Sodium Chloride-Induced Leaf Senescence in *Hydrocotyle bonariensis* Lam. *and Foeniculum vulgare* L.

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ABSTRACT

Leaf senescence induced by sodium chloride was studied in Hydrocotyle bonariensis Lam. and Foeniculum vulgare L. Both species belong to Umbelliferae family, however only H. bonariensis grows spontaneously in sandy soils of coastal regions (saline soils). Leaves of plants receiving nutrient solutions containing different concentrations of NaCl were evaluated for fresh and dry weights and chlorophyll content. The denaturating electrophoretic profiles of leaf proteins were also studied. Sodium chloride changed the protein profile of F. vulgare and hastened the leaf senescence of both the species. However, plants of H. bonariensis receiving 599 mM NaCl lasted longer than F. vulgare. Therefore, the occurrence of H. bonariensis in saline soils might be related with mechanisms of salinity tolerance.

Key words: chlorophyll; protein; salinity; senescence.

INTRODUCTION

Excess of salts in the soil may reduce the growth and production of many plants. Soil may be saline for several reasons including flooding of the coastline with sea water. The sea water has approximately 599 mM NaCl (Mc Intyre, 1982). Therefore, in coastline the salt negative effect in plant development may be mainly attributed to NaCl.

Based on their salt tolerance, the plants can be classified as halophytes or glycophytes. In general while halophytes can complete their life cycle in saline environment, glycophytes can not. The existence of some species in saline habitats does not necessarily mean that NaCl is essential for their growth and development, although some species can not survive without NaCl (Wainwright, 1984).

Besides inducing a decline in growth, high concentrations of NaCl hasten the senescence of several crops (Helmy *et a*l, 1994; Lutts *et al*, 1996; Munns *et al*, 1995; O'Leary & Prisco, 1970a; Prisco & O'Leary, 1972; Prisco, 1980; Sharma, 1996), most of them being glycophytes (Wainwright, 1984). Jackson and Drew (1984) described NaCl as one of the factors promoting leaf senescence.

Leaf senescence is a highly-controlled sequence of events comprising the final stage of development, from mature, fully expanded state until death. As a leaf passes the peak of assimilatory capacity, the photosynthetic apparatus is dismantled and the nutrients are exported to young growing tissues or storage organs (Smart, 1994). Moreover, there is a fall in the chlorophyll content (Matile *et al*, 1989, 1992), changes in the pattern of protein synthesis (Thomas *et* al, 1992) and a decrease in the protein concentration (Smart, 1994).

Hydrocotyle bonariensis Lam. is a member of the *Umbelliferae* family. It has an underground shoot that is connected with the leaves by long petioles. This species has a large distribution including saline coastal soils, what might be an indication that it is a halophyte.

Foeniculum vulgare L. is largely used as a spice. It also belongs to the *Umbelliferae* family. However, it is not found in saline coastal soils.

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The objective of this work was to compare the influence of sodium chloride on the leaf senescence of these two species.

MATERIALS AND METHODS

Plant material and growth conditions: Cuttings of *H. bonariensis* with only one leaf connected by the petiole to the part of an underground shoot were obtained from plants growing in clay soil (44% clay and 12.7% silt-Yamaoka-Yano & Mazzafera, 1998). These cuttings had very young leaves, still unfolded, with average leaf blade fresh and dry weights of 46 and 6 mg (average of 10 leaves), respectively. The petiole and rhizome length were 4.5 and 3 cm, respectively. The apical region of the rhizome was removed to avoid a sink effect of the nutrients from the leave to the active growing tissue that could accelerate the leaf senescence. All the leaves appearing later in these cuttings were removed for the same reason. The cuttings were washed in a 0.2% sodium hypochloride solution for 2 min before planting.

The *F. vulgare* plants grew from seeds. Only their primary leaves were submitted to chlorophyll, protein and growth analyses.

At the time of the experiments, *F. vulgare* plants were 15 days old and *H. bonariensis* cuttings were 10 days old. The plants, unless indicated, were grown in 1.5 L pots with sand, kept in the greenhouse. They were weekly irrigated with Hoagland nutrient solution (Hoagland & Arnon, 1938) up to the saturation point, or received the same nutrient solution with added NaCl so that the final salt concentration was 257 or 599 mM. One day before the application of the solutions, the sand of the pots was washed with tap water to avoid the accumulation of salt above the established values. The same was made with the pots receiving only nutritive solution (control treatment).

The plants used for the evaluation of the effect of sodium chloride on the protein electrophoretic profile were transferred from pots with sand to hydroponic solution under photoperiod of 12 hours (light/dark) and average temperature of 25°C. They grew up in Hoagland solution with or without added NaCl (259 mM). The solution was replaced weekly and between the changes, the solution levels in the pots were maintained by the addition of destilled water.

Leaf growth: The leaf dry and fresh weights were obtained every 10 days from 10 replicates growing in sand. Dry weight measures were obtained keeping the leaves at 80°C for 24 h.

Chlorophyll: The chlorophyll contents in the leaves were obtained every 10 days from 3 replicates growing in sand. Chlorophyll was extracted with 95% ethanol at 60°C and the absorbances at 645 and 665 nm were used to determine the chlorophyll concentration (Lichtenthaler & Wellburn, 1983).

Protein electrophoretic profile: Three replicates were used in this experiment. Leaves of both species were homogenized in a mortar with cold (4^{o}C) 50 mM phosphate buffer (pH 7), containing 5 mM 2-mercaptoethanol. The extracts were centrifuged at 27,200g for 15 min and the supernatants were recovered. The protein concentrations were determined (Bradford, 1976) and the extracts were diluted (1:1, v/v) with 50 mM phosphate buffer (pH 7), containing 5 mM 2-mercaptoethanol, 1% SDS and 5% glycerol. Protein denaturation was carried out at 100°C for 10 min and the extracts were stored at -20^{o}C for electrophoretic analysis. The proteins were separated by denaturating polyacrylamide gel electrophoresis - SDS-PAGE (Laemmlli, 1970), using 6% of polyacrylamide in the stacking gel and 12% in the main gel. The same amount of proteins was applied in the gels for both the species. Ovalbumin, bovine serum albumin, chymotrypsin and ribonuclease A were used as molecular weight markers. Proteins were stained with Comassie Blue R250.

Na and Cl concentration in the soil: Samples of sandy (quartz) coastal soils of two localities (São Sebastião and Ubatuba, São Paulo State) were analyzed for their Na and Cl contents (Bataglia *et al*, 1978). *H. bonariensis* was found growing naturally in both places. Sand was collect from the upper 20 cm layer. A sample of a clay soil (Yamaoka-Yano & Mazzafera, 1998) was also collected from a place where *F. vulgare* and *H. bonariensis* grew spontaneously.

In addition to this another analysis was made: 200 ml of deionized water were added to 200 g of each soil and vigorously stirred. After sedimentation of the soil particles, the conductivity was determined using a conductivimeter. The values obtained were applied to a standard curve obtained with the data of NaCl deionized aqueous solutions at different concentrations.

RESULTS

NaCl affected the first leaf life span of the 15 days old plants of *F. vulgare* (Figure 1A). The leaves of plants irrigated with 599 mM NaCl died before the first measurement at 10 days. Leaves of plants irrigated with 257 mM NaCl lasted longer (20 days), but with a marked decrease of fresh and dry weight. The death of leaves of the control plants occurred only at 60 days and the highest fresh weight (Figure 1A) and dry weight (figure 1B) were observed in these plants at 20 and 40 days, respectively.

When the cuttings of *H. bonariensis* were transfered to the sand pots the leaves had 46 and 6 mg of fresh and dry weight, respectively. After 10 days, they received the first salt irrigation.

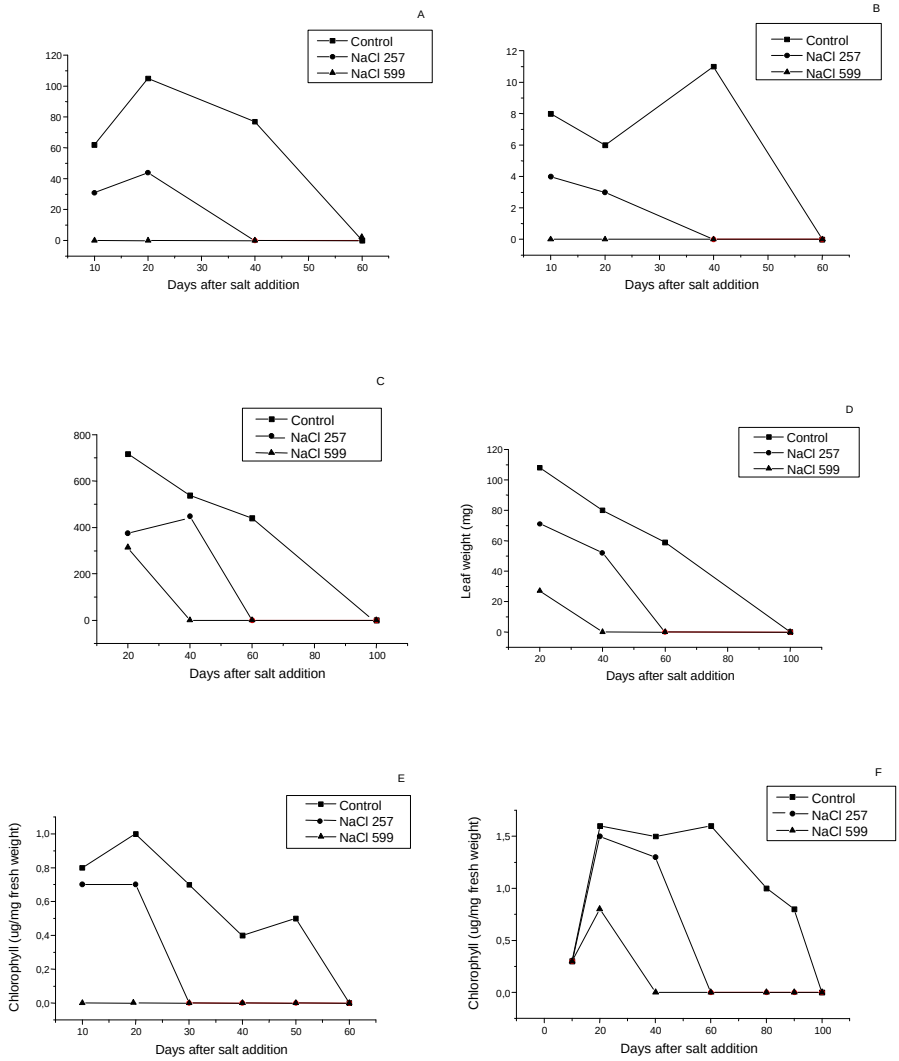

Figure 1: Fresh weight (A) and dry weight (B) of of leaves of *F. vulgare*, fresh weight (C) and dry weight of leaves of *H. bonariensis* and chlorophyll contents of leaves of *F. vulgare* (E) and *H. bonariensis* (F) treated or not with NaCl at 257 and 599 mM.

The highest fresh and dry weights of control leaves were observed at 20 days of the salt addition and death occurred at 100 days (Figure 1C and D, respectively). Plants of *H. bonariensis*, irrigated with salt showed reduction of leaf growth at both concentrations, however leaves of those receiving 257 mM NaCl lived longer than those of 599 mM. It was interesting to observe that leaves of plants irrigated with 599 mM survived more than 20 days after the first salt addition, resulting in an increase in fresh and dry weights when compared with the cutting leaves at the transplantation.

The highest chlorophyll content in the leaves of the control plants of *F. vulgare* was observed at 20 days (Figure 1E), indicating that after this period the senescence had been established in these plants. This is in agreement with the observed decrease in fresh weight (figure 1A), and happened before the decrease in dry weight (Figure 1B). The leaves of plants irrigated with 257 mM NaCl always showed lower chlorophyll contents than the control plants.

The chlorophyll contents in leaves of *H. bonariensis* were higher between 20 and 60 days (Figure 1F), although a decrease in the fresh and dry weights was observed in this period (Figure 1C and 1D, respectively). The opposite situation was observed in *F. vulgare.* In the leaves of plants receiving 599 mM NaCl (Figure 1F), the chlorophyll contents were lower than in the leaves of plants irrigated with 257 mM NaCl. Therefore, dry weight and chlorophyll content seem to be good indicators of salt-induced senescence.

The protein electrophoretic profiles were obtained from plants growing in hydroponics (Figure 2). The leaves of *H. bonariensis* were collected after 15 days of transfer to hydropony. On the other hand, the leaves of *F. vulgare* were collected just after 1 day, since at this time they already started to become yellow in the presence of 257 mM NaCl. While there was not any alteration in the number of bands of protein extracts from leaves of *H. bonariensis* irrigated with 257 mM NaCl, 3 bands desappeared in *F. vulgare* salt treated plants. Also, in these plants a new band was observed.

Salt induced alterations in the protein concentrations were also observed. In salt treated *H. bonariensis* plants, there was an increase in the protein concentration of 2 bands. On the other hand, in *F. vulgare* a reduction in the protein concentration of 4 bands was detected.

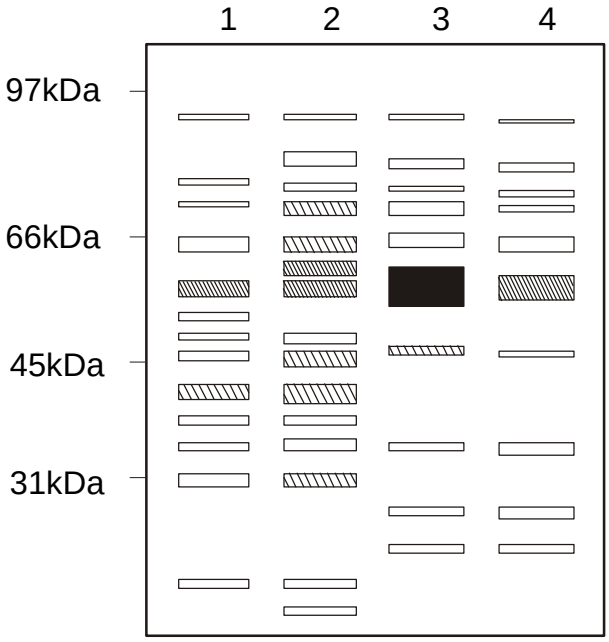

Figure 2: Schematic diagram of the eletrophoretic profile of proteins from *F. vulgare* primary leaves and *H. bonariensis* cuttings leaves, growing with or without 257 mM NaCl in the nutrient solution. 1 = *F. vulgare* + NaCl, 2 = *F. vulgare* - NaCl, 3 = *H. bonariensis* + NaCl, and 4 = *H. bonariensis* - NaCl. Molecular weight markers are indicated in the left side of the figure (ovalbumin = 97 kDa, bovine serum albumin = 66 kDa, chymotrypsin = 45 kDa, ribonuclease A = 31 kDa).

About the analysis of Na and Cl concentrations in the soil samples, although São Sebastião soil was collected from the sea-coast, it showed Na and Cl concentrations and conductivity similar to the clay soil (Table 1). The sandy soil from Ubatuba had 10 times more Na and Cl than the other two samples. The results of NaCl concentration in these same soil samples, obtained with the use of conductivimeter are in agreement with these results: sand soil from

Ubatuba presented 66 mM of NaCl, while the values for São Sebastião sand soil and the clay soil where 2 mM and 3 mM, respectively.

Table 1: Na, Cl and NaCl concentrations of three soil samples of two different kinds (sand and clay soil). São Sebastião and Ubatura are the places where sand soil was collected.

Soil Sample	**Ion Concentration (mM)**		**NaCl Concentration (mM)**
	Na	**Cl**	
Sand soil-Ubatuba	2850	4100	66
-S. Sebastião	250	390	2
Clay soil	220	385	3

DISCUSSION

NaCl induced a decrease of fresh and dry weights and of chlorophyll contents in the leaves of *H. bonariensis* and *F. vulgare*. Similar results were observed in bean (O'leary & Prisco, 1970a), wheat (Munns *et al,* 1995) and rice (Lutts *et al*, 1996). Therefore, our data supported the concept that the level of chlorophyll (Matile *et al*, 1992) and decrease of dry weight (Rabinowitch, 1951 *apud* Leopold & Kriedmann, 1975) were good indicators of leaf senescence.

Changes of the protein profile due to NaCl have been reported for several species. In cell suspensions of *Citrus sp* and *Nicotiana tabacum* adapted to high salt concentrations, several new proteins were observed (Singh *et al*, 1987).

Protein concentration could also change with the presence of salt. NaCl inhibited protein synthesis of embrio-axis of germinating bean seeds (O'Leary & Prisco, 1970b; Prisco, 1971) as well as induced decrease or increase of the proteins in leaves of different rice and soybean cultivars (Abd el-Samad & Shaddad, 1997; Lutts *et* al, 1996; Misra *et a.l.*, 1997). In some potatoes cultivars it was verified that NaCl induced a decrease of protein concentration, while at the same conditions, other ones showed an increase of proteins. The increase of proteins was associated with salt tolerance (Sasikala & Prasad, 1994). In the present work, there was an increase of protein concentration in salt treated *H. bonariensis* and three bands desappeared and one appeared in *F. vulgare*.

The sandy soils from the two coastal localities showed great difference in salt concentration, probably due to the frequency of flood tide and rain incidence, leaching the soil profile. It was difficult to compare the concentrations of NaCl used in our experiments with the values found in the soil analyses. A mixture of sand and water (1:1) indicated for the Ubatuba sand soil a value much lower than the NaCl concentrations used in this work. Alternatively, it must be considered that the conductivity might not be exclusively due to NaCl presence, since other ions present in the soil might have interfered.

Although death occurred with both species growing in NaCl solutions, plants of *H. bonariensis* lasted longer at the higher NaCl concentration than *F. vulgare*. Some halophytes grow better in saline environment. *Armeria maritima* grew better at 40 mM NaCl than with salt-free treatment and survived several months at 200 mM NaCl (Khol, 1997). Other halophytes do not need salt to grow, but are salt-tolerant species. Plants of *Phillyrea* species (*Oleaceae* family) survived 4 months at 500 mM NaCl and showed the first signs of injury in the leaves at concentration of 750 mM NaCl (Gucci *et al.*, 1997). On the other hand, *Olea* species, which belongs to the same family of *Phillyrea*, showed shoot growth inhibition at 100 mM NaCl (Gucci *et al.*, 1997). It was concluded that species of *Phillyrea* were found in saline soils because they could tolerate better the salt presence than *Olea* sp.

The occurrence of *H. bonariensis* in coastal regions may be determined by its capacity to tolerate saline environment, since this species has a large distribution, also growing in non saline soils. Therefore, it seems that NaCl is not essential for its growth. However, it is not possible to conclude from data of this work that *H. bonariensis* does not grow better with salt, since only two salt concentrations were used, which may be considered high when compared to other reports in the literature. Other experiments using lower salt concentrations have to be carried out to answer this question.

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RESUMO

Foi estudada a senescência de folhas, induzida por cloreto de sódio, em *Hidrocotyle bonariensis* Lam. e *Foeniculum vulgare* L. Ambas as espécies pertencem à família *Umbellifera.* Contudo, somente *H. bonariensis* cresce espontaneamente em solos arenosos da região costeira (solos salinos). Foram verificadas a massa fresa, massa seca e concentração de clorofila de folhas de plantas recebendo soluções de NaCl com diferentes concentrações. O perfil eletroforético denaturante de proteínas das folhas também foi avaliado. Aplicação de cloreto de sódio levou a alterações no perfil protéico de *F. vulgare* e acelerou a senescência das folhas das duas espécies. Todavia, plantas de *H. bonariensis* recebendo soluções de NaCl com 599 mM sobreviveram mais tempo que as de *F. vulgare*. Assim a ocorrência de *H. bonariensis* em solos salinos pode estar relacionada a mecanismos de tolerância à salinidade.

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