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Research Article

Effects of Fe_3O_4 Nanoparticle Stress on the Growth and Development of Rocket *Eruca sativa*

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Plants exposed to stress use the variety of gene regulatory mechanisms to achieve cellular homeostasis, including posttranscriptional regulation of gene expression where microRNAs (miRNAs) play a pivotal role. Since various environmental stress factors such as nanoparticles affect crop productivity and quality, the aim of the present study was to evaluate the genotoxicity level and to estimate miRNA expression level and chlorophyll *a* level in the magnetite (Fe_3O_4) nanoparticle-stressed rocket (*Eruca sativa* Mill.) seedlings grown in hydroponics. Rocket seedlings were exposed to 1 mg/L, 2 mg/L, and 4 mg/L Fe_3O_4 nanoparticles, and after 5 weeks, seed germination rate, root-shoot elongation, genotoxicity, chlorophyll *a*, and miRNA expression levels were evaluated. The obtained results indicated that 1 mg/L, 2 mg/L, and 4 mg/L concentrations of Fe_3O_4 nanoparticles induce low genotoxicity and have a positive effect on the growth and development of rocket seedlings and that nanoparticles may improve the ability of plants to stand against environmental stresses.

1. Introduction

Engineered iron oxide nanoparticles have unique electric and magnetic properties, such as large surface area, adjustable surface charge, and high reactivity that lead to increased production and use of these NPs [1, 2]. Fe_3O_4 NPs are effectively applied in medicine, wastewater remediation, biosensors, and electronics [3]. Iron oxide NPs are found in different product categories, such as paints, construction materials, cosmetics, catalysts, batteries, and plastics [2]. Intensive NP utilization leads to the accumulation of NPs in the environment including the aquatic environment [4]. Zuverza-Mena et al. [5] confirms that iron NP genotoxicity on plants is unknown. Additionally, toxicity threshold is species- and nanoparticle size-dependent. Therefore, it is essential to estimate the impact of iron NPs on plant genotoxicity. The randomly amplified polymorphic DNA (RAPD) technique has been efficiently used to evaluate mutagenic effects of NPs on various plant species [6–8].

Several biotic and abiotic stress factors significantly affect crop productivity and quality [9]. Plants exposed to stress use the variety of gene regulatory mechanisms to achieve cellular homeostasis, including posttranscriptional regulation of gene expression where microRNAs (miRNAs) play a pivotal role [10]. miRNAs are small noncoding RNA molecules, which consist of 20-24 nucleotides and are present in most eukaryotic organisms, including plants. Additionally, gene expression was negatively regulated in a sequence-specific manner by specifically binding and cleaving their target mature RNA or by inhibiting their translation [11]. miRNAs are involved in different regulating processes of plant growth and development. Moreover, changes in the expression of definitive miRNAs in plants under various environmental stresses, such as drought, salinity, cold, heat, pathogen

infection, heavy metal, and heavy metal nanoparticles (NPs), are reported [11–13]. Heavy metal toxicity is of interest around the world as they induce changes in various physiological and biochemical mechanisms of plants that cause significant crop loss. Heavy metals among other metals include iron that is toxic to plants [11]. According to previous studies, miR159 is important for plant growth and environmental stress response. Since miRNAs are plant species-dependent, they can be involved in different plant response mechanisms [14, 15]. For example, in *Arabidopsis*, miR159 was shown to be drought responsive [16], while in maize it is involved in response to hypoxia [12]. Moreover, in wheat, miR159 is involved in response to fungal infection [12].

Eruca sativa Mill., also known as arugula, garden rocket, or rucola, is an herbaceous vegetable whose consumption becomes increasingly popular worldwide [17]. Rocket salad is economically relevant, and rocket is cultivated in all continents thanks to its excellent nutritional properties, such as high content of glucosides, flavonols, mineral salts, vitamins A and C, and antioxidants [18, 19]. Additionally, rocket seed oil is valuable as it has antioxidant and antimicrobial activity and promotes inhibition of tumour growth proliferation [20].

The present study was aimed at evaluating the genotoxicity level and at estimating the miRNA expression level and chlorophyll level in the Fe_3O_4 NP-stressed rocket seedlings. To our knowledge, this is the first report of iron nanoparticle impact on *E. sativa*.

2. Materials and Methods

2.1. Nanoparticle Synthesis. Fe_3O_4 NPs were synthesized at Daugavpils University in G.Liberts' Innovative Microscopy Centre by a colloidal method at room temperature according to Martínez-Mera et al. [21]. The size of the derived nanoparticles was 25 nm.

2.2. Seedling Germination Rate. E. sativa Mill. known as "Sala" seeds was sterilised according to Kokina et al. [22]; rocket seeds ($n = 400$) of similar size were selected and placed in Petri dishes containing a filter paper. Seeds were divided into four groups ($n = 100$ each): three experimental groups were grown in 3 mL of different suspensions of Fe_3O_4 NPs (1 mg/L, 2 mg/L, and 4 mg/L) and control group plants were germinated in sterile water. All test groups were incubated at +25°C under a dark/light cycle (8/16 h), 2 Lx, and 80% humidity in a plant growth chamber. After 5 days, germination rate and shoot and root length were assessed. The seed germination rate was defined as the number of germinated seeds (radicle length ≥ 0.5 cm) [23] on the 5th day after culture initiation. Three replicates were performed for this experiment.

2.3. Hydroponic Culture. Separate experiments were conducted during the study: determination of morphological parameters, chlorophyll *a* fluorescence level, genotoxicity, and miRNA expression level. In each experiment, two-week-old rocket plants obtained as described in Kokina et al. [22] were divided into four groups (control group and three experimental groups) with an equivalent number of plants (for morphological parameters ($n = 50$), chlorophyll *a* ($n = 30$), genotoxicity ($n = 20$), and miRNA analysis ($n = 20$)) and transferred in tubes with three different Fe_3O_4 NP solutions described in Section 2.2 supplemented with Murashige and Skoog medium salt solution [24]. Control plants were transferred in tubes with only Murashige and Skoog medium salt solution. Plants were grown in a growth chamber at +20°C, 16/8 (day/night) photoperiod, 2 Lx, and 80% humidity for 5 weeks. After the exposure period, morphological parameters were assessed. Three replicates were performed for each experiment.

2.4. Morphological Parameters. Morphological parameters of control and experimental rocket plants were detected by counting the number of leaves and measuring the shoot and root length and mass. Additionally, the relative contribution of biomass to shoots and roots was calculated. Shoot length and root length were measured using the image processing program ImageJ (open-source software provided by the National Institute of Health (NIH), weblink: http://rsbweb.nih.gov/ij/download.html).

2.5. Analysis of Chlorophyll a Level by Confocal Laser Scanning Fluorescence Microscopy (CLSFM). CLSFM was performed with Nikon Eclipse Ti-E equipped with a digital sight DS-U3 camera and configured with a high-speed multiphoton A1R MP confocal system and motorized stage (Nikon, Japan) using Plan Apo 20x/0.75 and Plan Apo 40x/0.95 objectives to obtain imaging of chlorophyll *a* in the rocket leaves. Fluorescence was excited at 488 nm laser wavelength, and emission was detected using a spectral detector (523-743 nm) and analysed at wavelengths 654.43-662.43 nm. All confocal system parameters were similar (laser: 19.4, HV: 197, and pinhole: 120.4) with the exception of focus, which was customized for each leaf sample. Chlorophyll fluorescence was detected using NIS-Elements (Nikon, Japan) microscope imaging software.

2.6. RAPD Analysis to Evaluate Genotoxicity Level. Extraction of total genomic plant DNA was carried out using the Mini protocol (purification of total DNA from plant tissue (DNeasy Plant Mini Kit, Qiagen GmbH, Germany) with slight modifications by the QIAcube (Qiagen, Germany) extraction system). DNA was extracted from approximately 35 mg wet plant leaf. The final elution volume of DNA was 150 μL. DNA was quantified and qualified with a spectrophotometer (NanoDrop 1000, Thermo Scientific, USA). Stock DNA was diluted to make a working solution of 20 ng/μL for PCR analysis.

A total of 20 decamer primers were used for RAPD analysis [25]. PCR amplification was performed with a Veriti 96-Well Thermal Cycler (Applied Biosystems, USA). Each 20 μL reaction volume contained 5 μL of DNA as a template, 4 μL of 5x FIREPol® Master Mix with 7.5 mM $MgCl_2$ (Solis BioDyne, Estonia), 0.6 μL of primer, and 10.4 μL RNase-free water. For amplification, the reaction mixtures were treated as follows: 94°C for 1 min (initial denaturation)

followed by 35 cycles of 1 min at 94°C (denaturation), 1 min and 30 sec at 37°C or 50°C for OPC-08, OPD-08, and OPE-01 (annealing), and 2 min at 72°C (extension). The amplification was completed with the final extension at 72°C for 10 min. Deionized water was used as a negative control instead of a DNA template.

The PCR reaction products were electrophoresed with a QIAxcel Advanced (Qiagen, Germany) instrument utilizing a QIAxcel DNA high-resolution kit according to the protocol (determination of DNA fragment sizes using the QIAxcel ScreenGel Software (Qiagen, Germany)). To determinate DNA fragment sizes, QX Size Marker 100 bp-2.5 kb and QX Alignment Marker 15 bp/3 kb (Qiagen, Germany) were used. RAPD fragments were scored for the presence or absence of band products for all tested primers. The amplification reaction for each primer was repeated twice for each sample to ensure the reproducibility. Only clear and reproducible bands were considered for analysis.

2.7. Evaluation of Genomic Template Stability. Genomic template stability (GTS, %) was calculated according to the following formula:

$$\text{GTS}\,(\%) = \left(1 - \frac{a}{n}\right) \times 100, \quad (1)$$

where a is the average number of changes in each DNA profile of the experimental group and n is the number of total bands in control samples [26]. Disappearance of a normal band and appearance of a new band in RAPD profiles in comparison to control were observed as polymorphism. The average number of polymorphic bands was calculated for each experimental group.

2.8. Isolation of Total RNA and qRT-PCR Analysis of miRNA. Two-step qPCR analysis was performed to evaluate the expression of miRNA on the extended sampling of rocket plants ($n = 20$) grown under different concentrations of Fe_3O_4 NP conditions and control plants. Total RNA from rocket leaves was isolated using the miRNeasy Plant Mini Kit (Qiagen, Germany). RNA was quantified and qualified with a spectrophotometer (NanoDrop 1000, Thermo Scientific, USA). Only the samples with an A260/A280 ratio nearly 2.0 were used for further analysis.

miRNA target-specific primer lus-miR159c with locked nucleic acid was designed. The target miRNA lus-miR159c sequence was 5′-UUUGGAUUGAAGGGAGCUCUU-3′. In the relative quantification analysis, the elongation factor 1-alpha (*EF1α*) gene [27] was used as a reference gene in order to normalize expression values.

Reverse transcription for miRNA was performed using the Veriti 96-Well Thermal Cycler (Applied Biosystems, USA) and miRCURY LNA RT Kit (Qiagen, Germany) according to the manufacturer's protocol (first-strand cDNA synthesis). After reverse transcription, cDNA was diluted 60-fold by adding RNase-free water, and 3 μL of the sample was used to perform 10 μL qRT-PCR reaction according to the protocol (quantitative real-time PCR using individual miRCURY LNA miRNA PCR assays (miRCURY SYBR Green PCR Kit, Qiagen, Germany)).

Three biological replicates were performed for each gene within each treatment using the Rotor-Gene Q Real-time PCR system (Qiagen, Germany). The results were analysed using the ΔΔCT method.

2.9. Statistical Data Analysis. Results were expressed as an average for the measurement and were presented as mean ± SD. Statistical differences and significant means of the experimental data were examined by Student's t-test. The significant difference was assessed at a level of 0.05 (or 0.01) in all statistical analyses. Each of the experimental values was compared to their corresponding control.

Correlation between GTS and NP concentrations, seed germination rate, shoot/root length, and miRNA expression was determined by Pearson's correlation analysis using SPSS 21.0 (SPSS, Chicago, USA).

3. Results

3.1. Effects of NPs on the Germination Rate. To establish the Fe_3O_4 NP effects on the germination rate of *Eruca sativa* Mill. seeds and shoot and root length, three different concentrations of NPs were tested. Germination rate and shoot and root length were affected by Fe_3O_4 NP concentrations. A significant increase ($P < 0.01$) in the germination rate in rocket seeds exposed to tested concentrations was observed. Results are reflected in Figure 1(a). The maximum germination percentage (76%) was observed in seeds exposed to 4 mg/L while greatly lower germination percentage (39%) was detected in control seeds. Furthermore, there was a significant ($P < 0.01$) increase in shoot elongation from 22 mm in control seedlings to 26 mm and 27 mm at 2 mg/L and 4 mg/L, respectively (Figure 1(b)). Notably, root elongation tends to insignificantly ($P > 0.05$) decrease from 19 mm in control to 17 mm at the highest concentration. Germinated rocket seedling grown under different Fe_3O_4 NP stress conditions is presented in Figure 2(a).

3.2. Plant Morphological Parameters. To demonstrate the impact of NPs on rocket plants after five weeks of exposure, the number of leaves of rocket plants was counted and the shoot and root length and mass were measured. The relative contribution of biomass to shoots and roots was calculated (Table 1). Shoot and root length significantly ($P < 0.01$) differs between the control and experimental groups and between 1 mg/L and 4 mg/L. Shoot length was gradually increased with increasing NP concentration; that is, the length of rocket shoots varied from 18 ± 0.8 mm in the control group to 31 ± 0.24 mm in the experimental group (concentration of NPs at 4 mg/L). The longest shoots were observed in plants grown in the most concentrated NP solution. About three-, four-, and fivefold increase in root length was observed in experimental groups of 1 mg/L (168 ± 1.31 mm), 2 mg/L (200 ± 1.62 mm), and 4 mg/L (262 ± 2.91 mm), in comparison with control plants (59 ± 0.61 mm). Notably, after visual screening of plants,

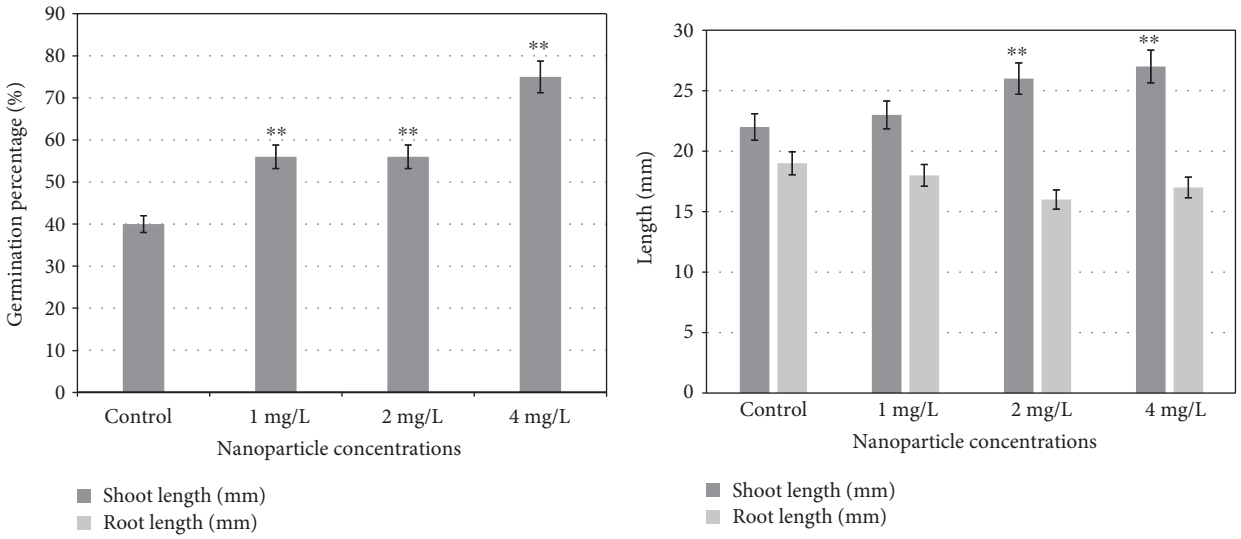


Figure 1: Germination rate (a) and shoot and root length (b) of seeds exposed to different Fe_3O_4 NP concentrations compared to control seeds on the fifth day. Values are mean of three replicates with SD. $*$ indicates significant difference from control ($P < 0.05$), $**$ indicates significant difference from control ($P < 0.01$).



Figure 2: Rocket germinated under Fe_3O_4 NP stress condition on the fifth day of exposure (a). Intensive accumulation of NPs on the root surface (b).

Table 1: Shoot and root length; shoot, root, and plant mass; and number of leaves of control and experimental rocket seedlings after five weeks of exposure.

	Control	1 mg/L	2 mg/L	4 mg/L
Shoot length (mm), mean ± SD	18 ± 0.18	26±0.21**	27±0.20**	31±0.24**
Root length (mm), mean ± SD	59 ± 0.61	168±1.31**	200±1.62**	262±2.91**
Number of leaves, mean ± SD	3.75 ± 0.03	4.00 ± 0.02	3.86 ± 0.03	4 ± 0.04
Shoot mass (mg), mean ± SD	22.79 ± 0.20	32.84 ± 0.22*	29.99 ± 0.21*	30.24 ± 0.20*
Root mass (mg), mean ± SD	14.28 ± 0.16	21.19 ± 0.18*	16.49 ± 0.11	17.18 ± 0.12
Plant mass (mg), mean ± SD	37.06 ± 0.31	54.03±0.48**	46.48 ± 0.40*	47.41 ± 0.39*

Values are mean of three replicates with SD. $*$ indicates significant difference from control ($P < 0.05$); $**$ indicates significant difference from control ($P < 0.01$).

intensive accumulation of NPs on the root surface was detected (Figure 2(b)).

Next, shoot and root mass was measured. Shoot mass significantly ($P < 0.05$) increased in all the experimental groups compared with the control. However, the largest mass of shoots was detected not in the group (4 mg/L) that has the longest plant shoots but in the group (1 mg/L) that has the shortest shoots. Root length in the experimental groups increased significantly ($P < 0.01$); nevertheless, root mass increased significantly ($P < 0.05$) in seedlings treated only with 1 mg/L of NPs in comparison with the control group. Significant changes in the number of leaves among control and experimental plant groups were not detected.

The relative contribution of rocket plant biomass to shoots and roots in all the experimental groups was calculated (Figure 3). The obtained data show negligible changes in relative contribution of biomass to shoots and roots in all the tested groups. The comparison between the control and experimental groups of plants revealed some differences, besides the fact that contribution to shoots significantly ($P < 0.05$) increased in seedlings treated with the highest NP concentration. However, contribution to roots showed converse results as the length significantly ($P < 0.01$) decreased at an NP concentration of 4 mg/L.

3.3. Chlorophyll Measurements. The fluorescence level of chlorophyll was detected at wavelengths 654.43-662.43 nm in rocket seedling leaves in a light-adapted state. Results showed differences in values of chlorophyll *a* fluorescence (Figure 4(a)). A significant increase in the fluorescence level was observed in 1 mg/L and 2 mg/L treatments (with values of 906 and 855, respectively), in comparison with the control (with a value of 621). Nevertheless, the smallest chlorophyll *a* fluorescence value of only 524 was detected in rocket leaves treated with 4 mg/L NPs.

3.4. Genotoxicity Analysis by RAPD Assay. The genotoxicity of Fe_3O_4 NPs was investigated by observing the band profile after the RAPD assay on 20 replicates per treatment obtained from rocket seedlings exposed to different concentrations of NPs. All of the 20 decamer primers were utilized for the RAPD analysis to amplify representative bands in control samples whose number varied from 2 to 6 (OPE-01 and OPD-02 and OPD-18, respectively). Amplification was highly reproducible since the same RAPD profile was observed within control replicates. All utilized primers ($n = 20$) generated a stable RAPD banding pattern for genotoxicity analysis. The differences in RAPD patterns referred to loss of normal bands and appearance of new bands in comparison with the control. Overall, 8 primers (CB-21, OPD-18, OPN-15, OPA-10, CB-19, OPD-07, OPC-08, and OPD-08) showed genomic changes. Unlike controls, two bands 100 bp and 200 bp (OPN-15 and OPC-08, respectively) disappeared in plants with NP treatment. Using OPD-18, CB-19, and OPD-07 primers, 280 bp, 440 bp, and 1.5 kb bands were amplified in the control, respectively, while they were absent in NP-treated plants. However, with OPA-10 and OPD-08 primers, an additional 150 bp and 500 bp bands, respectively, appeared in plants treated with the highest (4 mg/L) NP concentration. It is interesting to note that an additional 180 bp DNA band appeared with the CB-21 primer in plants exposed to 2 mg/L and 4 mg/L. Overall, 6 new bands and the absence of 2 normal DNA bands were observed.

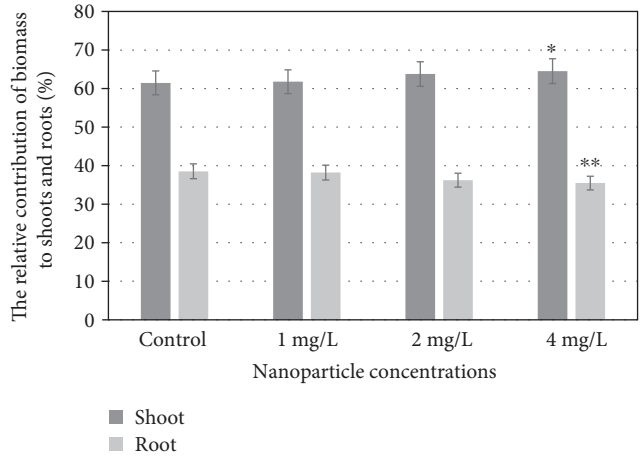


Figure 3: The relative contribution of biomass to shoots and roots of control and experimental rocket seedlings after five weeks of exposure. Values are mean of three replicates with SD. $*$ indicates significant difference from control ($P < 0.05$), $**$ indicates significant difference from control ($P < 0.01$).

3.5. Effect of Fe_3O_4 NPs on Genomic Template Stability (GTS). Diversity in the RAPD patterns generated by the treatment with different concentrations of Fe_3O_4 NPs was calculated as a qualitative measure reflecting changes or percentage of genomic template stability (GTS %) values for each 20 primers and is presented in Figure 4(b). It was observed that mean GTS values decreased from 93.9% to 87.8% with an increase in Fe_3O_4 NP concentration. The changes in the RAPD profile evaluated by the genomic template stability test were further correlated with other stress-related parameters.

3.6. miRNA Expression Analysis. To quantify the miRNA expression level, the qRT-PCR technique was used. Results showed that Fe_3O_4 NP treatment altered miRNA gene expression in a dosage-dependent manner (Figure 5). The miRNA expression level was upregulated at all tested concentrations; however, NP exposure insignificantly ($P > 0.05$) affected miRNA expression. The expression level of miR159c decreased with increasing NP concentrations. 1 mg/L concentration of NPs most of all increased the specific miRNA expression level (1.30-fold), while 2 mg/L and 4 mg/L concentrations increased the expression only to 1.19- and 1.04-fold, respectively.

3.7. Comparison of GTS and Germination Rate, Root-Shoot Growth, Chlorophyll, and miRNA Expression. The results of the present study indicated that GTS was affected by Fe_3O_4 NP concentrations; however, the correlation was low ($P = 0.07$). The same results were observed in the correlation between GTS and seed germination and shoot length ($P > 0.05$). However, a significant correlation was observed

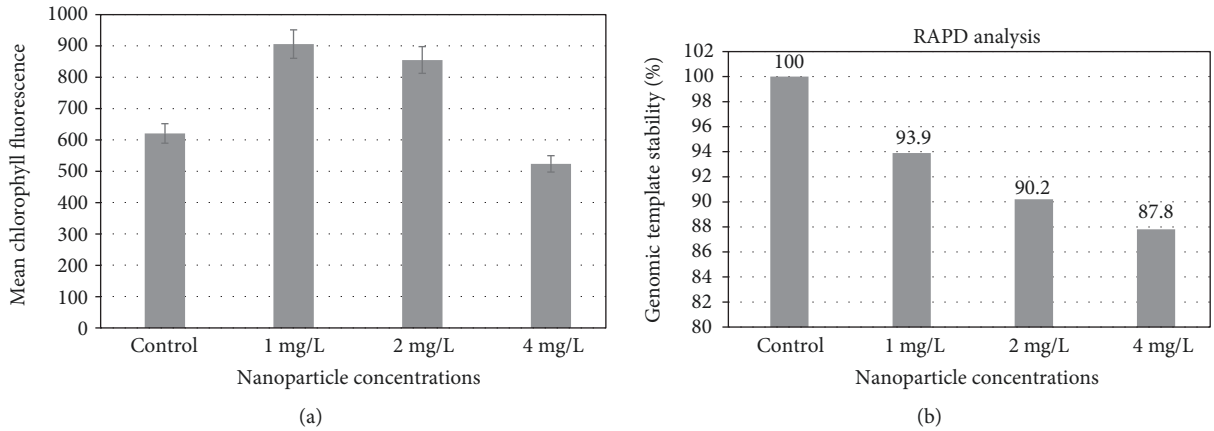


Figure 4: Mean chlorophyll *a* fluorescence values with SD in control and treated rocket leaves (a). Comparison of genomic DNA template stability in rocket seedlings exposed to different Fe_3O_4 NP concentrations (b).

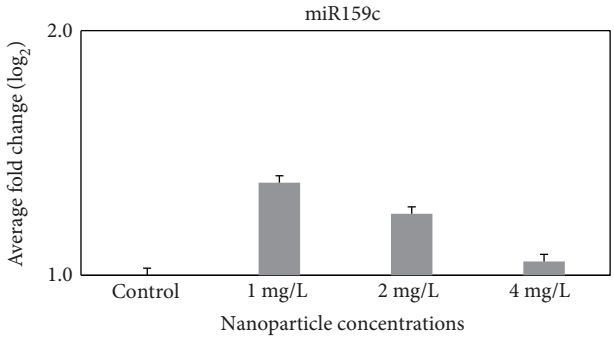


Figure 5: Expression of miRNA in control and treated (1 mg/L, 2 mg/L, and 4 mg/L) rocket seedlings. Values are mean of three replicates with SD.

between GTS and root length ($P < 0.01$) in seedlings after five-week exposure to NPs. Overall, with a decrease in GTS, rocket seed germination, root length, and chlorophyll level increased with the exception of 4 mg/L NPs where fluorescence notably decreased and was smaller than that in control leaves. A low correlation ($P > 0.05$) between GTS and miRNA expression level was detected.

4. Discussion

Nanotechnologies becomes more and more a popular field of industry, and nanomaterials can today be used in a large variety of products such as cosmetics, paints, and electronic devices [28]. As increased production of NPs leads to their release into the environment, the phytotoxicity of these nanoparticles on plants must be evaluated [3]. In this study, the effect of Fe_3O_4 NPs on the growth and development of rocket seedlings was investigated. Results indicate that the studied concentrations of Fe_3O_4 NPs induce low genotoxicity and have a positive effect on seed germination, growth, chlorophyll, and miRNA expression in rocket seedlings grown in hydroponics.

Currently, germination rate and shoot-root elongation are rapid tests that are actively used to determine the phytotoxicity of NPs on various plant species [29–31]. This test has several advantages such as sensitivity, simplicity, low cost, and suitability also for unstable NPs [32]. Results of the present study are similar to the results of other studies in which Ag NPs at a concentration of 30 μg/mL significantly increased rocket seed germination frequency. However, Cu NPs and Au NPs at the same concentration inhibited seed germination [33]. Although, in a similar study, significant changes in rocket seed germination by Ag NPs (0.5 mg/mL to 100 mg/mL) were not observed. According to Vannini et al. [31], Ag NPs significantly stimulated the radical growth of rocket; notably, 10-20 mg/mL concentrations maximally increased plant elongation. The present study also showed increased shoot elongation in Fe_3O_4 NP-treated rocket seedlings after seed germination. Overall, results suggest that Fe_3O_4 NPs have a positive impact on rocket seed germination.

In this study, an average shoot and root length of rocket seedlings were gradually increased with increasing NP concentration. As expected, shoot mass significantly increased in all the experimental groups. It was because of a shoot length increase. Surprisingly, the largest mass of shoots was detected not in the group (4 mg/L) with the longest plant shoots but in the experimental group (1 mg/L) with the shortest shoots. It may be described with assumption that all the tested concentrations of NPs were sufficient for shoot development, while at the same time, 4 mg/L concentrations of NPs showed a greater influence on shoot elongation but 1 mg/L showed a greater impact on increased biomass of shoots. It is interesting that the root length in all the experimental groups increased significantly; nevertheless, root mass increased only in seedlings treated with 1 mg/L of NPs. This suggests that under the impact of NPs, the thickness of rocket roots decreases meaningfully. Khashan et al. [34] mentioned that Fe_3O_4 NPs demonstrate high tendency to agglomerate in water solvents. The same phenomenon was also observed in this study. Moreover, it is proved that NPs aggregate to root pores and reduce the root hydraulic conductivity by inhibiting the water uptake [5]. It is possible that due to lack of necessary amount of water, root cell elongation increased [35]. These data are consistent with

the results of an independent study, where Fe_3O_4 NPs (50 to 200 mg/g in soil) also increased the growth and biomass of lettuce *Lactuca sativa* L. [36]. Moreover, magnetite NPs had a positive influence on soybean root elongation [37]. By contrast, tomato seedlings treated with 50–500 mg/L Fe_3O_4 NPs did now show any shoot morphology or growth differences [38]. Zaka et al. [33] claim that Ag NPs (30 μg/mL) stimulated root and shoot length; on the contrary, Cu and Au NPs at the same concentration inhibited root and shoot elongation in rocket. The obtained morphological parameters make it clear that the concentrations of NP solutions such as 1 mg/L, 2 mg/L, and 4 mg/L have an influence on the shoot size and mass and significantly increase root and shoot length in rocket plants grown in hydroponics.

According to literature, efficient detection of chlorophyll *a* is possible at wavelengths 660-663 nm [7, 20]. Therefore, the fluorescence level of chlorophyll was detected at wavelengths 654.43-662.43 nm in rocket seedling leaves in a light-adapted state. Results indicate that very small concentrations of Fe_3O_4 NPs, such as 1 mg/L and 2 mg/L, are able to slightly increase chlorophyll *a* fluorescence in rocked leaves; however, an NP concentration of 4 mg/L decreases fluorescence. Chlorophyll is a natural green pigment found in plants that plays a crucial role in photosynthesis [39]. Thanks to its different useful properties, such as antioxidant, antimutagenic, and antimicrobial activity, it is extensively used in food, cosmetics, and pharmaceutical industries [40]. Similarly, it was found that Fe_3O_4 NP treatments increased chlorophyll level in plants [41]. Moreover, it is important to promote chlorophyll accumulation in crop plants for biotechnological applications [40]. According to the results of the present study, a positive impact of smaller concentrations of Fe_3O_4 NPs on rocket chlorophyll level is related to the increase in photosynthetic carbon assimilation [42].

Detection of the genotoxic effect using the RAPD method allows simple and rapid analysis of a large number of samples and comparison of DNA profiles generated from unexposed and treated DNA samples [26]. Previous studies displayed that the RAPD assay is effective in evaluating the preliminary toxic effects on plants [43]. Recently, a large number of studies on genotoxicity caused by NPs in crop plants have been widely reported [32, 44–46]. The appearance of new DNA bands ($n = 6$) could be described as mutations, whereas the absence of normal DNA bands ($n = 2$) is possibly characterized as DNA disintegration or rearrangements of genetic materials [7] caused by iron oxide NP oxidative stress-induced genotoxicity. According to literature, genomic template stability is associated with the changes noticed in the RAPD profile. It was observed that mean GTS values decreased with an increase in Fe_3O_4 NP concentration. As expected, the highest concentration most of all decreased GTS (by ~12%) in rocket seedlings. Previous study also displayed low genetic toxicity induced by the same NPs in flax callus cultures [25]. Moreover, according to literature, exposure to NPs leads to increased reactive oxygen species (ROS) formation, destruction of cell homeostasis, lipid peroxidation, and reduction of mitochondrial function [47, 48]. Overall, the number of disappeared DNA bands was low and it appeared in all seedlings exposed to all tested NP concentrations. The results suggest that the impact of Fe_3O_4 NP-induced stress caused mutations in rocket seedlings, although the observed genotoxicity level of iron oxide NPs in rocket was very low.

Plant miRNAs, small noncoding molecules, have numerous biological functions that may be involved in the regulation of plant growth and development and environmental stress responses. Some plant miRNAs can downregulate the expression of target genes to get through the demands of growth and environmental stress [49]. The quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) technique is widely utilized in molecular biology investigations. This technique requires the selection of control or reference gene or genes that are stably expressed in given species. This allows the direct and sensitive comparison of transcript expression levels in different organisms [50, 51]. Therefore, to quantify the miRNA expression level, the qRT-PCR technique was used. Results indicated that lower concentrations of Fe_3O_4 NPs cause higher expression of miR159c in five-week-old rocket seedlings, which can be beneficial in increasing the miRNA expression level with small amount of NPs. These data are consistent with an independent study, where TiO_2 NPs also slightly increased the miR159 expression level in the same way in three-week-old tobacco seedlings [52]. The obtained results suggest that miR159c is slightly sensitive to Fe_3O_4 NP exposure. According to previous studies, miR159 is important for plant growth and environmental stress response. Unfortunately, data about the miR159 role in rocket is unknown; however, the obtained results and data of independent studies allow us to suggest that Fe_3O_4 NPs can be successfully used to increase plant resistance to biotic stress such as rust response [12].

It has been reported that iron oxide NPs are more toxic to crops than iron NPs [2]. Partly, it can be explained by the release of metal ions after oxidation and dissolution. The release of iron ions also results in the toxicity of iron oxide NPs [53]. Nevertheless, toxicity could be correlated with the type of suspension where NPs are dissolved. As noted previously, light can also induce the oxidation of Fe_3O_4 [53]; nevertheless, this factor can be eliminated in the present experiment, because rocket seedlings were protected from direct light. Moreover, the previous study on flax calluses indicated that large Fe_3O_4 NPs with a size of 25 nm can penetrate into flax callus tissues [25], and therefore it can be assumed that the same NPs also can penetrate in rocket seedling tissues. Furthermore, during the experiment, all seedling growing conditions were the same with the exception of the addition of different concentrations of NPs. This allows us to infer that all changes that occurred upon the experiment were induced by NPs.

On the whole, this data suggests that the studied concentrations of Fe_3O_4 NPs are capable of inducing changes in rocket seedlings grown in hydroponics. It was found that small concentrations of Fe_3O_4 NPs have a positive impact on the seed germination, growth, and development of five-week-old rocket seedlings. An overall significant increase in germination rates, root-shoot lengths, and biomasses was observed in seedlings exposed to 1 mg/L, 2 mg/L, and 4 mg/L of Fe_3O_4 NPs. Simultaneously, NPs induced slight

genotoxicity. This could be due to the induction of reactive oxygen species leading to oxidative DNA damage [48]. NP concentrations such as 1 mg/L and 2 mg/L increased the chlorophyll *a* level in treated rocket leaves. The same results are described in other studies [41]. However, the concentration 4 mg/L decreased the chlorophyll *a* fluorescence level. This could be described by the high toxicity of such concentration also for five-week-old rocket seedlings. Moreover, the miRNA expression level in rocket seedlings was also changed as a result of exposure to Fe_3O_4 NPs. Slightly increasing the miR159c expression may be tested in the future as an appropriate method to regulate the plant's ability to withstand environmental stresses.

More investigations are required to be executed to clarify the mechanisms of Fe_3O_4 NP toxicity and to assess if toxicity is caused by dissociation of the iron or iron oxide ion.

5. Conclusions

To our knowledge, this is the first report on the evaluation of magnetite effects on rocket seedlings grown in hydroponics with NPs. The results presented in the current multidisciplinary work indicate that even concentrations of Fe_3O_4 NPs such as 1 mg/L, 2 mg/L, and 4 mg/L can affect seed germination, root-shoot elongation, chlorophyll *a* level, genome stability, and miRNA expression. The obtained results allow us to conclude that the studied concentrations of Fe_3O_4 NPs may positively affect the yield and quality of rocket seedlings and probably these NPs could improve the ability of plants to stand against environmental stresses. The presented study might be useful to better understand the mechanisms of iron oxide NP effects on rocket and other crop plants as well as the impact of miRNAs on plant resistance against exogenous stress. Nevertheless, future investigations are required to clarify biochemical mechanisms of nanoparticle impact on plant genotoxicity, elongation, chlorophyll, and miRNA expression as well as identify the role and mechanisms of miR159 in mediating rocket biotic or abiotic stress responses and to test the possibility of regulating plant resistance using nanoparticles.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no conflict of interest.

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