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Improving Freezing Tolerance of 'Chambourcin' Grapevines with Exogenous Abscisic Acid

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Abstract*. The purpose of this study was to develop a protocol to increase freezing tolerance of field-grown 'Chambourcin' grapevines (*Vitis* spp.) using exogenous abscisic acid (ABA). The specific objectives were to determine the optimum concentration and timing for ABA foliar application in 'Chambourcin' and to evaluate morphological and physiological changes that lead to increased freezing tolerance in response to foliar ABA application. 'Chambourcin' grapevines were treated with a foliar ABA application of concentrations of 0, 100, 200, 300, 400, 500, 600, 700, and 800 mg·L^{-1} at 50% fruit set stage to evaluate ABA phytotoxicity under field conditions and identify the optimum concentration. In a subsequent experiment, 'Chambourcin' grapevines were treated with 400 and 600 mg·L^{-1} of ABA at different stages of development corresponding to 50% fruit set, 21 days after 50% fruit set, 50% veraison, 20, 30, 40, and 55 days postveraison. ABA concentrations of 700 and 800 mg·L^{-1} were phytotoxic and caused significant damage to leaves and flowers. Optimum concentrations of ABA did not affect yield components or basic fruit chemical composition, yet it promoted anthocyanin accumulation at harvest. Furthermore, ABA advanced bud dormancy, decreased bud water content, and eventually increased freezing tolerance under simulated freezing tests. The increased freezing tolerance of ABA-treated vines was confirmed by bud injury assessment after a natural freezing event in Jan. 2011. It was also determined that ABA was most effective when applied with an optimum concentration of 400 mg·L^{-1} 20 to 30 days postveraison. It is concluded that exogenous ABA enhanced dormancy and increased freezing tolerance; thus, it has the potential to protect grape cultivars from freezing injury.

'Chambourcin' (*Vitis* spp.) is a French–American hybrid cultivar with a higher disease and winter resistance than that in *Vitis vinifera* L. cultivars and produces quality wine (Read et al., 2004). Thus, it has been successfully grown and well adapted to the midwest and eastern U.S. environment. However, 'Chambourcin' requires a relatively long growing season to ripen its fruit; therefore, the vines are vulnerable to early fall frost in cool seasons and cannot acquire adequate cold acclimation, which results in winter injury. A five-year study with 'Chambourcin' demonstrated that the average fall frost date was 1 d later than harvest, resulting in inadequate acclimation and subsequent winter injury (Dami et al., 2006).

ABA is a phytohormone that plays an important role in plant cold acclimation by facilitating plant acquire freezing tolerance. At a genomic level, it has been found that three unique C-repeat/DRE Binding Factor genes were upregulated by low-temperature treatment or exogenous ABA; and these genes were conservative across both cold-tolerant (*Vitis riparia*) and cold-sensitive (*Vitis vinifera*) cultivars (Xiao et al., 2006). In addition, it has been reported that a mutant plant insensitive to endogenous ABA had reduced freezing tolerance (Llorente et al., 2000). At a physiological level, it has been reported that the endogenous ABA concentration positively correlates with the cold acclimation process and bud dormancy (Naor et al., 2008; Pagter et al., 2008). Furthermore, ABA treatment has increased freezing tolerance by reduced intracellular water content to avoid ice formation.

Exogenous ABA application to potted grapevines has delayed budburst for spring frost protection but was not effective on field-grown grapevines (Hellman et al., 2006). ABA has also been sprayed on table grape clusters during the veraison stage to promote anthocyanin accumulation and thus enhance color development (Peppi et al., 2007). Based on a greenhouse study, ABA has been reported to effectively induce growth cessation, leaf abscission, and increased periderm formation, which all are typically associated with dormancy and cold acclimation (Zhang et al., 2011). It has been reported that ABA treatment (100 mg·L^{-1}) increased dormancy extent of one-year-old buds of 'Chambourcin' (Gu, 2003). However, ABA treatment was applied by immersing the basal end of bud cuttings with ABA solution in a growth chamber (Gu, 2003). Therefore, the response of 'Chambourcin' grapevines to exogenous ABA is not known. The purpose of this study was to develop a protocol to increase freezing tolerance of field-grown 'Chambourcin' grapevines (*Vitis* spp.) using exogenous ABA. The specific objectives were to determine the optimum concentration and timing for ABA foliar application in 'Chambourcin' and to evaluate morphological and physiological changes that lead to increased freezing tolerance in response to foliar ABA application.

Materials and Methods

Plant materials, experimental design, and treatments. Grafted 'Chambourcin' (Seyve-Villard 12417 × Seibel 7053) grapevines on rootstock 'Couderc 3309' (*V. riparia* × *V. rupestris*) were planted in 1996 at the Research Vineyard in Wooster, OH (lat. 40°47′ N, long. 81°55′ W, elevation: 311 m a.s.l., Wooster silt-loam soil) and were used for this study. Vines were spaced 1.25 × 3 m (2722/ha), trained to a high-cordon system (height = 1.83 m), and spur-pruned to two buds per spur and 16 buds per meter of cordon followed by shoot and cluster thinning to 13 and 20 per meter of cordon, respectively, before ABA treatment. In 2009, two experiments were conducted on 'Chambourcin' grapevines to evaluate concentration and timing effect of ABA.

Year 1, Expt. 1: Concentration effect. The purpose of ABA concentration experiment was to evaluate the phytotoxicity of ABA application under field conditions and confirm the optimum concentration from the greenhouse study (Zhang et al., 2011). The experiment was conducted at 50% fruit set stage (FS), corresponding to Eichhorn and Lorenz (EL) stage 27 (Eichhorn and Lorenz, 1977) and nine concentrations of ABA solutions (0, 100, 200, 300, 400, 500, 600, 700, and 800 mg·L^{-1}) were applied to vines on a randomized complete block consisting of four blocks with two vines per plot unit.

Year 1, Expt. 2: Timing effect. The purpose of the ABA application timing experiment was to identify the optimum timing for ABA application. Ten treatments were assigned to vines on a randomized complete block consisting of three blocks with five vines per plot unit as follows: 0 (deionized water) and 400 mg·L^{-1} ABA sprayed at the FS stage (0FS and 4FS, respectively), 0 and 400 mg·L^{-1} ABA sprayed at 21 d after the FS stage (0FS21 and 4FS21, respectively), 0 and 400 mg·L^{-1} ABA sprayed at the 50% veraison stage (0V and 4V, respectively), 0 and 400 mg·L^{-1} ABA sprayed at 30 d after the 50%

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veraison stage (0V30 and 4V30, respectively), and multiple application of 0 and 400 mg·L^{-1} ABA at all four timing points (0Multiple and 4Multiple, respectively). A surfactant, Tween-20 (Acros Organic, Hampton, NH), was added to all treatments at the concentration of 0.05% (v/v).

Year 2, Timing effect. In 2010, ABA timing experiment was repeated with adjusted timing points based on the results of the first year and the same vines of Year 1 were used. Seven treatments were assigned to vines on a randomized complete block consisting of four blocks with five vines per plot unit as follows: control (deionized water), 400 and 600 mg·L^{-1} ABA sprayed at the 50% veraison stage (4V and 6V, respectively), 400 and 600 mg·L^{-1} ABA sprayed at 20 d after the veraison stage (4V20 and 6V20, respectively), and 400 and 600 mg·L^{-1} ABA sprayed at 40 d after the veraison stage (4V40 and 6V40, respectively).

The ABA sample (VBC-30051) was provided by Valent Biosciences Corporation (Libertyville, IL). The active ingredient was 20.0% (w/w) S-ABA. Whole vine canopies (leaves and clusters) were sprayed with ABA solutions to runoff with a 15-L back sprayer (Model SP0; SP System LLC, Santa Monica, CA) averaging a spray volume of 0.5 L per vine.

Abscisic acid phytotoxicity. The phytotoxicity of ABA application was evaluated on leaves and clusters. Visual observation of leaf damage followed the method by Zhang et al. (2011) and started 24 h after ABA application and continued until leaf damage was observed. The percent of damaged leaf area was visually estimated 3 weeks after ABA application and 1 week after ABA-damaged leaves were first detected. Visual observation of fruit damage (small green berries of 1 cm diameter) was conducted before harvest and the percent of green undeveloped berries was recorded.

Vegetative growth and vine size. The measurement of shoot growth started 1 d after ABA application in the ABA concentration experiment. One shoot was randomly selected per vine before ABA application and marked at 15 cm from the tip. The distance between the tip and marker was measured three times a week and the shoot growth rate (cm·d^{-1}) was calculated as the total new growth divided by the number of days. The results were averaged between two vines per replicate for each treatment. Vine size (pruning weight) was measured from the ABA timing experiment in Apr. 2010 and Apr. 2011. All one-year-old canes were weighed for each vine after pruning.

Yield components. Yield components including crop weight per vine, yield per node, cluster number per vine, and 50- or 100-berry weight were collected in both concentration and timing experiments. Cluster weight was calculated based on crop weight per vine divided by cluster number per vine. The berry number per cluster was calculated based on cluster weight divided by 50- or 100- berry weight and then multiplied by 50 or 100, respectively. Grapes were harvested on 21 Oct. 2009 and 18 Oct. 2010. The Ravaz Index was calculated based on the crop weight per vine divided by the pruning weight per vine.

Basic juice analysis. In 2009, a 50-berry sample was collected from each block weekly from veraison through harvest to determine soluble solids, titratable acidity (TA), and pH in the ABA timing experiment. One to three berries, representing top, middle, and bottom positions from random clusters, were sampled from each vine and totaling 50 or 100 berries per plot unit. Berries were weighed using an electronic scale (Denver Instrument, Bohemia, NY), and then the berry samples were juiced at room temperature. The juice was transferred to a 50-mL centrifuge tube and centrifuged at 8500 rpm for 5 min (accuSpin 400; Fisher Scientific, Pittsburgh, PA). A 10-mL supernatant was transferred to the titration work station (PH/EP Titration Workstation Model 350/352) with SAC80 sample change (Denver Instrument, Bihemia, NY) to measure pH and TA. TA was determined by titrating the 10-mL aliquot of juice sample to a pH 8.2 with 0.1 N NaOH solution. Soluble solids were measured with a digital refractometer (MISCO, Cleveland, OH) and expressed in Brix.

Anthocyanin analysis. Anthocyanin analysis was based on the method by Kleinhenz et al. (2003). At harvest, a 50-berry sample was collected from each treatment replicate and then frozen at –20 °C until analysis. The protocol of berry sampling was the same as that of juice analysis. Berries were thawed at room temperature and then crushed into slurry using a hand blender (Whirlpool Corp., Benton Charter Township, MI). Approximately 2 g slurry was placed into a 50-mL centrifuge tube (Fisher Scientific, Pittsburgh, PA) containing 40 mL of 1% HCl acidified methanol (Fisher Scientific) extraction solvent. Slurry suspensions were allowed to extract for 1 h at room temperature. Suspensions were then centrifuged for 15 min at 15,000 rpm and the supernatant was transferred into a 100-mL volumetric flask (NORMAX, New Milford, CT). The pellet was re-extracted using the procedure described previously, and then a third extraction was completed in 30 min using 15 mL 1% HCl acidified methanol. The solution was brought to volume with 1% HCl acidified methanol and equilibrated by inversion of the flask twice. One milliliter extract was diluted by the addition of 4 mL 1% HCl acidified methanol; the absorbance of the diluted solution was measured using a spectrophotometer (DU730; Beckman Coulter, Inc., Brea, CA) at 520 nm, the wavelength of maximum absorbance. In 2010, anthocyanin measurement was conducted in the skins only because most anthocyanin is located in berry skins (Jeong et al., 2004). Furthermore, a freeze-dry method was used to facilitate anthocyanin extraction (Ju and Howard 2003). Thus, anthocyanin concentration was expressed in mg·g^{-1} skin dry weight. For that purpose, the berry skins were manually separated from fleshy fruit tissues and freeze-dried using a lyophilizer (Labconco Corp., Kansas City, MO). The extraction procedure followed the same protocol as in 2009 except the dilution ratio was 1:20 for the absorbance measurement at the last step. The molar absorptivity of anthocyanin was reported by Giusti et al. (1999). The anthocyanin content was expressed in mg·g^{-1} and calculated as absorbance reading/molar absorptivity (mol·L^{-1}) × molecular weight (g·mol^{-1}) × dilution ratio (1:5 in 2009 samples, 1:20 in 2010 samples) × 0.1 L × 1000 (mg·g^{-1})/sample weight (g).

Periderm formation and leaf senescence. Periderm formation and leaf senescence were measured in the timing effect experiment and consisted of counting shoot internodes that changed color from green to tan or brown. In 2009 and 2010, two and five shoots were randomly tagged per treatment-replicate after the last spray application (V30 and V40), respectively. The evaluation was conducted on 30 Oct. 2009 and 15 Nov. 2010 and the numbers of lignified internodes and total internodes nodes were recorded. Periderm formation was expressed as the ratio of number of brown to total number of internodes per shoot.

Leaf senescence was assessed by monitoring chlorophyll content, which was measured using a SPAD-520 chlorophyll meter (Spectrum Technologies, Inc., East Plainfield, IL). Five random measurements were taken on the upper surface of the fifth basal leaf on two shoots per replicate. Weekly measurements were recorded in Wooster from late August (veraison stage) through early Nov. 2009 (killing frost). In 2010, 'Chambourcin' grapevine leaves were infected by downy mildew; thus, no chlorophyll measurement was conducted.

Bud dormancy. Bud dormancy assay was conducted in the timing effect experiment in both years. Two representative canes with a minimum of 12 to 15 lignified internodes were collected from each treatment-replicate and buds on node positions three to seven from each cane were used in the bud dormancy assay. A representative cane consisted of a one-year-old lignified cane with diameter between 0.5 and 1 cm and internode length between 10 and 12 cm. The periderm color was deep brown. In total, there were 10 nodes per replicate for dormancy assay. Canes were excised into one-node cuttings ≈5 cm long, then inserted into 2.5 cm × 2.5-cm foam medium (Smithers-Oasis, Kent, OH) and placed in 55 cm × 25 cm × 7-cm plastic trays (T.O. Plastics, Clearwater, MN) filled with water. Bud dormancy assay followed the protocol by Zhang et al. (2011). Budburst was recorded as EL Stage 5 (Eichhorn and Lorenz, 1977) and monitored three times a week until budburst of all treatments reached at least 50%. Dormancy was estimated as the number of days to 50% budburst (D50BB) (Wake and Fennell, 2000). In Year 1, bud samples were collected once on 18 Oct. In Year 2, bud samples were collected monthly from Oct. 2010 through Feb. 2011.

Spring budburst assessment. Budburst assessment was conducted three times a week

between April and May in 2010 and 2011. The date of 50% budburst reaching EL Stage 5 (Eichhorn and Lorenz, 1977) was recorded.

Laboratory and field determination of bud freezing tolerance. One representative one-year-old cane with a minimum of 12 to 15 lignified internodes was collected from each treatment-replicate and buds on node positions three to seven were used. The cane selection criteria were the same as for bud dormancy assay. There were five buds used per replicate with 15 and 20 buds per treatment in 2009 and 2010, respectively. Buds were excised and mounted on thermoelectric modules (MELCOR, Trenton, NJ), which were placed in a Tenney environmental chamber (Thermal Products Solutions, New Columbia, PA). The Tenney temperature was lowered from –2 to –50 °C at 4 °C·h^{-1}. Freezing tolerance of buds was determined using thermal analysis and was expressed as the average lethal temperature exotherm that kills 50% of the population or LT50 (Wolf and Pool, 1987). In Year 1, LT_{50}s were measured monthly from Sept. 2009 to Apr. 2010 and in 2010; in Year 2, it was repeated from Oct. 2010 to Feb. 2011.

On 22 Jan. 2011, air temperatures dropped to –19 °C in Wooster and a bud injury assessment of 'Chambourcin' grapevines was conducted. Ten canes with 10 buds (node positions three to 12) per replicate were collected from all treatments after the freezing event on 31 Jan. 2011. Canes were thawed for 24 h under room temperature in the laboratory and buds were excised with a razor blade and primary buds were visually assessed whether they were alive (green) or dead (brown). Winter injury was expressed as a percent of dead primary buds.

Bud water content. The water content in buds was measured in the timing effect experiment in 2010. One representative cane was collected from each treatment-replicate 24 h after ABA application from Oct. 2010 to Feb. 2011, corresponding to the same collection dates for the freezing tests. The selection criteria were the same as for bud dormancy assay and freezing tests. The canes were transported to the laboratory in a cooler filled with ice. Buds on node positions three to seven of each shoot were excised and weighed before and after placing in an oven at 70 °C for 1 week. Water content was expressed as percent of fresh weight.

Statistical analysis. The data of timing effect experiments were subjected to one-way analysis of variance using Minitab statistical software (Minitab Inc., State College, PA). The model tested for main effects of different treatments. When appropriate, means were separated using least significant difference ($\alpha = 0.05$). The correlation relationship between bud freezing tolerance and water content was determined using Pearson correlation analysis with Minitab statistical software (Minitab Inc.). The data of concentration effect experiment were subjected to linear regression analysis using Minitab statistical software (Minitab Inc.).

Results

Abscisic acid phytotoxicity. The leaf damage symptoms caused by ABA appeared as red spots and flecks ≈0.25 cm^2 in size and were similar to those observed in the greenhouse study (Zhang et al., 2011). However, the symptoms appeared 2 to 3 weeks after ABA application, which were much later than those in the greenhouse (24 h after application). In the ABA concentration experiment, leaf damage was observed on treated vines with ABA concentration of 300 mg·L^{-1} and above. The percent of damaged leaf area was 0.5%, 2.8%, 4.8%, 7.2%, 12.5%, and 18.2%, which corresponded to ABA concentrations of 300, 400, 500, 600, 700, and 800 mg·L^{-1}, respectively. The phytotoxicity of ABA treatment increased linearly with the increase of ABA concentration ($P < 0.001$, $R^2 = 0.797$). Only grapevines treated with ABA concentrations of 700 and 800 mg·L^{-1} had more than 10% of the leaf area damaged. At harvest, 3% and 6% undeveloped green berries were observed in clusters treated with ABA concentrations of 700 and 800 mg·L^{-1}, respectively, but none at lower concentrations. In addition, berry number per cluster averaged 85 in treated vines with ABA concentrations of 700 to 800 mg·L^{-1}, whereas the average number was 105 berries per cluster in treated vines with ABA concentrations of 0 to 600 mg·L^{-1} (Table 2). The berry number per cluster decreased linearly with the increase of ABA concentration (Table 2). There was no effect on time of budburst or bud fruitfulness (carryover effect) among all ABA treatments in the next season (data not shown).

Vegetative growth. In the ABA concentration experiment, shoot growth inhibition was observed on grapevines treated with ABA concentrations in the second week after application. Shoot growth inhibition increased with increased ABA concentrations. In fact, control vines averaged shoot growth rate of 1.3 cm·d^{-1}, similar to that in ABA-treated vines with concentrations of 100 to 500 mg·L^{-1}. However, the growth rate averaged 0.8 cm·d^{-1} in ABA-treated vines with the concentrations of 600 to 800 mg·L^{-1}.

Yield components and fruit composition. In the timing experiment, there were no differences among treatments in all yield components. In the concentration experiment, there was no significant linear regression between ABA concentration and all yield components except berry number per cluster (Tables 1 and 2). It was concluded that

Table 1. Timing effect of exogenous abscisic acid (ABA) on yield components in 'Chambourcin' grapevines.

Treatment[z]	Retained node number (per meter)	Yield (kg/vine)	Yield (g/node)	Cluster number (per vine)	Cluster wt (g)	50/100 berry wt (g)[y]	Berry number (per cluster)	Vine size (kg)	Ravaz Index
				2009					
Control	16	4.9	308	19	259	130	100	0.49	11
4FS	16	5.0	315	14	273	130	105	0.38	13
4FS21	16	5.5	343	13	241	130	93	0.38	15
4V	16	4.2	263	16	227	124	92	0.58	10
4V30	16	5.8	360	16	273	129	103	0.39	14
4Multiple	16	4.3	270	17	259	123	105	0.40	12
Significance[x]		NS	NS	NS	NS	NS	NS	NS	NS
				2010					
Control	16	5.7	292	30	188	226	83	0.37	16
4V	16	7.7	395	34	228	222	103	0.37	17
4V20	16	6.8	348	31	220	242	92	0.40	17
4V40	16	6.5	330	30	219	232	95	0.46	15
6V	16	6.7	344	30	226	228	100	0.37	18
6V20	16	6.2	316	30	211	222	95	0.34	19
6V40	16	6.5	334	29	228	226	102	0.42	16
Significance		NS	NS	NS	NS	NS	NS	NS	NS

[z]The values of control in 2009 are averages of five control groups, 0FS, 0FS21, 0V, 0V30, and 0Multiple, which corresponded to 0 mg·L^{-1} ABA solution sprayed at the 50% fruit set stage; 21 d after the 50% fruit set stage; 50% veraison stage; 30 d after the 50% veraison stage; and all four timing points, respectively. 4FS, 4FS21, 4V, 4V20, 4V30, and 4V40 = ABA concentration of 400 mg·L^{-1} sprayed at the 50% fruit set stage; 21 d after the fruit set stage; 50% veraison stage; and 20, 30, and 40 d after the 50% veraison stage, respectively. 4Multiple = ABA concentration of 400 mg·L^{-1} sprayed four times (FS, FS21, V, and V30). 6V, 6V20, and 6V40 = ABA concentration of 600 mg·L^{-1} sprayed at the 50% veraison stage and 20 and 40 d after the 50% veraison stage, respectively.

[y]In 2009 and 2010, 50-berry and 100 berry sampling were conducted, respectively.

[x]NS, *, **, and *** Nonsignificant, significant at $P \leq 0.05$, 0.01, and 0.001, respectively.

ABA treatment did not affect yield components of 'Chambourcin' grapevines in either experiment with the exception of reduced berries per cluster (Tables 1 and 2).

In 2009, there were no differences of Brix, pH, or TA among treatments on each collection date during the biweekly berry sampling for the ABA timing experiment (Table 3). There was no linear regression between ABA concentration and fruit composition either (Table 4). It was concluded that, based on Brix, pH, and TA, the fruit ripening process was unaffected by ABA. At harvest, there was no difference of fruit chemical compositions among treatments except for TA in 2009 (Table 3). In 2009, multiple application of ABA increased the anthocyanin concentration by 22% as compared with that in untreated grapevines (Table 3). In 2010, ABA applied at the V20 stage (both 4V20 and 6V20) consistently increased the anthocyanin concentration by 17% as compared with that in untreated grapevines (Table 3). In the ABA concentration experiment, there was no difference of fruit compositions among treatments.

Periderm formation and leaf senescence. In general, ABA treatments advanced periderm formation in 'Chambourcin' grapevines in both years. In 2009, ABA treatments increased the periderm formation by 100% compared with that in untreated grapevines. In 2010, ABA treatments increased periderm formation by 30% compared with that in untreated grapevines; and there was no difference among treatments with ABA concentrations of 400 and 600 mg·L^{-1} (Fig. 1). Furthermore, ABA sprayed at 4V30 and 4Multiple treatments advanced the progression of leaf senescence (Fig. 2). The leaf senescence, measured as a decline in chlorophyll content, started in all vines between late September and early October (Fig. 2). However, the senescence process was advanced by ABA treatment because the chlorophyll content of ABA-treated leaves was consistently lower than that of untreated leaves by 10% to 15% (Fig. 2). In late fall, leaves on ABA-treated grapevines abscised 2 weeks earlier than those on untreated ones.

Bud dormancy. In 2009, bud samples were collected only once on 18 Oct. and the dormancy assay was conducted on the same day. There was no difference of D50BB among all treatments (data not shown). In 2010, bud samples were collected monthly from Oct. 2010 to Feb. 2011. Budburst of ABA-treated vines at V and V20 was delayed by an average of 6 d from November through February (Fig. 3). The delay of budburst between ABA-treated and untreated vines during the period of November to February indicated that ABA application promoted a deeper dormancy status. There was no difference among treatments with ABA concentrations of 400 and 600 mg·L^{-1}. There was no difference of the date of 50% budburst among treatments in the next spring seasons.

Laboratory and field determination of freezing tolerance. During the 2009–10 dormant season, LT_{50}s were not different among treatments during the onset of cold acclimation in Oct. 2009 and during deacclimation in Mar. and Apr. 2010 (Fig. 4A). However, LT_{50}s of ABA-treated vines at the V30 stage decreased (i.e., freezing tolerance increased) in November, December, January, and February by 3, 3.5, 2.6, and 3.3 °C, respectively,

Table 2. Concentration effect of exogenous abscisic acid (ABA) on yield components in 'Chambourcin' grapevines in 2009.

ABA concn (mg·L^{-1})	Retained node number (per meter)	Yield (kg/vine)	Yield (g/node)	Cluster number (per vine)	Cluster wt (g)	100 berry wt (g)	Berry number (per cluster)
0 (Control)	16	5.6	287	15	290	267	108
100	16	4.8	246	13	231	269	109
200	16	4.1	210	15	268	271	107
300	16	3.6	184	15	259	264	105
400	16	3.5	179	14	231	269	103
500	16	4.1	210	14	290	264	109
600	16	4.9	251	16	290	269	102
700	16	3.7	190	12	227	269	88
800	16	4.1	210	14	218	254	82
P^z		NS	NS	NS	NS	NS	***
R^2							0.370

[z]NS, *, **, and ***, Level of significance for linear regression: nonsignificant, significant at $P \leq 0.05$, 0.01, and 0.001, respectively.

Table 3. Timing effect of exogenous abscisic acid (ABA) on fruit composition in 'Chambourcin' grapevines.

Treatment[z]	°Brix	pH	Titratable acidity (g·L^{-1})	Total anthocyanin
			2009	Whole berry (mg·g^{-1} fresh weight)
Control	21.5	3.25	11.8 ab[y]	0.93 b
4FS	21.5	3.27	11.6 b	1.00 ab
4FS21	20.6	3.21	11.8 ab	0.86 b
4V	21.0	3.20	13.6 a	0.98 ab
4V30	21.0	3.24	11.5 b	0.93 b
4Multiple	20.5	3.21	12.5 ab	1.15 a
Significance[x]	NS	NS	*	*
			2010	Berry skin (mg·g^{-1} dry weight)
Control	23.4	3.39	9.8	48 c
4V	23.0	3.41	9.6	50 bc
4V20	23.2	3.37	10.1	56 a
4V40	23.6	3.42	9.8	48 c
6V	23.2	3.46	9.7	54 ab
6V20	22.8	3.40	9.9	56 a
6V40	23.2	3.42	9.2	54 ab
Significance	NS	NS	NS	**

[z]The values of control in 2009 are averages of five control groups, 0FS, 0FS21, 0V, 0V30, and 0Multiple, which corresponded to 0 mg·L^{-1} ABA solution sprayed at the 50% fruit set stage; 21 d after 50% fruit set stage; 50% veraison stage; 30 d after the 50% veraison stage; and all four timing points, respectively. 4FS, 4FS21, 4V, 4V20, 4V30, and 4V40 = ABA concentration of 400 mg·L^{-1} sprayed at the 50% fruit set stage; 21 d after fruit set stage; 50% veraison stage; 20, 30, and 40 d after the 50% veraison stage, respectively. 4Multiple = ABA concentration of 400 mg·L^{-1} sprayed four times (FS, FS21, V, and V30). 6V, 6V20, and 6V40 = ABA concentration of 600 mg·L^{-1} sprayed at the 50% veraison stage and 20 and 40 d after the 50% veraison stage, respectively.

[y]Means in a column not followed by the same letter are significantly different at $P \leq 0.05$.

[x]NS, *, **, and *** Nonsignificant, significant at $P \leq 0.05$, 0.01, and 0.001, respectively.

Table 4. Concentration effect of exogenous abscisic acid (ABA) on fruit composition in 'Chambourcin' grapevines in 2009.

ABA concn (mg·L^{-1})	°Brix	pH	Titratable acidity (g·L^{-1})	Total anthocyanin whole berry (mg·g^{-1} fresh wt)
0 (Control)	21.1	3.22	12.3	0.86
100	21.5	3.23	12.8	0.90
200	21.7	3.21	11.4	0.71
300	21.3	3.23	12.0	0.86
400	21.5	3.25	12.1	0.66
500	21.3	3.23	11.8	0.70
600	21.8	3.27	11.1	0.58
700	21.3	3.23	12.0	0.84
800	21.3	3.23	12.1	0.72
P^z	NS	NS	NS	NS

[z]NS = level of significance for linear regression: nonsignificant.

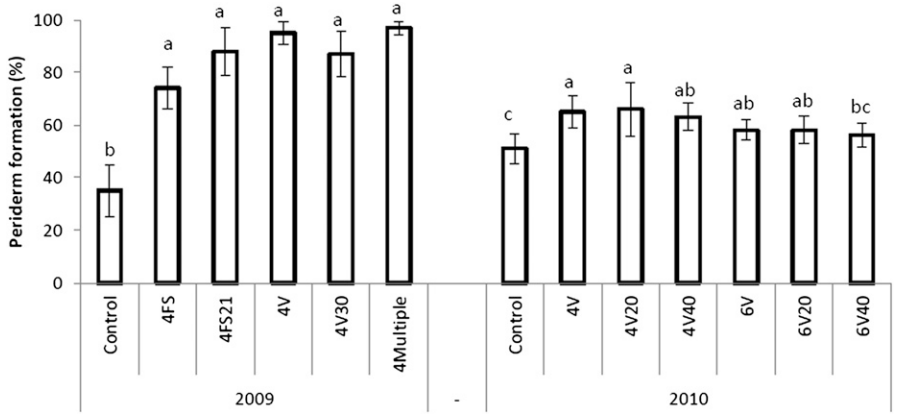

Fig. 1. Effect of exogenous abscisic acid (ABA) on periderm formation in 'Chambourcin' grapevines (40 and 50 d after ABA application in 2009 and 2010, respectively). For clarity, in 2009, the value of control was the average of five control groups, 0FS, 0FS21, 0V, 0V30, and 0Multiple, corresponding to 0 mg·L^{-1} ABA solution sprayed at the 50% fruit set stage, 21 d after the 50% fruit set stage, 50% veraison stage, 30 d after the 50% veraison stage, and all four timing points, respectively. Bars with the same letters are not significantly different at $P \leq 0.05$.

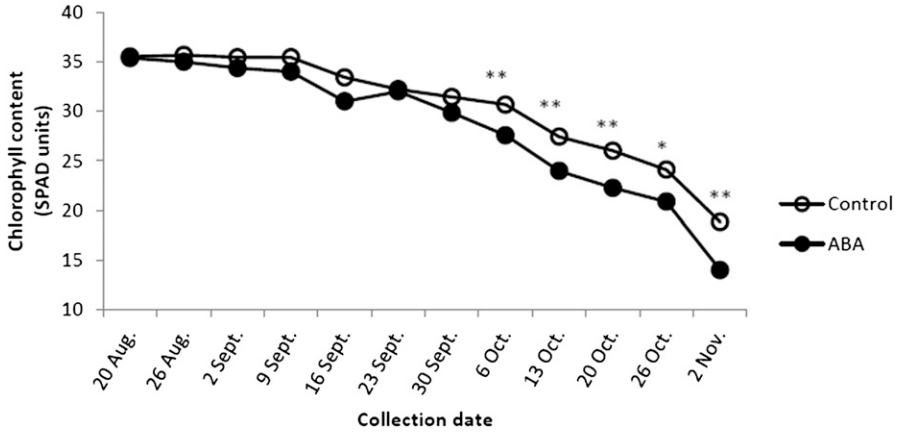

Fig. 2. Chlorophyll content progression in abscisic acid (ABA)-treated and untreated 'Chambourcin' grapevines in 2009. For clarity, only one ABA plot was presented, which was the average of 4V30 and 4Multiple treatments, corresponding to ABA application of 400 mg·L^{-1} sprayed 30 d after the 50% veraison stage and all four timing points, respectively. *, **, and *** indicate significance at $P \leq 0.05$, 0.01, and 0.001, respectively.

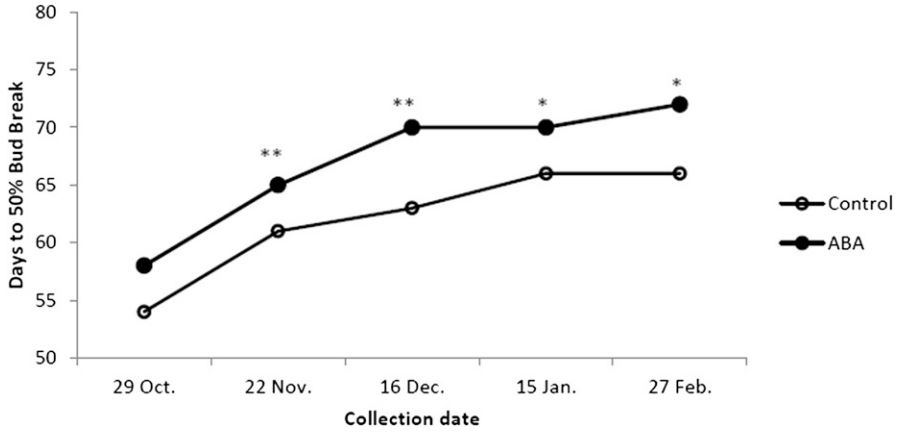

Fig. 3. Effect of abscisic acid (ABA) on bud dormancy (days to 50% bud break) of 'Chambourcin' grapevines during the 2010–2011 season. For clarity, only one ABA plot was presented, which is the average of 4V, 4V20, 6V, and 6V20 treatments. 4V and 4V20 correspond to ABA application of 400 mg·L^{-1} at the 50% veraison stage and 20 d after the 50% veraison stage, respectively. 6V and 6V20 correspond to ABA application of 600 at the 50% veraison stage and 20 d after the 50% veraison stage, respectively. *, **, and *** indicate significance at $P \leq 0.05$, 0.01, and 0.001, respectively.

as compared with those of control vines (Fig. 4A). Multiple applications did not increase the freezing tolerance on any collection date (data not shown).

During the 2010–11 dormant season, LT_{50}s were not different among treatments in Oct. 2010 (Fig. 4B). However, ABA treatment at the V and V20 stages consistently increased the freezing tolerance by an average of 3.5 °C. The freezing tolerance was also increased by ABA treatment at the V40 stage, but the effect was not consistent. There was no difference among treatments with ABA concentrations of 400 and 600 mg·L^{-1}. Additionally, it was observed in both years that LT_{50}s of ABA-treated vines decreased faster than those of untreated vines during the period from September through December, indicating that ABA treatment advanced cold acclimation in the fall. In other words, vines treated with ABA reached similar LT_{50} as in the control 3 weeks earlier (Fig. 4B). As a result of lack of availability of cane samples, LT_{50}s were not determined during the deacclimation stage in Year 2.

The assessment of bud death after exposure to –19 °C showed similar results to those obtained from LT_{50} determination. Bud death percent of ABA-treated vines (V and V20) and untreated vines was 11% and 15%, respectively. Therefore, ABA treatment (V and V20) sustained less bud death, i.e., had a higher freezing tolerance than control. However, there was no difference among treatments with ABA concentrations of 400 and 600 mg·L^{-1}.

Bud water content. It was hypothesized that ABA increases freezing tolerance of buds by inducing bud tissue dehydration and hence leading to less freezable water. Water content averaged 55% and 80% in bud and leaf tissues and was not different among all treatment 24 h after ABA application. However, there was a progressive bud dehydration that took place in all treatments including control from Nov. 2010 to Feb. 2011 (Fig. 5). The dehydration process was enhanced by ABA treatment, specifically on V and V20 treatment (Fig. 5). The buds from ABA-treated vines (V and V20) started to show lower water content than those from control on 22 Nov. 2010, or 80 d after ABA application at the V20 stage (Fig. 5). The water content in buds from V and V20 treatments decreased on average by 20% as compared with that in control (Fig. 5). ABA treatment at the V40 stage also reduced bud water content, but not consistently. There was no difference among treatments with ABA concentrations of 400 and 600 mg·L^{-1}. To support the hypothesis about the relationship between ABA-induced freezing tolerance increase and dehydration, correlation analysis was conducted between freezing tolerance (LT_{50}) and water content in buds. A positive correlation ($R = 0.342$, $P = 0.001$) between LT_{50} and water content was found, which indicated that decreased water content was associated with increased freezing tolerance (decreased LT_{50}) and vice versa.

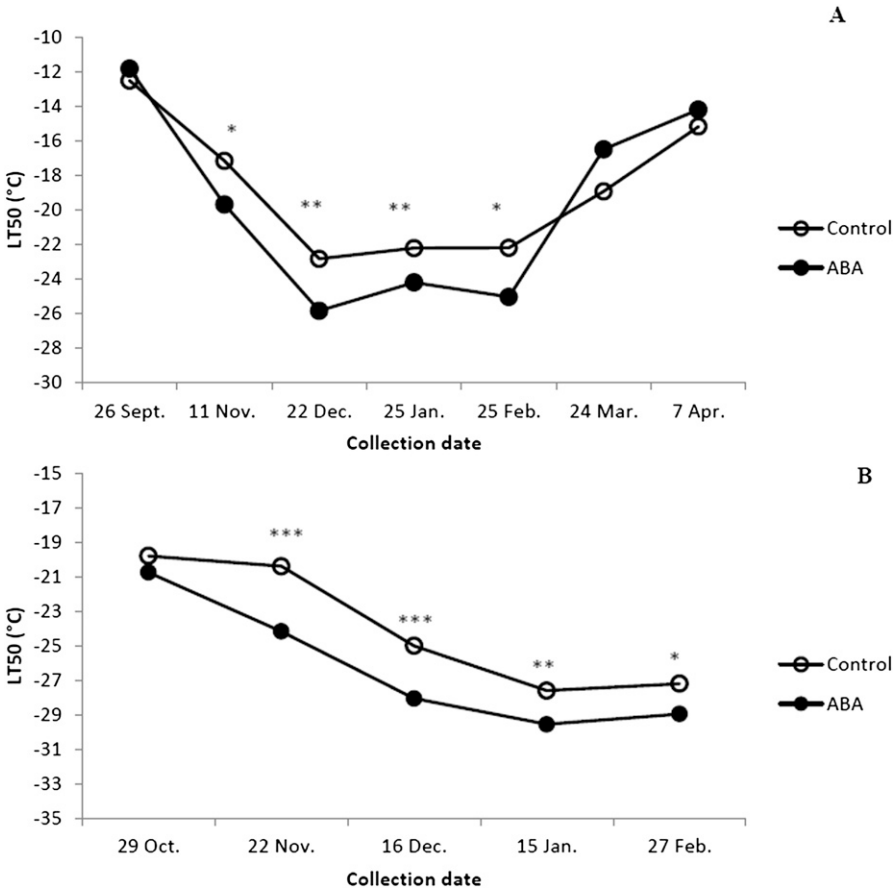

Fig. 4. Effect of abscisic acid (ABA) on freezing tolerance (LT_{50}) in 'Chambourcin' grapevines during the (**A**) 2009–2010 season and (**B**) 2010–2011 season. For clarity, only treatments with significant differences are shown. ABA plot is 4V30 in **A** and average of 4V, 4V20, 6V, and 6V20 in **B**. 4V, 4V20, and 4V30 correspond to ABA application of 400 mg·L^{-1} at the 50% veraison stage and 20 and 30 d after 50% the veraison stage, respectively. 6V and 6V20 correspond to ABA application of 600 at the 50% veraison stage and 20 d after the 50% veraison stage, respectively. *, **, and *** indicate significance at $P \leq 0.05$, 0.01, and 0.001, respectively.

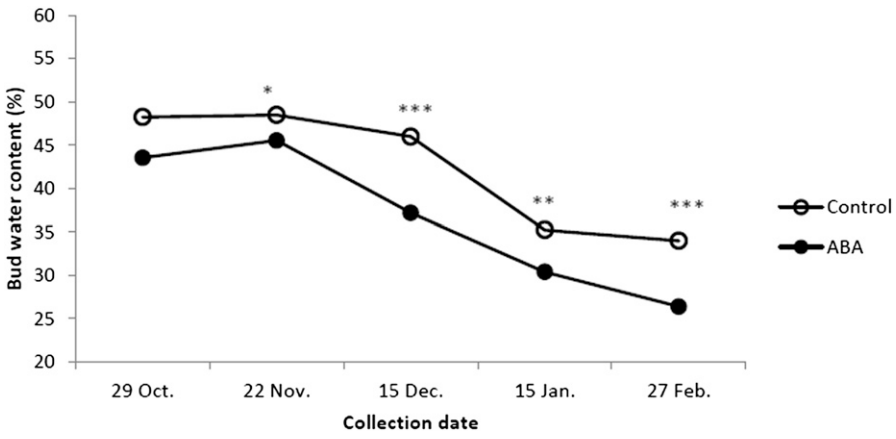

Fig. 5. Bud water content (percent fresh weight) progression in abscisic acid (ABA)-treated and untreated 'Chambourcin' grapevines during the 2010–2011 season. For clarity, ABA plot represents the average of 4V, 4V20, 6V, and 6V20; 4V and 4V20 correspond to ABA application of 400 mg·L^{-1} at the 50% veraison stage and 20 d after the 50% veraison stage, respectively; 6V and 6V20 correspond to ABA application of 600 at the 50% veraison stage and 20 d after the 50% veraison stage, respectively. *, **, and *** indicate significance at $P \leq 0.05$, 0.01, and 0.001, respectively.

Discussion

Based on this study, the highest ABA concentration with the least phytotoxicity for 'Chambourcin' grapevines should be less than 700 mg·L^{-1}. This result is similar to previous findings in a greenhouse study in which optimum concentration was between 400 and 600 mg·L^{-1} (Zhang et al., 2011). Because ABA was applied at the 50% fruit set stage, a portion of flowers was present and thus were injured resulting in reduced berry number per cluster and loose clusters. ABA toxicity can be attributed to an oxidative damage induced by the overproduction of ABA-inducible reactive oxidative species (Jiang and Zhang, 2001). The small berries that remained green at harvest were likely the result of flower damage and insufficient fertilization caused by high ABA concentrations (700 and 800 mg·L^{-1}). Similar flower abscission has been reported with apple and pear trees when ABA was applied as a growth thinning agent between bloom and fruit set (Greene, 2009; Greene et al., 2011).

Compared with a greenhouse study, the phytotoxicity and shoot inhibition effect were reduced under field conditions. The damaged leaf area percent in the field was 25% lower than that in the greenhouse with the same ABA concentration (800 mg·L^{-1}). Additionally, shoot growth inhibition was only observed on grapevines treated with ABA concentration of 600 mg·L^{-1} and above; whereas in the greenhouse, ABA treatment showed the effect at 200 mg·L^{-1}. The decreased ABA phytotoxicity under field conditions can be explained on the basis of leaf wax composition. It was reported that vines in the greenhouse have less cuticular wax than vines in the field because the former is typically well-watered (Bondada, 1996) and receive less ultraviolet radiation and light intensity (Gordon, 1998). Cuticular wax may reduce the efficiency of ABA penetration and absorbance.

In this study, the effects of ABA concentrations of 400 and 600 mg·L^{-1} were not different. Therefore, the optimum ABA concentration with the least phytotoxicity and most economical was 400 mg·L^{-1}. In a similar field trial with 'Cabernet franc' grapevines, responses to ABA treatment of 600 mg·L^{-1} were more consistent than those with 400 mg·L^{-1} (Zhang and Dami, 2012). It is suggested that the optimum concentration of ABA is cultivar-dependent and should be determined first before further evaluations.

In this study, ABA did not affect yield components or basic juice chemical compositions of 'Chambourcin'. This result is consistent with previous findings on table grapes in which yield and fruit chemical compositions were unaffected by ABA treatment at 400 mg·L^{-1} and higher (Lurie et al., 2009; Peppi et al., 2006, 2007). Because ABA was only sprayed on clusters at veraison stage, there was no report on leaf or flower damage from these studies.

The effect of ABA application on enhancing color development has been well documented. Research on table grapes showed that the expression of a key anthocyanin pathway gene was increased by exogenous ABA application, which promotes anthocyanin accumulation in berry skins (Peppi et al., 2008). It was also reported that the type of glucosides and anthocyanins that grapes contain had an influence on the effect of ABA

treatment (Kim et al., 1998). In the ABA timing experiment, the enhancement of anthocyanin accumulation was observed in both years. The effective treatment was 4Multiple and V20 (both 4V20 and 6V20) in 2009 and 2010, respectively. However, when applied during the V stage, ABA effect on color was not consistent, indicating the optimum timing for ABA application to enhance color development may be 3 weeks later than that in table grapes. It has been reported that on 'Cabernet Sauvignon', the most effective ABA applications were also those sprayed between 80% berry softening and 10 d after 100% berry softening (Gu et al., 2011). It is concluded that ABA application at the post-veraison stage can enhance color development by promoting anthocyanin accumulation even when basic fruit composition (Brix, pH, and TA) remains unchanged.

ABA also advanced periderm formation in field-grown 'Chambourcin'. In the field study, the leaf age of grapevines when applied ABA ranged between 70 and 130 d old since budburst. This finding is consistent with the greenhouse work in which it was reported that ABA induced periderm formation when applied on young (50 to 60 d) and old (100 to 120 d) leaves (Zhang et al., 2011). The extent of periderm formation in 2010 was lower than that of 2009, which may be associated with the higher cropload in 2010 than that in 2009. The average cropload of 2010 was 36% higher than that of 2009. Previous reports demonstrated that high cropload decreased periderm formation (Bates, 2008; Dami et al., 2005). It is noted that although clusters were thinned to the same level in both years, the cluster number in 2009 was lower than that of 2010 as a result of winter injury (86% primary bud death) recorded in Jan. 2009 (Dami et al., 2012). It is concluded that ABA application between fruit set and the post-veraison stage can enhance periderm formation of 'Chambourcin' grapevines.

The reduction of chlorophyll content occurred in mid-Sept. 2009 indicating a reduced photosynthesis activity and initiation of leaf senescence. Vines treated with ABA at the V30 stage (4V30 and 4Multiple, leaf age = 130 d) consistently had lower chlorophyll content compared with that of untreated vines. The significant difference was observed on 6 Oct. 2009, 1 week after ABA application. This result was similar to the findings in a previous greenhouse work, in which it was observed that a reduction of chlorophyll content occurred 2 weeks after ABA was applied to 120-d-old leaves and the leaf senescence process was advanced at the same time (Zhang et al., 2011). The role of ABA in leaf senescence was widely investigated in many plants and confirmed by both physiological and molecular evidences. It has been reported that the translocation of ABA from root to shoot triggered leaf abscission in cotton (Dong et al., 2008). Additionally, two senescence-associate mRNA, *pSEN4,* and *pSEN5* were upregulated after exogenous ABA application in *Arabidopsis thaliana*, which demonstrated a linkage between ABA and leaf senescence (Park et al., 1998)

In this study, ABA treatments at V and V20 stages effectively advanced the bud dormancy process and when placed under forcing conditions, budburst was delayed as compared with that of the control. ABA-induced bud dormancy has been reported in other plants and a positive relationship between the concentration of endogenous ABA and dormancy has been demonstrated (Guak and Fuchigami, 2001; Or et al., 2000). ABA has been considered to play a positive role in dormancy induction and maintenance (Kucera et al., 2005). Therefore, under the forcing conditions used in the dormancy assay, D50BB ranged between 50 and 75 in both years, indicating that vines already entered endodormancy (Gu, 2003). The type of ABA-induced dormancy is considered endodormancy. This is further confirmed because single-node cuttings (not paradormancy, resulting from inhibition from other plant tissues) were used under favorable growing conditions (not ecodormancy, resulting from inhibition from the environment) (Lang et al., 1987). The results of field trials were consistent with greenhouse findings (Zhang et al., 2011).

It has been reported that exogenous ABA application can increase freezing tolerance of several plants such as *Cicer arietinum* L. (Kumar et al., 2008), Cacti family (Loik and Nobel, 1993), *Secale cereal* L. (Churchill et al., 1998), and *Malus domestica* (L.) Borkh (Guak and Fuchigami, 2001). In this study, ABA treatment at V20 and V30 stages consistently increased the freezing tolerance of buds during midwinter. There was no difference during the early cold acclimation (September and October) and deacclimation (March and April) stages. Additionally, it was demonstrated that there was a negative relationship between bud water content and freezing tolerance. Plant cells are usually dehydrated under an extracellular freezing process; and the dehydration-induced freezing tolerance has been well illustrated (Pearce, 2001). In grapes, it has been demonstrated that water stress in late fall can make grapevines acquire an early cold acclimation and dormancy, which can also be a consequence of the induction of ABA synthesis caused by water stress (Keller, 2010). It has been reported that an ABA-responsive protein kinase was involved with dehydration and cold acclimation in *Triticum aestivum* L. (Holappa and Walkersimmons, 1995).

In summary, foliar ABA application with an optimal concentration of 400 $mg \cdot L^{-1}$ at 3 to 4 weeks postveraison was effective to induce cold acclimation and deep dormancy resulting in increased freezing tolerance of field-grown 'Chambourcin' grapevines. ABA treatment also increased anthocyanin content in berry skin without affecting yield components or basic juice chemical composition, which is a positive attribute for fruit and wine quality. It is concluded that exogenous ABA application can be considered as an additional protection tool against freezing stress of cold-sensitive grape cultivars grown in cold regions.

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