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EFFECT OF ANCYMIDOL AND PHLOROGLUCINOL ON THE NUMBER AND THE QUALITY OF SHOOTS IN THE MICROPROPAGATION OF DATE PALM (*PHOENIX DACTYLIFERA* L.)

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ABSTRACT

The disadvantages of practical date palm micropropagation are tissue browning, low callus proliferation rate, low multiplication efficiency, and vitrification. The aim of the study was to determine the effect of ancymidol (Ancy) and phloroglucinol (PG) on the growth and some biochemical components of the 'Barhee' date palm cultured *in vitro*. The combination of 0.75 mg·l^{-1} Ancy and 50 mg·l^{-1} PG was found to be the most effective in terms of callus regeneration rate (89%) and number of shoots (14.3). A reduction in browning was observed in tissues cultured on media supplemented with 0.75 mg·l^{-1} Ancy in combination with 25 or 50 mg·l^{-1} PG. The medium supplemented with 0.75 mg·l^{-1} Ancy and 50 mg·l^{-1} PG eliminated shoot vitrification. Effective micropropagation was associated with increased carbohydrate and protein content. In this study, the genetic stability of plants obtained by micropropagation was confirmed by DNA-based RAPD fingerprinting. The results may indicate that the micropropagation protocol used in this study was suitable and applicable to the production of genetically stable date palm plants on a mass scale.

Key words: callus browning, callus induction, endogenous hormones, genetic stability, RAPD, shoot vitrification

INTRODUCTION

The date palm (*Phoenix dactylifera* L.), belonging to the Arecaceae family, is a monocotyledonous, dioecious, perennial tree of great ecological, social, and economic importance. Date fruits contain many chemical components of high nutritional and medicinal value. Date palms can be propagated by seeds or vegetatively by offshoots; however, these methods are ineffective for large-scale propagation, making micropropagation the sole practical approach. Monocotyledonous shoot meristems are of basal origin, and stems lacking cambium tissue are particularly culture-responsive (Jasim et al. 2009). Tissue culture technology can produce the maximum possible number of plants in a limited time and space that are true to type and agronomically equal to or superior to conventionally propagated plants (Al-Mayahi 2022a, b; Al-Mayahi 2023). In plant tissue culture experiments, there is a constant need to search for new compounds whose addition to the culture medium can lead to better or more efficient *in vitro* growth (Al-Mayahi 2021).

Growth regulators that inhibit the biosynthesis of gibberellins are known as growth retardants or gibberellin biosynthesis inhibitors. For plant scientists, growth retardants, with their typical and additional biochemical effects, are valuable tools for better understanding the purposes of their action. Some of them, ancymidol, flurprimidol, and paclobutrazol, have an N-containing heterocycle. Ancymidol [α-cyclopropyl-α-(4-methoxyphenyl)-5-pyridinemethanol] is a pyrimidine analog. Its action blocks the monooxygenase enzyme that catalyzes the oxidation of *ent*-kaurene, an essential step in the pathway between *ent*-kaurene and gibberellins (Rademacher 2016). Ancymidol has a strong effect on various developmental processes, e.g., shoot proliferation, promotion of chlorophyll accumulation, and increased levels of endogenous cytokines (Rajiv et al. 2018).

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Phloroglucinol (1,3,5-trihydroxybenzene) is a phenolic compound and is applied in combination with growth regulators due to its activating effect on cell division, differentiation, and proliferation (Aremu et al. 2015; Londe et al. 2017). It also mitigates laboratory-induced physiological disorders such as vitrification and tissue browning (Naidoo et al. 2017). One of the main goals of micropropagation technology is to ensure the genetic fidelity of regenerated plants, which is necessary for commercial production. Molecular markers have shown promise in fingerprinting and genetic testing (Al-Mayahi 2022c). Additionally, they can be used for quality control in plant tissue culture at various stages of growth. Among the various molecular markers used to estimate the genetic fidelity of *in vitro* propagated plants, RAPD is one of the easiest, fastest, and most cost-effective methods and requires small amounts of DNA (Chaudhary et al. 2022). Despite its many advantages, *in vitro* propagation of date palm still faces many obstacles. However, no study has assessed the effects of ancymidol and phloroglucinol on *in vitro* propagation of date palms and genetic fidelity. Therefore, in the present study, the effect of ancymidol at different concentrations on its possible use in combination with phloroglucinol in tissue culture of the 'Barhee' date palm was examined.

MATERIALS AND METHODS

Both experiments in this study were conducted in the tissue culture laboratory of the Date Palm Research Centre at the University of Basrah, Iraq.

Initiation stage

Three- to four-year-old 'Barhee' date palm offshoots were separated from healthy mother plants grown in the Abu al-Khasib area south of Basrah. The outer leaves and fibrous tissues at their base were gradually removed until the shoot tip zone was exposed. Shoot tips were excised from the offshoots, trimmed to 0.8–1.0 cm wide and 1–2 cm long, and used as initial explants in this study. Explants were kept in antioxidant solution (100 $mg \cdot l^{-1}$ ascorbic acid and 150 $mg \cdot l^{-1}$ citric acid), sterilized with 70% ethanol for 1 min and 5% sodium hypochlorite for 20 min, and then rinsed three times with sterile distilled water. To stimulate callus induction, explants were cultured on MS basal medium (Murashige & Skoog 1962) supplemented with 100 $mg \cdot l^{-1}$ glutamine, 5 $mg \cdot l^{-1}$ thiamine HCl, 1 $mg \cdot l^{-1}$ biotin, 30 $g \cdot l^{-1}$ sucrose, and 2 $g \cdot l^{-1}$ activated charcoal, and then solidified with agar at a concentration of 6.0 $g \cdot l^{-1}$, with the addition of growth regulators: naphthaleneacetic acid (NAA) at a concentration of 30 $mg \cdot l^{-1}$ and 6-(γ,γ-dimethylallylamino)purine (2iP) at a concentration of 3 $mg \cdot l^{-1}$ (Al-Mayahi 2024). Cultures were kept in complete darkness at a temperature of 27 ± 2 °C. Cultures were transferred to fresh media of the same composition every six weeks until callus induction. Two experiments were conducted.

Effect of ancymidol and phloroglucinol on callus growth (experiment 1)

Ancymidol (Ancy) and phloroglucinol (PG) supplementation at different concentrations in the growth medium was evaluated to study their effects on callus growth. Treatments consisted of 8 media types as shown in Table 1. The pH of the medium was adjusted to 5.7 with 0.1 N NaOH or HCl before adding the agar. The media were distributed into culture containers and autoclaved for 20 min at 121 °C and 1.04 $kg \cdot cm^{-2}$. The induced callus was separated from the apical buds, weighed, and cultured (100 mg) in 340 ml glass jars containing 50 ml of medium supplemented with the growth regulators – NAA at 6 $mg \cdot l^{-1}$ and 2iP at 2 $mg \cdot l^{-1}$. After inoculation of callus into the medium, the cultures were incubated directly at a temperature of 27 ± 2 °C. Photoperiod (2000 lux) of 16 hours using cool white fluorescent tubes. The callus weight was measured eight weeks after from the initiation of callus culture.

Table 1. Treatments used in the study

	Treatment ($mg \cdot l^{-1}$)
T1	0.0
T2	0.75 Ancy
T3	1.5 Ancy
T4	3.0 Ancy
T5	25.0 PG
T6	50.0 PG
T7	0.75 Ancy + 25.0 PG
T8	0.75 Ancy + 50.0 PG

Ancy – ancymidol, PG – phloroglucinol

Effect of ancymidol and phloroglucinol on indirect organogenesis (experiment 2)

Callus cultures were transferred to modified MS medium fortified with the same composition mentioned above, but with a different combination of growth regulators: 1 $mg \cdot l^{-1}$ naphthaleneacetic acid (NAA), 0.5 $mg \cdot l^{-1}$ 6-benzyl adenine (BA), and 0.5 $mg \cdot l^{-1}$ kinetin (K), suitable for shoot formation. It was supplemented with the same concentrations of ancymidol and phloroglucinol to investigate their effects on shoots regeneration, multiplication, and some physicochemical parameters in *in vitro* cultured tissues. Cultures were incubated at 27 ± 2 °C and irradiated for 16 h with diffused light provided by daylight fluorescent lamps. There were 18 replicates of each treatment. The percentage of bud regeneration and the number of buds per jar were recorded 12 weeks after inoculation of the callus onto the medium.

Vitrification and browning

Vitrification was scored according to Ibrahim et al. (2016) (Table 2). Browning was assessed by counting the number of brown calluses and shoots relative to the total number of cultures.

Table 2. Vitrification evaluation

Scoring	Vitrification degree
-	no shoot vitrification
+	below moderate shoot vitrification
++	moderate shoot vitrification
+++	above moderate shoot vitrification
++++	severe shoot vitrification

Physiological and biochemical parameters

Physiological and biochemical analyses were performed at the shoot stage. Eighteen replicates of each analysis were performed.

Total soluble carbohydrates

The anthrone technique adopted by Watanabe et al. (2000) was used to determine the total carbohydrate content in the form of glucose.

Total soluble protein

Total soluble protein was determined by the procedure of Bradford (1976).

Endogenous IAA content

Auxins were extracted and quantified according to Nagar and Sood (2006). Five grams of tissue from all treatments were homogenized using 80% methanol. The extract was filtered through Whatman filter paper (no. 1) and evaporated under vacuum in the dark at 4 °C. The supernatant was withdrawn by 0.1 M potassium phosphate (pH 8.1). The eluate was obtained using 1 N hydrochloric acid (HCI) and by partitioning (4×) with diethyl ether, in dryness, in water at pH 2.5. Injection into reverse HPLC, C18 column, in isocratic elution mode through concentrate determined phytohormones using a portable acetone step (26 : 74) with 30 mM of phosphoric acids. A UV detector (2996 PDA detector) with 280 nm was passed through the column eluents and auxins (IAA) was characterized and quantified. An auxin standard was used as a reference.

Genetic stability among regenerated date palm seedlings

To study genetic similarities, several regenerated seedlings were analyzed at the molecular level using RAPD analysis. Total genomic deoxyribonucleic acid (DNA) was separated from propagated date palm seedlings using the CTAB technique described in Rogers and Bendich (1985). Polymerase chain reactions (PCR) were performed using a set of three arbitrary 4-mer primers (Operon Technology, USA) (Table 3).

Statistical data analysis

The experiments were conducted in a completely randomized design (CRD). All recorded data were subjected to analysis of variance (ANOVA) using SPSS version 18.0, and significant differences between treatments were assessed by the LSD test at $p \leq 0.05$.

Table 3. RAPD sequences used to assess genetic fidelity

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

RESULTS

Effect of ancymidol and phloroglucinol on callus growth and shoot regeneration

Ancymidol (Ancy) and phloroglucinol (PG) in the media enhanced the value of the parameters compared to the control, except for Ancy 3.0 mg·l^{-1}. Ancy at a concentration of 3.0 mg·l^{-1} prevented callus growth, wheras at a concentrations of 1.5 and 0.75 mg·l^{-1} callus growth was stimulated, which after subsequent transplanting to a new medium resulted in high bud induction (Fig. 1–2, Table 4) after 6 and 12 weeks. Cultures grown in medium containing 50 mg·l^{-1} PG resulted in 38% heavier callus, 48% greater shoot regeneration and almost doubled shoot regeneration compared to 25 mg·l^{-1} PG. The combination of Ancy and PG additionally influenced growth parameters. The medium of 0.75 mg·l^{-1} Ancy and 50 mg·l^{-1} PG proved to be the most optimal and gave the highest callus weight, almost 90% shoot regeneration, and a number of shoots from one explant higher by 36% (14.3 pcs.) than in the case of treatment with Ancy 0.75 mg·l^{-1} alone after 6 and 12 weeks on the respective medium.

Vitrification percentage and browning

The highest vitrification rate was recorded in the control. Ancy and PG reduced vitrification, and in medium containing 0.75 mg·l^{-1} Ancy + 50 mg·l^{-1} PG vitrification was eliminated (Table 5). More than 33% of the explants in the control turned brown (Fig. 1). Ancy at a concentration of 0.75 mg·l^{-1} and PG at a concentration of 50 mg·l^{-1} reduced browning to 6%, while the combination of Ancy and PG eliminated this phenomenon (Table 5).

Table 4. Effect of ancymidol (Ancy) and phloroglucinol (PG) on the growth of 'Barhee' date palm *in vitro*

Treatment		Callus weight (mg)	Shoot regeneration percentage	Shoot number
T1	0.0	119.0f ± 7.2	33.3e ± 4.1	4.9d ± 0.4
T2	0.75 Ancy	179.0c ± 9.4	66.7c ± 3.9	10.5b ± 0.9
T3	1.5 Ancy	147.0de ± 7.5	55.6d ± 3.1	7.1c ± 0.2
T4	3.0 Ancy	100.0g ± 7.2	27.8f ± 4.1	3.6e ± 0.5
T5	25.0 PG	128.0ef ± 7.3	44.5e ± 3.1	5.8d ± 0.8
T6	50.0 PG	194.0bc ± 7.5	72.2bc ± 6.4	10.9b ± 0.8
T7	0.75 Ancy + 25.0 PG	219.0b ± 7.3	77.8b ± 6.4	11.1b ± 0.8
T8	0.75 Ancy + 50.0 PG	271.0a ± 9.2	88.9a ± 6.4	14.3a ± 0.4

± standard error (n = 18); Values followed by the same letter are not significantly different (P < 0.05)

Table 5. Effect of ancymidol (Ancy) and phloroglucinol (PG) on *in vitro* shoot vitrification and shoot browning of 'Barhee' date palm

Treatment		Vitrification*	Browning percentage (%)
T1	0.0	+++	33.3 ± 4.0
T2	0.75 Ancy	+	5.6 ± 0.2
T3	1.5 Ancy	+	16.7 ± 1.9
T4	3.0 Ancy	++	27.8 ± 3.3
T5	25.0 PG	++	16.7 ± 1.9
T6	50.0 PG	+	5.6 ± 0.2
T7	0.75 Ancy + 25.0 PG	+	0.0 ± 0.0
T8	0.75 Ancy + 50.0 PG	-	0.0 ± 0.0

*(n = 18)

Note: see shoot vitrification key in Table 2

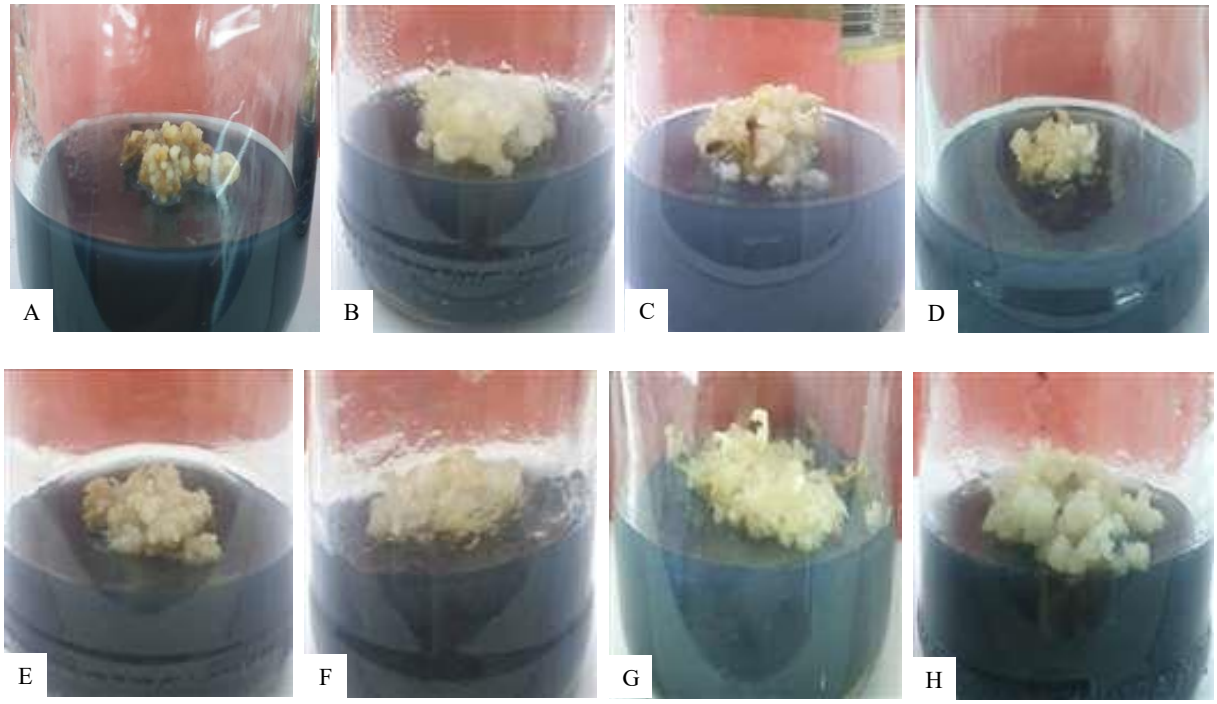

Figure 1. Callus induction on MS medium with control (A), 0.75 mg·l^{-1} Ancy (B), 1.5 mg·l^{-1} Ancy (C), 3.0 mg·l^{-1} Ancy (D), 25 mg·l^{-1} PG (E), 50 mg·l^{-1} PG (F), 0.75 mg·l^{-1} Ancy + 25 mg·l^{-1} PG (G), 0.75 mg·l^{-1} Ancy + 50 mg·l^{-1} PG (H); magnification 0.8×
Note: see Table 1

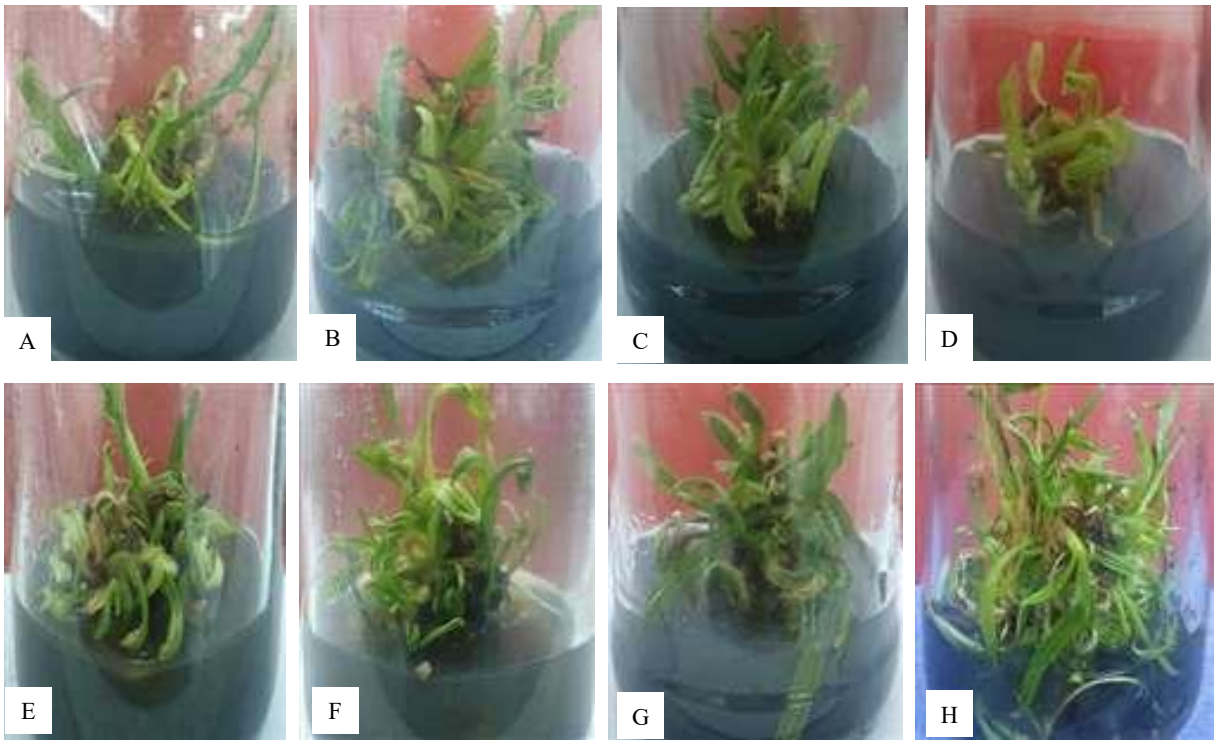

Figure 2. Bud induction on MS medium with control (A), 0.75 mg·l^{-1} Ancy (B), 1.5 mg·l^{-1} Ancy (C), 3.0 mg·l^{-1} Ancy (D), 25 mg·l^{-1} PG (E), 50 mg·l^{-1} PG (F), 0.75 mg·l^{-1} Ancy + 25 mg·l^{-1} PG (G), 0.75 mg·l^{-1} Ancy + 50 mg·l^{-1} PG (H) ; magnification 0.8×
Note: see Table 1

Effect of ancymidol and phloroglucinol on biochemical parameters carbohydrate content

Ancymidol and, to a lesser extent, phloroglucinol at a concentration of 50 mg·l^{-1} led to the accumulation of carbohydrates in date palm microshoots. This effect increased with the concentration but was not additive (Fig. 3A).

Protein content

Ancy and PG increased the protein content (%) compared with control (Fig. 3B). Ancy at a concentration of 0.75 mg·l^{-1} and 1.5 mg·l^{-1} doubled the protein concentration compared with control. PG was less effective but the combination of Ancy and PG almost tripled the protein content.

Endogenous IAA content

The addition of Ancy or PG had no effect on the content of IAA in microshoots (Fig. 4).

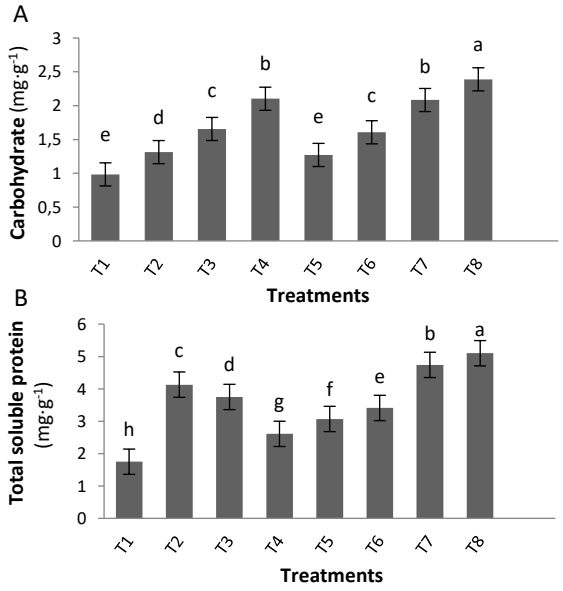

Figure 3. Effect of ancymidol and phloroglucinol on total soluble carbohydrates (A), total soluble protein in 'Barhee' date palm (B)
Note: see Table 1

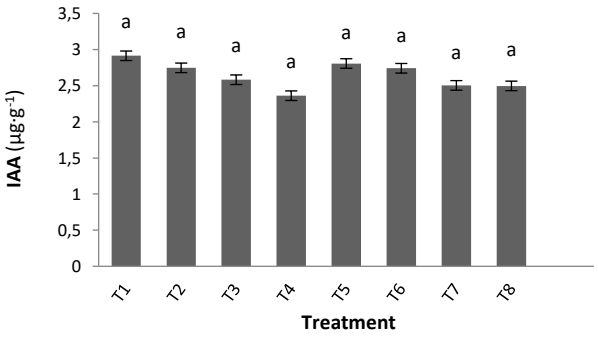

Figure 4. Effect of ancymidol and phloroglucinol on endogenous indole acetic acid (IAA) content (n = 15)
Note: see Table 1

RAPD analysis

PCR amplification showed no differences in the bands generated, suggesting genetic homogeneity of the *in vitro* propagation material, regardless of the medium composition.

DISCUSSION

Commercial *in vitro* propagation of date palm encounters certain limitations (Al-Mayahi et al. 2010; Awad et al. 2020; Al-Asadi et al. 2019). Our results indicated that the addition of ancymidol in combination with phloroglucinol had a beneficial effect on most of the growth parameters studied compared to the control or individual applications of these compounds. Ancy, as a final effect, increased the total number of shoots produced. The greatest increase was observed at a concentration of 0.75 mg·l^{-1} Ancy. One possible role of Ancy may be the disruption of apical dominance, which may be due to reduced levels of endogenous auxin production, as reported by Rajiv et al. (2018) in *Nerium oleander* (L.) Ancy has been shown to affect various plant growth hormones (Hofmannová et al. 2008). Ancy increased the level of carbohydrates, the concentration of which in the centers of meristematic tissue preceded bud regeneration. Similar results were obtained by Maki et al. (2005), who suggested that the addition of Ancy to the culture medium significantly augmented *Hosta* shoot induction.

Of the PG concentrations used in this study, 50 mg·l^{-1} was most helpful for organogenesis, compared with 25 mg·l^{-1}. PG is a simple phenolic characterized by 3 oxidryl groups and is the basic structure of many bioactive molecules that occur naturally in microorganisms, animals, and plants (Singh et al. 2010). PG acts as a hormone, stimulating callus induction and shoot organogenesis. The effect of PG on shoot regeneration may also be due to its effect in reducing vitrification of buds and shoots. The positive effect of PG on growth stimulation and bud regeneration has been observed in many plant species (Teixeira da Silva et al. 2013; Aremu et al. 2015; Londe et al. 2017; Al-Asadi et al. 2020; Palaz et al. 2020).

Micropropagation of many plant species faces serious problems due to abnormal shoot morphology, e.g., vitrification symptoms (overwatering), which reduce shoot proliferation and vigor and may

hamper the successful transfer of micropropagated plants to *ex vitro* conditions (Salem 2016; Hassanein et al. 2018). Vitrification also reduces cell division and leads to abnormal lignification of cell walls (Kevers et al. 2004).

Vitrification is a common physiological disorder in shoot formation under tissue culture conditions (Al-Mayahi & Ali 2021; Polivanova & Bedarev 2022). The main factors influencing the rate of *in vitro* vitrification of plants are the mineral and hormonal composition of the medium and the physical culture conditions. Chen & Ziv (2004), Sarkar et al. (2001); Maki (2005); Thakur (2006) reported that Ancy had significant effects on reducing vitrification and increasing shoot multiplication in narcissus, potato, *Hosta*, and *Lilium* cultures. Several studies have shown that PG in combination with other hormones reduces plant vitrification (Chandrika et al. 2015; Vélez-Mora et al. 2015; Chaudhary et al. 2022). As an essential phenolic compound, PG is thought to play a role in the cell wall and is therefore also a potential factor in tissue culture in reducing vitrification phenomena. Vitrification in plants is associated with reduced production of enzymes responsible for lignin production. Since PG enhances the lignin biosynthetic pathway, it effectively controls vitrification and improves the rate of shoot proliferation (Gururaj et al. 2004). Therefore, adding PG to the medium can solve vitrification problem by increasing enzyme production and thus increasing lignin synthesis in plants (Londe et al. 2017; Teixeira da Silva et al. 2019). The results of our study are consistent with those of Naidoo et al. (2017), who reported that PG supplemented medium reduced vitrification disturbance.

Browning of medium and tissue culture explants can be a serious problem hindering successful *in vitro* propagation, mainly of woody and perennial plants. Many attempts have been made to control the browning problem in these species (Al-Asadi et al. 2024). The effectiveness of these methods, however, varies and depends on the physiological conditions of the plant.

Addition of PG to the culture medium reduced tissue browning in *Ficus carica* leaf slices. PG increased the rate of morphogenesis and provided high survival rate (Kim et al. 2007; Chaudhary et al. 2022).

Various physiological changes were observed in the cultured tissues. The presence of carbohydrates is a necessary physiological adaptation to new conditions, contributing to the osmotic regulation of the plant cell and continuing its growth and metabolism (Manokari et al. 2021). Carbohydrate biosynthesis and metabolism are essential aspects of plant growth and reproduction and support energy for growth and development A potential role for Ancy may be to simultaneously disrupt apical dominance and alter carbohydrate metabolism. It is known that soluble sugars can affect leaf formation (Gibson 2005). Marković et al. (2020) confirmed that changes in carbohydrate content were associated with axillary bud regeneration and meristematic differentiation. Le Guen-Le Saos et al. (2002) showed that adding Ancy to the regeneration medium increases carbohydrate accumulation. According to Aremu et al. (2015) and Naidoo et al. (2017), the addition of PG to the culture medium can improve the quality and physiological condition of *in vitro* propagated shallots. Plant growth retardants have been reported to affect protein biosynthesis and cell division and enlargement, possibly by altering protein biosynthesis and membrane permeability (Chen & Ziv 2001). Our results also indicate an association between Ancy and protein and carbohydrate.

The possible role of Ancy action may be due to reduced levels of endogenous auxin production and thus shoot regeneration as reported by Rajiv et al. (2018) in *Nerium oleander* (L.).

Molecular techniques such as RAPD are useful in detecting somaclonal variants, which are often stimulated by chemical and physical factors *in vitro* (Bidabadi et al. 2010). RAPD technology has attracted the attention of many researchers in this field (Piątczak et al. 2015; Bhalang et al. 2018).

RAPD analysis of micropropagated cultures of 'Barhee' date palm showed that Ancy at all concentrations added alone and in combination with PG did not induce any detectable genetic variation in genomic DNA. It should be noted, however, that only three primer pairs were used in our study. Similarly, RAPD analysis did not detect any genetic variation in potato cultures stored in an Ancy-containing medium for 16 months. Ancymidol did not cause detectable genetic variation as assayed by RAPD in tissue culture potato microplants (Sarkar et al. 2001).

CONCLUSION

The present study demonstrates for the first time that ancymidol and phloroglucinol are important factors regulating callus growth and shoot regeneration rate in date palm micropropagation, as well as reducing the level of physiological disorders such as vitrification and browning. The most effective addition for the propagation of the 'Barhee' date palm was 0.75 $mg \cdot l^{-1}$ Ancy + 50 $mg \cdot l^{-1}$ PG. This study provides valuable results for practical micropropagation of date palm.

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