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Effect of Humic Acid Functionalized Bentonite on Heavy Metal Uptake by Spinach (*Spinacia oleracea* cv. All Green) Grown on Metal Contaminated Soil

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

A pot culture study was conducted to investigate the impact of applying humic acid functionalized bentonite (HA-B) on the uptake of heavy metals by spinach. When the soil was amended with 7.5 g kg^{-1} of humic acid intercalated bentonite, there was a significant improvement in plant growth. Specifically, plant growth increased by 180.7%, 212%, and 231% during the first, second, and third cuttings, respectively. Additionally, the concentration of metals in the spinach decreased substantially, with reductions of 62.8, 69.7, and 77.7% for Cd and 34.7%, 45.2%, and 64.7% for Ni

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during the first, second, and third cuttings, respectively, when 7.5 g kg^{-1} of humic acid functionalized bentonite was applied. Furthermore, the addition of humic acid intercalated bentonite at a rate of 7.5 g kg^{-1} to the soil significantly lowered the bioconcentration factor (BCF) of metals. The BCF decreased by 36.3%, 40%, and 55.2% for Cd and 15.2%, 13%, and 34.7% for Ni during the first, second, and third cuttings, respectively, indicating a reduced uptake of these metals by the plants. Moreover, the non-cancer health risk through hazard quotient (HQ) computation showed that the risk was significantly reduced due to consumption of spinach by the application of 7.5 g kg^{-1} of humic acid intercalated bentonite.

Keywords: Humic acid intercalated clays; heavy metal remediation; metal pollution; risk assessment.

1. INTRODUCTION

The existence of hazardous heavy metals in soil and water poses a substantial danger to both the ecosystem and human well-being. Among these heavy metals, cadmium (Cd) and nickel (Ni) warrant particular attention due to their acknowledged harmful impacts on the soil biological and physicochemical properties. This leads to impaired soil fertility, decreased organic matter, extreme pH and electrical conductivity (EC) levels, and hinderance in the availability of micronutrients for plants [1,2]. Cadmium and nickel are considered significant environmental contaminants which are released into the environment as a result of various various human-led activities such as lead smelters, battery manufacturing, paint production, paper manufacturing, mining operations, etc [3]. Being highly toxic they pose a serious threat to the human health by having serious ulterior effect on some major organs like kidneys, heart and liver [4]. The contamination of heavy metals also carries serious consequences for plant growth and physiological processes, making it a significant global environmental issue [5].

Heavy metals negatively affect plant growth by restricting nutrient availability, inhibiting root development, and diminishing photosynthetic rate. These outcomes ultimately result in reduced food production [5,6]. Critically toxic levels of heavy metals in plants entails complex interactions at the cellular level, significantly disrupting the normal metabolism, genetic processes, and potentially leading to mutations and cell death [7]. Alia and co-workers [8] observed a significant reduction in the shoot and root biomass, both in terms of fresh and dry measurements, of spinach crops when they were exposed to the highest concentrations of Cd and Ni in comparison to the control treatment. Cadmium (Cd) and Ni from the soil finds their way into the human food chain through consumption of plants grown in contaminated soils. Assessing the potential health risks associated with consuming food grown in environments with elevated metal levels is of paramount importance. The hazard quotient is a straightforward tool commonly utilized by researchers to grasp the chronic non-cancer health risks linked to leafy vegetables grown in soils polluted with metals [9]. Researchers face the pressing challenge of preventing the transfer of heavy metals, such as cadmium and nickel, from progressing through the soil-plant-human cycle. This issue demands immediate attention and action to safeguard both the environment and human health.

In recent times, various approaches have been employed to address the issue of heavy metal contamination in soil, water, and sediments. These strategies encompass a wide array of methods including thermal treatment, chlorination, chemical extraction, ion-exchange, reverse osmosis, etc. which are effective but tend to incur more cost [10,11]. Adsorption process has garnered significant attention for its effectiveness and cost-efficiency in extracting heavy metal ions from diverse sources. Aluminosilicate clay minerals like smectite clays belonging to the montmorillonite clay group, especially bentonite, have demonstrated higher adsorption capacity to adsorb heavy metal ions, thus making them suitable for use in purifying water, sewage, and soil [12, 13, 14]. These types of clay possess distinctly higher specific surface area exhibiting high swelling capacity when put in aqueous medium. Such attributes make them highly efficient at adsorbing inorganic and organic pollutants. Consequently, it was thought proper to synthesize functionalized bentonite clay products and evaluate these products for their efficacy in arresting the transfer of Cd and Ni from soil to plants. Additionally, the study assessed the potential health risks associated with the plants grown in these metal-polluted soils.

2. MATERIALS AND METHODS

A bulk soil sample was collected from a metal-contaminated area in Unnao district, Uttar Pradesh, India. The soil sample was air-dried, ground, and processed prior to analyzing its physico-chemical characteristics using standard methods. The DTPA extractable Cd and Ni content in soil was determined by standard procedures [15] and the corresponding values were 6.45 mg kg^{-1} and 4.46 mg kg^{-1} for Cd and Ni, respectively. Soil pH was measured in a 1:2 soil-to-water suspension using a digital pH meter [16]. The soil was acidic in nature with a pH value of 5.9. Organic carbon content in the soil was 7.2 g kg^{-1} which was determined by potassium dichromate wet oxidation method [17]. The mechanical analysis of soil was done by hydrometer method [18], and its texture was identified using the soil textural triangle. The soil was sandy clay loam in texture, with 55% sand, 15% silt, and 30% clay content. The cation exchange capacity of the soil was assessed to be 20.46 cmol (p+) kg^{-1} using the ammonium acetate method [19]. The bentonite clay used in this study was obtained from Minerals Limited, New Delhi. Humic acid modified bentonite was prepared according to established procedures by Wang and co-workers [20].

2.1 Pot Experiment

A pot culture experiment was conducted during the *rabi* season (October-December) of 2021-22 taking spinach as the test crop following completely randomized design with three replications. The experiment was conducted at the net house facility of the Division of Soil Science and Agricultural Chemistry, ICAR-IARI, New Delhi. Each pot contained 4 kg of air-dried, and processed soil. The different treatments included variable doses and types of functionalized clay products which are as follows: T_1: Control pot (without clay), T_2: 2.5 g kg^{-1} HA-B, T_3: 5 g kg^{-1} HA-B and T_4: 7.5 g kg^{-1} HA-B. In each pot, a population of five spinach plants was maintained. A uniform basal dose of N, P, and K @ 108 mg per pot were applied using urea, diammonium phosphate, and muriate of potash, respectively. An additional dose of 108 mg of nitrogen was applied 30 days after sowing. The plant and soil samples were collected at three stages, viz. 35, 65, and 95 days after sowing (DAS). These samples were then analyzed to determine the concentrations of Cd and Ni. The analysis involved the use of a microwave digester with concentrated (65%) suprapure nitric acid [21], and the measurements were carried out using ICP-OES (Inductively Coupled Plasma Optical Emission Spectrometry).

2.2 Bioconcentration Factor

The bioconcentration factor (BCF) serves as an indicator of the potential bioavailability of a specific metal within a particular plant component. To calculate the BCF, you compare the metal concentration in plant tissue (typically measured in mg kg^{-1} dry weight, Dw) such as stems, leaves, roots, etc., to the metal concentration in the soil (also measured in mg kg^{-1} dry weight, Dw) [22]. The BCF for Cadmium (Cd) and Nickel (Ni) is determined using the following equation:

$$BCF = \frac{\text{Cplant}}{\text{Csoil}} \quad (1)$$

Cplant= metal concentration in plant (mg kg^{-1}), Csoil= metal concentration in soil (mg kg^{-1}).

2.3 Hazard Quotient (HQ)

The study assumes that the daily intake of green vegetables is 0.2 kg day^{-1}. A factor of 0.082 was used to for leafy vegetables which accounts for the weight in dry weight basis [14]. Thus, the HQ for an adult was calculated as (Eq. (1)):

$$\text{HQ} = \frac{\text{MPlant x W x F}}{\text{RfD x 70}} \quad (2)$$

M_{Plant} is the metal content (mg kg^{-1}) of plant, W is the daily intake of green vegetable (kg kg^{-1} body weight) and F is the factor of conversion of fresh to dry weight. The allocated reference dose values for Cd and Ni were 0.001 mg kg^{-1} day^{-1} and 0.02 mg kg^{-1} day^{-1}, respectively. The average body weight for adult was assumed to be 70 kg.

3. RESULTS AND DISCUSSION

The dry weight of shoot biomass often serves as an indicator of a plant's ability to tolerate unfavourable environmental conditions. Fig. 1 illustrates that the quantity of clay mineral had a notable impact on the biomass production of spinach. Specifically, plant shoot biomass exhibited an increase as the level of clay application increased. The extent of this increase was most significant when bentonite was applied at a rate of 7.5 g kg^{-1} compared to the unamended control soil. During the first cutting, the dry matter yield increased from 2.18 g pot^{-1} in T1 (control) to 3.84 g pot^{-1}, 4.45 g pot^{-1}, and 6.12

g pot^{-1} in T_2, T_3, and T_4, respectively. During the second cutting, the dry matter yield experienced a significant boost, with increases of 100%, 135%, and 212% observed when bentonite was applied at rates of 7.5 g kg^{-1}, 5 g kg^{-1}, and 2.5 g kg^{-1}, respectively, in comparison to the unamended control soil. This trend persisted in the third cutting as well. These findings suggest that the use of humic acid-intercalated bentonite led to enhanced plant growth by adsorbing heavy metals in the soil, creating additional adsorption sites, and consequently reducing the stress caused by heavy metals on the plants. This improvement in plant growth was achieved by mitigating heavy metal stress through the amendment of humic acid-modified bentonite [13, 14, 23, 24]. Adding functionalized clay minerals increased microbial activity, but the mineralization of organic matter restricted metals and metalloids from being accessible to microorganisms. The potential enhancement of soil fertility might explain the observed rise in plant biomass in treatments where bentonite was added [1, 10, 25].

The accumulation of heavy metals in plants hinges on their bioavailability, which determines whether they can be taken up by plants. The application of humic acid functionalized bentonite significantly reduced the concentration of heavy metals in spinach shoots, as shown in Table 1. Specifically, the application of bentonite reduced the cadmium concentration in spinach from 5.52 mg kg^{-1} (T_1) in the control soil to 3.78 mg kg^{-1} (T_2), 2.98 mg kg^{-1} (T_3), and 2.05 mg kg^{-1} (T_4) during the first cutting. In the second cutting, these values were 3.15 mg kg^{-1} (T_2), 3.68 mg kg^{-1} (T_3), 1.69 mg kg^{-1} (T_4), and 5.58 mg kg^{-1} (T_1) in the control soil. Likewise, in the third cutting, cadmium content decreased to 2.80 mg kg^{-1}, 2.21 mg kg^{-1}, and 1.25 mg kg^{-1} in soil amended with 2.5, 5, and 7.5 g kg^{-1} of humic acid functionalized bentonite, respectively, compared to the control's 5.63 mg kg^{-1}. For nickel, its concentration in spinach decreased from 3.25 mg kg^{-1} in the control to 3.05 mg kg^{-1}, 2.88 mg kg^{-1}, and 2.12 mg kg^{-1} when humic acid functionalized bentonite was applied at rates of 2.5, 5, and 7.5 g kg^{-1}, respectively, in the first cutting. In the second and third cutting, nickel content was reduced to 2.78 and 2.55 mg kg^{-1}, 2.25 and 2.01 mg kg^{-1}, and 1.67 and 1.12 mg kg^{-1} in soil amended with 2.5, 5, and 7.5 g kg^{-1} of humic acid functionalized bentonite, respectively, compared to control values of 3.15 and 3.18 mg kg^{-1}. It's important to note that the bioavailability of heavy metals in soil depends on their concentration in the soil solution and the release of heavy metal ions from the solid phase of the soil.

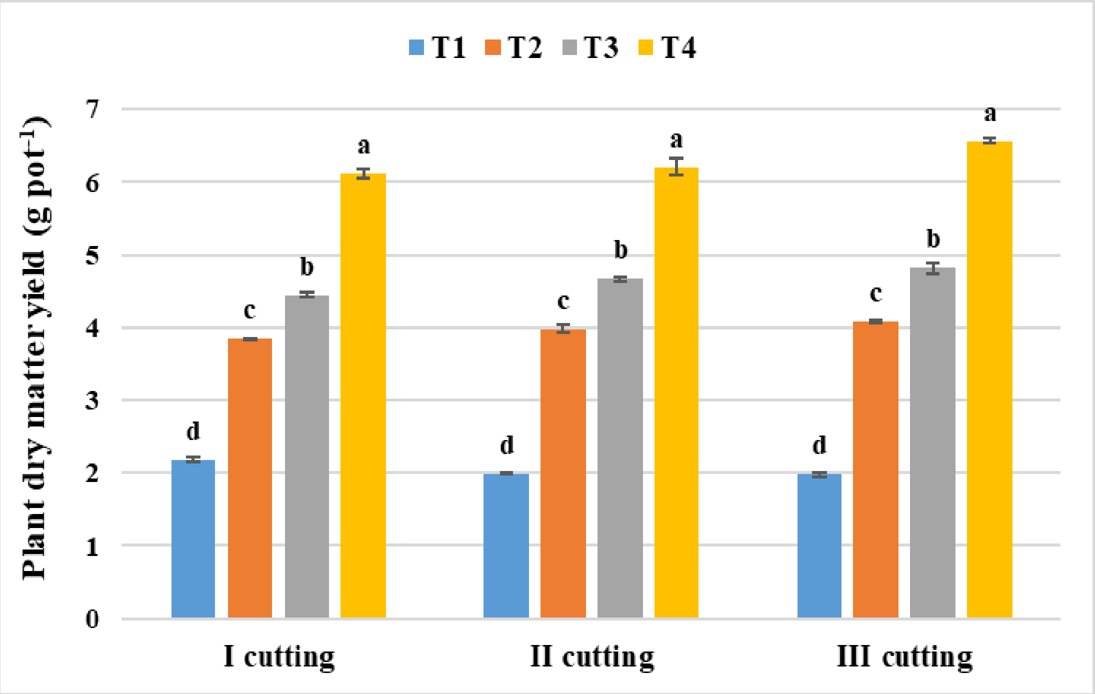

Fig. 1. Effect of humic acid functionalized bentonite levels on dry matter yield (g pot^{-1}) of spinach

Table 1. Effect of humic acid functionalized bentonite levels on metal concentration (mg kg^{-1} DW) in spinach

Treatments	Cd			Ni		
	Cutting I	Cutting II	Cutting III	Cutting I	Cutting II	Cutting III
T_1	5.52^a	5.58^a	5.63^a	3.25^a	3.15^a	3.18^a
T_2	3.78^b	3.15^b	2.80^b	3.05^{ab}	2.78^b	2.55^b
T_3	2.98^c	2.68^c	2.21^c	2.88^b	2.25^c	2.01^c
T_4	2.05^d	1.69^d	1.25^d	2.12^c	1.67^d	1.12^d
CD (P = 0.05)	0.22	0.11	0.10	0.20	0.11	0.12

The easily extractable fraction of heavy metals, as extracted by DTPA, exhibited a notable decrease due to the application of humic acid functionalized bentonite (as shown in Fig. 2). When the soil was amended with 7.5 g kg^{-1} of humic acid functionalized bentonite (T_4), there was a substantial reduction of 77.7% for DTPA-extractable cadmium (Cd) and a reduction of 64.7% for DTPA-extractable nickel (Ni) compared to the control (T_1). The application of clay minerals decreased the plant available of heavy metal fraction in soil which is mostly attributed to the higher specific surface area and enhanced sorption capacity. This causes reduction in solution metal concentration, consequently reducing the uptake of these metals by plants [24]. The bioavailable metal fraction in the soil is directly related to the soluble and extractable metal content in soil, and as well as the affinity with which the metal is held onto the solid phase. The use of clay products, such as pillared bentonite, can effectively decrease the bioavailability of heavy metals by adsorbing the solution metal physically through Van der Waals force of attraction and/or chemically by anion exchange. Consequently, there is a reduction in the concentration of cations in the soil solution, leading to a decreased uptake of these metals by plants [26]. Bentonite's ability to immobilize heavy metals in soil is aided by various factors, including isomorphic substitution, its negative charge, and its compatibility with the environment. These characteristics enhance the ability of bentonite to efficiently capture and retain heavy metals, effectively limiting their movement and reducing the likelihood of potential harmful consequences [5]. Bentonite's substantial surface area plays a pivotal role in its robust ability to adsorb metal ions, enabling it to capture heavy metal ions within its structure and enhance isomorphic substitution, as discussed in reference [6]. Vrinceanu et al. [7] revealed that the addition of bentonite to soil raised the soil pH, improving the retention of heavy metals in the solid phase of soil and substantially decreasing the uptake of Cd and Zn in the above-ground plant parts. This pH adjustment effect led to the binding of heavy metals, facilitating their long-term diffusion into the clay mineral layers. When comparing untreated soil with the functionalized clay treated (5% HA-Mont (humic acid-modified montmorillonite)), it was observed that there was a substantial decrease in bioavailable Cd and Hg content by 94.1 and 93.0%, respectively. This reduction brought these concentrations below the regulatory limits as per the Toxicity Characteristic Leaching Procedure. In contrast to soil treated with unmodified montmorillonite, the HA modification decreased in Cd and Hg by 69.5% and 65.9%, respectively in leachate [20].

The Bioconcentration Factor, which represents the ratio of the metal content in edible tissue to the total metal content in the soil, was used to evaluate the effectiveness of clay minerals in immobilizing heavy metals in the soil (Table 2). For Cadmium (Cd), the BCF values varied from 0.66 (T_1) in the control soil to 0.50 (T_2), 0.48 (T_3), and 0.42 (T_4) in soil amended with 2.5, 5, and 7.5 g kg^{-1} of humic acid functionalized bentonite, respectively, during the first cutting. In the second and third cuttings, the BCF of Cd decreased by 34.2% and 42.1%, 35.7% and 48.6%, and 40% and 55.2% when 2.5, 5, and 7.5 g kg^{-1} of humic acid functionalized bentonite were applied, respectively, compared to the control soil. Similarly, for Nickel, the BCF values for spinach ranged from 0.72 to 0.61, 0.67 to 0.58, and 0.69 to 0.45 at the first, second, and third cuttings, respectively. The addition of bentonites resulted in a significant reduction in the translocation of metals to the plants, as evidenced by the BCF values. Contaminated food consumption represents a significant and primary pathway (accounting for over 90%) through which humans are exposed to heavy metals, surpassing exposure through inhalation and dermal contact. When humans ingest heavy metals at toxic levels, it can lead to a range of physiological and metabolic disorders, as discussed in reference [27]. To evaluate the effectiveness of humic acid functionalized bentonite in metal immobilization and its impact on the health risk associated with vegetable consumption from both clay-amended and control soils, a hazard quotient (HQ) was calculated using the USPEA protocol (IRIS, 9). The results, as shown in Table 3, indicate that the HQ for Cadmium (Cd) decreased to 0.89, 0.70, 0.48, and 0.74, 0.63, 0.40, and 0.66, 0.52, 0.29 for soil amended with 2.5, 5, and 7.5 g kg^{-1} of humic acid functionalized bentonite from the initial values of 1.29, 1.31, and 1.32 in the control soil during the first, second, and third cuttings, respectively. The decrease in the hazard quotient (HQ) observed upon the application of humic acid functionalized bentonite can be attributed to the reduced uptake of heavy metals, resulting from the immobilization of these metals in the soil. HQ values equal to or greater than 1 indicate that the consumption of certain food materials may pose a hazard to humans due to the intake of specific metals. It's important to note that the consumption of leafy green vegetables is just one source of metal intake for humans. When considering other sources like drinking water and inhalation of dust, a safe HQ limit in risk assessments of contaminated soils can be considered as 0.5. Therefore, in the control soil, the HQ for Cadmium (Cd) exceeded 0.5, but the application of humic acid functionalized bentonite reduced the values below this safe limit of 0.5. The addition of bentonite enhances the chemical sorption of heavy metals, reducing their mobility due to complex formation. The results of the study suggest that humic acid functionalized bentonite @ 7.5 g kg^{-1} could effectively immobilise the heavy metals in soil. This method enables the reduction of plant metal content, thus decreasing the risk associated with consuming vegetables grown in metal-contaminated soil [13, 14, 24].

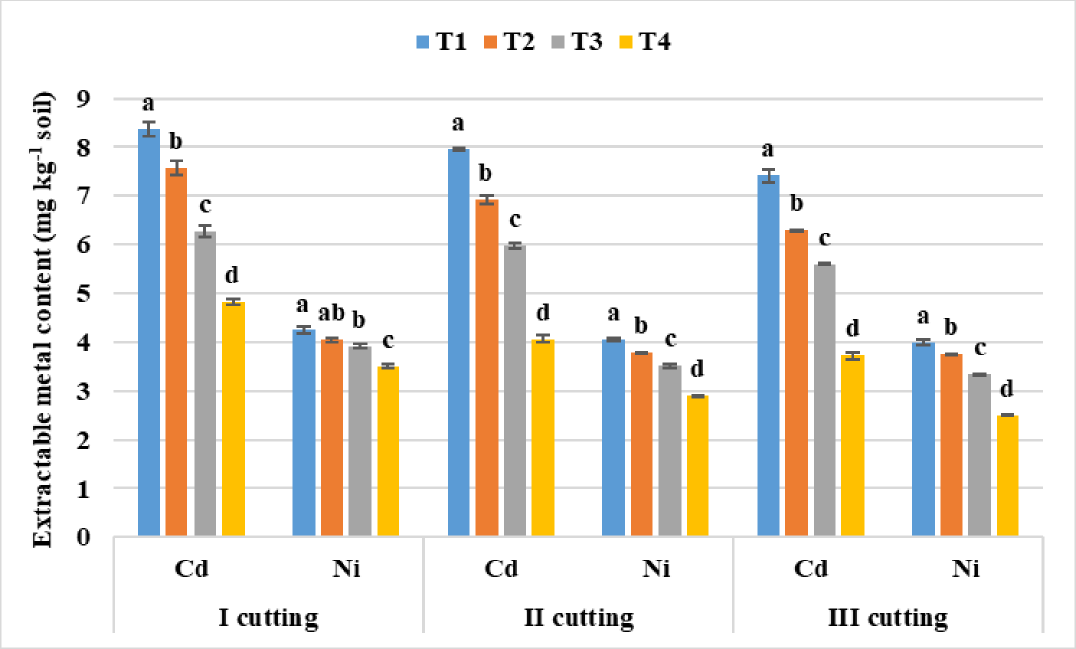

Fig. 2. Effect of humic acid functionalized bentonite levels on extractable heavy metals in soil

Table 2. Effect of humic acid functionalized bentonite levels on bioconcentration factor of metals in spinach

Treatments	Cd			Ni		
	Cutting I	Cutting II	Cutting III	Cutting I	Cutting II	Cutting III
T_1	0.66^a	0.70^a	0.76^a	0.72^a	0.67^b	0.69^a
T_2	0.50^b	0.46^b	0.44^b	0.70^a	0.70^a	0.68^a
T_3	0.48^c	0.45^b	0.39^c	0.73^a	0.64^b	0.60^b
T_4	0.42^d	0.42^c	0.34^d	0.61^b	0.58^c	0.45^c
CD (P = 0.05)	0.02	0.01	0.04	0.03	0.02	0.03

Table 3. Effect of humic acid functionalized bentonite levels on hazard quotient of metal through consumption of spinach

Treatments	Cd			Ni		
	Cutting I	Cutting II	Cutting III	Cutting I	Cutting II	Cutting III
T_1	1.29^a	1.31^a	1.32^a	0.04^a	0.04^a	0.04^a
T_2	0.89^b	0.74^b	0.66^b	0.04^{ab}	0.03^b	0.03^b
T_3	0.70^c	0.63^c	0.52^c	0.03^{bc}	0.03^b	0.03^b
T_4	0.48^d	0.40^d	0.29^d	0.02^c	0.02^c	0.02^c
CD (P = 0.05)	0.06	0.03	0.01	0.01	0.01	0.01

4. CONCLUSION

The current study highlights the positive effects of using humic acid intercalated bentonite clay to improve the growth and enhance the tolerance of spinach plants to heavy metals. The addition of functionalized bentonite had a significant and favourable impact on spinach biomass production during all three cuttings periods, resulting in a significant increase in plant growth compared to untreated soil. The research findings demonstrated that the most effective method for immobilizing heavy metals like cadmium (Cd) and nickel (Ni) was the application of humic acid functionalized bentonite at a rate of 7.5 g kg^{-1}. The introduction of functionalized bentonite improved the chemical adsorption of heavy metals, effectively reducing their mobility within plants. The substantial surface area and enhanced adsorption capacity of bentonite effectively trapped heavy metals within the soil, decreasing their availability for uptake by plants. The decrease in the hazard quotient for heavy metals in spinach can be attributed to the reduced uptake of these metals by plants, achieved by immobilizing them in the soil through the application of humic acid functionalized bentonite. The method described in this research provides a way to incorporate modified bentonite into soil to limit the movement and accessibility of heavy metals to plants. This, in turn, reduces the health risks associated with consuming vegetables grown in soils polluted with heavy metals.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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