Lipid Peroxidation as a Biomarker for Heavy Metal Stress in Plants

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Heavy metal contamination in soil poses a significant threat to plant health, agriculture, and ecological stability. This study investigates the potential of lipid peroxidation, specifically malondialdehyde (MDA) accumulation, as a biomarker for oxidative stress in plants exposed to heavy metals. Plants were subjected to varying concentrations of Copper Sulfate, and physiological parameters such as root length were recorded to assess growth inhibition. To quantify lipid peroxidation, a thiobarbituric acid reactive substances (TBARS) assay was performed, with a standard curve generated using 1,1,3,3-tetramethoxypropane (TMP) as an MDA equivalent. Results revealed a dose-dependent increase in MDA levels correlating with reduced root growth, indicating enhanced oxidative damage under metal stress. These findings support the use of lipid peroxidation as a reliable indicator of heavy metal toxicity and lay the groundwork for its application in phytoremediation studies and environmental monitoring.

Environmental contamination by heavy metals poses a significant threat to plant health and agricultural productivity. Copper, while essential in trace amounts, becomes toxic at elevated concentrations, leading to the generation of reactive oxygen species (ROS) via redox reactions with the metal. These ROS trigger oxidative stress, damaging cellular components such as proteins, nucleic acids, and lipids.[1]

Lipid peroxidation refers to the oxidative degradation of polyunsaturated fatty acids in cell membranes, a process initiated by ROS. A key byproduct of this reaction is malondialdehyde (MDA), which can be quantitatively measured using a method developed by Janero.[2] The accumulation of MDA thus provides a proxy for assessing oxidative damage in plant tissues.[3]

This study aims to evaluate whether lipid peroxidation, as measured through MDA levels using the thiobarbituric acid reactive substances (TBARS) assay, can serve as a reliable and sensitive biomarker for heavy metal stress in plants. By establishing a correlation between $CuSO_4$ concentrations and MDA accumulation, we seek to validate a cost-effective method for monitoring environmental toxicity.[4]

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Materials and Methods

Plant Material and Growth Conditions

Two plant species were selected for this study: *Pisum sativum* (pea) and *Raphanus sativus* (radish). Seeds were surface-sterilized by immersion in 70% ethanol for 1 minute followed by 1% sodium hypochlorite for 10 minutes, then rinsed three times with sterile distilled water to eliminate surface contaminants.[5] Sterilized seeds were germinated in a controlled environment on culture media or soil treated with varying concentrations of copper sulfate ($CuSO_4$).

For the root elongation assay, both pea and radish seeds were germinated on agar-solidified media supplemented with $CuSO_4$ at concentrations of 0 (control), 50, 100, 250, 350, and 500 µM.6 Seeds were spaced evenly in sterile Petri dishes and oriented such that root growth would occur down the length of the plate. Plates were placed at a 45° angle to encourage straight root growth.

For the MDA assay, only radish plants were used. Seeds were sown in soil contained in pots, each amended with six increasing concentrations of $CuSO_4$ (0–400 ppm). Plants were grown for 14 days under ambient light, with daily watering to maintain consistent moisture. One additional radish plant was grown in soil containing 250 ppm $CuSO_4$ for assay validation.

Root Elongation Assay

After 7 days of growth, roots of germinated seedlings were carefully removed from the plates and photographed. For each concentration, two plates containing six seedlings were analyzed. Average root length per treatment was calculated and plotted to evaluate dose-dependent effects of copper toxicity.

MDA Quantification via TBARS Assay

The level of lipid peroxidation in radish tissues was estimated by quantifying malondialdehyde (MDA) using a thiobarbituric acid reactive substances (TBARS) assay, adapted from the protocol described by Dhindsa *et al.*,[3] and followed by Baryla *et al*.[4] Leaf tissues (0.3 g) were harvested from each treatment. Samples were ground in a chilled mortar with 1.25 mL of 0.1% trichloroacetic acid (TCA) containing 1% sodium dodecyl sulfate (SDS) to ensure protein denaturation and lipid solubilization.

The homogenate was centrifuged at 12,000 g for five minutes, after which 300 µL of supernatant was mixed with 1 mL of 20% TCA containing 0.5% thiobarbituric acid (TBA). The mixture was incubated at 95°C for 30 minutes, then cooled rapidly in an ice bath to halt the reaction. Absorbance was measured at 532 nm using a UV-visible spectrophotometer. Non-specific absorbance at 600 nm was subtracted to correct for turbidity.[7]

MDA concentration was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}.[2] Standard curves were prepared using 1,1,3,3-tetraethoxypropane (TEP) as the MDA standard, allowing for quantitative comparisons. Although the TBARS assay is widely used, it should be noted that it is

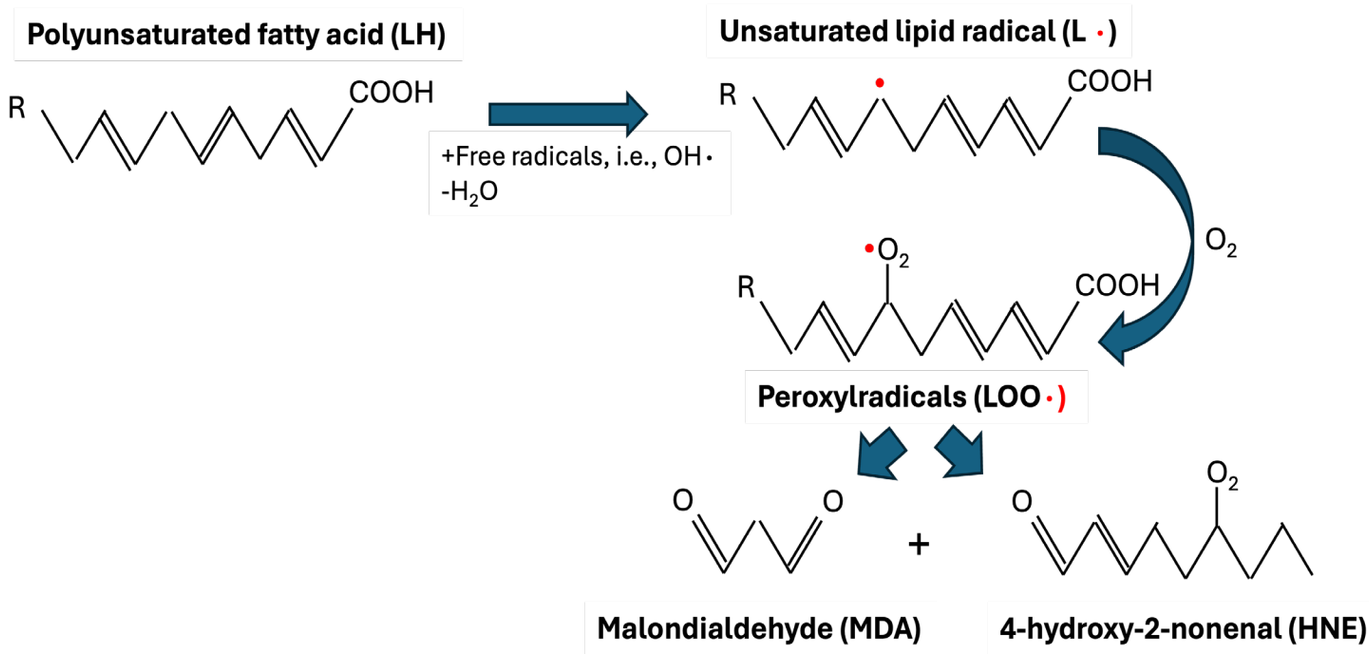

Figure 1 | Process of MDA formation as a byproduct from Lipid peroxidation reaction.

not wholly specific for MDA and may also detect other aldehydes or sugar degradation products. [4,8]

Assay Validation

To evaluate the accuracy of the MDA-based assay for estimating copper exposure, a standard curve was generated by correlating known soil concentrations of $CuSO_4$ with corresponding MDA levels measured in plant tissues. A test sample grown in soil containing 250 ppm $CuSO_4$ was then analyzed, and its MDA concentration was used to interpolate the Copper exposure from the established standard curve. A ±5% deviation threshold was predetermined as the target range for acceptable assay accuracy.

Results

Effects of Copper Sulfate on Root Elongation

The root elongation assay revealed a clear dose-dependent inhibition of growth in both Pisum sativum (pea) and Raphanus sativus (radish) seedlings exposed to increasing concentrations of $CuSO_4$. In control conditions (0 µM $CuSO_4$), both species exhibited healthy, elongated root systems, with average root lengths of 7–10 cm after seven days of growth (**Figure 2A&B**).

Upon exposure to 50 µM $CuSO_4$, a significant reduction in root length was observed, indicating early signs of metal-induced stress. More pronounced inhibition occurred at 100 µM. At 250 µM and above, root growth was significantly impaired (**Figure 2C&D**). At 500 µM $CuSO_4$, radish roots showed marked browning and necrosis at the tips—symptoms often associated with metal. This progressive reduction in root elongation reflects a dose-dependent phytotoxic response to copper exposure.

Quantification of Lipid Peroxidation via MDA Levels

The TBARS assay used to estimate MDA content in radish leaves showed a strong positive correlation between $CuSO_4$ concentration in soil and the degree of lipid peroxidation. In control plants, baseline MDA levels were minimal, indicating low levels of oxidative stress under non-toxic conditions.

With the introduction of 50 ppm $CuSO_4$ into the soil, a measurable increase in MDA content was observed, suggesting the onset of oxidative stress (**Figure 3**). At 100 ppm, MDA levels nearly doubled and continued to rise steadily with each subsequent concentration. The highest MDA concentration was recorded in the 300 ppm treatment group. The MDA concentration at 400 ppm was lower than that observed in 300 ppm, the cause for which could indicate failure of metabolic functions due to very high toxicity from copper.

The results align with those of Baryla *et al*.,[4] who observed similar increases in MDA content in *Brassica napus* exposed to Cu-enriched media. These findings support the hypothesis that copper-induced oxidative stress can be reliably detected by assessing lipid peroxidation in plant tissues.

Linear regression analysis demonstrated a statistically significant correlation (**Figure 3A**, $R^2 > 0.95$) between $CuSO_4$ concentration and MDA levels in plant tissues. The generation of a standard curve using 1,1,3,3-tetraethoxypropane (TEP) allowed for precise quantification of MDA, confirming the assay's sensitivity and reproducibility across a range of copper treatments.

The regression model confirmed the linear behavior of the TBARS assay within the tested concentration range, validating its use as a quantifiable indicator of stress severity.

Assay Validation and Accuracy

To evaluate the accuracy of the TBARS assay in estimating Cu-induced oxidative stress, a validation sample was cultivated in soil amended with 250 ppm $CuSO_4$. A target tolerance of ±5% deviation from the known concentration was established a priori. The assay estimated internal Cu exposure at 264.2 ppm, corresponding to a 5.6% deviation—slightly exceeding the predefined threshold (**Figure 3B**). While this result did not meet the established criterion

for accuracy, the minimal overage suggests the method may approach a practical level of reliability with refinement. These findings support further refinement of the assay protocol and warrant its re-evaluation in future studies aimed at field-applicable soil toxicity screening and bioavailability assessment.[4]

Visual and Physiological Symptoms

Beyond quantitative data, visual inspection of the radish plants **(data not shown)** revealed symptoms that further supported the biochemical findings. As $CuSO_4$ concentration increased, radish leaves exhibited curling, reduced leaf area, and, in severe cases, chlorosis and necrosis. These symptoms corresponded with MDA trends and are consistent with copper's role in disrupting photosynthesis and generating ROS.[1]

Discussion

The findings support the hypothesis that lipid peroxidation serves as a measurable marker of oxidative stress in plants exposed to heavy metal contamination. Despite the need for further refinement, the TBARS assay proved to be a straightforward and reproducible method for quantifying MDA levels.

Root elongation assays complemented the biochemical data by directly correlating copper toxicity with impaired plant health. Studies have consistently reported that copper accumulation significantly inhibits root growth—even at relatively low concentrations—serving as a sensitive indicator of metal-induced outcomes.[9,10] Our root assays confirm this well-established relationship, strengthening the validity of the TBARS results by linking oxidative damage to physiological impairment. Although the validation assay slightly overestimated Cu concentration—yielding a 5.6% deviation from the known value—this result remains near the predefined ±5% margin of acceptability. This minimal discrepancy underscores the assay's potential for practical use in environmental monitoring and preliminary diagnostic applications.

The tentative correlation between $CuSO_4$ concentration and MDA accumulation demonstrates that this method can be used for early and quantitative detection of environmental contamination. The simplicity and cost-effectiveness of the TBARS assay make it a valuable tool for agricultural and ecological applications.

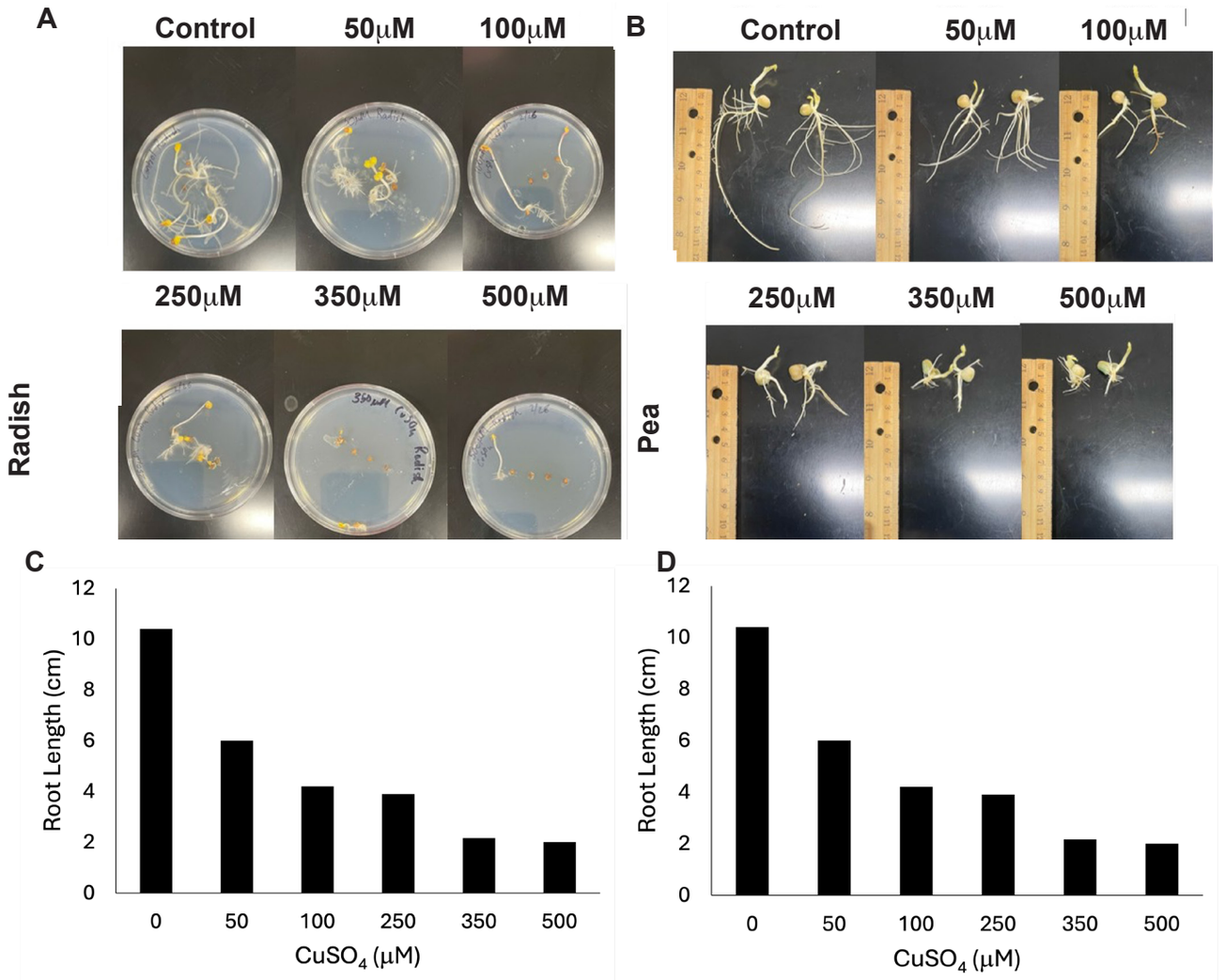

Figure 3 | Root Growth is inversely correlated with concentration of $CuSO_4$ in soil. Root Elongation Tests on *Pisum sativum* (pea) and *Raphanus sativus* (radish). A & B show Radish and Pea roots respectively grown in a petri dish with 1% Agar and $CuSO_4$. C & D show corresponding graphs of the A & B. Readings were taken after 7 days of sowing.

Future Directions

Given that the validation assay marginally exceeded the established accuracy threshold, the current methodology will be reviewed for potential improvements. While this study centered on copper exposure, future research should evaluate the specificity of MDA as a biomarker for other heavy metals, including cadmium, lead, and zinc. Expanding the assay's application to additional plant species and varied stress conditions will further assess its robustness and broaden its environmental relevance.

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Author Biography

Soham Kawade is arecent graduate of the Biotechnology program at the University of Kansas. His research focuses on plant stress responses and biomarker development. He plans to pursue a career in industrial biotechnology, developing innovative solutions to improve human and environmental health through applied research and technology development.

Author Contributions

S.K. contributed to the experimental work, design, and writing of this work; B.M., S.T., M.D., and J.F.T contributed to the design and editing of this work.

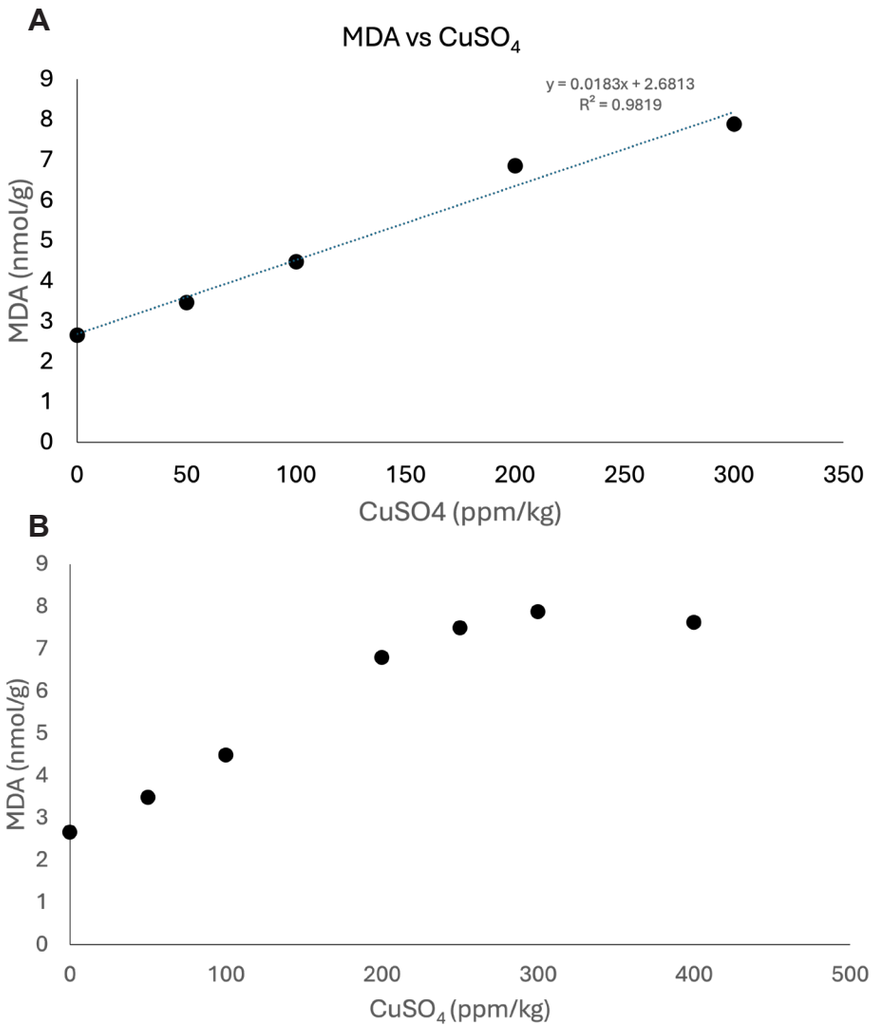

Figure 3 | MDA Calculation as a function of $CuSO_4$ in soil. A Shows a standard curve of MDA readings from plants grown in $CuSO_4$ -infused soil with regression analysis for standard curve generation (R^2 = 0.9819). B shows an 'unknown' plant sample plotted against the standard for assay validation. Samples were collected after 2 weeks of planting.

References

1. Halliwell, B. & Gutteridge, J. M. Oxygen toxicity, oxygen radicals, transition metals and disease. Biochem. J. 219, 1–14 (1984).
2. Janero, D. R. Malondialdehyde and thiobarbituric acid-reactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury. Free Radic. Biol. Med. 9, 515–540 (1990).
3. Dhindsa, R., Plumb-Dhindsa, P. & Thorpe, T. Leaf senescence and lipid peroxidation. J Exp Bot 32, 93–101 (1981).
4. Baryla, A., Laborde, C., Montillet, J. L., Triantaphylidès, C. & Chagvardieff, P. Evaluation of lipid peroxidation as a toxicity bioassay for plants exposed to copper. Environ. Pollut. Barking Essex 1987 109, 131–135 (2000).
5. D, S. & M, M. Standardizing the Methods for Breaking Seed Dormancy to Enhance Germination of Gloriosa superba Seeds. Expert Opin. Environ. Biol. 04, (2016).
6. Hajiboland, R. An evaluation of the efficiency of cultural plants to remove heavy metals from growing mediu. Plant Soil Environ. 51, (2005).
7. Heath, R. L. & Packer, L. Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. Arch. Biochem. Biophys. 125, 189–198 (1968).
8. Bird, R. P. & Draper, H. H. Comparative studies on different methods of malonaldehyde determination. Methods Enzymol. 105, 299–305 (1984).
9. Rooney, C. P., Zhao, F.-J. & McGrath, S. P. Soil factors controlling the expression of copper toxicity to plants in a wide range of European soils. Environ. Toxicol. Chem. 25, 726–732 (2006).
10. Copper-induced stress in *Solanum nigrum L.* and antioxidant defense system responses - Fidalgo - 2013 - Food and Energy Security - Wiley Online Library. https://onlinelibrary.wiley.com/doi/full/10.1002/fes3.20?utm_source=chatgpt.com.