

STIMULATING EFFECT OF CEFOTAXIME AND SILVER NANOPARTICLES ON PLANT REGENERATION IN DATE PALM TISSUE CULTURE

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ABSTRACT

The essential challenges in practical applications of date palm micropropagation include explant browning, reduced callus growth, low multiplication rate, and frequent tissue contamination. Our study aimed to evaluate the effects of cefotaxime (Cefo) and silver nanoparticles (AgNPs) on microbial contamination removal, callus growth, and shoot regeneration in date palm micropropagation. Cultures were initiated from bud tips of ‘Barhee’ date palm. Murashige and Skoog medium with auxins, cytokinins, and activated charcoal was amended with Cefo (50, 100, and 200 mg·l⁻¹), AgNPs (0.125 and 0.250 mg·l⁻¹), and a combination of both. The medium supplemented with 200 mg·l⁻¹ Cefo and 0.250 mg·l⁻¹ AgNPs resulted in the best callus growth (318 mg). In contrast, 200 Cefo + 0.125 AgNPs resulted in maximum organogenesis and shoot number per jar (83.4% and 14.1 shoots per jar, respectively). These two combinations resulted in contaminant-free cultures. The total amount of phenolic compounds was significantly reduced to 0.79 and 0.57 mg GAE·g⁻¹ DM in shoots cultured in the above media, which was reflected in the low browning rate. The data revealed that the maximum endogenous IAA content of shoots (2.681 µg·g⁻¹ and 2.345 µg·g⁻¹) was obtained in response to 200 mg·l⁻¹ Cefo + 0.250 AgNPs and 200 mg·l⁻¹ Cefo + 0.125 mg·l⁻¹ AgNPs, respectively. Therefore, the optimized compositions established in the present study could be applicable in reducing contamination and helping callus production and multiple shoot regeneration. To our knowledge, this is the first study of the antibacterial and growth-promoting effects of Cefo in combination with AgNPs in *in vitro* cultures of ‘Barhee’ date palm.

Key words: callus induction, contamination, micropropagation, plant regeneration, *Phoenix dactylifera*

INTRODUCTION

The date palm (*Phoenix dactylifera* L.) is one of the oldest fruit trees, the most widely consumed fruit in the world, and is considered an essential part of the diet in various cultures. The nutrients provided by date fruit are mainly glucose and fructose. Moreover, dates are a good source of necessary minerals such as potassium and magnesium (Al-Mssallem et al. 2020). Date palms are traditionally propagated by offshoots, which is inefficient because each tree produces several offshoots, especially in some genotypes, and fruiting can take up to seven years (Jasim et al. 2009; Gantait et al. 2018; Al-Mayahi 2022a, b). Therefore, rapid propagation of date palm by tissue culture is the most promising technique for producing large numbers of high quality date palm plantlets (Al-Asadi et al. 2020, 2024; Abdalla et al. 2022; Al-Mayahi 2022c).

However, the use of plant tissue culture for commercial propagation of woody plants can be associated with several challenges, mainly fungal and bacterial contamination (Rout et al. 2006; Al-Mayahi et al. 2010). The adverse effects of microbial contamination are deterioration, browning, and death of infected tissue due to the release of toxic substances such as degrading enzymes (e.g., cellulase or phenoloxidase) into the medium (Emoghene et al. 2020; Ahmed & Abass 2022). Several methods prevent or eliminate contamination (Jasim et al. 2021; Permadi et al. 2023). Among other things, new antimicrobial compounds are being sought that will be effective in combating microorganisms and at the same time harmless to plant explants. Abdel-Karim (2017) reported that the incorporation of fungicides or antibiotics into the medium significantly reduced the contamination rate during different stages of *in vitro* date palm culture.

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Low contamination and survival rates in ‘Barhee’ date palm cultures were obtained using silver nanoparticles (Rohim et al. 2020). Moreover, antibiotics have been shown to stimulate the growth and development of explants (Mittal et al. 2009; Shehata et al. 2010; Okoroafor 2022). It has been suggested that some β -lactams antibiotics have auxin-like activity (Ogawa & Mii 2004; Qin et al. 2011). New applications of antibiotics such as cefotaxime (Cefo) can induce *in vitro* differentiation and organogenesis in woody plants, as has been demonstrated in eucalyptus and citrus (Aggarwal et al. 2010; de Oliveira et al. 2010). Although several antibiotics have been described for effective control of *Agrobacterium* cells, Cefo, which belongs to the β -lactams group, has minimal toxicity to most plant tissues and has thus become the most widely accepted in *Agrobacterium*-mediated transformation (Grzebelus & Skop 2014). β -Lactam antibiotics, such as carbenicillin, cefotaxime, and co-ticarcav, are most commonly used to control bacterial growth in plant tissue cultures (da Silva Mendes et al. 2009; Qin et al. 2011). In cultured *Zea mays* tissues, Cefo has been shown to have effects similar to plant hormones (Danilova & Dolgikh 2004). The plant growth medium is not optimal for microorganism growth due to sucrose levels, pH, and temperature. When the culture conditions alter, they can become more suitable for microbial growth, and bacteria can propagate and even damage the growing plants (Wojtania et al. 2005).

Silver nanoparticles (AgNPs) were common nanoscale antibiotics inhibiting the growth of bacteria, fungi, and viruses (Ibraheem et al. 2022). AgNPs have been developed as plant growth promoters (Jasim et al. 2017) as well as plant protection products (Worrall et al. 2018). AgNPs can effectively eliminate microorganisms without negatively affecting plant growth and development (Lakshmi et al. 2021). Therefore, the present study was carried out to investigate the effect of Cefo and AgNPs on the growth rate of contaminated cultures and to determine the effect of these two compounds on the growth parameters of ‘Barhee’ date palm.

MATERIALS AND METHODS

Initiation stage

Shoot tips of 3–4-year-old ‘Barhee’ date palm offshoots were excised and trimmed to a size of approximately 0.8–1.0 cm in width and 1–2 cm in length as starting material for induction culture. Explants were sterilized with 70% ethanol for 1 minute

and 5% sodium hypochlorite for 20 minutes and then rinsed three times with sterile distilled water. The explants were transferred to MS basal medium (Murashige & Skoog 1962) with the addition of 100 mg·l⁻¹ glutamine, 5 mg·l⁻¹ thiamine HCl, 1 mg·l⁻¹ biotin, 30 g·l⁻¹ sucrose, 2 g·l⁻¹ of activated charcoal and solidified with agar at a concentration of 6.0 g·l⁻¹. The media were supplemented with growth regulators: naphthaleneacetic acid (NAA) at a concentration of 30 mg·l⁻¹ and 6-(γ,γ -dimethylallylamino)purine (2iP) at 3 mg·l⁻¹. Before adding the agar, the pH of the media was adjusted to 5.7. The media were dispensed into culture containers and autoclaved for 20 minutes at 121 °C and 1.04 kg·cm⁻². Cultures were incubated in the dark at 27 ± 2 °C. They were transferred to fresh media of the same composition every 6 weeks until callus initiation.

First experiment

Effect of cefotaxime and silver nanoparticles on callus growth

The stabilized callus was separated after six months and 100 mg were cultured in 340 ml glass jars containing 50 ml of MS basal medium, with NAA at 6.0 mg·l⁻¹ and 2iP at 1.5 mg·l⁻¹. To study the effect of Cefo and AgNPs on callus growth, these compounds were supplemented at different concentrations: 0, 50, 100, and 200 mg·l⁻¹ Cefo or 0, 0.125, and 0.250 mg·l⁻¹ AgNPs, 200 mg·l⁻¹ Cefo + 0.125 mg·l⁻¹ AgNPs, or 200 mg·l⁻¹ Cefo + 0.250 mg·l⁻¹ AgNPs. The control was callus grown on media without Cefo or AgNPs. After callus inoculation, cultures were transferred directly to an incubator at 27 ± 2 °C and a 16 h photoperiod (2000 lux) using cool white fluorescent tubes. Each treatment contained eighteen replicates (jars). Callus weight was recorded eight weeks after experiment initiation.

Second experiment

Effect of cefotaxime and silver nanoparticles on indirect organogenesis

Calli from the first experiment were transferred to media consisting of the basal MS described above, but with different growth regulators – 1 mg·l⁻¹ NAA, 0.5 mg·l⁻¹ 6-benzyladenine (BA), and 0.5 mg·l⁻¹ kinetin (K), suitable for shoot formation (Al-Mayahi 2023). Callus cultures were subjected to the same Cefo and AgNPs treatments as in the first experiment. The effect of these compounds on shoot multiplication and some physicochemical parameters was examined. Cultures were kept at 27 ± 2 °C and irradiated for 16 hours with diffused light provided by daylight fluorescent lamps. Each treatment contained eighteen

replicates (jars). The percentage of bud regeneration and the number of buds per jar were recorded 12 weeks after callus inoculation onto the media.

Browning

The degree of callus browning was categorized into three levels: no tissue browning (-), moderate tissue browning (+), and severe tissue browning (+++). Each treatment contained eighteen replicates (jars).

Percentage of contamination

The percentage of contamination was calculated by dividing the number of contaminated cultures (fungi, bacteria) by the total number of cultures grown. Each treatment contained twelve replicates (jars).

Total soluble phenolic compounds

Total phenolic contents (TPC) in callus extracts according to the method described by Singleton and Rossi (1965). Gallic acid was used as the reference standard; 0.2 ml of extract solution and 0.2 ml of Folin–Ciocalteu reagent were added, and the contents were mixed thoroughly. After 5 minutes, 1 ml of 15% sodium bicarbonate (NaHCO_3) was added and the mixture was left at room temperature for 2 hours. The absorbance was obtained in a spectrophotometer at a wavelength of 765 nm. Each treatment contained eighteen replicates (jars). Results were expressed as milligrams of gallic acid equivalent (mg of GAE per gram of DW).

Auxin (IAA) extraction and measurement

Auxins were quantified using the method of Nagar and Sood (2006). Two grams of shoots from different treatments were homogenized using 80% methanol ($20 \text{ ml} \cdot \text{g}^{-1}$). The homogenates were kept overnight at 4°C in the dark, filtered, and reextracted 4 times with 80% methanol. The solution was frozen at -20°C , thawed and centrifuged at 9000 rpm for 30 minutes at 4°C to remove suspended impurities. The supernatants were dried in a vacuum, taken up in 0.1 M potassium phosphate buffer (pH 8.1), and then applied to a polyvinylpyrrolidone (PVP) column. The obtained eluate was evaporated to dryness, taken up in water and the pH adjusted to 2.5 with 1 N HCl and then partitioned ($4\times$) with diethyl ether. The combined ether phases were evaporated in a vacuum and used in methanol (HPLC grade) to estimate bound auxins. Indole-3-acetic acid (IAA) standard was used as reference. Each treatment contained five replicates (jars).

Statistical data analysis

A completely randomized design (CRD) was used. The results were subjected to normality test and analysis of variance (ANOVA) using F test, and means

were compared using the least significant difference test (LSD, $p < 0.05$) using SPSS software (version 20).

RESULTS

Effect of cefotaxime and silver nanoparticles on callus growth and plant regeneration traits

Cefo and AgNPs stimulated callus growth and organogenesis in date palm cultures (Table 1). The effect of Cefo and AgNPs increased with concentration. The highest growth parameter values were obtained with a combination of $200 \text{ mg} \cdot \text{l}^{-1}$ Cefo and $0.250 \text{ mg} \cdot \text{l}^{-1}$ AgNO_3 . The number of shoots showed the same dependence (Table 1, Fig. 1, 2). The combination of $200 \text{ mg} \cdot \text{l}^{-1}$ Cefo and $0.250 \text{ mg} \cdot \text{l}^{-1}$ AgNPs doubled or tripled the control values. The percentage of organogenic calluses increased from 39% in the control to 83% in the treatment where Cefo and AgNPs were combined.

Percentage of contamination

Cultures grown in medium supplemented with $200 \text{ mg} \cdot \text{l}^{-1}$ Cefo combined with 0.250 or $0.125 \text{ mg} \cdot \text{l}^{-1}$ AgNPs were free from contamination. At the same time, 33% of control cultures were contaminated. Cefo alone reduced contamination to 25%, 17%, and 8% at concentrations of 50, 100, and $200 \text{ mg} \cdot \text{l}^{-1}$, respectively (Fig. 3). The efficacy of AgNPs alone was equal to that of Cefo at a concentration of $50 \text{ mg} \cdot \text{l}^{-1}$.

Browning

Cefo and AgNPs reduced the callus browning even at the lowest concentrations (Table 2). Cefo at $200 \text{ mg} \cdot \text{l}^{-1}$ combined with 0.250 or $0.125 \text{ mg} \cdot \text{l}^{-1}$ AgNPs reduced browning to zero (Fig. 1).

Total soluble phenolic compounds (TSPC)

The highest content of phenolic compounds was recorded in the control callus (Table 2). The reduction in TSPC was correlated with the increase in Cefo and AgNPs concentrations. The lowest TSPC values were found in callus from media supplemented with $200 \text{ mg} \cdot \text{l}^{-1}$ Cefo combined with 0.250 or $0.125 \text{ mg} \cdot \text{l}^{-1}$ AgNPs (Table 2).

Endogenous indole-3-acetic acid

Cefo and AgNPs modified the endogenous IAA level in shoots. Increasing the concentration of Cefo and AgNPs resulted in a proportional increase in endogenous IAA content. The highest IAA content in shoots ($2.681 \mu\text{g} \cdot \text{g}^{-1}$) was recorded at $200 \text{ mg} \cdot \text{l}^{-1}$ Cefo and with the combination of Cefo and AgNPs (Fig. 4), where the IAA concentration was about 3 times higher compared to the control.

Table 1. Effect of cefotaxime (Cefo) and silver nanoparticles (AgNPs) on callus weight, shoot regeneration frequency (%), and number of buds per 100 mg callus per jar of 'Barhee' date palm

Treatment (mg·l ⁻¹)	Callus weight* (100 mg per jar)	Shoot regeneration (%)	Number of shoot
0 (control)	111 ± 7.2g	38.9 ± 4.0 h	3.9 ± 0.5 f
50 Cefo	140 ± 7.4 f	44.5 ± 3.9 gh	4.6 ± 0.2 f
100 Cefo	171 ± 9.4 e	55.6 ± 4.3 fg	6.1 ± 0.4 e
200 Cefo	201 ± 13.2d	66.7 ± 6.9 cd	8.0 ± 0.1 c
0.125 AgNPs	220 ± 7.6d	72.2 ± 4.6 bc	9.1 ± 0.2c
0.250 AgNPs	265 ± 9.2 c	61.1 ± 4.7 de	7.9 ± 0.1 d
200 Cefo + 0.125AgNPs	291 ± 12.3 b	83.3 ± 3.3 a	14.1 ± 1.9 a
200 Cefo + 0.250AgNPs	318 ± 12.3 a	77.8 ± 4.6 ab	11.1 ± 0.2 b

* initial callus weight 100 mg per jar

Values followed by the same letter are not significantly different at $p < 0.05$; $\pm$ standard error (n = 18)

Table 2. Effect of cefotaxime (Cefo) and silver nanoparticles (AgNPs) on total soluble phenolic compounds (TSPC) and callus browning of 'Barhee' date palm

Treatment (mg·l ⁻¹)	TSPC (mg GAE·g ⁻¹)	Browning*
0 (control)	1.69 ± 0.21 g	+++
50 Cefo	1.48 ± 0.8 f	++
100 Cefo	1.29 ± 0.3 e	+
200 Cefo	1.07 ± 0.04 cd	+
0.125 AgNPs	1.18 ± 0.13 de	+
0.250 AgNPs	0.98 ± 0.07 c	+
200 Cefo + 0.125AgNPs	0.79 ± 0.05 b	-
200 Cefo + 0.250AgNPs	0.57 ± 0.09 a	-

*(-) no tissue browning (white), (+) moderate tissue browning, (+++) severe tissue browning

Values followed by the same letter are not significantly different at $p < 0.05$; $\pm$ standard error (n = 18)

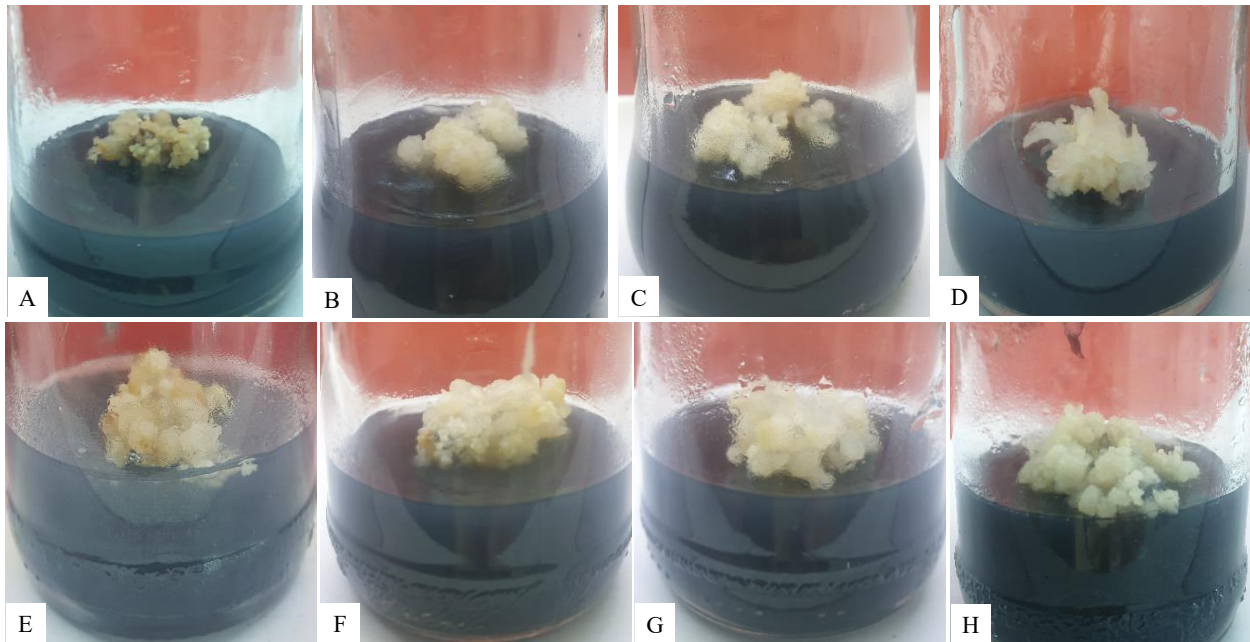


Figure 1. Growth of 'Barhee' date palm callus on MS media supplemented with cefotaxime (Cefo) and silver nanoparticles (AgNPs): control (A), 50 mg·l⁻¹ Cefo (B), 100 mg·l⁻¹ Cefo (C), 200 mg·l⁻¹ Cefo (D), 0.125 mg·l⁻¹ AgNPs (E), 0.250 mg·l⁻¹ AgNPs (F), 200 mg·l⁻¹ Cefo + 0.125 mg·l⁻¹ AgNPs (G), 200 mg·l⁻¹ Cefo + 0.250 mg·l⁻¹ AgNPs (H); magnification 0.8×

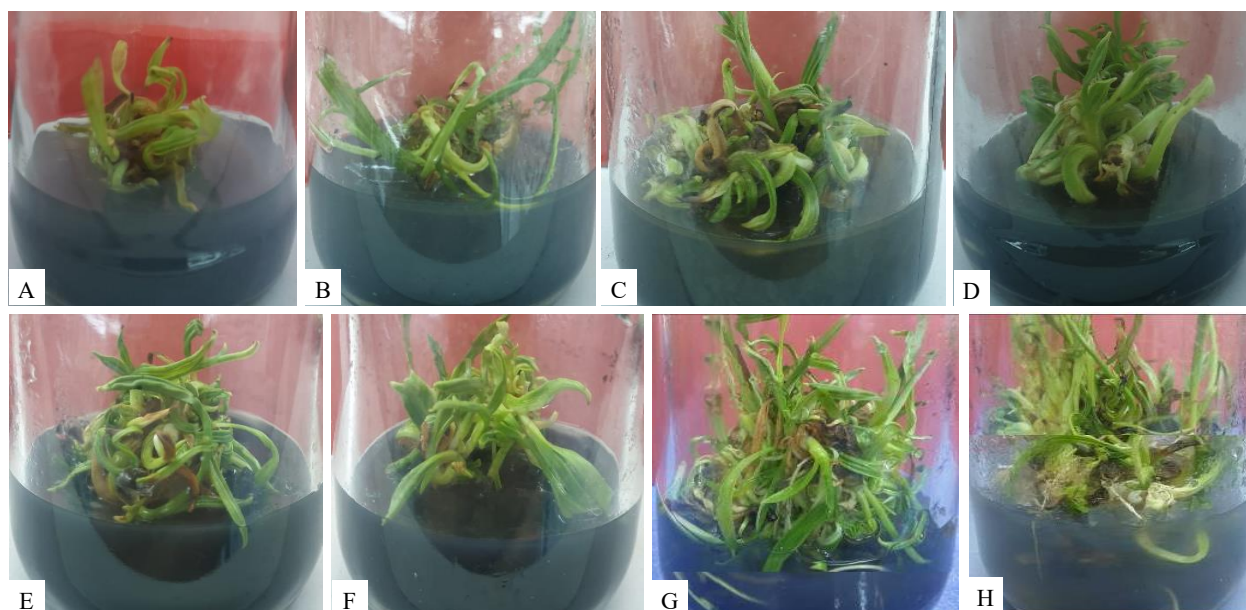


Figure 2. Bud induction of 'Barhee' date palm on MS media supplemented with cefotaxime (Cefo) and silver nanoparticles (AgNPs): control (A), 50 mg·l⁻¹ Cefo (B), 100 mg·l⁻¹ Cefo (C), 200 mg·l⁻¹ Cefo (D), 0.125 mg·l⁻¹ AgNPs (E), 0.250 mg·l⁻¹ AgNPs (F), 200 mg·l⁻¹ Cefo + 0.125 mg·l⁻¹ AgNPs (G), 200 mg·l⁻¹ Cefo + 0.250 mg·l⁻¹ AgNPs (H); magnification 0.8×

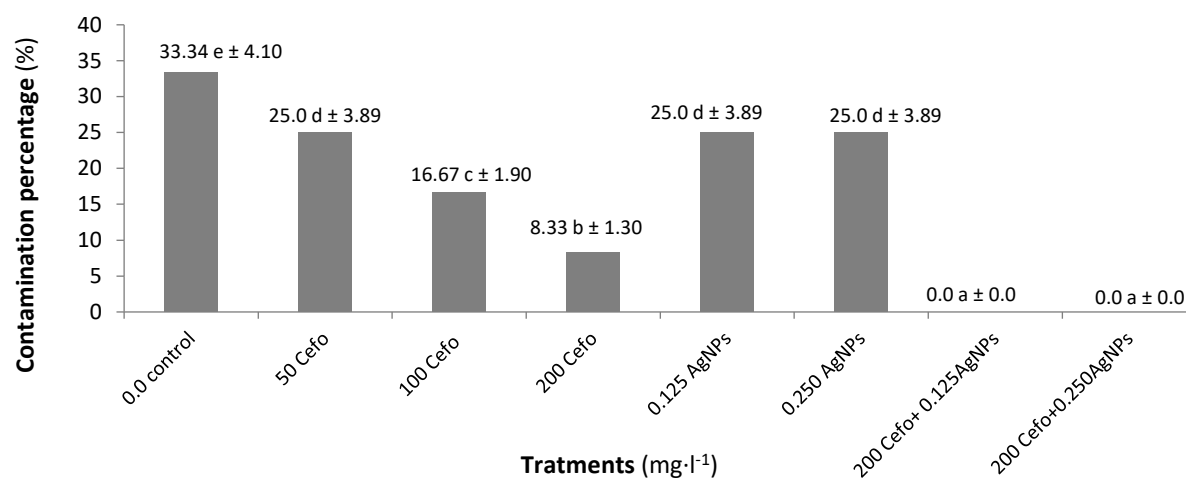


Figure 3. Effect of cefotaxime (Cefo) and silver nanoparticles (AgNPs) on contamination in *in vitro* cultures of 'Barhee' date palm

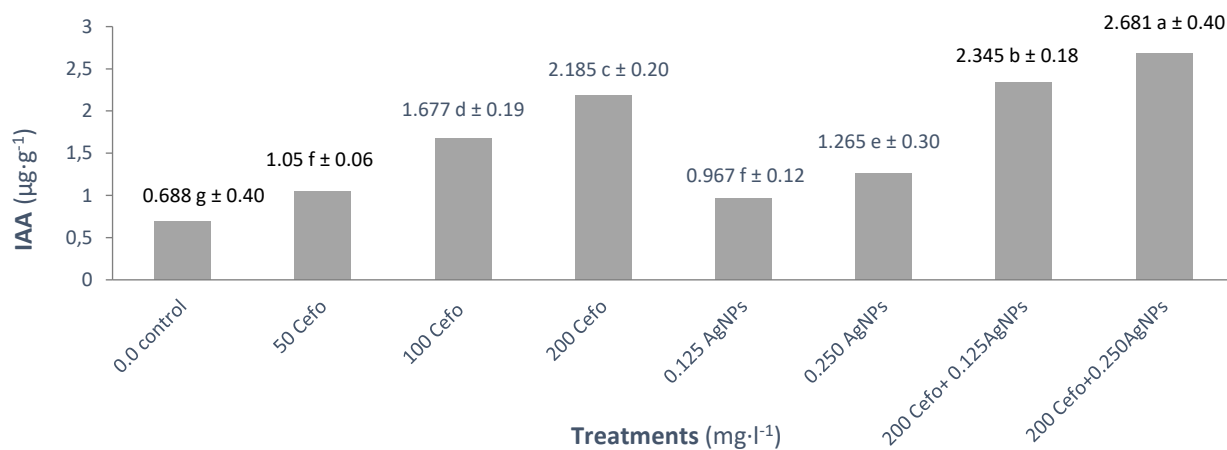


Figure 4. Effect of cefotaxime (Cefo) and silver nanoparticles (AgNPs) on IAA concentration in *in vitro* shoots of 'Barhee' date palm

DISCUSSION

Date palm micropropagation allows for mass production of plant material with appropriate attributes in a short time compared to conventional methods (Al-Mayahi 2024a, b). This method requires optimization of protocols and adaptation to specific cultivar requirements. Abdel-Karim (2017) reported that the incorporation of fungicides or antibiotics into the medium significantly reduces the contamination rate at different stages of date palm propagation. In this regard, Ahmed (2023) reported that products used to combat contamination, if improperly applied in date palm tissue cultures, can cause decreased regeneration capacity, reduced callus growth, inhibition of adventitious shoot growth, and even death of explants. The present investigation revealed the positive effects of Cefo and AgNPs on callus induction and shoot regeneration of date palm *in vitro*. Among the different treatments used, 200 mg·l⁻¹ Cefo combined with 0.250 mg·l⁻¹ AgNPs was found to be the most effective in enhancing callus production, while the combination with 0.125 mg·l⁻¹ AgNPs resulted in the highest number of shoots. This proves that Cefo and AgNPs have a callus-promoting effect and thus enhance plant regeneration. The stimulating effect of Cefo on callus growth and shoot regeneration may be associated with the inhibition of endogenous microbial growth (Manchanda et al. 2011). Cefo in shoot regeneration tissue culture may act at the level of chlorophyll synthesis (Kaur et al. 2008). Zuverza-Mena et al. (2016) suggested that AgNPs may affect plant growth by changing water and nutrient content. The positive effects of AgNPs may also be related to their role in reducing ethylene production in the culture medium, which positively influences plant differentiation (Rezvani et al. 2012). Several other mechanisms have been proposed to be behind the antibacterial activity of AgNPs, including intracellular ATP depletion via outer membrane destabilization, plasma membrane rupture, suppression of respiration, and DNA damage (Dong et al. 2017). On the other hand, AgNPs have remarkable antifungal activity. Kim et al. (2012) observed that AgNPs exerted strong antifungal activity against plant pathogenic fungi by destroying membrane integrity. Adding AgNPs to the media allowed for full control of

olive culture contamination and obtaining the highest number of shoots per explant, number of leaves per shoot and shoot length (Rostami & Shahsavari 2009; Hegazi et al. 2021). The results also showed that the presence of AgNPs significantly affected the IAA level in tissues compared to those grown on a medium without silver nanoparticles. Thus, the interaction between 200 mg·l⁻¹ Cefo with 0.250 or 0.125 mg·l⁻¹ AgNPs showed higher IAA content compared to treatments with no additives or one additive. IAA is the most abundant naturally occurring auxin and is involved in many aspects of plant growth and development, such as cell division, differentiation, and elongation (Chudasama & Thaker 2007; Mano & Nemoto 2012). AgNPs can stimulate auxin biosynthesis (Syu et al. 2014). AgNPs have been developed as plant growth promoters in plant tissue culture (Parveen & Rao 2015; Jasim et al. 2017). These effects are probably due to the influence of AgNPs on hormonal signaling, especially auxins (Syu et al. 2014). Vinković et al. (2017) reported that AgNPs affect plant hormones such as cytokinins and auxins. Callus induction requires the presence of auxins in the medium and is a prerequisite for *in vitro* plant regeneration.

In plant tissue culture, oxidative browning is a common problem that leads to reduced growth (Alagarsamy et al. 2018) and even plant death (Permadi et al. 2023). Jones and Saxena (2013) noted that the accumulation of phenolic compounds in culture media and specialized tissues causes tissue browning. Phenolic compounds are known to participate in programmed cell death in plants responding to pathogen stress. In this study, 200 mg·l⁻¹ Cefo combined with 0.250 or 0.125 mg·l⁻¹ AgNPs could inhibit the browning of cultures, reducing the abundance of polyphenols in the treated culture materials and significantly improving culture growth and development. Krishnaraj et al. (2012) found that AgNPs reduced the total phenolic content in *Bacopa monnieri*. SDS-PAGE revealed new polypeptide bonds in all cultures treated with Cefo and AgNPs. Changes in protein pattern synthesis may be due to changes in mRNA translation efficiency or regulation of RNA transcription, transport, and stability, according to the needs of the plant and its response to the type of contaminant to which it is exposed to ensure control under these conditions (Duressa et al. 2010).

CONCLUSIONS

The use of cefotaxime at a concentration of 200 mg·l⁻¹ in combination with silver nanoparticles a concentration of 0.125 or 0.250 mg·l⁻¹ increased callus production and the number of shoots three-fold and shoot regeneration twofold in an *in vitro* culture of date palm. The above treatments increased IAA content in shoots *in vitro*. Combined use of cefotaxime and silver nanoparticles eliminated callus contamination and browning. Hence, the regeneration system proposed in this study will facilitate the efficient production of contamination-free commercial planting material.

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