

***In Vivo* Efficiency of Silver Nanoparticles and Copper Nanoparticles on Date Palm Seedling (cv. Sewi) and Its Genetic Stability**

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Abstract

Date palm is one of the important fruits in Egypt and the Arabian Peninsula. Nanotechnology, a modern field of study, has been applied to enhance various aspects of agriculture. In this study, we utilized silver and copper nanoparticles (AgNPs and CuNPs) to evaluate their effects on the characterization of date palm plants. Both AgNPs and CuNPs were chemically synthesized, and their absorption spectra were measured, revealing peaks at 415 nm for AgNPs and 575 nm for CuNPs. Plantlets (cv. Sewi) were treated with different concentrations of AgNPs and CuNPs (25, 50, and 100 mg/L). Physiological analysis showed an increase in total phenols in plants treated with 100 mg/L of AgNPs, while no changes were observed in total indoles across all treatments. Amino acid levels significantly increased with CuNPs at concentrations of 50 and 100 mg/L. Additionally, antioxidant levels, as well as chlorophyll A and B, increased with CuNPs at 100 mg/L and with AgNPs at 25 mg/L. Interestingly, carotenoid levels were highest in the control group compared to all treatments. The genetic stability of the treated plants was assessed using ISSR-PCR with 10 ISSR primers, and the results indicated no significant genetic changes in the treated plants compared to the control explants. This study highlights the potential of nanoparticles to enhance the physiological traits of date palm plants while maintaining their genetic stability.

Keywords: Date palm, silver nanoparticles, copper nanoparticles, genetic stability.

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Introduction

One of the earliest plants ever domesticated by humans is the date palm (*Phoenix dactylifera* L.), a tropical and subtropical fruit tree indigenous to Egypt and the Arabian Peninsula (Abd Allah, 2018). Al-Shahib and Marshall (2003) state that the date palm is a monocotyledonous, dioecious plant belonging to the genus *Phoenix* and the family Arecaceae. Date trees are essential for culture, religion, medicine, ornamentation, and sustenance (Haq and Khan, 2020). According to FAO (2022), Egypt produced 1685035 tons of yield, compared to 9475420 tons produced worldwide. In many nations, date fruits are a staple diet that is rich in vital elements like fiber, carbs, vitamins, and minerals. For those who live in areas with few agricultural resources, they represent a very significant source of nutrition and energy (Ghnimi *et al.*, 2017). A contemporary scientific topic, nanotechnology finds use in practically every industry, including agriculture (Shukla *et al.*, 2019). Farmers use nano-pesticides, nano-fertilizers, nano-biosensors, and nano-metrological instruments to improve soil conditions and increase crop yield and

quality (Dimetry and Hussein, 2016). They also use sensors to automate the agriculture sector and forecast local weather (Khan and Upadhyaya, 2019). Copper (Cu) is a crucial naturally occurring micronutrient for plants that functions as both an enzyme and a co-enzyme in a variety of plant metabolic processes (Cumplido-Nájera *et al.*, 2019). Cu is essential for the lignification of cell walls through the metabolism of polyphenols, which in turn affects the water balance in plants. Additionally, it supports the processes of respiration, protein metabolism, photosynthesis, and glucose synthesis. It also plays a major role in the synthesis of chlorophyll (Taran *et al.*, 2017). Silver (Ag) is seen as advantageous, promotes the growth and development of plants, and makes a significant contribution to soil bioremediation (Javed *et al.*, 2020). Copper nanoparticles (CuNPs) and silver nanoparticles (AgNPs) make plants more resistant to drought (Yasmeen *et al.*, 2015) and They are crucial for increasing plant development, photosynthetic efficiency, seed germination, and plant body growth (Jhanzab *et al.*, 2019). In this investigation, study of different concentrations synthesized CuNPs and AgNPs on the growth of date palm plantlets (sewi cultivar) and studying of the genetic stability of treated plants.

Materials and methods

All experiments in this study were conducted at the Agricultural Research Center (ARC), Central Laboratory for Date Palm Research and Development (CLDPRD) in Giza, Egypt.

Synthesis of silver nanoparticles (AgNPs)

In accordance with Šileikaitė *et al.* (2006), silver nanoparticles (AgNPs) were created in the manner described below: The heater was used to bring 50 milliliters of 1 mM AgNO₃ (made in deionized water) to a boil. Then, while stirring, a 1% w/v sodium citrate solution was added by dropping it onto the boiling AgNO₃. The mixed solution's color changed to a light yellowish hue as a result of the preceding operation. Taking the mixture off of the heater, it was allowed to cool to room temperature. A solution with a concentration of 100 mg/ml was obtained by centrifuging the cooled mixture, collecting the pellet, weighing it, and re-suspending it in deionized water.

Synthesis of copper nanoparticles (CuNPs)

The chemical reduction method as mentioned by Biçer and Şişman (2010) was used to create CuNPs. L-ascorbic acid (0.11M) was added to an aqueous solution of CTAB (0.09M). The temperature was maintained at 85 °C while the pH was adjusted to 6.8 using a NaOH solution. As the CTAB and L-ascorbic acid combination was being stirred, a solution of copper sulfate pentahydrate (0.03M) was added. Until a reddish brown hue formed, the reaction was maintained. Centrifugation was then used to gather CuNPs for use and characterization.

UV-Vis spectroscopy of AgNPs and CuNPs

In accordance with Alfarraj *et al.* (2023), the absorbance of produced AgNPs and CuNPs was measured. Using a UV/Visible spectrophotometer (Orion-Aquamate 8000 UV/VIS), the absorbance was measured at intervals of 5 nm and in the wavelength range of 300 to 800 nm.

***In vivo* effect of AgNPs and CuNPs on plant growth**

Date palm plantlet preparation

Sewi cultivar seeds were made in the manner outlined by Abd Allah (2018): After being gathered and thoroughly cleaned with dH₂O, the seeds were completely immersed in a Rhizolex fungicide solution (3 grams per liter) for ten days, with the water being changed every two days. Following that, seeds were incubated at 37°C and 50% humidity for 50 days. Following seeding, the plantlets were thoroughly immersed in 3% hypochlorite (5% conc.) for ten minutes before being cleaned with sterile dH₂O. Ultimately, the plantlets were placed in sterile pots with sterile clay soil and peat moss (1:1 w/w) and allowed to mature for six months before being used.

Treatment *In vivo*

Three different concentrations of each AgNPs and CuNPs (25, 50, and 100 mg/l) were tested on date palm plantlets. Each pot has three plantlets, and there were 3 replicates of each treatment and watered with the concentrations of AgNPs and CuNPs. Each 15 days the pots were watered again with the previous concentrations for 90 days. Finally, plantlets were collected to study the efficiency of AgNPs and CuNPs.

Physicochemical changes

For the physicochemical estimation, three replicates of each treatment were used. To one gram of each treatment sample, five milliliters of 80% ethanol were applied. Following a pestle and mortar homogenization, each sample was stored in the dark at 4°C for a full day. Following centrifugation at 13000xg/5min, the supernatant of samples was put into an assay for total amino acids, total phenol, total indols and pigments.

Total phenol contents

One milliliter of each previously extracted sample, 5ml milliliters of dH₂O, and 0.5ml of the Folin-Ciocalteu reagent (F-C) were placed in a tube. The substance was well mixed after 5ml of vortexing. After adding 1.5 ml of 20% sodium carbonate (Na₂CO₃), the volume rose to 10 ml. The mixture was then allowed to settle in the dark for two hours at room temperature. At 765 nm, the samples were finally read on the Orion AquaMate 8000 spectrophotometer. This method was carried out according to Ainsworth and Gillespie (2007).

Total indol contents

Total indol contents were estimated using the methodology described by Selim *et al.* (1978) as follows: Four milliliters of Para di-amino benzoate (PDAB) were added to a

tube containing one milliliter of the aliquot sample. After that, the tubes were incubated at 37°C for one hour. The samples were read at 530 nm using the Orion AquaMate 8000 spectrophotometer.

Total amino acid assay

1ml of the extracted sample was added to the test tube, dH₂O was added to fill the remaining 4 milliliters, and then 1 milliliter of ninhydrin was added as a reagent. Covered, the tubes were placed in the water route for 15 minutes at 100 degrees. The tubes were then placed in a bath of cooled water, and one milliliter of 50% ethanol was added. Samples were finally read at wave-length 570 nm on the Orion AquaMate 8000 spectrophotometer. This procedure was carried out as mentioned by Mc.Grath (1972).

Determination of antioxidants

When assessing the antioxidant activity of extracted materials, 2, 2'diphenylpicrylhydrazyl (DPPH) was used. According to Burits and Bucar (2000), the antioxidant's activity was calculated as follows: After adding 1 milliliter of the extracted sample and 1 milliliter of 0.004% (w/v) DPPH dissolved in 95% methanol to a test tube, the combination was allowed to sit at room temperature for 30 minutes. The samples were then reddened at 517 nm using an Orion AquaMate 8000 spectrophotometer, and quercetin was used as a positive control to compare them to DPPH, which served as a blank. The following formula was used to determine the antioxidant's percentage activity (%I):

$$I\% = (A_{\text{blank}} - A_{\text{sample}} / A_{\text{blank}}) \times 100$$

I%: The percentage of antioxidant activity

A_{blank}: the absorbance of the positive control

A_{sample}, the absorbance of the tested sample

Pigments assessment

In accordance with Wettstein (1957), plant pigments (like carotenoids, chlorophyll a, and chlorophyll b) were extracted and examined as follows: Using a mortar and pestle, one gram of fresh leaf material was ground with 85% acetone, a small amount of CaCO₃, and sterilized sand. After filtering the ground, the residue was periodically cleaned with acetone until the filtrate was completely colorless. 15 ml of acetone to volume was used to finish the extracted solution. This extract's optical density was measured at 440, 640, and 660 nm using a spectrophotometer (Orion AquaMate 8000). The concentrations of carotenoids, chlorophyll a, and b were measured using the Weinstein formula as follows:

$$\text{Chlorophyll A} = 9.784 \times R_{660} - 0.99 \times R_{640} \text{ mg/g}$$

$$\text{Chlorophyll B} = 21.426 \times R_{640} - 4.65 \times R_{660} \text{ mg/g}$$

$$\text{Carotenoids} = 4.695 \times R_{440} - 0.268 \times C (\text{chl. A} + \text{chl. B}) \text{ mg/g.}$$

Stability of genes

Inter-simple sequence repeat (ISSR)-PCR was used to compare the fingerprinting of the treated samples with the control to assess the genetic stability of the treated plants.

DNA extraction

DNA was isolated from plant tissue in the manner described by Arif *et al.* (2010): 500 µl of lysis buffer (100 ml/pH 8.0 with 0.4 g Na₂EDTA, 1.21 g tris-base, 8.12 g NaCl, 2.0 g CTAB, and 2.0 g PVP) was used to grind 100 milligrams of plant tissue in a mortar. The pulverized tissue was moved into a 1.5 ml Eppendorf tube and maintained at 60°C for 30 minutes in a water bath. The samples were then centrifuged at 9500xg/5min, and the supernatant was moved to a fresh Eppendorf tube. After adding an equal amount of the chloroform:isoamyl-alcohol (24:1) combination to the transferred supernatant and vortexing thoroughly, the samples were centrifuged at 9500xg/5 minutes. Ten percent of the transferred volume from 3M potassium acetate and 500µl of cool isopropanol were added to the aqueous phase, which was then carefully mixed and centrifuged at 11500xg/10min. After discarding the supernatant and adding 500 µl of cold ethanol, the samples were centrifuged at 7000xg/5 minutes. Before being used, the pellet was stored at 4°C after being reconstituted in 100µl of sterile dH₂O.

PCR assay

As indicated in Table (1), ISSR primers (10 primers) were used for the DNA fingerprint of the treated plant. Two microliters of DNA (~250–400 ng), two microliters of each primer (10 p.mol), 12.5 microliters of TaqMan (OmniPCR, BIO-HELIX), and 6.5 microliters of dH₂O make up the 25 microliter PCR reaction. The MJ-Research thermo-cycler (PTC-200) was used to perform the PCR amplification protocol, which was 94°C/5min, 35 cycles (94°C/45sec, 50.5°C/45sec, 72°C/90sec, and 72°C/7min). Using agarose (1.2%), the samples were electrophoresed for 30 minutes at 120V, and the gel was stained with ethidium bromide dye (1 mg/ml).

Analysis of data

Amplified fragments of ISSR-PCR were scored as present (1) or absent (0) and only clear fragments were scored. Using Past program (version 4.03), a matrix of similarity using Jaccard's coefficient was used to analyze the obtained data.

Statistical analysis of data

All collected data were statistically examined utilizing SPSS version 23 and one method of analysis of variance (ANOVA), following Snedecor and Cochran (1980).

Table (1): List of ISSR primer sequences used in PCR

No.	Name of primer	Sequence 5'-3'	
1	ISSR-1	AGAGAGAGAGAGAGAGAYC	Abouseada <i>et al.</i> , (2023)
2	ISSR-2	AGAGAGAGAGAGAGAGAYG	
3	ISSR-3	ACACACACACACACACYT	
4	ISSR-4	ACACACACACACACACYG	
5	ISSR-5	GTGTGTGTGTGTGTGYG	
6	ISSR-6	CGCGATAGATAGATAGATA	
7	ISSR-7	GACGATAGATAGATAGATA	
8	ISSR-8	AGACAGACAGACAGACGC	
9	ISSR-9	GATAGATAGATAGATAGC	
10	ISSR-10	GACAGACAGACAGACAAT	

Results and discussion

UV-Vis spectroscopy

The absorbance spectra for manufactured AgNPs and CuNPs were recorded using a UV/visible spectrophotometer at wavelengths ranging from 300 to 800 nm with a 5-nm interval, and the peak was identified. The data obtained, as displayed in Fig. (1), indicated that the absorbance peaked at 415 nm with AgNPs and was at 775 nm in the case of CuNPs. According to the preparation technique and purity, the particle radius in theoretical calculations varied from 5 nm to 100 nm. The size, purity, and concentration of AgNPs determined the range of the peak that formed from the absorbance to AgNPs, which had to be between 400 and 420 nm (Christopher *et al.*, 2011) and in CuNPs in the 560–590 nm range (Henglein 2000). As mentioned by Guzmán *et al.* (2009), hydrazine hydrate was used as a reducing agent in the preparation of AgNPs, and the highest absorption band was located at 418 nm. Additionally, AgNP absorption peaked at 411 nm, according to Kumar *et al.* (2017). Alfarraj *et al.* (2023) observed that the highest absorption of synthesized AgNPs occurred at 415 nm. On the other hand, CuNPs Mohamed *et al.*, (2018) reported that the synthesized CuNPs was measured using spectroscopy and the peak was at 572nm. While Yaremchuk *et al.*, (2019) referred that the adsorption spectrum of CuNPs was at 594nm.

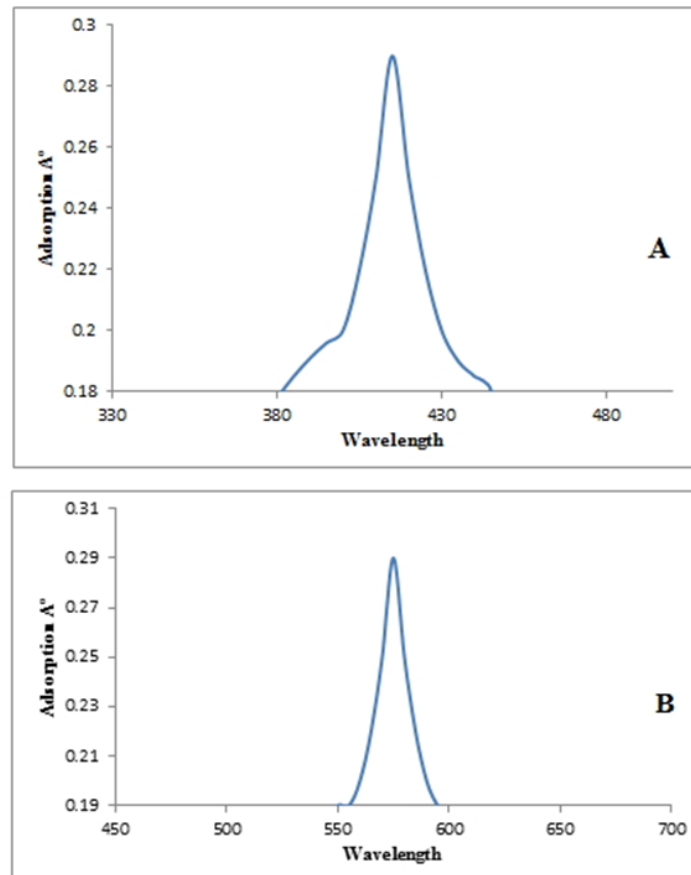


Fig (1): Absorbance spectra of synthesized nanoparticles A) AgNPs, B) CuNPs

Physicochemical changes

Some of the physicochemical changes (including total phenol, total indol, total amino acid, and antioxidants) on plantlets caused by the effect of different concentrations of nano-silver and nano-copper were evaluated (as shown in Table 2 and Fig 2). Also, pigment changes (including chlorophyll a, chlorophyll b and carotenoids) on the previous plantlets were estimated and tabled as shown in table (3) and illustrated in fig (3): Results showed that the high concentration of total phenol was in plants that were treated with 100mg/l of AgNPs. While the low concentration was in 25mg/l of AgNPs plantlets. CuNPs and AgNPs are considered effective factors in forming the phenol in the shikimic acid pathway. These results were in agreement with Mohamed *et al.*, (2018) when they stated that the level of phenol increased when using several concentrations of CuNPs up to 2g/l. While (Sadak, 2019) mentioned that AgNPs with a concentration of 40 mg/l worked on increasing the ratio of phenol in Fenugreek (*Trigonella foenum-graecum*). Total indol was evaluated on the treated plantlets, results showed there were non-significant between all treatments and control. Mohamed *et al.*, (2018) and Rodríguez *et al.* (2023) explained that CuNPs stimulate the plantlets for increasing the total indol. In the case of total amino acid, the obtained results referred to there were high levels of amino acid in the plants treated with CuNPs with concentrations 50 and 100mg/l and there were non-significant between other treatments.

Antioxidants in treated plantlets were measured and the high level of antioxidants was at CuNPs with 100mg/l and was at AgNPs with a concentration of 25 mg/l in treated plantlets. CuNPs may increase the total phenol which is considered one of the antioxidants factors. Mohamed *et al.*, (2018) mentioned that antioxidants increased in the high level of phenol and high concentration of CuNPs.

Table (2): Evaluation of physiochemical changes in treated plantlets

Treatments	Total phenol mg/g	Total indol mg/g	Total amino acid mg/g	Anti-oxidants %
Control	21.82 ^d	0.414 ^a	1.54 ^{ab}	83.17 ^c
AgNPs (25)	2.70 ^a	0.331 ^a	0.40 ^a	91.17 ^d
AgNPs (50)	6.22 ^b	0.336 ^a	0.88 ^a	84.25 ^c
AgNPs (100)	46.82 ^f	0.368 ^a	1.50 ^{ab}	83.00 ^c
CuNPs (25)	7.73 ^b	0.467 ^a	0.86 ^a	66.83 ^a
CuNPs (50)	18.63 ^c	0.396 ^a	2.29 ^b	68.67 ^b
CuNPs (100)	36.37 ^e	0.378 ^a	3.47 ^b	91.17 ^d
P-value	0.001	0.001	0.001	0.001
LSD	2.15	0.19	1.71	1.35

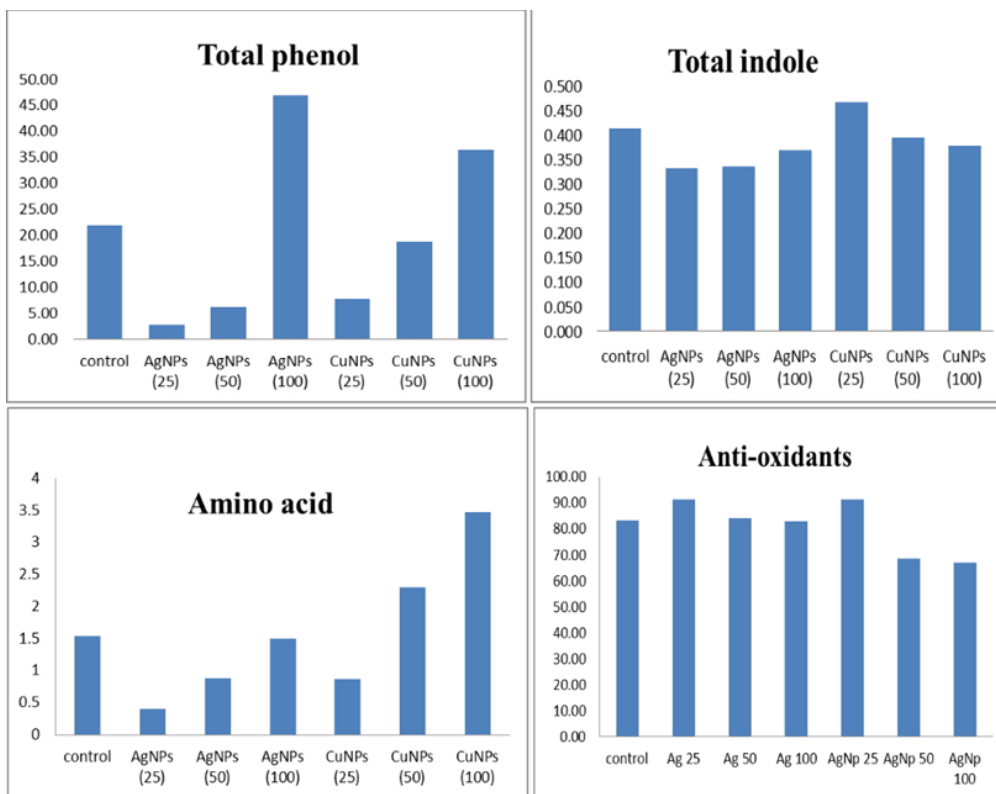


Fig. (2): Evaluation of physiochemical changes in treated plantlets

Pigments also were estimated in the treated plants, both chlorophyll a and chlorophyll b were increased in the treated plants with CuNPs and AgNPs with concentrations of 100 mg/l and 25 mg/l respectively compared with the control which was the low concentration. On the other hand, total carotenoid values were recorded at a high level in control and CuNPs with a concentration of 50 mg/l. when the low concentration in all treatments of AgNPs with a concentration of 25 mg/l. On the contrary in the case of total carotenoids decreased comparing with increasing of chlorophyll a and chlorophyll b. the previous results were agreement with Abd Allah (2018) noted that chlorophyll a and chlorophyll b increased in the high level of phenol and total carotenoids decreased in the same case. In case of total carotenoid values, high level was in control and CuNPs with a concentration 50 mg/l. when the low concentration in all treatments of AgNPs with a concentration 25mg/l. Total carotenoids decreased comparing with increasing of chlorophyll a and chlorophyll b. the previous results were agreement with Abd Allah (2018) noted that chlorophyll a and chlorophyll b increased in the high level of phenol and total carotenoids decreased in the same case.

Table (3): Evaluation pigments in treated plantlets

Treatments	Chlorophyll A mg/g	Chlorophyll B mg/g	Carotinoids mg/g
Control	13.03 ^b	18.54 ^a	5.19 ^c
AgNPs (25)	9.14 ^a	26.88 ^d	0.72 ^a
AgNPs (50)	10.03 ^a	17.45 ^a	2.77 ^b
AgNPs (100)	14.03 ^b	24.37 ^c	3.75 ^{bc}
CuNPs (25)	9.57 ^a	17.91 ^a	3.74 ^{bc}
CuNPs (50)	13.62 ^b	23.95 ^c	5.15 ^c
CuNPs (100)	21.26 ^c	21.16 ^b	4.10 ^{bc}
<i>P-value</i>	0.001	0.001	0.001
LSD	2.45	2.56	1.66

Stability of DNA

ISSR-PCR using 10 ISSR primers was used to test the genetic stability of the treated samples with varying doses of AgNPs and silver nitrate. The results of the PCR fragments on the electrophoresed gel for each primer were recorded, and the similarity and distance indices were computed. There was no change in the results of the fragments that were produced in the PCR gel, according to the ISSR-PCR test findings shown in Fig. (4), therefore the similarity result was stable at 100% in all treated samples.

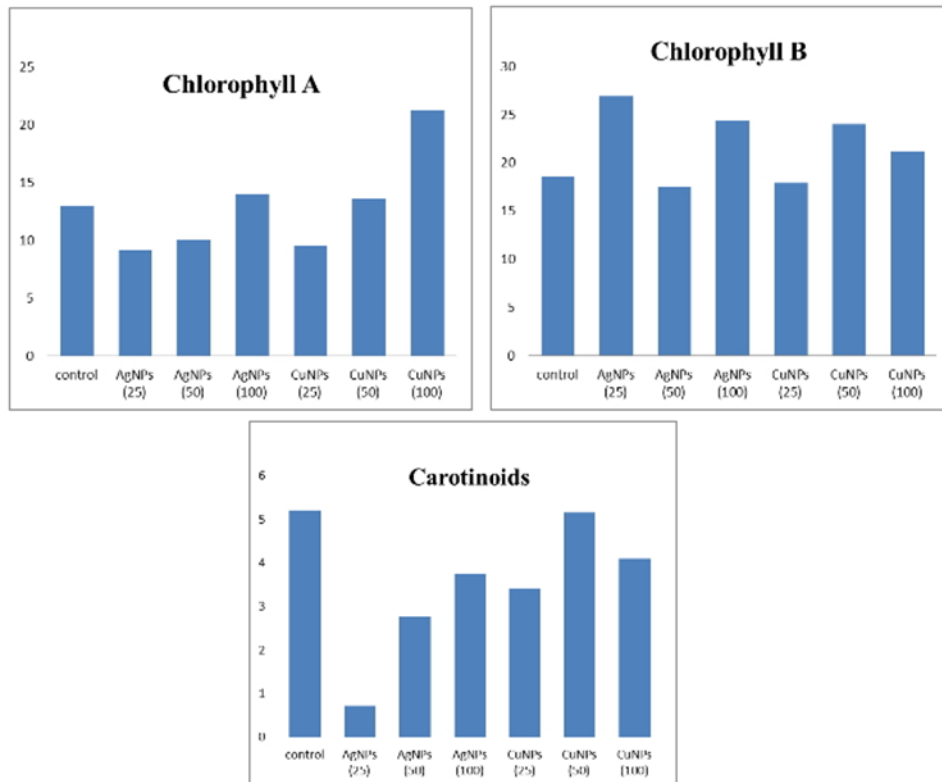


Fig (3): Evaluation pigments in treated plantlets

According to the data, the genetic structure of the treated plantlets remained unchanged when compared to the control date plantlet. In line with Al-Mayahi (2022), the genetic stability of date palm plants (cv. Barhee) was evaluated following the application of a chitosan and thidiazuron combination to the explants. Using random amplified polymorphic DNA (RAPD) and RAPD-PCR, genetic stability was assessed. The results showed that the explants' genetic stability remained unchanged when compared to the control. Additionally, Arafa *et al.* (2023a) reported that iron nanoparticles (FeNPs) were applied at several concentrations (0.5, 1, 2, and 4mg/l) to improve the growth of date palm (cv Bartmoda) explants in tissue culture technique. Seven RAPD primers were employed to assess the genetic stability of treated explants. The results of the RAPD-PCR approach showed that, when compared to the control, the genetic stability of the explants remained unchanged. Using the ISSR-PCR approach, Arafa *et al.* (2023b) examined the genetic stability of a date palm (cv Zaghlol) explant that was generated from tissue culture and contrasted it with the mother plant. The findings indicated that the mother plant and the explants were identical.

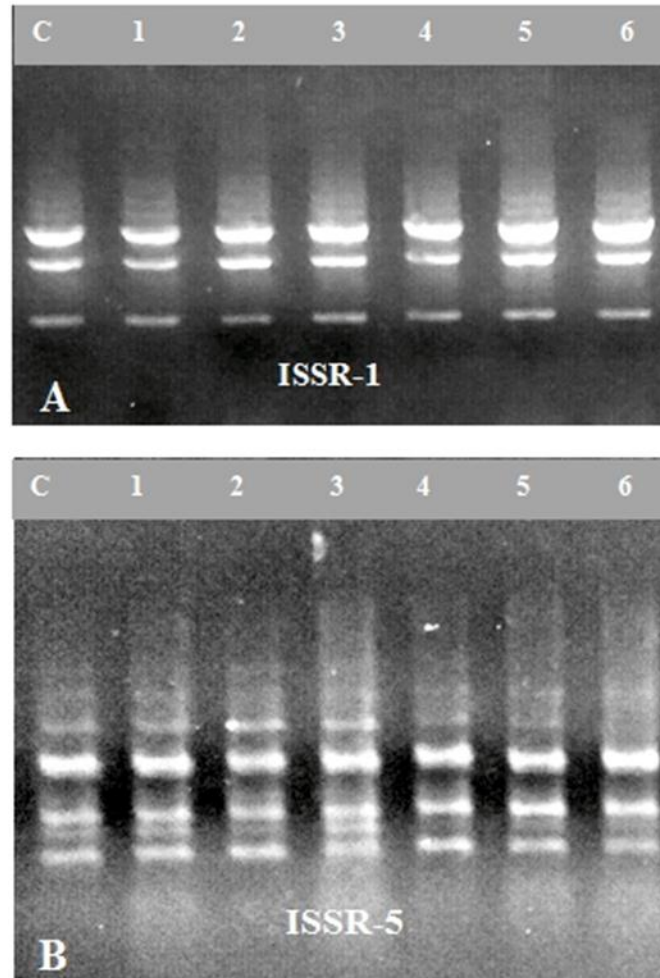


Fig. (4): Gel profiles of treated samples with various concentrations of AgNPs and CuNPs using the ISSR-PCR technique. Panel (A) shows results with the ISSR-1 primer, and panel (B) shows results with the ISSR-5 primer. C: control sample; 1: AgNPs (25 mg/L); 2: AgNPs (50 mg/L); 3: AgNPs (100 mg/L); 4: CuNPs (25 mg/L); 5: CuNPs (50 mg/L); 6: CuNPs (100 mg/L)

Conclusion

In conclusion, this study demonstrated the beneficial effects of silver and copper nanoparticles (AgNPs and CuNPs) on the physiological traits of date palm seedlings (cv. Sewi). Treatment with AgNPs significantly increased total phenol content, while CuNPs enhanced amino acid levels and antioxidant activity. Importantly, the genetic stability of treated plants remained unchanged compared to control samples, indicating that nanoparticle applications can improve plant characteristics without compromising genetic integrity. These findings support the potential of nanotechnology in agricultural practices for date palm cultivation.

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اختبار كفاءة جزيئات الفضة النانوية والنحاس النانوية على نباتات نخيل البلح (صنف سيوي) وكذلك على مدى الثبات الوراثي

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الملخص العربي

نخيل البلح هو واحد من محاصيل الفاكهة ذو الأهمية الاقتصادية في مصر والمنطقة العربية. تقنية النانو تعتبر واحدة من التقنيات الحديثة التي تستخدم في عدة مجالات مختلفة مثل مجال الزراعة. في هذه الدراسة، تم استخدام جزيئات الفضة النانوية وجزيئات النحاس النانوية في تحسين خواص نباتات النخيل. كل من جزيئات الفضة والنحاس النانوية تم تحضيرهما بطريقة كيميائية وتم دراسة طيف الامتصاص لهما وكانت قمة منحى طيف الامتصاص في النانوالفضة عند ٤١٥ نانومتر وكانت في النانو نحاس عند ٥٧٥ نانومتر. تم معاملة النباتات باستخدام تركيبات مختلفة من الفضة والنحاس النانوية (بتركيزات ٢٥، ٥٠، ١٠٠ مجم/لتر). تم بعد ذلك دراسة مدى تأثير تركيبات النانو المستخدمة على التغيرات البيوكيميائية على النباتات حيث ارتفع محتوى النباتات من الفينولات الكلية في النباتات المعاملة بتركيز ١٠٠ مجم/لتر من جزيئات النانو فضة. في حين لم تظهر أي تغييرات معنوية من مستوى الاندولات الكلية. الاحماض الامينية زادت بشكل معنوي في حالة النانو نحاس بتركيزات ٥٠ و ١٠٠ مجم/لتر. كل من مضادات الاكسدة، الكلوروفيل أ و الكلوروفيل ب زادت في حالة النباتات المعاملة بالنانو نحاس والنانو فضة بتركيز ١٠٠ مجم/لتر و ٢٥ مجم/لتر على التوالي. في حالة الكاروتينات، كانت في اعلى مستوياتها في النباتات الكنترول مقارنة بكل المعاملات. في دراسة مدى الثبات الوراثي، تم استخدام تقنية تفاعل البلمرة المتسلسل (PCR) وذلك باستخدام بادئات من النوع التكرار التسلسلي البيئي البسيط (ISSR) حيث تم استخدام ١٠ بادئات مختلفة، من خلال النتائج المتحصل عليها فقد وضح انه لا يوجد أي تغييرات حدثت في المدى الوراثي للنباتات وان درجة الثبات الوراثي في النباتات لم تتغير وذلك مقارنة مع النبات الكنترول.

الكلمات الدالة: نخيل التمر، جزيئات الفضة النانوية، جزيئات النحاس النانوية، الثبات الوراثي.