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Mediterranean Journal of Chemistry

Mediterranean Journal of Chemistry **2022**, 12(1), 11-18

Lead uptake, flavonoids, and proline relationship in Atriplex nummularia growing in a galena mining area

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Abstract: Phytoremediation is an emerging cost-effective remediation technology that uses plants to remove metals from contaminated soils. Average levels of Lead (Pb), total flavonoids content (TFC), and proline were assessed in 108 samples of Atriplex nummularia growing around an active mining area in southeastern Morocco.

From January to December 2018, three different locations were sampled within a radius of 0 to 800 meters from the central ore storage area to monitor the evolution of these content levels. These results were compared to a control group of plants.

Lead uptake ranged between 16-270 ppm and was found to be irregular during the sampling time frame or within the geographic location. Measurement results showed the expected effect of metal uptake on flavonoids and proline content. Thus, high levels of lead are likely correlated with increasing proline (r = 0.949**) and decreasing flavonoids (r = 0.972**) concentrations, respectively.

Keywords: Atriplex nummularia; phytoremediation; proline; lead uptake; flavonoid.

1. Introduction

Excessive heavy metal concentration from contamination of the environment represents an aggressive risk to human, animal, and plant health [1]. Several industries are responsible for this contamination, such as mining and the use of leaded paints and oil industries [2]. In plants, for instance, metal toxicity affects various physiological processes such as photosynthesis activity [3], nitrogen metabolism, and nutrient uptake [4].

Green plants were used for the rehabilitation of contaminated soils plants by reducing heavy metal contamination in a technique referred to as phytoremediation. This offers an alternative to engineering techniques of soil recovery, providing a cost-effective replacement that conserves soil structure and has minor adverse impacts on the environment [5]. Lead (Pb) is considered one of the heavy metals of environmental concern, although some remediation research is focused on this pollutant [5]. Although chemical and physical techniques to eliminate lead from soils have been developed [6], they are expensive and only applicable to small areas.

However, the efficiency of phytoextraction is limited by the low mobility and bioavailability of some heavy metals (especially Pb) in polluted soils [7]. Furthermore, most of the recent Pb-phytoextraction studies focused on fast-growing crops (e.g., Brassica juncea, Zea mays, Helianthus annuus) with high biomass yields combined with the enhancement of heavy metal mobility and bioavailability through the addition of synthetic chelators like EDTA [8].

Among metal-absorbing plants, the halophyte Atriplex genus was widely studied in laboratory experiments and showed an ability to absorb either lead (Pb) or cadmium (Cd) [9].

Halophytic plants have also been testified to tolerate and accumulate significant heavy metal levels in their tissues by triggering detoxifying/sequestering mechanisms [10]. A. nummularia is naturally present in an environment characterized by an excess of toxic ions and can accumulate large amounts of salts in harvestable parts.

In many plants, adverse environmental effects (such as dry weather conditions and heavy metal stress) cause the accumulation of proline levels, one of the universal polyfunctional stress-protective substances. Proline accumulates upon exposure to heavy metals such as lead and is thought to be involved in specific stress resistance mechanisms. The ability of plants to get proline under heavy metal stress could be caused by a direct effect of Pb ions and water deficiency [11].

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DOI: http://dx.doi.org/10.13171/mjc02204121619merzougui

Received January 26, 2022
Accepted March 22, 2022
Published April 12, 2022

Furthermore, few scientific studies have evaluated the correlation between the effect of Pb in soils on flavonoids content and its absorption by plants.

2. Material and methods

Therefore, this work aims to evaluate the effect of metal (Pb) uptake on total flavonoids content (TFC) and proline amount. In order to find a possible correlation between Pb-proline concentration and Pb-TFC concentration, a set of108 samples of A. nummularia growing around an active mining area within 0-800 meters of the central ore storage was monitored. These results were compared to those of a control group of plants.The study compares the evolution of monthly results (from January to December 2018) in A. nummularia at the entrance of an active galena mine in the southeastern region of Morocco (Tafilalet). These results were also compared to a control group containing a few A. nummularia plants growing far from the mining area.

2.1. Instrumentation and solutions

For background correction, all the atomic absorption measurements were carried out with UNICAM 929 with a Deuterium (D2) lamp. Lead was determined by the flame absorption atomic spectroscopy (FAAS) in an acetylene-air flame. All chemicals used were of analytical-reagent grade. All solutions were prepared with deionized water; glass was kept overnight in a 10% (V/V) HNO_3 solution and then rinsed with deionized water.

2.2. Sampling locations

The location of interest in this study is an active galena mining area located 800 meters from Hassan Addakhil dam in Errachidia, a semi-arid region located in southeastern Morocco. The mine has the following GPS point 32° 2’ 15, 26” N; 4° 27’ 0,6” W.

The plant A. nummularia was implanted in the region of Tafilalet by the High Commission for Water and Forests and the Fight against Desertification [12].

In this study, we evaluated the effect of Pb on three halophyte A. nummularia growing at the different points around a galena mining field. The result was the mean of three analyses; **location A** represents the mean of A_1, A_1’ and A_1’’, **location B** for A_2, A_2’ and A_3’’and the **location C** mean of A_3, A_3’ and A_3’’ (Figure 1**)**. In addition, we supervised lead content, flavonoids, and proline as plant responses for over a year, from January 2018 to December 2018.

Only aerial parts (leaves) of plants were examined. Locations A, B, and C are 100, 400, and 800 meters from the mine. 108 samples were taken from different sampling points over twelve months, as shown in Table 1.

Table 1. Monthly collection of samples from the studied locations.

	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sept	Oct	Nov	Dec
Location A	3	3	3	3	3	3	3	3	3	3	3	3
Location B	3	3	3	3	3	3	3	3	3	3	3	3
Location C	3	3	3	3	3	3	3	3	3	3	3	3

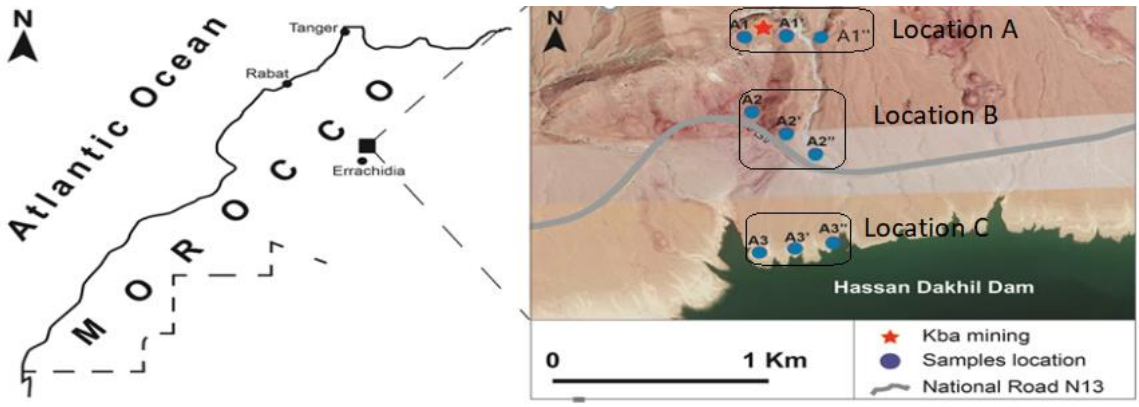

Figure 1. Map showing locations and sampling points of A. nummularia

2.3. Preparation of methanolic extracts

The leaves of A. nummularia were collected, washed twice with deionized water, dried at 55°C, and finally crushed by an agate stone mortar.

Plant material of A. nummularia was prepared using the ultrasound method; 2.5 g of leaves were dried and crushed, then placed in a 500 mL pointed flask where 25 mL of methanol was added. The flask and its contents were immersed into a 40 kHz bath and sonicated for 30 min [13]. The temperature was kept at 30°C. After that, the methanol extract was filtered by a Whatman filter (Millipore 0.45 µm) to eliminate particulate matter [14].

2.4. Total flavonoid content (TFC)

Total flavonoid content (TFC) of dried A. nummularia extract was spectrophotometrically determined by the aluminum chloride $AlCl_3$ method using quercetin as a standard [15]. Two milliliters of each extract (1mg/mL) were added to 2 mL of the $AlCl_3$ solution (10% in ethanol), and the mixture was vigorously agitated. After 10 min of incubation, the absorbance was measured at 430 nm. To quantify flavonoids, a calibration curve was established using quercetin (0-40 mg/l). The TFC was expressed in milligrams of quercetin equivalent per gram of extract (mg EQ/g of extract).

2.5. Calcination to determine Pb by FAAS

1 g of the powder sample was measured in a porcelain container, put in a muffle oven, and heated from room temperature to 450°C for 3 hours with a ramp rate of 2°C/min to enhance calcination. Then the white residue was dissolved in 1 mL cont. HCl, 1M HNO_3 was added, after filtration into a 25 mL measuring flask, the volume was made up to the mark with 1M $HN0_3$ "solution A" [16]. Low concentrations' analysis consists of preconcentrating the metal ions using cloud point extraction. This method was used with a calibration curve (0-20 mg/l) using lead nitrate to prepare different concentrations of Pb^{2+} [17].

The cloud point extraction method was developed for concentrations less than 100 mg/kg. After neutralization with 1M NaOH solution 10 mL of "solution A" was placed in a glass-centrifuged tube. The pH was then adjusted to 8.5 by adding 5 mL of phosphate buffer, then adding Na_2SO_4 to form the cloud point of Tween 80. The tube was heated at 65°C for 60 min after adding three milliliters of Tween 80 (4%, w/w) solution. This turbid solution was then cooled for 20 min at 4°C in a refrigerator to promote decantation. Finally, the solution was centrifuged for 10 min at 3500 rpm. Surfactant rich phase was separated from the aqua phase by simple decantation, which was dissolved with 1.0 mL 1M HNO_3 in methanol to decrease the viscosity. The final volume was made up to 5 mL with 1M HNO_3 solution. The metal concentration of the final solution was determined by flame atomic absorption spectrometer using a calibration curve of $Pb(NO_3)_2$ (0-8 mg Pb/l) [18].

2.6. Amount of proline

The amount of proline was calculated using ninhydrin reagent according to Alia et P. P. Saradhi procedure [19]. 2 ml of glacial acetic acid and 2 mL of ninhydrin reagent (2.5% of ninhydrin in glacial acetic acid and 6M orthophosphoric acid 6:4 v/v), were added to 2 mL of sulfosalicylic acid extract. Then the solution was incubated at 100°C for 1 hour and cooled for 30 min at +4°C in a refrigerator [20].The color developed was extracted with 4 mL of toluene and read at 520 nm. The amount of proline was calculated from a standard graph. The amount of proline in the sample was expressed in mg [proline]/g of dried weight, a calibration curve (0-20 mg/l) of D-Proline was used [21].

2.7. Statistical Analysis

Statistical analysis was performed using GraphPad Prism 8 software. The standard error (SE) of all experiments was represented in the tables (mean ± SE). Statistical significance was determined using the analysis of variance (one-way ANOVA) with a significance level of 0.05.

3. Results and discussions

3.1. Concentration of lead (Pb)

Pb concentration decreases significantly as the plant is far away from the ore storage area (Figure 2). About a mean of 252 mg Pb/Kg (DW) at the mine site to about 28 mg Pb/Kg (DW) at 800 m. Therefore, it remains approximately at constant levels close to the mean value in "B" and "C" (Figure 3). Location "A" may have its roots in direct contact with mineralized veins, and that explains the high concentrations values of the Pb.

The concentrations observed on plants in different locations "B" and "C" are below 71 mg Pb/Kg (DW), and are less irregular than "A". Therefore, Pb root absorption may be explained by the underground mineral infiltration lead process. Indeed, the vertical drop between the ore deposit area and plants in locations "B" and "C" is about 300 meters.

Concentration levels of location A were found to be very significant over the year compared to locations B and C. This could stem from the fact that aerial parts of these plants are often exposed to dust produced during irregular exploitation work. Therefore, some foliar absorption mechanism of Pb may perhaps behave occurred.

These results were compared to a group of control in which we found a mean of about 6.62 ± 0.82 mg Pb/Kg (DW). A significant difference was found (**$p<0.05$**) between each location (A, B, and C) compared to the control using the one-way ANOVA test. This result showed that the absorption of lead varies significantly when moving away from the mine, due to bioavailability of Pb in the soil.

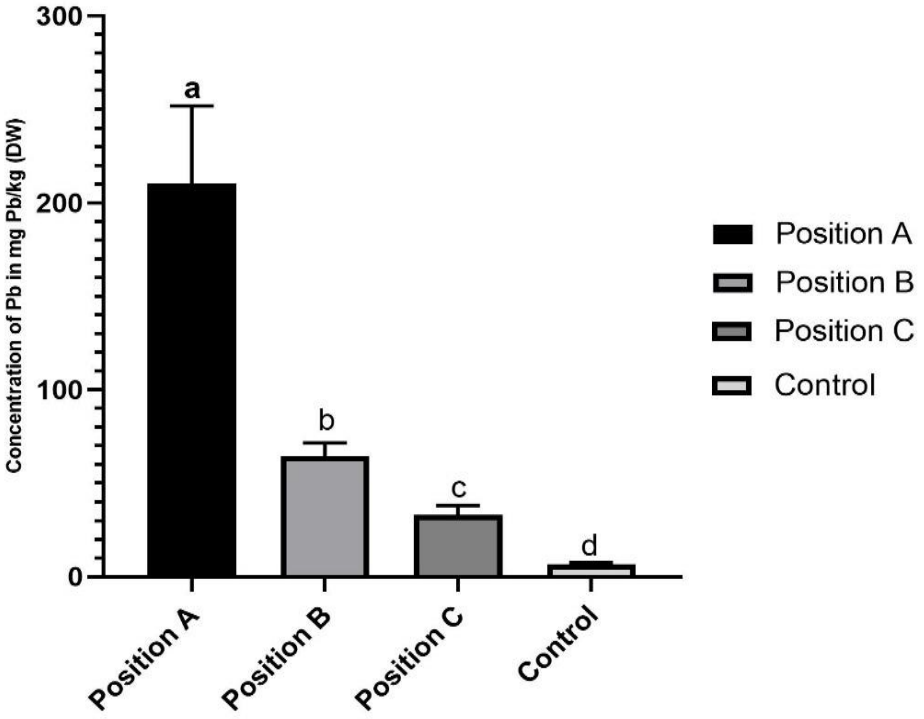

Figure 2. Variation of Pb concentration in different locations noted location A, location B and location C compared to the control

Figure 3. Irregular values of Pb uptake in A. nummularia during a year

3.2. Flavonoid concentration

The observed mean of flavonoid content increases from 17.40 mg EQ/g of extract for location "A", to 29.08 mg EQ/g of extract for "C" located at 800 meters. This variation may be related to Pb bioavailability in soil factors that affect the flavonoid concentration [22].

The level of flavonoids remains constant; however, it reaches the highest value in March, the flowering period, explained by the secondary metabolite development during this period [23]. On the other hand, the control shows a constant value during the harvest period.

Table 2. Total flavonoids content (TFC) in A. nummularia in mg equivalent of quercetin per gram of extract (mg EQ/g of extract); data are given with mean ± standard deviation (mean ± SD).

	TFC in location A (mg EQ/g of extract)	TFC in location B (mg EQ/g of extract)	TFC in location C (mg EQ/g of extract)	TFC in control samples (mg EQ/g of extract)
Jan	17.42 ± 1.01	22.35 ± 2.1	27,27 ± 2,98	45
Feby	17,63 ± 2,72	24,85 ± 0,05	23,57 ± 2,21	39
Mar	**19,57 ± 3,23**	**32,86 ± 2,39**	**40,82 ± 1,68**	**42**
Apr	18,34 ± 3,53	25,58 ± 0,02	36,29 ± 3,14	39,4
May	18,33 ± 2,48	26,85 ± 3,35	30,36 ± 0,96	42,2
Jun	16,51 ± 1,58	23,30 ± 3,95	31,77 ± 1,52	38,5
Jul	16,96 ± 4,46	20,9 ± 2,66	27,18 ± 4,6	44,05
Aug	17,3 ± 1,10	24,14 ± 2,31	27,8 ± 1,61	49,6
Sept	15,97 ± 1,79	25,23 ± 2,45	25,12 ± 5,13	55,15
Oct	14,74 ± 3,19	23,65 ± 1,18	23,87 ± 3,73	40,7
Nov	17,55 ± 1,80	22 ± 2,32	28,35 ± 0,26	46,25
Dec	18,52 ± 3,57	26,29 ± 1,46	26,55 ± 3,94	51,8
Mean	17,40	24,83	29,08	44,47

3.3. Proline content

In this study, the stress response of our candidate plants in different locations was quantified. The results show important values 1.64 ± 0.4 mg/g (DW) in June for the samples close to the mine "location A" with a mean of 1.37 ± 0.41 mg/g (DW), compared to the other's location, which the amount of proline was found about 1.06 ± 0.06 mg/g (DW) at 400 m with a mean of 0.81 ± 0.095 mg/g (DW) as shown in Table 3. As expected, the amount of proline decreases significantly as the plants are further away from the ore storage area (Figure 4) and have a different significant **p<0.05** from the control sample using a one-way ANOVA test. Compared to other studies, they show higher proline concentrations for plants under lead (Pb) stress [24].

A correlation of r = 0.949** was found between proline content and lead uptake. The stress production explains this in sampled plants. In general, the amount of proline observed in unstressed A. nummularia was 0.2 mg [proline]/g (DW), the stressed one ranges from 0.4 to 2.1 mg [proline]/g (DW) [25]. These results were compared to a blank sample where the proline level is 0.35 ± 0.018 mg [proline]/g (DW). This confirms that Pb is the main reason for this stress production in the present study.

Proline, an amino acid, plays a vital role in plants. It protects the plants from various stresses and helps plants recover from stress more rapidly. In addition, when applied exogenously to plants exposed to stress, proline increases plants' growth and other physiological characteristics [11].

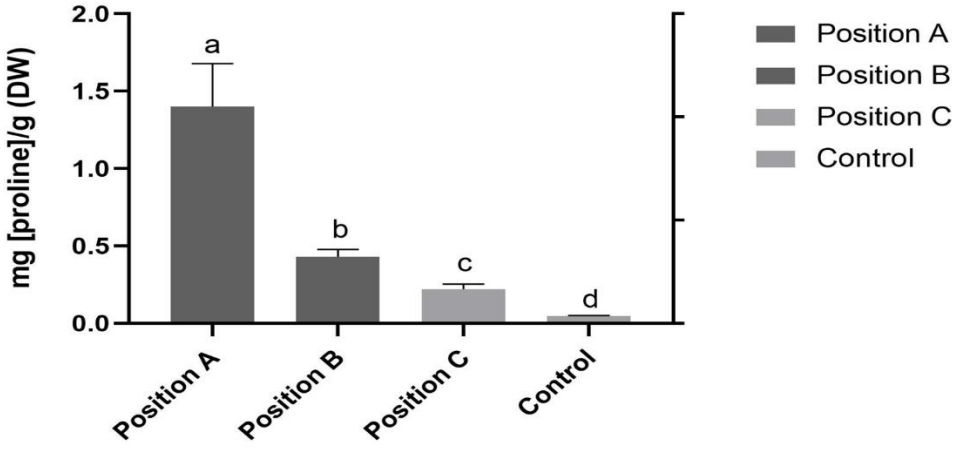

Figure 4. Amount of proline in mg/g (DW) during a year in A. nummularia

Table 3. Amount of proline (mean ± SD) in mg [proline]/g (DW) in three different locations during a year in A. nummularia.

	Proline in location A (mg/g (DW))	**Proline in location B (mg/g (DW))**	**Proline in location C (mg/g (DW))**	**Proline in control samples (mg/g (DW))**
Jan	1.51 ± 0.32	0.95 ± 0.01	0.76 ± 0.046	0.23
Feb	1.31 ± 0.46	0.75 ± 0.03	0.59 ± 0.11	0.19
Mar	1.09 ± 0.39	0.82 ± 0.13	0,39 ± 0.065	0.15
Apr	1.20 ± 0.30	0.76 ± 0.06	0.83 ± 0.1	0.32
May	0.97 ± 0.16	0.78 ± 0.09	0.77 ± 0.06	0.28
Jun	1.64 ± 0.40	0.95 ± 0.26	0.66 ± 0.095	0.30
Jul	1.41 ± 0.55	0.62 ± 0.17	0.41 ± 0.095	0.33
Aug	1.35 ± 0.42	0.56 ± 0.16	0.49 ± 0.07	0.35
Sept	1,51 ± 0.4	0.88 ± 0.01	0.72 ± 0.035	0.38
Oct	1.54 ± 0.59	0.64 ± 0.20	0.5 ± 0.06	0.40
Nov	1.44 ± 0.48	1.06 ± 0.06	0.5 ± 0.05	0.42
Dec	1.49 ± 0.51	1.02 ± 0.02	0.65 ± 0.04	0.44
Mean	1.37 ± 0.41	0.81 ± 0.095	0.60 ± 0.02	0.31

The linear correlation coefficient is a measure of the strength and the meaning of an association. Varying from -1 to +1, it is equal to 0 when there is no association. As more as this coefficient is close to -1 or +1, the stronger the association between the two variables, until it is perfect [26].

A good correlation was found r = 0,949**** between lead uptake and the amount of proline in different sampling locations, using the Pearson correlation test and Table 4.

Table 4. Pearson coefficient and possible correlation between different parameters Pb concentration, proline and TFC.

		Pb concentration	**Proline**	**Flavonoids**
Pb concentration	**Pearson correlation coefficient**	1		
	N	9		
Proline	**Pearson correlation coefficient**	**,949****	1	
	N	9	9	
Flavonoids	**Pearson correlation coefficient**	**-,972****	**-,930****	1
	N	9	9	9
****. The correlation is significant at the 0.01 (two-tailed) level.**				

Another correlation was found in this study r = 0,972** between TFC and Pb concentration; this correlation shows that as more as the plant is far away from the mine location, the TFC increases significantly.

Accumulation of proline has been observed in plant species when present under various types of stress. As reported in this study, as more as Pb concentration is high, the amount of proline increases due to heavy metal stress (Pb-Stress).

Heavy metal accumulation depletes low molecular weight antioxidants, such as glutathione, consumed under phytochelatin formation [27].

The majority of studies show that the level of flavonoids increased under metal stress [28], distinct from our research, which lasted a year to find a different result showing the decrease in the level of flavonoids under lead stress in our case.

4. Conclusion

This study showed a direct correlation between the increased lead concentration and proline compared to TFC. In addition, this study revealed a correlation between the plant location and the chemical uptake of the plant's concentration. The concentration of lead and proline increased significantly when getting nearer to the mine. Whereas TFC showed the opposite trend, these results were confirmed by a control group located in a different place from this study's locations, which showed a higher average of TFC and a lower average of proline and lead.

Acknowledgment

This work was conducted in the Laboratory of the research team: Chemical Analysis, Materials and Environment in the Faculty of Sciences and Technologies of Errachidia. The authors would like to thank the High Commission for Water and Forests and the Fight against Desertification in Errachidia for offering samples of the control plants of A. nummularia.

Formatting of funding sources

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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