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Lithium Toxicity in *Lepidium sativum* L. Seedlings: Exploring Li Accumulation's Impact on Germination, Root Growth, and DNA Integrity

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Abstract: The predicted increase in demand for minor metals for modern technologies raises major concerns regarding potential environmental concentration increases. Among the minor metals, lithium (Li) is particularly noteworthy due to growing demand for battery production. Concerns have been raised about the impact on biota of increasing Li concentrations in the environment. To expand the knowledge of the effects of Li on plants, garden cress (*Lepidium sativum* L.), a model plant for ecotoxicity assay, was tested in a 72 h test in Petri plates. The results showed a stimulation effect of Li at the lowest concentration (Li chloride 10 mg L^{-1}) on seed germination and primary root elongation. Conversely, higher Li concentrations (50 and 150 mg L^{-1}) caused a progressive impairment in both parameters. A genotoxic effect of Li on root cells, evaluated through the alkaline comet assay, was observed at each concentration tested, particularly at 150 mg L^{-1} Li chloride. Elemental analysis showed that Li accumulated in the seedlings in a dose–concentration relationship, confirming its ability to be readily absorbed and accumulated in plants. Given the likely increase in Li levels in the environment, further research is required to clarify the toxicity mechanisms induced by Li on growth and nucleic acids.

Keywords: alkaline comet assay; critical raw materials; ecotoxicity; garden cress; genotoxicity; minor metals; pollution

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1. Introduction

Modern industrial processes are increasingly demanding so-called "minor metals", depending on their occurrence in the Earth's crust. Among them, earth metals, precious metals, and special metals are particularly targeted [1]. An increase in their concentrations in the environment is then strongly predicted and, therefore, the interaction between minor metals and biota should be better studied. Lithium is a versatile element with a wide range of applications. While it is commonly known for its use in batteries for electric cars, it is also used in ceramics, glass, lubricating greases, air treatment, pharmaceuticals, and optics. In this regard, the increasing demand for lithium (Li) to produce Li-ion batteries [2] raises concerns about its potential accumulation in the environment through improper disposal [3] and its impact on organisms in aquatic and terrestrial ecosystems [4]. It is also worth noting that Li has been included in the list of critical raw materials approved by the EU Commission in 2023, including elements that have high economic importance for the

EU and a high supply risk (https://single-market-economy.ec.europa.eu/sectors/raw-materials/areas-specific-interest/critical-raw-materials_en (accessed on 26 March 2024)).

Lithium is present in freshwater at concentrations ranging from 0.07 μg L^{-1} to 40 μg L^{-1}. In lithium-rich sites, such as tailing dams, concentrations have been found to be as high as 13 mg L^{-1}, while in brines Li concentrations range from 20 mg L^{-1} in the Dead Sea to 1500 mg L^{-1} in Salar de Atacama. Lithium concentrations of 56 mg kg^{-1} have been measured in sediments, and in soils the content ranges from 3 mg kg^{-1} to 350 mg kg^{-1} [5,6]. Nevertheless, there is still a lack of information about how Li concentrations have changed in recent years considering growing global consumption of Li [7].

Compared to other cations in soil, Li exhibits relatively high mobility and may be leached into waters and readily taken up by plants [8]. As reported by Kastori et al. [9], the higher Li concentration in the leaves compared to the seeds of leafy plant species suggests an ascending translocation of Li in the xylem through the transpiration stream and its immobility in the phloem. Lithium absorption and accumulation have been described in many plant species, although no fundamental physiological processes have been directly associated with the presence of Li, indicating that this metal is not essential for life [10]. In addition to plant species, the accumulation of Li in plants is also influenced by the chemical form and substrate [11,12]. In this regard, Kalinowska et al. [13] have observed that lettuce plants exposed to Li chloride in nutrient solution were able to accumulate more than 3000 mg kg^{-1} of Li, while Zacchini et al. [14] have reported in *Cannabis sativa* that the supply of Li chloride to substrate resulted in the accumulation of more than 2000 mg kg^{-1} Li in in vitro culture microshoots. Finally, in soil spiked with Li chloride, Jiang et al. [15] detected 1800 mg kg^{-1} Li in leaf tissues of *Apocinum venetum*, while more than 1300 mg kg^{-1} Li was found by Kavanagh et al. [12] in the leaves of *Helianthus annus* exposed to Li sulphate.

An excessive presence of Li in plants may result in toxic effects [9,16,17]. Germination, shoot length, root length, and chlorophyll *a* and *b* content are the most studied toxicity endpoints in Li-exposed plants [18]. The reduction in growth observed in Li-treated plants is attributed to the decrease in water content and tissue turgidity resulting from Li exposure [10]. In addition to these traits, other physiological processes have been reported to be affected by Li accumulation in plants. Zacchini et al. [14] have observed an impairment in photosynthetic mechanisms and alteration in nutrient uptake in plants grown in the presence of high concentrations of Li in the substrate.

It has long been described in animals that Li increases the production of reactive oxygen species (ROS), leading to oxidative stress [19]. In plants, an increase in ROS production was reported by Dawood et al. [20] in *Brassica napus* exposed to 100 mg kg^{-1} Li carbonate. Furthermore, high levels of MDA were detected by Hawrylak-Nowak et al. [21] in different plant tissues that underwent Li toxicity, while Bakhat et al. [22] showed the activation of antioxidant enzyme activities in spinach plants grown on Li-supplemented soil. The toxic effects of Li carbonate on meristematic cells of *Allium cepa* were reported by Kuloğlu et al. [23] to be a result of a deterioration of antioxidant/oxidant dynamics. Further, this study reveals that Li can induce cytotoxic effects as determined by mitotic index (MI) ratio and genotoxic effects detected using micronucleus and chromosomal aberrations. In this context, it is important to note that information about Li toxicity on nucleic acids is yet to be elucidated, as pointed out by Tanveer et al. [16].

Lithium accumulation in plants may represent an important pathway for Li to enter humans or ecosystems [8], in addition to its direct effects on plant vitality. Therefore, it is important to increase our understanding of the toxic effects of Li accumulation in plants at both growth and nucleic acid levels. This will enable us to better evaluate the potential impact of the predicted increase in Li levels in the environment on ecosystems.

This study aims to study the toxicity of Li in plants, targeting both germination and root growth and nucleic acid integrity in garden cress (*Lepidium sativum* L.), a model plant for ecotoxicity studies on terrestrial plants [24]. To the best of our knowledge, only one study that has focused on this plant species is present in the literature—that by Adiloglu et al. [25],

focusing solely on the accumulation of Li and some other metals in plants grown on Li-supplied soil. There is no literature reporting on genotoxic studies on Li toxicity in *Lepidium sativum* plants. Therefore, this study aims at expanding the knowledge on the toxic effects of Li on plants, especially with regards to the potential genotoxicity of this metal. To this scope, a plant species recognised in official protocols [23] as suitable for these kind of studies, *Lepidium sativum,* was chosen, owing also to its well-known characteristics of uniformity in seed germination and root elongation resulting in satisfactory reliability and reproducibility of the results and thus allowing for the adoption of experimental schemes with a limited number of replicates.

2. Materials and Methods

2.1. Germination Toxicity Assay and Root Elongation Measurement

Seeds of garden cress (*Lepidium sativum* L.) were purchased from Ingegnoli seed company (Milan, Italy) and used to evaluate the effects of LiCl (Sigma-Aldrich, St. Louis, MO, USA) on seed germination and root elongation based on official toxicity assays [26,27]. In detail, ten garden cress seeds were placed in 9 cm diameter plastic Petri plates containing one foil of filter paper filled with 5 mL of deionised water supplied with 0 (control), 10 mg L^{-1}, 50 mg L^{-1}, or 150 mg L^{-1} of Li chloride, with five replicates per concentration. The Li concentrations to be tested were chosen within the range of the Li concentrations assayed in similar experiments [28] and refer to the levels found in natural soils [6]. At the end of the incubation time (72 h) in the growth cabinet (Eyela incubator, Tokyo, Japan) in darkness at 25 ± 1 °C, the following biometric parameters were evaluated by processing images using an imaging analysis software (ImageJ, IJ 1.46r, http://imagej.nih.gov/ij/ (accessed on 26 March 2024)): number of germinated seeds, length of total plantlet, and length of root. The data were used to calculate the percent germination index (GI%, [29]) and analyse the root growth as absolute values.

2.2. Genotoxicological Assessment (Alkaline Comet Assay)

Root apices (1 cm in length) were excised from germinated seeds exposed to solutions with varying concentrations of Li chloride for 72 h, as detailed in 2.1. The excised root apices were placed in 150 µL of chilled (4 °C) buffer (400 mM Tris buffer, pH 7.5) within a 60 mm glass Petri dish, which was kept on ice. Using a razor blade, the root apices were finely chopped into numerous small fragments to isolate the nuclei. The resulting nuclear suspension was mixed with an equal volume of 0.8% low-melting-point agarose (in phosphate-buffered saline (PBS) buffer) at 38 °C and distributed onto two GelBond sheets (20 × 50mm, Lonza (Basel, Switzerland)) previously coated with 1% normal-melting-point agarose. The alkaline comet assay, employed as a biomarker of exposure, was conducted following the methodology outlined by Pietrini et al. [30]. Each experiment was replicated twice. DNA damage was assessed by quantifying the migration of 100 randomly selected nuclei on each GelBond sheet, which were photographed at 40× magnification using a Digital HD camera (Leica ICC50HD (Wetzlar, Germany)) and analysed using LAS V4.9 software. The acquired images were further processed using TriTekCorp™ CometScore software, version 2.0, to measure the Tail Moment (TM). TM is defined as the product of the tail length and the fraction of total DNA in the tail, providing insights into both the size of migrating DNA and the number of broken DNA fragments [31].

2.3. Lithium Chemical Analysis

Samples of *Lepidium sativum* L. plantlets, at the end of the 72 h treatment with Li chloride, were oven-dried for 48 h at 60 °C, homogenized, and weighed by using an analytical balance (Gibertini Europe 60; Gibertini Elettronica Srl, Milano, Italy). Approximately 25 mg of dry weight (dw) for each sample was acid digested (HNO_3/H_2O_2, 2:1, *v*/*v*; Promochem, LGC Standards GmbH, Wesel, Germany) in a microwave oven (Ethos Touch Control with Q20 rotor, Milestone, Bergamo, Italy) for 30 min at 180 °C. Further details regarding the microwave-assisted acid digestion efficiency can be found in Astolfi et al. [32]. The ob-

tained solutions were diluted in 500 mL of deionized water and filtered using syringe filters (25 mm diameter, 0.45 µm pore size, GVS Filter Technology, England, UK). Li concentration was analysed by using an inductively coupled plasma mass spectrometer (ICP-MS; model 820-MS; Bruker, Bremen, Germany) equipped with a glass nebulizer (0.4 mL min^{-1}; Analytik Jena AG, Jena, Germany). Three replicates were performed for each measurement, showing standard deviations < 5%. The calibration curve was generated using solutions of Li at 5, 20, 50, 200, and 500 µg L^{-1} obtained by serial dilutions of a 1000 ± 2 mg L^{-1} Li standard stock solution (Merck Millipore Ltd., Billerica, MA, USA). Two internal standards, yttrium and rhodium (1000 ± 2 mg L^{-1}; Panreac Quimica, Barcelona, Spain), were used for all the measurements to control nebulizer efficiency. The limit of detection (LOD) for Li concentration (0.012 µg L^{-1}) was determined as the mean plus three times the standard deviation (SD) of five replicate blank determinations. Finally, the Li concentration (mg kg^{-1}) in the *Lepidium sativum* L. plantlets was calculated by dividing the Li content by the dw of each sample.

2.4. Statistics

The experimental trial was carried out by following the US EPA (1996) and APAT-RTI CTN_TES 1/2004 (2004) guidelines for ecotoxicological testing with *Lepidium sativum* L., with at least three replicates per treatment. For the germination toxicity assay and Li concentration analyses, one-way ANOVA was used to analyse normally distributed data using the SPSS v. 28.0.1 (Chicago, IL, USA) software tool. Percent data regarding seed germination rate were previously subjected to the arcsin square root transformation. The statistical significance of the mean data was assessed by using Tukey's test ($p \leq 0.05$), unless otherwise stated. For the genotoxicological assay, the results, represented as Tail Moment (TM) values, were reported as means with standard error (SE). Due to the non-normal distribution of the data after the Shapiro–Wilk test, a non-parametric test (Kruskal–Wallis) was employed to compare each treatment with its corresponding control group using the statistical analysis program PAST (version 4.06). Statistical significance was determined at a threshold of $p \leq 0.05$.

3. Results and Discussion

Figure 1 shows the results of the effects of Li presence in the medium used to germinate *L. sativum* seeds. The data are expressed as the germination index (GI%), which is a suitable parameter to evaluate the toxicity of chemical compounds on model plants such as *L. sativum* [33]. After 72 h of incubation, the presence of Li chloride had a different effect on the germination of *L. sativum* seeds compared to those germinated in distilled water. No toxicity or slight stimulation effects were observed in the germination of seeds exposed to the lowest concentration of Li chloride. However, the presence of higher amounts of Li in the medium caused a reduction in the GI% value to 74 at 50 mg L^{-1} Li chloride. Furthermore, the exposure of seeds to 150 mg L^{-1} Li chloride resulted in a more evident toxic effect, lowering the GI% value by 40% compared to the control. These results are consistent with the findings by Gayathri et al. [28], which showed no effects of Li sulphate at 10 mg L^{-1} on *Amaranthus viridis* seed germination. However, a similar reduction in germination rate to that reported in Figure 1 was observed at 50 and 100 mg L^{-1}. The toxicity of Li on seed germination was also evaluated by Kavanagh et al. [12], who exposed 34 plant species to various levels of Li sulphate in solutions. It was observed that, in most plants, the seed germination rate decreased with the increasing concentration of Li in the medium.

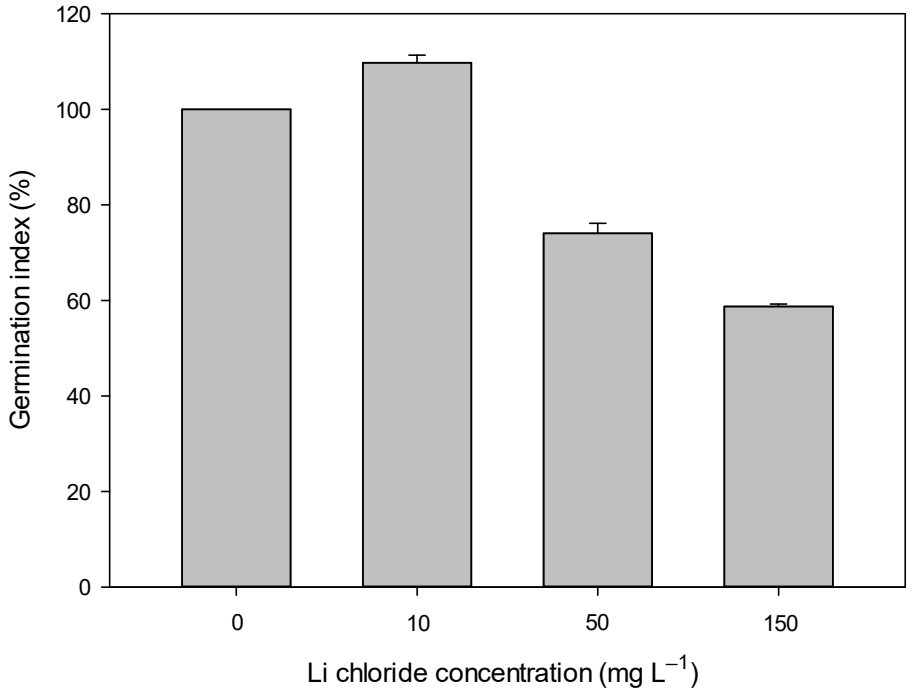

Figure 1. Germination index (GI %) evaluated in garden cress (*Lepidium sativum* L.) seedlings after 72 h of treatment with deionised water supplied with 0 (control), 10, 50, and 150 mg L^{-1} of Li chloride (LiCl). In each bar, percent mean data (n = 5 ± S.E.) are shown.

The exposure of plants to metals is reported to affect the root growth process in plants [34]. Specifically, root elongation is used as an early indicator of the toxic effects of chemical compounds on plants (US EPA, 1996; APAT-RTI CTN_TES 1/2004, 2004). In this regard, *L. sativum* is considered a model plant species to investigate the toxicity of minor metals using this trait [33]. Figure 2 shows the effects of increasing Li chloride concentration in the liquid medium on the primary root elongation of *L. sativum* seedlings. Similar to the data presented in Figure 1 on seed germination, a slight stimulation of root elongation was observed at 10 mg L^{-1} of Li chloride compared to control. Moreover, as the Li concentration in the medium increased, there was a significant decrease in root elongation. At 50 mg L^{-1} Li chloride, the decrease was 25% compared to control roots, and at 150 mg L^{-1} Li chloride, it was nearly 40%. This highlights the concentration-dependent toxic effect of Li on seedling growth. Gayathri et al. [28] have reported similar results in *Amaranthus viridis* seeds exposed to various Li sulphate concentrations. Lower concentrations (10 mg L^{-1} to 50 mg Li L^{-1}) stimulated root growth, while the highest Li concentration (100 mg Li L^{-1}) caused a marked reduction in root elongation. Overall, the results obtained for seed germination and root elongation in *L. sativum* exposed to different levels of Li chloride confirmed the growth-stimulating effects of Li at low concentrations (2–25 mg Li L^{-1}), as already reported for root and shoot biomass in different plant species by Naranjo et al. [35], Li et al. [36], Hawrylak-Nowak et al. [21], and Kavanagh et al. [12]. In this respect, a possible hormetic effect of Li in plants can be claimed, as suggested by Kalinowska et al. [13], since the responses of plants to the presence of low concentrations of Li are consistent with the stimulation of plant growth by sublethal doses of potentially toxic agents, characterising the process of hormesis in plants. The same authors highlighted how the toxicity effect of Li hydroxide on seed germination rate was higher than that of Li chloride at similar Li concentrations to those used in the present work. Therefore, even if an effect of salinity increase in the medium of plants cannot be completely ruled out, the work by Kalinowska et al. [13] suggests that, at the Li concentrations used in these experiments, such an effect can be considered negligible.

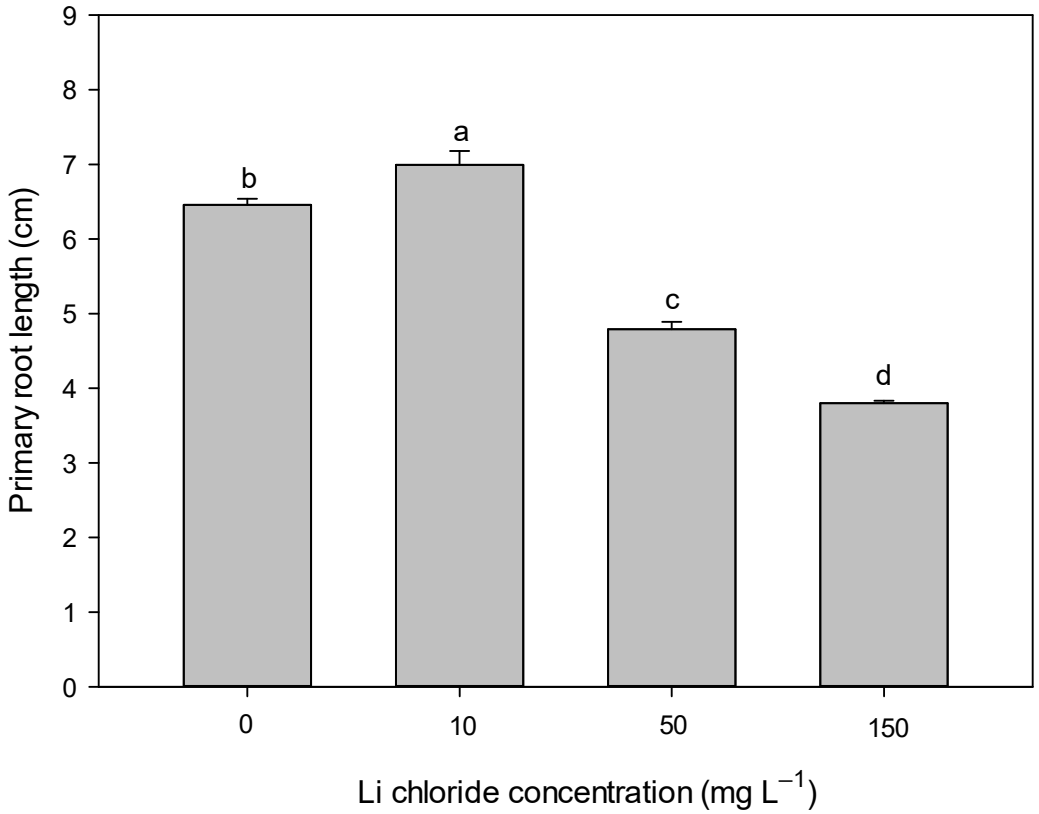

Figure 2. Primary root length measured in garden cress (*Lepidium sativum* L.) seedlings after 72 h of treatment with deionised water supplied with 0 (control), 10, 50, and 150 mg L^{-1} of Li chloride (LiCl). In each bar, mean data (n = 5 ± S.E.) are shown. Different letters correspond to statistical different values (Tukey's test, $p \leq 0.05$).

As part of the studies aimed at better characterising the toxic effects of Li in plants, biochemical processes related to the oxidative stress status in plants exposed to Li were investigated. In this regard, an increase in lipid peroxide content after Li treatments of plants was reported by Hawrylak-Nowak et al. [21]. The generation of an oxidative stress condition by Li might explain the cytotoxic and genotoxic effects found in the root tips of Allium cepa bulbs treated with Li sulphate by Kuloğlu et al. [23]. However, as stated by Tanveer et al. [16], more information is required on this subject to increase knowledge of the potential genotoxicity of Li in plants. Figures 3 and 4 show the results of the genotoxicological study carried out by using an alkaline comet assay to assess DNA damage in the roots of L. sativum seedlings exposed to increasing concentrations of Li chloride for 72 h. The extent of DNA damage in root cells was evaluated by calculating the Tail Moment (TM), and the results are shown in Figure 3. Statistically higher values of TM were observed in all Li-treated plants compared to the controls (untreated plants), highlighting a clear genotoxic effect of Li on L. sativum plants. Furthermore, this toxic effect was particularly dramatic in plants exposed to 150 mg L^{-1} Li chloride, with TM values almost 3 times higher than in the controls.

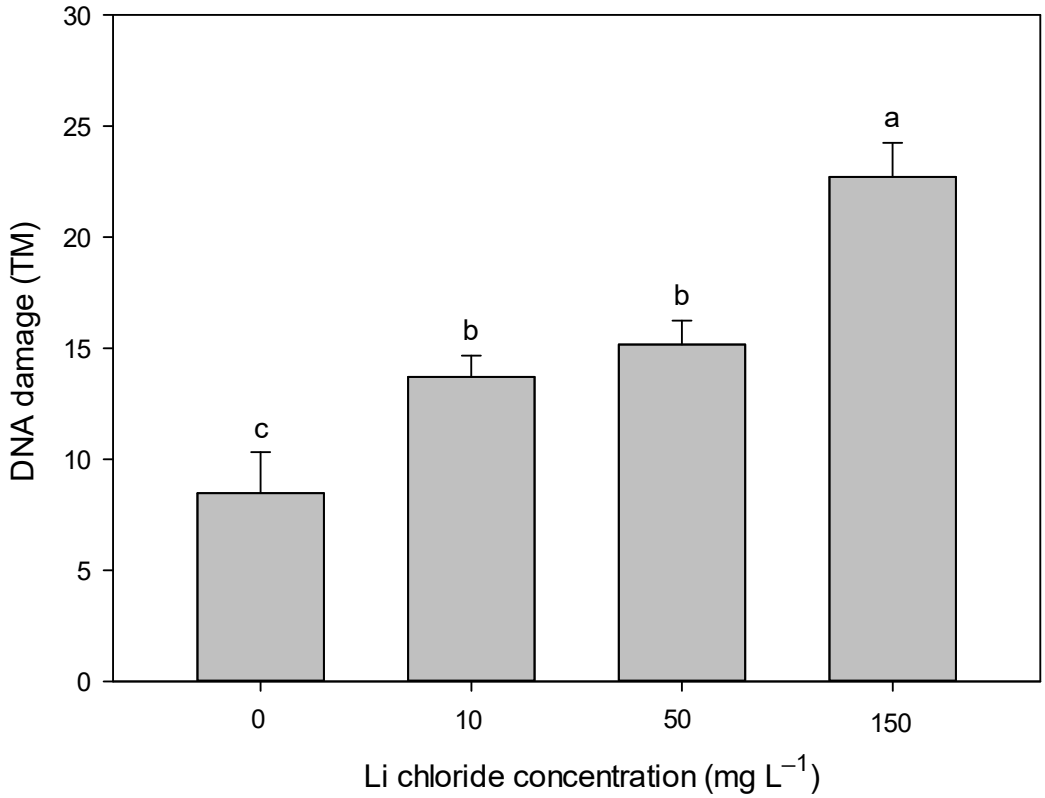

Figure 3. DNA damage expressed as Tail Moment, defined as the product of the tail length and the fraction of total DNA in the tail, in root apices of garden cress (*Lepidium sativum* L.) seedlings after 72 h of treatment with deionised water supplied with 0 (control), 10, 50, and 150 mg L^{-1} of Li chloride (LiCl). Data are reported as mean± S.E (n = 3). Different letters correspond to statistical different values (Kruskal–Wallis test, $p \leq 0.05$).

Representative images of DNA degradation in the form of comets observed in the roots of the control (Figure 4a) and Li-treated plants (Figure 4b) are shown. These results confirm the observation reported by Kuloğlu et al. [23], although a different Li formulation (carbonate vs. chloride) and plant species were tested. Remarkably, the study by Kuloğlu et al. [23] highlighted the possible mechanisms by which Li can affect DNA integrity, demonstrating the ability of this metal to intercalate by binding to the same and different strands in DNA and possibly by binding to regions rich in G-C, C-A, A-A, and T-A nucleotides. To the best of our knowledge, no other literature focuses on this issue for plants. In animal cells, although more literature is available [37], the genotoxicity of Li has only been demonstrated in amphibian tadpoles exposed to Li at concentrations of 2.5 and 20 mg L^{-1} [38]. It is worth noting that exposure to 10 mg L^{-1} Li chloride for 72 h caused significant DNA damage in seedlings, which is similar to the results observed in plants exposed to 50 mg L^{-1}. However, it had no effect or slightly stimulated seed germination and root elongation. This observation validates the alkaline comet assay as a reliable biomarker for detecting early biological impacts of minor metals on plants, as previously noted for bismuth by Liman [39] and Passatore et al. [33].

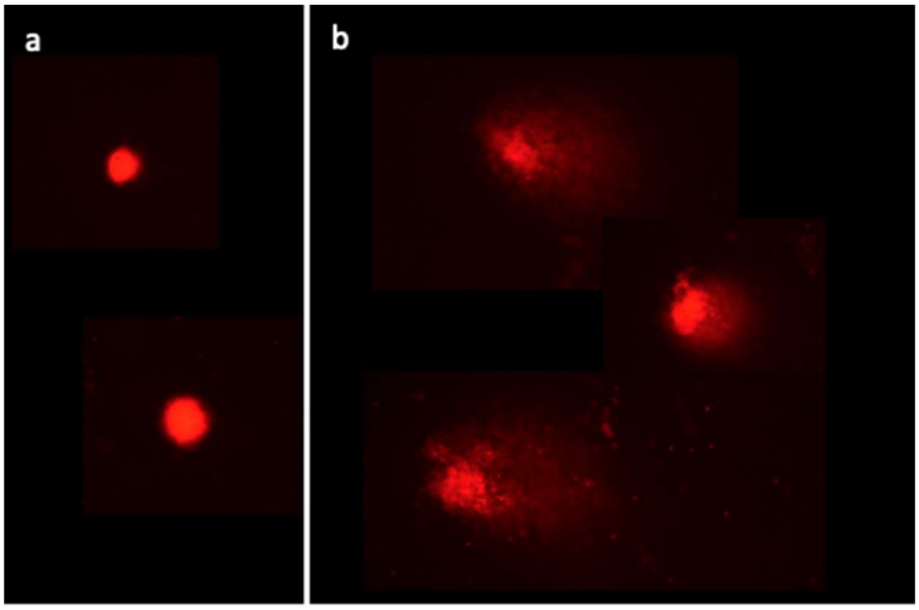

Figure 4. Representative images (after alkaline comet assay procedure and EtBr staining) of nuclei from root tissues of garden cress (*Lepidium sativum* L.) seedlings after 72 h of exposure, exhibiting different DNA damage levels: untreated (**a**) and 150 mg L^{-1} Li chloride-treated (**b**).

Lithium is readily absorbed and transported by plants [16] and accumulates at high levels in above-ground tissues under different experimental conditions [13–15], likely because of the similarity of this element to K and Na [12]. Figure 5 shows the concentrations of Li detected in *Lepidium sativum* seedlings treated with different levels of Li chloride for 72 h. The results highlight the ability of garden cress seedlings to accumulate Li in a dose-dependent manner, with plant Li concentration closely related to the metal concentration in the solution. In the control plants grown in solution without Li supply, the presence of Li is due to the metal accumulation from soil during the development of the seeds used in this experiment. Li accumulation in seeds, although lower than in other plant organs, is in accordance with the results of Kastori et al. [9]. The exposure of garden cress seedlings to higher Li concentrations (50 mg L^{-1} and 150 mg L^{-1} Li chloride) resulted in significant Li concentrations in the plants (i.e., 142 and 318 mg kg^{-1} dw), confirming that Li is readily accumulated by plants. These findings are consistent with those observed by Adiloglu et al. [25] in garden cress plantlets grown for 45 days in soil supplied with 0 to 8 mg kg^{-1} Li. In fact, in that study, an increase in Li concentrations in plant tissue was observed with an increase in the presence of Li in the soil. However, due to the different experimental conditions, the Li accumulations in plant tissue are not comparable between the two trials, mainly because of the different metal availabilities in the substrates. The accumulation and translocation of Li in plants have been reported to be dependent on the chemical formulation and type of medium, as described in several studies [12–14]. Finally, the results of this study indicate that garden cress plants are capable of significantly accumulating Li in their tissues, with a bioconcentration factor (BCF, see [40]) of nearly 13 when seedlings are exposed to 150 mg L^{-1} Li chloride in aqueous solution. This raises concerns about the safety of consuming this vegetable in the human diet, taking into account that Li concentrations in soil range between 2 and 200 mg kg^{-1} [6].

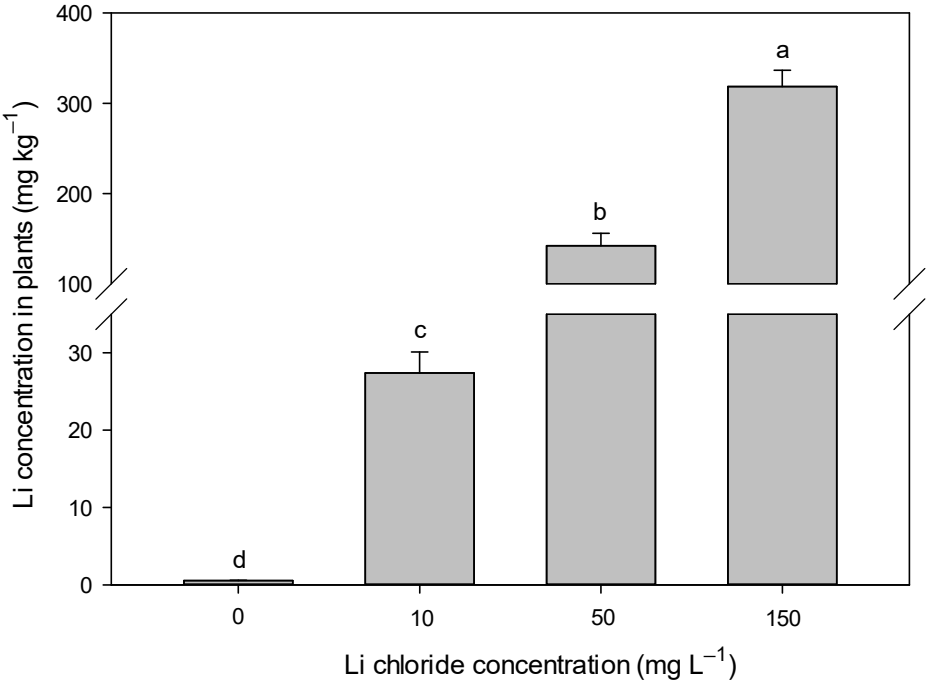

Figure 5. Lithium concentration in garden cress (*Lepidium sativum* L.) seedlings after 72 h of treatment with deionised water supplied with 0 (control), 10, 50, and 150 mg L^{-1} of Li chloride (LiCl). In each bar, mean data (n = 3 ± S.E.) are shown. Different letters correspond to statistically different values (Tukey's test, $p \leq 0.05$).

4. Conclusions

This study confirms the toxic effect of Li on the growth of *Lepidium sativum* L. at environmentally relevant concentrations. However, exposure to low concentrations of Li did not result in adverse effects and even had a slight stimulating effect, suggesting a possible hormetic effect of Li on plants. A significant accumulation of Li in plant tissue was detected in close relation to the Li concentration in the medium, confirming the ability of plants to readily absorb and transport this metal. Notably, evidence of the genotoxicity of Li in plants was found, even at the lowest Li concentration (10 mg L^{-1} Li chloride) in the medium, which was not toxic to growth. This further highlights the suitability of the alkaline comet assay for assessing DNA damage as an early endpoint to evaluate the stress condition caused by chemicals in plants, as already observed for both organic [30] and inorganic [33] pollutants.

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Data Availability Statement: The raw data supporting the conclusions of this article will be made available by the authors on request.

Conflicts of Interest: The authors declare no conflicts of interest.

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