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Glycinebetaine Enhances Osmotic Adjustment of Ryegrass under Cold Temperatures

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Abstract: Perennial (*Lolium perenne* L.) and annual (*L. multiflorum*) ryegrass are important species for landscape (e.g., turf) and agricultural (e.g., pasture systems) uses. Abiotic stresses limit the survival, growth, and/or appearance of these species. The synthesis and accumulation of quaternary ammonium compounds (QACs) such as glycinebetaine (GB) are an adaptive response to abiotic environmental stresses in some species. Both *L. perenne* and *L. multiflorum* are GB-accumulating species, and exogenous application of GB may enhance growth under less-than-optimal environmental conditions. We tested the effects of exogenous application of GB on growth and water relations of annual and perennial ryegrass growing under temperatures at the lower limits of their optimal growth. Osmotic stress resulted in increased GB accumulation in *L. perenne*, but exposure to cold temperatures did not result in increased GB accumulation in either species. Both species accumulated higher concentrations of GB in leaf and stem tissues when exogenous GB was supplied, regardless of growing temperature. Exogenous GB did contribute to lower osmotic potential in both species, but did not affect relative water content, although succulence was higher in some cases. Overall, exogenous GB did not affect growth under optimal growing temperatures, but did enhance growth of *L. perenne* growing under low temperatures.

Keywords: quaternary ammonium compound; cold tolerance; *Lolium perenne*; *Lolium multiflorum*; osmotic potential

1. Introduction

Annual (*Lolium multiflorum*) and perennial (*Lolium perenne*) ryegrass are important feed sources for a number of animals, and are important for their use in ornamental and sports turfs. Both species are widely grown, but growth is often limited under cold spring temperatures [1]. Traditional breeding is time-consuming, and agronomic approaches to enhancing growth under cold temperatures could be applied to multiple genotypes that have already been selected for growth under other environmental specifics (e.g., soil conditions) of a given region.

Osmoprotectants are an important component of the suite of traits utilized by plants to maintain growth under cold temperatures [2]. *Lolium perenne* and *L. multiflorum* both produce proline, and production is increased in response to abiotic stresses such as drought [3,4]. Biosynthesis of the quaternary ammonium compound glycinebetaine (GB) has been reported in *L. perenne* [5], and GB is thought to play a role in drought tolerance in *L. multiflorum* [6].

Glycinebetaine plays a role in plant stress tolerance via protection of proteins and membranes and as an osmoticum that acts to lower cell osmotic potential under various osmotic stresses [7]. Transformation of *L. multiflorum* with the betaine aldehyde dehydrogenase (BADH) gene (the final

step in GB synthesis from choline) from zoyzia grass resulted in tolerance to NaCl, although GB concentrations in plants were not reported [7]. Expression of both spinach choline monooxygenase (CMO) and BADH in transgenic *L. perenne* also resulted in enhanced salt tolerance [8].

Exogenous application of GB can enhance stress tolerance, including in several grass species [9–11], and exogenous application of GB enhanced water-use efficiency of wheat [12]. Several reports have demonstrated positive effects of exogenous application of GB on freezing [13], chilling [14], and frost tolerance [15].

Although there are many reports of beneficial effects of GB on stress tolerance, there are conflicting results on the effects of exogenous GB on pasture growth under cold temperatures [16,17]. Given the fact that increased GB concentrations may also have positive effects on animal health and milk yields in dairy cows [16], the application to ryegrass may result in multiple benefits. Our objective was to determine the effects of exogenous GB application on growth of annual and perennial ryegrass under cold temperatures. We determined the natural accumulation of GB under different temperatures relative to other abiotic stresses, and quantified the contribution of GB to osmotic adjustment of these species. Since previous studies with conflicting results were conducted under field conditions, we sought to assess the effects of GB under controlled conditions.

2. Materials and Methods

2.1. Plant Materials and Growth Conditions

For all experiments, seed of *Lolium multiflorum* 'Crusader' and *L. perenne* 'Kingston' were treated with a 10% bleach solution for 10 min, then imbibed with water overnight. Seeds were sown in 14.4 cm diameter containers, and thinned to 6 plants per container. The soil mix consisted of 80% composted pine bark (0–12 mm) and 20% pumice (3–6 mm), amended with agricultural lime (1 kg m^{-3}), and Hydraflo II granular wetting agent (1 kg m^{-3}). Half-strength Hoagland solution was used throughout the experiments.

2.2. Treatment Applications

2.2.1. Experiment 1—Accumulation of Glycinebetaine (GB) under Various Abiotic Stresses

For the quantification of changes in GB synthesis in response to various stresses (cold, heat, drought, salinity), plants were maintained in a growth room (Conviron) with a day and night constant temperature of 18 °C for 21 d. Light levels in this chamber and subsequent experiments were ca. 200 µmol m^{-2} s^{-1} with a photoperiod of 12 h. Cold-stressed plants were then moved to a 6 °C chamber for 20 d prior to sampling. Temperature was reduced by 2 °C d^{-1} for 4 d prior to exposure to 6 °C for 15 d. Heat-stressed plants were moved to a 40 °C chamber for 5 d prior to harvest. Water was withheld from water-stressed plants for 10 d prior to harvest. Salinized plants were treated with nutrient solution plus 50, 100, or 150 mM NaCl for 15 d prior to harvest. Salinity was increased from 50 (4 d) to 100 mM (4 d), and finally, 150 mM for respective treatment levels. $CaCl_2$ was added to the NaCl solution in a 5:1 Na:Ca molar ratio. For each species × treatment, three replicate containers were used. All plants were harvested on the same day when leaf relative water content (RWC) and GB concentrations were quantified.

2.2.2. Experiment 2—Accumulation of GB under Different Temperatures

To quantify GB accumulation in ryegrass growing under different temperatures, plants were maintained in a growth room with a day and night constant temperature of 18 °C for 10 d, after which half of the plants were transferred to a 6 °C chamber for 22 d. Tissue was harvested for GB quantification from plants growing under both temperatures at 0, 15, and 22 d after moving to 6 °C. Three replicate containers were used for each species × temperature × GB treatment.

2.2.3. Experiment 3—The Effect of Exogenous GB Application on GB Accumulation in Ryegrass

To determine if *L. multiflorum* and *L. perenne* have the ability to take up exogenously applied GB, and its effect on water relations, plants were grown in an 18 °C chamber for twelve weeks. Plants were sprayed to dripping once a week with 0 (water control) or 100 mM GB. After twelve weeks, plants were harvested and biomass, leaf GB, relative water content (RWC), succulence, and osmotic potential of the cell sap was measured. Three replicate containers were used for each species × temperature × GB × time treatment.

2.2.4. Experiments 4 and 5—The Effect of Exogenous GB Application on Growth under Cold Temperatures

Seeds were germinated and plants were grown in an 18 °C chamber for two weeks, at which point half of the plants were moved to a chamber set to identical conditions except regarding temperature, which was set at 6 °C. Plants were sprayed to dripping once a week with 0 (water control), 50, or 100 mM GB. Every 7 d (over 35 d total), plants were harvested, and biomass, stem and leaf GB, relative water content (RWC), succulence, and osmotic potential of the cell sap were measured. To confirm the effects of exogenous application of GB on growth of these species under cold conditions, another set of plants (n = 15) was grown for 2 weeks at a light-period temperature of 18 °C. After these two weeks, GB was applied at 0 or 100 mM, and then plants were grown at 18 or 6 °C for another 2 weeks. Plants were harvested and total above-ground tissue was dried and weighed (n = 15).

2.3. Quantification of Glycinebetaine

Samples were taken from five to six individual plants of each species. A representative sub-sample of the leaf and stem (in some experiments) tissue bulked from the individual plants (1 to 1.5 g FW) was collected. Tissue was extracted by immersion in pre-weighed vials containing 10 mL methanol. Methanol extracts were stored at 4 °C in the dark and then phase-separated with chloroform and water, as described previously [18–21]. The upper (aqueous) phase was concentrated to dryness under an air stream. Total QAC levels in the betaine fraction were determined by a spectrophotometric periodide assay [22,23].

2.4. Quantification of Growth

In Experiment 3, five to six plants per container were harvested and fresh and dry weight was obtained. In Experiment 4, this was also done but plants were harvested weekly to determine growth over the experimental period. Growth over time was linear, so linear functions were fit to the data and differences among slopes (increase in growth per day) were tested.

2.5. Water Relations Measurements

Leaf tissue was excised and placed in pre-weighed vials containing ca. 10 mL of deionized water. Leaf fresh weight (FW) was calculated as initial vial weight subtracted from the vial weight containing tissue. After weighing, vial caps were removed, and the vials were placed under low light conditions for ca. 10 h. Leaf tissue was then removed from the vials and blotted dry before weighing to obtain the turgid weight (TW) of the tissue. Leaf tissue was then dried to obtain dry weight (DW), and relative water content (RWC) was calculated as:

$$[(FW - DW)/(TW - DW)] \times 100. \quad (1)$$

Succulence was calculated as:

$$FW/DW. \quad (2)$$

Immediately after excision, a random sample of leaf tissue from the bulk sample was placed in a microfuge tube containing a cut pipette tip (ca. 5 mm) and a piece of mesh screen. The tube was

then frozen in liquid nitrogen and stored at −35 °C. Tubes were thawed for 20 min and spun for 5 min at 10,000× *g* in a microcentrifuge. Osmolality of the extracted cell sap was quantified with a vapor pressure osmometer (Wescor 5500, Wescor Inc., Logan, Utah, USA). Osmolality was converted to osmotic potential by the equation:

$$\Psi_\pi = -C_s RT, \quad (3)$$

where Ψ_π is osmotic potential, C_s is the osmolality, R is the gas constant, and T is temperature. The osmotic potential at full turgor ($\Psi_\pi{}^{100}$) was calculated as:

$$\Psi_\pi \times \text{RWC}. \quad (4)$$

2.6. Statistical Analyses

All analyses were conducted using SAS 9.4 software (SAS Institute, Cary, North Carolina, USA). Data were subjected to ANOVA and mean values were compared using Tukey's multiple range test ($p \leq 0.05$). For quantification of growth under different GB × temperature treatments, the slope of FW or DW over all weeks of growth for 50 or 100 mM GB-treated plants was tested for significant interaction with the same slope for 0 mM GB-treated plants.

3. Results

3.1. Glycinebetaine Concentrations in Two Lolium Species in Response to Various Stresses

To determine if GB is synthesized in response to abiotic stress, plants of *L. multiflorum* 'Crusader' and *L. perenne* 'Kingston' were exposed to salinity, a water deficit, and low and high temperatures. In both species, salinity of 100 and 150 mM NaCl and 10 d of water stress resulted in a lower RWC (Figure 1A). However, accumulation of GB increased in response to 150 mM NaCl and water stress only in *L. perenne* 'Kingston' (Figure 1B). In both species, exposure to high temperature resulted in increased accumulation of GB, whereas exposure to low temperature resulted in no change in accumulation of GB (Figure 1B).

To confirm the lack of accumulation of GB in response to cold temperatures in the two species, plants were grown for 20 d at 18 °C; then, plants were grown under 18 or 6 °C. After 15 d, plants of both species maintained at 18 °C had similar GB concentrations to day 0, whereas after 22 d, GB concentrations increased in *L. perenne*. 'Kingston' plants (Figure 2). Plants of both species had lower GB concentrations after 15 d of growth under 6 °C, although GB concentrations in these plants returned to day 0 values after 22 d. Exposure to cold temperatures, unlike some other abiotic stresses, do not result in an increase in endogenous levels of GB in either species.

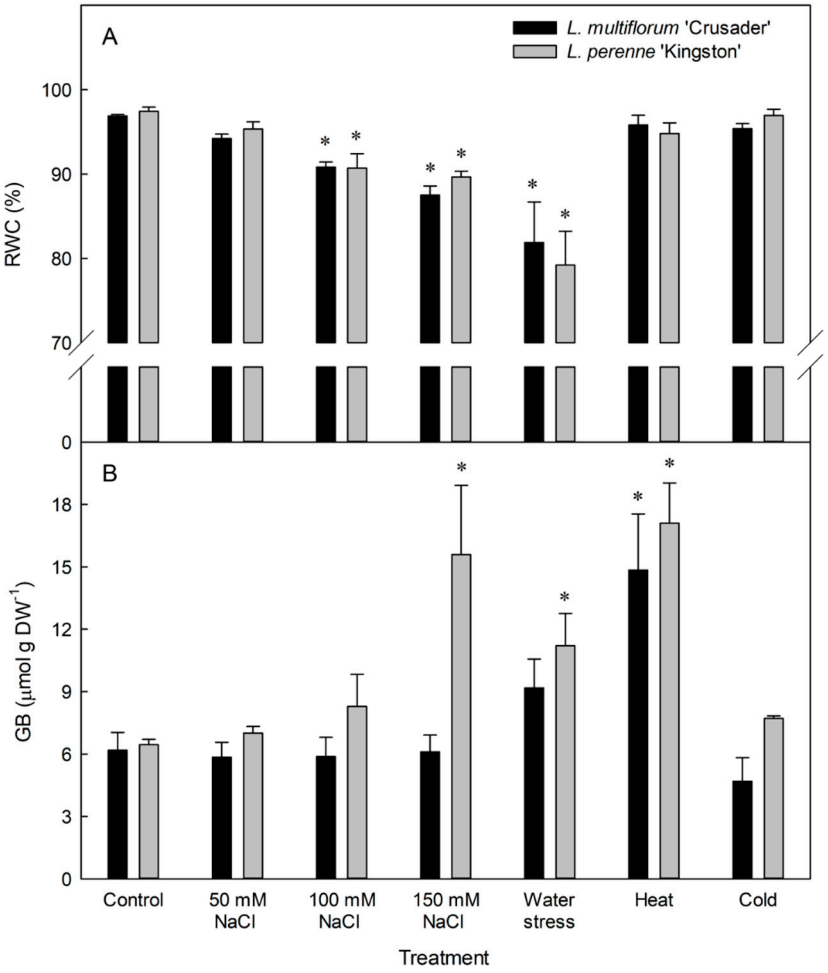

Figure 1. Relative water content (RWC, (**A**)) and leaf glycinebetaine (GB, (**B**)) concentrations of *Lolium multiflorum* 'Crusader' and *Lolium perenne* 'Kingston' growing at 18 °C (Control) or after exposure to 50, 100, or 150 mM NaCl, 10 d of water stress, 4 d at 40 °C (heat), or 20 d at 6 °C (cold). Within a species, treatment plants are lower (RWC) or higher (GB) than control plants at $p \leq 0.05$ (*), based on Dunnett's one-way test ($n = 3$).

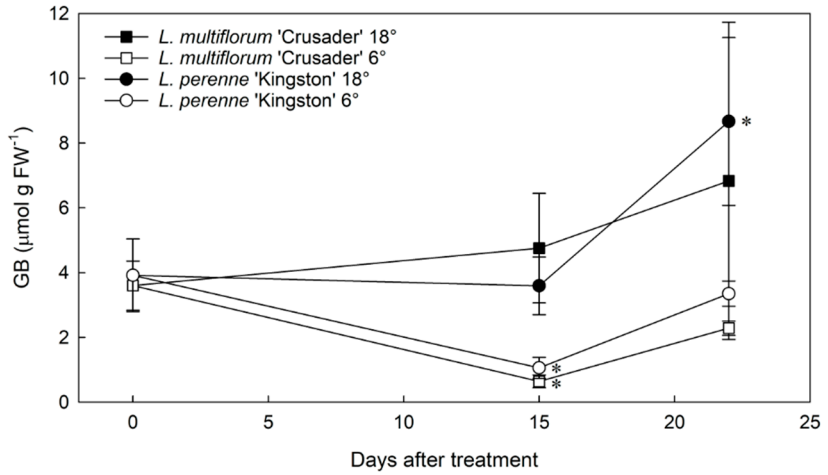

Figure 2. Accumulation of glycinebetaine (GB) in *Lolium multiflorum* 'Crusader' (squares) and *Lolium perenne* 'Kingston' (circles) grown at 18 (solid) or 6 °C (open). GB concentrations at 0, 15, and 22 d after treatment initiation are lower or higher than control plants at $p \leq 0.05$ (*), based on Dunnett's two-way test ($n = 3$).

3.2. Effects of Exogenous Glycinebetaine Application on Glycinebetaine Accumulation, Growth, and Water Relations

To determine if *L. perenne* and *L. multiflorum* were able to take up GB and the potential effects on growth and water relations under optimal temperatures, GB (100 mM) or water was applied weekly for twelve weeks. Both species accumulated GB, which was a significant contributor to osmotic adjustment based on $\Psi_\pi{}^{100}$ values (Table 1). Growth, RWC, and succulence were not affected by GB application under these conditions.

Table 1. The effects of glycinebetaine (GB) on relative water content (RWC), osmotic potential (Ψ_π), osmotic potential at full turgor ($\Psi_\pi{}^{100}$), leaf succulence, GB content, and the contribution of GB to Ψ_π (GBC) (n = 6).

Species	Applied GB	DW	RWC	Ψ_π	$\Psi_\pi{}^{100}$	Suc	GB	GBC
	mM	g	%	MPa	MPa		nmol	%
L. m.	0	1.64	91.8	−1.27	−1.15	6.50	1.23	2.74
	100	2.38	91.6	−1.40	−1.27 *	6.88	5.93 *	11.98 *
L. p.	0	1.53	93.5	−1.37	−1.28	6.12	1.42	3.34
	100	1.10	93.5	−1.50 *	−1.38 *	5.86	5.50 *	10.57 *
Sig [z]	GB	n.s.	n.s.	**	**	n.s.	***	***
	Species	*	**	**	**	***	n.s.	n.s.
	GB × Species	*	n.s.	n.s.	n.s.	**	n.s.	*

[z] Treatment means within a species and main (GB and species) or interaction (GB × Species) effects are not significantly different (n.s.) or significantly different at $p \leq 0.05$ (*), 0.01 (**), or 0.001 (***).

To test the effects of exogenous GB application on ryegrass growth under cold temperatures, plants were grown in 18 °C, and then grown under 18 or 6 °C while applying 0, 50, or 100 mM GB. Both species accumulated applied GB, and in most cases, GB uptake into leaf tissues was linearly correlated with the concentration of GB applied (Figure S1). The application of 100 mM GB resulted in an increased growth rate of *L. multiflorum* 'Crusader', but not of *L. perenne* 'Kingston' when grown at 18 °C (Table 2). When growing at low temperatures, application of 50 or 100 mM GB enhanced growth in *L. perenne* 'Kingston', but not *L. multiflorum* 'Crusader'. This difference in effect on growth was despite the fact that both species accumulated GB in leaf and stem tissues when GB was applied (Figures 3 and 4).

Table 2. The effects of exogenous glycinebetaine (GB) on the growth rates of *Lolium multiflorum* 'Crusader' (*L. m.*) and *L. perenne* 'Kingston' (*L. p.*) plants grown under 18 or 6 °C.

Temperature	Species	Applied GB	Growth Rate	Growth Rate
		mM	mg FW day^{-1}	mg DW day^{-1}
18 °C	*L. m.*	0	19.15	6.23
		50	13.76	4.41
		100	32.91 ***	7.04 **
	L. p.	0	14.19	4.89
		50	12.24	4.15
		100	22.13	5.94
6 °C	*L. m.*	0	6.32	2.61
		50	5.76	2.53
		100	10.33	2.10
	L. p.	0	5.84	1.22
		50	14.16 *	4.17 ***
		100	18.75 ***	4.30 ***

The slope of biomass over five weeks of plants treated with 50 or 100 mM GB is significantly different from the slope of plants treated with 0 mM GB at $p \leq 0.05$ (*), 0.01 (**), or 0.001 (***).

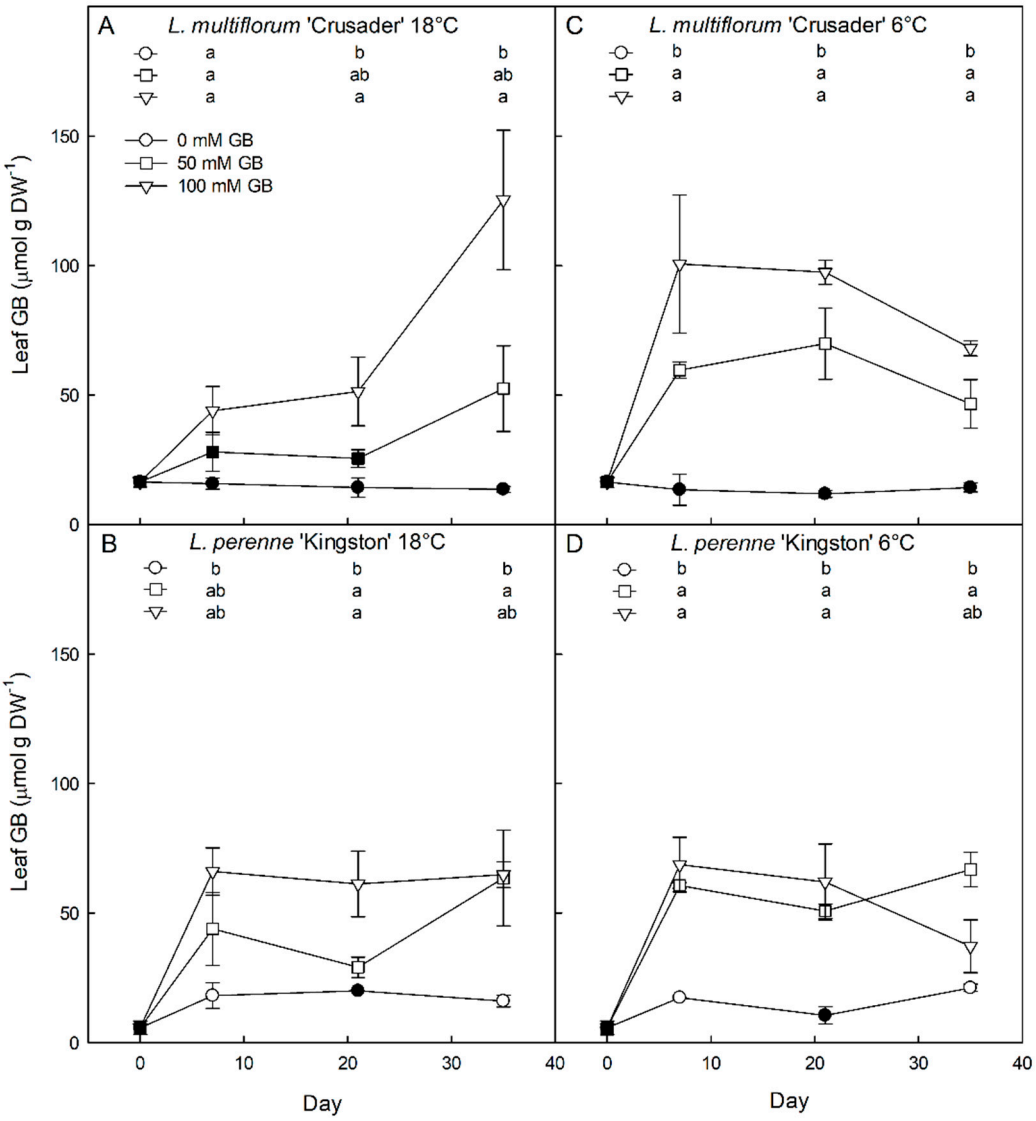

Figure 3. Leaf lamina glycinebetaine (GB) concentrations of *Lolium multiflorum* 'Crusader' (**A**,**C**) and *Lolium perenne* 'Kingston' (**B**,**D**) grown in 18 (A,B) or 6 °C (C,D) environments with application of 0 (circles), 50 (squares), or 100 (triangles) mM GB. Open symbols indicate significantly higher concentrations in plant tissue compared to day 0, based on Dunnett's one-way test. Within each species × temperature × day group, GB treatments with the same letter (presented in table at the top of each panel) are not significantly different from each other, based on Tukey's honest significant difference test ($p \leq 0.05$) ($n = 3$).

Application of GB had no effect on relative water content (RWC) of leaf tissue, and tissue succulence was only increased when 100 mM GB was applied to plants growing under 18 °C (Table 3). Exogenous GB resulted in a lowering of osmotic potential (Ψ_π) and a lowering of osmotic potential at full turgor (Ψ_π^{100}), suggesting that exogenous application of GB contributed to osmotic adjustment in these plants (Table 2 and Figure 5). The contributions of GB (on a molar basis) to leaf osmotic potential were ca. 10% and 8% in plants growing under 18 and 6 °C, respectively, but GB accounted for up to almost 40% of the solutes when it was applied exogenously. Overall, exogenous application of GB resulted in enhanced growth under cold conditions only in *L. perenne* 'Kingston' when applied at 100 mM, despite the fact that GB application had a positive effect on solute accumulation in both species under all conditions. Overall, there was no effect of exogenous GB application on the allocation of biomass between leaf and stem (Table S1).

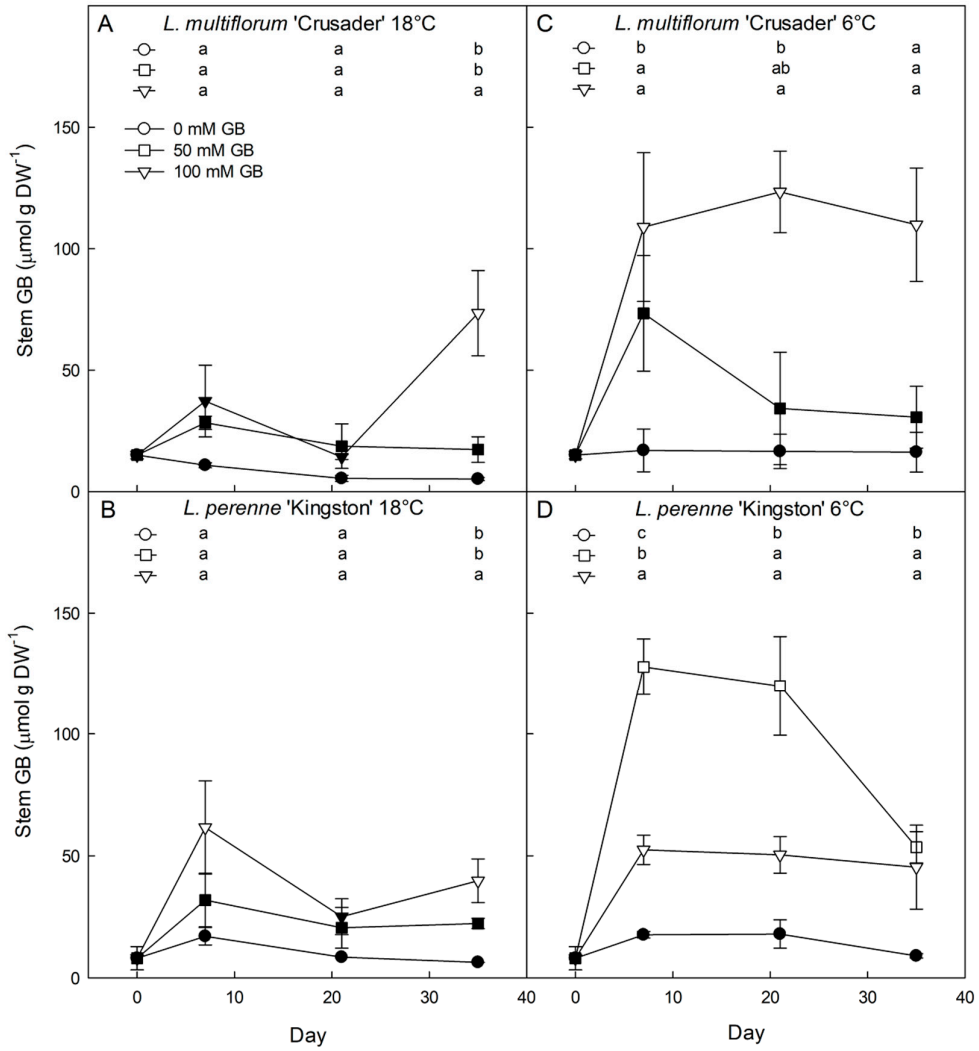

Figure 4. Stem glycinebetaine (GB) concentrations of *Lolium multiflorum* 'Crusader' (**A**,**C**) and *Lolium perenne* 'Kingston' (**B**,**D**) grown in 18 (A,B) or 6 °C (C,D) environments with application of 0 (circles), 50 (squares), or 100 (triangles) mM GB. Open symbols indicate significantly higher concentrations in plant tissue compared to day 0, based on Dunnett's one-way test. Within each species × temperature × day group, GB treatments with the same letter (presented in table at the top of each panel) are not significantly different from each other, based on Tukey's honest significant difference test ($p \leq 0.05$) ($n = 3$).

Table 3. The effects of applied glycinebetaine (GB) on relative water content (RWC), leaf succulence (Suc), osmotic potential (Ψ_π), osmotic potential at full turgor ($\Psi_\pi{}^{100}$), and the contribution of GB to Ψ_π (GBC) values of *Lolium multiflorum* 'Crusader' (*L. m.*) and *L. perenne* 'Kingston' (*L. p.*) plants grown under 18 or 6 °C ($n = 6$).

Temperature	Species	Applied GB	RWC	Suc	Ψ_π	$\Psi_\pi{}^{100}$	GBC
°C		mM	%		MPa	MPa	%
18	*L. m.*	0	92.8	3.98b	−1.12b	−1.03	10.0b
		50	92.6	4.19b	−1.22ab	−1.15	23.2a
		100	88.9	4.93a	−1.29a	−1.14	33.4a
	L. p.	0	92.3	3.91b	−1.28b	−1.17	11.4b
		50	89.0	4.17ab	−1.46a	−1.28	25.1ab
		100	86.7	4.84a	−1.48a	−1.26	31.0a

Table 3. *Cont.*

Temperature	Species	Applied GB	RWC	Suc	Ψ_π	Ψ_π^{100}	GBC
°C		mM	%		MPa	MPa	%
	Sig [z]	GB	**	*	***	*	***
		Species	n.s.	n.s.	***	***	n.s.
		GB × Species	n.s.	n.s.	n.s.	n.s.	n.s.
6	*L. m.*	0	85.5	4.07ab	−1.28b	−1.07b	7.8b
		50	84.4	3.95b	−1.47a	−1.21a	31.5a
		100	85.8	4.36a	−1.52a	−1.28a	38.0a
	L. p.	0	85.3	4.36	−1.35b	−1.13b	8.4b
		50	85.2	4.19	−1.51a	−1.26a	29.4a
		100	84.6	4.22	−1.51a	−1.25a	24.9a
	Sig [z]	GB	n.s.	n.s.	***	***	***
		Species	n.s.	n.s.	n.s.	n.s.	n.s.
		GB × Species	n.s.	n.s.	n.s.	n.s.	n.s.

[z] Means within a column are not significantly different (n.s.) or are significantly different at $p \leq 0.05$ (*), 0.01 (**), or 0.001 (***). Values with the same letter within a species × temperature level are not significantly different, based on Fisher's Protected LSD test.

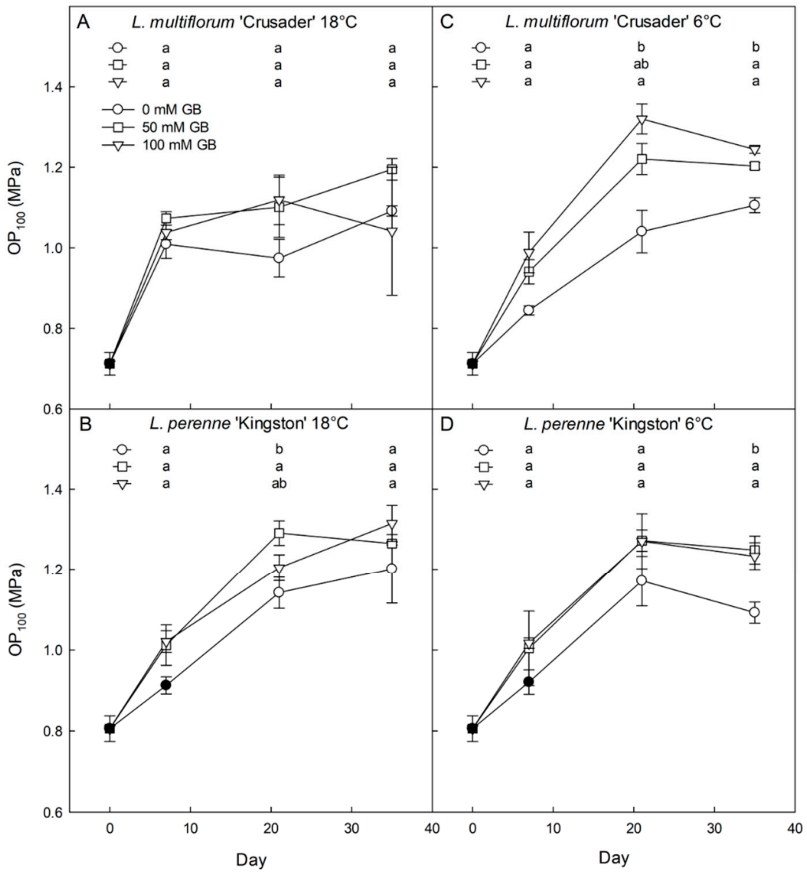

Figure 5. Leaf osmotic potentials at full turgor (OP_{100}) of *Lolium multiflorum* 'Crusader' (**A**,**C**) and *Lolium perenne* 'Kingston' (**B**,**D**) grown in 18 (**A**,**B**) or 6 °C (**C**,**D**) environments with application of 0 (circles), 50 (squares), or 100 (triangles) mM GB. Open symbols indicate significantly higher concentrations in plant tissue compared to day 0, based on Dunnett's one-way test. Within each species × temperature × day group, GB treatments with the same letter (presented in table at the top of each panel) are not significantly different from each other, based on Tukey's honest significant difference test ($p \leq 0.05$) ($n = 3$).

To confirm the effects of exogenous application of GB on growth of these species under cold conditions, plants were grown for 2 weeks at light-period temperature of 18 °C, GB was applied at 0 or 100 mM, and then half of the plants were moved to 6 °C. After 2 weeks, plants were harvested. In this experiment, the application of GB had no positive effect on plant dry weight (Figure S2).

4. Discussion

4.1. Glycinebetaine Concentrations Do Not Increase In Response to Cold Temperatures

Lolium perenne can accumulate exogenously applied GB under non-stress conditions [5,11,24], and accumulation increases in response to salinity [11,24]. There are no reports of GB accumulation in *L. multiflorum*. *Lolium perenne* and *L. multiflorum* also accumulate significant amounts of proline, and accumulation of this compatible solute is also increased under stress conditions [3]. Concentrations of GB in both species in this set of experiments (Figure 1B, Tables 1 and 3) are similar to concentrations previously reported for *L. perenne* [5,11] and cereals such as wheat [25].

When exposed to osmotic stress, only *L. perenne* 'Kingston' had higher concentrations of GB over controls (Figure 1B), consistent with previous reports of induced accumulation of GB in *L. perenne* in response to osmotic stress [11,24]. This could be due to the relatively young age of the plants used in this study [26] and/or differences in environmental conditions. Other grass species, such as *Festuca arundinaceae* [27] and wheat [28,29], also respond to water stress with increased concentrations of GB.

Only the highest level of NaCl (150 mM) elicited an increase in GB and only in *L. perenne* (Figure 1B). Short-term salt stress also resulted in only a mild accumulation of GB in tomato [30]. Genotypes of spring wheat also vary in their accumulation of GB in response to salt [25]. Longer-term exposure resulted in GB accumulation in *Anneurolepidium chinense* [31]. It is likely that GB accumulation in these ryegrass species is dependent on genotype, plant age, and length of stress exposure.

When exposed to temperatures above the optimal for growth of both species, accumulation of leaf GB greatly increased (Figure 1B). Likewise, GB accumulation in response to high temperatures was much greater than in response to salinity in tomato [30]. Cold stress does elicit an accumulation of GB in some species such as wheat [32]. When exposed to temperatures at the low end for growth in these species, GB accumulation did not occur (Figure 1B), and, in fact, concentrations initially decreased in response to low temperatures (Figure 2). Several field trials show no beneficial effects of GB on growth on crop plants under moderate temperatures [17,33]. However, given the lack of induction in GB synthesis in response to cold temperatures, we hypothesized that exogenous application may aid growth in both species.

4.2. Both L. perenne and L. multiflorum Have the Ability to Accumulate Exogenously Applied Glycinebetaine That Contributes to Reduced Osmotic Potential

Many plant species have the capacity to take up and translocate GB [25,34], although there is typically genetic variability in the ability to accumulate GB [15]. Therefore, GB could be exogenously applied to both annual and perennial ryegrass for potential enhancement of growth and/or stress tolerance. Accumulation of GB in leaves following exogenous GB application was similar in both species (Figures 3 and 4 and Tables 1 and 3), and was, in most cases, linearly correlated with the concentration applied (Figure S1). Application of GB resulted in very high tissue concentrations, similar to concentrations reported in other crops following exogenous application of GB [33]. Importantly, there were no signs of toxicity as observed in some other species [35].

Thomas [3] speculated that solute accumulation in the stem of *L. perenne* may be more important for drought tolerance than leaf tissue levels, which may also be the case with cold tolerance, as regrowth of tillers appears to be an important freezing tolerance mechanism [36].

Since osmotic adjustment is a key trait for drought tolerance in perennial ryegrass [37], it is likely that GB application may enhance drought tolerance in these species. GB significantly contributes to solute potentials in wheat [38] and *Anneurolepidium chinense* [31].

4.3. Exogenous Application of Glycinebetaine Results in Lowered Solute Potential, But Inconsistently Affects Growth

Accumulation of GB has been demonstrated in some species to enhance stress tolerance, notably via protection of the photosynthetic apparatus [29]. There is genetic diversity for cold tolerance in *L. perenne* [39] and *L. multiflorum* [36], so it may be that other genotypes would respond differently to exogenous GB. Furthermore, it is likely that genotypes within each species vary for the ability and magnitude for GB accumulation [26].

Others have reported increased relative growth rate of crops treated with foliar-applied GB [40]; however, we found mixed results. Exogenous application of GB had contrasting effects on the two species in the first experiment we conducted. When plants were grown at 18 °C, *L. perenne* 'Kingston' exhibited an enhanced growth rate when 100 mM GB was applied, whereas *L. multiflorum* 'Crusader' growth was not enhanced by GB application (Table 1). When grown under low temperatures, GB enhanced the growth rate of *L. multiflorum* 'Crusader' at both GB concentrations, but GB had no effect on *L. perenne*. In a previous experiment, although exogenous GB application increased N, S, and K concentrations in perennial ryegrass, it did not enhance growth [41]. Lee et al. [17] found no effect of GB application on perennial ryegrass-clover pasture growth. GB application varied among sugarcane genotypes from positive to negative effects [42]. GB application had different effects on different rice cultivars [43].

5. Conclusions

The objectives of this set of experiments was not to assess the effect of GB on frost or freezing tolerance in annual and perennial ryegrass, but rather to determine if GB application would improve growth of these species under low-temperature growing conditions. We determined that both *L. perenne* and *L. multiflorum* do not accumulate GB in response to cold temperatures, but do have the capacity to take up exogenously applied GB, resulting in higher concentrations in both leaves and stems. This GB contributes to a decrease in osmotic potential, but the effect on growth was variable across experiments. Given the fact that toxicity was not observed, it is possible that higher concentrations of GB may provide more consistent contributions to growth under cold temperatures.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/10/2/210/s1. Table S1: The effects of exogenous glycinebetaine (GB) on biomass, leaf area, and leaf:stem values of *Lolium multiflorum* 'Crusader' (*L. m.*) and *L. perenne* 'Kingston' (*L. p.*) plants grown under 18 or 6 °C for 35 d (n = 3). Figure S1: Uptake of glycinebetaine (GB) in response to applied concentrations of GB (x-axis) in *Lolium multiflorum* (A, C) and *Lolium perenne* (B, D) grown at 18 (A, B) or 6 °C (C, D). Figure S2: Dry weight of *Lolium multiflorum* and *Lolium perenne* grown at 18 °C for two weeks, and then transferred to 18 or 6 °C for another two weeks. Prior to transfer, 0 or 100 mM glycinebetaine was applied exogenously. Plants treated with 100 mM GB are significantly different than control plants at $p \leq 0.01$ (**), based on Dunnett's two-way test (n = 15).

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