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Nitrogen Effect on Growth-Related Parameters and Evaluation of *Portulaca oleracea* as a Phytoremediation Species in a Cr(VI)-Spiked Soil

Georgios Thalassinos, Elina Nastou, Spyridon A. Petropoulos * and Vasileios Antoniadis *

Department of Agriculture, Crop Production and Rural Environment, School of Agricultural Sciences, University of Thessaly, 38446 Volos, Greece; thalassinosgeorgios@hotmail.gr (G.T.); elina_90@windowslive.com (E.N.)

* Correspondence: spetropoulos@uth.gr (S.A.P.); antoniadis@uth.gr (V.A.); Tel.: +30-242-109-3196 (S.A.P.); +30-242-109-3241 (V.A.)

Abstract: In a pot experiment, we assessed the potential of purslane (*Portulaca oleracea*) as a phytoremediation species in Cr(VI)-contaminated soils. We focused on the evaluation of phytotoxic Cr(VI) effects at concentrations reaching 150 mg Cr(VI) kg^{-1} and the possible stress amelioration effect of nitrogen on Cr(VI)-stressed plants. Treatments were T-0 (control), T-1 (25 mg Cr(VI) kg^{-1}), T-2 = 50 mg kg^{-1}, T-3 = 100 mg kg^{-1}, and T-4 = 150 mg kg^{-1}. We measured Cr(VI) concentration in aerial and root tissues, a series of parameters related to photosynthesis and plant growth, phosphorus aerial plant tissue content, and we also calculated indices (ratios) related to leaf growth and above ground tissue water content. Cr(VI) almost exclusively was found in root tissues; all physiological and growth parameters studied were severely affected and plants selectively accumulated phosphorus in aerial plant tissues with increasing Cr(VI) soil concentrations. On the other hand, N amendment resulted in improved plant features in some of the measured parameters: chlorophyll index was improved with added N at T-2, plant height was significantly higher at T-0, T-1, and T-2, and aerial dry weight and leaf area was higher at T-0; these effects indicate that added N did increase P. oleracea potential to ameliorate Cr(VI) toxic effects. We conclude that purslane showed a potential as a possible species to be successfully introduced to Cr(VI)-laden soils, but more research is certainly necessary.

Keywords: hexavalent Cr; photosynthesis; phosphorus uptake; Cr(VI) tissue; leaf characteristics; purslane; soil contamination; heavy metals

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1. Introduction

Metal ions can be introduced to surface soils by natural or anthropogenic processes and their environmental impact is greatly affected by their mineralogical and geochemical form [1]. Cr is mainly found in two valence states, namely +3 (chromite (Cr(III)) and +6 (chromate Cr(VI)). However, in natural soil conditions trivalent chromium Cr(III) is the predominant state [2]. Hexavalent Cr compounds are found in wastes of numerous industrial activities (i.e., chromic acid and Cr-pigment production, leather tanning, cement production, metal plating, and stainless-steel production), and its anionic form results in increased possibility of Cr(VI) pollution dispersal [2–4].

Cr(III) is an essential element for some redox reactions that serve fundamental cellular functions relevant to sugar, protein and lipid metabolism in humans (recommended adult intake of 50 to 200 µg/d); however it is not an essential element for plants [5–7]. Hexavalent chromium (Cr(VI)) is of much higher toxicity (10 to 100 times) compared to Cr(III) for both acute and chronic exposure, posing serious health hazards for humans. Hexavalent Cr has been identified as one of the seventeen chemicals threatening human health and is classified as a human carcinogen causing a variety of cancer diseases in humans that result in increased overall mortality rates. Cr(VI) and Cr(III) in soil are in dynamic equilibrium

and the concentration of each form depends primarily on soil characteristics and redox conditions [5,7–9].

The particularly high toxicity of Cr(VI) in prokaryotes and eukaryotes is attributed to the high bioavailability of Cr(VI) oxyanions found in the soil environment (i.e., chromate (CrO_4^{2-}), hydrogen-chromate ($HCrO_4^-$), and dichromate ($Cr_2O_7^{2-}$). In common soil pH values, soil constituents bear mainly negative charges resulting in limited anion binding capacity [4,5,10].

Inside cells, the metabolic reduction of Cr(VI) through enzymatic and non-enzymatic processes leads to the formation of Cr(III) and in the parallel production of reactive oxygen species ROS, resulting in severe oxidative damage to plant cells. Cr(III) remains inside plant cells because of its low membrane permeability, forming stable complexes with proteins and nucleic acids resulting in the inhibition of DNA replication and RNA transcription [8,11–14].

In plants, translocation and accumulation of Cr largely depends on Cr speciation, plant species-specific stress alleviating mechanisms, concentration of Cr and Cr availability in the growth medium [11,15,16]. Cr(VI) enter plant cells via the cell membrane generic anion channels, due to structural similarities of chromate to sulfate and phosphate anions [12,13]. Contrary to that, Cr(III) ions can cross cell membrane at a much lower rate via simple diffusion or pinocytosis [17,18]. Several studies indicated that Cr primarily accumulated in the plant roots [19]. Cr(VI) is readily sequestered in root vacuoles and is poorly transported to aerial biomass in an effort of the plant to address Cr(VI) toxicity, thus avoiding exposure of important aerial organs for its physiological functions to elevated Cr(VI) [2,11].

It has been proposed that mechanisms developed from plant species tolerant to abiotic stresses, contribute to heavy metal tolerance. Cross-tolerance mechanisms between abiotic stresses and heavy metal tolerance mechanisms have been reported for various plant species [20]. Halophyte plant species have developed a series of mechanisms that confer tolerance to many metal ions, in concentrations prohibitive to the growth of most plant species [21,22]. The mechanisms implemented from halophytes include vacuole accumulation of metal ions, exclusion of metal ions from entering root cells and excretion of metal ions through the salt glands [23]. Furthermore, in halophytes various organic compounds may be accumulated, for cells to maintain their structure and protect the function of enzymatic mechanisms due to salt stress [24]. In halophytic plant species, increased concentrations of proline, total soluble sugars, and amino acids (such as leucine, isoleucine, valine, glutamine, glutamate, tyrosine, threonine, arginine, phenylalanine, and tryptophan) have been reported as a response to elevated toxic metal concentrations [25,26]. The above mechanisms result in the alleviation of heavy metal stress, rendering halophytes as potential candidates for phytoextraction and phytostabilization, as well as for saline agriculture [20,23,27,28].

Overall, high levels of Cr in plant tissues result in reduced plant height, root length, chlorophyll and pigment content in leaves, reduced photosynthetic rate, damaged root tissues, ultrastructural modifications of cell membranes, mineral nutrient imbalance and reduced enzymatic activity [6,14,29]. Chromium can limit the absorption of elements essential for plant growth such as N, P, K, Fe, Mg, Mn, Mo, Zn, Cu, Ca, and B [11,30,31]. Moreover, Cr(VI) has a negative effect on enzymes relevant to nitrogen metabolism, decreasing the activity of nitrate and nitrite reductase, glutamine synthetase, urease, and glutamate dehydrogenase [2,11,32].

Nutrient supply of micro- and macronutrients is an essential factor influencing plant growth that helps to alleviate the negative effects of biotic and abiotic stressors on plants. However, under field conditions only a limited number of nutrients have been found to alleviate biotic and abiotic stresses, among which, sulfate [33]. On the other hand, it is known that species exposed to a certain environmental restrictive agent, may become less effective in addressing other additional stresses [34,35]. In that sense, it should be expected that plants growing in unfertilized soils could be severely affected upon exposure to Cr(VI) contamination. However, to the best of our knowledge, this has never been tested

before in real soil conditions. Thus, it could be the case that well-fertilized plants address Cr(VI) exposure in a way that their developmental (i.e., root and shoot weight, and aerial part height), as well as physiological features (i.e., photosynthetic rate and chlorophyll content) are less severely affected compared to non-fertilized plants; however, due to a lack of evidence from the literature, this potentially beneficial effect of fertilization has to be elucidated [36].

Purslane (*Portulaca oleracea*) is a halophytic annual plant species, tolerant to several abiotic stresses [22]. This species adaptability is largely attributed to great morpho-cyto-physiologic variability that greatly contributes to the rapid growth and propagation under harsh environmental conditions. Key factors that contribute to purslane adaptability involve the production of secondary metabolites and the species ability to switch from C4 to CAM photosynthesis (carbon fixation-photosynthetic mechanism) under drought stress [21,37]. Salinity, drought and metal stress induce common physiological responses from plants [38]. Tolerant plants to other abiotic stresses can be possible candidates to be tested for phytoremediation purposes. To the best of our knowledge, despite the literature reports regarding the accumulation and toxic effects of chromium on purslane plants [36,39–41], there is a void in the literature concerning the ability of the species to grow under elevated Cr(VI) soil conditions, especially when N-added soils and compared to soil with non N addition.

Furthermore, it may be the case that nitrogen applied to Cr(VI)-stressed purslane could result in higher plant aerial biomass and thus in higher overall removal of Cr(VI) from soil. If this is the case, purslane could act faster as a phytoremediation species for the restoration of a Cr(VI)-laden area, when considering that metal uptake is affected both by plant tissue Cr(VI) content and the aerial biomass. However, this beneficial effect is also not elucidated by current literature evidence. Hence, the aims of this work were to study the developmental and physiological features of purslane, as well as its Cr(VI) content and possible toxicity symptoms resulting from Cr(VI) exposure in a soil well-fertilized with N and compare these effects with those in purslane plants grown in a non-fertilized soil. This study targeted specifically the evaluation of purslane as a potential phytoremediation species towards Cr(VI)-contaminated soils. To the best of our knowledge, although there are some works that have investigated purslane as a phytoremediation species towards Cr(VI) (e.g., [36]), there is no investigation in relation to the effects of added N on the phytoremediation capacity of the species.

2. Materials and Methods

A 10-treatment (2 levels of nitrogen × 5 levels of Cr(VI)) × 10 replicates) experiment was established. Overall, we had 100 replicates (each in 2-L pots) and for each replicate, a mixture of 1000 g of soil and 800 mL perlite was prepared. Soil was obtained from a field in the agricultural region between Volos and Larisa (39.394925 N, 22.756285 E), an area not affected from any known source of pollution. Soil spiking was performed using Cr(VI) solution of 10,000 ppm Cr(VI), by dissolving 19.22 g of CrO_3 in 1000 mL distilled water. Spiking solution was applied to the soil resulting in 5 Cr(VI) treatments (T-0: control; T-1: 25 mg Cr(VI) kg^{-1}, with 2.5 mL spiking solution per pot; T-2: 50 mg kg^{-1}, with 5 mL solution; T-3: 100 mg kg^{-1}, 10 mL solution; and T-4: 150 mg kg^{-1}, 15 mL solution.

For each Cr(VI) treatment, half of the replicates (10 out of 20) were amended in rates equivalent to 200 kg of nitrogen per hectare or 100 mg N per kg soil as NH_4NO_3 salt (thereafter named N-1 treatments and the non-added-N treatments are named N-0). The spiked soil treatments along with the un-amended control were placed in 2-L plastic pots, watered to their holding capacity and the spiked soil was left to equilibrate for 20 days. During the equilibration period, soil was thoroughly mixed three times per week and water was added as needed to keep soil to its water holding capacity.

At the end of the equilibration period, four samples per Cr(VI) treatment were obtained from the pots, air dried, passed through a 2-mm sieve in order to determine the initial (Day 0) hexavalent chromium (Cr(VI)) soil concentration.

2.1. Plant Establishment, Measurements and Soil and Plant Analysis

Cr(VI) was extracted from soil samples using 0.01 M KH_2PO_4 solution, color was developed by the diphenyl carbazide method and absorption values were determined using a Biochrom Libra S11 spectrophotometer at 540 nm [42]. For each treatment the initial hexavalent chromium (Cr(VI)) concentrations were estimated (T-0: 0.0 mg Cr(VI) kg^{-1} (control), i.e., un-amended soil; T-1: 20.65 mg Cr(VI) kg^{-1}; T-2: 49.92 mg Cr(VI) kg^{-1}; T-3: 106.43 mg Cr(VI) kg^{-1}; and T-4: 148.62 mg Cr(VI) kg^{-1}). Furthermore, three randomly acquired samples were analyzed for soil physiochemical parameters (pH = 7.8, ECe = 850 μS cm^{-1}, $CaCO_3$ = 10.4%, soil of loam texture) according to commonly used laboratory protocols [43].

Plants were grown in an unheated greenhouse. On Day 0, *P. oleracea* plants, already sown 25 days before Day 0 in peat-filled seedling trays, were transplanted in pots (one plant per pot). Transplantation took place when plants reached a height of 12 cm.

During the growth period, to compensate for any light and temperature differences in the greenhouse, plant positions were exchanged regularly, and water was applied to the plants according at regular intervals in amounts that depended on weather conditions (50–250 mL per pot). One month prior to harvest date, we measured plant height in cm, photosynthetic rate (μmol CO_2 m^{-2} s^{-1}) at a constant light intensity (250 μmol cm^{-2} s^{-1}) using the LI-Cor LI-6400XT Portable Photosynthesis System (LI-Cor, Lincoln, NE, USA)) and chlorophyll content (SPAD index) was measured using the OPTI-SCIENCES CCM-200 plus chlorophyll content meter (Opti-sciences, Hudson, NH, USA).

Plants were grown in the Cr(VI) spiked soil for 50 days—from 14 October 2019 (establishment of seedlings in the pots) to 4 December 2019, when plants were harvested. On harvest day we measured the weight of stems, the weight of leaves and the leaf area per plant. Then, aerial plant tissues were washed with deionized water and root tissues were meticulously washed so that no soil particles remained attached and further rinsed with deionized water.

Aerial and root plant tissues were dried in an air-forced drying oven at 70 °C for 96 h. Both aerial and root tissues were weighted and pulverized. Then, 1.00 g samples of plant tissue were dry-ashed at 500 °C for 4 h and ash was extracted using 10 mL of 20% HCl. Plant tissue K, P and Cr(VI) concentrations were estimated according to established laboratory protocols—dry ashing at 500 °C for 5 h, and then ash extraction with 20 mL of 20% HCl [44]. Due to the lack of sufficient plant tissue mass, especially in the high Cr(VI) treatments, out of the 10 replicates initially sown, 5 replicates for extraction and measurement were formed by combining tissues from every two pots. Furthermore, out of the primary data, we calculated a secondary index, i.e., tolerance index (TI), equal to dry aerial biomass in contaminated soil over that in control. Because of the fact that we had effectively two controls, typical to a two-factor experiment like ours, i.e., (a) no Cr(VI) with no N, and (b) no Cr(VI) with added N, we calculated TI as two independent factors, one for soils without N and one for soils with N.

2.2. Quality Assurance and Statistical Analyses

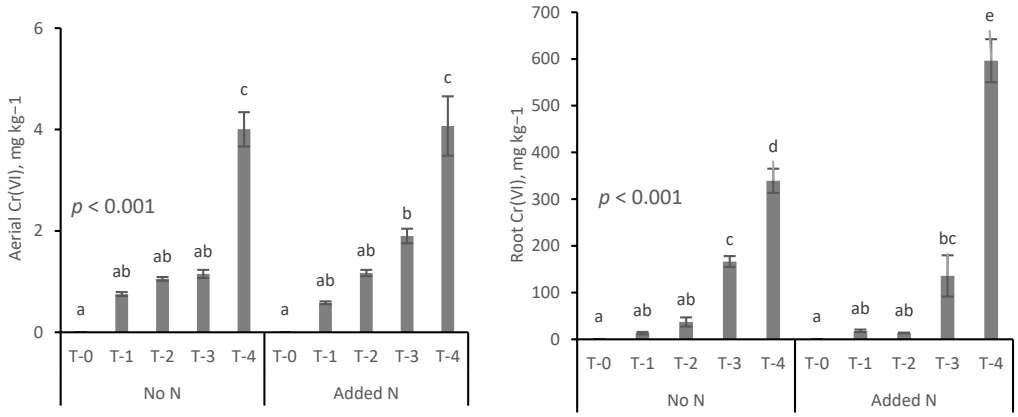
For data quality control purposes in-house plant and soil reference materials were used and recovery rates were within the range of 95% to 105% of the certified value. To rule out any possibility of cross-contamination, for every extraction batch blank samples were also measured. For Cr(VI) calibration curves, Merck standard solutions were used (Merck, Burlington, MA, USA).

Statistical analysis of the data was performed using IBM SPSS Statistics 25 and Excel 2019 software. One-way ANOVA and Duncan's multiple range tests were used to identify statistically significant differences between treatments and two-way ANOVA and Duncan's multiple range tests at $p = 0.05$ were used to monitor the effect that Cr and nitrogen had on the different parameters studied.

3. Results

3.1. Cr(VI) Concentration in Plant Tissues

Increasing soil Cr(VI), increased aerial tissue Cr(VI) content ($p < 0.001$), reaching 4.13 mg Cr(VI) kg^{-1} dry matter (T-4 (Cr(VI)) treatment with no added N), while nitrogen addition had no significant effect in the aerial tissue Cr(VI) content ($p = 0.915$) (Figure 1a). Furthermore, increasing Cr(VI) soil concentration resulted in significant increase ($p < 0.001$) in root tissue Cr(VI) concentration and nitrogen addition had no significant effect on root tissue Cr(VI) content ($p = 0.109$). In root tissues, Cr(VI) levels were orders of magnitude higher compared to aerial tissues, reaching 339 mg kg^{-1} dry matter at the highest Cr(VI) soil concentration with no added N, while at T-4 Cr(VI) with added N root Cr(VI) concentrations reached 596 mg kg^{-1} ($p < 0.001$). This finding indicates that in the highest tested soil Cr(VI) concentrations, N amendment resulted in increased root Cr(VI) concentrations (Figure 1b), significantly higher than the non-added-N treatment, whereas aerial contents of Cr(VI) remained low without being affected by N addition.



(**a**) (**b**)

Figure 1. Cr(VI) concentration in plant tissues: (**a**) Aerial tissue Cr(VI) concentrations (mg Cr(VI) kg^{-1} dry matter); (**b**) Root tissue Cr(VI) concentrations (mg Cr(VI) kg^{-1} dry matter). Different letters denote statistically significant differences at $p < 0.05$. Error bars represent standard error (T-1 = 25, T-2 = 50, T-3 = 100, T-4 = 150 mg Cr(VI) kg^{-1} soil).

3.2. Effects of Cr(VI) on Parameters Relative to Photosynthesis and Plant Growth

3.2.1. Chlorophyll Content Index and Photosynthetic Rate

As a result of Cr(VI) exposure, the purslane developmental and physiological parameters studied were significantly affected. Chlorophyll content (SPAD index) was found to gradually decrease ($p < 0.001$) from 12.35 ((T-0)-no added N) to 4.82 (T-4-no added N). Added nitrogen resulted in significantly higher chlorophyll content (Figure 2a). Similarly, with increasing Cr(VI) soil concentrations, reduced photosynthetic rate values were noticed ($p < 0.001$) and nitrogen addition resulted in increased ($p = 0.020$) photosynthetic rate values for every Cr(VI) level (Figure 2b).

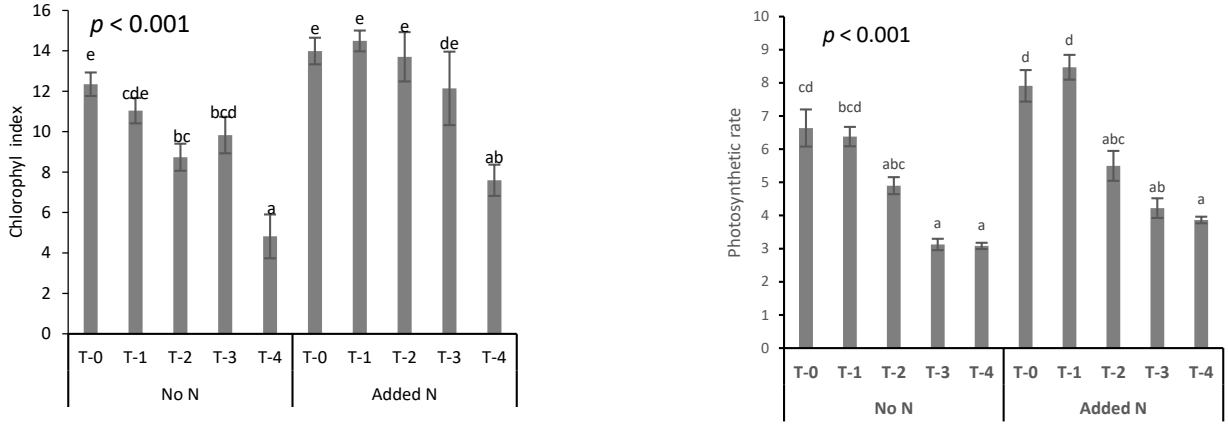


Figure 2. (**a**) Chlorophyll index; (**b**) Photosynthetic rate (μmol CO_2 m^{-2} s^{-1}). Different letters denote statistically significant differences at $p < 0.05$. Error bars represent standard error (T-1 = 25, T-2 = 50, T-3 = 100, T-4 = 150 mg Cr(VI) kg^{-1} soil).

3.2.2. Plant Height and Aerial Fresh Weight

Plant height and aerial fresh weight, as expected, followed the same trend observed for photosynthetic rate and chlorophyll content. Increasing Cr(VI) concentrations resulted in lower height values ($p < 0.001$) and nitrogen amendment had a positive effect in plant height in every Cr(VI) level tested ($p = 0.038$). For aerial fresh weight the trend was similar, with increasing Cr(VI) concentrations exerting negative effects on the measured values ($p < 0.001$) and in this case nitrogen addition had also a positive effect ($p < 0.001$) (Table 1).

Table 1. Plant height and aerial fresh weight at harvest date.

Treatments		Plant Height	Aerial Fresh Weight
		(cm)	(g pot^{-1})
No N	T-0	29.9 ± 1.09^{f}	13.4 ± 2.18
	T-1	18.9 ± 0.57^{d}	5.9 ± 0.58
	T-2	16.3 ± 0.91^{c}	4.4 ± 0.33
	T-3	15.0 ± 0.41^{bc}	4.5 ± 0.35
	T-4	11.2 ± 0.46^{a}	2.3 ± 0.31
Added N	T-0	31.2 ± 1.39^{d}	29.5 ± 3.21
	T-1	22.0 ± 0.67^{bc}	8.2 ± 0.52
	T-2	15.7 ± 0.48^{ab}	5.7 ± 0.30
	T-3	15.7 ± 0.99^{ab}	4.2 ± 0.38
	T-4	13.5 ± 0.46^{a}	3.5 ± 0.40
Treatment effect		$p < 0.001$	$p = 0.298$
Cr effect		$p < 0.001$	$p < 0.001$
Nitrogen effect		$p = 0.038$	$p < 0.001$

Mean ± S. E. Different letters denote significant ($p < 0.05$) difference between means in columns according to Duncan's multiple range test (T-1 = 25, T-2 = 50, T-3 = 100, T-4 = 150 mg Cr(VI) kg^{-1} soil).

3.2.3. Aerial Dry Weight and Root Dry Weight and Leaf Area per Plant

Cr(VI) increasing concentrations negatively affected aerial dry matter production ($p < 0.001$), and nitrogen addition resulted in higher values for every Cr(VI) level studied ($p < 0.001$). More specifically, in treatments where no nitrogen was added, values decreased gradually from 0.99 g (T-0) to 0.23 g (T-4) and in treatments where nitrogen was added, aerial dry weight values were significantly higher, reaching 2.73 g in the control treatment (T-0) and gradually decreased to 0.36 g for the highest soil Cr(VI) concentration used in the experiment (T-4). On the other hand, root dry weight showed an increasing trend with increasing Cr(VI) concentrations even from the lower Cr(VI) level (T-1) (0.39 g per pot),

despite the fact that differences failed to escalate in the higher Cr(VI) levels, resulting in marginally higher than 0.05 significance ($p = 0.053$); nitrogen addition resulted in higher root dry weight values ($p = 0.021$) (Table 2). Considering the results presented in Figure 1 and Table 2, the addition of N in plants treated with the highest Cr(VI) concentration may result in the removal of significantly higher amounts of Cr(VI) from contaminated soils. In particular, despite the similar contents of Cr(VI) in the aerial parts the increase in dry weight per pot (from 0.23 g pot^{-1} to 0.36 g pot^{-1} in no N and added N plants, respectively) indicates an increase in the removal of Cr(VI) from soils by 56.5%. On the other hand, in the case of roots we noticed an increase in both root dry weight per pot (from 0.5 g pot^{-1} to 0.96 g pot^{-1} in no N and added N plants, respectively) and Cr(VI) content in dried tissues (339 mg kg^{-1} and 596 mg kg^{-1} in no N and added N plants, respectively), meaning a cumulative increase of 237.6% in Cr(VI) removal from soils.

Table 2. Fresh and dry weight of aerial tissues, and dry weight of root tissues.

Treatments		Aerial Dry Weight (g pot^{-1})	Root Dry Weight (g pot^{-1})
No N	T-0	0.99 ± 0.167 ^{b}	0.30 ± 0.089 ^{a}
	T-1	0.53 ± 0.009 ^{ab}	0.39 ± 0.053 ^{ab}
	T-2	0.42 ± 0.015 ^{a}	0.56 ± 0.084 ^{abc}
	T-3	0.48 ± 0.049 ^{ab}	0.83 ± 0.093 ^{abc}
	T-4	0.23 ± 0.015 ^{a}	0.50 ± 0.127 ^{abc}
Added N	T-0	2.73 ± 0.339 ^{c}	0.38 ± 0.056 ^{ab}
	T-1	0.76 ± 0.107 ^{ab}	0.76 ± 0.169 ^{abc}
	T-2	0.50 ± 0.019 ^{ab}	1.02 ± 0.126 ^{c}
	T-3	0.45 ± 0.051 ^{a}	0.79 ± 0.095 ^{abc}
	T-4	0.36 ± 0.052 ^{a}	0.96 ± 0.146 ^{bc}
Treatment effect		$p < 0.001$	$p = 0.045$
Cr effect		$p < 0.001$	$p = 0.053$
Nitrogen effect		$p < 0.001$	$p = 0.021$

Mean ± S. E. Different letters denote significant ($p < 0.05$) difference between means in columns according to Duncan's multiple range test (T-1 = 25, T-2 = 50, T-3 = 100, T-4 = 150 mg Cr(VI) kg^{-1} soil).

3.2.4. Leaf Area per Plant, Leaf Weight/Total Aerial Weight Ratio and Aerial Tissue Dry Matter Content (Aerial Dry Weight/Aerial Fresh Weight Ratio)

Dry to fresh aerial tissue weight ratio increased significantly ($p = 0.002$) even from the lowest soil Cr(VI) (T-1) concentration (Table 3). Leaf area was significantly reduced ($p < 0.001$) even from the lowest level of Cr(VI) soil concentration (T-1), while nitrogen addition had a positive effect, increasing leaf area ($p < 0.001$) (Table 3). Apart from leaf area, leaf weight-to-total aerial weight ratio followed the same pattern as the leaf area, with increasing Cr(VI) concentrations resulting in lower ($p < 0.001$) ratio values and N addition resulting in higher ratio values ($p = 0.007$) (Table 3). Leaf weight/total aerial tissue weight ratio decreased gradually with increasing Cr(VI) concentrations ($p < 0.001$) and nitrogen addition resulted in significantly higher ratio values (Table 3).

Table 3. Leaf area, leaf/total aerial weight ratio and fresh weight to dry weight ratio of aerial tissues.

Treatments		Leaf Area	Leaf/Aerial Weight Ratio	Aerial Tissue (Dry Weight/Fresh Weight Ratio)
		(cm^2 Plant^{-1})	(g g^{-1})	(g g^{-1})
No N	T-0	114.5 ± 18.8 ^{b}	0.33 ± 0.020	0.0723± 0.0032 ^{a}
	T-1	59.6 ± 2.5 ^{ab}	0.28 ± 0.021	0.0936 ± 0.0015 ^{bc}
	T-2	43.3 ±2.8 ^{a}	0.27 ± 0.028	0.0884 ± 0.0041 ^{bc}
	T-3	48.3 ±4.1 ^{a}	0.30 ± 0.022	0.0934 ± 0.0042 ^{bc}
	T-4	41.0 ± 3.7 ^{a}	0.26 ± 0.025	0.0953 ± 0.0047 ^{bc}
Added N	T-0	381.5 ± 19.0 ^{c}	0.50 ± 0.070	0.0790 ± 0.0026 ^{ab}
	T-1	120.9 ± 4.1 ^{b}	0.31 ± 0.012	0.0875 ± 0.0009 ^{bc}
	T-2	98.3 ± 5.9 ^{ab}	0.33 ± 0.018	0.0927 ± 0.004 ^{bc}
	T-3	68.4 ± 2.4 ^{ab}	0.31 ± 0.027	0.0996 ± 0.004 ^{c}
	T-4	49.9 ± 1.9 ^{a}	0.26 ± 0.024	0.0998 ± 0.008 ^{c}
Treatment effect		$p < 0.001$	$p = 0.358$	$p = 0.015$
Cr effect		$p < 0.001$	$p < 0.001$	$p = 0.002$
Nitrogen effect		$p < 0.001$	$p = 0.007$	$p = 0.324$

Mean ± S. E. Different letters denote significant ($p < 0.05$) difference between means in columns according to Duncan's multiple range test.

3.2.5. Tolerance Index

Tolerance index values (aerial biomass in contaminated soil/aerial biomass in control), complemented the results of physiological and growth parameters. Cr(VI) increasing soil concentrations resulted in lower tolerance index values ($p < 0.001$) and nitrogen addition had a similar effect ($p < 0.001$). It seems that nitrogen addition resulted in higher plant growth potential in the control treatment, that was abruptly limited from the toxic effect of Cr(VI), even from the lower level of Cr(VI) applied to the soil (Figure 3).

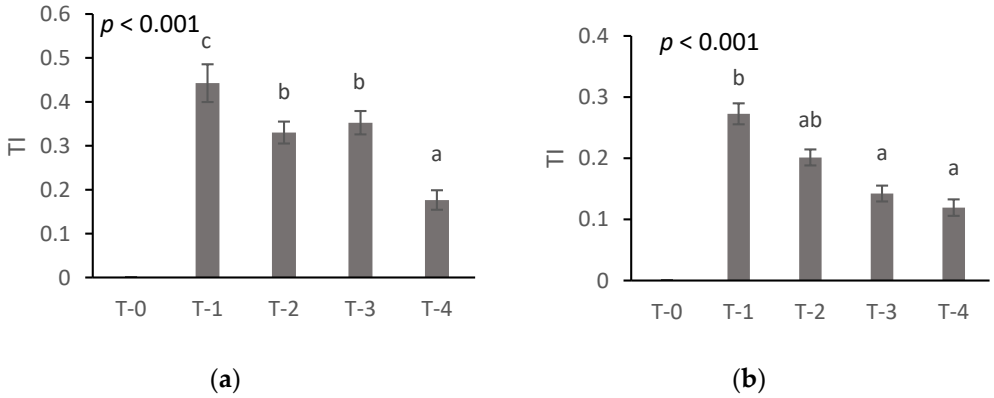


Figure 3. Tolerance index (TI) values for plants non-treated (**a**) or treated with nitrogen (**b**). Different letters denote statistically significant differences at $p < 0.05$. Error bars represent standard error (T-1 = 25, T-2 = 50, T-3 = 100, T-4 = 150 mg Cr(VI) kg^{-1} soil).

3.3. Phosphorus in Plant Tissues

In aerial plant tissues phosphorus content was measured and Duncan post hoc test results indicated that aerial tissue P concentrations increased ($p < 0.001$) with rising Cr(VI) soil concentrations, while nitrogen addition resulted in lower aerial tissue P concentrations when compared to the no N treated plants ($p = 0.034$) (Table 4). However, it must be noted that when the N effect was compared between same Cr(VI) additions, no differences were evident. For potassium content, despite the fact that the effect of Cr(VI) and N addition were significant ($p < 0.001$ and $p = 0.002$ respectively), the trend was not clear and further data are required to reach conclusive results (Table 4).

Table 4. Phosphorus and potassium content (g kg^{-1} DW) in the aerial part of purslane (*Portulaca oleracea*) aerial tissues (n = 5).

Treatments		Phosphorus Content	Potassium Content
No N	T-0	3.41 ^{ab}	39.50 ^{f}
	T-1	4.10 ^{abc}	28.75 ^{abc}
	T-2	5.10 ^{cd}	31.62 ^{bcde}
	T-3	4.72 ^{bcd}	34.65 ^{cdef}
	T-4	6.10 ^{d}	37.63 ^{ef}
Added N	T-0	3.99 ^{abc}	35.50 ^{def}
	T-1	2.95 ^{a}	24.04 ^{a}
	T-2	4.16 ^{abc}	25.92 ^{ab}
	T-3	4.40 ^{abc}	36.07 ^{ef}
	T-4	4.75 ^{bcd}	29.17 ^{abcd}
Treatment effect		$p < 0.001$	$p < 0.001$
Cr effect		$p < 0.001$	$p < 0.001$
Nitrogen effect		$p = 0.034$	$p = 0.002$

Mean ± S. E. Different superscripts denote significant ($p < 0.05$) difference between means in columns according to Duncan's multiple range test. (T-1 = 25, T-2 = 50, T-3 = 100, T-4 = 150 mg Cr(VI) kg^{-1} soil).

4. Discussion

Cr(VI) levels in root tissues were orders of magnitude higher compared to Cr(VI) concentrations found in aerial tissues, especially when plants were treated with the highest Cr(VI) concentration and fertilized with nitrogen. Increased Cr(VI) concentrations in root tissues have been noticed for a series of plant species, where plants limit the translocation of potentially toxic elements to the aerial plant tissues [2,10,11,15,45]. The physiological parameters studied (chlorophyll content and photosynthetic rate) were significantly affected with increasing Cr(VI) concentrations, while nitrogen amendment had a positive effect. Reduced values of total chlorophyll content, photosynthetic rate, and impediment of plant growth due to Cr(VI) stress have been documented for several plant species [2,8,20,26,45,46].

Values of all the parameters relevant to plant growth (aerial fresh weight, aerial dry weight, root dry weight, plant height, and leaf area) were significantly affected with rising Cr(VI) concentrations; nitrogen addition partly alleviated Cr(VI) toxic effects. Cr(VI) is known to impede several processes essential for plant growth such as photosynthesis, mineral uptake, enzyme and gene function, that inevitably result in reduced plant growth [6,11,45]. According to Kale et al. [47], plant growth of hydroponically grown *P. oleracea* was severely affected by increasing Cr contents, while plant accumulated significant amounts of Cr compared to other species (up to 190 mg kg^{-1} dry biomass). Various research articles have reported that nutrient addition to the growth medium alleviated to some degree Cr(VI) stress effects, as was the case with *Arabidopsis thaliana* [48,49]. In these investigations, the effect of N was non-significant, but the experimental settings were very different from ours (i.e., seedlings were watered with nutrient or Cr(VI) solutions in soilless culture). Contrary to such reports, our findings indicate that nitrogen amendment can support the growth of plants under Cr(VI) stress and partially compensate for the negative effects of Cr(VI) on plant physiological and metabolic processes. Leaf growth characteristics were proposed as bio-indicators of heavy metal stress. Cr stress is known to result in reduced leaf area, leaf size, and total leaf number per plant [2,45,50,51]. In the present experiment, a series of parameters such as leaf area and the ratio of leaf weight/total aerial weight were significantly affected with increasing Cr(VI) concentrations. Nitrogen amendment partly alleviated the effects of Cr(VI) stress. In parallel to the leaf growth restriction, significantly lower water content was noticed in above ground tissues. These results are in accordance with other works supporting that toxic effects of Cr(VI) in root tissues, alterations on the membrane structure of stomatal guard cells and the reduced diameter of tracheary vessels under Cr(VI) stress are the main factors that limit the water

supply to aboveground tissues and therefore inhibit plant growth [2,51]. It seems that root tissues are the most affected plant parts since heavy metals are usually accumulated in higher amounts in roots compared to other plants parts. This was also the case in the study of Kale et al. [47], who recorded higher amounts of Cr in root tissues, followed by a reduction in root length with increasing Cr content in nutrient solution. Similar results were reported by Dwivedi et al. [52] who evaluated two *Portulaca* species (e.g., *P. tuberosa* and *P. oleracea*) for their phytoremediation capacity of multiple heavy metals (e.g., Cu, Ni, Hg, and Pb) and suggested that roots accumulated the highest amounts of metals, followed by stems, leaves and flowers, regardless of the studied metal. Based on the findings of Anandi et al. [53], this selective accumulation of heavy metals in plant tissues could be due to differences in tolerance to toxic effects, as aerial tissues are more susceptible to stress than roots.

According to the literature, *Portulaca* species have been reported for the phytoremediation of heavy metal-polluted soils, since the species seems to be tolerant to toxic effects of increased contents of various metals. For example, Deepa et al. [54] suggested the efficiency of *P. oleracea* stem cuttings in removing Cu from two different types of soils (e.g., Alfisol and Vertisol), while plant uptake was higher for the Alfisol due to the lower availability of Cu in this particular soil type. Moreover, it is worth to highlight the potency of *Portulaca* species to hyperaccumulate different heavy metals, e.g., Cd, As and Cr; this indicates the presence of efficient defense mechanisms that alleviate heavy metal toxic effects [55]. The suggested mechanisms for stress alleviation include the biosynthesis of osmoregulators such as proline or the induction of antioxidant enzymes, e.g., guaiacol peroxidase (GPX) [47]. Finally Yang et al. [56] reported that purslane above and below ground plant parts showed a very high concentration in various trace elements, including chromium, and further suggested the use of the species as a potential biomonitor or phytoremediator.

Cr(VI) also limits the uptake of N, P, K, Mn, Fe, Cu, Zn and S. It is referred that Cr(VI) root uptake is mainly performed by phosphate and sulphate transporters due to the structural similarity of Cr(VI) to phosphate and sulfate ions [45,57]. Results of de Oliveira et al. [58] indicated that increasing Cr(VI) concentrations resulted in higher sulfate root uptake and elevated sulfur in aerial plant tissues. Elevated phosphorus plant tissue content exerts positive effects on enzymes involved in Cr(VI) reduction [59] and increased P uptake under Cr(VI) stress was noticed in *Citrullus vulgaris* [60]. In *Arabidopsis thaliana* seedlings, high phosphorus concentrations in plant tissues resulted in significantly reduced Cr(VI) tissue concentrations [48]. On the other hand, *Brassica napus* plants subjected to oxidative stress recorded higher phosphorus cell content and results indicated that elevated P content resulted in lower ROS stress [61]. These results are in accordance with the present experimental results, where purslane seemed to selectively absorb soil phosphorus when under Cr(VI) stress and nitrogen amendment had a positive effect on P accumulation in purslane aerial tissues with increasing Cr(VI) concentrations.

5. Conclusions

- Cr(VI) contents in root tissues were orders of magnitude higher than the concentrations found in aerial plant tissues.
- All physiological and growth parameters measured were severely affected and nitrogen in all cases resulted even partially in Cr(VI) stress alleviation.
- Under Cr(VI) stress purslane plants selectively accumulated phosphorus in aerial plant tissues.
- Cr(VI) stress resulted in lower water content in aerial plant tissues.
- Added N did not result in increased Cr(VI) content in aerial biomass compared to same Cr(VI)-amended treatments without N; however, the fact that added N improved plant's growth and physiological functions even when exposed to high Cr(VI) soil concentrations, means that sufficient N fertilization may be a satisfactory treatment to increased purslane tolerance against Cr(VI) toxicity.

- On the same lines, added N makes purslane a species to be further considered for phytoremediation of Cr(VI)-laden soils; however, we acknowledge that more research is necessary before conclusive decisions may be drawn.

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