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Effects of dicamba and casein hydrolysate on *in vitro* growthand shoot regeneration of date palm (*Phoenix dactylifera* L.) cv. Barhee

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Abstract

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The investigation was carried out to evaluate the influence of the dicamba (3,6-Dichloro-2-methoxybenzoic acid) (DIC) and casein hydrolysate (CH) on the callus growth, shoot multiplication, and some biochemical constituents of date palm cv. Barhee cultured *in vitro*. Both DIC and CH were required for callus growth and shoots regeneration. The medium supplemented with 4.0 mg l^{-1} DIC in combination with 1.0 g l^{-1} CH gave the highest callus weight (287 mg), while the maximum response rate and the number of shoots per jar (86.67% and 15.07 shoots/jar) were found in MS media equipped with 4 mg l^{-1} DIC and 0.5 mg l^{-1} CH combination. The total amount of phenolic compounds was significantly reduced to 0.82 and 0.79 mg GAE g^{-1} in shoots cultured in the medium equipped with 4.0 mg l^{-1} DIC with 0.5 and 1.0 g l^{-1} CH, which is reflected in the rate of browning. The results showed that the highest shoots content of endogenous IAA (3.71 and 3.50 µg g^{-1}), were obtained in response to 4 mg l^{-1} DIC + 1.0 g l^{-1} CH and 4.0 mg l^{-1} DIC + 0.5 g l^{-1} CH, respectively. The macronutrient K, P, Ca, and free amino acids content significantly increased in the *in vitro* shoots regenerated on the media supplemented with 4.0 mg l^{-1} DIC + 1.0 g l^{-1} CH. The genetic stability of this study was confirmed by the DNA-based fingerprinting method RAPD. The RAPD binding patterns indicated no variation among tissue culture-derived plants. The in vitro propagation protocol described herein can be introduced to the production of genetically stable date palm plants.

Keywords

amino acids, auxin, *in vitro*, macronutrient, RAPD, shoot regeneration

Introduction

Date palm (*Phoenix dactylifera* L.) is a flowering plant belonging to the monocotyledonous family Arecaceae and is an economically important tree species. Dates are a major food source and income source for local populations in the Middle East and North Africa (Al-Khalifah et al., 2013).

The traditional vegetative method of date palm propagation is by offshoots, what is inefficient because each tree produces only a few offshoots, especially in some elite varieties, and fruit-bearing can take up to seven years (Gantait et al., 2018). The second way of propagation is by seeds, but it has many limitations including low rate of germination and progeny variations (Chand and Singh, 2004). With all these facts, *in vitro* propagation is the most promising method for the rapid propagation of date palms to overcome the decline in the number of desirable cultivars (Al-Mayahi, 2019; Abdalla et al., 2022). Plant

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tissue culture effectivity as a means of plant propagation is greatly affected by selecting the proper culture medium (Al-Mayahi et al., 2010; Al-Mayahi and Ali, 2021).

Auxins are widely used in plant tissue culture, especially for callus induction and tissue and organ differentiation. Auxin and cytokinin ratio during *in vitro* tissue culture can play a critical role to induce the response in higher plants. Auxins are available in both natural and synthetic forms.

Auxinic herbicides are synthetic auxins and may also be described as active growth regulators with a growth-regulating effect. Dicamba, also known as 3, 6-Dichloro-2-methoxybenzoic acid (DIC), is an herbicide that acts as a phytohormone. It tends to show higher activity compared to other auxins, which promotes somatic embryonic development in tissue culture (Phillips and Garda, 2019). DIC was shown to surpass 2, 4-dichlorophenoxyacetic acid (2, 4-D) in inducing shoot regeneration in cereals tissue culture (Trifonov et al., 2001; Przetakiewicz et al., 2003). Filippov et al., 2006 reported that adding a higher level of DIC to the callus medium could greatly promote the frequency of the formation of callus and regenerated shoots of wheat. On the other hand, complex organic additives promote the growth and differentiation of plant cultures *in vitro*. It has been reported that culture medium supplemented with casein hydrolysate (CH) can improve callus growth and somatic embryogenesis in various plants including date palm (Khalda and Forkan, 2006; Ageel and Elmeer, 2011; Al-Khayri, 2011). High regeneration capacity from commercial date palm varieties is a primary requirement for efficient genetic modification. Molecular markers had become a promising method in fingerprinting and genetic analysis (Saad-Allah and Youssef, 2018). Out of various molecular markers applied to evaluate *in vitro* proliferated plants' genetic fidelity, RAPD is one of the simplest, quick and cost-effective techniques and require only small amounts of DNA (Chaudhary et al., 2015). Srivashtav et al., (2013) reported that RAPD markers are more efficient markers than ISSR for evaluating genetic variation in date palms. Despite many advantages, micropropagation of date palm still faces many problems, and research related to studying the effects of DIC and CH on the growth and development of date palm *in vitro* cultures is scarce. Therefore, the aim of this study was to evaluate the impact of these two compounds on callus growth, multiplication shoots, and some biochemical traits of the date palm cv. Barhee cultivated *in vitro*. Genetic stability was also analyzed in the tissue culture-derived plantlets, and Random Amplification of Polymorphic DNA (RAPD) indicators were used to determine the protocol effectiveness.

Materials and methods

Tissue cultures experiments

Impact of dicamba (DIC) and casein hydrolysate (CH) on growth and development of callus and shoot regeneration of date palm cv. Barhee

The experiments were carried out in the tissue culture laboratory for Date Palm Research Centre at Basrah University, Basrah, Iraq.

Young offshoots (2–3 years old) of date palm cv. Barhee were detached from the parent palm. Outer leaves and fibrous tissues at their bases were removed gradually until the shoot tip zone was exposed. Sterilization of explants was performed using 70% ethanol for 1 min and 2.5% sodium hypochlorite for 20 min. Explants were then rinsed three times with sterile distilled water. For callus induction, explants were cultured on the MS basal medium (Murashige and Skoog, 1962) with the addition of 3.0 mg l^{-1} 6-(dimethylallyl amino purine) (2iP), 30 mg l^{-1} of 1-naphthalene acetic acid (NAA), 30 g l^{-1} of sucrose, 2.0 g l^{-1} of activated charcoal, and solidified with 6.0 g l^{-1} agar–agar. The cultures were transferred to fresh media, with the same composition every 6 weeks until the callus began to initiate. All cultures were incubated in a culture room under darkness at 27 ± 2 °C for 180 days to initiate callus.

To study the effects of dicamba (DIC) and casein hydrolysate (CH), supplementation of these compounds at various concentrations in the growth media was assessed. The induced callus was separated from the apical buds, weighed, and cultured (100 mg per jar) on MS medium, with the addition of growth regulators NAA at 6.0 mg l^{-1} and 2iP at 2.0 mg l^{-1}. To study the effects of dicamba (DIC) and casein hydrolysate (CH) on callus growth, supplementation of these compounds at different concentrations in the growth medium was assessed. MS medium was supplemented with 0.0, 2.0, 4.0, and 6.0 mg l^{-1} DIC (Sigma-Aldrich, St. Louis, MO) or 0.0, 0.5, and 1.0 g l^{-1} CH, or added together at 4.0 mg l^{-1} DIC + 0.5 g l^{-1} CH, and 4.0 mg l^{-1} DIC + 1.0 g l^{-1} CH. The pH of the medium was adjusted to 5.7–5.8 before the addition of agar. Media were dispensed into culture containers and autoclaved at 121 °C and 1.04 kg cm^{-2} for 20 min. The cultures were kept in a culture room at 27 ± 2 °C with 16 h light and irradiance of 13.5 µmol m^{-2} s^{-1} provided by cool white fluorescent lamps at the light intensity of 2,000 lux.

For differentiation and multiplication, the callus on growth media was divided and subcultured on differentiation and multiplication media supplemented as mentioned above, except for the plant growth regulators 1 mg l^{-1} (NAA), 0.5 mg l^{-1} 6-benzyladenine (BA), and 0.5 mg l^{-1} kinetin (K). It was also supplemented with the same DIC and CH concentrations to study their effects on buds multiplication and some changes in phytochemicals properties. The cultures were maintained under room temperature 27 ± 2 °C, with a photoperiod of 16/8 h day/night. The light intensity was 2,000 lux provided by cool white fluorescent lamps. There were 18 replicates of each treatment. The percentage of bud regeneration and bud number per jar were recorded at 12 weeks from the inoculation of callus on the media.

Browning percentage (%)

The browning percentages were scored according to the

following: (–) No tissue browning; (+) Moderate tissue – browning; (+++) Severe tissue – browning.

Biochemical analysis

Total phenolic content

The total phenolics content was extracted according to the method described by Singleton and Rossi (1965). Gallic acid was used as a reference standard. The results were expressed in mg equivalent of gallic acid per gram (mg GAE g^{-1}).

Free amino acids

The free amino acids were determined according to the method of Lee and Takahashi (1966) by utilizing ninhydrine reagent.

Extraction and measurement of auxin Indole-3-acetic acid (IAA)

Auxin was extracted and quantified according to the method of Nagar and Sood (2006). Five grams of shoots after various treatments with DIC or CH and their combinations were homogenized using 80% methanol. The extract was filtered through the Whatman filter paper (no.1) and evaporated at 4 °C in dark conditions. The supernatant was dried in vacuum, and withdrawn by a 0.1 M potassium phosphate (pH 8.1). Eluate obtained was evaporated to dryness, taken up in water and pH adjusted to 2.5 with 1 N HCl and then partitioned (4×) with diethyl ether. Combined ether phases were evaporated to dryness in vacuum and the residue was dissolved in methanol for the estimation of free auxins up to 80%. The remaining aqueous phase was hydrolyzed at pH 7.0 for 3 h at 100 °C (Sundberg et al., 1994). The hydrolysate was cooled, neutralized with 1 M HCl up to pH 3.0 and finally partitioned against diethyl ether. The combined ether phases were evaporated in vacuum, and taken up in methanol (HPLC grade) for the estimation of bound auxins. For the estimation of endogenous contents of auxins, methanolic extracts ready for HPLC analysis were filtered through 0.45 µM Millipore filters. Elution was carried out with Methanol (40%) in 30 mM acetic acid (HPLC grade) at a flow rate of 1 ml min^{-1}. Column eluents were passed through a UV detector (2996 PDA detector) at 280 nm was passed through the column eluants and auxins were characterized and quantified. Standard auxins were used as reference (IAA).

Determination of macronutrient content

The content of potassium (K), calcium (Ca), and phosphorus (P) in shoots were measured by the method of Cresser and Parsons (1979). Phosphorus was measured by a spectrophotometer at 880 nm, according to Murphy and Riley (1962). K and Ca were determined by atomic absorption spectrometry, according to the method described by Black (1968). There were three replicates of each analysis.

DNA isolation and RAPD analysis

Total genomic deoxyribonucleic acid (DNA) was isolated from regenerated date palm shoots using the CTAB method as described in Rogers and Bendich (1985). Polymerase chain reaction (PCR) reactions were performed using a set of three arbitrary 3-mer primers. These primers and their sequences are presented in Table 1.

Table 1. RAPD primers and their sequences used for the genetic fidelity evaluation

Primer	Sequence	CG (%)
OPD-10	GGTCTACACC	60
OPO-07	CAGCACTGAC	60
OPA-02	TGCCGAGCTG	70

The PCR mixture

The reaction mixture (20 µl) contained 10 ng DNA, 200 µM deoxynucleotide triphosphates (dNTPs), 1 µM primer, 0.5 units of Red Hot Taq polymerase (AB-gene Housse, UK) and 10-X Taq polymerase buffer (AB-gene Housse, UK). For DNA amplification, a Perkin Elmer thermal cycler (2720) was used with following program: initial denaturation: 95 °C for 5 min followed by 94 °C for 0.45 min, annealing (35 cycles) 35 °C for 1 min, and extension step first at 72 °C for 1.5 min and 30 s and then at 72 °C for 7 min (Adawy et al., 2004). The amplification products were separated in 1% (w/v) agarose gel in 1× Tris/Borate/Ethylene diaminetetra acetic acid (TBE) buffer and visualized by staining with ethidium bromide. The reproducibility of DNA profiles was determined by replicating all RAPD re-

Table 2. Effect of dicamba (DIC) and casein hydrolysate (CH) on in *in vitro* growth of date palm cv. Barhee

Treatments	Callus weight (mg)	Frequency (%)	Shoot number
0.0	120.0 g ± 7.33	33.34 f ± 4.05	3.40 e ± 0.50
2 mg l^{-1} DIC	189.0 de ± 3.60	60.00 cd ± 3.89	7.44 c ± 0.19
4 mg l^{-1} DIC	227.0 c ± 2.21	73.34 b ± 4.60	9.36 b ± 0.22
6 mg l^{-1} DIC	201.0 d ± 2.21	66. 67bc ± 6.80	7.80 c ± 0.58
0.5 g l^{-1} DIC	145.0 f ± 3.60	53.34 de ± 3.05	5.38 d ± 0.80
1.0 g l^{-1} DIC	171.0 e ± 2.21	46.67 e ± 3.89	5.14 d ± 0.59
4.0 mg l^{-1} DIC + 0.5g l^{-1} CH	255.0 b ± 7.33	86.67 a ± 6.40	15.07 a ± 0.42
4.0 mg l^{-1} DIC + 1.0 g l^{-1} CH	287.0 a ± 4.79	80.00 a ± 6.40	13.83 a ± 0.40

± Standard error (n = 15). Values followed by the same letter are not significantly different at 5% level by (LSD) test.

actions at least three times using DNA markers. The primers were evaluated from wise pair comparison for the proportion of shared bands amplified (Nei, 1978). The similarity coefficient was calculated using the statistical software package STATISTICA-SPSS Version 18.0 (Stat Soft Inc.).

Statistical analysis

The experiments were conducted in a completely randomized design (CRD). Data were analyzed by one-way analysis of variance (ANOVA) using Statistical Package for

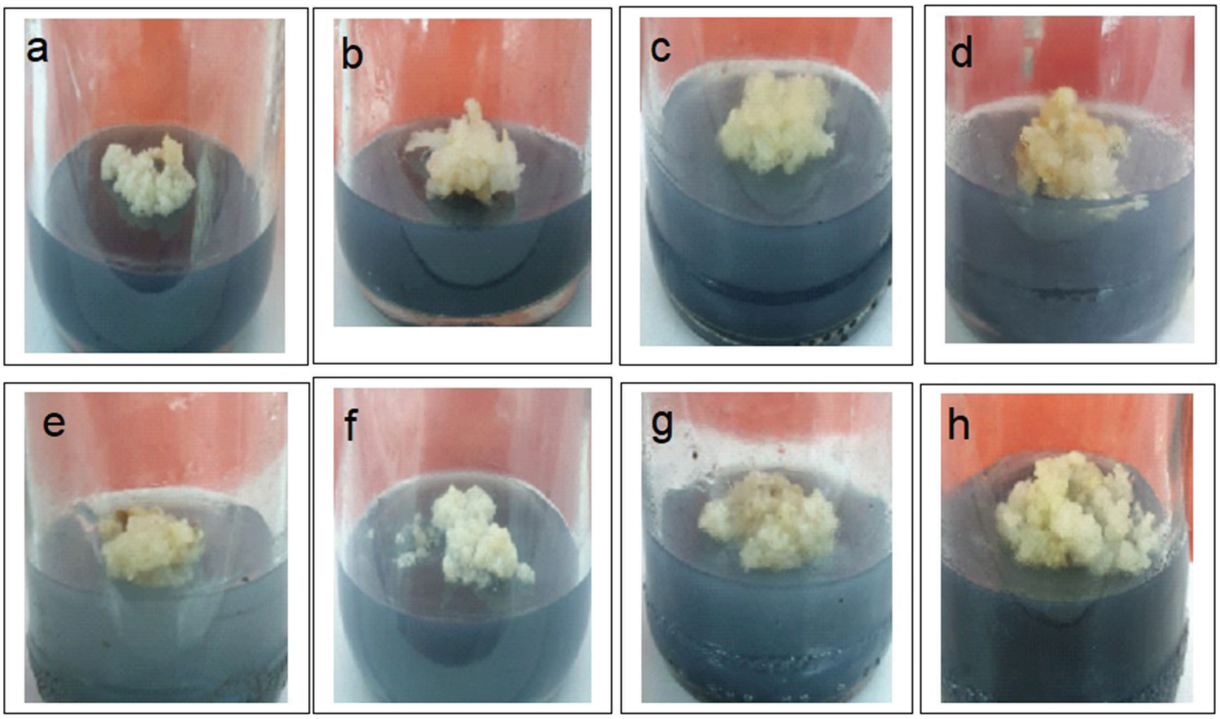

Fig. 1. Callus profileration on MS medium with (a) control, (b) 2.0 mg l^{-1} DIC, (c) 4.0 mg l^{-1} DIC, (d) 6.0 mg l^{-1} DIC, (e) 0.5 g l^{-1} CH, (f) 1.0 g l^{-1} CH, (g) 4.0 mg l^{-1} DIC + 0.5 g l^{-1} CH, (h) 4.0 mg l^{-1} DIC + 1.0 g l^{-1} CH, (× 0.8).

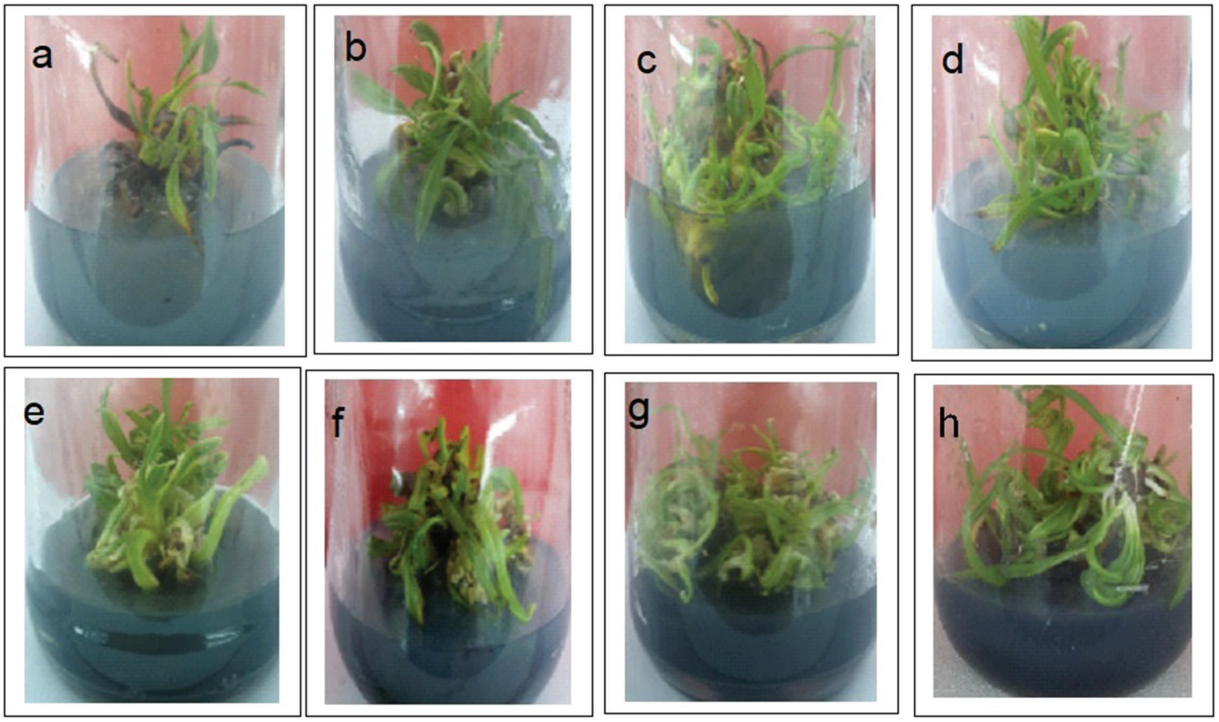

Fig. 2. Bud induction on MS media with (a) control, (b) 2.0 mg l^{-1} DIC, (c) 4.0 mg l^{-1} DIC, (d) 6.0 mg l^{-1} DIC, (e) 0.5 g l^{-1} CH, (f) 1.0 g l^{-1} CH, (g) 4.0 mg l^{-1} DIC + 0.5 g l^{-1} CH, (h) 4.0 mg l^{-1} DIC + 1.0 g l^{-1} CH, (× 0.8).

Table 3. Effect of dicamba (DIC) and casein hydrolysate (CH) on a browning degree and total phenolic compounds (mg GAE g^{-1}) of date palm buds cv. Barhee cultivated *in vitro*

Treatments	Browning degree	Phenolic compounds (mg GAE g^{-1})
0.0	+++	2.01 e ± 0.25*
2.0 mg l^{-1} DIC	+	1.03 c ± 0.02
4.0 mg l^{-1} DIC	+	0.92 b ±0.01
6.0 mg l^{-1} DIC	+	1.10 c ±0.05
0.5 g l^{-1} CH	+	1.45 d ±0.12
1.0 g l^{-1} CH	+	1.39 d ±0.10
4.0 mg l^{-1} DIC+ 0.5 g l^{-1} CH	−	0.82 a ±0.06
4.0 mg l^{-1} DIC+ 1.0 g l^{-1} CH	−	0.79 a ±0.09

*± Standard error, ** Means within each column followed by the same letter are not significantly different at 5% level by (LSD) test, *** − no browning response, + poor, ++ moderate, +++ high.

Social Sciences (SPSS) software version 18.0. Treatment means were compared using the least significant difference test (LSD, $P < 0.05$).

Results

Callus growth occurred at all DIC concentrations tested in this study although the significantly higher weight was at 4.0 mg l^{-1} (Table 2, Fig. 1c). The data show an increase in callus weight as a result of increasing CH concentration, which reached 171.0 mg of callus weight at a concentration of 1.0 g l^{-1} (Table 2, Fig. 1f). The combination of 4.0 mg l^{-1} and 1.0 g l^{-1} CH gave the significantly highest callus weight rate (287.0 mg l^{-1}) compared to the other treatments (Fig. 1) after 8 weeks on the respective medium.

Callus tissues implanted in 4.0 mg l^{-1} DIC combined with 0.5 g l^{-1} CH showed better results in the percentage of shoot production from callus (86.67%) with the highest number of shoots formation (15.07 shoots/jar), compared to the other treatments (Fig. 2). The maximum reduction in growth parameters was observed in cultures implanted in the control medium.

Changes of browning percentage and phenolic content of date palm cultures

The results in Table 3 showed that various concentrations of DIC and CH had significant effects on the browning percentage. The cultures grown on the medium supplemented with 4.0 mg l^{-1} DIC combined with 0.50 or 1.0 g l^{-1} CH, showed the best results in reducing the browning degree to zero (−), as compared with no additives (control treatment) or with their individual application (Fig. 3 and 4). The control treatment aggravated the occurrence of browning to (+++) (Table 3).

The highest phenolic compounds were recorded in the tissues of the cv. Barhee, in the control treatment, which had 2.01 mg GAE g^{-1}. At the same time, a significant decrease was recorded in a media supplemented with 4.0 mg l^{-1} DIC combined with 0.50 or 1.0 g l^{-1} CH, which were 0.82 and 0.79 mg GAE g^{-1}, respectively (Table 3).

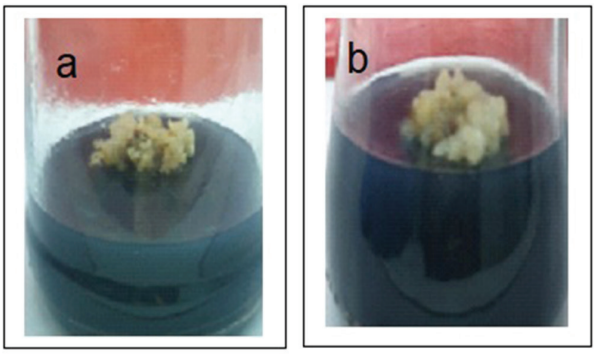

Fig. 3. Effect of dicamba (DIC) and casein hydrolysate (CH), control (a), 4.0 mg l^{-1} DIC + 1.0 g l^{-1} CH (b) on callus browning of date palm cv. Barhee.

Endogenous indole-3-acetic acid (IAA)

Figure 4a shows the effect of DIC and CH on endogenous IAA content under in vitro conditions. Increasing the DIC concentration of the culture medium from 0.0 to 6.0 mg l^{-1} resulted in a proportionally increasing content of endogenous IAA of tissues, as also observed when CH concentration was increased from 0.0 to 1.0 g l^{-1}. The highest IAA content (3.716 µg g^{-1}) in the shoots was obtained in the MS medium supplemented with 4.0 mg l^{-1} DIC + 1.0 g l^{-1} CH. The lowest content (0.91 µg g^{-1}) was recorded in shoots grown in the control medium.

Free amino acids content

According to the results (Fig. 4b), increasing the DIC concentration of the medium to 4.0 mg l^{-1} increased free amino acid content. However, free amino acids content was decreased with an increase in the concentration of DIC to 6 mg l^{-1} in the culture media (Fig. 4b). The opposite trend of results was seen with free amino acids content in treated cultures with CH, thus, was evident by the increase of free amino acids content from 1.91 mg g^{-1} to 2.21 mg g^{-1} for CH treatments at 0.50, and 1.0 g l^{-1} respectively. However, the combination of 4.0 mg l^{-1} DIC + 1.0 g l^{-1} CH application gave the highest free amino acids content (2.690 mg g^{-1}) (Fig. 4b), as compared with absence (control treatment) or individual application of additives after 6 weeks of culture.

Macronutrient content

Statistical analysis showed that DIC and CH treatments significantly affected the accumulation of macronutrients in shoot tissues of date palms implanted *in vitro*. A significant increase of K, P, and Ca (19.79% 3.80, and 4.16 mg g^{-1} DW, respectively) was recorded in the shoots tissues implanted on media equipped with 4.0 mg l^{-1} DIC + 1.0 g l^{-1} CH as compared with no additives (control treatment) or with their individual application (Table 4).

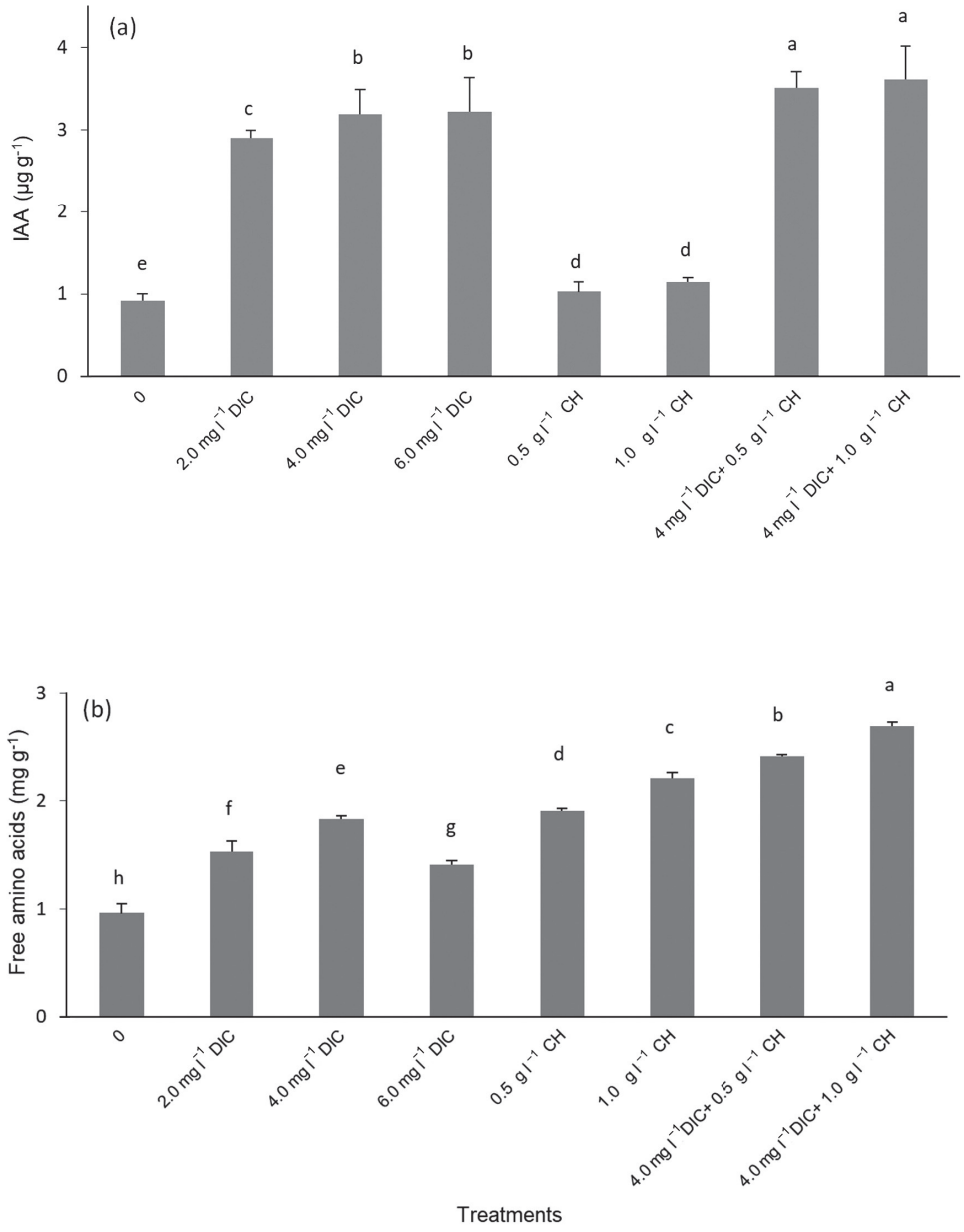

Fig. 4. Effect of dicamba (DIC) and casein hydrolysate (CH) on (a) endogenous indole acetic acid (IAA) content, (b) free amino acids, in the calli tissues of in vitro cultivated date palm cv. Barhee, bars with the same letter are not statistically different at the 5% level by (LSD) test. ± Standard error.

Molecular analysis

The PCR amplification results showed that all tested primers *in vitro* derived date palm plants produced a monomeric band, confirming the genetic uniformity of the micro-propagated material. Whereas, RAPD analysis of *in vitro* propagated plants (*P. dactylifera* L. cv. Barhee) revealed a profile similar to that of the control treatments that clearly demonstrated the genetic stability of those plants (Fig. 5) and the accuracy of the *in vitro* propagation protocol to produce true-to-type date palm plants, indicating that the use of DIC and CH during micropropagation stages did not cause any variation in the plants of this date palm cv. Barhee.

Discussion

According to the results obtained, using dicamba (DIC) and casein hydrolysate (CH) plays a synergistic role in enhancing callus growth and shoot formation of date palms cultured *in vitro*. Our experiments indicate that both DIC

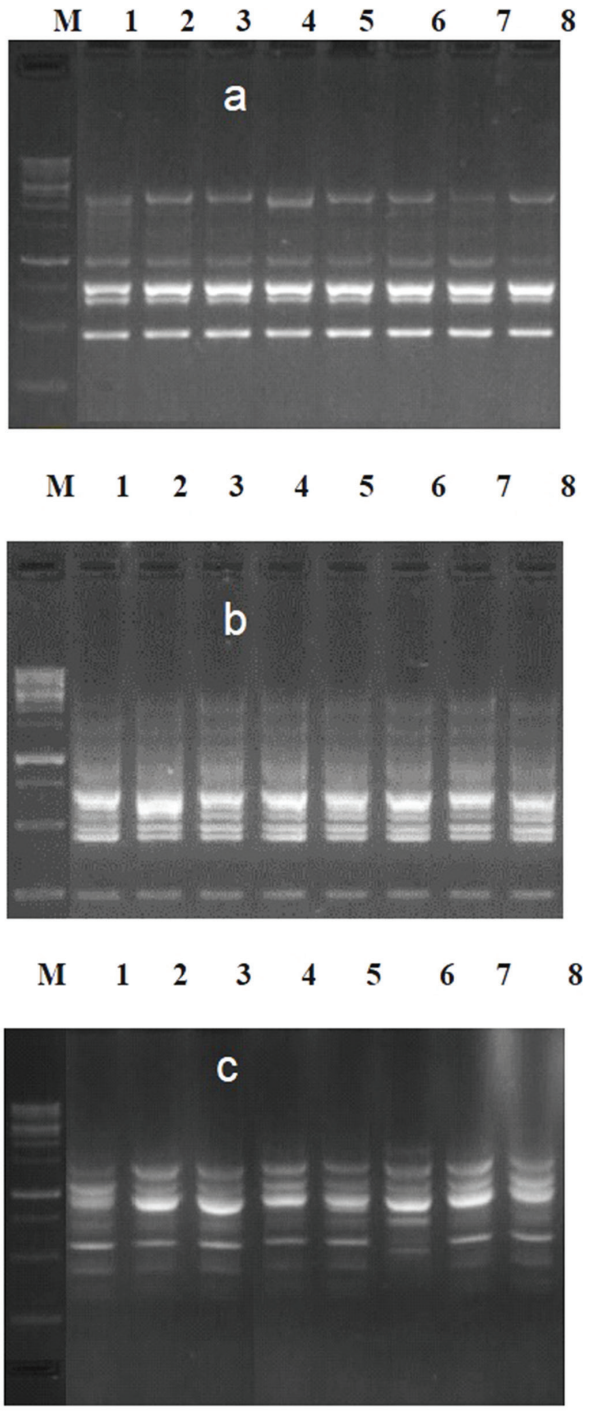

Fig. 5a, OPD 10; b, OPO 07; and c, OPA 02. RAPD pattern of regenerated plants of *Phoenix dactylifera* L. cv. Barhee on MS medium supplemented with dicamba (DIC) alone or in combination with casein hydrolysate (CH): M: Protein molecular marker; (1) control treatment; (2) 2 mg l^{-1} DIC, (3) 4.0 mg l^{-1} DIC, (4) 6.0 mg l^{-1} DIC, (5) 0.5 g l^{-1} CH, (6) 1.0 g l^{-1} CH, (7) 4.0 mg l^{-1} DIC + 0.5 g l^{-1} CH, (8) 4.0 mg l^{-1} DC + 1.0 g l^{-1} CH, (× 0.8).

and CH had a beneficial effect on most of the growth parameters studied compared with no additions (control treatment) or with their single application. The presence and concentration of DIC in the culture medium highly affect callus growth and shoot regeneration. DIC is considered a growth regulator in plant tissue cultures. In general, the concentration of PGRs varies to achieve optimal callus growth, development, and regeneration (Jasim et al., 2009, Ibrahim et al., 2013).

On the other hand, it is shown that CH can improve callus growth and regeneration frequencies. CH contains many substances such as carbohydrates, proteins, fats, and many vitamins, and it is a source of a mixture of up to 18 amino acids (Dehestani-Ardakani et al., 2017). The data of this study show an increase in callus weight as a result of increasing CH concentration, which reached 171.0 mg of callus weight at a concentration of 1.0 g 1^{-1}, as compared with no additives (control treatment) that resulted in 120.0 mg. A culture medium with CH was found to be the best medium composition for callus induction of sorghum (Indra and Krishnaveni, 2009). The effect of different CH supplements on date palm callus growth was determined by Abdel-Rahim et al., 1998 who observed that the weight callus rate gave the highest values during the cultivation period. Amino acids are organic sources of nitrogen that are rapidly absorbed by plants rather than N-inorganic (Al-Mayahi, 2010). CH can enhance the efficiency of callus formation and shoot regeneration of the callus *Phoenix dactylifera* L. cv. Bream (Khierallah and Hussein, 2013). These findings are in harmony with those of Afolabi et al. (2008), Shahsavari (2010) in which the positive effect of casein hydrolysate had been shown in different plant species such as, rice and date palm. The data indicated that DIC treatments of tissue cultures at lower concentrations were beneficial in delaying tissue browning and phenolic substances accumulation in implanted tissues (Table 3). The lowest phenolic content was recorded in MS culture media equipped with 4.0 mg l^{-1} DIC and 1.0 or 0.5 g l^{-1} of CH (Fig. 3), with significant differences compared to other treatments. Browning is one of the problems facing the practical applications of date palm propagation *in vitro*, and eliminating or minimizing this phenomenon is essential to establishing a successful culture (Al-Mayahi et al., 2020; Al-Mayahi and Ali, 2021). Phenolic compounds production is correlated with the browning intensity of date palm tissues cultures, and many authors proved these findings in their studies (Al-Mayahi et al., 2018; Shehata et al., 2014), however, Mustafa et al. (2013) demonstrated that high concentrations of auxins led to accumulating of phenolic compounds and inhibited the growth of cultures.

Tissue exposure to increasing levels of DIC (0.0 to 6.0) leads to IAA accumulation in shoots. The results also showed that the presence of CH had a positive effect on the IAA levels of the tissues compared to those implanted in a CH-free medium. Thus, the interaction between 4.0 mg l^{-1} DIC with 1.0 or 0.5 g l^{-1} of CH recorded the highest content of IAA (3.617 and 3.507 µg g^{-1}), compared with treatments with no additives or with one additive alone (Fig. 4a). Auxin is one of the plant hormones' essential classes for the growth and development of *in vitro* organs (Al-Mayahi, 2021). IAA is produced in response to many physiological or metabolic changes (Asami and Nakagawa, 2018). Additionally, IAA is the most abundant naturally occurring auxin and is involved in many aspects of plant growth and development, such as cell division, differentiation, and

elongation (Chudasama and Thaker, 2007; Mano and Nemoto, 2012). These results indicate that IAA is important for stimulating tissue growth.

The DIC and CH treatments led to significant effects on the accumulation of macronutrients and amino acids in the shoots. Shoots cultured in the medium supplemented with 4.0 mg l^{-1} DIC in combination with 1.0 g l^{-1} CH caused significant ($p \leq 0.05$) increase of K (4.160 mg g^{-1} DW), P (3.809 mg g^{-1} DW), Ca (19.790 mg g^{-1} DW), and amino acids (2.690 mg g^{-1}) in shoot tissue compared with no additives (control treatment) or with their individual application (Fig. 4b). Macronutrients play an important role in plant metabolism, and their availability at adequate levels is essential to achieving optimum physiological performance.

DIC and CH can have many effects on plant functions and some of them may modulate, directly or indirectly, ion uptake. CH contains several elements including calcium, phosphate, etc. (Ageel and Elmeer, 2011). It is noted that casein hydrolysate enhances growth in cultures where phosphate deficiency inhibits it, indicating that this deficiency is compensated by amino acids. They are constituents of proteins and play an essential role in plant metabolism and growth.

Our findings using the RAPD molecular markers indicated that there was no genetic variation among the treatments of cv. Barhee date palm plants production *in vitro*. The detected bands were 100% monomorphic, indicating that the use of DIC and CH during micropropagation stages did not cause any difference in tissue culture-derived cultures of this date palm genotype.

Most of the previously mentioned studies on field evaluation of tissue culture-derived date palms rely on morphological parameters, which are prone to changes, as a result of environmental factors and other agricultural treatments. Molecular techniques are more advantageous than other methods because they are not influenced by environmental factors and generate reliable and reproducible results. Clonal alteration is often induced by culture media and subculture cycles (Bidabadi et al., 2010). Micropropagation cannot be considered fully successful unless complete genetic fidelity is maintained. The utility of molecular analysis of regenerated cultures *in vitro* has been well-documented by several authors (Piatcza et al., 2015; Bhalang et al., 2018). Similarly, no variation appeared in genetic variation using RAPD has been reported in date palm plants derived from tissue culture (Al-Mayahi, 2022a, and 2022 b). A successful propagated in vitro method should give true-to-type plantlets without any genetic or morphological variation (Prakash et al. 2016; Safarpour et al., 2017; Khattab and Yusuf, 2018). It has been previously reported that micro-propagated plants maintain genomic stability (Borsai et al., 2020).

Conclusion

To the best of our knowledge, this is the first report on the efficacy of dicamba (DIC) and casein hydrolysate (CH) together on multiple-shoot developments of *in vitro* cultivated date palms. The present study demonstrates that DIC alone or in combination with CH plays a synergistic role in improving callus induction, differentiation, and multiplication of shoots. Adding 4.0 mg l^{-1} DIC to the medium equipped with 1.0 and 0.5 g l^{-1} CH was the best combination to promote the growth of date palm cv. Barhee as it resulted in the highest callus weight, response rate, and number of shoots. The macronutrient K, P, Ca, free amino acids, and IAA content significantly increased in the *in vitro* shoots regenerated on the media supplemented with 4.0 mg l^{-1} DIC + 1.0 g l^{-1} CH. Observations showed that combined use between 4.0 mg l^{-1} DIC and 0.5 or 1.0 g l^{-1} CH eliminated the browning. Furthermore, RAPD-PCR of DNA from treatments with DIC and CH showed genetic similarity among tissue culture-derived plants. The *in vitro* propagation protocol developed in this study could be applied for the large-scale production of genetically stable date palm cv. Barhee.

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