



BIOLOGICAL EFFECTS OF ZINC OXIDE NANOPARTICLES ON *IN VITRO* ASPARAGUS OFFICINALIS L. PLANT

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Abstract

This study was carried out at the Experimental Field and Plant Tissue Culture Laboratory, Botany Department, Faculty of Agriculture, Cairo University, Giza, Egypt, as well as the Plant Biotechnology Department, National Research Center, Dokki, Giza from the year 2017 to 2019, to evaluate the effect of Zinc Oxide nanoparticles (ZnO NPs) at different concentrations (0.0, 0.5, 1, 3, 5, 10, 30, 50 and 100 ppm) on callus biomass, cell viability (%) and flavonoids content in callus and suspension cultures of Asparagus plant. The results clarified that the treatment 10 ppm of ZnO NPs had the greatest callus fresh weight (8.01g) with a significant increase percentage of 352.5% compared with control plants (ZnO NPs-free). The best cell viability percentage was recorded at 10 ppm of ZnO NPs concentration on the 9th day, and 5 ppm on the 15th day with the same value (100%). In callus culture, using ZnO NPs decreased the callus content of Rutin and total flavonoids compared with their ZnO NPs-free counterparts. In suspension culture, the best flavonoid detected was Rutin compound (37.78 mg/100g) under 10 ppm of ZnO NPs concentration with the third rank of total flavonoids content (94.59 mg/100g). On the contrary, the treatment 5 ppm of ZnO NPs recorded the lowest content of Rutin compound (4.60 mg/100g), but it ranked second in total flavonoids content (161.64 mg/100g). On the other hand, the concentration 50 ppm of ZnO NPs ranked first in total flavonoids (291.48 mg/100g) with the third grade of Rutin compound content (27.28 mg/100g). Rutin compound content in ZnO NPs-free treatment was the last in ranking of total flavonoids (20.40 mg/100g).

Keywords: Asparagus, ZincOxide nanoparticles, callus, suspension culture, cell viability, flavonoids.

Introduction

Asparagus (*Asparagus officinalis* L.) is one of the promising horticultural crops in Egypt (Hassan, 2001). Asparagus is a rhizomatous perennial herb reproduces by rhizomes and seeds but germination rate is very low (Huang *et al.*, 2008). *Asparagus* is an important monocot medicinal plant (Mashitha *et al.*, 2011). MS medium supplemented with various combinations of growth regulators lead to biomass accumulation which correlated with saponin and other secondary metabolites. Production of secondary metabolites in cell suspension cultures has been reported from various medicinal plants for commercial scale (Hussain *et al.*, 2012). Rutin was the most important detected flavonoid in Asparagus plant, which represented 55-98% of the total flavonoids (Fuentes-Alventosa *et al.*, 2007). Zinc Oxide nanoparticles have an obvious effect on growth parameters and secondary metabolites accumulation such as flavonoids and phenols as antioxidants, and have medicinal, industrial and antimicrobial effect (Mierziak *et al.*, 2014 and; Javed *et al.*, 2016). MS medium at a lower concentration of ZnO NPs caused an increase in non-enzymatic antioxidative molecules (Zaraf *et al.*, 2016). Zinc Oxide nanoparticles have a significant effect on germination percentage, root length, shoot length and seedling length.

Germination percentage increased at high concentrations (Afrayem and Chaurasia, 2017). The aim of this study was to investigate the effect of ZnO NPs on callus biomass, cell viability (%) and flavonoids content in callus and suspension cultures of *Asparagus officinalis* L. plant.

Materials and Methods

Zinc Oxide nanoparticles preparation

Zinc acetate (ZnAc₂, 2H₂O, General purpose Reagent, minimum 98.5%) was used. The necessary quantities were firstly dissolved in 100 ml of ultra pure water, and then 20 ml of aqueous 1M NaOH solution was added under flow control and slow magnetic stirring at a temperature of 50°C. The reaction mixture was maintained at this temperature for 1 hour and then cooled to room temperature. The precipitate was at once filtered by centrifugation, then washed with ultra pure water and subsequently dried by lyophilization by freezing in ice. In contrast to Lee *et al.* (2014) and other authors, our procedure has the advantage to avoid ripening and annealing treatment. The size and shape of ZnO NPs were described using electron microscopy. (Zouihri *et al.*, 2011), after preparation of Negative Electron Microscopy Stains Protocol (Aebi and Pollard, 1987).

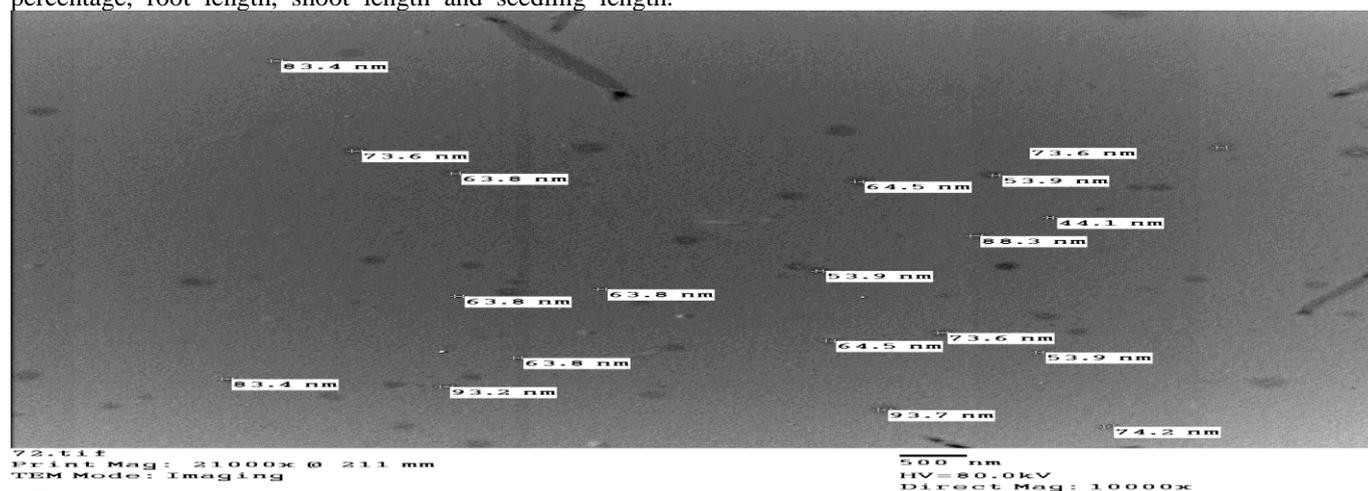


Fig. 1 : TEM characterization for ZincOxide nanoparticle sizes (ranged from 44.1 to 93nm).

Seedlings production

Asparagus seeds were sown in pots (40 cm diameter) filled with light loamy soil, and then irrigated regularly to produce seedlings.

Callus induction

30-days old seedlings produced from seeds were disinfected using 70% ethyl alcohol for 10 seconds and 15% sodium hypochlorite (Clorox) for 15 min and washed several times with sterile distilled water, then used as a source of explants. Explants (internodes with axillary buds) were cultured on MS-medium supplemented with different 36 combinations of (NAA and BAP). The combinations of both regulators were (0.0, 0.1, 0.5, 1.0, 2.0, 4.0 mg/l) plus 3% sucrose, 0.2% gelrite, and the pH of medium was adjusted at 5.8.

The culture medium was sterilized by autoclaving at 120°C for 20 min. The sterilized cultures were incubated under controlled conditions of temperature 24±2°C with 16 hr. photoperiod under 3000 Lux cool white fluorescent light intensity.

After 2 subcultures, the combination that induced the best callus biomass was selected then the different concentrations of ZnO NPs were added to the previous mentioned media. Then callus fresh weights were determined after 8 weeks of culturing.

Suspension culture

The callus induction treatment produced a compact callus, which not preferred for suspension culture. So we needed to overcome this problem by increasing auxin and reducing sugar content in the medium. Half gram of compact callus was used in 25 ml of liquid MS-medium + 30g/l sugar and pH 5.9 and kept on a shaker for one week, then the cells were collected by centrifuge at 3000 rpm for 10 min, and 2 ml of re-suspended cells were transferred to 25 ml of new liquid culture with the same composition plus NAA (2mg/l) + BAP (0.3mg/l) and with different concentrations of ZnO NPs (0.5, 1, 3, 5, 10, 30, 50, 100 ppm), then transferred to 150 ml of new liquid culture in 250 ml flasks. The flasks were agitated at 120 rpm and incubated at 25°C under continuous low light intensity. Cultures were maintained after 21 days and cells numbers were counted every 3 days using hemocytometer. This method was used to estimate the viability of cells in Asparagus suspension culture.

Viability measurements

Total No. viable cells

Total No. nonviable cells

Total number of cells = No. viable cells + No. nonviable cells

Average No. viable cells/square = Total No. viable cells/Number of squares

Dilution factor = (Volume of cell suspension sample + Volume of diluting Trypan blue solution)/Volume of cell suspension sample

Percentage of viability = (Total number of viable cells / Total number of cells) × 100

Concentration of viable cells/square × dilution factor × 10⁴ (Shokrzhadeh and Modanloo, 2017)

Scale up using flasks 250ml (150 ml liquid media) was carried out with the same component as mentioned before in the same procedure.

Statistical analysis

Data were subjected to appropriate statistical and conventional methods of analysis of variance according to Snedecor and Cochran (1989). Computer software designed (Microsoft Excel 2007) was used.

Flavonoids determination by HPLC for callus and suspension cultures

One gram of Asparagus callus culture (8 weeks old-compact callus) as well as 9 days old-suspension culture had been extracted with 10 ml ethanol 80% and soaked in brown bottle for 2 hrs. at room temperature, then sonicated using a KQ-200VDE ultrasonic bath (Kunshan Ultrasonic Instrument Co. Ltd., Kunshan City, Jiangsu Province, China) with the output power 200W for 10 min, the volume was adjusted up to 50 ml with 80% ethanol, then filtered through Whatman filter paper 42 (125 mm).

HPLC conditions

Agilent 1260 infinity HPLC series (Agilent, USA) equipped with quaternary pump, a Zorbax Eclipse plus C18 column 150mm × 4.6 mm i.d., (Agilent Technology, USA), operated at 30°C, Eluent methanol, H₂O with 0.5 % H₃PO₄, 50:50 with flow rate 1 ml/min, the injected volume was 20 µl. Detection: UV. Detector set at 210 nm, (Schneider, 2014).

Results and Discussion

Callus induction

Callus proliferation was observed within 8 weeks following culture initiation using segmental internodes with auxiliary buds. Callus formation was observed in all explants with all of the ZnO NPs treatments plus 2 mg/l BAP. Data in Table (1) showed that all ZnO NPs treatments significantly increased than the untreated control, except the lowest concentration (0.5 ppm) where the increase was insignificant (13%). The treatment 10 ppm of ZnO NPs had the best callus fresh weight (8.01g) with significant increase percentage of 352.5% compared to control (1.77 g) as shown in Figure (2), followed by the treatment 30 ppm which recorded 7.27 g callus fresh weight (310.7%). In general, there was a gradual increase in the fresh weight of callus by increasing the concentration of ZnO NPs up to a concentration of 10 ppm, and then there was a gradual decrease in the callus fresh weight to a concentration of 50 ppm of ZnO NPs. After that, there was a noticeable reduction (48.8%) in the callus fresh weight when using the highest concentration of ZnO NPs (100 ppm) compared to the previous concentration (50 ppm).

These results were in accordance with Alharby *et al.* (2016) and Choudhary *et al.* (2018), who noticed that the presence of ZnO NPs was incorporated into plant hormone synthesis such as auxin. Kouhi and Lahouti (2018) and Kavianifar *et al.* (2018) found that callus biomass decreased with increasing of ZnO NPs concentration but with low concentrations, it stimulated the callus growth and pointed out the nanoparticles role in decontamination, regeneration, organogenesis, callus formation and activated a protein that had a vital role in growth. Zafar *et al.* (2016) reported that ZnO NPs had an obvious effect more than Zinc Oxide bulk

particles and Zn²⁺.MS medium acidity led to high dissolution behavior of ZnO NPs into Zinc ion which acted as a cofactor for many enzymes such as oxidases, dehydrogenases, anhydrases, peroxidases, and had a function in auxin synthesis regulation.

Table 1: The effect of different concentrations of Zinc Oxide nanoparticles plus 2 mg/l BAP on callus fresh weight (g) derived from *Asparagus* segmental internodes after 8 weeks of culturing.

ZnO NPs(ppm)	Callus fresh weight (g) ± SE
0.0	1.77 ± 0.06
0.5	2.00 ± 0.12
1	2.55 ± 0.07
3	3.11 ± 0.12
5	5.87 ± 0.22
10	8.01 ± 0.33
30	7.27 ± 0.15
50	6.64 ± 0.19
100	3.40 ± 0.02
LSD(0.05)	0.41

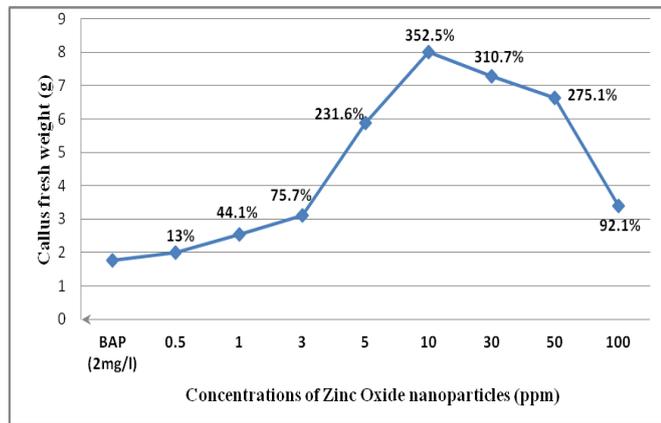


Fig. 2 : Differences in fresh weights (g) of *Asparagus* callus grown on different Zinc Oxide nanoparticles concentrations added to MS medium contained 2 mg/l BAP after 8 weeks of culturing.



Fig. 3 : Compact greenish yellow callus of *Asparagus* formed after 8 weeks of culturing explants (internodes with axillary buds) on MS medium contained 2mg/l BAP.

Suspension culture

The semi friable-greenish yellow callus of *Asparagus* was transferred to a liquid medium contained NAA(2 mg/l) +

BAP(0.3 mg/l) and sugar reduced to 20 g/l to establish the suspension culture as shown in Figure (4a and b).

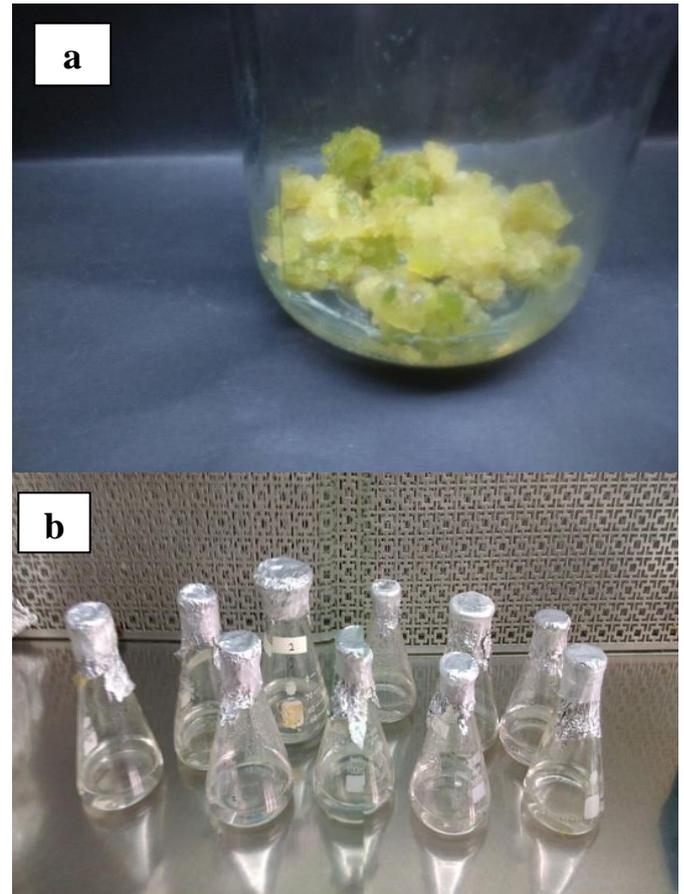


Fig. 4 : (a) Semi-friable greenish yellow callus of *Asparagus* which used in cell suspension culture established on a medium contained NAA(2 mg/l) + BAP(0.3 mg/l) and sugar reduced to 20 g/l. (b) Suspension culture after inoculation.

The results in Figure (5) showed the cell viability percentage of suspension cultures treated by different ZnO NPs concentrations (0.5, 1, 3, 5, 10, 30, 50 and 100 ppm) in addition the control treatment (ZnO NPs-free) at eight-time intervals (initial, 3rd, 6th, 9th, 12th, 15th, 18th, 21st day). In the control treatment, the cell viability percentage was the highest (66.6%) on the 3rd day of counting with an increase of (299.4%) than the initial count of viable cells (16.7%). Then, it declined significantly until the 9th day of counting and after that, it increased again until the 18th day of counting. The percentage of cell viability was the lowest (25%) on the 21st day of counting with an increase of 49.7% than the initial count of cells. On the third day of counting, when 0.5 ppm of ZnO NPs concentration was used, the viable cells increased to the highest percentage (87%) with an increase ratio of 46.7% comparing with the initial count (59.3%). The percentage of cell viability decreased until the 12th day related to the initial count and then, it increased again, reaching 83.3% with an increased ratio of 40.5% on the 15th day compared to the initial count. The vitality then diminished again until the 21st day of counting related to the initial count. Using 1 ppm of ZnO NPs concentration led to slightly increased cell viability from 88.9% in the initial count to 93% on the third day of counting with an increased ratio of 4.6%, and a subsequent cell viability contraction until 21st day of counting. The use of 3 ppm of ZnO NPs concentration led to the percentage of cell viability at the beginning of the count was 87.8%, and then the vitality of the cells decreased

until the 6th day of counting. The vitality of the cells increased to a maximum ratio of 91.6% on the 9th day of the count with an increase of 4.3% over the initial count. After that, cell viability decreased until 21st day related to the initial count. The use of 5 ppm of ZnONPs concentration led to the vitality of the cells at the beginning of the count was 55.5%, and then increased until the 9th day of the count (95.2%). Thereafter, cell viability decreased significantly to 53.3% on the 12th day and increased to the maximum (100%) on the 15th day of the count, with an increase of 80.2% related to the initial count. Cell viability significantly decreased (57.1%) on the 18th day of the count, although it was higher than the initial count with an increase of 2.9%, and then decreased again on the 21st day of the count. Cell viability was 43.3% at the beginning of the count when using a concentration of 10 ppm of ZnO NPs, and then decreased (33.3%) on the 3rd day of the count. Cell viability increased again until the 15th day of counting. The vitality of the cells reached the maximum (100%) on the 9th day of the count where it increased by 130.9% more than the beginning of the count (Figure, 6), and then decreased again less than the initial count until the 21st day of counting. The cell viability was (33.3%) at the beginning of the count when using a concentration of 30 ppm of ZnO NPs, and then stabilized at this percentage until the 3rd day of counting. The cell viability increased again until the 18th day of counting more than the initial count. The ratio was rolled back as it was at the initial count (33.3%) on the 21st day of the count. The cell viability was 30.5% at the beginning of the count when using a concentration of 50 ppm of ZnO NPs, and then decreased on the 3rd day of counting to 27.7%. After that, the cell viability increased until the 18th day of the count, reaching a maximum of 83.3% on the 9th day of the count with an increase of (173.1%) over the initial count. Cell viability decreased again on the 21st day of the count compared to the initial count. The viability of the cells was 25% at the beginning of the count when using a concentration of 100 ppm of ZnO NPs. There after, the viability ratio decreased on the 3rd day of counting (11.1%). The percentage of cell viability raised on the 6th day to the 15th day compared with the initial count. The cell viability reached the maximum (80.5%) on the 9th day of the count with an increase of 222% over the initial count. Cell viability decreased again (23.2 and 12.1%) on the 18th day and 21st day of the count, respectively, compared to the initial count. From the previously mentioned results we concluded that, the best cell viability percentages were at 10 ppm on the 9th day and 5 ppm on the 15th day. Toxic effect of Zinc Oxide nanoparticles on cell viability depends on concentration and time which led to the membrane and intracellular deterioration that observed at high concentration (200 mg/l) of Zinc Oxide nanoparticles and long treatment period in algae which led to viability loss due to Zinc Oxide nanoparticles accumulation. Using high concentration of Zinc Oxide nanoparticles caused a decrease in protein and polysaccharides as detected by Djearmane *et al.*, 2018. Nanoparticles at the concentration of 1000 µg/ml led to DNA destruction compared with control (Demir *et al.*, 2014). Nanoparticles showed a significant decline on cell viability at high concentration (100 mg/ml) after 120 hr High concentration of nanoparticles caused free ion formation to have a toxic effect on cell organelles (Krystofova *et al.*, 2013), but low concentration of Zinc Oxide nanoparticles induced growth and antioxidant defense system (Abbasi *et al.*, 2019).

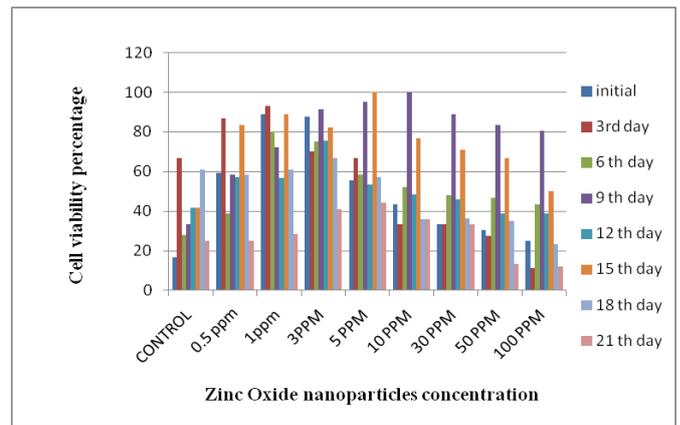


Fig. 5 : Concentration of viable cells /ml in Asparagus suspension culture at intervals on different concentrations of Zinc Oxide nanoparticles ($\times 10^4$) (150 ml liquid medium).

Flavonoids determination by HPLC for callus and suspension cultures

Data in Tables, 2 and 3 clarified HPLC results for different flavonoids in callus and suspension cultures treated by different concentrations of ZnO NPs.

(a) In callus culture

Data in Table (2) showed the HPLC flavonoids profile quantity in Asparagus callus culture after growing under (0.0 mg/l NAA) and (1, 2, and 4 mg/l BAP) plus different concentrations (0.0, 10, and 30 ppm) of ZnO NPs. Differences in the concentration of Asparagus flavonoid compounds can be observed. The best identified flavonoids substances were Rutin and Myricetin. The treatment 4 mg/l BAP showed the highest concentration of Rutin (67.930 mg/100g CFW) and ranked third in total flavonoids (90.663 mg/100g CFW), while 2 mg/l BAP gave the best concentration of Myricetin (22.121 mg/100g CFW) and ranked first in total flavonoids (102.303 mg/100g CFW). On the other hand, treatment with 1 mg /l BAP ranked second in Rutin, Myricetin and total flavonoids concentrations with values of 28.125, 17.223, and 96.724 mg/100g CFW, respectively. While the treatment 2 mg/l BAP, 0.0 mg/l NAA, and 30 ppm of ZnO NPs was the lowest one of Myricetin (5.407 mg/100g CFW) with low value of total flavonoids (13.917 mg/100g CFW). The obtained results were in agreement with Javed *et al.*, 2016 in Stevia, who found that the high concentration of Zinc Oxide nanoparticles led to phytotoxicity and feedback inhibition in the secondary metabolites production and had not been detected. Flavonoid concentration of *Echinacea purpurea* callus treated by different concentrations of Zinc Oxide decreased by increasing the concentration of Zinc Oxide nanoparticles (Karim *et al.*, 2018). Otherwise, the high concentration of Zinc Oxide nanoparticles had a direct role in increasing enzymatic and non-enzymatic antioxidants such as flavonoids and phenols as described by Josué *et al.* (2018) on *Capsicum annuum* L. Zinc Oxide nanoparticles not only affect phenols and flavonoids but also had an obvious effect on plant hormones such as auxin (Tirani *et al.*, 2018). Another nanoparticle such as silver caused accumulation of secondary metabolites in callus due to induction of oxidative stress in *Caralluma tuberculata* as mentioned by Ali *et al.* (2019).

Table 2 : HPLC result for different flavonoids in *Asparagus callus* treated by different combinations of plant growth regulators and Zinc Oxide nanoparticles.

ZnO* NPs Conc. (ppm)	BAP Conc. (mg/l)	NAA Conc. (mg/l)	Rutin (mg/100g) CFW	Myricetin (mg/100g) CFW	Quercetine (mg/100g) CFW	Naringenin (mg/100g) CFW	Kaempferol (mg/100g) CFW	Apigenin (mg/100g) CFW	Total Flavonoids (mg/100g) CFW
0.0	1	0.0	28.128	17.223	8.069	11.923	17.864	13.517	96.724
0.0	2	0.0	**ND	22.121	ND	15.500	20.532	44.150	102.303
0.0	4	0.0	67.930	12.719	ND	5.681	4.333	ND	90.663
10	1	0.0	25.375	15.999	ND	ND	ND	ND	41.374
10	2	0.0	ND	ND	ND	ND	ND	ND	ND
10	4	0.0	14.727	10.586	ND	5.732	7.567	17.973	56.585
30	1	0.0	15.517	ND	ND	ND	3.774	ND	19.291
30	2	0.0	ND	5.407	ND	ND	2.752	5.758	13.917
30	4	0.0	12.000	11.100	ND	ND	ND	ND	23.100

*ZnO NPs means Zinc Oxide nanoparticles**ND means not detected

(b) In suspension culture

The results in Table (3) showed the effect of ZnO NPs concentrations on *Asparagus* suspension culture to produce flavonoids fractions. It was observed that the best flavonoid detected was Rutin when the concentration of ZnO NPs used was 10 ppm with a value of 37.78 mg/100g CFW (Figure, 7) followed by 3 ppm with a value of 29.14mg/100g CFW nine-days after initial culturing compared with control and other ZnO NPs concentrations. Another flavonoid fraction such as Myricetin was detected only at 5ppm of ZnO NPs concentration in low concentration (0.74mg/100g CFW). Whereas, total flavonoids concentrations recorded first rank (291.48 mg/100g CFW), second rank (161.64 mg/100g CFW), and third rank (94.59 mg/100g CFW) when ZnO NPs of 50, 5, and 10 ppm were used, respectively. The use of high concentrations of ZnO NPs (50 and 100 ppm) led to the appearance of Rutin (27.28 and 25.74 mg/100g CFW, respectively), while most other flavonoids were not observed. The results showed different effects of ZnO NPs with a type of flavonoids fraction.

From the results in Table 3, Figures 6 and 7 it was observed that there is a relationship between the viability of cells in suspension culture and their Rutin and total flavonoids contents when using different concentrations of

ZnO NPs. The highest cell viability (100%) on the 9th day of counting with the highest Rutin content (37.78 mg/g CFW), and a high total content of flavonoids (94.59 mg/g CFW) was obtained when 10 ppm of ZnO NPs was used. While using 50 ppm of ZnO NPs led to the high cell viability (83.3%) on the 9th day of counting with the highest total flavonoids content (291.48 mg/g CFW) and high Rutin content (27.28 mg/g CFW). Previous results were consistent with findings reported by some researchers. Zinc Oxide nanoparticles induced accumulation of phenols and flavonoids in cell suspension culture of *Linum usitatissimum* L. in the limited period due to positive relationships with antioxidative effect as a defense mechanism against nanoparticles stress as noticed by Moharrami *et al.*, 2017 and Abbasi *et al.*, 2019. Metabolic pathway altered by Zinc Oxide nanoparticles such as transcriptional levels of HMG-CoA reductase gene which manipulate the mevalonate pathway (Bhardwaj *et al.*, 2018). There was a strong link between trigger in secondary metabolite production and reactive oxygen species induced by nanoparticles treatments, it is considered as a signaling pathway as observed by Marslin *et al.*, 2017. Zinc Oxide nanoparticles had multiple metabolic functions such as osmotic regulation which caused disruption in some biological processes such as photosynthesis, transpiration and enzyme activity as well as a regulatory cofactor.

Table 3 : HPLC result for different flavonoids in *Asparagus* suspension culture treated by different concentrations of Zinc Oxide nanoparticles and NAA(2mg/l) + BAP(0.3mg/l).

ZnO NPs Conc. (ppm)	Rutin (mg/100g)	Myricetin (mg/100g)	Quercetine (mg/100g)	Naringenin (mg/100g)	Kaempferol (mg/100g)	Apigenin (mg/100g)	Total Flavonoids (mg/100g)
Control	20.40	ND	ND	ND	ND	ND	20.40
0.5	21.19	ND	ND	1.50	ND	ND	22.69
1	22.04	ND	ND	ND	ND	ND	22.04
3	29.14	ND	ND	ND	ND	ND	29.14
5	4.60	0.74	ND	155.00	1.30	ND	161.64
10	37.78	ND	ND	56.81	ND	ND	94.59
30	26.62	ND	ND	ND	ND	ND	26.62
50	27.28	ND	ND	66.53	ND	197.67	291.48
100	25.74	ND	ND	57.32	ND	ND	83.06

Figure (6): Cell viability % in suspension culture on the 9th day

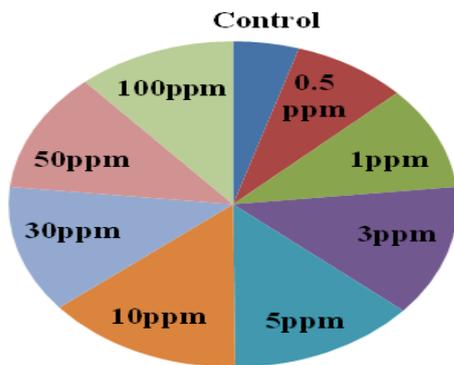
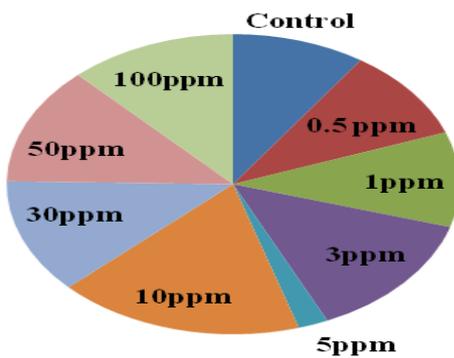


Figure (7): Rutin content in suspension culture (mg/100g CFW)



Flavonoids content increased with increasing Zinc Oxide nanoparticles concentration. Nanoparticles played a crucial role in signaling transduction induce gene responsible for jasmonate production which involved in important metabolite pathway (Sharafi *et al.*, 2013). Zinc Oxide nanoparticles concentrations at 0.75ppm and 1 ppm had high oxidative stress effect in *Bacopa monnieri* (L.) cell suspension culture (Bhardwaj *et al.*, 2018). Zinc Oxide nanoparticles improved flavonoids accumulation in plant cell at 20 mg/l by adding in MS medium (Chayaprasert and Sompompalin, 2017). The low concentration of Nano cobalt treatment in *Artemisia annua* caused positive regulation expressions of DBR2 gene and led to decrease in artemisin in content (Ghasemi *et al.*, 2015).

Conclusion

It could be concluded from the previous results that, Zinc Oxide nanoparticles (ZnONPs) at the concentration of 10 ppm gave the highest callus fresh weight and had a superior effect on Rutin production in suspension culture. The greatest cell viability percentages in suspension culture were recorded at 5 and 10 ppm of ZnONPs with the same value of 100% on the 15th and 9th days of counting, respectively. The highest concentration of Rutin (37.78 mg/100g CFW) was recorded at 10 ppm of ZnONPs with total flavonoids of 94.59 mg/100g CFW while the addition of 50 ppm of ZnO NPs gave the highest total flavonoids content (291.48 mg/100 g CFW).

Acknowledgement

The researchers express their sincere thanks to the Department of Agricultural Botany, Faculty of Agriculture, Cairo University and the Department of Plant Biotechnology, National Research Center for their full support to accomplish this work.

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