Effect of Salinity on *Puccinellia distans* (L.) Parl. Treated with NaCl and Foliarly Applied Glycinebetaine

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**ABSTRACT**

Turfgrasses general appearance is much affected by environmental stresses because the species used for this purpose are particularly exigent in terms of technical inputs and water need. In the Mediterranean area, sometimes irrigation is provided by using waste water which may contain high concentrations of dissolved salts which can cause salt stress injury and poor turf quality. *Puccinellia distans* (L.) Parl. is a halophyte cool season grass that seems to have a high salinity tolerance when cultivated in sodic soils or in NaCl-rich hydroponic cultures. We investigated the response of *P. distans* to salinity in a soil culture in a controlled growth environment. The effect of different concentrations of NaCl (85, 275 and 600 mM) on shoot and root growth and chloride content was assessed. After determining the resistance of *P. distans* to the highest salinity level, we tested the efficiency of the osmoprotectant glycinebetaine (GB) in reducing salt stress effects by measuring some physiological parameters. The results showed a very good adaptability of *P. distans* to salinity conditions. When irrigated with salt solution at the highest concentration (600 mM), plants showed a reduction in growth rate and biomass production that seemed to be relieved by GB application. With GB application, leaf relative water content and biomass production were similar to the control. However, GB application did not result in a generally better turf quality compared to untreated plants, so it did not give a relevant advantage in reducing technical and labor inputs with respect to the maintenance of a *P. distans* turf.

**Keywords:** chlorophyll content, glycinebetaine, *Puccinellia distans*, salt stress, turfgrass

**Abbreviations:** DW, dry weight; FW, fresh weight; GB, glycinebetaine; RWC, relative water content; TW, turgid weight

**INTRODUCTION**

Despite the economic importance and continuous public demand for turf areas, the current drinking water shortages in all Mediterranean areas clearly set limits on the amount and quality of water available for landscape irrigation, so non-potable water sources may be used for this purpose (Marcum 2004). However, effluent water or low quality groundwater can often contain high concentrations of dissolved salts.

The detrimental effects of salinity on turfgrass growth include osmotic stress, ion toxicity, and nutritional disturbances (Greenway and Munns 1980; Lauchli 1986; Cheeseman 1988). Soil salinity, in low to moderate concentrations, mainly reduces growth due to its osmotic effect (Greenway and Munns 1980; Munns and Termaat 1986). At higher concentrations, salts may accumulate in the leaves to a toxic level, resulting in ‘scorching’ or ‘firing’ of leaves (Batulks and Primo-Millo 1992; Storey and Walker 1987). The detrimental effects of stress on growth and leaf firing are a problem for turf maintenance, as density and color are important traits that characterize turf quality.

Sodium (Na+) and chloride (Cl-) are the two key ions responsible for both osmotic and ion-specific damage that significantly reduce growth and yield (Munns and Tester 2003).

Salt tolerant plants have the ability to minimize these detrimental effects with a series of anatomical, morphological, and physiological adaptations, such as an extensive root system and salt secreting glands on the leaf surface (Lipshitz and Waisel 1974; Nöss and Thomson 1982; Marcum and Murdoch 1990a; Marcum et al. 1998).

Some growth indicators have been related to salinity tolerance, such as root length and/or weight in turfgrasses cultivated in liquid medium (Marcum 1999; Alshammary et al. 2004; Marcum 2004). Some authors have found that salinity growth curves for shoot and root can be considered the most important parameter to classify salinity resistance of halophytes and their ecotypes (Lee et al. 2004, 2005); some others have measured gas exchange parameters and chlorophyll content to relate them with the reduction of biomass production (Hameed and Ashraf 2008). Limited information is available to compare salinity responses of shoot and root in solution culture versus soil culture systems.

Glycinebetaine (GB) is an osmoprotector produced naturally by many halophytes (Guy et al. 1984), which is involved in reducing cellular damage under stress conditions (Rajasekaran et al. 1997; Chen and Murata 2008).

Several authors have studied GB as an osmoprotectant in the adaptation to water, salt and cold stress (Guy et al. 1984; Kishitani et al. 1994; Xing and Rajashekar 2001; Girija et al. 2002). Mäkelä et al. (1996) reported that plants are able to use foliar-applied GB and translocate it to almost all plant parts, especially developing organs. Thus, foliar applications increase the levels of GB in plants that are unable to synthesize this compound (Agboma et al. 1997). *P. distans* is a halophyte cool season grass adapted to a wide range of soils and climatic conditions, able to settle also on salty soils (Tarasoff et al. 2007).

The choice of a turf species that responds well to salty water irrigation and of a natural osmoprotector that can be applied to reduce salt stress effects could be a key-point for turfgrass specialists. The aims of this study were: to determine the behavior of a turf of *P. distans* irrigated with saline water and to verify whether GB could be used to reduce effects of NaCl stress, achieving a better turf quality compared to untreated plants.

In a first experimental trial we measured leaf height,
fresh and dry weight, Cl− amount, total osmolyte amount in roots, shoots and leaves of *P. distans* grown in soil filled pots under controlled conditions, irrigated with different NaCl concentrations (85, 275 and 600 mM). In a second experimental trial we tested the effect of foliar application of GB on plants irrigated with 600 mM NaCl.

**MATERIALS AND METHODS**

**Plant material and treatments**

*P. distans* seeds (15 g m⁻²) were sown in 465.5 cm³ pots. The soil mixture used was 45% sand, 45% organic rich soil (C/N=25), 5% peat and 5% organic manure (swine and equine, Jolly Flor Pellet®, C/N=12.5) (pH 6.5). This organic rich soil is typically used in nurseries when optimum growth conditions are required, and with the addition of sand it was adapted for turfgrass. Maintenance fertilization was provided by adding a slow releasing fertilizer (OSMOCOTE plus, 15: 9: 11 w/w N: P₂O₅: K₂O; MgO traces of B, Ca, Cu, Fe, Mn, Mo, S and Zn; Scotts®) to the soil mixture. The growth-chamber was set up at 25 ± 1°C with 12 h photoperiod provided by neon fluorescent lamps (PAR 45.50 μmol m⁻² s⁻¹ at the leaf surface).

The first experimental cycle was carried out to test the response of *P. distans* to different NaCl concentrations. Pots were irrigated with tap water until the 40th day after sowing, when salt treatments were started. For salt treatments, pots were irrigated once a week with 20 ml of NaCl solution, until the 90th day after sowing. In the first experimental cycle, the three NaCl concentrations tested were: 85, 275 and 600 mM. Control pots were irrigated with tap water throughout the experiment.

In the second experimental cycle the effect of foliar application of GB was tested. Sowing and growth conditions were the same as above, and salt treatment was provided with 600 mM NaCl solution. GB treatment was provided by hand-spraying each pot with 10 ml of a 0.1 M GB solution (Agboma et al. 1997), once a week from the 40th to the 90th day after sowing.

Throughout the experiments, water stress was avoided by checking the weight of all pots daily and irrigating each one to field capacity with tap water if necessary. Irrigation with water or saline solution was always done slowly, to avoid percolation and leaching of NaCl from the pots.

**Destructive and non-destructive growth analysis**

All plants were cut once a week, at height of 5 cm from the soil surface, beginning from the 40th day after sowing. To determine growth rate, plant height was measured before each weekly cut; leaf biomass excised from individual pots during each cut was weighed for the determination of clipping fresh weight (FW), dried at 70°C overnight and weighed to determine clipping dry weight (DW).

The leaf relative water content (RWC) of leaf samples excised during the last cut for each pot was measured. The leaf portions were weighed (fresh weight FW), floated on water for two hours to allow turgidity to be regained, weighed again (turgid weight, TW) and dried overnight at 70°C to determine dry weight (DW). The relative water content was calculated as (FW − DW)/(TW − DW).

At the end of the experiment, leaves and shoots were removed and weighed for the determination of total biomass fresh weight. Root length was measured as root extension (in cm) from the stembases to the farthest extending root. Roots were washed with deionized water, blotted dry, weighed to determine fresh weight, and then dried at 70°C for 24 h to determine root dry weight.

General overall condition of plants was monitored and a percentage of leaf firing was attributed at the end of both the experimental cycles. The turf quality and leaf firing on pots were visually estimated with the attribution of a percentage of chlorotic leaves respect the total.

**Sap analysis and chloride content**

A 1 g sample of the biomass taken at the end of the experiment from each pot was used for the extraction of foliar sap. The leaf sample was put in a plastic hypodermic syringe, frozen in liquid nitrogen and after thawing sap was expressed 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. The total osmolality of cell sap (expressed per gram of leaf fresh weight) was measured with a cryoscopic osmometer (Osmomat 30 GONOTEC). The quantity of chloride ion (as meq per gram of leaf fresh weight), was determined by titration on the same sap samples (Vogel 1989).

A sample of 1g of fresh roots per pot was used for the extraction of sap as described above for leaves and the Cl− ion content of the sap was determined by titration. Soil of each pot was dried at 105°C and then used for the determination of Cl− by titration (Vogel 1989).

**Chlorophyll content**

At the end of the second experimental cycle, 0.1 g of leaf biomass for each pot were extracted in 80% acetone (Arnon 1949) and chlorophyll content was determined by spectrophotometric analysis (Beckman Coulter DUS800), absorbance of the supernatant was read at 652 nm. An identical quantity of leaves from each pot was taken and dried overnight at 70°C to determine dry weight that was used to express the total chlorophyll content results.

**SEM**

To check for the presence of salt glands, leaf surface was observed by scanning electron microscopy (Leica Cambridge – LEO 420) on 0.5 cm long leaf segments, dried overnight at 60°C, fixed on aluminum stubs and gold metalized under vacuum.

**Experimental design and data analysis**

Pots were arranged in a complete randomized design, with five replicates per thesis. Mean values and standard deviations were calculated and the significance (P<0.005) of differences between sets of data was tested using one-way ANOVA, followed by Tukey’s test in cases involving significant F-values (Systat software, SYSTAT Inc.).

Correlation between different parameters was carried out applying Pearson correlation (SPSS software, SPSS Inc.).

**RESULTS AND DISCUSSION**

A 90-day experimental cycle was set up to study the effects of different NaCl solutions on *P. distans* in terms of growth and Cl− ion accumulation. Measurements of the amount and distribution of Cl− were chosen because this is an essentially free ion in the circulating solution and is immediately available for the plants (White and Broadley 2001), while soil cation exchange capacity may reduce the uptake of Na⁺. Furthermore there is evidence that in glyphothes such as pepper, the tissue concentration of Na⁺ was lower than that of Cl (Silva et al. 2008) and that monocotyledonous halophytes tend to take up less Na⁺ in the shoot than dicotyledonous halophytes (Testor and Davenport 2003).

Variation in leaf firing, shoot and root weight are highly correlated, and are therefore useful parameters to predict salinity tolerance (Marcum and Murdoch 1990b; Marcum 2006). On the other hand, plant height, leaf relative water content, chlorophyll content and total produced biomass are physiological parameters that can give information on the overall general condition of plants and their metabolic efficiency.

**Destructive and non-destructive growth analysis**

Table 1 shows the plant height reached before each weekly cut and the weight of leaf biomass removed. On the 17th day after irrigation with salty water, corresponding to the second week of treatment, plants watered with 600 mM NaCl solution had a lower growth rate respect the control, while plants treated with 85 mM NaCl solution had a growth rate higher than the controls (P<0.005). This behavior is in
Table 1 Plant height reached before every weekly cut and leaf weight of removed biomass, data are the means of five replicates.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Leave shoots weighta (± SEM)</th>
<th>Plant heightb (± SEM)</th>
<th>Leave shoots weightb (± SEM)</th>
<th>Plant heightb (± SEM)</th>
<th>Leave shoots weightb (± SEM)</th>
<th>Plant heightb (± SEM)</th>
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<tr>
<td></td>
<td>85 mM</td>
<td>275 mM</td>
<td>600 mM</td>
<td></td>
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<tr>
<td>cut I</td>
<td>0.20 ± 0.08 13.4 ± 4</td>
<td>0.22 ± 0.01 14.1 ± 4</td>
<td>0.25 ± 0.06 14.2 ± 4</td>
<td>0.17 ± 0.04 11.1 ± 4</td>
<td></td>
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</tr>
<tr>
<td>II</td>
<td>0.12 ± 0.01 10.3 ± 0.7</td>
<td>0.13 ± 0.02 12.0 ± 1</td>
<td>0.11 ± 0.03 10.6 ± 0.8</td>
<td>0.06 ± 0.01 9.7 ± 0.4</td>
<td></td>
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</tr>
<tr>
<td>III</td>
<td>0.18 ± 0.05 12.4 ± 0.9</td>
<td>0.22 ± 0.04 11.9 ± 0.4</td>
<td>0.19 ± 0.05 12.7 ± 3</td>
<td>0.16 ± 0.03 10.1 ± 3</td>
<td></td>
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</tr>
<tr>
<td>IV</td>
<td>0.08 ± 0.03 9.0 ± 0.5</td>
<td>0.09 ± 0.03 9.0 ± 0.8</td>
<td>0.2 ± 0.4 9.3 ± 0.3</td>
<td>0.2 ± 0.5 9.1 ± 1</td>
<td></td>
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</tr>
<tr>
<td>V</td>
<td>0.11 ± 0.03 9.3 ± 0.3</td>
<td>0.4 ± 0.3 10.1 ± 1</td>
<td>0.17 ± 0.01 11.1 ± 1</td>
<td>0.12 ± 0.02 11.1 ± 1</td>
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</tr>
<tr>
<td>VI</td>
<td>0.100 ± 0.005 10 ± 1</td>
<td>0.3 ± 0.3 10.1 ± 1</td>
<td>0.12 ± 0.02 10.1 ± 1</td>
<td>0.12 ± 0.04 10 ± 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VII</td>
<td>0.05 ± 0.02 7 ± 1</td>
<td>0.07 ± 0.01 8.2 ± 0.6</td>
<td>0.08 ± 0.01 8 ± 1</td>
<td>0.06 ± 0.02 6.8 ± 0.8</td>
<td></td>
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</tbody>
</table>

*a g

b cm

* this value is significant for P<0.005

Fig. 1 Root to shoot fresh weight ratio of the plants at the end of the first experimental cycle. Data are the means of five replicates. Tukey’s paired test of the ratio was not significant.

Fig. 2 Mean weight of the fresh foliar (shoot + leaf) biomass harvested on the 90th day after sowing, at the end of the second experimental cycle, after 6 weeks of weekly treatments with salt and GB depending on the thesis. Different letters indicate significant differences at P<0.005 using Tukey’s test.

Fig. 3 Mean weight of the dry foliar (shoot + leaf) biomass harvested on the 90th day after sowing, at the end of the second experimental cycle after 6 weeks of weekly treatments with salt and GB depending on the thesis. Different letters indicate significant differences at P<0.005 using Tukey’s test.

Plants which maintain under salt stress conditions growth rates similar to those of control plants.

The root to shoot fresh weight ratio calculated at the end of the first cycle showed higher values with increasing NaCl concentrations (Fig. 1), though the differences were not statistically significant. Alshammary et al. (2004) reported a similar behaviour for both Puccinellia distans and Festuca arundinacea: increased rooting may be an adaptive mechanism to overcome the osmotic and nutrient deficiency stresses present under saline conditions by increasing root absorbing surface at the expense of above-ground biomass.

The results of the first experimental cycle showed that P. distans was able to tolerate irrigation with the highest NaCl concentration tested, showing a 30% of leaf firing that could be considered acceptable and interesting for further tests. For this reason a second 90 day experimental cycle was carried out to see if foliar applications of GB could reduce the effects of salt stress resulting from irrigation with 600 mM NaCl.

Fig. 2 shows that, at the end of the second experimental cycle, NaCl-treated plants had the lowest fresh biomass. GB treatment increased leaf and shoot fresh weight both in salt stressed and non-stressed plants. In non stressed plants, GB also increased dry biomass, while this did not occur in salt stressed plants (Fig. 3), where GB application appeared to affect tissue water content. This was also confirmed by the higher relative water content in the NaCl+GB treatment with respect to the salt treatment (Fig. 4). This effect of GB has been reported also in other species such as tobacco (Agboma et al. 1997) and Kidney bean (Lopez et al. 2002).

Treatment with GB of NaCl-irrigated plants, however, did not affect leaf firing, that was again about 30% respect to the control.
Foliar sap analysis and Cl⁻ content

Total osmolarity and Cl⁻ content of leaf sap are shown in Figs. 5 and 6 expressed both on a fresh weight and dry weight basis. The comparison of the total osmolarity with the quantity of chloride ion per gram of leaf fresh weight shows that plants treated with 600 mM NaCl had higher foliar sap osmolarity, but the amount of chloride ion was not significantly different from control plants. These data suggest that treated plants have the capacity to avoid accumulation of the toxic ion Cl⁻ in the leaves, through a mechanism of exclusion either by sequestration in or translocation to the roots (Rajendran et al. 2009). The increase in total osmolarity may counter the osmotic stress caused by irrigation with salty solution, so that salt treated plants may improve water uptake thanks to osmotic adjustment.

Table 2 shows the correlation among all measured parameters: NaCl concentration of the irrigation solution and osmolarity per gram of leaf fresh weight are highly correlated, as the amount of Cl⁻ mol g⁻¹ of dry soil with the total osmolarity per gram of leaf fresh weight (P<0.01) confirming the effect of increasing soil salinity on the osmotic adjustment of the leaves. Root fresh weight is also positively correlated with the total osmolarity per gram of foliar fresh weight (P<0.05).

No correlation was found between Cl⁻ ions in shoot fresh weight and NaCl concentration of the solutions applied to the soil, this could further support the hypothesis that in P. distans in Cl⁻ toxicity is avoided by exclusion from the leaves.

Total chlorophyll content at the end of the second experiment is shown in Fig. 7. In agreement with data reported by Hameed et al. (2008) for Cynodon dactylon, chlorophyll content was significantly lower in salt stressed plants and GB application did not increase chlorophyll content either in salt stressed or control plants.

Pictures with SEM were taken to check if salt glands were present on P. distans leaf surface and if the different treatments had any effect on leaf morphology. Salt glands were not found in any of the treatments, in agreement with Alshammary et al. (2004). Figs. 8 and 9 show the detail of a leaf from a control plant and a NaCl 600 mM+GB plant respectively, parallel veins are evident and no salt excreting structure can be seen in either sample.

CONCLUSIONS

Data obtained showed a very good adaptability of P. distans to different salinity levels; at the end of the first trial there were no significant differences in the growth parameters examined. The Cl⁻ amounts found in foliar sap were not significantly different among the four treatments, probably
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because of the ability of the plant to translocate and/or sequester Cl- in the root cells.

When treated with GB, *P. distans* showed a better overall physiological condition: plants had the capacity to produce more fresh biomass respect to the salt treatment and had a higher leaf relative water content. However, the percentage of leaf firing was not reduced and the chlorophyll content was not increased with respect to the salt stressed plants, so that the physiological benefits were not related to a better general aspect of the turf. Moreover, from a practical point of view, the fact that GB treated plants produced more biomass would be a disadvantage because of the need for more frequent cuts of a turf. GB applications therefore did not seem to give a relevant advantage in reducing technical inputs required by a *P. distans* turf.

*P. distans* shows a good response when irrigated with salty water, but the physiological mechanisms of this resistance still need to be investigated and defined. Ongoing field experiments are being carried out to validate our results and test the effects of GB on this species when subjected to a longer period of salt stress.

ACKNOWLEDGEMENTS

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Table 2

Correlation table of the measured parameters (Pearson’s).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>GB</th>
<th>NaCl+GB</th>
<th>NaCl</th>
</tr>
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<tbody>
<tr>
<td>Average root length</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Mean weight root</td>
<td>.146</td>
<td>1</td>
<td>.925(*)</td>
<td>.956(**)</td>
</tr>
<tr>
<td>Average shoot height</td>
<td>.871</td>
<td>-.033</td>
<td>.892</td>
<td>1</td>
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<tr>
<td>Mean weight cut</td>
<td>.875</td>
<td>.421</td>
<td>.460</td>
<td>.760</td>
</tr>
<tr>
<td>Total salt content per g of LFW</td>
<td>-.219</td>
<td>.925(*)</td>
<td>-.045</td>
<td>.568</td>
</tr>
<tr>
<td>CI moles per g of LFW</td>
<td>.375</td>
<td>.793</td>
<td>.460</td>
<td>.568</td>
</tr>
<tr>
<td>Mean weight shoot</td>
<td>-.246</td>
<td>.628</td>
<td>-.058</td>
<td>.273</td>
</tr>
<tr>
<td>CI moles per g of soil (DW)</td>
<td>-.466</td>
<td>.759</td>
<td>-.668</td>
<td>-.258</td>
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<tr>
<td>mMolarity</td>
<td>-.384</td>
<td>.725</td>
<td>-.685</td>
<td>-.285</td>
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<tr>
<td>Average root length</td>
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<td>CI moles per g of LFW</td>
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* Correlation is significant at the 0.05 level (1-tailed).
** Correlation is significant at the 0.01 level (1-tailed).

Fig. 7 Total chlorophyll content at the end of the second experimental cycle, expressed on a leaf dry weight basis. Different letters indicate significant differences at P<0.005 using Tukey’s test.

Fig. 8 Scanning electron microscopy photograph showing the leaf surface of a control plant.

Fig. 9 Scanning electron microscopy photograph showing the leaf surface of a NaCl + GB-treated plant.
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