Arab Universities Journal of Agricultural Sciences
(2024) 32 (2) 173-182
Website: http://ajs.journals.ekb.eg

Optimizing $CuSO_4$ and $CoCl_2$ for Superior Somatic Embryos Regeneration of Date Palm

Sara M Abdelmotagaly[1]*, Yasmin M R Abdellatif[1], Amal FM Zein El Din[2], Ola H Abdelbar[1], Ibrahim S Ibrahim[1]

1- Agricultural Botany Dept, Fac of Agric, Ain Shams Univ, P.O. Box 68, Hadayek Shubra 11241, Cairo, Egypt

2- The Central Laboratory for Date Palm Researches and Development, Agricultural Research Center, Giza 12619, Egypt

*Corresponding author: saraabdelmotagaly@agr.asu.edu.eg.

https://doi.org/10.21608/AJS.2024.270961.1557

Received 21 February 2024; Accepted 1 July 2024

Keywords:

Antioxidant enzymes, Biochemical components, *Phoenix dactylifera* L., Somatic embryogenesis

Abstract: The purpose of the research was to improve the morpho-anatomical and physiological features of somatic embryos (SEs) of date palms in tissue culture media that have the potential to produce green and healthy plantlets. Copper sulfate ($CuSO_4$) and cobalt chloride ($CoCl_2$) were supplemented to date palm maturation medium at various concentrations, separately or combined. Adding $CoCl_2$ to the media of maturation produced the greatest relative growth and differentiation percentages of date palm embryogenic calli, particularly at 0.52 mgL^{-1}. In comparison to the control, when combined treatments were administered alone, the quantity of the produced somatic embryos rose dramatically (17/jar). Total sugars and free amino acids were the highest in SEs grown on a maturation medium with $CoCl_2$ at 0.26 mgL^{-1}. Moreover, total phenols and indoles were picked significantly at their higher levels in the generated SEs that were cultured on a medium enhanced with 0.52 mgL^{-1}. When the maturation medium was administered along with both as a treatment, the antioxidant enzyme, ascorbate peroxidase, reached its maximum activity. Whereas, adding 0.52 mgL^{-1} of $CoCl_2$ to the culture medium enhanced the activities up to of highest levels of ascorbate peroxidase, polyphenol oxidase and phenylalanine ammonia-lyase.

1 Introduction

Date palm (*Phoenix dactylifera L.*) is a tree that has been cultivated for its sweet and edible fruit for a long time. Around the Middle East dry areas, the date palm has been cultured not only for its profitable fruits but also for fiber, fuel and ground crop shelter, putting it among the most important resources of industry (El Hadrami and Al-Khayri 2012). Its fruit is nourishing and obtains extreme amounts of fiber, vitamins, minerals, and carbs (Gaviria et al 2021). There are two approaches that exist for propagation, seeds for sexual propagation and offshoots for vegetative propagation (Al-Khalifah and Shanavaskhan 2012). *In vitro*

micropropagation, either somatic embryogenesis or organogenesis, are effective strategies to solve propagation challenges (Al-Khayri 2003, Mujib et al 2004, Bhattacharjee 2006). Somatic embryogenesis may be preferred over other techniques because of its high capacity for multiplication (Fki et al 2003).

One of the main variables influencing the speed of regeneration in date palms is the quality of the callus. According to reports, one of the important aspects of enhancing date palm embryogenic callus quality is the *in vitro* medium's composition (Al-Najm et al 2018).

It has recently been known that several heavy metals, acting as microelements, are crucial for plant tissue cultures to regenerate. According to Hussain et al (2010), metals such as iron, cobalt, zinc and copper are necessary for plant survival but only at very minute levels while at greater concentrations, they become poisonous. Using microelements improved the normality of callus formed on *in vitro* date palms. Plants require the microelement copper to promote healthy development and growth. It conducts crucial physiological and biochemical activities in plant species. Furthermore, copper acts as a co-enzyme, mostly for enzymes involved in electron transport, where it catalyzes the redox processes in the cytoplasm, mitochondria, cell walls and chloroplasts of plant cells (Marschner 2012). Studies on grains such as wheat and barley conducted by Nirwan and Kothari (2003) have demonstrated that increasing copper significantly enhances regeneration. According to Ghaemi et al (1994), making embryos synthetically from wheat anther cultures was dramatically increased when $CuSO_4$ (40 μM) was added to the medium. Still, there was little research done on the consequences of various formulations of these nutrients in tissue culture medium, and no obvious ideal amounts of Cu were identified.

A heavy metal that is thought to hinder the formation of ethylene is cobalt (Santana-Buzzy et al 2006). AL-Mayahi (2014) mentioned that the medium treated with combined cobalt chloride and copper sulfate at 2 μM had a greater rate of date palm callus growth cv. Ashgar ($p<0.05$) and obtained 7.12 shoots/explant. The most effective addition to the cooked medium was the combination of these two salts in increasing the frequency of regenerations and the number of shoots produced per each explant, moreover, the ability to regenerate shoots from callus. The goal of this study is to ascertain the influence of $CuSO_4$ and $CoCl_2$ on specific morphological criteria, anatomical characters of somatic embryos generated *in vitro*, and examine the alterations in their biochemical parameters (total sugars, free amino acids, phenols, and indoles). Additionally, this study assesses the effects of adding extra maturation medium with various concentrations of cobalt chloride and copper sulfate that enhances the ascorbate peroxidase, polyphenol oxidase and phenylalanine ammonia-lyase activities as well as improves the quality of date palm somatic embryos of Barhee cv.

2 Materials and Methods

The current study was carried out from 2019 to 2023 in the Central Laboratory for Date Palm Researches and Development, Agricultural Research Center (Tissue Culture Lab.), Giza, Egypt and in the Agricultural Botany Dept., Faculty of Agriculture, Ain Shams University, Cairo, Egypt.

2.1 *In vitro* Date palm

The standard procedure for date palm shoots apical dome explants (*Phoenix dactylifera* L.) callogenesis was established as described by Zein El Din and Ibrahim (2015). The nutrient medium for callus induction was Murashige and Skoog (1962) basic medium was fortified by 2,4-dichlorophenoxyacetic acid (2,4-D) at 10 mgL^{-1} plus isopentenyladenine (2ip) at 3 mgL^{-1}. The prepared cultures were incubated for 6 to 8 months at 27 °C ± 2 in a total darkness-controlled growth room, with sub-cultures being made 6 weeks intervals on similar freshly prepared medium. Afterward, friable calli were cultivated for two subcultures of six weeks each on MS media enhanced with 0.1 mgL^{-1} naphthalene acetic acid (NAA) to improve their viability for embryogenesis.

2.2 Examine the impact of $CuSO_4$ and $CoCl_2$ on somatic embryogenesis

The friable embryogenic calli **(Fig 1A)** were transferred into MS maturation media enhanced with 0.5 mgL^{-1} abscisic acid and 5 g l^{-1} polyethylene glycol (PEG-4000). During the current experiment, copper sulfate ($CuSO_4$) and cobalt chloride ($CoCl_2$) were separately added into the maturation medium at concentrations of (0.08, 0.32, 0.64 mgL^{-1}) and (0.065, 0.26, and 0.52 mgL^{-1}), respectively, these concentrations were used according to AL-Mayahi (2014). The prior treatments were compared with basal MS medium ($CuSO_4.5H_2O$ and $CoCl_2.6H_2O$ both at 0.025 mgL^{-1}. Additionally, the impact of supplying a combination of $CuSO_4$ and $CoCl_2$ at 0.32 and 0.26 mgL^{-1}, respectively were examined. In total darkness, incubate all of the

embryogenic callus cultures at 27°C±2 for eight weeks in a controlled growth room. After the maturation period, it was observed that there was asynchronous somatic embryogenesis. The fully developed somatic embryos were harvested for morphological, anatomical and biochemical analyses.

2.2.1 Morphological observations

The morphological data recorded during the experiments were relative growth percentage (%) and differentiation percentage (%) of completely formed somatic embryos and their number. The formula used to determine the relative growth percentage was (RG%): RG% = [(Wn - W0)/W0] × 100, according to Santoso and Thornburg (1992). Where, Wn is the callus' mass, after the period of incubation and W0, is the callus' mass at the beginning of the experiment. Observations and photomicrographs were recorded using LEICA stereomicroscope model EZ4D.

2.2.2 Anatomical studies

Samples of developed embryos from different treatments were kept for 24 hours in FAA (50% ethyl alcohol added to formalin and acetic acid by volume 90:5:5, identified as killed and fixed solution. The samples have been embedded in paraffin wax after being dried with ethanol, as stated by Abdelbar (2017). Using a rotary microtome LEICA type RM (2125) RTS, longitudinal slices with a thickness of 8 µm were created. Sections were stained using a mixture of Fast Green and Safranin. Using a digital camera and a LEICA light microscope model DM-500, observations and photomicrographs were recorded.

2.2.3 Biochemical analyses

Chemical analyses were conducted for fully developed embryos eight weeks following the start of enhancement trials on 0.4 g of fresh weight (FW). Total sugars were purposed using 3,5-dinitro salicylic acid (DNS) in accordance with Miller's (1959) methodology; glucose was used as standard. Extraction of free amino acids was estimated in accordance with Ackerson (1981), and evaluated spectrophotometrically according to Jayaraman (1981) using a ninhydrin solution; glycine was utilized as standard. The Folin-Ciocalteu colorimetric technique was used to determine the concentration of phenolic compounds and expressed in milligrams of gallic acid (Shahidi and Naczk 1995). Salkowski reagent was used to extract and measure indole acetic acid using Shindy and Smith's (1975) technique. Leaf samples were combined with a 100 mM potassium phosphate buffer (pH = 7.0) supplemented with polyvinyl pyrrolidone at 1% (W/V) and 0.1 mM EDTA at 4°C. The supernatants were regarded as crude extracts of enzymes and were used to quantify the activity of both ascorbate peroxidase (APX), polyphenol oxidase (PPO) and phenylalanine ammonia-lyase (PAL). Furthermore, the amount of soluble proteins in the crude enzyme extract was measured using Bradford's (1976) method for determining the specific activity of enzymes. Bovine serum albumin was utilized as a reference to measure the protein concentration.

The specific activity of APX (EC 1.11.1.11) in enzyme crude extract was determined at 470 nm per minute using Nakano and Asada's (1981) technique. An enzyme's unit is equal to 0.01 ΔOD min^{-1} and expressed as IU/mg protein.

The specific activity of PPO (EC 1.14.18.1) was assessed as reported by Oktay et al (1995). Increasing the absorbance by 0.001 each minute at 420 nm is regarded as a unit of PPO activity. The unit mg^{-1} protein of the enzyme activity was reported.

The specific activity of PAL (EC 4.3.1.5) was measured using the Lister et al (1996) method. An increase in absorbance by 0.01 each hour at 290 nm was defined as an enzyme activity unit. Unit mg^{-1} protein was used to express the enzyme activity. All biochemical determinations were applied by a UV–-Vis spectrophotometer, UV 9100 B, LabTec.

2.3 Statistical analysis

The one-way ANOVA method was employed using SAS (2020) software, using Duncan's multiple range test of Duncan (1955). The significance of the differences between the mean values for the treatments was examined at the 5% level (Snedecor and Cochran 1990).

3 Results and Discussion

3.1 Morphological observations

Both individual embryos and clusters of differentiated embryos in synchronized stages were formed. Data **(Table 1)** presented that date palm somatic embryos produced in media supplemented with either $CuSO_4$ or $CoCl_2$ characterized by high growth efficiency in all determined morphological parameters compared to the control medium as shown in **(Fig 1 B, C)**. Relative growth and differentiation percentage of embryogenic calli as well as the number of SEs in each jar increased

Table 1. Impact of different concentrations of ($CuSO_4$) and ($CoCl_2$) on relative growth %, differentiation % of embryogenic calli and the number of somatic embryos/ jar that regenerate on maturation medium for one subculture (8 weeks) of date palm cv. Barhee

Treatment	Conc. (mgL^{-1})	Relative growth% /Jar	Differentiation%	No. embryos/ Jar
Control	-	117^{f}±28.87	37^{f}±2.52	4^{f}±1
$CuSO_4$	0.08	283^{e}±28.87	56^{e}±5.13	7^{ef}±0.58
	0.32	400^{d}±50	64^{cd}±3.61	9^{de}±0.58
	0.64	433^{cd}±28.87	72^{b}±2.89	13^{bc}±1.53
$CoCl_2$	0.065	500^{bc}±50	61^{de}±3.21	$7d^{ef}$±1
	0.26	517^{b}±28.87	74^{ab}±3.61	10^{cd}±1
	0.52	600^{a}±50	80^{a}±5	14^{ab}±3.21
$CuSO_4$ +$CoCl_2$	0.32 + 0.26	450^{bcd}±50	69^{bc}±4.04	17^{a}±2.52

* Means within a column with different superscript letters differ significantly (P<0.05). SD: standard deviation

gradually with increasing the concentrations of $CuSO_4$ or $CoCl_2$. As Al-Mayahi (2014) noted, plants require the microelement copper to grow and develop properly. It conducts essential physiological and biochemical tasks in plant species. It participates in the processes of respiration, glucose transportation, and nitrogen compound conversion. The highest increment in relative growth was recorded when $CoCl_2$ at different concentrations was added individually in the media (500, 517 and 600%). This result agrees with Hu et al (2021), who reported that there are two advantages to using cobalt in plant growth medium, its ability to prevent oxidative processes caused by copper and iron ions, as well as its protection against metal chelate toxicity. Conversely, the medium treated with a mixture of $CuSO_4$ and $CoCl_2$ showed the greatest number of SEs, reaching 17 embryos/jar as shown in **(Fig 1F)**. Copper and cobalt are micronutrients that cells need to function normally. Energy is required for the process of callus multiplying. As a result, cells often respire more quickly to provide the energy needed for cell division and callus formation. Plants require micronutrients such as copper, zinc, iron and manganese, as they are vital for vital metabolism. Plants have a narrow range of normal amounts for each of these metals; while both excess and shortage of these elements lead to negative physiological effects. The coordinated actions of the transporters that facilitate cell import, correct distribution and utilization of metalloenzymes inside the cell are necessary to maintain a sufficient amount of metals acting as redox-active in the plant (Rai et al 2021). Cobalt is a necessary transition metal of numerous enzymes and coenzymes. The amount of cobalt present in the surrounding media determines the impact on plant development and metabolism (Palit et al 1994).

Histological observations of both individual embryos and clusters illustrated a normal distinct embryonic axis surrounded by the cotyledon. The procambial strand can be distinguished with a well-developed root apex at this stage **(Fig 1E).** Then the embryo follows up elongation. Applying $CoCl_2$ can prevent ethylene from being synthesized. Low levels of $CoCl_2$ block the enzyme amino-cyclo-propane-carboxylate oxidase (ACC oxidase; E.C. 1.14.17.4), which lowers the amount of ethylene produced naturally (Zhang et al 2021). Somatic embryogenesis in carrots was stimulated after the heavy metal-induced suppression of ethylene biosynthesis of cobalt and nickel (Roustan et al 1989).

In addition, Akeel and Jahan (2020) mentioned that metals including Co, Cr, Cu, Zn and Mn, when present at appropriate levels, are crucial for the metabolism of plants and significantly affect the development and productivity of the plants. Cobalt is necessary for several enzymes and coenzyme-related processes and is a part of cobalamin (Vitamin B_{12}). Additional benefits include bud formation, when applied exogenously, stem evolution and coleoptile elongation promoting both growth and yield of the plant. Consequently, combining copper and cobalt demonstrates how the additive effect may be used to accelerate cell-grown respiration and may have boosted the rate of callus growth due to synergistic effects. Ethylene production can be prevented by the cobalt ion. Adding $CoCl_2$ to the medium increased the regeneration frequency and the number of shoots produced per explant (Abdellatef and Khalafalla 2008). It has been observed that in addition to its impact

on either plant growth or development, the production of ethylene by *in vitro*-grown shoots in sealed containers delays plant regeneration and cell differentiation (Pua 1993).

Coper sulfate is one of the essential components of MS medium **(**Murashige and Skoog 1962). Copper is a necessary cofactor for several proteins in plants. In this investigation, the authors found that the addition of varied concentrations of $CuSO_4$ to MS medium verified a noteworthy impact on percentages of differentiation and the percentage of growth of matured somatic embryos. Malik et al (2021) corroborated these findings by confirming that $CuSO_4$ is necessary for efficient embryogenic callus and regeneration.

The individual SEs formed during the current investigation showed strong shoot and root systems when transferred to a medium of germination containing NAA (0.1 mgL^{-1}) and BA (0.05 mgL^{-1}) as shown in **(Fig 1 F & G)**

3.2 Biochemical analyses

Different amounts of copper sulfate and cobalt chloride applied to the maturation media (8 weeks) have an impact on the biochemical components of date palm somatic embryos (Barhee cv) that have been regenerated following a single subculture. When evaluating the physiological integrity of date palm somatic embryos, it is commonly known that the accumulation of storage compounds is essential. The absence of these compounds may affect the embryos' capacity to reach their final developmental stages and turn into viable plantlets Zein Eldin and Ibrahim 2015).

Table 2 indicates that the addition of $CoCl_2$ as an individual or combined with $CuSO_4$ increased the concentration of total sugars within the formed SEs during the maturation phase. The highest significant increment in total sugars was detected with $CoCl_2$ at 0.52 mgL^{-1} and with the interference of $CoCl_2$ and CuSO4 (0.69 mg g^{-1} fwt). This result coincides with those of AL-Mayahi (2014), who reported that the rate of callus growth may have risen because of the synergistic effects of the combination of $CoCl_2$ and $CuSO_4$ in the medium. As for the individual treatment of $CuSO_4$, an insignificant difference in total sugars was determined with $CuSO_4$ at 0.32 and 0.64 mgL^{-1} (0.59 and 0.53 mg g^{-1} f.wt.) in comparison to the controlled treatment (0.53 mg g^{-1} f.wt.). While adding $CuSO_4$ at 0.08 mgL^{-1} to the medium, decreased remarkably the total sugars in the produced embryos. According to Rabie et al (1992), the visualized decrease in the total sugars in relation to the high levels of micro-elements is due to its involvement in the enzyme activity connected to the carbohydrate catabolism cycles. The concentration of total free amino acids (TAA) in the regenerated SEs released during the maturation stage was raised by the treatments as shown in **Table 2,** whether $CuSO_4$ or $CoCl_2$ was applied separately or in combination. The maximum significant increase was obtained with $CoCl_2$ at 0.26 mgL^{-1}, where the concentration of free amino acids reached 5.73 mg g^{-1} f.wt. While the combination treatment ($CuSO_4$ + $CoCl_2$) produced 5.36 mg g^{-1} f.wt. *In vitro*-cultured plant embryos, the metal ions Cu and Co can affect the content of free amino acids, according to Shakya et al (2022), Santiago-Diaz et al (2023). Copper is considered a cofactor for several enzymes taking part in amino acid synthesis, such as glutamate dehydrogenase, glutamine synthetase and asparagine synthetase.

When the biochemical analysis of indole acetic acid (IAA) was estimated, it was noticed that all additive treatments of $CuSO_4$ and $CoCl_2$, whether individual or combined, increased the concentration of IAA in the analyzed SEs in comparison to the control treatment. The combination treatment of $CuSO_4$ and $CoCl_2$ caused the most significant increase in IAA concentration over all other treatments. Gong et al (2019) recorded that in higher plants, IAA is the main auxin; it is a crucial signaling molecule that serves as a stress tolerance component as well as a regulator of plant development. Auxins are plant growth regulators that play an indespinsable role in SEs, as they stimulate cell division, differentiation and morphogenesis.

Overall, varying micronutrient additives increased the total soluble phenol concentration in SEs compared with the control treatment. Cobalt chloride at 0.52 mgL^{-1} notably boosted the total soluble phenol in the regenerated SEs (5.17mg g^{-1} f.wt.), followed by concentrations 0.26 and 0.065 mgL^{-1} (4.09 and 4.08 mg g^{-1} f.wt., respectively). When treating the maturation medium with variable concentrations of $CuSO_4$, the soluble phenol was significantly lower than with $CoCl_2$ treatments. These acquired data coincide with those of Cvikrova´ et al (2003) who demonstrated that, in plants, the key function of phenylpropanoids is enrolled in lignin synthetic, a crucial structural material found in the tracheary walls. Both Cu and Co serve as stressors; they trigger the process of producing phenolic compounds as a protective strategy against metal toxicity or oxidative damage. Antioxidant-rich phenolic compounds can bind metal ions to lessen their availability and deleterious effects**.** Cu and Co serve as cofactors or regulators of the phenolic chemical-producing enzymes phenylalanine ammonia-lyase (PAL) and tyrosine ammonia-lyase (TAL).

Table 2. Effect of varying concentrations of $CuSO_4$ and $CoCl_2$ on total sugars (TS), total amino acid (TAA), total soluble phenols and total indole (mg g^{-1}FW) of formed date palm somatic embryos after 8 weeks incubation period

Treatment	Conc. (mgL^{-1})	TS	TAA	Total soluble phenols	Total indole
Control	-	0.53^{bc}±0.03	3.19^{e}±0.33	2.44^{d}±0.39	0.016^{c}±0.014
$CuSO_4$	0.08	0.45^{c}±0.03	4.24^{cd}±0.2	2.7^{cd}±0.50	0.0318^{c}±0.007
	0.32	0.59^{b}±0.09	4.37^{cd}±0.17	3.07^{cd}±0.56	0.101^{bc}±0.087
	0.64	0.53^{bc}±0.02	4.82^{b}±0.18	2.89^{cd}±0.38	0.114^{bc}±0.097
$CoCl_2$	0.065	0.58^{b}±0.02	4.09^{d}±0.15	4.08^{b}±0.58	0.076^{bc}±0.016
	0.26	0.75^{a}±0.09	5.73^{a}±0.08	4.09^{b}±0.40	0.104^{bc}±0.090
	0.52	0.68^{a}±0.04	4.52^{bc}±0.36	5.17^{a}±0.88	0.166^{b}±0.143
$CuSO_4$ +$CoCl_2$	0.32 + 0.26	0.69^{a}±0.02	5.36^{a}±0.09	3.44^{bc}±0.23	0.309^{a}±0.021

*Overall means within a column with different superscript letters differ significantly (P< 0.05).
#TS: Total Sugar, $CuSO_4$: copper sulphate, $CoCl_2$: Cobalt Chloride, and SD: standard deviation

Table 3. Effect of different ($CuSO_4$) and ($CoCl_2$) concentrations added to the date palm (Barhee cv.) maturation media on the activities of POD, PPO and PAL (unit mg $^{-1}$ protein) within the formed SE after 8 weeks incubation period

Tr	Conc. (mgL^{-1})	APX	PPO	PAL
Control	-	1.47^{ef}±0.29	20332^{e}±1,617	21609^{e}±4,375
$CuSO_4$	0.08	1.31^{f}±0.28	63829^{d}±20,607	35915^{cde}±7,845
	0.32	2.11^{def}±0.64	67574^{d}±9,340	41174^{bcd}±3,487
	0.64	2.97^{cde}±1.14	48952^{d}±5,907	31224^{de}±4,104
$CoCl_2$	0.065	3.51^{cd}±0.22	89486^{c}±3,176	49594^{bc}±2,347
	0.26	3.92^{c}±0.53	138251^{b}±13,155	54715^{b}±4,843
	0.52	6.13^{b}±0.67	162308^{a}±6,778	82131^{a}±6,312
$CuSO_4$ +$CoCl_2$	0.32 + 0.26	19.87^{a}±1.70	122746^{b}±13,006	76219^{a}±19,916

*Means within a column with different superscript letters differ significantly (P< 0.05).
#APX: Ascorbate peroxidase, PPO: Polyphenol oxidase, PAL: Phenylalanine ammonia-lyase, and SD: standard deviation.

As **Table 3** shows treatments with $CuSO_4$ and $CoCl_2$ at different concentrations, either individually or together, affect the activation of APX, PPO, and PAL. When $CuSO_4$ and $CoCl_2$ were combined, the highest significant increase in the activity of APX (19.87 mg g^{-1} protein) was achieved, followed by $CoCl_2$ treatments at 0.52, 0.26, and 0.065 mgL^{-1} (6.13, 3.92, and 3.51 mg g^{-1} protein, respectively), with a significant difference compared with the control treatment. The activity of the APX enzyme decreased when the embryos were supplemented with $CuSO_4$ at 0.64, 0.32 and 0.08 mgL^{-1} (2.9, 2.11, and 1.31 mg g^{-1} protein, respectively) compared to the $CoCl_2$ treatment. An insignificant reduction in APX activity was recorded with $CuSO_4$ at 0.08 mgL^{-1} in comparison with the control.

Moreover, remarkable increased activity was estimated in PPO enzyme with all additive treatments, either for $CoCl_2$ or $CuSO_4$, whether added individually or combined, compared to the control treatment. Some enzymes, including tyrosinase, amine oxidase, superoxide dismutase, cytochrome oxidase and polyphenol oxidase are composed of copper (Singh et al 2007).

Cobalt chloride treatment raised the PPO activity more than copper sulfate, especially when $CoCl_2$ was added individually at 0.52 mgL^{-1}. The same results were recorded in the activity of PAL where a significant increase was recorded with all additives compared to the control. The addition of $CoCl_2$, either as an individual or combined with $CuSO_4$, significantly achieved the highest PAL activity compared to all other treatments. Furthermore, an insignificant increase in PAL activity was recorded between $CuSO_4$ concentration treatments. PPO converts phenols to quinones, which condense with IAA, hence regulating free endogenous IAA (Leopold and Plummer 1961). Moreover, the removal of

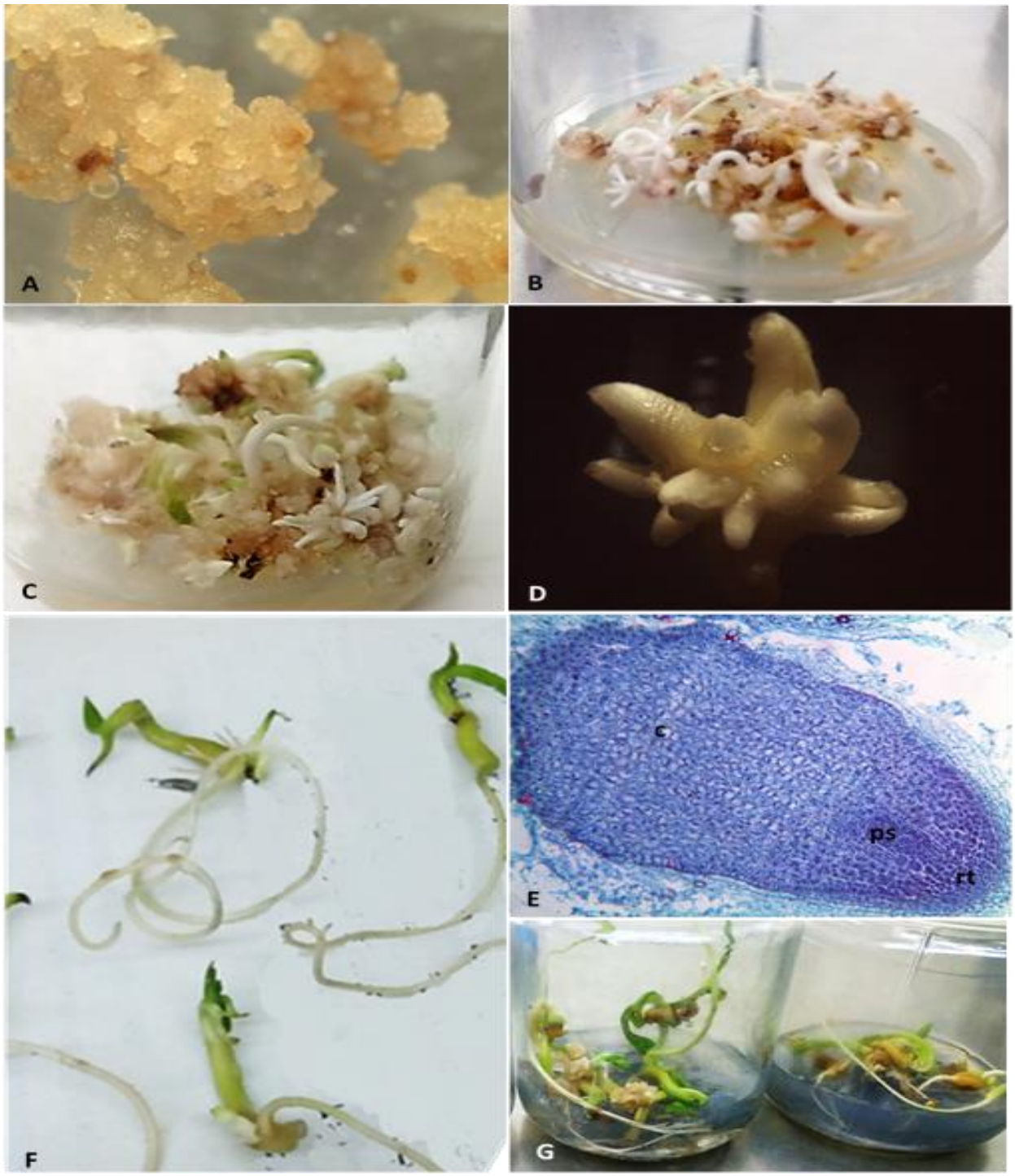

Fig 1. A) Morphology of the embryogenic callus of date palm cv. Barhee developed from the shoot tip, has a nodular appearance during culturing on maturation media supplemented with 0.5 mgL^{-1} ABA and 5 gL^{-1} PEG-4000. B& C. Different developing stages of somatic embryos from the embryogenic callus after 8 weeks of culturing on maturation medium. B) control medium. C) medium supplemented with ($CoCl_2$ + $CuSO_4$), note the obvious increase in growth and differentiation rates than the control medium. D) A cluster of differentiated embryos in synchronized stages. E) Longitudinal section in full-developed individual embryo showed distinct embryonic axis surrounded by cotyledon (c). The procambium strand (ps) and the well-developed root tip (rt). F&G) germinated somatic embryos cultured on germination medium (which contained 0.1 mgL^{-1}NAA and 0.05 mgL^{-1}BA)

free endogenous IAA is a consequence of auxin-phenol complex formation; this has possibly affected the future development of the embryogenic callus (Zein Eldin and Ibrahim 2015).

Miguel (2023) reported that Cu is known to include or activate many significant enzymes involved in the metabolism of polyphenols, protein and carbohydrate biosynthesis, electron transport, and other activities. PAL activity in leaves rose in response to Cu concentrations, suggesting that some Cu co-enzymes are necessary for plant regeneration. Palit et al (1994) also mentioned that the transition metal cobalt is essential for many enzymes and co-enzymes.

4 Conclusion

The combined application of $CuSO_4$ and $CoCl_2$ at 0.32 and 0.26 mgL^{-1}, respectively gave optimistic results in high relative growth and differentiation percentages, as well as a higher number of fully developed somatic embryos (SEs) of date palm. These SEs exhibited better biochemical markers. In date palm, the obtained results are useful in enhancing micropropagation protocol.

Acknowledgment

The authors would like to thank the Central Laboratory for Date Palm Researches and Development, Agricultural Research Center (Tissue Culture Lab.), Giza, Egypt, for providing the facilities to carry out the research work.

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