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Biomineralization in *Sarcocornia pruinosa* Growing in the Tidal Area of Río Tinto, an Extreme Acidic Environment

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Abstract: We present the localization of Na, K, Ca and Mg biominerals in *Sarcocornia pruinosa* (Chenopodiacaeae), a halophyte species growing in the estuarine area of Río Tinto basin. The estuarine soils of the Tinto basin are characterized by slightly acidic pH and high concentrations of salinity ions. They are exposed to the daily tides, with the correspondent increase in pH and Na and Mg concetrations. The aim of this work was to characterize the elemental composition and to identify the biominerals in cell tissues of *S. pruinosa*.

Analytical techniques ICP-MS, XRD and microscopy such as OM (optical microscopy) with histochemical staining, SEM (scanning electronic microscopy) coupled with EDX (energy dispersive X-ray) have been used to analyze the plant tissues and for mineral characterization.

Elemental composition in succulent stems and seeds of *S. pruinosa* shows high values of Na and K followed by Ca, Mg and Fe. We documented the occurrence of halite, sylvite, weddellite and glushinskite as biominerals in this halophyte species. It has to be underlined the scarcity of data related to the presence of biominerals in plants. Our data suggest the importance of plants in the biogeochemical cycles in the estuarine areas.

Keywords: *Sarcocornia pruinosa*; biominerals; Río Tinto; Biological Absorption Coefficient.

1. Introduction

Sarcocornia pruinosa is a succulent halophyte from the *Sarcocornia* genus (Chenopodiaceae family) rencently described from European Atlantic estuarine coastal salt marshes (France, Portugal and Spain). It is a perennial woody subshrub up to 50 cm tall with woody and erect main branches followed by succulent erect stems, characterized by reduced opposite amplexilicaule leaves, fused to the stem forming a collar like segment, and by inflorescens with opposite three [1].

S. pruinosa occurs in low- and mid-intertidal zones subjected to regular and partial flooding by daily tides in the European Atlantic saltmarshes from southern Spain to southern France. The Rio Tinto is located in the southwest of the Iberian Peninsula (Huelva province) and its last section, together with the Río Odiel, forms an estuary situated in Huelva city.

Regarding the estuarine area soils have been described as extremely acidic to slightly alkaline and slightly saline. They contain high concentration of S and Fe together with Na, Mg, P, Cu and Zn.

Mineralogy of this soils is composed mainly by quarz, phyllosilicates (kaolinite and illite), Ca-Na feldpars, K-feldspars, dolomite and calcite [2, 3]

Vegetation of this territory correspond to halophyte vegetation, in particular, to perennial gramminoids communities and succulent Chenopodiaceae shrubs. This last one includes *S. pruinosa*, which constitutes the main biomass of this succulent suffruticose communities subjected to daily tides influence.

As an halophyte, *S. pruinosa* grows and reproduce in saline environments (salt concentration ≥ 200 mM) [4]. To maintain a positive turgor pressure halophytes must adjust osmotically, thus their cells should have a greater total solute concentration than the soil solution. In order to achive this, halophytes accumulates saline ions in their cellular structure and this could led to the formation of different biominerals.

Plants are known to be able to produce biominerals. This has been reported in most organs and tissues, being the most common calcium biominerals, mainly as oxalate, carbonate but also observed as sulphatse and phosphates. Other known minerals generated by plants are amorphous silica or magnesium oxalate [5-9]. Also, Fe biomineralizations as jarosite and Fe-oxides (ferrihydrite, hematite and spinel phases) has been thoroughly studied in the grass *Imperata cylindrica* growing on the Rio Tinto and also under controlled hydroponic cultures [10, 11].

The aim of this work was to characterize the elemental composition, the description of the biomineral morphological characteristics and their indentification and distribution in different organs and tissues of the halophytic species of *S. pruinosa* in their natural habitats, using different microscopy methodologies.

2. Results

2.1. Elemental Composition

The Río Tinto soils in which *S. pruinosa* is found are exposed to the tidal flow. The characteristics of the estuarine soils in the Rio Tinto basin have been described with a mean pH of 6.3, slightly acid and an average electrical conductivity of 6.22 dS / cm. The analysis of the elemental composition of the soil show high concentrations of S, Fe, Na, Mg and P. They also present high concentration values for Cu, Zn, Pb and As [3].

As for *S. pruinosa* samples, most of the elements measured by ICP-MS in succulent stems had the normal ranges for vascular plants [12]. The highest concentration correspond to Na, which is found over 10000 mg/kg d.w for seeds and over 61000 mg/kg d.w. for succulent stems. The values obtained for the macronutrients Mg, Ca and K are over 4000 mg/kg d.w. The dasta for seeds follow the same pattern, highlighting the values of Ca, Mg and K. The other element with high concentrations is Fe with 418 mg/kg d.w for seeds and means values of 245 mg/kg d.w. for succulent stems (Table 1).

The values obtained for micronutrients Mn, Ni and Zn and the toxic elements As and Pb are found in normal ranges, while Cu has shown high concentrations for both, seeds and succulent stems. The Biological Absorption Coefficient (BAC) values for Na, Mg and K show that *S.pruinosa* actively absorbs and accumulates these elements in its tissues (BAC > 1). Our analyzes confirm the results already described for the Rio Tinto flora [13].

Table 1. Elemental composition of the soils and the seed (A) and succulent stem (B) of *Sarcocornia pruinosa* analyzed by ICP_MS, and the correspondent BAC values. Soils data have been extracted from Rufo et al. [3]. All data are expressed in mg/kg d.w. M: mean; DS: Standard deviation; nd: under the detection limit. Number of samples: Soils: n = 12; A: n = 1; B: n = 6.

	Na	Ca	Mg	K	Mn	Fe	Ni	Cu	Zn	As	Pb	Ba	Sr
Soil (M)	13150	7517	7015	3884	393	73142	24,4	1391	1501	857	826	166	199
DS	8241	5174	2715	1279	3767	381	99,5	627	918	559	613	82,6	170
Seed	16612	7645	6231	6865	13,7	418	5,32	191	56,3	2,48	5,30	6,06	450
Stem (M)	61107	6952	7281	7181	24,4	245	3,42	64,1	42,7	1,47	2,65	4,51	33,7
DS	19873	881	1521	4471	12,8	186	2,10	3,33	23,3	1,98	3,69	2,09	5,80
BAC	4,64	0,92	1,03	1,84	0,06	0,003	0,14	0,05	0,03	0,001	0,003	0,03	0,16

2.2. X-ray Diffraction Analysis

We present data of two representative samples located in high salt marsh area. In the first diffractogram the highest intensity diffraction peaks (Figure 1A) correspond to halite (NaCl) and calcium oxalate dihydrate weddellite ($C_2CaO_4{\cdot}2H2O$).

In the second spectrum, halite (NaCl), sylvite (KCl) and weddellite ($C_2CaO_4{\cdot}2H_2O$) have been detected. The peak of lower intensity corresponds to glushinskite magnesiuym oxalate dihydrate ($C_2MgO_4.2H_2O$). Peaks of lower intensity but not detected by other techniques have not been taken into account.

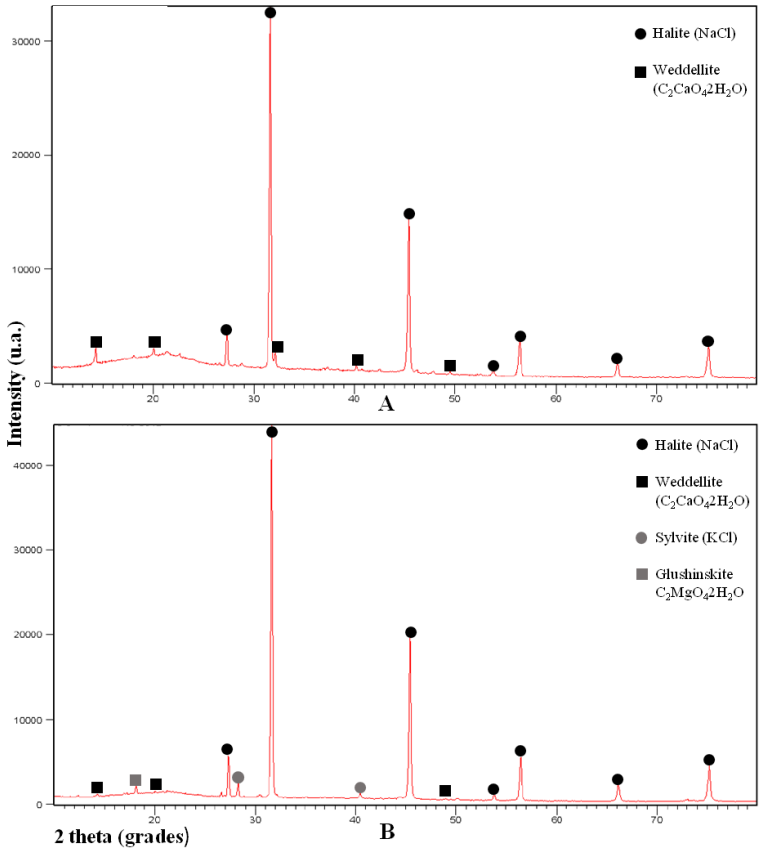

Figure 1. Representative X-ray diffraction spectra of succulent stems of *S. pruinosa*. **A** Halite and weddellite. **B** Halite, weddellite, sylvite and glushinskite.

2.3. Microscopic Analysis

Stems and leaves

The accumulation of biominerals in *S. pruinosa* has been studied under SEM microscopy in the different visible tissues in the transverse and longitudinal sections of their stems (epidermis, parenchyma and vascular bundle, Figure 2A). The greatest accumulation and variety of biominerals are found in the parenchymal tissue, followed by the vascular bundle and finally the epidermis, sometimes with large crystalline structures.

In the outermost layer of the stems, the epidermis, sodium and chlorine crystals predominate (Figures 2A, 2B and Figure 4a spectrum). Less frequently in this layer have been observed structures dominated by calcium crystals with chlorine and sodium. In the parenchyma of the succulent stems the main crystals are of NaCl (Figure 2D and Figure 4b spectrum), mixtures of K, Na, Cl crystals with other elements (Figure 2E and Figure 4c spectrum), and the presence of cubic Ca crystals (Figures 3A and 3B and Figures 4e and 4f spectra). Mg mineralizations have been found mostly in the parenchymal cells of the transverse sections of stems with flowers (Figures 3C and 3D and Figure 4g spectrum). Some particles formed by a mixture of Fe, S, Na, Cl, Si, Al, Mg and K have been less frequently observed in the walls of the parenchymal cells of the floriferous stems.

In the vascular bundle, mainly crystalline structures are found collapsing the vessels in mixtures with Na, K and Cl as major elements (Figure 2F, Figure 4d spectrum). Frequent predominant Ca mineralizations can be also found with other salts covering the ducts of the vascular bundles (Figure 3E and 3F and Figure 4h spectrum).

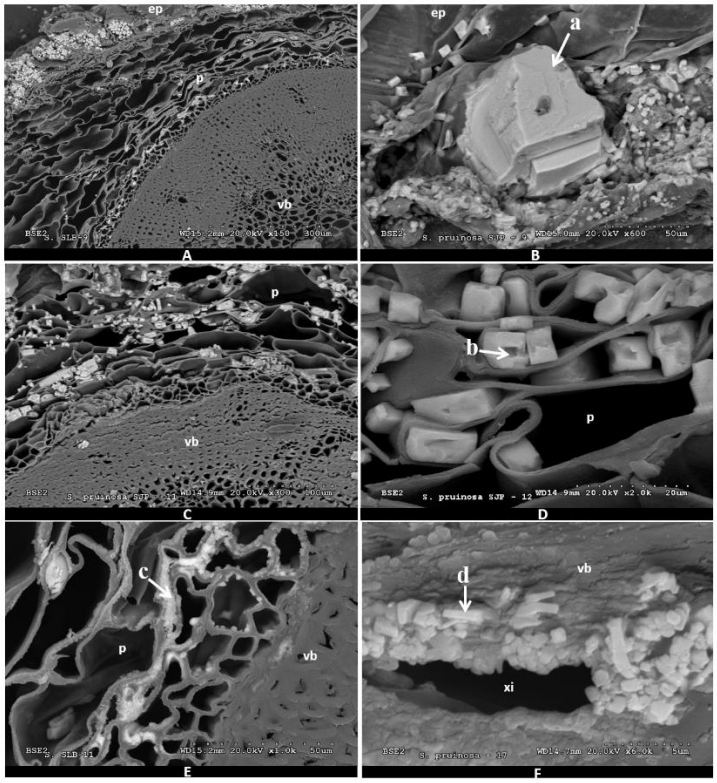

Figure 2: Representative SEM images of Na and K biominerals in *S. pruinosa*. **A** Stem cross section with Na and K salts. **B** Details of NaCl crystals in the epidermis. **C** Major salts of NaCl in parenchyma of transverse section of stem. **D** Detail of NaCl crystals in the parenchyma. **E** Detail of KCl salts in the parenchyma. **F** Xilema of floriferous stem collapsed by crystals of K, Cl and Na. Lowercase letters (a, b, c and d) of the parts B, D, E and F respectively correspond to the EDX spectra of Figure 4. Symbology: ep = epidermis; p = parenchyma; vb = vascular bundle; xi = xylem.

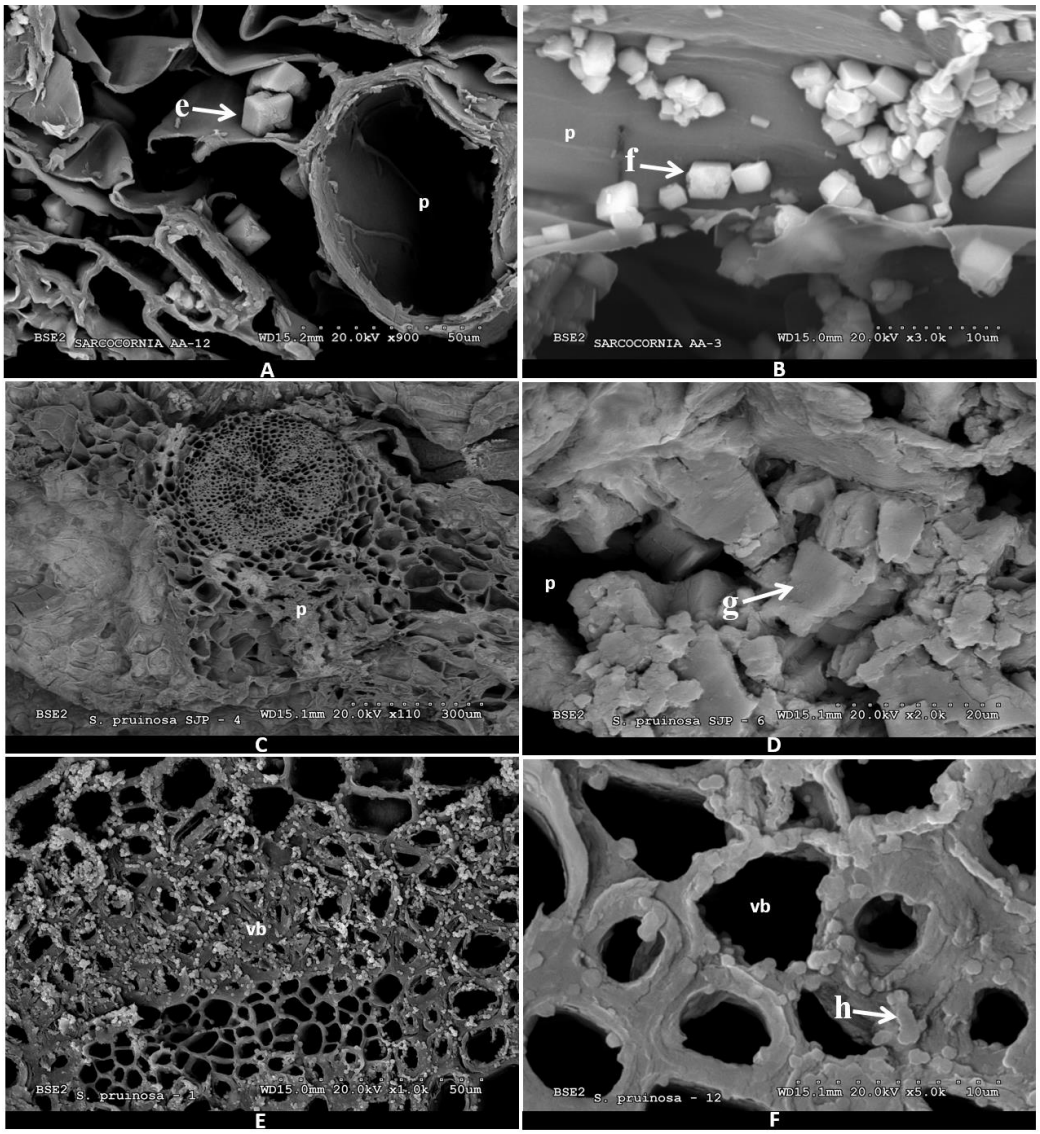

Figure 3. SEM images representative of Ca and Mg biominerals in succulent stems of *S. pruinosa*. **A** Cubic structures of Ca in parenchyma. **B** Ca crystals in the parenchyma cell walls in longitudinal section. **C** Location of Mg in parenchyma of flowering stem. **D** Detail of Mg in the parenchyma. **E** Vascular bundle showing abundant Ca bodies. **F** Detail of the Ca bodies. Lowercase letters (e, f, g and h) of the parts A, B, D and F respectively correspond to the EDX spectra of Figure 4. Symbology: p = parenchyma; vb = vascular bundle.

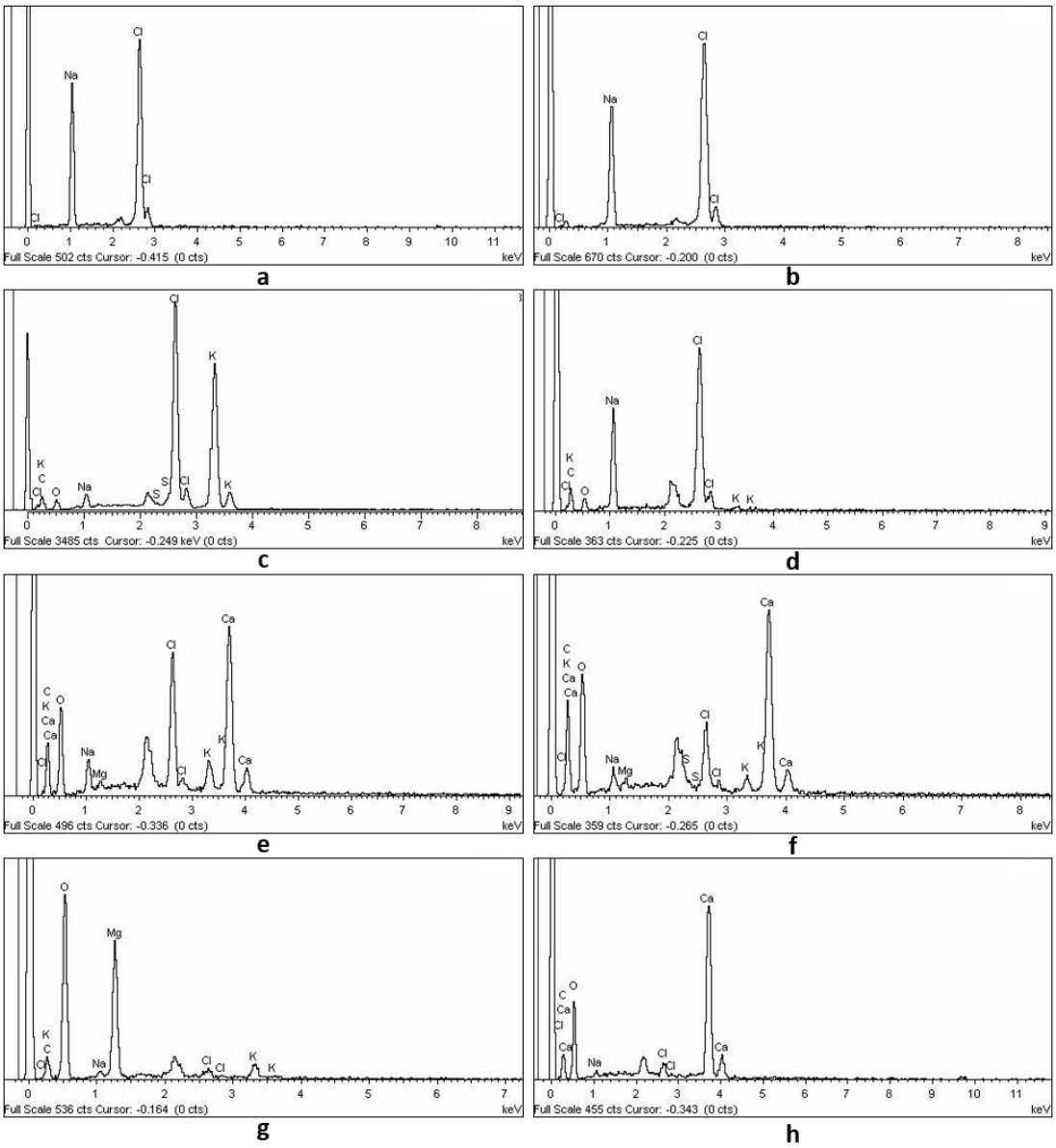

Figure 4. EDX spectra of the crystals and particles analyzed in figures 2 (a, b, c and d) and 3 (e, f, g and h).

3. Discussion

S. pruinosa, halophyte species typical of the Atlantic marsh ecosystems of Rio Tinto show high capacity to accumulate different minerals elements in relation to the soil where it grows.

The nutrient concentrations measured in *S. pruinosa* present similar ranges to those previously published for other halophytes that grow in this environment [13].

In the Tinto River salt marshes, Na, Ca, K and Mg are the ions that have higher concentrations inside the plant. These observations also correlate with the ionic values found in other species of Chenopodiaceae close to the genus *Sarcocornia* [14]. However, the dominance of each of the four elements in the plant tissues will depend on the combination of several factors such as soil materials or the different salinity degree produced by the tides [15] and it is not trivial to provide a specific order of predominance of these macro and micronutrients. On the other hand, should be underline the concentration of the Fe, which present important values both in succulent stems and in seeds.

The BAC values show that *S. pruinosa* follows an accumulation strategy for Na as other known succulent halophytes [4]. Other elements such as Ni, Cu, Zn, As and Pb are below 1, which could be

associated with an exclusion strategy, which has been described as the most common among the flora of Rio Tinto [13].

A semiquantitative XRD analysis of the majors crystal phases in samples material of *S. pruinosa* consisted in halite, weddellite, sylvite and glushinskite. These data are congruent with the crystal structures detected by SEM in the different and representative sections of the leaves and floriferous and non floriferous stems (Figures 2 and 3), where Na, Ca, K and Mg crystals have been found more frequently.

The accumulation of salts, especially Na of this halophyte species occurs in the cell vacuoles of virtually all tissues, although with greater presence in the tissues of the spongy parenchyma, is a strategy to adapt to growth in soil media with high concentrations. The way to maintain osmotic equilibrium at the cellular level is competed with K, whose concentrations are much higher than those needed for other species. On the other hand, the accumulation of Na occurs in biologically less active tissues and cells, such as the xylem vascular bundles of flowering stems, spongy parenchyma cells next to the epidermis. The concentration of halite and sylvite constitutes a biological process controlled by this species and we suggest that their storage in inactive forms may be a way to exclude ions that are toxic at a cellular level as Na. In the case of K, given its lower presence can be a reservoir to mobilize when it is necessary to balance ions.

Ca oxalate minerals (the mono-, di-, and tri-hydrates of $Ca(C_2O_4)$, are the most common family of organic minerals in natural environments and the accumulation and precipitation patterns of Ca and Mg are more species-specific than substrate-affected [8]. Ca oxalate biomineralization constitutes a good example of a biologically controlled process [16-18] and both, the chemical nature and crystal phenotype, as well as their localization within the plant body, are under strict genetic control [9]. Weddellite, $C_2CaO_4 \cdot 2H_2O$, is the biomineral found in greater proportion in this species being the most common of all in nature. The presence of this oxalate in the embryo constitutes an evidence of the genetic control of the species by facilitating the transport and subsequent accumulation of calcium in crystalline form as has been observed.

Together with wedellite we find in a smaller proportion the dihydrated oxalate $C_2MgO_4 \cdot 2H_2O$. This biomineral was also detected in the Cactaceae species *Opuntia ellisiana*, associated with whewellite and opal, constituting the first evidence of the presence of this oxalate in plants [8].

We highlight the first results of biominerals found in this species and in other halophytes of the Chenopodiaceae family. A semiquantitative XRD analysis of the majors crystal phases in samples material of *S. pruinosa* consisted in halite, weddellite, sylvite and glushinskite. These data are congruent with the crystal structures detected by SEM in the different and representative sections of the leaves and floriferous and non floriferous stems, where Na, Ca, K and Mg crystals have been found more frequently.

In this work a complete characterization of the biominerals present in one of the most representative species of the saline ecosystems of the Rio Tinto was made. Our data reveal the mineral composition of *S. pruinosa* and the importance that its use can have in different nutritional and biotechnological applications.

4. Materials and Methods

4.1. Plant Material

Complete individuals of *S.pruinosa* were collected directly from the riverside communities of Río Tinto, in high salt marsh (San Juan del Puerto, UTM 29SPB6122) and low salt marsh areas (Monumento a Colón, UTM 29SPB3820) (Huelva, Spain) selecting the best preserved and with similar size. Three wild samples were selected for elemental analysis from each locality.

4.2. Elemental Analysis

Plant samples were washed with distilled water, dried in an oven at 75 °C for 24 h and powdered for homogenization. Small portions of dry plant tissues powder (500 mg) were digested at high pressure in a mixture of 8 ml of HNO_3 65% and 2 ml of H_2O_2 30% in a MLS Ethos 1600 URM Milestone microwave digester, fol- lowing the protocol described by Zuluaga et al. [19]. Aliquots of the different samples were analyzed by ICP-MS using an ELAN-6000 PE-Sciex (Toronto, Ontario, Canada) instrument. The ICP-MS technique used has an inherent error of 15%.

4.3. X-ray Diffraction Analysis

X-ray diffraction patterns for each analysed sample were obtained using a Siemens-D5000 (Siemens AG, Karlsruhe, Germany), diffractometer with Cu Kα (8.04 keV) radiation and a SOL-X detector provided by Bruker. The samples were analysed using the unoriented powder method. The components were identified using the patterns registered in the crystallographic data base PDF-2 of the ICCD (International Centre for Diffraction Data). The diffractograms were carried out in the Servicio Interdepartamental de Investigación from the Universidad Autónoma de Madrid (UAM, Spain).

4.4. Scanning Electron Microscopy (SEM)

Plant material of *S. pruinosa* was analyzed by SEM complemented with an energy dispersive X-ray analyzer (EDX) following the protocol for elemental analysis and metal localiza- tion study in plant material [20-22]. The organs and tissues analyzed were: roots (epidermis, parenchyma, endodermis and pith), rhizome (epidermis, cortex, cen- tral cylinder and pith) and leaves (cuticle, epidermis, trichomes, parenchyma and vascular bundles). Samples were mounted onto conductive graphite stubs and sputters and gold-coated in a BIO-RAD SC 502 apparatus to ensure electrical conductivity and prevent charging under electron beams. Samples were examined with a Hitachi S-3000 N (Japan) SEM using an accel- eration voltage of 20 kV and a working distance of 15 mm. Analyses were performed at room temperature. The qualitative element composition of samples was determined using an INCAx-sight with a Si-Li Detector (Oxford, England) with a detection limit of 10% of the main element.

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