Learnovate-International
An International Open-Access Journal Published By Lapain Press Publications[LPP]
Content Available at www.lapinjournals.com E-ISSN: 3049-1592
Research Article Open Access

SELECTIVE HEAVY METAL UPTAKE AND TOLERANCE MECHANISMS IN BRASSICA NIGRA: IMPLICATIONS FOR SUSTAINABLE PHYTOREMEDIATION

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Article History: Received: 20 Feb 2026, Revised: 25 Mar 2026, Accepted: 08 Apr 2026

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ABSTRACT
Heavy metal environmental contamination is a pressing global concern, as buildup in soils and water presents substantial threats to human and ecological health. This study examines the phytoremediation capability of Brassica nigra for the extraction of iron (Fe), zinc (Zn), and molybdenum (Mo) from polluted soils. B. nigra seeds were grown under controlled conditions in soils artificially contaminated with Fe, Zn, or Mo, and their physiological and anatomical responses were assessed. Atomic absorption spectroscopy indicated considerable absorption of Fe, Zn, and Mo by B. nigra, accompanied by notable decreases in soil metal concentrations and matching increases in plant tissues. Physiological studies revealed increased activity of polyphenolics, superoxide dismutase, glutathione peroxidase, and polyphenol oxidase in metal-treated plants, indicating strong antioxidant responses. Anatomical studies revealed metal-specific modifications in vascular tissue diameter and stomatal dimensions, with exposure to Fe and Mo significantly decreasing vascular diameter and stomatal width. These findings emphasize the selective and efficient metal absorption of B. nigra and underscore its ability to endure and accumulate heavy metals. The findings endorse B. nigra as a viable option for sustainable phytoremediation in heavy metal-contaminated settings.

Keywords: *Heavy metals, soil contamination, Brassica nigra, phytoremediation, antioxidant enzymes.*

1. INTRODUCTION

Environmental contamination by heavy metals has emerged as a significant global issue. The extraction of heavy metals from ores and their subsequent processing for various uses has resulted in the release of these elements into the environment. The issue of heavy metal contamination is intensifying due to escalating industrialisation and the disruption of natural biogeochemical cycles [1]. In contrast to organic substances, heavy metals are fundamentally nonbiodegradable and hence accumulate in the environment. The accumulation of heavy metals in soil and water is a threat to environmental and human health. These elements accumulate in the tissues of living animals (bioaccumulation), and their concentrations escalate when they transition from lower to higher trophic levels (biomagnification). Heavy metals in soil induce toxicological effects on soil microorganisms, potentially resulting in a reduction of their populations and activity [2]. Heavy metals are categorised as essential and non-essential based on their function in biological systems. Essential heavy metals are required by living organisms in trace amounts for crucial physiological and biochemical processes. Essential heavy metals include Fe, Mn, Cu, Zn, and Ni [3]. Non-essential heavy metals are those that are not required by living organisms for any physiological or metabolic activity. Examples of nonessential heavy metals include Cd, Pb, As, Hg, and Cr [4].

Heavy metals infiltrate the environment from both natural and anthropogenic sources. The primary natural sources comprise mineral weathering, erosion, and volcanic activity, whereas anthropogenic sources encompass mining, smelting, electroplating, pesticide and phosphate fertilizer application, agricultural biosolids, sludge disposal, industrial effluent, and atmospheric deposition [5, 6]. Heavy metals adversely affect human health; thus, the contamination of the food chain by heavy metals warrants significant

consideration. Numerous heavy metals and metalloids are hazardous and can induce adverse effects and significant issues even at minimal doses [7]. Heavy metals induce oxidative stress through the generation of free radicals [8], which can surpass the cell's inherent antioxidant defenses, resulting in cellular damage or apoptosis [9]. Moreover, they can substitute critical metals in pigments or enzymes, impairing their functionality [10]. The most concerning heavy metals in terms of toxicity include Hg, Cd, Pb, As, Cu, Zn, Sn, and Cr [11, 12].

The permanence and toxicity of heavy metals necessitate the cleanup of polluted sites as an environmental and public health priority. Conventional remediation techniques, including soil excavation, chemical leaching, and solidification, are frequently expensive, environmentally intrusive, and may produce secondary contamination. These constraints have stimulated research in alternate, sustainable methods that are both efficient and environmentally friendly. Phytoremediation is a technique that utilizes the inherent capabilities of green plants and their associated rhizosphere bacteria to eliminate, stabilize, or decompose pollutants in situ [13]. Phytoremediation is defined as the utilization of plants to diminish the concentrations or harmful effects of contaminants, such as heavy metals, radionuclides, and organic compounds, in contaminated soils, sediments, and water. In contrast to traditional methods, phytoremediation is economically efficient, reliant on solar energy, environmentally sustainable, and can be executed with minimal site disruption [14, 15]. Moreover, plants typically manage pollutants without compromising topsoil, thus preserving its functionality and fertility. They can enhance soil fertility by the addition of organic matter [16]. Green plants have a significant capacity to absorb environmental contaminants and facilitate detoxification through multiple methods [17].

However, the choice of plant species has a significant impact on the effectiveness of phytoremediation. Effective phytoextractors must exhibit traits such as substantial biomass production, fast development, tolerance to elevated metal concentrations, and the capacity to accumulate metals in harvestable tissues [16]. Numerous species within the Brassicaceae family, including *Brassica juncea*, *Brassica napus*, and *Brassica nigra*, exhibit remarkable capacity for heavy metal accumulation and tolerance. These plants can sequester elevated levels of metals in nonmetabolic regions, therefore mitigating toxicity to essential physiological functions and preserving growth and productivity [17]. *Brassica nigra* (black mustard) is distinguished among the possibilities for its vigorous growth, substantial biomass yield, and exceptional capacity to withstand and accumulate metals [16]. Nonetheless, it is important to acknowledge that elevated concentrations of heavy metals might impede plant growth by disrupting nutrient absorption, constraining cellular development, and diminishing photosynthetic efficiency [18, 19]. This study aims to assess the phytoremediation capability of *Brassica nigra* for the extraction of Zn, Fe, and Mo metals from soil, along with the morphological and physiological alterations in *Brassica nigra*.

2. MATERIALS AND METHODS

Procurement of *Brassica nigra* Seeds

Healthy and young *Brassica nigra*seeds were collected the fields of local farmers of Vizianagaram (Long: 18°33' 52"to 18° 32' 11" N; Lat: 84° 21' 26" E to 84° 18' 22" E) Andhra Pradesh, India. Approximately, 500 gm of seed samples were collected for research study. The collected *Brassica nigra*seeds were aseptically transferred to the zip bags and transported to the laboratory.

Germination of Seeds

The seeds of *Brassica nigra*were washed with tap water and then distilled water. Washed seeds were surface sterilized with 70% ethanol and 2% sodium hypochlorite. Then the seeds were soaked overnight in water and kept for germination on moistened filter paper in sterile Petri dish for 7 days. After initiation of germination seedlings were transferred to plastic pots filled with autoclaved vermiculture. The pots were grown at 25°C under 16 h photoperiod in a green house.

Experimental Design

The *Brassica nigra* plants were divided into four experimental groups. In the 1st experimental group, the plants were irrigated with distilled water and it referred to as control group. In the 2nd experimental group, the plants were grown in a 100 mM $FeCl_3$ contaminated soils. In the 3rd experimental group, the plants were grown in a 100 mM $ZnSO_4$ contaminated soils. In the 4th experimental group, the plants were grown in a 100 mM Na_2MoO_4 contaminated soils. The 2nd, 3rd, and 4th experimental groups treated with respective salt solutions for every 3 days. After 2 weeks of growth, the soil and the plants were harvested for the further analysis including accumulation of Fe, Zn, and Mo, as well as plant physiological and anatomical changes.

Extraction and Quantification of Fe, Zn, and Mo

The mineral extraction was performed from the roots and leaves of all the four experimental groups as well as from soil samples of respective experimental groups. To extract metals, 5 gm of tissue and soil were taken from each treatment group and dried in a hot air oven. Then the dried material was powdered and placed into 25mL conical flask. Then, 6mL HNO_3 acid was added and kept at room temperature overnight to predigest the sample. The sample with HNO_3 acid into a digestion block port was then heated at 150°C for 60 minutes to remove red fume, Afterwards, it was cooled at room temperature. 2mL of $HClO_4$ acid was added to the sample and placed again into a digestion block port at a block temperature of 215°C for 2 hours. Then the sample was cooled in a hood for 20 minutes and 10mL de-ionized water was added on hot

plate (90°C). The solution was mixed up well using a vortex stirrer, next it was cooled, and diluted in a 50mL volumetric flask. Upon completion of the digestion process, the samples were filtered into a 50 ml flask using Whatman No. 44 filter paper. The concentration of Fe, Zn, and Mo ions in the roots and leaves of all the three experimental groups as well as from soil samples of respective experimental groups were determined using Atomic Adsorption Spectroscopy (AAS) in conjunction with flame photometry (Shimadzu AA-6800).

Screening of Physiological Responses

Estimation of Polyphenolics

The quantity of total phenolics in the leaf extract was determined according to the Folin-ciocalteu procedure [20]. 200 µl of the leaf and root tissue methanolic extracts from all the three treatment group plants were taken in test tubes. 1ml of Folin ciocalteu reagent and 0.8 ml of 7.5% sodium carbonate were added. The tubes were mixed and allowed to stand for 30 min. Absorption of the developed colour was measured by spectrophotometer at 765 nm. The total phenolic content was calculated using standard Gallic acid calibration curve. And the results were expressed as mg of gallic acid equivalents (GAE) per gm extract.

Extraction and Estimation of Superoxide Dismutase

0.1g fresh seedlings from all the three treatment group plants were homogenized with 3 ml of 0.1 M phosphate buffer. The homogenate was centrifuged at 2000rpm for 10 minutes at 4°C and the supernatant was used for the determination of superoxide dismutase [21]. To 0.5 ml of leaf extract, 1ml 125mM of Sodium Carbonate, 0.4 ml of 24µM NBT and 0.2 ml of 0.1mM EDTA were added. The reaction was initiated by adding 0.4 ml of 1mM Hydroxylamine hydrochloride. At zero-time, absorbance was taken at 560 nm using spectrophotometer, followed by recording the absorbance after 5 min at 25°C and the control was simultaneously run without leaf extract. The activity of an enzyme measured from inhibiting the reduction of NBT by 50%. The specific activity was expressed in terms of Units/mg of protein.

Estimation of Glutathione Peroxidase (GPX)

0.1g fresh seedlings from all the three treatment group plants were homogenized with 3 ml of 0.1 M phosphate buffer. The homogenate was centrifuged at 2000rpm for 10 minutes at 4°C and the supernatant was used to determine GPX activity by coupled enzyme method [22]. In this assay,the reaction mixture consisted of 1mMGSH, 1 U of GRD/mL, and 0.2mM NADPH in reactionbuffer, with a final volume of 1.39 mL. 100 µLof test sample or dH_2O (blank) was incubated with the reaction mixture for 4 minutes at 21°Cprior to addition of 10 µL of 17mM cumene hydroperoxide (final concentration, 0.1mM). Absorbance at340 nm was measured via spectrophotometer every 30seconds for 15 minutes. The concentration of oxidizedNADPH was calculated by use of the molar extinctioncoefficient for NADPH, 6.220 $m^{-1} \cdot cm^{-1}$.

Determination of PPO

The increase in the absorbance at 410 nm was used for determining the PPO activity. Catechol was used as a substrate in all the experiments. Different concentration of substrate (0.01, 0.03, and 0.05 M), at pH 7.0 and temperature 40°C were used. Each tube has 2.0 ml of the substrate solution, sodium phosphate buffer (0.9 ml) with pH 7.0, and crude plant extract (0.1 ml). The blank tube has only the substrate and the buffer. A change in the absorbance of 0.001 $minute^{-1}$ was considered as one unit of PPO activity [23].

Screening of Anatomical Changes

The anatomical changes in the leaf and stem from all the experimental groups was performed by observing under microscope. For anatomical analyses, stem and the first fully expanded leaves from all the experimental groups collected. The material was fixed in FAA (formaldehyde + acetic acid + ethyl alcohol 70 % 5:5:90 $L\ L^{-1}\ L^{-1}$) [24] for 72 hours and then kept in ethyl alcohol 70 %. Then the upper and lower epidermis of leaf was peeled and safranin staining mixture was applied [25]. For roots cross sections were taken and the sections were stained with a safranin solution at 1%. Glycerin 50 % was used to mount the slides. All slides were examined and photographed using a Lynx light microscope and a Capture 2.4 HDMI camera.

Statistical Analysis

All the results of present studies were given as Mean ± Standard Deviation (SD) obtained from three independent experiments, and the data was assessed by one way analysis of variance (ANOVA). The 'p' value between greater than 0.01 and less than 0.05 was considered as significant difference.

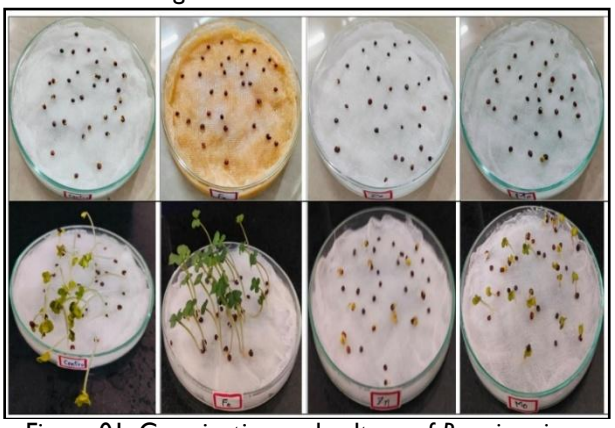

Figure 01: Germination and culture of *Brassica nigra* seeds with experimental conditions.

3. RESULTS AND DISCUSSION

Determination of Fe, Zn, and Mo Metal Accumulation

The Fe, Zn, and Mo quantities in the soil samples after experiment of three treatment groups were shown in Table 1. From these results, the concentrations of Fe, Zn, and Mo in control soil was found to be 0.23±0.01, 0.41±0.01, and 0.39±0.01 µM. While, the soil of 2nd

treatment group (Fe treated) has Fe, Zn, and Mo concentrations of 47.22±3.5, 0.22±0.01, and 0.37±0.01 µM. The soil of 3rd treatment group (Zn treated) has Fe, Zn, Mo concentrations of 0.31±0.01, 48.33±2.68, and 0.29±0.01 µM. As well as, the soil of 4th treatment group (Mo treated) has Fe, Zn, Mo concentrations of 0.31±0.01, 48.33±2.68, and 0.29±0.01 µM. From these results, it was found that the Fe, Zn, Mo concentration in soils were significantly reduced by plant growth.

Table 01:Fe, Zn, Mo quantities in the soil samples after the experiment.

S. No.	Soil group	Concentration of metals (µM)		
		Fe	Zn	Mo
1	Control soil	0.23±0.01	0.41±0.01	0.39±0.01
2	Treatment group II (100 µM Fe treated soil)	47.22±3.5	0.22±0.01	0.37±0.01
3	Treatment group III (100 µM Zn treated soil)	0.31±0.01	48.33±2.68	0.29±0.01
4	Treatment group IV (100 µM Mo treated soil)	0.35±0.01	0.29±0.01	56.41±1.67

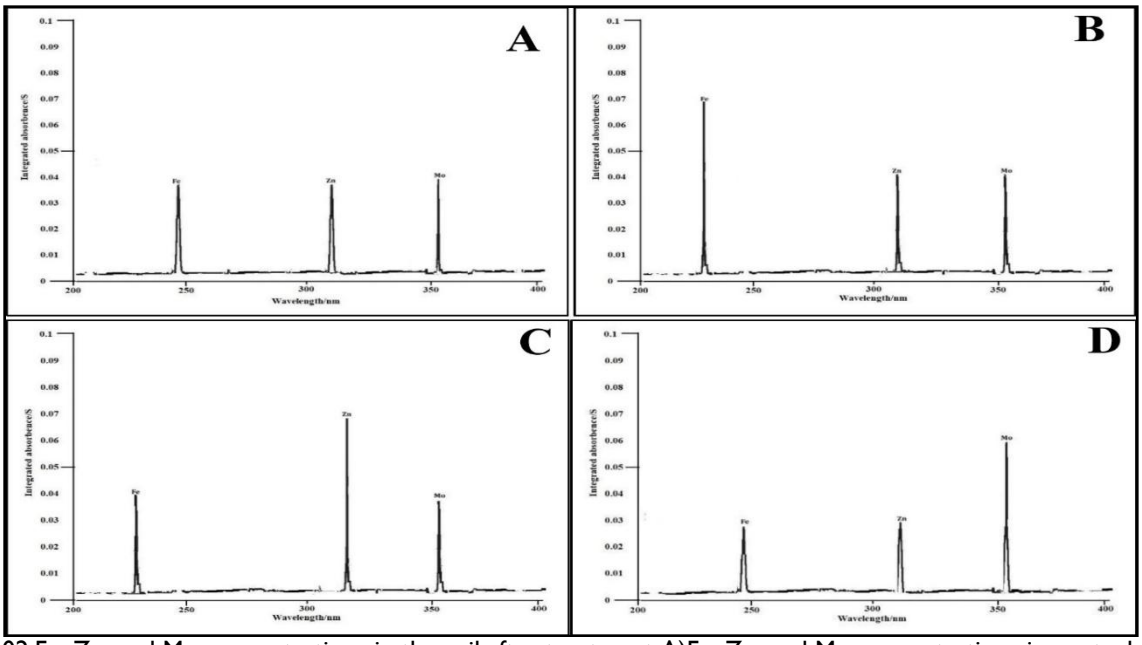

Figure 02:Fe, Zn, and Mo concentrations in the soil after treatment A)Fe, Zn, and Mo concentrations in control soil B) Fe, Zn, and Mo concentrations in 2nd treatment group (Fe-treated) C) Fe, Zn, and Mo concentrations in 3rd treatment group (Zn-treated) D) Fe, Zn, and Mo concentrations in 4th treatment group (Mo-treated).

Furthermore, the Fe, Zn, and Mo quantities in the seedlings of *Brassica nigra* plants after experiment of four treatment groups were shown in Table 2. From these results, the concentrations of Fe, Zn, and Mo in the seedlings of control plant were observed as 0.36±0.09, 0.02±0.01, and 0.01±0.01 µM. While, the concentrations of Fe, Zn, and Mo in the seedlings of Fe treated (2nd experimental group) plant were observed as 23.21±2.4, 0.02±0.01, and 0.01±0.01µM. Furthermore, the concentrations of Fe, Zn, and Mo in the seedlings of 3rd treatment group (Zn-treated) plant were observed as 0.03±0.01, 29.81±3.4, and 0.2±0.01 µM respectively. The concentrations of Fe, Zn, and Mo concentration in the seedlings of 4th treatment group (Mo-treated) plants were observed as 0.02±0.01, 0.02±0.01, and 32.18±2.1 µM. From these results, it was found that the Fe, Zn, and Mo metals were significantly absorbed by plant and exhibited substantial phytoremediation potential.

Table 02: Fe, Zn, and Mo quantities in the seedlings of *Brassica nigra* plants after experiment.

S. No.	Treatment group	Concentration of metals (µM)		
		Fe	Zn	Mo
1	Treatment group I (Control)	0.36±0.09	0.02±0.01	0.01±0.01
2	Treatment group II (100 µM Fe treated)	23.21±2.4	0.02±0.01	0.01±0.01
3	Treatment group III (100 µM Zn treated)	0.03±0.01	29.81±3.4	0.2±0.01
4	Treatment group IV (100 µM Mo treated)	0.03±0.01	0.02±0.01	32.18±2.1

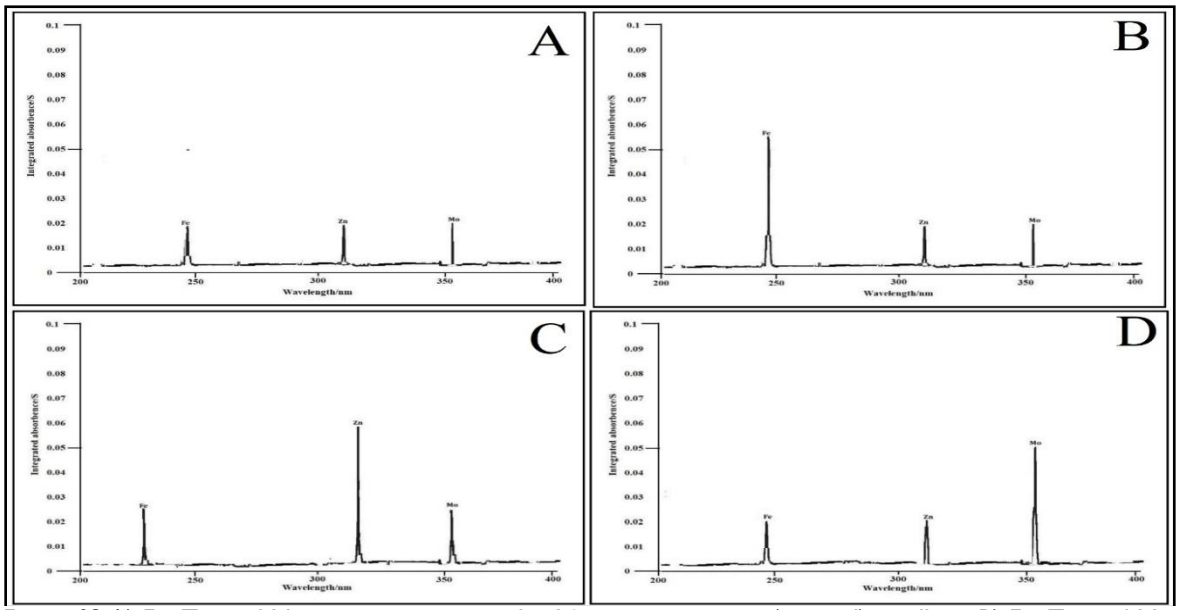

Figure 03:A) Fe, Zn, and Mo concentrations in the 1st treatment group (control) seedlings B) Fe, Zn, and Mo concentrations in the 2nd treatment group (Fe-treated) seedlings C) Fe, Zn, and Mo concentrations in the 3rd treatment group (Zn-treated) seedlings D) Fe, Zn, and Mo concentrations in the 4th treatment group (Mo-treated) seedlings.

In the present study, the phytoremediation potential of *Brassica nigra* by analyzing the uptake of Fe, Zn, and Mo across four different treatment groups. The control group exhibited baseline metal concentrations in seedlings at 0.36 ± 0.09 µM (Fe), 0.02 ± 0.01 µM (Zn), and 0.01 ± 0.01 µM (Mo). Notably, Fe-treated plants showed a dramatic increase in Fe content (23.21 ± 2.4 µM), while Zn and Mo levels remained similar to controls. This suggests a selective and efficient uptake of Fe when supplied in excess, consistent with earlier findings on metal-specific uptake in *Brassica* species [26].

In accordance with the present findings, Sharma and Dubey [27] mentioned that *Brassica juncea* is especially notable due to its high biomass, rapid growth, and exceptional ability to hyperaccumulate metals such as Pb, Cd, and Zn. Similarly, studies of Zhou et al. [28] demonstrated that *Brassica napus* exhibited moderate metal uptake but excels in tolerating contaminated conditions, making it suitable for phytoextraction in soils with mixed heavy metal pollution. Ali et al. [29] reported that *B. oleracea* efficient metal uptake, offers resilience against metal-induced phytotoxicity and can be used for phytostabilization strategies. Differences in phytoremediation efficiency among *Brassica* species are attributed to variations in root architecture, metal transport, and sequestration mechanisms. For instance, *B. juncea*'s roots release more exudates that mobilize metals, enhancing uptake. In contrast, *B. napus* tends to accumulate metals in roots rather than shoots, reducing the risk of metal entry into the food chain [28]. Sharma and Dubey [27] stated that the targeted uptake underscores the capacity of *Brassica nigra* to selectively accumulate specific heavy metals based on their availability in the growth medium, a characteristic that is highly valuable for phytoremediation applications.

Screening of Physiological Responses

Estimation of Polyphenolics

The quantities of polyphenolics showed significant deviation among the treatment groups of Fe, Zn, and Mo. The results of total polyphenolic content in all the four treatment groups such as control, Fe treated, Zn treated, and Mo treated were shown in Table 3. From these results, the seedlings of *Brassica nigra* under the stress of Fe treatment exhibit greater concentration of polyphenolics whereas, the control group shows the lowest concentration of polyphenolics. The total polyphenolic quantities in the seedlings of control, Fe treated, Zn treated, and Mo treated were found as 28.37±2.7, 79.83±4.3, 57.12±3.8, and 44.51±2.8 mg/gmFW.

Table 03:Total polyphenolics concentration in the seedlings of four treatment group plants.

S. No.	Treatment group	Polyphenolicsconcentration (mg/gmFW)
1	Control	28.37±2.7
2	Fe treated	79.83±4.3
3	Zn treated	57.12±3.8
4	Mo treated	44.51±2.8
Values reported as mean ± standard deviations of triplicates. Means with different superscripts are significantly different ($p < 0.05$).		

Estimation of Superoxide Dismutase (SOD)

The quantities of SOD showed significant deviation in the seedlings of all the treatment groups. The results of SOD content in the seedlings of four treatment groups such as control, Fe-treated, Zn-treated, and Mo-treated were shown in Table 4. From these results, the seedlings of *Brassica nigra* under the stress of Fe treatment exhibit greater concentration of SOD whereas, the control group shows the lowest concentration of SOD. The total SOD quantities in the seedlings of control, Fe-treated, Zn-treated, and Mo-treated were found as 173.6±19.6, 238.4±21.5, 193.7±23.4, and 218.5±17.8 U/mg protein respectively.

Table 04:Superoxide dismutase activities in the seedlings of four treatment group plants.

S. No.	Treatment group	Superoxide dismutase (U/mg protein)
1	Control	173.6±19.6
2	Fe treated	238.4±21.5
3	Zn treated	193.7±23.4
4	Mo treated	218.5±17.8
Values reported as mean ± standard deviations of triplicates.		

Estimation of Glutathione Peroxidase (GPX)

The quantities of GPX showed significant deviation in the leaves and roots of all the treatment group plants. The results of GPX content in the leaves and roots of three treatment groups such as control, Ni treated, and Cr treated were shown in Table 5. From these results, seedlings of *Brassica nigra* under the stress of Fe treatment exhibit greater concentration of GPX whereas, the control group shows the lowest concentration of GPX. The total GPX quantities in the seedlings of control, Fe-treated, Zn-treated, and Mo-treated were found as 13.8±2.3, 64.5±5.6, 38.4±3.8, and 42.5±4.2 U/mg protein respectively.

Table 05:Glutathione peroxidase activities in the seedlings of four treatment group plants.

S. No.	Treatment group	Glutathione peroxidase(U/mg Protein)
1	Control	173.6±19.6
2	Fe treated	238.4±21.5
3	Zn treated	193.7±23.4
4	Mo treated	218.5±17.8
Values reported as mean ± standard deviations of triplicates.		

Estimation of Polyphenol Oxidase (PPO)

The results of polyphenol oxidase in the seedlings of four treatment groups such as control, Fe-treated, Zn-treated, and Mo-treated were shown in Table 6. From these results, the seedlings of *Brassica nigra* under the stress of Fe treatment group exhibit greaterconcentration of polyphenol oxidase. whereas, the control group shows the lowest concentration of polyphenol oxidase. The polyphenol oxidase activities in the seedlings of control, Fe-treated, Zn-treated, and Mo-treated were found as 67.5±2.4, 187.6±4.3, 145.2±3.9, and 153.4±4.2 U/mg protein respectively.

Table 06:Polyphenol oxidase activities in the seedlings of four treatment group plants.

S. No.	Treatment group	Polyphenol oxidase(U/mg protein)
1	Control	67.5±2.4
2	Fe treated	187.6±4.3
3	Zn treated	145.2±3.9
4	Mo treated	153.4±4.2
Values reported as mean ± standard deviations of triplicates.		

The physiological responses of a plant to heavy metal stress such as Fe, Zn, and Mo are often complex and multi-faceted. In recent decades, the development of novel analytical approaches, such as high-throughput phenotyping and bioinformatics, has enabled the rise of disciplines such as metabolomics, which reveal complex physiological responses in plants under stress.

SOD is present in many organisms, such as bacteria, yeast, animals, and plants. Plants have multiple genes encoding SOD that can be regulated by development, tissue-specific and environmental signals [30]. Plants can control the ROS by scavenging them with antioxidant enzymes such as SOD, CAT, and POD [31]. Among antioxidant enzymes, SOD is one of the chief antioxidants, which can scavenge O_2^- . and generates H_2O_2 and O_2 [32]. Previous works showed that ion tolerance is intimately related to the efficiency of antioxidant enzymes [33]. The present research data show resemblance with the earlier reports of Gill and Tuteja [34] who reported the activities of antioxidants like SOD increases with the stress. The results of the present study show collinearity with the findings of Kumar et al. [35] in *Zizania aquatica*.

Glutathione peroxidase is a physiologically significant enzyme that uses NADPH to catalyse the conversion of oxidised glutathione (GSSG) into reduced glutathione (GSH) [36]. Glutathione is the prevalent endogenous antioxidant in the majority of cell types and is physiologically active in its reduced form [37]. In order to maintain the cellular redox potential, GR causes the conversion of oxidised glutathione (GSSG) to reduced glutathione (GSH). Hence, the GR is important for the glutathione redox cycle by maintaining appropriate quantities of GSH. Pang and Wang [38] stated that GSH is involved in the detoxification of organic peroxides, xenobiotics, and reactive oxygen species, as well as being a substrate for other antioxidant enzymes such as glutathione peroxidase and glutathione-s-transferase.

Polyphenol oxidase is one of the polyphenolic compounds redox catalyzing enzymes that is widely distributed throughout mammals, fungi, and plants, but several plant PPOs tend to be deficient in tyrosinase activity [39]. Dolatabadian et al. [40] reported that the oxidation of phenolic compounds mainly requires polyphenol oxidase, hence it was referred as key enzyme for phenolic compounds oxidation. The studies of Hutabarat and Halbwirth [41] revealed that higher quantities of phenolic compounds significantly induce the higher expression of polyphenol oxidases, since polyphenolics are the primary substrate for PPO. In addition, Vaughn and Duke [42] reported that the polyphenols oxidation is triggered by the contact between PPO and phenolic compounds during the cell damage. However, the expression of PPO is depending on the various conditions such as ionic stress [43], and pathogenic infections [44]. Agarwal and Pandey [45] reported that the enhanced activities of PPO under stress, probably come from an increased capacity of oxygen radical scavenging and maintenance of cellular membranes.

Anatomical Changes

The anatomical changes in the leaf and stem from all the experimental groups was performed by observing under microscope. For anatomical analyses, stem and the first fully expanded leaves from all the experimental groups collected. The transverse section of stem from all the treatment groups exhibited significant variation in the diameter of vascular tissue. The control plants show the vascular tissue with greater diameter, whereas lower diameter of vascular tissue observed in Mo-treated plants. The diameters of vascular tissue from the stems of control, Fe-treated, Zn-treated, and Mo-treated were found as 223.839, 133.353, 156.907, and 108.936 μm respectively. Figure 4 illustrated the microscopic images of stem anatomy.

Furthermore, the studies of leaf anatomy demonstrated that the treatment of metals significantly affect the stomata structure. The stomata with greater width were observed in control seedlings, whereas the least dimensions of stomata were found in Fe-treated plants. In addition, the length of stomata was elongated in metal treated plants, while the width was decreased. In the control plant the length and width of stomata was found to be 236.35 and 207.57 μm respectively. In the Fe-treated plant the length and width of stomata was found to be 187.61 and 98.34 μm respectively. In the Zn-treated plant the length and width of stomata was found to be 273.03 and 178.12 μm respectively. In the Mo-treated plant the length and width of stomata was found to be 282.99 and 179.25 μm respectively. Figure 5 illustrated the microscopic images of stomata. Table 7 shows the stomatal dimensions from all the treatment groups.

Table 07:Stomatal dimensions from all the treatment groups.

S. No.	Treatment group	Stomatal dimensions (μm)	
		Length	Width
1	Control	236.35	207.57
2	Fe treated	187.61	98.34
3	Zn treated	273.03	178.12
4	Mo treated	282.99	179.25

The anatomical alterations in *Brassica* seedlings subjected to heavy metal exposure demonstrate significant variations among treatments. Control plants displayed the greatest vascular tissue diameter (223.839 μm), whereas Mo-treated plants demonstrated the smallest diameter (108.936 μm), signifying a pronounced inhibitory effect of Mo on vascular development. Plants treated with Fe and Zn exhibited intermediate values, indicating a metal-specific influence on stem architecture. These findings corroborate the earlier research by Sharma and Dubey [46], which indicated that heavy metals diminish vascular tissue size, hence hindering water and nutrient transfer. Moreover, leaf anatomical examinations revealed that heavy metal treatments substantially modified stomatal anatomy. The control seedlings exhibited the largest stomatal dimensions (236.35 × 207.57 μm), while the Fe-treated plants displayed the smallest dimensions (187.61 × 98.34 μm). Plants treated with Zn and Mo exhibited increased stomatal length, especially in Zn-treated specimens (273.03 μm), while showing diminished width, corroborating findings that metal stress impairs stomatal development and gas exchange [47].

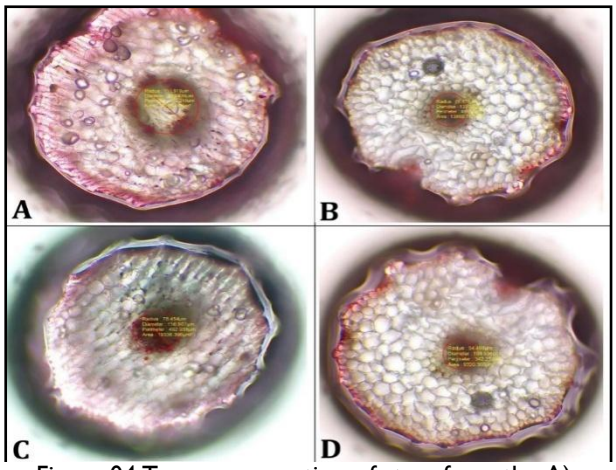

Figure 04:Transverse section of stem from the A) Control seedlings, B) Fe-treated seedlings, C) Zn-treated seedlings, D) Mo-treated seedlings.

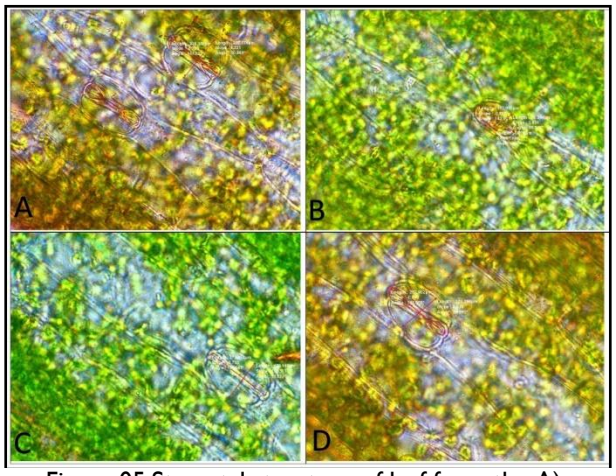

Figure 05:Stomatal structure of leaf from the A) Control seedlings, B) Fe-treated seedlings, C) Zn-treated seedlings, D) Mo-treated seedlings.

4. CONCLUSIONS

This study identifies *Brassica nigra* as an efficient plant species for the phytoremediation of soils contaminated with iron, zinc, and molybdenum. The plant exhibited considerable absorption of these metals, resulting in a marked reduction in their soil concentrations. In addition to its accumulation ability, *B. nigra* exhibited enhanced antioxidant enzyme activities and adaptive anatomical modifications, which contributed to its tolerance under metal stress. The modifications in vascular tissue and stomatal architecture indicate processes that facilitate sustained plant growth and physiological function under unfavourable environments. The amalgamation of high bioaccumulation, tolerance to metal toxicity, and adaptive physiological responses highlights the appropriateness of *B. nigra* for extensive, sustained phytoremediation initiatives. The results advocate for the integration of *B. nigra* into remediation programs aimed at restoring environments impacted by heavy metal contamination, thus supporting both environmental health and agricultural productivity.

5. ACKNOWLEDGMENTS

The authors are grateful to the Department of Botany and Department of Zoology, Government Degree College, Chodavaram, Andhra Pradesh, India, for providing the necessary facilities and support to carry out this research.

6. AUTHOR CONTRIBUTIONS

B. Sandhya Sri: Conceptualization, methodology, investigation, data collection, analysis, and manuscript preparation.

V. Ratna Barathi: Supervision, validation, review, editing, and final approval of the manuscript.

7. CONFLICT OF INTEREST

The authors declare that they have no conflict of interest regarding the publication of this manuscript.

8. ETHICAL STATEMENT

This study involved only plant materials and did not include human participants or experimental animals. Therefore, ethical approval was not required.

9. FUNDING

This research received no external funding.

10. INFORMED CONSENT STATEMENT

Not applicable. This study did not involve human participants or animals requiring informed consent.

11. DATA AVAILABILITY STATEMENT

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

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