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Article

The effect of chondroitin sulfate on the development of plants grown in roadside soil

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SUMMARY

Soil contamination with heavy metals, associated with the development of transportation, can lead to the destruction of vegetation cover and the formation of "technogenic" deserts, or traffic-induced barren areas. Heavy metals catalyze the formation of reactive oxygen species, inactivate enzymes, and disrupt photosynthesis. Chondroitin sulfate (CS) is a component of connective tissue in humans and animals and is involved in the chelation of metal ions. Therefore, we aimed to study the effect of CS on the growth of bioindicator plants grown in roadside soil. We hypothesized that the introduction of CS into contaminated roadside soil would lead to a reduction in soil phytotoxicity due to the chelation of transition metal ions by CS. We showed that on the seventh day of observation, plants growing in roadside soil were smaller than those in park soil but adding CS to the roadside plants improved their growth. Also, after adding CS, the amount of chlorosis in the leaves of plants grown in roadside soil decreased, and the intensity of the catalase reaction increased. Overall, these results demonstrate the potential of CS as an environmentally relevant agent for reducing heavy metal-induced phytotoxicity in roadside soil. The proposed approach may be further developed by researchers in soil remediation and environmental sciences and applied in nature-based strategies for the restoration of degraded urban and transport-associated ecosystems.

INTRODUCTION

Soil acts as a major geochemical barrier that limits the movement and bioavailability of toxic solutions and chemical elements, thereby separating them from plants and other biological components. However, soil cover is subject to constant contamination from various sources (1). Motor vehicles are the main source of soil contamination in roadside areas through their introduction of copper, iron, zinc, lead, nickel, mercury, and cadmium to the soil (2).

It is known that many metals are necessary in certain amounts for the normal functioning of higher plants (2–5). For example, copper is a structural component of the cytochrome oxidase complex of the mitochondrial respiratory chain, while zinc and nickel are cofactors of various enzymes (2, 6–8). However, the accumulation of heavy metals above the maximum permissible concentration in plants negatively affects their growth (2, 3, 6, 8). One of the phytotoxicity mechanisms of heavy metals is the binding of amino and sulfhydryl groups in proteins, resulting in a change in conformation and inactivation of enzymes. For example, nickel, zinc, and copper can replace the magnesium ion in ribulose 1,5-bisphosphate carboxylase. This replacement leads to enzyme inactivation, which impairs CO_2 assimilation during photosynthesis, resulting in reduced sugar synthesis, energy deficiency, and subsequent suppression of plant growth and productivity (2, 6–9). An increase in nickel concentration also leads to a decrease in the activity of δ-aminolevulinic acid dehydratase, which is involved in the formation of chlorophyll precursors (7). Additionally, cadmium can act as a zinc antagonist due to the similarity of their chemical properties and disrupt enzyme function, inhibiting plant growth (2).

It is important to note that most heavy metals belong to the group of transition elements, those that easily give or accept free electrons from or to their outer electron orbitals due to redox processes that occur both in the soil and in plant cells (10, 11). By interfering with the respiratory chain of mitochondria, such metals can disrupt electron transfer processes and further catalyze the formation of reactive oxygen species (ROS) (3, 5). As a result, the antioxidant system is depleted and oxidative stress can occur (4, 5, 10, 11). This process occurs in chloroplasts, mitochondria, and peroxisomes (5, 7, 8). Thus, elevated concentrations of zinc, iron and copper ions, due to their ability to accept and donate free electrons, disrupt the electron transport chain and oxidative phosphorylation. This results in a decrease in adenosine triphosphate (ATP) synthesis, an increase in the formation of ROS, and activation of lipid peroxidation reactions. Consequently, oxidative stress in mitochondria leads to growth inhibition and plant death (3, 7, 8).

The effect of metals on the antioxidant status of plants varies (3, 9, 11). Antioxidant enzymes such as superoxide dismutase (SOD), which catalyzes the dismutation of superoxide radicals, as well as peroxidase and catalase, which are involved in the detoxification of hydrogen peroxide, play a key role in protecting plants from oxidative stress (3, 9, 11). For instance, when peas were exposed to elevated concentrations of cadmium, SOD and peroxidase activity decreased (9, 11). Elevated concentrations of lead and mercury also inhibited SOD and catalase due to the interaction of these metals with specific protein components of these enzymes (3, 9, 11). On the other hand, a decrease in enzyme activity may indicate depletion of the antioxidant system (3, 11). However, when plants were treated with copper solutions, an increase in peroxidase, catalase, and SOD activities was observed (9).

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Oxidative stress initiated by transition metal ions can be reduced by using molecules that have chelating activity towards these metals (12). Metal ion chelation occurs through the formation of stable complexes between metal ions and functional groups of organic molecules, such as carboxyl, hydroxyl, or sulfate groups, which bind the metal ion at multiple sites. Examples of chelating molecules include chondroitin sulfate (CS), ethylenediaminetetraacetic acid, citric acid, humic substances (natural chelators in soils and aquatic environments), and amino acids such as histidine (13).

CS is one of the main components of connective tissue in animals and can be found in bones, cartilage, tendons, and ligaments. CS provides structural support and elasticity, retains water, participates in the transport and chelation of metal ions, regulates cellular interactions, and provides protective functions in the extracellular matrix (12, 13). Due to these qualities, CS has been used in medical practice for over 40 years. It is used mainly in the treatment of degenerative joint diseases, where it acts as a chondroprotective agent by preserving cartilage structure, inhibiting matrix degradation, reducing inflammation, and chelating metal ions involved in oxidative stress (12, 13–16). CS administration orally or intravenously reduces the severity of diseases involving free radical generation, in particular, osteoarthritis. It may act by reducing hydroperoxides and H_2O_2, by sequestering metal ions, scavenging active free radicals, or repairing damage (12, 13).

The CS molecule is a sulfated glycosaminoglycan consisting of long unbranched chains with repeating residues of N-acetyl galactosamine and glucuronic acid (17). The presence of sulfated and carboxyl fragments in CS determines its electrostatic activity, which is the basis for the molecule's involvement in the transport of water and nutrients, and chelation of metal ions (**Figure 1**). Thus, the sulfate groups at the 4-carbon position (CS-4) or 6-carbon position (CS-6) stabilize the interaction between the metal cation and the carboxyl group, creating a high negative charge density. CS-6 generally exhibits slightly greater conformational flexibility due to sulfation at C6, potentially facilitating more accessible coordination interactions compared to CS-4 (17).

Although CS is not naturally synthesized by plants, its strong chelating properties and biocompatibility make it a promising remediation compound for contaminated soils. CS is commercially obtained from the cartilage tissue of cattle and fish, a by-product of the meat industry, using established extraction and purification processes (18). This makes CS generally accessible for application at the scale of small sites and restoration projects along roadsides or industrial zones (18).

Numerous *in vivo* animal studies have shown that the antioxidant activity of CS is due to its ability to chelate transition metal ions, such as copper and iron, by binding the ion to carboxylate and sulfate groups within one or more chains (10, 12–14, 19, 20). It has also been shown that the content of CS significantly increased in the bronchi of rats after exposure to diesel exhaust particles, which also suggested that CS contributes to the inactivation of heavy metal cations during oxidative stress (13, 15). The effectiveness of oral CS-iron colloid has also been demonstrated experimentally for rats with severe anemia (13, 16, 20). The antioxidant activity of CS in rat models of collagen-induced autoimmune arthritis, acute hepatitis, and pancreatitis has also been demonstrated (13, 19).

These data led us to hypothesize that the introduction of CS into contaminated roadside soil may contribute to a reduction in soil phytotoxicity due to the chelation of transition metal ions. We aimed to study the effect of CS on the development of bioindicator plants grown in soil in roadside areas expected to be contaminated with heavy metals and in park zones serving as presumably uncontaminated controls (21). We also investigated the condition of the leaf blade and redox activity of bioindicator plants after the addition of CS to the soil. Our results showed that plants grown on roadside soil exhibited reduced growth, increased chlorosis, and lower catalase activity compared to those grown on park soil, whereas the addition of CS was associated with improved growth, reduced leaf damage, and partial restoration of catalase activity. Overall, our results demonstrate the promising potential of CS as an environmentally relevant agent for reducing heavy metal-induced phytotoxicity in roadside soils. The proposed approach may be further developed by researchers in soil

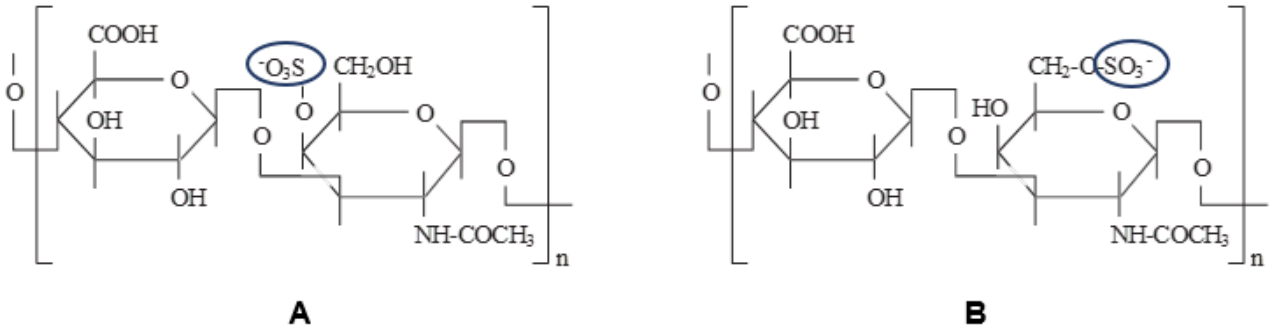

Figure 1: The structure of the chondroitin sulfate molecule determines its participation in the chelation of metal cations. Chemical structure of (A) chondroitin-4-sulfate (CS-4) and (B) chondroitin-6-sulfate (CS-6). The sulfate group in position 4 or 6 (circled) stabilizes the interaction between the metal cation and the carboxyl group, which is also facilitated by its location along the central line of the polymer, thus creating a high negative charge density. CS-6 generally exhibits slightly greater conformational flexibility due to sulfation at C6, potentially facilitating more accessible coordination interactions compared to CS-4.

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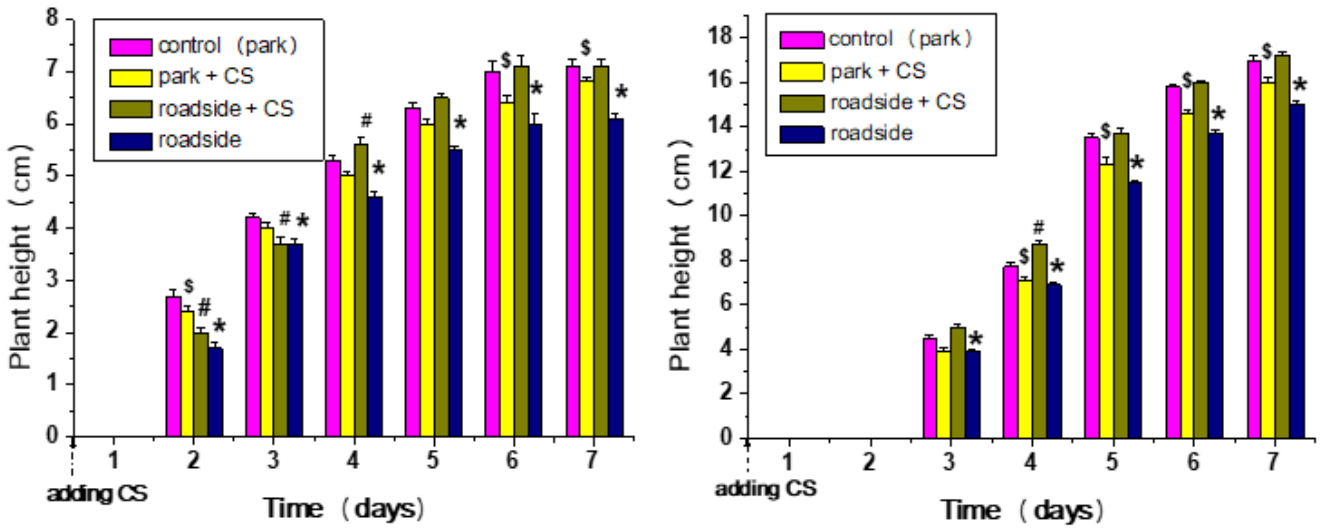

Figure 2: The addition of chondroitin sulfate to the contaminated soil improves the growth parameters of bioindicator plants. Effect of chondroitin sulfate (CS) on the growth of bioindicator plants in different soil types (n = 45). Growth dynamics of bioindicator plants (A) watercress and (B) Daikon radish grown for seven days in park soil (control; magenta), park soil supplemented with CS (yellow), roadside soil supplemented with CS (brown), and roadside soil (dark blue). CS was added at time of seeding. Seedling height was measured starting from day 2 after planting for watercress and day 3 for Daikon radish. Data are presented as mean ± SD. Statistical analysis was performed using a two-way repeated measures ANOVA followed by Bonferroni post hoc test. Symbols above bars indicate statistically significant differences ($p<0.05$): * = roadside soil; # = roadside soil supplemented with CS; $ = park soil supplemented with CS, each compared with the control (park soil).

remediation and environmental sciences and applied in nature-based strategies for the restoration of degraded urban and transport-associated ecosystems.

RESULTS

To evaluate the potential of CS to reduce soil phytotoxicity associated with roadside contamination, we grew watercress and Daikon radish in soil collected from roadside and park areas, with and without the addition of CS. These plants exhibit rapid seed germination and near 100% germination rate, which is significantly reduced in the presence of pollutants such as heavy metals (22–24).

We monitored plant development over a seven-day period under identical conditions: 24-hour artificial lighting, a temperature of 25°C, and watering twice a day.We evaluated differences due to soil origin and CS addition using growth parameters of the above-ground parts of the plants, visual and microscopic assessment of leaf condition, and semiquantitative analysis of catalase activity as an indicator of antioxidant response. For each experimental group, we performed measurements on 15 individual seedlings per container, with 3 independent biological replicates (n = 45 plants per treatment). Quantitative data are presented as a mean with standard deviation (± SD).

Growth indicators of the above-ground parts of bioindicator plants

First, we measured the mean height (± SD) of the above-ground parts of watercress and Daikon radish seedlings over the seven-day growth period under different soil conditions (**Figure 2**). Seedling height increased progressively over the observation period in all experimental groups (**Figure 2**). Therefore, differences between treatments were further evaluated at day seven as the final experimental time point. The heights of the above-ground parts of watercress and Daikon radish sprouts grown in roadside soil were, on average, 1.0 cm (15%) and 2.1 cm (12%) lower, respectively, compared to sprouts grown on park soil (**Figure 2**; roadside soil vs. park soil; $p < 0.05$). The addition of CS to roadside soil restored seedling height to values not significantly different from the park soil control (**Figure 2**; roadside soil + CS vs. park soil; $p > 0.05$). In contrast, the addition of CS to park soil resulted in a small but statistically significant reduction in seedling height by 5% in watercress and 9% in Daikon radish compared to plants grown in park soil without CS (**Figure 2**; park soil + CS vs. park soil; $p < 0.05$). Notably, the mean height of plants grown on roadside soil with CS were higher than the mean height of plants grown on park soil with CS (**Figure 2**). Overall, these data suggest that CS alleviates growth inhibition under contaminated soil conditions but may reduce growth when applied to non-contaminated soil.

The condition of the leaf plates of bioindicator plants

The proportion of plants with chlorosis and necrosis is an important diagnostic feature in biotesting (25). Chlorosis, visible as leaf yellowing due to chlorophyll breakdown, is associated with compromised photosynthetic capacity, whereas necrosis denotes the appearance of dead tissue and irreversible damage to leaf cells (25). These visual symptoms are widely used as indicators of plant stress and health status in phytotoxicity studies (25).

We observed various changes in leaf quality of watercress and Daikon radishes in the studied groups. Leaves of plants grown in park soil were well-developed and had a rich emerald color, with necrosis and chlorosis affecting less than 1% of plants (**Figure 3**). Plants grown on roadside soil had yellowed and curled leaves, and the proportion of plants with necrosis and chlorosis increased to approximately 15% (**Figure 4**).

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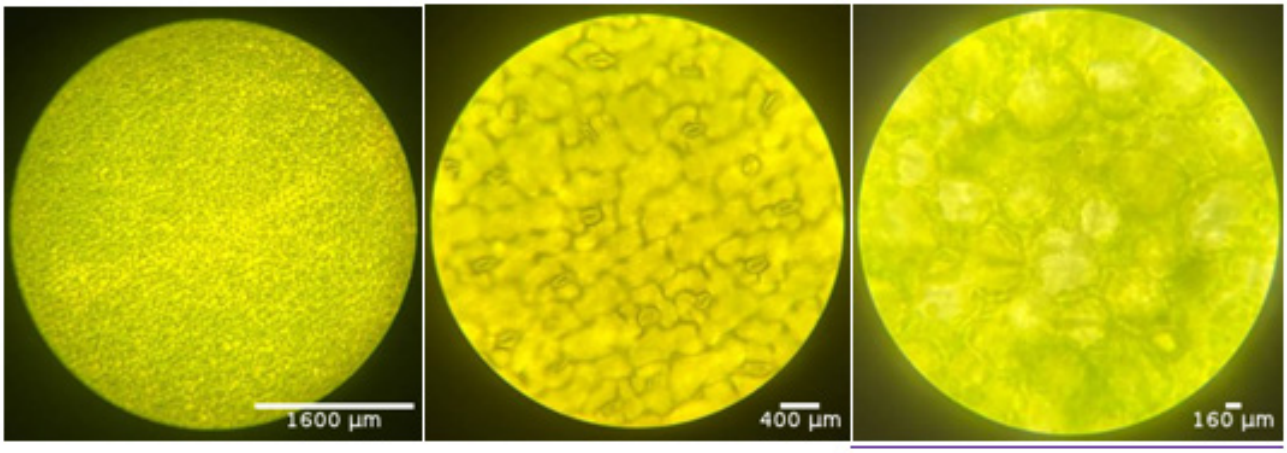

Figure 3: Intact leaf morphology of watercress grown in uncontaminated soil. Fragment of a watercress leaf grown in park soil in seven days under a microscope. (A) 100x, the leaf is evenly and intensely colored, with numerous stomata. (B) 400x, uniform bright coloring, black arrows indicate the well-colored open stomata, which are visible between the epidermal cells. (C)1000x, black arrows indicate the numerous chloroplasts, which can be seen through the epidermal cells along the perimeter of the rounded mesophyll cells.

Microscopically, we observed that plants grown in park soil exhibited intact, well-organized chloroplasts, whereas plants grown in roadside soil showed chlorotic features that may be associated with alterations in chloroplast structure and function (**Figure 4**). When CS was added to the contaminated roadside soil, the proportion of plants with necrosis and chlorosis decreased to approximately 8%. The increased incidence of chlorosis and necrosis observed in plants grown on roadside soil is consistent with impaired leaf function and reduced physiological health under contaminated conditions. The reduction of these symptoms following CS addition indicates improved leaf condition and reduced visible stress symptoms, suggesting a reduction in soil phytotoxicity.

Catalase activity in the leaves of bioindicator plants

We observed catalase activity in leaves of bioindicator plants under a microscope to evaluate oxidative stress responses, as catalase activity serves as an indicator of the plant antioxidant defense system and reflects the ability of plants to detoxify ROS generated under environmental stress conditions.

We assessed catalase activity using a semiquantitative macroscopic scoring system based on the intensity of oxygen bubble formation following the addition of 3% hydrogen peroxide to extracts from watercress and Daikon radish leaves. We scored the relative intensity of catalase-mediated H_2O_2 decomposition on a 0–4 scale (0 – no activity; 4 – intense bubble formation). The lowest catalase activity (1–2 points) was observed in the leaves of plants grown in roadside soil, with a more pronounced reduction in Daikon radish compared to watercress (**Table 1**). The highest catalase activity (3–4 points) was observed in the leaves of plants grown in park soil (**Table 1**). We found that the addition of CS to roadside soil resulted in an increase in catalase activity in both species, indicating a partial restoration of enzyme activity relative to plants grown in contaminated soil without CS (**Table 1**). Microscopic observation of oxygen bubble release directly from leaf tissue confirmed the same qualitative trends across treatment groups (**Figure 5**). Thus, roadside soil was associated with reduced catalase activity in bioindicator plants, whereas CS addition partially alleviated this effect.

DISCUSSION

In our study, we found that the addition of CS to roadside soil significantly improved the growth parameters of bioindicator plants, whereas the addition of CS to park soil resulted in a slight but statistically significant reduction in growth. Although we did not directly investigate the underlying mechanisms, these contrasting effects are consistent with our hypothesis that CS would mitigate soil phytotoxicity associated with heavy metal contamination, potentially through chelation.

Sulfated glycosaminoglycans like CS are known to possess metal-binding properties, as demonstrated in previous biochemical studies (12–14, 19). However, we did not quantify the concentrations and composition of heavy metals in the studied soils in the present work. Therefore, we cannot directly confirm the proposed chelation of metal ions by CS and consider it a plausible mechanism requiring further verification. The reduced growth observed in park soil amended with CS suggests that under conditions of low background contamination, CS may also interact with essential micronutrients, leading to subtle growth alterations. This finding underscores the importance of soil context when evaluating the application of chelating agents.

We observed that plants grown in roadside soil exhibited a higher incidence of chlorosis and necrosis compared to those grown in park soil, indicating increased stress under roadside conditions. We found that the addition of CS to roadside soil reduced the proportion of damaged leaves, suggesting an overall improvement in plant physiological status. While elevated concentrations of heavy metals are commonly reported in roadside soils due to traffic-related emissions and atmospheric deposition, we did not directly assess the presence and contribution of specific metal contaminants in the investigated soil (1, 2). Heavy metal stress represents one plausible explanation for the observed leaf damage, as chlorosis and necrosis are widely associated with metal-induced impairment of chlorophyll synthesis and

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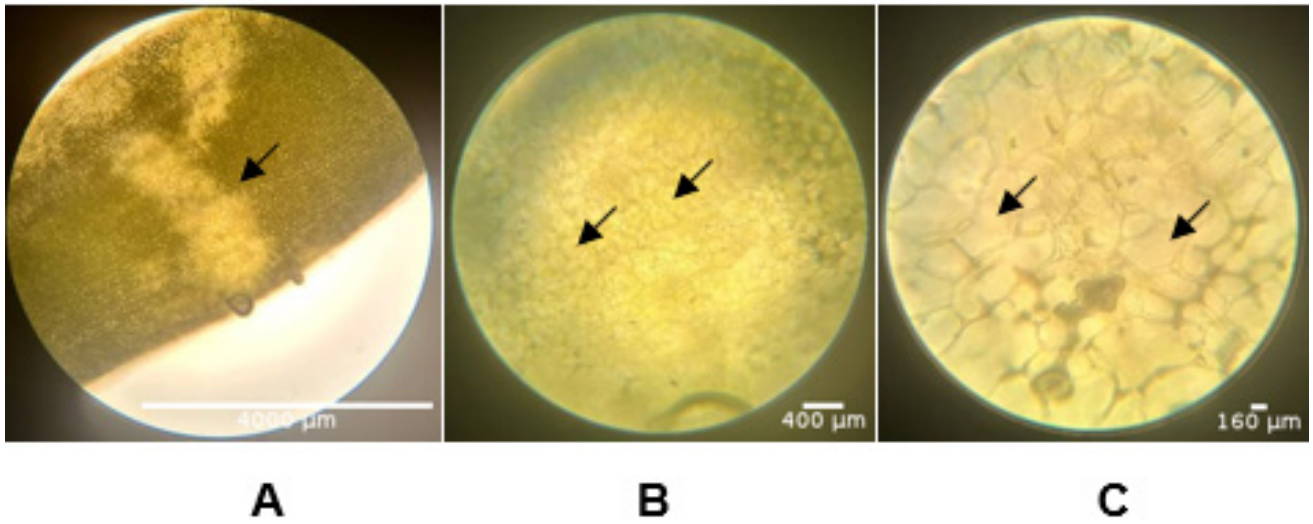

Figure 4: Altered leaf morphology of watercress grown in contaminated soil. Fragment of a watercress leaf grown on roadside soil in seven days under a microscope. (A) 40x, the leaf is brownish in color, black arrows indicate the areas of pigment loss. (B) 400x, the leaf is faintly and unevenly colored, black arrows indicate the stomata, which are pale and closed (C) 1000x, black arrows indicate the empty and colorless mesophyll cells, which are visible through the epidermal cells.

cellular integrity (3–5, 7, 10). Nevertheless, other uncontrolled factors typical of open-environment soils, including salinity, hydrocarbons, de-icing agents, and altered nutrient balance, may have also contributed to the observed effects (25).

We observed that catalase activity decreased in plants grown in roadside soil compared to park soil, with a more pronounced decrease in Daikon radish plants, indicating elevated oxidative stress under roadside conditions. Inhibition of catalase activity is commonly reported in plants exposed to environmental stressors, including heavy metals such as cadmium, lead, and mercury, although we did not measure metal concentrations in this study (9, 11). We found that catalase activity partially recovered following CS addition, suggesting an alleviation of stress conditions. While interactions between CS and metal ions represent one possible explanation, alternative mechanisms—such as changes in nutrient availability or interactions with non-metal stressors—cannot be excluded. Accordingly, changes in catalase activity should be interpreted as indicators of altered stress status rather than direct evidence of heavy metal chelation.

Overall, we found that CS addition improved growth performance and reduced stress-related symptoms in plants grown on roadside soil, whereas its application to park soil (presumed to be less contaminated with heavy metals) resulted in minor growth inhibition. Together, these findings suggest that CS may mitigate certain adverse soil conditions, although the specific mechanisms remain unresolved.

This study focused on early growth and stress-related responses of fast-growing bioindicator species. Watercress and Daikon radish are widely used in phytotoxicity assays due to their high sensitivity and rapid response, making them suitable for detecting early effects of soil-associated stress (22–24). However, these species may not fully represent the diversity of plants inhabiting roadside ecosystems, particularly perennial or woody species with different uptake strategies and stress tolerance mechanisms. We assessed plant performance using integrative morphological and physiological indicators that are commonly applied in biotesting studies and allow robust comparative evaluation between treatments, although additional physiological and biochemical parameters could further refine interpretation.

The use of soils collected from natural roadside and park environments reflects environmentally realistic conditions, where plants are exposed to complex and heterogeneous stress factors. While this approach enhances ecological relevance, it limits the ability to isolate individual stressors. The lack of information about heavy metal concentrations in the soil samples means that the measured effects in this study cannot be directly attributed to heavy metal-induced toxicity.

Within this experimental framework, the observed improvements in plant growth and stress-related traits following CS addition provide a basis for future research. Direct quantification of heavy metal concentrations and speciation in soil before and after CS application is essential

Plant species	Soil type	CS treatment	Catalase reaction activity, points
Watercress	Park	No	4
		Yes	4
	Roadside	No	2
		Yes	3
Daikon radish	Park	No	4
		Yes	4
	Roadside	No	1
		Yes	3

Table 1: The addition of chondroitin sulfate to the roadside soil increases the catalase activity in the leaf extracts. Catalase activity in the leaf extracts of plants grown in different soil types for seven days. 3% hydrogen peroxide was added to extracts from watercress and Daikon radish leaves grown in different soil types. Enzyme activity was assessed visually by the intensity of oxygen bubble formation, on a 4-point scale (0 – no activity; 4 – intense bubble formation).

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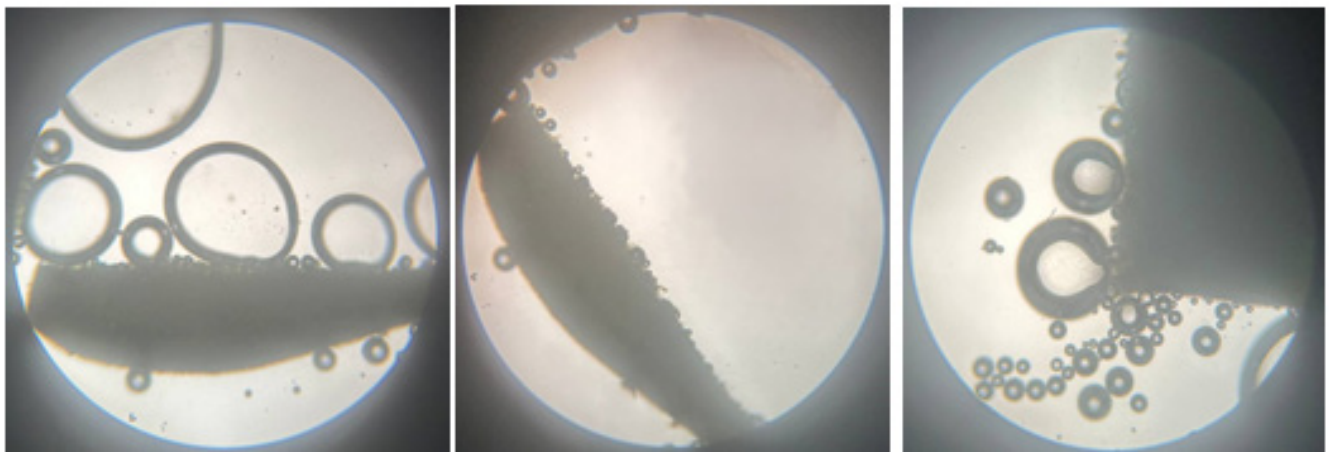

Figure 5: The addition of chondroitin sulfate to the contaminated soil increases catalase activity in the leaves. The intensity of the catalase reaction, assessed visually by the intensity of oxygen bubble formation in the leaf of Daikon radish in different soil types in seven days under a microscope at 40x magnification. 3% hydrogen peroxide was added on a leaf that was grown in (A) park soil, (B) roadside soil, or (C) roadside soil with the addition of CS.

to assess the influence of CS on metal mobility and bioavailability. Dose-response studies could help determine optimal CS concentrations and clarify potential inhibitory effects at higher doses. Longer-term experiments would allow evaluation of the persistence and stability of CS effects over time. Finally, controlled experiments using standardized soils supplemented with defined concentrations of individual heavy metals would enable clearer assessment of CS-mediated mechanisms.

Beyond the specific findings, this study contributes to the broader discussion on environmentally sustainable approaches to soil remediation in urban and transport-associated ecosystems. By demonstrating that a biologically-derived, biocompatible compound can influence plant performance under roadside soil conditions, this work provides an initial framework for exploring nature-inspired soil amendments and supports further investigation into low-impact strategies for the rehabilitation of degraded urban soils.

MATERIALS AND METHODS

Two plants were selected as bioindicators: watercress *(Lepidium sativum)*, a vegetable plant of the Brassicaceae family, and Daikon radish *(Raphanus raphanistrum subsp. sativus)*, a vegetable variety of radish belonging to the Brassicaceae family.

Soil samples were collected from a roadside area of a public motorway in Warsaw, Poland at a distance of 2 m from the road and were considered potentially contaminated with heavy metals. Soil was also collected from green park areas, serving as presumably uncontaminated controls. Soils were similar in appearance: dark in color, lumpy and grainy in structure, with no obvious admixtures of clay, sand, or gravel. Commercially available shark cartilage capsules (Healthyway Production, USA) were used as a source of CS. There were 750 mg of pure cartilage per capsule, containing 20% CS. In shark cartilage, CS-6 is generally the predominant form, although the CS-4/CS-6 ratio depends on species and tissue origin. Since the exact disaccharide composition of the commercial shark cartilage preparation used here was not independently quantified, the specific contribution of each sulfation pattern to the observed metal-binding behavior could not be resolved.

Four experimental groups were set up for each plant species: (1) soil from the park area and seeds; (2) soil from the park area, seeds, and CS; (3) soil from the roadside area, seeds, and CS; (4) soil from the roadside area and seeds. We added 150 g of soil to separate containers for each experimental group. Since the study of CS in soil has not been previously described in the literature, we chose to add 750 mg CS from one capsule to each 150 g container of soil. We thoroughly moistened the soil in groups 1 and 4 and sowed 5 g of seeds per container. In groups 2 and 3, we supplemented the soil with shark cartilage powder from capsules prior to sowing. After sowing, the watercress containers were tightly covered with cellophane and placed in a dark place, whereas the sown Daikon radish seeds were gently pressed into the soil to improve seed-to-soil contact, given their relatively large size, and were likewise kept in darkness. After 48 hours for watercress and 72 hours for the Daikon radish, we removed the crops, opened, watered and measured.

The plants were then grown under identical conditions: 24-hour artificial lighting and a temperature of 25°C. Plants were watered twice a day. The experiments were carried out for seven days from the time of planting the seeds, as a slowdown in plant growth was observed from day eight onward. All experiments were performed in three independent replicates: for each treatment, seedlings were grown in three separate containers prepared in parallel. In each container, the parameters of 15 individual seedlings were assessed during the experiment.

Plant growth measurement

Seedling height was measured daily on the same 45 plants for 6 days for watercress and 5 days for Daikon radish. Seedling height was recorded with a standard metric ruler graduated in millimeters. Precision of the measurements was ±0.5 mm.

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Statistical analysis of the results was performed using Microsoft Excel (MS Office 2021) and Origin 7.0 (Microcal Software Inc., USA). Data were analyzed using two-way repeated measures analysis of variance (ANOVA), with treatment as the between-subject factor and time as the within-subject factor. Data distribution was assessed for normality. Post hoc comparisons were performed using Bonferroni correction. Results are presented as mean ± standard deviation (SD). Differences were considered statistically significant at $p<0.05$.

Brightfield microscopy

A Levenhuk 320 Base optical microscope with a 10x eyepiece and 4x, 10x, 40x, and 100x objectives was used (total magnification 40x, 100*x*, 400*x* and 1000*x*) to image samples. To take an image of the samples, a camera was attached to the eyepiece of the microscope. Scale bars were calculated based on the nominal field number (FN) of the eyepiece (FN = 16 mm) and total magnification. Each specimen was examined at all four magnifications. Attention was paid to the signs of chlorosis, cell color changes, chloroplast density, and stomatal condition (25).

We quantified the occurrence of chlorosis and necrosis as the percentage of seedlings exhibiting visible symptoms relative to the total number of analyzed plants in each group (n = 45 per treatment), based on both macroscopic inspection and microscopic examination of leaf tissues. The content of chloroplasts was assessed semi-quantitatively based on the visual analysis of microscopic images, taking into account the relative density of chloroplasts compared to the control group. The state of stomata was determined visually by their position (open or closed) when analyzing several fields of view for each sample. Visual observations were recorded using a semi-quantitative assessment approach to assign subjects to the corresponding categories.

Catalase activity assay

To compare catalase activity, 0.5 g of leaves from each group was collected. The plant leaves were cut, ground in a mortar with the addition of water, and filtered into a test tube through a moistened folded filter (Whatman No. 1, pore size ~11 *μm*, Cytiva, Cat. No. 1001-125). Then, 2 ml of 3% hydrogen peroxide solution was added to the extract. As a result of the decomposition of hydrogen peroxide by catalase, oxygen bubbles were released, producing a clearly visible foam. The reaction of breaking down hydrogen peroxide by catalase, which is toxic to living cells, proceeds according to the equation: $2H_2O_2$ + catalase $\rightarrow$ $2H_2O + O_2$

Enzyme activity was assessed semiquantitatively by visual (macroscopic) evaluation using on a 4-point scale: intense foam (bubble) formation = 4 points, moderate = 3, weak = 2, very weak = 1 point, no activity = 0 points.

In parallel, catalase-mediated oxygen bubble formation was examined microscopically by applying a drop of 3% hydrogen peroxide solution directly onto a leaf fragment. Microscopic observations were used as a qualitative confirmation of the macroscopic assessment and for photographic documentation but were not used for scoring.

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