
Effects of foliar application of glycine betaine and chitosan on *Puccinellia distans* (Jacq.) Parl.

subjected to salt stress

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1. Summary

Using brackish water for irrigation may expose turfgrasses to salinity stress. Finding the best treatments to maintain high quality turfs under saline conditions is an important requirement for turfgrass management. We tested the response of a halophyte grass, *Puccinellia distans*, to irrigation with saline solutions and to foliar application of two osmoprotectants, glycine betaine (GB) or chitosan (CH). Plants were grown in pots under controlled conditions and irrigated with 200 mM or 600 mM NaCl solutions. The response to salinity treatments and osmoprotectant application was evaluated after 90 days by measuring leaf firing, leaf density, shoot length and biomass, root length and shoot water potential. Increasing salinity reduced shoot density, shoot and root length, shoot water potential and increased leaf firing and shoot solute potential at 200 mM NaCl. These effects were more pronounced at 600 mM NaCl. Application of GB greatly increased shoot growth traits at 200 mM NaCl and showed beneficial effects on most traits also at 600 mM. Application of CH showed positive effects only on leaf firing and leaf water potential at 600 mM. Our results show that *Puccinellia distans* can tolerate high levels of salt stress which can be best alleviated by GB treatment..

2. Introduction (330 words)

Turfgrass management often requires irrigation with poor quality water, to avoid the use of valuable fresh water resources [35] and this practice may expose turfgrasses to salt stress. In coastal areas, salt stress may also be due to direct exposure to salt spray. For this reason, the ability to maintain a positive water balance, growth, photosynthesis, chlorophyll content and therefore good visual quality

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is an important trait for the selection of turfgrass species [10, 29]. Moreover, the application of osmoprotectants that may alleviate drought stress is a further requirement to maintain turf quality when brackish water must be used for irrigation [28].

Puccinellia distans, or weeping alkali grass, is a C3 perennial, cool-season halophytic grass, that grows in saline environments throughout the world. Salt stress affects plant growth and metabolism mainly through ion toxicity and osmotic stress. To counteract osmotic stress, tolerant genotypes may accumulate compatible solutes such as polyamines, betaines and polyols [24]. One of the most widely present betaines is glycine betaine (GB), a quaternary ammonium compound that has a well known activity as a natural compatible solute [34, 6, 17]. In addition to endogenous GB accumulation, this osmoprotectant can be taken up after exogenous application and can be translocated through the phloem to the whole plant [19], so it has often been used as an osmoprotectant in agricultural practices. Exogenous foliar application of GB has been reported to increase plant resistance to drought and salt stress [e.g. 4, 27, 37]. Another compound with a protective effect is chitosan (CH), the deacetylated form of chitin, a non-toxic biodegradable polymer that has been widely used in agriculture due to several beneficial effects on plant growth and resistance to stress [16, 25, 33].

The aim of this work was to test the effect of foliar application of the osmoprotective compounds GB and CH on growth and appearance of potted *Puccinellia distans* plants subjected to two different levels of salt stress, achieved by irrigation with 200 and 600 mM NaCl solutions.

3. **Materials and Methods** (612 words)

Experimental design, plant material and treatments

Seeds of weeping alkaligrass, *Puccinellia distans* (Jacq.) Parl. (Bottos Sementi srl, Pordenone, Italy) were sown (0.25 g per pot) in plastic pots of 465.5 cm³ of volume (7 × 7 cm and 9.5 cm in depth) and placed in a controlled growth chamber at 25°C temperature with 12 h of illumination per day provided by cool white fluorescent tubes (photosynthetically active radiation (PAR) 150 μmol m⁻² s⁻¹). The soil mixture was 45% sand, 45% organic rich soil (C/N = 25), 5% peat and 5% organic manure. Maintenance fertilization was provided by adding 3 g of a slow release fertilizer (Osmocote Plus, Scotts) per litre of soil mixture. After sowing, pots were irrigated weekly to field capacity with either distilled water, 200 mM NaCl or 600 mM NaCl solutions. Starting 40 days after sowing, plants were kept at a cutting height of 6 cm by clipping weekly. A total of 60 pots were arranged in a total randomized design. Five pots were used as replicates for each treatment. The experiment ended 90 days after sowing, for a total of 13 saline treatments. For GB treatment, an aqueous 0.1 mol L⁻¹ solution of a commercial formulation (Greenstim Verdera, Finland, 97.5% GB) was prepared. For CH treatment, a 0.1 mol L⁻¹ solution was prepared dissolving 1 g chitosan (Sigma-Aldrich, 85% degree of deacetylation) in distilled water acidified with acetic acid, final pH 5.5. These concentrations of osmoprotectants were chosen based on literature data and previous experimental trials [5, 3, 28]. Osmoprotectant treatments were started 40 days after sowing. Each treated pot was sprayed weekly with 10 mL of the GB or CH solution, while controls were sprayed with distilled water. The effect of salinity, GB and CH treatments on several functional traits were assessed at the end of the experiment.

Growth measurements

The overall condition of plants was monitored and the degree of leaf firing was visually estimated with the attribution of a percentage of chlorotic leaves compared to the total of each pot. After measuring shoot density, plants were removed from the pots, roots were washed with deionized water, blotted dry, and root length was measured as root extension from the stem-bases to the farthest extending root. After measuring shoot length, leaves and shoots were removed and weighed for the determination of total shoot biomass fresh weight (FW). After recording fresh weight, shoots were dried to constant weight at 70°C for 48 hours and dry weight (DW) was determined.

Water potential measurements

At the end of the experiment, before removing plants from the pots, shoots were sampled for leaf water potential (Ψ_L) determination. Measurements were carried out with a pressure chamber (SKPM 1400, Skye Instruments Ltd., Powys, UK). To measure leaf solute potential (Ψ_s), samples of fresh

shoots were taken from each pot and used for the extraction of cell sap. Leaf samples were rinsed in distilled water, blotted dry and placed in plastic hypodermic syringes. After freezing in liquid nitrogen and thawing, sap was expressed by hand and collected in an Eppendorf vial. The sap samples were centrifuged (Eppendorf Microfuge) to precipitate cell debris and 50 μl of the supernatant were used for analysis of Ψ_s of cell sap. Osmolality of expressed shoot sap was measured with a cryoscopic osmometer (Osmomat 030, Gonotec, Germany). Leaf turgor potential (Ψ_p) was calculated as the difference between Ψ_L and Ψ_s .

Data analysis

All data are presented as mean values \pm standard deviation. Data were analysed with one-way analysis of variance (ANOVA), using the software package SigmaPlot 12 (Systat Software, Inc., San Jose, USA). Fisher's LSD multiple comparisons test ($\alpha = 0.05$) was used to compare means when ANOVA was significant.

4. **Results** (902 words)

Growth measurements

Leaf firing is commonly used in grasses as an easily measured index of the survival rate of leaves. A certain degree of leaf firing was observed in all treatments: control plants at the end of the experiment showed a leaf firing of about 30%. Saline treatment increased the level of leaf firing from 30 to 40% only at the highest concentration tested, 600 mM, while irrigation with 200 mM NaCl did not show any effect. GB application showed a beneficial effect on turf appearance even without saline treatment, slightly reducing leaf firing of the controls to 25% and of 600 mM treated plants to 35%. Plants sprayed with CH showed in all treatments a more intense pigmentation and a 30% level of leaf firing, so this osmoprotectant had a slightly greater effect on leaf firing reduction at 600 mM than GB.

Shoot density is related to wear tolerance, an important trait for recreational turfgrass use. Shoot density did not differ significantly in non-salt controls with or without application of either GB or CH, and was on average 17 shoots cm^{-2} (Fig. 1a). Under 200 mM saline treatment, shoot density was reduced to 11.8 shoots cm^{-2} , and spraying with GB significantly increased this value to 14 shoots cm^{-2} , while treatment with CH increased shoot density slightly but not significantly. Increasing saline concentration further decreased shoot density, which was reduced to 9.7 shoots cm^{-2} at 600 mM NaCl. Also in this case GB treatment significantly increased shoot density to 10.7 shoots cm^{-2} , while CH treatment had no significant effect.

Shoot length, root length and fresh or dry biomass are often used to evaluate growth responses of grasses. Shoot length at the end of the experiment, before clipping, was on average 8 cm in no-salt control plants (Fig. 1b). Application of GB greatly increased shoot length, up to 10 cm, while CH application reduced shoot length slightly but not significantly compared to controls. Similar results were found for 200 mM NaCl treated plants: saline treatment alone or combined with CH application reduced shoot length to 7 cm, while GB application increased shoot length to 9.6 cm. A further increase in saline concentration reduced growth in all treatments: shoot length at the end of the experiment was 6 cm and neither GB nor CH application affected this parameter at 600 mM NaCl (Fig. 2). The effect of saline treatment on shoot biomass depended on NaCl concentration (Fig. 3): at 200 mM there was no significant effect on fresh shoot biomass, while at 600 mM there was a reduction in fresh and dry weight of 42% and 27%, respectively. Application of GB significantly increased fresh weight in all treatments, 48% when compared to no-salt controls, 35% with respect to NaCl 200 mM treatment and up to 80% when compared to 600 mM NaCl treatment. There was also a slight increase in dry weight with GB application, particularly evident in the 600 mM treatment where a 40% increase was measured. Application of CH resulted in a small increase in fresh weight when compared to controls, between 10 and 20%, but a 20% decrease in dry weight in both saline treatments, which was significant for the 600 mM treatment.

Root length decreased under saline treatment, and this decrease was greater with rising NaCl concentrations, going from 10 cm in no-salt controls to 7 cm in the 600 mM treatment (Fig. 4). A reduction in root length with increasing saline concentration was observed also in GB treated plants; this reduction was significant when compared with no-salt controls, but not at 200 and 600 mM NaCl

treatments. The application of CH also reduced root length, but this did not appear to be related to saline concentration, as in all treatments root length was 6 cm (Fig. 5).

Water potential measurements

To adapt to saline environments, plants must be able to adjust their water potential in order to maintain water uptake from the soil. Shoot water potential was on average -0.3 MPa in no-salt control plants, and was not affected by GB or CH application (Fig. 6a). Under 200 mM saline treatment, Ψ_L decreased to -1.5 MPa. GB treatment allowed partial recovery of Ψ_L that reached -0.8 MPa, while CH application had no significant effect. A further drop in Ψ_L was measured under saline 600 mM treatment, down to -4 MPa, and also in this case GB treatment significantly improved plant water status. At this saline concentration, application of CH resulted in an increase in Ψ_L up to -2.6 MPa. Solute potential of expressed sap from the leaves was on average 2.0 MPa in no-salt controls, and application of GB or CH did not significantly affect this parameter (Fig. 6b). Solute potential increased significantly with saline concentration. At 200 mM Ψ_s was 3.7 MPa; GB application reduced Ψ_s to 2.7 MPa, while the effect of CH application on Ψ_s was not significant. At 600 mM, Ψ_s ranged from 4.6 to 4.3 MPa and no significant differences were evident with either GB or CH application. Pressure potential values were around 1.3 - 1.8 MPa in no-salt controls with or without GB or CH treatment. Turgor pressure was maintained at positive values also at 200 mM, while Ψ_p was closer to turgor loss point at 600 mM for controls (0.5 MPa), increased slightly with GB treatment (0.8 MPa) and reached values comparable to no salt controls with CH treatment (1.7 MPa).

5. **Discussion** (1064 words)

Leaf firing, shoot biomass, shoot and root length, have been widely used as traits to determine the response to salinity of turfgrasses [20, 14]. Healthy appearance and high shoot density are key factors in the evaluation of turfgrass quality for recreational purposes, and irrigation with brackish water or the direct effect of salt spray in coastal areas can cause leaf firing and shoot die-out. *Puccinellia distans* can tolerate relatively high saline concentrations [31, 2], and our data showed a significant increase in leaf firing only at the highest concentration tested, 600 mM NaCl. Leaf firing recorded in no-salt controls can be explained by the length of the experimental period, quite longer than for other reported data [e.g.14]. The degree of injury with GB or CH application was lower than that of a widely used halophyte grass, *Zoysia matrella*, which showed 39% leaf firing at a salinity level of approximately 480 mM [32]. Shoot density was negatively affected already at 200 mM, and while CH application had no effect on shoot density, GB increased it at all saline concentrations, a desirable effect as this trait is not only an important component of turf visual quality, but it is also related to wear tolerance [11]. On the other hand, the effect of CH on leaf firing was more pronounced than that of GB at 600 mM saline treatment, resulting in greener, less damaged shoots. Chitosan application has been reported to induce plant defence mechanisms, mediated by an oxidative burst response, and in several species chitosan application resulted in increased chlorophyll content [25].

One of the requirements of turf management is to reduce the frequency of cuts, so the cost-benefits trade-off of osmoprotectant application must be evaluated. GB consistently increased shoot length by 30% in control treatments and 200 mM saline treatment, thus increasing the frequency of cuts to maintain the desired turf height. At higher saline concentration, instead, there was no increase in shoot length, so the positive effects on turf color and density were obtained without further costs in terms of more frequent cuts. CH did not affect shoot length in any treatment, but on the other hand did not increase turf density under saline conditions.

P. distans showed osmotic adjustment with increasing salinity, as would be expected for a halophyte and as reported also for another species of the genus, *P. tenuiflora* [13]. The natural adaptability of *Puccinellia* to salt stress conditions was further enhanced by GB application. GB has a well documented effect as osmoprotectant, and maintaining a better water status allows greater gas exchange levels and photosynthesis, and higher turgor driving cell expansion and shoot growth. Furthermore, GB application has been reported to increase leaf nitrogen content [38], thus contributing to growth enhancement. Indeed, foliar application of GB resulted in higher levels of

fresh biomass, due to greater tissue water content of the shoots, which can reduce the toxic effects of high levels of NaCl [21]. The better water status was confirmed by less negative Ψ_L values than in the saline treatments, particularly at 200 mM. The effect of GB in increasing water content was evident also from the osmotic potential of expressed sap, which was lower under this treatment except for 600 mM. At the higher saline concentration, GB treatment did not avoid the decrease in average cell turgor pressure, which was comparable to that of the 600 mM NaCl control. The positive effect of GB on growth in all saline treatments was confirmed by the increase in dry biomass. The ability to maintain higher growth rates under saline conditions is a typical trait for salt-tolerant turfgrasses [22], and in *P. distans* GB application indeed re-established shoot growth back to control conditions. Many halophytes show a typical enhancement of growth with low levels of salinity [12] but in this case it was not evident. This is however in agreement with data reported for other species of *Puccinellia* that did not show stimulation of growth at low salt concentrations [9, 30, 7]. In previous experiments we found an increase in growth at low levels of salinity only up to 40 days after sowing [29], while data here reported were taken 90 days after sowing. CH application did not result in significantly increased growth in any of the treatments, either in terms of fresh or dry shoot biomass. CH treatment gave better results than GB for some traits only under 600 mM conditions, improving leaf firing and overall shoot colour, and increasing leaf water potential, which was found to be less negative than in the other treatments, possibly due to the effect of CH on the reduction of transpiration [5]. Halophytes are generally able to increase ROS scavenging systems under salt stress, but the extent of this response is dependent on genotype and salinity level [7]. As CH application has also been reported to increase antioxidant activity, the contribution to salt tolerance could occur through a protective effect on cell membrane integrity [35].

The relation between root growth and salt tolerance is not straightforward, as extensive root growth is often [8, 23] but not always [21] related to high salt tolerance. In *P. distans* we found that root length decreased gradually with increasing saline concentration, as reported for example in maize varieties [15]. Application of osmoprotectants determined a reduction of root length in the no-salt controls. In the case of GB application, this could be explained as a result of increased allocation of resources to the shoots, which increased in both length and biomass. This however could not explain the reduction of root length with CH application, as in this case there was only a slight and not significant increase in shoot biomass. Furthermore, the effect of CH application on root length was constant and did not vary with saline concentration. In *Paspalum* accessions grown under salt stress, water status traits were more strongly related to shoot than to root growth, suggesting that different factors were necessary to explain root growth [18].

Our data confirm that *Puccinellia distans* is a well suited choice for turfs exposed to salt stress. While CH was more effective at improving shoot water status and visual quality at the highest saline concentration tested, a comprehensive evaluation of the different traits showed that treatment with GB gave the best overall results as an osmoprotectant, improving health, growth and visual quality of the turf under both levels of salinity tested.

6. **Conclusion for Future Biology** (89 words)

Turfgrass industry requires sustainable management and efficient water and resource use. Availability and cost of irrigation water are serious threats to landscape development and to the expansion and maintenance of sport fields, golf courses and other turfgrass areas, especially in saline environments [1, 17]. Though extrapolation of results from controlled conditions to field conditions requires careful attention and is not always straightforward [34], screening for responses in controlled systems is a first step toward the selection of species, varieties and treatments best suited for turfs exposed to salt stress.

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Ethical Statement

None to declare.

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None to declare.

Data Accessibility

The article's supporting data and digital research materials are available on request from the corresponding Author.

Competing Interests

We have no competing interests.

Authors' Contributions

EO, GR and FG contributed to the conceptualisation and experimental design and to interpretation of the results; GR and EO contributed to data acquisition and analysis of data; FG and EO drafted and revised the article.

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Figure captions

Fig. 1 Effects of salt stress and GB or CH application on shoot density (a) and shoot length (b) of *Puccinellia distans* at the end of a 90 day experimental cycle. The values are means (\pm SD) of 5 replicates. Different letters indicate significant differences (Fisher's LSD, $p < 0.05$).

Fig. 2 Appearance of representative samples of *Puccinellia distans* at the end of the experiment, 90 days after sowing, treated with a) GB or b) CH.

Fig. 3 Effects of salt stress and GB or CH application on shoot biomass of *Puccinellia distans* at the end of a 90 day experimental cycle. The values are means (\pm SD) of 5 replicates. Different letters indicate significant differences within either DW or FW data (Fisher's LSD, $p < 0.05$).

Fig. 4 Effects of salt stress and GB or CH application on root length of *Puccinellia distans* at the end of a 90 day experimental cycle. The values are means (\pm SD) of 5 replicates. Different letters indicate significant differences (Fisher's LSD, $p < 0.05$).

Fig. 5 Representative samples of *Puccinellia distans* at the end of the experiment, 90 days after sowing, showing root and shoot length.

Fig. 6 Effects of salt stress and GB or CH application on shoot water potential (a) and shoot solute potential (b) of *Puccinellia distans* at the end of a 90 day experimental cycle. The values are means (\pm SD) of 5 replicates. Different letters indicate significant differences (Fisher's LSD, $p < 0.05$).

