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EFFECT OF HYDROXYLAPATITE AND ZINC OXIDE NANOPARTICLES ON SEED GERMINATION AND SEEDLING GROWTH

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ABSTRACT

The present study aims to investigate the effects of hydroxylapatite (HA-NPS) and zinc oxide (ZnO-NPs) Nanoparticles on seed germination of four plant species; radish (*Raphanus sativus*), tomato (*Solanum lycopersicum*), wheat (*Triticum aestivum* L.) and cucumber (*Cucumis sativus* L.). Seeds were treated with DI-water (control) and Nanoparticles suspensions (HA-NPS and ZnO-NPs) solutions, at six concentrations of 10, 20, 50, 100, 200 and 500 ppm. The treatment of HA-NPS showed inhibition effect on all treated seed germination with significant dose dependent. The inhibition effect was related positively with HA-NPS concentration and was significant at the rate of 50 ppm in radish and tomatoes, and at 100 ppm in cucumber and wheat seeds. In ZnO-NPs treatment, the inhibition effect was related positively with ZnO-NPs concentration and was significant started from 20 ppm in radish, tomatoes, and cucumber. Meanwhile, wheat seeds were weakly responded to ZnO-NPs inhibition showing significant effect when applied at 200 ppm. The germination index data (combined seed germination and root elongation), indicated that the response of the tested seeds against Hydroxylapatite Nanoparticles was evident at the highest applied rate

(500 ppm), and was as follows: **Radish > Tomatoes > wheat > Cucumber**. In ZnO-NPs treatment was **Radish > Tomatoes > Cucumber > wheat**.

Key words: Hydroxylapatite, Zinc Oxide, Nanoparticles, Seed germination.

INTRODUCTION

Nanotechnology has enormous potential uses and benefits. Nowadays tremendous research has been carried out to explore the positive impacts of Nanoparticles on the plant growth and development while few studies reported their negative effect. Nanoparticles are the atomic or molecular aggregates having size dimension between 1 to 100 nm. They have diverse and unique physic-chemical properties as compared to other bulk materials (**Nel et al., 2006**). The Nanoparticles, with their ultra-small size, specific shape, geometric structure, and unique properties, may have the potential for increased toxicity (**Arruda et al., 2015**).

Fertilizers are very important for plant growth and development. Most of the applied fertilizers are rendered unavailable to plants due to many factors, such as leaching, degradation by photolysis, hydrolysis, and decomposition (**DeRose et al., 2010**). Hence, it is necessary to minimize nutrient losses in fertilization, and to increase the crop yield through the exploitation of new applications with the help of Nanotechnology and Nanomaterials.

The presence of Nanoparticles on the root surface can change the surface chemistry of the roots and consequently affect the uptake of nutrients into the plant root (**Mirzajani et al., 2013**) thus; these have to be taken into consideration too.

Seed germination and seedling growth are being widely used to test phytotoxicity of many chemical species such as engineered Nanomaterials which may be released into the environment because it is crucial stage in plant growth and it is also an important phenomenon in agriculture because it is regarded as thread of life of plants that ensure its survival (**Iqbal et al., 2016**).

Seed germination, shoot and root elongation measurements are quite rapid for use on acute phytotoxicity tests with several advantages: sensitivity, simplicity, low cost, and suitability for reactive chemicals and contaminated soil samples (**Munzuroglu and**

Geckil, 2002). Therefore, this study aimed to compare the effect of different concentrations of selected Nanoparticles on seed germination of four plants when used as Nanofertilizers compared to untreated.

MATERIALS AND METHODS

Nanoparticles:

Two types of Nanoparticles (hydroxylapatite and zinc oxide) were used in this study.

Preparation of hydroxylapatite Nanoparticles:

Hydroxylapatite (HA-NPS) was synthesized from calcium hydroxide and orthophosphoric acid. Both chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hydroxylapatite prepared via wet-chemical precipitation method as described by **Bianco *et al.* (2007)**. In the present work, calcium ions react with phosphate ions based on a molar ratio of $\text{Ca/P} = 1.67$. Calcium hydroxide was dissolved in deionized water (Milli-Q, Millipore, USA) under vigorously stirring at 1000 rpm/min for 12 h at 25 °C. The prepared orthophosphoric acid solution was slowly added dropwise into the stirring suspension of calcium hydroxide in distilled water. The precipitated materials were allowed to settle overnight before filtration. The filtered precipitation was rinsed three times by using deionized water and then dried overnight in an oven at 100 °C for 2 h.

Physical, Chemical and Morphological Characterization:

The formation process of HA-NPS crystal was investigated by X-ray diffraction (XRD, X'pert Pro, PanAnalytical, Netherlands) in the 2 θ range 0° to 80° using CuK α 1 radiation ($\lambda=1.54056$ Å). The morphology and size distribution of the synthesized HA-NPS powder was characterized with a transmission electron microscope (HR-TEM, Tecnica G20, FEI, Netherlands), operating at 80 kV. An aspect ratio can be defined by the ratio length/width of the HA-NPS Nanocrystals. All preparation procedures and characterization of calcium Nanoparticles were done at the Nanotechnology & Advanced Materials Central Lab, ARC, Giza, Egypt.

Preparation of zinc oxide Nanoparticles:

Zinc oxide was synthesized by the precipitation method. In a typical procedure, 14.38 g of zinc sulfate heptahydrate was dissolved in 50 ml of deionized water (Milli-Q, Millipore, USA) and then, 4 g of sodium hydroxide in 50 ml of deionized water was added dropwise under magnetic stirring. After the addition was completed, the stirring was continued for 30 min. The precipitates were filtered and washed using pure water several times. Then the precipitates were dried at 60°C for 24 h and dried at 400°C for 2 h. The crystalline and phase structure of the synthesized ZnO NPs was studied by an X-ray diffractometer (XRD, X'Pert Pro, PanAlytical, Netherlands). The morphology and size were determined by the transmission electron microscopy ((TEM, Tecnai G20, FEI, Netherlands).

Seeds:

Seeds of four plant species; radish (*Raphanussativus*), tomato (*Solanum lycopersicum*), wheat (*Triticum aestivum* L.) and cucumber (*Cucumis sativus* L.) were used for the test as recommended by **Migliore et al. (2003)**.

Preparation of HA-NPS and ZnO NPs suspensions:

Weight of 1 g from HA-NPS and ZnO-NPs were suspended individually in 1 liter of deionized water (DI-water) and dispersed by ultrasonic vibration (100 W, 40 kHz) for 30 min to prepare 1000 ppm stock solution for each. The stock solutions were diluted by DI- water to make serial concentrations of 10, 20, 50, 100, 200 and 500 ppm. Small magnetic bars were placed in the prepared solution for stirring before use to avoid aggregation of the particles.

Germination assay:

Germination assay was carried out according to **Yang and Watts (2005)**. Seeds were immersed in sodium hypochlorite (10%) solution for 10 min to ensure surface sterility, then rinsed three times with DI-water. After rinsing, seeds were prepared for assay by soaking in DI-water for control treatment and in the suspension of HA-NPS or ZnO-NPs for 2 h., (**Kikui et al., 2005**). One piece of filter paper was put into a Petri dish (10 mm) and 10 soaked seeds were transferred onto the filter paper and 5 ml of DI-water or test Nanoparticles suspension were added/dish with three replicates for each treatment/concentration. Petri dishes were covered and placed in an

incubator for 7 days in the dark at $28 \pm 2^\circ\text{C}$. After the incubation period, root and shoot length, germination percentage, relative elongation %, relative germination rate and germination index were recorded. The results were compared with the untreated (control) for each seed type.

The relative elongation %, relative germination rate and germination index were calculated as follows:

$$\text{Relative germination rate} = \frac{\text{Seeds germinated in test sample} \times 100}{\text{Seeds germinated in control}}$$

$$\text{Relative root elongation} = \frac{\text{Mean root length in test sample} \times 100}{\text{Mean root length in control}}$$

$$\text{Germination Index} = \frac{\text{Relative germination rate} \times \text{Relative root elongation}}{100}$$

Statistical analysis

The statistical analysis was done by using SPSS statistical software (Landau and Everitt, 2004). The results were presented as mean $\pm$ SD (standard deviation). Each of the experimental values was compared with the corresponding control. Statistical significance was accepted when the probability of the result assuming the null hypothesis (p) is less than 0.05.

RESULTS AND DISCUSSION

1.Characterization of synthesized hydroxylapatite Nanoparticles

1.1.X-Ray diffraction (XRD):

The X-ray diffraction patterns of as-synthesized calcium phosphates are shown in **Fig. (1)**. The XRD spectra obtained have characteristic peaks consistent with the International Centre for Diffraction JCPDS files number 00-064-0738 files for HA-NPS. The HA-NPs exhibited several signals at $2\theta = 25.88, 31.77, 32.19, 32.91,$ and 49.49 , corresponding to the diffraction planes (0 0 2), (1 2 1), (1 1 2), (0 3 0), and (1 2 3), respectively.

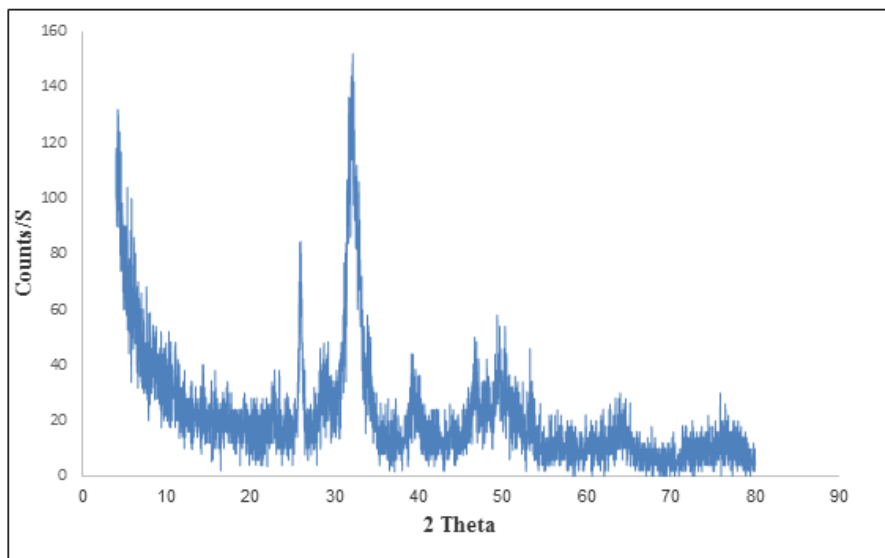


Fig. 1. XRD patterns of hydroxyapatite (HA) powders synthesized.

1.2. Transmission electron microscopy (TEM):

The TEM image of the HA-NPS Nanoparticles is shown in **Fig. (2)**. The needle-like morphology of the apatite particles with a width less than 10-16 nm and a length about 30–55 nm was clearly observed.

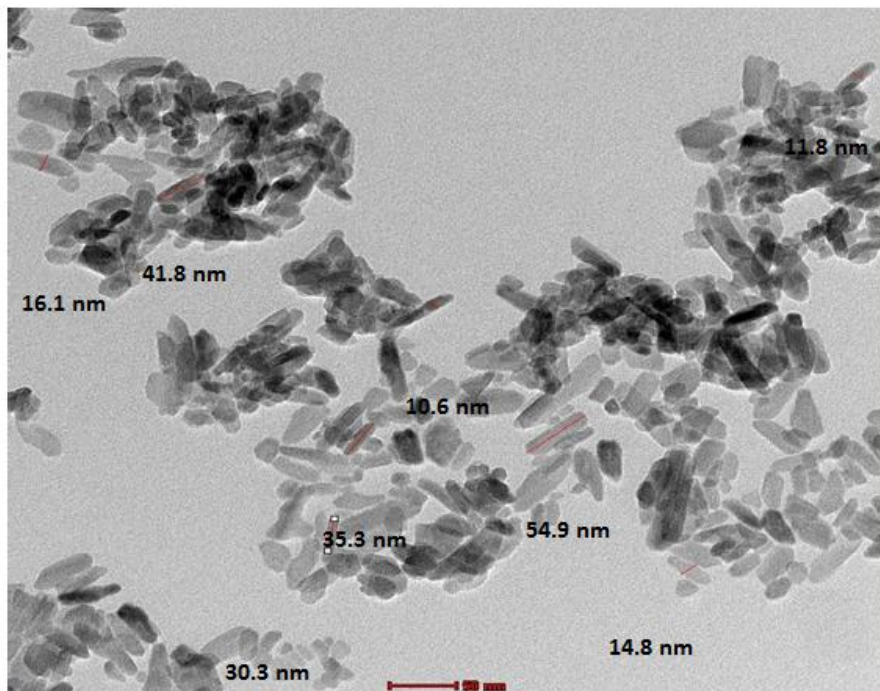


Fig.2. TEM image of HA-NPS powders synthesized.

1.3.XRD analysis:

The XRD pattern of the synthesized ZnO-NPs is shown in **fig. (3)**. The peaks at $2\theta = 31.77^\circ$, 34.4° , 36.26° , 47.54° , 56.60° , 62.86° , 66.38° , 67.95° , 69.09° , 72.57° , and 76.97° were assigned to (100), (002), (101), (102), (110), (103), (200), (112), (201), (004), and (202) of ZnO-NPs, indicating that the crystalline structure of synthesized ZnO-NPs presented a hexagonal phase structure of the wurtzite (Zincite, JCPDS 5-0664).

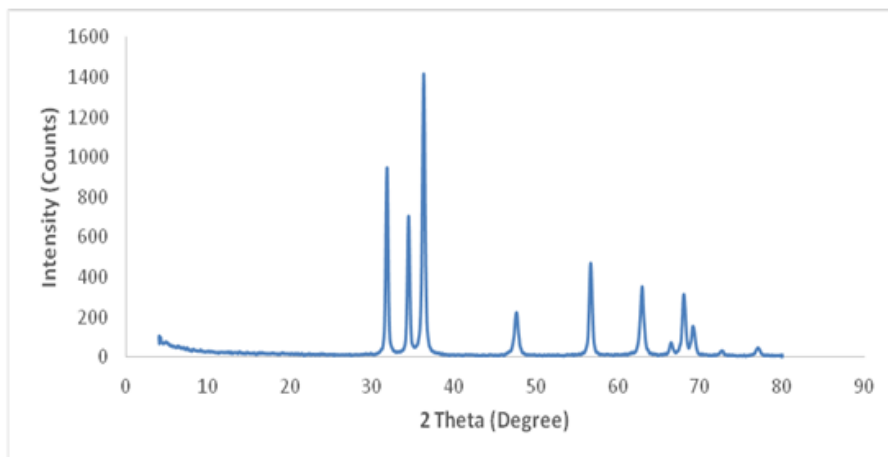


Figure3. The XRD pattern of the synthesized ZnO-NPs.

2.Characterization of synthesized zinc oxide Nanoparticles

2.1.TEM analysis:

High Resolution Transmission Electron Microscopic (HR-TEM) studies were carried out to find out exact particle size of synthesized ZnO-NPs. TEM images as illustrated at **Fig. (4)** ZnO-NPs which having particle size in the range of 20–32 nm with nearly spherical shaped particles.

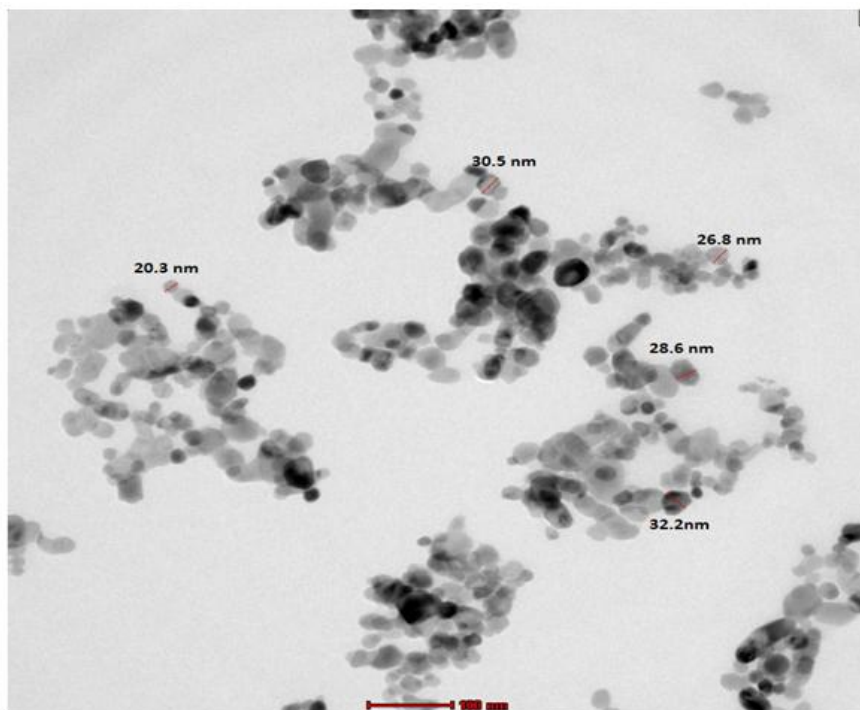


Figure 4. TEM image of the synthesized ZnO-NPs.

3. Effects of HA-NPS and ZnO-NPs suspensions on seed germination

3.1. Effects of HA-NPS:

Data in **Table (1a)** and **(1b)** showed that HA-NPS suspension decreased the root and shoot length of all treated seeds compared with control. The results showed inhibition effect on all treated seed germination with a clear significant dose dependent. The inhibition effect was related positively with HA-NPS concentration and was significant at the rate of 50 ppm in radish and tomatoes, and at 100 ppm in cucumber and wheat seeds. The concentration of 500 ppm showed the highest inhibition effect comparing to control and significantly decreased root and shoot length of all tested seeds being 4.32, 5.31, 3.02 and 5.32 cm for root and 5.25, 3.08, 1.27 and 5.01 cm for shoot of radish, tomatoes, cucumber and wheat seeds, respectively.

The data, revealed that the relative % root or shoot length at the concentration 500 ppm of HA-NPS recorded 48.16 or 87.06 % for radish, 78.32 or 68.29 % for tomatoes, 84.59 or 61.95% for cucumber,

81.60 or 77.78% for wheat, respectively. From the germination index data (combined seed germination and root elongation), its indicated that the sensitivity of the tested seeds against Hydroxylapatite Nanoparticles at the highest applied rate (500 ppm) was as follows:

Radish > Tomatoes > wheat > Cucumber

Table 1a. The Effect of HA-NPS suspensions on Radish and Tomatoes seeds.

Conc. (ppm)	Radish						Tomatoes					
	Root(cm)	Relative root length %	Shoot (cm)	Relative shoot length %	Relative Germ. %*	Germ. Index**	Root(cm)	Relative root length %	Shoot (cm)	Relative shoot length %	Relative Germ. %*	Germ. Index**
0.0 (Control)	8.97±0.04a	100.00	6.03±0.05a	100.00	100.00	100.00	6.78±0.07a	100.00	4.51±0.07a	100.00	100.00	100.00
10	8.80±0.03a	98.10	5.90±0.05a	97.84	95.79	93.97	6.75±0.04a	99.56	4.38±0.04a	97.12	97.83	97.39
20	8.53±0.05a	95.09	5.88±0.05a	97.51	93.68	89.09	6.71±0.03a	98.97	4.27±0.02a	94.68	95.65	94.66
50	5.58±0.09b	62.21	5.49±0.05b	96.02	91.58	56.97	5.71±0.01b	84.22	3.25±0.05b	72.06	93.48	78.73
100	5.49±0.09b	61.20	5.40±0.09b	89.55	89.47	54.76	5.61±0.03b	82.74	3.21±0.01c	71.18	86.96	71.95
200	5.21±0.09b	58.08	5.37±0.09b	89.05	84.21	48.91	5.51±0.01c	81.27	3.18±0.01c	70.51	76.09	61.83
500	4.32±0.1c	48.16	5.25±0.09b	87.06	63.16	30.42	5.31±0.02d	78.32	3.08±0.01c	68.29	67.39	52.78

Values are means of three replicates of each parameter ± standard deviation. Means within each column followed by the same letter are not significantly at $p > 0.05$.

* Relative germination rate comparing with control

**Germination Index = (Relative germination rate × Relative root length)/100

Table 1b. The Effect of HA-NPS suspensions on Cucumber and Wheat seeds.

Conc. (ppm)	Cucumber						Wheat					
	Root(cm)	Relative root length %	Shoot (cm)	Relative shoot length %	Relative Germ. % [*]	Germ. Index ^{**}	Root(cm)	Relative root length %	Shoot (cm)	Relative shoot length %	Relative Germ. % [*]	Germ. Index ^{**}
0.0 (Control)	3.57±0.06a	100.00	2.05±0.07a	100.00	100.00	100.00	6.52±0.07a	100.00	5.33±0.06a	100.00	100.00	100.00
10	3.56±0.05a	99.72	1.92±0.06a	93.66	96.67	96.40	6.42±0.05a	98.47	5.32±0.07a	99.81	95.56	94.09
20	3.53±0.04a	98.88	1.65±0.05a	80.49	97.78	96.68	6.32±0.05a	96.93	5.23±0.05a	98.12	95.56	92.62
50	3.49±0.04a	97.76	1.57±0.09a	76.59	88.89	86.90	6.22±0.06a	95.40	5.13±0.03a	96.25	93.33	89.04
100	3.32±0.01b	93.00	1.47±0.02b	71.71	87.78	81.63	6.12±0.01b	93.87	5.03±0.05ab	94.37	88.89	83.44
200	3.21±0.01b	89.92	1.36±0.02b	66.34	80.00	71.93	5.33±0.02c	81.75	4.48±0.01b	84.05	83.33	68.12
500	3.02±0.01b	84.59	1.27±0.02b	61.95	77.78	65.80	5.32±0.02c	81.60	4.45±0.01b	83.49	77.78	63.46

Values are means of three replicates of each parameter ± standard deviation. Means within each column followed by the same letter are not significantly at $p > 0.05$.

* Relative germination rate comparing with control

**Germination Index = (Relative germination rate × Relative root length)/100

3.2.Effect of ZnO-NPs

Root and shoot length:

Data presented in **Table (2a)** and **(2b)**, revealed that ZnO-NPs showed a similar pattern of HA-NPS. ZnO-NPs inhibited root growth and decreased the root length of all treated seeds compared with the untreated. The inhibition effect was related positively to ZnO-NPs concentration and was significant started from 20 ppm in radish, tomatoes, and cucumber. Wheat seeds were more tolerant effect to ZnO-NPs and were significant when applied at 200 ppm. At 500 ppm showed the highest inhibition effect comparing to the control and significantly decreased roots and shoots length of all tested seeds being 1.13, 1.01, 1.02, and 4.1 cm for root and 4.02, 1.61, 1.25 and 5.01 cm for shoot of radish, tomatoes, cucumber and wheat seeds, respectively.

Relative root and shoot length:

The data in **Table (2a)** and **(2b)** revealed that the relative % of root or shoot length were correlated negatively with increasing ZnO-NPs applied rate and showed the minimum relative % at the concentration of 500 ppm and recorded 12.60 or 66.67% for radish,

14.90 or 35.70% for tomatoes, 28.57 or 60.98% for cucumber and 62.88 or 94.00% for wheat seeds, respectively. According to the germination index data (combined seed germination and root elongation), the sensitivity of the tested seeds against Zinc oxide Nanoparticles at the highest applied rate (500 ppm), was as follows:

Radish > Tomatoes > Cucumber > wheat

Table 2a. The Effect of ZnO-NPs suspensions on radish and tomatoes seeds.

Conc. (ppm)	Radish						Tomatoes					
	Root(cm)	Relative root length %	Shoot (cm)	Relative shoot length %	Relative Germ. % *	Germ. Index **	Root(cm)	Relative root length %	Shoot (cm)	Relative shoot length %	Relative Germ. % *	Germ. Index **
0.0 (Control)	8.97±0.04a	100.00	6.03±0.09a	100.00	100.00	100.00	6.78±0.09a	100.00	4.51±0.09a	100.00	100.00	100.00
10	7.98±0.03a	88.96	5.99±0.07a	99.34	94.74	84.28	5.97±0.07a	88.05	4.08±0.05a	90.47	96.74	85.18
20	6.58±0.1b	73.36	5.81±0.05b	96.35	92.63	67.95	3.77±0.02b	55.60	3.73±0.04b	82.71	89.13	49.56
50	4.87±0.3c	54.29	5.75±0.05b	95.36	89.47	48.58	2.77±0.03c	40.86	3.46±0.04b	76.72	86.96	35.53
100	4.35±0.1d	48.49	5.32±0.05b	88.23	84.21	40.84	1.98±0.01d	29.20	3.11±0.04b	68.96	76.09	22.22
200	2.08±0.1e	23.19	4.62±0.03c	76.62	63.16	14.65	1.12±0.02e	16.52	3.01±0.03b	66.74	65.22	10.77
500	1.13±0.09f	12.60	4.02±0.01d	66.67	52.63	6.63	1.01±0.02e	14.90	1.61±0.01c	35.70	55.43	8.26

Values are means of three replicates of each parameter ± standard deviation. Means within each column followed by the same letter are not significantly at $p > 0.05$.

* Relative germination rate comparing with control

**Germination Index = (Relative germination rate × Relative root length)/100

Table 2b. The Effect of ZnO-NPs suspensions on cucumber and wheat seeds.

Conc. (ppm)	Cucumber						Wheat					
	Root(cm)	Relative root length %	Shoot(cm)	Relative shoot length %	Relative Germ. % *	Germ. Index **	Root(cm)	Relative root length %	Shoot(cm)	Relative shoot length %	Relative Germ. % *	Germ. Index **
0.0 (Control)	3.57±0.09a	100.00	2.05±0.06a	100.00	100.00	100.00	6.52±0.03a	100.00	5.33±0.04a	100.00	100.00	100.00
10	3.37±0.05a	94.40	1.98±0.06a	96.59	94.44	89.15	6.42±0.02a	98.47	5.31±0.05a	99.62	86.67	85.34
20	2.28±0.02b	63.87	1.53±0.02b	74.63	84.44	53.93	6.32±0.03a	96.93	5.29±0.04a	99.25	86.67	84.01
50	1.27±0.04c	35.57	1.52±0.02b	74.15	83.33	29.65	6.21±0.04a	95.25	5.21±0.02a	97.75	86.67	82.55
100	1.23±0.05c	34.45	1.31±0.02b	63.90	72.22	24.88	6.13±0.03a	94.02	5.15±0.05a	96.62	73.33	68.95
200	1.18±0.05c	33.05	1.28±0.02b	62.44	71.11	23.50	4.9±0.09b	75.15	5.09±0.09b	95.50	72.22	54.28
500	1.02±0.0c	28.57	1.25±0.01c	60.98	57.78	16.51	4.1±0.09b	62.88	5.01±0.08b	94.00	70.00	44.02

Values are means of three replicates of each parameter $\pm$ standard deviation. Means within each column followed by the same letter are not significantly at $p > 0.05$.

* Relative germination rate comparing with control

**Germination Index = (Relative germination rate $\times$ Relative root length)/100

Accordingly, its concluded that cucumber and wheat seed showed the lowest significant response against HA-NPS and ZnO-NPs Nanoparticles treatment comparing to radish and tomatoes seed, that might be depend on Nanoparticles type or the plant species (**Ngo et al., 2014 and Dewez et al. 2005**). **Figure 5**, indicated that (1) ZnO-NPs showed more inhibition effect than HA-NPS on seed germination index except wheat which showed a converse results. The inhibition effect of HA-NPs on seeds germination/growth may be due to that HA-NPs can reduce the plant uptake of water and element to plant, (**Li et al., 2016**).

The treatment with HA-NPs with high concentrations (1,000 and 2,000 $\mu\text{g/ml}$) could cause some negative effect on seeds germination/growth as reported by **Bala et al., (2014)**. Also, ZnO-NPs caused a concentration dependent inhibition of root length of garlic (*Allium sativum* L.) when treated with 50 mg L^{-1} , for 24 h where the

root growth was completely blocked, which could be due to the total percentage of abnormal cells increased with the increase of ZnO-NPs concentration and the prolonging of treatment time, (**Hossain *et al.*, 2016**).

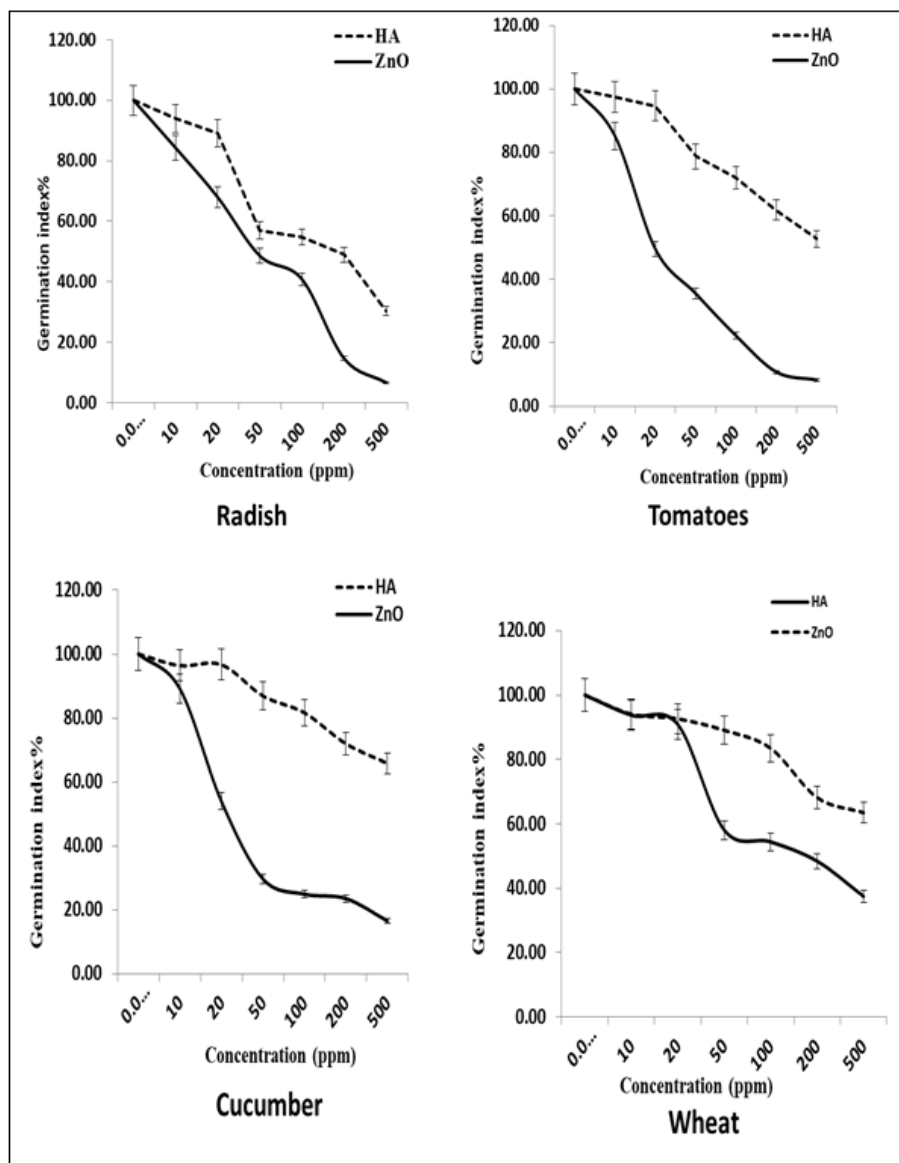


Figure (5). The effect of Hydroxylapatite and Zinc oxide nanoparticles suspensions on different treated seeds Germination Index.

The results of ZnO-NPs are in agreement with **Shaymurat *et al.* (2012)**, who indicated that ZnO-NPs have been shown to induce oxidative stress in soybean (*G. max*) seedlings at a concentration of 500 mg L⁻¹. Plant growth, rigidity of roots, and root cell viability were markedly affected by ZnO-NPs stress. Oxidation–reduction cascade related genes, such as GDSL motif lipase 5, SKU5 similar 4, galactose oxidase, and quinone reductase were down-regulated in ZnO-NPs treatment.

Moreover, some Nanoparticles are taken up by plant roots and transported to the above ground parts of the plant through the vascular system, depending on the composition, shape, size of Nanoparticle, and anatomy of the plant. Some Nanoparticles remain adhered to the plant roots. Toxicity of Nanoparticles may be attributed to two different actions: (1) a chemical toxicity based on the chemical composition, e.g., release of toxic ions; and (2) stress or stimuli caused by the surface, size and/or shape of the particles (**Lin, 2007**).

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تأثير جزيئات نانو هيدروكسيلايتات و اكسيد زنك علي إنبات البذور ونمو البادرات

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تهدف الدراسة لتوضيح تأثير جزيئات النانو لمركبات هيدروكسيلايتات و اكسيد الزنك علي انبات بذور اربع نباتات: الفجل ، الطماطم ، القمح و الخيار. تم معاملة جميع البذور بالماء و ستة تركيزات من محاليل مركبي النانو (10، 20، 50، 100، 200، 500 جزء في المليون). اعطت المعاملة بجزيئات نانو هيدروكسيلايتات تأثير تثبيطي لكل البذور التي تم معاملتها بمعنويه واضحه اعتماداً علي الجرعه او التركيز. التركيز 50 جزء في المليون كان له تأثير تثبيطي معنوي متساوي في الفجل والطماطم بينما تركيز 100 جزء في المليون كان له تأثير معنوي علي الخيار والقمح. اعطت المعاملة بجزيئات نانو اكسيد الزنك تأثير تثبيطي معنوي لجميع المعاملات بداية من تركيز 20 جزء في المليون لكل من الخيار والطماطم و الفجل بينما كانت بذور القمح اكثر مقاومه للتأثير التثبيطي لجزيئات النانو لأكسيد الزنك عند المعاملة بتركيز 200 جزء في المليون. كانت قيم معامل الانبات للنباتات المعاملة بتركيز 500 جزء في المليون من جزيئات نانو هيدروكسيلايتات كالآتي: الفجل < الطماطم < القمح < الخيار بينما عند المعاملة بجزيئات نانو اكسيد الزنك كانت قيم معامل الانبات كالآتي: الفجل < الطماطم < الخيار < القمح.