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EFFECT OF COPPER SULPHATE AND COBALT CHLORIDE ON GROWTH OF THE *IN VITRO* CULTURE TISSUES FOR DATE PALM (*PHOENIX DACTYLIFERA* L.) CV. ASHGAR

Ahmed Madi Waheed AL-Mayahi

Department of Plant Tissue Culture, Date Palm Research Centre, University of Basrah, Basrah, Iraq

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ABSTRACT

This study was carried out to investigate the effects of copper sulphate and cobalt chloride on propagation of date palm cv. Ashgar *in vitro*. The rate of callus proliferation was significantly higher in the medium supplemented with 2 µM copper sulphate and 2 µM cobalt chloride together ($p<0.05$). Addition of copper sulphate and Cobalt chloride to the medium was most effective for shoots regeneration from callus and enhanced regeneration frequency as well as number of shoots obtained per explant, the best result (7.12 shoot/explant) was obtained by using copper sulphate and Cobalt chloride at 2.0 µM. Also the results of this experiment indicates that the maximum induction of roots can be achieved in the media containing both additives at 0.5 µM. The callus exhibited a decline in carbohydrate contents, proteins, whereas total phenol content increased at high concentration of Cu and Co. The greatest formation of chlorophylls a, b and total chlorophyll was obtained in plantlets grown in the media containing both additives at 0.5 µM. As well as Maximum survivability was noticed for plants cultured in media that containing both copper sulphate and Cobalt chloride at 0.5 µM.

Keywords: Copper Sulphate, Cobalt Chloride, *In Vitro*, Ms, N6 Medium, Date Palm

1. INTRODUCTION

Date palm (*Phoenix dactylifera* L.) of the family Arecaceae is a key plantation crop of many countries of arid regions of West Asia and North Africa (Al-Khalifah *et al*., 2012). The propagation is of both types (sexual and vegetative) Sexual propagation is through seeds and vegetative propagation by offshoot (Al-Khalifah and Shanavaskhan, 2012). The vegetative multiplication of date palm is traditionally achieved by offshoots.This offshoot propagation has limitations such as slow propagation rate, transmission of disease-causing pathogens and insects and production of offshoots in a limited number for a certain period in the life time of a young palm tree (Gueye *et al*., 2009). Second sources of the propagation are seeds, but it has many limitations like low rate of germination and progeny variations (Chand and Singh, 2004). To overcome the propagation problems and to maintain the germplasm, the *in vitro* micropropagation (somatic embryogenesis and organogenesis) is the successful technique (Al-Khayri, 2003; Mujib *et al*., 2004; Bhattacharjee, 2006). Plant tissues and organs are grown *in vitro* on artificial media, which supply the nutrients necessary for growth. The success of plant tissue culture as a means of plant propagation is greatly influenced by the nature of the culture medium used. For healthy and vigorous growth, intact plants need to take up from the soil. It has become evident that several heavy metals as micro elements play important roles in the regeneration of plant tissue cultures. The metals like cobalt, iron, manganese, copper and zinc are essential for plant life but are required in a very small or trace amounts and become toxic at higher concentrations (Hussein *et al*., 2010). Copper is a microelement that is essential for normal growth and development of plants. In plant organisms it performs very important physiological and biochemical functions. It takes part in the processes of photosynthesis, respiration, conversion of nitrogen compounds, transport of

carbohydrates and also regulates the process of DNA formation (Podlesna and Wojcieska-Wyskupajtys, 1996). Also It is a constituent of the protein component of several enzymes in plants, mainly those participating in electron flow, catalyzing redox reactions in mitochondria, chloroplasts, cell wall and cytoplasm of plant cells (Lolkema, 1985). Ghaemi *et al*. (1994) reported that the addition of 40 µM $CuSO_4$ to the medium significantly increased the embryoids production from wheat anther cultures. Dahleen (1995) studied the effect of different concentrations of $CuSO_4$ on callus culture of two cultivars of barley and found that medium containing 50 µM copper regenerated significantly more plants with an average of 17 plants per embryo of the cultivar "Hector" in comparison with normal MS level which regenerated only 5 plants per embryo. The plant exhibited a decline in growth, chlorophyll contents, protein and DNA, RNA content carbohydrate, where as proline, total and phenol content increased at high concentration of Cu and Zn (Vinod *et al*., 2012). Cobalt is not regarded as an essential element Nevertheless, it was found to have been included in approximately half of a large sample of published plant culture media (George *et al*., 1987). Cobalt is consider of heavy metals, inhibitor of ethylene production (Chraibi *et al*., 1991; Santana-Buzzy *et al*., 2006). Propagation of date palm through tissue culture techniques would offset slow growth rates and limited vegetative propagation potential and would provide large number of desirable clones on demand (Bekheet *et al*., 2001). The quality of the callus of date palm is one of the major factors to determine the rate of regeneration. It has been reported that of mature factors to improve the quality of the callus is the composition of the *in vitro* media. Inorganic macronutrient and micronutrient levels used in most plant tissue culture media are based on levels established in the medium developed by Murashige and Skoog (1962) for tobacco tissue culture "MS medium". For several micronutrients. Chu *et al*. (1975) did not mention the effect of Cu and they did not use it in the N6 medium developed for rice anther culture. Some other more rarely used media contain a higher amount of Cu but the specific effect of Cu on morphogenesis has not been described (Litvay *et al*., 1985). However, no clear optimal levels were apparent and the effect of alternative formulations of these nutrients in tissue culture media was scarcely studied, until now in most cases the role of Cu and CO have not been investigated or it have not proved to be significant in spite of Copper and Cobalt added to certain media, but their requirements for cell growth has not been precisely established. Hence the aim of this study to evaluate the effect of copper sulphate and cobalt chloride supplementation on the performance of growth date palm cv. Ashgar and determine effect of elements on biochemical parameters (chlorophylls, sugars and total proteins) for tissue cultured *in vitro*.

2. MATERIALS AND METHODS

The experiments for this study were carried out in tissue culture Laboratory for Date Palm Research centre in Basra university during the period from 2012 and 2013. Explants Preparation and Sterilization Young offshoots of Ashgar cultivar (2-3 years old) were chosen and detached from the mother palm. Offshoots were dissected acropetaly until the shoot tips appeared. Shoot tips of 3 cm (apical meristems with soft inner leaves) (**Fig. 1**) were excised with immature fiber 2 cm in diameter and then applied in antioxidant solution consisting of 150 mg L^{-1} citric acid plus 100 mg L^{-1} ascorbic acid (Tisserat, 1991). Explants were sterilized in commercial bleach (sodium hypochlorite) 20% containing one-two drops of tween-20 as emulsifier for 20 min with vacuum and rinsed three times with sterile distilled water. Then they were transferred to Petri dishes and all leaf primordia were removed except two pairs surrounding the apical meristems.

2.1. Initiation Stage

The apical meristems were divided longitudinally into four equal segments and cultured on medium composed of (MS) (Murashige and Skoog, 1962) (**Table 1 and Fig. 2**), with additional 30 mg L^{-1} Naphthalene Acetic Acid (NAA), 3 mg L^{-1} 2iP and 3 g L^{-1} activated charcoal. The pH of the medium was adjusted to 5.8 with 0.1 N NaOH or HCl, before the addition of agar. Media were dispensed into culture test tube with 25 mL in each, then covered with Cotton and aluminum foil. All test tubes with media were autoclaved at 121°C and 1.04 kg/cm^2 for 15 min. All cultures were incubated in a culture room under darkness at 27±2°C for 23 days to initiate callus.

Effect of different formulations and concentrations of copper sulphate and cobalt chloride on growth and development of callus and shoot regeneration of date palm cv. Ashgar resultant callus was separated and cultured on the medium composed of N6 basal elements (Chu *et al*., 1975) (**Table 1**), with additional 10 mg L^{-1} Naphthalene Acetic Acid (NAA) 2 mg L^{-1} 2iP and 1.5g L^{-1} activated charcoal.

Fig. 1. Trimmed date palm's offshoot until arrival to apical bud

Table 1. Composition (in mg/L) of basic media used in this study

	MS*	N6**
Micronutrients components	Mg/L	Mg/L
KNO3	1900.00	2830.00
NH_4NO_3	1650.00	-
$(NH4)_2SO_4$	-	463.00
KH_2PO_4	170.00	400.00
$CaCl_2.2H_2O$	440.00	166.00
$MgSO4.7H_2O$	370.00	185.00
Macronutrients components		
$MnSO_4$. H_2O	16.90	3.33
H_3BO_3	6.20	1.60
$ZnSO_4.7H_2O$	8.60	1.50
KI	0.83	0.80
$Feso_4$. $7H_2O$	27.85	27.85
Na_2EDTA	37.25	37.25
Organic components		
Glyecin	2.00	2.00
Thiamine	0.40	1.00
Pyridoxine	0.50	0.50
Nicotinic acid	0.50	0.50
Myo-inositol	100.00	100.00
Carbon source (Sucrose)	30.00	30.00
Solidifying (Agar)	6.00	6.00

*MS medium (Murashige and Skoog, 1962) ** N6 medium (Chu *et al*., 1975)

Also the media were supplemented with different concentrations of copper sulphate or cobalt chloride (0.0 control, 0.5, 2.0, 4.0 and 8.0) µM or both additives at the concentration of 2 µM. The control was the medium without additional microelements. There were ten replicates of each treatment. Rate of proliferation was calculated at the end of the third subculture:

$$\frac{\text{Final weight of callus} - \text{Initial weight of callus}}{\text{Initial weight of callus}} \times 100$$

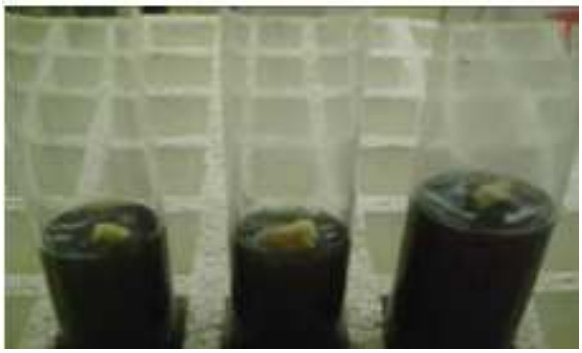


Fig. 2. Culture of Apical meristems in test tubes that containing "MS" medium and incubated under darkness at 27±2°C. for 23 days

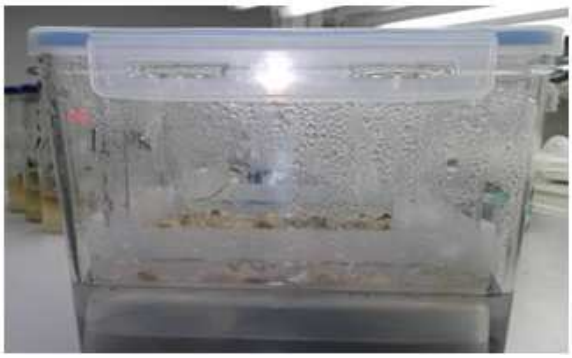


Fig. 3. Dehydration of callus by use bioreactor for 72 h

Callus of date palm cv. Ashgar obtained from the established protocol, where the basal medium containing 2 µM copper sulphate and 2 µM cobalt chloride were first dehydrated (Tsukahara and Hirosawa, 1992). Where used for this purpose in this

study bioreactor (**Fig. 3**). Were ratio lose about 25% of the fresh weight at incubate in the culture room for 72 h. About 100 mg callus dehydrated was cultured on 30 mL medium in one test tube. These dehydrated callus cultured on the N6 medium composed of 3% sucrose, 0.5 mg L^{-1} NAA, 3 mg L^{-1} 2iP and Polyvinyl Pyrrolidone (PVP) 500 mg L^{-1} inoculated in the regeneration media containing different combinations of additives of copper sulphate and cobalt chloride (0.0 control, 0.5, 2.0, 4.0 and 8.0) µM or both additives at the concentration of 2 µM and placed under 8/16 h dark/light provided by daylight fluorescent lamps at 27+2°C. evaluation of their effects on plant regeneration. Results of the experiments were evaluated 12 weeks after inoculation of callus in the regeneration medium. There were twelve replicates of each treatment.

2.2. Effect of Different Formulations and Concentrations of Copper Sulphate and Cobalt Chloride on Rooting of Plants *in vitro* for Date Palm cv. Ashgar

Micropropagated shoots of date palm cv. Ashgar with no visible signs of root development were inserted into rooting medium consisting of N6 medium supplemented with 0.2 mg L^{-1} NAA. The media were supplemented with different concentrations of copper sulphate or cobalt chloride (0.0 control, 0.5, 2.0, 4.0 and 8.0) µM or both additives at the concentration of 0.5 µM. Test tubes containing Micro-propagated shoots were incubated at 27±2°C and 16 h photoperiod. Six weeks later root induction was measured by the percentage of roots, number of roots regenerated per shoot and root elongation, as measured by the mean root length per shoot, as well as the length of shoots were recorded. There were nine replicates of each treatment.

2.3. Determination of Biochemical Parameters

2.3.1. Callus Stage

Content of callus of carbohydrates total soluble was analyzed by the way phenol-sulfuric acid according to the method described by (Herbert *et al*., 1971). The total carbohydrates were measured by Spectrophotometer at 485 nm.

Proteins were extracted according to Method described by (Lecouteux *et al*., 1993). Fresh callus material (250) mg was extracted with 2 mL of 0.25M Phosphate buffer PH7 and centrifuged for 3 min at 7000 g. The supernatant was used as the crude Protein extract. The total proteins were measured by Spectrophotometer at 595 nm according to (Bradford, 1976).

Phenolics compound were extracted according to Method described by (El-Hadrami, 1995; Macheix *et al*., 1990). The callus homogenized with 2 mL (80%) Methanol at 4°C and centrifuged for 3 times at 7000 g. for 3 min. About 100 µL of supernatant were recuperated each time. About 100 µL of supernatant was added to Folin-Ciocalteu reagent (250) µL and solid carbonate (20%). The mixture was incubated of 40°C for 30 times and the blue colour was determinate at 760 nm.

2.4. Plant Stage

2.4.1. Estimate the Amount of Chlorophyll in the Leaves of Date Palm cv. Ashgar

Take one gram of leaflets date palm tissue (Plant inside the tubes). The extent in which the amount of chlorophyll by the method described by Abbas and Abbas (1992). Where was added to the sample 50 cm^3 acetone (80%) and Grinded leaflets by mortar ceramic and returned extraction process that has become the sample colorless then taking part of the sample and placed in the centrifuge for 3 min, then take a part of the solution pure and put in a device the Spectrophotometer type Apel PD303-UV and who was Adjust by acetone 80% and took reading the optical density at a wavelength of 645 and 665 nm and then estimated the total chlorophyll quantity of the sample according to the following equation: Total chlorophyll mg/liter = 20.2×optical density at a wavelength 645 + 8.02×optical density at a wavelength 665 conversion of the amount of chlorophyll mg/L to mg/100 g according to the following equation:

$$\text{Mg} / 100\text{g} = \frac{\text{Mg} / \text{L}}{1000\text{cm}^3} \times \frac{100}{\text{sample weight } (\text{g})}$$

2.5. Acclimatization Stage

Plants for date palm cv. Ashgar which produced from rooting stage (about 10-12 cm in length) (**Fig. 4**). Where transferred from the test tubes under tap water to free the root from agar, then, the plantlets were washed with distilled water and treated with fungicide (Benlet 500 mg L^{-1}) for 20 min and transferred to plastic pots containing autoclaved a mixture of peat moss and perlite (2:1) (AL-Mayahi, 2008). Covered the plants with glass bottle to maintain humidity and were kept under culture room conditions (**Fig. 5**). After six weeks, glass bottles were removed gradually plants were irrigated with $^1/_2$ strength MS salts. The survival of acclimatized plants was then recorded.

After eight weeks calculated the percentage for plantlets acclimated as follows:

$$\text{Percentage of plantlets acclimated} = \frac{\text{Number of plantlets acclimated}}{\text{The total number of plantlets}} \times 100$$

2.6. Experimental Design and Statistical Analysis

Completely randomized design was used. The data was subjected to the analysis of variance and mean values were compared using revised LSD at 5% (Snedecor and Cochran, 1989).

Fig. 4. Rooted plantlets ready for transplanting to plastic pots

Fig. 5. Coverage of plant by glass jar for 6 weeks from transition to plastic pots

3. RESULTS

3.1. Effect of Different Formulations and Concentrations of Copper Sulphate and Cobalt Chloride on Growth and Development of Callus Date Palm cv. Ashgar

According to the results obtained , copper sulphate at (2 and 4) µM was found suitable for producing of callus. However, the rate of callus proliferation was decreased when the concentration of copper sulphate to 8 µM in the media. High quality granular callus and creamy in colour were produced in the media containing 2 µM copper sulphate (**Fig. 6B**). Comparing copper sulphate and Cobalt chloride, copper sulphate was found better for callus proliferation .The rate of callus proliferation was increased in the media contain Cobalt chloride at 2 µM however statistical analysis did not show significant differences between different concentrations of cobalt sulfate and Control (no additives) treatment on callus proliferation at the 0.05 level (**Fig. 6A, C and Table 2**). The results clearly showed that the rate of High quality granular callus proliferation in this experiment was significantly higher in the medium containing both additives at the concentrations of 2 mg L^{-1} $CuSO_4$ and 2 mg L^{-1} $CoCl_2$ (**Fig. 6D**). This value was higher than that in the media supplemented with no additives or with one additive alone.

3.2. Effect of Different Formulations and Concentrations of Copper Sulphate and Cobalt Chloride on Growth and Development of Callus and Shoot Regeneration of Date Palm cv. Ashgar

It seemed that the optimal concentration for multiplication “shoot regeneration” was 2 µM of copper sulphate (**Fig. 7A and B**). The addition of cobalt in the form of cobalt chloride did not show significant effect as control treatment in the “shoot regeneration at low concentrations. The poor regeneration abilities were obvious even in the medium supplemented with higher concentrations of copper sulphate or Cobalt chloride. combination of copper sulphate and Cobalt chloride at 2 µM was found to be beneficial as they significantly enhanced the percentage shoot regeneration and number of regenerated shoots per explant 75% and 7.12 shoots respectively (**Fig. 7C and Table 3**). Comparatively though the regeneration medium containing 2 µM copper sulphate show the better results in regeneration all other media containing one additive alone gives the poor results (**Table 3**).

(A) (B) (C) (D)

Fig. 6. Callus proliferation on N6 basal medium containing (A) Control (no additives) (B) 2 µM/l CuSO4 µM (C) 2.0µM /l CoCl2 µM (D) 2.0 µM/l CuSO4 +2.0 µM/l CoCl2 µM

(A) (B) (C)

Fig. 7. Shoots induction from callus cultured in N6 medium supplied (A) Control (no additives) (B) 2 µM for copper sulphate (C) 2 µM for both copper sulphate and Cobalt chloride after 12 weeks from culturing

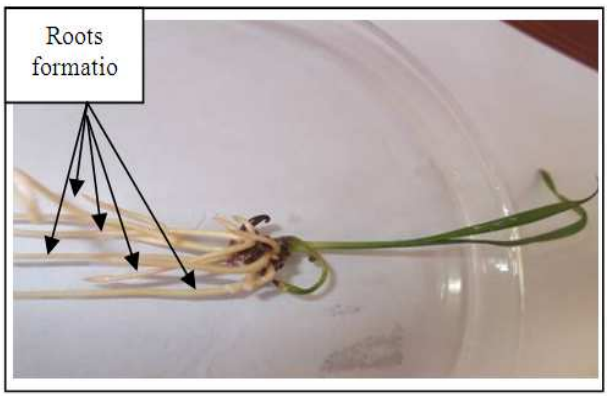


Fig. 8. Roots formation in N6 medium supplied with both copper sulphate and cobalt chloride at 0.5 µM after 6 weeks from culturing

Table 2. Effect of different combinations of copper sulphate and cobalt chloride on rate of callus proliferation of date palm cv. Ashgar at the end of the third subculture

Treatments	Rate of callus proliferation (%)
Control (no additives)	200±3.37*
0.5 µM L^{-1} $CuSO_4$	207±1.79
2 µM L^{-1} $CuSO_4$	312±4.1
4 µM L^{-1} $CuSO_4$	269±1.10
8 µM L^{-1} $CuSO_4$	175±2.05
0.5 µM L^{-1} $CoCl_2$	191±2.13
2 µM L^{-1} $CoCl_2$	241±3.6
4 µM L^{-1} $CoCl_2$	221±2.13
8 µM L^{-1} $CoCl_2$	153±2.6
2.0 µM L^{-1} $CuSO_4$ + 2.0µM L^{-1} $CoCl_2$	521±2.13
L.S.D.	19

*± Standard error

Table 3. Effect of different combinations of copper sulphate and cobalt chloride on a response percentage of desiccated callus for shoot formation and number of shoots/100 mg callus after 10 weeks from culturing for date palm cv. Ashgar

Treatments	Response of callus (100 mg weight) for shoot formation	Number of shoots/ 100 mg callus
Control (no additives)	41.67±8.34*	3.8±0.25
0.5 µM L^{-1} $CuSO_4$	41.67±8.34	4.0±0.29
2 µM L^{-1} $CuSO_4$	58.34±4.81	5.14±0.18
4 µM L^{-1} $CuSO_4$	33.34±4.81	4.5±0.33
8 µM L^{-1} $CuSO_4$	25.0±4.81	2.66±0.25
0.5 µM L^{-1} $CoCl_2$	33.34±4.81	3.25±0.68
2 µM L^{-1} $CoCl_2$	41.67±8.34	4.2±1.01
4 µM L^{-1} $CoCl_2$	33.34±4.81	3.75±0.78
8 µM L^{-1} $CoCl_2$	25.0±4.81	2.33±0.18
2.0 µM L^{-1} $CuSO_4$ + 2.0µM L^{-1} $CoCl_2$	75.0±4.81	7.12±0.29
L.S.D.	13.9	0.7

*± Standard error (n = 12)

Table 4. Regeneration of plants from desiccated callus cultured on the media containing different combinations of additives

Treatments	Rooting (%)	Numbers of roots	Lengths of roots	Lengths of plants
Control (no additives)	55.56	3.0±0.07*	3.8±0.11	5.11±0.6
0.5 µM L^{-1} $CuSO_4$	77.78	4.28±0.42	4.4±0.37	6.27±0.4
2 µM L^{-1} $CuSO_4$	44.45	3.0±0.07	3.6±0.7	4.79±0.2
4 µM L^{-1} $CuSO_4$	44.45	2.75±0.25	3.0±0.08	3.97±0.2
8 µM L^{-1} $CuSO_4$	22.23	1.5±0.30	2.6±0.32	3.40±0.1
0.5 µM L^{-1} $CoCl_2$	66.67	3.2±0.2	4.0± 0.5	5.70±0.1
2 µM L^{-1} $CoCl_2$	44.45	2.75±0.25	3.2±0.2	4.48±0.5
4 µM L^{-1} $CoCl_2$	33.34	2.66±0.42	2.8±0.04	3.63±0.2
8 µM L^{-1} $CoCl_2$	11.12	1.0±0.30	1.3±0.05	2.88±0.2
0.5 µM L^{-1} $CuSO_4$ + 0.5µM L^{-1} $CoCl_2$	88.89	5.37±0.10	4.2±0.5	8.17±0.1
L.S.D.	17.9	0.35	0.6	0.8

* ± Standard error (n = 9)

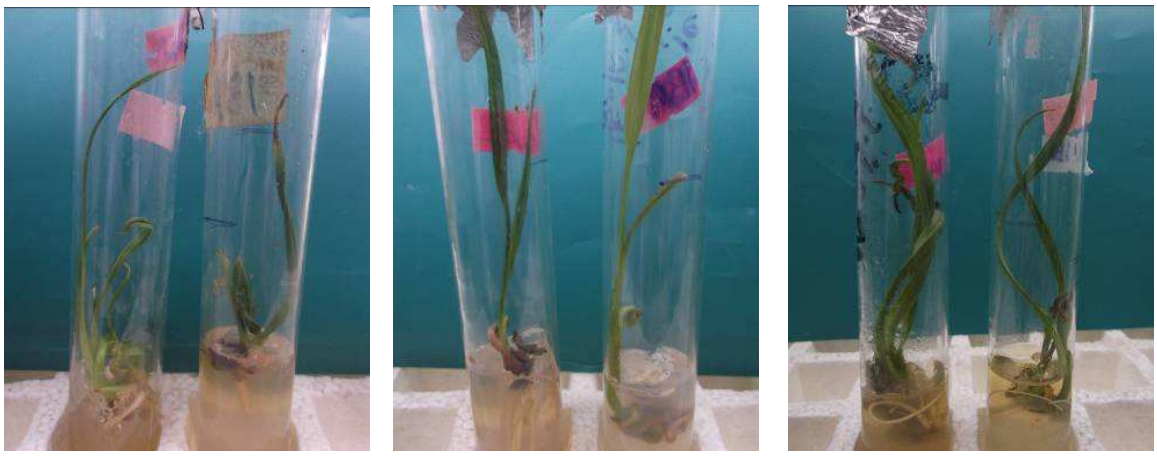
3.3. Effect of Different Formulations and Concentrations of Copper Sulphate and Cobalt Chloride on Rooting Plantlets of Date Palm cv. Ashgar *in Vitro*

According to the results obtained (**Table 4**), Optimal induction of roots which was observed in the media containing lower concentrations (0.5 µM) of copper sulphate. The addition of cobalt chloride to the rooting medium at low concentrations did not show significant effect as control treatment in the percentage of root. Also the results of this experiment indicates that the maximum induction of roots can be achieved in the media containing both additives at 0.5 mg L^{-1} (**Fig. 8**). A decline in induction of roots was observed as the concentration of copper sulphate or cobalt chloride were increased up to 2 µM (**Table 4**). Roots at 4 and 8 µM appeared thick and small.

The highest length of leafs of the plantlets was 12 cm with media containing equal concentration of Copper sulphate 0.5 µM and 0.5 µM Cobalt chloride (**Fig. 9C**). followed by 9.0 cm on the media with Copper sulphate 0.5 µM (**Fig. 9B**). While recorded less length of leafs of the plantlets cultured in control (no additive) (**Fig. 9A**).

3.4. Effects of Copper Sulphate and Cobalt Chloride on Callus Total Carbohydrates and Total Proteins Content

The biochemical analysis showed that copper sulphate at 2.0 µM increased content of callus from total carbohydrates and percentage of proteins compared with other treatments (**Table 5**). The addition of cobalt (2 µM) to the medium in the form of cobalt chloride did not show significant effect as control treatment in the content of callus from total carbohydrates and percentage of proteins. Increasing copper sulphate or cobalt chloride to 8 µM led to significant decrease of total carbohydrates and total proteins content .The highest values of total carbohydrates and percentage of proteins were observed in media that containing both additives copper sulphate and Cobalt chloride at 2 µM were significantly enhanced callus content of its compare with (control) no additives or with one additive alone treatments (**Table 5**).



(A) (B) (C)

Fig. 9. Regeneration of plants in the (A) control (no additives) (B) medium containing 0.5 µM copper sulphate only (C) 0.5 µM copper sulphate and 0.5 µM cobalt chloride

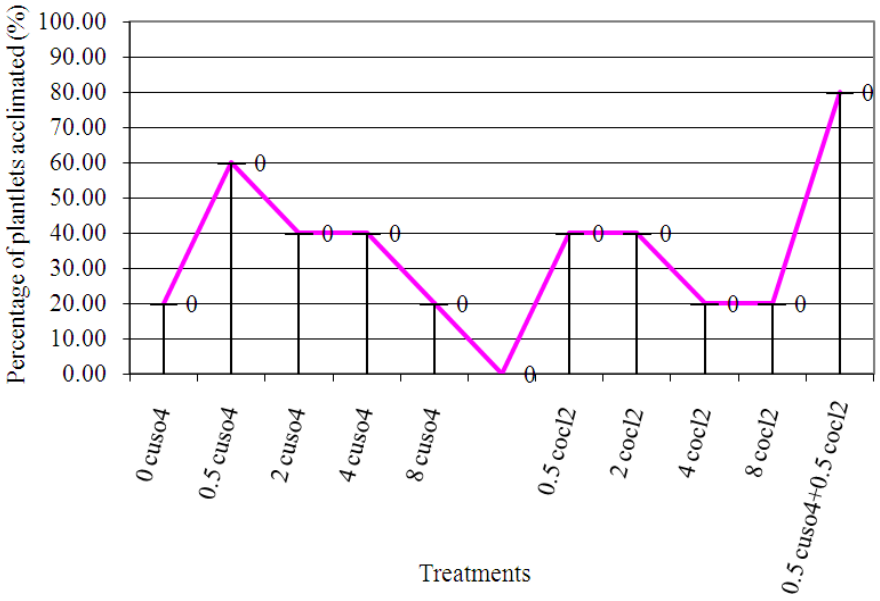


Fig. 10. Effect of copper sulphate and cobalt chloride on acclimatization of date palm cv. Ashgar after eight weeks from transition to plastic pots

3.5. Effects of Copper Sulphate and Cobalt Chloride on Callus Total Phenol Content

According to the results obtained (**Table 5**), Optimal treatment was observed in the medium that containing lower concentrations (0.5 µM) of copper sulphate or Cobalt chloride. Also the biochemical analysis found that phenolic content of callus were increasing with increasing levels of copper sulphate or Cobalt chloride to 8 µM. Also the results of this experiment indicates the minimum of Phenol content achieved in the media that containing both additives from copper sulphate and Cobalt chloride at 0.5 mg L^{-1} compared with control (no additives) or with one additive alone. These results are in harmony with those obtained by (Ganeva and Zozikova, 2007). Who reported that the content of free phenols was found to increase in wheat with increasing Cu^{2+} concentration in the medium.

Fig. 11. Date palm vitro plants after 60 and 75 days from transition of media that containing copper sulphate and cobalt chloride at 0.5 µM to greenhouse

Table 5. Effect of different concentrations of $Cuso_4$ and $Cocl_2$ in the total carbohydrates,total proteins, total Phenol content for callus and content of leaves from Chlorophylls a + b and total Chlorophyll of date palm cv. Ashgar

	Callus stage			Plants stage amount of chlorophyll mg/L to mg/100 g FW		
Treatments	Carbohydrates (Mg/gm FW)	Total protein (%)	Total phenol (%)	Chl a	Chl b	Chl t
Control (no additives)	0.619±0.023	26.228±1.53	7.68±0.21	0.71±0.06	0.17±0.04	0.88±0.04*
0.5 µM L^{-1} $CuSO_4$	0.628±0.022	27.800±0.99	6.79±0.73	0.81±0.04	0.23±0.03	1.04±0.06
2 µM L^{-1} $CuSO_4$	1.039±0.018	31.117±1.2	7.08±0.26	0.79±0.07	0.17±0.03	0.96±0.04
4 µM L^{-1} $CuSO_4$	0.753±0.014	29.080±0.7	7.37±0.23	0.58±0.01	0.13±0.03	0.71±0.07
8 µM L^{-1} $CuSO_4$	0.449±0.025	23.792±0.59	8.28±0.69	0.49±0.08	0.11±0.06	0.60±0.08
0.5 µM L^{-1} $CoCl_2$	0.493±0.021	26.323±1.59	6.88±0.65	0.80±0.04	0.17±0.03	0.97±0.06
2 µM L^{-1} $CoCl_2$	0.623±0.014	27.080±0.16	7.22±0.01	0.76±0.04	0.15±0.03	0.91±0.06
4 µM L^{-1} $CoCl_2$	0.611±0.019	26.119±1.53	7.48±0.16	0.53±0.07	0.13±0.03	0.66±0.04
8 µM L^{-1} $CoCl_2$	0.379±0.031	23.374±1.21	8.37±0.65	0.47±0.03	0.12±0.03	0.59±0.07
0.5 µM L^{-1} $CuSO_4$ + 0.5µM L^{-1} $CoCl_2$	-	-	-	1.03±0.06	0.33±0.06	1.36±0.04
2.0 µM L^{-1} $CuSO_4$ + 2.0µM L^{-1} $CoCl_2$	1.321±0.011	33.110±0.8	6.20±0.16	-	-	-
L.S.D.	0.129	2.225	0.30	0.33	0.027	0.073

3.6. Effects of Copper Sulphate and Cobalt Chloride on Leaves Chlorophylls Content

Data presented in **Table 5** show that application of 0.5 and 2.0 µM of copper sulphate increased Chlorophyll a content. Whereas higher copper sulphate concentrations (4 and 8 µM) led to significant reductions in chlorophyll a biosynthesis. Chlorophyll a content were slightly increased at low concentrations and inhibited by high cobalt chloride concentrations. A similar effect of copper sulphate or cobalt chloride on chlorophyll b biosynthesis was exhibited. Thus, low $Cuso_4$ and $Cocl_2$ concentrations increased the biosynthesis of chlorophyll b, while high concentrations led to reduction in its content. The highest rate of Chlorophyll a,b and total Chlorophyll was obtained in media containing both additives" copper sulphate and Cobalt chloride" at 0.5 µM were significantly enhanced plant content of Chlorophyll a,b and total Chlorophyll compare with (control) no additives or with one additive alone (**Table 5**).

3.7. Acclimatization

From the obtained data (**Fig. 10**) the addition of cobalt chloride to copper sulphate improved survival percentage. Maximum survivability was noticed for plants cultured in media that containing both copper sulphate and Cobalt chloride at 0.5 µM (**Fig. 11**),

followed plants cultured in media that containing copper sulphate alone. We found media no contain copper sulphate and cobalt chloride (control) and addition of cobalt chloride or copper sulphate at high concentrations were not suitable for date palm hardening where was survival percentage low.

4. DISCUSSION

The compositions of the macro and micro elements in most of the standard media were developed by evaluating the effects of different minerals on the callus growth. Various media formulations differing in basal salt composition are arbitrary selected to provide essential nutrients for plant *in vitro* cultures, the best callus growth was achieved in cv. Khusab using W and WPM media cv. Berny using SH and NN medium and cv. Barhee using SH, W and MS media (Al-Khayri, 2011). The Copper (Cu) is require to supply the within the culture of most plant because it is essential for plant cell and tissue growth either the cobalt (Schenk and Hildebrandt, 1972) obtained requirement in tests on a wide variety of plants, retained the element in their medium because they occasionally observed an apparent stimulation to the callus growth of some monocotyledons. Copper and cobalt are microelements essential to the physiological activities in cells. Callus proliferation is an energy consuming activity. Therefore, the rate of respiration in cells is normally higher during callus proliferation and cell division to produce required energy. Micronutrients in plant cells, especially copper, iron, manganese and zinc, are important in the process of respiration where iron and copper are the functional parts of some oxidative enzymes contained in plant tissues (Sumner and Somers, 1953). On the other hand, cobalt is a transition element which is an essential factor in many enzymes and co-enzymes. It affects the growth and metabolism of plants in various degrees depending on the concentration of cobalt in the surrounding medium (Palit *et al*., 1994). Therefore, copper and cobalt together show the additive effect to trigger the rate of respiration in cultured cells. Through which the rate of callus proliferation may have been improved with synergistic effects when they are together in the medium. Ouzounidou *et al.* (1992) reported that increasing Cu concentration in nutrient medium reduced the uptake of nutrient elements such as Ca, Mg, K and Fe.

It has become evident that several heavy metals as micro elements play important roles in the regeneration of plant tissue cultures. Cu is known to be a component or activator of many important enzymes involved in electron transport, protein and carbohydrate biosynthesis, Polyphenol metabolism and so on; it is therefore possible that some Cu enzymes might play an important role in plant regeneration (Purnhauser and Gyulai, 1993). Besides Cu other heavy metals such as Co (Roustan *et al*., 1989). Also growth regulators in the media may be the synergistic effects of these microelements on improving the metabolic activities in shoot initiation and development. There are evidences which clearly shows that cobalt interact with other microelements to form complexes (Palit *et al*., 1994). The competitive absorption and mutual activation of the elements like copper in these complexes influence and activate the action of cobalt on various phytochemical reactions showing the synergistic effects. Advantage from adding cobalt to plant culture media might be derived from the fact that the element can have a protective action against metal chelate toxicity and it is able to inhibit oxidative reactions catalysed by ions of copper and iron (Albert, 1958). Moreover the cobalt ion can inhibit ethylene synthesis (Chraibi *et al*., 1991; Eltayb and Khalafalla, 2008). Ethylene production by *in vitro* grown shoots in sealed containers has been observed to inhibit plant regeneration (Robinson and Adams, 1987) and cell differentiation (Chraibi *et al*., 1991) as well as affecting general plant growth and development (Pua, 1993).

The inhibition was stronger for high concentrations of copper and cobalt in roots than in the shoot because the plant roots are first point of contact with these toxic heavy metal species in the nutrient media. The reduction in plant growth during high stress may be due to low water potential, hampered nutrient uptake and secondary stress such as oxidative stress (John *et al*., 2009). The reason for reduced growth under high concentrations of metal treatment could be the reduction in meristematic cells present in this region and some enzymes. Same result was earlier reported by (Arduini *et al*., 1994; Bipasha *et al*., 2000). The reduction in root growth was explained by the previous authors by an inhibition either in cell division or cell elongation or both. moreover, (Mengel and Kirkby, 1982) demonstrated that root growth inhibition is often the first expression of Cu toxicity symptoms, probably because roots tend to bind Cu, while leaves and stems usually exhibit Cu toxicity symptoms at higher concentrations. The promotive effect of Co^{2+} on date palm elongation (and probably other Co^{2+} effects on plant development) is attributed to the inhibition of ethylene biosynthesis.

Cu is known to be a component or activator of many important enzymes involved in electron transport, protein and carbohydrate biosynthesis (Purnhauser and Gyulai, 1993). The observed decline in total sugar with respect to the high level of elements may be due to its role on the enzymatic reactions related to the cycles of carbohydrate catabolism (Rabie *et al*., 1992). These results are in harmony with those obtained by (Singh *et al*., 2007) who found that the Protein was appreciably reduced in wheat at the higher concentration of copper exposure.

When heavy metals are accumulated in excess in plant tissues, these may cause alteration in various physiological processes such as transpiration, photosynthesis and photosynthetic electron transport, biosynthesis of chlorophyll as well as cell membrane integrity (Hussein *et al*., 2010). While high oncentrations led to reduction in its content because of exposure to high concentration of Cu can cause a broad range of deleterious effects such as inhibition of photosynthesis and pigment synthesis, damage to plasma membrane permeability as well as other metabolic distur bances, *in vitro* grown plants (Gori *et al*., 1998). Moreover the Copper (Cu) is involved with chlorophyll synthesis and is found in some enzymes (Lolkema, 1985). The beneficial effect of cobalt on growth of plants could be due to an increase in the leaf water potential relative to compared those untreated plants. The higher leaf water potential could enhance the photosynthesis process (Rathsooriya and Nagarajah, 2003).

Acclimatized is a very important step to complete propagation process of date palm plantlets where was survival percentage low for many reasons, plantlets are grown in optimum conditions (moisture" which his often 90-100% *in vitro*", salts, sucrose and water). Therefore the cuticle layer of these plantlets is often poorly developed and extra water loss through evaporation when plantlets were transferred to soil in which the relative humidity is lower. Leaves of the *in vitro* plantlets are thin, soft, undeveloped plastids, the mesophyll air space and their stomata are not photo synthetically active and subsequently not adapted for in vivo conditions. may be due to beneficial effect of cobalt could be increase in the leaf water potential relative to compared those untreated with cobalt The leaf water potential could enhance the photosynthesis process directly by influencing the photosynthesis system or indirectly by decreasing the total leaf resistance to the diffusion of CO2 into the leaf (Nagarajah and Rathsooriya, 1977). Also found Radin (2004) that cobalt increased water content in cotton leaves and stomatal resistance was increased. Gad (2005) indicated that cuticle tissues of tomato leaves was increased as cobalt addition.

5. CONCLUSION

We are concluded from the present study that copper sulphate "$Cuso_4$" stimulatory for callus growth and it is effectively enhance shoot and roots regeneration in date palm especially additive together with $Cocl_2$. As well as obtained results in this study showed that $Cuso_4$ and $Cocl_2$ at high concentration decreased in carbohydrate content, protein and chlorophylls but total phenol content increased. While Maximum survivability was noticed for plants cultured in media that containing both copper sulphate and Cobalt chloride at 0.5 µM.

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