Impact of iron oxide nanoparticles on yellow medick (Medicago falcata L.) plants

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ABSTRACT
A large number of studies have explored the effects of various nanoparticles (NPs) on different economically important plant species. In this study, yellow medick plants were grown for five weeks using hydroponics with the addition of Fe3O4 NPs at 1, 2, and 4 mg/L. Plant morphology, chlorophyll a, genotoxicity and expression of miR159c, one of the most important plant miRNA that is involved in plant response to fungal infections, were investigated. The results indicated that Fe3O4 NPs significantly increased plant root length (9%–32%), chlorophyll a fluorescence (1.94–2.85-fold), miRNA expression (0.31–0.42-fold), induced genotoxicity and reduced genome stability (12.5%–13.3%), compared to those of the control. The study demonstrated that Fe3O4 NPs simultaneously induce genome instability in yellow medick and increase expression of miR159c. Therefore, Fe3O4 NPs can be used to increase plant resistance to fungal diseases, such as powdery mildew.

1. Introduction
The growing population and subsequent increasing food requirements are important problems globally due to the resulting deterioration of the environment and decreasing crop productivity (Miao et al. 2015). Plants are incapable of changing location to avoid environmental changes; therefore, various abiotic stresses may influence plant growth, development, and productivity (Qu et al. 2016). These stresses can alter the morphological, physiological, molecular, and biochemical responses in plants (Song et al. 2012; Gehan et al. 2015). This occurs because, under abiotic stress conditions, plant metabolism is disturbed which leads to the reorganization of the metabolic network to retain its most important processes and allow it to acclimatize in a changed environment (Obata and Fernie 2012). Investigations have shown that hundreds of plant genes are regulated differently in response to abiotic stresses which are related to transcription factors, epigenetic modifications, plant hormones, noncoding RNAs, etc. (Wang et al. 2003; Miao et al. 2015; Laloum et al. 2018).

MicroRNAs (miRNAs), a class of endogenous single-stranded small noncoding RNAs, are found in all eukaryotic organisms (Li et al. 2016). They play a crucial role in the post-transcriptional regulation of gene expression by binding to complementary sites in their target mRNAs (Desvignes et al. 2015; Zhang et al. 2018). MiRNAs regulate all biological processes in both plants and animals, including responses to abiotic stresses (Barciszewska-Pacak et al. 2015; Budak and Akpinar 2015). Therefore, this field of science has been actively studied (Okamura et al. 2013; Liu et al. 2015; Gismondi et al. 2017; Kashani et al. 2019). MiR159 is an attractive miRNA with its role in several plant response mechanisms such as drought, hypoxia, fungal infection and nanoparticle (NP) stress (Kantar et al. 2011; Khraiwesh et al. 2012; Plaksenkova et al. 2019).

Also known as yellow medick, lucerna, or alfalfa, Medicago falcata L. (M. falcata) is a perennial leguminous plant grown and cultivated worldwide (Liu et al. 2015). M. falcata is an economically and ecologically important legume herbage. As a legume, M. falcata is capable of fixing atmospheric nitrogen through symbiotic relations with rhizobia (Graham and Vance 2003; Miao et al. 2015). This ability together with having multiple ploidy levels promotes a strong tolerance against several abiotic stressors such as drought, cold, soil infertility, salt, and heavy metals (Zhang et al. 2011; Liu et al. 2015). Moreover, as yellow medick belonging to the genus Medicago has the ability to remove heavy metals from soil through the hyperaccumulation of heavy metals in its tissue, it can grow well in polluted soil (Amer et al. 2013; Panchenko et al. 2017).

Nanoparticles have unique properties and, therefore, inorganic NPs can be extensively used in industrial, agricultural, and consumer products, and medical applications (Schaumann et al. 2015; McGillicuddy et al. 2017). This results in direct or indirect emission of NPs into the environment. Indirect emissions are possible via wastewater treatment plants, the application of biosolids, etc. (Bundschuh et al. 2018). The largest emission of NPs was estimated globally to be in soil and landfills followed by aquatic environments and the air (Keller et al. 2013). The presence of NPs in the environment raises question about environmental contamination by NPs and the risk of adversely effecting communities, ecosystems, and ecosystem functions (Bundschuh et al. 2018). Scientists around the world have investigated effects of various types of NPs on the environment, but the results are still not conclusive regarding the comparative usefulness and harm of NPs.

The aim of this study was to investigate Fe3O4 NP effects on M. falcata L., particularly its seedling morphology, chlorophyll fluorescence, genotoxicity, and miRNA.
2. Materials and methods

2.1. NPs used

A sample of 25 nm of Fe₃O₄ NPs was kindly provided by G. Libert’s Center of Innovative Microscopy, Daugavpils University.

2.2. Plant materials and growth conditions

Medicago falcata seeds were purchased. The seeds were rinsed with tap water and transferred to Petri plates at 24°C and kept in the dark for 2 d. The seedlings were transferred to a hydroponic solution with ½ MS under a 16/8 h day/night photo-period at 23°C. For the treatment of NP stress, two-week-old seedlings were transferred to an aqueous hydroponic solution supplemented with 1, 2, or 4 mg/L of Fe₃O₄ NPs for five weeks. The plants were watered daily and fertilized with fresh ½ MS solution every day. For the analysis of plant morphology, chlorophyll a content, determination of genotoxicity and evaluation of miRNA level, fresh leaves were sampled.

2.3. Measurement of biomass, shoot and root length

The heights of the shoots and roots were measured, and then the medick plants were washed several times with flowing tap and deionized water. Next, the plants fresh weight was measured, and a fresh sample of leaves were used for chlorophyll a fluorescence detection.

2.4. Measurement of chlorophyll a fluorescence

A confocal laser scanning microscope (Nikon Eclipse Ti-E) was used for chlorophyll a fluorescence microscopy measurement. Fluorescence was excited at a 488 nm laser wavelength, and NP emission was detected using a spectral detector (523–743 nm) and analyzed at wavelengths 653.03–661.04 nm. All confocal system parameters were similar (laser: 19.8, HV: 175, and pinhole: 107.6) with the exception of focus which was customized for each leaf sample. Chlorophyll fluorescence was detected using NIS Elements (Nikon, Japan) microscope imaging software.

2.5. DNA isolation and randomly amplified polymorphic analysis

The genotoxic effects induced by Fe₃O₄ NPs were assessed using the randomly amplified polymorphic DNA (RAPD) technique. Genomic DNA was extracted from 200 samples of fresh rucoila leaves (50 in each group). Extraction was done with slight modifications using the purification of total DNA from the plant tissue Mini Protocol (DNeasy Plant Mini Kit, Qiagen GmbH, Hilden, Germany). The quantity and quality of genomic DNA were assessed using a spectrophotometer (NanoDrop 1000, Thermo Scientific, Waltham, USA).

A total of 10 decamer primers, OPA-02, OPA-03, OPA-05, OPA-07, OPA-10, OPA-11, OPN-15, OPD-18, OPV-07, and CB-21, were selected for the RAPD analysis. The RAPD PCR program was set to initially denature at 94°C for 3 min, followed by a 35-cycle denaturation step at 94°C for 1 min, an annealing step at 37°C for 1 min 30 s, an extension of products at 72°C for 2 min and the final extension set at 72°C for 10 min using the Veriti 96-Well Thermal Cycler (Applied Biosystems, USA). 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)). QX Size Marker 100 bp–2.5 kb and QX Alignment Marker 15 bp/3 kb (Qiagen, Germany) were used to determine DNA fragment sizes. RAPD fragments were scored based on the presence or absence of band products for all tested primers. The whole RAPD-PCR procedure was repeated twice as quality control.

2.6. Detection of genotoxicity by estimation of genomic template stability

Genomic template stability (GTS) was calculated for each primer using the equation as reported by Salarizadeh and Kavoussi (2015):

\[ \text{GTS} = \left(1 - \frac{a}{n}\right) \times 100\% \]

where \( a \) is the average number of polymorphic bands found in each treated group and \( n \) is the number of total bands in the control samples. The polymorphic bands observed in the RAPD analysis were defined as the gain or loss of bands in comparison to those of the control profile.

2.7. Expression validation of microRNA using real-time qPCR

Small RNAs were extracted from the leaves of the five-week-old plants using a miRNeasy plant mini kit (Qiagen, Germany), and first-strand cDNA was synthesized with a miRCURY LNA RT Kit (Qiagen, Germany). A miRCURY SYBR Green polymerase chain reaction (PCR) Kit (Qiagen, Germany) was used to perform a miRNA qRT-PCR analysis according to the manufacturer’s protocol for quantitative, real-time PCR using individual miRCURY LNA and miRNA PCR assays. The spike-in UniSp6 RNA was used as an internal control. Mature miRNA specific PCR forward primers (sense DNA oligoidentical to the entire mature miRNA sequence) and the miRNA target-specific PCR primer lus-miR159c with locked nucleic acid were designed according to the miRNA sequence (Plakenskova et al. 2019). 1-alpha (EF1α) was used as a reference gene for miRNAs expression data normalization (Cavaiuolo et al. 2017). The relative expression of the miRNAs in differently treated samples compared to that of the controls was calculated using the 2^{−ΔΔCt} method (Livak and Schmittgen 2001).

2.8. Statistical analysis

The experiment had a completely randomized design with three replications. A one-way analysis of variance (ANOVA) was conducted to test differences in plant morphology. Tukey’s Multiple Comparison test was used at 0.05 p level to compare the means. The student t-test was used for the RAPD analysis. P values less than 0.05 were considered to be statistically significant.
3. Results and discussion

3.1. Measurements of biomass, shoot, and root length

The results showed that different Fe3O4 NPs concentrations influenced plant morphology. All tested NP concentrations affected both the sample length and number of leaves. NPs increased the yellow medick root length, with higher NP concentrations increasing root length. Furthermore, 2 and 4 mg/L NP concentrations increased root length significantly from 5 ± 0.74 cm (control) to 6.27 ± 1.51 cm (25%) and 6.6 ± 1.6 cm (32%), respectively (Table 1). There were no significant differences between plant shoot length in control samples and treated plants. Length varied from 3.25 cm for the control to 3.35 cm for the samples treated with 4 mg/L of NPs. The total sample length was most affected by the 4 mg/L NP concentration, and this concentration significantly affected the total length (9.95 ± 1.09 cm) in comparison to the control (8.25 ± 0.79 cm). The number of leaves was significantly affected by all tested NP concentrations. The control plant samples had approximately seven leaves per sample, while the plant samples grown with different NP concentrations ranged from 10 leaves (4 mg/L) to 11 leaves (1 mg/L). The weight of the plants was most affected by a NP concentration of 2 mg/L.

These data are consistent with a recent study where the same NP concentrations were used to investigate the impact on rocket Eruca sativa morphology. The results were the same, and the length of the shoot and, root increased as the concentration increased. Nevertheless, the number of leaves were similar in all tested groups (Plaksenkova et al. 2019). Similarly, Elfeky et al. (2013) presented results where iron oxide NPs were used as a growth factor for Ocimum basilicum. The results showed that increasing concentrations of Fe3O4 NPs (1, 2 and 3 mg/L) also increased the total plant mass, root length, number of leaves, and weight. An enhancement in root elongation was also observed in ryegrass and pumpkin plants (Wang et al. 2011). Similar data were collected by Zahra et al. (2015) where shoot and root elongation increased after exposure to Fe3O4 NPs. However, Lee et al. (2010) declared the inhibition of root elongation in Arabidopsis under 400, 2000 and 4000 mg/L of Fe3O4 NPs (<50 nm). Also, Mushaq (2011) observed the same situation in cucumbers. A study was conducted by Ochoa et al. (2017) where the addition of CuO NPs into soil were used as a growth factor for Pisum sativum L. plants. Plants were grown for 45 d with 50 mg/kg of soil and 100 mg/kg of NPs in the soil. As a result, both concentrations of NPs also increased the stem length, root length and number of leaves. Moreover, Salama et al. (2019) showed that different concentrations (10, 20, 30 and 40 ppm) of zinc oxide NPs also affect dry bean (Phaseolus vulgaris) morphology. These NPs increased the bean shoot length, root length, and number of leaves. Results from similar research studies suggest that various concentrations of Fe3O4 and CuO NPs stimulate growth in plants of the Fabaceae family.

3.2. Measurement of chlorophyll a fluorescence

Iron oxide NPs increased the chlorophyll a level in all treated yellow medick plants. The mean value of chlorophyll fluorescence increased from 404.52 nm in the control samples to 784.26 nm (1.94-fold), 840.26 nm (2.08-fold), and 1134.15 nm (2.8-fold), for NP concentrations of 1, 2 mg/L, and 4 mg/L, respectively (Figure 1). All tested concentrations significantly affected the medick plants: 1 mg/L at the P < 0.05 level, 2 mg/L at the P < 0.01 level, and 4 mg/L at the P < 0.01 level.

For the same experiment with Eruca sativa, the NPs increased the chlorophyll a level compared to that of the control group; however, an increasing NP concentration decreased the amount of chlorophyll a (Plaksenkova et al. 2019). Tombuloglu et al. (2019) declared that concentrations 125, 250, 500, and 1000 mg/L of Fe3O4 NPs (~13 nm) increased chlorophyll a and b levels in barley through the dramatic upregulation of photosystem marker genes. Therefore, the increased level of chlorophyll a promoted the growth of the M. falkata. However, according to Sharma and Uttam (2017), copper oxide NPs also affect the wavelength of absorption of chlorophyll a in wheat plants. In the case of increasing copper oxide NP concentrations, the fluorescence level decreases.

3.3. RAPD analysis

The RAPD technique has been effectively utilized to detect genotoxic effects in several plants induced by various NPs (Remédios et al. 2012; Mattiello et al. 2015; Ghosh et al. 2019). This sensitive method is capable of detecting variations in plant genome profiles (Salama et al. 2019).

Ten decamer primers were used to study the genotoxic effects of Fe3O4 NPs for both control and NP treated samples. All utilized primers generated a stable RAPD banding pattern. The genomic changes were noted as (a) appeared or (b) disappeared bands in treated plant DNA as compared to those of the control. The appearance of new patterns can be explained by changes in genomic DNA template stability due to mutations, large deletions, homologous recombinations, or changes in priming sites leading to new annealing events (AlQuraidi et al. 2019). Nevertheless, the absence of normal DNA bands can be characterized as DNA disintegration or rearrangement (Venkatachalam et al. 2017). Band changes were detected in all experimental groups using the primers (Table 2). The number of total bands varied from 7 (OPA-07) to 47 (OPA-11). The largest number of polymorphic bands (n = 29) showed OPA-11 in plants treated with 4 mg/L of NPs. Nevertheless, the lowest number of polymorphic bands (n = 1) showed OPA-07 in plants treated with 1 mg/L of NPs. Overall, 79 new bands appeared in treated plants for NP concentrations of 1 and 2 mg/L and 67 new bands in plants treated with 4 mg/L of NPs. However, 72
(1 mg/L), 71 (2 mg/L) and 74 (4 mg/L) disappeared bands were detected compared to the case with the control samples. The example of electropherogram is presented in Figure 2.

Overall, the RAPD results demonstrated that Fe$_3$O$_4$ NPs significantly changed the genome of the yellow medick plants. Additionally, the GTS for all treated plants was calculated (Figure 3). The GTS for untreated (control) seedlings were fixed as 100%. There was a significant ($P < 0.01$) decrease in the GTS of all treated plants; however, genome stability increased by 13.3% in plants treated with 1 mg/L of NPs and by 12.5% in plants exposed to 4 mg/L of NPs. This indicates that the largest genome changes were induced by the lowest NP concentrations. It can be concluded from the results that the Fe$_3$O$_4$ NPs significantly reduce the stability of the yellow alfalfa genome.

Compared to the same study with *Eruca sativa* with the same concentrations of Fe$_3$O$_4$ NPs, the GTS decreased with an increase in the concentration of NPs from 93.9% (1 mg/L) to 87.9% (4 mg/L) (Plaksenkova et al. 2019). The present experiment showed that these NPs have a stronger effect on yellow medick compared to that on *Eruca sativa*. In contrast, barley with concentrations of 125, 250, 500, and 1000 mg/L of Fe$_3$O$_4$ NPs (~13 nm) did not show any toxic effects (Tombuloglu et al. 2019). Similarly, Wang et al. (2011) demonstrated that 25 nm large Fe$_3$O$_4$ NPs significantly induce oxidative stress in ryegrass and pumpkin plants grown in hydroponics. A previous study revealed that 0.5, 1, and 1.5 mg/L of Fe$_3$O$_4$ NPs (25 nm) induced genotoxicity in a flax callus culture. Nanoparticle genotoxicity in plants could be caused by direct NPs intercalation in DNA strand or indirect by releasing free ions which can cause DNA damage (Kruszewski et al. 2011). For example, Cu NPs (Zhang et al. 2017) or Au NPs (Baetsen-Young et al. 2018) can intercalate into the DNA strand. It is not yet known whether Fe$_3$O$_4$ NPs are able to intercalating between DNS strands, but we suppose that released Fe$^{2+}$ or Fe$^{3+}$ ions (Mahdavi et al. 2013) could directly affect DNA.

Data from RAPD analysis suggest that *M. falcata* is sensitive to the effects of Fe$_3$O$_4$ NPs based on the number of band alterations in the NP samples compared with the control.

### Table 2. Results of RAPD analysis: the primers used, number of polymorphic bands in plants treated with 1, 2 mg/L and 4 mg/L of Fe$_3$O$_4$ NPs, total number of bands for each primer and average number of polymorphic bands for every plant group.

| Primer ID | Primer sequences (5'-3') | Length (bp) | Number of polymorphic bands of 1 mg/L | Number of polymorphic bands of 2 mg/L | Number of polymorphic bands of 4 mg/L | Total number of bands |
|-----------|-------------------------|-------------|---------------------------------------|---------------------------------------|---------------------------------------|-----------------------|
| OPA-02    | TGCCGAGCTG               | 10          | 10                                    | 7                                     | 5                                     | 6                     |
| OPA-03    | GTCCGAGCCAC              | 10          | 10                                    | 5                                     | 8                                     | 6                     |
| OPA-05    | AGGCGCTTGT               | 10          | 10                                    | 7                                     | 7                                     | 1                     |
| OPA-07    | GAAACGCGGTG              | 10          | 10                                    | 1                                     | 4                                     | 1                     |
| OPA-10    | GTGATCGCAG               | 10          | 10                                    | 5                                     | 5                                     | 1                     |
| OPA-11    | CAATCGCCGT               | 10          | 10                                    | 7                                     | 1                                     | 6                     |
| OPA-15    | TAGCGACGTG               | 10          | 10                                    | 6                                     | 6                                     | 1                     |
| OPA-18    | GGAGCCCAAC               | 10          | 10                                    | 15                                    | 15                                    | 11                    |
| OPV-07    | GAAGCCAGCG               | 10          | 10                                    | 12                                    | 12                                    | 12                    |
| CB-21     | CAGACTGAC                | 10          | 10                                    | 14                                    | 11                                    | 15                    |

Note: $a$ – new bands; $b$ – disappeared bands.
3.4. microRNA analysis

The Rotor Gene Q Series Software was used to analysis the miR159c expression level. Each sample group had different results. According to the Gurjar et al. (2016), miRNA expression can be measured by the logarithmic formula Log2(treatment/control).

The results showed that all NP concentrations used in the study slightly increased the specific amount of miRNA in M. falcata L. plants. With an increase in NP concentration, the miRNA expression level also increased: 0.31-fold at 1 mg/L of NPs, 0.36-fold at 2 mg/L of NPs and 0.42-fold at 4 mg/L of NPs (Figure 4). This indicates that 25 nm Fe3O4 NPs can increase the expression of miR159c after five weeks of exposure. Interestingly, data from the same study conducted on rockets showed diverse results, where increasing NP concentrations decreased the miR159c expression level (Plaksenkova et al. 2019). This suggests that miR159c in different plant species responded to Fe3O4 treatment in different ways. One potential reason is that plant species may respond differently to abiotic stress, including with miRNAs. According to previous studies, miR159 is important not only for plant growth but also for environmental stress responses, including that of fungal infection such as powdery mildew in wheat (Khraiwesh et al. 2012; Venkat Rajam 2012). Unfortunately, data on the miR159 role in yellow medick is unknown; however, the obtained results and data of independent studies suggest that this approach may enhance some plants’ resistance to serious fungal diseases, such as powdery mildew.

The findings of this study demonstrate that the exposure of M. falcata plants grown on hydroponics to 25 nm Fe3O4 NPs affects their morphology, chlorophyll a level, specific miRNA expression level, and induces genotoxic effects. The only difference between the treated and control plants was the presence or absence of Fe3O4 NPs, which supports the theory that the changes in plants were caused by this effect of the NPs. Furthermore, Zhu et al. (2008) confirmed that 20 nm Fe3O4 NPs can

Figure 2. Examples of RAPD profile of genomic DNA isolated from Medicago falcata L. plants.

Figure 3. Genome template stability (%) in plants after 5 week exposure with different concentrations of Fe3O4 NPs. The mean is averaged from three replicates and error bars correspond to standard deviation of mean. *Indicates significant difference from control (P < 0.05); **indicates significant difference from control (P < 0.01).
penetrate into pumpkin cells and translocate and accumulate in the plant tissues. Moreover, it has been proven that 25 nm Fe₃O₄ NPs can penetrate flax callus culture cells (Kokina et al. 2017). As our best knowledge, there has not yet been studied the impact of Fe₃O₄ NPs on plant miR159c which is one of the most important plant miRNA that is involved in plant response to fungal pathogen. Obtained results could be used in future to develop new technology for successful increasing of plant resistance against fungal pathogen.

4. Conclusion

According to previous studies, Fe₃O₄ NPs positively enhance rocket, basil, ryegrass, and pumpkin root elongation. Many investigations also confirmed an increase in chlorophyll a level in several plants after being exposed to different sizes of Fe₃O₄ NPs. Similarly, this study demonstrated a significant enhancement of the chlorophyll a level. The NPs did not frequently induce genotoxic effects in plants, but the RAPD analysis of this study confirmed the genotoxic effect of Fe₃O₄ NPs which induced genomic DNA modifications in M. falcata. One important finding of this study was the increase in miR159c expression which may indicate that these NPs can be used for increasing plant resistance against fungal pathogens.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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