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Chromosomal and Nuclear Alteration Induced by Nickel Nitrate in the Root Tips of *Allium cepa var. aggregatum*

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ABSTRACT

Nickel nitrate is a heavy metal known as an environmental contaminant due to its toxicity, long environmental half-lives, and capacity for bioaccumulation. This study aims to determine chromosomal aberration, nuclear alteration, and cell death in *Allium cepa* var. aggregatum L. root caused by different nickel concentrations. Roots of *Allium cepa* var. aggregatum were induced by soaking bulbs in water, then transferred to a solution containing nickel (Ni) at a concentration of 20 ppm, 30 ppm, and 40 ppm for 72 hours. Root tip mitotic chromosome preparations were done by the squash method. The chromosome was stained with aceto-orcein and chromosomal damages were observed under a microscope. The results showed that the mitotic index decreased from 5.025% at control to 3.144%, 2.467%, and 2.181% at immersion with 20 ppm, 30 ppm 40 ppm nickel nitrate, respectively. Anaphase and telophase indexes in roots with Ni treatments were lower than in control, suggesting that nickel inhibits cell division. Nickel nitrate induced chromosomal damages and nuclear abnormalities, such as sticky chromosome, fragmented chromosome, chromosome bridge and chromosome laggard, micronuclei, binucleate and nuclear budding. The percentage of chromosomal damage increases with a higher concentration of Ni. In situ cell visualization showed that the higher the nickel concentration, the more coloured the root tips indicating high levels of cell death.

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INTRODUCTION

Heavy metals such as cadmium, lead, mercury, and nickel are environmental pollutants that harm the environment, including soil, plants, animals, and humans. There has been increasing attention to the ecological risk of heavy metal contamination in soil and water. High concentrations of heavy metals can be toxic to the environment and living things (Ali et al., 2019).

Nickel is an important trace metal for plants, animals, and humans (Poonkothai & Vijayavathi, 2012). Hence symptoms of deficiency or toxicity can occur when, respectively, too little or too much is absorbed. The range of nickel content in soils has been estimated to be between 3 and 1000 ppm (Iyaka, 2011). According to Canadian Soil Quality Guidelines for Protection of Environmental and Human Health (2007), the soil quality for Ni is 50 ppm. Nickel toxicity has

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emerged as a distinct concern due to pollution caused by its expanded industrial use, fossil fuel combustion, and excessive pesticide and fertilizer use in agriculture (Lešková et al., 2020). The critical toxicity level of nickel (Ni) is greater than 10 ppm dry mass (DM) in sensitive species, greater than 50 ppm DM in moderately tolerant species, and greater than 1,000 ppm DM in Ni hyper-accumulator plants species (Yusuf et al., 2011). Nickel at high concentrations is harmful not only to plants but also to humans who consume agricultural products contaminated with Nickel (Amari et al., 2017). In plants, heavy metals cause inhibition of several physiological processes, such as photosynthesis, respiration, cell elongation, and inhibition of mineral nutrient metabolism (Ghori et al., 2019). Excess nickel damages membrane functions, inhibits seed germination, plant growth, and development, and significantly reduces plant yield (Shahzad et al., 2018). In animals and human, it has been shown that nickel cause DNA damage, such as single-strand DNA breaks and double-strand DNA breaks (Morales et al., 2016; Guo et al., 2019). Furthermore, nickel inhibited DNA repair mechanism (Morales et al., 2016).

Physicochemical examination of water or sediment samples is the most typical method for detecting the presence of heavy metals (Akhtaret et al., 2016). However, environmental wastes are complex; therefore, phytotoxicity-based bioassays may increase the effectiveness of assessing risks and monitoring the environment contaminated with heavy metals (Parlak, 2016). The mutagenic activity of chemical compounds, including heavy metals, can be determined using plants as a test system. The test system is carried out using a cytogenetic test by observing the presence of chromosome aberrations and nuclear alterations. Also, the mitotic index in the meristematic area is beneficial for determining cells' health status and meristematic activity (Graña, 2018).

Among many plant species, the most commonly used to evaluate environmental pollution is *Allium cepa*. The Allium tests are based on observing morphological changes in chromosomes (Bonciu et al., 2018). Bioassay with the root tip of *A. cepa* is the simplest and most reliable way to observe the genotoxic potential of a chemical. This is due to a high percentage of cell division, large chromosome size, and well-coloured chromosomes (Mangalampalli et al., 2018). Other Allium species have also been used in chromosomal aberration assays such as *A. fistulosum* (Trofimov & Pyansina, 2005) and *A. sativum* (Kumar & Nagpal, 2015). This assay is efficient and cost-effective and no ethical concerns are needed for the use of animals in testing. Moreover, it was reported that *A. cepa* chromosomal aberration assay had similar results as compared to the assay using animal cell lines (Tedesco & Laughinghouse, 2012).

Nickel can have a stimulating or harmful influence on various physiological and biochemical processes depending on its concentration. Little is known about the mechanisms of nickel causing growth inhibition. Sarac et al. (2019) did a study on nickel's toxicity in *A. sativum* and several chromosomal aberrations were observed such as chromosome clumping, stickiness and bridges. The concentrations of nickel nitrate used by Sarac *et al.* (2019) were 50, 150 and 450 ppm, and all treatments resulted in chromosomal aberrations. Therefore, lower concentrations of nickel need to be tested. This study aimed to analyse chromosomal aberration, nuclear abnormalities and cell death induced by nickel at 20 ppm, 30 ppm and 40 ppm in root tips of *A. cepa* var. *aggregatum*.

MATERIALS AND METHODS

Equal-sized (diameter of approximately 2.5 cm) *A. cepa* var. *aggregatum* bulbs were obtained from a local market in Denpasar, Bali, Indonesia. Five bulbs were used for each treatment. The bulbs were washed in running water and transferred to small dark glass bottles containing tap water for rooting. The water was changed every 24 hours to prevent the growth of microorganisms. After the roots grew to 1 cm, the bulbs were transferred to the nickel nitrate hexahydrate ($Ni(NO_3)_2.6H_2O$) solution at 20 ppm, 30 ppm and 40 ppm for 72 hours. These

concentrations were used to test if lower concentrations than that used by Sarac et al. (2019) can induce chromosomal aberration. The 72 hours treatment period was also based on Sarac et al. (2019). As a control, the bulbs were kept in water.

The chromosome preparation was done following Sabeen et al. (2020) with modifications. Root tips of plants were cut in the morning and put in a microtube. Freshly prepared Carnoy's fixative solution (1:3 acetic acid: absolute ethanol) was added to the microtube and the root tips were incubated for 72 hours. After fixation, the roots were transferred to 70% ethanol and stored in the refrigerator until the observation was conducted.

The roots were placed into a glass watch, soaked in 1 N HCl, and then incubated for 5 min in an oven at 60°C. The roots were transferred using forceps to a glass slide and cut as long as 1–2 mm using a surgical blade. One to two drops of 2% acetoorcein was added and incubated for 5 min at room temperature. The excess dye was removed with filter paper. After that, the cover glass was put over the root tip and squashed by thumb. The chromosomes were observed using a microscope (Yazumi XSZ-107BN) with 400x magnification. Five slides were prepared for each treatment from different roots. As many as 500 to 600 cells from five microscope fields of view were observed in each slide. Observation was done on the number of cell divisions, type and number of chromosomal aberration and nuclear alteration. Photographs were taken using optilab (Miconos).

The reduction of cell viability was investigated using the Evans Blue staining technique (Vazhangat and Thoppil, 2017). Following treatments with Ni, three bulbs with intact roots were placed for 15 minutes in an aqueous Evans Blue solution containing 0.25% (w/v), and then rinsed for 30 minutes with distilled water. The roots were then macro-photograph to detect cell death qualitatively.

The cell aberrations and the mitotic index were calculated based on Kumar and Srivastava (2015) and Sabeen et al. (2020). The formulas are as follows:

(1) Mitotic index (MI) = (total number of dividing cell/total number of cell) x 100%

(2) Decrease of mitotic index to control: (MI treatment – MI control) x 100%

(3) The percentage of aberrant cell = (total number of aberrant cells/total number of cells) x 100%

(4) Phase mitotic index = (number of dividing cells of phases/total number of dividing cells) x 100%

The percentage of aberrant cell consisted of both chromosomal aberration and nuclear abnormalities. The data were then analysed using analysis of variance (ANOVA) using MINITAB 20. The differences between means were analysed using Tukey comparison test at 0.05 probability level.

RESULTS AND DISCUSSION

Treatment of Ni affected mitotic index of root tip cell of *A. cepa* var *aggregatum*. Determination of health status and meristematic activity of cells can be reflected in mitotic index (Graña, 2018). The mitotic index is used to analyse cytotoxicity in living things based on the increase or decrease in cell division (Debnath et al., 2016). As can be seen in Table 1, the mitotic indexes of root tip cells treated with Ni were lower significantly than in control. The indexes were significantly different among Ni concentrations of 20 ppm and 30 ppm, as well as 20 ppm and 40 ppm. However, the mitotic indexes were not significantly different between 30 ppm Ni and 40 ppm Ni. The effect of Ni on mitotic index was concentration-dependent. The higher the concentration of Ni, the lower the mitotic index. According to Nefic *et al.* (2013), a decrease in the mitotic index is a genotoxic indicator. This occurs because of a disruption in the cell cycle which is induced by the interaction of heavy metals such as Cr, Cu and Pb, with DNA (Mesi & Kopliki, 2014). Similar mechanism could apply to Ni. In human bronchial epithelial cells, substantial G2/M cell

Table 1. Mitotic index of root tip of *A. cepa* var. *aggregatum* due to Ni treatments

Characters	Treatment with Ni			
	Control	20 ppm	30 ppm	40 ppm
Mitotic index (%)	5.025[a]	3.144[b]	2.467[c]	2.181[c]
Decrease to control (%)	100	62.57	49.09	43.40

Means followed by different letters at the same column are significantly different (P<0.05)

Table 2. The phase indexes of the control cells and cells with Ni treatments

Treatment with Ni	Phase index (%)			
	Prophase	Metaphase	Anaphase	Telophase
Control	14.943[a]	34.732[a]	36.843[a]	9.587[a]
20 ppm	11[a]	74[b]	9.833[b]	5.167[a]
30 ppm	24.333[a]	58[ab]	14.333[b]	3.333[ab]
40 ppm	24[a]	64[ab]	12[b]	0[b]

Means followed by different letters in the same column are significantly different (P<0.05)

Table 3. The percentage of abnormal cells in root tips of *A. cepa* var. *aggregatum* due to treatments with Ni

Characters	Treatment with Nickel			
	Control	20 ppm	30 ppm	40 ppm
Aberrant cell (%)	0.339[a]	3.264[b]	6.620[c]	8.246[d]

Means followed by different letters are significantly different (P<0.05)

cycle arrest was observed after exposure to Ni (Ding et al., 2009).

Based on the calculation of the decrease of mitotic index as compared to control, Ni showed sub-lethal effect at concentrations 30 ppm and 40 ppm. According to Mesi and Kopliku (2014), a decrease below 50% of mitotic index is considered to have sub-lethal effect, while a reduction of mitotic index below 22% inducts lethal effect.

The phase index is the frequency of each phase in mitosis. Table 2 demonstrates that nickel affected metaphase index, anaphase index and telophase index. The metaphase indexes in Ni-treated *A. cepa* var. *aggregatum* root tips were higher than that in control, while the anaphase indexes and telophase indexes in roots treated with Ni were lower than the control (Table 2). Treatment with 40 ppm Ni resulted in 0% of telophase index. This percentage was significantly different to the percentage of telophase at control and 20 ppm Ni. As can be seen in able 2, the 0% telophase at 40 ppm Ni indicated that the cells were mostly arrested at metaphase. This caused the decrease of mitotic index.

Nickel treatments induced cell abnormalities. Table 3 shows that the percentages of aberrant cells were significantly increased when roots were treated with Ni at 20 ppm, 30 ppm and 40 ppm. The higher the concentration of Ni, the more abnormal cells occurred (Table 3).

The abnormal cells started to be observed at a concentration of 20 ppm with an incubation period of 72 hours. This concentration was lower than that tested by Sarac et al. (2019) who used 50 ppm Ni as the lowest concentration tested with 72 hours incubation on *A. sativum*. The response of *A. cepa* var. *aggregatum* root tips to Ni was dose-dependent. The percentage of cell abnormalities increased when the roots were exposed to a higher concentration of Ni in the exact duration of exposure. Treatment with 40 ppm Ni resulted in the highest percentage of cell abnormalities (8.246%). These results of cell abnormalities are comparable to previous study by

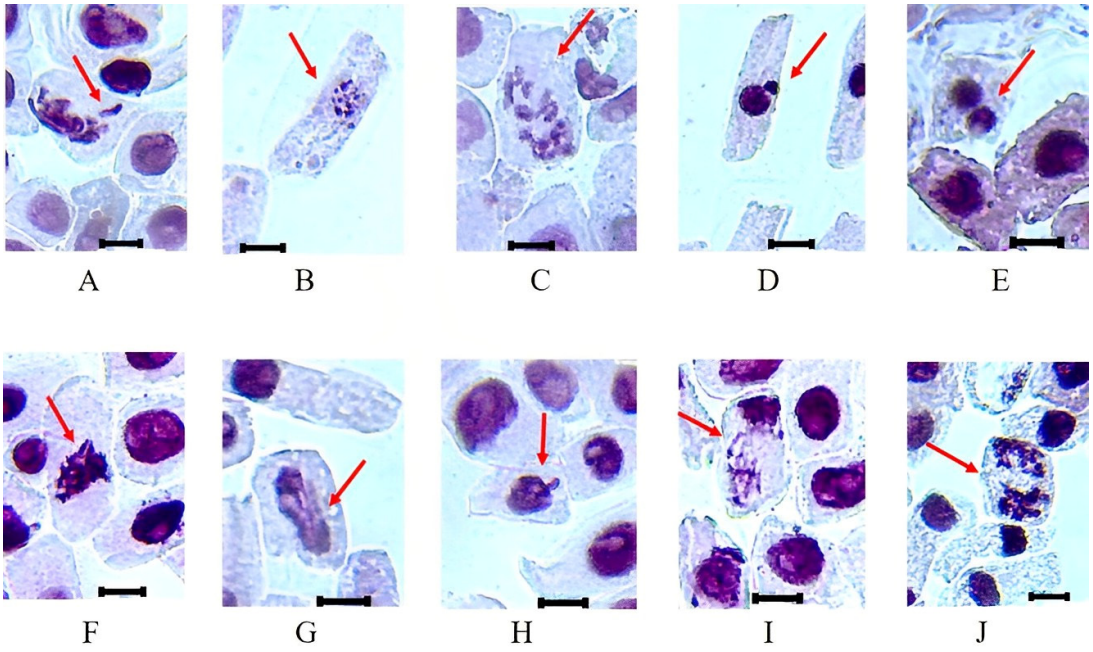

Fig. 1. Various chromosomal aberration and nuclear abnormalities observed due to immersion of roots with Ni for 72 hours. A. Chromosome fragment, B. Fragmented nucleus/apoptotic bodies, C. Chromosome laggard, D. Micronucleus, E. Binucleate, F. Stickiness at metaphase, G. Chromosome bridge, H. Nuclear budding, I. Chromosome vagrant, J. Chromosomes stickiness at anaphase. Bar = 10 µm

Table 4. The percentage of abnormal cells in root tips of *A. cepa* var. *aggregatum* due to Ni treatments
Sk=Sticky, Lg=Laggard, Vg=Vagrant, Bg=Bridge, Mn=Micronucleus, Fg=Fragment, Dm=Diagonal metaphase, Bd=Budding, Fn=Fragmented nucleus, Bn=Binucleate. Means followed by different letters within the same column are significantly different (P<0.05)

Treatment with nickel	Type of cell and chromosomal aberration (%)										Total
	Sk	Lg	Vg	Bg	Mn	Fg	Dm	Bd	Fn	Bn	
Control	0.256^{a}	0.083^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0.339
20 ppm	1.252^{ab}	0.756^{b}	0.346^{ab}	0.224^{a}	0.226^{ab}	0.221^{a}	0.108^{a}	0.131^{ab}	0^{a}	0^{a}	3.264
30 ppm	2.060^{bc}	1.240bc	0.281^{ab}	0.149^{a}	0.763^{b}	0.308^{a}	0.133^{a}	0.516^{b}	1.122^{ab}	0.048^{a}	6.620
40 ppm	2.883^{c}	0.646^{c}	0.631^{b}	0^{a}	0.044^{a}	0.214^{a}	0^{a}	0.044^{ab}	3.742^{b}	0.042^{a}	8.246

Sarac et al. (2019) who found that at 50 ppm Ni with 72-hours soaking period resulted in 8.61% abnormalities in *A. sativum* root tips.

Several types of chromosomal aberration and nuclear abnormalities were observed. Among them were sticky chromosome, fragmented chromosome, chromosome bridge, chromosome laggard, chromosome vagrant, fragmented nuclei, micronuclei, binucleate and nuclear bud (Figure 1 and Table 4). Most types of damage started to be observed at 20 ppm, while fragmented nucleus and bi-nucleus began to occur at 30 ppm treatment (Table 4). However, some abnormality types, such as vagrant chromosome, diagonal metaphase, nuclear budding and bi-nucleus, were not significantly different between control and treatments since they occurred in low percentages.

Chromosome stickiness is a chromosomal aberration with the highest percentages in nickel-treated root tip cells. Treatments with 30 ppm and 40 ppm Ni resulted in significantly higher

percentage of chromosome stickiness compared to control. The percentage of chromosome laggard was significantly higher in Ni-treated root tip cells than in control.

Phytotoxicity from nickel is associated with the formation of reactive oxygen species (ROS). It was reported that high concentration of nickel caused an increase in the concentration of ROS such as superoxide anions, hydroxyl radicals, nitric oxide and hydrogen peroxide (Georgiadou et al., 2018). At low concentrations, treatment with nickel increased the activity of antioxidant enzymes (Duman & Ozturk, 2010), indicating ROS scavenging activity as plant defense mechanism. A study on *Zea mays* showed the increase of malondialdehyde (MDA) content as lipid peroxidation marker after treatment with 20 ppm and 40 ppm $NiCl_2.6H_2O$ (Amjad et al., 2020). Excessive nickel decreased the activity of antioxidant enzymes (Gajewska et al., 2006). ROS are very reactive and interact directly or indirectly with DNA or histones which change the surface property of chromosomes and induce chromosome stickiness (Kumar & Rai, 2007).

In this research, Ni had a highly significant effect on chromosome stickiness. Moreover, chromosome stickiness was the most common type of abnormalities observed. This agrees with several findings which showed that chromosome stickiness was frequently observed in genotoxic studies such as in Kuchy et al. (2016) and Dada et al. (2018). Therefore, chromosome stickiness has become an indicator of toxic effects.

A low level of chromosome stickiness was found at control (treatment with no nickel). This may be due to spontaneous alterations of DNA. According to Nefic et al. (2013), the root tip cells have spontaneous aberration at a very low frequency. In other studies of genotoxic compounds, chromosome abnormalities were rarely observed in control or untreated root. However, it is possible to happen, such as reported by Rosculete et al. (2018) and Fattah and Omer (2021), where a low number of chromosome abnormalities found in control roots. Chromosome stickiness can cause a failure in anaphase that leads to the induction of chromosome bridge (Sabeen et al., 2020). In this study, chromosome bridge was observed at low percentage at 20 ppm and 30 ppm Ni.

Nucleus fragmentation that formed apoptotic bodies was observed at the Ni treatments of 30 ppm and 40 ppm. The number of nuclear fragmentations was significantly higher at 40 ppm than 30 ppm. According to Azad (2014), apoptotic bodies occur due to the accumulation of reactive oxygen species and oxidative stress. Oxidative stress is a signaling event which triggers programmed cell death in plants. A study focused on the effect of heavy metals on program cell death (apoptosis) in *A. cepa*, concluded that apoptosis is an overall response to metal treatment. This was proved by the increased activation of caspase-3, a protease that degrades protein because of nickel and chromium exposure (Cortés-Eslava et al., 2018).

Chromosome fragments occurred at 20 ppm, 30 ppm and 40 ppm Ni. However, there was no significant difference in the percentage of fragments observed between control and the three Ni concentrations. Fragments are formed from double-strand DNA breakage or inhibition of DNA synthesis. Heavy metals, including nickel, were reported to induce DNA double-strand break and inhibit DNA repair (Morales et al., 2016). DNA strand breaks occur due to oxidative stress through production of ROS by heavy metals (Das & Roychoudhury, 2014). Therefore, the production of ROS is the primary response to heavy metal toxicity.

Chromosome fragments can lead to the formation of micronuclei. In this study, micronuclei were also observed at 20 ppm, 30 ppm and 40 ppm Ni. Chromosome fragments or chromosomes that are not included in the daughter cell nucleus during telophase in mitosis arise to form micronuclei (Kwon et al., 2020).

The phase index is the frequency of each phase in mitosis. Anaphase and telophase indexes in *A. cepa* var. aggregatum roots with Ni treatments were lower than that in control. Immersion with Ni caused the mitotic phase stuck in metaphase. This is indicated by the high metaphase index in the roots immersed in Ni compared to the control. Low anaphase and telophase indexes can result in impaired daughter cells, thus inhibiting plant growth. The decrease of anaphase

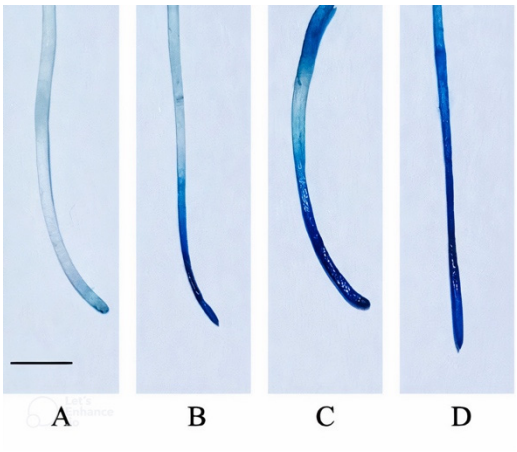

Fig. 2. Analysis of cell death activity using Evans Blue staining of root of *A. cepa* var. aggregatum treated with Ni. A=control, B=20 ppm Ni, C=30 ppm Ni, D=40 ppm Ni. Bar=5 mm

index telophase index was also reported in root tips of *A. cepa* treated with *Aloe vera* gel. In that study, metaphase index decreased, indicating the disturbance of spindle formation (Ilbas et al., 2011). However, in this present study, Ni treatment resulted in a high metaphase index. Although the metaphase index increased in nickel-treated root, it was observed that in metaphase there was high occurrence of sticky chromosomes with different levels of stickiness. This agrees with Palvova (2017), who found that in *Plantago lanceolata* treated with nickel, chromosome stickiness was most frequently observed and mostly found in metaphase and anaphase. This indicates that Ni inhibits the formation of spindle, leading to sticky chromosome, which in turn cause low anaphase and telophase indexes

Evans blue dye was utilized to analyse in situ cell death. Because cell membranes are semipermeable, living cells prevent the dye from entering. However, the dye cannot be removed from injured cells, which is why they are stained blue (Roy et al., 2019). Figure 2 displays the visualization of cell death caused by Ni using Evan's blue staining. Cell death was induced in Ni-treated roots. Higher concentrations of Ni resulted in more coloured roots indicating higher levels of cell death.

Cell death is a well-known biological reaction that cells display when they experience DNA damage. There are several categories of cell death, two of them are programmed cell death (PCD) or apoptosis and non-programmed cell death (necrosis). When plant cells are exposed to heavy metals, cell death PCD is usually activated (Sychta et al., 2021). In this present study, nuclear fragmentation as a sign of apoptosis was observed.

In this study ($Ni(NO_3)_2.6H_2O$) was used to evaluate the genotoxic of Ni. NO3 is reported to results in Ni accumulation in plants. NO3 increase the rhizospheric pH thus will increase accumulation of Ni in plants. In the presence of NO3, the antioxidant enzymes activities induced by Ni were more notable (Hu et al., 2013).

CONCLUSION

This study showed that Ni at the lowest concentration tested (20 ppm) with 72 hours of soaking duration had genotoxic effect on root tips of *A. cepa* var. *aggregatum*. The percentages of cell abnormality increased with increasing Ni concentrations. Nickel inhibits cell cycle shown by a decrease in the mitotic index and reduction in the percentage of anaphase and telophase indexes. In Ni-treated roots, cell death was generated and higher degrees of cell death was

produced by higher Ni concentrations. Studies on Ni bioremediation to reduce the genotoxic effect of Ni are needed.

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CONFLICT OF INTEREST

The authors declare that there is not any conflict of interest regarding the publication of this manuscript. In addition, the ethical issues, including plagiarism, informed consent, misconduct, data fabrication and/ or falsification, double publication and/or submission, and redundancy have been completely observed by the authors.

LIFE SCIENCE REPORTING

No life science threat was practiced in this research.

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