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Effect of Copper Sulfate and Zinc Oxide on the Growth of Vegetative Buds and Microbial Contamination in Date Palm Tissue Culture (cv. Barhi) *in vitro*

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ABSTRACT

Zinc oxide and copper sulfate can assist in plant growth and development if they are added appropriately to plant nutrition. In plant tissue culture, microbial contamination negatively affects bud quality and causes numerous issues in plant regeneration. Adding zinc oxide and copper sulfate can enhance bud characteristics and inhibit microbial contamination. This research aimed to investigate the effects of copper sulfate and zinc oxide as microbicides to inhibit microbial contamination while embarking on in vitro plant tissue culture. The objective was to further assess the effects of both compounds on growth characteristics. Both compounds were added to the culture medium at various concentrations (0, 30, 60, and 90 mg L^{-1}). Initial vegetative buds of the Barhi date palm variety were cultivated at six months of age. The results showed that zinc oxide was more effective than copper sulfate in stimulating plant vegetative growth and root development. In addition, higher concentrations of both compounds enhanced growth characteristics. Thus, zinc oxide and copper sulfate positively affected plant pigment content, mostly carotene. On the other hand, chlorophyll content was not significantly affected. Furthermore, zinc oxide was more effective than copper sulfate in reducing fungal and bacterial contamination, with the highest concentration (90 mg L-1) yielding optimal results. These results suggested that zinc oxide and copper sulfate can effectively enhance the success rate of date palm tissue culture.

Introduction

The date palm (*Phoenix dactylifera* L.) is one of the world's oldest and most significant horticultural crops, cultivated extensively across the Middle East, North Africa, and South Asia (Chao and Krueger, 2007). Its fruits are rich in diverse nutrients and have served as a staple food for centuries (Al-Shwyeh, 2019).

Recent advances have focused on propagating date palms through tissue culture techniques, such as micropropagation, somatic embryogenesis, and organogenesis (Rai et al.,

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2022). Plant tissue culture is crucial for propagating elite date palm cultivars, enabling the production of large numbers of healthy, uniform plants in a relatively short period (Rai et al., 2022). A successful tissue culture protocol requires effective steps for explant and media sterilization, making efficient sterilization techniques essential (Okoroafor, 2022). The quality of buds used in plant tissue culture is paramount to ensuring successful plant regeneration and increasing survival rates. However, microbial contamination can severely impact bud quality, leading to numerous issues in the plant regeneration process, as contamination by microbes, bacteria, and fungi is a significant challenge (Permadi, 2023). Microbial contamination in tissue culture can destroy explants and entire cultures, underscoring the importance of eliminating or reducing its occurrence (Abass, 2013; Okoroafor, 2022).

One effective method to control microbial contamination in tissue culture involves the use of copper and its derivatives, such as copper sulfate ($CuSO_4$). Copper is known for its fungicidal and bactericidal properties, playing a crucial role in inhibiting contamination (Grass et al., 2011; Gyawali et al., 2011; Noyce et al., 2007). Previous research has explored the use of copper sulfate in inhibiting microbial growth and reducing contamination during plant tissue culture (Seliem, 2021; Javed, 2017). For instance, adding copper sulfate at concentrations of 35, 70, and 140 mg L^{-1} reduced bacterial contamination in *Philodendron selloum* to 25%, 0.0%, and 0.0%, respectively (Seliem, 2021).

Zinc also plays a critical role in various cellular reactions in both microorganisms and higher organisms, even though only a low concentration is required (Leung et al., 2012). Zinc ions are generally nontoxic to higher organisms under physiological conditions, as several transporters regulate their concentration (Gaballa and Helmann, 1998). However, when provided in sufficient concentrations, zinc ions act as biocides or antimicrobial preservatives. Zinc salts dissolve in aqueous media, releasing Zn2+ ions, while zinc oxide (ZnO), which appears as a solid powder, also releases Zn^{2+} and exhibits antimicrobial activity through several mechanisms (Jones et al., 2008).

This research was based on the hypothesis that copper sulfate and zinc oxide positively affect the growth and quality of date palm buds. Additionally, it is hypothesized that these compounds can inhibit microbial contamination in tissue culture. Therefore, the primary objective of this study was to assess the effects of copper sulfate and zinc oxide on the vegetative characteristics of date palm buds (cv. Barhi) in plant tissue culture. Furthermore, the research aims to determine the effectiveness of these microbicides in inhibiting microbial contamination associated with vegetative buds *in vitro*.

Material and Methods

This experiment was conducted in the tissue culture laboratory at the Date Palm Research Centre, University of Basrah, Basra, Iraq. The study aimed to investigate the effects of copper sulfate and zinc oxide on microbial contamination in palm tissue culture and to evaluate their impact on the growth of vegetative buds. The Barhi cultivar, a widely cultivated and commercially significant variety in Iraq, was selected for these laboratory experiments.

Plant materials

Vegetative buds of the Barhi cultivar were obtained from the Date Palm Research Centre laboratories at the University of Basrah, Iraq. These buds were initially prepared by cultivating apical buds in Murashige and Skoog (MS) culture medium using the Direct Organogenesis technique for date palm tissue propagation.

Medium preparation

The medium for growing the vegetative buds was prepared by dissolving 4.4 g of Murashige and Skoog (MS) salts in 700 mL of distilled water, using a 1 L volumetric flask placed on a magnetic stirrer hot plate (Gamborg et al., 1968). The medium was supplemented with Gamborg's vitamin group, which included 100 mg L^{-1} myo-inositol, 1 mg L^{-1} nicotinic acid, 0.1 mg L^{-1} pyridoxine, and 10 mg L^{-1} thiamine (Phytotechnology Lab Com). Additional components included 4000 mg L^{-1} sucrose, 170 mg L^{-1} acid sodium orthophosphate, 40 mg L^{-1} adenine sulfate, 250 mg L^{-1} neutral activated charcoal powder, and 5 mg L^{-1} glycine. Growth regulators—Indole acetic acid (IAA), Benzyl adenine (BA), and Kinetin (Kn)—were added at a concentration of 0.5 mg L^{-1} each. IAA was dissolved in 0.1 M sodium hydroxide, while BA and Kn were dissolved in 0.1 M hydrochloric acid. The pH of the medium was adjusted to 5.7 using a pH meter (Lutron model 206) with HCl and NaOH 0.1 M solutions. The final volume was then increased to 1 L, and 8000 mg L^{-1} agar powder was added. The medium was heated to 95 °C to ensure complete dissolution.

Medium treatment with copper sulfate and zinc oxide

The sterilized medium was treated with varying concentrations of copper sulfate (General Drugs House Co. Ltd., India) and zinc oxide (General Drugs House Co. Ltd., India) at 0, 30, 60, and 90 mg L^{-1}, with four replicates for each concentration. The concentrations were prepared using sterilized distilled water, and 10 mL of each solution was added to 50 mL of medium in 250 mL bottles. The bottles were sealed with aluminum foil and metal caps. The cultivation tools and prepared medium were autoclaved at 121 °C and 1.05 kg cm^{-2} pressure for 20 minutes. After sterilization, the bottles were thoroughly shaken to homogenize the medium, allowed to cool, and stored in a refrigerator until use.

Cultivation of buds on the treated medium

Vegetative buds of the Barhi variety, selected at six months of age, were used to conduct the experiments. All treatment groups were incubated at 27 ± 2 °C for 90 days to allow the buds to multiply, elongate, and root (Fig. 1).

Fig. 1. Cultivation and propagation of the initial vegetative buds on (MS) culture medium.

Determination of vegetative characteristics

Bud fresh weight, count, length, and diameter of the buds were measured. Also, root length and root count were recorded. Pigment content was estimated in the plants. All of these characteristics were used as indicators of plant growth and health.

Fresh weight of vegetative buds

Bud fresh weight was calculated according to a relevant protocol (Saad, 2001). First, the flasks were weighed (first weight) immediately after adding the medium, and then the same flasks were weighed after the medium had dried (current weight). The same flasks were weighed immediately after planting the vegetative buds in them. The percentage of weight loss in the medium was calculated according to Equation 1:

$$\% \text{ of loss of medium} = \frac{(\text{current weight} - \text{first weight})}{(\text{first weight})} \times 100$$

The actual weight of each flask (for all contents) was determined by weighing each treated flask, taking into account the percentage of loss of the medium. Then, the weight of the vegetative buds at the beginning of the experiment was calculated according to Equation 2:

The weight of the vegetative buds at the beginning of the experimen
= the weight of the flask after planting
− the weight of the before planting

Finally, the weight of the vegetative buds was calculated as follows:

Weight of vegetative buds =
actual weight of the flask −
first weight of the buds at the beginning of the experiment

Vegetative buds count:

The number of vegetative buds for each flask was calculated separately, according to Equation 3:

Number of vegetative buds
= number of total buds at the end of the experiment
− number of buds at the beginning of the experiment

Length of vegetative buds

Bud length was measured using a ruler (mm),

with four replicates for each concentration.

Diameter of vegetative buds
Ten vegetative buds were extracted from each replicate and their diameter was measured (mm) using a Vernier.

Length of bud roots
Bud root length was measured (mm) using a ruler, with four replicates for each concentration.

Root count
The number of vegetative bud roots for each flask was calculated separately.

Leaf pigment content
The chlorophyll content of the leaves and carotene pigments was estimated based on Holden's method described by Howertiz (1975). Fresh leaves (0.5 g) were weighed, cut into small pieces using scissors, and grounded using a ceramic mortar with 15 mL of acetone 80% (acetone/water v/v). The filtrate was separated from the precipitate using a centrifuge at 1600 rpm for 10 min. The extract was repeated until the color of the precipitate was free of green pigments. Then, the volume of the extract was increased to reach 15 mL of acetone collected in test tubes covered with opaque paper to block the light and to prevent photo-oxidation of the pigments. Optical density absorbance of the filtrate was determined by a Shimadzu spectrophotometer (UV-1700) at 645, 665, and 480 nm. The concentration of chlorophyll and carotene pigments in the leaves was estimated, and calculated based on mg 100 gm^{-1} soft plant tissue, according to Equations 4 and 5:

$$\text{Total chlorophyll (mg L}^{-1}) = 20.2 \times \text{O. D. (645)} + 8.02 \times \text{O. D. (665)}$$

$$\boldsymbol{X} = \frac{\text{EY}}{(e\ 100)\times 1000\ mg}$$

Where:
O.D: Optical density of extracted chlorophyll
E: spectrophotometer reading at 480 nm
X: milligrams of carotene in 1 cm3 of solution
Y: volume of the final solution after dilution with acetone
e: carotene constant 2300
The units were converted to (mg 100 gm^{-1}) according to Zaehringer (1974).

$$\text{Mg 100 gm}^{-1} = \frac{(\text{mg L}^{-1})}{1000} \times \frac{100}{(sample\ weight)}$$

Percentage of contamination
The percentage of contamination was calculated according to Equation 6. Each concentration was replicated three times. Ten jars were used in each replicate.

$$\text{Mg 100 gm}^{-1} = \frac{(\text{mg/L})}{1000} \times \frac{100}{(sample\ weight)}$$

Data analysis
The experiments were conducted in a completely randomized design with four replications. Analysis of variance (ANOVA table) enabled data evaluation using the Statistical Package for the Social Sciences (SPSS) software v 22. The Least Significant Difference (LSD) facilitated the separation of mean values with significant differences ($P < 0.05$). The results appeared as mean values $\pm$ SE.

Results
Determination of vegetative characteristics
Based on the results (Fig. 2), zinc oxide significantly outperformed copper sulfate at all concentrations in promoting better vegetative and root growth. A comparison of mean values indicated a significant superiority of zinc oxide over copper sulfate across most of the tested concentrations when evaluating multiple parameters, including fresh bud weight, bud count, bud length, bud diameter, root count, and root length.

Both copper sulfate and zinc oxide demonstrated a positive correlation with bud vegetative and root growth as their concentrations increased. The highest fresh bud weight was recorded at a concentration of 90 mg L^{-1} for both zinc oxide and copper sulfate. Specifically, fresh bud weights were 24.3 g and 16.05 g in response to zinc oxide and copper sulfate, respectively.

Similarly, the highest bud counts were observed at a concentration of 90 mg L^{-1} for both compounds, outperforming the control treatment (0 mg L^{-1}). Zinc oxide treatment resulted in 25.00 buds, while copper sulfate produced 20.25 buds, compared to the control value of 7.32 buds for both treatment groups (Figs. 2b and 3).

Regarding bud length and diameter, both compounds at 90 mg L^{-1} produced significantly better results than the other concentrations. Bud lengths reached 16.75 cm with zinc oxide and 9.11 cm with copper sulfate (Figs. 2c and 4). Additionally, bud diameter measured 8.21 mm in the zinc oxide-treated medium, compared to 7.04 mm in the copper sulfate-treated medium (Fig. 2d). Furthermore, at 90 mg L^{-1}, both zinc oxide and copper sulfate resulted in higher root counts

and lengths compared to the other concentrations. The root count was 5.00, with a length of 7.45 cm in the zinc oxide-treated medium, while the root count was 1.50, with a length of 6.13 cm in the copper sulfate-treated medium (Figs. 2e, f, and 5).

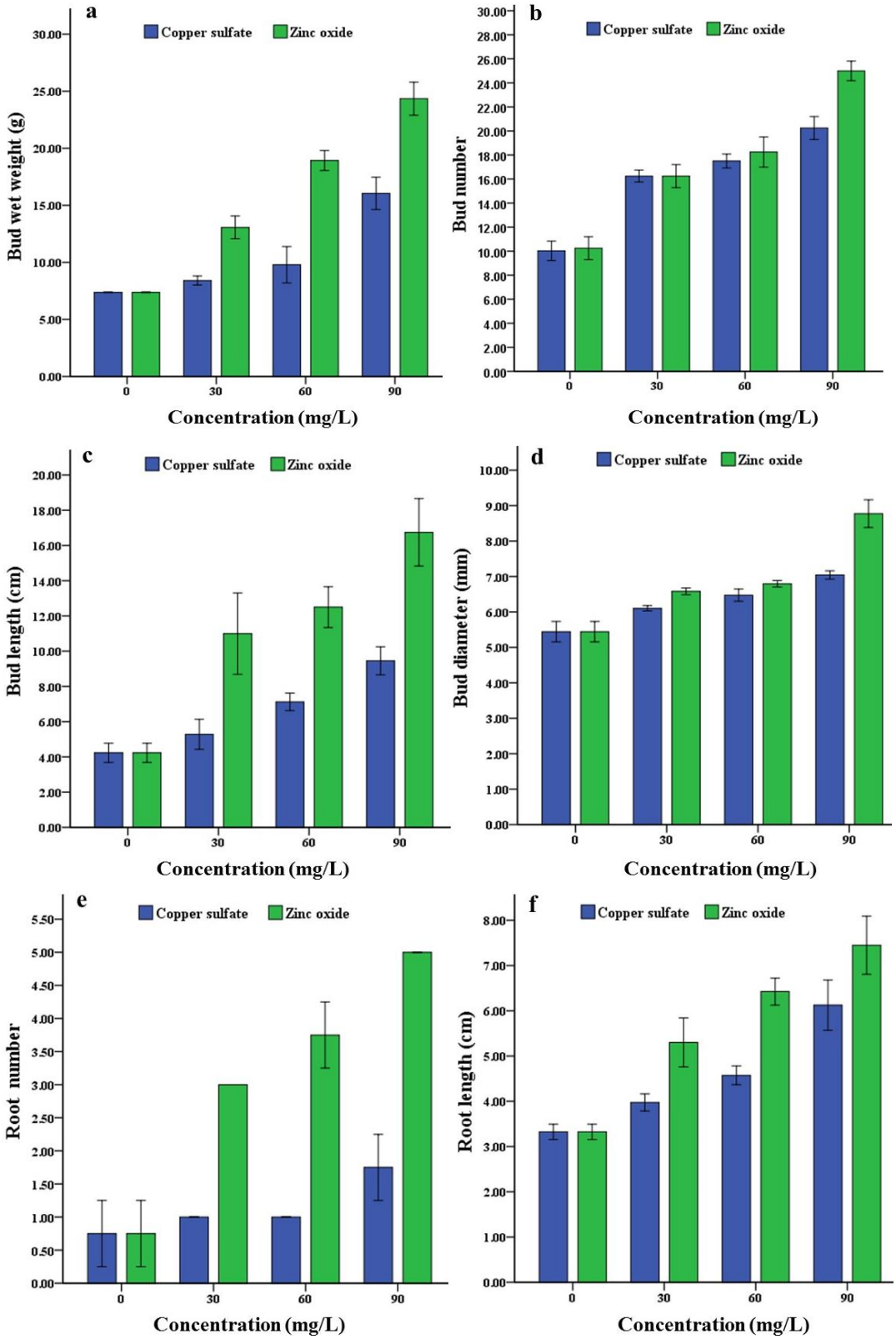

Fig. 2. Effect of zinc oxide and copper sulfate on bud and root growth. (a) Fresh bud weight, (b) bud count, (c) bud length, (d) bud diameter, (e) root count, and (f) root length. Bars indicate the standard error of mean values.

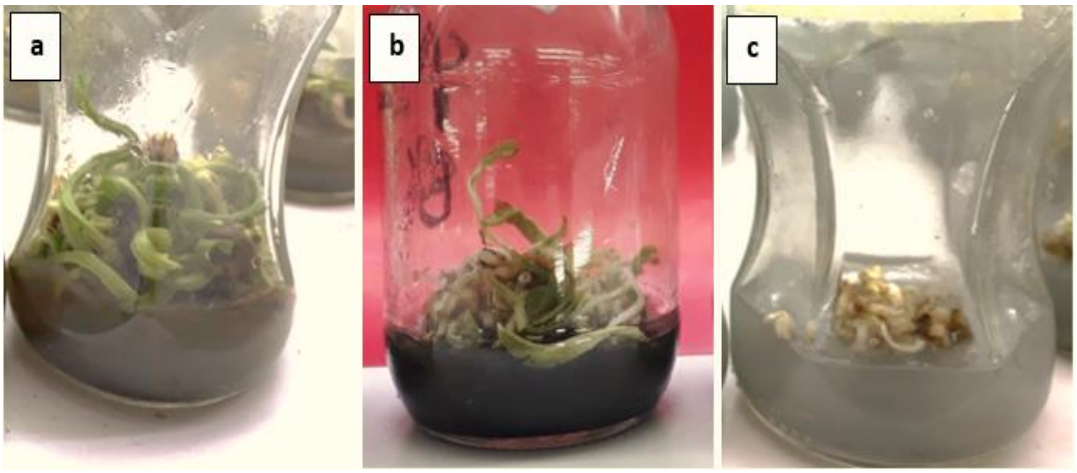

Fig. 3. Bud count after 30 d of cultivation. (a) Medium treated with copper sulfate. (b) Medium treated with zinc oxide. (c) Control treatment.

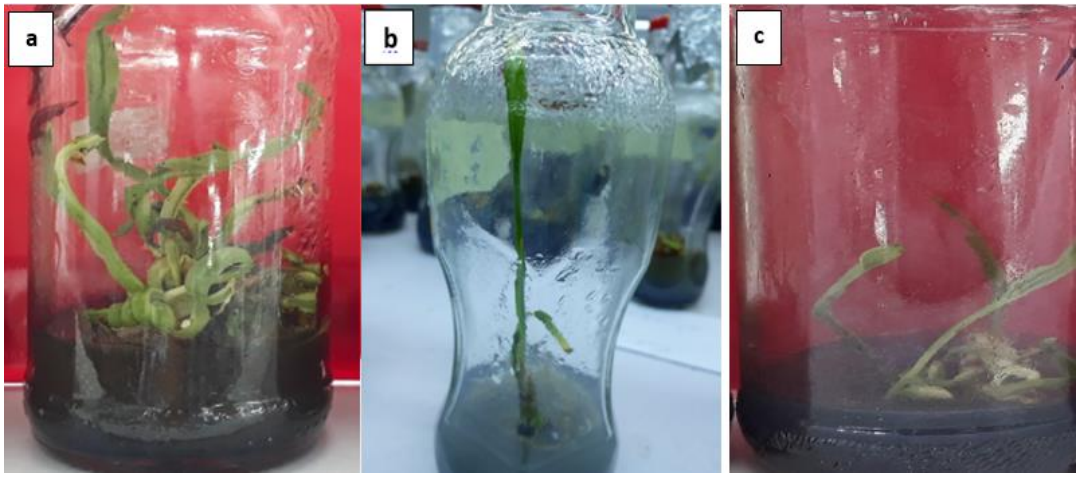

Fig. 4. Bud length after 60 d of cultivation. (a) Medium treated with zinc oxide. (b) Medium treated with copper sulfate. (c) Control treatment.

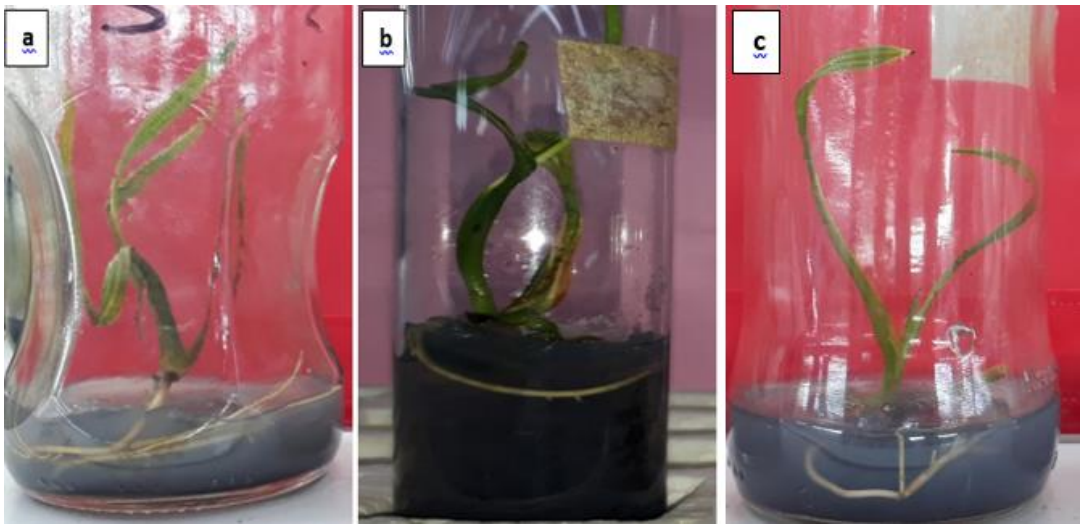

Fig. 5. Root length after 90 d of cultivation. (a) Medium treated with zinc oxide. (b) Medium treated with copper sulfate. (c) Control treatment.

The results indicated that both chemical agents, when used at a concentration of 90 mg L^{-1}, were significantly more effective than other tested concentrations in terms of carotene content. The

highest carotene content was observed at the 90 mg L^{-1} concentration for both treatments. Vegetative buds grown on a culture medium supplemented with copper sulfate exhibited a carotene content of 0.117 mg L^{-1}, while those grown on a medium with zinc oxide had a carotene content of 0.11 mg L^{-1}. In contrast, the control treatment (0 mg L^{-1}) resulted in the lowest carotene content at 3.66 mg L^{-1}. Regarding chlorophyll content, no significant effects were noted. At the 90 mg L^{-1} concentration of both copper sulfate and zinc oxide, chlorophyll content became higher compared to the other tested concentrations, although the differences were not statistically significant (Fig. 6).

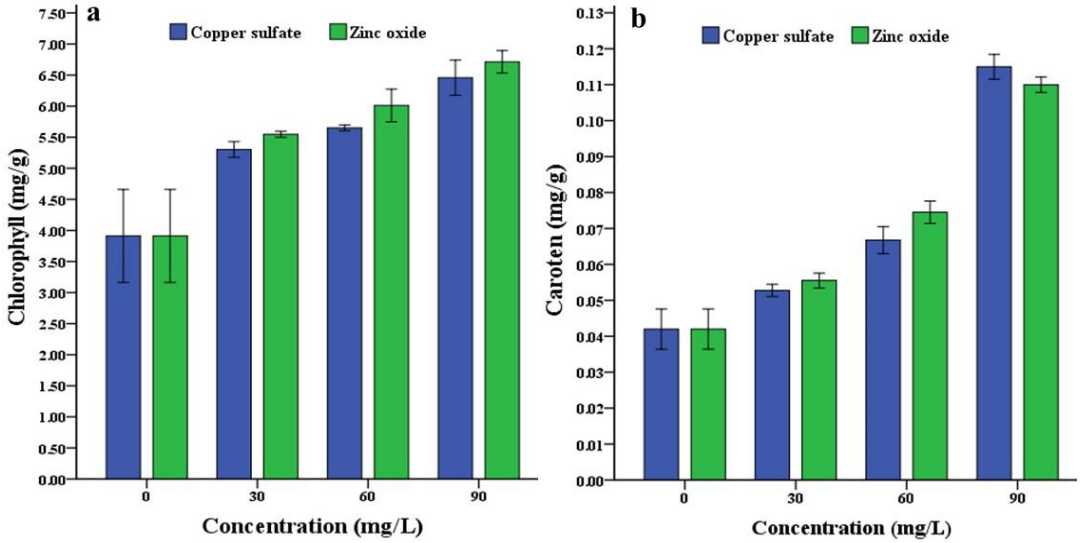

Fig. 6. Effect of zinc oxide and copper sulfate on plant pigment content. (a) leaf chlorophyll content. (b) leaf carotene content. Bars indicate standard errors of the mean values.

Effects of zinc oxide and copper sulfate on microbial contamination

Significant differences were observed among zinc oxide and copper sulfate treatments across all concentrations in reducing fungal and bacterial contamination. At a concentration of 90 mg L^{-1}, both chemical agents were most effective in reducing microbial contamination compared to other concentrations. Specifically, starting with initial contamination rates of 20.25% for bacteria and 62.00% for fungi at 0 mg L^{-1}, zinc oxide at 90 mg L^{-1} reduced bacterial contamination to 2.00% and fungal contamination to 16.00%. In comparison, copper sulfate reduced bacterial contamination to 6.75% and fungal contamination to 19.00% from the same initial contamination rates (Figs. 7 and 8).

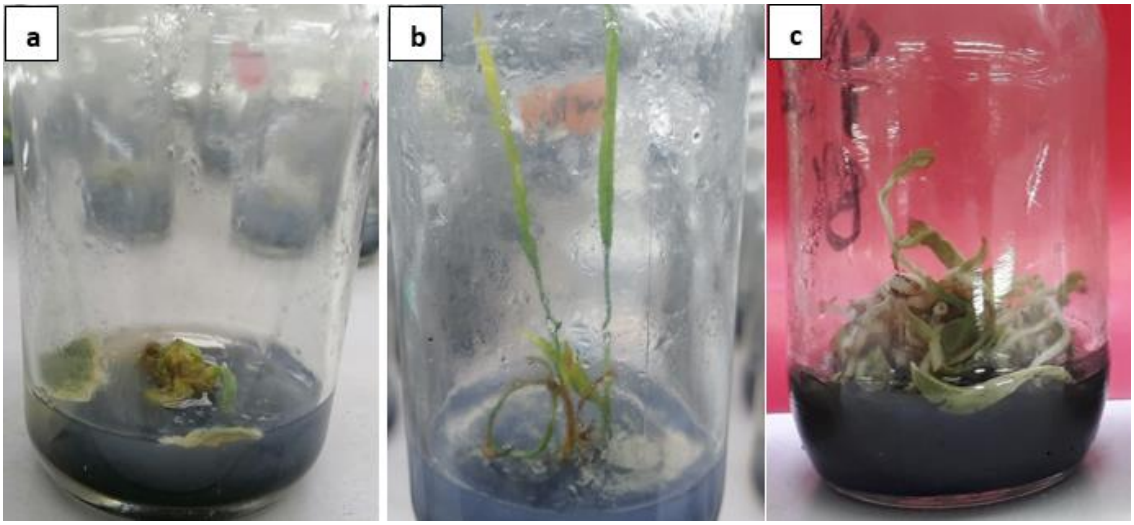

Fig. 7. Effect of zinc oxide and copper sulfate on microbial contamination. (a) percentage of fungal contamination. (b) percentage of bacterial contamination. Bars indicate standard errors of mean values.

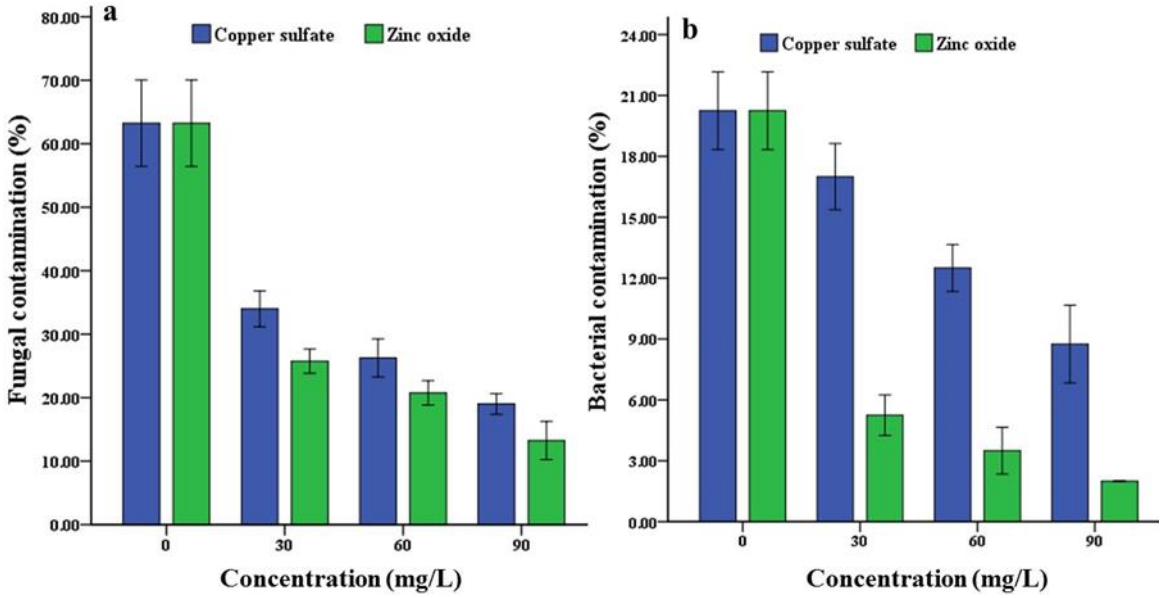

Fig. 8. Effect of zinc oxide on microbial contamination. (a) Fungal contamination in a control treatment. (b) Bacterial contamination in a control treatment. (c) Vegetative buds free from microbial contamination, cultured on medium with zinc oxide.

Discussion

The results of this study demonstrated that both zinc oxide and copper sulfate, even at low concentrations, effectively reduced microbial contamination and enhanced plant growth characteristics. Zinc and copper ions are essential micronutrients for plant growth (Nadeem et al., 2018; Kumar et al., 2021). Therefore, applying zinc oxide and copper sulfate at appropriate concentrations can significantly improve plant growth and development. These elements are crucial for various physiological processes, support plant metabolism, and compensate for deficiencies that may lead to stunted growth and increased susceptibility to disease (Nadeem et al., 2018; Kumar et al., 2021). The observed enhancements in vegetative and root growth following the application of zinc oxide and copper sulfate may reflect the gradual release of zinc and copper ions. These treatments likely affect root elongation and vegetative growth by participating in the synthesis of auxins (zinc) or influencing enzyme activity (copper), including enzymes involved in auxin metabolism (Suganya, 2020; Bhat et al., 2020). Consequently, adequate availability of zinc oxide and copper sulfate can promote the development of stronger root systems and more vigorous plant growth. Zinc and copper ions act as cofactors for many plant enzymes, and their presence is essential for maintaining healthy enzyme activity and metabolic balance. By providing zinc oxide and copper sulfate, plants can sustain necessary enzyme activities and metabolic processes vital for growth and development (Brown et al., 1993; Bhat et al., 2020).

Additionally, copper facilitates the uptake and utilization of other essential nutrients, including nitrogen and iron. It plays a role in nitrogen metabolism and aids in the conversion of nitrate into amino acids, the building blocks of proteins. Therefore, adequate copper levels are necessary for optimal plant growth, ensuring efficient nutrient uptake and utilization (Cui et al., 2022; Rai et al., 2021).

The study also found that both zinc oxide and copper sulfate enhanced plant pigments, such as chlorophyll and carotene. The zinc and copper ions released by these compounds participate in various physiological processes, including photosynthesis, enzyme activity, protein synthesis, and carbohydrate metabolism, resulting in increased levels of chlorophyll and carotene (Yruela, 2009; Solanki, 2021).

Growth enhancement and pigment production following the use of zinc oxide and copper sulfate may also be indirectly influenced by reduced microbial contamination and the prevention of subsequent damage. Previous studies have confirmed that both zinc oxide and copper sulfate exhibit inhibitory activity against a wide range of microbial contaminants. Zinc oxide, with its antimicrobial properties, is particularly effective in combating microbial challenges (Jin and Jin, 2021). Its antimicrobial activity is attributed to the release of zinc ions, which interact with microbial cell membranes, disrupting their

integrity and function (Lallo da Silva, 2019). Zinc oxide also inhibits fungal enzyme activity and growth (Zheng et al., 2014). Similarly, copper sulfate exhibits broad-spectrum activity and inhibits the growth and spread of pathogens such as fungi and bacteria by disrupting their cellular processes (Lamichhane et al., 2018). Furthermore, copper is essential for the synthesis of several plant-based compounds that help plants protect themselves against pathogens and pests. Copper contributes to lignin production, strengthening cell walls and fortifying physical barriers (Tripathi et al., 2022).

Conclusion

This research investigated the effects of copper sulfate and zinc oxide on microbial contamination associated with the vegetative buds of date palm (cv. Barhi) while propagating the explants via plant tissue culture *in vitro*. As evidenced by various growth parameters, zinc oxide was more effective than copper sulfate in enhancing plant vegetative features and root growth. Higher concentrations of zinc oxide and copper sulfate generally caused better growth characteristics. Thus, zinc oxide and copper sulfate increased plant pigment contents, particularly carotene. However, the chlorophyll content did not appear to be significantly affected. Moreover, zinc oxide performed better than copper sulfate in reducing fungal and bacterial contamination, with the highest concentration (90 mg L^{-1}) being the most effective.

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Conflict of Interest

The authors indicate no conflict of interest in this work.

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