Microanalysis of leaves of *Atriplex canescens* (Pursh) Nutt. under saline conditions

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ABSTRACT: Water deficit and salinity are the sources of abiotic stress that impose more limits on the utilization of plant resources. In the Chenopodiaceae family, the species of the genus *Atriplex* possess adaptations that provide them with advantages in saline environments. In Mexico, this genus is considered an important ecological resource; however, there is little information on its physiology. With the aim of contributing to the knowledge of the adaptations of this genus, 30-day seedlings of *A. canescens* were subjected to salinity for 12, 24 and 48 hours, and analyzed by scanning electron microscopy and energy dispersive X-ray spectrometry (SEM-EDS). The images showed that the surface of the leaves had abundant trichomes with vacuoles of 10-70 µm, which both promote and delimit the formation of crystals. Microanalysis suggests that these vacuoles store water and ions in an orderly fashion; the most abundant ions are Cl⁻, Na⁺ and K⁺. Magnesium was the only element present at all exposure times, suggesting its importance for the function of the gland. Cl⁻ and K⁺ were detected after 12 hours of exposure; however, the concentration of K⁺ decreased after 48 hours and was gradually replaced by Na⁺.

Keywords: *Atriplex canescens*, microanalysis, salt stress, SEM, Trichomes

INTRODUCTION

Abiotic stress causes morphological, physiological and biochemical changes that affect the growth and productivity of plants. Water deficit and soil salinity are considered major sources of stress that restrict the use of plant resources. However, plants are able to perceive stress and to respond to it with various survival strategies; tolerance to stress is controlled by sets of genes, proteins, metabolites and specialized cells and structures that, when expressed in a certain pattern, result in tolerant phenotypes. However, at present these mechanisms are not fully clarified (Yamaguchi-Shinozaki and Shinozaki, 2006).

In plants, salt stress causes physiological drought and osmotic stress due to the increased amount of intracellular ions, mainly Na⁺ and Cl⁻, which can cause damage at the cellular level and nutritional deficiency (Hasegawa, 2000). Halophyte plants are able to complete their life cycle under toxic salinity conditions because they have mechanisms that confer tolerance by selective accumulation or exclusion of ions. To restore ionic homeostasis it is necessary a fine regulation of the expression of transporters of Na⁺ and K⁺, as well as of Na⁺/H⁺ pumps, both in the plasma and the vacuolar membrane. There are also highly efficient eco-physiological adaptations such as the presence of salt secretory glands and trichomes and changes in carbon metabolism (Shi and Zhu, 2002).

The Chenopodiaceae family is one of the most interesting from an ecological point of view, since the genera and species that comprise it are characterized by a great diversity of life forms and structures with unique tolerance...
(Voznesenskaya, 1999). The genus Atriplex, which belongs to this family, has about 400 species; Atriplex canescens is one of the best represented in arid and saline areas of northeastern Mexico (Echavarria-Chairez., 2009). Although this species has the unique ability to tolerate extremely saline soils with electrical conductivity of 27.96 mS/cm (Moreno-Limon., 2014), there are few studies about the mechanisms involved. In order to contribute to the knowledge of this species, this study analyzed the morphology and chemical composition of leaves of A. canescens exposed to salinity for different periods of time using scanning electron microscopy and energy dispersive X-ray spectrometry analysis (SEM-EDS). Understanding the morpho-physiological adaptations of this plant and their interaction with the environment is crucial for understanding the ecology and distribution of the species, allowing the improvement and implementation of strategies for the appropriate management of plant resources.

MATERIALS AND METHODS

Collection site
The collection of biological material of A. canescens (mature fruit and herbarium specimens) was performed in the Ejido San Felipe (100° 17' 57" W, 24° 06' 03" N) of the Municipality of Doctor Arroyo, Nuevo Leon, Mexico.

Plant material and growth conditions
The ripe fruits of A. canescens were dissected to extract the seeds. Five hundred seeds were measured and weighed with a digital vernier (Surtek) and an analytical balance (HR-120; AND), respectively. The seeds were then disinfected with sodium hypochlorite (3%) for 10 min, followed by ethanol (70%) for 10 seconds and washed with distilled water. A total of 100 seeds were germinated using germination trays (10x30 cm) containing peat-moss. They were then placed in a bioclimatic chamber (26 °C±1 °C and 12/12 photoperiod) and watered every 72 hours with distilled water for 30 days after sowing (DAS). Four groups of 25 plants each were formed; the first group served as control, watered with distilled water only; the other groups were treated with a 200 mM salt solution containing chlorides (MgCl₂, NaCl, CaCl₂ and KCl) for 12, 24 and 48 hours, respectively.

Morphological Analysis
Leaf samples were collected from each of the previously described treatments. Cross sections of 5 x 5 mm² were made in the central part of the leaves and fixed in 2.5% glutaraldehyde to preserve their structure. Transverse and adaxial observations of leaf surface were performed using a scanning electron microscope (NOVA NANOSEM 200, FEI) at low vacuum and low voltage (10kV).

Microanalysis
We determined qualitatively and semi-quantitatively the elementary chemical composition and atomic percentage of the elements present on the leaf surface of A. canescens. We used for that an energy dispersive X-ray detector (Octane Silicon Drift Detector; EDAX) coupled to a scanning electron microscope (NOVA NANOSEM 200; FEI). The spectra obtained were processed and analyzed using the software TEAM™ EDS. We used the following working conditions: voltage of 10 kV at a distance of 4.5 mm, 500X magnification, acquisition time of 60 sec, Spot of 3.5 mm and a low vacuum detector (LVD).

Statistical Analysis
The microanalysis data were analyzed using one-way analysis of variance followed by a multiple comparison of means using the Tukey test (P <0.01).

RESULTS AND DISCUSSION

Germination and growth of A. canescens under salinity conditions
The average size and weight of the seeds of A. canescens was 2.61x1.82 mm and 2.14 mg respectively, with a germination percentage of 87.5. The 30 DAS seedlings showed no visible signs of stress compared with the control group after applying the chloride salt solution at 12, 24 and 48 hours.

Morphology and response to salinity of the leaf surface of A. canescens
The leaf surface of the control group showed a large quantity of clearly pedicellate glandular trichomes in collapsed state, with no presence of crystals inside or outside. After 12 hours of exposure to salt, about 50% of the
glands were in semi-swollen state with an average size between 100 and 200 µm. After 24 hours of exposure, 90% of the glands were in swollen state, with no presence of crystals inside. After 48 hours of exposure, we could observe that the glands had collapsed, showing a large amount of sediments and crystals. It is important to note that the size of the epithelial cells associated with the glandular trichome increased considerably in size from 50 to 100 µm. In addition, we observed that exposure to salt for 12 hours was sufficient to activate stomatal closure, passing from exposed stomata to stomata sunken in the epidermis (Figure 1). Leaf analysis of A. canescens revealed the presence of Kranz anatomy due to the presence of bundle sheath cells completely or incompletely surrounding the vascular bundles, as well as abundant lacunar parenchyma (Figure 2).

Exclusion mechanism and chemical composition of the glandular trichomes of A. canescens

The glandular trichomes of A. canescens showed one or more vacuoles of irregular shape and size ranging from 10 to 70 µm, which may or may not be present in the epithelial cells or trichomes. Using SEM-EDS analysis, it was found that these subcellular compartments contained the total amount of the elements present in the salinity treatments; the most abundant were Cl⁻, Na⁺ and K⁺. In addition, it was observed that the formation of crystals was promoted and delimited by these areas (Figure 3). Using the information obtained by SEM-EDS semi-quantitative analysis of the leaf surface of A. canescens, we determined by ANOVA that, with the exception of carbon and potassium, the other elements present in the glands of A. canescens showed significant differences (P <0.01) between treatments. Carbon and oxygen were present in higher concentration; the concentration of the carbon was directly proportional to the salinity treatment, while the concentration of the oxygen was inversely proportional. These elements reached little more than 50 and 60% of the atomic content, respectively (Figure 4). As shown in Figure 5, magnesium was the only element present during the four times of exposure to salinity, while chlorine and potassium
began to appear after 12 hours; however, the concentration of the potassium decreased after 48 hours. Moreover, calcium and sodium were detected only after 48 hours of exposure.

Figure 3. Microanalysis of the surface of the glandular trichome of A. canescens after 48 hours of exposure to salinity. A) Vacuolar area and X-ray scattering spectrum. B) Non-vacuolated area and X-ray scattering spectrum. C: carbon; O: oxygen; Zn: zinc; Na: sodium; Mg: magnesium; Al: aluminum; Si: silicon; S: sulfur; Mo: molybdenum; Cl: chlorine; K: potassium; Ca: calcium

Figure 4. Percentage content of carbon and oxygen on the surface of the glands of A. canescens under different exposure times to salinity. C: carbon; O: oxygen. Minimum detectable value, > 0.01%
In this study, A. canescens showed no visible signs of stress when subjected to salinity shock for 12, 24 and 48 hours of exposure, which is consistent with several reports demonstrating the ability of the genus to grow even at higher salt concentrations. In this regard, Aslam. (1986) and Faycal and Mounir (2011) showed, respectively, that A. amnicola and A. halimus achieve to develop in a range of 400-800 mM NaCl. The results obtained through analysis of leaf morphology allowed us to show that the adaptive strategies to salinity of A. canescens might be centered around two main morpho-physiological mechanisms; on the one hand, the exclusion of ions through the increased expression of the glandular trichomes present on the leaf surface; on the other, Kranz anatomy, about which some authors such as Sage and Monson (1999) have said that C4 metabolism is as an important mechanism for coping with water scarcity in arid regions and with physiological drought caused by salinity. This ecological strategy allows A. canescens and other species with C4 metabolism, such as A. coriacea, A. rosia, A. spongiosa, A. undulata and A. patula (Kaderit., 2003), to respond to ionic and osmotic stress (Mitsch and Gosselink, 1993). The response to salinity must be a quick and organized process, as it requires the coordinated activation of several molecular, biochemical, physiological and morphological mechanisms. Our results suggest that the movement of water, the activation of glands and the compartmentation of ions in A. canescens requires at least 12 hours of exposure to stress, which is consistent with that reported by Munns (2002), who suggests that glycophytic plants previously exposed to salinity require up to 27 hours to restore the damage caused by osmotic stress. Previous studies have reported the presence and the chemical composition of crystals and salts on the leaf surface and the outside of the glands of A. hastata (Apóstolo, 2005); however, our results suggest that, in A. canescens, the compartmentation of ions and their transport through glandular trichomes seem to be a very organized process. In this study, we used SEM-EDS analysis to show that the glandular trichomes of A. canescens have a number of vacuoles of irregular size and shape that had never been reported for the genus Atriplex; these vacuoles allow the selective compartmentation of ions in space and time, a process that requires a considerable expenditure of energy, highlighting the importance of this mechanism. The results suggest that the primary role of these subcellular structures would be to sequester and crystallize the most toxic ions (chlorine and sodium), reducing cell damage and the spread of these ions to more sensitive tissues such as mesophyll cells; in addition, these subcellular structures provide great help in the recycling of water, an essential process in arid regions with salinity problems. Apóstolo (2005) reported magnesium oxalate crystals and calcium crystals for A. hastata and Chenopodium macrosporum. However, for A. canescens we found only sodium chloride crystals. Moreover, SEM image analysis allowed us to distinguish the difference between the mechanism of the glandular trichomes of A. canescens from that reported for A. hastata and A. halimus (Mozafar and Goodin, 1970; Breckle,., 1990); in the case of A. canescens, the gland does not burst or excretes salts into the environment. The absence of crystals on the outer part of the leaf surface, the decrease in size of the gland and the oxygen content inside (which is inversely proportional to the exposure time) support the hypothesis that water might be recycled after close to 48 hours of exposure to salt. In agreement with the literature, magnesium is an indispensable element for the dynamics and functionality of the trichomes of A. canescens; our leaf microanalysis showed that magnesium was present in all treatments with an atomic percentage of 0.6-1.5%. In this regard, it has been revealed that Mg\(^{2+}\) regulates the transport of Na\(^{+}\), K\(^{+}\), Cl\(^{-}\) and influences the movement of ions through their channels (Flatman, 1991). Another important function of Mg\(^{2+}\) is to stabilize membranes by forming complexes with phospholipids. In contrast, the deficit of Mg\(^{2+}\) increases the permeability of the plasma membrane, increasing the intracellular levels of Ca\(^{2+}\) and decreasing those of K\(^{+}\) (Planells., 1993); this may explain why calcium was detected only after 48 hours and in concentrations very close to the minimum detection limit. Furthermore, K\(^{+}\) and Cl\(^{-}\) were detected in trichome vacuoles once the plants were exposed to salinity, making up 1.1 and 1.7% of the total atomic percentage and tendency to increase towards 24 and 48 hours, respectively. This process is absolutely necessary to make way for the compartmentation of Na\(^{+}\) after 48 hours of exposure, at which time Na\(^{+}\) reached an atomic percentage of 1.8%, similar to that found for Cl\(^{-}\), encouraging the formation of crystals in the vacuole. These results provide some evidence on the morpho-physiological adaptations of A. canescens to extreme climates. However, due to the complexity and multigenic nature of the response to abiotic stress, we believe that more research is needed to help clarify the mechanisms involved.

REFERENCES


