG) grafted on 140 Ru rootstock were selected for the experiment. A standard annual fertilization program was used for all cultivars (Kandilli et al., 2023). After the GB application, cuttings were taken at three different periods (two acclimation stages and one deacclimation stage) for differential thermal analysis (DTA) testing and biochemical laboratory testing. After sampling, the cuttings were stored in polyethylene bags in an airtight manner and at +4°C until the DTA test time. The buds were reserved for biochemical analyses and were kept at -80°C.

GB application

Liquid containing GB was applied by spraying to six grapevines of each cultivar. In the applications, a product which containing 22% GB and 0.01% *Yucca schidigera* was used. The concentration recommended (3 mL L^{-1}) was used. GB was applied at three different periods (three different stages of dormancy) 72 h before the vine samples were collected (recommended to be applied 72 h before frost damage occurs in the product brochure). Each test group vine was treated once for each period (two different vines for the 1st stage acclimation, two different vines for the 2nd stage acclimation and two different vines for the deacclimation stage). The cuttings were taken 72 h after the application and immediately before the DTA test and laboratory tests were carried out. No application was made to the control group vines.

Differential thermal analysis (DTA)

The low temperature exotherm (LTE) was measured using a DTA programmable freeze chamber (Tenney Junior Environmental Test Chamber) in Atatürk University, Agricultural Faculty, Department of Horticulture, Erzurum. The buds were carefully excised using a razor blade from canes leaving 1-2 mm woody tissue next to the buds (Wolf and Pool, 1987). Eight buds were placed on each TEM (thermo electric module). Each test performed had three replications, or a total of 24 winter buds were tested in three TEMs. The freeze chamber was programmed to cool at a rate of 4°C h^{-1} (Mills et al., 2006; Kaya and Kose, 2019). The cooling program was started at +4°C in all DTA tests and ended at -30°C. The lethal temperature at which 50% of the buds died (LTE_{50}) was determined from the measured peak values (Wolf and Pool, 1987).

Measurement of proline

The buds reserved for biochemical analysis were mixed with liquid nitrogen, crushed into powder, and homogenized by mixing with 3% wt/vol sulfosalicylic acid. Then, ninhydrin reagent (1% w/v ninhydrin in 60% acetic acid) was added followed by filtering through no. 2 filter paper (Whatman no. 2, Beckman Coulter Biyomedikal, Instanbul, Turkey). This mixture was incubated at 100°C for 1 h and then cooled on ice. Four mL of toluene was added to the mixture and shaken vigorously for 15 s. The chromophore containing toluene was aspirated from the aqueous phase and 520-nm absorbance readings were obtained in the spectrophotometer (Hitachi U-2900 Japan) following the method described by Bates et al. (1973). In order to create a calibration curve, proline at different dosages were subjected to the same chemical reaction as the samples, the absorbance was read at 520 nm, and the proline concentration in the samples were determined by creating a calibration curve with these values.

Measurement of MDA

For the MDA analysis, 0.1 g of grapevine bud sample was taken and placed in an empty mortar and crushed into powder by adding liquid nitrogen to it. A 2-mL aliquot of 5% TCA was added to the crushed buds and mixed well so that the MDA content in the bud was transferred to the buffer solution. Then, the mixture was placed in 2-mL eppendorf tubes and centrifuged at 12000 rpm for 30 min. There after the samples were then removed and 1 mL of supernatant was taken and 1 mL of 0.6% TBA was added to it, and the glass tubes were closed and kept in a 90°C water bath for 30 min. The samples were then centrifuged at 12000 rpm for 15 min and the absorbance of the resulting supernatant was recorded at 450, 532 and 600 nm to determine the amount of MDA using Equation 1 as described by Zhang and Dami (2012). For the control, 2 mL of buffer (without sample) and 1 mL of TCA and 1 mL of TBA were used (Heath and Packer, 1968). Analyses were performed for three replications.

$$(\text{nmol g}^{-1}\text{ FW}): [(6.45\times(532\text{ nm} - 600\text{ nm}) - (0.56\times450\text{ nm})] / 0.1\text{ (FW)} \quad (1)$$

Statistical analysis

The Q-Q plot analysis (quantile-quantile plot) were used to check LTE values data set normal distribution. Those values were expressed by using Box-and-Whiskers plot analysis using Excel 2016 (Microsoft Corp., etc). JMP statistical software (manufacturer and manufacturer's location) was used in both biochemical and LTE values. Istatistical analyzes (version. 7.0, SAS Institute Inc., Cary, NC) (SAS, 2003).

RESULT AND DISCUSSION

The effect of GB on frost tolerance

According to the Q Plot analysis, all cultivars frost degrees exhibited less difference in 2021 at all three dormancy stages than 2020. 'Trakya İlkeren' (Tİ) grafted on Kober 5BB rootstock, which is considered an early cultivar, had LTE_{50} values that varied more in 2020 depending on the dormancy stage, when compared to the LTE_{50} values and the same dormancy stages in 2021 (Figure 1). In 2021, the autumn (1st stage) and spring (3rd stage) freezing degrees are lower than the same periods in 2020. It is possible to say that GB application in Tİ has no effect on frost tolerance on the basis of years and periods. There was no statistical difference between the applications both of two years.

According to the Q Plot analysis of 'Michele Palieri' (MP), which is considered a late cultivar on 1103 Paulsen rootstock, the interaction of dormancy stage and GB application differed for LTE_{50} values (Figure 2). The LTE_{50} values at the three dormancy stages in 2021 varied less when compared to 2020. The autumn (1st stage) and spring (3rd stage) LTE_{50} values were lower in 2021 than values that occurred in the same periods in 2020. The GB application lower the LTE_{50} by 3.07° compared to the control in the 3rd stage in 2020. In contrast, the GB application lowered the LTE_{50} during the 1st stage by 1.72°C in 2021.

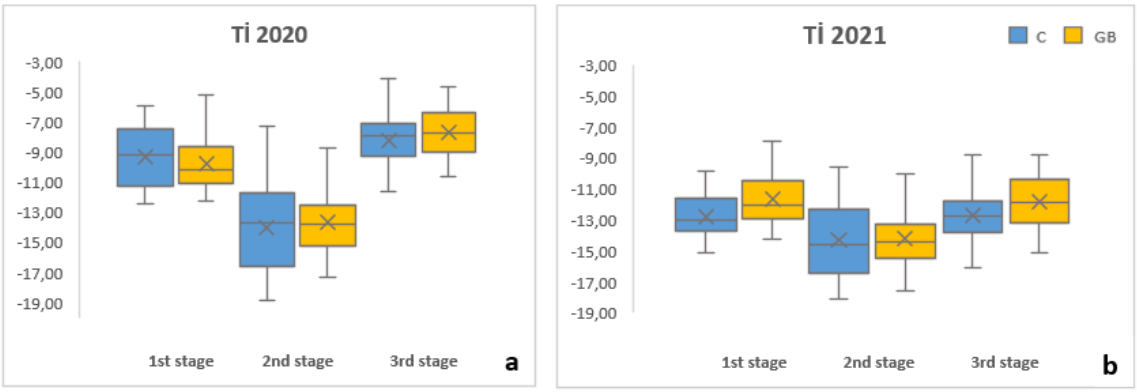


Figure 1. The average LTE_{50} (low temperature exotherm) in 'Trakya İlkeren' buds (°C) during 2020 and 2021 at three dormancy stages. C: Control, GB: Glycine betaine application.

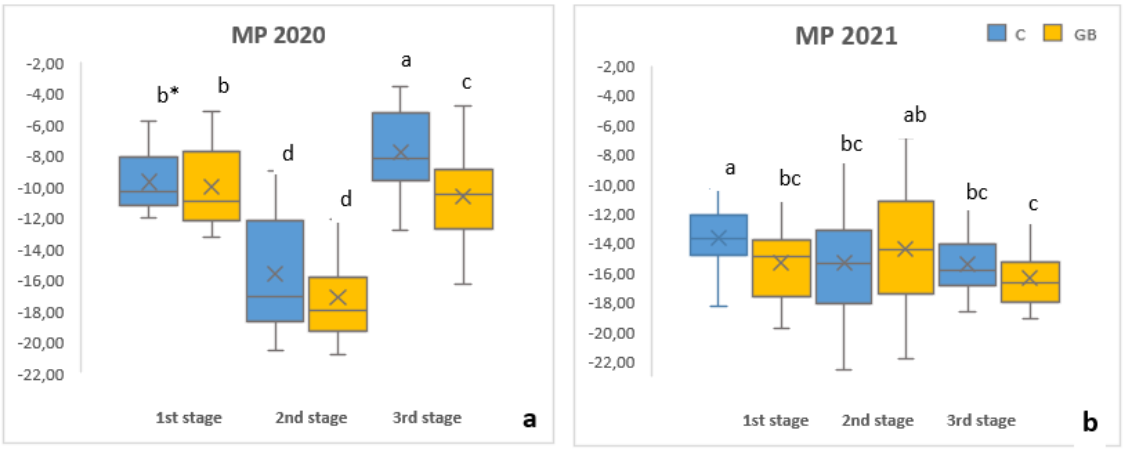


Figure 2. The average LTE_{50} (low temperature exotherm) in 'Michele Palieri' buds (°C) during 2020 and 2021 at three dormancy stages. *Means for each year with the same letter are not significantly different according to the LSMeans Student's multiple test at, 0.05 level.

During the cold acclimation treatment to *Arabidopsis thaliana*, the GB level in the leaves increased sharply as the plant gained frost tolerance (Xing and Rajashekar, 2001). In other studies, it has been determined that the treatment of GB in many plants provides a significant increase in frost tolerance (Zhao et al., 1992; Allard et al., 1998; Rajashekar et al., 1999; Somersalo et al., 1996; Karabudak et al., 2014). The GB biosynthetic genes from plants or bacteria have been cloned and used to genetically regulate GB biosynthesis for a series of species and the resultant GB accumulation in most cases showed improved tolerance to low temperatures (Hayashi et al., 1997; Holmström et al., 2000).

The osmotic role of GB in grapevine and its effect on stress tolerance have not been investigated yet. There is ample evidence that GB plays an important role in stress tolerance (Mickelbart et al., 2006). Foliar GB applications for the accumulation of low GB concentrations in plants is a promising approach to reduce the adverse effects imposed by various abiotic factors (Chen and Murata, 2008). GB foliar application was a recommended and functional method in cases where low solutes provided tolerance in plants against stress conditions (Bhatti et al., 2013).

The Q Plot analysis of 'Red Globe' (RG), which is considered a late cultivar grafted on 140 Rugeri rootstock, the interaction of dormancy stage and GB application differed for LTE_{50}

values (Figure 3). Similar to the other two cultivars in the study, the change in LTE_{50} values during the three stages varied more in 2020 than in 2021 throughout the dormant season. Although the winter LTE_{50} values (2nd stage) were lower 2020 than 2021, the LTE_{50} values that occurred the autumn (1st stage) and spring (3rd stage) were lower in 2021 than the same stages in 2020. The most obvious LTE_{50} difference was the lower LTE_{50} of 1.75°C by the GB application in the spring of 2020 (Figure 3a). The control had lower LTE_{50} values for both the 1st and 2nd stages in 2021 (Figure 3b). There was no significant difference between the applications in the spring period (3rd stage).

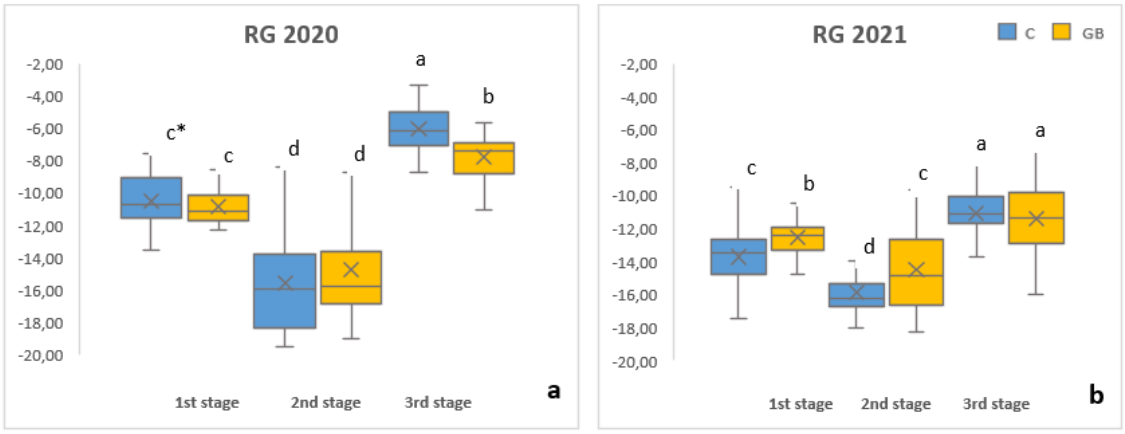


Figure 3. The average LTE_{50} (low temperature exotherm) in 'Red Globe' buds (°C) during 2020 and 2021 at three dormancy stages. *Means for each year with the same letter are not significantly different according to the LSMeans Student's multiple test at, 0.05 level.

In Tİ, which is an early cultivar, no effect on frost tolerance was observed when GB was applied in both years (Figure 1), while the GB application increased frost tolerance (lower LTE_{50}) in spring periods for RG in 2020 and both years for MP (Figures 2a, b and 3b). Application of GB to vines during critical periods (e.g., before spring frosts) may protect plants from frost damage and prevent yield loss (Mickelbart et al., 2006). Xing and Rajashekar (2001) have shown that GB plays a role in the induction of cold adaptation, ABA and frost tolerance in response to water stress in *Arabidopsis* plants. It has been determined that GB protects thylakoids, a sac-like membrane in the chloroplasts of plant cells in spinach from frost damage (Coughlan and Heber, 1982). It was also determined that exogenous application of GB to the plant increased frost tolerance in alfalfa (Zhao et al., 1992), wheat (Allard et al., 1998), strawberry (Rajashekar et al., 1999) and tomato (Park et al., 2006).

The effect of GB application on biochemical contents

1. Proline.

Proline was not detected in all cultivars during the 3rd stages of 2020 (Table 1). In 2020, the highest amount of proline was detected in Tİ and MP cultivars in winter, but in the spring for the RG cultivar. The proline amount of the control group buds were higher than GB application group in all varieties for each stage except for RG during the 2020 winter stage. On the other hand, the highest amount of proline in all cultivars was during the spring stage in 2021. Although the effect of GB treatment on the amount of proline varied according to the cultivar and dormancy stage, there was no evidence that the GB application decreased or increased the proline concentration (Table 1).

Table 1. Influence of year, dormancy stage, and cultivar on the proline content in winter buds (μg g^{-1}).

Year	Stages	Application	Tİ	MP	RG
2020	1	GB	Nd	Nd	Nd
		C	Nd	Nd	Nd
	2	GB	8.3±0.07 b	9.25±0.10 b	5.78±0.04 c
		C	18.93±0.72 a	12.25±0.17 a	5.92±0.13 c
	3	GB	6.04±0.31 c	8.24±0.23 c	12.67±0.11 b
		C	8.31±0.73 b	9.51±0.25 b	14.98±0.15 a
CV			0.122	0.02	0.012
2021	1	GB	2.95±0.04 d	1.18±0.27 e	1.76±0.13 c
		C	1.51±0.35 e	0.93±0.33 e	1.89±0.02 c
	2	GB	4.57±0.31 c	1.81±0.02 d	1.2±0.04 d
		C	1.22±0.04 e	3.39±0.06 c	2.91±0.13 a
	3	GB	6.51±0.52 b	6.58±0.14 b	2.7±0.11 a
		C	8.12±0.18 a	7.85±0.14 a	2.27±0.15 b
CV			0.061	0.053	0.058

Means in the same column for a specific year with the same letter are not significantly difference according to the LSMeans Student's multiple test at, 0.05 level. Undetected values were not included in the means separation.
C: control, GB: glycine betaine application, CV: coefficient of variation, Nd: non-detected.

2. MDA.

The dormancy stage and cultivar influenced the amount of MDA (Table 2). It has been determined that GB application and dormancy stage statistically had no affect on the amount of MDA content in 2020. The difference was determined in MP and RG cultivars, but no significant difference was found in Tİ cultivars in 2021. The highest amount of MDA was determined in the spring period for the MP cultivar and in the winter period for the RG cultivar. At the end of the two-year statistical evaluation, there was no evidence that GB application had an effect on the amount of MDA (Table 2).

Table 2. Influence of year, dormancy stage, and cultivar on the content in winter buds (nmol g^{-1}).

Year	Stages	Application	Tİ	MP	RG
2020	1	GB	3.64±0.61	3.64±0.05	3.34±0.04
		C	4.47±0.36	3.49±0.24	2.53±0.08
	2	GB	3.84±0.44	2.26±0.35	3.27±0.35
		C	3.84±0.42	2.15±0.52	2.15±0.52
	3	GB	1.93±0.06	2.64±0.69	2.82±0.05
		C	3.59±0.53	3.36±0.41	1.77±0.01
CV			NS	NS	NS
2021	1	GB	4.76±0.12	5.31±0.17 c	3.44±0.09 d
		C	5.22±0.23	3.85±0.19 d	3.67±0.26 d
	2	GB	4.83±0.63	5.59±0.06 c	5.02±0.14 b
		C	5.21±0.3	4.90±0.29 c	5.66±0.14 a
	3	GB	3.99±0.13	9.63±0.63 a	4.18±0.05 c
		C	3.80±0.16	6.61±0.35 b	3.99±0.22 c
CV			NS	0.058	0.038

Mean separation within columns by LSMeans Student's multiple test at, 0.05 level.
C: control, GB: glycine betaine application, CV: coefficient of variation, NS: non-significant.

Rapid accumulation of GB occurs during exposure to salt, drought and low temperature stresses in natural accumulators such as spinach (*Spinacia oleracea*), maize (*Zea mays*), sugar beet (*Beta vulgaris*) and barley (*Hordeum vulgare*) (Kishitani et al., 1994; Bohnert et al., 1995). Some economically important crops cannot accumulate GB, including rice (*Oryza sativa*), potato (*Solanum tuberosum*) and tomato (*Solanum lycopersicum*). Therefore, these plants are potential targets for engineering betaine biosynthesis (McCue and Hanson, 1990). In some genetic studies, it has been determined that GB biosynthesis in COD-transgenic *Arabidopsis* (Hayashi et al., 1997) and COD-transgenic tomato (Park et al., 2006) confers increased tolerance to low temperatures at various stages of plant development.

CONCLUSIONS

It is a difficult task to determine the protective effect of the GB application during plant stress which biochemically affected in plant physiology, and more studies are needed to determine this in the future. This study not only refers to the protective effect of GB application in grape frost damage, but it also presents a significant approach to demonstrate its limited effect on some biochemical parameters.

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