Research Square

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Controlled ecotoxicological bioassays reveal contrasting cadmium and zinc tolerance and accumulation in Cannabis sativa and Medicago sativa

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Abstract

Metal contamination of agricultural soils requires remediation strategies that combine pollutant removal with plant tolerance and biomass production. This study compared the early-stage responses of *Cannabis sativa* (hemp) and *Medicago sativa* (alfalfa) to cadmium (Cd) and zinc (Zn) using controlled ecotoxicological bioassays. In vitro germination assays were conducted with nominal elemental-metal concentrations of 0, 125, 250, 500 and 1000 mg L − 1, followed by soil-based seedling-tray assays and atomic absorption spectrometry (AAS) of plant material. A qualitative Vicia faba root-tip assay was additionally used to screen for cytogenetic effects. Cd caused a much stronger loss of viability in M. sativa than in C. sativa: at 500 and 1000 mg L − 1, alfalfa averaged < 1 germinated seed per 40-seed replicate, whereas hemp maintained approximately 16–18 germinated seeds per 20-seed replicate. Hemp shoot length also declined under Cd, from 6.1 cm in the control to 1.2 cm at 1000 mg L − 1, but remained greater than the strong inhibition observed in alfalfa. AAS showed higher Cd concentrations in C. sativa than in M. sativa across the tested exposures, reaching 201.8 ± 116.2 versus 151.8 ± 6.5 mg kg − 1 at 1000 mg L − 1. In contrast, Zn accumulation was consistently higher in M. sativa, reaching 130.4 ± 4.0 mg kg − 1 compared with 66.2 ± 47.5 mg kg − 1 in C. sativa at the highest treatment. Soil-tray biomass data further indicated stronger overall growth retention in hemp. Qualitative microscopy detected micronuclei/chromosomal abnormalities following Cd exposure in V. faba, whereas no comparable aberrations were recorded for Zn. These findings support species-specific remediation roles: C. sativa appears comparatively tolerant and effective for Cd accumulation, whereas M. sativa shows greater Zn accumulation. Field-scale remediation efficiency, metal translocation and long-term soil depletion remain to be established.

1 Introduction

Metallic trace elements can persist in soils for long periods and may impair plant establishment, crop productivity and ecosystem functioning. Cadmium is of particular concern because it has no known biological requirement in higher plants and can interfere with cell division, photosynthesis and nutrient homeostasis. Zinc is an essential micronutrient, but excess Zn can also disturb germination and growth. Consequently, plant-based remediation is attractive where a species can tolerate contamination while accumulating or stabilizing metals in harvestable biomass.

Phytoremediation offers a comparatively low-disturbance option for contaminated soils, but its effectiveness depends on several linked traits: contaminant bioavailability, root uptake, translocation, tissue tolerance, biomass production and the safe management of contaminated biomass. Hemp (Cannabis sativa L.) has received attention because of its rapid growth, extensive root system and ability to accumulate several trace elements. Earlier studies reported Cd tolerance and uptake by hemp under contaminated-soil conditions (Citterio et al. 2003; Linger et al. 2005). Under Mediterranean field conditions, hemp and alfalfa have also been evaluated as candidate phytoextractors, illustrating that uptake efficiency can differ strongly among elements and species (Guidi Nissim et al. 2018).

Medicago sativa L. (alfalfa) provides a useful comparator because it is a perennial, high-biomass forage species with a deep root system and agronomic relevance. A direct comparison of hemp and alfalfa under controlled metal exposure can therefore help distinguish tolerance from accumulation capacity. This distinction is important: a plant may survive exposure without accumulating large quantities of a target element, whereas another may accumulate an element efficiently but suffer strong growth inhibition.

Ecotoxicological bioassays can complement chemical measurements by linking exposure to biological responses. Germination and early seedling growth are sensitive endpoints for phytotoxicity, and Vicia faba root tips are widely used for cytogenetic screening because their chromosomes are large and readily observable. Previous work has shown that Cd can reduce mitotic activity and induce micronuclei in V. faba roots (Béraud et al. 2007).

The objective of this study was to compare C. sativa and M. sativa under controlled Cd and Zn exposure using multiple endpoints: seed germination, early growth, soil-based biomass production and metal accumulation measured by AAS. A complementary qualitative V. faba root-tip assay was used to screen for visible cytogenetic abnormalities. We hypothesized that the two species would differ not only in tolerance but also in element-specific accumulation, with implications for selecting plants for remediation of Cd- and Zn-affected soils.

2 Materials and methods

2.1 Plant material and experimental design

Three plant species were used. Cannabis sativa and Medicago sativa were tested for germination, early growth and metal accumulation, while Vicia faba was used for the cytogenetic screening assay. For the in vitro bioassays, each treatment was performed in triplicate according to the source laboratory protocol, using 20 C. sativa seeds or 40 M. sativa seeds per Petri dish. Distilled water served as the control. The experimental design is summarized in Table 1.

Table 1
Overview of the controlled ecotoxicological assays. Concentrations are expressed as nominal elemental-metal equivalents reconstructed from the 1 g L − 1 stock solutions.

Assay	Species	Matrix	Exposure levels	Duration	Replicates / unit
In vitro germination	C. sativa	Filter paper	0, 125, 250, 500, 1000 mg L − 1 Cd or Zn	8 d	3; 20 seeds/dish
In vitro germination	M. sativa	Filter paper	0, 125, 250, 500, 1000 mg L − 1 Cd or Zn	8 d	3; 40 seeds/dish
Soil-based tray growth	C. sativa, M. sativa	Air-dried, 2-mm-sieved soil/peat	0, 250, 500, 1000 mg L − 1; Cd, Zn or Cd + Zn+Fe mixture	14 d	3 treatment rows in source protocol
Qualitative cytogenetic screen	V. faba	Cotton / aqueous solution	0, 125, 250, 500, 1000 mg L − 1 Cd or Zn	10 d	Source protocol; cell counts not retained

2.2 Preparation of metal solutions

Nominal Cd and Zn stock solutions were prepared to provide 1 g L − 1 of elemental metal. The source protocol used cadmium nitrate (2.75 g L − 1 as prepared in the laboratory) for Cd and zinc sulfate (4.34 g L − 1) for Zn. Working solutions were obtained by dilution to 50%, 25% and 12.5% of the stock, corresponding to nominal elemental concentrations of 500, 250 and 125 mg L − 1, respectively. The 100% treatment corresponded to 1000 mg L − 1, and sterile distilled water was used for the 0 mg L − 1 control. For the soil-based assay, Cd, Zn and Fe stock solutions were also combined in equal volumes to prepare the Cd-Zn-Fe treatment mixture. The source laboratory record did not retain the hydration state or analytical grade of the salts; these reagent details should be verified against the original laboratory labels before submission.

2.3 In vitro germination bioassays

Seeds were surface-sterilized using 5 mL household bleach in 100 mL distilled water and thoroughly rinsed with sterile water. They were then soaked in 150 mL distilled water, refrigerated for approximately 24 h, and floating seeds were discarded. For each metal and exposure level, seeds were placed on filter paper in Petri dishes and exposed to 2 mL of treatment solution. Petri dishes were maintained for 8 d and moistened with approximately 2 mL sterile distilled water as required. Germinated seed number, germination percentage and representative seedling/shoot length were recorded. The laboratory records used both 'root length' and 'shoot length' for the same graphical endpoint; to avoid over-interpreting organ identity, this endpoint is referred to as 'seedling length' in the present analysis.

2.4 Soil-based seedling-tray assay

Soil was air-dried and passed through a 2-mm sieve. Seeds were pre-germinated for 48 h on moist filter paper, after which three pre-germinated seedlings were transferred to each tray cell. Soil was initially

moistened with distilled water. Cd, Zn or the combined Cd-Zn-Fe solution was subsequently applied during daily irrigation for 14 d at nominal 250, 500 or 1000 mg L − 1 stock-equivalent treatment levels; control cells received distilled water. Growth was monitored daily and final biomass was recorded. Although the source thesis described this phase as 'in situ', it was performed in seedling trays under controlled conditions; it is therefore referred to here as a soil-based tray assay rather than a field in situ experiment.

2.5 Mineralization and atomic absorption spectrometry

After the 8-d germination exposure, plant material was dried and ground. A 1-g subsample was weighed into a crucible and ashed at 450°C for 4 h, with the furnace temperature increased progressively by approximately 50°C every 30 min. The ash was treated with a small volume of 10% HNO3 and dried on a hot plate, followed by 5 mL H2O2 and evaporation. The residue was then digested in 5 mL aqua regia (1:1 HCl:HNO3 in the source protocol) for approximately 30 min on a hot plate, brought to 10 mL with distilled water and filtered. Three analytical replicates were prepared for each exposed treatment, together with a blank. Cd and Zn concentrations were quantified by atomic absorption spectrometry (AAS) and are reported as mg kg − 1 dry material.

2.6 Qualitative Vicia faba cytogenetic screening

Vicia faba seeds were sterilized for 10 min in 5 mL bleach per 100 mL distilled water, rinsed, and soaked in 150 mL distilled water for 24 h under refrigeration. After pre-imbibition in Cd or Zn solutions (0, 125, 250, 500 or 1000 mg L − 1), seeds were placed on cotton pads moistened with 4 mL treatment solution, covered with parafilm and germinated for 10 d at room temperature until radicles were approximately 2 cm long. Following 48 h refrigeration, root tips were excised and stained in cold aceto-carmine for approximately 5 min on a sand bath, then mounted for microscopic observation. The source dataset retained representative micrographs but not systematic cell counts; consequently, micronuclei and chromosomal abnormalities are reported qualitatively and no frequency, mitotic index or inferential statistic is assigned to this endpoint.

2.7 Data treatment

Replicate-level germination and AAS data were compiled from the laboratory records. Data are reported descriptively as means and, where triplicate observations were available, standard deviations. For some germination controls and soil-tray biomass endpoints, only treatment-level values were retained in the available dataset. Accordingly, inferential significance claims are not made for endpoints lacking complete replicate-level observations. Statistical inference should be performed from the complete original raw dataset, with the statistical model, assumptions, exact n values, test statistics and P values reported explicitly.

3 Results

3.1 Germination responses to cadmium and zinc

The two species showed markedly different responses to Cd (Table 2). Cannabis sativa retained relatively high germination across the full exposure range. The mean number of germinated hemp seeds was 18.0 ± 2.0 at 125 mg L − 1, 15.7 ± 2.5 at 250 mg L − 1, 18.3 ± 2.1 at 500 mg L − 1 and 16.0 ± 2.6 at 1000 mg L − 1, compared with a compiled control value of 19/20 seeds. The non-monotonic increase at 500 mg L − 1 indicates substantial biological/experimental variability rather than a simple dose-response relationship.

Medicago sativa was much more sensitive to Cd. Germination remained high at 125 mg L − 1 (33.3 ± 5.5 seeds out of 40) but fell to 8.7 ± 3.1 at 250 mg L − 1 and to 0.7 ± 0.6 at both 500 and 1000 mg L − 1. The compiled control value was 33/40. Thus, severe inhibition in alfalfa occurred between 125 and 250 mg L − 1, whereas hemp maintained substantial germination even at the highest Cd exposure.

Responses to Zn were less monotonic. Hemp germination remained between 16.3 and 19.0 seeds per 20-seed replicate across the treatment range and reached 19.0 ± 0.0 at 1000 mg L − 1, compared with a compiled control value of 16/20. Alfalfa showed high germination at 125, 500 and 1000 mg L − 1 (38.3 ± 1.5, 37.0 ± 1.7 and 36.3 ± 2.5 seeds, respectively), but a pronounced reduction at 250 mg L − 1 (18.3 ± 3.5). Because this isolated trough was not dose-dependent, it should be interpreted cautiously.

Table 2
Germination after 8 d. Values are mean ± SD for retained triplicates. *Control is the compiled treatment-level value; replicate-level control observations were not available in the analyzed dataset.

Metal	Nominal exposure (mg L − 1)	C. sativa germinated /20	C. sativa (%)	M. sativa germinated /40	M. sativa (%)
Cd	0	19.0*	95.0	33.0*	82.5
Cd	125	18.0 ± 2.0	90.0	33.3 ± 5.5	83.3
Cd	250	15.7 ± 2.5	78.3	8.7 ± 3.1	21.7
Cd	500	18.3 ± 2.1	91.7	0.7 ± 0.6	1.7
Cd	1000	16.0 ± 2.6	80.0	0.7 ± 0.6	1.7
Zn	0	16.0*	80.0	37.0*	92.5
Zn	125	16.3 ± 2.1	81.7	38.3 ± 1.5	95.8
Zn	250	16.7 ± 0.6	83.3	18.3 ± 3.5	45.8
Zn	500	16.3 ± 0.6	81.7	37.0 ± 1.7	92.5
Zn	1000	19.0 ± 0.0	95.0	36.3 ± 2.5	90.8

3.2 Early seedling growth under Cd exposure

Cadmium reduced the representative seedling length of both species, but the decline was more severe in alfalfa. In hemp, the recorded length decreased from 6.1 cm in the control to 4.8, 4.3, 2.4 and 1.2 cm at

125, 250, 500 and 1000 mg L − 1 Cd, respectively. In alfalfa, the corresponding values were 6.3 cm in the control and 2.0, 1.2, 0.2 and 0.2 cm under increasing Cd exposure. These measurements reinforce the germination result: C. sativa maintained early growth under Cd levels that nearly arrested M. sativa development. Zn-related length measurements were internally inconsistent across the source spreadsheets and narrative and are therefore not used as a primary quantitative endpoint in the present reconstruction.

3.3 Cadmium and zinc accumulation measured by AAS

AAS revealed element- and species-specific accumulation patterns (Table 3). For Cd, C. sativa had higher mean tissue concentrations than M. sativa at every non-zero exposure level. Hemp increased from 111.9 ± 100.0 mg kg − 1 at 125 mg L − 1 exposure to 201.8 ± 116.2 mg kg − 1 at 1000 mg L − 1. Alfalfa increased from 87.9 ± 45.6 to 151.8 ± 6.5 mg kg − 1 over the same range. Variability among hemp analytical replicates was high, particularly at 125, 500 and 1000 mg L − 1, so the magnitude of between-species differences should not be overstated without confirmatory statistics.

The pattern was reversed for Zn. M. sativa accumulated substantially more Zn than C. sativa at all tested exposure levels. Alfalfa ranged from 83.6 ± 7.5 mg kg − 1 at 125 mg L − 1 to 130.4 ± 4.0 mg kg − 1 at 1000 mg L − 1. Hemp ranged from 42.1 ± 4.3 to 82.0 ± 4.3 mg kg − 1, with the highest mean occurring at 500 mg L − 1 rather than 1000 mg L − 1. Thus, the results indicate an element-specific pattern: a Cd accumulation advantage for hemp and a Zn accumulation advantage for *alfalfa*.

Table 3
Metal concentrations in mineralized plant material measured by AAS. Values are mean ± SD (n = 3 analytical replicates per exposed treatment). Control/blank values were recorded as 0.

Metal	Exposure (mg L − 1)	C. sativa (mg kg − 1)	M. sativa (mg kg − 1)	Higher mean
Cd	125	111.9 ± 100.0	87.9 ± 45.6	C. sativa
Cd	250	156.7 ± 38.1	91.0 ± 33.9	C. sativa
Cd	500	161.0 ± 135.0	136.1 ± 18.1	C. sativa
Cd	1000	201.8 ± 116.2	151.8 ± 6.5	C. sativa
Zn	125	42.1 ± 4.3	83.6 ± 7.5	M. sativa
Zn	250	50.5 ± 5.4	113.3 ± 3.8	M. sativa
Zn	500	82.0 ± 4.3	121.1 ± 11.5	M. sativa
Zn	1000	66.2 ± 47.5	130.4 ± 4.0	M. sativa

3.4 Biomass response in the soil-based tray assay

The 14-d tray assay showed a strong species contrast (Table 4). Cannabis biomass under the metal treatments ranged from 0.4058 to 1.0517 g, while its recorded control biomass was 0.5279 g. The

largest hemp biomass value occurred under the 500 mg L − 1 Zn treatment (1.0517 g). Therefore, the tray data do not show a uniform biomass depression in hemp with increasing exposure. In contrast, alfalfa biomass under all metal treatments (0.0124–0.0854 g) was well below the recorded control value (0.2623 g), consistent with greater growth sensitivity. Because complete replicate-level tray biomass values were not available for this endpoint, these observations are descriptive.

Table 4
Recorded biomass after the 14-d soil-based tray assay. Recorded control biomass was 0.5279 g for C. sativa and 0.2623 g for M. sativa. The “mix” treatment combined Cd, Zn and Fe.

Exposure (mg L − 1)	C. sativa Cd	C. sativa Zn	C. sativa mix	M. sativa Cd	M. sativa Zn	M. sativa mix	Unit
250	0.5834	0.7531	0.4058	0.0180	0.0402	0.0854	g
500	0.7625	1.0517	0.7768	0.0530	0.0124	0.0425	g
1000	0.7615	0.7745	0.5940	0.0621	0.0356	0.0429	g

3.5 Qualitative cytogenetic observations in Vicia faba

Microscopic examination of V. faba root-tip preparations showed micronuclei and chromosomal abnormalities in Cd-exposed material. Comparable aberrations were not reported in the Zn treatments. Because the archived dataset contains representative images but no systematic counts of total cells, micronucleated cells, mitotic index or aberration frequency, these findings should be interpreted as qualitative evidence of Cd-associated genotoxic stress rather than a quantitative genotoxicity estimate.

4 Discussion

4.1 Hemp shows substantially greater early-stage tolerance to cadmium

The clearest result is the contrasting Cd response of the two species. M. sativa germination collapsed at 250–1000 mg L − 1, while C. sativa retained 78–92% mean germination over the same nominal range and continued to produce measurable seedlings. This relative tolerance agrees with previous reports that hemp can maintain growth in metal-contaminated substrates and can accumulate Cd, particularly in roots (Citterio et al. 2003; Linger et al. 2005). The current experiment extends that pattern to a controlled early-development comparison with alfalfa.

The non-monotonic hemp germination response, including recovery at 500 mg L − 1 relative to 250 mg L − 1, argues against treating the dataset as a simple linear dose-response. Such patterns may reflect biological heterogeneity, seed-lot variability, solution chemistry, or limited replication. Consequently, the strongest inference is comparative tolerance between species rather than a precise Cd toxicity

threshold. A future study should increase replication and use concentration-response modelling with measured, rather than nominal, exposure concentrations.

4.2 Accumulation is element-specific: Cd favors hemp, Zn favors alfalfa

The AAS results demonstrate an important element-specific distinction. Hemp did not show universally greater accumulation of both metals: it contained more Cd than alfalfa at each exposure level, whereas alfalfa contained more Zn at every corresponding level. This distinction is scientifically meaningful because phytoremediation performance is element-specific. It also illustrates why tolerance and uptake must be evaluated together rather than inferred from germination alone.

The greater Cd concentration in hemp is consistent with prior work demonstrating Cd uptake by C. sativa and its capacity to tolerate substantial root Cd burdens (Citterio et al. 2003; Linger et al. 2005). Conversely, the strong Zn accumulation in M. sativa is compatible with the broader observation that species differ in transporter activity, rhizosphere interactions and metal partitioning. Studies of Cd/Zn hyperaccumulators show that Cd and Zn can share or compete for transport pathways, while some genotypes possess more selective Cd transport systems (Lombi et al. 2001; Zhao et al. 2002). The present experiment did not measure transporter expression, rhizosphere chemistry or tissue partitioning, so these mechanisms remain hypotheses rather than demonstrated explanations.

4.3 Implications for phytoremediation and phytomanagement

A useful phytoremediation candidate must combine contaminant uptake with sufficient biomass and tolerance to permit repeated cultivation and harvest. The tray assay suggests that hemp maintained more biomass than alfalfa under the tested treatments, while the AAS data show a Cd accumulation advantage. Together, these traits support further evaluation of hemp for Cd-oriented phytomanagement. Field studies under Mediterranean conditions have already demonstrated that hemp can participate in trace-element phytoextraction, although overall removal depends strongly on biomass yield and translocation to harvestable tissues (Guidi Nissim et al. 2018).

For Zn, alfalfa's higher tissue concentrations could be advantageous for extraction, but its growth response was less stable in the present assays. The Zn germination trough at 250 mg L − 1 and low tray biomass indicate that uptake alone is insufficient to define suitability. A rigorous field assessment should quantify total metal removal per unit area, not only tissue concentration, and should calculate bioconcentration and translocation factors from paired soil, root and shoot measurements.

4.4 Cytogenetic observations reinforce the environmental relevance of Cd

The qualitative V. faba observations are directionally consistent with established Cd genotoxicity. Béraud et al. (2007) reported reduced mitotic activity and micronucleus induction in V. faba root cells after Cd

exposure. In the present study, visible micronuclei/chromosomal abnormalities were observed in Cd-treated roots but not in the examined Zn treatments. However, the lack of systematic cell counts prevents estimation of micronucleus frequency or a concentration-response relationship. Future assays should score a predefined number of cells per replicate and report mitotic index, micronucleus frequency and major aberration classes.

4.5 Study limitations and priorities for validation

- The metal treatments were defined from nominal stock-solution concentrations; measured Cd and Zn concentrations in the exposure solutions and soils were not reported.
- The exact hydration states/purity of cadmium nitrate and zinc sulfate should be verified from reagent labels to ensure reproducible stock-solution calculations.
- Replicate-level control data were not available consistently for all endpoints, and replicate-level tray biomass data were incomplete; formal inferential statistics therefore require the complete original raw dataset.
- AAS was performed on bulk mineralized plant material, without separate root and shoot measurements; translocation factors and harvestable-metal removal cannot be calculated.
- The 8- to 14-d experiments assess early-stage tolerance and accumulation, not long-term field remediation efficiency or soil-metal depletion.
- The V. faba assay was qualitative; standardized cytogenetic counts are needed for a quantitative genotoxicity conclusion.
- Any regulatory authorization associated with Cannabis sativa seed acquisition/cultivation should be documented in the submitted manuscript where applicable.

5 Conclusions

Controlled bioassays demonstrated a clear species-by-metal contrast. Cannabis sativa was much more tolerant than Medicago sativa to Cd during germination and early growth and showed higher Cd concentrations in mineralized plant material. Medicago sativa, however, accumulated more Zn than hemp across the tested exposures, showing that phytoremediation traits are element-specific. Soil-tray biomass data were also more favorable for hemp, whereas alfalfa biomass remained below its control under all recorded metal treatments. Qualitative Vicia faba microscopy supported the greater cytogenetic hazard of Cd relative to Zn but requires quantitative validation. Overall, the results justify further evaluation of hemp for Cd-oriented phytomanagement and of alfalfa for Zn uptake, while emphasizing that field-scale recommendations require complete statistical validation, tissue-specific metal partitioning, soil-to-plant transfer metrics, long-term trials and safe post-harvest biomass management.

Declarations

Authors contributions declaration:

Author contributions: Afaf Belabhir, Hajar Sebban, Hind ait Belcaid, Abdelilah El Abbassi, and Sedki Azeddine contributed to the study and manuscript preparation. All authors reviewed the manuscript, approved the final version, and agree to be accountable for the work. A.B. and H.S. wrote the main manuscript text and H.B. prepared all the figures and S.A. and E.M. reviewed and corrected the manuscript.

Ethics and consent to participate:

Human- and animal-research ethics approval was not applicable, as this study did not involve human participants or animals. The study was not a clinical trial; therefore, clinical trial registration and informed consent to participate were not applicable.

The collection, acquisition, cultivation, and use of plant materials (*Cannabis sativa* and *Medicago sativa*) were conducted in accordance with applicable local and national guidelines and regulations governing plant research. All experimental procedures involving plant materials followed the relevant institutional and national guidelines.

Consent for publication:

Not applicable.

Consent to participate:

Not applicable. This study did not involve human participants.

Funding:

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Author Contribution

A.B. and H.S. wrote the main manuscript text and H.B. prepared all the figures and S.A. and E.M. reviewed the manuscript.

Data availability statement:

The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Figures

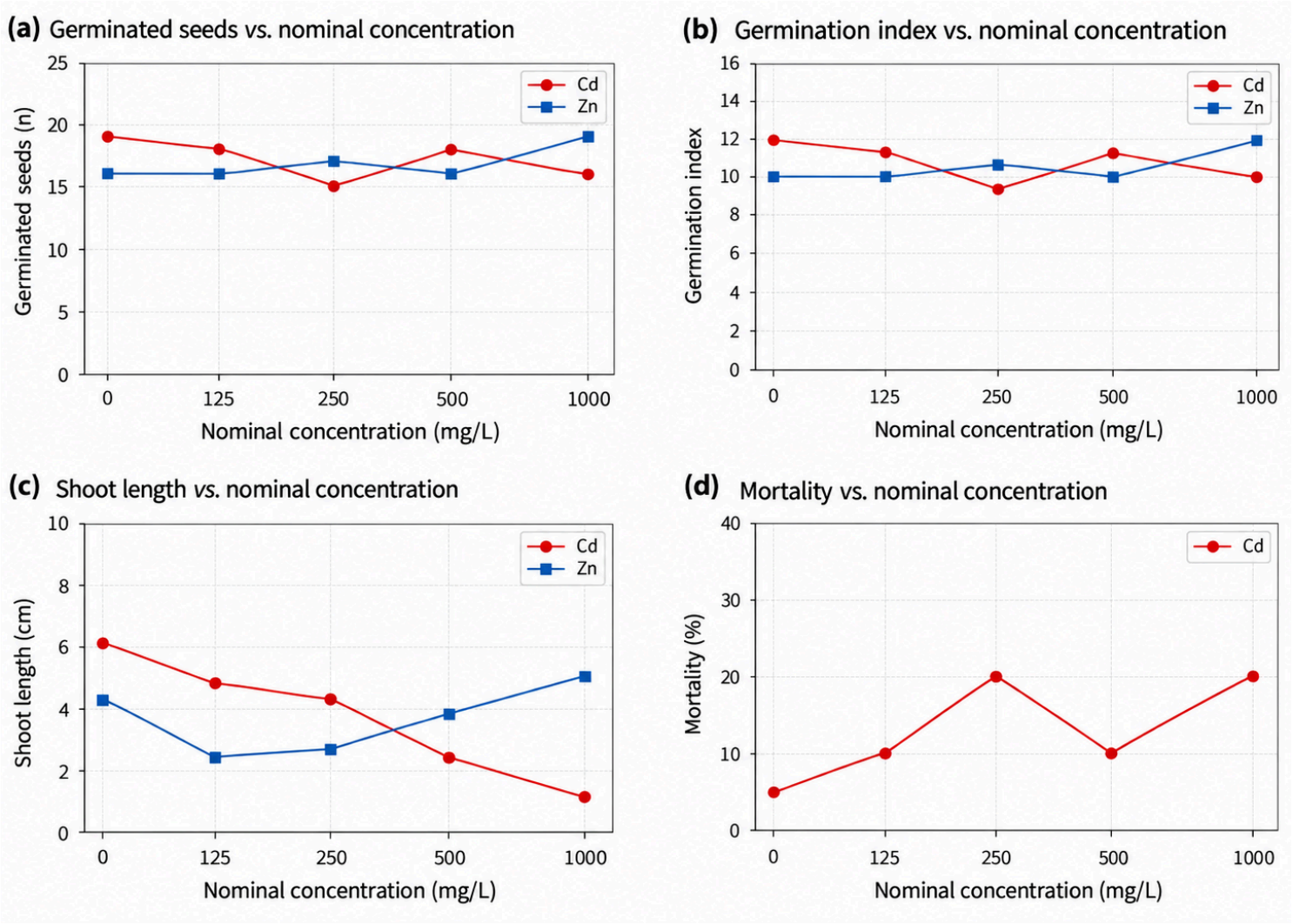


Figure 1

In vitro response of Cannabis sativa to cadmium and zinc. Germinated seeds, germination index, shoot length, and mortality under nominal Cd and Zn

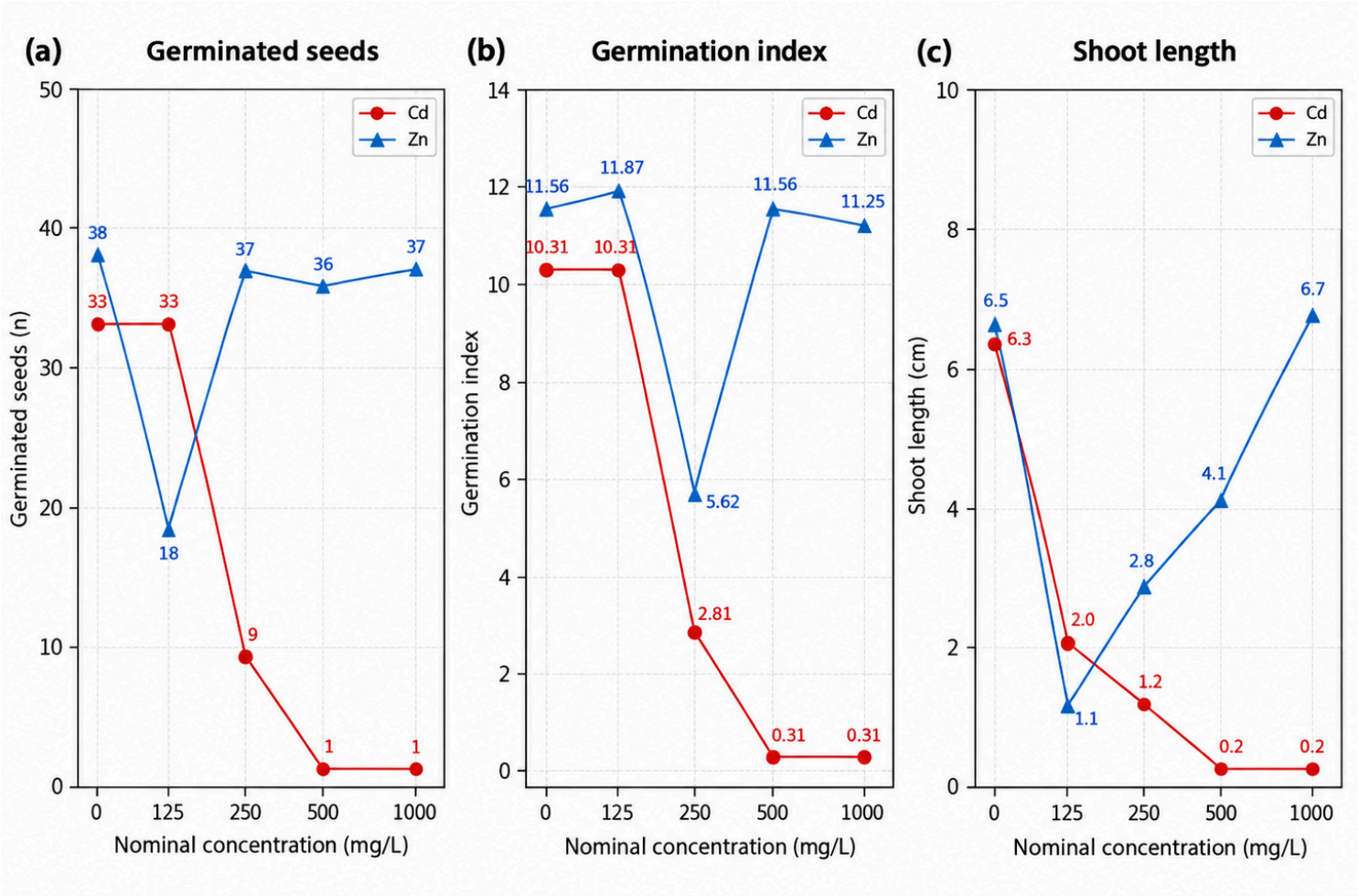


Figure 2

In vitro response of Medicago sativa to cadmium and zinc. Germinated seeds, germination index, and shoot length under nominal Cd and Zn concentrations.

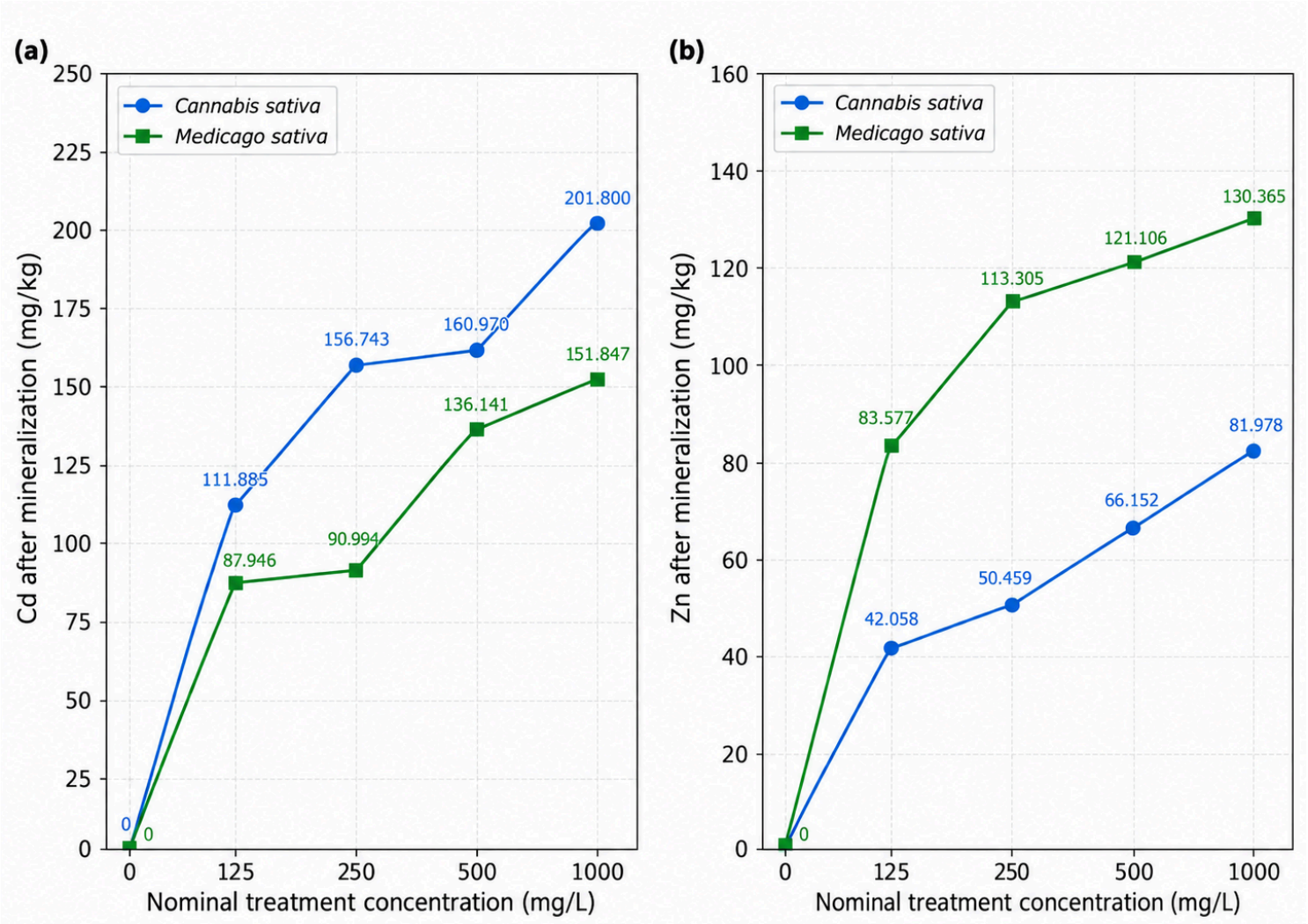


Figure 3

Metal accumulation after mineralization. Cadmium and zinc concentrations measured by AAS in mineralized plant material of Cannabis sativa and Medicago sativa across nominal treatment concentrations.

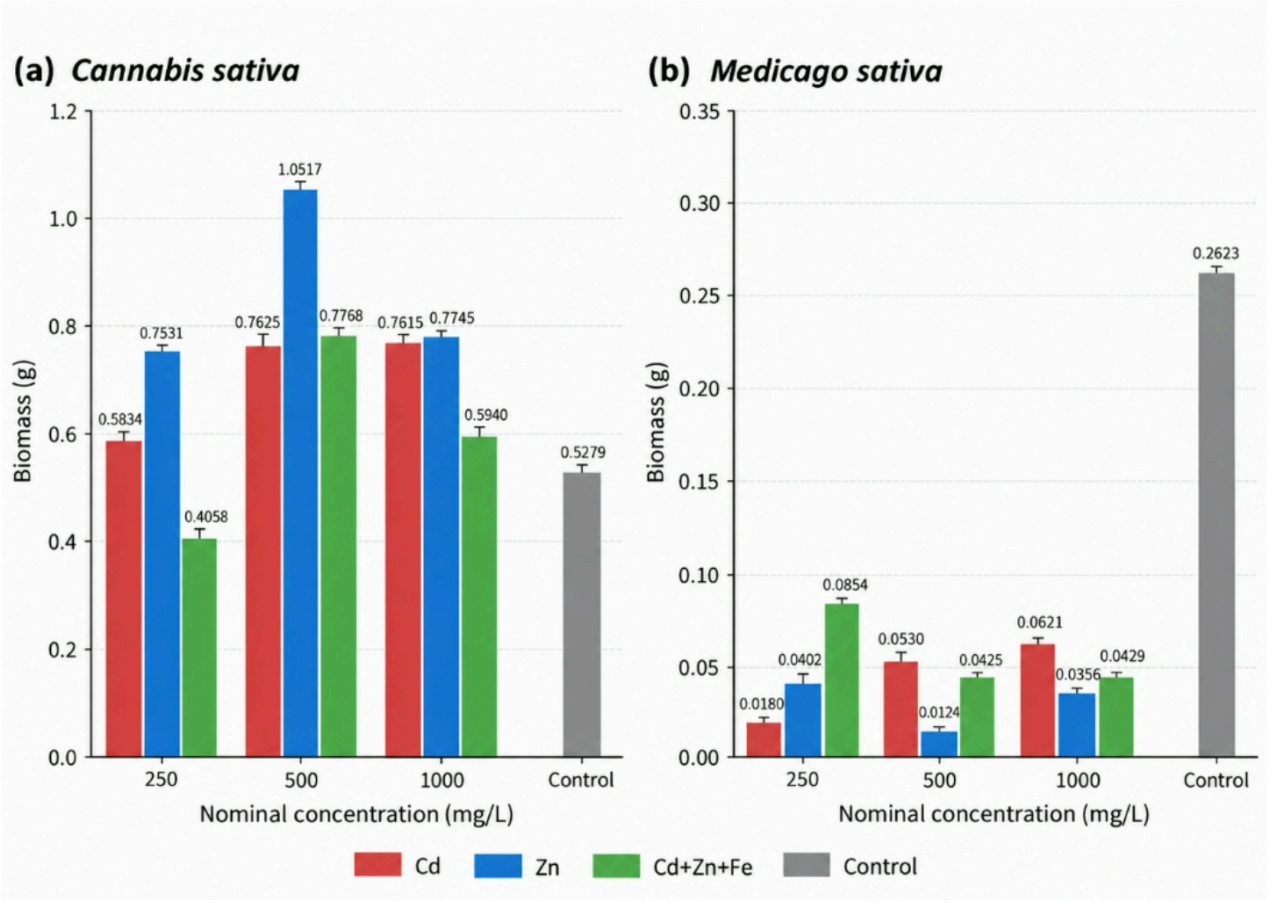


Figure 4

Biomass in the soil-based seedling-tray assay. Biomass response of Cannabis sativa and Medicago sativa under Cd, Zn, and Cd+Zn+Fe treatments, with control values shown separately.

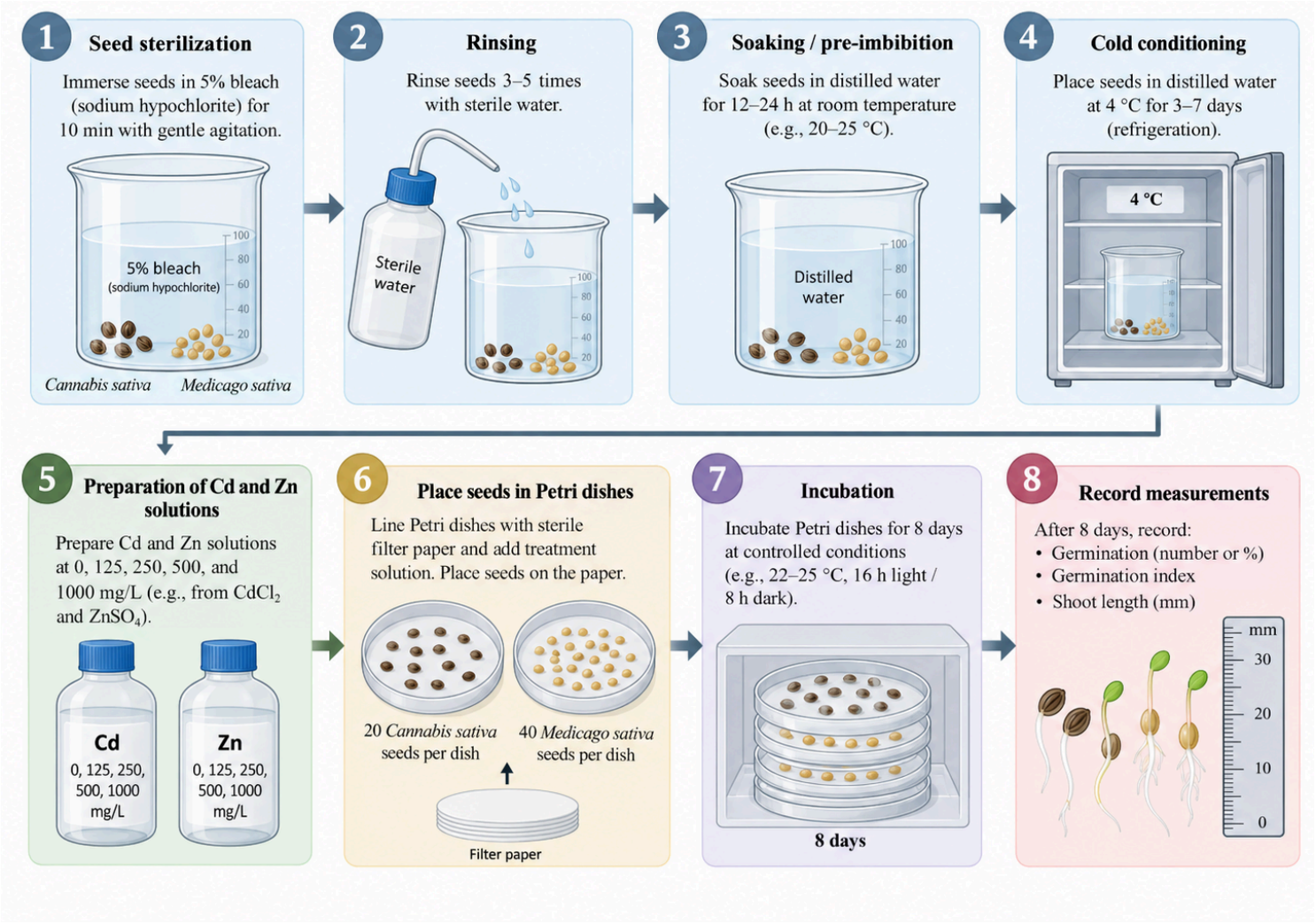


Figure 5

Workflow of the in vitro germination assay, including seed sterilization, pre-treatment, incubation, and endpoint recording. Conceptual schematic generated by Gemini Google.

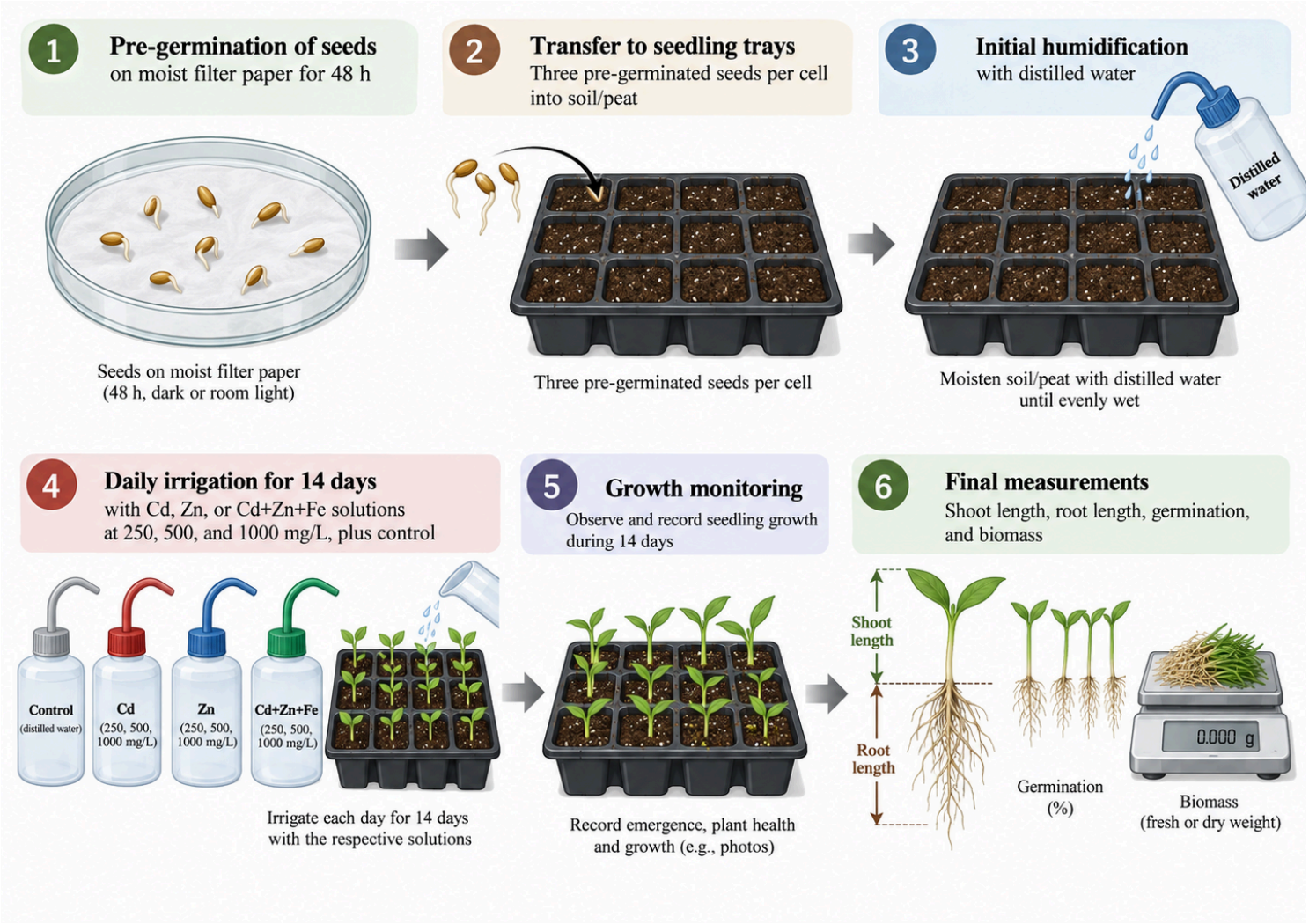


Figure 6

Workflow of the soil-based seedling-tray assay used to monitor seedling growth, germination and biomass under daily irrigation with the different metal treatments. Conceptual schematic generated by Gemini Google.

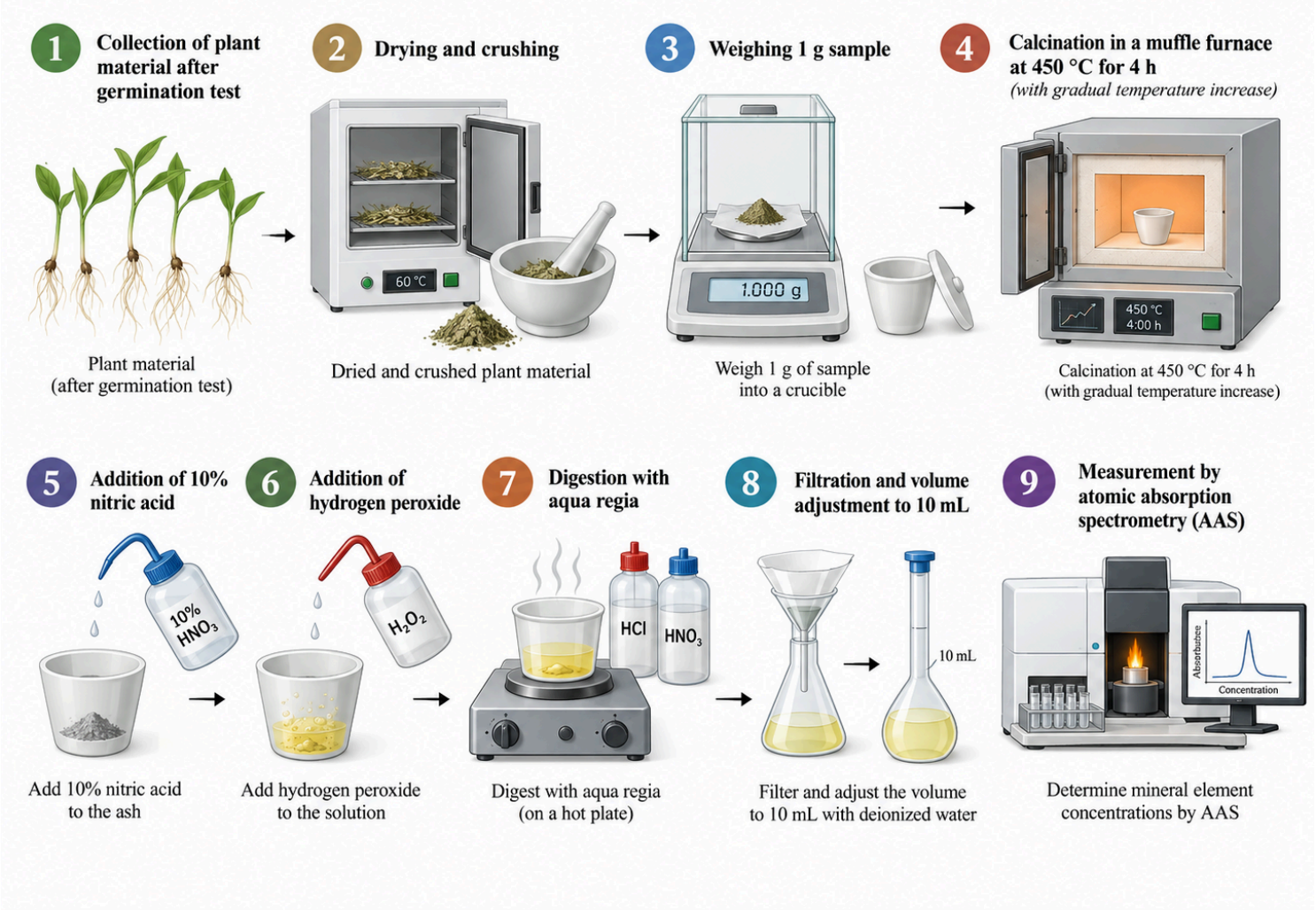


Figure 7

Workflow of plant sample mineralization and atomic absorption spectrometry (AAS) analysis. Conceptual schematic generated by Gemini Google.

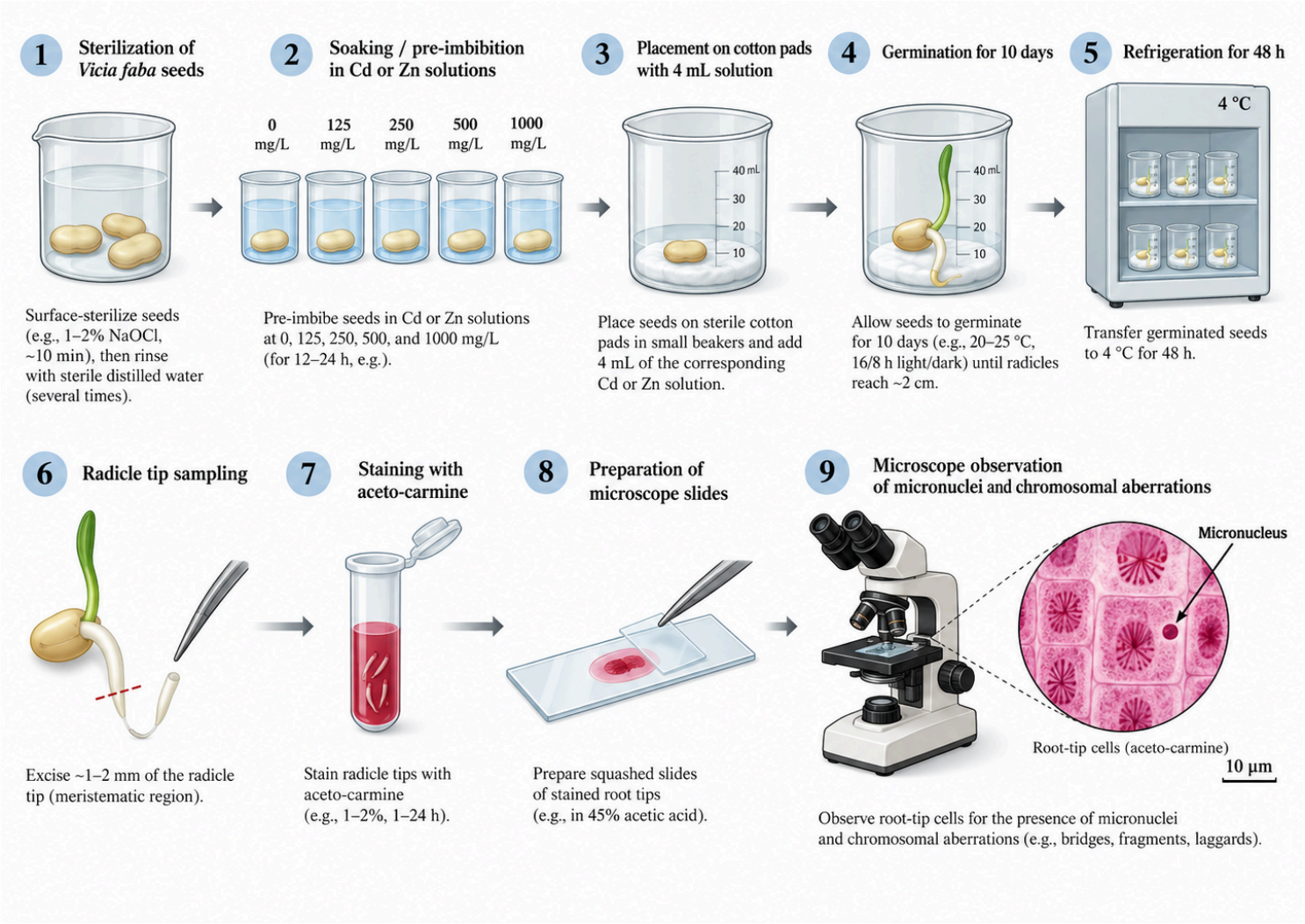


Figure 8

Workflow of the Vicia faba genotoxicity assay, from seed sterilization and exposure to cytological observation of micronuclei and chromosomal aberrations. Conceptual schematic generated by Gemini Google.