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BOOSTING ANTIOXIDANT ACTIVITY AND PHENOLIC CONTENT IN HASSAWI DATE PALM CALLUS THROUGH CHELATED IRON

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ABSTRACT

TThe date palm (*Phoenix dactylifera* L.) is a vital crop in Saudi Arabia, the Arabian Peninsula, and numerous Arab countries, valued for its exceptional resilience to harsh environmental conditions and its profound nutritional, economic, and cultural importance. Tissue culture techniques have been utilized to preserve plant genetic integrity and pass on desirable qualities, allowing for regulated growth of plant tissues to safeguard certain genetic traits. Chelated iron, which is made up of iron ions attached to organic molecules, makes iron more soluble and bioavailable. It is an important part of many physiological processes in plants, including the production of chlorophyll, metabolic activity, and the response to stress. This study investigates the influence of chelated iron on the production of phenolic compounds and the activation of antioxidants in date palm callus cultures, focusing on three elite cultivars native to the Al-Ahsa Oasis: Khalas, Ruziz, and Shishi. Statistical analysis using analysis of variance (ANOVA), coupled with heat map visualizations, revealed a strong correlation between chelated iron concentration and the enhancement of phenolic content and antioxidant activity. Results demonstrated that a double concentration of chelated iron significantly improved the formation of phenolic compounds and boosted antioxidant activity across all cultivars, with the Shishi cultivar exhibiting the highest response. Notable differences were observed between cultivars and treatment levels, emphasizing the critical role of cultivar-specific responses in optimizing tissue culture protocols. These findings underscore the importance of chelated iron in enhancing the biochemical properties of date palm callus cultures and provide a foundation for refining tissue culture practices to support sustainable agriculture and genetic preservation in date palm cultivation.

Keywords: *Phoenix dactylifera* L., antioxidants, oxidative stress, phenolic compounds, somatic embryogenesis.

INTRODUCTION

Al-Ahsa Oasis, located in Saudi Arabia, is the largest green oasis globally and serves as a cornerstone of food security for the Kingdom. It is home to over 4.1 million date palm trees from more than 15 Hassawi cultivars, with Khalas, Ruziz, and Shishi collectively

contributing more than 70 % of the oasis's total date production (Almadini *et al*., 2021; MEWA, 2023). These cultivars are valued for their adaptability to extreme conditions such as drought and high temperatures, as well as their nutritional, economic, and cultural significance. Dates from these trees are rich in dietary fiber, minerals, and sugars beneficial for human health (Al-Saikhan, 2006).

The date palm (*Phoenix dactylifera* L.) plays a pivotal role in environmental sustainability and is integral to Saudi Arabia's Vision 2030, which emphasizes the preservation of genetic resources and the sustainable use of natural assets for future generations. Despite its long lifecycle and limited production of offshoots (20–30 over a lifetime), the palm is a significant source of antioxidants, primarily concentrated in its fruits during the ripening stage, which occurs 5–8 years after planting (Shehata *et al*., 2014). These antioxidants have been shown to support immune health, combat oxidative stress, and reduce the risks of chronic diseases, including cancer and neurodegenerative disorders (Al-Shwyeh, 2019).

Micronutrients, although required in small amounts, are vital for plant growth and development, contributing to processes such as cell division, elongation, and the synthesis of secondary metabolites like phenolic compounds and antioxidants (Rency *et al*., 2018; Sinkovič *et al*., 2023). Iron is critical as it facilitates enzymatic reactions in respiration and photosynthesis and aids in mitigating oxidative stress by reducing free radicals (Amente and Chimdessa, 2021).

Given the high antioxidant potential of date palms and the importance of phenolic compounds for plant and human health, this study investigates the role of chelated iron in enhancing the production of antioxidants during the callus formation stage. By utilizing somatic embryogenesis in tissue culture, the research aims to optimize the concentration of chelated iron in the Murashige and Skoog (MS) nutrient medium to maximize antioxidant and phenolic compound production in Khalas, Ruziz, and Shishi cultivars. This work complements prior studies by exploring the biochemical and physiological impacts of chelated iron, paving the way for advancements in date palm micropropagation.

MATERIALS AND METHODS

Callus initiation

This experiment aimed to induce callus formation through indirect somatic embryogenesis using explants from shoot tips, leaf primordia, and axillary buds, focusing on three key Hassawi date palm cultivars (Khalas, Ruziz, and Shishi) due to their agricultural importance and consumer preference. The study investigated the relationship between phenolic content and browning during callus development and morphogenesis. Young offshoots, aged 4–5 years, measuring 70–100 cm in length and weighing 5–7 kg, were carefully selected between 2021 and 2022 from mature trees at the King Faisal University farm and meticulously separated from the parent trees to ensure the integrity of the plant material.

Sterilization protocol

To minimize contamination, the explants underwent a stringent sterilization process. First, they were washed three times with sterile distilled water. Next, they were immersed in a 60 % Clorox solution (containing 5.25 % sodium hypochlorite) for 20 min, followed by a 2–3-min soak in 100 % ethyl alcohol. Subsequently, the explants were treated with a 1.5 g L^{-1} mercury chloride ($HgCl_2$) solution for 3–5 min. After each step, the explants were rinsed at least three times with sterile distilled water to ensure complete removal of sterilizing agents (Alturki *et al*., 2013; Aldaej *et al*., 2014; Shehata *et al*., 2014).

Nutrient medium preparation

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). The medium was prepared by mixing 20 mL each of stock solutions A and B and 5 mL each of stocks C, D, E, F, and G with approximately 1000 mL of distilled

Table 1. Impact factors of chelated iron (Stock F) and supplementary components used in the Murashige and Skoog (MS) medium for date palm (*Phoenix dactylifera* L.) callus formation.

Stock solution	Constituents	Concentration (g L^{-1})	Final concentration in MS medium (mg L^{-1})	To make up 1 L of MS medium (mL L^{-1})	Concentration difference	Code
A	NH_4NO_3	82.50	1650.00	20	Full	---
B	KNO_3	95.00	1900.00	20	Full	---
C	H_3BO_3	1.240	6.20	5	Full	---
	KH_2PO_4	34.00	170.00			
	KI	0.166	0.83			
	$Na_2MoO_4 \cdot 2H_2O$	0.050	0.25			
	$CoCl_2 \cdot 6H_2O$	0.005	0.025			
D	$CaCl_2 \cdot 2H_2O$	88.00	440.00	5	Full	---
E	$MgSO_4 \cdot 7H_2O$	74.00	370.00	5	Full	---
	$MnSO_4 \cdot 4H_2O$	4.460	22.30			
	$ZnSO_4 \cdot 7H_2O$	1.720	8.60			
	$CuSO_4 \cdot 5H_2O$	0.005	0.025			
F*	$Na_2 \cdot EDTA$	7.450	37.25	5	Zero	F-1
					Half	F-2
	$FeSO_4 \cdot 7H_2O$	5.570	27.85		Full	F-3
					Double	F-4
G	Thiamine·HCl	0.200	0.10	5	Full	---
	Nicotinic acid	0.100	0.50			
	Pyrodoxine·HCl	0.100	0.50			
	Glycine	0.400	2.00			

*The standard Stock F levels (chelated iron F-1: zero; F-2: half; F-3: full; F-4: double).

water under continuous stirring. This medium provided a consistent foundation for the experimental procedures, ensuring successful callus initiation and enabling the investigation of phenolic compounds' role in browning and antioxidant production in date palm tissue cultures.

Experimental design and measurements

The experiment evaluated varying concentrations of Na_2·EDTA and $FeSO_4·7H_2O$ (Stock F solution) to improve the growth medium for *in vitro* date palm tissue culture. Key parameters, including explant survival rates, callus initiation, and browning, were systematically assessed after each subculture. Biochemical assays were used to quantify the total phenolics and antioxidant enzyme activities to assess the efficacy of the medium. The study aimed to determine the optimal balance of iron and chelating agents, enhancing plant growth and antioxidant production while avoiding toxicity or nutrient deficiencies. Since excessive iron can be detrimental, Na_2·EDTA was used carefully to prevent over-chelation and maintain a balanced nutrient profile (Table 1). This stock solution was prepared by dissolving each constituent in 200 mL of distilled water. The Na_2·EDTA·$2H_2O$ solution was heated, and the $FeSO_4·7H_2O$ 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 refrigeration. Modified media used for cultures were supplemented with different concentrations of phytohormones, carbohydrates, vitamins, and other addenda (Murashige and Skoog, 1962).

Preparation of nutrient medium

The pH of the nutrient medium was adjusted to 5.7 using 1N KOH or 1N HCl. The medium was then autoclaved at 121 °C (1.2 kg cm^{-2} pressure) for 20 min before the addition of the gelling agent. To assess the impact of varying levels of Na_2·EDTA and $FeSO_4·7H_2O$ on synthetic antioxidant production, the medium was prepared with concentrations set at zero, half, full, and double the standard Stock F levels. It was further supplemented with specific compounds (mg L^{-1}): 170 $NaH_2PO_4·2H_2O$, 80 adenine sulfate, 100 myo-inositol, 30 000 sucrose, 2000 gerlite, 2000 activated charcoal, 2.5 thiamine-HCl, 2 biotin, 100 2,4-dichlorophenoxyacetic acid, and 3 6-benzylaminopurine (Sigma-Aldrich, USA). The final medium was dispensed into jars containing 25 ml each and sterilized again under the same autoclaving conditions (Alturki *et al*., 2013; Shehata *et al*., 2014; Aldaej *et al*., 2014).

Callus cultivation and incubation conditions

Sterilized explants were cultured on the prepared medium and incubated under dark conditions at 25 ± 2 °C. Cultures were refreshed with modified media every two months over an eight-month incubation period. Data were collected at the end of three subcultures (8, 16, and 24 weeks) for each date palm cultivar, recording the number of surviving explants, callus initiation, and the extent of browning. The visual scoring system used by Pottino (1981) was used to read callus data and brown discoloration on the explants of each variety under study, with five replicates for each treatment.

Methods of antioxidant analysis

After the callus formation stage for all explants from the three date palm cultivars, samples of the callus (3 g) from each treatment were collected for analysis to determine their total phenolic content (mg g^{-1}), expressed as gallic acid equivalents, and antioxidant activity using the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) method. The resulting data were analyzed and compared to identify significant differences between the treatments, using the least significant difference (LSD) test at the 5 % probability level.

Antioxidant analysis was conducted using 3 g of callus tissue, which was ground and homogenized for 10–12 min. The homogenized tissue was extracted with 100 mL of methanol at 20 °C for 5 h using an orbital shaker (LSI-LabTECH, Korea). After filtration, the mixture was centrifuged at 4000 rpm for 10 min under reduced pressure at 40 °C. The supernatant was concentrated using a rotary evaporator for 3 h, yielding the methanol crude extract, which was stored in dark glass bottles at freezer temperatures for three days prior to analysis.

Reagents for the analysis included catechin, sodium carbonate, gallic acid, ascorbic acid, sodium nitrate, trichloroacetic acid, methanol, aluminum chloride, and Folin-Ciocalteu's phenol reagent, which were sourced from Merck (Germany). Additional chemicals, such as 2,4,6-tripyridyl-S-triazine (TPTZ), ABTS, Trolox, $FeCl_3 \cdot 3H_2O$, potassium persulfate, sodium carbonate, and sodium acetate, were procured from Sigma-Aldrich (USA). Antioxidant activity was assessed using the enhanced ABTS method (Cai *et al*., 2004), with Trolox standard solutions (0–15 μM in 80 % ethanol) used to generate a standard curve. Sample absorbance was measured against the Trolox standard, and antioxidant activity was expressed in Trolox equivalents. Total phenolic content was determined using the Folin-Ciocalteu method (Singleton and Rossi, 1965), with results expressed as mg of gallic acid equivalents (GAE) per 100 g of sample, following the protocol by Shui and Leong (2006).

Data analysis

Data analysis was performed using analysis of variance (ANOVA) following a completely randomized design, as outlined by Gomez and Gomez (1984). Treatment means were compared using the least significant difference (LSD) test at a 5 % significance level to determine statistically significant differences. Statistical analyses were conducted using the SAS software (SAS Institute Inc., 2001). Additionally, multivariate analysis, including heat map visualization, was carried out using Orange Data Mining software (Demsar *et al*., 2013) to evaluate the correlation between antioxidant activity and total phenolic content in the explants.

RESULTS AND DISCUSSION

Effect of chelated iron (Stock F) on callus initiation of Hassawi date palm

Increasing the concentration of chelated iron positively influenced explant survival across all cultivars (Figure 1). Higher concentrations, particularly full and double, resulted in improved survival rates (3.67 and 3.33, respectively). Among the three cultivars, Shishi (Sh) exhibited the highest explant survival under increased iron concentrations (3.08), followed by Khalas (Kh) (2.34), while Ruziz (Ru) (2.25) demonstrated the lowest survival rates overall. These findings suggest that iron availability is a key factor in explant viability and callus initiation, likely due to its role in oxidative stress management (Briat *et al*., 2007; Xiao *et al*., 2021).

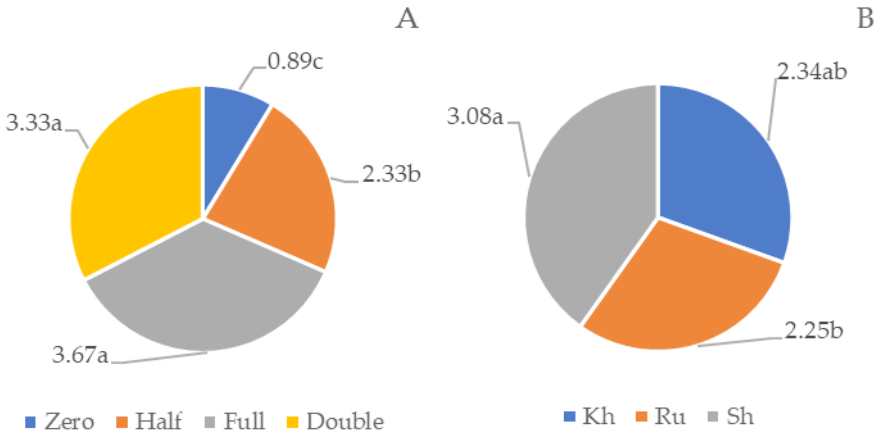

Figure 1. Effect of different concentrations of chelated iron (Stock F solution) (A) and three date palm cultivars (*Phoenix dactylifera* L.) (B) on the number of surviving explants during callus *in vitro* initiation. Kh: Khalas; Ru: Ruziz; Sh: Shishi. Values with different letters are statistically different ($p \leq 0.05$).

Data indicates that survival rates were significantly influenced by both iron concentration and cultivar type (Figure 2). Among the iron treatments, higher concentrations (full and double) resulted in greater explant survival across all cultivars, confirming the essential role of iron in promoting cell viability and reducing oxidative stress during early tissue culture stages. In contrast, explants treated with zero and half iron concentrations showed lower survival rates, indicating that insufficient iron availability affects cell division and metabolic activities (Al-Shwyeh, 2019). When comparing cultivars, Sh showed the highest survival rate at all iron concentrations, indicating a strong adaptive response to iron supplementation. Kh showed intermediate survival rates, while Ru had the lowest survival rates, even at high iron concentrations. This variation can be linked to the viability of the cultivar grown in iron absorption.

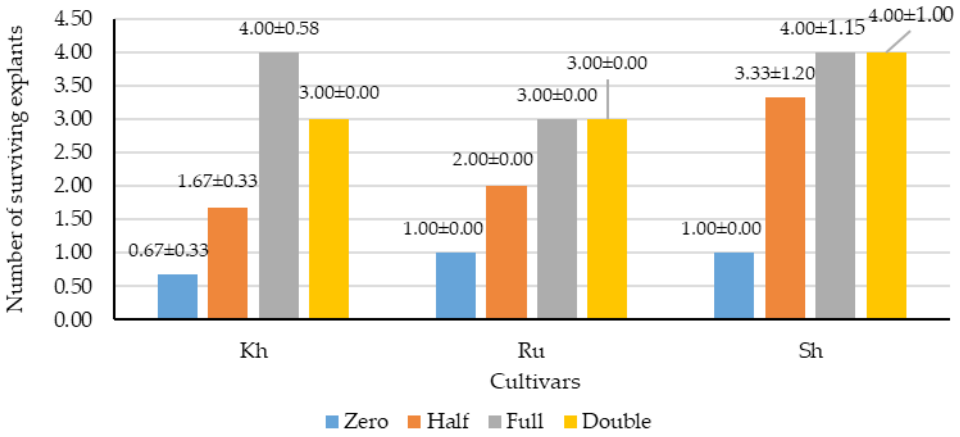

Figure 2. Interaction effects of different concentrations of chelated iron (Stock F solution) and date palm cultivars (*Phoenix dactylifera* L.) on the number of surviving explants during callus *in vitro* initiation. Kh: Khalas; Ru: Ruziz; Sh: Shishi. Significance level (S = 0.0001, T = not significant (NS), S × T = NS).

Increasing the concentration of chelated iron significantly enhanced callus initiation, with explants exposed to full and double iron levels exhibiting the highest callus formation at the same rates (1.56) (Figure 3A). In contrast, explants cultured with half and zero iron concentrations demonstrated slower and lower callus induction (1.11 and 1.00, respectively), suggesting that iron availability is a limiting factor in early-stage callus development. The positive effect of higher iron concentrations could be attributed to its role in cell division, enzyme activation, and oxidative stress reduction, all of which are critical for successful callus initiation (Rency *et al*., 2018).

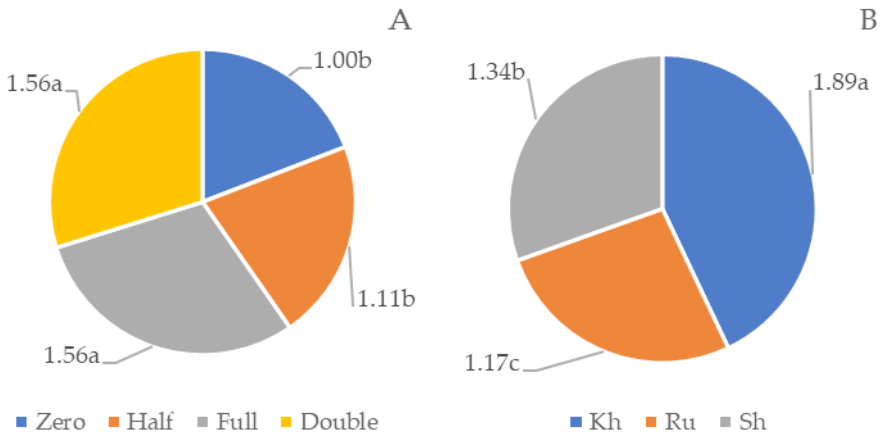

Figure 3. Effect of different concentrations of chelated iron (Stock F solution) (A) and three date palm cultivars (*Phoenix dactylifera* L.) (B) on callus initiation rates during *in vitro* culture. Kh: Khalas; Ru: Ruziz; Sh: Shishi. Values with different letters are statistically different ($p \leq 0.05$).

For the callus initiation responses among the three cultivars, Kh and Sh exhibited superior callus induction rates (1.89 and 1.34, respectively), particularly under full and double iron treatments, whereas Ru displayed relatively lower callus formation across all iron concentrations (1.17) (Figure 3B). This callus induction variation through different date palm rootstocks suggests that different cultivars may have distinct iron uptake mechanisms, influencing their responsiveness to iron supplementation in tissue culture (Al-Shwyeh, 2019).

The results indicated that higher iron concentrations (full and double) positively influenced callus initiation across all three cultivars (Figure 4). Notably, Kh and Sh achieved the highest callus induction rates under full and double iron treatments, with an average value of 1.67. In contrast, Ru showed a relatively lower response, particularly under zero and half iron concentrations, where callus initiation remained at 1.00.

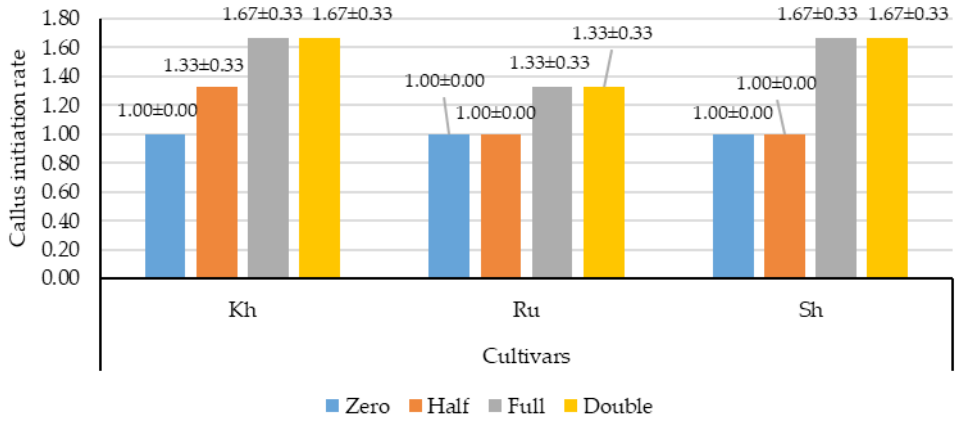

Figure 4. Interaction effect of different concentrations of chelated iron (Stock F solution) and three different date palm cultivars (*Phoenix dactylifera* L.) on callus initiation rate during *in vitro* culture. Kh: Khalas; Ru: Ruziz; Sh: Shishi. Significance level (S = not significant (NS), T = NS, S × T = NS).

These findings emphasize the importance of optimizing iron supplementation in tissue culture media to enhance callus induction, particularly for cultivars with lower inherent responsiveness, such as Ru. Future studies could further investigate the molecular mechanisms underlying cultivar-specific differences in iron metabolism to improve large-scale propagation strategies for elite date palm cultivars.

The varying iron concentrations on browning severity showed the highest browning observed at the double iron level (3.45), followed by the half (2.44) and full (1.78) treatments, while the lowest browning occurred under zero iron conditions (1.33) (Figure 5A). These results suggest that higher iron concentrations accelerate oxidative stress, leading to increased phenolic oxidation and tissue browning (Gill and Tuteja, 2010).

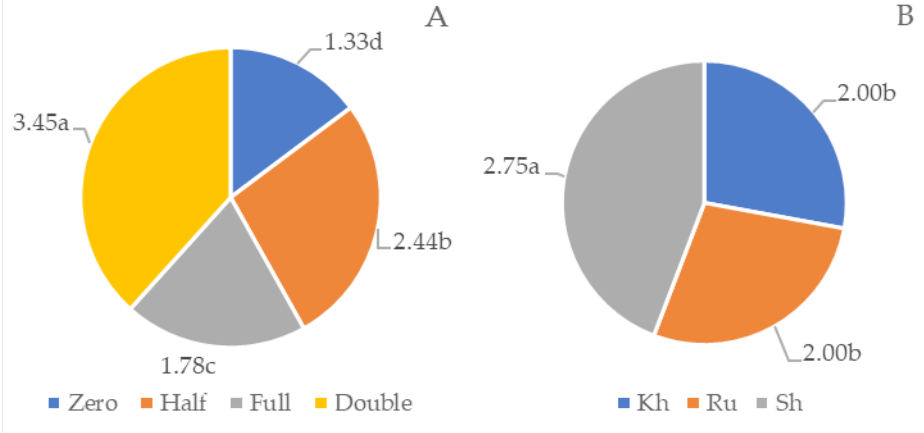

Figure 5. Specific effect of different concentrations of chelated iron (Stock F solution) (A) and three date palm cultivars (*Phoenix dactylifera* L.) (B) on the browning intensity during callus *in vitro* initiation. Kh: Khalas; Ru: Ruziz; Sh: Shishi. Values with different letters are statistically different ($p \leq 0.05$).

The cultivars showed an effect on the browning severity (Figure 5B). Sh exhibited the highest browning intensity (2.75), significantly greater than Kh and Ru, both of which showed similar browning levels (2.00). The higher browning observed in Sh may indicate a greater accumulation of phenolic compounds, which, upon oxidation, contribute to tissue browning. In contrast, the relatively lower browning in Kh and Ru suggests a better ability to regulate antioxidant enzyme activity, or potentially due to differences in iron uptake efficiency among cultivars, as previously indicated by Al-Saikhan (2006).

These results highlight the importance of cultivar selection when optimizing tissue culture conditions, as excessive browning can negatively affect explant viability and callus formation. Future studies should explore strategies to mitigate browning, such as the use of antioxidants, polyphenol oxidase inhibitors such as ascorbic acid, polyvinylpyrrolidone (PVP), and iron supplementation to minimize browning to enhance tissue culture success and elevate callus formation efficiency (Jones and Saxena, 2013; Amente and Chimdessa, 2021).

Both the iron concentration and cultivar significantly influenced browning intensity (Figure 6). Double-strength chelated iron resulted in the highest browning across all cultivars, with values of 3.67 in Kh and Sh and 3.00 in Ru, suggesting that excess iron may promote oxidative stress, leading to increased browning. The zero-iron treatment showed the lowest browning, with values of 1.00 in Kh and Ru and 2.00 in Sh, indicating that iron is involved in browning but excessive amounts accelerate the process. Meanwhile, the half- and full-strength iron treatments produced moderate browning levels, with cultivar-specific differences. For example, in Kh, half-strength (2.33) caused more browning than full-strength (1.00), while in Ru and Sh, full-strength iron led to slightly higher browning than half-strength.

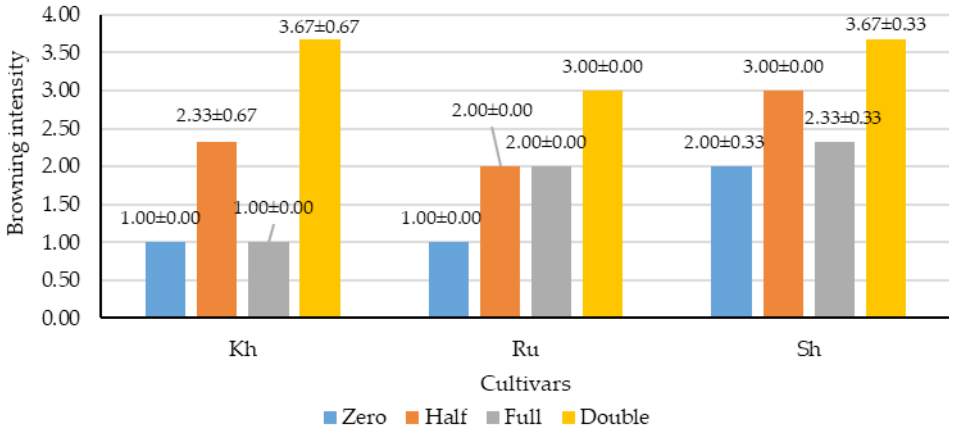

Figure 6. Interaction effect of different concentrations of chelated iron (Stock F solution) and three date palm cultivars (*Phoenix dactylifera* L.) on the browning intensity during callus *in vitro* initiation. Kh: Khalas; Ru: Ruziz; Sh: Shishi. Significance level (S = 0.001, T = 0.005, S × T = not significant).

These results suggest that the lack of chelated iron negatively affected cell growth and elongation, possibly due to insufficient enzyme formation required for respiration and photosynthesis, which are vital for cellular functions. Iron is involved in the synthesis of these enzymes and the reduction of free radicals, which helps mitigate oxidative stress (Gill and Tuteja, 2010; Amente and Chimdessa, 2021).

Effect of chelated iron on antioxidant activities of Hassawi date palm

After eight months of *in vitro* culture, increasing the iron concentration enhanced both phenolic accumulation and antioxidant activity, though the extent of this effect varies among cultivars (Table 2). In terms of total phenolic content, the data reveals a strong positive correlation between iron concentration and phenolic compound production. Sh exhibited the highest total phenolic content, reaching 2.987 mg g^{-1} under the double-strength iron treatment (F-4), followed by Kh, which showed a maximum phenolic content of 1.949 mg g^{-1} at the same iron level. In contrast, Ru exhibited the lowest phenolic accumulation, with its highest recorded value being 1.274 mg g^{-1} at F-4. These differences suggest that certain cultivars, particularly Sh and Kh, may have a stronger capacity for phenolic biosynthesis in response to iron supplementation, while Ru appears to be less responsive to increased iron availability.

Similarly, antioxidant activity, as measured by ABTS inhibition percentage and Trolox equivalent (μM Trolox), followed the same trend. Sh demonstrated the highest antioxidant activity, recording 96.75 % inhibition and 981.66 μM Trolox under the F-4 treatment, whereas Kh reached 74.95 % inhibition and 739.03 μM Trolox at the same iron concentration. Ru again exhibited the lowest antioxidant capacity, with its maximum values being 58.04 % inhibition and 554.81 μM Trolox at F-4. Explants cultured without chelated iron (F-1) had the lowest phenolic content and antioxidant activity, confirming the essential role of iron in secondary metabolite production.

Table 2. Impact of different concentrations of chelated iron and date palm cultivars (*Phoenix dactylifera* L.) on total phenolic content and antioxidant activity during eight months of *in vitro* culture.

Cultivar	Chelated iron	*Total phenolic content (mg g^{-1})	Antioxidant activity by ABTS Inhibation (%)	Antioxidant activity by ABTS UMTrolox
Kh	F-1	0.843 ± 0.004^{i}	37.650 ± 0.080^{h}	333.568 ± 2.853^{h}
	F-2	1.339 ± 0.003^{d}	58.079 ± 0.201^{d}	556.785 ± 3.742^{d}
	F-3	0.998 ± 0.020^{i}	42.853 ± 0.090^{f}	391.654 ± 1.458^{f}
	F-4	1.949 ± 0.007^{c}	74.959 ± 0.279^{c}	739.030 ± 2.873^{c}
Ru	F-1	0.784 ± 0.004^{k}	32.352 ± 0.214^{i}	277.364 ± 4.720^{i}
	F-2	$1.257 \pm 0.005^{e,f}$	51.167 ± 0.235^{e}	482.674 ± 2.076^{e}
	F-3	0.653 ± 0.002^{l}	28.444 ± 0.130^{k}	233.401 ± 3.001^{j}
	F-4	1.274 ± 0.004^{e}	58.048 ± 0.190^{d}	554.812 ± 4.720^{d}
Sh	F-1	0.955 ± 0.010^{h}	39.324 ± 0.287^{g}	359.867 ± 5.173^{g}
	F-2	2.560 ± 0.003^{b}	95.174 ± 0.060^{b}	972.308 ± 0.449^{b}
	F-3	0.837 ± 0.003^{j}	28.945 ± 0.197^{j}	234.278 ± 1.112^{j}
	F-4	2.987 ± 0.010^{a}	96.754 ± 0.211^{a}	981.661 ± 3.704^{a}
Least significant difference at $p \leq 0.05$		1.754	0.404	4.825

*Total phenolic concentration expressed as mg of gallic acid equivalents (GAE) per 100 g of sample. ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) method; Kh: Khalas; Ru: Ruziz; Sh: Shishi. Stock F (F-1: zero; F-2: half; F-3: full; F-4: double).

Additionally, cultivar differences were evident, as Sh consistently outperformed Kh and Ru in phenolic accumulation and antioxidant capacity across all iron treatments (Al-Saikhan, 2006). These findings highlight the importance of iron availability in tissue culture media, not only for promoting callus formation but also for enhancing the production of bioactive compounds, likely due to its role in activating antioxidant enzymes and promoting phenolic biosynthesis (Amente and Chimdessa, 2021).

The heat map (Figure 7) generated from the experimental data illustrates the significant effect of chelated iron on both total phenolic content and antioxidant activity across different date palm cultivars (Kh, Ru, and Sh). The data showed a clear increase in phenolic content and antioxidant activity (measured by inhibition percentage and μM Trolox) as the concentration of chelated iron rises. Specifically, Sh exhibited the highest levels of phenolic content (2.987 mg g^{-1}), inhibition (96.754 %), and μM Trolox (981.661) when exposed to the highest iron concentration (F-4). In contrast, Ru showed relatively lower phenolic content and antioxidant activity, indicating cultivar-specific responses to iron supplementation. Iron plays an important role in plant physiology, especially in enhancing secondary metabolite production and activating antioxidant defense mechanisms. Iron is crucial for the synthesis of antioxidant enzymes, such as catalase and peroxidase, which help mitigate oxidative stress by neutralizing reactive

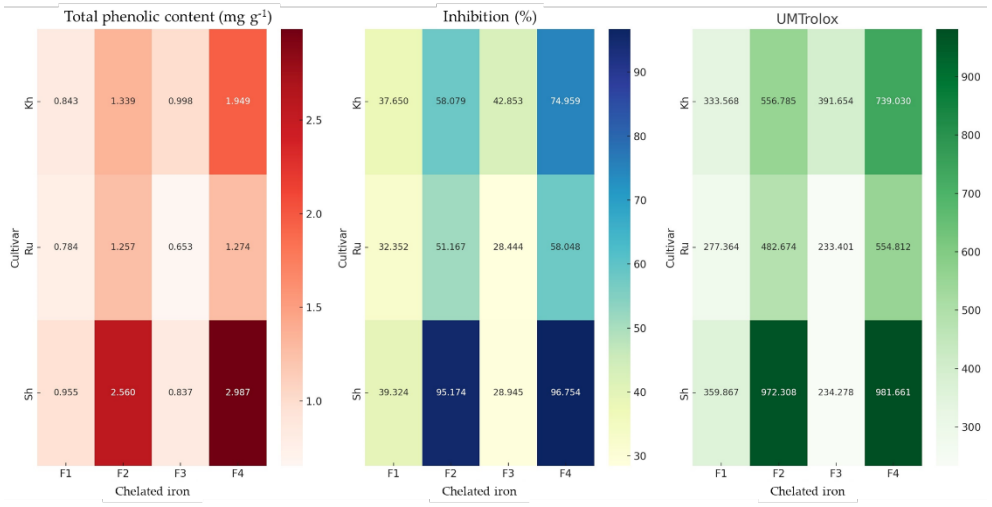

Figure 7. Heat map analysis of chelated iron effects on phenolic content, antioxidant activity, and Trolox equivalent levels (μM Trolox) in Khalas, Ruziz, and Shishi date palm cultivars (*Phoenix dactylifera* L.). Kh: Khalas; Ru: Ruziz; Sh: Shishi. Stock F (F-1: zero; F-2: half; F-3: full; F-4: double).

oxygen species (ROS) (Gopinathan *et al*., 2020). Furthermore, phenolic compounds, which contribute to a plant's antioxidant properties, are often produced in response to increased nutrient availability, including iron (Shui and Leong, 2006).

Iron is a vital component of chlorophyll, which is necessary for photosynthesis. Adequate iron levels, provided by $FeSO_4 \cdot 7H_2O$, can enhance photosynthetic efficiency in date palms. Increased photosynthesis can indirectly support the production of antioxidants. Additionally, iron acts as a cofactor for several enzymes involved in key metabolic processes, including antioxidant defense (Hell and Stephan, 2003). $FeSO_4 \cdot 7H_2O$ can activate enzymes such as catalase (CAT), superoxide dismutase (SOD), and peroxidase (POD), which help scavenge reactive oxygen species (ROS) and protect the plant from oxidative damage (Xiao *et al*., 2021). The availability of sufficient iron is crucial for the synthesis of antioxidants like vitamins C and E, which neutralize ROS and maintain cellular integrity (Abdellatif *et al*., 2022). By ensuring an adequate supply of iron, date palms may show enhanced tolerance to environmental stress, leading to a potential increase in antioxidant production (Sinha *et al*., 1997).

Iron is usually added to the MS nutrient medium in the form of $FeSO_4 \cdot 7H_2O$. It is prepared after dissolving it with the compound $Na_2 \cdot EDTA$ (sodium ethylenediaminetetraacetate) that has significant effects on plant growth, development, and antioxidant production. It plays a crucial role in plant metabolism by participating in univalent redox reactions, where it alternates between its oxidized form (ferric, Fe^{3+}) and its reduced form (ferrous, Fe^{2+}) (Gopinathan *et al*., 2020). These two oxidation states of iron can influence plant physiology, including the production of antioxidants, which are essential for protecting plants from oxidative stress (Xiao *et al*., 2021).

Iron is an integral component of chlorophyll, the molecule responsible for photosynthesis. Adequate levels of iron enhance photosynthetic efficiency, potentially boosting energy production and the synthesis of antioxidants (George *et al*., 2008). A deficiency in iron can result in chlorosis, a condition that hinders plant health and may disrupt antioxidant production (Krohling *et al*., 2016; Zhang *et al*., 2019). Ferric iron (Fe^{3+}), being the oxidized form, is generally less soluble in water compared to ferrous iron and is less accessible to plants, particularly in high pH environments (Marschner *et al*., 2011). As a result, plants may struggle to absorb ferric iron, leading to deficiencies that negatively impact growth and antioxidant formation (Briat *et al*., 2007 Al-Mayahi, 2021).

Ferrous iron (Fe^{2+}) is the reduced form of iron, meaning it has gained electrons. It is generally more soluble in water than ferric iron (Zhang *et al*., 2019). Ferrous iron is more readily absorbed by plantlet roots due to its higher solubility. However, under iron-deficient conditions, ferric iron is usually reduced to ferrous iron, transporting across cellular membranes (Jain *et al*., 2014). Adequate levels of ferrous iron support are essential for plant functions, including chlorophyll synthesis and various enzymatic activities. Sufficient ferrous iron availability can positively influence antioxidant formation in plants, since it is directly involved in the synthesis and activation of enzymes that contribute to antioxidant defense mechanisms (Al-Mayahi, 2021).

On the other hand, sodium ethylenediaminetetraacetate (Na_2·EDTA) is a chelating agent commonly used to improve the availability of essential micronutrients, particularly iron, in nutrient media by forming stable, water-soluble complexes with metal ions, enhancing their uptake by plants (Ramos *et al*., 2009). This process can influence antioxidant production in date palms by facilitating the absorption of key micronutrients like iron, zinc, copper, and manganese, which serve as cofactors for enzymes such as catalase (CAT), peroxidase (POD), and superoxide dismutase (SOD), all vital for combating oxidative stress (Abdellatif *et al*., 2022).

By improving micronutrient uptake, Na_2·EDTA can also support the synthesis of photosynthetic pigments like chlorophyll, indirectly boosting antioxidant production and enhancing the plant's ability to respond to reactive oxygen species (ROS) (Al-Mayahi, 2021; Abdellatif *et al*., 2022). However, its use must be carefully managed, as environmental factors like light intensity and temperature can affect its efficiency (Aebi, 1983). Furthermore, while Na_2·EDTA offers benefits in nutrient management, it poses potential environmental risks due to its ability to form stable complexes with heavy metals, which may become mobilized in the environment if improperly handled (Al-Mayahi, 2021). Therefore, Na_2·EDTA should be applied cautiously, with careful consideration of its environmental impact and its influence on nutrient balance, especially in *in vitro* preparations where conditions such as light and temperature must be precisely controlled.

CONCLUSIONS

This study highlights the potential of date palm tissue culture as an efficient and sustainable method for producing antioxidants year-round, independent of fruit ripening or offshoot availability. The findings confirm that Murashige and Skoog medium supplemented with chelated iron supports optimal callus formation, even at low concentrations, while higher iron levels (double-strength) significantly enhance antioxidant production. Among the tested cultivars, Shishi, particularly under double-strength iron treatment, demonstrated superior potential for antioxidant biosynthesis, suggesting its suitability for nutritional and pharmaceutical applications. These results emphasize the role of optimized *in vitro* culture conditions in improving the production of secondary metabolites such as flavonoids and phenols, which enhance the antioxidant capacity of date palm explants.

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