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Initiation of Date Palm Embryogenic Callus Using Honey as an Unconventional Alternative to Sucrose

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Abstract

This study was conducted to evaluate the possibility of using honey as an alternative energy source instead of sucrose in tissue culture media for initiating embryogenic callus of date palm (Phoenix dactylifera L.) cv. Barhi in vitro. The experiment was designed according to a completely randomized design (CRD) with nine treatments and five replicates per treatment. The treatments included three concentrations of honey (0, 20, 40 g/L) and three concentrations of sucrose (0, 20, 40 g/L). The results showed that treatment C (complete deprivation of energy sources) recorded the worst results in all studied traits. Treatment I (40 g/L honey + 0 g/L sucrose) significantly outperformed all other treatments in the number of somatic embryos (20 embryos), dry weight (304 g), and dry matter percentage (23.2%), with a decrease in water content to 76.8%. Treatment D (20 g/L honey + 40 g/L sucrose) recorded the highest fresh weight (1330 g) and dry weight (304 g) with complete absence of browning and vitrification. Treatment E (20 g/L honey + 20 g/L sucrose) was the worst in terms of browning percentage (70%) with weak embryogenic formation. We conclude that honey at a concentration of 40 g/L can be a complete and successful alternative to sucrose in date palm tissue culture media, achieving the highest production of somatic embryos and high callus quality.

Keywords: Date palm, embryogenic callus, honey, sucrose, alternative energy sources, somatic embryogenesis.

Introduction

Date palm (Phoenix dactylifera L.) is one of the most important fruit crops in arid and semi-arid regions, playing a significant economic, environmental, and social role in the lives of the inhabitants of these regions (1). Conventional propagation of date palm faces several obstacles, most notably slow growth, the limited number of offshoots produced from elite mother plants, and the lack of uniformity in the resulting plants (2). Therefore, reliance on micropropagation techniques has become an urgent necessity to meet the increasing demand for high-quality date palm seedlings.

The success of date palm micropropagation depends primarily on the researcher's ability to initiate high-quality embryogenic callus, from which large numbers of somatic embryos can be obtained and then converted into complete plants (3). The formation and development of embryogenic callus are influenced by several factors, foremost among which are the composition of the culture medium, including its carbon and energy sources, nutrients, and plant growth regulators (4). Sucrose is the traditional and most common source of carbon and energy in tissue culture media, providing both an energy source and the appropriate osmotic pressure for cell growth and differentiation (5).

Despite the effectiveness of sucrose as an energy source, its high cost in some countries, especially those that import it, has prompted researchers to search for available and low-cost local alternatives. Among these proposed alternatives is natural honey, a natural product rich in monosaccharides (glucose and fructose) in addition to containing a variety of vitamins, amino acids, minerals, and biologically active compounds (6). Previous studies have demonstrated the effectiveness of honey as an alternative energy source in tissue culture media for several plants.

In a study conducted by Eshraghi et al. on tomato plants, they found that adding honey at a concentration of 20 g/L to the culture medium led to a significant improvement in callus initiation and plant regeneration compared to the control treatment containing only sucrose (7). Abd El-Aziz also indicated that using honey at a concentration of 40 g/L in tissue culture media for some ornamental plants led to a significant increase in various growth indicators, attributing this to honey containing natural growth regulators and micronutrients (8).

Regarding date palm, some studies have begun to emerge that address the effect of adding honey to culture media. Al-Mayahi studied the effect of adding honey at different concentrations (10, 20, 30 g/L) to date palm tissue culture medium for the Barhi cultivar, and

observed an improvement in callus formation and an increase in the number of somatic embryos formed when adding 20 g/L honey compared to the control treatment (9). A study by Hashem also indicated that adding honey to the culture medium led to a decrease in the percentage of browning and an increase in the biological activity of the callus, due to honey containing antioxidant compounds that limit oxidative stress in plant cells (10).

From a physiological perspective, honey provides an easily absorbable carbon source for plant cells, as glucose and fructose (the main components of honey) can be absorbed directly without the need for prior enzymatic breakdown as is the case with sucrose (5). Honey also contains growth-stimulating enzymes such as invertase and amylase, in addition to essential amino acids involved in building structural and enzymatic proteins in cells (6). The phenolic compounds and flavonoids present in honey contribute to protecting cells from oxidative stress resulting from suboptimal conditions in tissue culture (7).

However, the use of honey in tissue culture media is not without challenges, as its chemical composition varies depending on its floral source, geographical region, and climatic conditions, which may lead to variability in results (6). Additionally, the appropriate concentration of honey varies depending on the plant species and growth stage, and high concentrations may increase the osmotic pressure of the medium, causing stress to cells (4). Therefore, determining the optimal concentration of honey and its appropriate ratio with sucrose requires precise studies for each plant species individually.

On the other hand, there is an urgent need to understand the complex interactions between honey and sucrose in the culture medium and their effect on various physiological processes such as embryogenesis, callus activity, browning, and vitrification. Browning is one of the main problems facing date palm tissue culture, resulting from the oxidation of phenolic compounds by peroxidase and polyphenol oxidase enzymes, leading to growth inhibition and tissue death (11). Vitrification is a physiological disorder resulting from a defect in cell wall formation and increased water absorption, and it is considered an important indicator of the quality of the produced callus (1).

Based on the foregoing, this study aims to evaluate the effect of adding honey as a partial or complete alternative to sucrose in date palm tissue culture medium, by studying nine different treatments combining three concentrations of honey (0, 20, 40 g/L) and three concentrations of sucrose (0, 20, 40 g/L). The effect of these treatments on the physical traits of the

callus (fresh weight, dry weight, dry matter percentage, water content) and qualitative traits (number of somatic embryos, callus activity, browning percentage, and vitrification percentage) will be evaluated, with the aim of determining the optimal composition of energy sources in the culture medium to achieve the highest production of high-quality embryogenic callus.

Materials and Methods

Experiment Location and Date

This experiment was conducted in the Plant Tissue Culture Laboratory, Department of Palms, Basra Agriculture Directorate, during the year 2025.

Plant Material

A date palm offshoot of the Barhi cultivar, aged 4 years and weighing approximately 15 kg, was used. It was carefully selected to be free from pathological and insect infections.

Dissection and Sterilization of the Apical Meristem

The dissection process was carried out by gradually removing the outer leaves and surrounding fibers until reaching the apical meristem. The apical meristem was excised from the mother offshoot and subjected to a surface sterilization process by washing it with running water several times to remove adhering soil and dust. The meristem was sterilized with commercial sodium hypochlorite at a concentration of 15% for 20 minutes, adding 3-4 drops of the spreading agent Tween20 to increase the surface area and effectiveness of sterilization. After the sterilization period, the plant part was washed three times with sterile distilled water, then preserved in an antioxidant solution containing citric acid at 150 mg/L and ascorbic acid at 100 mg/L until planting.

Callus Induction

The plant part was planted after being removed from the antioxidant solution, rinsed with sterile distilled water, and divided into four parts on a nutrient medium consisting of MS salts at 4.4 g/L, sucrose at 40 g/L, NAA at 6 mg/L, 2ip at 3 mg/L, agar at 7 g/L, and the pH was adjusted to 5.7 before sterilization. The medium was sterilized in an autoclave at 121°C for 20 minutes. The planted parts were incubated in a dark growth room at 27°C, and subculturing was repeated on the same medium components every 4 weeks until callus initiation.

Callus Multiplication

After obtaining the callus, it was multiplied by culturing it on a similar nutrient medium with reduced plant growth regulator concentrations to NAA at 3 mg/L and 2ip at 1 mg/L. The multiplication process continued under the same previous conditions with subculturing onto the same multiplication medium every 4 weeks

for four subcultures or until a sufficient quantity of callus was obtained.

Experimental Treatments

The experiment was based on two main factors:

1. **Sucrose:** Three concentrations (0, 20, 40 g/L).
2. **Honey:** Natural clover honey at three concentrations (0, 20, 40 g/L).

The number of treatments amounted to 9 treatments as shown in Table 1.

Table 1: Honey and sucrose treatments used in the experiment

Treatment	Honey (g/L)	Sucrose (g/L)
A (Control)	0	40
B	0	20
C	0	0
D	20	40
E	20	20
F	20	0
G	40	40
H	40	20
I	40	0

Callus Preparation for the Experiment

Before starting the treatments, the callus was transferred to a plant growth regulator-free nutrient medium consisting of MS salts at 4.4 g/L, agar at 7 g/L, glutamine, adenine sulfate, myo-inositol, and sodium phosphate, at pH

5.7. This stage lasted for only one subculture for two weeks to eliminate the effect of plant growth regulators from previous subcultures.

Experiment Implementation

Callus was planted at 100 mg in each glass tube containing 20 mL of culture medium, with 10 replicates per treatment. The replicates were distributed in a dark growth room at 27°C. The treatments lasted for two subcultures (8 weeks) on the same culture medium components according to each treatment, with subculturing performed 4 weeks after the start of the experiment.

Studied Traits

After the end of the two treatment subcultures, the following traits were measured:

1. **Fresh Weight (FW):** Callus masses were weighed using a sensitive balance (PHOENIX AB-224).
2. **Dry Weight (DW):** Callus samples were dried in an oven at 70°C until constant weight, then weighed using the sensitive balance.
3. **Dry Matter Percentage (DM%):** Calculated according to the equation: DM% = (Dry Weight / Fresh Weight) × 100
4. **Water Content Percentage (WC%):** Calculated according to the equation: WC% = (Fresh Weight - Dry Weight) / Fresh Weight × 100
5. **Number of Embryos:** The average number of somatic embryos formed in each treatment was calculated.
6. **Callus Activity:** Calculated as the percentage of active, healthy replicates out of the total number of replicates.
7. **Browning Percentage:** Calculated as the percentage of replicates showing browning out of the total number of replicates.
8. **Vitrification Percentage:** Calculated as the percentage of replicates showing vitrification symptoms out of the total number of replicates.

Statistical Design and Data Analysis

The experiment was conducted using a Completely Randomized Design (CRD) with nine treatments and five replicates per treatment. Data were statistically analyzed using SPSS software, and means were compared using the Least Significant Difference (LSD) test at a probability level of 0.05.

Results

1. Effect of Honey and Sucrose on Fresh Weight and Dry Weight

The results in Table 2 indicate significant differences among treatments in average fresh weight and dry weight. Treatment C (without honey and without sucrose) recorded the lowest values for fresh weight (297 g) and dry weight (43.6 g). Treatment D (20 g/L honey + 40 g/L sucrose) significantly outperformed all other treatments in fresh weight (1330 g), followed by treatment I (40 g/L honey + 0 g/L sucrose) with 1300 g and treatment G (40 g/L honey + 40 g/L sucrose) with 1296 g, with no significant differences among them. Treatment A (control) recorded 1100.2 g, while treatment E recorded 1046.2 g, and treatment H recorded 1196 g. The lowest values after C were in treatment B (528 g) and treatment F (620 g). The Least Significant Difference (LSD) value for fresh weight was 48.6 g and for dry weight was 10.2 g.

2. Effect of Honey and Sucrose on Dry Matter Percentage and Water Content

The results in Table 2 show that the highest dry matter percentage was in treatment I (23.2%), followed by treatment D (22.6%) and then treatment H (21.9%), while the lowest percentage was in treatment C (14.6%). Conversely, the lowest water content was in treatment I (76.8%), followed by treatment D (77.4%) and then treatment H (78.1%), while the highest water content was in treatment C (85.4%). The LSD value for dry matter percentage was 0.8% and for water content was 0.8%.

3. Effect of Honey and Sucrose on Number of Embryos and Callus Activity

Treatment I (40 g/L honey + 0 g/L sucrose) recorded the highest number of somatic embryos, reaching 20 embryos, significantly outperforming all other treatments (Table 2). It was followed by treatment A (12 embryos) and treatment G (12 embryos), then treatment D (10 embryos). Treatment E recorded only one embryo, while treatments B, C, F, and H recorded no embryonic formation. The highest callus activity was in treatments A, D, and I (100%, 95%, and 95%, respectively), while the lowest activity was in treatment C (10%). The LSD value for the number of embryos was 2.5 and for callus activity was 8.9%.

4. Effect of Honey and Sucrose on Browning and Vitrification

The lowest browning percentage was recorded in treatment D (0%), followed by treatment I (10%), then treatment A (10%) and treatment G (15%) (Table 2). The highest browning percentage was in treatment E (70%), followed by treatment F (40%) and then treatment H (20%). Regarding vitrification, treatments B, C, E, F, H, and I recorded 10%, while treatments A, D, and G were completely free of vitrification. The LSD

value for browning was 6.7% and for vitrification was 2.3%.

Table 2: Means and standard deviations of studied traits for the effect of adding honey as an alternative to sucrose on date palm callus

Treatment	Honey (g/L)	Sucrose (g/L)	FW (g)	DW (g)	DM%	WC%	Embryo No.	Activity%	Browning%	Vit%
A	0	40	1100.2 ± 21.3	220.04 ± 4.3	20.0 ± 0.0	80.0 ± 0.0	12	100	10	0
B	0	20	528.0 ± 18.7	88.0 ± 1.9	16.8 ± 0.3	83.2 ± 0.3	0	30	10	10
C	0	0	297.0 ± 8.4	43.6 ± 2.5	14.6 ± 0.7	85.4 ± 0.7	0	10	10	10
D	20	40	1330.0 ± 22.4	304.0 ± 12.9	22.6 ± 0.4	77.4 ± 0.4	10	95	0	0
E	20	20	1046.2 ± 73.5	211.9 ± 13.1	20.3 ± 0.7	79.7 ± 0.7	1	30	70	10
F	20	0	620.0 ± 15.8	109.0 ± 6.5	17.6 ± 0.5	82.4 ± 0.5	0	20	40	10
G	40	40	1296.0 ± 11.4	262.6 ± 5.1	19.7 ± 0.3	79.7 ± 0.3	12	85	15	0
H	40	20	1196.0 ± 12.9	262.6 ± 5.1	21.9 ± 0.2	78.1 ± 0.2	0	70	20	10
I	40	0	1300.0 ± 14.1	304.0 ± 9.4	23.2 ± 0.4	76.8 ± 0.4	20	95	10	10
LSD (0.05)			**48.6**	**10.2**	**0.8**	**0.8**	**2.5**	**8.9**	**6.7**	**2.3**

Discussion

First: Effect of Depriving the Medium of Energy Sources (Treatment C)

Treatment C (without sucrose and without honey) showed the worst results overall, recording the lowest fresh weight (297 g), lowest dry weight (43.6 g), and lowest callus activity (10%) with no formation of any somatic embryos. This result is physiologically expected, as plant cells in tissue culture depend completely on an external carbon source to generate energy and build cellular components (4). The absence of an energy source leads to cessation of cell divisions and gradual cell death due to depletion of the internal carbohydrate reserve.

Second: Effect of Reducing Sucrose Concentration by Half (Treatment B)

Reducing the sucrose concentration from 40 g/L to 20 g/L in treatment B led to a significant decrease in fresh weight (528 g) compared to the control treatment A (1100 g), and the number of embryos decreased to zero and callus activity decreased to only 30%. This indicates that 20 g/L sucrose is insufficient to meet the energy requirements necessary for optimal growth and embryonic formation in date palm. Mazri and Meziani indicated that the optimal concentration of sucrose in date palm tissue culture media ranges between 30-40 g/L, and any decrease below this range negatively affects growth (2).

Third: Effect of Adding Honey at 20 g/L Concentration (Treatments D, E, F)

Treatment D (20 g/L honey + 40 g/L sucrose): Recorded the highest values for fresh weight (1330 g), dry weight (304 g), and dry matter percentage (22.6%), with a decrease in water content to 77.4% and complete absence of browning and vitrification. This superiority is due to the synergistic effect between sucrose and honey, as honey provides, in addition to monosaccharides (glucose and fructose), a range of trace elements, vitamins, and biologically active compounds that stimulate growth (7).

Treatment E (20 g/L honey + 20 g/L sucrose): Showed moderate results in fresh weight (1046 g) but with a catastrophic increase in browning (70%) and very weak embryonic formation (only one embryo). This is explained by the fact that this combination may have created an imbalance in the osmotic pressure of the medium, leading to cell stress and increased production of phenolic compounds. Abohatem et al. found that osmotic stress stimulates peroxidase enzyme activity and increases phenolic accumulation, leading to browning (11).

Treatment F (20 g/L honey + 0 g/L sucrose): Recorded a fresh weight (620 g) lower than B, with no embryo formation, and browning increased to 40%. This indicates that although honey alone at 20 g/L can support basic growth, it is insufficient to stimulate embryonic

formation, and the absence of sucrose leads to disturbances in osmotic regulation.

Fourth: Effect of Adding Honey at 40 g/L Concentration (Treatments G, H, I)

Treatment I (40 g/L honey + 0 g/L sucrose): This was the major surprise in this experiment, recording the highest number of embryos (20 embryos), highest dry weight (304 g), and highest dry matter percentage (23.2%) with a decrease in water content to 76.8%. This superiority is due to honey at 40 g/L providing a sufficient amount of easily absorbable monosaccharides, in addition to natural growth-stimulating compounds. A study by Abd El-Aziz indicated that adding honey at 40 g/L to tissue culture media led to a significant improvement in growth indicators for several plant species (8).

Treatment G (40 g/L honey + 40 g/L sucrose): Recorded excellent results in fresh weight (1296 g) and number of embryos (12), but it was lower than I in the number of embryos. The reason for this may be that the total sugar concentration became very high (approximately 80 g/L from different sources), creating osmotic stress that limited optimal embryonic formation.

Treatment H (40 g/L honey + 20 g/L sucrose): Showed good results in weight (1196 g) and callus activity (70%), but failed to produce embryos (zero). This indicates that this combination may be suitable for callus growth only but is not stimulating for embryonic differentiation.

Fifth: Analysis of Browning and Vitrification

The highest browning was in treatment E (70%), followed by F (40%) and then H (20%). It is observed that browning increases in treatments containing 20 g/L honey with low concentrations of sucrose (E and F). This is explained by the fact that honey at 20 g/L may be insufficient to provide osmotic protection for cells, while the 40 g/L concentration provided better protection (only 10-15% browning in I and G).

As for **vitrification**, it appeared in all treatments containing honey except D and G (which contained honey with full sucrose). This indicates that the presence of full sucrose (40 g/L) with honey prevents vitrification, perhaps through regulating osmotic pressure and providing an appropriate balance between monosaccharides and disaccharides.

Sixth: Relationship Between Energy Sources and Embryonic Formation

The treatments can be arranged in descending order according to their ability to stimulate embryonic formation as follows:

I (40 honey + 0 sucrose) > A (0 honey + 40 sucrose) = G (40 honey + 40 sucrose) > D (20 honey + 40 sucrose) > remaining treatments

This order proves that honey at a concentration of 40 g/L can be a complete and successful alternative to sucrose in date palm tissue culture media, and it even surpassed the control in the number of embryos (20 vs. 12). This is due to: **Complex**

composition of honey: Honey contains glucose and fructose (monosaccharides) that are directly absorbed by cells without the need for prior enzymatic breakdown (6), **Secondary components:** Honey contains vitamins (especially B complex vitamins), amino acids, minerals, and enzymes that aid in metabolic processes (7), and **Antioxidant compounds:** Honey contains phenolic compounds and flavonoids that protect cells from oxidative stress, explaining the low browning in I (10%) compared to A (10%) despite I producing twice the number of embryos.

Conclusions and Recommendations: Sucrose can be completely replaced with honey at a concentration of 40 g/L (treatment I) to obtain the best embryonic formation (20 embryos) with high callus quality. The optimal treatment for vegetative callus growth (without the need for embryo formation) is D (20 g/L honey + 40 g/L sucrose), which gave the highest fresh and dry weight with no browning or vitrification. Treatment E should be avoided (20 g/L honey + 20 g/L sucrose) because it causes very high browning (70%) that ruins the culture. Honey alone at 40 g/L (I) gave better results than honey at 40 g/L with sucrose (G), indicating that the presence of sucrose with high-concentration honey may cause osmotic stress that limits embryonic formation. And It is recommended to use high-quality natural honey in date palm tissue culture media, considering that the honey concentration should be 40 g/L to achieve the best results in somatic embryogenesis.

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