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Evaluation of Physiological and Biochemical Parameters and Some Bioindicators of Barium Tolerance in *Limbarda crithmoides* and *Helianthus annuus*

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Abstract: Soils and water resources of our ecosystems may contain Barium (Ba), a toxic metal naturally existent in the Earth's crust and also can be derived from recycled wastes produced of several anthropogenic activities. As a result of this fact, the accumulation of Ba in agriculture soils would increase to reach the crops and eventually end up in the human food chain. The purpose of this work was to study tolerance and accumulation abilities in *Limbarda crithmoides* and *Helianthus annuus* treated with increasing concentrations of barium (from 0 to 500 µM) for 45 days. In order to evaluate the response of these species to Ba stress, the biomass production, the water status, and the accumulation of the secondary metabolites, macronutrients, total inorganic nitrogen (TIN), and Ba in shoots and roots, as well as chlorophyll levels, and metal tolerance index of the entire plant were assessed. Results showed an increase in plant biomass production and tolerance index in the two species with increasing Ba concentration. A significant increase in polyphenols and flavonoids levels was also shown with no negative effect on the macronutrients and TIN; however, the latter were found reduced in roots of *L. crithmoides*. Chlorophylls also were not affected. An average of 3000 µg·g⁻¹ DW of Ba was accumulated in each organ of *L. crithmoides* while *H. annuus* accumulated up to 1350 µg·g⁻¹ DW in the shoots. Our findings proved that *L. crithmoides* and *H. annuus* were susceptible to tolerate Ba-induced stress with high levels of Ba accumulation in the aboveground parts as well as in the roots during the 45 days of the experiments.

Keywords: barium stress; biomass production; tolerance; chlorophylls; macronutrients; polyphenols



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1. Introduction

Barium (Ba) is one of the alkaline trace metallic elements (TME) that constitutes roughly 0.05% of the Earth's crust and is found present in the environment in relatively high levels [1,2]. Barite (barium sulphate) and witherite (barium carbonate) are the main common barium compounds that can be found in nature, specifically in sedimentary rocks, as underground ore deposits [3,4]. Due to the low water solubility of these two compounds, Ba is not predicted to be very mobile in soils, unless in acid soils [2,5,6]; this property may reduce the environmental risk in aerated soils [7]. However, the most harmful forms of water soluble barium compounds are barium acetate, barium hydroxide, barium sulphide, barium nitrate, and barium chloride, which are abundantly used in several industries [3].

Generally, Ba is used in wide production fields including glass, bricks, cement, ceramics, electronics, soaps, fluorescent lamps, insecticides, fertilisers and soil amendments, plastic stabilisers, railroad flares, paints, fireworks, explosives, fine chemicals, drilling fluids, lubricating oil additives, sugar refining, and paper coating [2,3,8]. Barium could be also

discharged in wastewater from industrial and metallurgical processes, and the resulting wastes such as drilling fluids waste or mud are frequently disposed in farmlands [2,9]. The increased availability of Ba in various anthropogenic fields may induce an inappropriate accumulation of this metal in our ecosystems to reach water and agricultural soils [10]. It has been mentioned that the concentration of Ba in agricultural soils ranges from 10 to 5000 mg·kg⁻¹, with an average abundance of about 500 mg·kg⁻¹ [3].

As an essential element for all living organisms including humans, animals, and plants, Ba exposure can be toxic in high concentrations [11]. Human ingestion of Ba may induce various health issues including cardiac and renal failure, pulmonary edema, respiratory paralysis, and gastric and intestinal haemorrhages; Kravchenko et al. also stated that the consumption of BaCl₂ causes vomiting, diarrhoea, liver and kidney failure, cardiac arrhythmia, anxiety, disorders of the nervous system, brain swelling, and even paralysis [12]. However, few studies have been performed to investigate the effect of barium on plant species and information concerning Ba phytotoxicity is also limited [10].

Marisamy et al. reported that photosynthetic pigments, including chlorophylls a and b and carotenoids, decreased in *Amaranthus caudatus* L. and *Cyamopsis tetragonoloba* L. with the increase of Ba concentrations in the growth medium; moreover, inhibitory effects of Ba on the growth of these two species were noted where root and shoot lengths, leaf area, and fresh and dry biomass productions were significantly reduced [13,14]. On the contrary, other plants grown in Ba-contaminated medium showed adaptation abilities to elevated Ba concentrations throughout the enhancement of plant biomass and the accumulation of high Ba amounts in the plant's tissues, such as *Typha domingensis* [7], mustard, sunflower, and castor bean [15].

It has been mentioned that numerous halophytes are capable of tolerating metallic stress [16]. In this context, among halophilic species, *Limbarda crithmoides* L. (synonym *Inula crithmoides* L. Dumort.) is known for its extremely high tolerance to salinity [17] and was chosen in this study as the test halophyte to assess the behaviour of this plant against Ba-induced stress, when compared with *Helianthus annuus* (glycophyte). The present study was conducted to evaluate metal phytotoxicity and tolerance ability in these two plant species throughout the assessment of Ba effect on the growth, mineral nutrition, and chlorophyll synthesis and how antioxidants such as polyphenols and flavonoids were involved to defend against Ba stress. On the other hand, Ba contents were also determined, in the shoots and roots, to investigate barium accumulation potential in the present species and explore new Ba-hyperaccumulators species that could be used as an ecological alternative for the remediation of Ba-polluted soils.

2. Material and Methods

2.1. Plant Culture and Treatments Experimentations

Seeds of *Limbarda crithmoides* were collected from the north shore of the lagoon of Bizerte in Menzel Jemil, Bizerte, Tunisia (37°14'19" N, 9°54'59" E). Seeds of *Helianthus annuus* were obtained from a land farm of sunflower situated in the north of Tunisia (36°49'90" N, 9°13'77" E). Collected seeds were naturally dried before starting the culture.

Plants were grown in plastic pots containing a mixture of perlite and gravel substrate (2:1; v/v), in a semi-controlled greenhouse (natural photoperiod, temperature of 25 ± 5 °C, relative humidity ranging between 60% and 90%). After the germination, seedlings were regularly watered with Hewitt nutritive solution [18]. Subsequently, plants were divided into 5 groups of 10 plants and regularly treated with a volume of 100 mL/pot of nutritive solution supplemented with Ba at 0, 100, 200, 300, and 500 µM (until the water flowed out of the pot), 3 times a week for 45 days. Barium chloride (BaCl₂, Sigma-Aldrich St. Louis, CA, USA) was used as a source of Ba.

Shoot and root fresh weights were separately determined immediately after the collection of the treated plants, which were subsequently dried in the oven to constant weight (70 ± 2 °C). After the drying process, dry weights were taken to determine the water con-

tent and metal tolerance index [19–22]. Tolerance index and water content were calculated according to the following equations, respectively:

$$TI (\%) = \frac{\text{dry weight treated plants}}{\text{dry weight untreated plants}} \times 100, \quad (1)$$

$$WC (\text{mL} \cdot \text{g}^{-1}) = \frac{\text{fresh weight} - \text{dry weight}}{\text{fresh weight}}, \quad (2)$$

2.2. Chlorophyll Fluorescence

Chlorophyll *a* fluorescence parameters were measured using a FluoroPen FP100-MAX (Photon systems Instruments, Dràsov, Czech Republic) and the measurements were performed in *L. crithmoides* and *H. annuus* old leaves after 45 days of Ba exposure [23]. The maximum primary yield of photochemistry of Photosystem II (Fv/Fo), the maximum quantum efficiency of Photosystem II (Fv/Fm), the photochemical efficiency of Photosystem II (Fv'/Fm'), and the Electron transport rate (ETR) were determined. Absorbed (ABS), trapped (TRo), dissipated (Dio), and transported (ETo) energy fluxes per reaction centre (RC) were also measured [24].

2.3. Chlorophyll Content Determination

Chlorophyll *a* and *b* extraction was performed following Bankaji et al.'s protocol [25]. Briefly, approximately 250 mg of fresh leaves tissue were mixed in 10 mL of ethanol 80%. The prepared mixture was placed in a water bath at 80 °C for 40 min. The absorbance measurements of the extracts were read at 645 and 663 nm using a spectrophotometer (Genesys 10S UV-Visible, Thermo Scientific, Madison, WI, USA). Chlorophyll *a* and *b* concentrations were expressed in milligrams per gram of fresh weight (mg·g⁻¹ FW) and calculated following Ayvaz et al. [26].

2.4. Minerals and Barium Accumulation

Minerals (K, Ca, and Mg) and Ba were quantified in the shoots and roots of the treated plants by atomic absorption spectrometry (Perkin Elmer PinAAcle900T, Waltham, MA, USA); after the digestion of 30 mg of dry samples (shoots or roots) in 3 mL of an acid mixture (containing HNO₃:H₂SO₄:HClO₄; 10:1:0.5; v/v/v) at 110 °C for 2 h, the extracts were diluted with 0.5% HNO₃ (v/v) [21,22,27].

2.5. Total Inorganic Nitrogen

Total inorganic nitrogen (TIN) content was determined following Kjeldahl's method [28] where 30 mg of the dry sample (shoots or roots) was gradually heated (from 150 to 350 °C for 2 h) in pure sulfuric acid (95%) to transform the organic nitrogen into its mineral form. The TIN content was determined in milligrams per gram of the dry weight of each organ.

2.6. Polyphenols and Flavonoids

Polyphenols and flavonoids contained in 30 mg of plant dry tissues (shoots or roots) were extracted in methanol 80% and determined following Velioglu et al. and Lamaison and Carnat's methods [29,30], respectively. To determine polyphenols level, 150 µL of the extract was added to 500 µL of Folin–Ciocalteu reagent (10%, v/v) and 400 µL of Na₂CO₃ (7.5%, w/v); then, the mixture was incubated for 90 min in dark and the absorbance was read at 765 nm. A mixture consisting of 500 µL of the extract and 500 µL of AlCl₃ (2%, w/v) was incubated for 30 min to determine flavonoids and the absorbance was read at 430 nm.

2.7. Statistical Analysis

Each analysis used 10 biological replicates and all the values were presented as means ± standard deviation (SD). Statistics were evaluated using ANOVA analysis and Tukey honest significant difference (HSD) tests by STATISTICA software. Significant differences among treatments were shown at *p* < 0.05 and *p* < 0.001. Associations analysis between the

studied parameters was performed by correlation circle from principal component analysis (PCA) using STATISTICA 8.0 software.

3. Results and Discussion

3.1. Plant Growth

Although Ba is considered a non-essential metal for living organisms [8,12], research on the toxicity induced by this metal in plants is limited, where most of the studies reported its inhibitory effect on plant growth [31]. Among the physiological changes that could be induced by Ba stress is the inhibition of the photosynthetic process through the depletion of CO₂ assimilation, resulting in a reduction in plant growth [32–34].

Contrarily to the findings stated in the previous studies, the results shown in Table 1 showed positive effects of Ba on the biomass production of the studied species. When compared with the control, Ba significantly increased shoots DW in *L. crithmoides* by increasing Ba concentration in the nutritive solution ($p < 0.05$). The roots showed also an elevation in DW with the increase of Ba levels; however, the increase was only significant at 500 μM Ba ($p < 0.05$; Table 1). Moreover, a similar trend was detected in the FW of the shoots and roots but the noted increase was only significant in the shoots of 500 μM Ba ($p < 0.05$; Table 1). Regarding plants morphology, *L. crithmoides* did not show any phytotoxicity signs after Ba exposure (Figure S1). In contrast, a slight increase was shown in the DW and FW of the shoots and roots of *H. annuus* in all Ba concentrations; however, the variation was not significant as compared with the untreated plants (Table 1). In addition, only plant height reduction was observed in all Ba-treated plants (Figure S2).

Table 1. Assessment of the growth (DW: dry weight, FW: fresh weight, WC: water content) and the metal tolerance index (TI %) in the shoots and roots of *Limbarda crithmoides* and *Helianthus annuus* after 45 days of Ba exposure. Data are shown as means \pm SD; values with lowercase denote significant differences at $p < 0.05$.

<i>Limbarda crithmoides</i>							
Ba (μM)	DW (mg)		FW (mg)		WC ($\text{mL}\cdot\text{g}^{-1}$ DW)		TI (%)
	Shoots	Roots	Shoots	Roots	Shoots	Roots	Entire Plant
0	437.5 ^c \pm 43.8	68.0 ^b \pm 14.4	5887.1 ^b \pm 541.1	835.3 ^b \pm 123.5	0.926 ^a \pm 0.002	0.909 ^a \pm 0.008	
100	576.8 ^{bc} \pm 76.7	82.6 ^b \pm 11.4	7182.1 ^b \pm 1025.8	812.8 ^b \pm 120.5	0.919 ^a \pm 0.001	0.897 ^a \pm 0.003	136.3 \pm 20.3
200	669.6 ^b \pm 87.7	110.9 ^b \pm 24.0	7816.2 ^b \pm 873.3	976.2 ^b \pm 207.3	0.916 ^a \pm 0.004	0.887 ^a \pm 0.004	164.9 \pm 21.2
300	761.4 ^{ba} \pm 57.9	119.0 ^a \pm 13.3	8283.4 ^{ba} \pm 1651.3	1000.4 ^b \pm 117.7	0.917 ^a \pm 0.003	0.878 ^a \pm 0.007	188.4 \pm 16.4
500	875.9 ^a \pm 72.2	168.1 ^a \pm 18.8	9769.7 ^a \pm 694.7	1500.2 ^a \pm 134.3	0.911 ^a \pm 0.002	0.889 ^a \pm 0.006	227.0 \pm 21.1
<i>Helianthus annuus</i>							
Ba (μM)	DW (mg)		FW (mg)		WC ($\text{mL}\cdot\text{g}^{-1}$ DW)		TI (%)
	Shoots	Roots	Shoots	Roots	Shoots	Roots	Entire Plant
0	1807.7 ^a \pm 105.3	539.3 ^a \pm 31.7	12,664.5 ^a \pm 554.6	8520.8 ^a \pm 794.8	0.858 ^a \pm 0.003	0.935 ^a \pm 0.003	
100	1924.6 ^a \pm 168.6	603.1 ^a \pm 71.6	13,428.9 ^a \pm 1046.5	9764.9 ^a \pm 1081.9	0.867 ^a \pm 0.006	0.938 ^a \pm 0.002	102.4 \pm 11.1
200	1931.2 ^a \pm 156.6	491.5 ^a \pm 34.8	14,100.7 ^a \pm 1024.5	8363.1 ^a \pm 942.0	0.863 ^a \pm 0.005	0.939 ^a \pm 0.004	103.6 \pm 6.8
300	2097.5 ^a \pm 134.4	548.5 ^a \pm 59.2	14,543.0 ^a \pm 865.6	7764.8 ^a \pm 1023.8	0.856 ^a \pm 0.001	0.927 ^a \pm 0.005	114.2 \pm 8.2
500	2169.5 ^a \pm 85.5	645.3 ^a \pm 53.6	14,035.1 ^a \pm 905.6	10,181.4 ^a \pm 667.4	0.855 ^a \pm 0.003	0.937 ^a \pm 0.002	116.5 \pm 8.9

These findings were in agreement with Bouslimi et al.'s results [11], where the supplied Ba increased the fresh biomass production in *Brassica juncea* species shoots and roots. Other studies reported that shoots of *Helianthus annuus*, *Brassica juncea*, and *Ricinus communis* plants grown in a Rhodic Hapludox soil containing 150 and 300 $\text{mg}\cdot\text{kg}^{-1}$ of BaSO₄ did not show any signs of plant intoxication induced by Ba stress [35]. Ribeiro et al. also mentioned the absence of Ba morphological toxicity signs in *Oryza sativa*, *Eleocharis interstincta*, and *Cyperus papyrus* species with non-significant variation in shoots and roots dry biomass in the two first species, while *Cyperus papyrus* plants treated with varying concentrations of Ba significantly increased the dry biomass of its shoots and roots [36]; it was reported that low metal concentrations can stimulate the increase of mitotic index, leading to the increase in plant growth [21].

In response to metallic stress, plants may develop adaptation characteristics through the increase of tolerance strategies to reduce or avoid the adverse effects of toxic metals. For

example, aluminium induced the increase of chaperone proteins in the plant cells of soybean and citrus, resulting in the maintenance of cellular and protein homeostasis, and, therefore, this enhanced the tolerance of the plant to harmful environmental conditions [19,37,38]. This particularity can be evaluated by the determination of the metal tolerance index in plants [39,40]. Results showed (Table 1) that the TI of the entire plants of *L. crithmoides* and *H. annuus* treated with Ba were higher than 100% and it was found to increase with the increase of Ba concentration. This confirmed that both *L. crithmoides* and *H. annuus* species highly tolerated the different supplied concentrations of Ba in the growth medium. Additionally, our results were supported by Bouslimi et al., who observed that *Brassica juncea* was able to tolerate Ba stress [11]. Regarding the obtained results, Ba might be beneficial for plants; Srivastava et al. reported that Ba has been classified among the essential micronutrients, which remains associated with some metallo-enzymes; nonetheless, this metal is toxic at high concentrations [41].

The hydration state in stressed plants could be assessed by the estimation of water content [42]. It was reported that heavy metals such as Cd and Pb introduced the disturbance of water balance and transpiration rate in plants; indeed, a decrease in relative water content and transpiration was observed in *Atriplex canescens* [43]. In this study, Ba treatments did not induce any significant effect ($p > 0.05$; Table 1) on the water content of *L. crithmoides* and *H. annuus* when compared to the control (a negligible decrease was noted in WC in both shoots and roots under the effect of all Ba concentrations in *L. crithmoides*). It was also reported that the water status was not significantly affected by the application of Ba in *Glycine max* and *Cucumis sativus* plants [31,32]. This behaviour might be due to the accumulation of proline in plant cells, acting as a cytoplasmic osmotic solute, to preserve water and avoid the internal water deficit [44]. Thus, plants could develop an adaptive response to stress through the accumulation of this amino acid in order to increase the resistance to metals [44,45].

According to the results discussed above, it is possibly admitted that *L. crithmoides* and *H. annuus*, treated with different Ba concentrations, interestingly acquired tolerance to barium, which could be a result of the stimulation of some growth phytohormones such as auxin, abscisic acid, gibberellic acid, and cytokinin, etc. These phytohormones are known by their effective implication in metal toxicity mitigation and growth inhibition prevention in retaining plant growth plasticity during development [46]. A previous study reported that a metallophyte species (moss *Scopelophila cataractae*) maintained a good plant growth under high copper concentration and this was due to the accumulation of auxin that activated genes required for optimal growth and cell differentiation [47].

3.2. Chlorophyll Fluorescence

After 45 days of Ba treatments, the maximum primary yield of photosystem II (PSII) photochemistry (F_v/F_o), the maximum quantum efficiency of PSII (F_v/F_m), and the photochemical efficiency of PSII (F_v'/F_m') in *L. crithmoides* and *H. annuus*-treated plants remained statistically the same as the control ($p < 0.05$; Table 2); the electron transport rate (ETR) of *L. crithmoides* significantly increased only under 500 μM Ba ($p < 0.05$). Contrarily, *H. annuus* plants showed no significant variation in all Ba concentrations ($p > 0.05$) when compared to the control. It is well known that the photosynthetic apparatus is sensitive to adverse environmental constraints [48]; thus, the assessment of the photosynthetic quenching parameters can provide estimation on plant leaf photosynthetic capacity to evaluate the adaptation potential of plants to abiotic stress [24]. Results showed that Ba exposure did not induce significant impacts on PSII photochemical performance including F_v/F_o , F_v/F_m , and F_v'/F_m' in *L. crithmoides* and *H. annuus* (Table 2); these findings are in line with Reboledo et al. and Sghaier et al., who reported that metal stress induced by arsenic did not affect F_v/F_m and F_v'/F_m' in *Eucalyptus globulus* [49] and *Tamarix gallica* [23] plants. Similar results were also reported in *Elsholtzia argyi* species treated with high Cd concentrations (F_v/F_o did not differ as well under the stress) [50].

Table 2. Chlorophyll fluorescence parameters of *Limbarda crithmoides* and *Helianthus annuus* under the effect of different Ba concentrations after 45 days of the treatment. Data are shown as means \pm SD; values with lower-case denote significant differences at $p < 0.05$. Fv/Fo: maximum primary yield of photochemistry of Photosystem II, Fv/Fm: maximum quantum efficiency of Photosystem II, Fv'/Fm': photochemical efficiency of Photosystem II, ETR: electron transport rate, ABS/RC: absorption flux, TRo/RC: trapped energy flux, ETo/RC electron transport flux, DIo/RC: dissipation flux, RC: active reaction center.

<i>Limbarda crithmoides</i>								
Ba (μ M)	Fv/Fo	Fv/Fm	Fv'/Fm'	ETR	ABS/RC	TRo/RC	ETo/RC	DIo/RC
0	1.67 ^a \pm 0.15	0.62 ^a \pm 0.02	0.70 ^a \pm 0.04	62.50 ^b \pm 4.51	3.02 ^b \pm 0.08	1.88 ^a \pm 0.10	0.26 ^a \pm 0.02	1.14 ^b \pm 0.07
100	1.68 ^a \pm 0.04	0.63 ^a \pm 0.01	0.72 ^a \pm 0.05	69.51 ^b \pm 1.50	3.14 ^{ab} \pm 0.09	1.97 ^a \pm 0.04	0.26 ^a \pm 0.03	1.17 ^{ab} \pm 0.05
200	1.62 ^a \pm 0.18	0.58 ^a \pm 0.01	0.68 ^a \pm 0.07	65.21 ^b \pm 1.73	3.13 ^{ab} \pm 0.11	1.96 ^a \pm 0.03	0.26 ^a \pm 0.03	1.17 ^{ab} \pm 0.08
300	1.69 ^a \pm 0.08	0.63 ^a \pm 0.01	0.71 ^a \pm 0.07	71.03 ^{ab} \pm 4.70	3.29 ^{ab} \pm 0.30	1.99 ^a \pm 0.07	0.29 ^a \pm 0.04	1.30 ^{ab} \pm 0.07
500	1.66 ^a \pm 0.05	0.61 ^a \pm 0.03	0.70 ^a \pm 0.06	73.10 ^a \pm 2.38	3.60 ^a \pm 0.05	2.02 ^a \pm 0.01	0.29 ^a \pm 0.02	1.53 ^a \pm 0.05
<i>Helianthus annuus</i>								
Ba (μ M)	Fv/Fo	Fv/Fm	Fv'/Fm'	ETR	ABS/RC	TRo/RC	ETo/RC	DIo/RC
0	2.53 ^a \pm 0.13	0.72 ^a \pm 0.01	0.80 ^a \pm 0.03	85.00 ^a \pm 3.79	2.07 ^b \pm 0.05	1.48 ^a \pm 0.02	0.41 ^a \pm 0.03	0.59 ^b \pm 0.03
100	2.45 ^a \pm 0.20	0.68 ^a \pm 0.02	0.78 ^a \pm 0.05	86.50 ^a \pm 2.06	2.13 ^{ab} \pm 0.10	1.58 ^a \pm 0.04	0.40 ^a \pm 0.02	0.55 ^{ab} \pm 0.02
200	2.50 ^a \pm 0.08	0.71 ^a \pm 0.01	0.77 ^a \pm 0.06	72.51 ^a \pm 6.65	2.05 ^{ab} \pm 0.08	1.46 ^a \pm 0.05	0.36 ^{ab} \pm 0.04	0.59 ^{ab} \pm 0.04
300	2.66 ^a \pm 0.08	0.73 ^a \pm 0.01	0.79 ^a \pm 0.07	81.52 ^a \pm 2.50	2.19 ^a \pm 0.02	1.45 ^a \pm 0.02	0.36 ^b \pm 0.00	0.63 ^a \pm 0.06
500	2.45 ^a \pm 0.20	0.71 ^a \pm 0.02	0.76 ^a \pm 0.06	81.60 ^a \pm 1.50	2.34 ^a \pm 0.10	1.50 ^a \pm 0.04	0.33 ^b \pm 0.02	0.76 ^a \pm 0.07

Regarding energy fluxes, *L. crithmoides* showed a significant increase in the absorbed (ABS) and dissipated (DIo) energy fluxes in 500 μ M Ba-treated plants only ($p < 0.05$), when compared with that of the control; however, no changes were marked in the trapped (TRo) and transported energy fluxes (ETo) in all treated plants ($p > 0.05$). *H. annuus* showed that the energy absorption and dissipation significantly increased in the highest Ba concentrations (300 and 500 μ M) while the transported energy was significantly reduced at the same concentrations ($p < 0.05$) in comparison with the control. The trapped energy was not modified under Ba ($p > 0.05$; Table 2). The increase in ABS/RC under the effect of abiotic stress could be explained by the inactivation of some parts of PSII reaction centers (RCs) and that might be due to the inactivation of the oxygen-evolving complexes (OEC), or by the increase of antenna size [51–53]. Liu et al. also reported the significant increase in absorbed and dissipated energy fluxes in *Melia azedarach* treated with manganese [53]. Additionally, Pb stress induced an increase in energy absorption and dissipation within the PSII with a decline in electron transport [54]. It has been revealed that some plant species like halophytes (e.g., *Tamarix gallica*) are able to dissipate excessive energy in order to overcome the accumulation of excessive reducing energy that is known as the primary source of free radicals generation, and this could avoid the photo-destruction of the photosynthetic apparatus [23].

Kaustky plots derived from the OJIP transient analysis only showed an evident modification in (O–J) and (J–I) sides of the curve of *L. crithmoides* plants exposed to 200 μ M Ba (Figure 1). In contrast, *H. annuus* did not show different behaviour in the Kaustky curves of all Ba treatments when compared to the control (Figure 2).

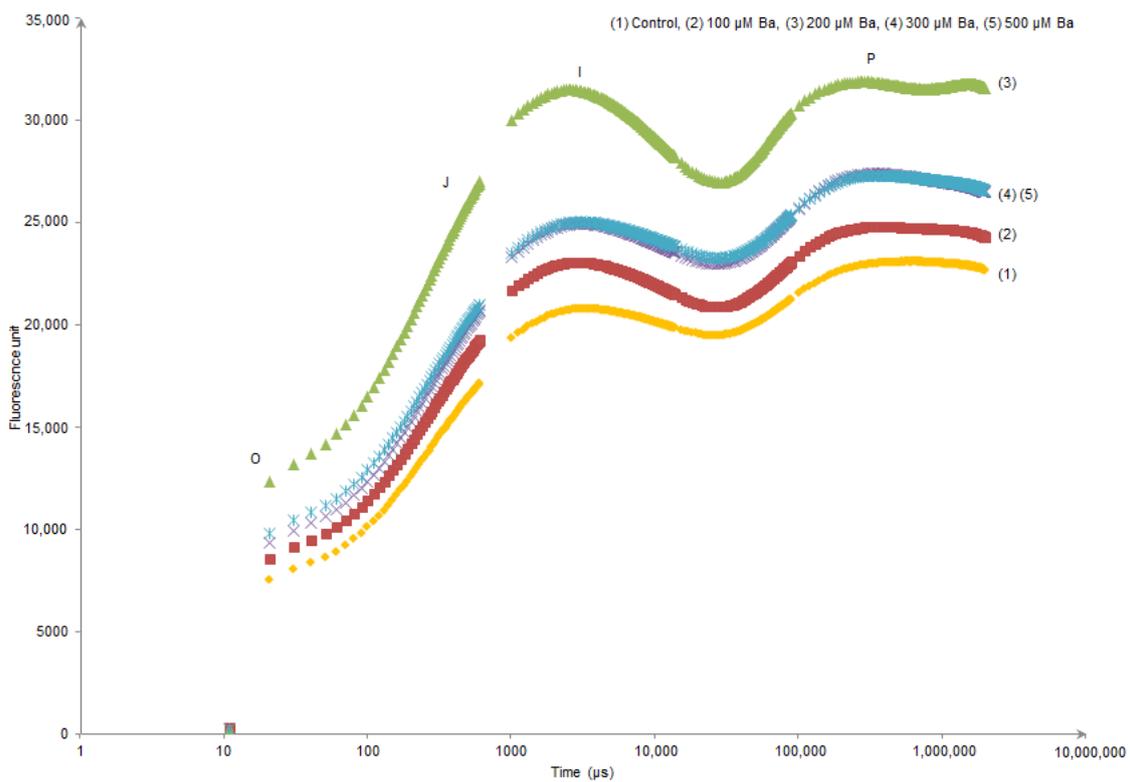


Figure 1. Kautsky curves derived from the OJIP transient analysis in dark-adapted leaves of *Limbarda crithmoides* after 45 days of Ba exposure.

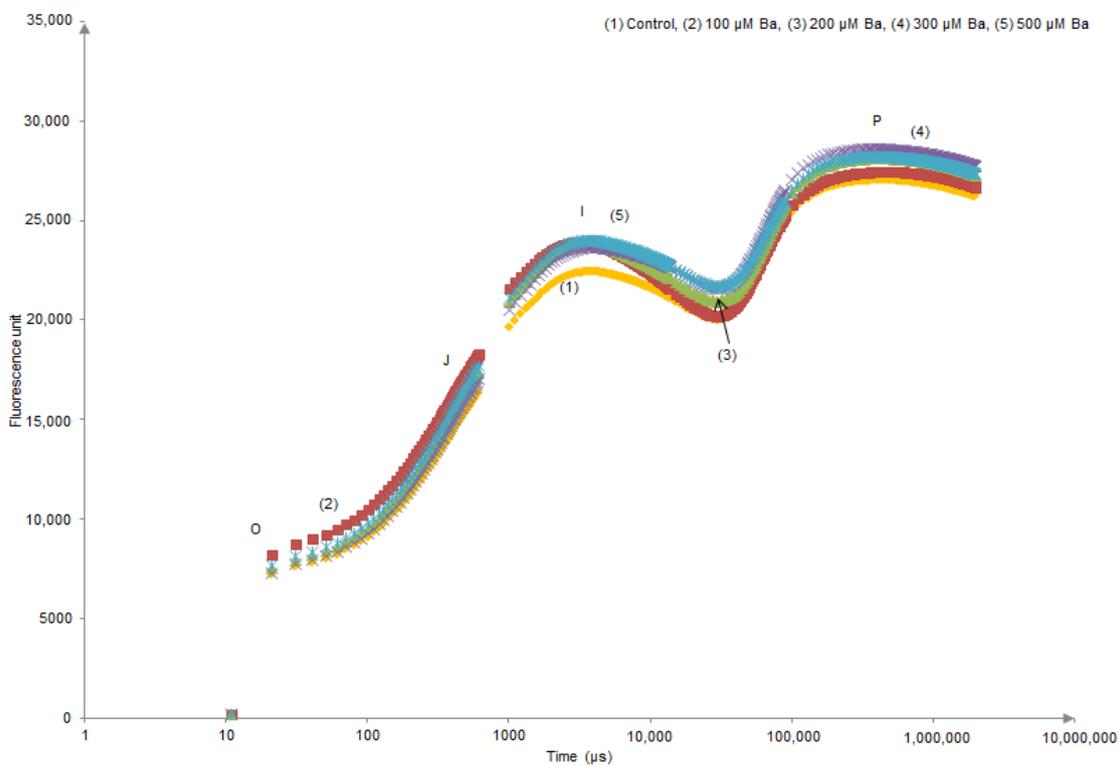


Figure 2. Kautsky curves derived from the OJIP transient analysis in dark-adapted leaves of *Helianthus annuus* after 45 days of Ba exposure.

OJIP transient of dark-adapted leaves presents a polyphasic chlorophyll a fluorescence induction curve. In the results presented in Figures 1 and 2, the OJIP curve was only higher in *L. crithmoides* of 200 μM Ba with no considerable changes observed in *H. annuus*. Indeed, the increase in chlorophyll fluorescence in the O–I part of the plot might be due to the net photochemical reduction of QA (first electron acceptor of PSII) to QA $^-$, while the increase of J–I typically depends on a disturbance in the structure and function of OECs inducing the alteration of the rate of oxygen evolution [23,55]. Moreover, the rise in the I–P part is attributed to the reduction of electron transporters (e.g., ferredoxin, intermediary acceptors, NADP) of the Photosystem I acceptor side [56].

3.3. Chlorophylls Contents

In addition, chlorophyll level can also reflect the sensitivity of plants to abiotic stresses where the decrease in these pigments presents a common symptom of metal toxicity in plants [25]. Despite the slight changes obtained in some chlorophyll fluorescence parameters under the effect of Ba, the results presented in Figure 3 showed that chlorophylls determined in plant leaves of *L. crithmoides* and *H. annuus* were not negatively affected by Ba-induced stress; Chl *a* content was found increased only in the highest Ba concentration (500 μM) in *L. crithmoides*, when compared with the control ($p < 0.05$). Furthermore, no variation in Chl *b* levels in all Ba concentrations was shown in the two species ($p > 0.05$). Our results are in line with several studies, for example, a study realised on *Cyamopsis tetragonoloba* plants, treated with high doses of BaCl₂ (going from 2 to 10 mM Ba), showed that Chl *a* and Chl *b* contents were not affected in the lowest applied Ba concentration (2 mM), but a decrease in the level of these pigments was noted in the highest Ba ones [13]. This fact may clearly explain that the application of Ba might not affect photosynthesis in plants; however, at a very high Ba dose, the photosynthetic activity could be reduced. Lamb et al. also mentioned that Ba can be phytotoxic only when applied at very high doses [8]. In addition, Sosnowski and Drozłowska reported that the amount of chlorophyll was found to increase in marine algae treated with BaCl₂, proving that these algae remained productive despite the toxic effects of Ba [43]. Overall, the maintenance of the photosynthetic efficiency and the biosynthesis of chlorophylls in *L. crithmoides* and *H. annuus* under varying Ba concentrations was in agreement with the increased biomass production in the present species throughout the experiment. Moreover, plant tolerance to Ba stress might be associated with a high ability of these two species to remove ROS through the activation of such protective mechanisms, e.g., increase in antioxidant enzymes activity and stimulation of the antioxidant active compounds synthesis [56].

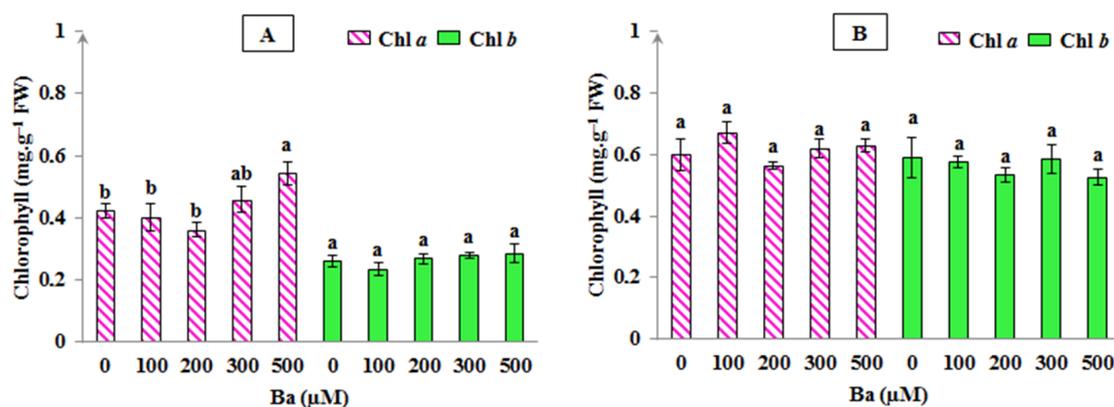


Figure 3. Chlorophyll *a* and *b* levels in *Limbarda crithmoides* (A) and *Helianthus annuus* (B) leaves after 45 days of Ba exposure. Data are presented in mean values \pm SD, $n = 10$. Bars marked with lower-case letters were significantly different at $p < 0.05$.

3.4. Polyphenols and Flavonoids Levels

The stimulation of the production of secondary metabolites such as polyphenols and flavonoids in plant cells is a response to a stress factor, which can be a metallic stress induced by barium [43]. The generation of reactive oxygen species (ROS) is the primary response of plants when exposed to toxic heavy metals (even to elevated concentrations of essential metals) [57,58]. The secondary metabolites, belonging to the non-enzymatic antioxidant system, are involved to scavenge free radicals and also to chelate metals to reduce their toxicity in the plant and eventually avoid the occurrence of plant damage [59,60]. Indeed, when exposed to metal stress, the antioxidant system is activated by the stimulation of the phenylpropanoid pathway using the phenylalanine to biosynthesise phenolic compounds (phenolic acids, flavonoids, rosmarinic acids, phytoalexins, and lignins) [61] and this is due to the increase of enzymes such as phenylalanine ammonia-lyase, polyphenol oxidase, shikimate dehydrogenase, and chalcone synthase involved in the biosynthetic pathway of phenolic compounds. Moreover, the chelation of metal ions with phenols can enable metal sequestration and reduce metal ions uptake to aerial parts of the plant [62]. Kisa et al. reported that phenolic compounds allow plants to adapt to different abiotic stress conditions in the environment, including metal toxicity [59].

In our experiment, the addition of all Ba concentrations to the growth medium (from 100 to 500 μM) significantly promoted the increase of polyphenols in the shoots of *L. crithmoides* and *H. annuus* ($p < 0.05$), whereas an increase of more than 30% and 50% was detected, respectively, in comparison with the control (Figure 4A,B). The accumulation of polyphenols in the roots of *L. crithmoides* was not changed in the presence of Ba in the irrigation solutions when compared with the control (Figure 4A). In contrast, polyphenols in roots of *H. annuus* significantly increased with the increase of Ba ($p < 0.05$; Figure 4B). Moreover, the flavonoids biosynthesis was significantly stimulated in the shoots of all Ba-treated plants ($p < 0.05$); the concentrations of 100, 200, 300, and 500 μM Ba induced an increase of 1.5-, 0.85-, 1-, and 1.6-fold in *L. crithmoides*, and an increase of 2-, 1.8-, 2.6-, and 2-fold in *H. annuus*, respectively, in comparison with the untreated plants (Figure 4). However, a slight increase in flavonoids of the roots was observed by increasing Ba concentration but the enhancement was only significant at 500 μM Ba ($p < 0.05$). *H. annuus* did not show significant variation in flavonoids content in roots under Ba.

Our results are in accordance with Sosnowski and Drozłowska, who reported an increase in polyphenols in algae treated with BaCl_2 [43]. Bouslimi et al. Also demonstrated that Ba stress exhibited polyphenols production in the shoots and roots of two *Brassicaceae* species, i.e., *Brassica juncea* and *Cakile maritima*, and no significant variation was reported in flavonoids level in roots of *Brassica juncea* [11].

3.5. Nutrients and Total Inorganic Nitrogen Contents

Table 3 showed different variations in the determined concentrations of K, Ca, Mg, and TIN in the shoots and roots of *L. crithmoides* and *H. annuus* under Ba stress. Regarding K levels, all Ba treatments significantly enhanced the content of this element in the upper parts of the two studied species and only in the roots of *H. annuus* in comparison with the control ($p < 0.05$). Conversely, the underground parts of *L. crithmoides*-treated plants accumulated less K than the untreated ones; this decrease was significant in all Ba treatments ($p < 0.05$). Moreover, Ba stress did not affect calcium levels in the shoots; however, as compared with the control, a significant reduction in Ca' roots (up to 20%) from 100 to 500 μM Ba was marked ($p < 0.05$). In contrast, Ca contents in the shoots and roots of *H. annuus* were not affected by Ba when compared to the control ($p > 0.05$). Results also showed that Mg content was significantly increased only in the shoots of *L. crithmoides* in 500 μM Ba, and decreased in the roots of 100, 200, 300, and 500 μM Ba (1.4-, 1.4-, 1.2- and 1.5-fold decrease, respectively), as compared with the control ($p < 0.05$). Regarding *H. annuus* plants, a significant increase in Mg was noted only in shoots of 100 and 200 μM Ba ($p < 0.05$).

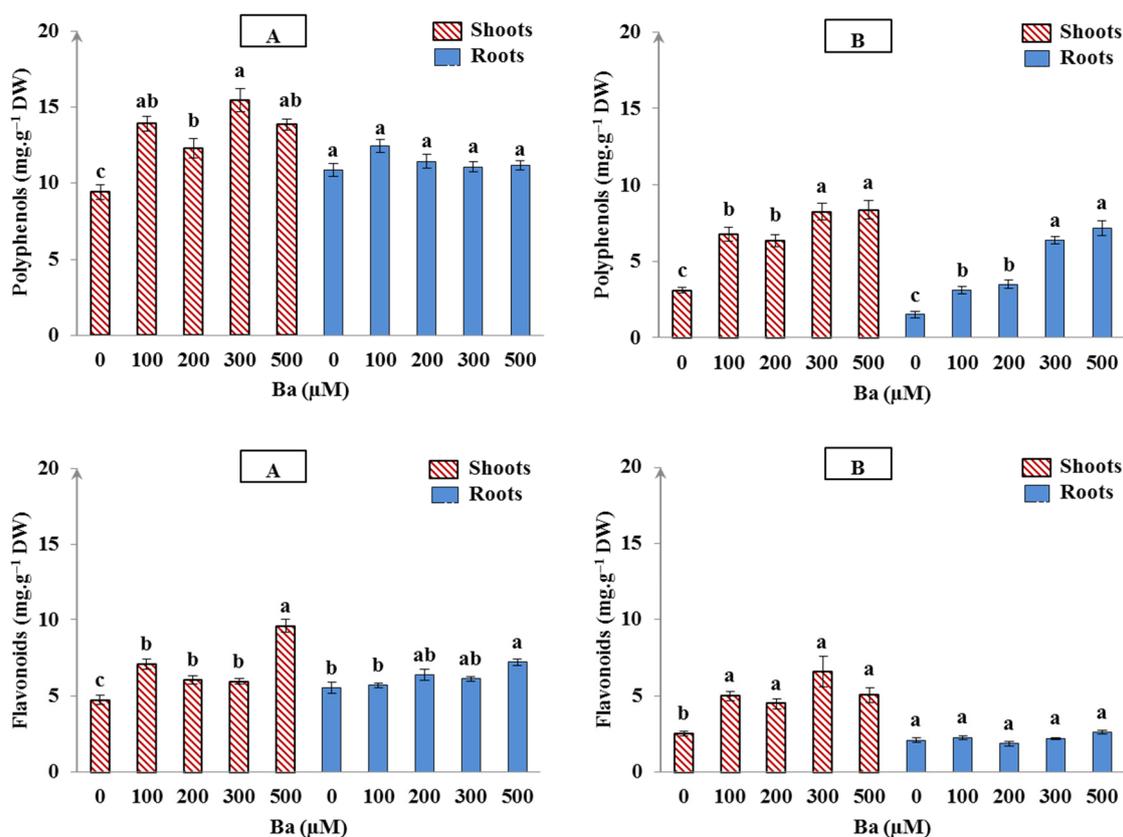


Figure 4. Accumulation of polyphenols and flavonoids in shoots and roots of *Limbarda crithmoides* (A) and *Helianthus annuus* (B) plants after 45 days of Ba treatments. Data are presented in mean values \pm SD, $n = 10$. Bars marked with different lower-case letters were significantly different at $p < 0.05$.

Table 3. Nutrient elements uptake in the shoots and roots of *Limbarda crithmoides* and *Helianthus annuus* over 45 days of Ba exposure (K: potassium, Ca: calcium; Mg: magnesium, TIN: total inorganic nitrogen). Data are shown as means \pm SD; values with lowercase denote significant differences at $p < 0.05$.

<i>Limbarda crithmoides</i>								
Ba (μ M)	K ($\text{mg}\cdot\text{g}^{-1}$ DW)		Ca ($\text{mg}\cdot\text{g}^{-1}$ DW)		Mg ($\text{mg}\cdot\text{g}^{-1}$ DW)		TIN ($\text{mg}\cdot\text{g}^{-1}$ DW)	
	Shoots	Roots	Shoots	Roots	Shoots	Roots	Shoots	Roots
0	47.1 ^c \pm 1.6	31.8 ^a \pm 2.1	31.6 ^a \pm 1.7	24.0 ^a \pm 0.5	19.5 ^b \pm 0.5	8.1 ^a \pm 0.4	40.1 ^b \pm 1.3	40.6 ^a \pm 1.5
100	60.2 ^a \pm 1.9	24.2 ^b \pm 0.2	32.5 ^a \pm 2.4	19.4 ^b \pm 0.6	17.9 ^b \pm 0.7	5.7 ^b \pm 0.4	50.8 ^a \pm 2.2	35.0 ^b \pm 1.0
200	63.1 ^a \pm 0.9	20.6 ^c \pm 1.6	30.8 ^a \pm 1.4	20.9 ^b \pm 0.5	19.0 ^b \pm 0.7	5.8 ^b \pm 0.3	48.6 ^a \pm 1.4	37.7 ^a \pm 1.3
300	52.9 ^b \pm 2.2	23.6 ^b \pm 0.5	31.9 ^a \pm 1.2	20.4 ^b \pm 0.4	21.0 ^b \pm 0.7	6.8 ^b \pm 0.2	51.3 ^a \pm 1.2	35.5 ^b \pm 0.9
500	52.8 ^b \pm 1.9	25.8 ^b \pm 1.1	30.5 ^a \pm 1.0	20.4 ^b \pm 0.5	23.4 ^a \pm 1.4	5.5 ^b \pm 0.5	46.4 ^a \pm 1.1	31.2 ^c \pm 1.1
<i>Helianthus annuus</i>								
Ba (μ M)	K ($\text{mg}\cdot\text{g}^{-1}$ DW)		Ca ($\text{mg}\cdot\text{g}^{-1}$ DW)		Mg ($\text{mg}\cdot\text{g}^{-1}$ DW)		TIN ($\text{mg}\cdot\text{g}^{-1}$ DW)	
	Shoots	Roots	Shoots	Roots	Shoots	Roots	Shoots	Roots
0	20.5 ^b \pm 1.0	10.7 ^b \pm 0.7	21.1 ^a \pm 1.1	13.4 ^a \pm 0.7	8.9 ^b \pm 0.2	7.3 ^a \pm 0.3	22.7 ^b \pm 1.1	14.7 ^b \pm 1.0
100	32.9 ^a \pm 1.4	16.5 ^a \pm 1.0	24.7 ^a \pm 1.3	17.3 ^a \pm 1.9	11.5 ^a \pm 0.5	8.4 ^a \pm 0.3	30.9 ^a \pm 1.3	21.4 ^a \pm 0.9
200	29.9 ^a \pm 1.4	14.6 ^a \pm 1.1	23.7 ^a \pm 1.1	14.6 ^a \pm 1.7	11.1 ^a \pm 0.5	7.4 ^a \pm 0.8	37.8 ^a \pm 2.0	31.9 ^a \pm 1.8
300	30.1 ^a \pm 1.9	10.4 ^b \pm 1.1	22.3 ^a \pm 0.7	17.5 ^a \pm 1.4	10.3 ^b \pm 0.5	7.0 ^a \pm 0.3	30.2 ^a \pm 1.2	15.0 ^b \pm 0.7
500	24.4 ^b \pm 1.0	8.9 ^b \pm 0.8	21.5 ^a \pm 0.8	17.6 ^a \pm 0.7	9.0 ^b \pm 0.4	6.8 ^a \pm 0.2	19.4 ^b \pm 0.6	13.0 ^b \pm 0.6

Regarding the obtained results, nutrients uptake was negatively affected only in the roots of *Limbarda crithmoides* in the presence of Ba. These results are in agreement with Suwa et al. and Monteiro et al., who stated the decrease of K in the roots of soybean and *Tanzania Guineagrass* plants treated with barium [32,43]. Suwa et al. also reported that the potassium ratio (epidermal cells K^+ / guard cells K^+) in soybean decreased with increasing Ba concentrations [32]. This decline in potassium levels in these plants was explained by the fact that Ba is a competitive antagonist for K-channels, which can block the passive efflux of intracellular K [9], and by the inhibition of the opening of K channels in the membrane, K absorption would be reduced [32]. Similarly to the results of *L. crithmoides*, the concentrations of Ca and Mg were also reduced in roots of *Tanzania Guineagrass* when Ba rates increased, which may reflect an antagonistic relationship between these divalent cations [43], whereas Ba did not change Ca and Mg uptake levels in soybean species [32]. Regarding our evaluation, it was demonstrated that the reduction in K, Ca, and Mg contents in roots had no critical effects on the growth of *L. crithmoides*; this can be explained by the fact that Ba could play a crucial role in increasing plant growth and different physiological functions, or that the reduced nutrients was not a negative effect but rather a decrease in the luxury consumption. Plants have the ability to accumulate nutrients as an insurance strategy in order to survive such sudden environmental abiotic stress [63,64]. In addition to that, the increase in K content in the shoots of *L. crithmoides* and in both shoots and roots of *H. annuus* Ba-treated plants could be explained by the stimulation of Abscisic acid synthesis, the key hormone implicated in tuning responses to abiotic stresses [65,66]. The enhancement in Mg levels in shoots of *L. crithmoides* (500 μ M Ba) and *H. annuus* (100 and 200 μ M Ba) can be due to the involvement of this nutrient in metal toxicity alleviation via the maintenance of iron status, the increase in antioxidative activity, and the conservation of chlorophyll biosynthesis [21].

Moreover, when compared with the control, *H. annuus* showed a significant enhancement in TIN content in shoots of 100, 200, and 300 μ M Ba and in roots of 100 and 200 μ M Ba-treated plants ($p < 0.05$). A similar trend was observed in shoots of *L. crithmoides* in all applied Ba concentrations; however, TIN content decreased in roots of this species under Ba, but the decline was only significant at 100, 300, and 500 μ M ($p < 0.05$). Marisamy et al. and Marisamy et al. reported that nitrate leaves contents in *Cyamopsis tetragonoloba* and *Amanranthus caudatus* treated with Ba were significantly increased to 78% and 119%, respectively, and the authors found that the increase in nitrate levels in these species was relative to the inhibition of nitrate reductase activity caused by Ba treatments [13,14]. Nitrogen was able to reduce or avoid metal toxicity, enhanced the photosynthetic activities through the increase of chlorophyll synthesis in plants, and increased the antioxidant enzymes activity [67]; therefore, this fact might also justify the increase of the studied species biomass. However, the decrease in the TIN in roots of *L. crithmoides* in this study can be a result of the increase of amino acids synthesis (from the nitrate sources) such as proline and peptides as GSH to tolerate and detoxify the metal through the elimination of hydroxyl radicals, the maintenance of osmoregulation, and the prevention of enzyme destruction [14,68,69].

3.6. Barium Content

Several plant species, growing on metal-contaminated soils, showed an adaptation to high concentrations of TME through the stabilisation, the translocation, and the extraction of metals such as Cd, Pb, Ni, Cu, Zn, Cr, and Ba, inducing as a result a sustained accumulation that mainly depends on biomass production and growth [31,36,70].

As can be seen in Figure 5, high levels of Ba were accumulated in *L. crithmoides* and *H. annuus* species after 45 days of Ba exposure. The accumulation of this metal was more notorious in *L. crithmoides* where Ba content was approximately distributed in equal proportions between the shoots and the roots of this plant in all Ba treatments. Adversely, *H. annuus* plants accumulated Ba in their roots more than in the shoots.

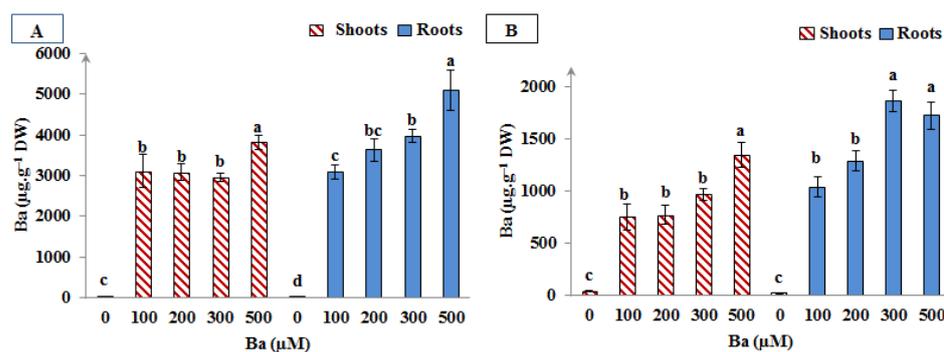


Figure 5. Barium content in shoots and roots of *Limbarda crithmoides* (A) and *Helianthus annuus* (B) after 45 days of Ba treatment. Data are presented in mean values \pm SD, $n = 10$. Bars marked with different lower-case letters were significantly different at $p < 0.001$.

Similarly, various species showed their capability to survive in barite-contaminated medium and also to accumulate Ba in their different organs such as *Cucumis sativus* species, which has accumulated an amount of $6 \text{ mg}\cdot\text{g}^{-1}$ DW of Ba in the roots and a similar quantity was accumulated in the shoots as well [31]. Ribeiro et al. also demonstrated high accumulating rates of Ba in the aerial parts or in roots or in both organs of *Oryza sativa*, *Eleocharis acutangula*, *Eleocharis interstincta*, *Cyperus papyrus*, and *Typha domingensis* after 120 days of BaCl_2 treatment [43].

The translocation of metals to the shoot and the accumulation of these elements in non-toxic form are the most adopted resistance mechanisms by plants facing a metallic stress [21]. Kee et al. mentioned that metal hyperaccumulation mechanisms may rely on phytochelatins and metallothioneins, defined as metal binding-peptides, which might also be related to detoxification and vacuolar sequestrating of cationic metals [70].

Although several species have been identified as accumulators of Ba in previous studies, no specific definition exists to classify plant species as Ba hyperaccumulators [71]. However, taking into consideration similar toxic metals as Ba, the threshold values of metal concentrations retained in the upper parts of plants used as metal hyperaccumulators were fixed at $100 \text{ mg}\cdot\text{kg}^{-1}$ for Cd and $1000 \text{ mg}\cdot\text{kg}^{-1}$ for Pb, Al, Co, Ni, Cu, and As [72–74]. Thus, the fact that *L. crithmoides* and *H. annuus* were able to accumulate around 3000 and $1000 \text{ }\mu\text{g}\cdot\text{g}^{-1}$ DW of Ba, respectively, in their aerial parts makes it possible for them be identified as Ba-hyperaccumulator species.

3.7. Correlation Analysis

The correlations between the studied parameters were statistically analysed using principal component analysis (PCA) to reveal the effect of barium on the physiological and biochemical parameters in the present plant species. The statistical analysis showed that the multifactorial analysis of Ba influence indicated that axis were Factor 1 and Factor 2, where Factor 1 explained the variance of 60.93% and 55.17% in the shoots and roots of *L. crithmoides* (Figure 6A) and 42.41% and 51.04% in the same organs of *H. annuus* (Figure 6B), respectively, while Factor 2 represented only 24.03% and 28.95% of variance in shoots and roots of *L. crithmoides*, and *H. annuus* showed a variance of 37.42% and 27.14% in shoots and roots, respectively. The two components explained 84.96% and 84.12% in *L. crithmoides* and 79.89% and 78.18% in *H. annuus* of the total variance in the shoots and roots, respectively. These results were considered highly significant for this study. Shoots of *L. crithmoides* showed that Ba was positively correlated to DW, FW, Mg contents, flavonoids, polyphenols, and chlorophylls *a* and *b*, and negatively correlated to WC (Figure 6A(a)). A positive correlation between Ba and DW, FW, and flavonoids was marked in roots of the same plant (Figure 6A(b)). *H. annuus* showed that Ba was positively correlated to DW, FW, polyphenols, and flavonoids in shoots (Figure 6B(a)), and also positively correlated to Ca, polyphenols, and flavonoids in roots (Figure 6B(b)).

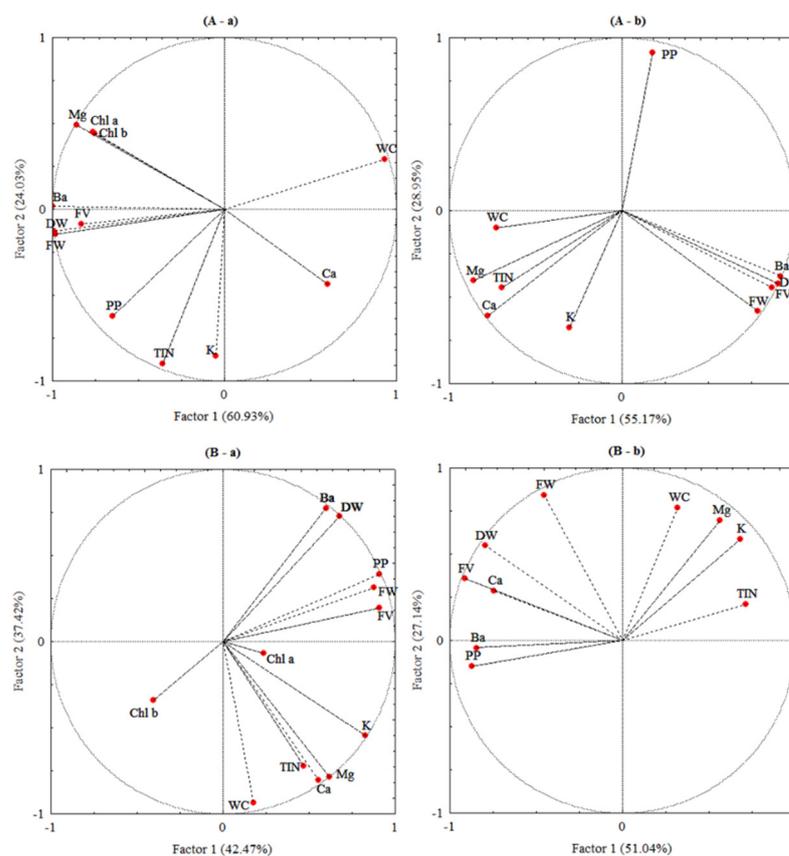


Figure 6. Correlation circle from the principal component analysis (PCA) of barium concentration (Ba), dry biomass (DW), fresh biomass (FW), water content (WC), chlorophylls (Chl *a* and Chl *b*), polyphenols (PP), flavonoids (FV), and nutrient contents (potassium (K), calcium (Ca), magnesium (Mg), and total inorganic nitrogen (TIN)) data of the shoots (a) and roots (b) of *Limbarda crithmoides* (A) and *Helianthus annuus* (B).

4. Conclusions

Regarding the huge availability of Ba in various components of our ecosystems, the exploitation of new plant species that can survive, accumulate, and safely remove this metal from polluted area would be certainly a part of the solution to reduce all related Ba toxicity risks occurred in various living organisms up to humans. The present study showed the high tolerance susceptibility of *L. crithmoides* and *H. annuus* to barium-induced stress; indeed, Ba did not harmfully affect the physiological and biochemical processes of the plants, which maintained good mineral nutrition of the aerial parts as well as the photosynthetic activity assessed by chlorophylls levels in plant leaves. Despite the reduced amounts of K, Ca, and Mg determined in the roots of *L. crithmoides*, the plants did not show any deficiency of these nutrients followed by an increase in biomass production overall for the treated plants. The competitive antagonistic propriety of Ba for K, Ca, and Mg might explain the retention of Ba in the roots to replace these macronutrients without reducing biomass production. The increase in polyphenols and flavonoids biosynthesis in the two species indicated the efficiency of the antioxidant system against Ba stress. Results also showed that at 500 μM , the highest applied Ba concentration, the present plant species could accumulate a rate of 3800 and 1350 $\mu\text{g}\cdot\text{g}^{-1}$ DW of Ba in *L. crithmoides* and *H. annuus* aerial parts, respectively, during the 45 days of Ba exposure. Therefore, the accumulation potential of barium in aerial parts and roots of *L. crithmoides* and *H. annuus* associated with high plant biomass production (specifically *L. crithmoides*) allows these species to be exploited for phytoremediation schemes in barium-contaminated soils. Nonetheless, studying molecular responses could help to better understand the mechanisms involved to tolerate Ba constraint. In addition, experiencing higher Ba concentrations in the growth

medium is recommended to assess the tolerance limit of Ba in *L. crithmoides* and *H. annuus*. According to the finding results, Ba could be a beneficial metal for some plant species when applied at low concentrations; however, the effect of barium on the physiological, biochemical, and molecular behaviours of other plant species grown in soils containing Ba needed to be further investigated.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijpb13020012/s1>, Figure S1. Morphological aspect of *Limbarda crithmoides* after 45 days of Barium exposure; Figure S2. Morphological aspect of *Helianthus annuus* after 45 days of Barium exposure.

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