RESEARCH ARTICLE

Impact of Excess Nickel on the Seed Germination, their Growth and Other Physiological Characteristics of Spinach

Girish Chandra Pathak[1*], Nilu Singh[1], Rajiv Dwivedi[2]

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Abstract

From the beginning of Industrial Revolution, heavy metal concentration changed dramatically in the environment and it, led to metal toxicity. Contamination of soil and groundwater by heavy metals becomes a serious threat to the environment and human health. In trace amounts, certain heavy metals are required for the normal growth and development of plants, and in excess amounts, they cause toxicity to plants, humans and animals. In this study, we conduct a test on spinach (*Spinacia oleracea* L.) to find out the toxic effect of nickel on seed germination, root and shoot growth and antioxidant enzymes. A Set of four solution culture experiments was done with different concentrations of Ni (control, 10, 100 and 200 µM). Nickel toxicity leads to a reduction in germination (no. of seeds), shoot and root length, as compared to seeds germinated in low nickel (control). It also reveals the antioxidative defense mechanisms of plants, first increasing enzyme (catalase) activity at 10 µM Ni but later getting inhibited on increasing the Ni concentration 100, 200 µM. The increase in Ni toxicity it leads to the breakdown of the antioxidative defense mechanisms of plants.

Keywords: Antioxidative defense, Catalase, Micronutrients, Nickel toxicity, Physiological parameters, Seed germination.

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Introduction

Nickel is now considered an essential micronutrient for the proper growth of plants and also for their development in low concentration (da Silva *et al.,* 2012). As mentioned by Mulrooney and Hausinger (2003) Ni plays a major role in metabolic processes, like methane biogenesis, hydrogen metabolism, ureolysis and acitogenesis and also in many physiological processes, like seed germination, seedling growth and biomass production (Torres *et al.,* 2016).

Due to industrialization and urbanization, there is a gradual increase in the concentration of pollutants like chemical fertilizers, pesticides, heavy metals and petroleum products in our natural resources like soil, water and air, which degrade their quality and also affect both plants and animals (Singh, 2020).

Nickel is a hard transition element that shows a silverish-white color and, is present in a solid state and is mainly used for making coins, jewelry and stainless steel. Ni is a natural heavy metal that is present in water, air, sediments and soil (Kieling-Rubio *et al.,* 2012).

There are many compounds of nickel, like Ni $(CH_3CO_2)_2$, $NiCO_3$, Ni $(OH)_2$ and NiO, which are commonly used in various industrial processes (WHO 1991). In fresh water generally contains approximately 300 ng dm^{-3} of nickel. The concentration of Ni in an agricultural field is about 3 to 1000 mg of Ni/Kg of soil but it may vary in soil near industries or oil refineries and dried sludge up to 24000 and 53000 mg kg^{-1} of Ni, respectively. At pH 6.5, some compounds of nickel show higher solubility in soil, but it is mostly found in insoluble hydroxides format pH 6.7 (Bhalerao *et al.,* 2015). There are some studies that found that Ni shows toxic effects on the mitosis processes by disturbing the spindle formation in both serpentine and non-serpentine seedlings and it also causes aberrations of root-meristem cell division on chromosomes (Pavlova, 2018).

Ni shows different ecotoxicities in soil, which depend upon its various salt forms (due to their anionic partner). Their toxicities rank as follows: $NiSO_4$< Ni (CHCOO) < Ni (II)-citrate <NiCl< Ni (II)-EDTA (Nie *et al.,* 2015).

In excess Ni concentration, it shows toxic effects in plants by inhibiting seed germination, photosynthesis processes, metabolism (Pandey and Pathak, 2006), and normal plant growth (Parlak, 2016). Nickel in higher concentration can change many metabolic processes of plants like the ratio of water and mineral uptake, nutrients dynamics, inhibit the enzymatic rate and alter enzyme structure, open and closing processes of the stomata, degrading chlorophyll molecules and disrupt the electron transport in photosynthesis processes, reduce its photosynthesis rate, decrease the chlorophyll content and decrease the productivity of plants (Yusuf *et al.,* 2011; Bybordi and Gheibi, 2009).

As reported by Ahmad *et al.,* (2011) that Ni causes toxicity to most plant species by affecting some enzymes activity like protease, amylase and ribonuclease which may lead to a reduction in the percentage of seed germination and the biomass production of crops. In some study, it was found that excess Ni may impair the digestion of proteins and

[1]Plant Nutrition and Stress Physiology Laboratory, Department of Botany, University of Lucknow, Lucknow 226007, Uttar Pradesh, India.

[2]Deen Dayal Upadhyay, Government PG College, Sitapur, Uttar Pradesh, India.

***Corresponding author:** Girish Chandra Pathak, Plant Nutrition and Stress Physiology Laboratory, Department of Botany, University of Lucknow, Lucknow 226007, Uttar Pradesh, India., Email: gc.pathak@gmail.com

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carbohydrates and their mobilization in germinating seeds, reduction in root and shoot length, reduce chlorophyll content, reduction in biomass production and alter the activity of enzyme carbonic anhydrase (Siddiqui, *et al.,*2011). In mung beans, nickel toxicity also affects the photosynthetic pigments, reduces their yields and leads to the accumulation of other ions like $Ca^{2+,}$ Na^{+} and K^{+} in mung bean (Ahmad *et al.,*2007). Heavy metal toxicity and abiotic stress affect many plant processes, including as germination of seeds, their growth, osmotic homeostasis, photosynthesis and, carbohydrate metabolism, etc. (Sethy and Ghosh, 2013). In soil, excess Ni shows negative impacts on the growth and yielding of mung beans at various concentrations of Ni (Ali *et al.,* 2015).

In all wheat cultivars, the percentage of seed germination gradually decreases as an increase in the $NiCl_2$ concentration, while $NiCl_2$ at 0.05 mg/L shows normal seed germination. It shows that a high concentration of $NiCl_2$ had a negative effect on seed germination of wheat cultivars and it drastically depressed root growth (Kumar and Verma 2018). The study has found 14-day-old seedlings of *Triticum aestivum* cv. Vergina, by increasing the concentration, shows that most symptoms appear on roots and decreased shoot growth. A study on soya beans by Prasad et al. (2005) shows growth of seedlings was highly reduced due to the accumulation of Ni in leaves, which may inhibit some important biochemical and physiological processes.

In coriander and milk thistle seedlings, different concentrations of nickel nitrate $Ni(NO_3)_2$ show a negative influence on their growth traits. High concentration Ni $(NO_3)_2$ reduced radicle growth during the experiment, but radicle growth was stopped in the final days of the experiment, which led to their death. A high concentration of Ni negatively influences the seed vigor index and their germination. It is found that Ni in high concentration shows inhibitory effects on the growth traits of Milk thistle and coriander seedlings, but the impact is more dramatic on milk thistle seedlings (Poozesh*et et al.,* 2014; Batool 2018).

Spinach is a green, leafy vegetable that is highly rich in vitamins (vitamins A, C and K) and minerals (K, Fe, Ca, Mg, nitrate), lutein, an antioxidant, folate and fibers. It has various health benefits like lowering blood pressure levels, reducing oxidative stress, helping in preventing the growth of cancer cells, eye health, etc. Because of the enriched nutrients make it great staple vegetable for a diet. Consumption of vegetables is an important pathway through which heavy metals enter in food chain. As reported by Genchi et al. (2020), when a human is exposed to exposure of heavy metals, it causes severe diseases like cardiovascular disease, respiration-related disease (lung fibrosis, lung and nasal cancer), allergies and kidney diseases.

In this study, we consider Ni a toxic substance and an important environmental pollutant due to its availability and persistence in soil. This experiment is set to examine the effect of excess Ni on seed germination, root and shoot length and activity of antioxidant enzymes in spinach. While heavy metal stress on plants has been widely studied, the specific impact of nickel toxicity on spinach (*Spinacia oleracea* L.) might be less documented. Spinach is an important leafy vegetable, and understanding how nickel accumulation affects its growth and safety for consumption has practical importance. Spinach is an important leafy vegetable, and understanding how nickel accumulation affects its growth and safety for consumption has practical importance.

Material and Methods

We perform this experiment to detect the negative effect of excess Ni on seed germination and on their growth and development by using the solution culture technique.

Solution Culture Technique

A set of solution culture experiments were planned to estimate the phytotoxic impact of Ni on early seedling germination and biochemical changes in its components, on the seeds of mustard and spinach. First, we sterilize the seeds by using 5% (v/v) $HgCl_2$ solution and wash them properly by using deionized amnesty still water (MSW) before their germination. At room temperature, first, the petri dishes with threefold filter paper and then soak the treated seeds properly in MSW in Petri dishes. Glass/distilled water is used for culture work and also for supply of nutrition. We prepared standard nutrients solution for proper seed germination and their growth by using different nutrients like 4 mM KNO_3, 4 mM $Ca(NO_3)_2$, 2 mM $MgSO_4$, 0.4 mM $(NH_4)_2SO_4$, 30 µM NaCl, 0.33 µM HBO_3, 2 µM $MnSO_4$, 0.3 µM $CuSO_4$, 0.8 µM $ZnSO_4$, 0.1 µM Na_2MoO_4, 0.1 µM $CaSO_4$, 0.1µM $NiSO_4$ and 20 µM Fe EDTA (Hogland and Arnon, 1950). $NiSO_4$ is used for the supply of Ni at various concentrated 0.1 µM $NiSO_4$, 10, 100 and 200 µM, as indicated in different experiments. Four slots of Petri dishes containing 50 seeds were set up for each treatment. We used AR-grade salt for the preparation of macronutrients (without $NiSO_4$) and micronutrients stock solution and acid-washed Pyrex glass reagents bottles were used to store the stock solution. By diluting the stock solution 10 times, we prepared inter stock solution that provide the various macronutrients and micronutrient elements at a required concentration (pH = 6.5 approximately). To maintain the level of nutrients in the culture solution, we changed the solution on an alternate day.

Antioxidant Enzymes

Extraction

we homogenized collected fresh leaves with 2 mL of 150 mM K_3PO_4 buffer at pH 7.0, which contains 2% PVP and 1 mM EDTA (we also added 1 mM of ascorbate for APX). We centrifuged the prepared homogenate at 15000 rpm for 10 minutes and we used obtained supernatant for the enzyme preparation. The temperature was maintained at 4℃ for the preparation of all enzymes.

Superoxide dismutase

Superoxide dismutase has the ability the inhibit the activity of NBT by photochemical reduction. By using this ability, we determined the SOD activity. In 3 mL of the reaction mixture, it contains 50 mM K_3PO_4 buffer (pH 7.8),75 µM NBT, 0.1 mM EDTA,13 mM methionine, 2 µM riboflavin and 0 to 50 µL enzyme extract. At last, we added riboflavin and on test tubes, bright light was supplied for 10 minutes. In blanks, bright light was not supplied and above-prepared reaction mixtures with no enzyme extract

show the highest color peak at 560 nm. We inhibit the activity of the reaction mixture by using 3mM KCN to determine the Zn/Cu SOD and subtract its amount from the total SOD. By using the percentage of inhibition, enzymes were qualified (Beauchamp and Fridovich, 1971).

Catalase

We used Euler and Josephson's (1927) method for the analysis of catalase by adapting the permanganate ($KMnO_4$) titration method. The temperature was maintained at 25°C for an enzyme reaction. We prepared a reaction mixture of 0.025M K_3PO_4 buffer, which contained 0.005M H_2O_2 and pH was maintained at 7.0 and we used 0.1N $KMnO_4$ for standardizing the reaction mixture. By adding 1-mL of enzyme extract (diluted), the reaction was started. After 5 minutes, add 2 mL of 2N H_2SO_4 to stop the reaction. In blanks, before adding the enzyme extract, add H_2SO_4 to the prepared reaction mixture and run the reaction simultaneously. The remaining H_2O_2 in the prepared reaction mixture was titrated By using 0.1 N $KMnO_4$ for titration of the remaining H_2O_2 in the prepared reaction mixture and using H_2O_2 to express the activity of catalase.

Peroxidase

We used Luck's (1963) modified method for assaying peroxidase. At 25°C reaction was performed. In the prepared reaction mixture, it contains 2 mL 0.1M K_3PO_4 buffer (pH = 6.0), 1-mL 0.5% p-phenylene diamine and 1-mL of 0.01% H_2O_2, which was added. To start the reaction, add 1-mL of enzyme extract to the prepared reaction mixture and keep it for 5 minutes to proceed with the reaction. To stop the reaction, we use 2 mL 4N H_2SO_4. In blanks, before adding the enzyme extract add 2 mL H_2SO_4 and simultaneously run the reaction. After that, refrigerate the reaction mixture for 20 minutes and later, at 4000 x g, centrifuge it. For reading the colour intensity, we used a spectrophotometer at 485 nm.

Ascorbate peroxidase

We used Nakano and Asada's (1981) method to measure the activity of APX. Prepare a reaction mixture by adding 50 mM K_3PO_4 buffer at pH = 7.0, 0.5 mM ascorbate and 0.1 mM H_2O_2 at 290 nm. Oxidation of ascorbate was measured as absorbance per minute.

Presentation of Data

We used ANOVA for data analysis. In the table we give the least significant difference (LSD at $p = 0.05$) and mean value. We present our results in the form of bar diagrams for better understanding and visuals.

Results

Seed germination

Nickel in trace amounts is necessary for normal growth and development of plants, but the high concentration of it causes nickel toxicity which results in chlorosis and necrosis in plants. Germination percentage gradually reduced in all spinach seeds as elevating the level of nickel in the solution. In contrast, 10 µM of nickel concentration of nickel had adverse effects on seed germination of spinach. Seeds soaked in 100 and 200 µM nickel concentrations showed minimum germination as compared to the control set, as recorded in Table 1. The petri plates having nickel concentrations of 100 and 200 µM reduced seed germination by 72%. It is clear from the present findings that excess nickel show negative effects on germination of seed and their growth.

We found that excess Ni shows an inhibitory effect on seedling growth. Ni in high concentration gradually reduces the length of root and shoot in Spinach plants, as recorded in Table 2. The minimum root length and shoot length were noted at 200 µM of Ni 0.7 and 0.9 cm, respectively. Nickel in high concentration inhibits the plant growth by reducing the supply otheressential ions.

10 µM Ni stimulated the activity of catalase, but 100 and 200 µM Ni inhibited its activity, as shown in Fig. 1(b). The activity of peroxidase was inhibited by 10 µM Ni but increased in response to 100 and 200 µM Ni supply, as shown in Fig. 1(c). Antioxidant enzyme SOD shows a marked increase in its activity and increased concentration of antioxidants, supply of Ni at 10 and 100 µM Ni as shown in Fig. 1(a). Ascorbate peroxidase activity increased at 10 µM, but later, it decreased as an increase in Ni concentration, as shown in Fig. 1(d). However, with a constant supply of Ni, especially in seedlings supplied 100 and 200 µM, the toxicity effects were enhanced and were reflected in the breakdown of the antioxidative defenses of plants. This was manifest as the appearance of the visible symptoms death of plants initial stage. The tissue concentration of Fe decreases with increases in Ni toxicity Table 3.

Table 1: Toxic effect of Ni on seed germination of spinach

Seed germination days after treatment	*Number of seeds that germinate*			
	Control	*Ni (10 µM)*	*Ni (100 µM)*	*Ni (200 µM)*
3 Days	32 ± 1.5	20 ± 1.4	3 ± 0.5	2 ± 0.3
6 Days	42 ± 2.5	29 ± 1.3	8 ± 0.5	6 ± 0.4
12 Days	44 ± 2.3	31 ± 1.3	11 ± 1.0	9 ± 0.7

Table 2: Toxic effect of Ni on shoots and root length of spinach plant 12 days after treatment.

Ni treatment	*Shoot length (in cm)*	*Root length (in cm)*
Control	3.4 ± 0.3	2.9 ± 0.2
10 µM Ni	2.6 ± 0.6	1.7 ± 0.2
100 µM Ni	1.1 ± 0.2	0.9 ± 0.1
200 µM Ni	0.9 ± 0.2	0.7 ± 0.1

Table 3: Effect of Ni toxicity on tissue iron (leaves, stem, root) in spinach 3, 6 and 12 days after Ni treatment.

Tissue Fe µg g^{-1} dry wt.	*Treatment*			
	Control	*Ni (10 µM)*	*Ni (100 µM)*	*Ni (200 µM)*
3 Days	32 ± 2.6	20 ± 1.7	3 ± 0.2	2 ± 0.1
6 Days	42 ± 2.8	29 ± 2.5	8 ± 0.5	6 ± 0.3
12 Days	44 ± 2.5	31 ± 1.9	11 ± 0.5	9 ± 0.4

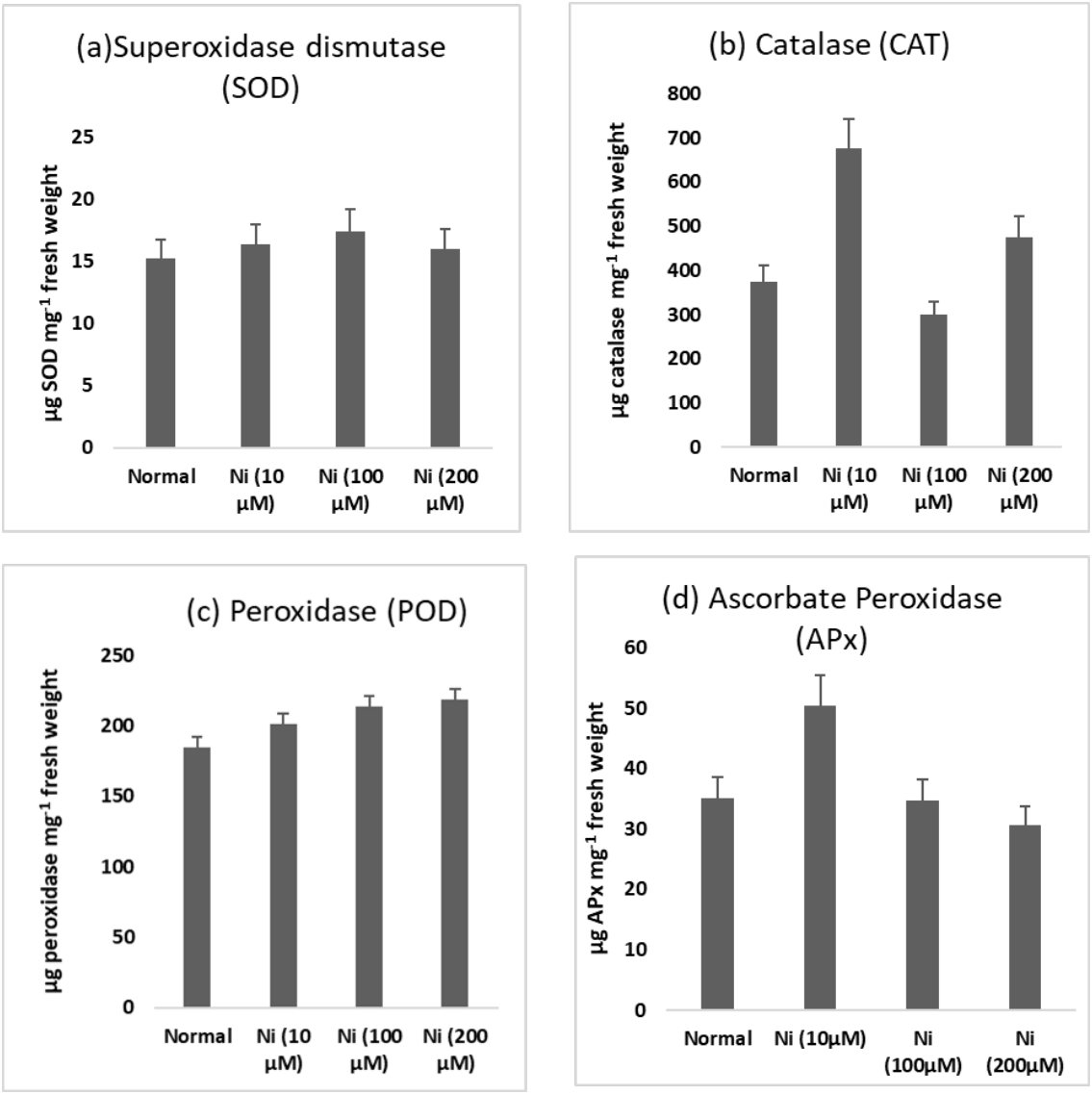

Fig. 1: Effect of $NiCl_2$ on the antioxidant enzymes (a) SOD content, (b) CAT content, (c) Peroxidase (POD) content and (d) APX in spinach 12 days after treatment.

Discussion

This study described the negative impact of nickel in excess amounts, particularly on the germination of seeds and the growth of spinach. The result shows that Ni contamination significantly decreased the number of seed germinates and seedling growth with comparison to the seed germinating in low Ni concentration (control). As mentioned by Pandey and Sharma (2002), this significant decrease in the percentage of seed germination and their growth rate is because of nutrient imbalance of Mg^{2+} ions by excess Ni, which inhibits chlorophyll biosynthesis. $NiCl_2$ in low concentration (5 mg/L) shows a positive effect on the percentage of seed germinates, their growth rate and fresh and dry weight in all wheat cultivars (Kumar and Verma, 2018; Hassan *et al.,* 2019). By increasing $NiCl_2$ concentration, the percentage of seed germination is reduced in all wheat cultivars, while low $NiCl_2$ concentration (0.05mg/L) stimulates seed germination in all wheat cultivars (Kumar and Verma 2018) and similar results were found in Guar (Prajapati *et al.,* 2022), finger millet, pearl millet and oats (Gupta *et al.,* 2017). In guar, a maximum reduction in number of seed germination was observed at 500 ppm of Ni (Prajapati *et al.,* 2022).

High concentrations of nickel 100µM and 200µM show a significant decrease in root length of spinach plants in comparison with 10 µM concentration. Plant growth shows a negative effect under the supply of Ni in high concentrations that lead to metal toxicity, but nickel is a micronutrient that, in low concentrations, can improve the growth of rice plants like seedling growth, fresh and dry weight and seedling index (Khan *et al.,* 2020). Nickel in low concentration is necessary for the proper growth and development of plants (Uruc*et al.,* 2016; Sreekanth *et al.,* 2013). Reduction in root growth was observed by 37 and 53% in wheat seedlings at 100 and 200 µM

of Ni, respectively (Ain *et al.,* 2016) and same similar result was found in field beans, lettuce and maize by Antonkiewicz *et al.,* (2016). The shoot length of mung bean is highly decreased in all high-concentration nickel treatments in comparison to the shoot length of mung bean in low concentration or control (Anjum *et al.,* 2023). Nickel in high concentration decreases plant growth by inhibiting the cell division at root meristem (Ivanov *et al.,* 2021). $NiCl_2$ in high concentration shows a gradual decrease in root length of all wheat cultivars (Kumar and Verma 2018). Accumulation of Ni get a decrease in root when $NiCl_2$ concentration is more than 50µM (Wang *et al.,* 2009). Nickel shows adverse effects on root length under high concentrations. In wheat cultivars at high Ni concentration (500 ppm), root length was 1.33 cm long and in control (at low conc.), it was 2.52 cm long (Prajapati *et al.,* 2022).

As mentioned by Hassan *et al*., (2019), Nickel toxicity causes necrosis and chlorosis and causes oxidative damage in plants by inhibiting many physiological processes. Ni toxicity induces changes in the activity of peroxidase, Catalase and Superoxide dismutase enzymes (Bhalerao *et al.,* 2015). At 60 mg/L of Ni treatment, activity and concentration of catalase increase significantly in sweet potatoes. The SOD and peroxidase enzymes activity increases at 15 mg/L of Ni and later at 30 mg/L and 60 mg/L Ni treatment, its activity reduced, but their activity was still higher than the control (Kumar *et al.,* 2022) and similar result found in maize (Amjad 2020). The Ni in high concentration results in a high amount of H_2O_2 (Naz *et al.,* 2022). SOD shows higher activity in the shoot and root length of the finger millet. The activity of catalase in root length and shoot length of finger millets, pearl millets and oats get reduced with an increasing concentration of Ni. The activity of peroxidase first increased and then decreased (Gupta *et al.,* 2017).

As the concentration of Ni increases, the activity of ascorbate peroxidase also increases in spinach seedlings. A similar result was found in the sponge gourd; an excess supply of Ni leads to an increase in APX activity. In comparison with control plants, APX activities get reduced in excess Ni-treated leaves and shoot from 62.5 to 30.4%. On supply of excess Ni, activities of antioxidant enzymes like APX, CAT, POD, and SOD were increased (Awasthi *et al*., 2013) and similar results found by Pandey *et al*., (2006) in green gram. APX activity was increased under Ni stress at 1oth and 25th day of treatment from 119 and 5 to 141% and 86%, respectively, compared to the control plant (Dubey and Pandey 2011).

Conclusion

Excess Ni has significant effects on spinach, decreasing the number of seed germination, their growth and various physiological parameters. Nickel toxicity severity depends on their concentration, period of exposure, and the specific environmental conditions. From this study, we concluded that low Ni had a stimulatory effect on the germination of seeds and seedling growth on spinach seeds. Using Ni more than 10 µg/L had greatly affected the germination and growth, resulting in chlorosis (yellowing of leaves) and reduced root and shoot growth. Ni concentration higher than 10 µg reduces their radicle growth and leads to their death on the final day of the experiment. Nickel contamination highly influenced seed vigor index and seed germination. Understanding these effects can help in developing strategies to manage nickel toxicity in spinach cultivation.

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Author's Contributions

Author Nilu Singh is involved in the experiments, data compilation and writing of the article. Rajiv Dwivedi and G C Pathak are involved in the overall idea, conception and design of the article and for giving crucial inputs and supervision from time to time.

Conflict of Interest

The authors declare no conflict of interest.

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