

# ENHANCING ANTIOXIDANT ACTIVITY AND PHENOLIC CONTENT IN DATE PALM (*Phoenix dactylifera* L.) CALLUS CULTURES THROUGH TRACE ELEMENT SUPPLEMENTATION

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## ABSTRACT

This study investigates the impact of trace element supplementation on phenolic content, antioxidant activity, and callus initiation in Hassawi date palm (*Phoenix dactylifera* L.) cultivars (Khalas, Ruziz, and Shishi) grown on Murashige and Skoog (MS) medium. Varying concentrations of trace elements ( $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ,  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ ,  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , and  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  at zero, half, full, and double Stock E) were tested to evaluate their effects on secondary metabolite production and oxidative stress management. Results demonstrated that double concentrations of Stock E significantly enhanced phenolic content ( $2.68 \text{ mg g}^{-1}$  GAE) and antioxidant activity (96.28 % inhibition and  $978.69 \text{ mg g}^{-1} \mu\text{M}$  Trolox), with the Shishi cultivar showing the most pronounced response. The improved phenolic accumulation and antioxidant capacity were attributed to the activation of enzymatic pathways, including phenylalanine ammonia-lyase (PAL), and the regulation of reactive oxygen species (ROS) through enhanced antioxidant enzyme activity. Additionally, reduced browning in callus tissues indicated effective modulation of polyphenol oxidase (PPO) activity. The study underscores the potential for optimizing trace element concentrations to enhance bioactive compound production in date palms. These findings contribute to sustainable agricultural practices, offering practical applications in food, pharmaceutical, and nutraceutical industries. Future research should focus on molecular analyses to elucidate the precise pathways influenced by trace elements and explore large-scale production systems for commercial antioxidant extraction.

**Keywords:** Antioxidants, browning, callus initiation, date palm, total phenolic, trace element,  $\mu\text{M}$  Trolox.

## INTRODUCTION

Al-Ahsa Oasis, the largest green oasis in Saudi Arabia and the world, is a critical source of food security for the kingdom. It hosts approximately 4.1 million date palm trees (*Phoenix dactylifera* L.). Hassawi cultivars constitute the main crop alongside other plants unique to the region (MEWA, 2023). The oasis's primary cultivars—Khalas, Ruziz, and Shishi—account for over 70 % of date palm tree production (Almadini *et*

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*al.*, 2021; Ismail *et al.*, 2022). These cultivars are particularly notable for their resilience to drought and heat, contributing significantly to environmental sustainability in line with Saudi Arabia's Vision 2030.

Dates from these cultivars are rich in fiber, minerals, and beneficial sugars, enhancing immunity and preventing diseases (Al-Saikhan, 2006). Furthermore, dates are distinguished by their high antioxidant content, such as phenolic compounds and dactylifera acid (Al-Shwyeh, 2019). This positions date palms as a valuable source for enhancing human health and combating chronic diseases (Zhang and Sun, 2021). It also contributes to cell regeneration and repair (Qiu *et al.*, 2022).

Despite their long growth cycle, the ability to harness antioxidants from date palms through advanced tissue culture techniques presents a sustainable solution. Plant tissue culture, particularly callus culture, offers a controlled platform for producing bioactive compounds, essential for food, pharmaceutical, and nutraceutical industries. The increasing demand for antioxidants has driven research into optimizing tissue culture systems to enhance phenolic compound production (Batista *et al.*, 2018).

This study aims to optimize the concentrations of trace elements in Murashige and Skoog (MS) medium to enhance phenolic production and antioxidant activity in callus cultures of Khalas, Ruziz, and Shishi cultivars. Trace elements such as zinc, manganese, and copper serve as cofactors for enzymes in the phenylpropanoid pathway, crucial for phenolic biosynthesis (Vazquez-Marquez *et al.*, 2024). Iron, a key trace element, supports the synthesis of enzymes related to respiration and photosynthesis, reducing oxidative stress by mitigating free radicals (Amente and Chimdessa, 2021). The experimental design incorporates controlled treatments and robust statistical analyses to validate the effects of trace elements on callus initiation, phenolic content, and antioxidant activity. By systematically assessing these variables, the study provides practical insights for enhancing tissue culture protocols and scaling antioxidant production (Zhang, 2023).

Aligning with global priorities for sustainable agriculture, this research explores the potential of date palm tissue culture to improve phenolic biosynthesis and antioxidant activity. The findings contribute to developing efficient, large-scale production systems, such as bioreactors, and assessing the economic feasibility of these protocols for commercial use (Liu *et al.*, 2018; Muszyńska and Labudda, 2019; Dai *et al.*, 2023). This approach underscores the broader relevance of optimizing trace element concentrations in tissue culture systems to support sustainable food systems and agricultural resource utilization.

## MATERIALS AND METHODS

### Callus initiation

The plant material for this study consisted of three primary date palm cultivars (Khalas, Ruziz, and Shishi) from Al-Ahsa Oasis, collected during the period from October 2021 to April 2022. Young offshoots (50–70 cm in height and weighing 5–7 kg) were separated from adult *Phoenix dactylifera* L. trees.

Under sterile conditions, explants underwent surface sterilization. First, they were rinsed three times with sterile distilled water, followed by a 20-min soak in a 3.2 % sodium hypochlorite solution (60 %; v/v commercial bleach with three drops of Tween, 20 per 100 mL), and then rinsed again three times with sterile distilled water (Alturki *et al.*, 2013). The explants were then transferred to sterile Petri dishes and cut into 5 × 5 mm pieces. Subsequently, all explants were soaked in 70 % ethanol for 3 min, rinsed three times with sterile water, and immersed in a 1.5 g L<sup>-1</sup> mercuric chloride (HgCl<sub>2</sub>) solution for 3–5 minutes. Finally, explants were rinsed three times with sterile distilled water and cultured under laminar flow conditions (Shehata *et al.*, 2014).

The explants were cultured on a basal Murashige and Skoog (MS) nutrient medium (Murashige and Skoog, 1962), formulated with specific concentrations of inorganic salts (Table 1). To prepare the final medium, 20 mL of stock solutions A and B and 5 mL of stock solutions C, D, E, F, and G were added to 1 L of distilled water and

**Table 1.** Minor mineral element concentrations (Stock E) and additional components used in Murashige and Skoog (MS) medium for date palm (*Phoenix dactylifera* L.) explant culture.

Stock solution	Constituents	Concentration in MS medium (g L <sup>-1</sup> )	To make up 1 L of MS medium (mL L <sup>-1</sup> )	Concentration difference	Code
A	NH <sub>4</sub> NO <sub>3</sub>	82.500	20	Full	---
B	KNO <sub>3</sub>	95.000	20	Full	---
C	H <sub>3</sub> BO <sub>3</sub>	1.240	5	Full	---
	KH <sub>2</sub> PO <sub>4</sub>	34.000			
	KI	0.166			
	Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O	0.050			
D	CoCl <sub>2</sub> ·6H <sub>2</sub> O	0.005	5	Full	---
	CaCl <sub>2</sub> ·2H <sub>2</sub> O	88.000			
E	MgSO <sub>4</sub> ·7H <sub>2</sub> O	74.000	5	Zero	E-1
	MnSO <sub>4</sub> ·4H <sub>2</sub> O	4.460		Half	E-2
	ZnSO <sub>4</sub> ·7H <sub>2</sub> O	1.720		Full	E-3
	CuSO <sub>4</sub> ·5H <sub>2</sub> O	0.005		Double	E-4
F*	Na <sub>2</sub> ·EDTA	7.450	5	Full	---
	FeSO <sub>4</sub> ·7H <sub>2</sub> O	5.570			
G	Thiamine·HCl	0.200	5	Full	---
	Nicotinic acid	0.100			
	Pyridoxine·HCl	0.100			
	Glycine	0.400			

\*This stock solution was prepared by dissolving each constituent in 200 mL-distilled water. The Na<sub>2</sub>·EDTA·2H<sub>2</sub>O solution was heated, and the FeSO<sub>4</sub>·7H<sub>2</sub>O solution was added with continuous stirring. After cooling, it was diluted to 1000 mL with distilled water. All stocks were stored in amber colored bottles in refrigerator. Modified media used for cultures were supplemented with different concentrations of phytohormones, carbohydrates, vitamins, and other addenda (Murashige and Skoog, 1962).

stirred. The mix was supplemented as follows (in mg L<sup>-1</sup>): 170 NaH<sub>2</sub>PO<sub>4</sub>·2H<sub>2</sub>O, 80 adenine sulfate, 100 myo-inositol, 2 biotin, 2.5 vitamin B<sub>1</sub> (thiamine-HCl), 100 2,4-dichlorophenoxyacetic acid, 5 6-benzylaminopurine, 30 000 sucrose, 7000 agar, and 2000 activated charcoal (Sigma Chemical Co., St. Louis, MO, USA). Before adding the agar and activated charcoal, the pH was adjusted to 5.7. The medium was autoclaved at 1.2 kg cm<sup>-2</sup> at 121 °C for 20 min, then dispensed into small jars with 25 mL of media each (Shehata *et al.*, 2014).

The explants were transferred to a modified MS medium with different stock E (MgSO<sub>4</sub>·7H<sub>2</sub>O, MnSO<sub>4</sub>·4H<sub>2</sub>O, ZnSO<sub>4</sub>·7H<sub>2</sub>O, and CuSO<sub>4</sub>·5H<sub>2</sub>O) concentrations, i.e., zero, half, full, and double (0, 2.5, 5, and 10 mL L<sup>-1</sup>, respectively). Cultures were incubated at 25 ± 2 °C in a totally dark growth room for six months and maintained by transferring to fresh media every two months. Data was recorded for all cultivars at the end of each subculture. The visual scoring system by Pottino (1981) was used to read the callus data and brown discoloration on the explants of each cultivar under study, with five replicates per treatment. The surviving and browning explant jars were numbered, and cultivars with callus initiation were registered to determine the effects of microelements on callus initiation and phenolic compounds on browning.

#### Antioxidant analysis

The following chemicals and reagents were used: Folin-Ciocalteu's phenol reagent, gallic acid, sodium carbonate, ascorbic acid, trichloroacetic acid, catechin, sodium nitrite, sodium nitrate, aluminum chloride, and methanol (all from Merck, Darmstadt, Germany), along with 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) diammonium salt, 2,4,6-tripyridyl-S-triazine (TPTZ), and 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) from Sigma-Aldrich (USA). Additionally, FeCl<sub>3</sub>·3H<sub>2</sub>O, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), sodium acetate, sodium carbonate, and potassium persulfate from Sigma-Aldrich were used as analytical-grade reagents for analyzing antioxidant compounds (Alturki *et al.*, 2013).

For sample preparation, 3 g of callus tissue were crushed and dry-blended for 10 min, then extracted with 100 mL methanol using an orbital shaker (LSI-LabTECH, Korea) at 20 °C for 5 h. The extract was filtered and centrifuged at 4000 rpm for 10 min, then concentrated using a rotary evaporator (Heidolph-Laborota, Germany) under reduced pressure at 40 °C for 3 h. Extracts were stored in dark glass bottles at -20°C until further analysis (Shehata *et al.*, 2014).

Antioxidant activity was measured using the ABTS method as described by Cai *et al.* (2004). ABTS radical cations were prepared by reacting 7 µM ABTS with 2.45 µM potassium persulfate in the dark at 23 °C for 16 h, then diluting with 80 % ethanol to an absorbance of 0.7 ± 0.005 at 734 nm. To each 0.1 mL test sample, 3.9 mL of ABTS were added, and absorbance was measured at 734 nm after 6 min at room temperature. A Trolox standard curve (0–15 µM in 80 % ethanol) was used for comparisons, and results were expressed in Trolox equivalents (Aldaej *et al.*, 2014).

Total phenolics were determined using Folin-Ciocalteu reagent (Singleton and Rossi, 1965). Every 40 mL explant extract or gallic acid standard was mixed with 1.8 mL of 10-fold prediluted Folin-Ciocalteu reagent, incubated for 5 min at room temperature, and combined with 1.2 mL of 7.5 % sodium bicarbonate. Absorbance was measured at 765 nm after 60 min, and results were expressed as milligrams of gallic acid equivalents (GAE) per 100 g of sample (Shehata *et al.*, 2014).

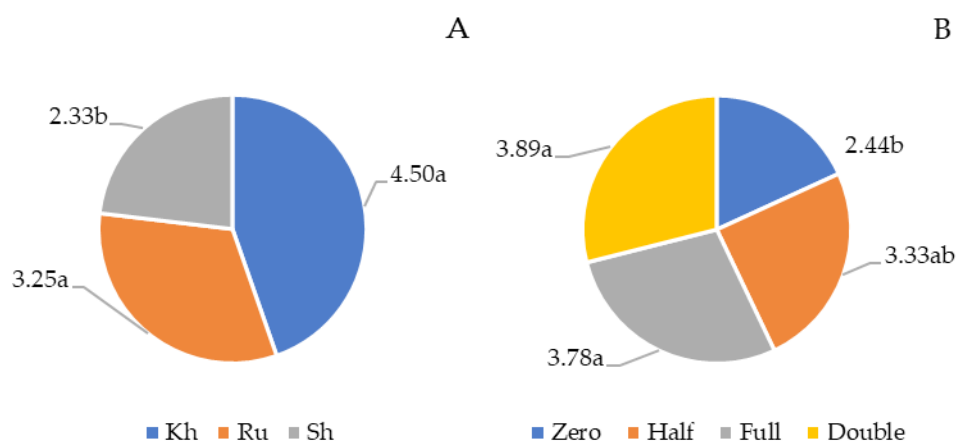
### Statistical analysis

Data were analyzed using analysis of variance (ANOVA) for a completely randomized design as per (Gomez and Gomez, 1984). Treatment means were compared using the least significant difference (LSD) at a 5 % probability level. All computations and analyses were performed using SAS software (SAS Institute Inc., 2001). Multivariate analysis, including heat map analysis via the Orange Data Mining software (Demsar *et al.*, 2013), was used to illustrate correlations between antioxidant activity and total phenolic content.

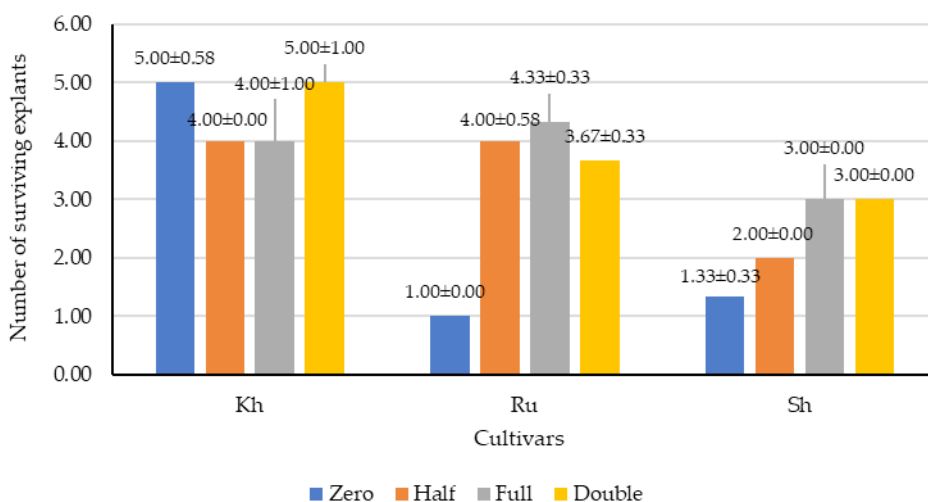
## RESULTS AND DISCUSSION

### Effect of microelements on callus initiation

The number of surviving explants (Figures 1 and 2) significantly varied among cultivars and Stock E (SE) concentrations ( $p < 0.01$ ). The survival rate of explants increased with higher SE concentrations, with the highest survival observed at E-4 (mean = 3.89) and the lowest at E-1 (mean = 2.44). Among the cultivars, Khalas (Kh) exhibited the highest overall survival (mean = 4.5), followed by Ruziz (Ru) (mean =



**Figure 1.** Specific effect of three date palm (*Phoenix dactylifera* L.) cultivars (A) and different Stock E concentrations (B) on the number of surviving explants during callus stage of *in vitro* culture. Cultivars (Kh: Khalas; Ru: Ruziz; Sh: Shishi); Stock E solution (Zero: E-1; half: E-2; full: E-3; double: E-4).



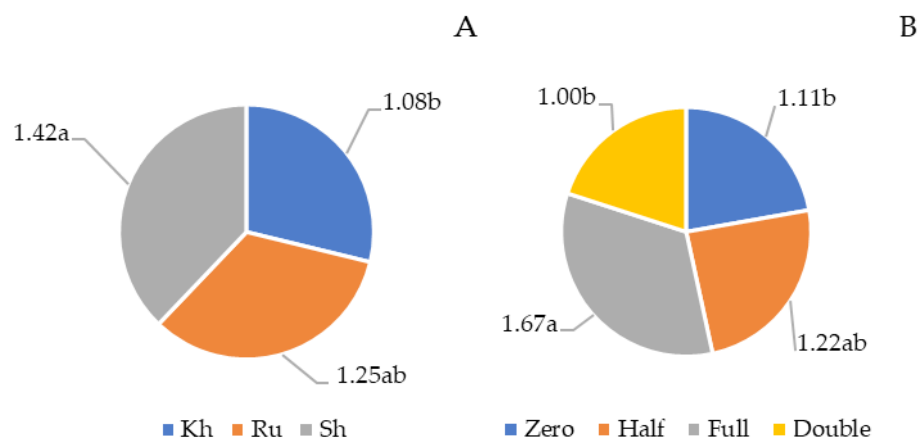
**Figure 2.** Interaction effect of different Stock E concentrations and three date palm (*Phoenix dactylifera* L.) cultivars on the number of surviving explants during the callus stage of *in vitro* culture. Cultivars (Kh: Khalas; Ru: Ruziz; Sh: Shishi); Stock E solution (Zero: E-1; half: E-2; full: E-3; double: E-4). Significance level (S = 0.006, T = 0.008, S \* T = 0.005).

3.25), while Shishi (Sh) had the lowest survival (mean = 2.33), suggesting a cultivar-dependent response to microelement availability. The statistical analysis confirmed that SE concentration (S), Treatment (T), and their interaction (S\*T) were all highly significant ( $p < 0.01$ ), indicating that both nutrient composition and cultivar type play a crucial role in explant viability.

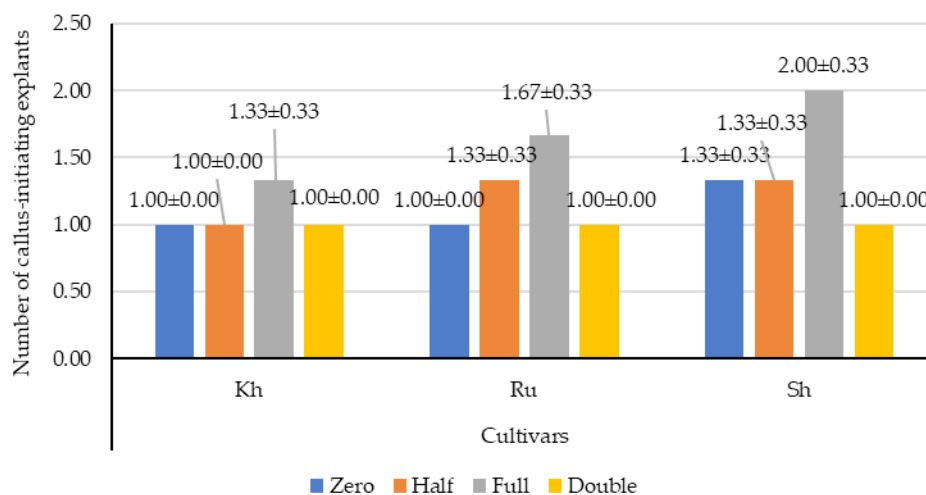
The positive correlation between SE levels and survival suggests that trace elements such as manganese, zinc, and iron play a crucial role in maintaining cellular integrity, enzymatic activity, and oxidative stress reduction, thereby promoting explant survival in plant tissue cultures (Vazquez-Marquez *et al.*, 2024; Amente and Chimdessa, 2021). Studies on *Triticum aestivum* L. have shown similar trends, where microelement supplementation enhances survival by minimizing oxidative stress and supporting cellular metabolism (Amente and Chimdessa, 2021). However, Shishi consistently exhibited the lowest survival rate, which may indicate a cultivar-specific sensitivity to microelements due to metabolic differences or a heightened oxidative stress response (Zhang, 2023). This highlights the need for optimized nutrient supplementation strategies, particularly for sensitive cultivars like Shishi, to improve viability and enhance tissue culture performance.

The number of surviving explants varied significantly depending on both the cultivar and the concentration of SE. For instance, higher concentrations of stock E generally led to a higher number of surviving explants, but this effect was also influenced by the specific cultivar. This is consistent with findings that microelements such as SE can significantly impact tissue culture success rates (Al-Khayri *et al.*, 2019).

The callus initiation response (Figures 3 and 4) was also significantly influenced by Stock E concentrations ( $p = 0.01$ ), with the highest callus formation observed at E-3 (mean = 1.67), while the lowest occurred at E-1 and E-4 (1.0 and 1.11, respectively). Among cultivars, Sh exhibited the highest overall callus initiation (1.42), followed by Ru (1.25), while Kh had the lowest callus formation (1.08). Statistical analysis confirmed that SE concentration (S) had a significant effect ( $p = 0.01$ ), whereas Treatment (T) and



**Figure 3.** Specific effects of date palm (*Phoenix dactylifera* L.) cultivars (A) and different Stock E concentrations (B) on callus initiation during six months of *in vitro* culture. Cultivars (Kh: Khalas; Ru: Ruziz; Sh: Shishi); Stock E solution (Zero: E-1; half: E-2; full: E-3; double: E-4).

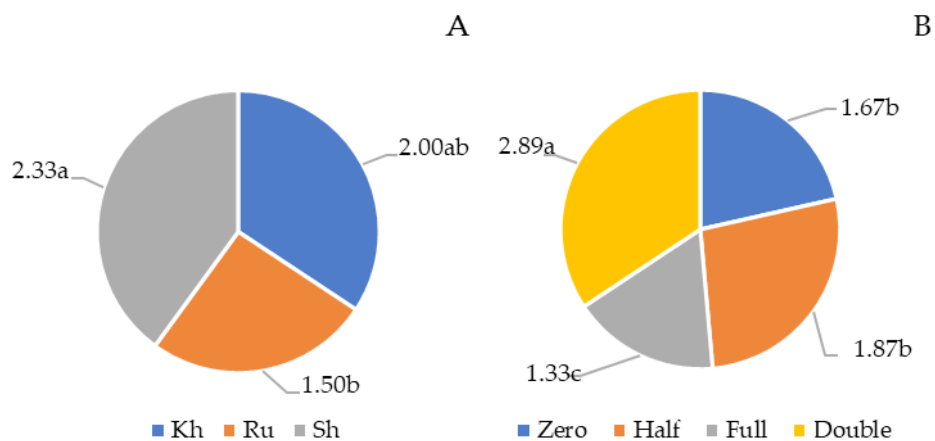


**Figure 4.** Combined influence of stock E concentrations and date palm (*Phoenix dactylifera* L.) cultivars on callus initiation during six months of *in vitro* culture. Cultivars (Kh: Khalas; Ru: Ruziz; Sh: Shishi); Stock E solution (Zero: E-1; half: E-2; full: E-3; double: E-4). Significance level (S = 0.01, T = not significant (NS), S \* T = NS).

the interaction (S\*T) were not significant, indicating that microelement availability played a more crucial role in callus induction than cultivar differences. However, the full SE concentration yielded the highest callus initiation rates, with Sh ( $2.00 \pm 0.33$ ), Ru ( $1.67 \pm 0.33$ ), and Kh ( $1.33 \pm 0.33$ ) outperforming other concentrations. With half SE, Sh, and Ru *cvs* exhibited similar initiation rates ( $1.33 \pm 0.33$ ), as did Shishi in the absence of microelements ( $1.33 \pm 0.33$ ). The lowest callus formation was observed with double E-4 concentration ( $1.0 \pm 0.0$ ).

Callus formation was optimal at E-3 but declined at E-4, likely due to excessive microelement accumulation disrupting cellular metabolism (Dai *et al.*, 2023). Moderate trace element supplementation at E-3 appears to enhance phenolic metabolism and enzyme activation, promoting cell differentiation and division (Liu *et al.*, 2018; Muszyńska and Labudda, 2019). Microelements such as zinc, copper, iron, and manganese serve as essential cofactors in enzymatic processes regulating tissue growth and oxidative stress balance (Zhang, 2023). Studies on *Capsicum annuum* L. demonstrated that copper supplementation enhances callus formation by stimulating peroxidase activity, which plays a key role in oxidative stress regulation and tissue development (Liu *et al.*, 2018; Muszyńska and Labudda, 2019). However, at E-4, excessive copper or manganese may induce toxicity, disrupting oxidative balance and limiting callus growth (Amente and Chimdessa, 2021). These findings emphasize the importance of precisely controlling trace element concentrations to optimize callus initiation while preventing toxicity-related metabolic imbalances in plant tissue cultures. This is consistent with studies showing that genetic factors play a significant role in callus formation, alongside the influence of trace elements (Al-Khayri *et al.*, 2019).

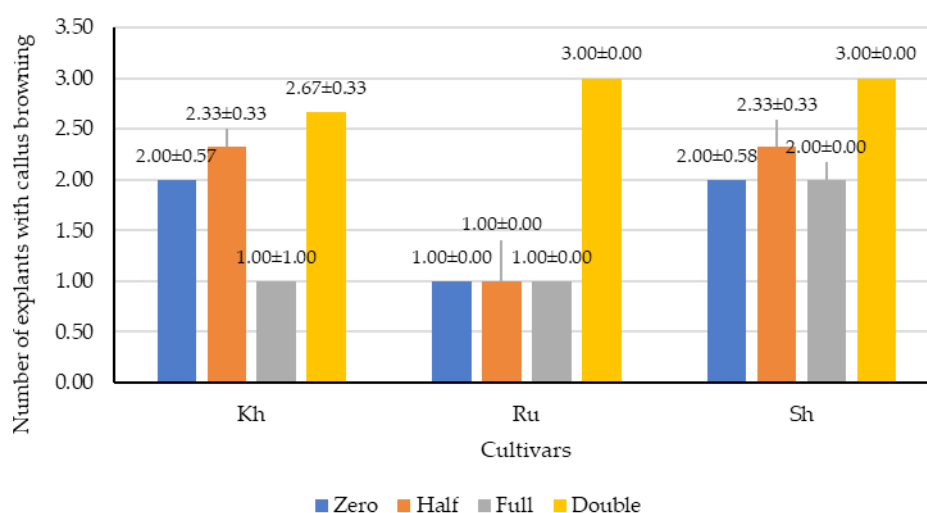
Browning (Figure 5) intensity varied significantly across SE concentrations, with the highest observed at E-4 (mean = 2.89) and E-2 (1.89), while the lowest occurred at E-3



**Figure 5.** Specific effects of date palm (*Phoenix dactylifera* L.) cultivars (A) and different Stock E concentrations (B) on callus browning during six months of *in vitro* culture. Cultivars (Kh: Khalas; Ru: Ruziz; Sh: Shishi); Stock E solution (Zero: E-1; half: E-2; full: E-3; double: E-4).

(1.33). Among the cultivars, Sh exhibited the highest browning (2.33), followed by Kh (2.00) and Ru (1.50). Statistical analysis confirmed that the SE concentration (S) significantly affected browning ( $p = 0.007$ ), whereas Treatment (T) and the interaction (S\*T) were not significant, suggesting that browning was primarily influenced by nutrient levels rather than cultivar differences.

Cultivars Sh and Ru displayed the most intense browning at the double (E-4) concentration ( $3.00 \pm 0.0$ ), followed by Kh ( $2.67 \pm 0.33$ ) (Figure 6). At half concentration (E-2), Sh and Kh also showed pronounced browning ( $2.33 \pm 0.33$ ). Callus browning was



**Figure 6.** Interaction effect of stock E concentrations and date palm (*Phoenix dactylifera* L.) cultivars on callus browning during six months of *in vitro* culture. Cultivars (Kh: Khalas; Ru: Ruziz; Sh: Shishi); Stock E solution (Zero: E-1; half: E-2; full: E-3; double: E-4). Significance level (S = 0.007, T = not significant (NS), S \* T = NS).

minimal at the full concentration (E-3), as recommended by Murashige and Skoog, which reduced discoloration across cultivars (Shehata *et al.*, 2014). Overall, Shishi exhibited the greatest browning sensitivity to both high and low SE concentrations, suggesting it has a higher phenolic compound content. This indicates Shishi's potential for greater antioxidant, fiber, and mineral content compared to other cultivars, supporting findings on its distinct nutritional profile (Al-Saikhan, 2006).

Increased browning at high Stock E concentrations (E-4) can be attributed to polyphenol oxidation by polyphenol oxidase (PPO), a common challenge in plant tissue cultures caused by oxidative stress or imbalanced nutrient availability (Amente and Chimdessa, 2021). Excess iron and copper at E-4 may trigger PPO and peroxidase activity, accelerating phenolic oxidation and tissue discoloration (Zhang and Sun, 2021). In contrast, browning was minimized at E-3, suggesting that a balanced microelement supply helps regulate oxidative stress by enhancing antioxidant enzyme

activity, thereby improving tissue stability (Zhang, 2023). These findings align with research indicating that iron and manganese influence PPO activity, directly affecting browning intensity in plant tissue cultures (Zhang, 2023). Therefore, optimizing Stock E concentrations is essential for minimizing browning and maintaining tissue quality in plant micropropagation systems.

The findings suggest that E-3 (full-strength) is the most effective concentration for maximizing explant survival, enhancing callus initiation, and minimizing browning, whereas E-4 (double-strength) may induce oxidative stress and metabolic imbalances. Optimizing trace element concentrations is crucial for improving phenolic biosynthesis and antioxidant activity, with potential applications in commercial propagation and bioactive compound production (Dai *et al.*, 2023). Similar trends have been observed in *Solanum lycopersicum* L., where balanced trace element supplementation reduced oxidative browning by stabilizing phenolic metabolism and regulating PPO activity (Vazquez-Marquez *et al.*, 2024). Future research should focus on molecular pathways regulating oxidative stress and enzymatic activity in response to trace element variations to further enhance tissue culture performance.

#### Effect of microelements (Stock E) on total phenolic contents and antioxidant production

The antioxidant analysis results for the three Hassawi date palm cultivars highlight the role of varying micro-mineral (SE) concentrations on antioxidant production during *in vitro* callus formation (Table 2). The total phenolic content in date palm

**Table 2.** Effect of different stock E concentrations and date palm (*Phoenix dactylifera* L.) cultivars on total phenolic content and antioxidant production during six months of *in vitro* culture.

Cultivars	Stock E	Total phenolic content (mg GAE g <sup>-1</sup> )*	Antioxidant activity by ABTS Inhibition (%)	μM Trolox
Kh	E-1	0.389 ± 0.004 <sup>l</sup>	14.579 ± 0.070 <sup>l</sup>	81.707 ± 2.147 <sup>l</sup>
	E-2	1.021 ± 0.070 <sup>e</sup>	42.853 ± 0.090 <sup>e</sup>	391.654 ± 1.458 <sup>e</sup>
	E-3	0.744 ± 0.003 <sup>h</sup>	36.061 ± 0.244 <sup>h</sup>	317.608 ± 3.435 <sup>h</sup>
	E-4	1.237 ± 0.007 <sup>b</sup>	54.467 ± 0.240 <sup>c</sup>	437.489 ± 1.391 <sup>c</sup>
Ru	E-1	0.395 ± 0.002 <sup>k,l</sup>	14.756 ± 0.060 <sup>k</sup>	80.427 ± 1.466 <sup>l</sup>
	E-2	0.998 ± 0.02 <sup>f</sup>	42.194 ± 0.325 <sup>f</sup>	324.336 ± 3.965 <sup>g</sup>
	E-3	0.653 ± 0.002 <sup>j</sup>	28.945 ± 0.197 <sup>j</sup>	233.401 ± 3.001 <sup>j</sup>
	E-4	1.139 ± 0.003 <sup>d</sup>	47.195 ± 0.050 <sup>d</sup>	382.457 ± 2.236 <sup>d</sup>
Sh	E-1	0.711 ± 0.003 <sup>i</sup>	29.726 ± 0.280 <sup>i</sup>	243.270 ± 2.388 <sup>i</sup>
	E-2	1.246 ± 0.004 <sup>b,c</sup>	55.305 ± 0.329 <sup>b</sup>	518.794 ± 4.301 <sup>b</sup>
	E-3	0.955 ± 0.010 <sup>g</sup>	39.324 ± 0.287 <sup>g</sup>	359.867 ± 5.173 <sup>f</sup>
	E-4	2.676 ± 0.005 <sup>a</sup>	96.283 ± 0.171 <sup>a</sup>	978.692 ± 3.122 <sup>a</sup>
Least significant difference (p ≤ 0.05)		1.754	0.404	4.825

Cultivars (Kh: Khalas; Ru: Ruziz; Sh: Shishi). Stock E solution (Zero: E-1; half: E-2; full: E-3; double: E-4). \*Expressed as milligrams of gallic acid equivalents (GAE) per 100 g of sample.

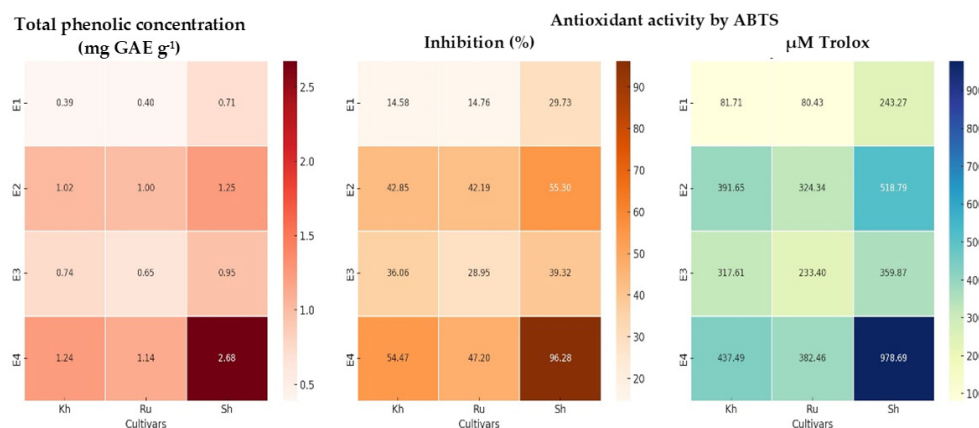
cultivars shows a clear trend of increasing with higher concentrations of Stock E. In Khalas, phenolic content rose from 0.389 mg g<sup>-1</sup> at E-1 to 1.237 mg g<sup>-1</sup> at E-4, indicating enhanced biosynthesis with more microelements. Ruziz followed a similar pattern, increasing from 0.395 mg g<sup>-1</sup> at E-1 to 1.139 mg g<sup>-1</sup> at E-4, suggesting a positive response to microelement supplementation. Shishi exhibited the highest phenolic content, peaking at 2.676 mg g<sup>-1</sup> at E-4, highlighting its superior capacity for phenolic production in response to higher microelement concentrations, which is crucial for its antioxidant properties.

The antioxidant activity demonstrates a positive correlation with the concentration of Stock E. In Khalas, the inhibition percentage increased from 14.579 % at E-1 to 54.467 % at E-4, with  $\mu$ M Trolox values peaking at 437.489 mg g<sup>-1</sup> under E-4. Ruziz followed a similar pattern, with inhibition percentages rising from 14.756 % at E-1 to 47.195 % at E-4, accompanied by an increase in  $\mu$ M Trolox values. Shishi exhibited the highest antioxidant activity, with inhibition reaching 96.283 % and  $\mu$ M Trolox values at 978.692 mg g<sup>-1</sup> under E-4, highlighting its potential for significant antioxidant production and suitability for further research and agricultural development. Additionally, double SE concentration was found to be the most effective for phenolic production, though it caused callus discoloration from brown to black, while half SE concentration led to lighter browning. The callus cultured in full MS medium or without SE microelements retained a tan or yellowish-white color.

ABTS inhibitory activity in adventitious callus tissues decreased with increasing SE concentration from zero to half to full to double SE strength (Table 2). A strong positive correlation ( $R^2 = 0.99$ ,  $p < 0.05$ ) was observed between total phenolic content and ABTS radical scavenging in callus extracts with all SE concentrations in media, which is consistent with findings by other researchers (Shehata *et al.*, 2014). Tawaha *et al.* (2007) also noted a positive correlation between total phenolic content and antioxidant activity in methanolic extracts of 51 plant species, including *P. dactylifera*.

The heat maps provide a detailed visualization of how the different microelement concentrations (E-1, E-2, E-3, and E-4) influenced total phenolic content, inhibition percentage, and  $\mu$ M Trolox (an antioxidant capacity measure) in the evaluated date palm cultivars. Shishi demonstrated the highest phenolic content (2.676 mg g<sup>-1</sup>) and antioxidant capacity ( $\mu$ M Trolox value of 978.692 mg g<sup>-1</sup>) under the E-4 treatment, highlighting its superior phenolic accumulation and antioxidant activity. Khalas and Ruziz showed moderate levels, with both reaching peak phenolic content under E-4. In terms of inhibition percentage, Shishi led at 96.283 %, followed by Khalas at 54.467 % and Ruziz at 47.195 % (Figure 7).

These results indicate that the E-4 microelement combination significantly boosts antioxidant properties, especially in Shishi. Phenolic compounds and antioxidants are essential for plant defense and human health, as they neutralize free radicals and mitigate oxidative stress (Amente and Chimdessa, 2021). Shishi's enhanced response to E-4 supports existing research on the positive effects of specific micronutrient formulations in promoting phenolic and antioxidant responses in date palms (Al-Shwyeh, 2019).



**Figure 7.** Heatmap visualization of the impact of microelements on phenolic content, antioxidant activity, and  $\mu\text{M}$  Trolox levels in date palm (*Phoenix dactylifera* L.) cultivars. Cultivars (Kh: Khalas; Ru: Ruziz; Sh: Shishi). Stock E solution (Zero: E-1; half: E-2; full: E-3; double: E-4).

These findings align with prior studies showing the impact of microelements like zinc, manganese, and copper in activating enzymatic pathways involved in phenolic biosynthesis, enhancing antioxidant capacity in plants (Vazquez-Marquez *et al.*, 2024). Overall, E-4, enriched with essential microelements, significantly improved both phenolic content and antioxidant activity across cultivars, with Shishi demonstrating the most robust response. This makes Shishi a promising candidate for further research and agricultural initiatives focused on optimizing antioxidant production. Trace elements play a crucial role in enhancing phenolic biosynthesis and antioxidant activity. For instance, studies on *Glycine max* L. (soybean) and *Triticum aestivum* L. (wheat) have shown similar trends where trace element supplementation leads to increased secondary metabolite production and stress resistance (Zhang, 2023; Amente and Chimdessa, 2021).

The positive correlation between microelement concentration and phenolic content in callus tissues supports the hypothesis that trace elements act as essential cofactors in enzymatic pathways, particularly those involved in phenylpropanoid metabolism. Enhanced activity of enzymes such as PAL and PPO in response to higher microelement concentrations could explain the increased phenolic production and reduced browning in the callus tissues (Vazquez-Marquez *et al.*, 2024).

Previous research examined the role of MS medium components in antioxidant formation (Alturki *et al.*, 2013; Shehata *et al.*, 2014; Aldaej *et al.*, 2014). Findings suggest a relationship between medium nutrient strength and the accumulation of secondary metabolites in callus cultures, as full-strength nutrient media primarily promote primary metabolism and cellular growth, which can inhibit tissue differentiation at the morphological and biochemical levels (Hamza *et al.*, 2016). Earlier studies have also explored related aspects, such as the effects of various growth regulators and

nutrient media on callus tissue regeneration (Al-Khayri *et al.*, 2019), highlighting how nutrient and hormonal environments shape secondary metabolite production.

Mineral elements in the MS medium, such as those in Stock E, are often bound to sulfur compounds (SO<sub>4</sub>), highlighting sulfur's essential role in antioxidant formation, cell division, growth, and tissue development in cultured plants (Narayan *et al.*, 2022). Sulfur is crucial for plants, as it contributes to amino acids, vitamins, and coenzymes involved in synthesizing proteins, enzymes, and other organic molecules (Al-Mayahi, 2021). Sulfur-containing compounds, including glutathione, are key antioxidants that aid in reducing oxidative stress in plants (Cao *et al.*, 2023).

In the MS medium, sulfur is supplemented as adenine sulfate (AdSO<sub>4</sub>) at 40–80 mg L<sup>-1</sup>, which, alongside cytokinin, promotes bud and branch formation and supports cell elongation (Rency *et al.*, 2018). Trace elements like copper, zinc, and manganese are also introduced as sulfates in Stock E, while iron is provided separately in a chelated form (Stock F) to prevent precipitation, as free iron ions can become toxic to tissues (Xiao *et al.*, 2021). Sulfate assimilation enhances sulfur-based antioxidant defenses in plants. Sulfate uptake influences antioxidant production, though its effects on date palm tissues may vary with factors such as physiological state, environmental conditions, and nutrient balance (Drira *et al.*, 2022).

Antioxidants are vital in protecting plants from oxidative stress caused by pollutants, UV radiation, and pathogens by neutralizing reactive oxygen species (ROS) (Hasanuzzaman *et al.*, 2020). Maintaining balanced micronutrient levels in the medium is essential for optimal antioxidant production in date palm callus tissues. Environmental factors, such as pH and organic matter, can also impact micronutrient availability (Xiao *et al.*, 2021; Abd Elaziem *et al.*, 2022). Stock E in the MS medium provides key micro-mineral elements, including ZnSO<sub>4</sub>·7H<sub>2</sub>O, CuSO<sub>4</sub>·5H<sub>2</sub>O, MnSO<sub>4</sub>·4H<sub>2</sub>O, and MgSO<sub>4</sub>·7H<sub>2</sub>O, each playing a specific role in callus growth, secondary metabolite production, and antioxidant formation.

Zinc is a crucial cofactor for enzymes that regulate oxidative stress and support antioxidant defenses. Zinc deficiency can compromise these defenses, underscoring its importance for plant health and antioxidant synthesis (Amiri *et al.*, 2021; Abdellatif *et al.*, 2022). Zinc is also fundamental in physiological processes like enzyme activation, photosynthesis, and the synthesis of phenolic compounds and flavonoids, which enhance antioxidant capacity (Lee, 2018). As a cofactor for key antioxidant enzymes, such as superoxide dismutase (SOD) and peroxidase, zinc aids in neutralizing reactive oxygen species (ROS) and reducing oxidative damage. Additionally, its role in chlorophyll structure and photosynthetic efficiency indirectly supports antioxidant production (Lee, 2018; Abdellatif *et al.*, 2022). Adequate zinc levels enable the plant to better manage oxidative stress and activate antioxidant defenses as part of its stress response mechanisms.

Copper (Cu) in the form of copper sulfate pentahydrate (CuSO<sub>4</sub>·5H<sub>2</sub>O) is an essential micronutrient present in Stock E of the MS medium. Copper is vital for antioxidant defense and various physiological processes in plants, including photosynthesis,

enzyme activation, and secondary metabolite synthesis. As a cofactor for antioxidant enzymes like SOD and ascorbate peroxidase, copper plays a key role in neutralizing ROS. However, copper levels must be balanced carefully, as excess can be toxic (Djamila *et al.*, 2022). In the electron transport chain of photosynthesis, copper contributes to energy production. It also affects the synthesis of secondary metabolites, such as phenolic compounds, which can lead to browning in cultured tissues. Oxidation enzymes, such as polyphenol oxidase (PPO) and peroxidase, both of which contain copper, aid in the conversion of phenolics into quinones, causing tissue discoloration from yellow to brown or black.

Browning is caused by PPO-mediated oxidation and may impair explant viability by binding phenols to proteins, thus reducing enzyme effectiveness and ultimately leading to tissue death (Zein El-Din and Ibrahim, 2015). PPO, a copper-dependent enzyme localized in plastids, catalyzes the oxidation of phenolics to quinones, producing pigments in wounded tissue. This reaction, triggered by cellular disruption, is part of the plant's defense against insects and pathogens and plays roles in pigmentation, oxygen scavenging, and chloroplast function (Djamila *et al.*, 2022).

Manganese (Mn), provided in the MS medium as manganese sulfate tetrahydrate ( $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ ), is essential for plant growth and plays a crucial role in activating enzymes related to antioxidant defense (Zein El-Din *et al.*, 2022). Manganese acts as a cofactor for superoxide dismutase (SOD), an enzyme that neutralizes superoxide radicals and supports photosynthesis by participating in the water-splitting reaction, thereby aiding the conversion of light energy into chemical energy. Through these processes, manganese contributes indirectly to antioxidant production and the synthesis of phenolics and flavonoids, which enhance the plant's antioxidant capacity (Bagnoli *et al.*, 2002). However, excessive manganese can be toxic, emphasizing the need for balanced levels.

The SE nutrient solution also contains magnesium sulfate heptahydrate ( $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ), or Epsom salt, as a source of magnesium (Mg) and sulfur (S). Magnesium is central to chlorophyll structure and is essential for photosynthesis, as well as activating antioxidant enzymes like SOD, catalase (CAT), and peroxidase (POD), which help to scavenge ROS in plant cells (Al-Shamsi *et al.*, 2021; Alkhoori *et al.*, 2022). Sulfur supports the synthesis of amino acids, proteins, and antioxidants, contributing to overall plant health and resilience. Thus,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  in the medium can positively impact antioxidant production in date palm cultures.

Other essential mineral elements in the MS medium, such as boron and molybdenum, are crucial for antioxidant formation in date palm callus cultures. Boron was supplied as boric acid ( $\text{H}_3\text{BO}_3$ ) and molybdenum as sodium molybdate ( $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ ), both part of the medium's third group (Stock C) (Table 1). Boron (B) is essential for carbohydrate metabolism, cell wall maintenance, and the synthesis of phenolic compounds with antioxidant properties. Sufficient boron levels support antioxidant production and cell function, while boron deficiency can reduce antioxidant activity (Al-Mayahi, 2020; Gilani *et al.*, 2021). Molybdenum (Mo) acts as a cofactor for enzymes involved

in nitrate reduction to ammonia, a key step in nitrogen metabolism that indirectly supports antioxidant production. Optimal molybdenum levels are important for plant health and may contribute to antioxidant synthesis (Schwarz *et al.*, 2009).

### CONCLUSIONS

Antioxidants can be efficiently produced from date palm explants through tissue culture, providing a year-round, scalable source without relying on fruit ripening or sacrificing cuttings. The Murashige and Skoog medium, enriched with minor mineral elements, supports optimal callus formation even in trace amounts; however, doubling these microelements enhances antioxidant production in cultured explants. Among the cultivars, Shishi, particularly with double Stock E concentration (E-4 treatment), showed strong potential for nutritional and pharmaceutical studies aimed at boosting antioxidant properties. This makes Shishi a promising candidate for future research and agricultural initiatives aimed at increasing antioxidant production.

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