Metal Uptake and Distribution in Cultured Seedlings of *Nerium oleander* L. (Apocynaceae) from the Río Tinto (Huelva, Spain)

Alejandro Franco · Lourdes Rufo · Javier Zuluaga · Vicenta de la Fuente

Abstract *Nerium oleander* L. (Apocynaceae) is a micro-nano phanerophyte that grows in the riverbanks of the Río Tinto basin (Southwest Iberian Peninsula). The waters and soils of the Río Tinto area are highly acidic and have high concentrations of heavy metals. In this environment, *N. oleander* naturally grows in both extreme acidic (EA) and less extreme acidic (LEA) water courses, excluding, and bioindicating certain metals. In this work, we compared and evaluated the accumulation preferences and capacities, the distribution and processes of biomineralization of metals (Fe, Cu, Zn, Mn, Mg, Ca) in the first stages of growth of EA and LEA oleanders by means of inductively coupled plasma–mass spectrometry, scanning electron microscopy, and energy dispersive X-ray analyzer analysis. Seeds from both environments were grown and treated with a self-made solution simulating the most extreme red waters from the Río Tinto. LEA plants drastically reduces the metal uptake at the beginning, but later reactivates the uptake reaching concentration values in the same range as the EA plants. The results showed high Mn, Zn and Mg concentrations, accumulation of Fe and Cu in plants from both environments, differing from the metal concentrations of field-grown oleanders. Iron bioformations with traces of other metals were present inside and over epidermal cells and inside vascular cells of stems and roots. They were absent of leaves. The accumulation properties of *N. oleander* in its early stages of development make it a species to take in consideration in phytoremediation processes but optimized conditions are needed to ensure enough biomass production.

Introduction

Nerium oleander L. (Apocynaceae) is a micro-nano phanerophyte which forms woodland communities in initial soils of river beds and creeks subdued to intense summer droughts in all the thermomediterranean area. *N. oleander* together with *Erica andevalensis* Cabezudo & Rivera constitutes the main vegetation of streams and rivers on the mining area of Río Tinto (Huelva, Spain) and the Iberian Piritic Belt [1].

Two kinds of water flaws are found in this territory: the extreme acidic water (EA) and the less extreme acidic water (LEA). The Río Tinto characteristic red water (EA) has a mean pH of 2.2 and high concentrations of SO_4^{2-}, Fe, Cu, Zn, As, and Pb [2]; similar features have been found in the acid mine drainages and other red water flows of Huelva [3]. LEA colorless water flaws are also found in the territory and clear differences in the vegetation of both kinds of waters have been reported [4]. *N. oleander* species has been found growing in both kinds of water forming riverine woodlands [1].

There are several studies about the mechanisms that allow the survival of *N. oleander* in territories with high metal concentrations, and its biotechnological applications. *N. oleander* leaves have been reported to be a good biomonitor and bioindicator for atmospheric and soil contamination with metals [5–9]. *N. oleander* also excludes Al, Ba, Cr, Fe, and Pb of the soil [8] and it is suitable to use in mine landscape phytostabilization [10].

As it grows in both EA and LEA water courses we decided to focus on the study of the ecological responses of this species to acidic water with high metal concentration and the differences between the individuals of both environments (EA and LEA). In previous works, we analyzed adult plants

A. Franco · L. Rufo · V. de la Fuente (✉)
Departamento Biología, Facultad de Ciencias, Universidad Autónoma de Madrid, 28049 Madrid, Spain
e-mail: vicenta.fuente@uam.es

J. Zuluaga
Departamento Química Física Aplicada, Facultad de Ciencias, Universidad Autónoma de Madrid, 28049 Madrid, Spain

growing on Río Tinto EA and LEA waters by means of inductively coupled plasma–mass spectrometry (ICP-MS) and scanning electron microscopy (SEM)–energy dispersive X-ray analyzer (EDX) techniques comparing individuals from both environments. No significant difference was found in total metal concentration and distribution in the plant tissues, but differences in Na, As, Fe, and Pb concentrations were found between root and leaves within each group [10]. To continue with this line of research, we decided to expose seedlings of *N. oleander* from both waters to a continuous stress with similar conditions to those of the most extreme waters of the Río Tinto. Our aim is to compare and evaluate the accumulation preferences and capacities, the distribution and processes of biomineralization of metals in the plant tissues of both study groups from the same species, and the reaction of LEA plants to extreme conditions.

Materials and Methods

Materials

Samples composed of fruits and seeds of *N. oleander* were collected from wild individuals growing in both types of water flaws (EA and LEA) along the river basin, in the same sampling localities studied in Franco et al. [10].

The seeds were extracted, cleaned and put onto germination paper inside sterilized petri plates for 20 days in a germination chamber at 25 °C. The resulting seedlings were transplanted to commercial substrate and left to grow for two months in a greenhouse.

A selection of 60 uniform plants from EA waters and 60 from LEA waters were taken to have enough data for the statistical analysis. The plants were cleaned and transplanted in sets of 30 individuals to a hydroponic system in plastic containers with Perlite as substrate.

Table 1 Composition of the self-made solution

Reagent	Metal concentration
$FeSO_4 \cdot 7H_2O$	2,300 mg/kg (Fe)
$MgSO_4 \cdot 7H_2O$	327 mg/kg (Mg)
$CuSO_4 \cdot 5H_2O$	257 mg/kg (Cu)
$ZnSO_4 \cdot 7H_2O$	114 mg/kg (Zn)
$Mn(SO_3)_2 \cdot 4H_2O$	55 mg/kg (Mn)
KCl+KBr	7 mg/kg (K)
$CoCl_2 \cdot 6H_2O$	6 mg/kg (Co)
$NiSO_4 \cdot 6H_2O$	2 mg/kg (Ni)
$CrCl_3 \cdot 6H_2O$	1 mg/kg (Cr)
KBr	1 mg/kg (Br and K)

Thirty plants coming from EA (T1) and LEA waters (T2) were exposed to a treatment with 500 mL of a self-made solution (SMS). The solution was designed based on elemental concentrations of the Río Tinto waters previously studied by López-Archilla et al. [2] and Rufo et al. [4], to simulate the more extreme acid waters of the Río Tinto, including the lack of some basic nutrients, such as nitrogen, phosphorous and calcium. The composition of this solution is shown in Table 1. The pH was adjusted to 1.8 with H_2SO_4 [1 M]. All the plants were grown in a greenhouse at 25 °C. The solution of each container was renewed every 4 days. Thirty plants coming from EA (C1) and LEA waters (C2) were left as control test. These control groups were watered with tap water. Once a week for 2 months, three random plants from each group were taken, cleaned and kept frozen at −20 °C until the analysis.

Plant Elemental Analysis

Plant samples were analyzed for total Ca, Mg, Fe, Zn, Mn, and Cu, using inductively coupled plasma–mass spectrometry technique (ICP-MS).

The samples were washed with distilled water and dried in an oven at 75 °C for 24 h. Small portions of dry plant tissues (500 mg) were digested in a mix of 8 ml of HNO_3 65 % and 2 ml of H_2O_2 30 % in a microwave digestor MLS Ethos 1600 URM Milestone, at high pressure, following the protocol described by Zuluaga et al. [11]. 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 %.

Microscopic Analysis

A micromorphological and semiquantitative elemental analysis was performed using SEM complemented with an EDX analyzer. Three samples of *N. oleander* from every harvest of each group of the experiment were selected and cleaned with a dry brush to prevent dissolution of the root iron plaque. Analyzed organs and tissues were leaves (cuticle, epidermis, trichomes, parenchymas, and vascular bundles), stems (epidermis, cortex, central cylinder, and pith) and roots (epidermis, parenchymas, endodermis, and pith).

Samples were mounted onto conductive graphite stubs and sputters and gold-coated in a BIO-RAD SC 502 apparatus for electrical conductivity and to prevent charging under electron beams. Samples were examined with a Hitachi S-3000N (Japan) scanning electron microscope using an acceleration voltage of 20 kV and a working distance of 15 mm. Analysis was 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.

Data Analysis

Statistical analysis was carried out using Statistica release 6.0 (Statsoft Inc., Tulsa, USA). Means, medians, standard deviations, and standard errors were calculated. Data were log transformed after being tested for normality with the Shapiro–Wilk test ($p > 0.05$). In order to test differences between groups we used Student's test and ANOVA. When suitable ($r^2 > 0.90$) linear regressions were calculated for each metal concentration variation in time. Absorption ratios were calculated as:

$$[\text{weekly mean absorption}]/[\text{Concentration in SMS}]$$

Results

Plant Elemental Analysis

Set T1: EA Water Plants Watered with SMS

Values of mean concentrations and standard deviations are showed in Table 2.

The Fe initial concentration was 594 mg/kg d.w. and it reached 41,818 mg/kg d.w. at the end of the experiment (eighth week). Data of the first three weeks (from 594 to 28,139 mg/kg d.w.) could be fitted to a linear regression model (Fig. 1). The slope of the fitted line was 9,516 mg/kg per week (Table 3). The mean concentration in the fourth week showed a decrease. A second line could be adjusted for the data from the fourth to the seventh week (16,257–45,503 mg/kg d.w.) (Fig. 1). The slope obtained was

9,955 mg/kg per week (Table 3). Fe concentration reached the maximum in the seventh week.

The same pattern of variation of the concentration in time (weeks 1–3 and weeks 4–7) was found for Cu, Mn, and Zn. In all these cases, the maximum concentration was measured in the seventh week of the experiment, with values of 5,120 mg/kg d.w. for Cu, 1,242 mg/kg d.w. for Mn, and 1,937 mg/kg d.w. for Zn.

The Mg initial concentration was close to 2,600 mg/kg d.w. and was maintained during the first week of the experiment. From that point on, Mg concentration showed two linear increases similar to those of the other metals (until the third week and weeks 4 to 7). In this case, the maximum concentration (7,215 mg/kg d.w.) was measured at the end of the experiment in the eighth week.

The slopes and absorption ratio (referenced to the concentration of each metal in the solution) for these linear periods of these five elements are shown in Table 3. We observed that the weekly absorption rates (V1 and V2 in Table 3) were proportional to the concentration of each element added in the treatment solution (between three and four times) and coincide for the two slopes.

For Ca, the initial concentration was 13,403 mg/kg d.w. and decreased during the first week until 9,250 mg/kg d.w. During the rest of the experiment the concentration continued decreasing slowly until 5,309 mg/kg d.w.

Set T2: LEA Water Plants Watered with SMS

The Fe initial concentration was 262 mg/kg d.w. During the first week, the concentration showed a fast increase until 7,120 mg/kg d.w. and then it was more or less constant until

Table 2 Evolution of Fe, Cu, Mn, Zn, Mg and Ca mean ($n=3$) concentrations (in milligram per kilogram dry weight) and standard deviation (SD) of EA water plants watered with SMS. Table also includes 8th week mean value of the C1 control plants

EA waters (weeks)											8 control
		0	1	2	3	4	5	6	7	8	
Fe	Mean	594	8,460	20,982	28,139	16,257	23,496	35,306	45,503	41,818	345
	SD	168	707	10,075	11,626	4,864	15,996	12,162	15,819	21,097	42.4
Cu	Mean	13.9	865	1,959	2,846	1,669	2,621	3,351	5,120	4,352	38.6
	SD	5.84	73.6	1,018	1,283	477	2,351	1,648	2,645	1,750	9.28
Mn	Mean	226	370	541	731	526	629	876	1,242	1,187	70.6
	SD	70.9	11.1	208	275	221	410	235	255	316	10.8
Zn	Mean	85.4	495	902	1,271	768	1,083	1,532	1,937	1,868	55.5
	SD	28.1	54.3	440	614	319	918	636	715	642	8.98
Mg	Mean	2,612	2,661	4,195	5,003	3,491	4,493	5,319	7,126	7,215	3,419
	SD	277	313	1,368	1,546	978	2,745	1,479	1,834	1,762	584
Ca	Mean	13,403	9,250	9,391	8,207	8,689	6,908	6,700	6,266	5,309	10,865
	SD	2,334	21	291	1,096	625	302	812	543	310	554

Fig. 1 Graphical representation of Fe (**a**), Mn (**b**), Cu (**c**), Mg (**d**), Zn (**e**), and Ca (**f**) mean (*n*=3) concentrations (in milligram per kilogram dry weight) in time (weeks) of *N. oleander* from EA water plants. *Points* are means and *whiskers* the standard error. *Dotted lines* represent the straight line of the adjusted linear regression of each 4 week period
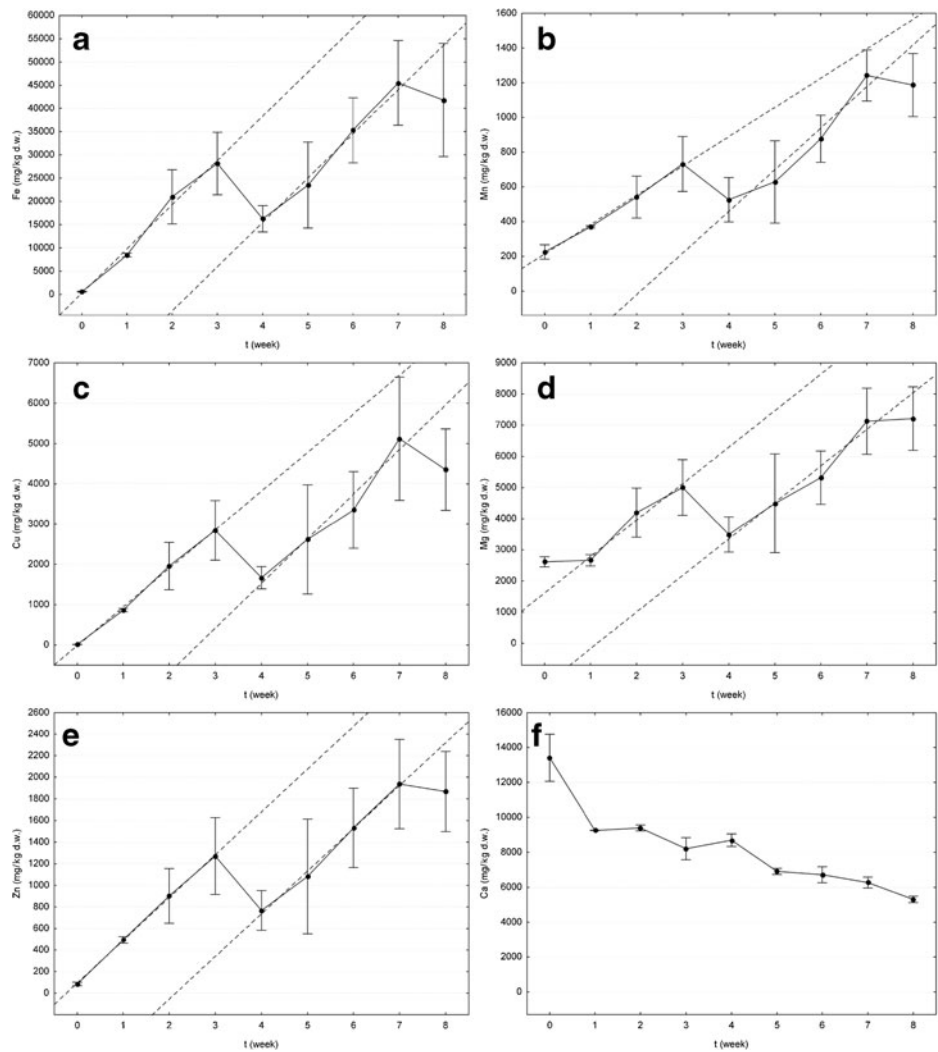
the third week (8,573 mg/kg d.w.). From the third to the sixth week, the concentration increased to 28,933 mg/kg d.w. In the seventh week, it decreased to 17,708 mg/kg d.w. and in the eighth week, it reached the maximum value (30,325 mg/kg d.w.) (Table 4, Fig. 2).

The variation of the concentrations in time for Cu, Mn, and Zn was similar to that obtained for Fe (Fig. 2, Table 4). The initial values and the maximum concentrations of each of these metals were 7.19 and 2,931 mg/kg d.w. for Cu; 227

and 819 mg/kg d.w. for Mn; and 71.6 and 1,199 mg/kg d.w. for Zn.

The Mg concentration at the beginning of the experiment was 1,937 mg/kg d.w. The concentration of this element showed a similar progression to the other metals, but the maximum concentration appeared in the fifth week (5,459 mg/kg d.w.). The concentration fell during two weeks until 2,952 mg/kg d.w., and then increased again until 4,875 mg/kg d.w. in the last week.

Table 3 Concentration added in solution (C), weekly absorption rate in milligram per kilogram of metal in plant (dry weight) for the first (V1) and the second (V2) linear periods

Metal	C	V1	Ratio V1/C weeks 0 to 3	V2	Ratio V2/C weeks 4 to 7
Fe	2,300	9,516	4.14	9,955	4.33
Cu	257	959	3.73	1,109	4.31
Mn	55	169	3.07	240	4.36
Zn	114	397	3.48	396	3.47
Mg	327	1,171	3.58	1,173	3.59

Table 4 Evolution of Fe, Cu, Mn, Zn, Mg and Ca mean ($n=3$) concentrations (in milligram per kilogram dry weight) and standard deviation (SD) of LEA water plants watered with SMS. Table also includes 8th week mean value of the C2 control plants

LEA Waters (weeks)											8 control
		0	1	2	3	4	5	6	7	8	
Fe	Mean	262	7,120	6,985	8,573	12,737	16,464	28,933	17,708	30,325	269
	SD	188	382	1,464	1,297	2,611	16,920	17,140	146	4,432	106
Cu	Mean	7.19	757	562	726	1,172	1,669	2,679	1,461	2,931	31.2
	SD	1.81	114	182	168	243	1,824	1,484	153	763	10.6
Mn	Mean	227	302	269	294	367	530	676	441	819	66.7
	SD	26.4	34.2	86.6	76.6	55.2	310	278	8.62	105	14.4
Zn	Mean	71.6	333	273	317	478	821	1,108	538	1,199	47.4
	SD	124	137	91.3	109	157	818	615	220	397	5.37
Mg	Mean	1,937	2,239	2,195	2,337	2,616	5,459	4,834	2,952	4,875	2,580
	SD	275	482	463	262	314	2,314	1,699	30.8	989	269
Ca	Mean	12,870	9,900	8,237	7,998	7,716	12,148	7,007	7,420	6,784	7,892
	SD	541	635	1,502	393	408	8,378	1,142	753	118	2,633

Fig. 2 Graphical representation of Fe (**a**), Mn (**b**), Cu (**c**), Mg (**d**), Zn (**e**), and Ca (**f**) mean ($n=3$) concentrations (in milligram per kilogram dry weight) in time (weeks) of *N. oleander* from LEA water plants. Points are means and whiskers the standard error
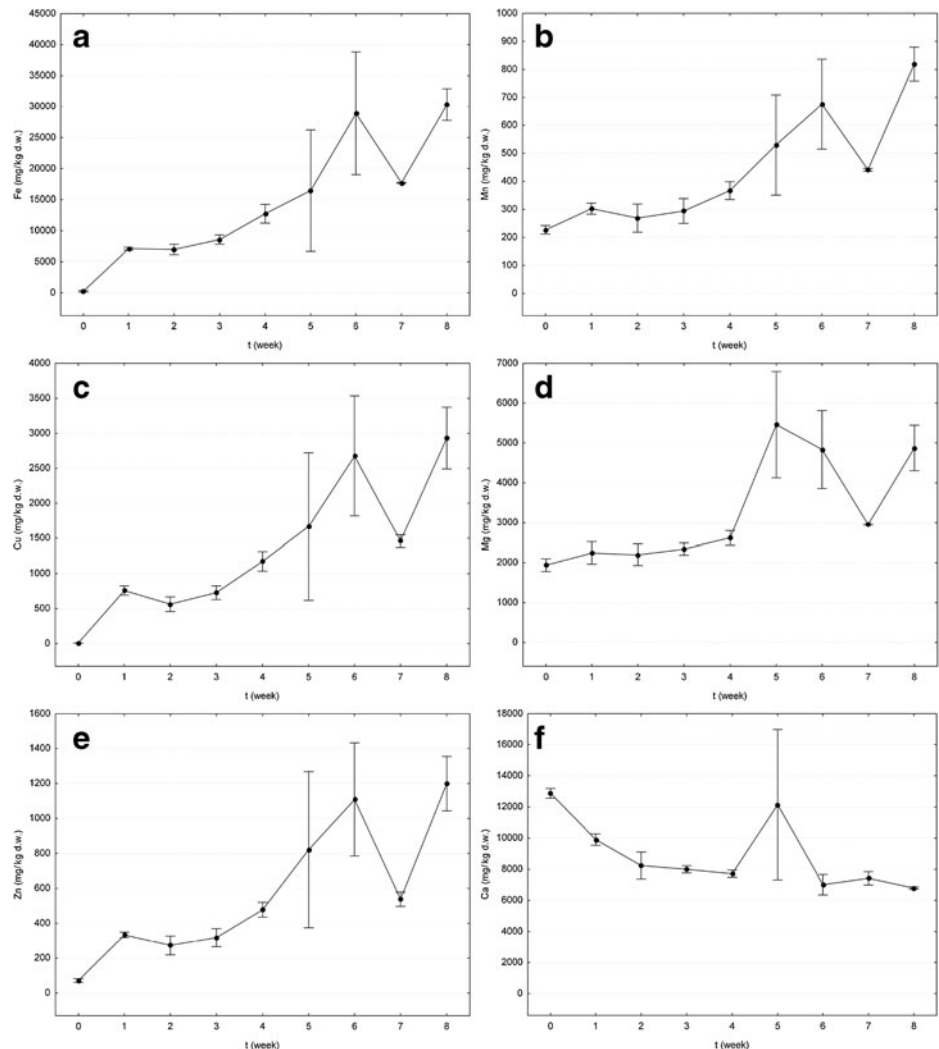
The initial Ca concentration was 12,870 mg/kg d.w. The concentration fell slowly until the end of the treatment, with a 6,784 mg/kg d.w. value in the eighth week.

Sets C1 and C2: Control Plants

No important variations were found in the control groups, the element concentrations were maintained almost constant or with a slight decrease in both sets. Only Cu and Mg presented a slight increase at the end of the experiment. Eighth week concentrations of the EA and LEA plants are shown in Tables 2 and 4.

Scanning Electron Microscopy

Sets T1 and T2: EA and LEA Water Plants Watered with SMS

Iron appeared in both root and stem, but it was not detected in the leaves for both EA and LEA water plants (Figs. 3a, e and 4a, e). It was detected over the epidermis, inside epidermal cells, cortex, and mostly accumulated in the central cylinder tissues of the roots and stems. In the root surface, patches composed mainly of Fe, K, S, and P, sometimes with Si and Al, were observed in the last weeks of the experiment. Inside the roots, Fe appeared forming amorphous deposits of the same composition covering the inner walls of the xylem vascular cells (Figs. 3b and 4b). In the first weeks of exposure some vascular cells and tracheas of the stem central cylinder appeared collapsed by Fe deposits with the same main composition (Fig. 3f). In later stages, fewer deposits were observed, but more Fe particles were detected included in the cell walls of the central cylinder (Fig. 4f and g).

There were no differences in the distribution of the Fe accumulations in both groups of study, but the Fe, S and P deposits were more frequently observed in the individuals coming from EA waters (Figs. 3b, c, f and 5a, b, d), while in those from LEA waters these deposits appeared with a higher concentration of Ca and S (Figs. 4b and 6a).

Calcium had been detected in stem and leaves and with less frequency in root samples, mainly forming oxalate druses and prismatic crystals composed of Ca, C, and O, sometimes with traces of K and Mg. In the roots, Ca appeared as isolated crystals in the cortex cells, and associated with other elements in the epidermal surface. In the stems Ca appeared in parenchymatous tissues (cortex and pith). In the first stages of the experiment, Ca and S were present as amorphous accumulations (Figs. 3g and 5e), while in later stages Ca was only observed as druses and prismatic crystals in the cortex and pith (Fig. 3h, i). Calcium druses were present in the palisade parenchyma tissues of the leaf
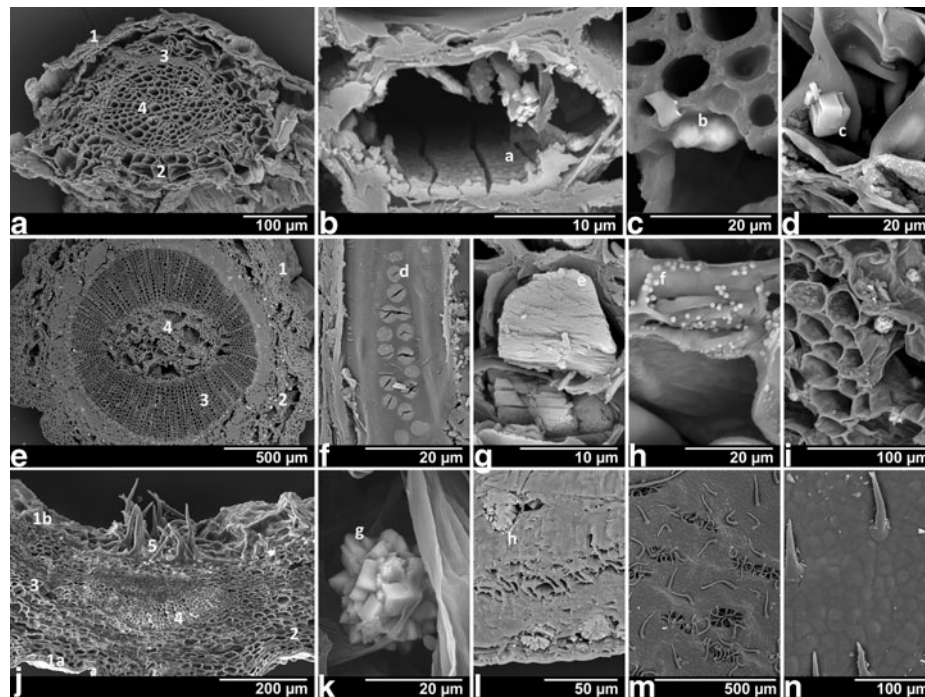

Fig. 3 Representative SEM micrographs of *N. oleander* from set T1 (EA water plants). **a** Transversal section of a root (*1* epidermis, *2* cortex, *3* endodermis, *4* central cylinder). **b** Root central cylinder bundle cell with Fe, S, K, and P coating. **c** Fe accumulation in the root cortex. **d** Mg crystals in root cortex. **e** Transversal section of a stem (*1* epidermis, *2* cortex, *3* central cylinder, *4* pith). **f** Stem vascular vessel tracheas collapsed with Fe deposits. **g** Detail of a collapsed stem central cylinder cell. **h** Ca crystals in the stem epidermis. **i** Ca druses inside the pith. **j** Transversal section of leaf blade (*1a* adaxial epidermis, *1b* abaxial epidermis, *2* palisade parenchyma, *3* mesophyll, *4* vascular tissue, *5* stomatical cavity). **k** and **l** Druses and location under the blade epidermis. **m** and **n**: abaxial and adaxial epidermis surfaces. *Lower case letters* indicate the location of the EDX analysis depicted in Fig. 5
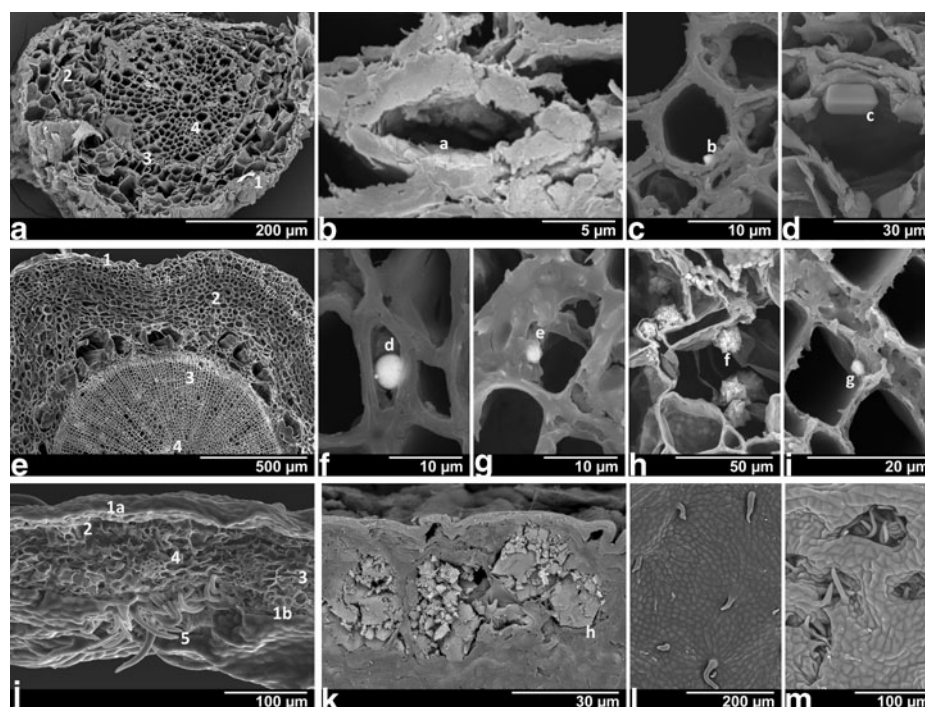
Fig. 4 Representative SEM micrographs of *N. oleander* from set T2 (LEA water plants). **a** Transversal section of a root (*1* epidermis, *2* cortex, *3* endodermis, *4* central cylinder). **b** Root central cylinder bundle cell with Fe, S, K, Ca, and P coating. **c** Particle of Fe attached to a cell wall in the central cylinder of the root. **d** Mg and K prismatic crystal in the root cortex. **e** Transversal section of a stem (*1* epidermis, *2* cortex, *3* central cylinder, *4* pith). **f** Fe sphere in the stem central cylinder. **g** Fe particle attached to the cell wall in the stem central cylinder. **h** Ca druses inside the pith. **i** Si particle in the central cylinder. **j** Transversal section of leaf blade (*1a* adaxial epidermis, *1b* abaxial epidermis, *2* palisade parenchyma, *3* mesophyll, *4* vascular tissue, *5* stomatical cavity). **k** Ca druses under the leaf adaxial epidermis. **l** and **m** adaxial and abaxial epidermis surfaces. *Lower case letters* indicate the location of the EDX analysis depicted in Fig. 6

blade under both adaxial and abaxial epidermis, and around the vascular bundle (Figs. 3l, 5h and 4k, 6h). No differences between EA and LEA plants were observed.

The other more frequently observed element in the SEM study was Mg, forming prismatic crystals in the root and stem cortex occasionally with Ca (Figs. 3d, 5c and 4d, 6c). It was also found in other metal crystallizations with Fe and Si. In the last stages of the experiment more Mg crystals were detected. No differences between EA and LEA were observed.

Other elements like Si, Al, P, K, Cl, and Na were detected in the EDX analysis. K, Cl, and Na usually appeared as background elements in all the plant tissues with C and O. Potassium appeared frequently in a low percentage as a component of other metal crystallizations. Occasional NaCl crystals were observed in roots, and crystals of Si and Al were found in the epidermal cells of roots and stems (Figs. 4i and 6g).

Sets C1 and C2: Control Plants

The SEM observations did not show any accumulations beyond Ca druses in the palisade parenchyma tissues of the leaf blade under both epidermis, and in the stem cortex and pith (Figs. 3j, m, n and 4j, l, m). Ca druses were equally distributed between set groups.

Discussion

N. oleander showed the same pattern of absorption in time for all the studied metals (Fe, Zn, Cu, Mn), although this pattern varied depending on the origin of the plants (EA or LEA waters). This may imply that *N. oleander* has similar absorption mechanism for all these elements.

Although no differences ($p > 0.05$) in total metal concentration were found within the two plant groups (EA and LEA) at the end of the experiment, from the first week the progression of the concentration in time showed a different pattern of absorption, being the LEA plants the group that suddenly reduced the absorption rate. This fact may indicate a difference in tolerance between both groups, as reported in Qureshi et al. [12]. But unlike non-tolerant individuals, in our experiment the LEA plants started increasing its metal concentration after four weeks of treatment, reaching similar values as the EA plants.

The EA water plants showed a linear increase of the metal concentration proportional to the concentration of elements added in the solution. The LEA plants did not present that linear relation, because of a sudden stop of the metal uptake during the first 3 weeks, before start increasing its metal concentrations. Taking that in consideration, we could say

Fig. 5 EDX spectra of *N. oleander* from EA water corresponding to *lower case letters* in Fig. 3
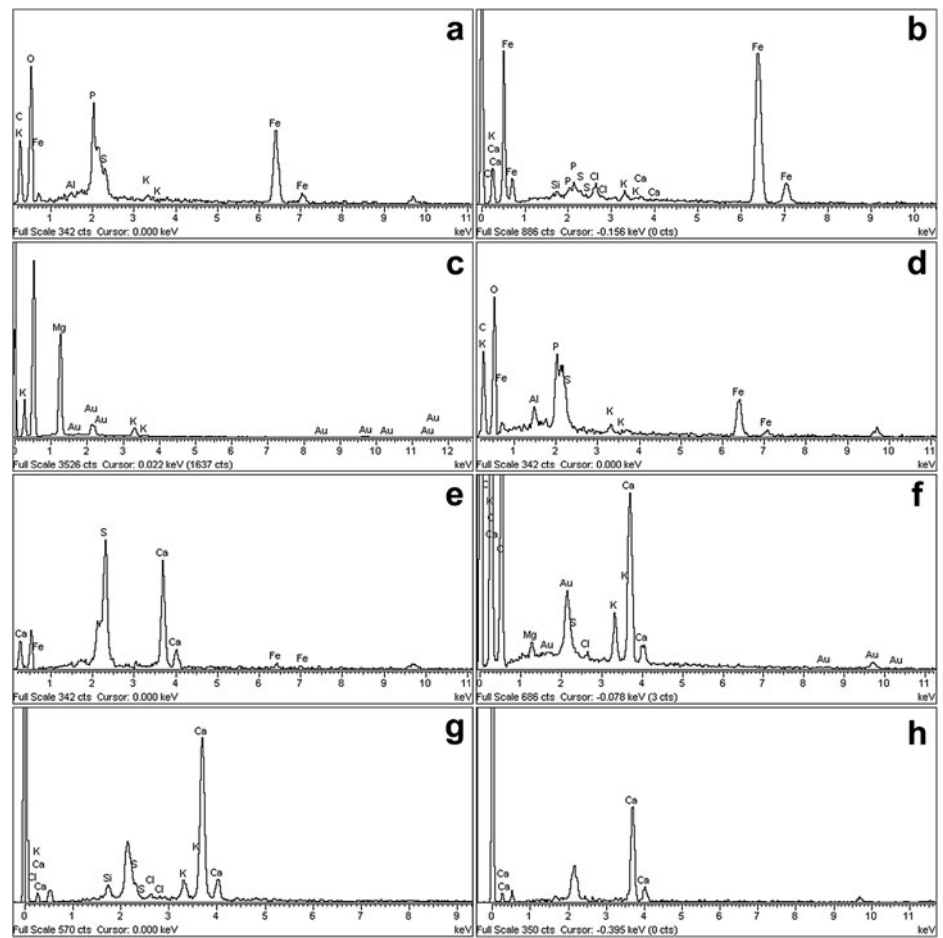
that the continuous exposure to the extreme conditions of the SMS caused the expression of some ecological adaptations to the highly acidic and metal enriched environment in the LEA plants, already present in EA plants from the beginning.

Although the chemical analysis indicated different absorption processes between groups, the appearance and growth were identical for both groups. From the first week until the fourth, the plants showed a normal growth of roots, stems and leaves, but from that point until the end, both groups showed a progressively degradation of the aerial tissues, losing leaves, buds, and upper sections of the stem. The roots and lower sections of the stem did not show signals of deterioration. The roots continued growing until the end of the experiment.

Comparing with the elemental concentrations for natural oleanders reported by Franco et al. [10], the concentration values for Mn, Zn, Ca, and Mg at the beginning of the experiment were in the same range of that of field-grown oleanders, while Fe and Cu concentrations were slightly lower. Shortly after putting into treatment, the absorption of Mn, Zn, Mg, Fe, and Cu exceeded that of field-grown oleanders. That difference in concentrations of metals with the

natural populations could be due to the fact that experimental conditions do not completely reflect real field conditions, such as plant phenology, soil chemistry, or seasonal bioclimatic changes and its interactions.

Even from the first week of treatment, the Fe concentration was higher than the normal values for vascular plants (600–2,500 mg/kg) and for plants living in metal rich soils given by Reeves and Baker [13]. At the end of the experiment, the concentration reached values as high as those of other species known for their Fe accumulation capacities like *Imperata cylindrica* (L.) P. Beauv in the Río Tinto [14, 15]. This is common to both EA and LEA water plants.

The SEM observations showed that Fe is generally presented as deposits, composed mainly of Fe, K, S, and P, found in the interior of the vascular cells of stem and roots. It also appears as an amorphous thin layer in the surface and inside the epidermal cells. The composition of this incipient plaque was also Fe, S, K, and P, with traces of Si and Ca. In previous studies on field-grown oleanders, we found a thicker iron plaque composed mainly of Fe, Si, and Al covering the root and lower sections of stem surfaces in which P was not detected [10]. The same difference in composition was found in the external plaque of *Phragmites*

Fig. 6 EDX spectra of *N. oleander* from LEA water corresponding to *lower case letters* in Fig. 4
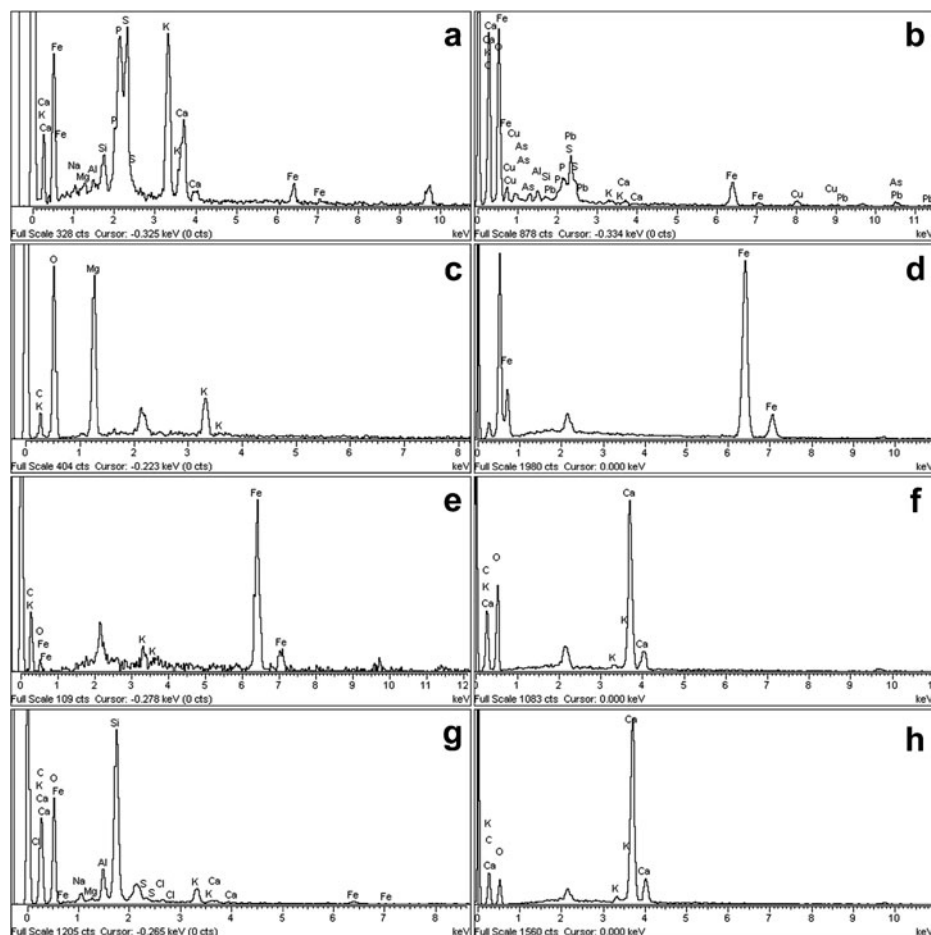
australis (Cav.) Steud. grown in laboratory conditions [16]. These authors explained that the presence of P in the experimental plants coating may be due to P adsorption, and it is not observed in the field plants due to the lack of P in acidic mine drainage environments. This composition of the initial plaque on roots of young individuals indicates that the initial stages of the plaque are due to a biological process of Fe biomineralization forming Fe oxides and hydroxides. Later, other elements as Si and Al in the field and K, S, and P in the laboratory are included in the plaque structure as long as they are available in the environment.

The Cu concentration exceeded the normal values given by Reeves and Baker [13] for vascular plants and for plants living in metal enriched soils (25–100 mg/kg). At the end of the treatment the concentration reached values of hyperaccumulation in both EA and LEA water plants. This fact does not correspond with the field observations of Mingorance et al. [8], and our previous studies of *N. oleander* in the Río Tinto [10, 17]. In the microscopy study, Cu was found together with Fe and P deposits in vascular cells of both roots and stems and over the plaque patches in the epidermis. It was also found occasionally with Ca oxalate crystals in the stem cortex.

In EA plants, Mn and Zn concentrations exceeded normal values (400–2,000 mg/kg in both cases) from the second and the first week respectively, but did not reach accumulation values along the experiment. In the LEA plants, Mn and Zn concentration were higher than normal values for vascular plants from the fifth and fourth week respectively, but also did not reach accumulation values at any point of the experiment. In the microscopic observations, Mn and Zn were found associated with Fe deposits in root central cylinder vascular vessels and stem cortex. In Franco et al. [10], we observed Mn and Zn particles also associated with Fe in LEA field-grown plants, but in that case those particles were included in the iron plaque.

Magnesium slightly exceeded the normal values for vascular plants in the last 2 weeks for the EA plants but stayed in normal values for the LEA plants during all the experiment. Its initial concentration was higher than other elements because as shown in Mingorance et al. [8] and Fuente et al. [17], it is a macronutrient normally present in high concentrations in *N. oleander*. We found Mg forming prismatic crystals alone or with Ca in the root and stem cortex parenchymatous cells. Those crystals were also observed in the field-grown oleanders in Franco et al. [10]. These crystals

could be magnesium oxalate as the ones observed by Monje and Baran [18] in *Opuntia ellisiana* Griffiths.

There was a slight increase in Mg and Cu concentration in both control sets probably due to the element concentrations in the tap water used as control solution. Both Mg and Cu are nutrients and thus are needed by the plant, and if available, the plants will uptake them until certain levels. In either case (C1 and C2 set groups) the concentrations did not exceed normal values for vascular plants and no accumulations of these elements were observed in the SEM analysis.

Calcium concentration, as reference for a nutrient not added during the experiment, showed the same pattern for EA and LEA water plants. This fact may indicate that Ca concentration decrease shown in the IPC-MS analysis was due to the lack of Ca in the substrate and the solution while the plant grows. Calcium is an important macronutrient that constitutes a part of the cell wall and other cellular structures, and is involved in transport processes and root development. SEM observations showed that the initial presence of a great quantity of druses in the stem cortex and pith was later substituted by a great quantity of smaller prismatic crystals and less and smaller druses. That may be related with a Ca reutilization mechanism in low Ca substrates described by Nakata [19]. We also observed Ca rich plaques and accumulations covering the cell walls of the stem cortex parenchyma, together with other elements as Cu or Fe, mainly in the initial stages of the experiment. As said in Mazen [20], Ca could be used as an immobilization mechanism for metals. That seems to be this case, at least while the concentration of Ca was still high enough.

Rodríguez et al. [14] found Fe biominerals inside *I. cylindrica* and characterized them reporting the presence of jarosite and ferritine. In previous works, we studied metal accumulation in *Cynodon dactylon* (L.) Pers. submitted to an experimental treatment and found that this species also seemed to create internal bioformations, mainly Fe oxides (Unpublished results). As in the case of *C. dactylon*, the Fe bioformations we found in both *N. oleander* EA and LEA plants are likely to be of a different mineral composition, as neither the EDX spectra nor the form of these accumulations match with the bioformations found in *I. cylindrica*.

This study has assessed the kinetics of Fe, Cu, Zn, Mn, and Mg absorption, transport, and bioformations in seedlings of *N. oleander* under a controlled laboratory experiment. High rates of Fe and Cu were absorbed despite the wild oleanders are Fe and Cu excluders as we mentioned in Franco et al. [10], a fact that is more related to the results obtained for the first weeks of the LEA water plants.

Those results could be of interest from a technical reclamation perspective. Doležalová et al. [21] said that primary spontaneous successions generate preferable environments and more biodiversity enriched ecosystems. As this study is related to extreme and toxic substrata, spontaneous successions may not be effective [22]. We propose that using autochthonous vegetation in mine landscape reclamations minimizes the impact, and at the same time enriches the landscape without altering the ecosystem. According to the results obtained in the experiment we can conclude that young *N. oleander* plants could be used indistinctively in Fe and Cu phytoextraction and blastofiltration in extremely acidic environments like the acidic mine drainages. This is supported by the easy germination and the relatively fast growth of this species, and adds another valuable use to the species, as grown oleanders are good candidates to phytostabilization. But if adult plants are to be used in restoration projects, it is necessary to ensure that the medium has sufficient nutrient levels to favor the development of the plant and the production of greater aerial biomass. Nevertheless, further studies should be taken to explain in detail the different absorption rates between EA and LEA plants.

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