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**Understanding plant–cadmium interactions to improve risk
assessment and mitigation**

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To all of you, thank you

Dedication

*To my beloved parents,
for their unconditional love, endless sacrifices, patience, and constant support.
No words can fully express my gratitude, respect, and deep affection for everything
you have done for me.
You have always been my strength, my refuge, and my greatest example of courage,
perseverance, and love.*

*To my dear sister,
for her affection, encouragement, and presence throughout my journey.
I wish you a life full of happiness, success, and fulfillment.*

*To my family,
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With love, gratitude, and deep appreciation, I dedicate this thesis to you

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Preface

This doctoral research was developed within the funded project **SAMOTHRACE – PNRR Missione 4, Componente 2, Investimento 1.5 (ECS00000022)**.

At the beginning of my PhD journey, I was particularly interested in understanding how environmental stressors alter plant physiological performance. As the work progressed, it became clear that stress responses cannot be fully interpreted through isolated measurements but rather require an integrative perspective that connects water relations, metabolism, biochemical defences, and functional outcomes. Cadmium contamination provided a compelling model to explore this complexity. Although widely studied, cadmium stress is often investigated through fragmented approaches that overlook the interaction between hydraulic behaviour, metabolic reprogramming, and the biological relevance of plant-derived compounds. The broader framework of plant health encouraged me to examine these processes not as independent responses, but as interconnected components of plant functioning.

Throughout the PhD, the research evolved progressively. What began as a mechanistic investigation of plant hydraulics under cadmium exposure expanded to include metabolic profiling, antioxidant dynamics, species comparisons, and mitigation strategies. This development reflects both the scientific objectives of the project and my own growing awareness of the need for system-level analyses.

The work presented in this thesis therefore represents not only a study of cadmium toxicity, but also an attempt to interpret metal stress within the broader conceptual framework of plant health. By integrating physiological, metabolic, and functional dimensions across species and contamination levels, this research aims to contribute to a more realistic and applied understanding of how plants cope with environmental challenges.

The doctoral research was carried out primarily at the University of Messina, Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, under the supervision of Professor Patrizia Trifilò and Professor Francesco Cacciola. In addition, I had the opportunity to conduct a five-month research period at the Laboratory of Growth Regulators, Palacký University, Olomouc (Czech Republic), where I further developed my technical and analytical expertise. The collaborative atmosphere and interdisciplinary exchanges within the project played a key role in shaping both the scope and the scientific depth of this work.

Introduction

Heavy metal contamination has become one of the most persistent and concerning environmental issues worldwide due to its toxicity, long-term stability, and capacity for bioaccumulation in ecosystems. Owing to their persistence and high toxicity, heavy metals are among the primary contributors to environmental degradation and constitute a serious hazard to plant development, human health, and the global food system. Their accumulation in edible plant tissues leads to dietary exposure, which can adversely affect multiple human organs, including the kidneys, liver, lungs, gastrointestinal tract, and central nervous system (Ondrasek et al., 2025; Mustapha et al., 2025). Historically, the term “heavy metals” referred to elements with a density greater than 5 g cm^{-3} . However, in modern environmental contexts it broadly includes metals and metalloids that may be harmful to organisms and ecosystems. Heavy metals encompass a broad group of elements that differ in biological function and chemical behaviour. They are commonly classified as non-essential metals, such as Cd, Pb, Hg and As, which have no known beneficial physiological role and exert toxicity even at very low concentrations, and essential metals, including Fe, Cu, Mn, Mo, Ni and Zn, which are required in trace amounts for normal metabolic processes, acting as cofactors in enzymatic reactions, facilitate electron transfer processes, and contribute to biochemical and structural stability. As an example, iron plays a key role in the functioning of photosynthetic and respiratory electron transport chains, contributes to redox homeostasis, and supports essential metabolic activities at the cellular level. However, when the concentration of these metals exceeds physiological requirements, both essential and non-essential metals become detrimental. Elevated accumulation disrupts cellular functions, interferes with metabolic pathways, and induces oxidative stress, ultimately resulting in cytotoxicity and impaired physiological performance in plants and humans alike. At the molecular level, heavy metals can bind to proteins, enzymes, and nucleic acids, forming stable complexes that alter biomolecular structure, inhibit catalytic activity, and interfere with normal biochemical reactions (Kiran et al., 2022; Mishra et al., 2019; DalCorso et al., 2013; Emamverdian et al., 2015). The widespread occurrence of heavy metals in soil and water environments is largely attributed to anthropogenic activities, including industrial production, mining operations, intensive agricultural practices, fossil fuel combustion, and improper waste disposal. Metals such as Cd, Pb, Hg, As, and Cr are commonly released through the use of fertilizers and pesticides, industrial effluents, atmospheric deposition, and poorly managed waste streams. These inputs favour the accumulation of heavy metals in agricultural soils, increasing their availability for plant uptake and facilitating their transfer into the food chain (Sumiahadi & Acar, 2018).

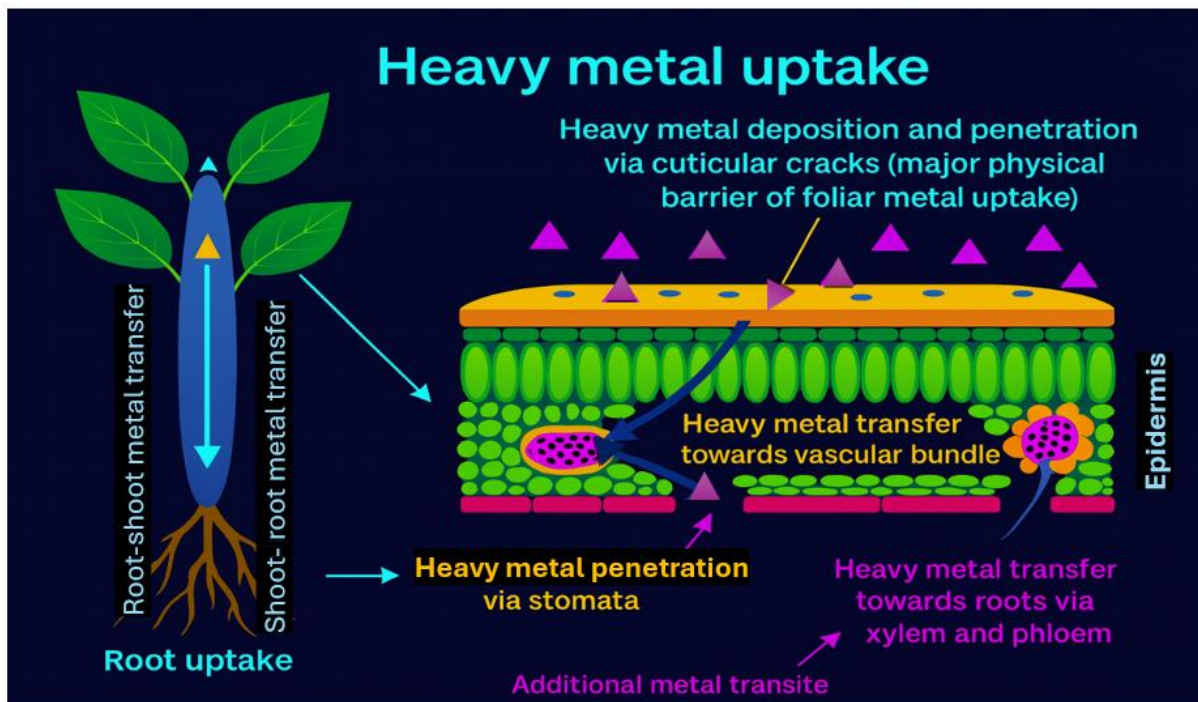


Figure 1: Heavy metal uptake and transport in plants via roots and leaves, with penetration through cuticle cracks or stomata and long-distance movement via xylem and phloem.

Exposure to elevated concentrations of heavy metals induces a wide range of physiological disturbances in plants. Growth inhibition represents one of the earliest and most evident responses, particularly at the root level, which constitutes the first site of contact with toxic ions. In addition, heavy metals interfere with key physiological processes, including photosynthesis, water relations, and stomatal regulation, often resulting in leaf chlorosis and reduced photosynthetic efficiency (Mohamed et al., 2025; Hu et al., 2023). Many of these effects are closely associated with excessive production of reactive oxygen species (ROS), leading to oxidative damage of cellular membranes, proteins, and pigments, and ultimately reducing overall plant vitality (Gallego et al., 2012). In particular, increasing evidence indicates that heavy metal exposure can alter stomatal structure and guard cell function, thereby modulating stomatal aperture and conductance, with consequences for transpiration and CO₂ assimilation; these responses are frequently associated with ROS- and abscisic acid mediated signalling, highlighting stomatal regulation as a relevant component of the integrated plant response to metal stress (Guo et al., 2023). Heavy metals typically decrease chlorophyll content and significantly inhibit photosynthetic rate, and chlorophyll a fluorescence analyses indicate negative effects on both photosystems II and I, with reductions in key photochemical parameters (Küpper and Andresen, 2016). Excess heavy metals can also disturb plant water relations, commonly causing strong reductions in transpiration rate, stomatal conductance, and water use efficiency (Hall, 2002). At the same time, heavy metal stress promotes excessive reactive oxygen species production, which drives lipid peroxidation and damages nucleic acids, proteins, and carbohydrates, often accompanied by increased malondialdehyde levels and changes in the activity of ROS-scavenging enzymes such as catalase,

superoxide dismutase, peroxidases, ascorbate peroxidase, and glutathione reductase (Małkowski et al., 2019). Contaminants can enter plants through both foliar and root pathways (Fig. I). Foliar uptake represents an important route of entry, especially under conditions of atmospheric deposition, where particulate matter and dissolved elements accumulate on leaf surfaces. Following deposition, metallic contaminants may penetrate the cuticle and gain access to internal tissues (Li et al., 2019) through stomata, cuticular discontinuities, and other aqueous pathways, allowing their movement toward mesophyll and vascular tissues (Kovár et al., 2023). In this context, plant canopies function as effective filters of atmospheric pollution, capturing airborne particles and associated metals, some of which may subsequently be internalized. Roots, however, remain the primary interface between plants and contaminated soils. During root uptake, elements dissolved in the soil solution are transferred into root tissues through a complex combination of apoplastic and symplastic transport pathways (Robbins et al., 2014; Enstone et al., 2002). The soil solution initially enters the plant via root hairs and moves through the apoplast until reaching the endodermis, where selective membrane transport facilitates entry into the symplast. Ions are then transported cell-to-cell through plasmodesmata toward the stele and subsequently loaded into the xylem. Once absorbed, these elements may be retained within root tissues or translocated to aerial organs. Long-distance transport is driven by transpiration-induced mass flow and is often facilitated by complexation with organic ligands, particularly organic acids such as citrate, which enhance metal mobility within the xylem and promote their distribution throughout the plant (Shahid et al., 2017; Jan & Parray, 2016; Sharma & Agrawal, 2005).

Cadmium as an environmental pollutant and its effects on plants

Cadmium is a non-essential and highly toxic metal that has become a major environmental contaminant due to anthropogenic activities such as industrial discharge, mining, fossil fuel combustion, and the excessive use of fertilizers and pesticides (Fig. II). Cd can also enter ecosystems through natural processes including rock weathering, volcanic activity, and atmospheric deposition. Once present in soils, Cd is readily absorbed by plant roots and efficiently translocated to aerial organs, where it disrupts numerous physiological, biochemical, and molecular processes. A well-documented effect of Cd exposure is its interference with the uptake, transport, and utilization of essential nutrients such as calcium, magnesium, potassium, phosphorus, iron, and zinc, often resulting in severe nutritional imbalances (Haider et al., 2021; Çeçen et al., 2025). Cd impairs water relations, alters membrane integrity through lipid peroxidation, and disturbs chloroplast metabolism, including the inhibition of chlorophyll biosynthesis. These disruptions manifest as reduced photosynthesis, stomatal dysfunction, leaf chlorosis, inhibited root and shoot growth, and overall biomass decline. Cd toxicity also triggers pronounced oxidative stress by stimulating excessive production of reactive oxygen species, which damage lipids, proteins, and nucleic acids (Bashir et al., 2015; Gallego et al., 2012; Riyazuddin et al., 2021). Although plants activate antioxidant defenses to mitigate this stress, sustained exposure leads to metabolic impairment and compromised development from seed germination through reproductive

stages. Because of its strong interference with mineral nutrition and its ability to accumulate in edible tissues, Cd poses serious risks not only to plant health but also to food safety and human well-being. (Asati et al., 2016; Kiran et al., 2022; Sharma & Agrawal, 2005; Hafeez et al., 2023).

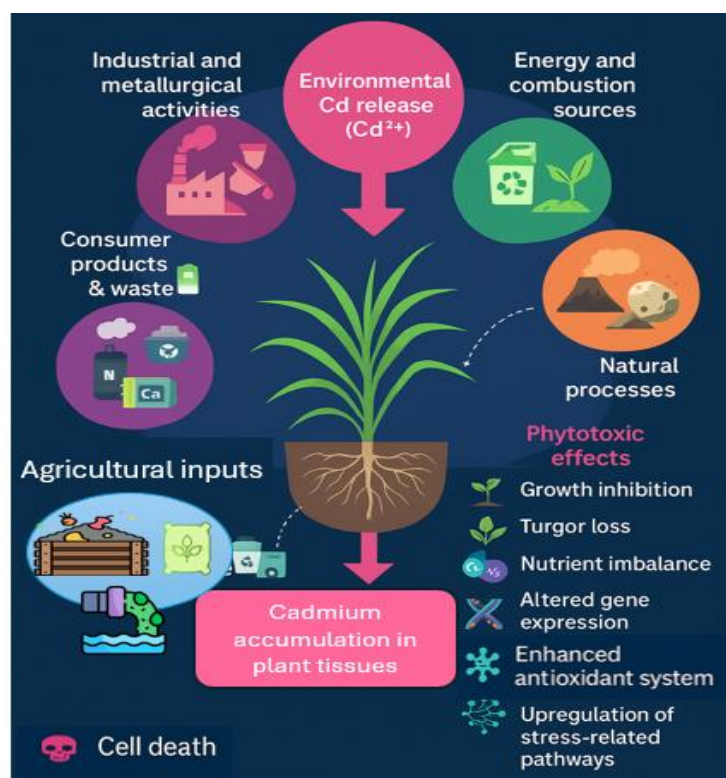


Figure II: Sources of environmental cadmium (Cd^{2+}) and its accumulation in plant tissues, causing phytotoxicity and potential cell death.

Plants have evolved a complex and highly coordinated network of defence mechanisms to mitigate cadmium-induced toxicity. These responses operate at multiple organizational levels and aim to restrict Cd entry, detoxify internalized metal, and alleviate Cd-induced oxidative damage. As an initial line of defence, plants may adopt avoidance strategies that limit Cd uptake from the soil. These include immobilization of Cd in the rhizosphere through the exudation of organic compounds, sequestration at the cell wall, and associations with symbiotic organisms such as arbuscular mycorrhizal fungi, which can bind Cd to fungal hyphae and reduce its bioavailability to the host plant (Haider et al., 2021; Hu et al., 2025). Once Cd enters plant tissues, tolerance and detoxification mechanisms are activated. A major detoxification strategy involves intracellular chelation of Cd ions by low-molecular-weight ligands, particularly phytochelatins and metallothioneins, followed by sequestration of Cd–ligand complexes into vacuoles, thereby preventing interaction with sensitive cellular targets. Additional chelators, including organic acids, glutathione, and specific amino acids such as proline, further contribute to intracellular Cd binding and detoxification (Cobbett, 2000; García De La Torre et al., 2022). Cadmium

stress is closely associated with excessive production of reactive oxygen species, leading to oxidative damage to membranes, proteins, and nucleic acids. To counteract this imbalance, plants activate an antioxidant defence system composed of both enzymatic components, such as superoxide dismutase, catalase, and ascorbate peroxidase, and non-enzymatic molecules, including ascorbate, glutathione, carotenoids, and phenolic compounds (Gill & Tuteja, 2010). Phenolic metabolites play a dual protective role by scavenging reactive oxygen species and chelating metal ions, thereby reducing metal-catalyzed oxidative reactions (Goncharuk & Zagorskina, 2023; Liu et al., 2011). In many species, Cd exposure stimulates the accumulation of flavonoids and anthocyanins, which are often compartmentalized in vacuoles and act as effective antioxidants and metal-binding agents. The activation and efficiency of these defence strategies depend on plant species, developmental stage, Cd concentration, and exposure duration. Together, these coordinated responses determine plant tolerance to cadmium stress and ultimately influence growth, productivity, and survival under contaminated conditions (Emamverdian et al., 2015; Goncharuk & Zagorskina, 2023).

Aim of the Thesis

Cadmium pollution is increasingly intensifying in agricultural ecosystems, yet its effects on plants are highly variable and strongly species-dependent, reflecting differences in physiological performance, antioxidant capacity, and secondary metabolite profiles. To limit environmental and food-chain risks, regulatory frameworks, including Italian legislation, define threshold concentrations of Cd in agricultural soils that are considered safe. However, these limits are primarily based on human exposure criteria and do not fully account for sublethal or long-term effects on plant physiological and metabolic functioning. Consequently, growing evidence suggests that even Cd concentrations within legally permitted ranges may interfere with key plant processes without causing visible toxicity symptoms.

Although substantial research has focused on Cd toxicity at high contamination levels, comparatively little attention has been given to the potential sub-lethal effects of Cd concentrations allowed by current regulations. In particular, it remains unclear whether Cd concentrations allowed by current regulations are sufficient to induce sub-lethal alterations in plant physiology and metabolism, especially in defensive pathways such as antioxidant systems and phenolic metabolism. Clarifying these responses is crucial for understanding how plants cope with Cd stress across different contamination levels, for identifying species-specific tolerance mechanisms, and for evaluating the potential of selected plant systems to both withstand and counteract Cd toxicity. Such knowledge provides the necessary biological basis for exploring plant-based remediation approaches and for testing strategies aimed at mitigating Cd-induced stress under environmentally relevant conditions.

In this context, the present thesis was designed to provide a comprehensive, multi-level assessment of Cd-induced toxicity across different plant models by testing both Cd concentrations compliant with Italian soil quality standards and higher, supra-threshold levels. This approach allowed the evaluation of whether and to what extent legally permitted Cd concentrations affect plant physiological

performance, defensive metabolism, and tolerance mechanisms, in comparison with more severe contamination scenarios.

In the first study, the effects of Cd were investigated in tomato plants (*Solanum lycopersicum* L.), with a specific focus on plant hydraulics, a key determinant of plant growth and productivity. Particular attention was paid to leaf hydraulic conductance in response to Cd exposure and dehydration, with the aim of elucidating how Cd stress interferes with water transport processes and how this interaction is further modulated under drought conditions. This approach addresses the urgent need to better understand the combined effects of heavy metal contamination and water limitation in the context of current climate change scenarios.

Building on the physiological insights obtained in tomato, the second phase of the research extended the investigation to two *Salvia* species, namely the widely cultivated *Salvia officinalis* L. and the rare endemic *Salvia ceratophylloides* Ard., allowing a deeper exploration of Cd-induced responses at both physiological and metabolic levels. In a preliminary step (Study 2), the phenolic profile and antioxidant potential of *S. ceratophylloides* were characterized to evaluate its suitability as a source of bioactive compounds. Subsequently, the effects of Cd exposure at different concentrations were assessed in both *Salvia* species grown under the same environmental conditions. In addition, a bioassay using *Artemia salina* was conducted on leaf extracts to evaluate cytotoxicity, comparing extracts from Cd-treated and untreated plants. This approach provided an integrated view of how Cd affects not only physiological and metabolic traits, but also the biological activity of plant secondary metabolites. The comparative study enabled the identification of species-specific tolerance strategies and clarified how phenolic metabolism and antioxidant responses are modulated under both regulatory-compliant and elevated Cd stress levels.

Building upon these findings, the final phase of the thesis (i.e., Study 4 and Study 5) focused on evaluating the potential of iron–citrate nanoparticles to mitigate cadmium (Cd)-induced stress. Given the impact of cadmium pollution on iron uptake and the role of this element in several physiological functions, iron–citrate nanoparticles were tested as a potential tool to improve plant resilience under Cd exposure. Their effects on plant growth, physiological performance, phytohormone levels, and phenolic composition were evaluated across the tested Cd concentrations, providing insight into their effectiveness as protective agents and their potential application in Cd-contaminated environments.

Overall, this integrated research fills a critical gap in the literature by examining plant responses to Cd stress across different species and contamination levels, including concentrations currently permitted by law. By clarifying the physiological and metabolic consequences of both regulatory and supra-regulatory Cd exposure, this thesis contributes to a better understanding of plant-Cd interactions, supports the refinement of environmental risk assessments, and provides new insights into strategies aimed at limiting Cd uptake, improving plant performance, and protecting human health through safer agricultural production systems.

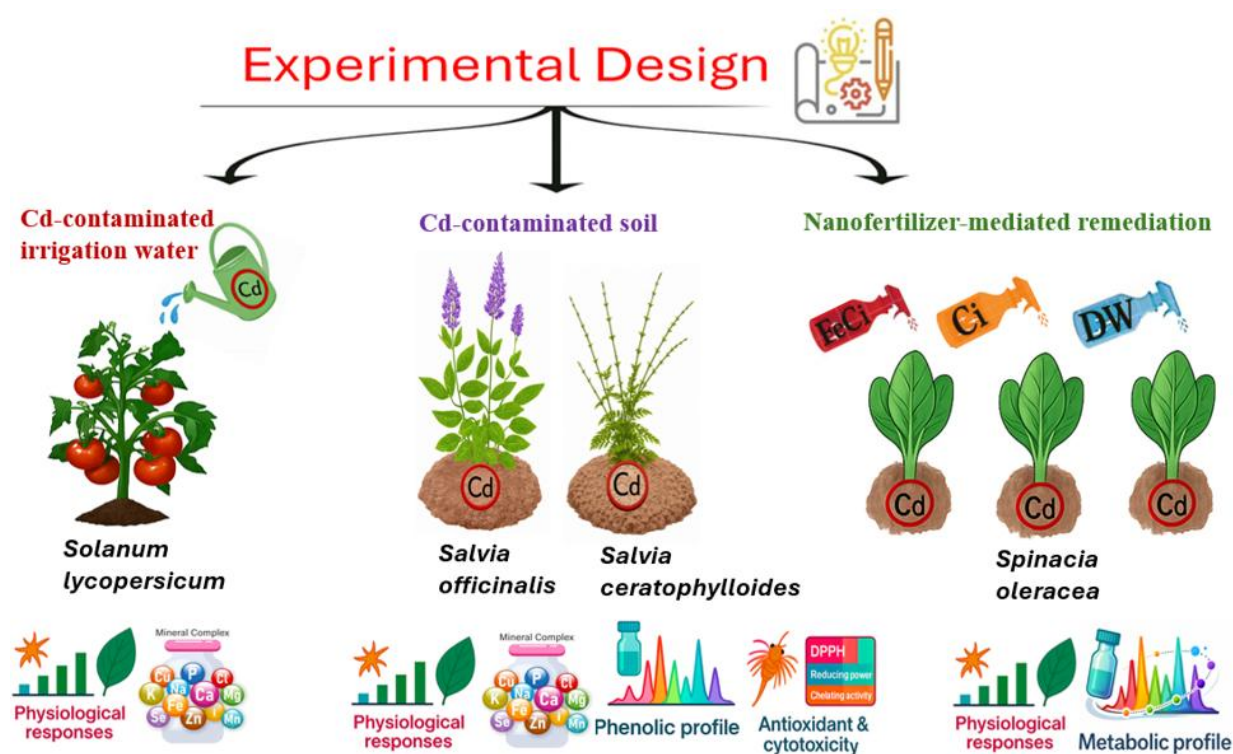


Figure III: Schematic overview of the experimental studies conducted in this thesis: cadmium exposure via contaminated irrigation water in *Solanum lycopersicum*, cadmium-contaminated soil in *Salvia officinalis* and *Salvia ceratophylloides*, and remediation trials in *Spinacia oleracea* using foliar sprays (iron citrate, citrate, and distilled water), with assessment of physiological responses, phenolic profile, and antioxidant/cytotoxicity endpoints.

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Chapter 1

Chronic mild cadmium exposure increases the vulnerability of tomato plants to dehydration

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1. Introduction

Toxic metal pollution is a major environmental and ecological issue that has received growing attention, particularly over the past two decades. In addition to the direct impacts of anthropogenic pollution on water and soil, the increasing occurrence of extreme climate events, such as rising temperatures, droughts, and floods, presents further threats to water quality. These events intensify the runoff of pollutants and sediments and heighten the risk of saltwater intrusion (Whitehead et al., 2009; Michalak, 2016). Consequently, climate change is significantly affecting food security by increasing the risk of plant die-off due to extreme events and compounding the dangers posed by water pollution and the use of low-quality water for irrigation. Cadmium (Cd) is a heavy metal naturally present in small quantities in air, water, and soil. However, harmful concentrations of Cd in soil and groundwater are frequently recorded due to various anthropogenic activities, such as industrial discharge and improper waste management (Kubier et al., 2019). Although cadmium is a non-essential element for plants, they are unable to prevent its uptake, as it occurs through essential metal transporters (Verbruggen et al., 2009; Cheng et al., 2018; Huang et al., 2020). To the best of our knowledge, both past and recent literature have extensively highlighted the severe alterations in plant metabolism caused by cadmium exposure (DalCorso et al., 2008; Gallego et al., 2012). Cadmium uptake negatively impacts plant biomass by inhibiting photosynthesis, reducing chlorophyll content, causing chloroplast degradation (He et al., 2011; Kieffer et al., 2009), and inducing oxidative stress (Smeets et al., 2008; Grobelak et al., 2019). It also disrupts nutrient balance (Naeem et al., 2019) and interferes with phytohormone metabolism (Elobeid et al., 2012). Surprisingly, to date, no studies have investigated the impact of cadmium, or other heavy metals, on leaf water transport efficiency, particularly in response to additional environmental stresses like drought. Given the critical role that water transport, especially leaf hydraulic conductance, plays in terrestrial plant productivity and drought resilience (Brodribb 2009; Scoffoni and Sack, 2017; Brodribb and Buckley, 2018; McDowell et al., 2019; Trifilò et al., 2021) understanding how cadmium affects these processes is essential for predicting plant responses in pulled environments. The structure and function of the plant water transport system are currently recognized as key determinants of productivity (Brodribb 2009; Smith and Sperry, 2014; McDowell et al., 2019). Hydraulic failure, the substantial loss of hydraulic function that prevents sufficient water transport to hydrate the plant, is considered one of the major causes of drought-driven vegetation mortality (Brodribb & Cochard, 2009; Blackman et al., 2016). Consequently, an expanding database of hydraulic traits, including how plants regulate water transport, is helping modelers more accurately capture not only how vegetation influences global carbon and water vapor fluxes but also the risks to vegetation posed by climate change (i.e., Christoffersen et al. 2016; Choat et al., 2018; Rosas et al., 2019; Hartill et al., 2023). Leaf hydraulic conductance, K_L , plays a key role in overall plant hydraulics and, consequently, in plant productivity (Brodribb and Holbrook, 2004; Scoffoni et al., 2016; Skelton et al., 2017; Brodribb and Buckley, 2018; Xiong and Nadal, 2020; Wolfe et al., 2023). Indeed, K_L significantly influences carbon assimilation due to the shared pathway of CO₂ uptake and water loss through stomata.

From this perspective, understanding the impacts of heavy metal pollution on leaf hydraulics in response to environmental stress can shed light on the potentially heightened vulnerability of species growing in heavy metal-contaminated soils.

In this study, we investigated the effects of chronic cadmium exposure on several plant hydraulics traits of *Solanum lycopersicum* L., cultivar Piccadilly. Special attention was given to leaf hydraulic conductance (K_L) in response to cadmium pollution and dehydration, due to the critical role of leaf hydraulic vulnerability in both carbon economics and drought tolerance.

We hypothesize that chronic exposure to even low concentrations of this toxic metal can impair plant physiology by negatively affecting K_L , particularly when combined with drought.

1.1 Material and Methods

1.1.1. Growth conditions

Experiments were performed from April to July 2023 on 60 plants of *Solanum lycopersicum* L., cultivar Piccadilly grown in a greenhouse at the Department CHIBIOFARAM of the University of Messina. The greenhouse was oriented northwest and received natural light. Supplementary lighting ($450 \mu\text{mol m}^{-2} \text{s}^{-1}$) was provided by LED lamps (Led Spectrum Grow 6, I-GROW s.r.l., Italy), in order to ensure a 16-hours light/8-hour dark photoperiod. The temperature ranged between 27°C/20°C day/night. Seeds were planted in greenhouse trays and, after cotyledons expanded, the seedlings were transplanted in 1 Kg soil (2.3 L) pots filled with nutrient-rich soil specifically formulated for vegetable growth, eliminating the need for supplementary fertilization (Comodo, Tercomposti S.P.A., Italy). Each pot contained one seedling, and the plants were divided into two groups: control samples (C) and cadmium-treated samples (Cd). Control and Cd-treated samples were regularly watered on the same schedule throughout the experimental period. Specifically, the plants received 70 mL of high-quality water every three days during the first month of growth, and subsequently, every two days.

To induce mild and chronic cadmium stress, we periodically contaminated the soil with cadmium at a concentration equal to approximately 10% of the maximum level allowed by Italian law for agricultural land (i.e., 5 mg Kg^{-1} , Law n. 46, 22 June 2019). Therefore, at four intervals (i.e., when plants were 10 days old and at 4, 7, and 10 weeks old), the Cd-treated samples were irrigated with 70 ml of water contaminated with $50 \mu\text{M CdCl}_2$ (equivalent to 0.4 mg of Cd per Kg of soil for irrigation), while the control (C) samples received an equal volume of good quality water (Fig. 1.1). To investigate the impact of drought on cadmium-treated plants, the leaf vulnerability to dehydration was measured. Moreover, measurements on drought-reirrigated plants were performed.

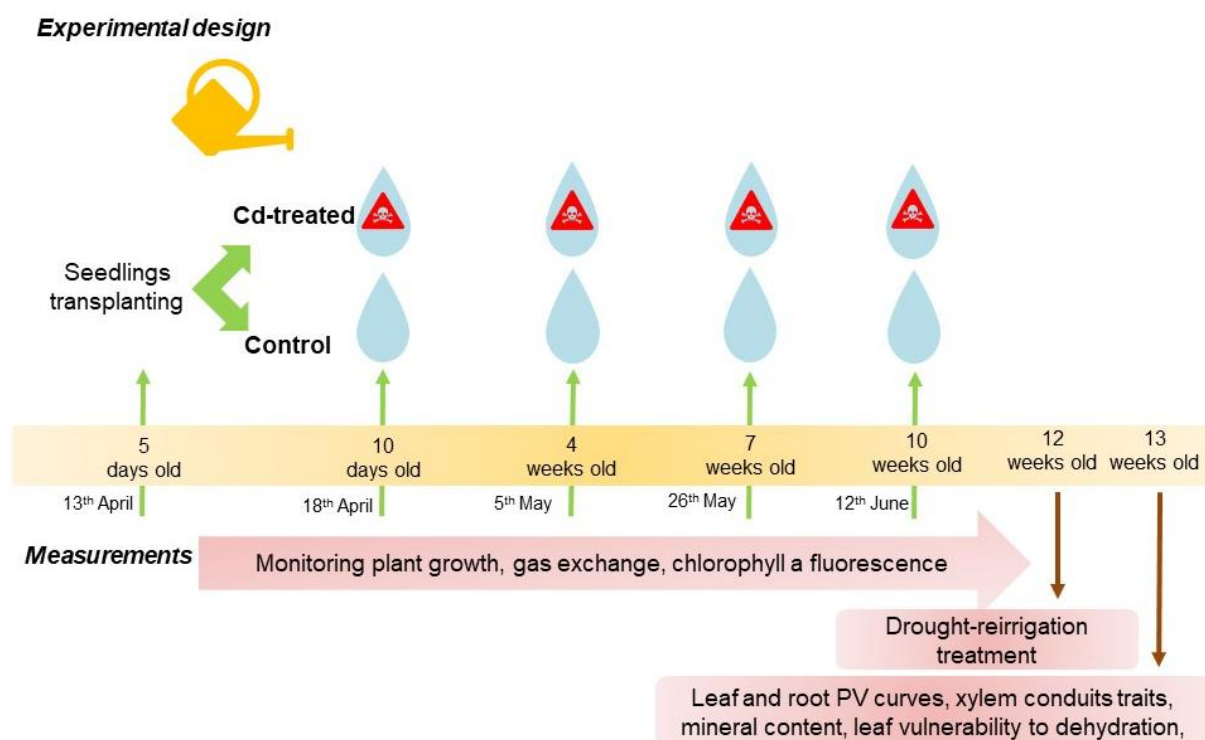


Figure 1.1: Experimental planning and performed measurements on *Solanum lycopersicum* L. plants submitted to a mild chronic cadmium stress.

1.1.2. Morphological and anatomical measurements

To monitor plant growth, measurements of shoot length (l), stem diameter at the root neck and the number of leaves were performed weekly in 5 samples per treatment. At the end of the experimental period, when the plants were 3 months old and some had developed red tomatoes, this set of C and Cd-treated plants was utilized to estimate plant biomass. Specifically, the plants were partitioned into roots (after gently rinsing to remove the soil), stem, leaves, and fruit and the fresh mass of each organ was recorded. Additionally, the diameter of the fruit, the leaf surface area (A_L) and the thickness (T_L) of three apical leaves per sample per treatment were measured. A_L was estimated by capturing leaf images with a scanner (HP Scanjet G4050, USA) and using Image J software (<http://imagej.nih.gov/ij/>). T_L was estimated by averaging values recorded at the bottom, middle, and top of the leaf using a digital caliper with an accuracy ± 0.01 mm. Subsequently, the plant material was dried in an oven at 80°C for three days, except for the fruit, which was kept for a week, to determine the dry weight (DW). Following this, the mineral content was measured (refer to section 2.5 for further details). Leaf mass area (LMA) was calculated as DW/A_L . Leaf density was estimated as: LMA/T_L and leaf volume was calculated as $T_L \cdot LMA$. The diameter of the fruit was measured with a caliper.

In addition, 5 control and 5 Cd-treated samples were used to evaluate potential changes in xylem anatomical traits. Hand transverse sections of root (approximately 2 cm below the root collar), stem (10 cm from the apex) and petiole (at its basal end) were prepared and stained with 0.1% safranin. Images were captured using a microscope (Laborlux S, Leitz GmbH, Stuttgart, Germany) connected to a PC via digital camera (OMAX camera, A35180U3, China) and analyzed using Image J software.

The following parameters were measured:

- xylem area, A_x ;
- total number of xylem conduits, N_c ;
- xylem vessel density, V_d , estimated as: N_c/A_x ;
- hydraulically weighted mean xylem conduit diameter (Kolb and Sperry, 1999), D_h , estimated as: $\Sigma D^5/\Sigma D^4$ where D is the xylem conduit diameter;
- theoretical water flow estimated as Sr^4 according to the Hagen–Poiseuille equation (a proxy of water transport efficiency)
- theoretical water flow per unit xylem area, $\Sigma r^4/A_x$

1.1.3. Gas exchange and water relations

During the experimental period, stomatal conductance to water vapour (g_L), transpiration rate (E_L) and photosynthesis rate (A_n) were measured twice a week in at least 5 leaves from different plants per treatment using a portable LCI Analyzer System (ADC Bioscientific Ltd., Herts, UK). Additionally, once a week, photosynthetic efficiency was estimated by measuring chlorophyll a fluorescence with a portable fluorometer (Handy Pea, Hansatech, Norfolk, UK). Leaves were darkened for 30 min and F_v/F_m was calculated as a proxy for quantum yield of PSII (Maxwell and Johnson, 2000). Three leaves from different plants per treatment were measured.

All measurements began approximately 5 hours after the lamps were turned on and were completed within 80 minutes. At the end of the experimental period (Fig.1), leaf and root water potential isotherms were performed using a pressure bomb (Soilmoisture Equipment Corp., California, USA) following the method outlined by Tyree and Hammel (1972), to obtain the water potential at the turgor loss point (Ψ_{tlp}), the osmotic potential at full turgor (π_0), the modulus of elasticity (e), the capacitance at full turgor (C_{ft}) and at turgor loss point (C_{tlp}) in both the leaf and root of C and Cd-treated samples. Leaves were sampled by cutting their petiole with a razor blade; they were then rehydrated for 1h with their petioles immersed in distilled water. Root samples were collected by gently pulling them out of the pot and immersing the root system into a container filled with water. The soil was then carefully rinsed to avoid damage to the root system. This procedure, which took approximately 30 minutes, ensured complete hydration of the samples, as confirmed by the initial water potential values. Following that, a root sample with a diameter of approximately 0.8 mm at its cut end, devoid of any nearby branching, was carefully selected. Excess water was removed by gently blotting with a paper towel. Subsequently, the sample was connected by its cut end to the pressure bomb. Pressure volume curves (PV) were obtained measuring water potential (Ψ) and the corresponding water loss at regular intervals during progressive leaf and root dehydration. Five different C and Cd-treated plants were measured. Ψ_{tlp} was estimated graphically as the water potential at which the relationship between water content and $-1/\Psi$ transitioned from curvilinear to linear. π_0 was calculated by the y-intercept of the linear region of this relationship;

e was calculated as $\Delta P_t/(\Delta W/W)$, where ΔP_t is the change of turgor pressure and $\Delta W/W$ is the relative change of the leaf water content. The capacitance at full turgor (C_R) and at the turgor loss point (C_{tlp}) were estimated based on the slope of the relationship between water loss and Ψ before and after Ψ_{tlp} , respectively (Scoffoni et al., 2014).

1.1.4. Leaf hydraulic conductance and leaf membrane damage during dehydration

Leaf hydraulic conductance (K_L) was estimated using the rehydration kinetic method (RKM) (Brodribb and Holbrook, 2003). Shoots were cut in the early morning from well-hydrated plants ($\Psi_L < -0.01$ MPa) and subjected to bench dehydration for varying time intervals ranging from 0 to 50 hours. This experimental procedure allowed us to compare the vulnerability of K_L to dehydration in control versus cadmium treated plants. During the dehydration period, shoots were enclosed in a black plastic bag containing a piece of moistened filter paper. From each shoot and at the same dehydration time, four leaves were collected. One leaf was used to estimate the initial water potential (Ψ_i). A second adjacent leaf was detached and rehydrated for 30–90 s maintaining the petiole immersed in 10 mM KCl solution. Following rehydration, the final leaf water potential (Ψ_f) was measured. Leaf hydraulic conductance was then calculated as: $K_L = C \cdot t^{-1} \cdot \ln(\Psi_i/\Psi_f)$ where C is the leaf capacitance (estimated by PV curves), t is the rehydration time. All K_L measurements were performed at laboratory irradiance ($PAR < 10 \mu\text{mol m}^{-2} \text{s}^{-1}$) and normalized at 25 °C.

The remaining two leaves were used to estimate the percentage loss of rehydration capacity (PLRC) and leaf membrane damage (Abate et al., 2021b; Trifilò et al., 2023). Specifically, the third leaf was weighed to determine its fresh weight (FW), then it was rehydrated for about 10 hours by immersing its petiole in a beaker containing deionized water to estimate its turgid weight (TW). Subsequently, the sample was oven-dried at 80 °C for three days to obtain the dry weight (DW). This experimental protocol enabled the calculation of the saturated water content of dehydrated samples, $SWC_d = (TW - DW)/DW$, i.e., of samples collected at different times of dehydration. PLRC was estimated as: $100 \times (1 - SWC_d/SWC_w)$, where SWC_w is the saturated water content of well-watered samples. SWC_w was estimated as the mean SWC value recorded in 5 leaves cut from well-watered plants in the early morning (before the lamps were switched on) and 5 leaves sampled at time 0. In all these 10 samples, the initial weight was considered the turgid weight. To monitor potential issues with overhydration, the weight of some samples was monitored hourly.

The fourth leaf was utilized to evaluate potential differences in cell membrane integrity between control and cadmium-treated samples through electrolyte leakage measurements (Abate et al., 2021b; Trifilò et al., 2023). Specifically, leaf pieces of about 0.5 cm² were placed in a test tube containing 8 mL of distilled water. After shaking the samples for 30 min, the initial electrical conductivity of the solution (EC_i) was recorded using a conductivity meter (Cond5, XS instruments, Italy). Subsequently, the samples were subjected to three freeze–thaw cycles (i.e., $T = -20^\circ\text{C}$, $+20^\circ\text{C}$) to induce complete

membrane disruption. Following the same processing steps as described above, the final electrical conductivity of the solution (EC_f) was measured. The relative electrolyte leakage (REL) was calculated as (EC_i/EC_f)*100.

1.1.5. Estimating gas exchange and leaf hydraulic conductance in response to drought-re-irrigation treatment

To investigate the combined impact of drought and cadmium pollution on leaf hydraulics and the plants' ability to recover from a drought event, additional measurements were conducted. Two weeks after the fourth irrigation with cadmium-contaminated water (Fig. 1), 20 control and cadmium-treated plants were divided into two groups: one group continued to be regularly irrigated at field capacity (5 C and 5 Cd), while the second group (5 C_{drought} and 5 Cd_{drought}) was subjected to drought stress by suspending irrigation until leaf water potential reached Ψ_{tlp} , as measured before lamps turned on. The Ψ_{tlp} was reached within 5-6 days after irrigation was suspended. At 10:00 am, gas exchange measurements were recorded from C, Cd, C_{drought} and Cd_{drought} leaves, which were then collected for leaf hydraulic conductance (K_L) measurements using the RKM. Additionally, another set of leaves from the same plants used for K_L measurements was collected to perform an electrolyte leakage test. After sample collection, the plants were immediately irrigated to field capacity and measured again after three days (C_{rec} and Cd_{rec}).

1.1.6. ICP-OES mineral element analysis

Three leaf, root and fruit samples, which were utilized for estimating root and shoot biomass were also employed to determine the cadmium and macro- and micronutrient content.

Each sample (0.5 g) was placed into an acid-prewashed vessel to which 7 mL of HNO₃ at 65% was added. A first step of digestion by controlled heating was carried out. Subsequently, 1 mL of 30% H₂O₂ was added to the acidic mixture using a closed-vessel digestion system. At the end of this procedure, the digested samples were brought to a volume of 25 mL with ultrapure water. Mineral content analysis was performed using an Avio200 ICP-OES instrument (Perkin Elmer, Waltham, MA, USA) equipped with a vertical DualView optical system and an S10 autosampler (Perkin Elmer, Waltham, MA, USA). Table 1.1 shows the recommended analytical lines length used to perform element analysis; the Argon line at 420.069 nm was used as an internal standard. The operational conditions applied are listed in Table 1.2. Data processing was performed using PerkinElmer Syngistix™ for ICP software (Perkin Elmer, Waltham, MA, USA).

Table 1.1: Analytical lines length (nm) used for analysis of mineral content. Ca: calcium; Cd: cadmium; Cu, copper; Fe: iron; K: potassium; Mg: magnesium; Mn: manganese; P: phosphorus; Zn: zinc.

Element	nm	Element	nm	Element	nm
Ca	317.933	Fe	238.204	Mn	257.610
Cd	228,802	K	766.490	P	213,617
Cu	327.393	Mg	285.592	Zn	213.857

Table 1.2: Operational conditions for ICP-OES.

Parameter	Conditions
Radiofrequency power (W)	1500
Plasma gas flow (L/min)	9
Auxiliary gas (L/min)	0.2
Nebulizer gas (L/min)	0.7
Sample uptake (mL/min)	1

The position of the torch was optimized before to the analytical phase using the optical optimization procedure of Syngistix™ ICP software with the Mn analytical line. All quantitative measurements were made against external calibration curves constructed using a Certified Reference Material multielement standard solution ISO 17034 purchased from CPAchem (Bulgaria). A Milli-Q ultrapure water system (Merck Millipore, Merck KGaA, Darmstadt, Germany) was used to produce water at 1.8 MΩ/cm for solutions preparation and samples dilution purposes. Calibration curves for all elements listed in Table 1 were established using the calibration blank and the reagent blank, all of which resulted in correlation coefficients (r^2) better than 0.999. The Detection Limits (DLs) of this procedure were determined by analyzing a matrix blank, which consisted of the same reagents and quantities as those used for sample preparation.

1.1.7. Statistical analysis

The data were analyzed with the SigmaStat 12.0 (SPSS, Inc., Chicago, IL, USA) statistics package. Differences in g_L , E_L , A_n , F_v/F_m (data shown in Fig. 1.2), biomass-related traits (data shown in Table 1.3), PV-related parameters (data shown in Table 1.4), xylem conduits traits (data shown in Table 1.5) and mineral content (data shown in Fig. 1.4) measured in control versus cadmium treated samples were tested through Student's t-test ($\alpha = 0.05$) after checking for normality and homoscedasticity assumptions.

To test the differences among treatment (C and Cd) and organs (root, stem, leaf and fruit) on cadmium content (Fig. 1.2) and among treatment (C and Cd) and watered availability (watered, drought, recovery)

on g_L , A_n , K_L and REL (Fig. 1.6) a two-way ANOVA test and a post hoc Holm-Sidak test were performed.

Simple linear regressions were used to determine the relationships between K_L and Ψ_L , REL and $PLRC$ values for control and cadmium-treated leaves independently. All regressions were considered significant at $P < 0.05$.

1.2. Results

1.2.1. Impact of cadmium pollution

The first irrigation with Cd-polluted water resulted in the death of less than 10% of the treated plants, and no further instances of plant death were observed following subsequent irrigations with cadmium-polluted water (data not shown). The tomato plants showed cadmium uptake. Indeed, at the end of the experimental period, a cadmium content of about $75 \text{ mg g}^{-1} \text{ DW}$ in the roots, $30 \text{ mg g}^{-1} \text{ DW}$ in the stem and about $25 \text{ mg g}^{-1} \text{ DW}$ in the leaves and fruit was recorded in Cd-treated plants (Fig. 1.2).

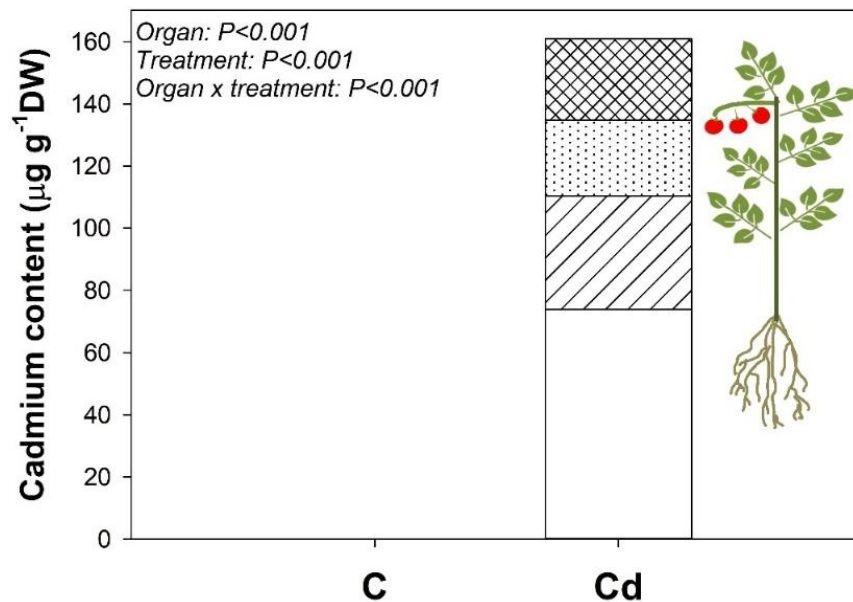


Figure 1.2: Cadmium content as recorded in control (C) and cadmium-treated (Cd) root (white bar), stem (slanting bar), leaves (dotted bar) and fruit (meshed dashed bar) of 13-week -old-well-watered *Solanum lycopersicum* (i.e., after 4 irrigations with cadmium contaminated water over three months).

Until the plants reached approximately 1 month of age (i.e., following the first irrigation with Cd-polluted water), Cd-treated plants showed comparable values of g_L , E_L , and A_n (Fig. 1.3). Approximately two weeks after the second irrigation with cadmium-polluted water, higher values of g_L and E_L were observed in Cd-treated plants compared to control (C) plants, but there were no significant differences in photosynthesis rate and efficiency of photosystem II (F_v/F_m). After the fourth irrigation with contaminated water, which occurred on June 12th, Cd-treated samples showed lower values of g_L and E_L , along with decreased A_n and efficiency of photosystem II, compared to control (C) plants.

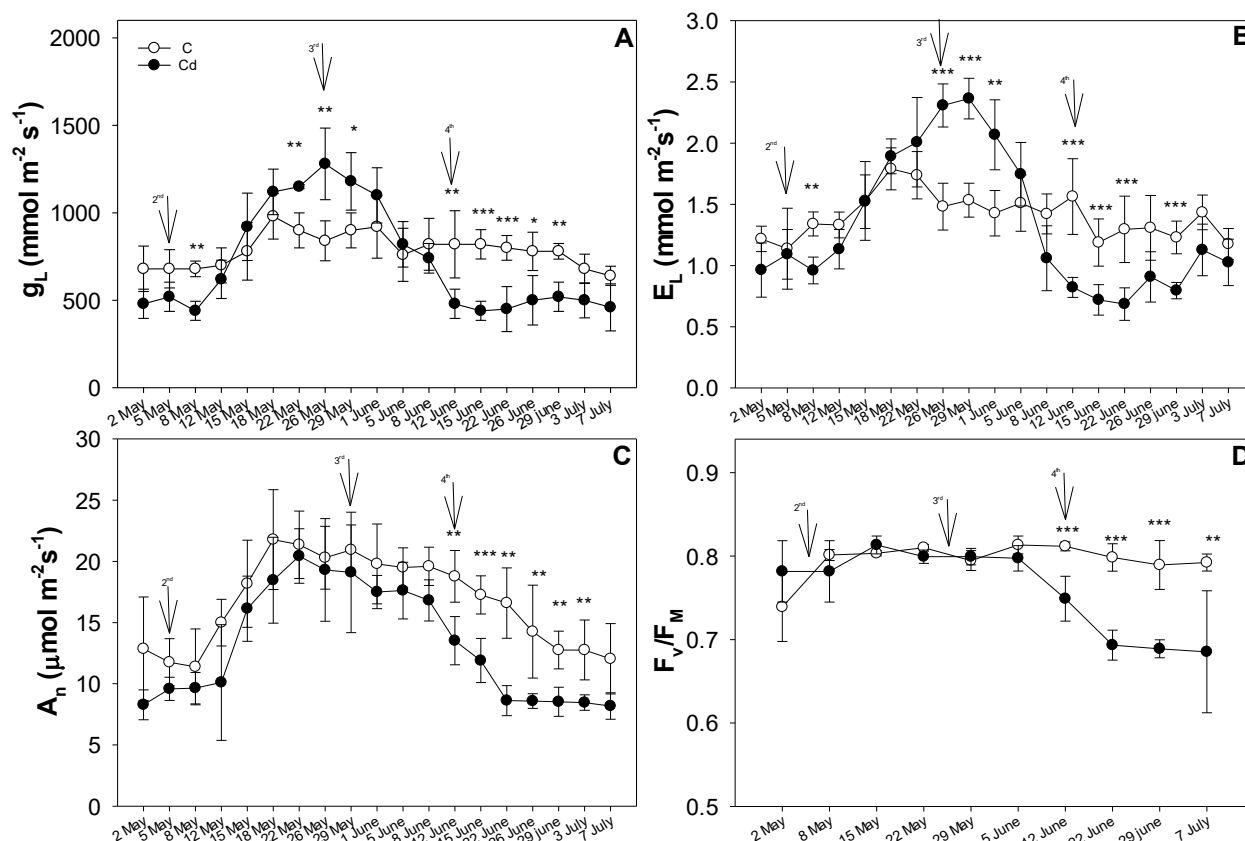


Figure 1.3: Mean values $\pm$ SD of A) stomatal conductance to water vapour, g_L , B) transpiration rate, E_L , C) photosynthesis rate, A_n , and D) photosynthetic efficiency, F_v/F_m , recorded twice a week (g_L , E_L and A_n) and once a week (F_v/F_m) in control (white circle) and cadmium treated (black circle) *Solanum lycopersicum* plants during the experimental period. The irrigations with cadmium-contaminated water are indicated by the arrows *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$.

No differences in morphological and biomass traits were recorded in control stem, root and fruit compared to cadmium-treated plants after 4 irrigations with cadmium contaminated water over three months (Table 1.3). However, visible but not alarming morphological alterations occurred in the leaves of Cd-treated samples. Specifically, despite a similar number of leaves per plant and comparable leaf dry weight, Cd-treated samples showed higher values of leaf surface area (A_L) and leaf thickness (T_L) and lower values of LMA compared to C samples.

Table 1.3: Mean values $\pm$ SD of biomass traits recorded in 13-weeks-old control (C) and cadmium-treated (Cd) *Solanum lycopersicum* plants (n=5). P values are reported. A_L : leaf area; LMA: leaf mass area; DW: dry weight; FW: fresh weight.

	C	Cd	P-value
Leaf number	13.0 $\pm$ 3.5	14.8 $\pm$ 6.1	0.585
Leaf DW (mg)	785.3 $\pm$ 359.8	817.9 $\pm$ 331.3	0.885
Apical A_L (cm ²)	4.3 $\pm$ 0.4	5.5 $\pm$ 0.7	0.015
Apical leaf thickness (mm)	0.15 $\pm$ 0.04	0.22 $\pm$ 0.02	0.008
A_L (cm ²)	13.5 $\pm$ 1.7	20.6 $\pm$ 3.2	0.005
LMA (g m ⁻²)	100.8 $\pm$ 11.7	81.8 $\pm$ 5.0	0.01
Apical leaf volume	0.07 $\pm$ 0.03	0.1 $\pm$ 0.02	0.07
Apical leaf density (g cm ⁻³)	0.43 $\pm$ 0.11	0.29 $\pm$ 0.05	0.032
Shoot length (cm)	39.6 $\pm$ 3.6	44.8 $\pm$ 7.6	0.204
Shoot diameter (mm)	0.40 $\pm$ 0.10	0.39 $\pm$ 0.09	0.827
Stem DW (mg)	798.6 $\pm$ 370.8	721.7 $\pm$ 188.0	0.690
Root DW (mg)	349.8 $\pm$ 115.2	513.1 $\pm$ 291.5	0.278
Fruit number	1.9 $\pm$ 1.2	2.3 $\pm$ 0.4	0.474
Fruit diameter (cm)	1.7 $\pm$ 0.6	1.9 $\pm$ 0.3	0.524
Fruit FW (mg)	5352.4 $\pm$ 3500.1	5725.0 $\pm$ 1514.2	0.575
Fruit DW (mg)	1852.3 $\pm$ 962.5	1768.9 $\pm$ 414.3	0.863

Cadmium pollution affected leaf and root water relations differently (Table 1.4). Leaves of Cd-treated plants showed a similar osmotic pressure at full turgor (p_o) but a less negative turgor loss point (Y_{tlp}), possibly due to lower wall stiffness (ϵ), in comparison to the C samples. This reduction in ϵ resulted in higher values of capacitance at full turgor (C_{ft}) in Cd-treated leaves compared to the control. In the root, Cd-treated samples showed more negative values of Y_{tlp} and p_o , lower values of ϵ and higher C_{ft} and C_{tlp} compared to control roots. It can be noted that leaves and root cells of Cd-treated plants showed higher values of saturated water content (SWC) compared to control plants.

Table 1.4: Mean $\pm$ SD ($n=5$) values of water potential at turgor loss point (Y_{tlp}), the osmotic potential at full turgor (p_o), the modulus of elasticity (e), the capacitance at full turgor (C_{ft}) and at turgor loss point (C_{tlp}) and the saturated water content (SWC_w) recorded in well-watered leaf and root samples of control (C) and Cd-treated (Cd) plants of *Solanum lycopersicum*. P-values are reported.

	Leaf		
	C	Cd	P-value
-Y_{tlp} (MPa)	1.48 $\pm$ 0.13	1.27 $\pm$ 0.06	0.006
-p_o (MPa)	1.18 $\pm$ 0.15	1.03 $\pm$ 0.12	0.146
e (MPa)	15.08 $\pm$ 2.32	11.21 $\pm$ 1.90	0.001
C_{ft} (MPa⁻¹)	0.13 $\pm$ 0.03	0.17 $\pm$ 0.01	0.006
C_{tlp} (MPa⁻¹)	0.37 $\pm$ 0.02	0.35 $\pm$ 0.04	0.153
SWC_w (g g⁻¹)	5.86 $\pm$ 0.98	7.48 $\pm$ 0.64	0.001
	Root		
-Y_{tlp} (MPa)	1.11 $\pm$ 0.09	1.34 $\pm$ 0.07	0.002
-p_o (MPa)	0.81 $\pm$ 0.07	0.95 $\pm$ 0.05	0.007
e (MPa)	7.36 $\pm$ 0.77	4.55 $\pm$ 0.68	<0.001
C_{ft} (MPa⁻¹)	0.18 $\pm$ 0.03	0.23 $\pm$ 0.04	0.037
C_{tlp} (MPa⁻¹)	0.35 $\pm$ 0.02	0.57 $\pm$ 0.05	<0.001
SWC_w (g g⁻¹)	7.38 $\pm$ 0.37	9.11 $\pm$ 0.76	0.001

The cadmium treatment (i.e., 4 irrigations with cadmium contaminated water over three months) resulted in an increase in xylem area and a greater number of vessels in all three measured organs compared to the control samples (Table 1.5). Nevertheless, C and Cd samples showed a similar vessel density. Moreover, similar mean xylem diameter and Dh values were recorded in the two groups of plants. The higher number of vessels in Cd stem and petiole led to a statistically higher value of water transport efficiency compared to the control. However, a different behavior was recorded at root level, where the increase in the number of vessels was not sufficient to enhance root water transport efficiency. When the theoretical flow was normalized by xylem area, a similar water transport efficiency occurred in both experimental groups for each measured organ.

Table 1.5: Mean values $\pm$ SD of xylem anatomical traits recorded in stem, petiole and root of control (C) and Cd-treated (Cd) samples of *Solanum lycopersicum* (n=5). P values are reported. Ax: xylem area, Nc: number of xylem conduits; Vd: vessel density; Dh: hydraulically weighted mean xylem conduit diameter; Sr^4 : efficiency of water transport. Sr^4/Ax : efficiency of water transport normalized by the xylem area.

	C	Cd	P-value
Petiole			
Ax (mm²)	0.251 $\pm$ 0.09	0.439 $\pm$ 0.12	0.026
Nc	71.0 $\pm$ 12.4	128.9 $\pm$ 11.05	<0.001
Vd (mm⁻²)	245.8 $\pm$ 74.4	339.1 $\pm$ 82.1	0.09
Dh (mm)	22.5 $\pm$ 1.6	23.3 $\pm$ 3.9	0.646
Sr^4 E-6 (mm⁴)	0.57 $\pm$ 0.18	1.18 $\pm$ 0.09	<0.05
Sr^4/Ax E-6 (mm²)	2.07 $\pm$ 0.76	2.86 $\pm$ 0.42	0.078
Stem			
Ax (mm²)	0.87 $\pm$ 0.28	1.36 $\pm$ 0.21	0.0176
Nc	160.9 $\pm$ 23.9	220.1 $\pm$ 27.1	0.006
Vd (mm⁻²)	213.4 $\pm$ 41.7	166.6 $\pm$ 33.9	0.088
Dh (mm)	41.3 $\pm$ 3.2	42.5 $\pm$ 3.0	0.558
Σr^4 E-4 (mm⁴)	0.14 $\pm$ 0.04	0.22 $\pm$ 0.06	0.038
$\Sigma r^4/Ax$ E-4 (mm²)	0.17 $\pm$ 0.04	0.16 $\pm$ 0.04	0.703
Root			
Ax (mm²)	0.42 $\pm$ 0.02	0.47 $\pm$ 0.03	0.004
Nc	62.3 $\pm$ 15.1	83.7 $\pm$ 11.7	0.018
Vd (mm⁻²)	151.5 $\pm$ 40.2	179.5 $\pm$ 35.1	0.137
Dh (mm)	40.6 $\pm$ 6.7	38.6 $\pm$ 8.4	0.688
Σr^4 E-4 (mm⁴)	0.06 $\pm$ 0.01	0.05 $\pm$ 0.01	0.122
$\Sigma r^4/Ax$ E-4 (mm²)	0.14 $\pm$ 0.02	0.11 $\pm$ 0.02	0.07

The Cd treatment also induced significant changes in mineral balance (Fig. 1.4). A higher concentration of potassium (K), copper (Cu), zinc (Zn), and iron (Fe), along with a lower concentration of calcium (Ca), were observed in Cd-treated fruit compared to the control (C). In leaves, Cd treatment resulted in a statistically higher concentration of magnesium (Mg), potassium (K), manganese (Mn), and copper (Cu), along with a reduced zinc (Zn) content compared to the control samples (C). Stems of Cd-treated plants exhibited higher levels of calcium (Ca), copper (Cu), zinc (Zn), and iron (Fe) than those irrigated

with good quality water. Additionally, roots of Cd-treated plants displayed higher potassium content, but lower zinc concentration compared to the control plants (C).

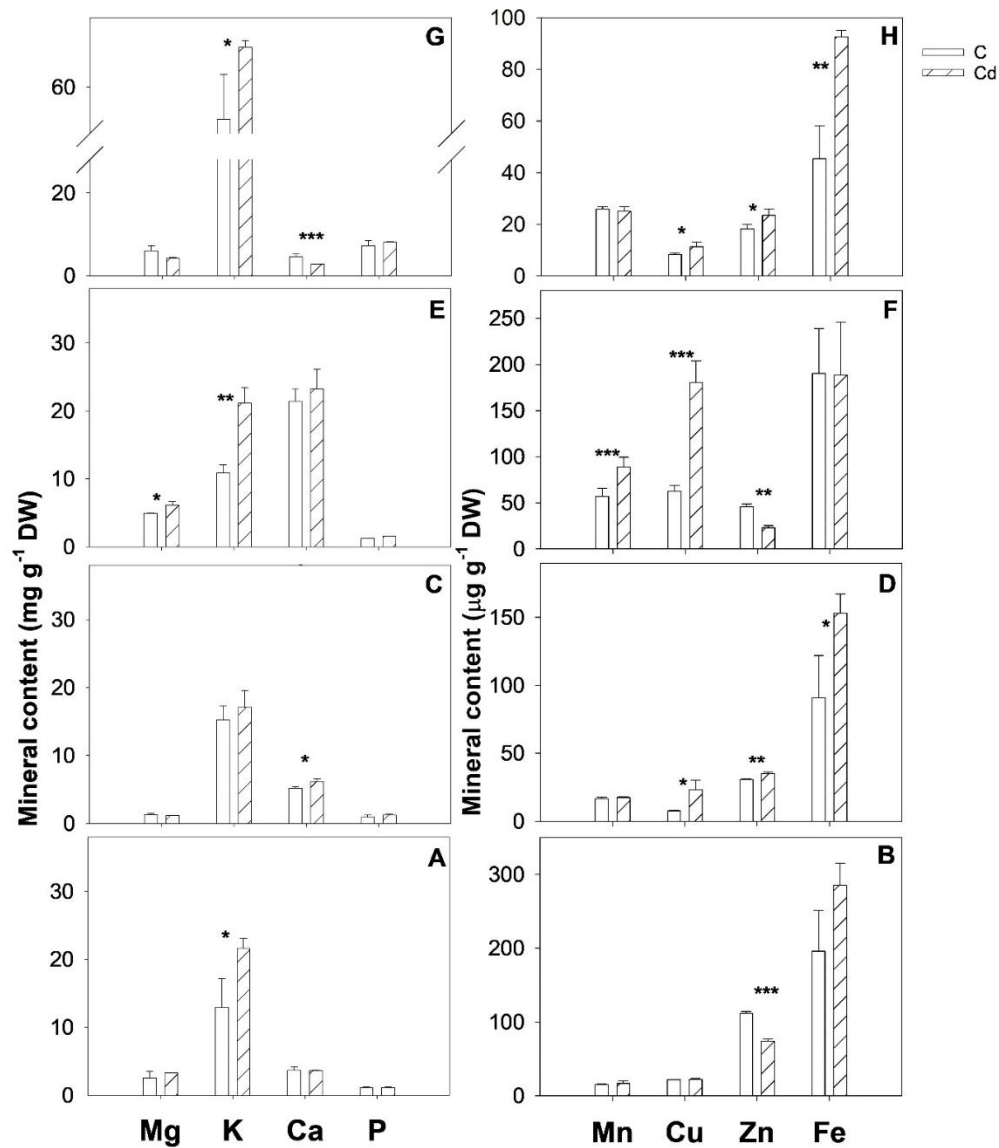


Figure 1.4: Mineral content of control (white bar) and cadmium-treated (dashed bar) root (A, B), stem (C, D), leaf (E, F) and fruit (G, H) of *Solanum lycopersicum* plants. Values are mean $\pm$ SD ($n=3$). Mg: magnesium; K: potassium; Ca: calcium; P: phosphorus; Mn: manganese; Cu, copper; Zn: zinc; Fe: iron. *: $P<0.05$; ** $P<0.01$; ***: $P<0.001$.

1.2.2. Impact of combined cadmium pollution and drought

The decline in leaf hydraulic conductance during dehydration was similar in both the control (C) and cadmium-treated (Cd) samples (Fig. 1.5). Indeed, values of leaf water potential leading to 50% (P50) and 80% (P80) loss of hydraulic conductance (K_L) were statistically comparable between the two experimental groups, i.e., approximately at -1.3 MPa and -2.1 MPa, respectively (Table S1.1).

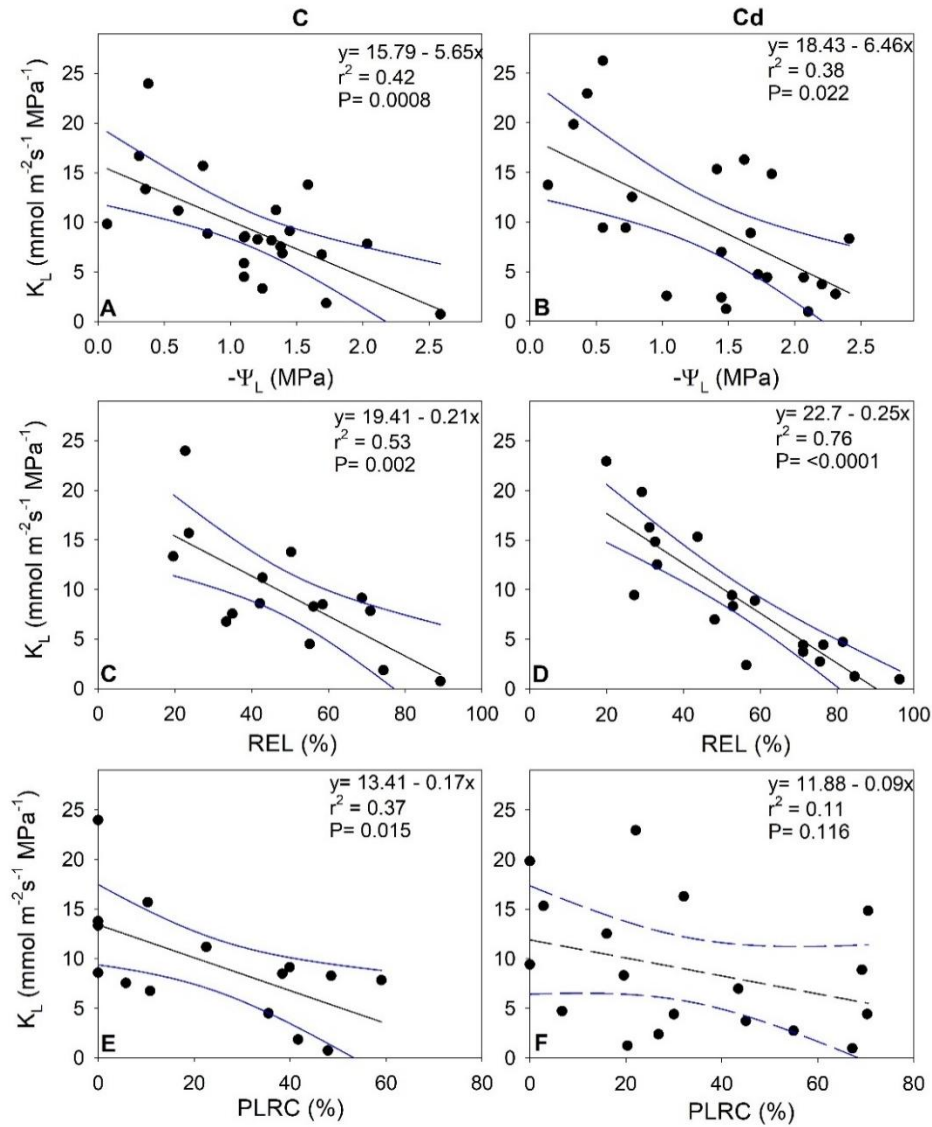


Figure 1.5: Relationships between the leaf hydraulic conductance, K_L and (A, B) the leaf water potential, Ψ_L , (C, D) the relative electrolyte leakage (REL) and (E, F) the percentage loss of rehydration capacity (PLRC) recorded during dehydration in control (C) and cadmium treated (Cd) plants of *Solanum lycopersicum*. Black and blue lines show, respectively, the regression curve and associate 95% confidence intervals. Regression equation, correlation coefficient (r^2) and P value are reported.

Both groups of leaves showed a similar relationship between K_L and relative electrolyte leakage (REL) which acts as an indicator of membrane integrity and, consequently, the functioning of the extra-xylem water pathway. However, higher variability was in the relationships recorded in Cd-treated samples compared to data recorded from control leaves. Moreover, despite the robust correlation recorded in control leaves between K_L and the percentage loss of rehydration capability, no statistically significant correlation was found between K_L and PLRC in cadmium treated samples.

In response to drought, as expected, a significant decrease in g_L , A_n , and K_L , along with an increase in REL values, was observed in both cadmium-treated and control leaves compared to well-watered samples (Fig. 1.6). Notably, g_L and A_n were lower, while REL values were higher in cadmium-treated

leaves (Cd) compared to control leaves (C), even under well-watered conditions, likely due to cadmium pollution.

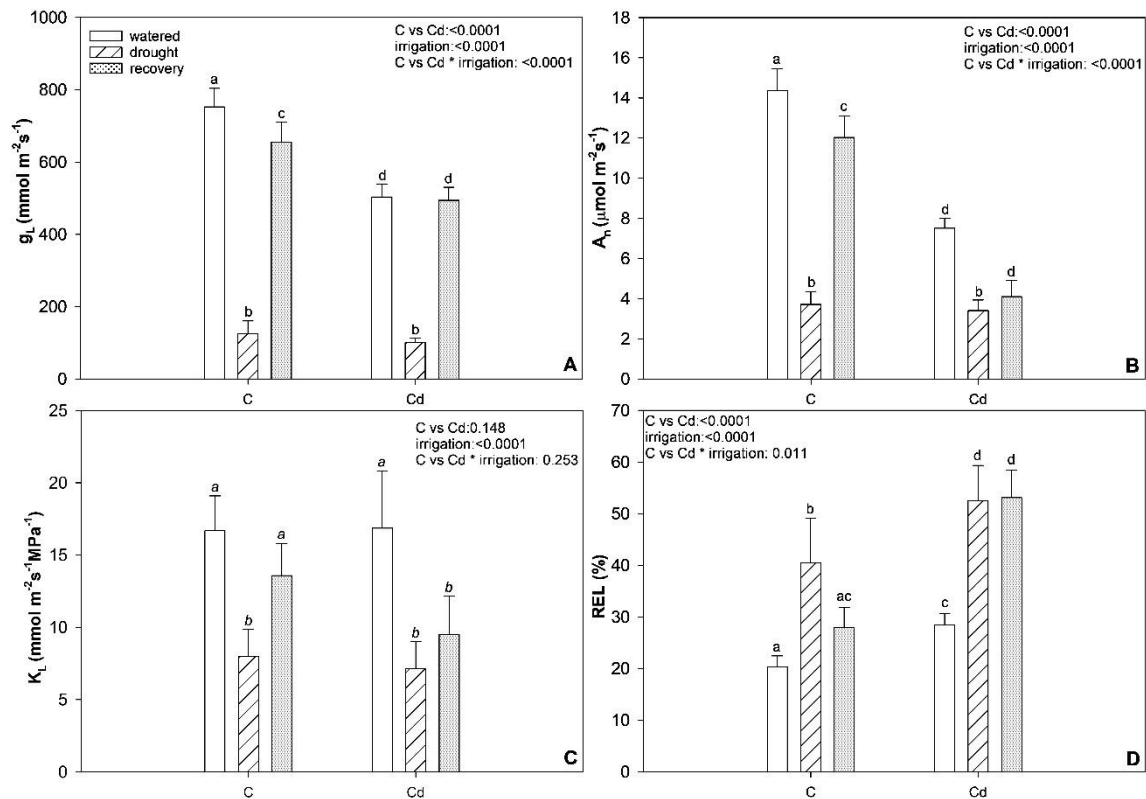


Figure 1.6: Mean values $\pm$ SD of A) stomatal conductance to water vapour (g_L), B) photosynthesis rate (A_n), C) leaf hydraulic conductance values (K_L) and C) relative electrolyte leakage (REL) recorded in control C and cadmium-treated leaves (Cd) under well watered (white columns), drought (coarsed columns) and three days after re-irrigation (gray columns) *solanum lycopersicum* plants. Different letters indicate statistically significant differences among groups. P values as resulted by two-way ANOVA of measured parameters by control and cadmium-treated leaves (C vs Cd) and irrigation treatment (watered, drought, re-irrigation) are reported.

Three days after re-irrigation, g_L , A_n , and K_L values of control leaves returned to levels comparable to those observed in well-watered samples. In cadmium-treated leaves, g_L values were recorded as higher than those in both well-watered and control leaves. In contrast, A_n and K_L values did not recover, resulting in values lower than those in both control and well-watered cadmium-treated leaves, likely due to the higher relative electrolyte leakage (REL) values.

1.3. Discussion

Tomato plants demonstrated a notable capacity for cadmium uptake, displaying a relatively high tolerance to heavy metal exposure. As long as the plants were well-watered, cadmium stress did not result in severe visible symptoms or significantly affect plant productivity, despite cadmium accumulation in various tissues, including the fruit. Both control and cadmium-treated leaves exhibited

similar susceptibility to dehydration, as shown by comparable P50 and P80 values. However, cadmium treatment influenced pressure-volume (PV) characteristics, such as the turgor loss point, and induced membrane damage even in well-watered leaves. Consequently, the ability of the leaves to restore water transport efficiency after drought was compromised, heightening the risk of plant decline under frequent or severe drought conditions.

1.3.1. Plant growth and productivity under well-watered conditions

Chronic, mild exposure to cadmium did not have a detrimental impact on the growth and productivity of tomato plants. Indeed, C and Cd-treated samples showed similar height, number of leaves and fruit yield, as well as comparable dry weights for the root, stem, leaf and fruit. Previous studies have suggested that the presence of cadmium can hinder the productivity of various plant species (Aslam et al., 2023; Li et al., 2023). However, many of these studies involved plants grown in hydroponic solutions, which are known to enhance mineral uptake compared to soil-based cultivation methods (Jeon et al., 2019). Furthermore, different species and even cultivars, within the same species, exhibit varying levels of tolerance to cadmium stress (Shi and Cai, 2009; Carvalho et al., 2018; Zhang et al., 2022). Our findings demonstrate that *S. lycopersicum* cultivar Piccadilly, a commonly cultivated variety, has a notable ability to withstand mild cadmium stress. These results underscore the importance of evaluating the tolerance of various tomato cultivars to cadmium and other heavy metals. Indeed, the observation that chronic cadmium stress resulted in the accumulation of cadmium in edible fruits without triggering visible signs of distress raises concerns for global food security.

Furthermore, it should be noted that exposure to cadmium resulted in alterations in the mineral profile of all measured organs, as this metal can interfere with the uptake of essential nutrients. Studies investigating the mineral content of plants exposed to cadmium pollution have yielded conflicting results, highlighting our current limitations in this area. As example, Lima et al. (2019) recorded a decrease in manganese content in the leaves of the tomato genotype *high pigment-1* but not in those of the tomato cultivar *Micro-Tom*. Conversely, Carvalho et al. (2018) reported similar manganese content in the leaves of two different tomato cultivars. These discordant results could stem from differences in the cadmium tolerance of various cultivars and/or variations in experimental growth conditions. Further research is needed to explore this hypothesis and elucidate the effects of cadmium uptake on mineral balance in both sensitive and tolerant species or cultivars.

It is notable that cadmium-treated samples showed higher leaf surface area and leaf thickness, yet lower leaf mass area and leaf density compared to control plants. Literature data suggest that leaf thickness correlates positively with leaf water status (Afzal et al., 2017; Scoffoni et al., 2014; Trifilò et al., 2016) and an increase in leaf thickness has been observed in response to salt stress in some species (Qadir and Shams, 1997; Longstreth and Nobel, 1979; Boughalleb et al., 2009; Paz et al., 2014; Rozema et al., 2015). The higher T_L recorded in Cd-treated compared to control leaves was primarily due to increased

succulence in these samples (see also comments below) rather than changes in tissue thickness or anatomical traits of cells, including xylem (Nardini, 2022). Indeed, lower values of LMA and leaf density were recorded in plants exposed to cadmium pollution. In other words, our findings suggest that tomato plants responded to the cadmium stress similarly to the more extensively studied sodium chloride stress, by increasing leaf succulence.

Cadmium exposure also led to alterations in xylem traits in the root, stem and petiole. However, overall, water transport efficiency was similar in cadmium-treated and control samples. Wang et al. (2019) measured no difference in xylem vessel diameter in Cd-treated stems of *Populus × euramericana* clone, although changes in xylem thickness and cell layers occurred in samples grown in cadmium-polluted soil. More recently, Zhang et al. (2022), measured no changes in stem hydraulic efficiency of *Acacia auriculiformis* and *Syzygium hainanense* growing in cadmium-polluted soil. Our results, in conjunction with existing literature, suggest that xylem traits in cadmium-tolerant species are minimally affected, especially when plants are grown under adequate water and mineral supply (Wang et al., 2019).

1.3.2. Plant-water relations and leaf water transport efficiency during dehydration and rehydration

Exposure to cadmium induced succulence in tomato leaf and root cells. Succulence is an adaptative trait observed in some halophytes that aids in the regulation of internal ion concentrations, including heavy metals (Grigore and Toma, 2021; Shang et al., 2020). Typically, at the leaf level, high saturated water content is associated with lower leaf density and a less negative turgor loss point (Nadal et al., 2023). Consistent with previous findings, in the present study, tomato leaves under cadmium treatment, increased their saturated water content and showed lower leaf density. This alteration may increase the risk of cell damage under low water availability, due to the less negative turgor loss point (Bartlett et al., 2012). On the other hand, higher water content might enhance the stability of cell membranes during dehydration, thereby delaying damage to the photosynthetic machinery (Guadagno et al., 2017; Trueba et al., 2019; Abate et al., 2021a, 2021b; Azzarà et al., 2023). In this context, the increased cell water content could be interpreted as a protective mechanism employed by tomato plants under cadmium stress, aimed at mitigating damage to the leaf extra-xylem water pathway during dehydration. However, cadmium treatment induced cell membrane damage, as evidenced by higher REL values in well-watered cadmium-treated samples compared to the corresponding controls. This membrane damage impaired the cells' ability to rehydrate after dehydration, as indicated by the lack of a relationship between PLRC and K_L in cadmium-treated leaves. Such relationships were present in control plants and have been documented in other species, suggesting an association between K_L decline and PLRC with leaf membrane integrity during dehydration (Abate et al., 2021b; Trifilò et al., 2021; Azzarà et al., 2023; Trifilò et al., 2023). In this context, our data strongly suggest that cadmium treatment caused irreversible damage to the leaf extra-xylem water pathway, which in turn compromised water transport efficiency and photosynthetic rates, particularly under drought conditions. As a result, despite exhibiting similar

vulnerability to dehydration, cadmium-treated plants were less able to recover from drought compared to the controls. These findings align with recent studies demonstrating that the extra-xylem water pathway plays a critical, though not fully understood, role in the decline of K_L under drought conditions, significantly affecting a leaf's hydraulic capacity to recover upon rehydration (Trifilò et al., 2016, 2021; Scoffoni et al., 2017).

It is evident that exposure to cadmium resulted in a reduction in cell wall rigidity, leading to a higher saturated water content at both root and leaf cell levels. Heavy metal pollution has been demonstrated to alter cell wall properties by interacting with the negative charges of cell wall polysaccharides, such as pectins (Cui et al., 2020, Li et al., 2022). Indeed, the negative charges of these polysaccharides can bind cations, thereby acting as the primary physiological barrier to prevent heavy metals from entering the cell. Notably, decreased cell wall rigidity can contribute to water storage and survival time, by increasing capacitance after the turgor loss point (Meinzer et al., 2004; Bartlett et al., 2019).

However, this was not observed in the measured tomato leaves. Indeed, both control and cadmium-treated leaves showed a similar capacitance after the turgor loss point. Consequently, the potential benefits of lower ϵ values did not translate into measurable advantages. It can be speculated that in root cells, once the binding capacity with pectins was saturated, the entry of cadmium led to an increase in osmotic compounds (both organic and inorganic) to counterbalance/bind this heavy metal. Conversely, at the shoot level, the distribution of cadmium among the three different organs (stem, leaf, and fruit) limited its presence within the cells. This could explain the similar osmotic potential at full turgor recorded in control and cadmium-treated leaves. Further studies, however, may be needed to elucidate more details about these intriguing mechanisms.

1.4. Conclusions

Our findings indicate that even mild but chronic exposure to cadmium alters various hydraulic traits, increasing the plants' risk of potential damage from co-occurring water stress. Indeed, it significantly impacted the water relations of tomato plants, particularly affecting the leaf cells' ability to recover from dehydration and, therefore, plants' ability to recover from drought.

Overall, these results highlight the intricate relationship between environmental pollution, climate change and their impacts on agricultural systems, particularly concerning water quality and plant stress responses.

1.5. Supplementary Material

Table S1: a) Leaf water potential values inducing 50% (P_{50}) and 80% (P_{80}) loss of leaf hydraulic conductance (K_L), b) relative electrolyte leakage values inducing 50% (REL_{50KL}) and 80% (REL_{80KL}) loss of K_L , c) percentage loss of rehydration capacity inducing 10% ($PLRC_{10KL}$) and 50% ($PLRC_{50KL}$) as recorded in control and cadmium treated tomato leaves. Confidence intervals (CI) are also reported.

Parameter	Treatment	Estimate	2.5% CI	97.5% CI
a) Water potential thresholds for loss of leaf hydraulic conductance (MPa)				
P_{50}	C	-1.4	-1.15	-2.0
P_{50}	Cd	-1.5	-1.3	-2.1
P_{80}	C	-2.1	-1.8	-3.0
P_{80}	Cd	-2.4	-1.9	-3.0
b) Relative electrolyte leakage associated with 50% and 80% loss of K_L (%)				
REL_{50KL}	C	58	48	78
REL_{50KL}	Cd	52	47	61
REL_{80KL}	C	80	66	100
REL_{80KL}	Cd	81	73	97
c) Percentage loss of relative capacitance associated with 10% and 50% loss of K_L (%)				
$PLRC_{10KL}$	C	5	0	20
$PLRC_{10KL}$	Cd	n.d.	n.d.	n.d.
$PLRC_{50KL}$	C	33	18	60
$PLRC_{50KL}$	Cd	n.d.	n.d.	n.d.

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Chapter 2

Phenolic Profile and Biological Evaluation of *Salvia ceratophylloides* Ard.: a Novel Source of Rosmarinic Acid

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2. Introduction

The excessive accumulation of free radicals in the human body is a key factor in the development of degenerative conditions such as chronic inflammation and neurodegenerative disorders (Farhat et al., 2013; Krishnaiah et al., 2011). Oxidative stress, which arises from an imbalance between reactive oxygen species (ROS) and the body's antioxidant defense, compromises cellular integrity and normal physiological functions. In this context, plant-derived polyphenols have attracted considerable attention due to their ability to counteract oxidative damage and to modulate biological processes, including inflammation, cell signaling, and apoptosis (Hassan et al., 2017; Rasouli et al., 2017). Due to their chemical diversity and multifunctional effects, polyphenols represent an important focus in natural product research. Consequently, the identification of polyphenol-rich plant species and the evaluation of their chemical composition and biological activity remain central goals in pharmacognosy and phytochemistry. The genus *Salvia* L. (Lamiaceae), comprising approximately 1000 species, distributed across tropical and temperate regions, is among the most diverse genera within the family. In Italy, 20 species contribute significantly to national plant biodiversity. Many *Salvia* species are valued for their culinary, medicinal, and ornamental uses. Among these, *Salvia officinalis* L. (common sage, *So*) is the most extensively studied, with numerous studies reporting antioxidant, anti-inflammatory, antibacterial, and anticancer properties (Jakovljević et al., 2019; Behradmanesh et al., 2013). While early investigations primarily focused on its essential oils, increasing attention has been directed toward polar constituents, particularly phenolic acids and flavonoids, as key contributors to its biological activities (Ghorbani & Esmaeilzadeh, 2017; Ben Akacha et al., 2024). In contrast, *Salvia ceratophylloides* Ard. (*Sc*) is a rare and endangered species, endemic to a restricted hilly area near Reggio Calabria in southern Italy. Despite its ecological significance and distinctive morphological traits, this species has received little attention from a phytochemical and pharmacological perspective. To date, research has mainly addressed its ecophysiology (Abate et al., 2021; Abate et al., 2021) and volatile organic compound profile (Vescio et al., 2021), leaving its phenolic composition and biological potential unexplored. The present study aims to fill this gap by providing the first detailed characterization of the polyphenolic profile, antioxidant activity, and preliminary safety evaluation of *S. ceratophylloides*. A comparative analysis with *S. officinalis*, used as a reference species, was conducted under controlled cultivation conditions to minimize environmental variability. Beyond its potential biological relevance, *S. ceratophylloides* represents an important component of southern Italy's endemic flora. Therefore, the characterization of its bioactive compounds contributes not only to natural product research but also to the valorization and conservation of this underexplored species.

2.1. Material and Methods

2.1.1. Plant material and growth conditions

Experiments were performed on five-month-old plants of *Salvia ceratophylloides* Ard. and *Salvia officinalis* L. Seeds of both species, kindly provided by the Botanical Garden of the University of

Messina, were soaked in distilled water for 24 hours and sown in greenhouse trays in October 2023. One month after germination, individual seedlings were transplanted into 3.5 L plastic pots filled with a nutrient-rich potting substrate formulated for vegetable cultivation (Comodo, Tercomposti S.P.A., Italy). Plants were grown in a greenhouse at the Department of Chemical, Biological, Pharmaceutical and Environmental Sciences (ChiBioFarAm), University of Messina. The greenhouse, oriented northwest, received natural daylight supplemented with LED lamps (Led Spectrum Grow 6, I-GROW s.r.l., Italy), providing a 16 h light/8 h dark photoperiod and a photosynthetic photon flux density of 450 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Temperature was maintained between 24 °C during the day and 18 °C at night. In February 2024, plants were transferred to an open terrace at the ChiBioFarAm Department, where they were exposed to natural environmental conditions and regularly irrigated to maintain field capacity. Leaf sampling for chemical analyses was conducted in April 2024.

2.1.2. Morpho-physiological measurements

To monitor plant physiological status throughout the experiment, stomatal conductance to water vapour (g_L), transpiration rate (E_L), and net photosynthetic rate (A_n) were measured weekly from February to April. Measurements were performed on at least five fully expanded leaves per species using a portable photosynthesis system (LCi Analyzer, ADC Bioscientific Ltd., Herts, UK). At the end of the experimental period, plant biomass was assessed. Roots (carefully rinsed to remove soil), stems, leaves, and flowers were oven-dried at 80 °C for three days to determine their dry weight (DW).

2.1.3. Extraction procedure

For the preparation of the extracts, the leaves of *S. ceratophylloides* and *S. officinalis* were lyophilized, finely ground, and subjected to extraction using 70% ethanol (1:10 w/v). A preliminary maceration at 25 °C was performed for 1 hour, followed by ultrasonic-assisted extraction at 50 °C for 15 minutes, repeated twice. The combined extracts were filtered and concentrated to dryness using a rotary evaporator. Extraction yields were calculated based on 100 g of lyophilized plant material and amounted to 42% for *S. ceratophylloides* (Sc) and 23% for *S. officinalis* (So). The yield obtained for So agrees with that previously found by (Glisic et al., 2011) in the 70% ethanol leaf extract of *S. officinalis* (1:12 w/v) obtained using ultrasound-assisted extraction for 45 min at 40 °C, resulting approximately 20%.

2.1.4. Determination of Polyphenolic Compounds by HPLC-PDA/ESI-MS

The qualitative and quantitative characterization of the polyphenolic components of Sc and So extracts was performed using high-performance liquid chromatography with photodiode array detection and electrospray ionization mass spectrometry (HPLC-PDA/ESI-MS). The dried extracts were redissolved in 70% ethanol and filtered through a 0.45 μm Acrodisc nylon membrane filter (Merck Life Science, Merck KGaA, Darmstadt, Germany) before to analysis. LC-MS grade solvents, including water,

acetonitrile, and formic acid, along with analytical standards (caffeic acid, apigenin, salvianolic acid B, luteolin-7-O-glucoside, and quercitrin) were also purchased from Merck Life Science.

Chromatographic analysis was carried out using a Shimadzu HPLC system (Kyoto, Japan) equipped with a CBM-20A controller, two LC-30AD dual-plunger parallel-flow pumps, a DGU20A3R degasser, a CTO-20AC column oven, a SIL-30AC autosampler, an SPD-M30A photodiode array detector, and an LCMS-2020 mass spectrometer. The system employed an ESI source operating in negative ionization mode. Separations were performed on an Ascentis Express C18 column (150 × 2.1 mm; 2.7 μm) obtained from Merck Life Science.

The mobile phase consisted of water (solvent A) and acetonitrile (solvent B), both acidified with 0.1% formic acid (v/v), under the following gradient elution conditions: 0 min: 0% B - 10 min: 10% B - 20 min: 11% B - 30 min: 15% B - 50 min: 18% B - 65 min: 23% B.

The flow rate was set at 0.5 mL/min, and the injection volume was 2 μL. Photodiode array (PDA) detection was applied over the 190–450 nm range and monitored at 330 nm (sampling frequency: 40 Hz, time constant: 0.050 s).

Mass spectrometry (MS) conditions were as follows: Scan range: m/z 100–1200; Scan speed: 7500 amu/s; Event time: 0.3 s; Nebulizing gas (N₂) flow rate: 1.5 L/min; Drying gas (N₂) flow rate: 15 L/min; Interface temperature: 350°C; Heat block temperature: 300°C; Desolvation line temperature: 300°C; Desolvation line voltage: 1 V; Interface voltage: -4.5 kV.

Quantification of the polyphenolic content in the extracts was based on calibration curves constructed for five standards: caffeic acid, apigenin, salvianolic acid B, luteolin-7-O-glucoside, and quercitrin (Table 4). Data acquisition and processing were performed using Shimadzu LabSolution software version 5.97.

Table 2.1: Analytical parameters of the HPLC–PDA method used for the quantification of selected polyphenolic compounds, including detection wavelength, calibration curves, coefficients of determination (R^2), limits of detection (LoD), limits of quantification (LoQ), repeatability, and accuracy evaluated at 100 mg L⁻¹

Compound name	Wavelength (nm)	Calibration curve	R^2	LoD	LoQ	Repeatability (%) 100 mg L ⁻¹	Accuracy (%) 100 mg L ⁻¹
Caffeic acid	330	$y = 4068.7x + 19686$	0.9996	0.053	0.161	0.25	100.30
Apigenin	330	$y = 22357x - 1527.1$	0.9999	0.037	0.111	0.26	100.50
Salvianolic acid B	330	$y = 3670.4x + 1415.2$	0.9999	0.145	0.439	0.14	100.82
Luteolin-7-O-glucoside	350	$y = 12881x + 54935$	0.9995	0.048	0.016	0.31	99.58
Quercitrin	350	$y = 8054x + 27465$	0.9997	0.034	0.103	0.12	99.85

2.1.5. Antioxidant activity

To comprehensively assess the antioxidant capacity of the extracts, three in vitro assays were performed: (i) 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, (ii) reducing power assay, and (iii) ferrous ion chelating activity assay.

2.1.5.1. DPPH radical scavenging assay

The DPPH test was carried out to estimate the free radical scavenging activity of *Sc* and *So* hydroalcoholic extracts, using the method reported by (Ohnishi et al., 1994). The extracts were tested at concentrations ranging from 0.0625 to 2 mg/mL. For each sample, 0.5 mL of the extract solution was mixed with 3 mL of freshly prepared 0.1 mM DPPH methanolic solution. The mixture was incubated in the dark at room temperature for 20 minutes, after which the absorbance was recorded at 517 nm using a UV-1601 spectrophotometer (Shimadzu). Butylated hydroxytoluene (BHT) was used as the reference standard. Results were expressed as the mean radical scavenging activity (%) $\pm$ standard deviation (SD) and the mean 50% inhibitory concentration (IC₅₀) $\pm$ SD, derived from three independent experiments.

2.1.5.2. Reducing power assay

The reducing power of *Sc* and *So* extracts was determined using the Fe³⁺-Fe²⁺ transformation method, as described by (Oyaizu, 1986). Extract concentrations ranged from 0.0625 to 2 mg/mL, with BHT and ascorbic acid serving as reference standards. To perform the assay, a reaction mixture was prepared by combining 2.5 mL of 0.2 M phosphate buffer (pH 6.6), 2.5 mL of 1% potassium ferricyanide, and 1 mL of the extract solution. The mixture was incubated at 50°C for 20 minutes and then rapidly cooled. Subsequently, 2.5 mL of 10% trichloroacetic acid was added, and the mixture was centrifuged at 3000 rpm for 10 minutes at 4°C. The supernatant (2.5 mL) was combined with 2.5 mL of distilled water and 0.5 mL of 0.1% ferric chloride. The reaction mixture was incubated in the dark at room temperature for 10 minutes, and the absorbance was measured at 700 nm. Results are presented as mean absorbance values $\pm$ SD and ascorbic acid equivalents per milliliter (ASE/mL) $\pm$ SD, based on three independent experiments.

2.1.5.3. Ferrous ion (Fe²⁺) chelating activity assay

The Fe²⁺ chelating activity of *Sc* and *So* extracts were determined spectrophotometrically by measuring the formation of the Fe²⁺-ferrozine complex, following the protocol described by (Kumar et al., 2008) concentrations ranged from 0.0625 to 2 mg/mL, with ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA) as the reference standard. In this assay, 0.05 mL of 2 mM ferrous chloride solution was mixed with 1 mL of the extract solution and 0.5 mL of methanol. Then, 0.2 mL of 5 mM ferrozine solution was added, and the mixture was shaken vigorously before being incubated in the dark at room temperature for 10 minutes. The absorbance was recorded at 562 nm using a spectrophotometer. Results are reported as the mean inhibition of the ferrozine-Fe²⁺ complex formation (%) $\pm$ SD and IC₅₀ $\pm$ SD, based on the average of three independent experiments.

2.1.6. *Artemia salina* Leach (brine shrimp) lethality bioassay

The potential toxicity of *Sc* and *So* extracts was determined using the brine shrimp lethality bioassay (Meyer et al., 1982). Brine shrimp eggs were incubated under a 60 W lamp that provided direct light and warmth (24–26 °C) in artificial seawater (33 g sea salt/L deionized water). To achieve final

concentrations in the range of 10–1000 µg/mL, ten brine shrimp larvae were incubated for 24 hours at 24–26 °C in artificial seawater mixed with varying volumes of the extracts dissolved in DMSO. Control groups included artificial seawater alone and artificial seawater added with 2% DMSO, corresponding to the highest solvent concentration used in the assay. After counting the number of surviving nauplii, GraphPad Prism 10 was used to calculate the median lethal concentration (LC₅₀) values. The test was performed in triplicate. The extracts' level of toxicity was evaluated using Clarkson's toxicity criterion, which states that an extract is non-toxic if its LC₅₀ is greater than 1000 µg/mL (R. Hamidi et al., 2014).

2.1.7. Statistical analysis

Statistical analyses were performed using SigmaStat 12.0 (SPSS Inc., Chicago, IL, USA). Differences between species in g_L, E_L, An (n = 40), DW (five plants per species), and antioxidant assay results (three independent extracts per species, n = 3) were assessed using Student's t-test. Data are expressed as mean ± SD, and differences were considered statistically significant at P < 0.05.

2.2. Results and Discussion

2.2.1. Morpho-physiological measurements

According to literature data (Abate et al., 2021; Abate et al., 2021; Vescio et al., 2021), *S. ceratophylloides* and *S. officinalis* differed significantly in gas exchange, and consequently, in plant biomass (Table 2.2).

Table 2.2: Mean values ± SD of total plant biomass (DW plant) recorded at the end of the experimental period (n = 5), and of stomatal conductance to water vapour (g_L), transpiration rate (E_L), and photosynthetic rate (An), recorded weekly from February to April in *Salvia ceratophylloides* (Sc) and *Salvia officinalis* (So). P-values as resulted by a t-student test are reported.

	Sc	So	P-value
DW plant (g)	3.62 ± 0.95	5.91 ± 0.73	0.002
g_L (mmol m⁻²s⁻¹)	314.3 ± 57.2	393.9 ± 65.2	0.006
E_L (mmol m⁻²s⁻¹)	2.44 ± 0.47	2.24 ± 0.53	0.373
An (mmol m⁻²s⁻¹)	4.21 ± 1.12	6.71 ± 1.88	0.001

In detail, despite being cultivated under identical optimal conditions, *So* showed higher stomatal conductance and photosynthetic rate than *Sc*, while both species showed comparable transpiration rates. Consequently, *S. officinalis* accumulated significantly greater biomass than *S. ceratophylloides* by the end of the experimental period.

2.2.2. Determination of polyphenolic compounds by HPLC-PDA/ESI-MS

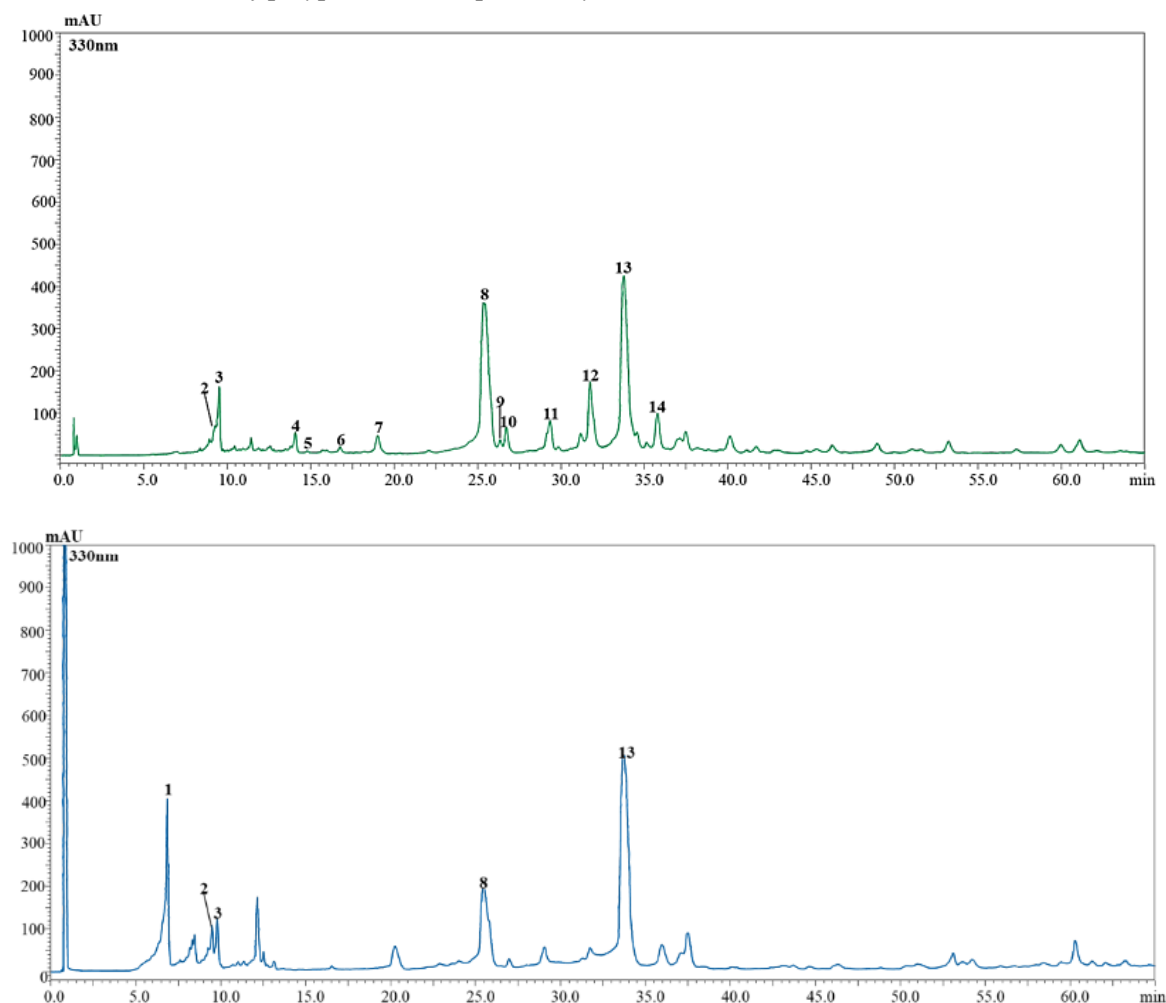


Figure 2.1: Chromatographic profile of hydroalcoholic extracts from leaves of *Salvia officinalis* (So, top) and *Salvia ceratophylloides* (Sc, bottom) at $\lambda = 330$ nm.

The two *Salvia* species under investigation exhibited distinct qualitative and quantitative phenolic profiles. In total, 14 phenolic compounds were tentatively identified across both extracts: 13 in *So* and 5 in *Sc*. These included 7 phenolic acids and 7 flavonoids, as illustrated in Figure 2.1 and detailed in Table 2.2. Interestingly, despite significant differences in the extraction yields of *Sc* and *So*, approximately double in the former (42%) compared to the latter (23%), as well as in the composition and number of compounds, the polyphenolic amount was remarkably similar between the two species: 274.59 mg/ in *So* and 274.27 mg/g in *Sc*. In *So* extract rosmarinic acid was the most abundant compound (142.00 ± 3.75 mg/g), followed by luteolin-7-O-glucuronide (53.40 ± 3.67 mg/g). In contrast, *Sc* was characterized by higher level of rosmarinic acid (183.18 ± 7.54 mg/g), accompanied by caffeic acid derivative (58.99 ± 1.06 mg/g) as the second most abundant phenolic. This substantial presence of rosmarinic acid in *Sc* suggests that this rare, endemic species may serve as a valuable and novel natural source of this compound, with potential nutraceutical or pharmaceutical applications. To the best of our knowledge, this is the first report on the phenolic composition of the aerial parts of *S. ceratophylloides*.

The identification of rosmarinic acid as its dominant phenolic compound is particularly noteworthy, especially considering that *S. officinalis*, a widely studied member of the *Lamiaceae* family, is commonly recognized as one of the richest natural sources of rosmarinic acid. When compared with previously published data on other *Lamiaceae* species, the levels of rosmarinic acid found in *S. ceratophylloides* are especially promising. For instance, *S. officinalis* has been reported to contain between 12.2-296 mg/Kg of rosmarinic acid in infusion preparations (Zimmermann et al., 2011). In a separate study, (Sharma et al., 2020) reported concentrations ranging from 67.41 ± 2.03 mg/Kg to 18.82 ± 150.20 mg/Kg in different extracts (Soxhlet methanol extract, and ultrasound water and methanol extracts) obtained from the leaves of cultivated *S. officinalis*. These reports, considered within their respective analytical frameworks, indicate that *Salvia* species exhibit a marked capacity for rosmarinic acid accumulation. In this context, the high accumulation observed in *S. ceratophylloides* supports its classification as a particularly rich natural source of this bioactive compound. Phenolic compounds in both species were identified using HPLC-PDA at 330 nm, based on the comparison of retention times, UV absorption spectra, and mass spectra, with those of available standards or literature data. The predominance of phenolic acids and flavonoids observed here is consistent with chromatographic investigations indicating that these compound classes constitute the principal bioactive fraction in *Salvia* spp. (Afzal et al., 2025). As expected, our analysis confirmed the presence of rosmarinic acid as the principal phenolic acid in *S. officinalis*, consistent with numerous previous studies (Farhat et al., 2013; Zimmermann et al., 2011; Ben Farhat et al., 2009; Dent, 2015).

Qualitatively, our findings are largely consistent with those reported by (Martins et al., 2015), with a few exceptions. Compounds such as sagecoumarin and sagerinic acid, previously detected in *S. officinalis*, were not found in our samples. Notably, significant quantitative differences were also observed, which may be attributed to variations in extraction protocols, environmental growing conditions, or plant phenological stages at the time of sampling. These differences underscore the importance of standardized methods when comparing phytochemical profiles across studies. Given the comparable phenolic content evidenced for *Sc* and *So*, the much higher amount in the extraction yield of *Sc* appears to be due to other polar compounds belonging to different chemical classes contained in the leaves of *S. ceratophylloides*, extracted with 70% ethanol, which paves the way for further studies aimed at obtaining a thorough characterization of the phytochemistry of this unexplored species.

Table 2.2: Characterization of polyphenol compounds in *Salvia officinalis* (So) and *Salvia ceratophylloides* (Sc) leaf extracts through HPLC-PDA/ESI-MS analysis

Peak No.	Name	t _R (min)	UV _{max}	[M-H] ⁻	So mg/g dry extract ± SD	Sc mg/g dry extract ± SD	Standard employed for quantification	Ref
1	Caffeic acid derivative	6.8	326	415, 191	-	58.99 ± 1.06	Caffeic acid	-
2	1-O-Caffeoyl glucose	9.3	298 sh, 326	341	8.49 ± 0.47	4.29 ± 0.99	Caffeic acid	(Chen et al., 2011)
3	Caffeic acid	9.4	322	179	19.08 ± 0.87	11.02 ± 0.89	Caffeic acid	(Chen et al., 2011)
4	Luteolin rutinoside	14.1	270, 335	593, 285	1.42 ± 0.08	-	Luteolin-7-O-glucoside	(Martins et al., 2015)
5	Lithospermic acid A	14.8	327	537	0.19 ± 0.02	-	Caffeic acid	(Maliki et al., 2021)
6	Quercetin 3-O-glucuronide	16.8	280 sh, 343	477, 301	0.75 ± 0.02	-	Quercitrin	(Maliki et al., 2021)
7	Apigenin 7-O-Allosyl-(1->2)-glucoside	19.2	340	593, 269	1.80 ± 0.06	-	Apigenin	(Maliki et al., 2021)
8	Luteolin-7-O-glucuronide	25.6	253, 347	461, 285	53.40 ± 3.67	16.79 ± 2.02	Luteolin-7-O-glucoside	(Martins et al., 2015)
9	Quercetin 3-O-glucuronide isomer	26.5	343	477, 301	0.63 ± 0.02	-	Quercitrin	(Maliki et al., 2021)
10	Salvianolic acid C	26.9	344	491	12.46 ± 0.26	-	Salvianolic acid B	(Maliki et al., 2021)
11	Chrysoeriol-7-O-rutinoside	29.3	276, 334	607, 299	5.99 ± 0.06	-	Luteolin-7-O-glucoside	(Maliki et al., 2021)
12	Apigenin-O-pentoside	32.0	335	445, 269	7.22 ± 0.32	-	Apigenin	(Maliki et al., 2021)
13	Rosmarinic acid	34.1	328	359, 179	142.00 ± 3.75	183.18 ± 7.54	Caffeic acid	(Chen et al., 2011)
14	Salvianolic acid k	36.1	287, 323	555	21.16 ± 0.23	-	Salvianolic acid B	(Maliki et al., 2021)
Total					274.59	274.27		

Values are expressed as the mean of mg/g of dried extract ± SD, sh: wavelength shoulder.

2.2.3. Antioxidant activity

Single-electron transfer (SET) and hydrogen-atom transfer (HAT) represent two major categories encompassing the primary chemical mechanisms underlying antioxidant activity. The SET mechanism involves the transfer of a single electron, to reduce oxidant species. Conversely, the HAT mechanism consists of hydrogen atom donation to neutralize free radicals. Although many antioxidant reactions are characterized as following either SET or HAT pathways these mechanisms can operate simultaneously. Consequently, the application of multiple complementary assays is recommended to obtain a comprehensive evaluation of the antioxidant properties of plant extracts (Pisoschi et al., 2016). In this study, the DPPH test, which involves both HAT and SET mechanisms, and the reducing power assay, based on the SET mechanism, were used to evaluate the primary antioxidant activity of *Sc* and *So* extracts. In addition, ferrous ion chelating activity was used to evaluate the secondary antioxidant capacity. The results of the DPPH test are shown in Figure 2.2. *Sc* extract exhibited notable radical scavenging activity compared with the standard antioxidant BHT, and higher activity than *So* extract at the lower tested concentrations (0.0625-0.25 mg/mL). Both extracts displayed a similar concentration-dependent trend, reaching maximum inhibition values of approximately 87% at 0.5 mg/mL. The strong ability of *Sc* to neutralize the stable free radical DPPH, demonstrates its potential to mitigate oxidative stress effectively. The IC₅₀ value confirmed the higher scavenging activity of the *Sc* extract compared with *So*, although both were less active than BHT (Table 2.3).

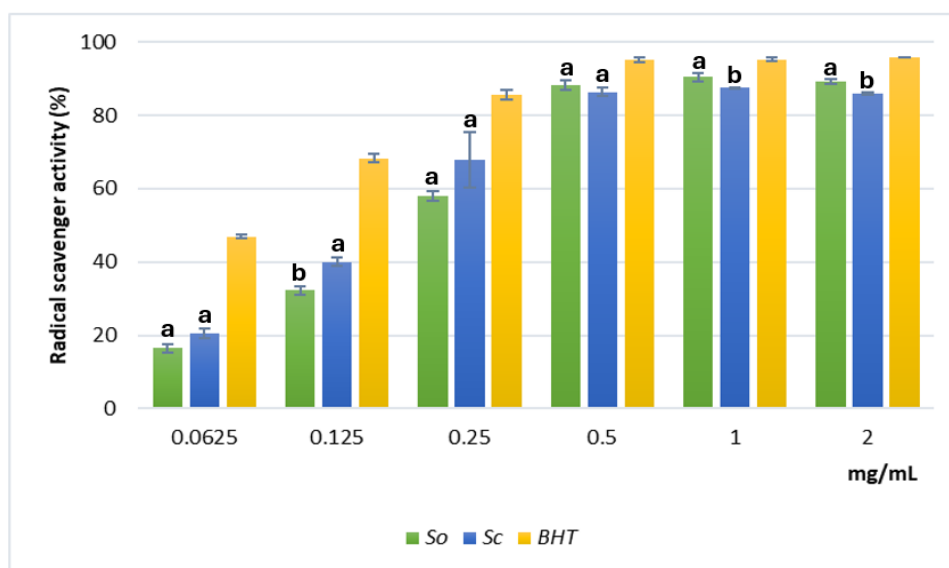


Figure 2.2: Free radical scavenging activity (DPPH test) of the hydroalcoholic extracts from leaves of *Salvia officinalis* (*So*) and *Salvia ceratophylloides* (*Sc*). The absorbance was recorded after 20 minutes of incubation at 517 nm. The percentage of scavenging activity was measured as the decrease in absorbance of the samples versus control. Data are expressed as the mean $\pm$ SD of three independent experiments ($n = 3$). Different letters indicate significant differences between *S. officinalis* and *S. ceratophylloides* within the same concentration according to one-way ANOVA followed by Holm–Sidak multiple comparison test ($P < 0.05$). BHT was used as a reference antioxidant standard for comparative purposes only and was therefore excluded from the statistical comparison among plant extracts.

The reducing power assay (Figure 2.3), which evaluates the ability to interrupt radical chain reaction through Fe^{3+} - Fe^{2+} reduction, revealed a moderate but concentration-dependent activity for the *Sc* extract, reaching an absorbance value of 1.8 at the highest concentration assayed. Nonetheless, the ASE/mL values calculated for the extracts were quite similar (Table 2.3).

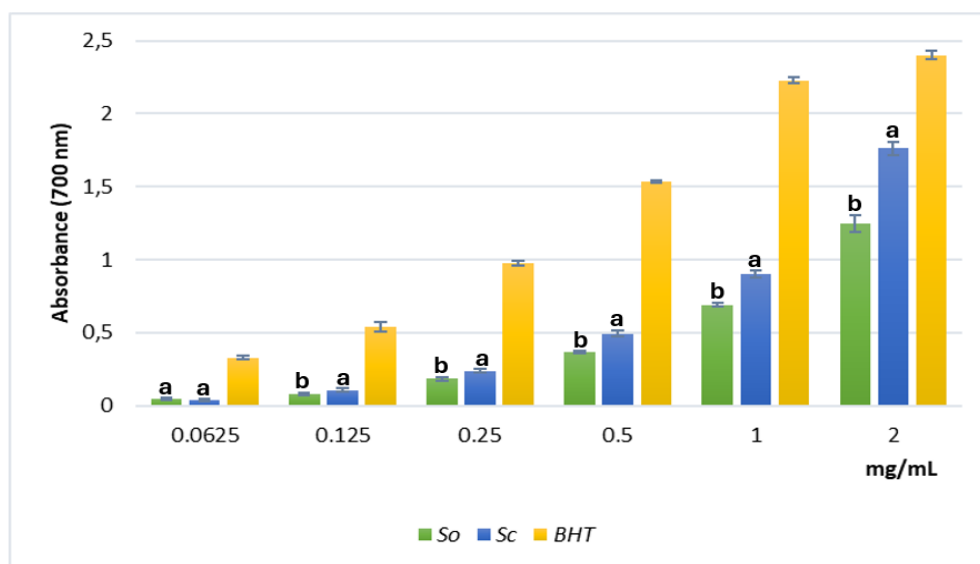


Figure 2.3: Reducing power of the hydroalcoholic extracts from leaves of *Salvia officinalis* (So) and *Salvia ceratophylloides* (Sc) evaluated by spectrophotometric detection of Fe^{3+} - Fe^{2+} transformation method. The absorbance was recorded after 10 minutes of incubation at 700 nm. Data are expressed as the mean $\pm$ SD of three independent experiments ($n = 3$). Different letters indicate significant differences between *S. officinalis* and *S. ceratophylloides* within the same concentration according to one-way ANOVA followed by Holm–Sidak multiple comparison test ($P < 0.05$). BHT was used as a reference antioxidant standard for comparative purposes only and was therefore excluded from the statistical comparison among plant extracts.

The results of the ferrous ion (Fe^{2+}) chelating activity (Figure 2.4) further indicated a stronger secondary antioxidant capacity for the *Sc* extract compared with *So*. Chelating activity increased with concentration, reaching about 80% at 1 and 2 mg/mL for *Sc*. The IC_{50} value for *Sc* was much lower than that for *So*, although both extracts showed weaker chelating ability than EDTA (Table 2.3).

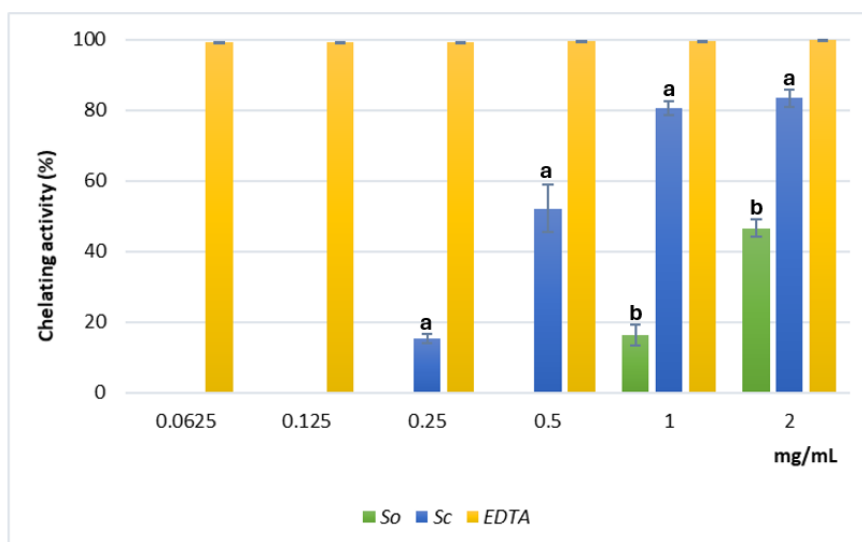


Figure 2.4: Fe²⁺ chelating activity of hydroalcoholic extracts from leaves of *Salvia officinalis* (So) and *Salvia ceratophylloides* (Sc). The absorbance was recorded after 10 minutes of incubation at 562 nm. The percentage of inhibition of ferrozine-Fe²⁺ complex formation was measured as the decrease in absorbance of the samples versus control. Values are expressed as the mean $\pm$ SD of three independent experiments ($n = 3$). Different letters indicate significant differences between *S. officinalis* and *S. ceratophylloides* within the same concentration according to one-way ANOVA followed by Holm–Sidak multiple comparison test ($P < 0.05$). EDTA was used as a reference antioxidant standard for comparative purposes only and was therefore excluded from the statistical comparison among plant extracts.

Table 2.3: Mean 50% inhibitory concentration (IC_{50}) determined by DPPH free radical scavenging activity and by Fe²⁺-chelating activity assays, and ascorbic acid equivalents (ASE/mL) determined by the reducing power assay of leaf extracts from *Salvia ceratophylloides* (Sc) and *Salvia officinalis* (So).

	IC_{50} , DPPH (mg/mL)	Reducing power (ASE/mL)	IC_{50} , Fe ²⁺ chelating activity (mg/mL)
Sc	0.174 ± 0.007^a	10.169 ± 0.508	0.471 ± 0.011^a
So	0.225 ± 0.007^b	9.174 ± 0.186	$> 2 \text{ mg/mL}^b$
Standard	BHT 0.07 ± 0.001	BHT 1.443 ± 0.021	EDTA 0.0067 ± 0.0003
P value	<0.001	0.06	<0.0001

Data are expressed as the mean $\pm$ SD ($n = 3$). Standards: BHT for DPPH and reducing power; EDTA for Fe²⁺ chelation. Differences between Sc and So were evaluated using Student's t-test. Different letters within the same column indicate statistically significant differences ($p < 0.05$). The corresponding p values are also reported .

Overall, these findings indicated that the *S. ceratophylloides* leaf extract possesses appreciable primary and secondary antioxidant activities under the experimental conditions adopted. These effects may be linked with the its higher content of rosmarinic acid, together with caffeic acid and its derivatives, compounds whose antioxidant properties are well documented. Consistent with this interpretation, studies on other medicinal plants have shown that variation in antioxidant performance is largely attributable to differences in the abundance of highly active phenolic acids, particularly rosmarinic acid and structurally related derivatives

(Harindranath et al., 2025). Previous investigations have shown the effectiveness of rosmarinic acid in DPPH scavenging, reducing power and chelation assays (Aldoghachi et al., 2021; Kowalczyk et al., 2024). In any case, the possible additive or synergistic effects of the different flavonoid derivatives and phenolic acids contained in the phytocomplexes that may contribute to the observed antioxidant activities should also be taken into account. For example, moderate synergism has previously been demonstrated for a combination of rosmarinic acid and luteolin-7-*O*-glucuronide in the DPPH assay (Trajčiková et al., 2020). However, the biological relevance of these antioxidant properties remains preliminary and requires further validation in cellular and in vivo models.

2.2.4. *Artemia salina* lethality bioassay

The toxicity *Sc* and *So* extracts was preliminarily evaluated using the *Artemia salina* lethality bioassay, a widely adopted screening method for assessing the toxicity of plant extracts. This assay offers several advantages, including simplicity, low cost, reproducibility, and ease of implementation, making it suitable for early-stage safety evaluation (Ntungwe et al., 2020). The results of the assay are shown in Figure 5. According to Clarkson's toxicity criterion, the *Sc* extract was classified as non-toxic to brine shrimp larvae, as the LC₅₀ value was found to be over 1000 µg/mL after 24 hours of exposure. In contrast, the *So* extract exhibited marked toxicity, with an LC₅₀ value lower than 100 µg/mL, equal to 79.27 ± 11.62 µg/mL. No mortality was observed in the control groups.

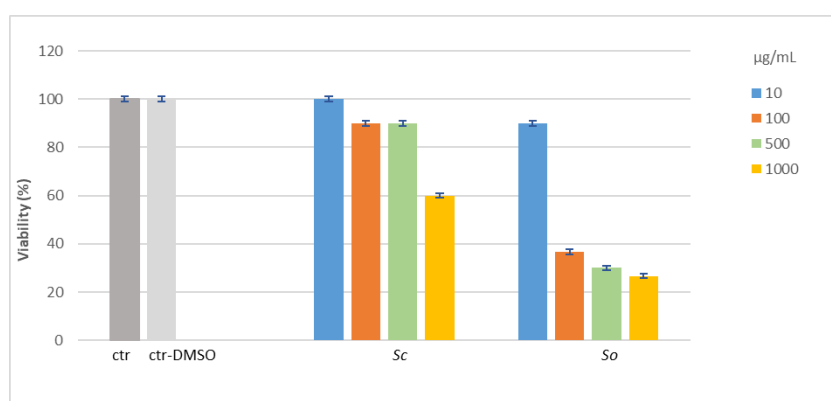


Figure 2.5: Preliminary toxicity assessment by the *Artemia salina* lethality bioassay of the hydroalcoholic extracts from leaves of *Salvia officinalis* (*So*) and *Salvia ceratophylloides* (*Sc*). The number of surviving nauplii was counted after 24 h treatment, and the vitality percentage was recorded in treated and control groups. ctr: artificial seawater; ctr-DMSO: artificial seawater added with 2% DMSO. Values are expressed as the mean $\pm$ SD of three independent experiments ($n = 3$).

The marked toxicity highlighted for *So* is an unexpected result, since some literature data report the safety of *S. officinalis* preparations. The lack of acute toxicity in rats was shown for an 80% ethanol extract obtained from leaves and flowers of *S. officinalis* by maceration (Bouteldja et al., 2023). Another recent study demonstrated the absence of acute toxicity in mice for a 70% ethanol leaf extract obtained from leaves of *S. officinalis* by 24-hour maceration (Sefah et al., 2025). Our disagreeable result could be explained by the

selected extraction procedure. Ultrasound-assisted extraction is an alternative and more efficient extraction method compared to conventional maceration, which can ensure improved recovery of bioactive components. It can be hypothesized that the use of this technique, in combination with a moderate temperature and using 70% ethanol as solvent, led to the extraction of phytoconstituents in high concentration, which may also include toxic compounds, such as the terpenes thujone and camphor (Walch et al., 2011). Interestingly, the *Sc* extract obtained with the same procedure was found to be non-toxic; this finding would seem to indicate a lower content of toxic constituents in the leaves of *S. ceratophylloides*. The different toxicity of *Sc* and *So* extracts could be clarified by further phytochemical investigations.

2.3. Conclusion

This study provides the first phytochemical characterization and preliminary biological evaluation of the rare endemic *Salvia ceratophylloides*. Despite its restricted geographical distribution, this species was found to accumulate high levels of rosmarinic acid and to exhibit notable antioxidant capacity in a set of vitro assays, together with an absence of toxicity in preliminary *Artemia salina* bioassay. In comparison, *S. officinalis* displayed lower antioxidant performance and higher toxicity under the same experimental conditions.

Taken together, these findings suggest that *S. ceratophylloides* is a promising source of bioactive phenolic compounds. However, the biological effects reported here should be regarded as preliminary, as they are based exclusively on in vitro antioxidant assays and a simple toxicity screening model. Further investigations involving cellular systems and in vivo studies are necessary to substantiate its potential relevance for nutraceutical or pharmaceutical applications. Additionally, the results highlight the importance of conserving this underexplored endemic species as part of Italy's phytochemical biodiversity (Laface et al., 2024).

Future research could also investigate the use of *S. ceratophylloides* extracts as natural antioxidant additives in biodegradable or other eco-friendly packaging materials. The phenolic compounds may enhance the functional properties of such films, although this remains a preliminary hypothesis requiring experimental validation (e.g., Koc-Bilican, 2025; Bilican, 2024).

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Chapter 3

Differential Cadmium Responses in Two Salvia Species: Implications for Tolerance and Ecotoxicity

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3. Introduction

Environmental pollution is one of the most critical global challenges, and heavy metals represent a major class of persistent and hazardous contaminants. These elements are released through industrial activities, intensive agriculture, and improper waste disposal, and their accumulation in soil and water severely affects plant physiology. Heavy metals interfere with photosynthesis, disrupt mineral nutrition, and induce oxidative stress, ultimately reducing plant growth and productivity (Goyal et al., 2020). Among them, cadmium (Cd) is particularly dangerous due to its high mobility, environmental persistence, and strong toxicity even at low concentrations (Shanmugaraj et al., 2019; Haider et al., 2021). Cd is readily absorbed by plants and disturbs cellular homeostasis by inducing reactive oxygen species (ROS) overproduction, lipid and protein oxidation, DNA damage, and alterations in antioxidant defense pathways (Hu et al., 2025). These biochemical disruptions often translate into marked changes in leaf structure, gas-exchange performance, and secondary metabolism. In non-hyperaccumulator species, tolerance to Cd often relies on exclusion and immobilization mechanisms, including preferential root retention and reduced translocation to shoots (Sterckeman & Thomine, 2020). These strategies are central to phytostabilization-based approaches, which aim to limit metal mobility and bioavailability while maintaining ecosystem functionality. Interestingly, heavy metal stress may not only damage plants but can also act as an abiotic elicitor in medicinal and aromatic species, enhancing the biosynthesis of valuable secondary metabolites (Maleki et al., 2017; Thakur & Vasudev, 2022). Several metals, including cadmium, copper and lead, have been reported to increase phenolic antioxidants or modify essential oil composition (Kunwar et al., 2015; Magray et al., 2023). In this context, phenolic compounds play a dual role by contributing to reactive oxygen species scavenging and by chelating metal ions, thereby reducing their cellular toxicity and ecological bioavailability. These responses have important implications for both phytoremediation and the valorization of plant-derived bioactive compounds. However, the magnitude and direction of these effects vary widely according to species, metal type, dose and exposure duration. Depending on these factors, heavy metals may either stimulate or suppress secondary metabolism. For instance, *Ocimum basilicum* showed shifts in essential oil composition under metal exposure, whereas *Mentha spicata* remained unaffected (Kunwar et al., 2015). Within this framework, the genus *Salvia* represents a relevant model because of its high antioxidant properties and diverse secondary metabolite profiles (Farhat et al., 2013; Aghaei Jeshvaghani et al., 2015). While *Salvia officinalis* L. is widely studied, the rare endemic *Salvia ceratophylloides* Ard. remains largely unexplored. Current research has focused mainly on drought tolerance and biotic interactions (Abate et al., 2021; Vescio et al., 2021), whereas its responses to heavy metal exposure are still unknown. Recent phytochemical data indicates high rosmarinic acid content under non-stressed conditions, suggesting strong antioxidant potential (Bekkai et al., 2026). To address this knowledge gap, this study compares *Salvia ceratophylloides* and *Salvia officinalis* under controlled cadmium exposure. Morphological traits, gas-exchange parameters, phenolic profiles, and in vitro antioxidant capacity (DPPH scavenging, reducing power, and Fe²⁺ chelation) were analyzed, together with a preliminary ecotoxicological assessment of leaf extracts using the *Artemia salina* lethality bioassay. The study aims to elucidate species-specific physiological and metabolic plasticity under Cd stress and to evaluate the ecological and

pharmacological implications of metal exposure, contributing to the mechanistic understanding of plant-based mitigation strategies and environmental risk assessment in Cd-contaminated soils.

3.1. Materials and Methods

3.1.1. Plant Material and Growth Conditions

Experiments were carried out using eight-month-old plants of *Salvia ceratophylloides* Ard. (Sc) and *Salvia officinalis* L. (So) (Supplementary Figure S3.1). Seeds of both species, supplied by the Botanical Garden of the University of Messina, were soaked in distilled water for 24 h and sown in greenhouse trays in October 2023. Approximately one month after germination, seedlings were individually transplanted into 3.5 L plastic pots containing 0.8 Kg of nutrient-enriched potting mix suitable for vegetable cultivation (Comodo, Tercomposti S.P.A., Italy), with pH 7.3 ± 0.1 , organic carbon $311 \pm 13 \text{ g kg}^{-1}$, cation exchange capacity $14.6 \pm 3.1 \text{ cmol kg}^{-1}$, and electrical conductivity $412 \pm 67 \text{ }\mu\text{S cm}^{-1}$, as reported by (Mileto et al., 2026). Plant growth took place in the greenhouse facilities of the Department of Chemical, Biological, Pharmaceutical and Environmental Sciences (ChiBioFarAm), University of Messina. The greenhouse was illuminated by LED lamps (Led Spectrum Grow 6, I-GROW s.r.l., Italy), ensuring a 16 h light/8 h dark regime and a photosynthetic photon flux density of $450 \text{ }\mu\text{mol m}^{-2} \text{ s}^{-1}$. Daytime and nighttime temperatures were maintained at approximately 24 °C and 18 °C, respectively. In February 2024, plants were transferred to an open-air terrace at the ChiBioFarAm Department. After one month of acclimation, they were divided into three experimental groups per species (Figure 3.1). The first group, used as control, was regularly irrigated with uncontaminated water. The second and third groups were irrigated with water containing cadmium (Cd) to achieve soil contamination levels corresponding to the maximum limit allowed by Italian law for agricultural land (5 mg kg^{-1} , hereafter referred to as Cd100) and twice that concentration (10 mg kg^{-1} , Cd200), respectively (Law No. 46, 22 June 2019). Specifically, plants assigned to the Cd100 treatment were irrigated with 40 mL of water containing $890 \text{ }\mu\text{mol L}^{-1} \text{ CdCl}_2$, corresponding to approximately 5 mg Cd per kg of soil, while plants in the Cd200 treatment received the same volume of water containing $1790 \text{ }\mu\text{mol L}^{-1} \text{ CdCl}_2$, equivalent to 10 mg Cd per kg of soil. To ensure homogeneous exposure, the solution was applied slowly and evenly over the surface of each pot, allowing cadmium to infiltrate naturally throughout the soil. A subset of control plants of *S. officinalis* and *S. ceratophylloides* was previously analyzed to characterize polyphenolic composition and antioxidant capacity. In the present study, these results served as reference for evaluating Cd-induced changes. Data from these control plants are provided in Supplementary Table S3.4, Supplementary Figures S3.2 and S3.3 of this study and in (Bekkai et al., 2026).

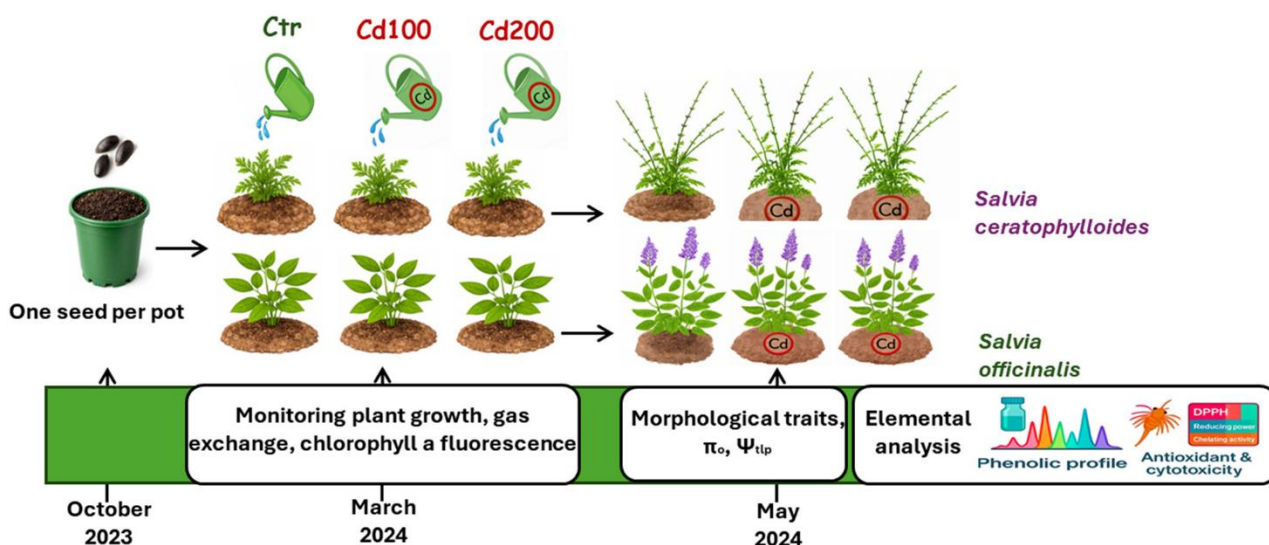


Figure 3.1: Experimental design. Seeds of *Salvia ceratophylloides* and *Salvia officinalis* were sown in October 2023. Approximately one month after germination, seedlings were individually transplanted into pots and, when they were about 4 months old (March 2024), they were divided into three experimental groups per species. The first group, used as the control, was regularly irrigated with uncontaminated water. The second and third groups were irrigated with water containing cadmium (Cd) to achieve soil contamination levels corresponding to the maximum limit allowed by Italian law for agricultural land (5 mg kg⁻¹, hereafter referred to as Cd100) and twice that concentration (Cd200), respectively. From March until the end of the experimental period, gas exchange and chlorophyll a fluorescence were measured. In May, before leaf sampling for osmotic potential at full turgor (π_o), turgor loss point (Ψ_{tip}), mineral content analysis, phenolic profiling, and leaf extract toxicity assays, morphophysiological traits were also measured.

3.1.2. Morphological Measurements

To evaluate the impact of Cd pollution on plant growth, at the end of the experimental period, once the plants had developing flowers, these samples were utilized to assess plant biomass ($n = 5$). The plants were divided into roots (gently rinsed to remove soil), stems, leaves, and flowers, and subsequently oven-dried at 80 °C for three days to determine their dry weight (DW). This dried material was used to estimate the mineral content of the organs, including Cd content (see below).

Prior to oven-drying, the leaf surface area (A_L) and thickness (T_L) of five leaves from different plants and species were measured. A_L was determined by scanning leaf images using an HP Scanjet G4050 scanner (USA) and analyzing them using Fiji (Fiji Is Just ImageJ), an open-source platform for biological-image analysis based on ImageJ. T_L was calculated as the average measurements taken at the base, middle, and tip of the leaf using a digital caliper with an accuracy of ± 0.01 mm. Leaf mass per area (LMA) was calculated as DW/A_L . Leaf density was estimated using the ratio LMA/T_L , while leaf volume was derived from the product $T_L \times LMA$.

3.1.3. Gas Exchange, Chlorophyll a Fluorescence and Water Relations Measurements

During the experimental period, the Ciras 4 Portable Photosynthesis System (Amesbury, MA, USA), set at $\text{CO}_2 = 400 \mu\text{mol m}^{-2}\text{s}^{-1}$, was used to assess the stomatal conductance to water vapor (g_L), transpiration rate (EL), and photosynthetic rate (A_n) twice a week in at least five leaves from different plants per treatment. Measurements of chlorophyll a fluorescence were performed using a portable fluorometer (Handy PEA+, Hansatech, Norfolk, UK) and F_v/F_m was measured as a proxy for the quantum yield of PSII of dark-adapted leaves (Chiofalo et al., 2025). At the end of the experimental period, the osmotic potential at full turgor (π_0) and the leaf turgor loss point (Ψ_{tlp}) were determined using a dewpoint hygrometer (Model WP4, Decagon Devices Inc., Pullman, WA, USA) on five plants per species and treatment. Leaves were collected in the early morning (7:00 a.m.) from well-watered plants and their petioles were immersed in distilled water for approximately 1 h to ensure full hydration. Leaves were then sealed in cling film, immersed in liquid nitrogen for 2 min, ground, and stored in sealed plastic containers at -20°C . Prior to measurement, samples were thawed at room temperature, and the osmotic potential of the expressed sap (π_{0_osm}) was measured with the dewpoint hygrometer.

The osmotic potential at full turgor (π_0) and the leaf water potential at the turgor loss point (Ψ_{tlp}) were calculated according to (Petrizzelli et al., 2019) using the following equations:

$$\pi_{0_fit} = 0.506 \pi_{0_osm} - 0.002 \cdot \text{LDMC}$$

$$\Psi_{\text{tlp}} = 1.313 \pi_{0_fit} - 0.032$$

where LDMC (mg g^{-1}) is the leaf dry matter content measured on five leaves different from those used for osmotic potential determination but collected from the same plants. Leaves used for LDMC determination were rehydrated for 1 h, and their turgid weight (TW) was measured using an analytical balance. Samples were then oven-dried at 80°C for 72 h to obtain the dry weight (DW), and LDMC was calculated as: DW/TW .

3.1.4. ICP-OES Mineral Element Analysis

Stem, leaf, root, and flower samples previously used for biomass measurements were also analyzed for cadmium and macro- and micronutrient content. Approximately 2 g of each sample was placed in an acid-prewashed digestion vessel, to which 7 mL of 65% HNO_3 was added. Samples were introduced into a microwave digestion system (Ethos 1; Milestone, Bergamo, Italy) and treated with a warm-up program of 20 min at 1000 W of microwave power followed by the addition of 1 mL of 30% H_2O_2 and further digested for 20 min at 1000 W. After digestion and cooling processes, samples were diluted to a final volume of 25 mL with ultrapure water.

Mineral content was measured using an Avio200 ICP-OES instrument (PerkinElmer, Waltham, MA, USA) equipped with a vertical DualView optical system and an S10 autosampler (PerkinElmer, Waltham, MA, USA). Supplementary Table S1 lists the recommended analytical wavelengths for each element, and the argon line at 420.069 nm was used as an internal standard. Operational parameters for ICP-OES are reported in Supplementary Table S2. Data acquisition and processing were performed using PerkinElmer Syngistix™ for ICP software (version 5.1 PerkinElmer, Waltham, MA, USA). Torch positioning was optimized prior to analysis using the optical optimization routine with the Mn analytical line. Quantification was performed

against external calibration curves prepared from a Certified Reference Material multielement standard solution (ISO 17034, CPAchem, Bogomilovo, Bulgaria). Ultrapure water (1.8 MΩ·cm, Milli-Q, Merck Millipore, Darmstadt, Germany) was used for solution preparation and sample dilution. Calibration curves for all the elements were generated using both calibration and reagent blanks, with correlation coefficients (r^2) exceeding 0.999. Detection limits (DLs) were determined by analyzing matrix blanks containing the same reagents and volumes as the samples and calculated as $DL=3.3 \times \sigma/S$, where σ is the standard deviation of the response and S is the slope of the calibration curve.

3.1.5. Leaf Extraction Procedure

Leaves of *S. officinalis* and *S. ceratophylloides* (control, Cd100, and Cd200) were collected in May 2024. After harvesting, the plant material was lyophilized and finely ground. The powdered leaves were first subjected to a preliminary maceration at 25 °C in 70% ethanol (1:10 w/v) for 1 h. Extraction was then carried out using 70% ethanol (1:10 w/v) in an ultrasonic bath at 50 °C for 15 min, and the procedure was repeated twice. The combined filtrates were subsequently evaporated to dryness using a rotary evaporator. The extraction yields of *S. ceratophylloides* (Sc_Ctr, Sc_Cd100, Sc_Cd200) and *S. officinalis* (So_Ctr, So_Cd100, So_Cd200) extracts, calculated with respect to 100 g of lyophilized plant material, are reported in Supplementary Table S3.5.

3.1.6. Determination of Polyphenolic Compounds by HPLC-PDA/ESI-MS

Each dried extract was re-dissolved in 70% ethanol and filtered through a 0.45 μm Acrodisc nylon membrane (Merck Life Science, Merck KGaA, Darmstadt, Germany) prior to chromatographic analysis. LC–MS grade water (H₂O), acetonitrile (ACN), formic acid, and the polyphenolic standards caffeic acid, apigenin, salvianolic acid B, luteolin-7-O-glucoside, and quercetrin were purchased from Merck Life Science (Merck KGaA, Darmstadt, Germany). For each compound, whose reference material was not available, a semi-quantitative approach was performed, considering the chemical structure and comparable absorptivity coefficients. Polyphenolic profiles of samples were determined using a Shimadzu HPLC system (Kyoto, Japan) including a CBM-20A controller, two LC-30AD dual-plunger parallel-flow pumps, a DGU-20A3R degasser, a CTO-20AC column oven, a SIL-30AC autosampler, an SPD-M30A photodiode array detector, and an LCMS-2020 mass spectrometer fitted with an electrospray ionization (ESI) source operating in negative ion mode (Shimadzu Kyoto, Japan).

Separations were performed on an Ascentis Express C18 column (150 × 2.1 mm, 2.7 μm; Merck Life Science, Merck KGaA, Darmstadt, Germany). The mobile phase consisted of water (solvent A) and acetonitrile (solvent B), both containing 0.1% formic acid (v/v). The gradient program was as follows: 0 min–0% B, 10 min–10% B, 20 min–11% B, 30 min–15% B, 50 min–18% B, and 65 min–23% B. The flow rate was set at 0.5 mL min^{−1}, and the injection volume was 2 μL. PDA detection was carried out in the range of 190–450 nm, with chromatograms monitored at 330 nm (sampling frequency: 40 Hz; time constant: 0.050 s).

Mass spectrometry parameters were as follows: scan range, m/z 100–1200; scan speed, 7500 amu s^{−1}; event time, 0.3 s; nebulizing gas (N₂) flow, 1.5 L min^{−1}; drying gas (N₂) flow, 15 L min^{−1}; interface temperature,

350 °C; heat block, 300 °C; desolvation line, 300 °C; desolvation line voltage, 1 V; and interface voltage, -4.5 kV. Quantification of polyphenolic compounds in the extracts was performed using calibration curves constructed for the five standard compounds. Data acquisition and processing were performed using Shimadzu LabSolution software (version 5.97).

3.1.7. *In Vitro* Antioxidant Assays

3.1.7.1. DPPH radical scavenging assay

The free radical scavenging activity of *S. ceratophylloides* (Sc_Ctr, Sc_Cd100, Sc_Cd200) and *S. officinalis* (So_Ctr, So_Cd100, So_Cd200) extracts was evaluated using the DPPH assay (Ohnishi et al., 1994). Sample concentrations ranged from 0.0625 to 2 mgmL⁻¹, with butylated hydroxytoluene (BHT) serving as the reference standard. Briefly, 0.5 mL of each extract solution was mixed with 3 mL of 0.1 mmol L⁻¹ DPPH methanol solution and incubated in the dark at room temperature for 20 min. Absorbance was measured at 517 nm using a UV-1601 spectrophotometer (Shimadzu, Milan, Italy). Results were expressed as the mean radical scavenging activity (%) ± standard deviation (SD) and the mean 50% inhibitory concentration (IC₅₀) ± SD.

3.1.7.2. Reducing power assay

The reducing power of the extracts was determined by the Fe³⁺ Fe²⁺ transformation method (Oyaizu, 1986). Sample concentrations ranged from 0.0625 to 2 mg mL⁻¹, with BHT and ascorbic acid as reference standards. Each assay involved mixing 1 mL of extract with 2.5 mL of 0.2 mmol L⁻¹ phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide, followed by incubation at 50 °C for 20 min. After rapid cooling, 2.5 mL of 10% trichloroacetic acid was added and the mixture centrifuged (10 min, 3000 rpm, 4 °C). The supernatant (2.5 mL) was combined with 2.5 mL of distilled water and 0.5 mL of 0.1% ferric chloride and incubated in the dark at room temperature for 10 min. Absorbance was measured at 700 nm. Results are presented as mean absorbance values ± SD and mean ascorbic acid equivalents per milliliter (ASE mL⁻¹) ± SD, based on three independent experiments.

3.1.7.3 Ferrous ion (Fe²⁺) chelating activity assay

The Fe²⁺ chelating ability of the extracts was evaluated by spectrophotometric measurement of the Fe²⁺-ferrozine complex (Kumar et al., 2008). Sample concentrations ranged from 0.0625 to 2 mg mL⁻¹, with EDTA as the reference standard. To 1 mL of extract, 0.05 mL of 2 mmol L⁻¹ ferrous chloride and 0.5 mL of methanol were added, followed by 0.2 mL of 5 mmol L⁻¹ ferrozine. The mixture was shaken vigorously and incubated in the dark at room temperature for 10 min. Absorbance was measured at 562 nm. Results are reported as the mean inhibition of the ferrozine-Fe²⁺ complex formation (%) ± SD, based on the average of three independent experiments, and as IC₅₀ ± SD

3.1.8. *Artemia Salina* Lethality Bioassay

The extract toxicity was preliminarily assessed using the brine shrimp (*Artemia salina* Leach) lethality bioassay (Meyer et al., 1982). Brine shrimp eggs were hatched in artificial seawater (33 g L⁻¹ sea salt in

deionized water) under a 60 W lamp at 24–26 °C. Twenty-four hours after hatching, groups of ten larvae were exposed for 24 h to varying concentrations of extracts dissolved in DMSO (10–1000 µg mL⁻¹) in artificial seawater. Surviving nauplii were counted, and median lethal concentration (LC₅₀) values were calculated using GraphPad Prism 10. Each assay was performed in triplicate. Extracts were classified according to Clarkson's toxicity criterion, considering non-toxic those exhibiting LC₅₀ > 1000 µg mL⁻¹ (R. Hamidi et al., 2014).

3.1.9. Statistical Analysis

Data was analyzed using the SigmaStat 12.0 statistical package (SPSS Inc., Chicago, IL, USA). A two-way ANOVA followed by a Holm–Sidak post hoc test was used to assess the effects of species (Sc and So) and cadmium treatments (Ctr, Cd100, Cd200) on biomass related traits, leaf traits and osmotic potential at full turgor and at the turgor loss point (Table 1), total mineral content (Supplementary Table S3.3) and data of the antioxidant assays (Table 2). Moreover, the same test was performed to assess the effects of organs (root, stem, leaf, flower) and cadmium treatments (Ctr, Cd100, Cd200) on cadmium accumulation (Figure 3.2) were also evaluated using a two-way ANOVA. A three-way ANOVA followed by a Holm–Sidak post hoc test was applied to test the effects of species, cadmium treatment, and exposure time on gL and An (Figure 3.3).

3.2. Results

In the control plants, cadmium concentrations in all organs were negligible, remaining below 1.5 µg g⁻¹ DW (Figure 3.2). Under cadmium-contaminated soil, the highest accumulation occurred in the roots, reaching approximately 100 µg g⁻¹ DW in Cd100 plants and over 120 µg g⁻¹ DW in Cd200 plants. At the Cd200 dose, *S. ceratophylloides* accumulated significantly more cadmium in the roots than *S. officinalis* (166.9 ± 24.7 vs. 125.0 ± 19.3 µg g⁻¹ DW, respectively). By contrast, leaf cadmium concentrations were relatively similar between species under both treatments, averaging around 10 µg g⁻¹ DW (Figure 3.1 and Table 3.1).

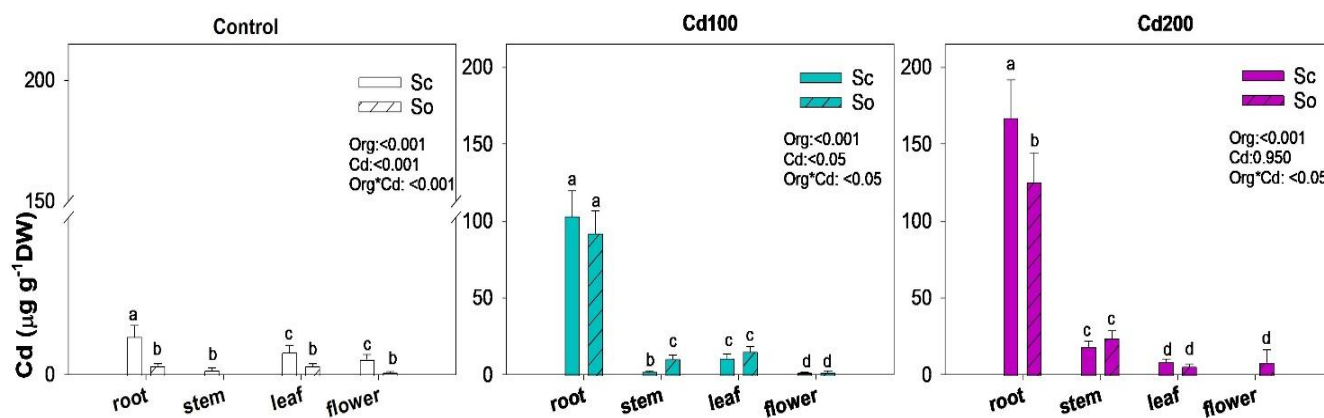


Figure 3.2: Mean ± SD (n = 3) of cadmium concentration in the root, stem, leaf and flower of *Salvia ceratophylloides* (Sc) and *Salvia officinalis* (So) plants grown in good-quality soil (Control) or in the same soil contaminated with two concentrations of cadmium (Cd100 and Cd200). Different letters indicate significant

*differences between means ($p < 0.05$) based on two-way ANOVA followed by Holm–Sidak post hoc test. Reported p values refer to the effects of organ (Org), cadmium concentration (Cd) and their interaction ($Sp \times Cd$). * denotes interaction between factors.*

Table 3.1: Mean $\pm$ SD of morpho-physiological traits measured *Salvia ceratophylloides* (Sc) and *Salvia officinalis* (So) plants grown in good-quality soil (Ctr) or in the same soil contaminated with two concentrations of cadmium (Cd100 and Cd200). Different letters indicate significant differences between means ($p < 0.05$) based on two-way ANOVA followed by Holm–Sidak post hoc test. Reported p values refer to the effects of species (Sp), cadmium concentration (Cd) and their interaction (Sp*Cd). For plant height (Hplant) and dry weight parameters (DWflower, DWleaf, DWstem, DWroot), values are based on five biological replicates ($n = 5$ plants, one plant per pot). For leaf functional and water relation traits (T_L : leaf thickness; LMA: leaf mass area; LDMC: leaf dry matter content; SWC: saturated water content; π_o : osmotic potential at full turgor; Ψ_{tlp} : leaf water potential at turgor loss point. values are based on five leaves collected from different plants ($n = 5$). * denotes interaction between factors.

	Sc_Ctr	Sc_Cd100	Sc_Cd200	So_Ctr	So_Cd100	So_Cd200	Sp	Cd	Sp*Cd
H_{plant} (cm)	66.2 $\pm$ 2.8 a	63.0 $\pm$ 14.8 a	33.3 $\pm$ 4.8 b	43.0 $\pm$ 4.2 c	36.7 $\pm$ 0.5 bc	47.0 $\pm$ 2.2 c	0.048	0.009	<0.001
DW_{flower} (g)	0.4 $\pm$ 0.1	0.6 $\pm$ 0.6	0	0.6 $\pm$ 0.1	0.72 $\pm$ 0.3	1.9 $\pm$ 1.2	0.044	0.554	0.073
DW_{leaf} (g)	3.2 $\pm$ 0.8 a	4.6 $\pm$ 0.1 a	1.6 $\pm$ 0.3 b	4.5 $\pm$ 0.8 ac	3.8 $\pm$ 0.7 ac	5.2 $\pm$ 1.4 c	0.011	0.373	0.006
DW_{stem} (g)	2.3 $\pm$ 0.5	4.0 $\pm$ 0.9	2.0 $\pm$ 0.4	5.9 $\pm$ 0.5	5.0 $\pm$ 2.3	3.3 $\pm$ 0.5	0.009	0.074	0.222
DW_{root} (g)	1.4 $\pm$ 0.2	2.0 $\pm$ 1.1	1.4 $\pm$ 0.1	1.8 $\pm$ 1.0	2.1 $\pm$ 1.1	1.9 $\pm$ 0.3	0.465	0.683	0.960
T_L (mm)	0.33 $\pm$ 0.01 a	0.39 $\pm$ 0.01 a	0.49 $\pm$ 0.02 b	0.27 $\pm$ 0.01 c	0.42 $\pm$ 0.01 a	0.69 $\pm$ 0.10 ac	0.996	<0.001	0.026
Leaf density (g cm⁻³)	0.19 $\pm$ 0.03 a	0.10 $\pm$ 0.01 b	0.07 $\pm$ 0.01 c	0.33 $\pm$ 0.08 d	0.16 $\pm$ 0.03 a	0.06 $\pm$ 0.01 c	<0.001	<0.001	0.002
LMA (g m⁻²)	64.5 $\pm$ 9.6	39.8 $\pm$ 2.9	32.5 $\pm$ 3.8	89.4 $\pm$ 20.8	67.5 $\pm$ 11.5	41.9 $\pm$ 6.1	<0.001	<0.001	0.24
LDMC (mg g⁻¹)	0.16 $\pm$ 0.02 a	0.11 $\pm$ 0.01 b	0.09 $\pm$ 0.00 b	0.23 $\pm$ 0.03 c	0.21 $\pm$ 0.03 c	0.15 $\pm$ 0.01 a	<0.001	<0.001	0.003
SWC (g g⁻¹)	5.54 $\pm$ 0.84 a	8.45 $\pm$ 0.19 b	9.04 $\pm$ 0.27 b	3.41 $\pm$ 0.53 c	3.94 $\pm$ 0.83 c	5.51 $\pm$ 0.31 a	<0.001	<0.001	<0.001
π_o (MPa)	-0.67 $\pm$ 0.04	-0.82 $\pm$ 0.08	-0.81 $\pm$ 0.03	-0.99 $\pm$ 0.04	-1.12 $\pm$ 0.03	-1.14 $\pm$ 0.04	<0.001	<0.001	0.753
Ψ_{tlp} (MPa)	-0.91 $\pm$ 0.05	-1.11 $\pm$ 0.1	-1.08 $\pm$ 0.03	-1.33 $\pm$ 0.06	-1.50 $\pm$ 0.03	-1.53 $\pm$ 0.05	<0.001	<0.001	0.596

Interestingly, cadmium was also detected in the flowers of *S. officinalis* under the Cd200 treatment (approximately $8.2 \mu\text{g g}^{-1}$ DW), while the same exposure inhibited flower production in *S. ceratophylloides*. Cadmium exposure significantly impaired the growth of *S. ceratophylloides*, particularly under the Cd200 treatment, which resulted in markedly reduced plant height, lower dry weights of leaves, and complete inhibition of flowering compared with both the control and Cd100 plants (Table 3.1). A different pattern was observed in *S. officinalis*, where cadmium exposure did not significantly alter biomass production. Leaf traits were strongly influenced by Cd in both species. Cadmium exposure resulted in thicker leaves, but with lower leaf density and reduced leaf mass per area (Table 1). LDMC values also decreased in both species, indicating that leaves became structurally less compact and more succulent under Cd stress. Water-relation traits supported this interpretation: both *S. officinalis* and *S. ceratophylloides* showed increased saturated water content when exposed to cadmium, a response that likely contributed to diluting Cd within leaf tissues (Table 3.1). Finally, cadmium exposure induced osmotic adjustments that lowered the turgor loss point (Table 3.1). The gas exchange of the two species exposed to cadmium showed a similar temporal pattern (Figure 3.3). After three weeks of exposure, cadmium in the soil caused stomatal closure and a reduction in photosynthetic rate compared with the control, regardless of metal concentration or species. From the fourth to the sixth week, both species exhibited a significant increase in gas exchange under cadmium exposure. In *S. ceratophylloides* values even approached those of the control plants. After the seventh week, however, gas exchange declined again in both species, independently of Cd concentration, with Sc_Cd200 and So_Cd200 showing the lowest values among all treatments (Figure 3.3). Conversely, Fv/Fm, used as a proxy of photosystem II efficiency, remained unchanged in both species and across all treatments (≈ 0.8).

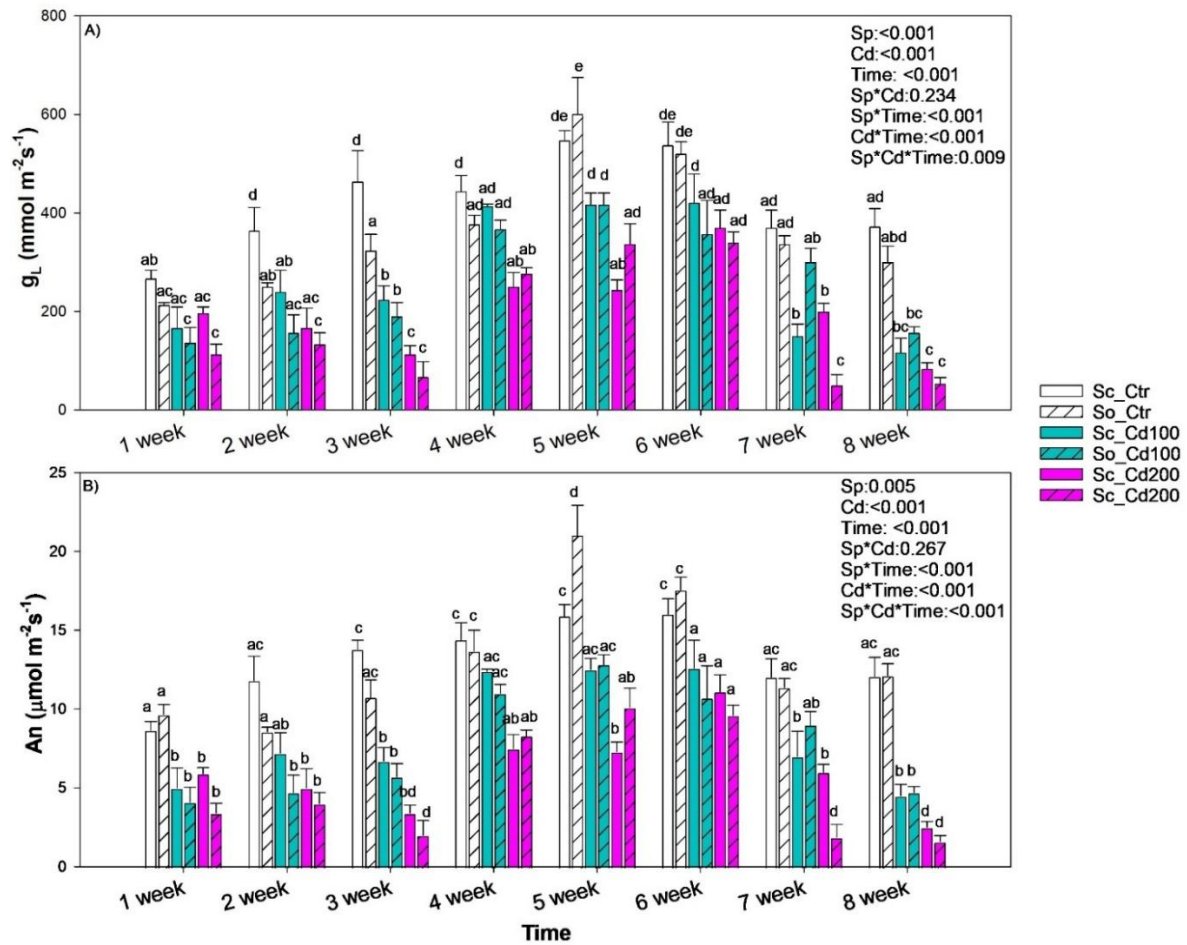


Figure 3.3: Mean $\pm$ SD of (A) stomatal conductance to water vapor (g_L) and (B) photosynthesis rate (An) recorded in *Salvia ceratophylloides* (Sc) and *Salvia officinalis* (So) plants grown in good quality soil (Ctr) or in the same soil contaminated with two concentrations of cadmium (Cd100 and Cd200). Different letters indicate significant differences between means ($p < 0.05$) based on three-way ANOVA followed by Holm–Sidak post hoc test. Reported p values refer to the effects of species (Sp), cadmium concentration (Cd), time of exposure (Time) and their interaction (Sp*Cd; Sp*Time; Cd*Time, Sp*Cd*Time). * denotes interaction between factors.

The presence of cadmium in the soil affected not only total nutrient content but also nutrient partitioning among plant organs, with *S. ceratophylloides* (Sc) and *S. officinalis* (So) showing distinct allocation strategies under heavy metal stress (Figure 3.4, Supplementary Table S3.3). In Sc, phosphorus increased under cadmium exposure, whereas iron, zinc and manganese decreased, particularly in roots and stems at the higher Cd level. In contrast, So showed an opposite trend: macronutrients P and Ca decreased under cadmium treatment. Among micronutrients, Fe, Mn increased markedly especially in the roots and under the Cd200 treatment—whereas zinc decreased in response to cadmium.

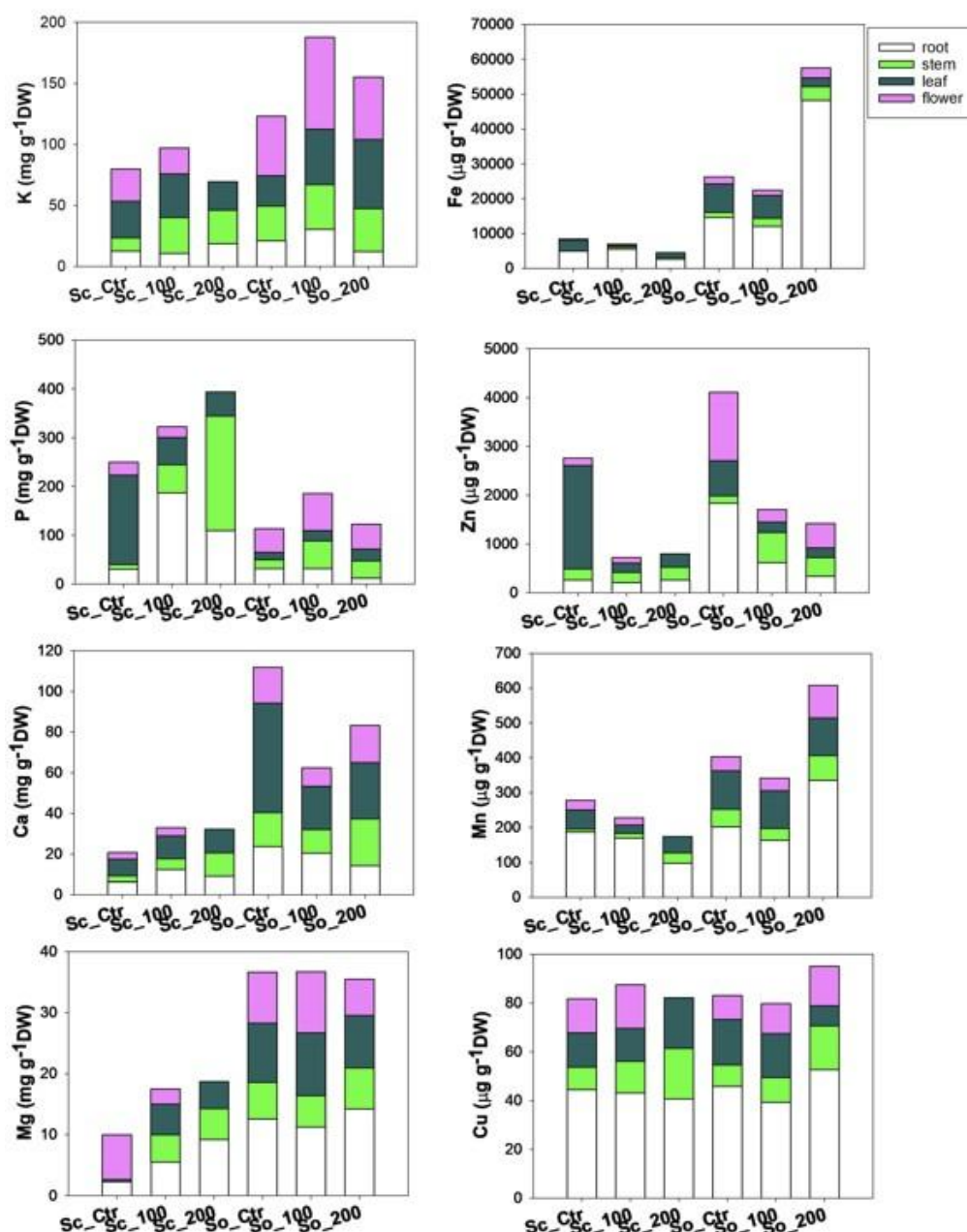


Figure 3.4: Values of concentration of macronutrients (K, P, Ca, Mg) and micronutrients (Fe, Zn, Mn, Cu) across different plant organs (root, stem, leaf, flower) measured in *Salvia ceratophylloides* (Sc) and *Salvia officinalis* (So) plants grown in good-quality soil (Ctr) or in the same soil contaminated with two concentrations of cadmium (Cd100 and Cd200).

The two *Salvia* species differed markedly in their phenolic profiles (Figure 3.5, Supplementary Table S3.4). Cadmium exposure induced distinct, compound-specific changes in the phenolic composition of both *S. officinalis* and *S. ceratophylloides*, compared with the values reported in plants grown in good quality soil by (Bekkai et al., 2026). Sc showed a concentration-dependent biphasic response in several phenolic compounds. Under Cd100, all five identified metabolites, including rosmarinic acid, caffeic acid derivatives, 1-O caffeoyl glucose, luteolin-7-O-glucuronide and quercetin glucuronides, showed a marked decline. However, at the higher cadmium concentration (Cd200), caffeic acid and caffeic acid derivative increased significantly relative

to the control. Moreover, rosmarinic acid also increased in this species under Cd200 compared with Cd100 (Figure 3.5, Supplementary Table S3.4).

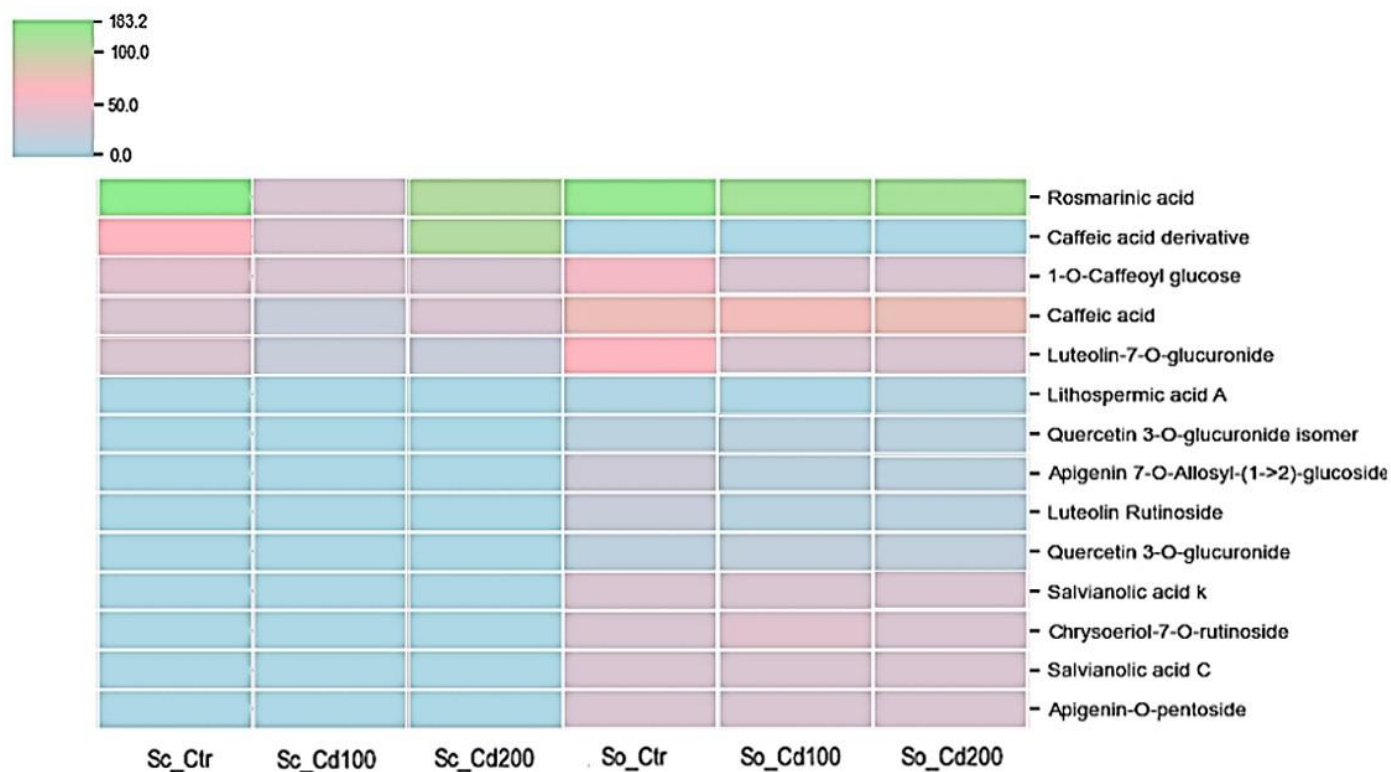


Figure 3.5: Heat map of phenolic compound variation measured in *Salvia ceratophylloides* (Sc) and *Salvia officinalis* (So) plants grown in good-quality soil (Ctr) or in the same soil contaminated with two concentrations of cadmium (Cd100 and Cd200). Concentration increases from blue to green, according to the color scale.

In *S. officinalis*, cadmium treatment caused pronounced reductions in most of the 13 quantified metabolites, with decreases ranging from approximately 20% to 70% relative to the control (Supplementary Table S3.4, Figure 3.5). The largest declines were observed for luteolin rutinoside and quercetin-3-O-glucuronide isomer. However, some metabolites, including caffeic acid, lithospermic acid, quercetin-3-O-glucuronide and apigenin O-pentoside accumulated to higher levels under cadmium stress, particularly at Cd200. Notably, lithospermic acid A decreased to 73% of the control value under Cd100 but doubled in concentration under Cd200.

The radical scavenging capacity of the extracts was evaluated by the DPPH assay (Supplementary Figure S3.2). According to (Bekkai et al., 2026), the control extracts (So_Ctr and Sc_Ctr) showed strong antioxidant activity. All the *S. ceratophylloides* extracts retained measurable radical scavenging activity, though with distinct differences across treatments. Sc_Ctr demonstrated the strongest activity, achieving ~86% inhibition already at 0.5 mg mL⁻¹, with an IC₅₀ of 0.174 ± 0.007 mg mL⁻¹ (Table 3.2). By comparison, Cd exposure caused a marked decline in activity: Sc_Cd200 maintained moderate performance (IC₅₀ = 0.345 ± 0.019 mg mL⁻¹), while Sc_Cd100 was the most affected, with the highest IC₅₀ (0.652 ± 0.035 mg mL⁻¹). For *S. officinalis* extracts, both So_Ctr and So_Cd100 exhibited comparable radical scavenging activity, reaching approximately

90% inhibition at the highest tested concentrations (0.5–2 mg mL⁻¹), a performance like the synthetic antioxidant BHT (Supplementary Figure S3.1). This trend is also reflected in their IC₅₀ values, which were nearly identical (0.225 ± 0.007 and 0.275 ± 0.005 mg mL⁻¹, respectively) (Table 3.2). In contrast, So_Cd200 showed reduced efficacy, with a higher IC₅₀ value (0.376 ± 0.024 mg mL⁻¹), indicating that cadmium stress at this concentration impaired the antioxidant potential.

Table 3.2: Mean 50% inhibitory concentration (IC₅₀) by DPPH free radical scavenging activity and by Fe²⁺-chelating activity assays, and ascorbic acid equivalents (ASE mL⁻¹) by reducing power assay of leaf extracts from *Salvia ceratophylloides* (Sc) and *Salvia officinalis* (So) grown under control (Ctr) or cadmium-contaminated soil (Cd100, Cd200). Data are expressed as mean ± SD (n = 3). Standards: BHT for DPPH and reducing power; EDTA for Fe²⁺ chelation. Different letters within the same column indicate significant differences between mean values (two ANOVA followed by Tukey's multiple comparisons test, p < 0.05). * denotes interaction between factors.

	IC ₅₀ , DPPH (mg mL ⁻¹)	Reducing Power (ASE mL ⁻¹)	IC ₅₀ , Fe ²⁺ Chelating Activity (mg mL ⁻¹)
Sc_Ctr	0.174 ± 0.007 a	10.169 ± 0.508 a	0.471 ± 0.011 a
Sc_Cd100	0.652 ± 0.035 b	24.785 ± 2.866 b	0.371 ± 0.003 b
Sc_Cd200	0.345 ± 0.019 c	4.267 ± 0.146 c	0.205 ± 0.004 c
So_Ctr	0.225 ± 0.007 d	9.174 ± 0.186 a	>2 d
So_Cd100	0.275 ± 0.005 e	25.146 ± 1.585 b	>2 d
So_Cd200	0.376 ± 0.024 c	12.536 ± 0.778 a	>2 d
Standard	BHT 0.070 ± 0.001	BHT 1.443 ± 0.021	EDTA 0.0067 ± 0.0003
Sp	<0.001	0.015	<0.001
Cd	<0.001	<0.001	<0.001
Sp*Cd	<0.001	0.004	<0.001

The reducing power capacity of the extracts was evaluated through the Fe³⁺-Fe²⁺ transformation assay (Supplementary Figure S3.3). Both species showed mild to moderate reducing activity, which increased with concentration but remained consistently lower than the BHT standard. In *S. officinalis*, cadmium exposure resulted in a clear reduction in activity, with So_Cd100 and So_Cd200 performing below the control extract across the tested range. Similarly, in *S. ceratophylloides*, activity was attenuated under stress, with Sc_Cd100 showing the weakest response, followed by Sc_Cd200. When comparing the two species, *S. ceratophylloides* demonstrated higher reducing power overall, although its sensitivity to cadmium stress was more pronounced than that of *S. officinalis*. These findings highlight a common trend of stress-induced reduction in ferric reducing capacity, with species-specific differences in the magnitude of decline.

The ferrous ion chelating activity of the extracts is shown in Supplementary Figure S3.4. In *S. ceratophylloides* growing into soil with cadmium (Sc_Cd100, Sc_Cd200), chelating activity was consistently higher respect to the plants growing in good quality soil. Extracts from heavy metal stressed leaves outperformed the control at most concentrations, with IC₅₀ values of 0.371 ± 0.003 mg mL⁻¹ (Sc_Cd100) and 0.205 ± 0.004 mg mL⁻¹ (Sc_Cd200), compared to 0.471 ± 0.011 mg mL⁻¹ for the control (Table 2). Only at the highest concentration

(2 mg mL⁻¹) the activities converge. Conversely, *S. officinalis* growing under cadmium pollution (So_Cd100, So_Cd200), a weak ferrous ion chelating activity of about 28% occurred only at 2 mg mL⁻¹. IC₅₀ values above 2 mg mL⁻¹ confirmed the limited chelating capacity of this species, further reduced under cadmium stress. Interestingly, free radical scavenging activity (measured as IC₅₀ in the DPPH assay) was tightly correlated with the total phenolic content in the leaves (Figure 3.6).

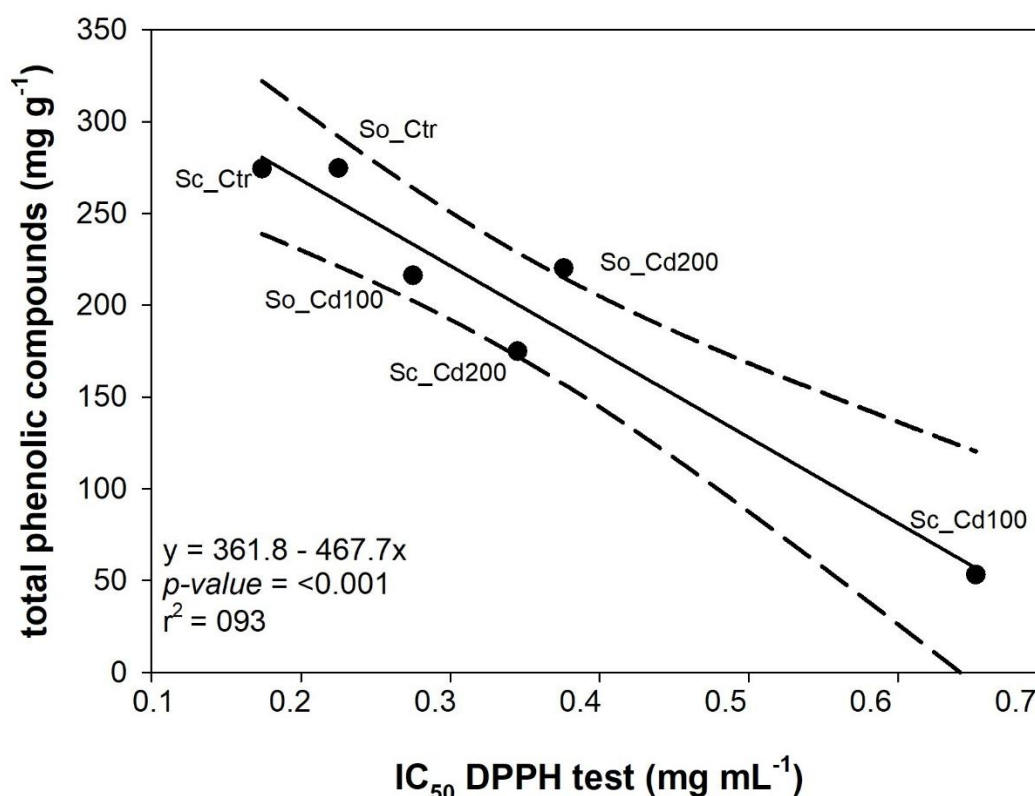


Figure 3.6: Relationship between the total amount of phenolic compounds detected by HPLC-PDA/ESI-MS analysis and the concentration of extract required to scavenge 50% of DPPH free radicals (IC₅₀ DPPH test) measured in *Salvia ceratophylloides* (Sc) and *Salvia officinalis* (So) grown under control (Ctr) or cadmium-contaminated soil (Cd100, Cd200). Linear regression and 95% confidence interval are shown by the solid line and dotted lines, respectively. The equations of the fitted linear regression and the corresponding p -value and R^2 value are reported.

The toxicity of the extracts was assessed by the *Artemia salina* leach bioassay. The obtained results of the bioassay carried out for *S. ceratophylloides* extracts (Sc_Ctr, Sc_Cd100 and Sc_Cd200) showed the absence of toxicity against brine shrimp larvae for all the extracts based on the Clarkson's toxicity criterion applied for the assessment of the degree of toxicity (LC₅₀ > 1000 µg mL⁻¹) after 24 h of exposure to the extracts (Table 3.3).

Table 3.3: *Artemia salina* lethality bioassay (median lethal concentration, LC_{50}) of leaf extracts from *Salvia ceratophylloides* (Sc) and *Salvia officinalis* (So) grown under control (Ctr) or cadmium-contaminated soil (Cd100, Cd200). Data are expressed as mean $\pm$ SD ($n = 3$).

	LC_{50} ($\mu\text{g mL}^{-1}$)
Sc_Ctr	>1000
Sc_Cd100	>1000
Sc_Cd200	>1000
So_Ctr	79.27 ± 11.61
So_Cd100	346.5 ± 0
So_Cd200	92.99 ± 9.91

On the other hand, *S. officinalis* extracts (So_Ctr, So_Cd100 and So_Cd200) showed toxicity in the following order: So_Ctr > So_Cd200 > So_Cd100, with LC_{50} values equal to 79.27 ± 11.61 , 92.99 ± 9.91 and 346.5 ± 0 $\mu\text{g mL}^{-1}$, respectively (Table 3.3).

3.3. Discussion

3.3.1. Morpho-Physiological Traits

The two *Salvia* species showed distinct morpho-physiological responses to Cd exposure. In both species, Cd accumulated predominantly in the roots, with limited translocation to aerial organs, a pattern typical of non-hyperaccumulator species and generally interpreted as a protective mechanism that restricts Cd movement toward photosynthetic and reproductive tissues (Page et al., 2006; Rizvi & Khan, 2018). *S. ceratophylloides* accumulated significantly higher Cd concentrations in the roots than *S. officinalis*, suggesting greater extraction capacity but also higher sensitivity to metal stress. This susceptibility manifested as substantial growth inhibition and complete suppression of flowering at the highest Cd concentration. In contrast, *S. officinalis*-maintained biomass across treatments, indicating a more efficient detoxification or compartmentalization strategy that mitigates Cd interference with key metabolic processes. Despite the reduction in growth and photosynthetic performance observed under Cd exposure, chlorophyll fluorescence parameters remained unaffected in both species, indicating that the photochemical efficiency of PSII was preserved at the applied Cd levels. The decline in net photosynthetic rate was therefore not associated with photochemical dysfunction but was accompanied by a reduction in CO_2 assimilation, suggesting that photosynthesis was mainly limited by restricted CO_2 diffusion rather than by damage to the photosynthetic apparatus. This pattern is consistent with a predominantly stomatal limitation of photosynthesis under moderate metal stress, where reduced CO_2 availability constrains carbon fixation while photochemical processes remain functional.

Notably, despite reduced growth, *S. ceratophylloides* exhibited no mortality even at the highest Cd level and showed increases in some phenolic compounds (see below comments), indicating that biochemical plasticity may partially compensate for morphological impairment. From an ecotoxicological perspective, the strong

root sequestration of Cd observed in both species reduces the immediate risk of metal transfer to aerial biomass and higher trophic levels; however, the contrasting growth sensitivity and metabolic responses highlight species-specific differences in stress cost and potential environmental impact.

Cd exposure produced significant structural and functional alterations in leaves. Both species developed thicker but less dense leaves with reduced leaf dry matter content (LDMC), suggesting increased succulence, a common response in plants exposed to heavy metal stress, also observed in tomato and other species (Chiofalo et al., 2025; Bekkai et al., 2024). Increased leaf thickness and succulence enlarge cellular volume, reducing the local concentration of Cd and helping shield sensitive organelles from damage. These anatomical changes were associated with modifications in water relations: both species showed higher saturated water content. Nevertheless, more negative osmotic potential and turgor loss point values were recorded under Cd stress, indicating enhanced osmotic adjustment likely driven by the accumulation of compatible solutes and/or metal-chelating molecules (Rucińska-Sobkowiak, 2016). While increased succulence may reduce intracellular Cd concentration, this structural adjustment alone does not necessarily translate into reduced ecotoxicological risk, which depends on metal speciation, compartmentalization, and the chemical nature of plant secondary metabolites. Notably, under heavy metal stress, some species increase leaf succulence, which can help dilute the concentration of potentially toxic ions in the tissues, without activating active osmotic regulation (Chiofalo et al., 2025; Bekkai et al., 2024). In contrast, more tolerant species actively accumulate compatible solutes such as proline, soluble sugars, and phenolic compounds, thereby lowering Ψ_{tlp} while maintaining cellular function under metal stress (Alia & Saradhi, 1991; Sharma & Dietz, 2006). This response allows tolerant species to cope with higher metal concentrations by combining structural (succulence) and biochemical (solute accumulation) strategies, whereas species less able to accumulate solutes rely predominantly on water uptake and tissue swelling.

Interestingly, total iron concentration decreased in Sc but increased in Cd-treated So plants. This result may initially appear inconsistent with literature reports describing Cd-induced iron deficiency (Gao et al., 2011; Zhang et al., 2023). However, these studies mainly refer to Fe deficiency in leaves. In line with this, in our study leaf Fe concentrations were indeed lower in Cd-exposed plants of both species compared to the controls. Similar responses have been reported in several plant species, in which Cd exposure reduces Fe translocation to the shoots while promoting Fe retention in the roots (Fodor et al., 2005; Mendoza-Cózatl et al., 2011). The enhanced Fe retention observed in roots under Cd exposure suggests an active restriction of long-distance Fe transport. Cadmium is known to impair Fe loading into the xylem, thereby limiting its translocation to aerial tissues (Sterckeman & Thomine, 2020; Meda et al., 2007; Mendoza-Cózatl et al., 2008). In addition, soil properties and interactions among micronutrients may have contributed to the increased Fe concentrations detected in roots. By immobilizing Fe within root tissues, plants may limit Cd co-translocation to the shoots; however, this protective strategy inevitably disrupts Fe homeostasis in aerial organs, highlighting the close physiological interplay between Cd detoxification mechanisms and Fe nutrition. Moreover, Cd exposure can modify rhizosphere chemistry, increasing the mobility and availability of Fe as well as other micronutrients such as Mn, Cu and Ni (Nagajyoti et al., 2010), which may further contribute to Fe accumulation in root tissues.

Thus, Fe retention in roots under Cd exposure represents a protective mechanism against metal translocation but simultaneously induces secondary nutritional stress in shoots, which may indirectly influence plant fitness and the ecological performance of Cd-exposed populations.

Overall, although Cd exposure induces substantial morpho-physiological alterations, particularly severe in *S. ceratophylloides*, both species activate compensatory biochemical mechanisms. These processes, explored in the next section, help explain the survival of both *Salvia* species under high Cd loads and are relevant to assessing their ecological resilience and potential applications.

3.3.2. Phenolic Metabolism and Antioxidant Responses

S. ceratophylloides showed a biphasic, hormetic response to cadmium. Moderate Cd exposure (Cd100) caused a decline in most phenolics and a temporary reduction in radical scavenging, reflecting initial stress. At higher Cd (Cd200), specific metabolites, particularly caffeic acid derivative, increased, restoring antioxidant activity and enhancing Fe²⁺ chelation, while reducing power remained moderate. These results indicate an inducible phenolic response that effectively counteracts Cd-induced oxidative pressure. In this context, the recovery of antioxidant capacity at higher Cd levels likely contributed to maintaining redox homeostasis and preventing excessive ROS accumulation at the chloroplast level, thereby preserving the functional integrity of the photosynthetic apparatus, as reflected by the stability of chlorophyll fluorescence parameters observed under Cd exposure.

These findings highlight that phenolic induction under metal stress can represent a double-edged sword, enhancing antioxidant protection while potentially generating compounds with different ecological and toxicological profiles. Toxicity bioassay confirmed that these metabolic adjustments did not produce harmful compounds, highlighting functional resilience despite stress. In contrast, *S. officinalis* showed a more conservative response: several phenolics decreased, but key metabolites such as rosmarinic acid, lithospermic acid A, apigenin-O-pentoside, and quercetin-3-O-glucuronide remained relatively abundant, preserving moderate antioxidant capacity. Radical scavenging declined slightly, and Fe²⁺ chelation remained limited, indicating less inducible plasticity. These results are consistent with previous studies in *Salvia sclarea* and other species, where Cd exposure increased non-enzymatic antioxidants, including total phenolics, anthocyanins, and carotenoids, enhancing ROS detoxification and stress tolerance (Kapoor et al., 2019; Dobrikova et al., 2021). Phenolic upregulation is linked to the phenylpropanoid pathway and increased PAL activity, often supporting enzymatic antioxidant systems (Sharma et al., 2019; Sytar et al., 2013). High phenolic and anthocyanin production under metal stress is also considered advantageous for phytoremediation and phytostabilization (Vidal et al., 2020).

The *A. salina* bioassay highlighted ecotoxicological relevance: although the *A. salina* assay represents a simplified screening tool, it provides an integrative indication of the biological effects of complex plant extracts, complementing chemical and physiological analyses. *S. ceratophylloides* maintained antioxidant defenses without producing toxic metabolites, indicating environmental safety, whereas *S. officinalis* generated moderately toxic compounds despite preserving antioxidants.

In summary, *S. ceratophylloides* relieve dynamic, inducible metabolic adjustments, conferring Cd tolerance and low ecotoxicological risk, making it a promising candidate for phytoremediation and safe cultivation on contaminated soils. *S. officinalis* employs a less flexible strategy, preserving essential defenses but with potential environmental implications. These species-specific responses underline the importance of linking plant stress physiology with ecotoxicological outcomes in evaluating plants for environmental remediation (Kapoor et al., 2019; Dobrikova et al., 2021). Collectively, these results show that metal-induced shifts in phenolic metabolism underline both the resilience of *Salvia* species to Cd stress and the potential ecological impact of their bioactive compounds.

3.4. Conclusions

Salvia ceratophylloides displays a dynamic and inducible phenolic response under cadmium stress, enhancing antioxidant capacity and metal-chelating potential while remaining non-toxic to *Artemia salina* larvae. These traits support its suitability for phytostabilization and for the environmentally safe cultivation of medicinal plants on Cd-contaminated soils. By contrast, *S. officinalis* adopts a more conservative metabolic strategy, maintaining key antioxidant compounds; however, control leaf extracts exhibited the highest ecotoxicity among treatments. This suggests that the production of potentially ecotoxic metabolites may represent a species-specific trait rather than a Cd-induced response. Overall, these findings highlight the importance of integrating plant stress physiology with ecotoxicological assessment when evaluating the environmental safety of medicinal plant cultivation.

3.5. Supplementary Materials

Supplementary Table S3.1: Analytical lines length (nm) used for analysis.

Element	nm	Element	nm	Element	nm
Cd	228,802	K	766.490	P	213,617
Cu	327.393	Mg	285.592	Zn	213.857
Fe	238.204	Mn	257.610	Ca	317.933

Supplementary Table S3.2: Operational conditions for ICP-OES analysis

Parameter	Conditions
Radiofrequency power (W)	1500
Plasma gas flow (L/min)	9
Auxiliary gas (L/min)	0.2
Nebulizer gas (L/min)	0.7
Sample uptake (mL/min)	1

Supplementary Table S3.3: Mean values $\pm$ SD ($n=3$) of macronutrients (K, P, Ca, Mg) and micronutrients (Fe, Zn, Mn, Cu) concentration measured in *Salvia ceratophylloides* (Sc) and *Salvia officinalis* (So) plants grown in good-quality soil (Ctr) or in the same soil contaminated with two concentrations of cadmium (Cd100 and Cd200). Different letters indicate significant differences between means ($p < 0.05$) based on two-way ANOVA followed by Holm-Sidak post-hoc test. Reported p values refer to the effects of species (Sp), cadmium concentration (Cd) and their interaction (Sp* Cd). * denotes interactions between factors.

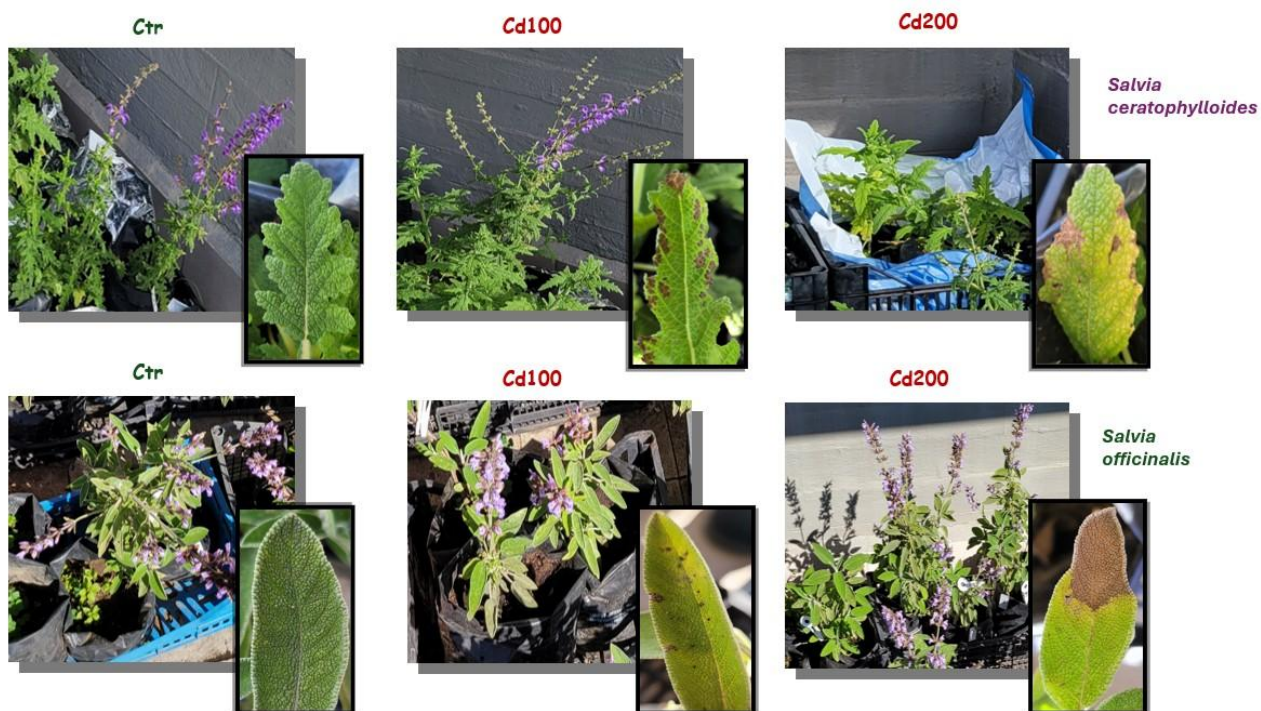
	K (mg g DW ⁻¹)	P (mg g DW ⁻¹)	Ca (mg g DW ⁻¹)	Mg (mg g DW ⁻¹)	Fe (mg g DW ⁻¹)	Zn (mg g DW ⁻¹)	Mn (mg g DW ⁻¹)	Cu (mg g DW ⁻¹)
Sc Ctr	79.7 $\pm$ 13.1	224.5 $\pm$ 32.8a	20.7 $\pm$ 6.2a	10.1 $\pm$ 2.5	8442.2 $\pm$ 794.6a	2759.9 $\pm$ 305.7a	278.1 $\pm$ 44.2a	81.7 $\pm$ 16.1
Sc 100Cd	97.1 $\pm$ 15.6	379.7 $\pm$ 60.2b	32.9 $\pm$ 9.1a	17.5 $\pm$ 4.5	6950.4 $\pm$ 665.5b	721.0 $\pm$ 105.2b	228.5 $\pm$ 37.1ab	87.5 $\pm$ 17.2
Sc 200Cd	69.3 $\pm$ 11.1	394.1 $\pm$ 59.1b	32.2 $\pm$ 8.8a	18.7 $\pm$ 4.5	4533.5 $\pm$ 472.3c	796.4 $\pm$ 109.9b	174.8 $\pm$ 29.9b	82.1 $\pm$ 15.9
So Ctr	123.0 $\pm$ 19.0	119.7 $\pm$ 22.6 c	111.9 $\pm$ 21.3b	36.5 $\pm$ 8.3	26321.5 $\pm$ 1942.2d	4104.4 $\pm$ 448.3c	403.3 $\pm$ 62.9c	83.0 $\pm$ 16.3
So 100Cd	187.7 $\pm$ 27.3	218.3 $\pm$ 37.4d	62.4 $\pm$ 13.1 c	36.7 $\pm$ 8.4	22449.7 $\pm$ 1888.1e	1706.5 $\pm$ 216.4d	342.1 $\pm$ 54.6c	79.7 $\pm$ 15.9
So 200Cd	155.1 $\pm$ 23.0	92.7 $\pm$ 18.3e	83.4 $\pm$ 16.8c	35.4 $\pm$ 8.0	417516.7 $\pm$ 19573.3f	1417.6 $\pm$ 186.2d	607.5 $\pm$ 88.8d	95.1 $\pm$ 18.3
Sp	<0.01	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.810
Cd	0.071	<0.001	0.659	0.099	<0.001	<0.001	0.153	0.810
Sp*Cd	0.290	<0.001	0.01	0.08	<0.001	0.026	<0.001	0.590

Supplementary Table S3.4: Concentration of phenolic compounds in leaf extracts of *Salvia ceratophylloides* (Sc) and *Salvia officinalis* (So) grown in soil contaminated with two cadmium concentrations (Cd100 and Cd200). Data are reported as absolute concentration values and as percentage changes relative to the levels observed in plants grown in uncontaminated soil.

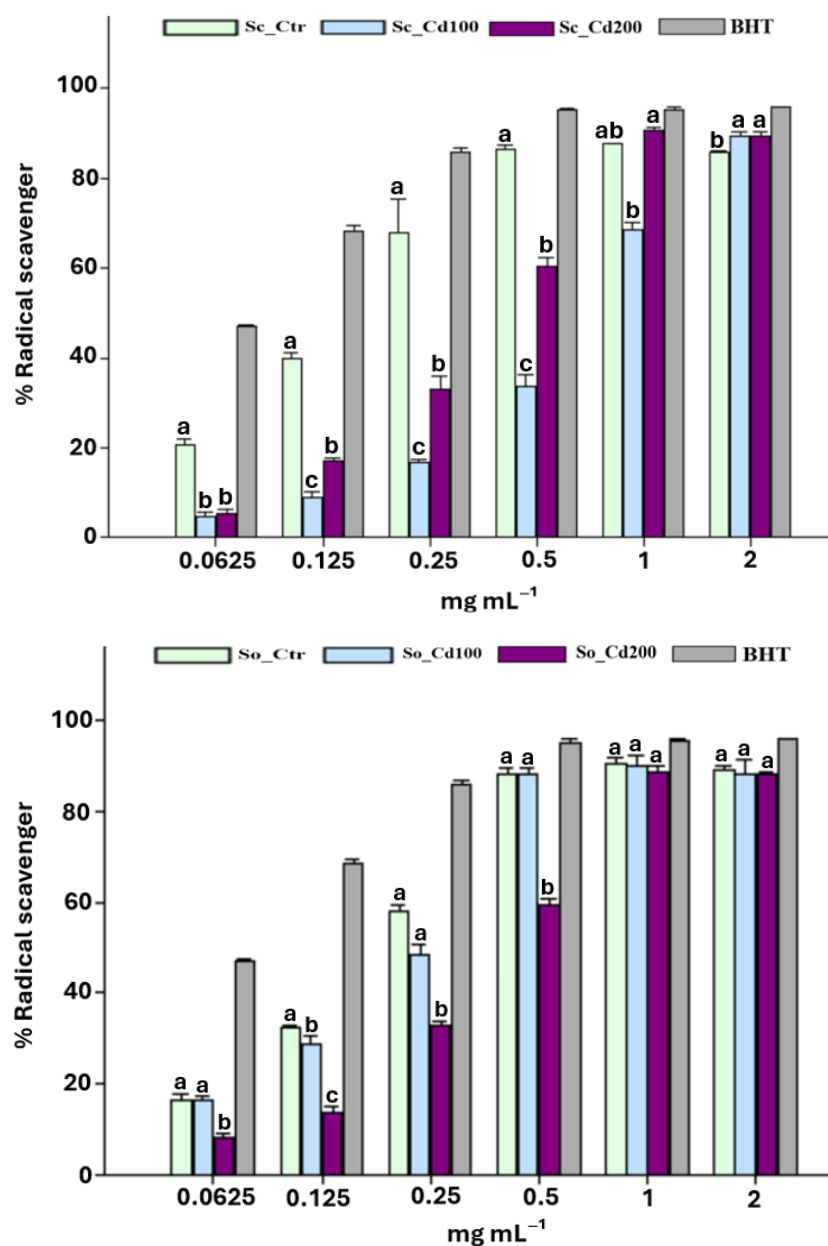
Compound	Employed Standard for quantification	Sc (mg g ⁻¹ DW)	Sc_Cd100 (mg g ⁻¹ DW)	DSc_Cd100 (%)	Sc_Cd200 (mg g ⁻¹ DW)	DSc_Cd200 (%)	So (mg g ⁻¹ DW)	So_Cd100 (mg g ⁻¹ DW)	DSo_Cd100 (%)	So_Cd200 (mg g ⁻¹ DW)	DSo_Cd200 (%)
Caffeic acid derivative	Caffeic acid	58.99	16.08	27.2	77.40	131.2	-	0	0	0.0	0
1-O-Caffeoyl glucose	Caffeic acid	4.29	3.14	73.2	2.84	66.2	8.49	7.48	88.1	7	82.4
Caffeic acid	Caffeic acid	11.02	6.30	57.2	12.30	111.6	19.08	17.51	91.8	21.04	110.3
Luteolin Rutinoside	Luteolin-7-O-glucoside	-	-	-	-	-	1.42	0.47	33.1	0.61	42.9
Lithospermic acid A	Caffeic acid	-	-	-	-	-	0.19	0.14	73.7	0.39	205.3
Quercetin 3-O-glucuronide	Quercitrin	-	-	-	-	-	0.75	0.95	126.7	1.01	134.7
Apigenin 7-O-Allosyl-(1->2)-glucoside	Apigenin	-	-	-	-	-	1.80	1.28	71.1	1,2	66.7
Luteolin-7-O-glucuronide	Luteolin-7-O-glucoside	16.79	5.22	31.1	3.76	22.5	53.40	42.78	80.1	43.76	81.9
Quercetin 3-O-glucuronide isomer	Quercitrin	-	-	-	-	-	0.63	0.19	30.2	0.34	53.9
Salvianolic acid C	Salvianolic acid B	-	-	-	-	-	12.46	9.1	73.0	9.4	75.4
Chrysoeriol-7-O-rutinoside	Luteolin-7-O-glucoside	-	-	-	-	-	5.99	4.01	66.9	3.18	53.1
Apigenin-O-pentoside	Apigenin	-	-	-	-	-	7.22	7.68	106.4	9.59	132.8
Rosmarinic acid	Caffeic acid	183.18	22.42	12.2	78.57	42.9	142.00	105.42	74.2	104.13	73.3
Salvianolic acid K	Salvianolic acid B	-	-	-	-	-	21.16	19.15	90.5	18.48	87.3

Supplementary Table S3.5: Percentage yields of *S. ceratophylloides* (Sc) and *S. officinalis* (So) leaf extracts. Plants were grown either in good-quality soil (Sc_Ctr and So_Ctr) or in soil contaminated with cadmium at two different concentrations (Sc_Cd100, Sc_Cd200 and So_Cd100, So_Cd200). For further details, see the main text.

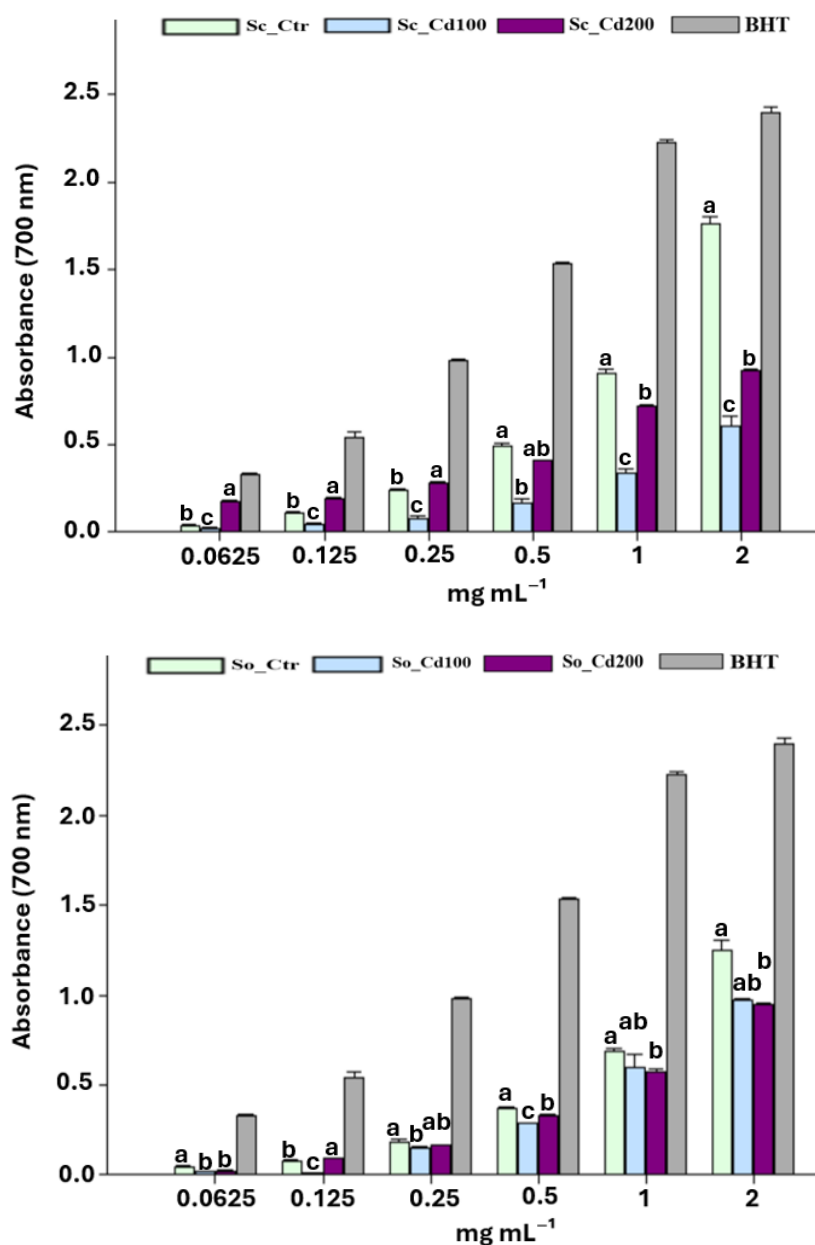
	Yield (w/w) %
Sc_Ctr	42
Sc_Cd100	30
Sc_Cd200	31
So_Ctr	23
So_Cd100	27
So_Cd200	30



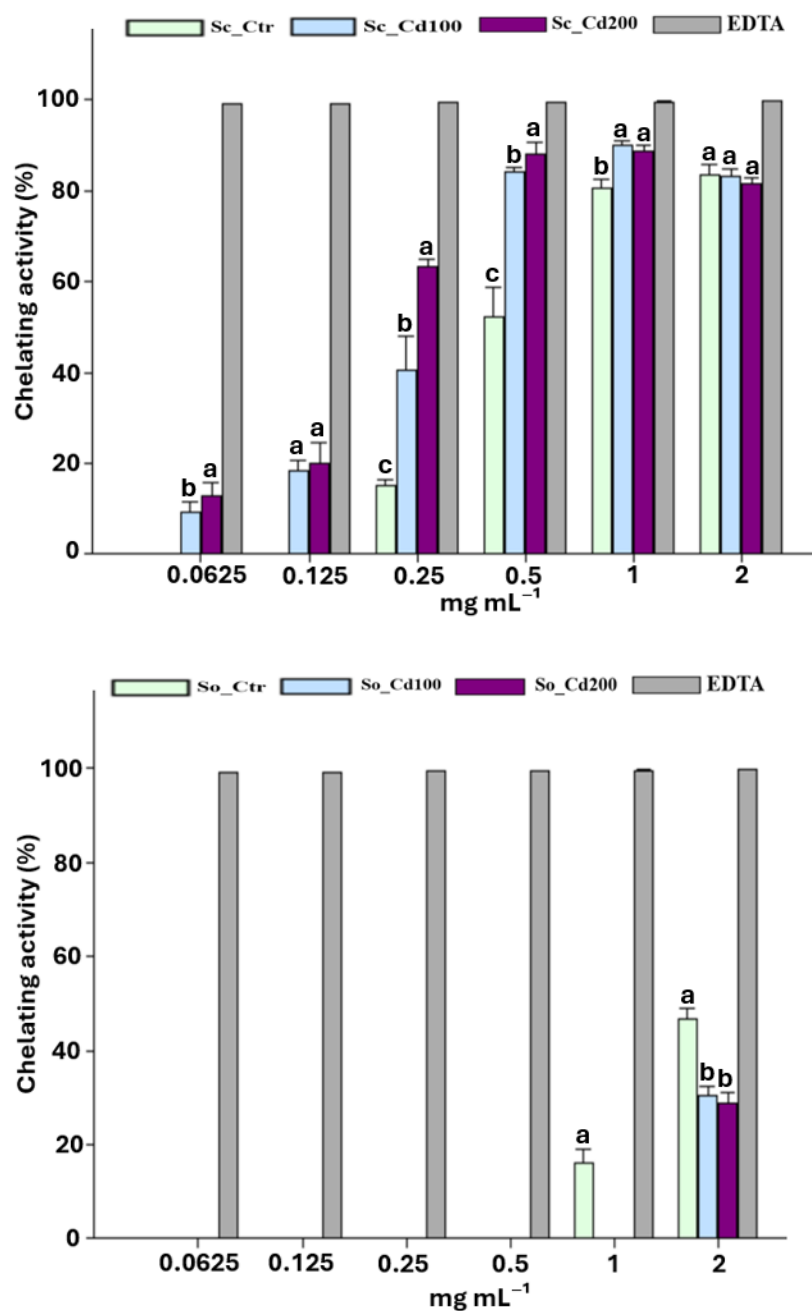
Supplementary Figure S3.1: Photographs of *Salvia ceratophylloides* and *Salvia officinalis* and details of their leaves recorded at the end of the experimental period. C: plants grown in uncontaminated soil. Cd100 and Cd200: plants grown under two cadmium contamination levels (for details, see Materials and Methods).



Supplementary Figure S3.2: DPPH radical scavenging activity of hydroalcoholic leaf extracts from *Salvia ceratophylloides* (Sc) and *Salvia officinalis* (So) under control, Cd100, and Cd200 treatments. Data are expressed as mean $\pm$ SD ($n = 3$). Different letters indicate significant differences among cadmium treatments within the same concentration according to one-way ANOVA followed by Holm-Sidak multiple comparison test ($P < 0.05$). BHT was used as a reference standard and was excluded from the statistical analysis.



Supplementary Figure S3.3: Reducing power activity, expressed as absorbance at 700 nm of hydroalcoholic leaf extracts from *Salvia ceratophylloides* (Sc) and *Salvia officinalis* (So) under control, Cd100, and Cd200 treatments. Data are expressed as mean $\pm$ SD ($n = 3$). Different letters indicate significant differences among cadmium treatments within the same concentration according to one-way ANOVA followed by Holm-Sidak multiple comparison test ($P < 0.05$). BHT was used as a reference standard and was excluded from the statistical analysis.



Supplementary Figure S3.4: Fe^{2+} -chelating activity of hydroalcoholic leaf extracts from *Salvia ceratophylloides* (Sc) and *Salvia officinalis* (So) under control, Cd100, and Cd200 treatments. Data are expressed as mean $\pm$ SD ($n = 3$). Different letters indicate significant differences among cadmium treatments within the same concentration according to one-way ANOVA followed by Holm-Sidak multiple comparison test ($P < 0.05$). EDTA was used as a reference standard and was excluded from the statistical analysis.

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Chapter 4

Study 4

Alleviating cadmium stress in spinach by iron oxide nanofertilizers

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4. Introduction

Over the past decades, the mitigation of cadmium (Cd) stress in plants has become increasingly important. Cadmium is a non-biodegradable and persistent heavy metal that can cause acute and chronic health disorder in humans and induce irreversible damage to natural ecosystems, even at low concentration. Several studies have identified conventional mineral fertilizers as a main source of Cd contamination in agricultural soils. In addition, intensive cropping systems contribute to multiple long-term cascading effects, including soil degradation and loss of organic matter (Marini et al., 2020).

Due to its high-water solubility, mobility, and toxicity, Cd is absorbed by plant roots, resulting in pronounced alterations in plant morphology and physiology. Cd exposure disrupts essential metabolic pathways, limits nutrient uptake, and inhibits seed germination and root elongation, ultimately leading to reduced biomass production. In addition, Cd triggers oxidative stress by promoting the excessive accumulation of reactive oxygen species (ROS), including hydrogen peroxide and superoxide anions, which cause oxidative damage to lipids, proteins, and nucleic acids. Moreover, cadmium-induced oxidative stress and nutrient imbalance further exacerbate plant growth inhibition. In particular, Cd interferes with iron (Fe) homeostasis not only by inhibiting Fe uptake and translocation but also by exploiting Fe transport systems to enter plant cells. This competition for Fe transporters limits Fe availability in chloroplasts, where Fe-dependent proteins such as ferredoxin, an essential electron carrier in photosystem I, are synthesized. Consequently, chlorophyll biosynthesis and photosynthetic efficiency are impaired, ultimately restricting plant growth and development (Sheikh et al., 2025).

In this context, nanotechnology-based approaches have emerged as promising strategies to mitigate heavy metal stress in plants. Many studies have demonstrated the positive impact of nanoparticles (NPs) due to their nanoscale size and large specific surface area. NPs can alleviate heavy metal by decreasing metal bioavailability and enhancing plant defense mechanisms against metal stress (Soni et al., 2024). Among them, iron oxide nanoparticles have attracted considerable attention as effective sources of bioavailable iron, capable of counteracting Cd uptake (Manzoor et al., 2021) and supporting photosynthetic processes. In addition, iron oxide NPs play key roles in enzyme activity and RNA synthesis. Iron oxide NPs, including Fe₂O₃- based formulation, have been successfully applied to reduce Cd accumulation in crops such as rice and muskmelon (Ghouri et al., 2025; Zou et al., 2023). However, the development of stable colloidal NP suspensions suitable for plant application remains a major challenge. For this purpose, maghemite (γ -Fe₂O₃) NPs represent particularly promising iron-based candidates, as their high dispersibility in aqueous media enhances their availability for plant uptake (Abu Noqta et al., 2019). Moreover, surface functionalization of iron oxide NPs surface can further improve colloidal stability and increase iron concentration in aqueous solutions. Several coating techniques have been reported including one-step and multistep coatings (Laurent et al., 2008). The first technique involves the NPs coating during their synthesis, whereas the multi-step approach involves the attachment of stabilizing molecules onto the iron oxide NPs surface after their synthesis (Kim et al., 2007; Gogoi et al., 2017). Citrate is a commonly used stabilizing and capping agent for metal oxide NPs. Its

negatively charged chemical structure facilitates anchoring onto Fe_2O_3 or Fe_3O_4 NPs, enhancing particles dispersion colloidal stability (Soares et al., 2014; Zhang and Wu., 2010).

Citrate is widely employed due to the presence of hydroxyl (-OH) and carboxylate (-COOH) groups (Szekeres et al., 2013), which adsorb onto the NP surface and prevent agglomeration through repulsive interactions between charged ions (Hui et al., 2008). Moreover, citrate is non-toxic, biodegradable, highly soluble in several solvents, hydrophilic, and biocompatible (Ramírez-Ceja et al., 2016; Sadeghi and Ziamajidi, 2007; Henintsoa et al., 2017). Notably, only one study has reported a synergistic effect of the combination of Fe_2O_3 NPs and citric acid in reducing arsenic stress in soybean (Akhter Bhat et al., 2022), suggesting potential for broader applications in mitigating heavy metal stress.

To evaluate functional potential of trisodium citrate dihydrate (TCD)-stabilized $\gamma\text{-Fe}_2\text{O}_3$ nanoparticles under environmentally and agronomically realistic conditions, we performed a foliar application on spinach plants grown in either optimal soil or cadmium-contaminated soil. Cadmium was supplied at two concentrations: (i) the maximum level permitted by Italian legislation for agricultural soils (Italian Law No. 46, June 22, 2019), representing a realistic regulatory scenario, and (ii) a concentration corresponding to twice this threshold. While the former ensured agronomic relevance, the latter served as a stress-amplification level to assess dose-dependent responses and define the operational limits of the nanofertilizer under increasing metal pressure.

To unequivocally attribute the observed responses to the nanofertilizer itself, the experimental design included two additional spray controls: a citrate-only treatment and a distilled water-only treatment. Citrate control accounted for potential physiological effects of the stabilizing agent, whereas the water control isolated responses related solely to the foliar application procedure. This design allowed us to specifically ascribe the biological effects to the iron–citrate nanoformulation.

4.1. Material and Methods

As part of a collaboration with the CNR group (Catania), citrate-stabilized maghemite iron oxide nanoparticles and the corresponding citrate formulation were prepared and characterized using standard physicochemical approaches, including colloidal stabilization and purification to remove excess citrate and non-functionalized material, followed by verification of particle size and morphology and quantification of Fe concentration using techniques such as dynamic light scattering, electron microscopy, and ICP-based analysis.

4.1.1. Foliar application of TCD-stabilized $\gamma\text{-Fe}_2\text{O}_3$ nanoparticles in spinach plants

The experimental trial began in October 2024 and involved 84 *Spinacia oleracea* L. plants cultivated in a greenhouse at the Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina (Italy). Three-week-old seedlings were purchased from a local supplier and transplanted into 2.3-L pots containing approximately 1 kg of substrate.

Plants were allocated to three soil treatments: uncontaminated soil (control, C) and soil experimentally spiked with cadmium at two concentrations corresponding to the maximum level permitted by Italian legislation for agricultural soils (Cd100, 5 mg kg⁻¹) and twice this level (Cd200, 10 mg kg⁻¹). A commercial nutrient-enriched

growth medium for vegetable cultivation (Comodo, Ter Composti S.P.A., Italy) was used. Cadmium was incorporated into the substrate prior to transplanting and thoroughly homogenized.

Within each soil treatment, plants were further assigned to four foliar regimes: a no-spray group, trisodium citrate dihydrate (TCD)-stabilized γ -Fe₂O₃ nanoparticles (FeCi), a citrate solution (Ci) matching the TCD concentration present in the nanoparticle formulation, and distilled water (DW) (Fig. 4.1). The inclusion of both citrate-only and distilled water controls allowed us to distinguish the specific effects of the nanoformulation from those attributable to the stabilizing agent or to the foliar spraying procedure itself, with DW controlling for mechanical and hydration-related effects.



Figure 4.1: Experimental design of the spinach trial. *Spinacia oleracea* L. plants were transplanted into pots containing either uncontaminated soil (C) or soil artificially contaminated with cadmium at two levels, corresponding to the Italian agricultural-soil threshold for Cd contamination (Cd100) and twice this threshold (Cd200). Within each soil condition, plants were divided into four foliar treatment groups: no foliar application, TCD-stabilized γ -Fe₂O₃ nanoparticles (FeCi), citrate solution (Ci), and distilled water (DW). Overall, the experiment consisted of 12 treatment combinations, each comprising seven plants. Foliar treatments began approximately three weeks after transplanting and were applied weekly for seven consecutive weeks.

In summary, the experiment consisted of 12 treatment groups, each comprising seven plants, resulting from the combination of three cadmium soil levels (control, Cd100, Cd200) and four foliar treatments (γ -stabilized γ -Fe₂O₃ nanoparticles NPs, citrate solution, distilled water, and no foliar application). Foliar treatments began approximately three weeks after transplanting and were applied weekly for seven consecutive weeks.

4.1.2. Gas exchange and water relations measurements

During the experimental period, stomatal conductance to water vapour (g_L) and photosynthesis rate (A_n) were monitored two days after spray treatments once per week at midday on five different plants per experimental

group using the Ciras 4 Portable Photosynthesis System (PPsystems.com), with CO₂ concentration set at 400 $\mu\text{mol mol}^{-1}$ and measurements performed under ambient greenhouse light conditions. The osmotic potential at full turgor (π_0) and the turgor loss point (Ψ_{tlp}) were assessed using a dewpoint hygrometer (WP4C series, Decagon Devices Inc., USA) on leaves. Samples were harvested maintained with their petiole immersed in distilled water for about 1 h to ensure full hydration but avoid over hydration. Then, leaf material was rapidly frozen in liquid nitrogen, homogenized, and stored at low temperature until measurement (Petrizzellis et al., 2019). Before instrument readings, samples were allowed to equilibrate at room temperature, and their osmotic potential (π_{0_osm}) was then determined with the dewpoint hygrometer.

The values of π_0 and Ψ_{tlp} were calculated following the empirical relationships proposed by Petrizzellis et al. (2019):

$$\pi_0 = 0.506 \pi_{0_osm} - 0.002 \text{ LDMC}$$

$$\Psi_{\text{tlp}} = 1.313 \pi_0 - 0.032$$

where LDMC is the leaf dry matter content and was determined using a different set of leaves collected from the same plants used to estimate the turgor loss point. For each leaf, the mass at full hydration (TW) and the dry mass (DW), obtained after oven-drying for 72 h at 80 °C, were used to calculate LDMC as DW/TW and the saturated water content (SWC) as (TW-DW)/TW. The same leaves were also used to determine the leaf mass per area (LMA), computed as DW/AL where AL is the leaf surface area and was measured immediately after recording TW by scanning each leaf (HP Scanjet G4050, USA) and analysing the images with ImageJ software (<http://imagej.nih.gov/ij/>). Moreover, at the end of the experimental period, three plants per treatment were dried in an oven at 80 °C for three days to determine the dry weight (DW).

4.1.3. Minimum cuticular conductance

To evaluate residual water loss through the cuticle and to assess potential cadmium-induced stress effects as well as the protective role of foliar $\gamma\text{-Fe}_2\text{O}_3$ nanofertilizer on leaf water retention, minimum leaf conductance to water vapor (g_{min}) was measured during the seventh week of spray treatments. g_{min} was determined using a gravimetric method based on minimum transpiration rates (John et al., 2018). Measurements were conducted on five fully expanded leaves per treatment. Leaves were initially rehydrated for at least 8 h, after which their area and fresh mass at full turgor were determined. Leaves were then suspended by their petioles in front of a fan for a minimum of 2 h to ensure complete stomatal closure. Leaf mass was recorded every 15 min during dehydration to quantify minimum transpiration. Ambient temperature and relative humidity were continuously monitored at 30 s intervals using a thermohygrometer (Velleman, Gavere, Belgium). At the end of the measurements, leaf area was re-measured, and leaves were oven-dried at 70 °C for 72 h to determine dry weight. Minimum cuticular conductance was calculated by normalizing the minimum transpiration rate to the leaf-to-air water vapor mole fraction deficit and to twice the mean leaf area.

4.1.4. Measuring leaf cadmium accumulation

In order to evaluate if nanofertilizer application affected the leaf cadmium accumulation, at the end of experimental period, 0.5 g of dried leaf per experimental group were digested in acid-washed vessels with 7 mL of 65% HNO₃ under controlled heating, followed by the addition of 1 mL of 30% H₂O₂ in a closed-vessel system. Digested samples were diluted to 25 mL with ultrapure water.

Cadmium concentration was determined by ICP-OES using an Avio200 instrument (PerkinElmer, Waltham, MA, USA) equipped with a DualView optical system. Quantification was performed at 228.8 nm, using the Ar line at 420.1 nm as an internal standard. External calibration was carried out using a certified reference standard (ISO 17034; CPAchem, Bulgaria), yielding a correlation coefficient (r^2) > 0.999. The method detection limit was determined using a matrix blank.

4.2. Results

Bare γ -Fe₂O₃ nanoparticles were not stable in water (pH 6.5) and rapidly precipitated; therefore, a citrate coating was used to obtain a stable aqueous colloidal suspension suitable for foliar application. The citrate-stabilized dispersion showed a monomodal hydrodynamic diameter centered at 60 ± 1 nm with a polydispersity index of 0.21 ± 0.01 (Figure 4.2a), a negative ζ -potential of approximately -33 mV, and an iron concentration of about 60 ppm. Stability monitoring over the application period, up to eight weeks, indicated no relevant changes in iron concentration or ζ -potential, confirming formulation stability during storage and use.

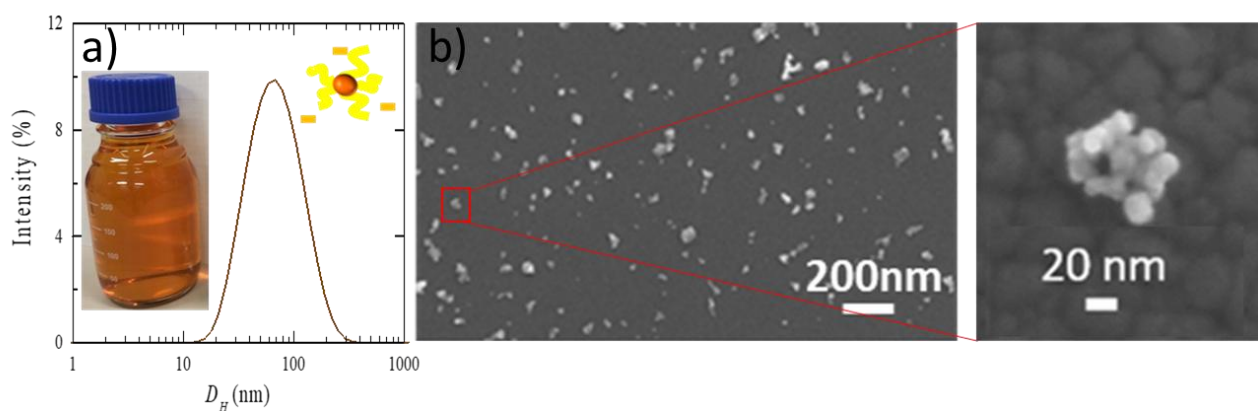


Figure 4.2: Physicochemical characterization of citrate-stabilized γ -Fe₂O₃ nanoparticles. a) Hydrodynamic diameter distribution of the colloidal suspension measured by dynamic light scattering. b) Electron microscopy images showing nanoparticle morphology and size.

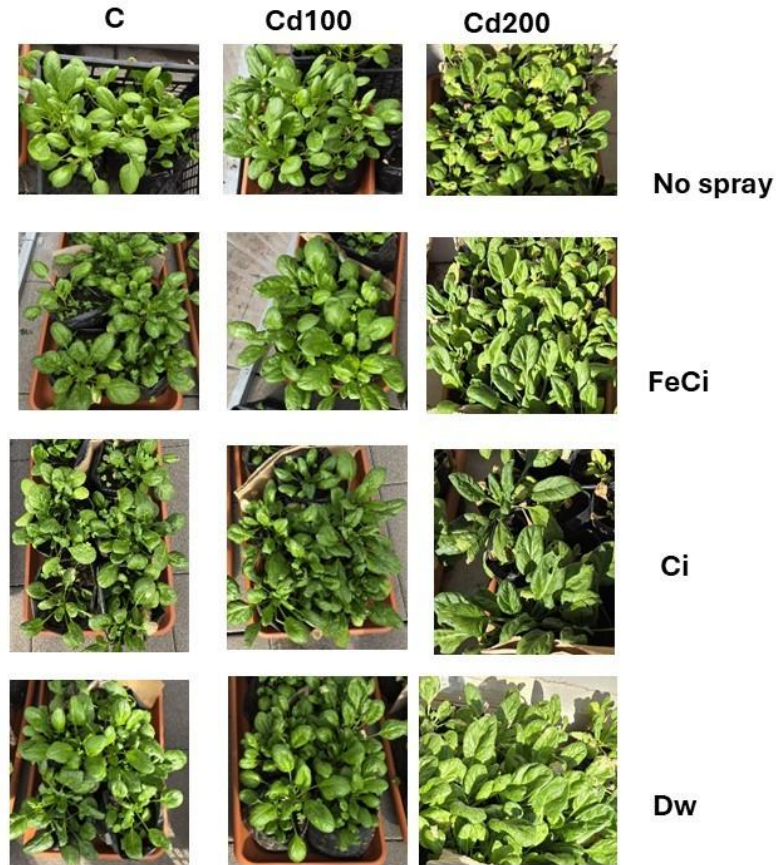


Figure 4.3: Photographs of *Spinacia oleracea* L. plants grown in uncontaminated soil (C) or in the same soil artificially contaminated with cadmium at 5 mg kg^{-1} (Cd100) or 10 mg kg^{-1} (Cd200). For each soil treatment, plants were either left without foliar application (untreated control) or sprayed with $\gamma\text{-Fe}_2\text{O}_3$ -citrate nanoparticles (FeCi), citrate alone (Ci), or distilled water (Dw).

Cadmium significantly affected plant height, whereas leaf area and LMA were not influenced by Cd alone (Table 4.1). Specifically, Cd reduced leaf production but increased plant height compared to controls, indicating a shift in biomass allocation rather than generalized growth inhibition. Leaf structural investment per unit area was maintained, with LMA around 35 g m^{-2} across control and Cd-treated plants.

Spray treatments influenced most growth parameters (Table 4.1, Fig. 4.3). FeCi tended to increase leaf area, particularly in Cd-treated plants, while reducing leaf number and plant height under Cd100, but not under Cd200. Citrate treatment produced high variability in leaf area and plant height, with values similar to controls. Interestingly, citrate consistently increased LMA across all experimental groups, reaching $\sim 55 \text{ g m}^{-2}$ in control and Cd100, and $\sim 48 \text{ g m}^{-2}$ in Cd200. Distilled water (Dw) treatment increased leaf area but reduced both leaf number and plant height in all groups and additionally decreased LMA in Cd200.

Cadmium exposure caused pronounced changes in all measured water relation parameters (Table 4.1). With increasing Cd concentration, saturated water content (SWC) increased, while osmotic potential (π_0) and turgor loss point (Ψ_{tlp}) became more negative. Concurrently, minimum cuticular conductance (g_{min}) decreased.

Citrate-alone foliar application induced pronounced changes in control and cadmium-polluted leaves. Citrate-alone application induced pronounced, context-dependent effects. In Cd100 and Cd200 plants, citrate reduced

SWC, while in all experimental groups it caused a more negative Ψ_{tp} . Additionally, in control plants, citrate markedly increased minimum cuticular conductance (g_{min}), reaching values of approximately $19 \text{ mmol m}^{-2} \text{ s}^{-1}$. Dw treatment had minimal effects, although under Cd200 it slightly reduced SWC.

Gas exchange measurements showed that photosynthetic rate (A_n) and leaf conductance (g_L) were not drastically suppressed under Cd treatments but not anyway reduced (Fig. 4.4). More in detail, in non-sprayed plants, control plants showed g_L of $\sim 400 \text{ mmol m}^{-2} \text{ s}^{-1}$ and A_n of $\sim 16 \mu\text{mol m}^{-2} \text{ s}^{-1}$, whereas g_L in Cd100 and Cd200 did not exceed $300 \text{ mmol m}^{-2} \text{ s}^{-1}$. A_n declined to $\sim 13 \mu\text{mol m}^{-2} \text{ s}^{-1}$ in Cd100 and $\sim 11 \mu\text{mol m}^{-2} \text{ s}^{-1}$ in Cd200, significantly lower than controls ($P < 0.05$).

Leaf spraying did not improve gas exchange in control plants. Dw and citrate treatments reduced both g_L and A_n compared to non-sprayed controls. FeCi reduced g_L in control leaves but maintained A_n . Under Cd stress, however, FeCi improved physiological performance. Cd100, and to a lesser extent Cd200, exhibited higher g_L and A_n when treated with FeCi compared to non-sprayed plants. This beneficial effect was not observed in citrate- or Dw-treated plants, which remained similar to their non-sprayed counterparts.

It can be highlighted that no spray treatment reduced the cadmium accumulation into the leaves that was 15 mg DW^{-1} in all measured cadmium-polluted samples (data not shown).

Table 4.1: Mean values $\pm$ SD ($n=5$) of leaf area (A_L), number of leaves per plant (N_{leaves}), plant height (H_{plant}), leaf mass area (LMA), saturated water content (SWC), osmotic potential at full turgor (p_o), turgor loss point (Y_{tlp}) and minimum cuticular conductance (g_{min}) recorded in *S. oleracea* L. plants growing in good quality soil (C) or in soil contaminated with cadmium at 5 mg kg^{-1} (Cd100) or 10 mg kg^{-1} (Cd200). Reported P-values refer to two-way ANOVA testing the effects of cadmium contamination (Cd) and spray treatment (Spray), followed by a Holm–Šidak post hoc test. Different letters denote statistically significant differences among treatments.

	A_L (cm^2)	N_{leaves}	H_{plant} (cm)	LMA (g m^{-2})	SWC (g g^{-1})	$-\pi_o$ (MPa)	$-\Psi_{\text{tlp}}$ (MPa)	g_{min} ($\text{mmol m}^{-2}\text{s}^{-1}$)
C	25.8 ± 2.3	22.0 ± 1.7	$27.0 \pm 3.4a$	$31.3 \pm 5.5a$	$12.2 \pm 0.7a$	1.02 ± 0.07	$1.29 \pm 0.05a$	$13.4 \pm 2.4ab$
Cd100	22.9 ± 2.1	20.0 ± 1.0	$37.0 \pm 2.8b$	$34.5 \pm 4.5a$	$13.2 \pm 0.7ab$	1.15 ± 0.09	$1.35 \pm 0.07a$	$11.9 \pm 1.4bc$
Cd200	29.7 ± 2.2	18.0 ± 0.8	$37.0 \pm 1.8b$	$37.2 \pm 3.9a$	$13.1 \pm 0.9b$	1.30 ± 0.10	$1.49 \pm 0.1ab$	$9.3 \pm 1.4c$
C_{FeCi}	20.6 ± 2.6	19.0 ± 2.1	$25.0 \pm 3.3a$	$34.1 \pm 2.4a$	$11.4 \pm 0.8a$	1.02 ± 0.06	$1.38 \pm 0.06a$	$10.3 \pm 1.7bc$
$\text{Cd100}_{\text{FeCi}}$	31.2 ± 4.7	17.5 ± 3.1	$24.0 \pm 4.5a$	$38.6 \pm 4.6a$	$15.3 \pm 1.8b$	1.18 ± 0.18	$1.51 \pm 0.13ab$	$14.3 \pm 1.2a$
$\text{Cd200}_{\text{FeCi}}$	31.0 ± 4.2	16.0 ± 4.5	$35.0 \pm 1.8b$	$34.1 \pm 3.7a$	$13.9 \pm 1.4ab$	1.39 ± 0.15	$1.63 \pm 0.14ab$	$10.2 \pm 1.6bc$
C_{Ci}	21.5 ± 6.1	19.0 ± 1.9	$30.0 \pm 6.3ab$	$53.2 \pm 4.4b$	$10.2 \pm 0.8ac$	1.03 ± 0.16	$1.42 \pm 0.20a$	$19.0 \pm 2.3d$
Cd100_{Ci}	23.5 ± 10.9	18.0 ± 2.3	$30.0 \pm 7.3ab$	$55.4 \pm 8.6b$	$12.1 \pm 0.6ac$	1.37 ± 0.13	$1.95 \pm 0.17c$	$10.1 \pm 0.9bc$
Cd200_{Ci}	27.0 ± 5.8	16.0 ± 4.1	$26.0 \pm 3.8c$	$47.8 \pm 7.7b$	$10.6 \pm 1.0 c$	1.22 ± 0.20	$1.63 \pm 0.15b$	$8.5 \pm 0.9c$
C_{Dw}	31.6 ± 2.6	16.0 ± 1.0	$27.0 \pm 5.6a$	$32.7 \pm 4.1ac$	$11.1 \pm 1.3ac$	1.07 ± 0.09	$1.48 \pm 0.13a$	$10.5 \pm 1.8bc$
Cd100_{Dw}	31.8 ± 7.8	14.0 ± 2.1	$29.0 \pm 4.9ab$	$40.0 \pm 2.4ac$	$12.9 \pm 0.7ac$	1.09 ± 0.06	$1.44 \pm 0.07ab$	$9.1 \pm 0.8c$
Cd200_{Dw}	31.2 ± 8.1	16.0 ± 3.9	$28.0 \pm 2.2 ac$	$24.8 \pm 4.4c$	$8.5 \pm 1.3c$	1.17 ± 0.15	$1.54 \pm 0.25ab$	$9.5 \pm 1.1c$
<i>P-value</i>								
Cd	0.086	0.002	0.006	0.127	<0.001	<0.001	<0.001	<0.001
Spray	0.019	<0.001	0.016	<0.001	<0.001	0.306	0.005	0.002
Cd*Spray	0.163	0.696	0.004	0.035	<0.001	0.164	0.036	<0.001

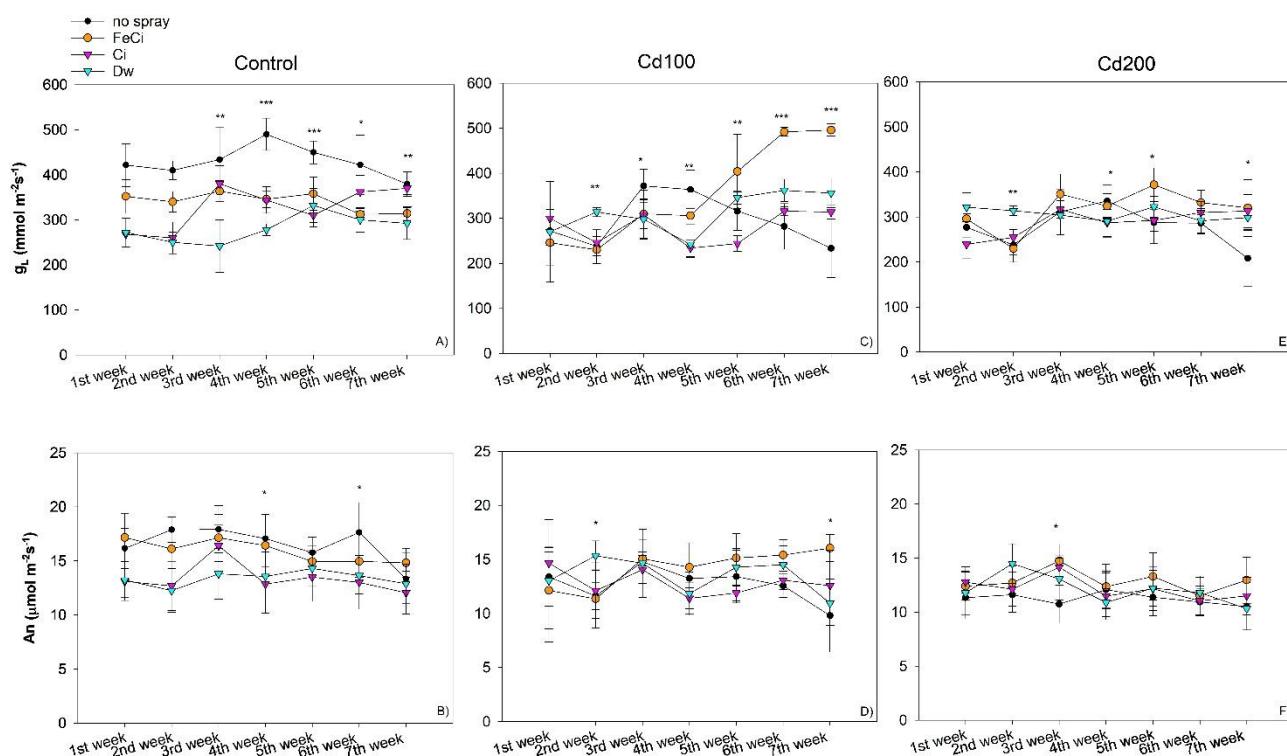


Figure 4.4: Mean $\pm$ SD values of stomatal conductance to water vapor (g_L) and photosynthesis rate (A_n) measured throughout the experimental period in control (C) and cadmium-treated (Cd100 and Cd200) leaves, following foliar application of the three different solutions. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$

4.3. Discussion

Cadmium exposure induced a clear shift in biomass allocation rather than generalized growth inhibition in *Spinacia oleracea*. Even under moderate Cd exposure, leaf number decreased while plant height increased, whereas leaf area and LMA remained unchanged. The maintenance of LMA ($\sim 35 \text{ g m}^{-2}$) together with only moderate reductions in photosynthetic rate suggests that structural investment per unit leaf area and carbon assimilation capacity were largely preserved. Thus, Cd did not impair tissue construction *per se* but rather altered developmental allocation patterns.

An increase in plant height in response to cadmium has been reported in other species (Bekkai et al., 2024, 2026), although opposite responses have also been observed depending on species and experimental context (Zhang et al., 2021). Importantly, *S. oleracea* exhibits a rosette growth form; therefore, measured plant height mainly reflects elongation of the flowering axis rather than overall shoot biomass. The observed pattern likely represents a stress-induced developmental shift prioritizing reproductive axis elongation over leaf organogenesis. Such reallocation is consistent with resource redistribution strategies frequently described under heavy metal stress (Ryser et al., 2007; Audet and Charest, 2008; Adomako et al., 2023), where plants redirect assimilates toward structures that maximize fitness under suboptimal conditions. The stability of LMA and the absence of severe photosynthetic collapse further support the interpretation of developmental reprogramming rather than generalized toxicity.

Beyond growth responses, cadmium markedly altered leaf water relations. Increasing Cd concentration resulted in higher saturated water content, more negative osmotic potential and turgor loss point, and reduced minimum cuticular conductance. The more negative π_0 and $\Psi_{t\bar{p}}$ indicate osmotic adjustment, allowing maintenance of cell turgor at lower water potentials. Concurrently, the reduction in g_{min} suggests tighter control of non-stomatal water loss. Together, these changes indicate a conservative hydraulic strategy that enhances drought-like tolerance under Cd stress.

The increase in SWC may reflect modifications in apoplastic water fraction or alterations in cell wall properties induced by cadmium, potentially linked to enhanced metal binding within the cell wall matrix. Heavy metals are known to affect wall elasticity and water partitioning, thereby influencing tissue hydration status. Similar osmotic adjustment and cuticular regulation mechanisms have been reported under metal stress (Chiofalo et al., 2025), where they contribute to maintaining hydraulic functionality despite reduced root efficiency or vascular disturbances (Lanning et al., 2020; Cardoso et al., 2025; Liu et al., 2025). Overall, our results indicate that hydraulic regulation represents a primary tolerance mechanism in spinach exposed to Cd.

FeCi foliar application modulated both growth and water relations, particularly under moderate Cd stress (Cd100). FeCi increased leaf area while slightly reducing plant height, shifted $\Psi_{t\bar{p}}$ to more negative values, and modestly affected SWC and g_{min} , suggesting enhanced hydraulic buffering without excessive structural overcompensation. These adjustments indicate that the iron–citrate nanoformulation contributed to stabilizing leaf water balance under Cd stress. In contrast, citrate alone induced pronounced but context-dependent responses. In control plants, citrate markedly increased g_{min} and LMA, indicating greater structural investment accompanied by increased cuticular permeability. Under Cd100 and Cd200, citrate enhanced osmotic adjustment and further lowered $\Psi_{t\bar{p}}$, particularly at Cd100. However, these hydraulic modifications did not translate into improved gas exchange. This suggests that citrate-induced osmotic adjustment does not necessarily enhance carbon gain and may even entail permeability-related costs under non-stress conditions. Previous studies have shown that chelating agents can modify metal availability and plant physiological responses, but their effectiveness strongly depends on environmental context and formulation (Kiu et al., 2005; Lestan et al., 2008). Distilled water treatment had minimal effects overall, confirming that foliar hydration alone does not substantially improve structural or hydraulic resilience under Cd stress.

Despite substantial adjustments in water relations, photosynthetic rate and leaf conductance were only moderately reduced under Cd treatments, even at Cd200. This indicates that stomatal regulation remained functional and that hydraulic compensation mechanisms effectively buffered the photosynthetic apparatus from severe impairment. The maintenance of A_n relative to the magnitude of osmotic adjustment supports the interpretation that hydraulic regulation successfully preserved leaf functionality.

Under Cd stress, FeCi partially mitigated the negative effects on gas exchange, particularly at Cd100. FeCi-treated plants exhibited higher g_L and A_n compared to non-sprayed counterparts, whereas citrate-alone and Dw treatments did not produce similar improvements. Notably, FeCi did not enhance gas exchange in control plants, suggesting that its beneficial effect emerges specifically under Cd-induced physiological constraint.

The improvement observed with FeCi is likely attributable to enhanced iron availability within leaf tissues. Cadmium is known to interfere with Fe uptake, transport, and homeostasis (Nakanishi et al., 2003; Zhang et al., 2023), often leading to functional Fe deficiency that compromises chlorophyll synthesis and photosynthetic electron transport. Given the antagonistic interaction between Cd and Fe, the nanoformulated iron–citrate complex may have alleviated Cd-induced Fe limitation, thereby sustaining photosystem activity and carbon assimilation under moderate stress. The absence of comparable effects with citrate alone confirms that the benefit derives from iron delivery rather than from chelation or mechanical spraying.

4.4. Conclusions

Overall, Cd tolerance in *Spinacia oleracea* appears to rely primarily on hydraulic adjustment and osmotic regulation rather than on complete avoidance of growth constraints. Cadmium induced a developmental reallocation toward axis elongation, accompanied by osmotic adjustment and reduced cuticular conductance, allowing the maintenance of photosynthetic functionality despite stress.

FeCi treatment did not fully restore leaf production nor reverse all growth limitations, but it stabilized water relations and partially recovered photosynthetic performance under moderate Cd stress (Cd100). This response was likely attributable to the enhanced availability of Fe within leaf tissues supplied by the nanoformulation, which may have supported photosynthetic functioning and water-status regulation under Cd stress. Under more severe contamination (Cd200), the beneficial effect diminished, indicating that the nanoformulation is most effective within a moderate stress window.

Citrate alone acted as a context-dependent modulator: it enhanced osmotic adjustment under Cd exposure but increased cuticular permeability under non-stress conditions and failed to improve photosynthetic performance. Therefore, the positive response observed with FeCi cannot be attributed to citrate alone, but is more likely related to the role of the Fe-containing nanoformulation in improving Fe availability and supporting physiological performance under Cd stress.

Thus, the TCD-stabilized γ -Fe₂O₃ nanofertilizer provides a functional advantage under regulatory-relevant Cd contamination levels by improving hydraulic balance and sustaining gas exchange, whereas citrate or simple hydration alone are insufficient. More broadly, the results emphasize that the effectiveness of nano-enabled fertilization strategies depends on stress intensity and on the integration of hydraulic and photosynthetic processes in determining plant resilience.

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Chapter 5

Iron-Citrate Nanoparticles Reprogram Hormonal Networks and Secondary Metabolic Pathways in Spinach under Cadmium Stress

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(Under preparation)

5. Introduction

Cadmium (Cd) is a highly toxic trace metal widely present in agricultural soils and represents a major concern for food safety (Clemens et al., 2013; Cuypers et al., 2010). Due to its high mobility and bioaccumulation potential, Cd can easily enter the food chain, particularly through leafy vegetables such as spinach (*Spinacia oleracea* L.), which efficiently accumulate metals in edible tissues (Study 4). For this reason, regulatory limits for Cd concentrations in soils and crops have been established at national and international levels. However, plant responses to Cd concentrations close to, or exceeding, legal thresholds under realistic agricultural conditions remain incompletely understood.

Exposure to Cd induces severe physiological and biochemical alterations in plants, including oxidative stress, nutrient imbalance and extensive metabolic reprogramming (Clemens 2006; Mihucz et al., 2012; Hocaoglu-Özyiğit and Genç, 2020). In particular, Cd stress strongly affects secondary metabolism and phytohormone homeostasis, leading to significant changes in the levels of stress-related hormones such as abscisic acid (ABA), salicylic acid (SA), jasmonic acid (JA) and brassinosteroids (BRs) (Shukla et al., 2017; Zhang et al. 2024; Zhou et al. 2025). Rather than acting independently, these hormones form an interconnected signalling network that coordinates growth, defence and stress tolerance. The balance among these pathways is therefore critical for plant adaptation to metal toxicity, especially under chronic exposure conditions (Rahman et al. 2023; Riyazuddin et al. 2021). Iron (Fe) plays a central role in plant responses to Cd stress, as Cd and Fe compete for uptake, transport and cellular binding sites. Cadmium exposure frequently results in functional Fe deficiency, exacerbating oxidative stress and impairing key metabolic processes (Bao et al., 2012; McInturf et al., 2022; Zhang et al., 2023). Citrate, a key organic acid in primary metabolism, contributes to metal chelation, long-distance transport and intracellular sequestration of both Fe and Cd. Through these functions, citrate can directly influence metal bioavailability and toxicity. Consequently, iron–citrate complexes may affect Cd stress responses by simultaneously improving Fe availability and modulating metal chelation and redox-related processes.

In recent years, increasing attention has been devoted to foliar-based strategies aimed at rapidly modulating leaf physiology and stress responses, particularly under conditions where root uptake is impaired by metal toxicity. Among these approaches, foliar application of iron-based formulations has emerged as a promising tool to influence plant nutritional status and stress-related signalling pathways (Vaghar et al., 2020; Wang et al., 2021).

In a previous study, we showed that foliar application of citrate-stabilized iron oxide nanoparticles is feasible under environmentally and agronomically realistic conditions and can positively influence spinach growth when plants are exposed to Cd at the maximum concentration permitted by Italian legislation (Study 4). While that work focused primarily on formulation optimization and growth-related outcomes, the metabolic and hormonal mechanisms underlying plant responses to foliar iron–citrate application under Cd stress remain largely unexplored.

In particular, it is still unclear how foliar iron-citrate modulates stress-related phytohormone networks, whether its effects differ from those of citrate alone, and how these responses change under higher Cd concentrations

and prolonged exposure. Addressing these aspects is essential to understand whether iron–citrate application promotes stress mitigation or, rather, a fine-tuning of hormonal signalling leading to acclimation under chronic metal stress.

Therefore, the aim of this study was to investigate the effects of foliar application of iron–citrate nanoparticles, citrate alone and water on selected phenolic compounds and stress-related phytohormones (ABA, SA, JA and brassinosteroids) in spinach grown in soils containing Cd at the maximum concentration allowed by Italian legislation and at twice this concentration. By extending the analysis to a higher Cd level and a temporal scale, this study builds on our previous work to provide mechanistic insight into hormonal and metabolic reprogramming induced by foliar iron–citrate under Cd stress.

We hypothesised that foliar application of iron–citrate nanoparticles would differentially modulate Cd-induced hormonal and metabolic responses compared with citrate alone, through combined effects on iron availability, metal chelation and redox-related signalling pathways. Specifically, this study addressed the following research questions: (i) Does foliar application of iron–citrate and citrate alone differentially reprogram stress-related phytohormone profiles in spinach exposed to environmentally relevant Cd concentrations? (ii) Are Cd-induced hormonal responses to foliar treatments dependent on both Cd dose and duration of exposure? (iii) Does foliar-applied iron–citrate promote a shift from acute stress signalling towards hormonal profiles associated with acclimation and tolerance under prolonged Cd exposure?

5.1. Material and Method

5.1.1. Growth conditions

The experiment was conducted from October 2024 to January 2025 using *Spinacia oleracea* L. plants cultivated under greenhouse conditions at the Department of Chemical, Biological, Pharmaceutical and Environmental Sciences (CHIBIOFARAM), University of Messina, Italy. Three-week-old seedlings were purchased from a local commercial nursery and transplanted into 2.3-L plastic pots filled with a nutrient-enriched commercial growing medium formulated for vegetable cultivation (Comodo, Ter Composti S.p.A., Italy).

Plants were assigned to three soil treatments: uncontaminated soil (control, C); soil contaminated with cadmium at a concentration of 5 mg kg⁻¹, corresponding to the maximum Cd concentration permitted by Italian legislation for agricultural soils (Cd100); and soil contaminated with twice this concentration (10.0 mg kg⁻¹; Cd200), according to Law No. 46 of June 22, 2019. After a four-week acclimation period following transplanting, plants were subjected to three foliar treatments: iron oxide nanoparticles complexed with citrate (FeCi), citrate alone (Ci), and distilled water (DW, foliar control). Experimental groups were therefore arranged according to cadmium level (C, Cd100, Cd200) and foliar treatment (FeCi, Ci, DW) (Figure 5.1). In addition, a set of untreated control plants (no foliar application) Cd200 was maintained.

Foliar applications were initiated after the acclimation period and applied weekly for seven consecutive weeks. Leaves from all treatment groups were collected at three time points: before the first foliar application (T0), after the third application (T3), and at the end of the experimental period, after the seventh application (T7).

Three independent biological replicates were used for each treatment, in accordance with previous studies focusing on targeted phytohormone and secondary metabolite analyses.

Throughout the experiment, plants were regularly irrigated to maintain adequate soil moisture and monitored for physiological and morphological traits. Collected leaf samples were immediately frozen and subsequently lyophilized for biochemical analyses.

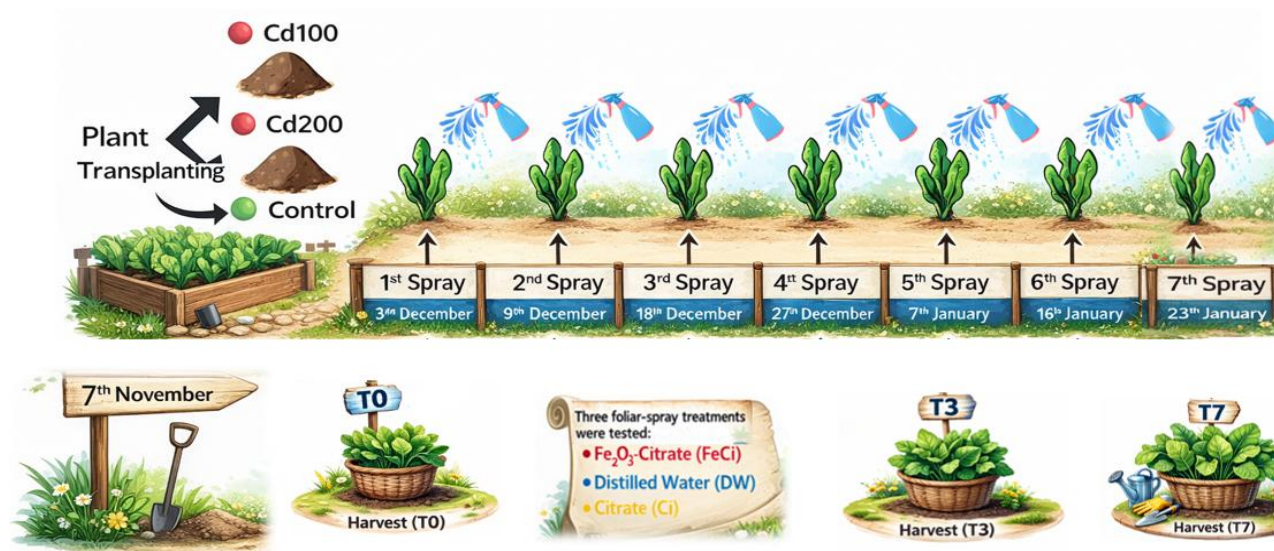


Figure 5.1: Experimental planning: Plants were transplanted on 7 November and divided into three soil conditions: Control (green marker), Cd100 (red marker; soil contaminated with cadmium at 100% of the legal limit), and Cd200 (red marker; soil contaminated with cadmium at 200% of the legal limit). After transplanting, plants were subjected to seven consecutive foliar spray applications performed between December and January. Three foliar treatments were tested: Fe₂O₃-citrate nanoparticles (FeCi), distilled water (Dw), and citrate (Ci).

Iron–citrate nanoparticles were prepared and characterized as described in Study 4 of the present Thesis and impact of spinach growth and water relation in response to cadmium pollution and spray treatment are described in the same study.

5.1.2 Extraction Procedure

Simultaneous quantification of ABA, SA, JA and JA-Ile was carried out by LC-MS/MS as described by Karady et al., 2024. The examined plant tissues were homogenized with zirconium oxide beads in 10 % acetonitrile with 0.5 % formic acid (v/v), internal standards were added, and samples were centrifuged at 30 000 × g. The collected supernatant of individual samples was further evaporated to dryness and subsequently analyzed by LC-MS/MS on a Kinetex Polar column (150 × 2.1 mm, 2.6 μm; Phenomenex). All solvents were of analytical grade or higher (Merck/Sigma-Aldrich, Darmstadt, Germany).

Stress and defense-related phytohormones included abscisic acid (ABA), jasmonic acid (JA), jasmonoyl-isoleucine (JA-Ile), and salicylic acid (SA), which was quantified as a signaling phytohormone involved in plant defense responses.

Brassinosteroids were represented by multiple biosynthetic intermediates and bioactive forms, including castasterone, 28-homocasterone, 28-norcastasterone, 24-epicastasterone, brassinolide, 28-norbrassinolide,

24-epibrassinolide, and 28-homobrassinolide. Endogenous BRs were extracted and quantified according to the protocol described by Oklestkova et al. (2017). Spinach samples (approximately 5 mg fresh weight) were extracted with ice-cold 60% acetonitrile. Each sample was supplemented with 25 pmol of deuterium-labelled BR internal standards (Olchemim Ltd, Olomouc, Czech Republic). The samples were incubated for 12 hours at 4°C and subsequently centrifuged. The supernatants were purified using 50 mg Discovery™ DPA-6S solid-phase extraction cartridges (Supelco®, Bellefonte, PA, USA). The purified extracts were evaporated to dryness and dissolved in 40 µL of methanol. Quantification of BRs was conducted using ultra-high-performance liquid chromatography coupled with tandem mass spectrometry (UHPLC–MS/MS) with an ACQUITY UPLC® I-Class System (Waters, Milford, MA, USA) and a Xevo™ TQ-S triple quadrupole mass spectrometer (Waters MS Technologies, Manchester, UK). Detailed analytical conditions for UHPLC–MS/MS are described in Oklestkova et al. (2017) and Tarkowská et al. (2016).

In order to estimate phenolic compounds, dried spinach leaf material (20 mg) was extracted with 80 % methanol containing internal standards of salicylic (3,4,5,6-²H₄) and 4-hydroxybenzoic (2,3,5,6-²H₄) acids at a final concentration of 5×10^{-5} M, using an oscillation ball mill operating at a frequency of 25 s⁻¹. The mixture was ultrasonicated for 15 min and centrifuged for 10 min at 19 000 g. The supernatants were transferred into Eppendorf tubes, evaporated under nitrogen, redissolved in 20 % methanol, filtered through 0.45 µm nylon membrane filters (Alltech, Breda, Netherlands), and analyzed with a UHPLC-MS/MS system (Micromass Quattro micro™, Waters). Phenolic acids associated with secondary metabolism and antioxidant responses included salicylic acid, 4-hydroxybenzoic acid, ferulic acid, and 4-hydroxycoumaric acid. In this case, salicylic acid was quantified as a phenolic compound within the phenylpropanoid-related pool, reflecting its dual biochemical nature and functional roles.

5.1.3 Chemicals

Standards of gallic, 3,5-dihydroxybenzoic, protocatechuic, chlorogenic (5-caffeoylquinic), gentisic, 4-hydroxybenzoic, caffeic, vanillic, syringic, 3-hydroxybenzoic, 4-coumaric, sinapic, ferulic, 3-coumaric, 2-coumaric, salicylic and trans-cinnamic acids were purchased from Sigma–Aldrich Fine Chemicals (St. Louis, MO, USA). Deuterium-labeled standards of 4-hydroxybenzoic acid (2,3,5,6-D₄) and salicylic acid (3,4,5,6-D₄) were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). Formic acid and methanol used for preparing mobile phases were purchased from MERCK (Darmstadt, Germany). Deionized water was prepared using a Simplicity 185 system (Millipore, Bedford, MA, USA). 2.2. Preparation of standard solutions Standard solutions were first prepared in methanol at a concentration of 10⁻³ mol L⁻¹ and then serially diluted to working concentrations. Internal standards (IS) of deuterium-labeled 4 hydroxybenzoic and salicylic acids were added to a final concentration of 10⁻⁵ mol L⁻¹ in all standard solutions.

5.2.4 UPLC–MS/MS instrumentation and conditions

UPLC–MS/MS analyses were carried out using an ACQUITY Ultra Performance LCTM system (Waters, Milford, MA, USA) linked simultaneously to both a PDA 2996 photo diode array detector (Waters, Milford, MA, USA) and a Micromass Quattro micro™ API benchtop triple quadrupole mass spectrometer (Waters MS Technologies, Manchester, UK), equipped with a Z-spray electrospray ionisation (ESI) source operating

in negative mode. MassLynxTM software (version 4.0, Waters, Milford, MA, USA) was used to control the instruments, and for data acquisition and processing. Sample solutions were injected into a reversed phase column (BEH C8, 1.7 μ m, 2.1 $\times$ 150mm, Waters, Milford, MA), which was maintained at 30 °C. The mobile phase consisted of the following 9.5-min sequence of linear gradients and isocratic flows of solvent B (acetonitrile) balanced with aqueous 7.5 mM HCOOH (solvent A) at a flow rate of 250 μ Lmin⁻¹: 5% B for 0.8min, 5–10% B over 0.4 min, isocratic 10% B for 0.7 min, 10–15% B over 0.5 min, isocratic 15% B for 1.3 min, 15–21% over 0.3 min, isocratic 21% B for 1.2 min, 21–27% B over 0.5 min, 27–50% B over 2.3min, 50–100% B over 1min, and finally 100–5% B over 0.5min. At the end of this sequence the column was equilibrated under initial conditions for 2.5 min. The pressure ranged from 4000 to 8000psi during the chromatographic run. The effluent was introduced into a PDA detector (scanning range 210–600 nm, resolution 1.2 nm) and subsequently into an electrospray source (source block temperature 100 °C, desolvation temperature 350 °C, capillary voltage 2.5 kV, cone voltage 25 V). Argon was used as collision gas (collision energy 16eV) and nitrogen as desolvation gas (500Lh⁻¹). The retention windows used for quantification were as follows: 0.00–3.00 min; 3.00–4.25 min; 4.25–4.70 min; 4.70–5.15 min; 5.15–5.80 min; 5.80–6.30 min; 6.30–7.30 min; 7.30–7.80 min; 7.80–8.90 min; 8.90–10.00 min.

5.2. Results

5.2.1 Hormonal and phenolic profiles at T0 under different Cd concentrations

Before foliar application of nanofertilizers or control solutions, spinach plants grown under different cadmium regimes already exhibited distinct basal hormonal and metabolic profiles (Table 5.1). These differences indicate that Cd exposure per se induced substantial physiological reprogramming at the metabolic level.

Abscisic acid (ABA) levels were significantly higher in plants grown at Cd200 compared with both control and Cd100 conditions ($P = 0.006$). Salicylic acid (SA) did not differ significantly among treatments. In contrast, jasmonic acid (JA) and its bioactive conjugate JA-Ile showed a non-linear response to Cd, with significantly higher levels at Cd100 and reduced concentrations at Cd200 (JA, $P = 0.014$; JA-Ile, $P = 0.008$).

Brassinosteroid profiles were significantly affected by Cd exposure, with compound-specific and dose-dependent responses. Several brassinosteroids, including 28-homocastasterone, 28-norcastasterone, 24-epicastasterone and 28-homobrassinolide showed significant differences among treatments.

Specifically, 28-homocastasterone was significantly higher in Cd200 plants compared with controls, whereas no differences were observed at Cd100. Conversely, 24-epicastasterone increased under Cd100, while 28-norcastasterone decreased at Cd100 relative to controls; neither metabolite differed from controls at Cd200. No significant differences were detected for castasterone, brassinolide, 28-norbrassinolide, or 24-epibrassinolide.

At baseline (T0), 4-OH-coumaric acid remained unchanged across treatments, whereas ferulic acid and 4-OH-benzoic acid increased only in Cd200 plants, indicating that high Cd exposure selectively triggered phenolic accumulation.

Table 5.1: Mean $\pm$ SD of metabolites measured in control and cadmium treated leaves (Cd100 and Cd200) before starting spray treatments (T0). P values from one-way ANOVA test are reported.

Metabolite	C	Cd100	Cd200	P-value
ABA (pmol/g)	361 $\pm$ 27.25	321 $\pm$ 32.9	443.7 $\pm$ 14.1	0.006
SA (pmol/g)	93.6 $\pm$ 22.8	133 $\pm$ 0	49.0 $\pm$ 30.8	0.163
Ja (pmol/g)	67.2 $\pm$ 27.6	321 $\pm$ 144.2	85.3 $\pm$ 39.7	0.014
JA-Ile (pmol/g)	47.16 $\pm$ 19.3	132 $\pm$ 47.11	14.8 $\pm$ 12.7	0.008
28-homocastasterone (pmol/g)	1391.4 $\pm$ 82.6	1455 $\pm$ 46.2	2550.4 $\pm$ 930.9	0.025
Castasterone (pmol/g)	11.0 $\pm$ 2.0	5.0 $\pm$ 1.4	18.3 $\pm$ 6.0	0.081
28-norcastasterone (pmol/g)	37.5 $\pm$ 0.86	12.8 $\pm$ 3.36	35.8 $\pm$ 12.2	0.019
24-epicastasterone (pmol/g)	11.0 $\pm$ 2.2	21.0 $\pm$ 3.2	13.0 $\pm$ 3.5	0.020
Brassinolide (pmol/g)	6.6 $\pm$ 2.1	6.5 $\pm$ 1.4	6.9 $\pm$ 1.5	0.994
28-norbrassinolide (pmol/g)	15.6 $\pm$ 1.2	15 $\pm$ 4.54	13 $\pm$ 4.4	0.908
24-epibrassinolide (pmol/g)	2.0 $\pm$ 1.1	2.8 $\pm$ 1.2	2.2 $\pm$ 0.5	0.931
28-homobrassinolide (pmol/g)	15.0 $\pm$ 8.2	9.1 $\pm$ 0.7	3.6 $\pm$ 1.0	0.050
salicylic acid (pmol/g)	0.24 $\pm$ 0.07	0.41 $\pm$ 0.02	0.44 $\pm$ 0.08	0.150
4-OH-benzoic acid (pmol/g)	4.6 $\pm$ 0.1	4.8 $\pm$ 0.1	8.8 $\pm$ 0.2	<0.001
Ferulic acid (pmol/g)	46.5 $\pm$ 4.8	36.6 $\pm$ 1.5	302.4 $\pm$ 16.4	0.011
4-OH-coumaric acid (pmol/g)	79.2 $\pm$ 6.7	90.4 $\pm$ 7.4	75.5 $\pm$ 4.7	0.231

5.2.2 Evaluating spraying on hormonal and phenolic profiles

To evaluate the impact of the spraying procedure itself, untreated plants were compared with those sprayed with distilled water after seven applications (Table 5.2).

Water spraying induced significant changes in a limited subset of metabolites, including SA, JA, JA-Ile, 28-norcastasterone, 28-homobrassinolide, 4-OH-ferulic acid, and 4-hydroxybenzoic acid. No significant differences were observed for the remaining compounds (Table 5.2).

Table 3: Mean $\pm$ SD of metabolites after 7 weeks in control and Cd-treated (Cd200) samples, in non-sprayed or Dw-sprayed leaves. P values from two-way ANOVA with Holm-Sidak test for effects of cadmium (Cd), spray, and their interaction.

Metabolite		Not-sprayed	Dw-sprayed	Cd	spray	Cd*Spray
ABA (pmol/g)	C Cd200	168.6 $\pm$ 5.1 306.6 $\pm$ 33.9	187.1 $\pm$ 16.9 278.8 $\pm$ 16.2	<0.001	0.352	0.161
SA (pmol/g)	C Cd200	142.4 $\pm$ 19.6 119.2 $\pm$ 11.09	481.2 $\pm$ 181.9 547.6 $\pm$ 59.4	0.890	<0.001	0.702
Ja (pmol/g)	C Cd200	1019.1 $\pm$ 42.9 821.8 $\pm$ 71.07	1068.7 $\pm$ 3 21.2 20.05 $\pm$ 96.02	0.003	0.007	0.058
JA-IIe (pmol/g)	C Cd200	126.0 $\pm$ 28.2 185.4 $\pm$ 9.1	64.6 $\pm$ 26.2 17.3 $\pm$ 31.2	0.113	<0.001	0.037
28-homocastasterone (pmol/g)	C Cd200	2668.5 $\pm$ 437.4 2831.9 $\pm$ 517.2	2940.4 $\pm$ 284.6 2880 $\pm$ 315.2	0.905	0.208	0.379
Castasterone (pmol/g)	C Cd200	9.4 $\pm$ 2.7 16.5 $\pm$ 3.3	6.5 $\pm$ 2.1 18.6 $\pm$ 10.5	0.038	0.631	0.905
28-norcastasterone (pmol/g)	C Cd200	44.1 $\pm$ 7.7 50.4 $\pm$ 3.6	24.7 $\pm$ 10.3 7.6 $\pm$ 2.17	0.308	<0.001	0.018
24-epicastasterone (pmol/g)	C Cd200	7.1 $\pm$ 3.05 12.4 $\pm$ 4.4	11.9 $\pm$ 2.6 7.3 $\pm$ 0.9	0.886	0.712	0.137
Brassinolide (pmol/g)	C Cd200	2.3 $\pm$ 0.7 1.8 $\pm$ 0.3	2.7 $\pm$ 0.5 1.1 $\pm$ 0.3	0.010	0.452	0.246
28-norbrassinolide (pmol/g)	C Cd200	15.6 $\pm$ 2.8 25.8 $\pm$ 15.2	25.8 $\pm$ 2.3 16.2 $\pm$ 2.23	0.892	0.875	0.095
24-epibrassinolide (pmol/g)	C Cd200	4.8 $\pm$ 1.2 4.2 $\pm$ 1.2	5.5 $\pm$ 1.5 2.9 $\pm$ 1.1	0.166	0.931	0.280
28-homobrassinolide (pmol/g)	C Cd200	21.0 $\pm$ 2.9 23.4 $\pm$ 3.2	10.5 $\pm$ 2.4 11.3 $\pm$ 2.14	0.465	<0.001	0.675
salicylic acid (pmol/g)	C Cd200	0.3 $\pm$ 0.1 0.6 $\pm$ 0.4	0.8 $\pm$ 0.2 0.9 $\pm$ 0.3	0.270	0.133	0.729
4-OH-benzoic acid (pmol/g)	C Cd200	2.2 $\pm$ 0.1 3.7 $\pm$ 0.2	7.9 $\pm$ 4.1 6.9 $\pm$ 0.3	0.994	0.011	0.340
Ferulic acid (pmol/g)	C Cd200	83.3 $\pm$ 46.2 48.2 $\pm$ 12.2	297.0 $\pm$ 47.2 18.8 $\pm$ 0.8	<0.001	0.007	0.002
4-OH-coumaric acid (pmol/g)	C Cd200	69.6 $\pm$ 8.12 67.9 $\pm$ 22.5	50.9 $\pm$ 31.8 40.6 $\pm$ 4.0	0.929	0.190	0.777

5.2.3 Effect of foliar Fe-citrate and citrate application (DW vs treatments)

The effects of repeated foliar applications of Fe-citrate and citrate were evaluated relative to water-sprayed plants (Figs. 5.2, 5.3), allowing treatment effects to be distinguished from the mechanical impact of spraying.

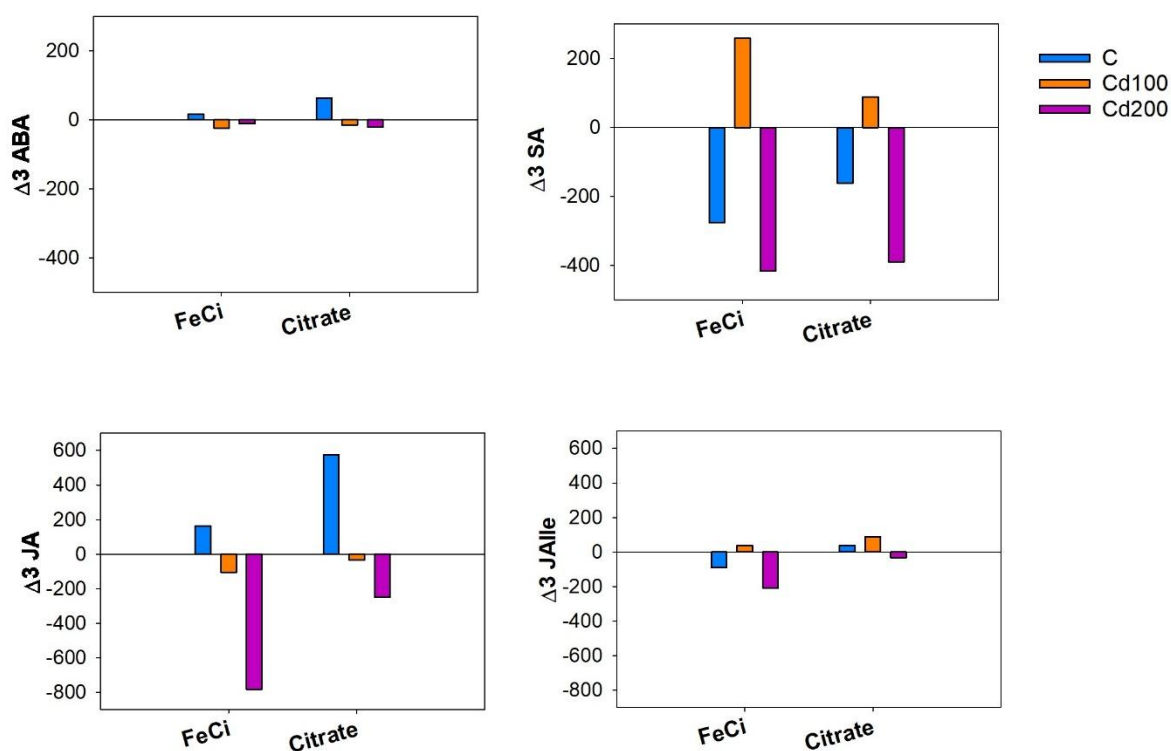


Figure 5.2: Variation in abscisic acid (ABA), jasmonic acid (JA), jasmonoyl-isoleucine (JA-Ile), and salicylic acid (SA) levels in control and cadmium-treated leaves (C, Cd100, and Cd200) after three ($\Delta 3$) foliar spray applications of Fe-citrate (FeCi) or only citrate, expressed relative to the corresponding values measured in leaves sprayed with distilled water

After three applications (T3), FeCi and citrate-alone treatments elicited largely similar phytohormonal responses (Fig. 5.2). In control plants, both treatments slightly increased ABA and more markedly increased JA, while SA decreased. In Cd-treated plants, both FeCi and citrate induced comparable responses at Cd100 and Cd200: ABA and JA decreased at both Cd concentrations, whereas SA and JA-Ile increased at Cd100 but declined at Cd200.

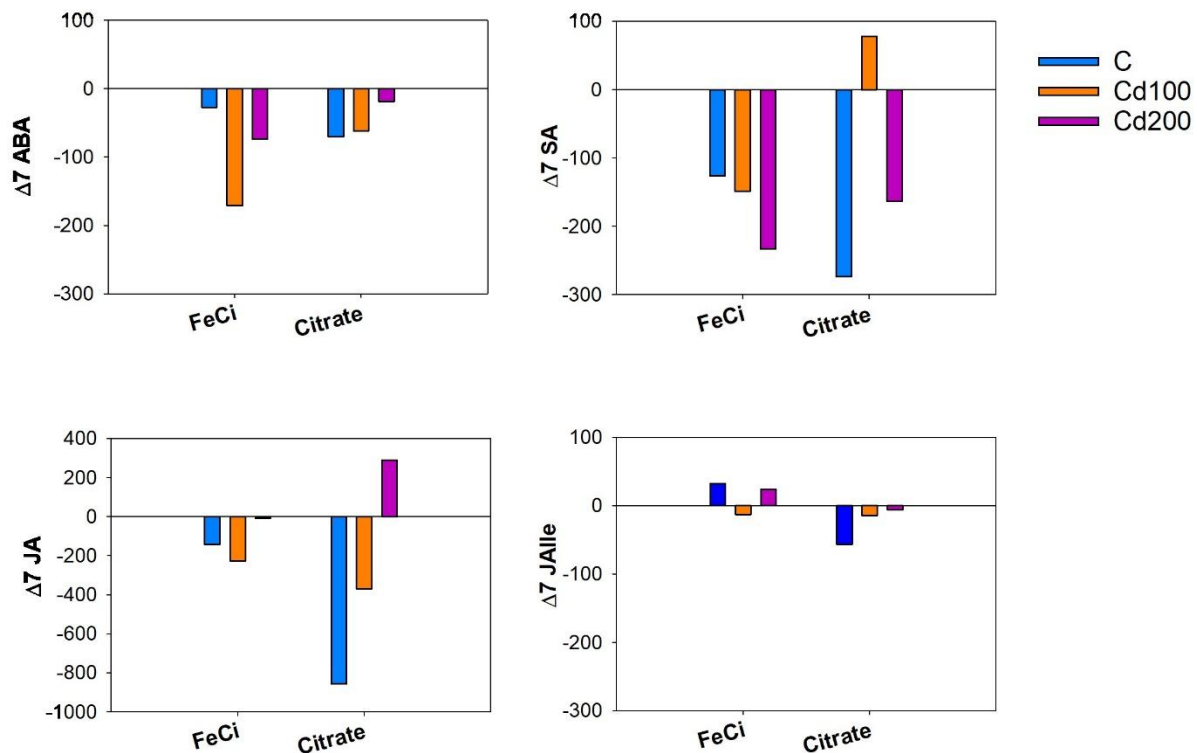


Figure 5.3: Variation in abscisic acid (ABA), jasmonic acid (JA), jasmonoyl-isoleucine (JA-Ile), and salicylic acid (SA) levels in control and cadmium-treated leaves (C, Cd100, and Cd200) after seven ($\Delta 7$) foliar spray applications of Fe–citrate (FeCi) or only citrate, expressed relative to the corresponding values measured in leaves sprayed with distilled water

After seven applications (T7), FeCi consistently attenuated the accumulation of major stress-related hormones (ABA, SA, and JA) in both control and Cd-exposed plants (Fig. 5.3). In contrast, JA-Ile showed a distinct pattern, increasing in control and Cd200 plants but slightly decreasing under Cd100. Citrate alone reproduced this pattern only for ABA, indicating differential modulation of hormone signaling pathways depending on the foliar treatment.

Baseline-normalized data (Supplementary Fig. S5.1) revealed hormone-specific temporal trends consistent with long-term acclimation rather than strictly treatment-driven effects.

Due to the absence of distilled water controls for brassinosteroids at T3, treatment comparisons at this time point were limited to major phytohormones and phenolic compounds.

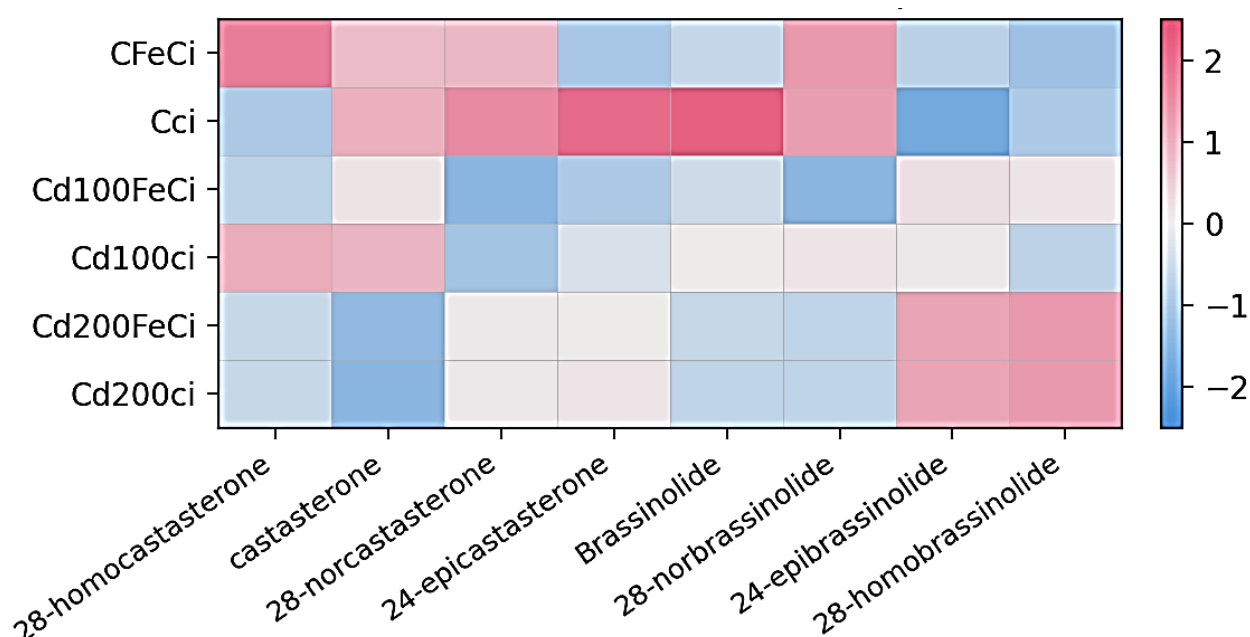


Figure 5.3: Heatmap representation of changes in brassinosteroid levels in leaves following foliar treatments, expressed as Δ values relative to water-sprayed controls (Dw). Rows represent experimental treatments (Control, Cd100 and Cd200 plants sprayed with iron–citrate or citrate alone), while columns correspond to individual brassinosteroids. Color intensity indicates the magnitude and direction of change, with red and blue denoting increases and decreases relative to Dw, respectively, according to the diverging colour scale shown. Due to the large dynamic range among brassinosteroids, the heatmap highlights relative modulation patterns within the pathway rather than absolute hormone abundance.

Heatmap visualization of brassinosteroid changes relative to water-sprayed controls revealed a non-uniform modulation of the BR pathway across treatments and cadmium levels (Fig. 5.3). The response was largely driven by marked variations in 28-homocastasterone, which showed the greatest amplitude of change, suggesting a substantial redistribution of metabolic flux within the brassinosteroid biosynthetic pathway. In contrast, late BR intermediates and bioactive forms, including brassinolide, epibrassinolide and homobrassinolide, displayed more moderate but coordinated changes, indicative of a fine-tuned regulation rather than a generalized accumulation.

Notably, iron–citrate treatment induced a more structured and treatment-specific BR profile compared with citrate alone, particularly under cadmium stress, whereas citrate-treated plants showed more heterogeneous and less consistent responses. Under high cadmium exposure, several late-stage BRs showed convergent modulation across treatments, suggesting that severe stress conditions may override treatment-specific effects and promote common adaptive adjustments of brassinosteroid metabolism.

Among the analyzed BRs, 28-homobrassinolide exhibited the most consistent response to Cd exposure (Supplementary Table S5.1).

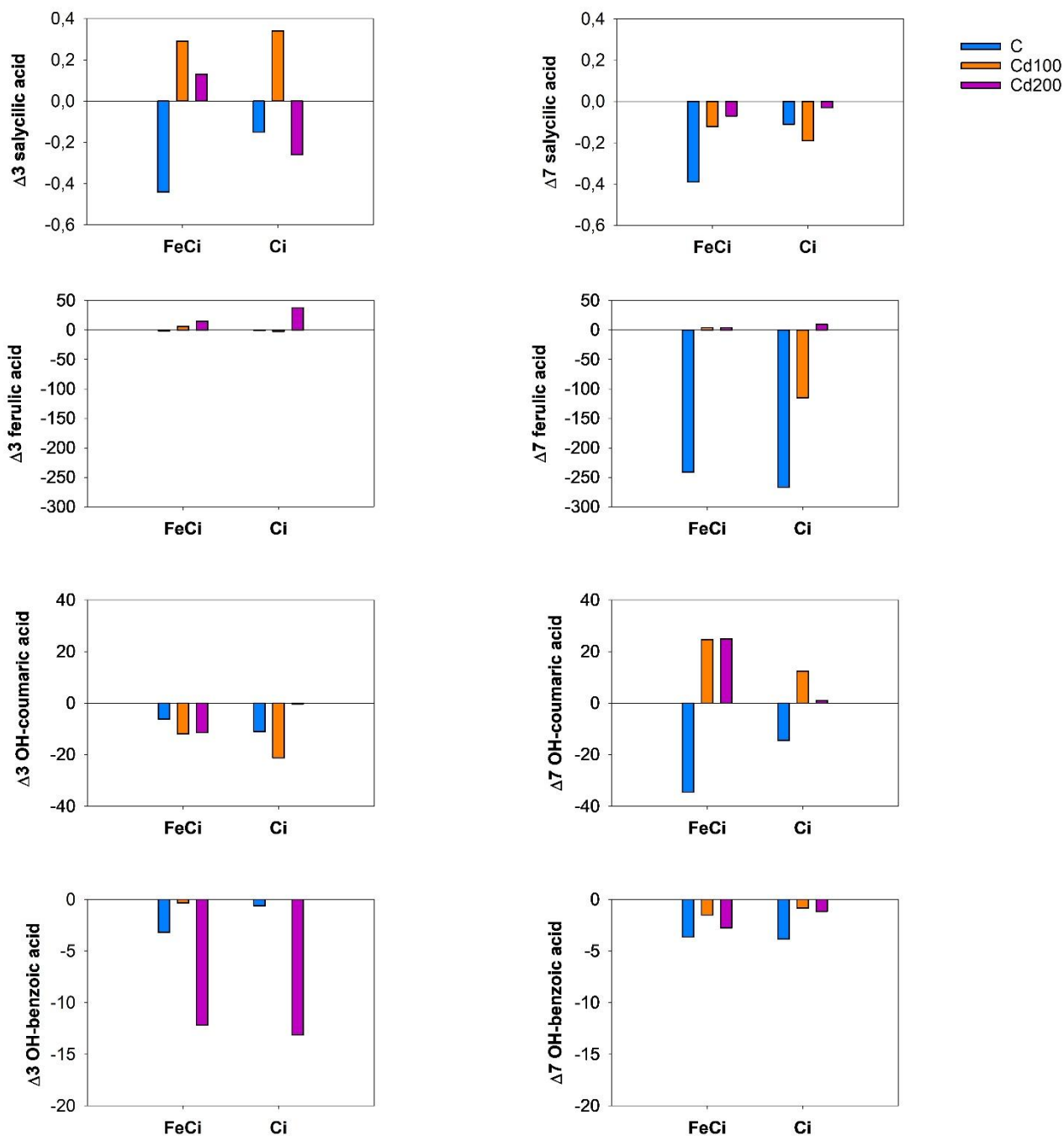


Figure 5.4: Variation in salicylic acid, ferulic acid, 4-OH coumaric acid and 4-OH benzoic acid levels in control and cadmium-treated leaves (C, Cd100, and Cd200) after three (Δ3) and seven (Δ7) foliar spray applications of Fe-citrate (FeCi) or only citrate, expressed relative to the corresponding values measured in leaves sprayed with distilled water

FeCi induced time- and Cd-dependent modulation of phenolic acids. After three applications (T3), FeCi increased salicylic acid in control plants but reduced it in Cd100 and Cd200 plants (Fig. 5.4). After seven applications (T7), salicylic acid decreased across all experimental groups, with the strongest reduction in controls. Citrate alone caused a transient increase in salicylic acid A in Cd100 at T3, followed by a decrease in all groups at T7.

Ferulic acid showed a distinct pattern, significantly increasing at T3 in Cd200 plants regardless of foliar treatment (Fig. 5.4). At T7, ferulic acid markedly decreased in FeCi-treated controls, remained stable in Cd-treated plants, and decreased in control and Cd100 plants treated with citrate alone.

Both FeCi and citrate reduced 4-OH-coumaric acid at T3 in all groups. At T7, a decrease persisted in control plants irrespective of treatment. However, FeCi induced accumulation of 4-OH-coumaric acid in Cd-treated plants, whereas citrate alone increased it only in Cd100 plants.

Finally, both treatments reduced 4-OH-benzoic acid across all groups, with stronger effects at T3 than at T7.

5.3. Discussion

5.3.1 Cadmium-induced hormonal reprogramming and phenylpropanoid pathway stimulation at baseline

Cadmium exposure profoundly altered both hormonal and phenolic profiles in spinach, reflecting a broad physiological reprogramming consistent with previous reports (Kováčik et al., 2011; Matayoshi et al., 2022; Neyshabouri et al., 2024). Notably, moderate (Cd100) and high (Cd200) cadmium levels elicited qualitatively different responses, indicating the existence of threshold-dependent regulatory mechanisms that determine the plant's defence strategy.

At T0, ABA accumulated selectively under Cd200 conditions, suggesting the activation of classical stress-signalling pathways associated with chronic and severe stress (Hsu and Kao, 2005; Liu et al., 2024). ABA is a key mediator of growth restraint and resource allocation under adverse conditions, and its exclusive induction at high cadmium levels indicates that only severe stress triggers a full ABA-mediated stress response. In contrast, jasmonic acid (JA) and its bioactive conjugate JA-Ile displayed a non-linear response, with elevated levels at Cd100 but reduced concentrations at Cd200. This pattern likely reflects an adaptive JA-mediated defence response under moderate stress, which becomes repressed or impaired under excessive cadmium exposure. Literature supports the protective role of JA in mitigating cadmium-induced stress (Singh and Shah, 2014; Yan et al., 2015; Zhao et al., 2016). Meanwhile, salicylic acid (SA) remained largely unchanged across treatments, suggesting that SA-dependent pathways are either not strongly engaged by cadmium in spinach or are maintained under tight homeostatic control.

Brassinosteroid profiles were also strongly affected in a compound-specific and dose-dependent manner, indicating fine-tuned modulation of the biosynthetic pathway rather than uniform changes in BR content. The accumulation of 28-homocastasterone at Cd200, without corresponding changes in bioactive end-products such as brassinolide, suggests a redistribution of metabolic flux within the pathway, potentially reflecting bottlenecks at later biosynthetic steps. This selective modulation highlights the role of BR metabolism as an integrator of growth and stress responses, balancing developmental processes with survival under heavy metal stress.

Basal phenolic profiles further corroborated the presence of a stress threshold. Ferulic acid and 4-hydroxybenzoic acid increased selectively at Cd200, while remaining unchanged at Cd100. This indicates that the phenylpropanoid pathway is primarily activated under severe stress, functioning as a secondary defence system when primary hormonal mechanisms are insufficient. Ferulic acid contributes to cell wall

reinforcement, whereas 4-hydroxybenzoic acid acts as an antioxidant and metal chelator, reducing cadmium-induced oxidative damage (Saini et al., 2024). Their selective induction underlines a strategic deployment of metabolic resources; whereby secondary metabolism is engaged only when stress intensity exceeds the buffering capacity of hormonal defences. The absence of changes at Cd100 implies that moderate stress can be managed primarily via hormonal adjustments, without invoking energetically costly secondary defences. Together, these observations support a model in which spinach plants fine-tune their hormonal and metabolic responses according to cadmium concentration, engaging primary stress signalling under moderate exposure (JA-mediated adaptation) and recruiting secondary metabolic defences, including phenolics, only under high-stress conditions (ABA-driven survival mode). This integrated response illustrates the dynamic interplay between primary hormonal regulation and secondary metabolite accumulation in plant adaptation to heavy metal stress.

5.3.2 Modulation of stress signalling by Fe-citrate and citrate treatments

The comparison between non-sprayed and water-sprayed plants revealed that the spraying procedure itself elicited measurable hormonal and metabolic responses, underscoring the sensitivity of spinach to even mild physical perturbations. Specifically, the observed modulation of JA, JA-Ile and SA is consistent with a wounding-like or mechanostimulus, in line with previous reports demonstrating that cadmium and/or minor mechanical stress can activate stress-related signalling pathways in plants (Llugany et al., 2013; Benikhlef et al., 2013). These changes likely reflect a transient activation of defence mechanisms, including early signalling cascades, ROS production, and secondary metabolite priming, which prepare the plant to respond to potential damage.

From an experimental standpoint, these findings validate the use of water-sprayed plants as an appropriate reference for evaluating the effects of foliar applications. By accounting for the mechanical impact of spraying, the design ensures that subsequent differences observed after treatments with Fe-citrate or citrate alone can be confidently attributed to their chemical and nutritional properties, rather than to the physical act of spraying. This emphasizes the importance of incorporating mechanical controls in foliar application studies, particularly when investigating sensitive stress-responsive hormones and metabolites.

Nevertheless, a central finding of this study is that Fe–citrate exerts regulatory effects that cannot be fully explained by citrate alone. Both Fe-citrate and citrate-alone treatments affected hormonal profiles, with their influence being particularly evident during the early phase of foliar application. After three treatments, the two formulations elicited largely similar responses, suggesting that citrate itself contributes substantially to initial signalling effects. This may occur through multiple mechanisms, including chelating capacity, which can modulate metal availability at the leaf surface, or alterations of leaf surface chemistry and nutrient uptake, thereby affecting hormone biosynthesis and signalling (Avellan et al., 2019; Karimi and Goli, 2021). In cadmium-exposed plants, the observed reduction of ABA and JA levels at this stage indicates a partial attenuation of stress perception, particularly under moderate cadmium stress, suggesting that foliar-applied citrate can modulate early defence signalling even in the absence of iron. In contrast, citrate alone reproduced these effects only partially, mainly affecting ABA levels, which underscores that iron availability, rather than

chelation alone, is essential for full alleviation of cadmium-induced stress. Citrate may improve metal mobility and early signalling, but without iron it cannot sustain the enzymatic and antioxidant processes required for long-term stress mitigation.

The distinct behaviour of JA-Ile, which showed treatment- and cadmium-dependent modulation, further highlights the complexity of jasmonate signalling under combined heavy metal stress and foliar nutrition. These observations suggest that Fe-citrate not only mitigates oxidative and hormonal perturbations but also contributes to a coordinated, durable adaptation, whereas citrate alone can only partially support this recovery. Distinct differences between treatments became more pronounced after seven foliar applications, highlighting the critical role of iron in long-term stress mitigation. Fe-citrate consistently reduced the accumulation of major stress-related phytohormones, including ABA, JA, and SA, across all cadmium regimes. This sustained down-regulation suggests that Fe-citrate enhances plant stress tolerance by restoring iron-dependent enzymatic activity, improving antioxidant defences, and promoting hormonal homeostasis (Briat et al., 2007; Greenshields et al., 2007; Alam et al., 2025; Wu et al., 2025).

One of the most intriguing aspects of our data is the non-uniform modulation of the brassinosteroid pathway. The analysis of brassinosteroid profiles revealed a highly regulated and non-uniform response to foliar treatments and cadmium exposure, consistent with reports showing that BR biosynthesis is stress-sensitive and compound-specific (Kour et al. 2021; Miao et al. 2024). The pronounced modulation of 28-homocastasterone suggests that this intermediate functions as a key control node within the BR network, reflecting shifts in metabolic flux rather than total pathway output (Joo et al., 2015; Bajguz et al. 2020). Iron-citrate treatment promoted more coherent and treatment-specific BR profiles, particularly under cadmium stress, whereas citrate alone led to more heterogeneous responses. This indicates that iron availability stabilizes BR metabolism, likely by supporting Fe-dependent enzymatic activity and maintaining redox homeostasis, as reported for other metal stress and iron nutrition studies (Wang et al. 2015; Ravet and Pilon 2013). Under high cadmium exposure, convergence of BR responses across treatments suggests that severe stress constrains metabolic plasticity, overriding treatment-specific modulation (Singh et al. 2020; Zulfiqar et al. 2022).

Phenolic acids exhibited time and treatment-dependent dynamics, reflecting a transition from early stress signalling to longer-term metabolic adjustment. The transient modulation of salicylic acid and progressive reduction of ferulic acid following Fe-citrate application is in line with studies showing that alleviation of metal stress reduces the need for sustained secondary metabolite accumulation (Kumar et al. 2023; Jańczak-Pieniążek et al. 2022). Conversely, the persistence of ferulic acid in cadmium-treated plants indicates residual stress that is not fully compensated, as observed in other studies on heavy metal exposure (Chen et al. 2020). The differential responses of 4-hydroxycoumaric and 4-hydroxybenzoic acids highlight flexible reallocation of phenylpropanoid fluxes, which allows plants to balance defence and metabolic cost depending on stress intensity and nutrient-mediated recovery (Sharma et al. 2019).

Collectively, these results support the concept that BRs and phenolic acids act in a coordinated manner, with brassinosteroids fine-tuning growth and stress signalling, while phenolic metabolism provides a secondary, adjustable layer of defence. This integrated regulation aligns with literature suggesting multi-layered

adaptation mechanisms under heavy metal stress, with iron nutrition playing a key role in restoring metabolic and hormonal homeostasis (Kosakivska et al., 2021; Shao et al., 2007; Choppala et al., 2014).

5.3.3 Conceptual model: Fe–citrate as a metabolic recalibration agent under cadmium stress

The overall evidence supports a conceptual framework in which cadmium stress and Fe–citrate application operates at distinct regulatory layers of plant physiology.

Cadmium exposure triggers a hierarchical stress program. Under moderate concentration (Cd100), cadmium activates jasmonate-centered signalling and selective brassinosteroid redistribution, reflecting an inducible defense phase. Under severe stress (Cd200), the response shifts toward ABA dominance and enhanced phenolic accumulation, indicative of survival-oriented metabolic reallocation and intensified redox buffering. Thus, Cd drives a transition from defence activation to stress endurance mode, characterized by hormonal disequilibrium and increased investment in secondary metabolism.

In contrast, Fe–citrate application was associated with attenuation of sustained stress-related hormone accumulation, coordinated redistribution of BR intermediates, and temporal modulation of phenolic compounds, consistent with progressive metabolic stabilization under prolonged Cd exposure. These adjustments suggest that Fe–citrate contributes to restoring regulatory balance within hormone networks and secondary metabolism, likely through improved iron availability and redox-dependent metabolic coordination. By comparison, citrate alone induced partial and less coordinated responses, indicating that iron bioavailability is a central determinant of systemic metabolic recalibration. Within this framework, Fe–citrate nanoparticles function not as stress elicitors, but as metabolic modulators that rebalance hormonal crosstalk and redirect secondary metabolism toward sustained acclimation under heavy metal pressure.

5.4 Conclusions

This study demonstrates that iron–citrate nanoparticles reshape hormonal and secondary metabolic networks in spinach exposed to cadmium, promoting adaptive recalibration rather than overstimulation of stress responses. By attenuating prolonged accumulation of major stress hormones and inducing coordinated remodelling of brassinosteroid and phenylpropanoid pathways, Fe–citrate enhances physiological stability under metal pressure. The differential response compared with citrate alone highlights the central role of iron bioavailability in regulating redox-sensitive metabolic circuits. These findings position Fe–citrate nanofertilization as a promising strategy to mitigate heavy metal stress through systemic metabolic optimization, offering new insights into how nanonutrients can fine-tune plant stress physiology beyond conventional fertilization paradigms.

5.5. Supplementary Materials

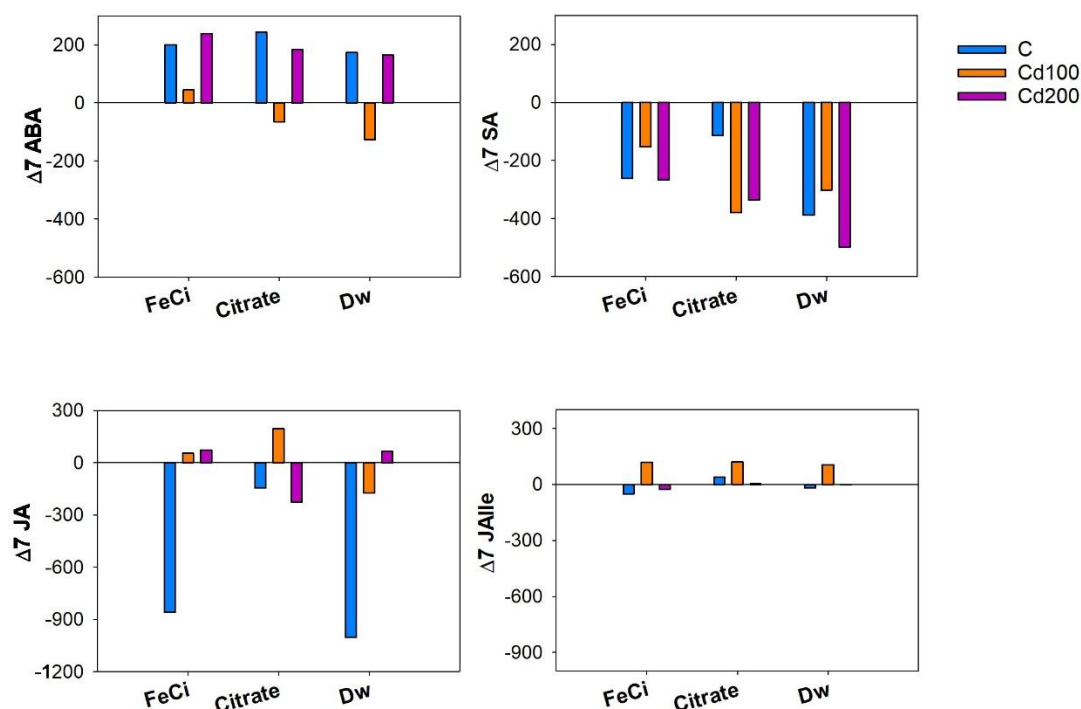
Supplementary Table S5.1: Mean values $\pm$ SD of metabolites measured in control (C) and cadmium-treated leaves (Cd100 and Cd200) after (A) three spray applications (T3) and (B) seven spray applications (T7) of iron citrate (FeCi), citrate alone, or distilled water (Dw).

A)

Metabolite	Treatment	FeCi -sprayed	Ci -sprayed	Dw -sprayed
ABA (pmol/g)	C	163.9 ± 6.6	210.3 ± 28.4	147.0 ± 5.0
	Cd100	148.1 ± 1.4	156.9 ± 15.6	172.2 ± 21.8
	Cd200	149.22 ± 10.1	138.6 ± 2.7	160 ± 6.8
SA (pmol/g)	C	287.7 ± 21.8	402.6 ± 102.3	564.0 ± 115.7
	Cd100	503.4 ± 41.2	333.0 ± 92.0	245 ± 41.5
	Cd200	378.3 ± 11.4	405.3 ± 36.5	794 ± 64.7
Ja (pmol/g)	C	1064 ± 125.6	1476 ± 106.1	901.6 ± 55.7
	Cd100	1407.7 ± 7.3	1479.9 ± 191.5	1512.0 ± 231.3
	Cd200	517.6 ± 76.9	1050.2 ± 49.3	1300 ± 104.1
JA-IIe (pmol/g)	C	210.35 ± 11.4	339.2 ± 42.14	300 ± 26.6
	Cd100	310.3 ± 24.6	360.7 ± 13.7	272.4 ± 16.8
	Cd200	209.3 ± 99.5	383.9 ± 40.3	416.3 ± 53.612
28-homocastasterone (pmol/g)	C	2565.2 ± 245.9	1332.5 ± 245.4	2100 ± 163.3
	Cd100	1939.3 ± 211.3	1900.0 ± 472.5	2013.7 ± 177.2
	Cd200	2412.6 ± 34.2	2193.2 ± 90.4	2800.0 ± 442.6
Castasterone (pmol/g)	C	9.8 ± 2.6	2.6 ± 0.33	9.3 ± 0.8
	Cd100	6.0 ± 0.8	9.2 ± 1.0	11.62 ± 0.4
	Cd200	15.3 ± 0.9	18.0 ± 5.8	5.5 ± 1.0
28-norcastasterone (pmol/g)	C	31.2 ± 3.0	29.8 ± 2.0	25.0 ± 2.2
	Cd100	33.8 ± 4.5	19.0 ± 2.93	17.7 ± 2.9
	Cd200	45.9 ± 5.1	28.5 ± 3.1	22.0 ± 2.2
24-epicastasterone (pmol/g)	C	6.8 ± 2.7	13 ± 1.6	
	Cd100	5.7 ± 4.1	7.3 ± 0.85	16.05 ± 5.61
	Cd200	19.0 ± 7.5	8.4 ± 3.4	13.2 ± 0
Brassinolide (pmol/g)	C	2.7 ± 1.3	3.7 ± 0	
	Cd100	2.8 ± 0.9	0.9 ± 0.2	1.31 ± 0
	Cd200	3.8 ± 1.8		1.3 ± 0
28-norbrassinolide (pmol/g)	C	23.1 ± 2.0	12.5 ± 0	
	Cd100	28.3 ± 3.7	18.9 ± 1.4	32.03 ± 0.6
	Cd200	34.0 ± 10.8	5.6 ± 0.6	38.83 ± 0
24-epibrassinolide (pmol/g)	C	1.4 ± 1.8	2.06 ± 0	
	Cd100	1.8 ± 0.3	1.7 ± 0.13	2.6 ± 1.74
	Cd200	5.3 ± 0.9	5.05 ± 2.006	0.7 ± 0
28-homobrassinolide (pmol/g)	C	12.6 ± 1.3	6.23 ± 0	
	Cd100	17.5 ± 2.9	8.3 ± 0.5	13.6 ± 11
	Cd200	15.5 ± 8.7	7.1 ± 0.2	11.8 ± 0
salicylic acid (pmol/g)	C	4.8 ± 2.06	7.34 ± 0.5	7.95 ± 1.64
	Cd100	5.7 ± 1.21	6.9 ± 0.34	6.0 ± 0.7
	Cd200	8.4 ± 13.5	7.43 ± 0.13	20.6 ± 0
4-OH-benzoic acid (pmol/g)	C	9.1 ± 2.5	9.4 ± 0.8	10.3 ± 1.3
	Cd100	18.2 ± 2.0	9.4 ± 0.9	12.2 ± 0.9
	Cd200	42.2 ± 2.4	65.6 ± 6.5	27.8 ± 0
Ferulic acid (pmol/g)	C	66.9 ± 19.4	62.04 ± 4.44	73.06 ± 13.43
	Cd100	74.3 ± 1.7	64.95 ± 3.3	86.2 ± 10.42
	Cd200	56.1 ± 15.6	67.23 ± 5.5	67.55 ± 0
4-OH-coumaric acid (pmol/g)	C	0.5 ± 0.1	0.74 ± 0.11	0.89 ± 0.2
	Cd100	0.6 ± 0.3	0.67 ± 0.2	0.33 ± 0.07
	Cd200	0.91 ± 1.25	0.52 ± 0.23	0.78 ± 0

B)

Metabolite	Treatment	FeCi -sprayed	Ci -sprayed	Dw -sprayed
ABA (pmol/g)	C	160.0 ± 17.0	116.8 ± 9.45	187.1 ± 16.9
	Cd100	275.8 ± 9.2	385.6 ± 12.7	447.1 ± 21.1
	Cd200	205.0 ± 10.4	260 ± 33.6	278.8 ± 16.2
SA (pmol/g)	C	355 ± 41.7	207.5 ± 120.3	481.2 ± 181.9
	Cd100	286.1 ± 32.2	512.3 ± 80.6	434.35 ± 49.8
	Cd200	315.0 ± 42.2	384.6 ± 44.4	547.7 ± 59.43
Ja (pmol/g)	C	926 ± 115.9	211.5 ± 30.8	1068.7 ± 321.3
	Cd100	267.5 ± 40.3	126.1 ± 15.9	494.3 ± 70.1
	Cd200	12 ± 1.4	310.05 ± 41.1	20.1 ± 96.0
JA-IIe (pmol/g)	C	97.0 ± 19.0	8.33 ± 1.8	64.6 ± 26.2
	Cd100	12.63 ± 1.54	11 ± 1.14	25.4 ± 3.62
	Cd200	41.0 ± 3.8	11.53 ± 34.4	17.4 ± 31.2
28-homocastasterone (pmol/g)	C	6315.9 ± 217.5	1349.4 ± 266.5	2940.5 ± 284.6
	Cd100	2594 ± 74.7	5784.1 ± 373.1	3700 ± 738.5
	Cd200	2008.1 ± 428.6	2017.7 ± 158.6	2880.0 ± 315.3
Castasterone (pmol/g)	C	6.6 ± 0.5	7.25 ± 3.8	6.5 ± 2.1
	Cd100	2.9 ± 3.5	5.23 ± 2.0	4.72 ± 0.8
	Cd200	12.0 ± 0.7	11.7 ± 0.8	18.6 ± 10.5
28-norcastasterone (pmol/g)	C	39.1 ± 4.6	44.9 ± 9.6	24.7 ± 10.3
	Cd100	26.7 ± 2.9	29.8 ± 7.2	31.2 ± 1
	Cd200	15.5 ± 3.6	15.5 ± 3.6	7.6 ± 2.2
24-epicastasterone (pmol/g)	C	6.9 ± 0.9	20.6 ± 2.24	11.97 ± 2.64
	Cd100	0.8 ± 0.1	3.6 ± 1.34	5.5 ± 0.84
	Cd200	7.0 ± 1.0	7.3 ± 1.05	7.3 ± 0.89
Brassinolide (pmol/g)	C	2.0 ± 0.1	4.2 ± 1.4	2.7 ± 0.53
	Cd100	1.4 ± 0.1	1.8 ± 0.2	1.9 ± 0.7
	Cd200	0.5 ± 0.05	0.44 ± 0.03	1.1 ± 0.3
28-norbrassinolide (pmol/g)	C	34.9 ± 2.5	34.7 ± 3.5	25.8 ± 2.3
	Cd100	20.6 ± 4.4	26.7 ± 4.7	22.3 ± 2.1
	Cd200	17.5 ± 5.2	17.5 ± 5.2	16.2 ± 2.23
24-epibrassinolide (pmol/g)	C	3.1 ± 1.1	1.2 ± 0.33	5.5 ± 1.5
	Cd100	2.2 ± 0.3	1.99 ± 0.8	2.9 ± 0.8
	Cd200	3.9 ± 1.9	3.92 ± 1.85	2.9 ± 1.11
28-homobrassinolide (pmol/g)	C	4.8 ± 0.5	7.2 ± 0.6	10.5 ± 2.44
	Cd100	12.7 ± 0.9	3.6 ± 1.4	4.0 ± 0.2
	Cd200	33.4 ± 12.6	33.44 ± 12.6	11.3 ± 2.1
salicylic acid (pmol/g)	C	4.25 ± 0.42	4.05 ± 1.7	7.9 ± 4.1
	Cd100	2.89 ± 0.23	3.57 ± 0.83	4.4 ± 0.5
	Cd200	4.18 ± 0.65	5.8 ± 0.3	6.92 ± 0.3
4-OH-benzoic acid (pmol/g)	C	55.8 ± 4.7	30.2 ± 4.9	297.0 ± 47.3
	Cd100	194.4 ± 1.1	75.7 ± 3.5	190.7 ± 26.0
	Cd200	22.5 ± 5.0	28.2 ± 0.6	18.8 ± 0.8
Ferulic acid (pmol/g)	C	16.3 ± 1.4	36.5 ± 19.61	50.9 ± 31.82
	Cd100	46.4 ± 2.5	34.25 ± 13.68	21.8 ± 5.81
	Cd200	65.5 ± 17.9	41.64 ± 0.33	40.57 ± 4.01
4-OH-coumaric acid (pmol/g)	C	0.47 ± 0.07	0.75 ± 0.3	0.86 ± 0.22
	Cd100	0.43 ± 0.11	0.36 ± 0.06	0.55 ± 0.13
	Cd200	0.84 ± 0.13	0.88 ± 0.20	0.91 ± 0.35



Supplementary Figure S5.1: Variation in abscisic acid (ABA), jasmonic acid (JA), jasmonoyl-isoleucine (JA-Ile), and salicylic acid (SA) levels in control and cadmium-treated leaves (C, Cd100, and Cd200) after seven (D7) foliar spray applications of Fe–citrate (FeCi), citrate or distilled water (Dw) expressed relative to values measured in leaves at T0.

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General Conclusions

This thesis provides an integrated view of how cadmium impacts plant physiology, metabolism, and extract bioactivity across species, and evaluates mitigation strategies based on iron oxide citrate nanoparticles.

Key findings include:

- **Physiological vulnerability under Cd:** Even at concentrations allowed by legislation, Cd compromises plant hydraulic integrity and recovery capacity, indicating that functional safety margins can be reduced without immediate yield losses.
- **Species- and compound-specific metabolic responses:** Cd exposure triggers selective remodelling of phenolic metabolism, affecting antioxidant capacity and the biological activity of plant extracts. Endemic species, such as *Salvia ceratophylloides*, may maintain bioactivity without toxicity despite stress, highlighting the value of studying phytochemical diversity.
- **Mitigation versus food safety:** Foliar application of Fe-citrate nanoparticles improves plant physiological performance under Cd stress by modulating hormonal networks and secondary metabolism. However, it does not reduce Cd accumulation in edible tissues, demonstrating that enhanced growth does not automatically translate into improved food safety.
- **Regulatory implications:** The observed physiological and metabolic effects under regulatory-compliant Cd concentrations suggest that current limits may still impose measurable stress costs, underscoring the need for integrated risk assessment frameworks that consider metabolism, water relations, and ecotoxicological endpoints.

Looking forward, these results suggest several directions for future research. Optimizing mitigation approaches to simultaneously sustain crop function and limit Cd accumulation in edible tissues will be critical. Field validation is needed to confirm the effectiveness of such interventions under realistic agricultural conditions. Expanding ecotoxicological screenings across species and bioactive compounds will help translate plant chemical changes into meaningful outcomes for human and ecosystem health. Moreover, the study of endemic or specialized species may uncover novel bioactive metabolites with inherent resilience to heavy metal stress. Finally, deeper investigation into the mechanistic interactions between metal stress, hormonal crosstalk, and secondary metabolism could provide a foundation for precision strategies aimed at improving plant tolerance while safeguarding food quality.

Overall, this work demonstrates that Cd stress is multifaceted, influencing physiological performance, metabolic networks, and bioactivity in complex and species-specific ways. Targeted interventions, such as Fe–citrate nanofertilization, can enhance functional resilience, but their application should be carefully integrated with strategies that ensure food safety and ecological sustainability.

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