

Assessing the impact of copper oxide nanoparticles on growth attributes and regenerative potential in date palm (*Phoenix dactylifera*) micropropagation

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Abstract

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Copper oxide nanoparticles (CuO-NPs; 0–40 mg L⁻¹) were applied in MS medium to evaluate microbial contamination, shoot regeneration, and multiplication in date palm (*Phoenix dactylifera* L. cv. Barhee). Compared to the untreated control (0 mg L⁻¹), copper oxide nanoparticles at 10 mg L⁻¹ eliminated microbial contamination without phytotoxicity, yielding the highest regeneration frequency (88.60%) and maximum shoot number (14.25 shoots jar⁻¹), whereas 40 mg L⁻¹ caused 21.6% culture mortality. Supplementation with 10 mg L⁻¹ CuO-NPs significantly increased shoot nutrient accumulation (N, P, K, Fe, Zn, Cu) relative to the control and other treatments ($p \leq 0.05$). Antioxidant enzyme activities (SOD, CAT, POD), total soluble carbohydrates (152.10 mg g⁻¹ DW), and free amino acids (8.05 mg g⁻¹ DW) reached maximum values at 10 mg L⁻¹, compared to the control, whereas soluble protein declined to 10.40 mg g⁻¹ DW at 40 mg L⁻¹, dropping below the untreated control. Root induction was highest at 10 mg L⁻¹ CuO-NPs, while higher concentrations (≥ 20 mg L⁻¹) suppressed rooting and induced root cellular modifications compared to the control. Plantlets derived from the 10 mg L⁻¹ CuO-NPs treatment achieved the highest ex vitro acclimatization survival rate (88%). Genetic fidelity analysis using monomorphic RAPD and ISSR markers confirmed genetic stability among regenerated plantlets relative to mother plants and control cultures.

Keywords

antioxidant activity, carbohydrate and nitrogen metabolites, copper oxide nanoparticles, date palm, genetic stability, histological analysis, micropropagation, nutrient uptake

Introduction

Date palm (*Phoenix dactylifera* L.) is a dioecious perennial monocotyledonous plant belonging to the Arecaceae family. Date fruit contains a wide variety of chemical constituents with valuable nutritive and medicinal properties (AL-MAYAH, 2025a; 2026a). Furthermore, the date palm represents the “tree of life,” creating a microclimate in arid and semi-arid regions that mitigates drought effects, maintains low-salinity soil moisture, and fosters a biodiversity hotspot for various plant and animal species under harsh desert conditions.

Conventionally, date palms are propagated by seeds or offshoots. However, seed-derived plants yield approximately 50% male palms which cannot be identified until flowering and exhibit high genetic heterogeneity. Consequently, to ensure orchard uniformity, traditional propagation relies on offshoots. Nevertheless, offshoot propagation is insufficient for large-scale commercial demand, making in vitro propagation the most practical alternative. Plant tissue culture provides a promising solution by overcoming traditional propagation bottlenecks, and its success largely depends on choosing the appropriate culture medium (AL-MAYAH,

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2022a, b; AL-ASADI et al., 2024). In recent years, nanotechnology has emerged as a transformative tool in plant biotechnology, offering novel ways to regulate growth, enhance metabolic functions, improve nutrient delivery, and protect cultures against phytopathogens. Numerous studies have demonstrated the positive effects of nanoparticles (NPs) on callus initiation, shoot growth, and multiplication (ALI et al., 2021; ELSAYH et al., 2022; AL-MAYAH, 2023). Furthermore, NPs have been used effectively to eliminate microbial contaminants in tissue cultures (HASANIN et al., 2023). However, plant performance under nanoparticle exposure is significantly influenced by genotype, as well as the type, size, and concentration of the nanoparticles (ASGARI-TARGHI et al., 2018). Among nanoparticles, copper oxide nanoparticles (CuO-NPs) have emerged as promising agents in agriculture due to their unique physicochemical properties, biocompatibility, low cost, and ease of manufacture (GRIGORE et al., 2016). CuO-NPs serve as essential nutrient sources and exhibit strong antimicrobial properties against bacteria, fungi, viruses, and algae, with a longer shelf life than other inorganic antimicrobials like gold and silver (AMIN et al., 2021; BUKHARI et al., 2021). Previous studies confirm that CuO-NPs (10 mg L^{-1}) successfully promoted organogenesis and shoot regeneration while reducing infection rates in rice, *Stevia rebaudiana*, poplar, and birch cultures (ANWAAR et al., 2016; JAVED et al., 2017; FEDOROVA et al., 2021), in addition to boosting phytochemical production (SIVANESAN et al., 2025). Despite these advances, *in vitro* rooting remains a challenging stage in date palm propagation due to slow natural root formation (IBRAHIM et al., 1999). Although some reports have addressed medium composition and growth conditions for certain cultivars (AL-MAYAH, 2024; 2022b), success remains strongly genotype-dependent. HASANIN et al. (2023) noted that 9 mg L^{-1} of St@CuO-NPs improved growth parameters, rooting efficiency, and chlorophyll content. However, research on monocotyledonous

responses to CuO-NPs remains scarce, with outcomes varying by concentration, exposure duration, and species (FEIGL, 2023). To prevent somaclonal variation a major issue impacting tissue culture utility molecular markers like RAPD and ISSR are routinely utilized to verify genetic fidelity (PHARMAWATI et al., 2021; TIKENDRA et al., 2021; AL-MAYAH, 2026b). Moreover, to comprehensively understand the physiological and metabolic mechanisms underlying morphogenic responses, metabolic parameters (carbohydrate dynamics, nitrogen metabolites, and antioxidant enzyme activities) were analyzed to elucidate how CuO-NPs modulate cellular redox status and nutritional assimilation during morphogenesis. In view of these challenges, this study aims to evaluate the effect of CuO-NPs on *in vitro* shoot regeneration and propagation processes in date palm culture media, alongside biochemical analyses and an assessment of genetic stability using RAPD and ISSR markers.

Materials and methods

Initiation stage

Three-year-old 'Barhee' date palm offshoots were separated from healthy parent plants grown in the Abu al-Khasib district south of Basrah (Fig. 1a). The station has a latitude of $30^{\circ}26' \text{ N}$, a longitude of $48^{\circ}06' \text{ E}$, and an elevation of 3 m above sea level. A total of five individual offshoots were used as donor plants. Shoot tips were excised from the offshoots and used as explants (Fig. 1b). The explants were taken and kept in an antioxidant solution (100 mg L^{-1} ascorbic acid, 150 mg L^{-1} citric acid) for 30 min. Explants were sterilized utilizing 70% ethanol for 1 min and 5% sodium hypochlorite for 20 min, then rinsed three times with sterile distilled water for 1 min each time. For callus induction, explants were implanted on MS basal medium (MURASHIGE and SKOOG, 1962) with 100 mg L^{-1} gluta-

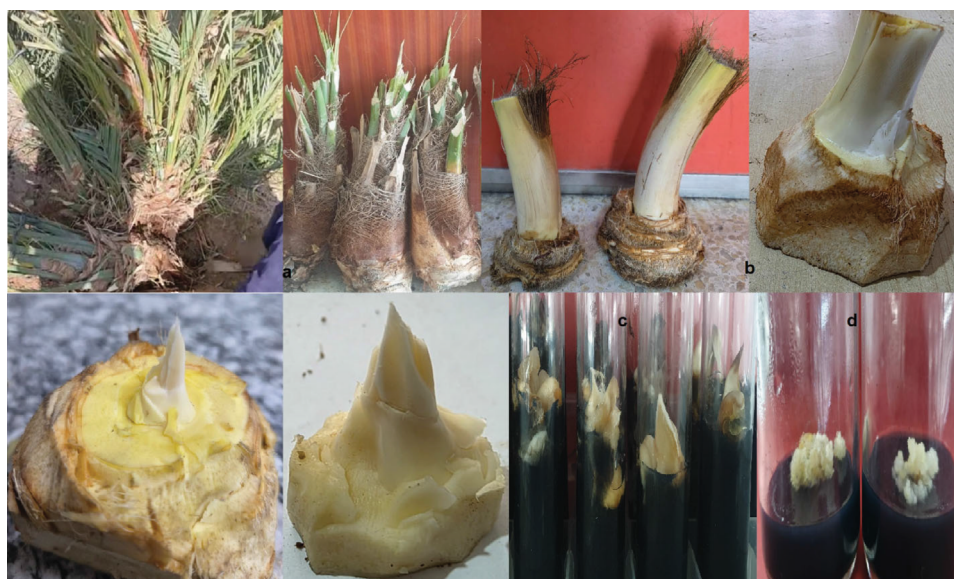


Fig.1. Induction of callus in date palm (*P. dactylifera* L. cv. Barhee) from apical buds of offshoot: (a) Offshoot; (b) The outer leaves were removed until the shoot tip area was exposed; (c) Tissue development; (d) Callus growth, magnification 0.8 $\times$.

mine, 5 mg L⁻¹ thiamine HCl, 1 mg L⁻¹ biotin, 30 g L⁻¹ sucrose, and 2 g L⁻¹ activated charcoal, along with the addition of growth regulators naphthaleneacetic acid (NAA) at 30 mg L⁻¹ and 6-(γ,γ -dimethylallylamino) purine (2iP) at 3 mg L⁻¹, solidified with 6 g L⁻¹ agar. The pH of the medium was adjusted to 5.7–5.8 before the addition of agar. Media were dispensed into culture containers and autoclaved at 121 °C and 1.04 kg cm⁻² for 20 min. Cultures were incubated in complete darkness at 27 ± 2 °C (Fig. 1c). The cultures were transferred to fresh media, with the same composition, every 6 weeks up to callus initiation (Fig. 1d).

Effect of copper oxide nanoparticles (CuO-NP) treatment on indirect organogenesis

To evaluate the effect of copper oxide nanoparticles on *P. dactylifera* growth, *in vitro* callus obtained after two sub-culture cycles (at 6-week intervals) was transferred to new MS media supplemented with CuO-NPs at concentrations of (0, 5, 10, 20, and 40 mg L⁻¹). The CuO-NPs used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA). The product consisted of high-purity (≥99.995%) CuO-NPs with an average particle size < 50 nm. The medium was also supplemented with 1 mg L⁻¹ (NAA), 0.5 mg L⁻¹ 6-benzyladenine (BA), and 0.5 mg L⁻¹ kinetin (K) to promote shoot development. The control treatment was cultured on MS medium with the above plant growth regulators, but without CuO-NPs. The media were sonicated in water bath for 30 min for thorough dispersion of nanoparticles, followed by the addition of agar (6 g L⁻¹). The agar was dissolved by heating and media were dispensed as 30/100 mL conical flask while thorough mixing. The pH of the medium was adjusted to 5.7–5.8 with 0.1 N NaOH or HCl before adding the agar. The culture media were then autoclaved for 15 min at 121 °C and 1.06 kg cm⁻² pressure. All cultures were maintained at 25 ± 2 °C under ambient light of 1,500–2,000 lx by cool white fluorescent lamps and a photoperiod of 16 h light/8 h dark. The results of the experiments regarding the effect of CuO-NPs concentrations on the percentage of shoot regeneration and the number of shoots per jar were evaluated 12 weeks after inoculating callus in the media. There were 24 independent replicates (jars) per treatment, with each jar inoculated with a callus mass weighing approximately 100 mg.

Effect of CuO-NPs on shoot rooting

Root induction (RI)

After the proliferation of adventitious shoots, 25 healthy individual shootlets (5 cm) derived from each different CuO-NPs treatment and control were excised and cultured in jars containing 25 ml of the MS medium supplemented as mentioned above, except that the NAA concentration was adjusted to 1 mg L⁻¹, and were maintained at room temperature (27 ± 1°C) with a 16 h light/8 h dark photoperiod. The percentage of RI (%), the number of roots (Root/shoot), and the average root length (cm) were recorded after 45 days of culture. The root length was determined

by measuring the length of all emerged roots per shootlet using a graduated ruler, and the mean value was calculated for each replicate. Each treatment consisted of 25 replicates.

Percentage of contamination

The percentage of contamination was evaluated based on daily visual inspection; fungal contamination was identified by visible mycelial growth, while bacterial contamination was characterized by media turbidity or bacterial halos around the explant. To ensure a robust assessment of initial contamination rates, each treatment contained 50 replicates (jars). However, only completely healthy, non-contaminated cultures were subsequently maintained and used for tracking shoot proliferation and rooting parameters.

Biochemical parameters

Total soluble carbohydrates

The total carbohydrates were quantified by the anthrone technique adopted from WATANABE et al. (2000). Total carbohydrates were measured at 620 nm, using glucose as a standard.

Free amino acids

Free amino acids were determined using the ninhydrin reagent according to the method of LEE and TAKAHASHI (1966).

Total soluble protein

Total soluble proteins were quantified using the procedure of BRADFORD (1976).

Antioxidant enzyme activities

For enzyme extraction, frozen plant tissue (0.5 g) was homogenized in ice-cold potassium phosphate buffer (50 mM, pH 7.0) containing 1% polyvinylpyrrolidone (PVP). The homogenate was centrifuged at 12,000 × g for 20 min at 4 °C, and the resulting supernatant was used as the crude enzyme extract for subsequent assays. The activity of the studied enzymes was expressed as enzyme units per gram of dry weight (U g⁻¹ DW).

Superoxide dismutase (SOD; EC 1.15.1.1) activity was assayed by measuring the inhibition of superoxide-driven nitrite formation from hydroxylamine hydrochloride, according to the method of DAS et al. (2000). Catalase (CAT) activity was measured spectrophotometrically at 240 nm by monitoring the decrease in H₂O₂ concentration (CAKMAK and MARSCHNER, 1992). Peroxidase (POD) activity was determined according to the method of CAKMAK and MARSCHNER, (1992).

Nutrient content in *in vitro* grown shoots

Shoots were digested using the acid digestion method described by CRESSER and PARSONS (1979). Following digestion, chemical analyses of individual nutrients were performed as follows: total N was measured by the dis-

tillation method according to Kjeldahl (PAGE et al., 1982); P was measured by spectrophotometer at 880 nm according to MURPHY and RILEY (1962); K was determined by atomic absorption spectrometry according to the method described by BLACK (1965); and Fe, Zn, and Cu were determined by atomic absorption spectrophotometry. There were six replicates of each analysis.

Chlorophyll content

Fresh leaf samples (0.1 g) were homogenized in 80% (v/v) ice-cold acetone to extract photosynthetic pigments. The homogenate was centrifuged at $10,000 \times g$ for 10 min. The absorbance of the supernatant was measured spectrophotometrically at 645 nm and 663 nm. Concentrations of chlorophyll a, chlorophyll b, and total chlorophyll were calculated and expressed as mg g^{-1} fresh weight (FW), according to the method described by LICHTENTHALER (1987).

Acclimatization

For acclimatization, well-rooted plantlets were carefully removed from the culture vessels, washed with sterile distilled water to remove residual medium, and transplanted into plastic pots filled with a mixture of peat moss and perlite (2:1 v/v). The plants were maintained under controlled glasshouse conditions ($25 \pm 2^\circ\text{C}$, 70–80% relative humidity) for 6 weeks before evaluating survival percentages.

Histology

Anatomical examinations were performed on roots in contact with the nutrient medium at concentrations of 0 and 10.0 mg L^{-1} of CuO-NPs, which were selected for anatomical evaluation to compare the control with the highest concentration treatment. Observations were performed using paraffin wax according to the method of CARLETON et al. (1967). Root tissues were fixed in FAA (formaldehyde-acetic acid-ethanol) solution, dehydrated through a graded ethanol series, embedded in paraffin wax, and sectioned at a thickness of $10 \mu\text{m}$ using a rotary microtome. Sections were stained with safranin and fast green. The prepared slides were examined under a compound optical microscope (Olympus, Tokyo, Japan)

equipped with a high-resolution camera, and scale bars ($100 \mu\text{m}$) were included in the micrographs.

Assessment of the genetic stability of plantlets using RAPD and ISSR markers

DNA isolation and analysis

Genomic DNA was isolated from fresh leaf tissues of the mother plant (MP, used as a reference) as well as regenerated plantlets (lanes 1–5, representing control (0), 5, 10, 20, and 40 mg L^{-1} CuO-NPs treatments, respectively) at the acclimatization stage using the CTAB method described by DOYLE and DOYLE (1987). PCR amplification for RAPD and ISSR markers (details of both primers are explained in Tables 1 and 2, respectively) was conducted using the reaction conditions described by WILLIAMS et al. (1990) and WELSH and McCLELLAND (1990), respectively.

Table 1. The list of RAPD and ISSR primers selected for genetic stability analysis

Primer	Sequence	CG %
RAPD OPB07	GGTGACGCAG	70
RAPD OPA02	TGCCGAGCTG	70
RAPD OPD10	GGTCTACACC	60
ISSR 814	CTCTCTCTCTCTCTAC	50
ISSR HB10	CTCTCTCTCTCTCTTG	50
ISSR 6	GACAGACAGACAGACA	50

Agarose Gel Electrophoresis

The PCR products were isolated on 1.5% agarose gel in 1X TAE by electrophoresis at 100 V for 3h, and bands were revealed by ethidium bromide staining. A 3 kb standard molecular weight marker of MBI, Fermentas, and Richlands B.C. was utilized. Resolved and unambiguous bands were registered visually for their presence or absence with each RAPD and ISSR marker. The DNA fragment profiles were recorded, reflecting the presence and absence of bands in each accession.

Table 2. Polymerase chain reaction RAPD and ISSR-PCR

Marker	Number of cycles	Time period	Temperature	Step	Phase
RAPD	1	5 min	Denaturation, 95°C	1	1
RAPD	35	1 min	Denaturation, 95°C	1	2
RAPD	35	1 min	Annealing, $32\text{--}34^\circ\text{C}$	2	2
RAPD	35	2 min	Extension, 72°C	3	2
RAPD	1	10 min	Final extension, 72°C	1	3
ISSR	1	5 min	Denaturation, 94°C	1	1
ISSR	35	1 min	Denaturation, 94°C	1	2
ISSR	35	1 min	Annealing, $44\text{--}54^\circ\text{C}$	2	2
ISSR	35	2 min	Extension, 72°C	3	2
ISSR	1	10 min	Final extension, 72°C	1	3

Statistical analyses

The data were analyzed using one-way analysis of variance (ANOVA) to assess the effects of different treatments used in this study. Statistical analyses were performed with SPSS software version 20, and treatment means were compared using the least significant difference (LSD) at a significance level of $p \leq 0.05$.

Results

Effect of CuO-NPs on shoot proliferation

The MS medium supplemented with 10 mg L CuO-NPs was confirmed to be the most effective treatment, resulting in the highest shoot regeneration frequency (88.60%) and the maximum number of shoots per jar (14.25), which were significantly higher than the control (48.50 % and 3.75, respectively) (Table 3). On the other hand, the 40 mg L⁻¹ CuO-NPs treatment resulted in a shoot regeneration

frequency (51.10%) that showed no significant difference compared to the control (48.50%), while showing a slight but significant increase in shoot number (4.80) compared to the control (3.75) (Table 3; Fig. 2).

Percentage of contamination

Cultures grown in media supplemented with 10, 20, and 40 mg L⁻¹ CuO-NPs were completely free from contamination (0.00%), demonstrating a significant antimicrobial effect at higher concentrations. At the same time, 25.50% of the control cultures were contaminated with bacteria, and 18.40% were contaminated with fungi. Treatment with 5 mg L⁻¹ CuO-NPs reduced bacterial and fungal contamination to 8.20% and 5.10%, respectively (Table 4).

Effect of CuO-NPs on in vitro rooting of date palm plantlets

Our findings revealed that CuO-NPs treatments significantly enhanced the in vitro rooting and growth performance of

Table 3. Effect of copper oxide nanoparticles (CuO-NPs) on shoot regeneration frequency (%), and the number of shoots/jar mg callus for date palm, cv. Barhee

Treatments (mg L ⁻¹)	Frequency (%)	Shoot numbers
0 control (no CuO-NPs)	48.50 d ± 2.65	3.75 e ± 0.14
5 CuO-NPs	74.20 b ± 2.89	8.60 b ± 0.38
10 CuO-NPs	88.60 a ± 3.10	14.25 a ± 0.75
20 CuO-NPs	65.40 c ± 2.40	7.20 c ± 0.50
40 CuO-NPs	51.10 d ± 1.80	4.80 d ± 0.29

± Standard error (n24). Values followed by the same letter are not significantly different at $P \leq 0.05$.

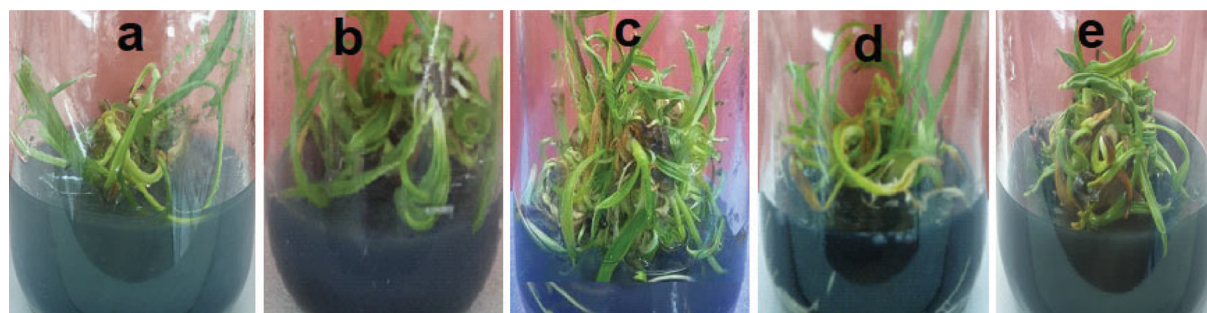


Fig. 2. Shoot induction on MS media supplemented with copper oxide nanoparticles (CuO-NPs) (mg L⁻¹) of date palm 'Barhee': (a) control: 0.0; (b) 5.0; (c) 10.0; (d) 20.0; (e) 40.0, magnification 0.8×.

Table 4. Effect of copper oxide nanoparticles (CuO-NPs) on bacterial and fungal contamination ratio and survival rate (%) of cultures *in vitro*

Treatments (mg L ⁻¹)	Bacterial contamination (%)	Fungal contamination (%)	Survival rate (%)
0 control (no CuO-NPs)	25.50 a ± 1.20	18.40 a ± 0.8	56.10 e ± 2.50
5 CuO-NPs	8.20 b ± 0.39	5.10 b ± 0.30	86.70 c ± 3.89
10 CuO-NPs	0.00 c ± 0.00	0.00 c ± 0.00	100.0 a ± 0.00
20 CuO-NPs	0.00 c ± 0.00	0.00 c ± 0.00	92.30 b ± 2.80
40 CuO-NPs	0.00 c ± 0.00	0.00 c ± 0.00	78.40 d ± 4.60

± Standard error (n50). Values followed by the same letter are not significantly different at $P \leq 0.05$.

Table 5. Rooting of in vitro plants derived from shoots treated with copper oxide nanoparticles (CuO-NPs) and grown in rooting medium supplemented with 1.0 mg L⁻¹ NAA, at 45 days of culture

Treatments (mg L ⁻¹)	Rooting percentage (%)	Number of roots (Root/shoot)	Root length (cm)
0 control (no CuO-NPs)	42.30 e ± 1.20	2.20 d ± 0.15	1.45 d ± 0.12
5 CuO-NPs	76.50 b ± 2.40	5.85 b ± 0.12	3.40 b ± 0.20
10 CuO-NPs	94.10 a ± 3.20	8.65 a ± 0.20	5.10 a ± 0.35
20 CuO-NPs	68.45 c ± 2.10	4.20 c ± 0.30	2.65 c ± 0.18
40 CuO-NPs	55.20 d ± 1.90	2.55 d ± 0.19	1.60 d ± 0.14

± Standard error (n25). Values followed by the same letter are not significantly different at P ≤ 0.05.



Fig. 3. Development of adventitious roots on MS medium supplemented with 10 mg L⁻¹ copper oxide nanoparticles (CuO-NPs), 45 days after culturing, magnification 0.8×.

date palm plantlets compared to the control. The 10 mg L⁻¹ CuO-NPs treatment was confirmed to be the optimal composition, achieving the highest rooting percentage (94.10%), root number (8.65 roots/shoot), and root length (5.10 cm), which were significantly higher than the control 42.30%, 2.20 roots/shoot, and 1.45 cm, respectively (Table 5; Fig. 3).

Effect of CuO-NPs treatments on some biochemical parameters

Total soluble carbohydrates

All CuO-NPs treatments increased the content of total

soluble carbohydrates in 'Barhee' plantlets compared to the control (85.40 mg g⁻¹ DW) (Table 6). However, the maximum concentration was recorded at 10 mg L⁻¹ CuO-NPs (152.10 mg g⁻¹ DW), representing a significant increase of approximately 78% outperforming all other treatments.

Free amino acids

CuO-NPs significantly increased the content of free amino acids in 'Barhee' plantlets compared to the control (4.50 mg g⁻¹ DW). The highest content was observed in plantlets treated with 10 mg L⁻¹ CuO-NPs (8.05 mg g⁻¹

Table 6. Effect of copper oxide nanoparticles (CuO-NPs) on total soluble carbohydrates, free amino acids, starch, and total soluble protein in regenerated shoots in 'Barhee' date palm

Treatments (mg L ⁻¹)	Total soluble carbohydrates (mg g ⁻¹ DW)	Free amino acid (mg g ⁻¹ DW)	Total soluble protein (mg g ⁻¹ DW)
0 control (no CuO-NPs)	85.40 e ± 2.15	4.50 e ± 0.15	18.20 e ± 0.65
5 CuO-NPs	112.60 b ± 3.40	6.20 b ± 0.22	24.50 b ± 0.82
10 CuO-NPs	152.10 a ± 4.80	8.05 a ± 0.35	32.45 a ± 1.31
20 CuO-NPs	102.35 c ± 2.95	5.60 c ± 0.20	21.80 c ± 0.70
40 CuO-NPs	92.15 d ± 2.39	4.95 d ± 0.18	10.40 d ± 0.60

± Standard error (n6). Values followed by the same letter are not significantly different at P ≤ 0.0.

Table 7. Effect of copper oxide nanoparticles (CuO-NPs) on the macronutrients and micronutrients content in the shoots of date palm cv. Barhee *in vitro*

Treatments (mg L ⁻¹)	N (mg g ⁻¹ DW)	P (mg g ⁻¹ DW)	K (mg g ⁻¹ DW)	Fe (μg g ⁻¹ DW)	Zn (μg g ⁻¹ DW)	CU (μg g ⁻¹ DW)
0 control (no CuO-NPs)	18.20 e ± 0.55	2.35 e ± 0.10	22.10 e ± 0.85	120.5 e ± 3.2	24.8 e ± 0.8	8.45 e ± 0.35
5 CuO-NPs	25.10 b ± 0.72	3.22 b ± 0.15	30.80 b ± 0.95	168.2 b ± 4.2	34.5 b ± 1.2	14.15 d ± 0.58
10 CuO-NPs	32.55 a ± 0.95	4.15 a ± 0.18	39.20 a ± 1.20	214.5 a ± 6.1	44.2 a ± 1.7	18.30 c ± 0.72
20 CuO-NPs	22.30 c ± 0.68	2.95 c ± 0.12	27.45 c ± 0.88	145.8 c ± 4.2	30.2 c ± 1.1	25.60 b ± 0.95
40 CuO-NPs	19.85 d ± 0.43	2.58 d ± 0.08	24.15 d ± 0.70	130.4 d ± 3.5	27.1 d ± 0.9	38.25 a ± 1.30

± Standard error (n6). Values followed by the same letter are not significantly different at $P \leq 0.0$.

DW), followed by 5 mg L⁻¹ (6.20 mg g⁻¹ DW) and 20 mg L⁻¹ (5.60 mg g⁻¹ DW) (Table 6).

Total soluble proteins

CuO-NPs significantly increased the content of total soluble proteins in 'Barhee' plantlets compared to the control (18.20 mg g⁻¹ DW). The maximum soluble protein content was observed in plantlets treated with 10 mg L⁻¹ CuO-NPs (32.45 mg g⁻¹ DW), representing a significant increase of approximately 78% over the control (Table 6).

Nutrient content

CuO nanoparticles significantly affected the accumulation of macronutrients and micronutrients in the shoot tissues of date palm cultured *in vitro*. The highest accumulation of nitrogen (32.55 mg g⁻¹ DW), phosphorus (4.15 mg g⁻¹ DW), potassium (39.20 mg g⁻¹ DW), iron (214.5 μg g⁻¹ DW), and zinc (44.2 μg g⁻¹ DW) was recorded in shoots cultured in

medium supplemented with 10 mg L⁻¹ CuO-NPs, showing a significant increase compared to the control (18.20, 2.35, 22.10 mg g⁻¹ DW, and 120.5, 24.8 μg g⁻¹ DW, respectively) (Table 7). In contrast, copper (Cu) accumulation increased progressively with higher treatments, reaching its maximum value at 40 mg L⁻¹ CuO-NPs (38.25 μg g⁻¹ DW) compared to the control (8.45 μg g⁻¹ DW) (Table 7).

Enzymatic activity

The activity of antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), and peroxidase (POX), increased significantly in plantlets treated with CuO-NPs compared to the control (11.20, 7.50, and 14.80 U g⁻¹ DW, respectively) (Table 8). Maximum enzyme activities were recorded at 10 mg L⁻¹ CuO-NPs (SOD: 19.95, CAT: 13.35, and POX: 26.40 U g⁻¹ DW), representing an overall increase of approximately 78% over the control. Higher concentrations (20 and 40 mg L⁻¹) resulted in a progressive decline in

Table 8. Effect of copper oxide nanoparticles (CuO-NPs) on Superoxide dismutase (SOD), Catalase (CAT) and Peroxidase (POX) activity in date palm cv. Barhee plant leaves *in vitro*

Treatments (mg L ⁻¹)	Superoxide dismutase (SOD) (U g ⁻¹ DW)	Catalase activity (CAT) (U g ⁻¹ DW)	Peroxidase activity (POX) (U g ⁻¹ DW)
0 control (no CuO-NPs)	11.20 e ± 0.35	7.50 e ± 0.05	14.80 e ± 0.45
5 CuO-NPs	15.80 b ± 0.48	10.60 b ± 0.32	21.20 b ± 0.65
10 CuO-NPs	19.95 a ± 0.61	13.35 a ± 0.45	26.40 a ± 0.85
20 CuO-NPs	13.50 c ± 0.48	9.10 c ± 0.28	18.10 c ± 0.55
40 CuO-NPs	12.10 d ± 0.39	8.20 d ± 0.22	16.20 d ± 0.48

± Standard error (n6). Values followed by the same letter are not significantly different at $P \leq 0.05$.

Table 9. Effect of copper oxide nanoparticles (CuO-NPs) on the chlorophylls a+b and total chlorophyll content (mg g⁻¹ FW) in date palm 'Barhee'

Treatments	Chl a	Chl b	Chl t
0 control (no CuO-NPs)	0.65 e ± 0.03	0.32 e ± 0.01	0.97 e ± 0.05
5 CuO-NPs	1.15 b ± 0.08	0.58 b ± 0.04	1.73 b ± 0.12
10 CuO-NPs	1.48 a ± 0.10	0.76 a ± 0.05	2.24 a ± 0.15
20 CuO-NPs	0.92 c ± 0.06	0.45 c ± 0.02	1.37 c ± 0.09
40 CuO-NPs	0.72 d ± 0.04	0.38 d ± 0.01	1.10 d ± 0.07

± Standard error (n6). Values followed by the same letter are not significantly different at $P \leq 0.05$.

enzyme activities compared to the 10 mg L⁻¹ peak, although they remained significantly higher than the untreated control (Table 8).

Chlorophyll content

Different treatments of CuO-NPs significantly affected ($p \leq 0.05$) the photosynthetic pigments content in the leaves of date palm 'Barhee' plantlets (Table 9). The addition of 10 mg L⁻¹ CuO-NPs to the culture medium recorded the highest values for chlorophyll a (1.48 mg g⁻¹ FW), chlorophyll b (0.76 mg g⁻¹ FW), and total chlorophyll (2.24 mg g⁻¹ FW), showing a significant increase compared to the untreated control (0.65, 0.32, and 0.97 mg g⁻¹ FW, respectively) (Table 9). Although higher CuO-NPs concentrations (20 and 40 mg L⁻¹) resulted in a decline in pigment levels compared to the 10 mg L⁻¹ peak, all treated plantlets maintained significantly higher chlorophyll contents than the control (Table 9).

Acclimatization

The acclimatized plantlets showed successful establish-

ment and high vigor when transferred to pots containing a peat moss and perlite (2:1 v/v) mixture. The highest survival rate of 88.0% was recorded for plantlets derived from the 10 mg L⁻¹ CuO-NPs treatment supplemented with 1.0 mg L⁻¹ NAA, as visually demonstrated in Fig. 4. This was followed by plantlets derived from the 5.0 mg L⁻¹ CuO NPs treatment and with the same concentration of NAA, which recorded a survival rate of 76% (data not shown).

Histological analysis of roots

Histological analysis revealed notable anatomical modifications in the root tissues of date palm plantlets cultured in media supplemented with CuO-NPs compared to the control. The epidermis of treated roots exhibited active live cells with a significantly higher density and elongation of root hairs (Fig. 5a, b). Furthermore, cross-sections showed enhanced development of the cortical parenchyma, characterized by increased cell proliferation and more prominent intercellular air spaces (aerenchyma) within the inner cortical layers (Fig. 5b). At the optimal concentration of 10 mg L⁻¹ CuO-NPs, the vascular cylinder (stele) displayed superior development, with greater dimensions and



Fig. 4. Acclimatization and successful establishment of date palm plantlets derived from 10 mg L⁻¹ copper oxide nanoparticles (CuO-NPs) treatment with 1.0 mg L⁻¹ NAA in a peat moss and perlite (2:1 v/v) substrate mixture, magnification 0.8×.

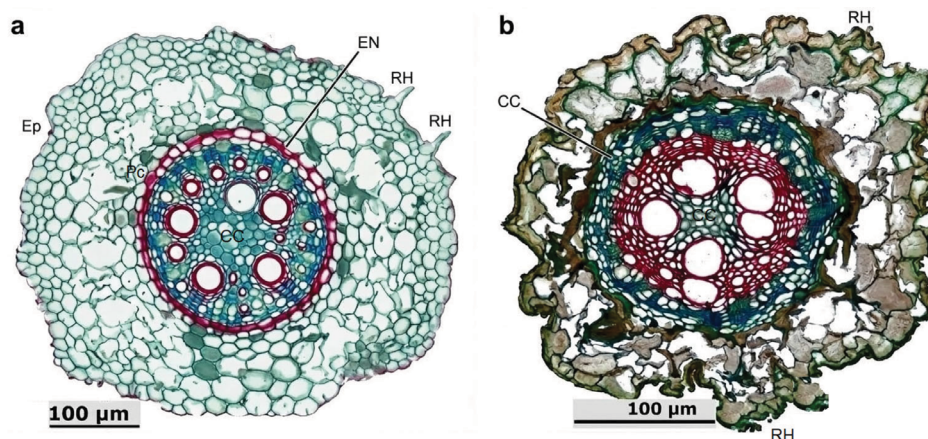


Fig. 5. Cross-section of a date palm root under two levels of medium supplemented with copper oxide nanoparticles (CuO NPs), (a) control, and (b) 10 mg L⁻¹ of CuO-NPs. RH: root hairs, Ep: epidermis, En: endoderm, Pc: pericycle, CC: central cylinder.

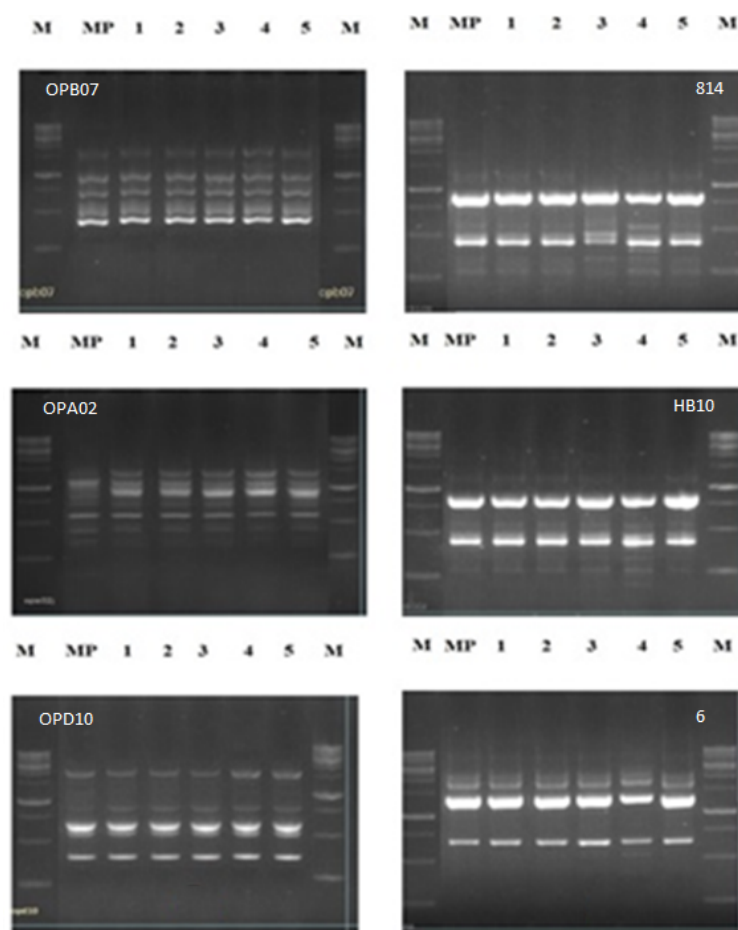


Fig. 6. Molecular analysis of date palm 'Barhee' regenerants treated with CuO-NPs: a) RAPD profiles generated using primers OPB07, OPA02, and OPD10; b) ISSR profiles generated using primers 814, HB10, and 6. For both gels, lanes are as follows: M, DNA marker; MP, mother plant; lanes 1–5, regenerants from 0.0, 5.0, 10.0, 20.0, and 40.0 mg L⁻¹ CuO-NP treatments, respectively.

cellular expansion in both xylem and phloem elements relative to the untreated control.

Genetic fidelity of micropropagated plantlets

PCR amplification using RAPD and ISSR markers demonstrated a consistent banding pattern across the studied samples, suggesting a high degree of genetic stability and true-to-type uniformity among tissue culture-produced plants compared to the mother plant (Figs 6a, b). These results indicate that the micropropagation protocol, including the application of CuO-NPs combined with NAA, did not induce detectable somaclonal variations under the tested molecular markers in the 'Barhee' date palm cultivar.

Discussion

The success of date palm micropropagation depends on optimizing culture media. Here, we tested the effects of different concentrations of copper oxide nanoparticles (CuO-NPs) on the micropropagation of the date palm Barhee cv. While CuO-NPs are economically important (GRIGORE et al., 2016), their specific effects on date palm

tissue culture have not been documented previously. Our results demonstrated that CuO-NPs affected indirect shoot regeneration in a concentration-dependent manner (Table 3, Fig. 2). The highest shoot proliferation was achieved at 10 mg L⁻¹ CuO-NPs, whereas higher concentrations (40 mg L⁻¹) exerted an inhibitory effect. This dual impact relates to reactive oxygen species (ROS) generation; at moderate concentrations, controlled ROS production acts as a signaling mechanism, whereas excessive concentrations disrupt cellular balance (NAZIR et al., 2021). The use of 10 mg L⁻¹ CuO-NPs positively affected growth parameters, as copper acts as an essential nutrient and cofactor in metalloenzymes, increasing Cu²⁺ bioavailability and accelerating endogenous nutrient mobilization (RAJPUT et al., 2018; SINGH et al., 2023). Furthermore, CuO-NPs effectively reduced microbial contamination in culture media. Complete freedom from contamination (0.00%) was achieved at concentrations (10, 20, and 40 mg L⁻¹ CuO-NPs), reflecting significant antimicrobial potency driven by ROS-induced oxidative stress on microbial cell membranes and DNA, compared to control cultures showing high bacterial and fungal infection rates (SAWANT et al., 2023; GIANNOUSI et al., 2017). Regarding rooting parameters CuO-NPs treatments significantly

enhanced the *in vitro* rooting and growth performance of date palm plantlets compared to the control. The optimal concentration of 10 mg L⁻¹ CuO-NPs (supplemented with 1.0 mg L⁻¹ NAA) achieved the maximum rooting percentage, root number, and root length, reflecting the stimulatory role of moderate copper levels in root morphogenesis and cellular expansion (XIONG et al., 2021; FEIGL, 2023). Regarding biochemical parameters, application of CuO-NPs significantly increased soluble sugar and free amino acid contents compared to the control, aligning with SHAFIQ et al. (2024). A reduction in soluble protein content at 40 mg L⁻¹ reflects the stress-induced metabolic variations (WANG et al., 2020). In terms of nutrient content, CuO-NPs significantly affected the accumulation of macro- and micro-elements in shoot tissues. The highest accumulation of nitrogen, phosphorus, potassium, iron, and zinc was recorded at 10 mg L⁻¹ CuO-NPs compared to the control (SHAFIQ et al., 2024). This enhanced uptake stems from nanoparticle-stimulated modifications in root ion transport mechanisms and cell membrane permeability (CHEN, 2024). In contrast, copper (Cu) accumulation increased progressively with higher treatments, reaching a maximum at 40 mg L⁻¹ CuO-NPs, aligning with the direct supply of copper from the nanoparticles (RAJPUT et al., 2018). Regarding enzymatic activity, antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT), and peroxidase (POX) increased significantly in plantlets treated with CuO-NPs, compared to the control, peaking at 10 mg L⁻¹ as an efficient cellular defense mechanism against oxidative stress (YASMEEN et al., 2017; FARAZ et al., 2023). Conversely, higher concentrations (20 and 40 mg L⁻¹) resulted in a progressive decline in enzyme activities as excessive exposure overwhelmed natural antioxidant scavenging efficiency (PELLEGRINO et al., 2020; MURSHED et al., 2014). For chlorophyll content, photosynthetic pigments (chlorophyll a, b, and total chlorophyll) were significantly affected by CuO-NPs, with the highest values recorded at 10 mg L⁻¹. This increase is attributed to the capacity of CuO-NPs to stimulate enzymes involved in chlorophyll biosynthesis (FARAZ et al., 2023; HASANIN et al., 2023), thereby improving overall photosynthetic capacity and maintaining metabolic homeostasis (JAHAN et al., 2023; HAMEED et al., 2021). Although higher concentrations declined relative to the peak, all treatments remained significantly higher than the control.

During acclimatization, plantlets showed successful establishment and high vigor when transferred to a peat moss and perlite mixture, with the highest survival rate (88.0%) achieved in plants derived from the 10 mg L⁻¹ CuO-NPs treatment combined with 1.0 mg L⁻¹ NAA, reflecting an improved physiological status and robust root development (FEIGL, 2023). Histological analysis of roots revealed notable anatomical modifications, including active live cells with higher density and elongation of root hairs, enhanced cortical parenchyma proliferation, and prominent intercellular air spaces (aerenchyma) compared to the control. The vascular cylinder (stele) also displayed superior development at the

optimal 10 mg L⁻¹ concentration, facilitating enhanced water and gas exchange. Finally, regarding genetic fidelity of micropropagated plantlets, RAPD and ISSR markers showed high pattern similarity among regenerated plantlets with no detectable somaclonal variations. Given the reliability of these molecular techniques, our findings confirm high preliminary genetic stability in the micropropagated tissues (AL-MAYAH, 2025b, 2026c).

Conclusion

This study demonstrates that the application of 10 mg L⁻¹ CuO-NPs effectively enhances the efficiency of date palm micropropagation, significantly improving shoot proliferation, rooting parameters, and survival rates during acclimatization. Furthermore, optimal CuO-NPs concentrations effectively eliminated culture contamination and positively modulated biochemical parameters, nutrient accumulation, and antioxidant enzyme activities. Conversely, higher concentrations (40 mg L⁻¹) exerted suppressive effects. These findings confirm that CuO-NPs can be reliably integrated into date palm tissue culture protocols as an effective agent for growth promotion and contamination control, without inducing genetic instability.

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