EFFECT OF CuO NANOPARTICLES ON SEED GERMINATION AND SEEDLING GROWTH IN *ECHINACEA PURPUREA IN VITRO.*

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ABSTRACT

This study was aimed to examin the effect of CuONPs on both seeds germination, seedling growth and comparing the method of soaking and adding to the culture medium, The research was implemented at PTC. Lab. College of Biotechnology - Al Nahrain University, during 2022 and 2023. The experimental design was factorial within CRD. It was included five experments and ten replicates (4X3). first experment was by using Sodium Hypochlorite (0.0, 1, 2 and 3%) with (5, 10, 15min) duartion time. The second experment was CuONPs (0, 25, 50, 75mg,L^{-1}) combined with (3,6,9 and 12 day) Time duration, same factors wre examined after soaking seeds with CuONPs which represented the third experment, fourth and fifth experments soaked before culture seeds were cultured respictively with CuONPs (0.0, 25, 50, 75mg,L^{-1}) for 1hour then culturing them on MS media. Results showed full reduction in the contamination rate of the selected *E. purpurea* explant recorded in 3% sodium hypochlorite at 10 and 15min. the highest rate of seeds germination were a chieved with CuONPs of 50 mg, L^{-1} for 9 days rated 7.80 germinated seeds in MS media culture method, at the soaking method the results clarify the highest CuONPs 75 mg, L^{-1} combined with 6,9 and 12 days of soaking a chieved the highest germination rate 10.0 seeds (100% germination).The results also showed that 50% of CuONPs increases shoot numbers 8.6 explant and dry weight 198 mg. 25% of CuONPs achieve the best shoot length 14.5 cm. in seeds soaking results showed the best shoot Nu. 8.7, shoot length 9.7 cm. and dry weight 204 mg. when seeds was soaked in 75 mg, L^{-1} of CuONPs.

Key words: tissue culture; agricultural applications of nanoparticles, medicinal plants.

تأثير جزيئات أوكسيد النحاس النانوية في إنبات البذور ونمو البادرات في نبات القنفذية خارج الجسم الحي

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المستخلص

هدف البحث الى تأثير أوكسيد النحاس النانوي على انبات البذور ونمو البادرات ومقارنة طريقة النقع بطريقة الاضافة الى الوسط الزرعي، نفذ البحث في مختبر زراعة الانسجة النباتية – كلية التقنيات الاحيائية– جامعة النهرين للفترة 2022 – 2023. كانت التجارب عاملية ضمن تصميم تام التعشية CRD باستعمال خمسة تجارب وبعشرة مكررات. التجربة الأولى هو تركيز هيبوكلورات الصوديوم (0.0، 1، 2 و 3٪) بالتداخل مع المدة الزمنية (5، 10، 15 دقيقة) ، التجربة الثانية هي تراكيز مختلفة من اوكسيد النحاس النانوي CuONPs (0.0، 25، 50، 75 ملجم، لتر$^{-1}$) بالتداخل مع (3،6، 9 و 12 يوم) وزراعتها في وسط MS ، تم فحص العوامل نفسها بعد نقع البذور بـ CuONPs والذي يمثل التجربة الثالثة، وتمت الزراعة بعد نقع البذور بـ CuONPs (0.0، 25، 50، 75 ملجم.لتر$^{-1}$) لمدة ساعة واحدة ثم زراعتها على وسائط MS. أظهرت النتائج انخفاض معدل التلوث لنبات القنفذية *E.purpurea* الى 0% عند تركيز 3% من هيبوكلورات الصوديوم بالتداخل مع المدد 10 و 15 دقيقة، واظهر أعلى معدل للبذور النابتة بلغ 7.80 بذرة باستعمال CuONPs بمقدار 50 ملغم، لتر$^{-1}$ لمدة 9 أيام بطريقة اضافة النانو الى الوسط ، وفي طريقة النقع أوضحت النتائج بان تركيز75 ملغم. لتر$^{-1}$ من CuONPs ولمدة 6 يوم اعطت أعلى معدل للانبات بلغ 10.0 بذرة (100% إنبات). كما أظهرت النتائج أن 50 ملغم. لتر$^{-1}$ من CuONPs زاد عدد الأفرع الى 8.6 فرع كذلك الوزن الجاف بلغ 198 ملغم، اما 25% من CuONPs حقق أفضل معدل لطول الفرع بلغ 14.5 سم. مقارنة بتجربة نقع البذور كان افضل عدد للافرع بلغ 8.7 فرع وطول9.7 سم. بوزن جاف بلغ 204 ملغم تحقق عند نقع البذور في 75 ملغم. لتر$^{-1}$ CuONPs.

الكلمات المفتاحية: زراعة الأنسجة، التطبيقات الزراعية للجسيمات النانوية، النباتات الطبية.

Received: 13/7/2023, Accepted:25/10/2023

INTRODUCTION

The bioactive components of *Echinacea purpurea* include polysaccharides, phenolics, flavonoids, and alkylamides. a Polyphenolic phytomolecules including phenolic acids and flavonoids are responsible for the echinacea plant extracts' antioxidant properties (12, 18). Monocytes and natural killer cells, the body's initial line of immune defense against infection, could be strengthened by echinacea extract (13). Cross-pollination led to the heterozygosity of genotype in *E. purpurea* seeds, which couses in complex dormancy, low rates of seeds sprouting, and challenging seeds collection. Additionally, there were noticeable disparities in the cultivated population's plant shape, growth, and development processes, which hindered the advancement of field management, quality stability, and yield improvement (2). The study of matter with dimensions on the order of 9-10 is known as nanotechnology, and it is a rapidly expanding field that has profound effects on all areas of biological sciences (1, 12, 15, 17, 24). although of the rapid development of nanotechnology, the health impacts due to exposure at different levels of nanoparticles was vary on both levels' environment and human (5). Nano particles approved to have a positive impact as anti-inflammatory and antibacterial effects **(8).** Copper nanoparticle absorption and accumulation are directly correlated with the concentration used (16) and are easily made in the lab as dark brownish-red powdered items(3, 4, 6, 20). Although the practices of growing Echinacea plants from seeds was common, it has a number of drawbacks, such as the uncertainty that the plants produced will be free of diseases due to their lengthy growth cycles and the dormancy of Echinacea seeds (22,26). Due to the ability to regulate the environmental factors and the elements of the nutrient medium required by the plant, tissue culture technology has created numerous opportunities for the production of secondary metabolites continuously and without being limited to a particular season (21). Most medicinal plants only contain a small percentage of therapeutically useful compounds, which prompted researchers to look for methods to boost the amount of therapeutically useful compounds and encourage the plant to produce them. These methods included using catalysts to help build the active compounds and the formation of primary or intermediate compounds that enter the structural pathways leading to the production of a particular compound (7). This study was aimed to investigase effect of copper nanoparticles (CuONPs) on in-vitro seed germination of *Echinacea purpurea* seeds on MS media supplemented with different concentrations of (CuONPs) and comparing the results with soaking *Echinacea purpurea seeds* in different concentrations of (CuONPs) then culturing them in MS media

MATERIALS AND METHODS

Plant sample collection

Seeds of *E. purpurea* collected from the local market at Baghdad city, calssified according to the National Herbal Commission / General Commission for Agricultural Research, Baghdad, Iraq.

Preparation of copper oxide nanoparticles solution and detection: Copper oxide nanoparticles (CuO.NPs) was obtained from Segma Company. The solution of nanomaterial was prepared by dissolving 100 mg of the material in 1000 ml of distilled water with continuous stirring using a hot plate magnetic stirrer to ensure its solubility, an ultrasonic probe was used at a frequency of 60Hz for 15min by placing a small drop of these samples on a glass slide to ensure the dispersion and disintegration of the nano-elements and ensure did not agglomerate and return them to their normal size, these samples left at the room temperature for experimental purposes. The drying sample of nanomaterial was examined with an (AFM) Atomic Force Microscope type Angstrom Advanced (AA) 2000 made in the United States of America, and the examination pattern used was the contact pattern and at room temperature (19).

Preparation the CuONPs concentrations .

Different concentrations of nanomaterial were prepared (0.0, 25, 50 and 75mg,L^{-1}) using sterilized distalled water for dissolving and dilution, then its kept in vials with dark situations at the room temperature for the purposes of using it in seed germination experiments later.

Sterilization of *E. purpurea* plant seeds: *E. purpurea* seeds with same size were randomly

collected, and in washed carfully with tab water, then they were soaked in Ethanol (90%) for 5sec, and sterillized using 2.5% sodium hypochlorite solution (NaOCl) about 20min's. The seeds were then rinsed five times with sterilized DDWH_2O for 5–10 minutes each time, continuously, using a magnetic stirrer tool.

Plants treatment with copper oxide nanoparticles.: *E. purpurea* seeds were cultured in Murashige and skooge free hormone media. the addition of Copper oxide nanoparticles (CuO.NPs) to the germination media was manged in Two experments as bellow:-

T1: Seeds were cultured in Murashige and skooge free hormone media, without any (CuO.NPs) concentrations as controle negative, 10 replicates used for this experment.

T2: Seeds were cultured in Murashige and skooge free hormone media supplemented with (CuO.NPs) mg,L^{-1} in concentrations (25,50,75,).10 replicates used for each concentration of this experment.

T3: Seeds were cultured in Murashige and skooge free hormone media after they were soacked for an hour in (CuO.NPs) solution using concentrations (25,50,75,)%. 10 replicates used for each concentration of this experment.

Universal vials 10 rep./ treatment were kept in growth chamber (1000 lux light) (14). Survival rate/time determination was collected along 12 days. Germination percentage and Shoot length were determined about six weeks after germination under stress, also, dry wights of the dried treated seedlings (drying in an oven in 45 °C) were obtained using sensetive balance.

Experimental design and statistical analysis. The obtained data from the factorial experiment were examined using the statistical application Genstat. Completely Randomized Design (CRD) was used in the experiment, and 10 replicates of each treatment were used (p=0.05).

RESULTS AND DISCUSSION

Copper oxide nanoparticles 3D examination of nanomaterials was shown in Figure 1 exhibited by the Atomic Force Microscope (AFM) that measure the surface topography, particle size, diameter and roughness of copper oxide nanoparticles. three-dimensional images of the surface topography of copper nanoparticles, where the size of the copper oxide nanoparticles is 51.81 nm, and the same figure shows the homogeneous distribution of copper nanoparticles on the surface. As for the accumulation of copper oxide nanoparticles, it is noticed that the average diameter of the copper oxide particles is 31.22 nm and the RMS surface roughness of the copper oxide nanoparticles was 4.96 nm. These results were agreed with those obtained by (4) who reported The analysis using the atomic force microscope (AFM) that was used in several researches to study the surface properties, size of nanoparticles and surface roughness of copper nanoparticles (CuONPs). The two- and three-dimensional images of all measured nanoparticles showed that they were all of regular and uniform shape and size.

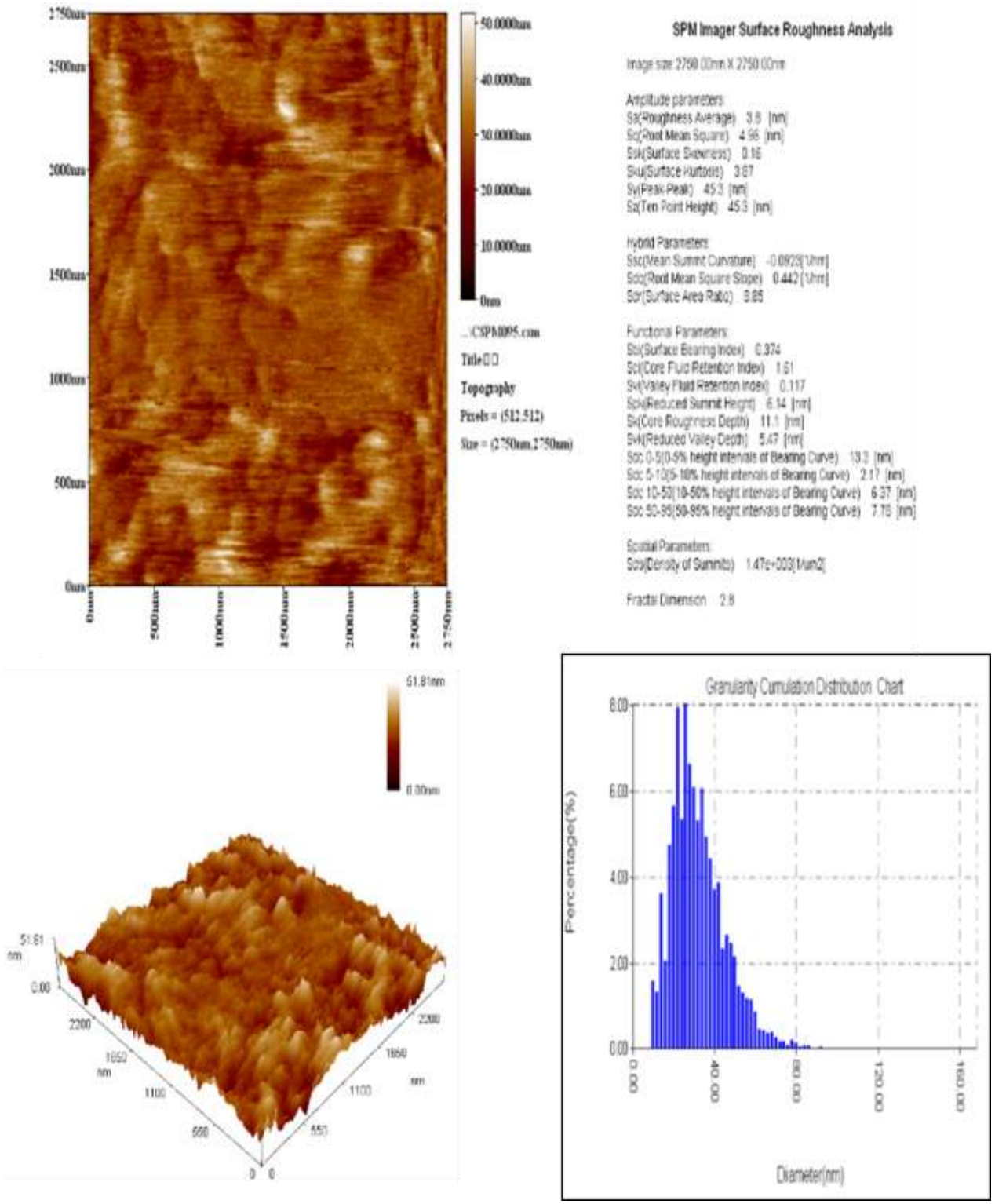

Figure 1. Three dimentional image of the surface morphology for CuONPs using AFM

Data in Table 1 shows a significant decrease in the contamination rate of *E. purpure*a seeds, the concentration of (1, 2 and 3)% sodium hypochlorite recorded (83, 38 and 3.4)% respectively compared to 100% contamination rate that control treatment records, and the lowest rate recorded in the concentration of 3% sodium hypochlorite, according to the treatment duration time, A significat decrease were obtained with the increasing of treatment duration time recording 45.3, 53.6 and 69.8 % through 5, 10 and 15 min respectively. While the interaction between the duration time of treatment and sodium hypochlorite conc. exhibited that the full reduction in the contamination rate recorded in 3% sodium hypochlorite at 10 and 15min recording 0.0 contamination rate respectively, for that the *E. purpure*a seeds were treated using 3% sodium hypochlorite for 15min to ensure 100%

sterilization of *E. purpure*a seeds during germination period. in their study, they mentioned that the nanoparticles manufactured *In vitro*, as well as the essential oils of the clove plant in its normal or nano-processed form, showed an anti-fungal effect against *A. solani*, *F. solani* or *F. oxysporum*. Also, laboratory treatments with laboratory-purified flavonoids or nanoparticles manufactured from green sources led to an increases in the production of polyphenol oxidase in all fungi used in the experiment. Alwash et al. (6), demonstrated in their study that M-CuONPs are selective as an antifungal treatment against Saprolegniasis, where they (M-CuNP) were used as a fair alternative treatment to chemotherapeutic compounds used in aquaculture feeding to achieve a cost-effective, hygienic and environmentally friendly benefit.

Table 1. Effect of sodium hypochlorite concentrations on the contamination rate of *E. purpurea* seeds grown in MS medium for a period of seven days. n=10

Sodium hypochlorite concentration %	Contamination rate % Treatment duration time (min)			Means
	5	10	15	
0	100	100	100	100
1	100	83	67	83
2	69.0	31	14	38
3	10.0	0.0	0.0	3.4
L.S.D. (0.05)		3.35		1.93
Mean	69.8	53.6	45.3	
L.S.D. (0.05) time		1.67		

Reselts in Table2 show a significant effect of CuONPs at 50 mg.L⁻¹ concentration in term of germinated seeds which had the highest rate 6.75 germinated seeds, compared to the control treatment which had the lowest germination rate 0.0 seeds (No germination). The time factor affected germination rate, 6 and 9 days treatments had the highest rate (4.35 and 4.45) germinated seeds, respectively. the two treatments (6 and 9 days) and did not differed significantly from each other, the lowest rate of germinated seeds was in the control treatment 0.0 seeds, no germination at all replicates observed. In the two-ways interaction between CuONPs concentration and time, the highest rate of germinated seeds was achieved when seeds were grown on MS nutrient medium containing CuONPs of 50 mg,L⁻¹ for 9 days and rated 7.80 germinated seeds, which was significantly higher than the rest of the treatments. As for the lowest rate of the germinated seeds, 0.0 seeds (no germination), it was achieved in the interaction between CuONPs 0.0 mg, L⁻¹ and 3 days treatment The survival rate of soaked seeds with CuONPs during 12 days was higher than the addition of CuONPs to the medium as shows in Table3, 75 mg, L⁻¹ CuONPs concentration resulted about 9.60 germinated seed. compared with both control and 25 mg, L⁻¹ con. Of CuONPs which did not germinated (0.0 seeds). Soaking time factor results also showed the highest germination rate in 6 days soaking time 3.95 seeds. The results also showed no significant differences between 3, 9 and 12 days which achieves the lowest seeds germination rate 3.25,3.45 and 3.15 germinated seeds, respectively. The interaction between both Nano CuONPs concentrations and soaking time factors results, clarify that the highest Nano CuO concentration 75 mg. L⁻¹ combined with 6,9 and 12 days of soaking with CuONPs which achieved the highest germination rate 10.0 seeds (100% germination) comparing with the lowest germination rate which was conducted in the control treatment (0.0 mg. L⁻¹) and its combinations with 3,6,9 and 12 days soaking time, the same rate 0.0 germinated seeds was observed from the combinations of Nano CuONPs 25 mg, L⁻¹ and 3,6,9 and 12 days soaking period.

Table 2. Effect of different concentrations of copper oxide nanoparticles mg, L^{-1} on seed germination of *E. purpurea* grown on MS medium . n=10

Concentration of CuO NPs (mg. L^{-1})	Time (days) 3	6	9	12	Means
	Germinated seeds				
0.0	0.00	0.00	0.00	0.00	0.00
25	4.20	5.60	5.80	6.40	5.50
50	4.80	7.20	7.80	7.20	6.75
75	5.20	4.60	4.20	3.20	4.10
L.S.D 0.05		1.15			0.57
Mean	3.55	4.35	4.45	4.00	
L.S.D 0.05		0.57			

The reason behind the increases in seeds germinatation could be the stimulating action of nanoparticles in urging to break the dormancy of seeds, which then results in stimulating the cells division of seeds under treatments. Researchers pointed out the importance of the role played by nanoparticles for plant growth and seeds germination. Texturally, it increases and speed up the germination of seeds. an experiment conducted to determine the effect of nano-gold element with a size of 24 nm (10 - 80 micrograms per ml) added to the nutrient MS medium on the germination of *Arabidopsis thaliana* L. seeds, the Nano Agent improved the seeds Germination, it increased threefold compared to the control treatment, in addition to the improvement in plant growth (14). It was explained by Siddiqui et al. (23) that treating tomato seeds with 8 g, L^{-1} SiO_2NPs led to an increase, in the percentage of seeds germination, germination time, germination coefficient, and seed germination strength coefficient, as well as the fresh and dry weights.

Table 3. Effect of soaking *Echinacea purpurea* seeds with different concentrations of CuO NPs after 12 days of culture, n=10

CuO NPs con. (mg, L^{-1})	Time (days) 3	6	9	12	Means
	Germinated seeds				
0.0	0.00	0.00	0.00	0.00	0.00
25	0.00	0.00	0.00	0.00	0.00
50	4.60	5.80	3.80	2.60	4.20
75	8.40	10.00	10.00	10.00	9.60
L.S.D 0.05		0.91			0.45
Means	3.25	3.95	3.45	3.15	
L.S.D 0.05		0.45			

The Data in Table 4 indicats a significant increase in the shoot number up to 8.60 for concentration 50 mg,l^{-1} of CuONPs that concedered the highest rate comparing with (0.0) that recorded on vials with concentration of CuONPs in control . while the shoot length increased significantly in 25mg.l^{-1} recording 14.50 cm then decreased in 50 and 75 mg.l^{-1} recording 12.90 and 10.50cm respectively. The seedlings dry weight increased significantly up to 198.2 mg in 50mg,l^{-1} CuONPs that recording the highest dry weight, then it decreased significantly in 75mg,L^{-1} CuONPs (83.9mg), and these results were disagreed with those obtained by Eliene et al.(10), Yang et al. (25) who recommended that 62.5, 125 or 250mg/L CuO-NPs dissolved in nutrient solution (Yoshida's) could negatively affect the rice seedlings growth and the contents of chlorophyll in rice plants. Aslo, oxidative effect was observed in rice shoots that exposed to CuO-NPs at different concentrations, at the time, and the electrical conductivity or the content of MDA significantly upregulated incomparasion to the control, that the activity of SOD was significant in rice seedlings roots that exposed to 125 mg/L CuONPs (25). This differences in the results could be due to the lowest concentrations used in this study. Also it was shows that with increasing the concentration of nanoparticles, the readings begin to decrease. However, root length, germination rate, and biomass decreased, while Cu uptake was increased in roots and shoots with high concentrations of CuO NPs. It was observed

that copper nanoparticles (CuO NPs) aggregated in plant cells especially in chloroplasts, and was accompanied by fewer thylakoids per granome. The photosynthetic rate, stomatal conductance and transpiration rate and the maximum quantitative yield of PSII photochemistry and photosynthetic pigment contents decreased significantly (7). Faraz et al.(11) reported that plants treated with CuO NPs recorded an increases in growth and biomass over their control. Among the different concentrations of CuO NPs (0, 2, 4, 8, 16 mg, L^{-1}) had a clear effect on the plant as 8 mg, L^{-1} proved to be the optimal treatment using the foliar spray method and increased the chlorophyll content, proline content, and assimilation rate. net photosynthesis in leaves, and antioxidants as enzyme activity. It could be concluded that CuO NPs interact dystrophic cells triggering conductive biochemical pathways to enhance growth traits.

Table 4. Shoot number , shoot length, and dry weight of *E. purpurea* grown on MS medium supplemented with different concentrations of copper oxide nanoparticles mg.l^{-1} four weeks after cultivation. n=10

CuO NPs con. (mg, L^{-1})	Shoot number	Shoot length (cm)	dry weight (mg)
0	0.0	0.0	0.0
25	4.50	14.50	92.50
50	8.60	12.90	198.20
75	3.30	10.50	83.90
Mean	4.10	9.47	93.60
L.S.D. (0.05)	0.88	1.26	21.41

The results in Table 5 show the effect of treatment 3 as the seeds were soacked in different concentrations of copper oxide nanoparticles, shoot number shoot length and dry weight recorded the highest numbers in concentration 75% as they were, 8.70, 9.70 cm and 204mg, respectivly compered with concentration of copper oxide nanoparticles in 50% (6.30,7.70cm and 153.20mg) while no data were recorded in concentration of copper oxide nanoparticles in 25% and the control negative treatment. It was clear that using saoking seeds method with different concentrations of CuO NPs has different results from adding CuO NPs directly to MS medium, because the higher concentrations enhanced seed germination on MS medium while lower concentrations didn't inhance germinathion at all, the seedlings were shorter and has yallowish green leaves. this disagreement with (thakur) who showed that higher concenrations of CuO NPs have given negative impacts like retarded growth in wheat seeds in vitro In addition, physiological indexes associated with antioxidants, including membrane damage and antioxidant enzyme activity, were also detected. Saoking seeds for an hour with CuO NPs a trend of plant recovery after 1h of NPs exposition. Based on these findings, we can conclude that the formation and operation of the photosynthetic apparatus of the seedlings were impacted when submitted to CuO NPs, which affects the development and growth of the plants. These results agreed with Ying et al.(26) weare the low concentrations didn't enhance germination rate as for the chlorophyll content of the cotyledon leaves of S. virgata, after 120 h of germination, there was an increase in chlorophyll fluorescence intensity in photosystem II at lower concentrations of nanoparticles and a suppression of chlorophyll activity at higher concentrations of nanoparticles, which suggests that CuO NPs were able to penetrate the seeds(9,11).

Table 5. Shoot Number, shoot length, and dry weight of *E. purpurea* grown on MS medium (after saoking for 1 hour with different concentrations of copper oxide nanoparticles) mg.l^{-1} four weeks after cultivation. n=10

CuO NPs con. (mg.l^{-1})	Shoot number	Shoot length (cm)	dry weight (mg)
0	0.0	0.0	0.0
25	0.0	0.0	0.0
50	6.30	7.70	153.20
75	8.70	9.70	204.0
Mean	3.75	4.35	89.30
L.S.D. (0.05)	0.64	1.30	20.51

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