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Cytological assessments and a methodological approach to observe the apoptotic effect of nickel sulfate ($NiSO_4$) on *Allium cepa* L. root germination using EB/AO fluorescence staining

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Abstract

Ethidium bromide–acridine orange (EB/AO) is one of the fastest, most economically valid methods for separating living and dead cells in plant root tips. The present study aimed to investigate the apoptotic effect, nuclear abnormalities and cell division index using an *Allium cepa* test assay with EB/AO staining and an ImageJ program. Nickel sulphate ($NiSO_4$) concentrations (1.75, 3.5, 7 and 14 ppm) were exposed to the root germination of *A. cepa* for 48 and 72 h to observe mitotic abnormalities, cytotoxic effects and 5-day exposure to apoptotic effects. It was found that amorphous nuclei, vacuolisation and C-mitosis were the most observed abnormalities, which increased at 14 ppm $NiSO_4$ exposure after 48 and 72 h. The total number of nuclear abnormalities significantly increased at all concentrations and exposure periods. The ImageJ program was used to determine apoptosis rates. The data obtained showed that high concentrations of $NiSO_4$ caused significantly more cell death in root tips than in the control group, resulting in root growth inhibition and increased apoptosis with increased concentration and exposure period. $NiSO_4$ caused toxic activity on root growth, identified as apoptosis, especially at the cortex and vascular region in the root tips. The study found affected damaged and apoptotic areas on the root tips using an EB/AO staining method, which can be used as a marker of damaged tissue areas.

Keywords: acridine orange; *Allium cepa*; apoptosis; ethidyum bromide; nickel; nuclear abnormalities

Introduction

Acridine orange [3,6-bis(dimeth-yl) acridinium chloride hemi (zinc chloride salt)] (AO) and ethidium bromide [(3,8-diamino-5-ethyl-6-phenyl phe-nanthridinium bromide)] (EB) are used as fluorescent dyes and are known as intercalating agents that bind DNA and RNA between base pairs (LePecq and Paoletti, 1967; Bugs and Cornelio, 2001). EB/AO are used to differentiate dead and live cells in plant and animal cells. AO penetrates every cell, either alive or dead, and emits green fluorescence, while EB penetrates only dead cells and emits red fluorescence. At the apoptosis stage, the cells' nuclear membrane permeability changes, and EB penetrates cells undergoing apoptosis fluoresces in colours ranging from yellow–orange to red. Although AO penetrates dead cells, the colour of EB suppresses the colour of AO, making it distinguishable under fluorescence microscopy. The degree of fluorescence intensity can show live and dead cells, and the stage of

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apoptotic cells (Byczkowska *et al.*, 2013). Another indicator that living, dead and necrotic cells are separated from each other is cell morphology. The condensation of chromatin material in the nucleus, the fragmentation of nuclear DNA, the shrinkage of cells and nucleus, cytoplasm vacuolization and cytoplasm fragmentation are morphological changes in apoptotic cells (Vanyushin *et al.*, 2004). In apoptotic cells, the cell's membrane integrity begins to deteriorate in the early stages of apoptosis, allowing EB to penetrate the cell membrane and to be observed in fluorescence microscopy from its morphological characteristics and colour differences in the early or late stages of apoptosis. Necrotic cells, however, have normal cell morphology and emit orange-red.

EB/AO dual staining is a fast, useful method for assessing the apoptotic effect of some chemicals on plant and animal cells. This method is used to investigate the apoptotic effect of some plant extracts on cancer cell lines (Manosroi *et al.*, 2015; Khursheed and Jain, 2021; Bozali *et al.*, 2022) also effect some chemicals on healthy tissues (L. Wang and Lu, 2007; AlKahtani *et al.*, 2014). Although this staining has been used more frequently in animal cells, the disintegration and chromatin condensation of apoptosis in plants are similar to those in animals (O'brien *et al.*, 1998). Petriccione *et al*. (2013) used EB/AO to distinguish apoptotic and necrotic cells in the root cells of *A. cepa* (Petriccione *et al.*, 2013). Kaźmierczak (2008, 2010) also studied cell atrophy in gametophytes using EB/AO (Kaźmierczak, 2008; Kazmierczak, 2010). Yassin *et al* (2019) also detected morphological and molecular evidence of apoptosis (Yassin *et al.*, 2019), Kunikowska *et al.* (2013) demonstrated kinetin-induced cell death on *Vicia faba ssp*. minor seedlings by using EB/AO dual fluorescence staining method (Kunikowska *et al.*, 2013).

The dual staining method is preferred for evaluating apoptotic effects in plants due to its low cost and ease of application (Ciniglia *et al.*, 2010). However, it can be time consuming to examine the cells separately under a microscope. Vertical sections of the root tips of plant tissue can provide information about the intensity of apoptosis and the detection of apoptotic regions. Apoptosis evaluations can also be made by preparing and examining tissue sections. As a result of fluorescent staining, the amount of apoptosis can be quickly determined using software that detects different wavelengths of live and dead cells in the tissue. It can also provide information about size changes in plant root tips.

Nickel accumulation in the environment has increased due to industrial and agricultural drainage (Yan *et al.*, 2018). Although nickel is an essential element for plants on biological pathways (Eskew *et al.*, 1983; Rahman *et al.*, 2005) and functions as a metalloenzyme, the increased accumulation of nickel in soil causes oxidative damage, DNA damage and the production of reactive oxygen species, resulting in a toxic impact on cells (Wang *et al.*, 2012). It has been shown that Ni disrupts DNA strands, crosslinks, and DNA repair (Klaunig and Kamendulis, 2004; Valko *et al.*, 2006). Previous studies have also indicated that Ni leads to impaired plant growth and development. The reason for this inhibition is metabolic downregulation (Murch *et al.*, 2003), impaired cell division and elongation (Demchenko *et al.*, 2005), nuclear abnormalities (Liu *et al.*, 1995; Sresty and Rao, 1999). High concentrations of Ni lead to toxic effects, including reduced root growth (Rahman *et al.*, 2005), inhibition of germination (Aggarwal *et al.*, 1990), mitotic root tip abnormalities (Mcilveen and Negusanti, 1994). It has been determined that nickel is effective on plant growth and metabolism, reduces growth, causes senescence, chlorosis in leaves and meristems, changes N metabolism, and decreases Fe intake (Ahmad and Ashraf, 2011).

The present study aimed to study the apoptotic and cytotoxic effects of $NiSO_4$ on *A.cepa* roots at the germination stage to investigate apoptotic areas, the apoptosis ratio, mitotic abnormalities and mitotic inhibition by using EB/AO fluorescence staining and the ImageJ program.

Materials and Methods

Preliminary study

The preliminary study aimed to determine the test concentrations of $NiSO_4$ for the experiment. The growth inhibition of the *A. cepa* root meristematic tissue was determined after exposure to 5, 10, 20, 40 and 50 ppm $NiSO_4.6H_2O$ concentrations. Root lengths of germinated bulbs were measured after the fifth, sixth and seventh days of exposure, and the IC_{50} concentrations were calculated according to the probit analysis. The 5-day IC_{50} concentration and its decreasing concentrations were used as test concentrations.

Treatment and analyse procedure for to estimate cell death

Clean, healthy *A. cepa* L. bulbs were chosen for the experiments, and five bulbs were used in each of the exposure groups and the control group. Before starting the experiments, the dry scales of the bulbs were removed, and the roots were exposed to different concentrations of $NiSO_4.6H_2O$ (1.75 ppm, 3.5 ppm, 7 ppm and 14 ppm) for five days. The control group was exposed to distilled water. The detection of cell death in the root tips of *A. cepa* bulbs induced by $NiSO_4$ was conducted according to the following procedure: apical fragments of roots were cut off and washed with a 0.01 M phosphate buffer saline (PBS) (pH 7.4). Then, the roots were stained with an EB/AO fluorescence staining mixture containing 100 μgml^{-1} AO (Sigma Cas No:260-94-6) and 100 μgml^{-1} EB (Sigma Cas No:1239-45-8 in PHB) for 3 min, washed with PBS and fixed with 1.0% gultar–dialdehyde (Merck Cas No 111-30-8) in PBS for 15 min. After fixation, the roots were cut along their axes with a razor blade. After washing with PBS, the root tips were immediately analysed using fluorescence microscopy with a blue filter (Byczkowska *et al.*, 2013).

Analysis of apoptosis by using ImageJ program

At the root tips, the green areas showed viable cells and were stained with AO. The red areas show the apoptotic cells stained with EB. To analyse apoptosis in root tips after exposure, fluorescent images were taken using an Olympus BX51 fluorescence microscope. The fluorescence intensity of the root tip images was measured using the ImageJ program. The fluorescence intensities of two different stains were determined in pixel size on equally sized photographic images of selected root tips. Green and red areas were determined by the program and apoptotic cell percentages were calculated in percentages as apoptotic index % = (a/b)×100, where a is the pixel size of dead cells in b, and b is the pixel size of live and dead cells.

Use of ImageJ program is as follows:

1. The image was opened using File and Open tabs in the menu of ImageJ program.

2. Selecting all the images in the same regions was essential. A line from the tip of the root to the elongation and maturation zones of the root was marked to measure the scale was the length of the area near the vascular system of the roots. This program gave the length of the selected line in pixels. In our study, the distance was 578 pixels. This number was used for further selections of the root tips while choosing the affected root tip areas because the dimensions of the root tips varied. For this step, a straight button was used to select the length of the root tip. Analyse> set scale marked the distance in pixels.

3. The Polygon selection button was used to select the root tip area. The edges of the area to be measured were delimited. The tab was used to clear the outside. The image button was used to adjust the colour threshold. In the threshold colour window, threshold colour red, colour space HSB and adjust brightness in red colour were selected. The selected areas were coloured in red using the brightness selection button to measure all the selected regions in pixel form. The Select button was used before analysing and measuring the tabs.

4. Threshold colour brightness was used to differentiate red and green areas, or apoptotic areas. In this study, threshold colour brightness was set at 110° on the bar. The Select button was tabbed and analysed and measured. The measurement in pixel forms from window>Results was taken. The first measurement was the width of the

selected area of the root tip, and the second measurement was the width of the apoptotic area. The ratio of the red-coloured area to the selected area provided the apoptotic ratio.

Treatment and analyse procedure for to observe cytogenetic abnormalities

$NiSO_4$ concentrations of 1.75, 3.5, 7 and 14 ppm were exposed to the root germination of *A. cepa* for 48 and 72 h to observe mitotic abnormalities and cytotoxic effects. For the microscopic analyses, root tips were hydrolysed with 1N HCl + 2% Aceto orcein (1:9) for 5 min, followed by the preparation of crushed material with the Aceto orcein dying method. For each group, 5,000 cells were evaluated for cytogenetic abnormalities. The mitotic index, nuclear aberrations and mitotic phase index were determined by examining 3,000 cells and were calculated as follows:

Mitotic index = (number of dividing cells × 100)/3,000 (number of counted cells)

Nuclear aberration index = (number of nuclear abnormalities × 100)/3,000 (number of counted cells)

Mitotic phase index = (number of mitotic phase cells × 100)/3,000 (number of counted cells)

Statistical analyses

IC_{50} concentration was determined with probit analysis using SPSS22 Statistics according to the results of the preliminary study. The analysis was performed by comparing the apoptotic values of control with exposure group values using the T-test. Cytogenetic abnormalities and mitotic index analysis were performed using Fisher's exact X2 test ($p = 0.05$). The Pearson correlation coefficient was used for dose-response relations.

Results

Apoptotic effect results

The IC_{50} concentration was determined by a probit analysis of growth inhibition measurements obtained on the fifth day of exposure to $NiSO_4$ from *A. cepa* root tip germination. The preliminary study results are shown in Table 1. After five days of exposure, the IC_{50} concentration was calculated as 13.99 ppm, and decreasing concentrations of the IC_{50} value in multiples were established as test concentrations.

Table 1. Growth inhibition ratios of *A. cepa* root tips after exposed with $NiSO_4$ concentrations between 0-50 ppm after 5, 6 and 7 days

Concentration	Growth inhibition (%) 5th day	Growth inhibition (%) 6th day	Growth inhibition (%) 7th day
Control (-)	100/0	100/0	100/0
5 ppm	21.87	42.25	44.65
10 ppm	45.98	48.67	61.83
20 ppm	59.81	57.11	72.08
40 ppm	69.77	76.4	80.97
50 ppm	83.93	83.33	92.07
IC_{50}	IC_{50}=13.99	IC_{50}=9.46	IC_{50}=6.36

In the present study, the apoptotic effect was observed by EB/AO dual staining method in that the green areas were accepted as live cells and stained with acridine orange, and the orange–red areas showed the cells that have undergone apoptosis and were stained with EB (Figure 1). Although the root cap and epidermal regions of *A. cepa* root tips were affected at lower concentrations of $NiSO_4$, the meristematic region, vascular cylinder, pericycle, endodermis and cortex were more affected after 7 and 14 ppm $NiSO_4$ exposure (Figures 1-2).

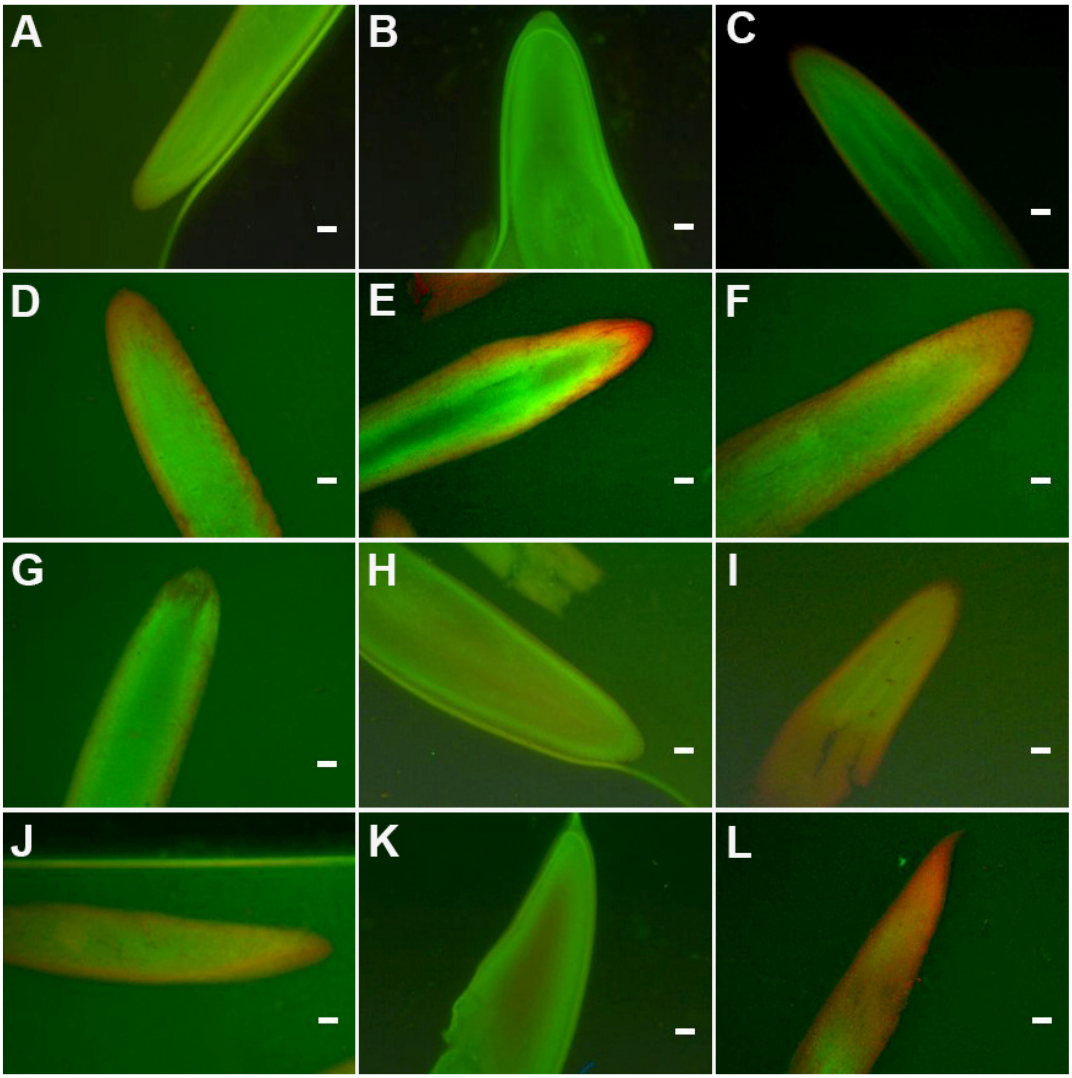

Figure 1. Fluorescence microscope images of *A. cepa* root tips at 5th day of root germination after exposed with different concentrations of $NiSO_4$ by using EB/AO staining method

Root tips show green and red colour with dual staining. Green coloured areas are the viable cells with normal cell morphology and orange-red coloured areas are the apoptotic cells. **(A,B,C)** control groups, **(D,E,F)** 3.5 ppm, **(G,H,I)** 7 ppm, **(J,K,L)** 14 ppm $NiSO_4$ exposure groups. Scale bar = 100 μm.

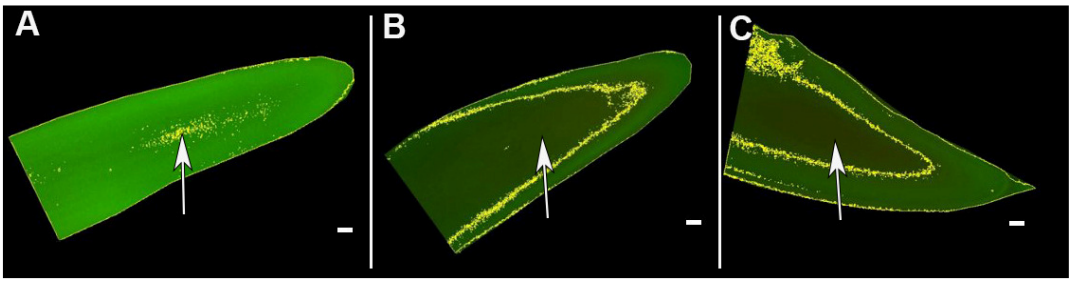

Figure 2. Viable and dead cell areas on the root tips were determined by ImageJ program distinguishes red and green colour areas and measured in pixel sizes

Apoptotic areas were near vascular system of roots in general. The calculation was done by calculating the ratio of apoptotic area to all the measured area. (A) Control group sample and (B, C) 7 and 14 ppm exposure groups. Arrow shows apoptotic regions. Scale bar = 100 μm.

A. cepa root tips were germinated at 1.75, 3.5, 7 and 14 ppm concentrations of $NiSO_4$ for five days. Apoptosis ratios were determined using the imageJ program by measuring fluorescence intensities. It was found that all tested concentrations induced apoptosis in root tips ($p \leq 0.001$) (Table 2). Significant cell death at root tips was observed at higher concentrations of $NiSO_4$ than in the control group, resulting in growth inhibition at the plant root tips. The apoptosis observed at the root tips increased as the exposure to $NiSO_4$ concentration increased (Pearson correlation coefficient, 867 *p = 0.028) (Figure 3).

Table 2. Apoptosis frequency observed on the 5th day of *A. cepa* root tips germinated at different concentrations of $NiSO_4$ (***p<0,001)

Concentration	Apoptosis Mean (%) ± SD
Control (-)	15.86±5.1
1.75 ppm	***35.19±3.28
3.5 ppm	***43.39±9.55
7 ppm	***48.28±11.41
14 ppm	***56.68±3.11

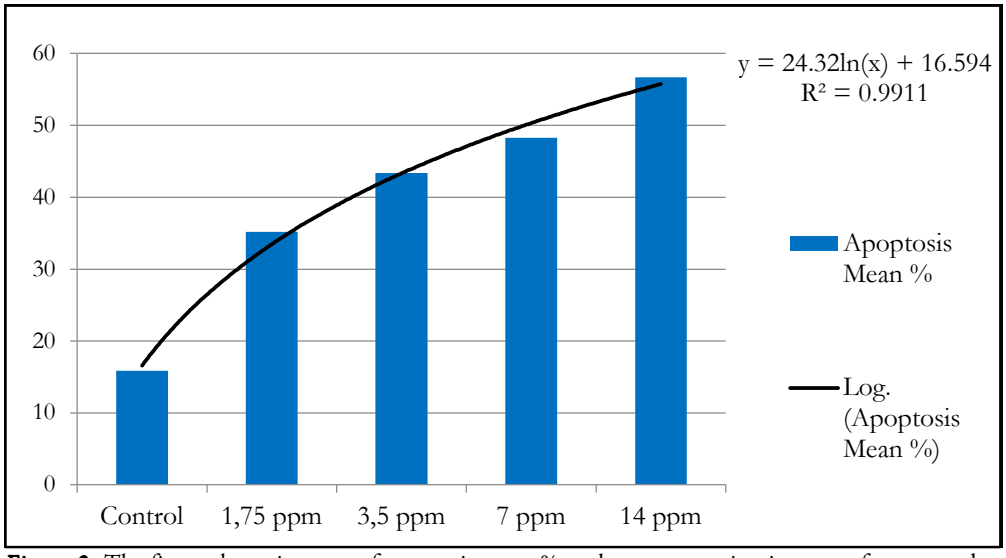

Figure 3. The figure shows increase of apoptosis mean % as the concentration increase after exposed at 1.75, 3.5, 7 and 14 ppm $NiSO_4$ on root tips
(Pearson correlation + ,867 *p=0.028; X-axis: $NiSO_4$ concentrations of; Y-axis: Apoptosis mean %)

The results of the present study showed that an increased concentration of $NiSO_4$ led to significant cell death on the root tips compared to that of the control group and that low concentrations of $NiSO_4$ led to growth inhibition.

Mitotic effect and cytogenetic abnormalities results

Mitotic abnormalities and cytotoxic effects on the meristematic tissue of *A. cepa* root tips were investigated after exposure to 1.75, 3.5, 7 and 14 ppm $NiSO_4$ for 48 and 72 hours using the *A. cepa* test assay (Table 3). It was observed that amorphous nuclei, vacuolisation and C-mitosis were the most commonly observed abnormalities and were increased at 14 ppm $NiSO_4$ exposure after 48 and 72 hours ($p < 0.001$) (Table 3). Total nuclear abnormalities were significantly increased at all concentrations and exposure periods. However, the results showed that the abnormalities were less after 72 h of exposure than after 48 h of exposure.

The results indicated a positive correlation between increases in nuclear abnormalities (NA) and concentrations after 48 hours of exposure (Pearson correlation coefficient +,921, p = 0.026) (Figure 4).

Table 3. Mitotic abnormalities on meristematic tissue of *A.cepa* root tips after exposed with 1.75, 3.5, 7 ve 14 ppm $NiSO_4$ for 48 and 72 hours

	TNA (%) ±S.D.	C-Mitosis	Stickiness	Bridge	MDMA	Vagrant	Fragment	AN	Vacuole
48 h									
Control	0.12±0.02	0.02±0.01	0.02±0.01	0.0±0.0	0.08±0.031	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
1.75 ppm	^{c}0.62±0.05	0.14±0.06	^{c}0.24±0.07	0.0±0.0	0.12±0.04	0.12±0.05	0.0±0.0	0.0±0.0	0.0±0.0
3.5 ppm	^{c}1.18±0.14	^{c}0.38±0.09	^{c}0.3±0.1	0.02±0.01	^{a}0.30±0.16	0.089±0.05	0.0±0.0	0.0±0.0	0.0±0.0
7 ppm	^{c}1.42±0.08	^{c}0.74±0.1	^{a}0.16±0.07	0.02±0.01	0.176±0.09	^{c}0.32±0.11	0.0±0.0	0.0±0.0	0.0±0.0
14 ppm	^{c}1.86±0.19	^{c}0.78±0.14	0.14±0.06	^{c}0.4±0.22	^{a}0.28±0.14	^{c}0.26±0.1	0.0±0.0	^{c}1.2±0.45	^{c}1.8±0.7
72 h									
Control	0.22±0.025	0.06±0.022	0.04±0.02	0.0±0.0	0.12±0.05	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
1.75 ppm	^{c}0.92±0.15	^{b}0.26±0.12	^{c}0.36±0.12	^{c}0.06±0.02	0.176±0.1	0.0±0.0	^{c}0.06±0.0	0.0±0.0	0.0±0.0
3.5 ppm	^{c}0.6±0.01	^{b}0.24±0.1	^{b}0.2±0.08	^{a}0.02±0.01	0.12±0.03	0.0±0.0	0.02±0.0	0.0±0.0	0.0±0.0
7 ppm	^{c}0.54±0.05	^{c}0.30±0.08	0.06±0.03	^{a}0.02±0.01	0.12±0.06	0.02±0.01	0.0±0.0	0.0±0.0	0.0±0.0
14 ppm	^{c}1.4±0.16	^{c}0.88±0.07	0.14±0.05	^{c}0.04±0.02	0.1±0.04	^{c}0.18±0.08	0.0±0.0	^{c}5±0.79	^{c}2±0.41

TNA; Total nuclear abnormalities, MDMA; Multipolar and disoriented metaphase and anaphase, AN; Amorphous nuclei. Mean values with superscripted letters of values are significantly different (^{a}p<0.05; ^{b}p<0.01; ^{c}p<0.001) based on Fisher's exact X^2 test significant difference comparisons.

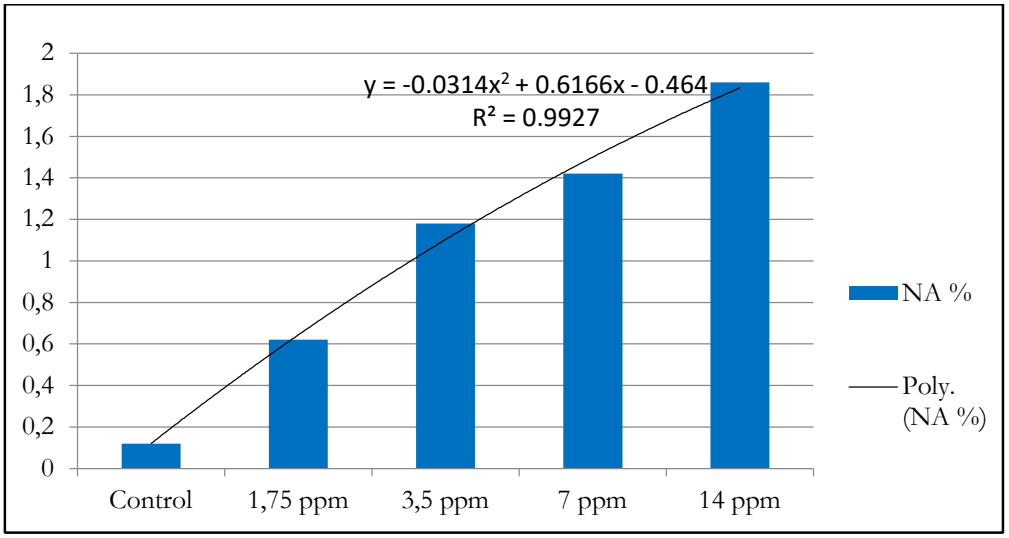

Figure 4. Positive correlation is shown between concentration and nuclear abnormalities after 48 hours' exposure period of 1.75, 3.5, 7 and 14 ppm $NiSO_4$ on root tips
(Pearson correlation; +,921 *p=0,026; X-axis: $NiSO_4$ concentrations; Y-axis: Nuclear abnormality %; NA: Nuclear Abnormalities)

The mitotic index and each mitotic phase were investigated and found to decrease significantly for all the tested concentrations for the 48- and 72-hour exposure periods (Table 4). There was a negative correlation between the mitotic index decrease and the concentration increase after the 72-hour exposure period (p = 0.03) (Figure 5).

Table 4. Mitotic index inhibition and mitotic phases observed on meristematic tissue of *A. cepa* root tips after exposed with 1.75, 3.5, 7 ve 14 ppm $NiSO_4$ for 48 and 72 hours. (*p<0.05; **p<0.01; ***p<0.001)

Exp. period	Conc.	Mitotic Index (%) ±S.D.	Prophase (%) ±S.D.	Metaphase (%) ±S.D.	Anaphase (%) ±S.D.	Telophase (%) ±S.D.
48 h	Control (-)	6.68±0.84	2.48±0.19	2.02±0.38	1.04±0.25	1.16±0.22
	1.75 ppm	4.02±0.79***	1.62±0.22***	0.96±0.16***	0.78±0.14***	0.66±0.18***
	3.5 ppm	3.82±0.62***	1.92±0.29**	0.94±0.158***	0.5±0.13***	0.46±0.22***
	7 ppm	2.87±0.63***	1.46±0.22***	0.66±0.08***	0.36±0.07***	0.4±0.16***
	14 ppm	2.9±0.74***	1.34±0.15***	0.75±0.15***	0.32±0.12***	0.56±0.45***
72 h	Control (-)	6.32±0.43	2.96±0.35	1.24±0.14	1.16±0.22	1,18±0.14
	1.75 ppm	3.84±0.26***	1.88±0.19***	0.82±0.07***	0.6±0.24***	0.54±0.14***
	3.5 ppm	3.4±0.57***	1.67±0.64***	0.86±0.12***	0.64±0.17***	0.34±0.08***
	7 ppm	2.58±0.45***	1.32±0.16***	0.56±0.12***	0.3±0.079***	0.4±0.16***
	14 ppm	0.98±0.35***	0.62±0.22***	0.08±0.02***	0.1±0.03***	0.18±0.09***

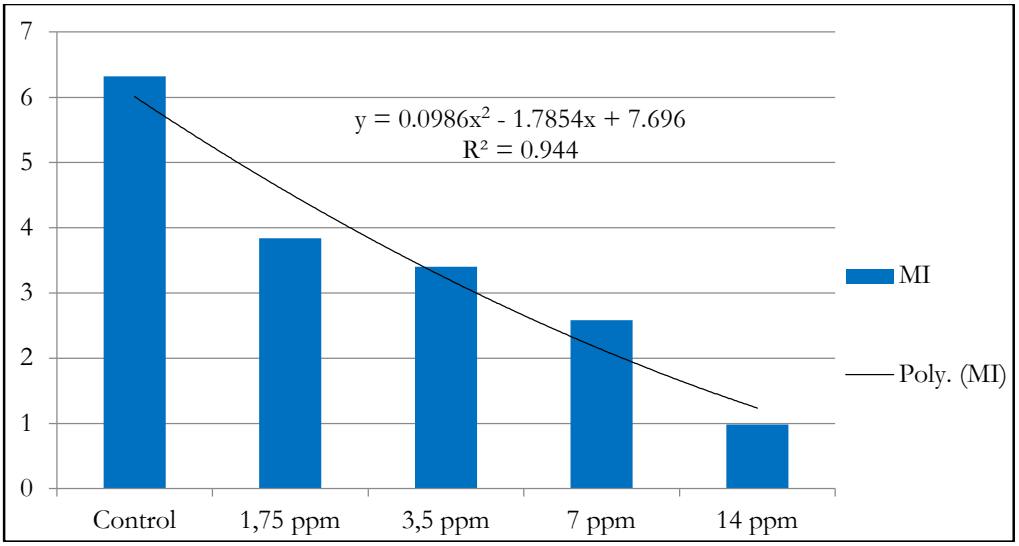

Figure 5. Negative correlation is shown between mitotic index decrease and concentration increase after 72 hours' exposure period of 1.75, 3.5, 7 and 14 ppm $NiSO_4$ on root tips. (Pearson correlation -,915, p=0.03; X-axis: $NiSO_4$ concentrations; Y-axis: Mitotic Index; MI: Mitotic index).

Discussion

Nickel, a phytotoxic metal, is easily taken up by plant root tips. It has been determined that Nickel,which may be found in large amounts in soil, cause programmed cell death in root tips (Samadi and Behboodi (2005). Previous studies have revealed that nickel inhibits root and stem growth as the concentration and duration period increase and also induces abnormal root tip mitosis (Liu *et al.*, 1994). Samadi and Behboodi (2005) also demonstrated that nickel-induced cell death at root tips shows a different cell morphology and identified apoptotic bodies in the apoptotic stages of cells Samadi and Behboodi (2005). A study by Cortes–Eslava *et al.* (2018) indicated that Ni activated Caspase-3-like proteins and increased the release of Cyt-C, resulting in apoptosis in *A. cepa* root tips (Cortes-Eslava *et al.*, 2018). It has been found in most studies that apoptosis is induced by DNA damage (Caicedo *et al.*, 2008; Jia and Chen, 2008).

The present study established that $NiSO_4$ significantly increased cell death in root tips as the concentration increased (Pearson correlation coefficient [$p < 0.000$]), resulting in root growth inhibition. All the tested concentrations induced apoptosis depending on the concentration increase in root tips ($p \leq 0.001$). Although 1.75 ppm $NiSO_4$ exposure induced cell death at the epidermal region of the root tips, exposure with

a concentration of over 3.75 ppm to the vascular region of the roots was more affected. Cell death was likely the result of accumulating a high percentage of Ni (over 80%) in the root vascular cylinder and less than 20% in the corticular region (Page and Feller, 2005; Riesen and Feller, 2005). In this vascular region, the pericycle, endodermis and cortex of the root tips were affected at higher concentrations. Live and apoptotic cells were observed in the root tips, and these regions were differentiated using an EB/AO dual staining method. Ni accumulation in the affected cells might have impaired cell division and the proliferation rate, resulting in cell death. Our results were in accordance with other studies showing that Ni penetrates the endodermal barrier and accumulates in pericycle cells (Seregin and Kozhevnikova, 2006).

In the present study, mitotic abnormalities showed that amorphous nuclei, vacuolisation and C-mitosis were the most commonly observed abnormalities and increased at 14 ppm $NiSO_4$ exposure after 48 and 72 hours ($p < 0.001$). Total NA were significantly increased at all concentrations and exposure periods. Other researchers have obtained similar results. For instance, Sarac *et al*. (2019) found that nickel nitrate induced several types of chromosomal aberrations at concentrations of 50, 150 and 450 ppm for 72 hours by using *Allium sativum*, indicating the aneugenic effects of nickel nitrate (Sarac *et al*., 2019). Nickel has been reported to cause nucleus abnormalities in onion root cells due to the toxic effect of high concentrations (Liu *et al*., 1994) and nuclear morphological alterations: irregular nuclei, chromosomal breaks, bridges, laggards and micronuclei with 20 to 100 µM concentrations of nickel ions (Ni)(Gantayat *et al*., 2018). Nickel nitrate has an aneugenic effect due to an increase in chromosome aberration in all treatment variants in the meristematic cells of *A. sativum* (Sarac *et al*., 2019). Oxygen radical generation, induced by metals, attacks DNA and other cellular components sensitive to oxidation (Valko *et al*., 2006). The reason for the generation of chromosome abnormalities is an increase in ROS-induced DNA damage and base alterations (Doreswamy *et al*., 2004). Chronic nickel exposure increases ROS generation involved in the cytotoxicity of *in vitro* and *in vivo* models (Kargacin *et al*., 1993; Kang *et al*., 2005). The induction of oxidative damage due to heavy metal exposure showed a significant reduction in root length and the mitotic index in *A. cepa* root tips (Gantayat *et al*., 2018). Nickel sulphate also induced chromosomal aberrations in the V79 Chinese hamster cell (Maehle *et al*., 1992).

It is known that the disorganisation of nuclear structures results in cell division inhibition. The root tips of *Cajanus cajan* grown in 1.5 mM concentration $NiSO_4 \cdot 6H_2O$ showed condensed chromatin, nuclear membrane disruption and two nucleoli developing in the nucleus (Sresty and Rao, 1999). High concentrations of nickel are known to induce NA in the root tips of *A. cepa* because of their toxic effect (Liu *et al*., 1994; Gantayat *et al*., 2018). Previous studies have also reported that nickel inhibited root and stem growth by increasing the concentration and duration of treatment and induced abnormal root tip mitosis (Liu *et al*., 1994; Akbas *et al*., 2016; Gantayat *et al*., 2018) and is known to have a significant mitotic inhibitor effect in the meristematic cells of *A. sativum* (Sarac *et al*., 2019). Cytotoxic results indicated that vacuolisation was the most observed effect in cells. Ni distribution is known to be higher in the cytoplasmic fluid and in vacuoles (over 87%) than in other organelles (Brooks *et al*., 1981). Nickel compounds are phagocytised in vacuoles (Cameron *et al*., 2011). Nickel compounds being phagocytised might be the reason for the higher vacuolisation observed in the present study.

In our preliminary study, root germination inhibition was obvious after a high concentration and a long exposure period of $NiSO_4 \cdot 6H_2O$. Also, MI inhibition showed the same result, although at lower concentrations (3.5, 7 and 14 ppm) and duration periods (48 and 72 h). In the present study, this inhibition was also supported by a significant decrease in mitotic phase ratios compared to the control group at all tested concentrations for both 48 and 72 h exposure periods. This germination and growth inhibition has also been demonstrated in other studies that found that increasing concentrations of Ni inhibited seed germination and seedling growth (Espen, 1997; Leon *et al*., 2005), because of the downregulation of protein synthesis and enzyme systems (Foy, 1978; Bishnoi *et al*., 1993). The roots that were directly exposed to Ni resulted in reduced growth and proliferation (Wong and Bradshaw, 1982; Kopittke *et al*., 2007; Gantayat *et al*., 2018) that

supports our results. MI inhibition and arrest were also indicated after $NiCl_2$ and $NiSO_4$ exposure on *Vicia faba* and *Triticum aestivum* roots (Demchenko *et al.*, 2005). The presence of the toxic effect of Ni on the cell and nucleus explains the mitotic inhibition observed in our study. The cells affected by the toxic effect of nickel not entering mitosis were observed as mitotic inhibition.

The present study found a positive correlation between concentration and NA after 48-hours of exposure, but no correlation after 72-hours of exposure as the aberrations decreased. A negative correlation was also determined between concentration and MI after 72 h exposure. In this case, we speculated that as the duration of exposure increased, abnormalities were not observed because the mitotic index decreased. Therefore, NA could not be observed because of a decrease in MI. The reason the abnormalities were not observed at a higher frequency was the long exposure time, meaning that they could not be detected because of cell death. After 72 hours of treatment, the cytological indications were the increase of amorphous nucleus and cell shape and the increase of cellular vacuolization, which might be due to an increase in affected cells and the inhibition of growth, leading to cell death. Therefore, the frequency of NAs after 72 h exposure was less than it was after 48 h exposure.

Conclusions

This study involved exposing different concentrations of nickel on *A. cepa* root tips to investigate cell death, mitotic inhibition and NA. The affected areas on root tips demonstrated that EB/AO staining and ImageJ program can be used as markers of affected areas. The results of the study indicated that nickel caused toxic activity on root growth, determined to be apoptosis, especially in the cortex and vascular region of the root tips. Cell division may be inhibited due to the toxic effects of $NiSO_4$, resulting in nuclear and cytological abnormalities depending on the concentration and germination period. Further studies with different application methods are needed to determine the toxic effects of $NiSO_4$ on plants and to ensure the avoidance of significant effects on ecosystem functions and animal and human health through food chains.

Authors' Contributions

FDG: Conducted the study, data analysis, writing - original draft, review and editing; MA: Completed the fluorescence microscopy analyzes; DG: Completed the application of the cytotoxicity method of the study; ÖM: Completed the data analysis part.

All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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