## Sprouting and Plant Regeneration Capability in Saline Conditions of Seashore paspalum, Manilagrass and Hybrid Bermudagrass Stolons.

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**Abstract**

Some of the most highly rated warm season turfgrass species [*Cynodon dactylon* x *C. transvaalensis* (Cdxt), *Zoysia matrella* (Zm) and *Paspalum vaginatum* (Pv)] can only be established via vegetative propagation (sodding, sprigging, hydrostolonizing, transplant of single plants) as no seed is commercially available (or genetically possible). These species are often used for creating high-end turf surfaces in golf courses, sports grounds, parks and gardens, in coastal areas affected by salt or in non-coastal areas where irrigation with low-quality water is carried out. Much is known on their adaptation to saline environment as a mature sward, and on similar species’ (*Cynodon dactylon* and *Zoysia japonica*) seed germination in saline conditions, but precious little data is available on the plant regeneration capabilities of their vegetative propagation organs (stolons and rhizomes) under saline conditions. A greenhouse trial was setup to evaluate stolon sprouting inhibition, aerial apex growth inhibition, root apex growth inhibition and root tip elongation inhibition of three varieties of the three above mentioned species in progressively increasing saline solutions (0%, 1%, 2%, 3%, 4%). All species performed very well at 0% salinity, while virtually no stolon sprouting was witnessed at the higher salinity level (4%). As salinity increases, Zm is the first to lose sprouting capability, followed by Cdxt, while Pv continues to show sprouting power up to 3% salinity. In accordance with its origin from waterlogged and coastal areas, Pv showed a clear adaptation to 2% salt concentrations (polyhaline brackish water), with higher vegetative growth, root growth and root tip elongation, compared to 1% and 3% salinity. As such Pv emerges as the clear choice for the vegetative establishment of turfgrass in saline conditions.

**Key words:** *Paspalum vaginatum* Swartz., *Zoysia matrella* (L.) Merr., *Cynodon dactylon* x *C. transvaalensis* Burtt.-Davey, roots, salinity, turfgrass.

**Introduction**

In temperate climate areas (i.e. Mediterranean climate, Humid subtropical climate, Oceanic climate), the increasing budgetary and environmental concerns are starting to dictate a shift towards the adoption of the more environmentally-friendly and aesthetically pleasing C4 species for the establishment of new turfgrass areas or for the conversion of existing C3 turfgrass areas to C4 species (Peel et al., 2007). Among the many environmental strong points of C4 turfgrass species, there is the capability of adapting to soil and/or irrigation water salinity. Salt affected soils impact nearly 10% of the land surface and 50% of all irrigated land in the world (Carrow and Duncan, 1998). Naturally, the distribution of these salt-affected sites is also reflected in a multitude of turfgrass sites for recreation (golf, football, horse racing, parks) and ornamental purposes (public and private gardens).

Salinity stresses are major factors limiting plant growth and productivity, with detrimental effects on plant growth resulting from direct effects of ion toxicity (Hasegawa et al., 1986) or indirect effects of saline ions on the soil water potential. Avoidance of toxicity may involve ion exclusion at the root cortex (Jeschke, 1984), redistribution of excess ions to senescing leaves or other plant parts (Yeo and Flowers, 1984) and, in some halophytic plants, secretion or sequestration of ions into salt glands or bladders (Marcum and Murdoch, 1990).

Salinity tolerance differs among crop species with growth of most crops adversely affected when electrical conductivity of soil (saturated paste extract, ECe) is 1 to 3 dS m-1, while moderately salt tolerant crops may continue to grow up to 8 dS m-1 (Maas, 1987). Only halophytes, however, can survive at higher salinity >30 dS m-1 (Flowers et al., 1977; Flowers and Yeo, 1986; Maas, 1987). As such, halophytes are defined as salt-tolerant, salt-loving, or saltwater plants that are genetically morphologically, and physiologically well-adapting in high salinity conditions, where most of domesticated crops (salt-sensitive glycophytes) rarely survive (Glenn et al., 1999; Khan and Duke, 2001). Halophytes generally accumulate Na+ and Cl- ions in vacuoles and synthesize organic osmolytes in the cytoplasm, which facilitates water uptake into the plant and turgor-driven enhancement at low to moderate salinity levels (Flowers et al., 1977; Flowers and Yeo, 1986; Bell and O’Leary, 2003).

Up to date many results have demonstrated that salinity stress produces different morphological changes in all the organs of the plant, especially in roots. Root system can exhibit high plasticity in response to local environmental conditions. This plasticity is critical for a plant’s ability to find nutrients and water in the soil and enhances the fitness of plants (Grime et al., 1986; Lynch, 1995, Sultan, 2000; Rewald et al., 2012). Root system modifications under salinity are a trade-off between the capacity to exclude excess ions and sustained water or nutrient uptake in response to different environmental parameters such as water and nutrient availability (Sorgonà et al., 2007; Wang et al., 2009; Gruber et al., 2011) or excess ions (Deak and Malamy, 2005; Zolla et al., 2010; Rewald et al., 2011a, b). Most of these modifications have been studied in several systems and are referred to be changes in root order frequency, morphology and anatomy: increased root:shoot ratios (Zekri and Parsons, 1989); reduced root branching (Pregitzer et al., 2002; Valenzuela-Estrada et al., 2008); modified axial root conductivity (Rewald et al., 2011b); a well-developed Casparian strip closer to the root apex (Walker et al., 1984; Lux et al., 2011); a shorter distance from the root tip to the closest protoxylem tracheary element (Reinhardt and Rost, 1995; Lux et al., 2011; Stoláriková et al. 2012; Vaculik et al., 2012).

Osmotic adjustment associated with saline ion accumulation has been noted to occur under saline conditions in a number of C4 turfgrasses (Marcum and Murdoch, 1990; Peacock and Dudeck., 1985). Seashore paspalum (*Paspalum vaginatum* Swartz), a halophytic warm season grass, has gained interest for use on saline turfgrass sites, drainage water reuse schemes, and land reclamation under saline conditions (Duncan and Carrow, 2000; Semple et al., 2003; Grattan et al., 2004; Rogers et al., 2005). Ecotypes of seashore paspalum grow on coastal sites subjected to seawater (34,486 mg L-1 total soluble salts or electrical conductivity of ECw of 54 dS m-1) (Duncan 1996, 1999). Osmotic adjustment through inorganic ion uptake or synthesis of organic compounds has been postulated to have a significant role in salt tolerance in seashore paspalum by Marcum and Murdoch (1994).

Salinity tolerance in other high quality C4 turfgrasses, *Cynodon dactylon* x *transvaalensis* and *Zoysia matrella*, is largely due to ion exclusion via sequestration into leaf salt glands (Marcum and Murdoch, 1994). Indeed in these species salinity tolerance is negatively correlated with leaf tissue Na+ concentration and positively correlated with leaf salt gland density (Marcum and Murdoch, 1990; Marcum and Pessarakli, 2006).

Traditionally salinity tolerance assessments on C4 turfgrass species, were based mainly on shoot growth parameters (Ackerson and Youngner, 1975; Maas, 1987; Hoffman et al., 1977, Lee et al., 2004), and on seed germination and seedling emergence (Serena et al. 2012; Johnson et al. 2007; Raymer et al., 2007). However, given the lack of commercially available seed of most high quality C4 turfgrass cultivars, be it due to their interspecific and hence sterile nature (i.e. *Cynodon dactylon* x *C. transvaalensis* ‘Tifway 419’), or due to the difficulty of carrying out commercially viable seed production (i.e. *Paspalum vaginatum* ‘Salam’, *Zoysia matrella* ‘Zeon’), the establishment of these species is usually carried out by stolonizing (Fagerness et al., 2002; Johnson and Duncan, 1998; Richardson and Boyd, 2001), plugging (Ruemmele et al., 1993) or by the transplant of single potted plants (Volterrani et al., 2008).

##### The objectives in this study were to assess the potential for germination and growth in an increasingly saline environment of stolons of *Cynodon dactylon* x *C. transvaalensis* (cv Tifway), *Paspalum vaginatum* (cv Salam) and *Zoysia matrella* (cv Zeon) in a greenhouse trial, and subsequently determine their plant regeneration capabilities and root tip architecture and elongation as affected by salinity.

**Materials and Methods**

##### The stolon germination trial was carried out between June 07 and July 06, 2010 at the Department of Agriculture, Food and Environment (DAFE) of the University of Pisa (43° 40’ N, 10° 19’ E; 6 m a.s.l.) in a greenhouse with controlled environment (max T° 35±4 °C; min T° 25±3 °C; 80±5 % relative humidity). Treatments consisted of three vegetatively-propagated turfgrass species [*Cynodon dactylon* x C. *transvaalensis* (cv Tifway = Cdxt), *Paspalum vaginatum* (cv Salam = Pv), *Zoysia matrella* (cv Zeon = Zm)] assessed for stolon germination in 6 ? 5 levels of saline (0%, 1%, 2%, 3%, 4%) environment. Hence the treatments studied were: Cdxt 0% (control), Cdxt 1%, Cdxt 2%, Cdxt 3%, Cdxt 4%, Pv 0% (control), Pv 1%, Pv 2%, Pv 3%, Pv 4%, Zm 0% (control), Zm 1%, Zm 2%, Zm 3%, Zm 4%. All treatments were carried out in 3 replicates for a total of 45 experimental units arranged in a completely randomized block design.

*Preparation of donor plants*

In September 07, 2009 three 50 cm2 plugs per species were harvested from mature swards of Cdxt, Pv and Zm grown on a silt loam soil at the research station of the Centre for Research on Turfgrass for Environment and Sports - University of Pisa. Plugs were washed clean from native soil and transferred to 5000 ml pots containing peat (Brill Type 3 Special: 50% white peat – 50% black peat; pH: 5.5-6.0; salt content: 0.7-1.2 g L-1; N: 100-160 mg L1; P2O5: 120-200 mg L-1; K2O: 140-240 mg L-1). The pots were arranged in a completely randomized block with three replications in a greenhouse environment and allowed to grow as stolon donor plants (plants suspended 1.5 m above ground) for 40 weeks. After initial rooting in the pots, only one mowing at 1 cm height was carried during the trial (November 01-10, 2009) in order to uniform vegetation. During the 40 weeks of greenhouse growing the identical management practices for all species were: daily irrigation with 5 mm of water; six fertilizations (September 07, October 12, November 09, February 08) each with 5 g m-2 of N, 1 g m-2 of P2O5 and 2 g m-2 of K2O; no fungicide or weed control application; no trimming of resulting stolons.

*Cutting explant*

In order to minimize effects deriving from stolon tissue age, single-node phytomers (cuttings) were prepared by selecting stolon nodes from donor plants after the 4th node (numbered from stolon apex) (Roche and Loch, 2005), and by cutting them leaving 1 cm of internode tissue fore and aft of the node. For each species 330 stolon cuttings were prepared, for a trial total of 990 cuttings.

*Saline solutions*

Saline solutions were prepared by gradually adding table marine salt to tap water until the required salinity level was attained (0%, 1%, 2%, 3%, 4%). During the preparation of saline solutions ECw was monitored using a Orion 160 conductivity meter (Orion, Boston, MA), which was also used to monitor salinity levels in the experimental units during the greenhouse trial. The 5 salinity levels generated the following electric conductivity levels (dS m-1): 0%=1, 1%=16, 2%=39, 3%=53, 4%=70). Tap water was used to correct EC throughout the trial as influenced by evapotranspiration.

*Cutting transplant, substrate and float system*

The experimental units were 9.7 L polyethylene vases (20 x 44 x 11 cm), each filled with 4 L of saline solution, in which polystyrene slabs containing 22 alveoli of 10 ml were left to float. The alveoli were filled with 10 ml of silica sand (0-2 mm Ø particle size, pH 6.9) as a growing medium. The single alveoli had a drainage hole at the bottom which was plugged with cotton cloth to allow water entrance and propagation through the substrate by capillarity. A single cutting was then inserted into each alveoli, with the node fully inserted in the silica sand.

Cutting sprouting and biometric assessments

The following assessments were carried out daily during the first week after transplant (1 WAT), three times during 2 WAT, two times during 3 WAT and once during 4 WAT:

* Sprouting percentage: by manual count of sprouted cuttings.
* Maximum aerial apex height / maximum root apex length: a cutting per experimental unit was sampled and placed on millimetric paper, and the maximum height of vegetation and depth of roots was measured.
* At 4 WAT, a cutting per replica was sampled and preserved in a FAA (10% formaldehyde, 5% acetic acid, 45% ethanol) fixing agent, in order to prepare it for laboratory assays on root tip elongation.

*Light Microscopy*

Cdxt, Zm and Pv isolated roots were fixed in FAA, dehydrated through a graded ethanol series (25, 50, 75, 95%, 5 hours each step), incubated overnight in 100% ethanol, infiltrated and embedded in LR White resin (Sigma). The material was polymerized at 60 °C for 24 h. Three μm seriated sections were cut with a LKB Ultratome Nova and stained with 0.05% toluidine blue O (TBO, BDH Chemicals) in 0.1 M acetate buffer, pH 4.4 (Feder and O'Brien 1968) to obtain the staining of the different tissues.

*Statistical analysis*

All data was subject to one-way analysis of variance (ANOVA). Fisher’s Protected LSD for P≥0.05 was used to detect differences between means. Aerial apex measurements and root apex measurements (mm) during the 8 weeks of trial were used to calculate **a)** the cumulative growth for each treatment (by summing the growths measured at each assessment date) and then **b)** the growth inhibition (as a percentage of the growth obtained by the 0% salinity controls) induced by the salinity level. Stolon sprouting count (n) at 4 WAT was used to calculate sprouting inhibition (as a percentage of the sprouting obtained by the 0% salinity controls) induced by the salinity level. Root tip elongation (mm) at 4 WAT was used to calculate root tip elongation inhibition (as a percentage of the root tip elongation obtained by the 0% salinity controls) induced by the salinity level. All percentage data was log-transformed before carrying out ANOVA.

Correlations via Pearson’s Product Moment Correlation Coefficient (r) were carried out between EC levels (dS m-1) and phytomer (stolon) sprouting inhibition, aerial apex growth inhibition, root apex growth inhibition and root tip elongation inhibition. For all statistical analysis the COSTAT 6.400 program (Costat 2008) was used.

**Results**

*Stolon sprouting*

Stolon sprouting inhibition (Fig. 1) at 4% salinity level as percentage of the control was next to 100% at 4 WAT. Zm is consistently the worst sprouting species at all salinity levels, and its sprouting is virtually inhibited at salinity levels above 1%. At lower salinity level (1%), Cdxt showed lower sprouting inhibition, the increase of salt concentration decreases its sprouting at 2% salinity level with inhibition percentages statistically not different from Pv, but at 3% salinity level the sprouting inhibition is high and not different from 4% salinity. In Pv sprouting inhibition is equal for the first three salinity levels.

*Vegetative growth*

At 4 WAT vegetative growth (Fig. 2) is strongly influenced by salinity levels. While Zm emerges as the most salinity-sensitive species, with a sudden inhibition in aerial apex growth from the lowest salinity level (1%), the response of Pv and Cdxt is more gradual and very similar between these species, with appreciable vegetative growth until the 3% salinity level.

*Root growth*

Root apex growth inhibition results (Figure 3) confirm the findings on vegetative growth with, apart from the slow-growing and salt-sensitive Zm, similar results obtained by Cdxt and Pv, although the inhibition in root growth between 1% and 2-3% salinity is less abrupt for Pv. This is probably the salinity level at which the halophytic behaviour of Pv begins to show in root growth, and indeed this is in accordance with the origin of this species that appears to have differentiated from swampy coastal areas (Duncan and Carrow, 2000) rich in brackish water.

*Root tip elongation*

The distance of the area of root tracheid differentiation from the root tip was considered a histological measure of salinity response. At 4 WAT the inhibition of tracheid differentiation from root tip (Fig. 4) increased with salinity increase for Cdxt and Zm, but Pv exhibited a different behavior, with even an increase in root tip elongation at salinity levels of 2%, as root tip elongation inhibition at 1% salinity was not statistically different from 3%.

Another aspect that emerged from root tip sections was the gradual response to salinity by Cdxt, as opposed to the abrupt tracheid differentiation inhibition arrest of Zm already from 1% salinity level. Only Zm and Cdxt showed statistically significant correlation between saline solution electric conductivity and root tip elongation inhibition.

*Correlations*

The correlations in the range of 0-3% salinity levels (Table 1) between electric conductivity (dS m-1) and 4 WAT cumulative percent stolon sprouting inhibition (%), aerial apex cumulative growth inhibition (%), cumulated root apex growth inhibition (%) and root tip elongation inhibition (%) showed strict correlations in Cdxt and Zm for all parameters, thus indicating consistent sensitivity to saline environment. Conversely, Pv showed a strong correlation between salinity levels and stolon sprouting inhibition (r=0,76) and aerial apex growth inhibition (r=0.80) and a weaker correlation with root apex growth inhibition (r=0,58), while no correlation was found for root tip elongation inhibition.

**Discussion**

Stolon sprouting, aerial apex growth and root apex growth inhibition at 4% salinity level confirm the 3% salinity level as the maximum operating level for vegetative establishment for the three species, beyond which point neither tolerance or avoidance mechanisms (Duncan and Carrow, 1999; Marcum and Murdoch, 1990) can allow plant regeneration.

Zm is the species with the worst performance at all salinity levels, indeed its sprouting is virtually inhibited at salinity levels above 1%, while aerial apex growth and root apex growth are inhibited since the lower salinity concentration.

The progressive increase of sprouting inhibition in Cdxt and its sprouting similar to Pv at 2% and the gradual and very similar to Pv response of this species to salinity level for aerial apex growth, could be explained by the high starch levels found in a study (Lulli et al., 2011) on the stolons of this species, high starch levels in Cdxt could explain this behaviour that is similar to that of a halophytic plants.These responses of bermudagrass even for the root apex growth, are in agreement with earlier studies (Marcum and Murdoch, 1990, 1994; Marcum, 1999) where bermudagrass cultivars responded to increasing salinity levels in a manner intermediate between that of glycophytes and halophytes.

Pv exhibited a different behavior, with even an increase in root tip elongation at salinity levels of 2%, as root tip elongation inhibition at 1% salinity was not statistically different from 3%.

Results show that the root anatomy is altered when plants are exposed to high NaCl concentrations. In particular, we observed changes in the size of the roots and all the primary root tissues developed closer to the root apex when compared with the control roots. There are many data showing that morphological and anatomical traits influence the uptake processes (Dunabin et al., 2004; Trubat et al., 2006; Rieger and Litvin, 1999; Rewald et al., 2012) and that the acceleration of primary tissues development might be related with the strategy of the plant root to reduce the uptake and the translocation of high amount of ions (Martinka and Lux 2004; Enstone et al. 2002; Reinhardt and Rost 1995; Karahara et al., 2004; Krishnamurthy et al., 2009, Vaculík et al., 2012b). These observations are in agreement with our results in which the earlier root modification observed could confer a higher tolerance to NaCl stress.

The weaker correlations between salinity levels and root apex growth inhibition and the absence of correlation for root tip elongation inhibition in Pv, confirmed a high degree of salinity tolerance and seemed to have adaptation mechanisms for superior salinity tolerance (Lee et al 2004).

**Conclusions**

The results of this trial prompt the following conclusions: a) *Zoysia matrella* is the most salt-sensitive of the three evaluated C4 turfgrass species, with little scope for technical vegetative propagation in situations above 1% salinity; b) *Paspalum vaginatum* is the clear choice for vegetative propagation in saline conditions, with even a pronounced sprouting stimulation at around 2% salinity, which seems to be the species’ preferred salinity level; c) hybrid bermudagrass (*Cynodon dactylon* x *C. transvaalensis*), despite not being a true halophyte, does show a remarkable adaptation up to 2% salinity levels and is hence very well suited to vegetative propagation in mildly-saline conditions; d) C4 turfgrass stolon sprouting dynamics in saline environment do differ markedly from seed germination dynamics, and they deserve to be the object of further studies in order to clarify the potential for turfgrass vegetative establishment in salt-affected soils.

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**Table 1.** Cdxt, Zm, Pv: Pearson Product Moment Correlation coefficient (r, n=12) between saline solution electric conductivity (dS cm-1) and the following data at 4 WAT (0-3% salinity): stolon sprouting inhibition, cumulative aerial apex growth inhibition, cumulative root apex growth inhibition, root tip elongation inhibition (% of 0% salinity control for each species). \*, \*\*, \*\*\* statistically significant differences respectively for P≤0.05, P≤0.01, P≤0.001; ns = not significant.

|  |  |  |  |
| --- | --- | --- | --- |
| **Species** | **Parameter** | **(r)** | **P** |
| **Cdxt** | Stolon sprouting | 0.97 | \*\*\* |
| Aerial apex growth | 0.81 | \*\* |
| Root apex growth | 0.88 | \*\*\* |
| Root tip elongation | 0.98 | \*\*\* |
| **Zm** | Stolon sprouting | 0.93 | \*\*\* |
| Aerial apex growth | 0.78 | \*\* |
| Root apex growth | 0.77 | \*\* |
| Root tip elongation | 0.88 | \*\*\* |
| **Pv** | Stolon sprouting | 0.76 | \*\* |
| Aerial apex growth | 0.80 | \*\* |
| Root apex growth | 0.58 | \* |
| Root tip elongation | - | ns |

**Figure. 1.** (*Cynodon dactylon* x *C. transvaalensis* (Cdxt), *Zoysia matrella* (Zm) and *Paspalum vaginatum* (Pv) stolon sprouting inhibition [% of control (0% salinity) for each species] at increasing salinity levels (4 WAT). Data columns followed by the same letters do not differ significantly at P≤0.05 (one-way ANOVA, with Fisher’s Protected LSD, P≤0.005).

**Figure. 2.** Cdxt, Zm and Pv cumulative aerial apex growth inhibition [% of control (0% salinity) for each species] at increasing salinity levels (4 WAT). Data columns followed by the same letters do not differ significantly at P≤0.05 (one-way ANOVA, with Fisher’s Protected LSD, P≤0.005).

**Figure. 3.** Cdxt, Zm and Pv cumulative root apex growth inhibition [% of control (0% salinity) for each species] at increasing salinity levels (4 WAT). Data columns followed by the same letters do not differ significantly at P≤0.05 (one-way ANOVA, with Fisher’s Protected LSD, P≤0.005).

**Figure. 4.** Cdxt, Zm and Pv root tip elongation inhibition [% of control (0% salinity) for each species] at increasing salinity levels (4 WAT). Data columns followed by the same letters do not differ significantly at P≤0.05 (one-way ANOVA, with Fisher’s Protected LSD, P≤0.005).