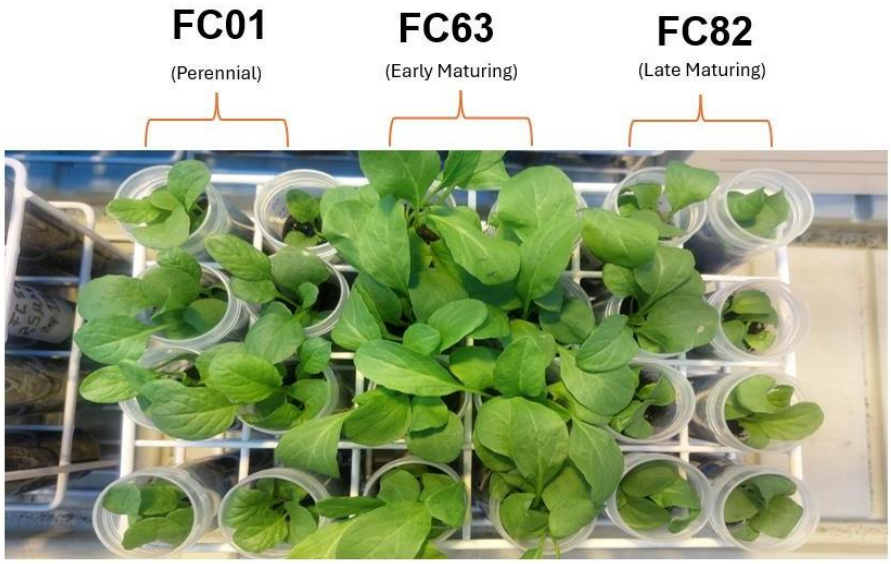
Evaluating cadmium tolerance of the novel bioenergy crop *Lepidium campestre* for its potential use in phytoremediation of agricultural soils



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Swedish University of Agricultural Sciences, SLU
Faculty of Landscape Architecture, Horticulture and Crop Production Science
Department of Plant Breeding
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Evaluating cadmium tolerance of the novel bioenergy crop *Lepidium campestre* for its potential use in phytoremediation of agricultural soils

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Abstract

Soil contamination by heavy metals, such as cadmium (Cd), has a substantial environmental and agricultural risk due to its persistence, toxicity, and tendency to accumulate in living organisms. Cadmium primarily enters the soil through human activities, such as mining, industrial emissions, and the use of phosphate fertilizers, contributing to soil degradation, decreased crop yields, and increased health risks via the food chain. This study explores the potential of *Lepidium campestre* (field cress) as a phytoremediation solution for Cd-contaminated agricultural soils. *Lepidium campestre* was selected for its potential adaptability and close genetic relationship to known cadmium (Cd)- accumulating species, such as *Arabidopsis thaliana* and *Lepidium sativum*. For comparison, durum wheat, a widely grown crop with low Cd tolerance, was also examined. Three *Lepidium campestre* genotypes, including two biennial types (FC63 and FC82) and a perennial type (FC01), were selected to establish a theoretical baseline for future assessments aimed at identifying the best-performing genotype for remediating cadmium-contaminated soils. *Lepidium campestre* and durum wheat germination rates in cadmium solution were assessed to highlight potential germination inhibition due to cadmium toxicity. The relative chlorophyll content, fresh weight, and dry weight of plants exposed to different concentrations were measured over 30 days. The results showed that durum wheat germination was significantly suppressed under Cd stress, whereas *Lepidium campestre* exhibited no substantial germination inhibition. However, notable genotypic differences emerged in biomass production, with FC01 and FC82 demonstrating higher tolerance to Cd toxicity. These results suggest that specific *Lepidium campestre* genotypes, particularly FC01 and FC82, may be promising candidates for phytoremediation due to their resilience and ability to accumulate biomass in the presence of cadmium (Cd). Future experiments should consider lengthening the evaluation period to facilitate the manifestation of cadmium effects on the plants. Additionally, using soil from contaminated fields can provide insights into the real cadmium concentration amounts in the soil and be used to validate laboratory experiments.

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1. Background

Soil is a fundamental non-renewable resource that supports terrestrial ecosystems and sustains agricultural productivity, land development, and quality surroundings (Oh et al., 2013). However, industrial emissions, improper waste disposal, mining, and the application of inorganic fertilizers on land are among the factors that have contributed to the increased accumulation of heavy metals in the soil (Kubier et al., 2019). These pollutants contain toxic metals that disrupt normal soil functions, inhibit crop growth, and pose a serious threat to human health. Most of the toxic metals found in emissions are categorized as heavy metals, which are defined as elements with a specific density greater than 5 g/cm³ (Järup, 2003). Heavy metals are considered harmful when they exceed the required environmental threshold due to their ability to persist in the environment for extended periods.

1.1 Sources and Distribution

The most common heavy metals include arsenic, cadmium, chromium, lead, zinc, and copper (Lambert et al., 2000). These metals primarily enter the soil through human activities, such as mining, industrial effluents, urban runoff, and the weathering of the Earth's crust (Morais et al., 2012). These processes alter the soil ecosystems, leading to soil degradation and adversely impacting the productivity of most arable soil.

The average cadmium concentration in soils globally is estimated to range from 0.1 to 0.5 mg kg-1 (McLaughlin et al., 1996, Smolders and Mertens, 2013). However, actual levels may vary depending on the abundance of the parent material, input through atmospheric deposition, industrial or agricultural activities, and minus output through leaching, erosion, and harvested crops (Six and Smolders, 2014). According to Ballabio et al. (2024), the Land Use/Land Cover Area Frame Survey (LUCAS), a project that monitors variations in land use and land cover in the European Union, revealed differences in Cd amounts among the soil samples collected from countries in the European Union (Figure 1). The soils in areas with excessive cadmium levels in the soil (>1 mg kg) were recorded to be as a result of

past emissions, specifically when smelters were operating under less stringent conditions (Ballabio et al., 2024).

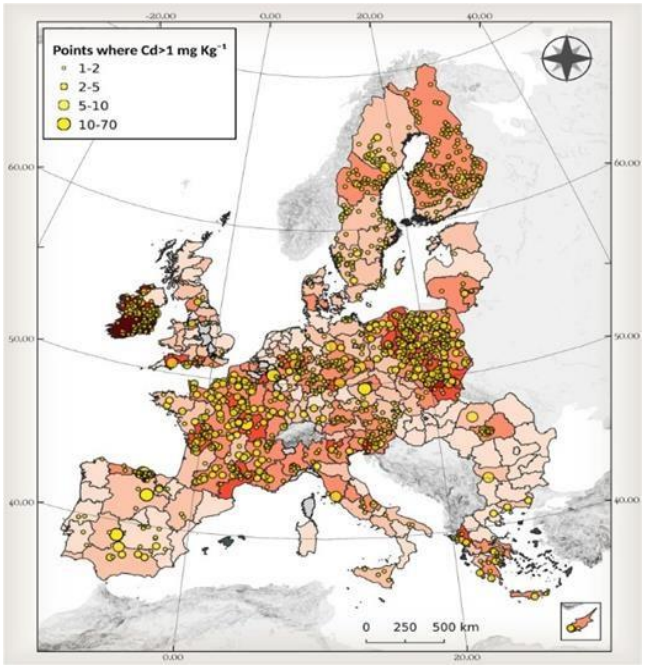

Figure 1. Soil Mapping showing the distribution and magnitude of the 1191 samples of the LCAS samples with Cd values above 1 mg·kg. Image Source.(Ballabio et al., 2024)

In Sweden, Cd emission levels have declined significantly since 1990, with aggregate emissions recorded at 500 kg as of 2023 (Naturvårdsverket, 2025). However, most arable soils in Sweden contain a significant amount of cadmium. Berndes et al. (2004) indicate that accumulated cadmium is primarily due to applying cadmium-containing phosphatic fertilizers or sludge on farms' soils. Additionally, agricultural soils in southern Sweden, specifically in Skåne, were found to contain significant amounts of cadmium, which was attributed to the presence of Cd-rich soil parent material (Söderström and Eriksson, 2013). The European Union regulations, stipulate the maximum permissible concentration of Cd in most types of cereals is 100 µg kg−1 wet weight, including winter wheat and cereal-based baby food, the limit is 40 µg kg. An assessment of cadmium contamination in agricultural fields, measured in the topsoil (0–20 cm depth) in Sweden's Skåne county, reveals that Cd concentrations in winter wheat grain harvested from the county tend to be higher than the national average (Eriksson et al., 2010). This

has been attributed to the influence of sedimentary rock, anthropogenic activities, and soil properties, such as clay content.

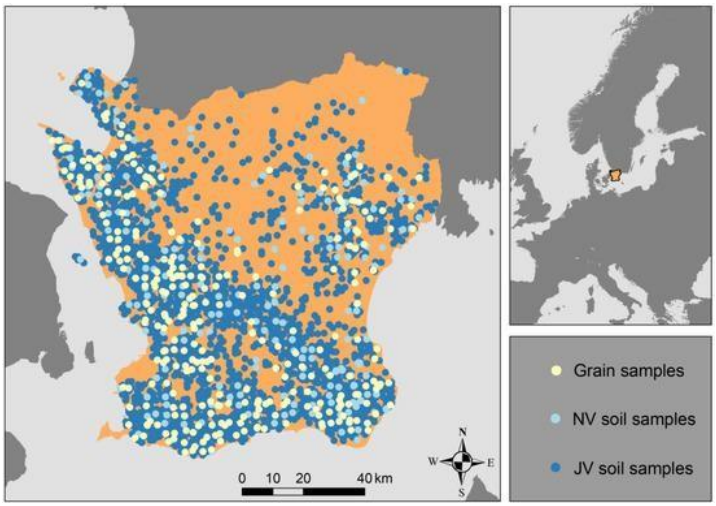

Figure 2. The map illustrates the study area in Skåne County, southern Sweden, along with various data-collection points. Light blue dots represent 304 NV (Naturvårdsverket) soil-sampling sites used to calibrate portable X-ray fluorescence and digital soil mapping (DSM) models. Dark blue dots mark 2,097 JV (Jordbruksverket) sampling sites. Combined NV and JV data were used for DSM model calibration. Additionally, light yellow dots show 307 sites with laboratory-measured cadmium concentrations in wheat grain samples. Image Source. (Adler et al., 2023)

The removal of cadmium from the soil remains low, resulting in continuous accumulation, which poses a risk to the habitability of soil microorganisms and the essential functions they undertake in the soil ecosystem, such as nitrogen fixation (Berndes et al., 2004). Mitigating these risks has necessitated effective remediation of contaminated soils to safeguard the environment.

Several remediation strategies have been established to address cadmium contamination in soil, categorized into physical, chemical, and biological methods. Physical remediation involves reversing damage to the soil through physical processes, such as soil replacement (Khalid et al., 2017). Polluted soil can be replaced with clean soil to dilute metal concentration and restore functionality (Khalid et al., 2017). To extract heavy metals, chemical remediation involves reducing the toxicity and migration ability of metals, such as through electroplating and soil

flushing (Sun et al., 2018). However, physical and chemical methods are costly, time-consuming, and often only temporarily effective, limiting their large-scale application (Lata et al., 2021; Sun et al., 2018). This has led to increased attention to biological methods, such as phytoremediation and bioremediation, which are considered eco-friendly and safe.

Bioremediation involves utilizing microorganisms to break down heavy metals in contaminated soils (Sun et al., 2018). However, several limitations hinder the effectiveness of this approach since there are limited microorganisms that can accumulate heavy metals, and they may also face competition from indigenous strains (Sun et al., 2018). Phytoremediation involves using plants to reduce or eliminate heavy metals in the soil and has been a key research area for developing sustainable alternatives for growers to remediate contaminated soil. This research study evaluates the tolerance of three *Lepidium campestre* genotypes to cadmium, aiming to gain insight into the phytoremediation potential of this novel bioenergy and cover crop for restoring cadmium-contaminated arable land.

1.2 Cadmium: Its uptake and effect on plants

The movement and uptake of heavy metals, such as cadmium, in the soil are influenced by several factors, including organic matter, mineral composition, and prevailing environmental conditions (Subašić et al., 2022). According to Shiyu et al. (2020), cadmium is relatively water-soluble under acidic conditions while exhibiting neutral solubility in alkaline soils. The uptake of cadmium from the soil to the plant shoots is a highly regulated process involving metal transporters in the plasma membrane of the root cell and translocation through the xylem and phloem of the plant (Ismael et al., 2018). Cadmium uptake in plant roots can occur through two major pathways of water flow: the apoplastic and symplastic pathways (Ismael et al., 2018). In the apoplastic pathway, metal ions accumulate in the root apoplast due to electrostatic interactions between positively charged metal cations and deprotonated, negatively charged carboxyl groups (Ismael et al., 2018). On the other hand, symplastic uptake depends on metabolic activity and is

considered a slower process (Ismael et al., 2018). The process may vary depending on the plant species, the concentration of heavy metals, or metal ions such as Fe^{2+}, Mg^{2+}, and Zn^{2} (Ismael et al., 2018).

The potential effects of plant cadmium uptake mainly impact the plant's growth and physiology. Cadmium accumulation in plant tissues can lead to plant death by disrupting essential functions such as enzyme activity, respiration, and photosynthesis (Subašić et al., 2022). Even more concerning, cadmium can be easily absorbed by plant roots (Subašić et al., 2022; Shiyu et al., 2020) and translocated along the food chain, eventually leading to bioaccumulation in the human body and an increase in health-related diseases. Clemens et al. (2001) also indicate that plants' absorption of other mineral elements, such as calcium, iron, and magnesium, can be restricted due to competition between cadmium and other cations in the pathway's mineral uptake from the soil to the root. Consequently, plants often become deficient in these nutrients, which negatively impacts their productivity. The damaging effects of cadmium in the soil and plants have created a need for soil remediation.

1.3 Phytoremediation of Cd-contaminated soils

Over the years, extensive research has been done on the phytoremediation of polluted soils. To date, the practical application of phytoremediation remains limited despite research studies highlighting its potential and benefits in experiments conducted on a small scale. Nevertheless, the call to adopt sustainable practices has led to enhanced exploration and assessment of plant species that can be utilized in soil remediation.

Phytoremediation is an environmentally friendly approach that reduces heavy metals in contaminated soil by assimilation or immobilization using plants (Dai et al., 2024). Depending on the plant type used, phytoremediation can occur through various mechanisms, including phytostabilization, phytoextraction, and phytovolatization (Dai et al., 2024). Phytostabilization involves using plants that restrict the uptake of heavy metals only at the roots and hinder their transportation to the aerial parts of the plant (Cioica et al., 2019). Phytoextraction involves absorbing the contaminant through the roots and transporting it to the aerial parts

without negatively impacting their growth and development until the plant is harvested. These plants can concentrate large quantities of heavy metals in their above-ground plant parts and have thus been found suitable for remediating contaminated soils (Cioica et al., 2019; Oh et al., 2013). The phytoextraction technique enables plants to extract heavy metals from water and soil media by forming complexes through chelation with these elements and their metabolites, thereby reducing toxicity (Khan et al., 2023).

Phytovolatization involves plants absorbing volatile metallic contaminants, processing them into less toxic compounds within the plant, and releasing them into the atmosphere as vapour through transpiration (Cioica et al., 2019). The volatile contaminants include substances such as Arsenic (As) and mercury (Hg), which can be evaporated from plant parts (Khan et al., 2023). This process aims to detoxify both hazardous inorganic and organic contaminants.

1.3.1 Plant Tolerance

Phytoremediation primarily relies on plant species that can thrive in metal-rich soils and accumulate heavy metals in their above-ground tissues at concentrations exceeding normal levels (Sytar et al., 2021). Additionally, defining characteristics such as fast growth, high biomass production, tolerance when grown in contaminated soil, and ease of harvest are essential for successful phytoremediation (Khan et al., 2023). The effectiveness of plants in extracting heavy metals is closely related to a variety of genes, whose expression products mainly include metal transporters (Pence et al., 2000), phytochelatin synthase (PCS), metallothioneins (MTs) and metal reductase (Ellis et al., 2006). These proteins are essential in plants' absorption, transport and partition of heavy metals.

Cadmium concentration amounts vary significantly across different types of soil and different areas. A soil survey conducted for European topsoil demonstrated the mean Cd concentration in EU topsoils is 0.20 mg/kg, with croplands averaging 0.17 mg/kg and grasslands 0.24 mg/kg with only around 5.5 % of samples collected exceed 1 mg/kg, considered the risk threshold (Ballabio et al., 2024). In research studies, cadmium concentration varies depending on the exper-

imental setup, where cadmium concentrations used tend to be higher than the recommended threshold of 0.1 to 0.5 mg kg-1 that should be found in soil. In this study, cadmium concentrations (25µM, 50µM, and 100 µM) were used to investigate the effects of different Cd concentrations on the growth and development of *Lepidium campestre* and durum wheat genotypes. Research studies by Jiao et al. (2024) and Jia et al. (2016), which entailed hydroponic experiments, serve as good examples, highlighting the similar cadmium concentrations used in the study evaluations. According to a research study by Jiao et al. (2024), a cadmium concentration greater than 5 µM was considered a high Cd treatment compared to a low Cd treatment, which ranged between 0.1 and 0.5 µM. Since research studies have shown that high cadmium concentrations can have a negative impact on plants, it would be prudent to evaluate whether they have a substantial effect on *Lepidium campestre* and durum wheat genotypes.

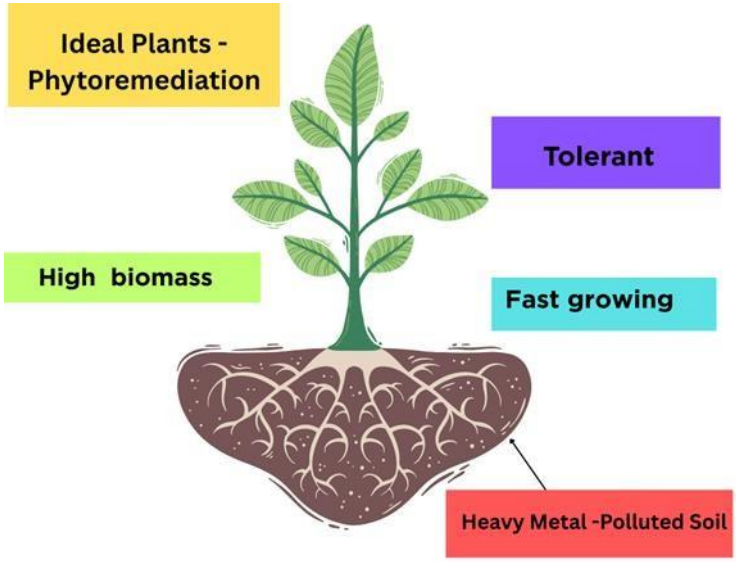

Figure 3. Highlights the key traits that ideal plants should possess for effective phytoremediation of cadmium-contaminated soil. Image source: Own conceptualization.

1.3.2 Plant Species

The absorption, translocation, and distribution of metals within a plant have been observed to differ across plant species, even when they are planted in the same contaminated site (Wan et al., 2024). *Lepidium campestre* and durum

wheat were utilized in this study to evaluate potential differences in cadmium accumulation from contaminated soil, as well as to evaluate the impact of cadmium exposure on plant growth and development.

1.3.2.1 *Lepidium campestre*

Lepidium campestre, also known as field cress, is an oilseed plant belonging to the Brassicaceae family (Gustafsson et al., 2018). It is native to Europe and Asia but has also naturalized in many parts of North America. The plant is typically biennial, exhibiting some annual growth. *Lepidium campestre* is considered resistant to pollen beetles, as Merker and Nilsson (1995) observed from field trials that the pollen beetle (*Meligethes aeneus*), which is an important insect pest in Brassica oilseed crops, is attracted to the inflorescences of *L. campestre* but does not cause any damage to the buds. Börjesdotter (1999) indicated that the reason could be attributed to the small buds, which are (<2 mm), making it an inappropriate host plant for this insect. One of the plant's most notable features is the flower raceme that emerges from its stems. The plant is also densely covered with tiny hair. The plant is recorded to have approximately a 30% higher yielding potential than the average winter oilseed rape (Ivarson et al., 2013). *Lepidium campestre* is cold-hardy and can be grown in areas where other oil crops cannot (Ivarson et al., 2013).

The plant has great potential to become a new oil crop. Ongoing research studies on *L. campestre* focus on its domestication and aim to promote its adoption as a catch, cover, and oil crop (Gustafsson et al., 2018). This is because nutrient leaching is a challenge that affects most arable lands in Sweden. One contributing factor is that most farms rely on tillage practices that enhance soil leaching. The plant can be undersown with spring cereal, which is harvested in the first year, and field cress, used as an oil crop, in the second year (Gustafsson et al., 2018). A study by Merker et al. (2010) found that undersowing *L. campestre* with barley has a positive effect on yield. This can be beneficial in the agricultural sector by reducing nitrogen (N) leaching into the soil (Ulén and Aronsson, 2018), thereby potentially increasing N uptake for subsequent crops. Its multifunctionality makes it a valuable economic crop that can be a cost-effective and sustainable method for growers and plant production companies to restore soil.

Lepidium campestre selection in potentially remediating cadmium-contaminated soil was due to the close linkage to *L. sativum*, both from the same genus *(Lepidium*) and family (Brassicaceae), as it demonstrated potential in remediating Heavy metal-contaminated soil. Studies on *L. sativum* L have recorded it as a hyperaccumulating plant that can be used in extracting cadmium (Cd) and lead (Pb) (Cioica et al., 2019).

1.3.2.2 Durum Wheat

Durum wheat, identified as *Triticum durum*, is commonly cultivated in North and East Africa, West Asia, India, and Mediterranean Europe. Due to its desirable traits, the expanding value chain of its industrial products and increased demand for food products such as pasta have significantly contributed to its preference for farm production. Durum production depends on many abiotic, chemical, and physical factors that vary across different environments. Fluctuations or alterations of the present environmental factors may lead to adverse physiological and morphological changes in the plant. The selection of durum wheat in this experiment was to illustrate the adverse effect of cadmium on a non-tolerant cultivar compared to field cress species, which are considerably tolerant when exposed to heavy metals.

1.3.2.3 Functionality

The model plant *Arabidopsis thaliana,* closely related to *Lepidium campestre*, which is derived from the Brassicaceae family, is recorded as tolerant to cadmium (Cd) toxicity, a trait likely attributed to the presence of specific Cd transporter genes. The phylogenetic proximity of *L. campestre* to *A. thaliana* provides a valuable framework for evaluating Cd tolerance. Comparing *L. campestre* to durum wheat may provide insights into the growth performance and adaptability of both plants to cadmium stress. These insights may support the use of *L. campestre* not only as an oilseed and catch crop but also as a sustainable candidate for phytoremediation efforts aimed at restoring environments contaminated with heavy metals.

1.4 The aim and objective

The primary objective of this research is to assess the tolerance of *L. campestre* to cadmium-contaminated soil, thereby contributing to its development as an oilseed crop and providing a sustainable solution for soil remediation. In addition, to assess the potential use of *Lepidium campestre* in phytoremediation, analysing the amount of cadmium extracted by L. campestre genotypes in comparison to durum wheat is fundamental to verify if there are substantial differences in heavy metal uptake by the plant.

The specific objectives were

- Evaluate the Cd tolerance of three *L. campestre* genotypes exhibiting differences in the life cycle (biennial or perennial) and maturity (early or late maturing).
- Compare the Cd tolerance level of *L. campestre* genotypes with durum wheat.
- Sequence analysis and functional prediction of Cd transporting genes in *L. campestre* and durum wheat.

2. Materials and Methods

2.1 Plant Material

Three genotypes of *L. campestre* were selected: two biennial types, FC63 and FC82, and a perennial type, FC01. The biennials were chosen for their defined early maturity (FC63) and late maturity (FC82) characteristics. The genotypes were obtained from the ongoing crossbreeding-based domestication of *L. campestre.* On the other hand, durum wheat (TD061), primarily cultivated for food production, was used as a non-tolerant crop compared with *L. campestre*.

2.2 Soil Components

The growing media used for planting was supplied by SW Horto, a company dedicated to refining the future of plant landscapes in cultivation and lush green spaces. The soil consisted of green materials and clay, providing a nutrient-rich substrate (https://swhorto.se/); however, the proportions of the soil components were neither specified nor measured. The soil is certified by KRAV for use in organic farming.

2.3 Experiment Setup

Seeds of *L. campestre* and Durum wheat genotypes were provided and sown in the greenhouse in SLU Alnarp. The greenhouse conditions ranged between 23°C during the day and 18°C at night, with artificial lighting from 6:00 to 18:00. Seeds of *L. campestre* and Durum wheat genotypes were germinated in petri dishes in the dark for three days and then exposed to light. After seven days, the seedlings were transplanted into 50 mL Falcon tubes, labelled according to plant genotype, and filled with soil up to the 45 mL mark.

The experiment consisted of three genotypes of *L. campestre* (FC63, FC82, FC01), one genotype of durum wheat (TD061), and four treatments: T1 (Control), T2 (watered with 25 µM of Cd solution), T3 (watered with 50 µM of Cd solution), and T4 (watered with 100 µM of Cd solution). The different cadmium concentrations were defined, as research studies have indicated that plants exhibit toxicity symptoms through physiological changes, such as alterations in photosynthesis and plant morphology. The exposure of *L. campestre* to different

cadmium concentrations would facilitate the assessment of translocation, biochemical stress and plant morphological change as the basis of cadmium tolerance. Each treatment per genotype consisted of eight biological replicates arranged in a completely randomized design (CRD), resulting in each genotype comprising 32 plants, and a total of 128 plants were assessed in the experiment. The experimental setup was repeated in triplicate.

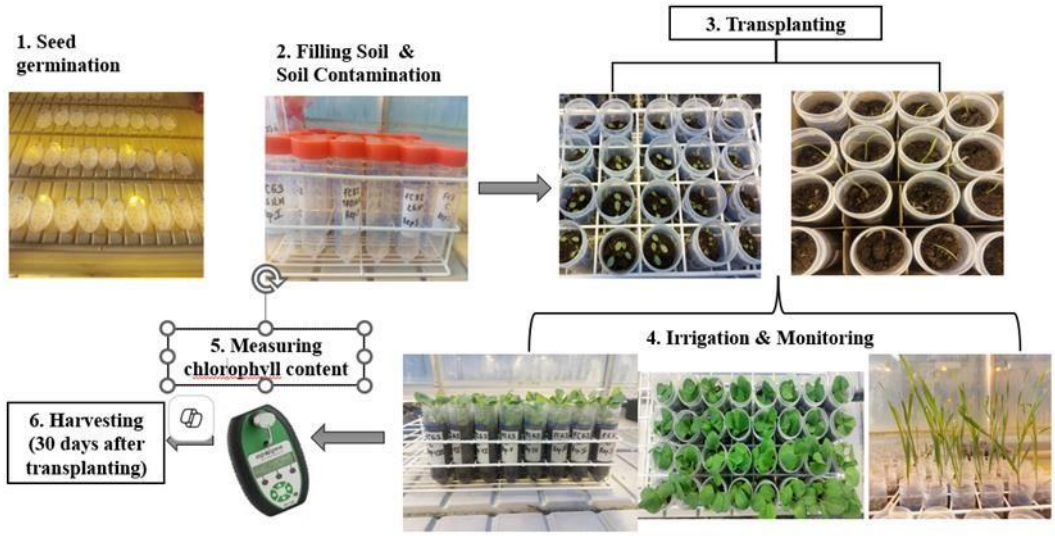

Figure 4. Illustrates experimental setup initiated from seed germination of *Lepidium campestre* and durum wheat genotypes, followed by transplanting and subsequent monitoring of growth parameters. The plants were irrigated at defined intervals with cadmium solutions and Milli-Q water until harvest.

2.3.1 Preparing cadmium solutions

The Cd stock solution of 1 molar (1M) was prepared using cadmium nitrate tetrahydrate, $Cd(NO3)_2.4H_2O$ (MW = 308.48), which was dissolved using 1 litre of MilliQ water using the formula below. The Milli-Q® EQ 7000 Ultrapure Water Purification System dispensed purified water used in formulating the cadmium solutions. The flask was gently shaken until it dissolved completely. The cadmium stock solution was used to create different concentrations of the cadmium solution (25 µM, 50 µM, and 100 µM), representing the experimental treatments described above. The concentration of the stock solution was determined through the molar dilution equation.

$$C = \frac{m}{V} \times \frac{1}{MW}$$

Where C is the final stock concentration, m is the mass of the solute, V is the final volume, and MW is the molecular weight of the solute.

The preparation of the different $Cd(NO3)_2.4H_2O$ solution treatments, at 25 µM, 50 µM and 100 µM, was calculated based on the dilution factor C1V1=C2V2. Considering that the stock solution has a concentration of 1000 µM, the quantity required to make 100 mL of a 100 µM $Cd(NO3)_2.4H_2O$ solution was 10 mL. Accordingly, these treatments were prepared in a final volume of 500 ml by diluting the stock solution in Milli-Q water, as shown in Table 1.

Table 1. The dilution rate of the different concentrations of cadmium solution

Cadmium Solution Concentration	Volume of Stock Solution (ml)	The volume of Water (ml)	Final volume (ml)
25 µM	12.5	487.5	500
50 µM	25	475	500
100 µM	50	450	500

2.3.2 Application of Cd treatments

The soil was watered by pipetting 5 ml of $Cd(NO3)_2.4H_2O$ solution 24 hours before seedling transplantation. The soils were irrigated by pipetting 5 ml of the respective treatments on the day after transplanting. Post-transplantation irrigation was performed every 48 hours, except on Fridays, when 7.5 ml was administered across all treatments to accommodate the long weekend.

After a three-week growth period, the above-ground parts were harvested, and the fresh weight was measured. Plant samples were then oven-dried for two days at 65°C using the Memmert GmbH & Co. KG incubator from Germany. The dry plant and soil samples were randomly selected for further processing to assess the amounts of $Cd(NO3)_2 \cdot 4H_2O$ extracted from the soil compared to those in the above-ground parts.

2.4 Germination Test

2.4.1 Seed Viability

The seeds were germinated in petri dishes with a double layer of filter paper moistened with Milli-Q water, with each population consisting of 10 *L. campestre* seeds and approximately 7-8 Durum wheat seeds. The petri dishes were placed in the SLU greenhouse at 23°C with a light duration of 10-12 hours. The optimum temperatures for germinating *L. campestre* seeds have been recorded to range between 16°C and 18°C (Mohammed and Mummenhoff, 2025). The seeds were kept in the dark for 2-3 days and then exposed to light. The germination test ended after a few days, with seedlings in good condition transplanted into their respective Falcon tubes. The number of seeds used in the three replicates varied, with 156 seeds for TD-061 (Durum wheat), 206 for FC82, and 270 for each of the FC63 and FC01 *L. campestre* genotypes.

2.4.2 Seed germination to Cadmium exposure

Lepidium campestre and Durum wheat seeds were germinated in petri dishes with a double layer of filter paper moistened with 2500 µL Milli-Q water (Control) and $Cd(NO3)_2.4H_2O$ solutions (25 µM, 50 µM, and 100 µM). Fifty seeds of each *L. campestre* genotype and 15 seeds of durum wheat were used per treatment and germinated in the dark in Petri dishes containing different concentrations of $Cd(NO3)_2.4H_2O$ solution. After three days, the petri dishes were exposed to light. The petri dishes were moistened with 1500µL of Milli-Q water and cadmium solutions corresponding to their respective treatments every 48 hours. The total volume of both solutions irrigated was 8.5 mL (8500 µL). The number of germinated seeds for both *L. campestre* genotypes and durum wheat genotypes was recorded following a 7-day growth period.

2.5 Measurement of Chlorophyll Content

One of the crucial indicators for evaluating a plant's physiological mechanism and productivity is its leaf chlorophyll content, which indicates the plant's photosynthetic capacity, development, and nutritional status (Liu et al., 2019). The chlorophyll content of the leaves was estimated 14 days after transplanting using the Apogee Instruments portable chlorophyll concentration meter, which obtained

SPAD values. The SPAD (Soil Plant Analysis Development) portable chlorophyll meter was used for its non-destructive measurements. Five large leaves were sampled from each tube in the respective treatments. Readings were not taken in tubes with tiny leaves. This was conducted for all experiment replicates.

2.5.1 Chlorophyll Meter Measurements

The estimation of SPAD values using the chlorophyll concentration meter is based on leaf transmittances at 650 nm and 940 nm. The chlorophyll concentration meter measures the ratio of red and near-infrared transmittance with a sample rate of less than 3 seconds, resulting in non-destructive and nearly instantaneous measurements. (Apogeeinstruments, 2025). The leaf transmittances measured at three sampled points on the leaf are averaged to obtain a SPAD value. The SPAD value is based on the ratio of transmittance at 940 nm to transmittance at 650nm, which measures relative chlorophyll content. In most SPAD chlorophyll meters, the relationship between the leaf transmittances and the SPAD values is translated with a coefficient of determination (R2) of 0.998 (Raymond Hunt Jr and Daughtry, 2014).

The Equation

$$SPAD = 37 * \log10(T940/T650) - 2.68$$

SPAD is the SPAD value, and $T940$ and $T650$ are leaf transmittances at 940 nm and 650 nm, respectively.

2.6 Above-ground plant biomass

After a 24-day growth period, the above-ground parts of all plants were harvested and placed in measuring envelopes. The fresh weight was measured using a Mettler Toledo Balance XSR105-Dual Range. Subsequently, the plant samples were oven-dried at 65°C for 48 hours using a Memmert GmbH & Co. KG incubator from Germany. Once dried, their weights were again measured using the same balance, and the individual dry weights were recorded. This was done for all experiment replicates.

2.7 Cadmium extracted

The dried plant and soil samples were retained for analysis to quantify cadmium accumulation in above-ground plant tissues and to compare it with the cadmium concentration in the soil. These data will be integrated into the study upon completion of the analysis and the availability of the results.

2.8 Statistical Analysis.

The germination rate (GD) for each genotype in the two tests was calculated using the equation.

GD (%) = (Number of germinated seeds/number of total seeds) × 100

The data were plotted using Excel to illustrate the variances among genotypes in seed viability and their responses to different cadmium treatments.

Data was prepared and plotted in Excel for all variables measured (chlorophyll content, fresh weight and dry weight). The normality of the data was evaluated using the Shapiro-Wilk test, which revealed that all variables produced a p-value greater than 0.05, confirming that the assumption of normal distribution was satisfied. One-way and Two-way ANOVA analyses were conducted in R Studio for each trait measured on durum wheat and *L. campestre*, respectively. Tukey's post hoc analysis of each trait was performed through pairwise comparisons, and estimated marginal means (emmeans) were obtained for statistically significant factors. Additionally, correlation analysis was conducted in R Studio to examine the relationships between variables.

2.9 Sequence analysis of cadmium-transporting genes

The protein sequence of NRAMP1 (Natural Resistance-Associated Macrophage Protein 1) from *Arabidopsis thaliana* was retrieved from the NCBI GenBank database found on the (https://www.ncbi.nlm.nih.gov) website. A keyword search for "*Nramp1 Arabidopsis thaliana*" revealed a total of 18 ref sequences relating to the *Nramp* protein. Among them, the *Nramp1 Arabidopsis thaliana* sequence with Accession No. AAF36535, consisting of 532 amino acids, was selected for further analysis. This sequence was used as a query for blastp analysis against a *Lepidium* genome and *Triticum durum*. The sequence with the highest similarity was selected for further comparative analysis.

2.8.1 Phylogenetic Tree

The aligned sequences of Nramp1-6 of *Arabidopsis thaliana* were used to construct a phylogenetic tree in Mafft (v7.511) using the Neighbour-Joining (NJ) method with six sequences. The default setting of Mafft was used for sequence alignment. The phylogenetic tree was based on 461 conserved sites with a JTT substitution model and 1000 bootstraps. The tree was visualized using Phylo.io values above 50%, which were indicated on major branches.

2.8.2 3D Model Predictions and Multiple Sequence Alignment of Nramp1 Proteins

The identified *Nramp* protein sequences were submitted to the SWISS-MODEL Expasy web tool (https://swissmodel.expasy.org) for comparative 3D structure models of the proteins. The predicted models were evaluated for structural quality and alignment using GMQE values, and the model with the highest sequence identity coverage was selected for *Arabidopsis, Lepidium*, and durum wheat sequences. Multiple sequence alignment was performed using the CLUSTAL Omega website (https://www.ebi.ac.uk/jdispatcher/msa/clustalo) to evaluate sequence conservation and assess the phylogenetic proximity of AAF36535.

NRAMP1 protein (*Arabidopsis thaliana*) to BBH56030 natural resistance-associated macrophage protein 3 (*Lepidium virginicum*) and VAI79988 unnamed protein product (*Triticum turgidum* subsp. durum). The aligned sequences were further analyzed to identify conserved and structural variations in the cadmium-transporting gene in these species.

3. Results

3.1 Germination potential without cadmium treatment

The seed viability for *Lepidium campestre* genotypes (FC63, FC82, FC01) and Durum Wheat (TD-061) was examined to compare intra-species variation among three *L. campestre* genotypes and contrast them with a single durum wheat genotype as an inter-species comparison. The variance in seed viability among the genotypes was evaluated by comparing the total number of seeds germinated with those that did not. Seeds were considered germinated through the emergence of the radicle from the seeds. FC63 exhibited the highest overall germination percentage (97%), while FC82 showed the lowest germination (56 %) across the *L. campestre* genotypes, as illustrated in Figure 5.

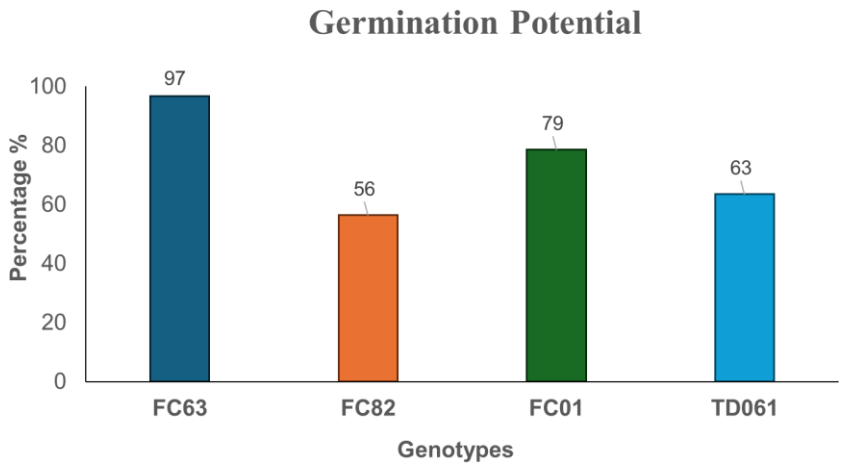

Figure 5. Overall germination percentage of *Lepidium campestre* and durum wheat genotypes

3.1.1 Germination in cadmium Solution

The Anova analysis of the germination rate revealed there was no significant impact of the cadmium treatment on the germination rate. *Lepidium campestre* and Durum wheat seeds were further assessed through exposure to different concentrations of Cd(NO3)2.4H2O solution, with the germination rate illustrated in Figure 6. Numerical observations indicated an increasing trend in germination rate for genotypes FC63, FC01, and FC76 under 25 µM and 50 µM cadmium treatments compared to the control. However, in the 100 µM treatment, FC63 and

FC01 demonstrated a decline in germination rate compared to the control. FC76 showed an increase in germination within the treatment compared to the control. TD-061 decreased germination at 25 µM and 100 µM, while increased germination was observed at 50 µM compared to the control.

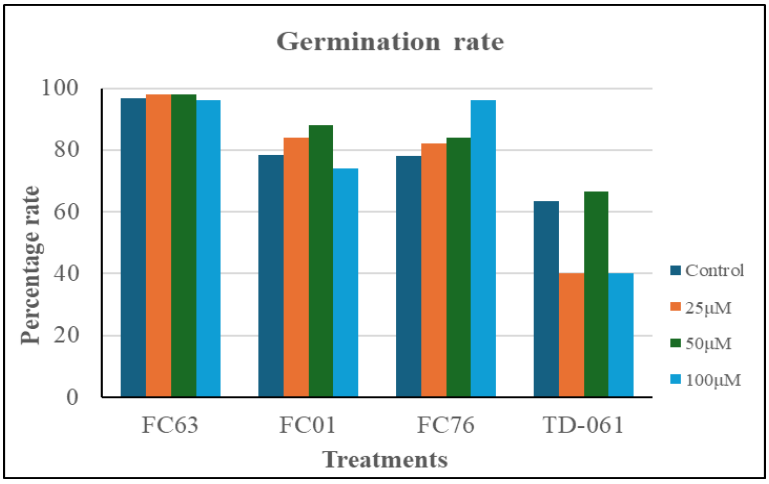

Figure 6. Germination rate of *Lepidium campestre* genotypes (FC63, FC82, FC01) and durum wheat genotype (TD-061) grown to different concentrations of cadmium solution (25 µM, 50 µM, and 100 µM) and control.

Table 2. Summary of ANOVA results for treatment effect.

Source	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	3	1606	535.2	1.259	0.332
Residuals	12	5103	425.3		

Estimation of Chlorophyll Content

The analysis of variance (ANOVA) revealed that there was no significant difference ($P > 0.05$) in relative Chlorophyll Content (CC) attributable to genotype, treatment, or the interaction of genotype with treatment (Table 3). On a numerical level, genotypes FC63 and FC01 exhibited higher mean chlorophyll content under cadmium treatments of 25 µM, 50 µM, and 100 µM compared to the control (*Figure 7*). Specifically, FC63 obtained chlorophyll content of (32.26 ± 0.43), (32.97 ± 0.51), and (32.37 ± 0.60), respectively, under the three cadmium treatments (25 µM, 50 µM, and 100 µM), compared to 30.95 ± 0.51 in the control.

Similarly, FC01 obtained 33.06 ± 0.68, 32.83 ± 0.73, and 32.62 ± 0.79, respectively, under cadmium treatments (25 µM, 50 µM, and 100 µM), compared to 32.24 ± 0.67 in the control. The FC82 genotype, however, showed a declining trend in fresh weight under cadmium exposure: 34.50 ± 1.48 (25 µM), 35.09 ± 1.37 (50 µM), and 33.17 ± 0.82 (100 µM), all of which were lower than the control value of 36.11 ± 1.33 (Figure 7). The observed increase in fresh weight for FC63 and FC01 under cadmium treatments may indicate a potential adaptive strategy employed by these genotypes in response to cadmium stress.

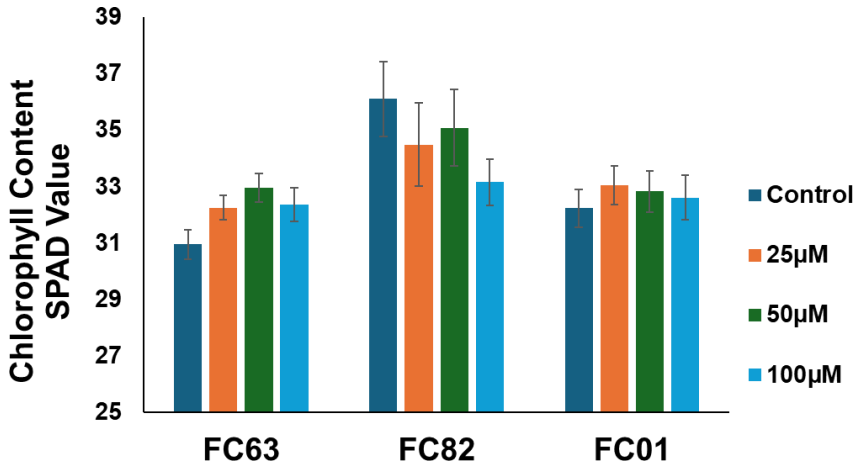

Figure 7. Estimated Chlorophyll content of three *Lepidium campestre* genotypes evaluated in different cadmium treatments and control conditions; Error bars represent standard error.

Table 3. Two-Way ANOVA Sum of Squares and Mean Squares of estimated chlorophyll content of the *Lepidium campestre* genotypes in cadmium treatments and control

Source of variation	Df	Sum Sq	Mean Sq	F value	Pr(>F)	Significance
Genotype	2	25.57	12.785	1.616	0.220	ns
Treatment	3	0.66	0.222	0.028	0.994	ns
Genotype × Treatment	6	25.00	4.167	0.527	0.782	ns
Residuals	24	189.88	7.912	—	—	—

Df; Degree of freedom, SS; Sum of Squares, MS: Mean Square, ns; non-significant.

3.2 Plant Biomass for *Lepidium campestre* genotypes

3.3.1 Fresh Weight

ANOVA analysis indicated that the genotype factor had a significant effect on fresh weight ($p = 0.000399$) (Table 4). In contrast, treatment ($p = 0.538664$) and the genotype-treatment interaction ($p = 0.660906$) did not significantly influence fresh weight, indicating that neither the cadmium treatment type nor its interaction with genotype had a significant impact.

On a numerical level, genotypes FC82 and FC01 exhibited higher mean fresh weights under cadmium treatments of 25 µM, 50 µM, and 100 µM compared to their respective control conditions (Figure 8). Specifically, FC82 obtained fresh weights of 443.03 ± 44.63mg, 484.00 ± 44.63mg, and 416.50 ± 47.21 mg, respectively, under the three cadmium treatments (25 µM, 50 µM, and 100 µM), compared to 378.84 ± 47.46 mg in the control condition. Similarly, FC01 recorded 588.53 ± 31.62 mg, 560.11 ± 33.76 mg, and 577.96 ± 27.99 mg, respectively, under cadmium treatments (25 µM, 50 µM, and 100 µM), compared to 549.64 ± 33.12 mg in the control. The FC63 genotype, however, showed a declining trend in fresh weight under cadmium exposure: 598.33 ± 12.85 mg (25 µM), 615.48 ± 29.30 mg (50 µM), and 537.58 ± 24.73 mg (100 µM), all of which were lower than the control value of 653.15 ± 14.70 mg (Figure 8).

Analysis of the significant effect attributed to the genotype factor through pairwise comparisons revealed significant differences between genotypes FC01 and FC82 ($p = 0.0042$) and between FC63 and FC82 ($p = 0.0005$), both of which had p-values below the 0.05 significance level (Table 5*)*. However, no significant difference was observed between FC01 and FC63 ($p = 0.6779$), indicating similar fresh weight performance between these two genotypes. The estimated marginal means (emmeans) for *L. campestre* genotypes were extracted to support the results. The genotype FC82 recorded the lowest mean value (425 ± 28.1), compared to FC63 (Mean = 601 ± 28.1) and FC01 (Mean = 567 ± 28.1), which had higher values. There was no significant difference between FC63 and FC01, which were assigned to group "b", compared to FC82, which was assigned to group "a" (Figure 9*)*.

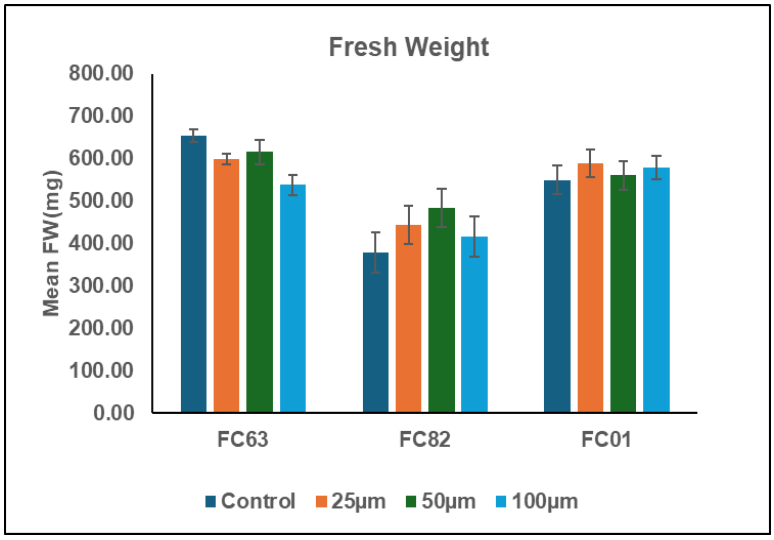

Figure 8. Mean Fresh weight (FW) of above-ground plant parts for three *Lepidium campestre* genotypes exposed to different cadmium treatments and control conditions; Error bars represent the standard error.

Table 4. Two-way ANOVA (Sum of Squares and Mean Squares) of fresh weight of *Lepidium campestre* genotypes in cadmium treatments and control

Source of variation	Df	Sum Sq	Mean Sq	F value	Pr(>F)	Significance
Genotype	2	209507	104754	11.038	0.000399	***
Treatment	3	21065	7022	0.74	0.538664	n.s.
Genotype × Treatment	6	39201	6534	0.688	0.660906	n.s.
Residuals	24	227771	9490	—		

Df; Degree of freedom, SS; Sum of Squares, MS: Mean Square,
Significance levels: p >0.05 non-significant (ns), *** p < 0.001.

Table 5*:* Tukey HSD Pairwise Comparisons *Lepidium campestre* genotypes

Contrast	Estimate	Std. Error	df	t-ratio	p-value
FC01 – FC63	-33.69	39.77	24	-0.847	0.6779 ns
FC01 – FC82	142.33	39.77	24	3.579	0.0042 **
FC63 – FC82	176.02	39.77	24	4.426	0.0005 ***

significance levels: p >0.05 not significant (ns), ** p < 0.01, *** p < 0.001

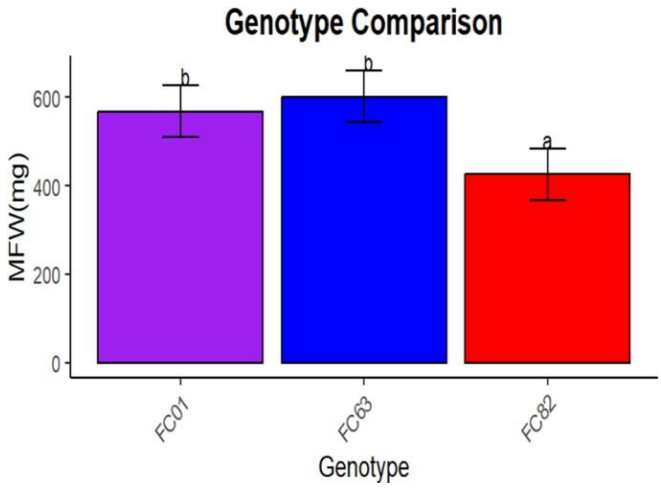

Figure 9. Genotype Comparison on the mean fresh weight (MFW) for *Lepidium Campestre* genotypes with error bars highlighting significant differences between the genotypes

3.2.1 Dry Weight

ANOVA revealed that the genotype factor significantly affected fresh weight ($p = 0.000134$). However, treatment ($p = 0.974544$) and the genotype with treatment interaction ($p = 0.464478$) did not have a significant impact on dry weight, as demonstrated in Table 6.

On a numerical level, genotypes FC82 exhibited higher mean fresh weights under cadmium treatments of 25 µM, 50 µM, and 100 µM compared to their respective control conditions (Figure 10). Specifically, FC82 obtained dry weights of 80.47 ± 10.79 mg, 89.00 ± 11.84 mg, and 90.11 ± 12.00 mg, respectively, under the three cadmium treatments (25 µM, 50 µM, and 100 µM), compared to 63.92 ± 10.55 mg in the control. Similarly, FC01 obtained a high dry mass of 128.11 ± 6.58 mg and 131.47 ± 6.23 mg, respectively, under cadmium treatments (25 µM and 100 µM), compared to 126.16 ± 10.43 mg in the control. However, a slight decline in dry weight is observed at 50 µM (114.39 ± 8.01 mg) compared to the control. The FC63 genotype showed a declining trend in fresh weight under cadmium exposure, with values of 131.28 ± 5.52 mg (25 µM), 124.05 ± 5.32 mg (50 µM), and 122.91 ± 5.34 mg (100 µM), all of which were lower than the control value of 154.33 ± 4.53 mg (Figure 10).

Analysis of the significant effect attributed to the genotype factor through pairwise comparisons showed significant differences between genotypes comparison FC01 and FC82 ($p = 0.0015$) and FC63 and FC82 ($p = 0.0002$); However, no significant difference was observed between FC01 and FC63 ($p = 0.6879$), indicating similar dry weight performance between these two genotypes (Table 7). The estimated marginal means (emmeans) for *L. campestre* genotypes were extracted to support the results. FC82 had the lowest mean value (83.2 ± 7.34) compared to both FC63 (Mean = 133.1 ± 7.34) and FC01 (Mean = 124.5 ± 7.34). No significant difference was observed in FC63 and FC01 grouped "b" compared to FC82 grouped "a" (Figure 11).

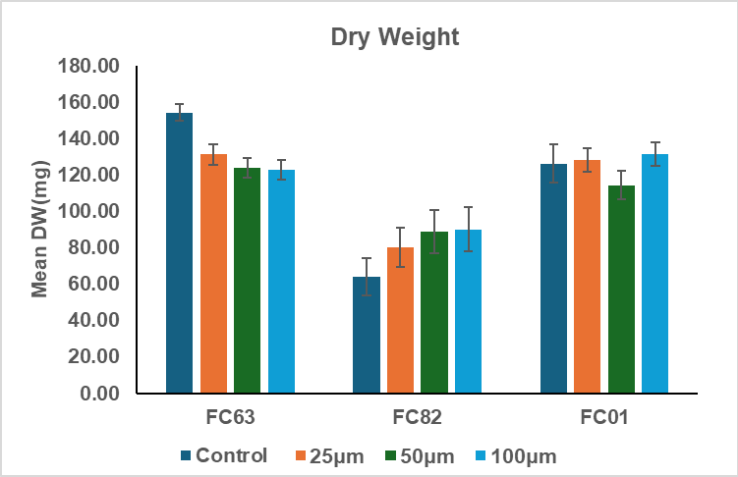

Figure 10. The mean dry weight (DW) of above-ground plant parts for three *Lepidium campestre* genotypes exposed to different cadmium concentrations and control conditions; Error bars represent standard error.

Table 6: Two-way ANOVA (Sum of Squares and Mean Squares) of the dry weight of the *Lepidium campestre* genotypes in cadmium treatments and control

Source of Variation	Df	Sum Sq	Mean Sq	F Value	Pr(>F)	Significance
Genotype	2	17,133	8,566	13.238	0.000134	***
Treatment	3	139	46	0.072	0.974544	ns
Genotype × Treatment	6	3,778	630	0.973	0.464478	ns
Residuals	24	15,531	647			

Df; Degree of freedom, SS; Sum of Squares, MS: Mean Square

Significance codes: *** p < 0.001, p >0.05 ns = not significant.

Table 7:Tukey HSD Pairwise Comparisons for genotypes

Contrast	Estimate	Std. Error	df	t-ratio	p-value
FC01 – FC63	-8.62881	10.38528	24	-0.831	0.6879 ns
FC01 – FC82	41.35575	10.38528	24	3.982	0.0015 **
FC63 – FC82	49.98456	10.38528	24	4.813	0.0002 ***

Significance levels: p >0.05 not significant (ns), ** p < 0.01, *** p < 0.001

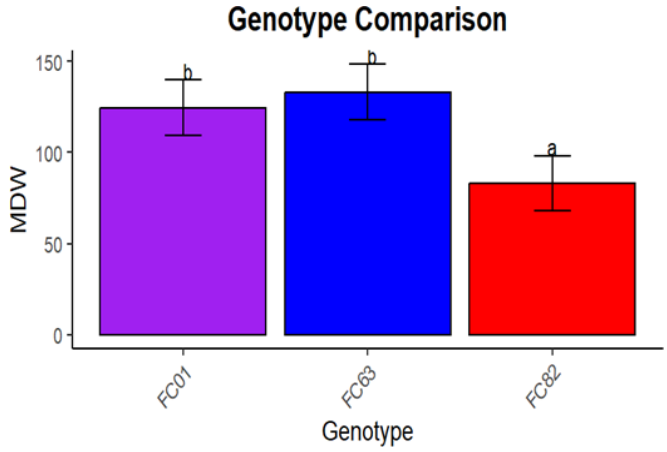

Figure 11. Genotype Comparison on the Mean Dry Weight (MDW) for *Lepidium campestre* genotypes with error bars highlighting significant differences between the genotypes

3.3 Correlation Analysis of the Growth Parameters

Pearson's correlation analysis revealed a significantly strong positive correlation between fresh weight (FW) and dry weight (DW), with a correlation coefficient of r = 0.95. In contrast, the chlorophyll content (CC) showed significantly strong negative correlations with all plant biomass variables measured across the three genotypes: FW (r = –0.81) and DW (r = –0.92), respectively (Figure 12).

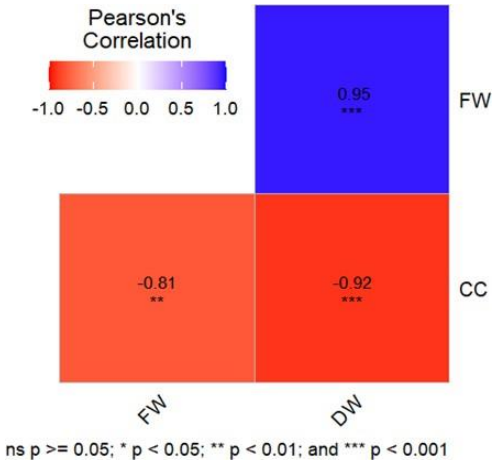

Figure 12. Pearson correlation analysis of growth parameters across the three *Lepidium campestre* genotypes (FC63, FC01, FC82) computed using the R software.

The genotypes were analyzed individually, yielding the observations below. A strong positive correlation is observed between FW and DW in FC63 (r = 0.77) and FC82 (r = 0.75), respectively. FC63 and FC82 genotypes exhibited a strong negative correlation between CC and (DW), where (r = –0.95) and (r = –0.80), respectively. The interaction of all factors had no significant effect (Figure 13).

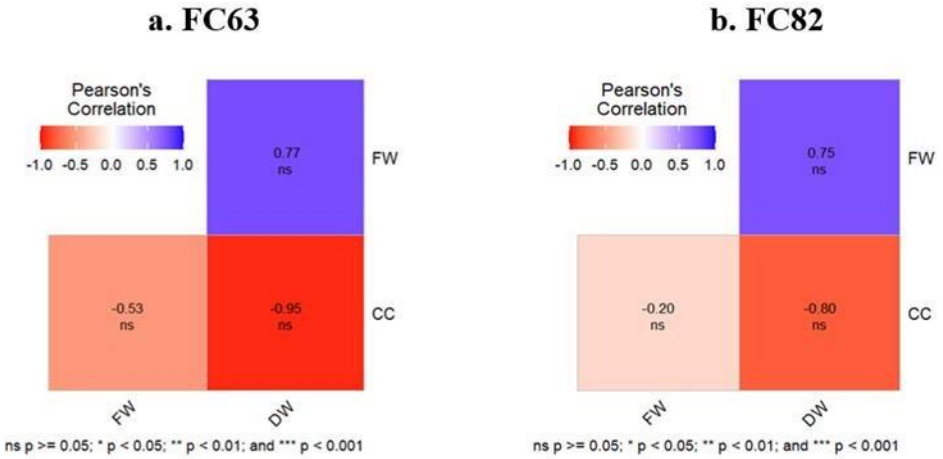

Figure 13. Pearson correlation analysis for growth parameters for *Lepidium campestre* a. FC63 and b. FC82 genotypes

The results in Figure 14 illustrate the correlation analysis of FC01, where a strong positive correlation was also observed between CC and FW (r = 0.77). A slightly weak negative correlation was demonstrated between CC and DW (r = -0.17). All factors showed no significant relationship, as the P value was greater than 0.05.

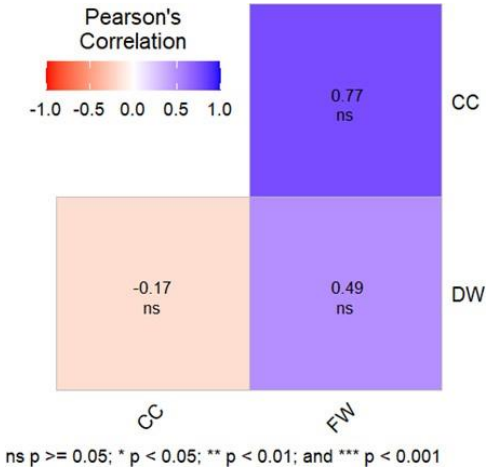

Figure 14. Pearson correlation analysis of growth parameters of *Lepidium campestre* genotype FC01

3.4 Durum Wheat

The one-way ANOVA for FW and DW revealed no statistically significant difference between the treatment concentrations, as the p-value was greater than 0.05 (Tables 8 and 9). This indicated that the treatments had no considerable influence on either fresh or dry weight. FW and DW measurements of *L. campestre* genotypes under different treatments were plotted in Excel (Figure 15). The overall assessment of the fresh weight of durum wheat exhibited a declining trend, which varied with increasing cadmium concentrations. The control treatment demonstrated the highest fresh weight of 543.33 mg, with the 50 µM treatment having the lowest fresh weight of 518.32 mg, as shown in Figure 15. On the other hand, the dry weight of the durum wheat plants varied across treatments, with plants from the 100 µM concentration retaining the highest dry matter (115.21 mg), while those from the control treatment had the lowest (103.74 mg).

Table 8. One-way ANOVA (Sum of Squares and Mean Squares) of the fresh weight of durum wheat in cadmium treatments and control.

Source of variation	Df	Sum Sq	Mean Sq	F value	Pr(>F)	Significance
Treatment	3	1678	559	0.156	0.923	ns (not significant)
Residuals	8	28751	3594			

Df; Degree of freedom, SS; sum squares, MS: Mean Square

Significance codes: ns = not significant

Table 9. One-way ANOVA (Sum of Squares and Mean Squares) of the dry weight of durum wheat in cadmium treatments and control.

Source of variation	Df	Sum Sq	Mean Sq	F value	Pr(>F)	Significance
Treatment	3	222	73.99	0.288	0.833	ns (not significant)
Residuals	8	2053	256.62			

Df; Degree of freedom, SS; sum squares, MS: Mean Square
Significance codes: ns = not significant

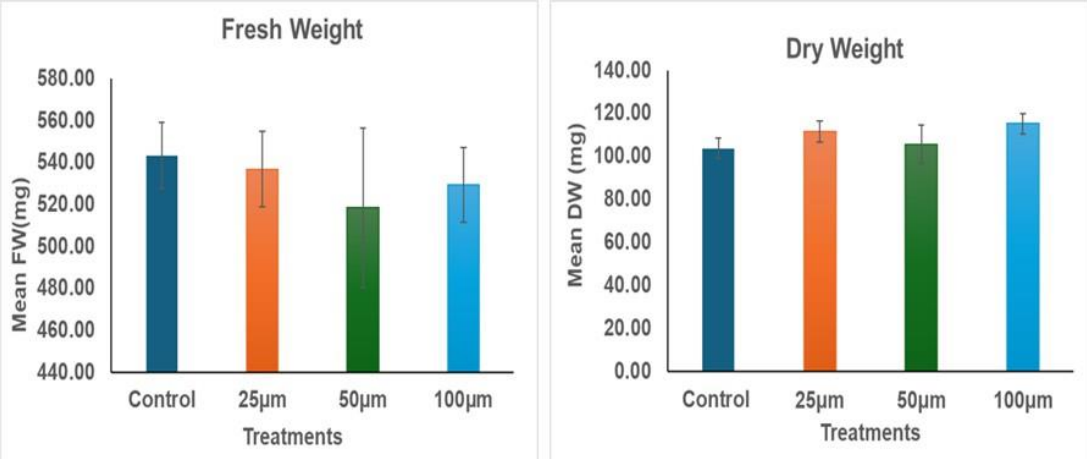

Figure 15. Represents the Mean FW and DW of durum wheat genotype -TD-061 across different cadmium treatments and control condition. Error bars represent the standard error of FW and DW values.

3.5 Comparison of *Lepidium campestre* genotype performance to durum wheat

A comparative analysis of fresh and dry weight was conducted between Durum wheat and the three *L. campestre* genotypes (FC63, FC82, and FC01). Tables 9 and 10 present a percentage summary of the changes in fresh and dry weights across different cadmium treatments relative to the control.

An evaluation of the fresh weight results (Table 9) demonstrated the percentage change in fresh weight for each genotype under increasing cadmium concentrations (25 µM, 50 µM, and 100 µM) relative to the control treatment. The FC63 genotype exhibited a decrease in fresh weight across all cadmium treatments, with the highest reduction (–17.67%) observed at 100 µM.

FC82 and FC01, in contrast, demonstrated a substantial increase in dry weight across all treatments, with the highest gain of +27.78% observed under 50 µM for FC82 and +7.08% observed under the Control for FC01. TD-061 (Durum wheat) demonstrated a decrease in dry weight, particularly at 50 µM cadmium concentration (–7.37%). Based on the results, FC63 and TD01 appeared to demonstrate sensitivity to cadmium stress.

Table 10:Fresh Weight Percentage Change Relative to Control

Genotype	Control (mg)	25 µM (%)	50 µM (%)	100 µM (%)
FC63	653.15	-8.39%	-5.76%	-17.67%
FC82	378.84	+16.39%	+27.78%	+9.95%
FC01	549.64	+7.08%	+1.91%	+5.15%
TD-061	543.33	-1.19%	-7.37%	-2.58%

An evaluation of the dry weight results (Table 10) demonstrates the percentage change in dry weight for each genotype under increasing cadmium concentrations (25 µM, 50 µM, and 100 µM) relative to the control treatment. The FC63 genotype exhibited a decrease in dry weight across all cadmium treatments, with the highest reduction (–13.69%) observed at 100 µM.

FC82, in contrast, demonstrated a substantial increase in dry weight across all treatments, with the highest gain of +32.76% observed at 100 µM. The rising trend could indicate some potential genotype tolerance to cadmium stress. FC01 exhibited a fluctuating response, with a slight increase (+1.55%) at 25 µM, followed by a decrease at 50 µM (–9.33%) and an increase (+4.21%) at 100 µM. The variation in the treatments indicates partial adaptability and sensitivity to cadmium stress. TD-061 (Durum wheat) demonstrated an increase in dry weight, particularly at 100 µM—cadmium concentration (+11.04%). Compared to FC82, both indicate the ability to tolerate cadmium stress.

Table 11: Dry Weight Percentage Change Relative to Control

Genotype	Control (mg)	25 µM (%)	50 µM (%)	100 µM (%)
FC63	142.42	-7.83%	-12.92%	-13.69%
FC82	67.86	+25.33%	+31.16	+32.76%
FC01	126.16	+1.55%	-9.33%	+4.21%
TD-061	103.74	+7.58%	+1.81%	+11.04

3.6 Sequence Analysis of NRAMP family metal-ion transporting genes

3.6.1 Phylogenetic Tree

The natural resistance-associated macrophage protein (NRAMP) family is a group of metal transporters that have been associated with the transportation of several cations (Ismael et al., 2018), such as Fe^{2+}, Zn^{2+}, Cd^{2+}, Cu^{2+}, and $Ni^{2.+}$ (Nevo and Nelson, 2006). In *Arabidopsis,* the NRAMP family, comprising six members (NRAMP1-6), has been identified. (Ismael et al., 2018) to complement Fe, Mn, and Cd uptake in plants (Cailliatte et al., 2009; Thomine et al., 2003).

The tree was constructed based on NRAMP1-6 protein sequences from *Arabidopsis thaliana.* The tree topology revealed the evolutionary relationship of the NRAMP proteins in *Arabidopsis thaliana* (Figure 16). NRAMP1 and NRAMP6 clustered together, with a 100% bootstrap value indicating they are closely related. NRAMP3 and NRAMP4 also cluster together, with 85% similarity. NRAMP2, NRAMP5, and the NRAMP3 and NRAMP4 clade form another larger group, validated by a 100% bootstrap value.

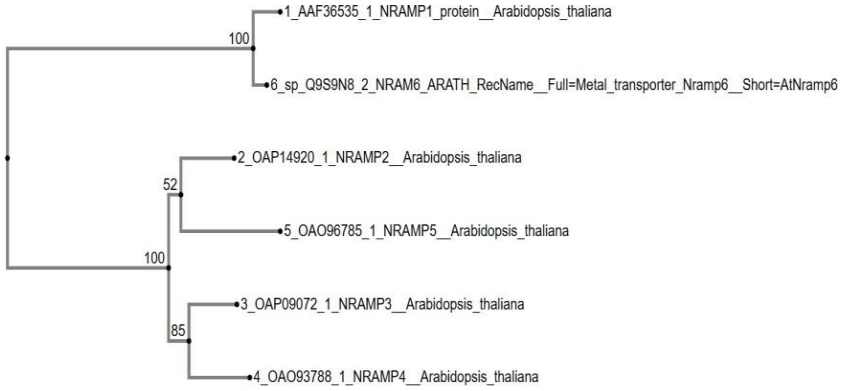

Figure 16. Phylogenetic relationships of NRAMP proteins from *Arabidopsis thaliana*

3.6.2 Cadmium metal transporters –(NRAMP)

In this study, the *Nramp1* protein sequence of *Arabidopsis thaliana* was obtained from the NCBI and used for a comparative analysis of closely related Nramp sequences from the *Lepidium* genome and Durum wheat. Due to limited information on *Lepidium* plant species*, Arabidopsis thaliana* was used as a close relative of *Lepidium campestre*, belonging to the Brassicaceae family. A BLASTp search using the Nramp1 protein sequence (*A. thaliana*) identified the closest homolog in the *Lepidium* genome *to be* Natural Resistance-Associated Macrophage Protein3 (*Lepidium virginicum*) (accession ID: BBH56030), with a 43.37% sequence identity, protein length of 375 amino acids. Similarly, a BLASTp search against durum wheat revealed the closest match to be an unnamed protein product (accession ID: VAI79988), with 72.88% sequence identity and a protein length of 548 amino acids.

The proteins were modelled using the Swiss Model, which demonstrated predicted 3D model structures of *Arabidopsis thaliana, Lepidium virginicum*, and *Triticum turgidum* subsp durum protein sequences (Figure 17). *Arabidopsis thaliana* sequence alignment was modelled on Template Q9S9N8.1.A Metal transporter, Nramp6, was an AlphaFold DB model of NRAM6_ARATH (gene: NRAMP6, organism: *Arabidopsis thaliana* (Mouse-ear cress). The sequence identity coverage was 88.74%, with a GMQE value of 0.79.

The results of *Lepidium virginicum* alignment were modelled on Template Q9SNV9.1. A Metal transporter, Nramp3, was an AlphaFold DB model of NRAM3_ARATH (gene: NRAMP3, organism: *Arabidopsis thaliana* (Mouse-ear cress). The sequence identity coverage was 95.20%, with a GMQE value of 0.88. The results of *Triticum turgidum* subsp. durum alignment was modelled on Template A0A0K9P6C1.1.A Manganese transport protein mntH, which was an AlphaFold DB model of A0A0K9P6C1_ZOSMR (gene: A0A0K9P6C1_ZOSMR, organism: *Zostera marina* (Eelgrass). The sequence identity coverage was 76.50%, with a GMQE value of 0.77.

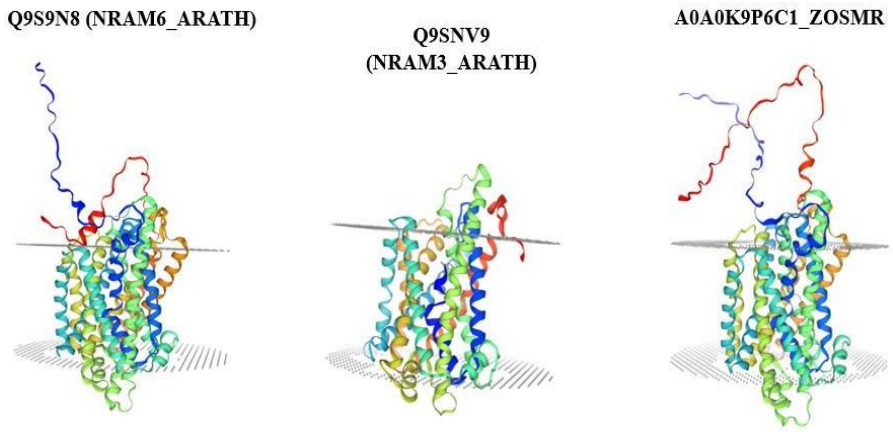

Figure 17. Predicted 3D structure of NRAMP proteins by Swiss Model.Q9S9N8 (NRAM6_ARATH) model represents *Arabidopsis thaliana* sequence, Q9SNV9 (NRAM3_ARATH) model represents *Lepidium virginicum* sequence, A0A0K9P6C1_ZOSMR model represents *Triticum turgidum* subsp. durum sequence

3.7.3 Multiple Sequence Alignment of NRAMP1 Proteins from *Arabidopsis thaliana, Lepidium virginicum* and *Triticum turgidum* subsp. Durum.

Alignment performed using CLUSTAL Omega https://www.ebi.ac.uk/jdispatcher/msa/clustal showed conserved and variable regions between *the Arabidopsis thaliana Nramp1* protein, with 532 amino acids in length and *Lepidium virginicum*, which has 375 amino acids, as well as *T. turgidum* subsp. Durum protein, which was 548 amino acids in length. The alignment revealed conservation in the central regions of the protein, marked by stretches of strongly similar residues (denoted by asterisks) (Figure 18). The N terminal revealed variability in the three alignments, especially in BBH56030 (*Lepidium virginicum*), with several deletions relative to the other two sequences. The C-terminal region exhibited relatively higher conservation between

AAF35635.1 and VAI79988.1 compared to BBH56030.1, as demonstrated in Figure 18. Overall, the alignment reveals the conservation of metal transporter genes across various plant species.

```
BBH56030.1      ------------------------------------------------------------   0
AAF36535.1      --MAATGSGRSQFISSSGGNRSFSN---SPLIENSDSNQIIVSEKKSWKNFFAYLGPGFL   55
VAI79988.1      MSGPRQGSSQPQFMTSVGQNNNLSNGPGTPLIDSIDVDQIVIPEKNSWKNLFSYIGPGFL   60

BBH56030.1      MSIAFLDPGNLEGDLQAGAIAGYSLLWLLMWATVMGLLVQLLSARLGVATGRHLAELCRD   60
AAF36535.1      VSIAYIDPGNFETDLQAGAHYKYELLWIILVASCAALVIQSLAANLGVVTGKHLAEQCRA   115
VAI79988.1      VSIAYIDPGNFETDLQAGAQYKYELLWIILIASCAALVIQSLAASLGVVTGKHLAEHCRD   120
                :***::****:* ******   *.***::: *:  .*::* *:* ***.**:**** **

BBH56030.1      EYPTWARMVLWIMAELALIGSDIQEVIGSAIAIKILTNGILPLWAGVVITALDCFVFLFL   120
AAF36535.1      EYSKVPNFMLWVVAEIAVVACDIPEVIGTAFALNMLF--SIPVWIGVLLTGLSTLILLAL   173
VAI79988.1      EYPKVTNFILWILAELAVVACDIPEVIGTAFALNMLF--KIPIWCGVLITGLSTLMLLFL   178
                ** .  .::**::**:*::..** ****:*:*:::*    :*:* **::*.*. :::* *

BBH56030.1      ENYGIRKLEAVFAVLIATMAVSFAWMFGQAKPSGSELLVGILVPKLSSRT-IQKAVGVVG   179
AAF36535.1      QKYGVRKLEFLIAFLVFTIAICFFVELHYSKPDPGEVLHGLFVPQLKGNGATGLAISLLG   233
VAI79988.1      QQYGVRKLEFLIAFLVFLIATCFLVELGYSKPNSSEVVRGLFVPEIKGDGATGLAISLLG   238
                ::**:**** ::*.*:  :* .*   :  :**. .*:: *::**::..      *:.::*

BBH56030.1      CIIMPHNVFLHSALVQSREVDKKQRYRVQEALNYYTIESTLALFVSFIINLFVTTVFAKG   239
AAF36535.1      AMVMPHNLFLHSALVLSRKIPRSA-SGIKEACRFYLIESGLALMVAFLINVSVISVSGAV   292
VAI79988.1      AMVMPHNLFLHSALVLSRKVPRSV-HGIKEACRFYMIESAFALTVAFLINISIISVSGAV   297
                .::****:******* **:: :.    ::** .:* *** :** *:*:**: : :* .

BBH56030.1      FYNTELA-------DSIGLVNAGQYLQDKYGGGVFPILYIWGIGLLAAGQSSTITGTYAG   292
AAF36535.1      CNAPNLSPEDRANCEDLDLNKASFLLRNVVGK-W--SSKLFAIALLASGQSSTITGTYAG   349
VAI79988.1      CSADNLNPEDRMNCNDLDLNKASFLLKNVLGN-W--SSKVFAIALLASGQSSTITGTYAG   354
                    :*        :.:.* :*.  *::  *        ::.*.***:************

BBH56030.1      QFIMGGFLNFKMKKWLRALITRSCAIIPTIIVALVFDSSEATLDILNEWLNVLQSIQIPF   352
AAF36535.1      QYVMQGFLDLRLEPWLRNLLTRCLAIIPSLIVALIGGSAGAGKLII--IASMILSFELPF   407
VAI79988.1      QYVMQGFLDLRMTPWLRNLLTRSLAIVPSLIVSLIGGSSAAGKLII--IASMILSFELPF   412
                *::* ***::::  *** *:**. **:*::**:*: .*: *   *:    .:: *:::**

BBH56030.1      ALIPLLCLVSKEQIMGGFKIGPV-------------------------------------   375
AAF36535.1      ALVPLLKFTSCKTKMGSHVNPMAITALTWVIGGLIMGINIYYLVSSFIKLLIHSHMKLIL   467
VAI79988.1      ALVPLLKFTSSKTKMGPHTNSRFISVLTWAIGSFIMVINIYFLITSFVRLLLHSGLSTVS   472
                **:*** :.* :  ** .

BBH56030.1      ------------------------------------------------------------   375
AAF36535.1      VVFCGILGFAGIALYLAAIAYLVFRKNRVATSLLISRDS----------QNVETLPRQD   516
VAI79988.1      QVFSGIFGFLGMLIYIAAILYLVFRKNRKCTLPLLESDAKLGDAGHTEGEGSLGHLPRED   532

BBH56030.1      ----------------        375
AAF36535.1      IVNMQLPCRVSTSDVD        532
VAI79988.1      ISSMQLPHQRPASDLD        548
```

Figure 18. Sequence alignment of *Nramp1* of *A. thaliana* (AAF36535), Natural Resistance-Associated Macrophage Protein3 *Lepidium virginicum* (BBH56030), and an unnamed protein product *T. turgidum* subsp. durum (VAI79988) protein sequences with Clustal Omega. Identical residues are denoted with *. Missing highlighted as – and conservative substitutions (: or.)

4. Discussion

Seed germination is a critical phase in a plant's life cycle, closely linked to breaking dormancy and enabling the plant to adapt to environmental conditions and achieve optimal growth (Šikuljak et al., 2024). In the present study, germination patterns revealed apparent genotypic differences that significantly influenced overall crop performance. Previous research has shown that early-flowering plants typically exhibit weaker seed dormancy than late-flowering varieties, which can translate into faster or more uniform germination (Gu et al., 2018). This relationship may explain the higher germination rates observed in the early maturing genotype (FC63), consistent with the results shown in Figure 5.

Variability in germination may arise from intrinsic seed characteristics such as embryo development, seed coat permeability, or physiological maturity, as well as external environmental factors, including temperature, light, and moisture availability (Šikuljak et al., 2024). Additionally, seed quality plays a crucial role; thus, the potential presence of underdeveloped embryos or low seed vigour can prolong the time required for germination (Abubakar & Attanda, 2022).

When evaluating the potential effects of cadmium (Cd) on seed germination, the results showed that cadmium treatments had no significant impact on germination (Table 2). However, Safari et al. (2020) reported that cadmium (Cd) significantly inhibits seed germination and early plant growth. The inhibitory effects of Cd are primarily due to its interference with water uptake and embryo development (Huybrechts et al., 2019; Haider et al., 2021). Additionally, Cd may impede starch degradation in the endosperm, thereby disrupting the mobilization of soluble sugars to the embryonic axis, which results in nutrient deficiencies that delay or prevent germination. The differences in seed germination likely stem from genetic variation, which may confer differing levels of tolerance or susceptibility to cadmium toxicity (Ahmad et al., 2012). Additionally, intrinsic seed characteristics condition of the seeds may be a potential factor contributing to the varying differences observed in the seeds.

Chlorophyll Content

Cadmium toxicity is known to negatively impact plant growth through various morphological and physiological disruptions, although the threshold for phytotoxicity differs across species and cultivars. In this study, the results showed no significant effect ($p > 0.05$) of increasing cadmium concentrations on chlorophyll content (CC); however, numerical genotypic differences were present (Figure 7). Since the measurement of chlorophyll content was conducted 14 days after transplanting, with a one-week interval for each experimental replicate, the numerical differences observed may have been attributed to temporal variations in the greenhouse. Interestingly, correlation analysis (Figure 12) revealed a significant negative relationship between CC and both fresh and dry weight in *L. campestre* genotypes, suggesting that higher chlorophyll content does not necessarily translate to increased biomass. It highlighted the possibility that the genotype's response to the environment played a dominant role in determining chlorophyll content under both cadmium-stressed and control condition.

Fresh Weight

The ANOVA results revealed that genotype had a significant influence on fresh weight ($p = 0.000399$), while cadmium treatment and the interaction between genotype and treatment had no significant effect. FC82 and FC01 genotypes exhibited increased fresh weights under cadmium treatments compared to the control (Figure 8). This may be attributed to plant responses that may involve the efficient sequestration of heavy metals in less toxic compartments (Benavides et al., 2005; Haider et al., 2021). These mechanisms support the improved growth observed in these genotypes in cadmium exposure compared to control conditions.

In contrast, genotype FC63 showed a consistent reduction in fresh weight with increasing cadmium concentrations. These results align with previous research that cadmium stress can negatively impact biomass production through oxidative stress, disruption of nutrient uptake, and inhibition of photosynthesis in susceptible genotypes. (Gallego et al., 2012).

Dry Weight

The ANOVA results for dry weight revealed a significant effect of genotype ($p = 0.000501$), while cadmium treatment and the interaction between genotype and treatment were not significant. Notably, genotype FC82 exhibited increased dry weight under all cadmium treatments (25 µM, 50 µM, 100 µM) compared to its control. Similarly, FC01 showed stable or slightly increased dry weight under 25 µM and 100 µM cadmium, although a minor reduction was observed at 50 µM (Figure 10). These results align with previous research findings that certain tolerant genotypes can maintain biomass under cadmium exposure through mechanisms such as metal chelation, vacuolar sequestration, and activation of antioxidant systems (Benavides et al., 2005; Haider et al., 2021).

Additionally, the illustration of FC82 (late maturing) being significantly different from FC63 and FC01 (early maturing and perennial, respectively) shows that late-maturing plant varieties tend to lower biomass accumulation compared to cases where enhanced tolerance is demonstrated in comparison to pioneer species. (Nogueira et al., 2004, Reich et al., 1994).

Sequence Analysis

The selection of the NRAMP (Natural Resistance-Associated Macrophage Protein) gene family was essential in evaluating the plant's ability to take up Cd from the soil, as their play is known to be involved in the transport and homeostasis of metal ions such as Cu^{2+}, Fe^{2+}, and Cd^{2+} (Nevo and Nelson, 2006). The close phylogenetic relationship between *Lepidium campestre* and *Arabidopsis thaliana*, both of which belong to the Brassicaceae family, made it prudent to examine NRAMP genes, which are well-studied and defined.

The analysis of NRAMP (Natural Resistance-Associated Macrophage Protein) sequences from *Arabidopsis thaliana, Lepidium virginicum,* and *Triticum turgidum* subsp. durum provides insights into the potential functional relevance of this metal transporter family across diverse plant species. *Nramp* is well-characterized in plants for its roles in the uptake of metal ions, particularly Fe^{2+} and Mn^{2+}, and Cd^{2+} (Cailliatte et al., 2009).

The observed 43.37% sequence identity between *A. thaliana* and *L. virginicum Nramp* proteins, despite both belonging to the Brassicaceae family, indicates some level of divergence. This may reflect functional specialization or evolution within this lineage, potentially influenced by environmental pressures or metal availability in soil. (Krämer, 2010). The high sequence similarity (72.88% identity) between *A. thaliana* and *T. turgidum Nramp* homologs was noteworthy, considering the plants are derived from different species. This suggests that the functional domains of Nramp transporters have been strongly conserved.

Regions of high conservation observed in the alignment, particularly in the central domain of the proteins, are likely associated with transmembrane helices and metal-binding motifs, consistent with previous studies describing the structure-function relationships in plant Nramps (Lanquar et al., 2005; Koen et al., 2013). These residues are known to play essential roles in coordinating metal ions during transport across cellular membranes, contributing to the uptake of important elements such as Fe and Mn.

The divergence in the N-terminal regions, particularly in *L. virginicum*, may represent species-specific regulatory adaptations, including localization signals or post-translational modifications (Cailliatte et al., 2009). The results reinforce the relevance of using *A. thaliana* as a model species for functional studies of metal transporters in the Brassicaceae family, particularly for less-characterized genera such as *Lepidium*, where genomic resources are currently limited. Additionally, the close relationship between *A. thaliana* and the Nramp homolog in *T. turgidum* subsp. durum highlights the context of cadmium accumulation in edible plant tissues, which has implications for food safety and agricultural sustainability.

Strengths of the study

Some of the revealed strong insights of this study included

- Study crop: The study examines *L. campestre*, an undomesticated and underutilized crop, for its cadmium tolerance and phytoremediation potential, providing new insights into sustainable soil remediation strategies.
- Genotypic Diversity: Assessing multiple *L. campestre* genotypes (FC63, FC76, FC01, FC82) provides valuable data on intra-species variation, enabling the selection of tolerant lines for future phytoremediation use.
- Comparative Approach: The use of durum wheat (TD-061) as a reference crop enables valuable comparisons under cadmium stress, thereby enhancing the relevance of the results.
- Sustainability efforts: The experiment aligns with broader environmental goals by identifying crops that have the potential to extract or tolerate heavy metals, thereby supporting land restoration efforts.

Weaknesses of the study

This study provided valuable insights into the cadmium tolerance of *Lepidium campestre* and durum wheat genotypes; several limitations may have influenced the results:

- Short evaluation period: The experiment's duration was approximately 30 days, which may have limited the ability to observe the full physiological and morphological effects of cadmium toxicity.
- Incomplete genotypic data: The late introduction of the FC76 L*epidium campestre* genotype resulted in missing data for FC82 germination in cadmium solution, a core component of the study's evaluation. This gap reduced the consistency of the data. However, the FC76 and FC82 are generated from a single parental line; therefore, we do not expect considerable genetic variation between them, as *Lepidium campestre* is a selfing species.
- Limited data on chlorophyll assessment: The chlorophyll content could not be measured in durum wheat due to insufficient leaf area, which prevented the comparison of *Lepidium campestre* genotypes with durum wheat in cadmium treatments and control conditions.

- Exclusion of root biomass: Biomass measurements focused solely on above-ground tissues, omitting root systems despite research studies indicating the effects of cadmium on root growth.

Opportunity to explore

The disposal of plants loaded with heavy metals is often overlooked in most research studies. Disposal methods currently utilized include heat treatment, extraction treatment, microbial treatment, synthesis of nanomaterials, and compression landfill, each with its own set of potential advantages and disadvantages. (Liu and Tran, 2021). Secondary pollution is a significant risk factor that can contribute to potential re-entry into the environment, as documented, which can originate from heat treatment, microbial treatment, and compression landfills (Liu and Tran, 2021). This presents a new opportunity to explore methods for safely disposing of plants used to extract cadmium from contaminated soils, thereby limiting its re-entry into the environment.

5. Conclusion

This study evaluated the cadmium tolerance of novel *L. campestre* genotypes to assess their potential application in the phytoremediation of cadmium-contaminated soils. The results revealed notable genotype differences in response to cadmium exposure, particularly in seed germination, chlorophyll content, and biomass accumulation. During the germination phase, FC76 demonstrated numerically stable growth under increasing Cd concentrations compared to the other genotypes. Overall, cadmium had a limited effect on overall biomass and chlorophyll content across genotypes; the observed genotype-dependent responses highlight the importance of targeted selection in identifying efficient phytoremediators. Notably, the results also indicated that *L. campestre* genotypes (FC82 and FC01) exhibit the biomass stability and physiological resilience necessary for use in phytoremediation, particularly in cadmium-contaminated soils. These traits align with the characteristics needed for plants used in phytoremediation.

Future research should focus on extending the study period to validate the use of *L. campestre* in phytoremediation and enhance its viability as a dual-purpose crop for both environmental restoration and renewable biomass production.

Recommendations

This study provided valuable insights into the cadmium tolerance of *L. campestre* and durum wheat genotypes; several limitations may have influenced the results:

- Experiment using soils known to be contaminated with cadmium. This would require measuring the cadmium content in soil samples to determine the amount of cadmium in the soil. Depending on the results, soil samples with varying concentrations can be used to assess the effect of cadmium on plant growth. It provides real-world contamination scenarios and can be used to validate lab-based results, thereby enhancing the study results to determine which genotypes can effectively tolerate and accumulate cadmium.
- Assess if *Lepidium campestre* can extract large amounts of Cd or not. Additionally, a comparison of *L. campestre* genotypes can be *conducted* to evaluate which one absorbs the most, and this can be further explored.

- Extended Study Evaluation Period: It would be prudent to lengthen the study duration from 30 days to approximately 60 days, providing more time for cadmium effects to manifest in the plant.
- The use of identical genotypes throughout the experiment can enhance the consistency of the data analyzed.
- Timely measure of chlorophyll content: Chlorophyll content should be measured at a stage when both plants have developed sufficient leaf area, allowing for accurate and representative quantification of chlorophyll content amounts.
- Inclusion of root biomass: Biomass measurements should include both above-ground and root biomass, indicating the effects of cadmium on both root growth and above-ground plant parts.
- Future studies can also evaluate the translocation of cadmium within different plant tissues, with a particular focus on potential transfer into seed tissues and seed oil.
- Conducting an expression analysis of *L. campestre* cadmium-transporting genes is necessary to understand their function in the species' cadmium tolerance.

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Appendix

Raw Data

Table 12. Estimated Mean Chlorophyll Content for *Lepidium campestre* Genotypes under different cadmium concentrations with standard error (±SE).

	Treatments			
Genotypes	**Control**	**25µM**	**50µM**	**100µM**
FC63	30.95 ± 0.51	32.26 ± 0.43	32.97 ± 0.51	32.37 ± 0.60
FC82	36.11 ± 1.33	34.50 ±1.48	35.09 ± 1.37	33.17 ± 0.82
FC01	32.24 ± 0.67	33.06 ±0.68	32.83 ± 0.73	32.62 ± 0.79

Data of the mean CC of all the experimental replicates and standard error (±SE)

Table 13. Mean Fresh Weight-*Lepidium campestre* Genotypes under different cadmium concentrations and control with standard error (±SE).

	Treatments			
Genotypes	**Control**	**25µM**	**50µM**	**100µM**
FC63	653.15 ± 14.70	598.33 ± 12.85	615.48 ± 29.30	537.58 ± 24.73
FC82	378.84 ± 47.46	443.03 ± 44.63	484.00 ± 44.63	416.50 ± 47.21
FC01	549.64 ± 33.12	588.53 ± 31.62	560.11 ± 33.76	577.96 ± 27.99

Data of the mean fresh weight(mg) of all the experimental replicates and standard error (±SE

Table 14. Mean Dry Weight-*Lepidium campestre* Genotypes under different cadmium concentrations and control condition with standard error (±SE).)

	Treatments			
Genotype	**Control**	**25µM**	**50µM**	**100µM**
FC63	142.42 ± 7.77	131.28 ± 5.52	124.05 ± 5.32	122.91 ±5.34
FC82	67.86 ± 10.13	85.08 ± 9.98	89.00 ± 11.84	90.11 ± 12.00
FC01	126.16 ± 10.43	128.11 ± 6.58	114.39 ± 8.01	131.47 ± 6.23

Data on Mean Dry Weight of all Experimental Replicates and Standard Error (± SE)

NCBI Sequence Retrieval (Nramp1)

>AAF36535.1 NRAMP1 protein [Arabidopsis thaliana]
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>BBH56030.1 NRAMP1 protein [Lepidium virginicum]
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>VAI79988.1 unnamed protein product [Triticum turgidum subsp. durum]
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Nramp Protein Sequence for *Arabidopsis thaliana*

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>OAP14920.1 NRAMP2 [Arabidopsis thaliana]
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>OAO96785.1 NRAMP5 [Arabidopsis thaliana]
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>sp|Q9S9N8.2|NRAM6_ARATH RecName: Full=Metal transporter Nramp6; Short=AtNramp6
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