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Application of nanoparticles (ZnO, TiO_2 and CuO), a new opportunity for the stimulation of cell growth and azadirachtin production in cell suspension culture of *Azadirachta indica*

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Abstract

Nanoparticles have unique physicochemical properties and provide great opportunities in plant science studies. In this study, we investigated the impact of ZnO, TiO_2 and CuO nanoparticles (0, 20, 40, 60, and 80 mg/L) and sampling times (2, 4, and 6 days) on cell suspension growth and azadirachtin accumulation and production. Factorial experiments based on a completely randomized design with three replications were used. Results demonstrated that different nanoparticles had a different effect on the studied characters. When ZnO nanoparticles were used, the highest fresh (540.73 g/L), dry cell weight (15.93 g/L), azadirachtin accumulation (5.15 mg/g DW) and production (68.27 mg/L) were obtained at control condition, 80 and 40 mg/L ZnO nanoparticles, and control condition after 6 days, respectively. The highest amount of fresh (526.95 g/L) and dry (17.05 g/L) cell weight and azadirachtin production (82.21 mg/L) and accumulation (5.93 mg/g DW) were observed in 20 mg/L TiO_2 nanoparticles, 40 mg/L of TiO_2 nanoparticles after 2 days, 20 mg/L TiO_2 nanoparticles in 4 days and 60 mg/L of TiO_2 nanoparticles, respectively. With applying CuO nanoparticles, the highest fresh cell weight and azadirachtin accumulation were 422.59 g/L and 4.00 mg/L, achieved in control conditions respectively. Also, the highest amount of azadirachtin production was 68.27 mg/L, observed in control conditions on the 6th day of treatment. It seems that suitable cell growth, except in some cases, occurred in the absence of elicitors, but azadirachtin accumulation and production were stimulated by nanoparticles treatment. However, the results showed that the CuO nanoparticles caused a decrease in overall azadirachtin accumulation and production in the cells.

***Key words*:** Azadirachtin, Elicitor, High performance liquid chromatography, Nanoparticles, Secondary metabolites.

INTRODUCTION

Nanoparticles are materials with<100 nm in diameter and derived from nanomaterials. According to the core material, nanoparticles are divided into organic and inorganic nanoparticles (Rajput *et al.,* 2018b; Giorgetti, 2019). Inorganic nanoparticles are divided into metals such as Zn, Ti and Cu and metal oxides such as ZnO, TiO_2 and CuO (Rajput *et al.,* 2018a). Nanoparticles are used in many areas including medicine, cosmetics, and agriculture (Khan *et al.,* 2017). Studies reported that the highest levels of nanoparticles had a certain degree of phytotoxicity and depending on the size, they can cross the apoplast and plasma membrane via endocytosis and enter the plant cells (Rico *et al.,* 2011). Therefore, nanoparticles can enter the cellular organelles including the nucleus, plastids, and vacuoles (Da Costa and Sharma, 2016). Nanoparticles can affect biochemical, physiological, and molecular levels by changing mineral nutrition and genotoxicity (Rizwan *et al.,* 2017).

The annual production of zinc oxide (ZnO) nanoparticles is between 550 to 33400 tons (Connolly *et al.,* 2016). The environmental levels of this nanoparticle in the soil are 3.1-31 µg/kg and in the water is 76-760 µg/L (Ghosh *et al.,* 2016). ZnO nanoparticles are used in many areas and their toxic effects on plants are not well investigated. Its toxic effects were observed in common onions after exposure to different concentrations (Kumari *et al.,* 2009). ZnO nanoparticles have the physical, optical and antimicrobial properties and improve the cultures. In many studies, the ZnO nanoparticles have been shown to increase plant growth (Raskar and Laware, 2014).

The TiO_2 nanoparticles are used in cosmetics, food, paints and drug-delivery systems (Clément *et al.,* 2013; Pulit-Prociak and Banach, 2016). The global output of TiO_2 nanoparticles was estimated to be between 60000 to 150000 tons in 2014 (Pulit-Prociak and Banach, 2016). TiO_2 nanoparticles have toxicity effects on plants by ROS generation and induction of enzymatic antioxidant defenses (Cox *et al.,* 2016; Yang *et al.,* 2017). In monocot plants such as corn and dicot plants such as narbon bean, the toxicity effects of TiO_2 nanoparticles were observed (Castiglione *et al.,* 2011).

Copper is an important microelement for plants and its low and high amounts in the soils have negative effects on the plant (Bellani *et al.,* 2014). CuO nanoparticles have biocidal activity and are used against biological agents (Ren *et al.,* 2009). CuO nanoparticles had many adverse effects on germination, shoot growth and root elongation, biomass production and changes in photosynthesis and the activity of some enzymes (Adhikari *et al.,* 2012; Rajput *et al.,* 2018a). In radish, perennial ryegrass, and annual ryegrass the oxidative damage of CuO nanoparticles was reported on plant genomic DNA during the seed germination (Giorgetti, 2019).

The *A. indica* has been used in traditional medicine. Different parts of this plant are used for the treatment of diseases such as malaria, infections, and skin diseases. The important limonoid of neem is azadirachtin, used as a pesticide (Gupta *et al.,* 2017; Blum *et al.,* 2019). The present study aimed to look for the impact of ZnO, TiO_2, and CuO nanoparticles on the cell growth parameters and azadirachtin accumulation and production of cell suspension culture of *A. indica* in *in vitro* conditions.

MATERIALS AND METHODS

Plant material, callus induction, and establishment of cell suspension culture

The leaves of the neem tree were obtained from Bandar Abbas, Iran (27°11′41.1″N+56°20′14.0″E) and surface-sterilized based on our previous study. The callus and cell suspension cultures were obtained according to the optimization of callus induction and cell suspension culture in our previous study in solid and liquid MS medium with 1 mg/L picloram, 2 mg/L kinetin, respectively (Farjaminezhad and Garoosi, 2019).

Treatment of cell suspension culture with different concentrations of ZnO, TiO_2 and CuO nanoparticles

The suspension cells were transferred into a 100-mL Erlenmeyer flask containing 25 mL of liquid MS medium supplemented with 1 mg/L picloram and 2 mg/L kinetin with 2.6×10^5 cells per mL initial cell density. According to the growth curves of our previous study (Farjaminezhad and Garoosi, 2019), 10 days after the culture, the different concentrations of ZnO (30-45 nm, US Research Nanomaterials, USA, Figure 1A), TiO_2 (20 nm, US Research Nanomaterials, USA, Figure 1B) and CuO (25-55 nm, US Research Nanomaterials, USA, Figure 1C) nanoparticles (control, 20 mg/L, 40 mg/L, 60 mg/L and 80 mg/L) were added to the media and the sampling was carried out 2, 4 and 6 days after treatment.

Fresh cell weight and dry cell weight

Fresh and dry cell weights were measured at the end of the experiment (Farjaminezhad and Garoosi, 2019). The cells were collected by Whatman No.1 filter paper using Büchner funnel under vacuum and weighed for fresh cell weight determination. Dry cell weight was obtained by oven-drying of the fresh cell at 50 °C for 72 h.

Azadirachtin extraction and analysis by HPLC

Intracellular azadirachtin was extracted (Farjaminezhad and Garoosi, 2019) and the amount of intracellular azadirachtin of samples was obtained using a Knauer HPLC system (UV detector, Germany) with Tosoh C-18 column (TSKgel-ODS C-18, 5µm, 4.6×250 mm, Japan) as a stationary phase. The mobile phase was 10% acetonitrile and 90% water, the flow rate was 0.9 mL/min, and the azadirachtin absorbance was calculated at 214 nm. The overall azadirachtin production of azadirachtin was measured by the Srivastava and Srivastava (2012) following mathematical formula:

(1) Azadirachtin production (mg/L)=biomass (g/L)× azadirachtin accumulation (mg/g DW)

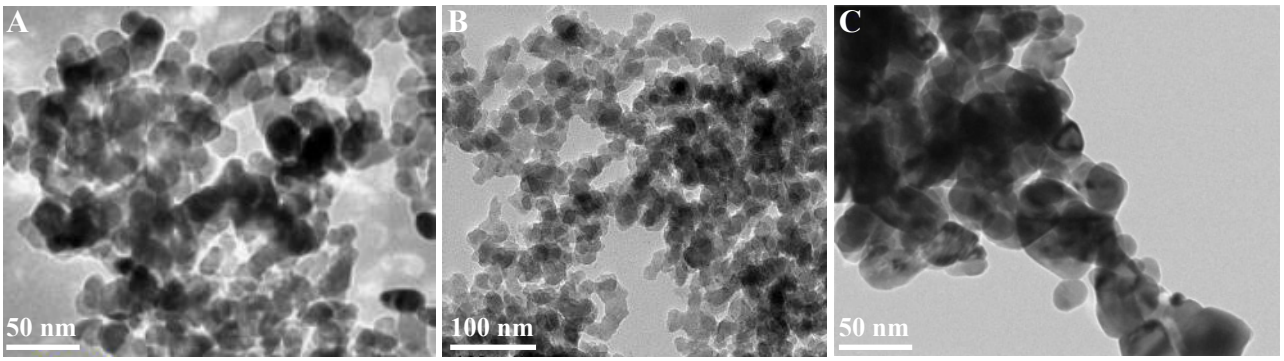

Figure 1. TEM-images of **A:** ZnO, **B:** TiO_2 and **C:** CuO nanoparticles (www.us-nano.com/nanopowders).

Table 1. Analysis of variance of the effect of different concentrations of ZnO nanoparticles and sampling times on neem cell suspension culture.

Source of variation	df	Mean of square			
		Fresh cell weight	Dry cell weight	Azadirachtin accumulation	Azadiractin production
ZnO concentration (ZnO)	4	7031.201^{ns}	8.406*	9.115**	1847.174**
Sampling times (T)	2	42265.431**	4.816^{ns}	3.260**	741.206**
ZnO×T	8	16326.839**	17.274**	3.138**	272.483**
Error	30	4341.208	2.728	0.370	49.386
Coefficient of variation (%)		16.21	12.98	16.19	14.53

^{ns}, * and **: non-significant, significant at 5% and 1% probability.

Statistical analysis

Factorial experiments based on a completely randomized design with three replication was used. For data analysis, the IBM SPSS Statistics 25.0 software was applied. Means comparisons were performed using Duncan's multiple range test.

RESULTS

Effects of different concentrations of ZnO nanoparticle

The variance analysis showed that different levels of ZnO nanoparticles, the sampling times, and their interactions had a significant effect on fresh and dry cell weight and azadirachtin accumulation and production (Table 1). By the addition of 20 mg/L and 40 mg/L ZnO nanoparticles to the cell suspension cultures, the fresh cell weight was increased, but was not different from the control. The amount of fresh weight increased by increasing sampling time and the highest amount of fresh cell weight was obtained 6 days after treatments. The maximum amount of fresh cell weight was 540.73 mg/L, obtained in the control, after 6 days of treatment. This was 1.43, 1.23, 1.25, and 1.01 fold higher than the amounts obtained for 6 days treatment with 20 mg/L, 40 mg/L, 60 mg/L, and 80 mg/L ZnO nanoparticle, respectively (Figure 2A). Application of 40 mg/L and 80 mg/L increased dry cell weight compared to the control, but the addition of 20 mg/L and 60 mg/L ZnO nanoparticles reduced dry cell weight. Between different concentrations of ZnO nanoparticles, 80 mg/L produced 13.53 mg/L dry cells, which was not statistically different to the control and 40 mg/L ZnO nanoparticles. The highest dry cell weight was 15.93 mg/L observed 6 days after treatment with 80 mg/L ZnO nanoparticles. This was 1.01, 1.46, 1.31, and 1.31 fold higher than the amounts obtained for control, 20 mg/L, 40 mg/L, and 60 mg/L, in the same day treatment, respectively (Figure 2B). The addition of 20 mg/L and 40 mg/L ZnO nanoparticles to the cell suspension cultures stimulated azadirachtin accumulation in cells but the use of 60 mg/L and 80 mg/L ZnO nanoparticles had an inhibitory effect on azadirachtin accumulation and caused a reduction in its content. Among different concentrations of ZnO nanoparticles, 40 mg/L had the most stimulatory effect on azadirachtin accumulation with 4.25 mg/g of DW. In terms of sampling time, the accumulation of azadirachtin increased over time. Overall, the highest azadirachtin accumulation (5.15 mg/g DW) was obtained 6 days after treatment with 40 mg/L ZnO

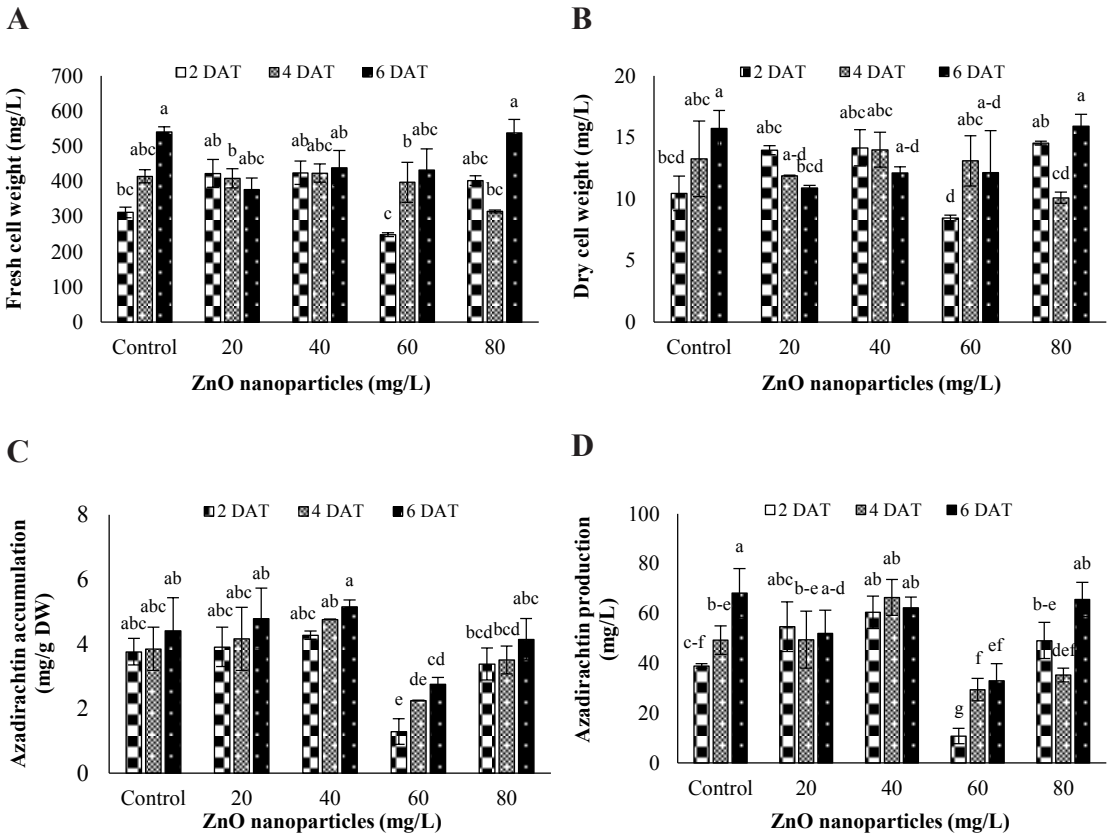

Figure 2. Effect of different concentrations of ZnO nanoparticles and sampling times on neem cell suspension culture. **A:** Fresh cell weight, **B:** dry cell weight, **C:** azadirachtin accumulation, and **D:** azadirachtin production. DAT: Day After Treatment. Different letters indicate a difference in the probability level of 1%.

nanoparticles, which was 1.17, 1.07, 1.87, and 1.24 fold higher than the amounts obtained for control, 20 mg/L, 60 mg/L, and 80 mg/L, in the same day treatment (Figure 2C). The 40 mg/L ZnO nanoparticles increased azadirachtin production but adding 20 mg/L, 60 mg/L, and 80 mg/L reduced its production. Therefore, the higher concentrations of ZnO nanoparticles (60 mg/L and 80 mg/L) had a negative and inhibitory effect on azadirachtin production. As a previous index, the azadirachtin production also increased over time and its maximum amount was observed on the 6th day. The highest amount the azadirachtin production was 68.27 mg/L obtained in the control condition after 6 days. This amount was 1.31, 1.09, 2.07, and 1.04 fold higher than the amounts obtained for 20 mg/L, 40 mg/L, 60 mg/L, and 80 mg/L ZnO nanoparticles treatment, respectively (Figure 2D).

Effects of different concentrations of TiO_2 nanoparticle

The results showed that different concentrations of TiO_2 nanoparticles had a significant effect on fresh cell weight, azadirachtin accumulation, and azadirachtin production. Different sampling time had a significant effect on fresh cell weight and azadirachtin accumulation. Also, the dry cell weight and azadirachtin production were significantly affected by the interactions between different concentrations of TiO_2 nanoparticles and sampling times ($P<0.01$) (Table 2). By the addition of 20 mg/L TiO_2 nanoparticles the fresh cell weight increased 1.25 fold compared to the control, but by adding 40 mg/L, 60 mg/L, and 80 mg/L TiO_2 nanoparticles, the fresh cell weight reduced 1.13, 1.11, and 1.22 fold compared to the control. The results showed that with increasing the TiO_2 nanoparticles concentrations the fresh cell weight decreased and the maximum amount of fresh cell weight (526.95 g/L) was obtained by the application of 20 mg/L TiO_2 nanoparticles (Figure 3A). Investigation on the effect of different sampling times showed that the highest fresh cell weight was observed on the 6th day (458.46 mg/L) (Figure 3B). By applying various concentrations of TiO_2 nanoparticles and taking the different sampling times, the highest dry cell weight, 17.05 g/L, was obtained two days after the addition of 40 mg/L TiO_2 nanoparticles to the media. This was 1.63, 1.11, 1.41, and 1.62 fold higher than the amounts obtained for

Table 2. Analysis of variance of the effect of different concentrations of TiO_2 nanoparticles and sampling times on neem cell suspension culture.

Source of variation	df	Mean of square			
		Fresh cell weight	Dry cell weight	Azadirachtin accumulation	Azadiractin production
TiO_2 concentration (TiO_2)	4	44878.279**	11.101^{ns}	9.694**	2018.869**
Sampling times (T)	2	41059.978**	14.199^{ns}	3.090**	152.722^{ns}
TiO_2×T	8	5484.313^{ns}	18.194**	0.102^{ns}	385.648*
Error	30	2700.870	5.546	0.372	130.089
Coefficient of variation (%)		12.67	18.29	13.18	19.22

^{ns}, * and **: non-significant, significant at 5% and 1% probability.

Table 3. Analysis of variance of the effect of different concentrations of Cu nanoparticles and sampling times on neem cell suspension culture.

Source of variation	df	Mean of square			
		Fresh cell weight	Dry cell weight	Azadirachtin accumulation	Azadiractin production
Cu concentration (Cu)	4	47601.452**	12.505^{ns}	11.835**	2116.521**
Sampling times (T)	2	1622.230^{ns}	25.537^{ns}	2.772**	368.793**
Cu×T	8	17298.431^{ns}	17.982^{ns}	0.105^{ns}	214.117**
Error	30	9644.814	8.843	0.405	52.925
Coefficient of variation (%)		29.20	25.51	21.51	20.24

^{ns}, * and **: non-significant, significant at 5% and 1% probability.

the control, 20 mg/L, 60 mg/L, and 80 mg/L TiO_2 nanoparticles treatments, respectively (Figure 3C). The addition of 20 mg/L, 40 mg/L, and 60 mg/L TiO_2 nanoparticles caused an increase in azadirachtin accumulation in the cells, but using 80 mg/L had an inhibitory effect and reduced the azadirachtin accumulation. Among different concentrations of TiO_2 nanoparticles, applying 60 mg/L had a most stimulatory effect and caused the accumulation of 5.93 mg/g DW azadirachtin in the cells (Figure 3D). In terms of sampling time, the azadirachtin accumulation increased over time and the highest amount of azadirachtin accumulation was 5.11 mg/g DW obtained in the 6 day of treatment (Figure 3E). Application of 20 mg/L, 40 mg/L, and 60 mg/L TiO_2 nanoparticles increased the azadirachtin production, but using 80 mg/L decreased its production. Therefore, the higher concentrations of TiO_2 nanoparticles had a negative effect on azadirachtin production in cell suspension culture of neem. By applying 20 mg/L TiO_2 nanoparticles the azadirachtin production increased 1.42 fold in comparison to the control. Overall, the highest amount of azadirachtin production, 82.21 mg/L, was obtained in the medium containing 20 mg/L TiO_2 nanoparticles, 4 days after treatment. This was 1.67, 1.42, 1.08, and 1.73 fold higher than the amounts obtained for the control, 40 mg/L, 60 mg/L, and 80 mg/L treatments, respectively (Figure 3F).

Effects of different concentrations of CuO nanoparticles

The ANOVA analysis showed that the fresh cell weight and azadirachtin accumulation and production were significantly affected by different concentrations of CuO nanoparticles. Also, the different sampling times had a statistically significant effect on azadirachtin accumulation and production. The interactions of different concentrations of CuO nanoparticle and sampling times had a significant effect on azadirachtin production ($P<0.01$) (Table 3). The different concentrations of CuO nanoparticles and the sampling time had no significant effect on dry cell weight. By the addition of various concentrations of CuO nanoparticles, the fresh cell weight was reduced. With the increase of the CuO nanoparticles concentration, the fresh cell weight also was decreased. Therefore, the CuO nanoparticles had a negative effect on fresh cell weight. The maximum amount of cell growth was 422.59 g/L achieved in the control conditions. By applying 20 mg/L, 40 mg/L, 60 mg/L, and 80 mg/L of the CuO nanoparticles, the fresh cell weight was 1.16, 1.15, 1.45, and 1.78 fold, lower than the control (Figure 4A).

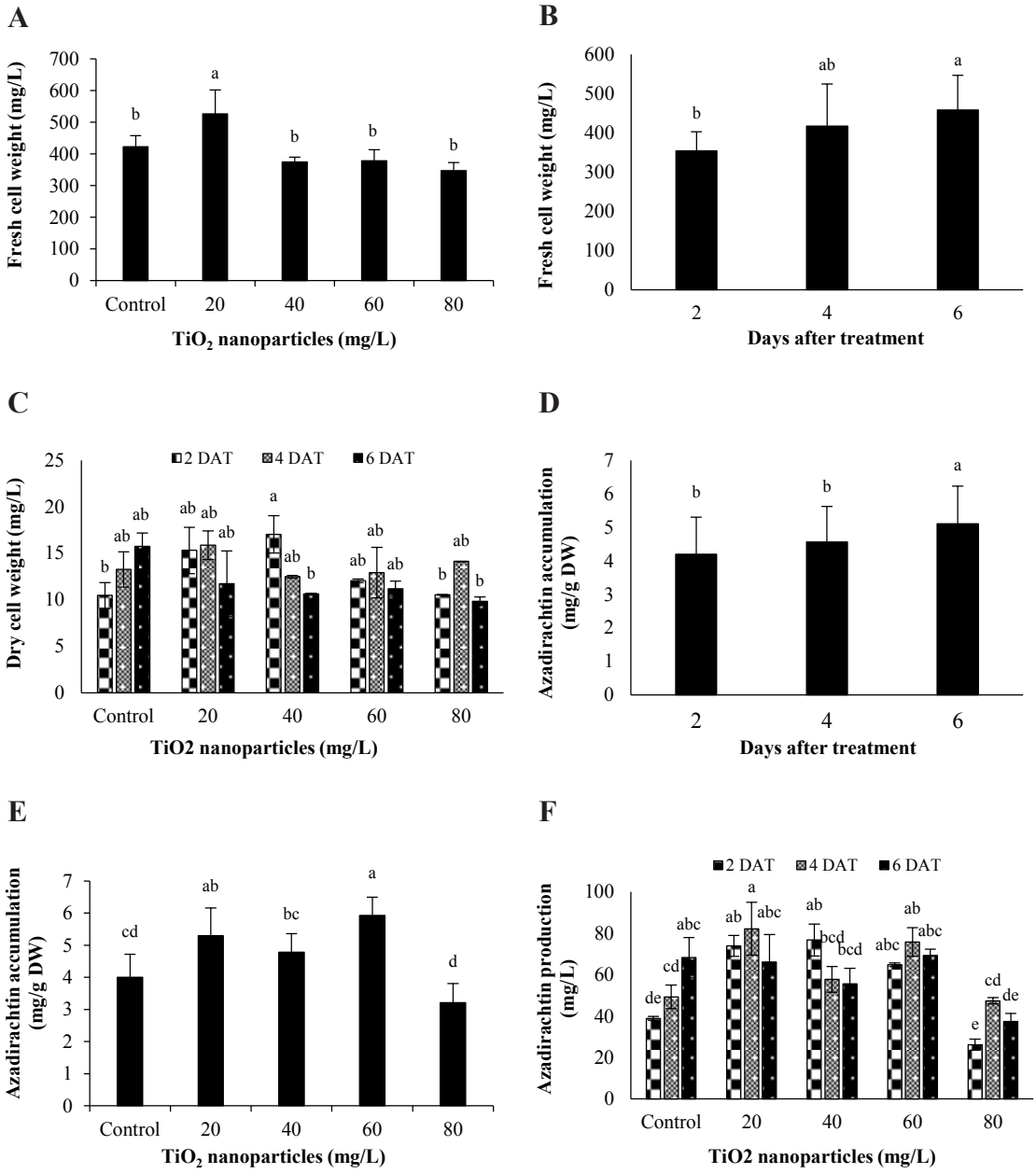

Figure 3. Effect of different concentrations of TiO_2 nanoparticles and sampling times on neem cell suspension culture. **A-B:** Fresh cell weight, **C:** dry cell weight, **D-E:** azadirachtin accumulation and **F:** azadirachtin production. DAT: Days After Treatment. Different letters indicate a difference in the probability level of 1% for Fresh and dry cell weight and azadirachtin accumulation and in the probability level of 5% for azadirachtin production.

The results showed that the CuO nanoparticles decreased the azadirachtin accumulation in the cells. By using the 20 mg/L, 40 mg/L, 60 mg/L, and 80 mg/L of CuO nanoparticles the azadirachtin accumulation decreased 1.23, 1.07, 1.49, and 3.58 fold compared to the control. Therefore, the highest amount of azadirachtin accumulation (4 mg/g DW) was obtained in the control conditions (Figure 4B). Over time, the accumulation of azadirachtin in the cell increased and its highest amount, 3.40 mg/g DW, was obtained in the 6th day (Figure 4C). According to the results, the azadirachtin production was increased by the application of CuO nanoparticles. In the control conditions, the produced azadirachtin, 52.17 mg/L, was obtained and its production increased over time. Therefore, the highest amount of azadirachtin production, 68.27 mg/L, was observed at control conditions after the 6 days, which was 2.35, 1.82., 2.17, and 3.67 fold higher than the amounts obtained for 20 mg/L, 40 mg/L, 60 mg/L, and 80 mg/L CuO nanoparticles treatment, respectively (Figure 4D).

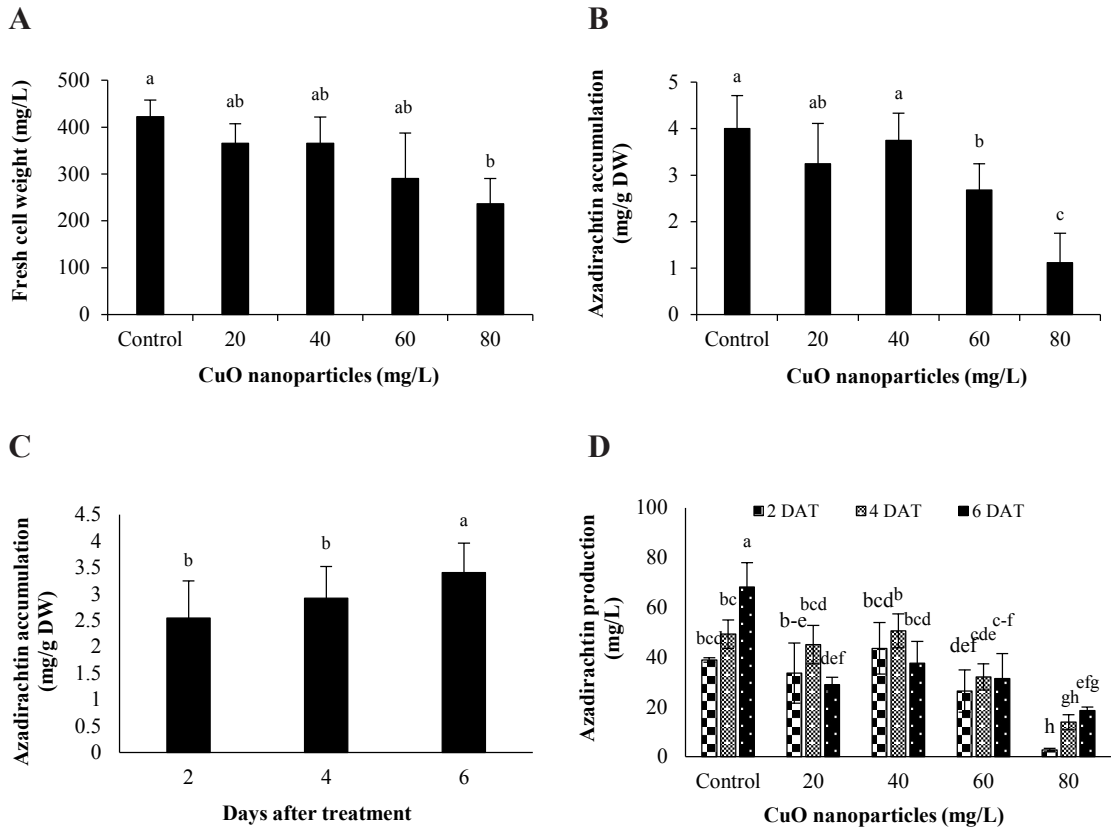

Figure 4. Effect of different concentrations of CuO nanoparticles and sampling times on neem cell suspension culture. **A:** Fresh cell weight, **B-C:** azadirachtin accumulation and **D:** azadirachtin production. DAT: Days After Treatment. Different letters indicate a difference in the probability level of 1%.

DISCUSSION

Nanoparticles have different effects on plant growth, depending on the concentration, size, physical and chemical properties, and nature of the plant (Siddiqi and Husen, 2016; Siddiqi and Husen, 2017). In most studies the external application of nanoparticles has been shown to have negative effects on plant growth (Kumari *et al.,* 2011). It has been reported that the initial responses of the plants to the nanoparticles included increased levels of reactive oxygen species, cytoplasmic calcium, and an increase in the MAPK cascade, which also occurs in other biological stresses. Studies have shown that in the Arabidopsis, nanoparticles are attached to the receptors on the membrane and targeted the calcium explosions and induction of ROS (Sosan *et al.,* 2016). It has been suggested that nanoparticles influence cell metabolism by releasing ions and binding to calcium receptors, calcium channels, and calcium/sodium ATPases (Mirzajani *et al.,* 2014). In this study the maximum amount of fresh and dry cell weight and azadirachtin accumulation and production were obtained in the control conditions after 6 days of treatment, 6 days after treatment with 80 mg/L ZnO nanoparticles, 6 days after treatment with 40 mg/L ZnO nanoparticles and control conditions after 6 days, respectively. Based on other studies, ZnO nanoparticles had different effects on growth and biochemical profiles of *Silybum marianum* and *Eruca sative* (Zaka *et al.,* 2016; Nazir *et al.,* 2018). Nalci *et al.* (2019) reported that 3×ZnO nanoparticles enhanced callus production in the mature embryo culture of wheat. Mahajan *et al.* (2011) found that the optimum concentration of ZnO nanoparticles displays good growth over control in seedlings of mung and gram. Another study found that ZnO nanoparticles induced oxidative stress and inhibited plant growth (Li *et al.,* 2016). For example, a study on the impacts of ZnO nanoparticles at 10-2000 mg/L on buckwheat showed that this nanoparticle caused the reduction of biomass, damage to the root surface, and induction of abnormal defense systems against the ROS (Lee *et al.,* 2013). Ghosh *et al.* (2016) reported that use of ZnO nanoparticles caused a reduction in the membrane stability, increases in chromosomal deviations, formation of small nuclei, and the breakage of the DNA. In sesame seedlings, the addition of various

concentrations of ZnO nanoparticles affected the root and shoot growth and fresh and dry cell weight. The lower concentrations of ZnO nanoparticles increased the fresh and dry weight, while its high concentrations reduced biomass (Narendhran *et al.,* 2016). In this study, the addition of 40 mg/L ZnO nanoparticles to the medium increased azadirachtin accumulation. This increase may be related to the oxidative stress, lipid peroxidation, and activity of the catalase enzyme (Marslin *et al.,* 2017). In some studies it has been reported that the use of nanoparticles affects plants secondary metabolism. For example, treatment of hairy roots of sweet wormwood with 900 mg/L Ag-SiO_2 nanoparticle increased the amount of artemisinin production (Zhang *et al.,* 2013).

Titanium acts as a photo-catalyst and used in the production of pigments (Sang *et al.,* 2014). Titanium induces the carbohydrate synthesis and helps the growth and photosynthesis of plants (Chen *et al.,* 2014). In this study, the highest amount of the fresh and dry cell weight and azadirachtin accumulation and production were observed by adding 20 mg/L TiO_2 nanoparticles, 2 days after addition of 40 mg/L TiO_2 nanoparticles, 60 mg/L TiO_2 nanoparticles, and 4 days after adding 20 mg/L TiO_2 nanoparticles. Similar to other nanoparticles, TiO_2 nanoparticles have also different effects on plants. Recently, Silva *et al.* (2017) demonstrated that in wheat, prolonged exposure to different levels of TiO_2 nanoparticles induced toxic effects and reduced the plant growth. TiO_2 nanoparticles control the activity of glutamate dehydrogenase, nitrate reductase, glutamine synthase and glutamic-pyruvic transaminase enzymes that are involved in nitrogen metabolism. These enzymes help the plants to absorb nitrate and synthesize chlorophyll, and raise the fresh and dry weights (Mishra *et al.,* 2014). TiO_2 nanoparticles increased plant growth of wheat and increased almost all agronomic traits including gluten and starch components under water stress condition (Jaberzadeh *et al.,* 2013).

Copper is available in two forms including Cu^{1+} and Cu^{2+}. Copper has a reducing or oxidizing activity and involves in free radicals production and oxidative stress (Rajput *et al.,* 2018a). In this study, by applying CuO nanoparticles the highest fresh cell weight and azadirachtin accumulation and production were obtained in control conditions. Therefore, CuO nanoparticle had a toxic effect on neem cell suspension culture. The genetic study on Arabidopsis showed that lower concentrations of CuO nanoparticles do not significantly affect the expression of oxidative stress- related genes, sulfur assimilation, glutathione, and proline biosynthesis (Nair and Chung, 2014). Recent studies showed that, the CuO nanoparticles had negative effects on wheat (Dimkpa *et al.,* 2012), cucumber (Moon *et al.,* 2014), rice (Peng *et al.,* 2015), onion (Deng *et al.,* 2016), black mustard (Zafar *et al.,* 2017), tomato and wild cabbage (Singh *et al.,* 2017).

CONCLUSION

Nanoparticles as a part of human life are widely used. According to the literature, excess levels of nanoparticles are harmful to plants, but in a low amount, they can be suitable for plants. The present study showed the significant effects of ZnO, TiO_2 and CuO nanoparticles on cell growth and azadirachtin production in *A. indica*. It was found that moderate concentrations of ZnO and lower concentrations of TiO_2 nanoparticles can improve cell growth and azadirachtin production, but CuO nanoparticles had toxic effects and reduced cell growth and azadirachtin production in cell suspension culture of *A. indica*.

According to these results, it is suggested that the concentrations of lower than 20 mg/L nanoparticles can be studied for azadirachtin and other metabolites synthesized upstream of the azadirachtin synthesis pathway, such as mevalonic acid and squalene, as well as the production of enzymes encoded by the genes involved in azadirachtin synthesis pathway, such as squalene synthase and squalene epoxidase.

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