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NaCl tolerance in maize *(Zea mays* ssp. *mays)* x *Tripsacum dactyloides* L. hybrid *calli* and regenerated plants

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Abstract

Since corn has been classified as one of the most sensitive crops to soil salinity, the evaluation of NaCl tolerance of a hybrid of *Zea mays* ssp. *mays* (2n=40) and *Tripsacum dactyloides* (2n=72), was considered of interest to determine the possibility of using this germplasm in corn tolerance improvement. Long-term organogenic calli, obtained from immature hybrid embryos, have shown high rates of multiplication and plant regeneration. After *in vitro* treatment of these calli with different levels of salinity, the survival and regeneration percentages and the daily relative weight increments were calculated. Regenerated plants were transplanted to pots and watered with NaCl solution (6.1 dS m^{-1}) in order to assess tolerance at a whole plant level. Measurements of height, number of leaves per plant, and fresh and dry weights were taken. Exposure to 170 mM NaCl *in vitro* during 35 days and *in vivo* during 20 days produced a fresh weight decrease of 51% in calli and 31% in plants, respectively. These results indicate an improved tolerance of the maize/*Tripsacum* hybrid to salinity stress, *in vitro* and *in vivo*, compared with results from previous reports using other corn genotypes under similar conditions.

Key words: intergeneric hybrid, salinity stress, organogenic callus, corn, gamagrass.

Resumen

Tolerancia a NaCl en callos y plantas regeneradas de un híbrido de maíz *(Zea mays* ssp. *mays)* x *Tripsacum dactyloides* L.

Dado que el maíz ha sido clasificado como uno de los cultivos más sensibles a la salinidad en el suelo, se consideró interesante evaluar la respuesta a la salinidad causada por NaCl *in vitro* e *in vivo* de un híbrido entre un maíz tetraploide (*Zea mays* ssp. *mays*, 2n=40) y *Tripsacum dactyloides* (2n=72), con el fin de determinar la utilidad de este material como base de partida para mejorar la tolerancia del maíz. A partir del rescate de embriones inmaduros se obtuvieron callos organogénicos que mantuvieron una alta tasa de multiplicación y regeneración de plantas por largos períodos. Dichos callos se sometieron a distintos niveles de salinidad, y se calcularon los porcentajes de supervivencia y regeneración, así como el incremento en peso fresco relativo diario. A partir de callos organogénicos del mismo híbrido, se obtuvieron plantas que se transplantaron a macetas y se regaron con solución salina (6,1 dS m^{-1}), a fin de evaluar la tolerancia *in vivo.* Se midieron las variables altura de planta, número de hojas por planta, peso fresco y peso seco. La exposición a 170 mM de NaCl durante 35 días *in vitro* y 20 días *in vivo*, produjo una disminución del peso fresco del 51% en callos y del 31% en plantas, respectivamente. Los resultados obtenidos muestran una mayor tolerancia del híbrido, tanto *in vitro* como *in vivo*, respecto a distintos genotipos de maíz cultivados en condiciones similares.

Palabras clave: híbrido intergenérico, estrés salino, callo organogénico, maíz.

Introduction

High salt concentrations in soils negatively affect corn growth and, consequently, produce a large drop in yield (Ashraf and McNeilly, 1989; Pasternak *et al.*, 1995). Although Maas and Hoffman (1977) classified corn among the crops most sensitive to soil salinity, in related reports a wide phenotypic variation was observed in response to this stress (Ashraf and McNeilly, 1989; Epstein, 1985).

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The effects of saline stress on corn have been well studied. Firstly, it has been shown to affect water relations (Cramer, 1992), which generates plant osmotic stress (Cramer *et al.*, 1994). Recently, it has been found that the presence of salt not only reduces the available water in the soil but also affects the permeability of root cortex, by altering cell membrane water channels (Hasegawa *et al*., 2000).

Other corn plant physiological parameters affected by soil salinity include cell wall rheological properties (Cramer, 1992; Pimentel, 1999), protein synthesis (Ramagopal, 1986; Yang *et al,* 1995; Saneoka *et al*, 1995), caryopsis soluble carbohydrate concentration (Pasternak *et al*, 1995), leaf bioelectrical activity (Shabala *et al*, 1998), and ionic balance (Hassan *et al.*, 1970; Ashraf and McNeilly, 1989; Shabala *et al.*, 1998). However, there was no consistent correlation between any of these parameters and salinity tolerance, measured as vegetative growth or yield. Therefore, the change in plant growth or yield compared with a control is the most reliable indicator of the tolerance to saline stress (Cramer *et al.*, 1994).

Hybridization of two phylogenetically distant species offers a great potential to increase the genetic variability, whether by the introgression of desirable characters in the cultivated species (introduction of simple genes or the addition/substitution/translocation of chromosomes or chromosome segments), or by the generation of new allopolyploids with one or more genomes and several useful characteristics of both parental species. The *Maydeae* tribe includes the genera *Zea*, *Tripsacum* (in America) and *Coix* (in India). *Tripsacum* has interesting characteristics to be used in crossings to generate genetic variability in corn. It is a complex genus that includes 16 species (Li *et al.*, 1999; Kindiger and Dewald, 1997) all of which are perennial but with different morphology and adapted to different environmental conditions (Wilkes, 1979). Although *Zea* and *Tripsacum* are reproductively isolated by gametophytic and sporophytic barriers, fecundation occurs cutting corn stigmas before hand pollination (Mangelsdorf and Reeves, 1931). Hybrid plants can be obtained by immature embryo rescue (James, 1979; Bernard and Jewell, 1985) and induction of somatic embryogenesis (Furini and Jewell, 1995; García and Molina, 1997).

Long-term organogenic calli were induced from immature embryos obtained from crosses between tetraploid maize and *T. dactyloides* (García and Molina, 1997). Several authors have observed that chromosomic stability *in vitro* is affected by factors such as the explant, the genotype and, mainly, the culture age (McCoy and Phillips, 1982; Molina and García, 1998). Therefore, long-term organic calli could be useful to induce chromosomic alterations that increase the exchange frequency between the chromosomes of the parental species. Moreover, induction of saline stress in long-term organogenic callus cultures and, especially during the *in vitro* regeneration process, is a convenient way of selecting tolerant genotypes (Johnson *et al.*, 1991). However, little is known about the response of maize x *Tripsacum* hybrids to saline stress, which is essential to implement an improvement programme.

The selection of tolerant genotypes *in vitro* opens up the possibility of accelerating the improvement process, but there are some restrictions such as the difficulty of regenerating plants from the selected material (McCoy, 1987a; Lutts *et al.*, 1999), and the occasional lack of correlation between tolerance *in vitro* and the response of the whole plant *in vivo* (McCoy, 1987b). Although the comparative effect of NaCl and mannitol on protein synthesis has been studied in corn (Ramagopal, 1986), there are no published data of the effects of saline stress on plant regeneration *in vitro,* or on the correlation between *in vitro* and *in vivo* tolerance, factors that would be essential to assess new genetic combinations obtained from embryo rescue.

The objectives of this work were: i) to determine the *in vitro* effects of NaCl stress on fresh weight increase, regeneration capacity and survival of organogenic calli of the hybrid *Zea mays* ssp. m*ays* (2n=40) x *Tripsacum dactyloides* L. (2n=72), and ii) to evaluate the growth of the hybrid regenerated plants exposed to 170 mM NaCl, a saline concentration considered to be harmful to cultivated maize.

Material and methods

Maize x *Tripsacum* (N107BxT) hybrid callus and plants were obtained as follow: the tetraploid maize line N107B (2n=40, supplied by the Corn Genetic Coop. Stock Center, Missouri, U.S.) was hand pollinated with *Tripsacum dactyloides* (2n=72), and 12 days old immature embryos were isolated and cultured in presence of 4 μM 2,4-dichlorophenoxyacetic acid (2,4-D) (García and Molina, 1997).

Maize x *Tripsacum* organogenic calli were grown *in vitro* for three years by monthly subcultures to the basic medium of García *et al.* (1992) supplemented

with 4 μM 2,4-D. On average, 11 calli of approximately 200 mg each, were exposed to each salinity concentration (0, 70, 140, 170, 210 and 250 mM NaCl). The experiment was repeated four times. The initial weight (Wi) of each callus was recorded just before the beginning of treatments and the final weight (Wf) was recorded after 35 days of growth at 30ºC, with 13 h of photoperiod. The daily increase in relative weight (RWI) was calculated using the following formula: $RWI = (Wf-Wi)\ Wi^{-1}\ d^{-1}$. The survival and regeneration percentages were calculated by considering green calli as live ones and calli with regeneration capacity as those with at least one shoot.

Regenerated shoots were subcultured to a basic medium without plant growth regulators to induce rooting. After 45 days, the plantlets were transplanted into 200 ml pots with a mixture of equal parts of earthworm compost and sterile soil and covered with plastic bags. In the first watering, Benomyl (1 g L^{-1}) was used as a systemic fungicide. When the plants reached an average height of 30 cm they were transplanted to 1700 ml pots with soil and irrigated with tap water for seven days. On the eighth day, 50 plants were randomly selected, of which 25 were watered for 20 days with saline solution (170 mM NaCl; 6.1 dS m^{-1}) and the rest with tap water (control; 1.5 dS m^{-1}). Both groups were watered every two days with 100 ml per pot. The experiment was done in a greenhouse. Three, eight, fifteen and twenty days after the first watering, four plants were randomly selected for evaluation, the height was measured, the number of leaves per plant was counted and the shoot was cut to determine their fresh weight (FW) and dry weight (DW) after heating at 70ºC for three days.

Statistical analysis

The results were analysed with the statistical software Statistix for Windows, Copyright 1985. More specifically, variance analysis, Tukey's Least Significant Differences (LSD), determination coefficient (linear regression) and F test for comparison of means were applied.

Results

Survival and regeneration of the hybrid N107BxT organogenic calli declined as the medium saline concentration increased (R^2=0.99 and R^2=0.96, respectively). However, an increase of 50% in NaCl concentration (from 70 mM to 140 mM) only produced a drop of 15% in plant regeneration (Table 1).

Callus relative daily weight increment (RWI d^{-1}) was strongly affected by the saline concentration (R^2=0.95). Tukey's LSD (5%) separated treatments into four homogenous groups, with no significant differences between the means within the groups (Table 1).

At the whole plant level, the increase in fresh and dry weight per plant declined, compared to control plants (0 mM NaCl), when watered with a 170 mM NaCl solution (Figure 1). Assuming a normal distribution with a significance level of 0.05, the significant differences between fresh weights, of plants watered with NaCl and control plants, appeared 15 days after the first watering (Figure 1A). However, there were no significant differences in dry weights (α: 0.05) between treated and control plants, during the 20 days of the experiment (Figure 1B).

Plant water content evolution clearly decreased in NaCl-treated plants compared to control ones (Table 2), which explains why significant differences were detected in the increases in plant fresh weight and not in dry weight.

Discussion

Organogenic calli of the maize/*Tripsacum* hybrid studied in this work, exhibited a 51% fresh weight re-

Table 1. Regeneration and survival percentages, and relative daily weight increase of hybrid N107BxT calli exposed to different NaCl concentrations

	NaCl concentration (mM)					
	0	**70**	**140**	**170**	**210**	**250**
Regeneration (%)	61.5 a	40 b	30 c	—	0 d	—
Survival (%)	92.3 a	60 b	40 c	—	8.3 d	—
RWI/day (g g^{-1} d^{-1})	0.123 a	0.087 b	0.071 bc	0.057 cd	0.059 bcd	0.033 d

Different letters in a row indicate significant differences between the means (Tuckey, α: 0.05).

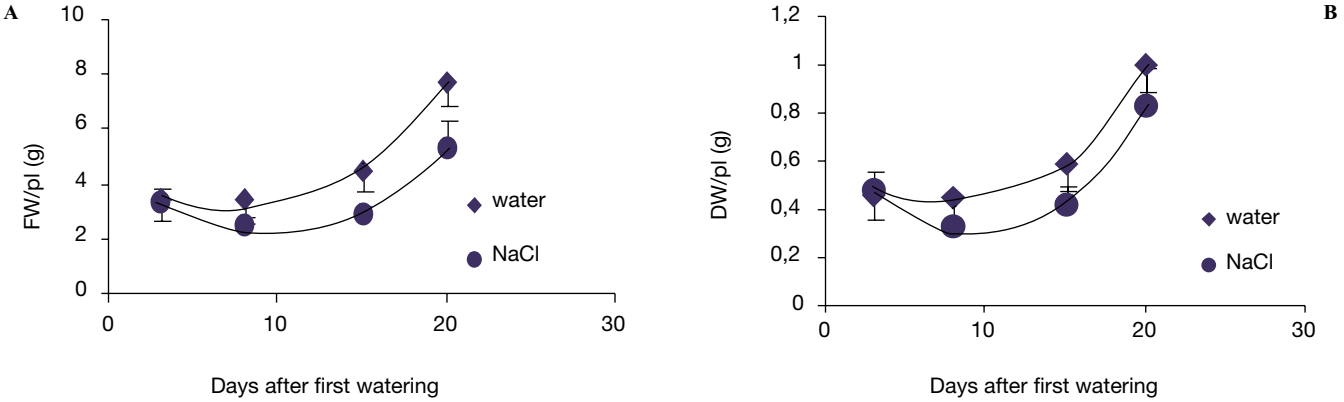

Figure 1. Fresh weigth (A) and dry weigth (B) per plant of the hybrid N107BxT, watered with tap water and 170 mM NaCl. The vertical bars represent the standar error.

duction relative to the control when cultured in 170 mM NaCl during one month; on the other hand, Black Mexican Sweet corn, grown with 100 mM NaCl over the same period, showed a reduced growth relative to the control of 81.54% (Ramagopal, 1986), which indicates a markedly greater tolerance of the hybrid *in vitro*.

The medium NaCl concentration is a key factor to select salinity tolerance *in vitro*; for that reason, the best relationship between a maximum selection pressure and the regeneration rate of plants must be determined. Although there are no reports about the effects of NaCl on plant regeneration from maize callus, in rice, for example, a 100 mM NaCl concentration completely inhibited this process (Lutts *et al.*, 1999). However, 30% of N107BxT calli maintained their regeneration capacity even in presence of 140 mM NaCl (Table 1). Regenerated plants were normal and phenotypically homogeneous, in contrast to the results of McCoy (1987a) who obtained abnormal plants with 45% dwarfism, from *Medicago sativa* cell lines exposed to NaCl.

Ashraf and McNeilly (1989) compared the response of nine corn cultivars watered with 180 mM NaCl for 30 days, and they observed that only five cultivars kept growing, with a maximum of 18%, of both fresh and dry final weight compared to the controls, in Sultan cultivar. Furthermore, the relative fresh and dry weights of Sultan plants (about 45% of control plants) in presence of 120 mM NaCl were still lower than those of the N107BxT hybrid cultured with 170 mM NaCl (69% and 84% of the control plants, respectively).

Cramer (1992) studied the responses of two corn cultivars with a different capacity to exclude sodium, in an attempt to determine which mechanisms were involved in the inhibition of growth by salinity, and concluded that the main cause of reduced corn growth in the presence of NaCl was osmotic stress. The results obtained for the hybrid in this work indicate that salinity also negatively affects water absorption. However, within the NaCl concentrations studied, the hybrid was able to maintain an increase in dry matter that did not significantly differ from the control, in spite of the less water content, suggesting the presence of some mechanisms that confer it the observed salinity tolerance.

The occasional lack of correlation between tolerance *in vitro* and the response of the whole plant is an important limitation to take into account when selecting tolerant genotypes *in vitro*. For example, in genus *Medicago*, almost all the genotypes which showed tolerance *in vitro* were sensitive to salinity at a whole plant level, and the opposite occurred in *Medicago maritime,* which showed salinity tolerance *in vivo*, but was the most sensitive at the cellular level (McCoy, 1987b). Nevertheless, the maize/*Tripsacum* hybrid showed greater salinity tolerance, *in vitro* and *in vivo,* if compared with corn. Exposure of the N107BxT hybrid to 170 mM NaCl produced fresh weight increase reductions of 51% *in vitro* and 31% *in vivo*, which are lower than those observed for corn exposed to similar conditions,

Table 2. Water contents (%) of hybrid N107BxT plants, watered with tap water and with 170 mM NaCl

	Days after first watering			
	3	**8**	**15**	**20**
Tap water	86.6 a	86.8 a	86.9 a	87.0 a
170 mM NaCl	85.5 a	86.9 a	85.4 b	84.3 b

Different letters in the column indicate significant differences between the means (Tuckey, α: 0.05).

i.e. 81.54% *in vitro* (Ramagopal, 1986) and 85% *in vivo* (Ashraf and McNeilly, 1989).

Given the tolerance found in the hybrid, it is an interesting material to investigate the mechanisms that confer this tolerance.

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