

The Effect of Some Micro-Elements on Free Amino Acids, Indols and total Phenols Production from Embryogenic Callus of Tow Date Palm Cultivars (Sakkoty and Bartamuda)

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Abstract. Effect of microelements on some chemicals analysis of secondary metabolites such as free amino acids, total indols content and total phenols content of date palm cultivars (Sakkoty and Bartamuda) were studied in this work. Different concentrations of manganese sulfate ($\text{MnSO}_4 \cdot 2 \text{H}_2\text{O}$) (22.3, 44.6 and 66.9 mg/l), zinc sulfate ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$) (8.6, 17.2 and 25.8 mg/l) and copper sulfate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) (0.025, 0.050, 0.075 mg/l) were added into nutrient medium of embryogenic callus stage. The results illustrated that, addition of manganese sulfate at (22.3 mg/l) to culture medium of embryonic callus of Bartamoda cv. gave the highest significant values of total free amino acids (1.75 mg/g fresh weight) and (0.33 mg/g fresh weight) of total indols. Where the addition of manganese sulfate at (66.9 mg/l) to nutrient medium of growing embryogenic callus of Bartamuda cv. gave the highest significant value of total phenols (1.17 mg/g fresh weight). The addition of zinc sulfate at (17.2 mg/l) to culture medium of embryogenic callus of Sakkoty cv., recorded the highest significant values of total amino acids (1.64 mg/g fresh weight) and Indoles (0.40 mg/g fresh weight). While the highest significant values of total phenol content was (1.24 mg/g fresh weight) when embryonic callus of Sakkoty cv. grown on medium contained zinc sulfate at (25.8 mg/l). Data showed also the highest significant values of total free amino acids and total indols content (1.36 and 0.40 mg/g fresh weight respectively) were achieved when embryogenic callus of Bartamuda cv. was grown on medium containing of copper sulfate at (0.025 mg/l), whereas the highest significant value of total phenols content (1.83 mg/g fresh weight) were recorded when embryonic callus of Sakkoty cv. was grown on nutrient medium supplemented with copper sulfate at (0.075 mg/l).

Introduction

Date palm has indispensable utilization in the economy and domestic life of growing countries. It is considered one of the most important commercial crops in the Middle East and Arab World [1].

Secondary metabolites are considered as chemicals that are produced by plants and these chemicals are diverse, Identification of them made into many classes. Each species or plant family has its own mixture of secondary metabolites and that's considered a main advantage in classification of plants. These chemicals could be used for medicinal purposes for humans [2]. Date palms can accumulate many chemicals in their tissues, as a primary metabolites containing carbohydrates and proteins, and secondary metabolites which are produced from primary ones



such as phenolics [3]. Secondary metabolite production can be induced by medium optimizations [4,5]. Microelements have many diverse roles and they are required in trace amounts for plant growth and development [6]. Culture conditions play an important role in the quality and quantity of the material obtained through secondary metabolites [7]. Optimization of the culture condition is effective in improving the accumulation of the desired product. External factors such as carbon source, nitrogen source, growth regulators, medium pH, temperature, light and oxygen are considered easy to regulate the expressions of plant secondary metabolite pathways [8]. Constituents in plant cell culture medium are determinants of growth and production of secondary metabolites. The specific roles for essential micronutrients in the production of active principles is due to their function as components or activators of enzymes of the secondary metabolism. Moreover, metals can chelate certain phytochemicals in plant tissues [9]. The aim of this work is to study the effect of microelements on production of (free amino acids, total indols content and total phenols content) in embryogenic callus stage of in vitro date palm (Bartamuda and Sakkoty cultivar).

Materials and Methods

Callus explants of two cultivars Bartamuda and Sakkoty were produced from indirect protocol of date palm micropropagation described by [10,11].

In this study received embryonic callus explants for both cultivars were cultured on basic nutrient medium for callus formation which composed of MS basal medium [12], supplemented 30 g/l sucrose and 3.0 g/l activated charcoal with 40 mg/L adenine – sulfate, 200 mg/l glutamine, 100 mg/l myo-inositol, 0.1 mg/l biotin, 170 mg/L NaH₂PO₄, 0.1 mg/l thiamine HCL 0.5 mg/l pyridoxine, 0.5 mg/l nicotinic acid, 3.0 mg/L 2- isopentenyl adenine (2iP) + 10.0 mg/l 2,4 –D dichlorophenoxy acetic acid (2,4 – D).

Micro elements compounds, Manganese sulfate (MnSO₄.4H₂O), Zinc sulfate Heptahydrate (ZnSO₄.7H₂O) and Cupric Sulfate (CuSO₄.5H₂O) were added to previous basic nutrient medium for both Bartamuda and Sakkoty cv. callus cultures, in three different separated treatments for each as follows:-

- 1- Manganese sulfate (MnSO₄.4H₂O) were added at (22.3, 44.6 and 66.9 mg/l)
- 2- Zinc sulfate Heptahydrate (ZnSO₄.7H₂O) were added at (8.6, 17.2 and 25.8 mg/l)
- 3- Cupric Sulfate (CuSO₄.5H₂O) were added at (0.025, 0.050 and 0.075 mg/l)

6.0 g/L agar were used to solidified Culture medium which were distributed in culture jars (250 ml); each jar contained 25 ml of culture nutrient medium. Culture jars were immediately capped with polypropylene closure autoclaved at 121°C at 1.05 kg/cm² for 20 min. The cultured jars were incubated under total darkness at 27±1°C and data were recorded every (6 weeks) for three subcultures on total steroids content (mg/g dry weight).

Callus samples were collected from all studied treatments of the micro elements compounds, Manganese sulfate (MnSO₄.4H₂O), Zinc sulfate Heptahydrate (ZnSO₄.7H₂O) and Cupric Sulfate (CuSO₄.5H₂O) for both Bartamuda and Sakkoty cv. for the following assay

1. Determination of free amino acids

Total amino nitrogen or free amino acids were determined according to Rosein [13]. For assay, one ml of sample was pipetted out into a series of test tubes, and then total volume made up to 4 ml with distilled water. One ml of ninhydrin reagent (4 %, 4 g ninhydrin was dissolved in 50 ml acetone and 50 ml acetate buffer) was added to each tube, mixed well, and the tubes were kept in a boiling water bath for 15 min. Then, the tubes were cooled and the volume was made up to 10 ml in measuring flask with ethanol 50 %. The pink color developed was measured using a

spectrophotometer at 570 nm DL-alanine. The concentration of total amino nitrogen as DL-alanine were calculated from the standard curve.

2. Extraction of Indoles and Phenols

One gram of fresh samples in three replicates were sectioned into minute pieces and extracted with 5 ml cold methanol 80 % and stored in cold condition for 24h. The combined extracts were collected and filtered. Then, the volume of sample was raised up to known volume with cold methanol.

A Determination of Total Indoles

The total indoles were determined in the methanolic extract using p-dimethyl amino benzaldehyde (PDAB) reagent, 1 g was dissolved in 50 ml HCl conc. and 50 ml ethanol 95 %) test according to Larsen et al., [14]. One ml of aliquot methanolic extract was pipetted into a test tube, then 4 ml of PDAB reagent was added and incubated at 30 – 40 °C for 1 h. The intensity of the resultant color was spectrophotometrically measured at 530 nm. A standard curve was established which refer to the relationship between different concentrations of IAA and their corresponding absorbance values.

B Determination of Total Phenols

Phenols determination was carried out according to Danial and George [15]. For estimation of total phenols, 1 ml of the methanol tissue extract was added to 0.5 ml of Folin-Ciocalteu's Phenol Reagent and shaken 3 min. Then, 1 ml saturated Na₂CO₃ (25 % w/v) plus 17.5 ml distilled water added. The mixtures were left for one hour at 30- 40 °C. Optical density of these samples was measured by a colorimeter using wavelength 730 nm. Concentrations of total phenols in different samples were calculated as mg phenol/100g FW. Amount of total phenolic compounds was calculated according to standard curve of pyrogallol (99.5 %).

Statistical analysis

The obtained data were subjected to analysis of variance. The mean values were compared using LSD test at the 5% level of probability. The data were tabulated and statistically factorial analysed according to the randomized complete block design with three replicates Snedecor & Cochran [16].

Results and Discussion

Effect of manganese sulfate (MnSO₄.4H₂O) concentration on some chemical component (total of amino acids, Indoles and Phenols) in embryogenic callus stage of in vitro date palm (Sakkoty and Bartamuda cultivar)

Effect of manganese sulfate on total amino acids content (mg/g fresh weight)

Data in Table (1) clearly showed that no significant differences were found between the two cultivars under investigation (0.90, 0.90 mg/g fresh weight), was for Bartamuda and Sakkoty respectively. The manganese sulfate concentration 22.3mg/l was the most effective forming the highest significant value (1.73 mg/g fresh weight).Concerning the interaction between cultivars and manganese sulfate concentrations, the results illustrated that the highest significant value (1.75 mg/g fresh weight) was for Bartamuda cultivar embryogenic callus grown on medium contained 22.3mg/l manganese sulfate. The lowest value (0.29 mg/g fresh weight) was for Bartamuda cultivar embryogenic callus grown on medium contained 66.9 mg/l.

Table 1: Effect of manganese sulfate on total amino acids content (mg/g fresh weight).

Cultivar (A)	Manganese sulfate mg/ l (B)			
	22.3	44.6	66.9	Mean (A)
Bartamuda	1.75	0.67	0.29	0.90
Sakkoty	1.71	0.58	0.42	0.90
Mean (B)	1.73	0.62	0.35	
L.S.D 0.05: A=N.S, B=0.30, AB=0.43				

Effect of manganese sulfate on total indoles content (mg/g fresh weight)

Table 2: Effect of manganese sulfate on total indoles content (mg/g fresh weight).

Cultivar(A)	Manganese sulfate mg/ l (B)			
	22.3	44.6	66.9	Mean (A)
Bartamuda	0.33	0.16	0.15	0.21
Sakkoty	0.29	0.13	0.08	0.16
Mean (B)	0.31	0.15	0.11	
L.S.D 0.05: A=0.046, B=0.057, AB=0.081				

Data in Table (2) clearly showed that significant differences were observed between the two cultivars under investigation (0.21, 0.16 mg/g fresh weight, Bartamuda ,Sakkoty respectively), the manganese sulfate concentration 22.3 mg/l was the most effective, forming the highest significant value (0.31 mg/g fresh weight), concerning the interaction between cultivars and manganese sulfate concentrations, the results illustrated that the highest significant value (0.33 mg/g fresh weight) was recorded by Bartamuda cultivar embryogenic callus which was grown on medium contained 22.3mg/l manganese sulfate. The lowest value (0.08 mg/g fresh weight) was for Sakkoty cultivar embryogenic callus grown on medium contained 66.9 mg/l.

Effect of manganese sulfate on total phenols content (mg/g fresh weight)

Table 3: Effect of manganese sulfate on total phenols content (mg/g fresh weight).

Cultivar (A)	Manganese sulfate mg/ l (B)			
	22.3	44.6	66.9	Mean (A)
Bartamuda	0.48	0.80	1.17	0.82
Sakkoty	0.37	0.83	1.07	0.76
Mean (B)	0.42	0.82	1.12	
L.S.D 0.05, A=N.S, B=0.13, AB=0.19				

Data in Table (3) clearly showed that no significant differences were found between the two cultivars under investigation (0.82, 0.76 mg/g fresh weight, Bartamuda, Sakkoty respectively).The manganese sulfate concentration 66.9 mg/l was the most effective. The highest significant value (1.12 mg/g fresh weight), concerning the interaction between cultivars and manganese sulfate concentrations, the highest significant value (1.17 mg/g fresh weight) was for

Bartamuda cultivar embryogenic callus grown on medium contained 66.9 mg/l manganese sulfate. The lowest value (0.37 mg/g fresh weight) was for Sakkoty cultivar embryogenic callus grown on medium contained 22.3 mg/l.

Effect of Zinc sulfate Heptahydrate (ZnSO₄.7H₂O) concentration on some chemical component (total of amino acids, Indoles and Phenols) in embryogenic callus stage of in vitro date palm (Sakkoty and Bartamuda cultivar)

Effect of zinc sulfate on the total amino acid content (mg/g fresh weight)

Table 4: Effect of zinc sulfate on total amino acid content (mg/g fresh weight).

Cultivar (A)	zinc sulfate mg/ l (B)			
	8.6	17.2	25.8	Mean (A)
Bartamuda	0.77	1.63	0.42	0.94
Sakkoty	0.57	1.64	0.36	0.86
Mean (B)	0.67	1.64	0.39	
L.S.D 0.05: A=N.S, B=0.30, AB=0.43				

Data in Table (4) showed that, no significant differences were noticed between the two cultivars under investigation (0.94, 0.86 mg/g fresh weight respectively) zinc sulfate concentration 17.2 mg/l was the most effective as it induced, the highest significant value was(1.64 mg/g fresh weight), concerning the interaction between cultivars and zinc sulfate concentrations, the highest significant value (1.64 mg/g fresh weight) was produced by for Sakkoty cultivar embryogenic callus grown on medium contained 17.2 mg/l zinc sulfate. The lowest value (0.36 mg/g fresh weight) was for Sakkoty cultivar embryogenic callus grown on medium contained 25.8 mg/l zinc sulfate.

Effect of zinc sulfate on the total indoles content (mg/g fresh weight)

Table 5: Effect of zinc sulfate on total indoles content (mg/g fresh weight).

Cultivar (A)	zinc sulfate mg/ l (B)			
	8.6	17.2	25.8	Mean (A)
Bartamuda	0.076	0.376	0.113	0.188
Sakkoty	0.090	0.400	0.133	0.207
Mean (B)	0.083	0.388	0.123	
L.S.D 0.05: A=N.S, B=0.070, AB=0.099				

Data in Table (5) showed that no significant differences were formed between the two cultivars under investigation (0.18, 0.20 mg/g fresh weight respectively), zinc sulfate concentration(17.2mg/l) was the most effective as it produced ,the highest significant value was(0.38 mg/g fresh weight), concerning the interaction between cultivars and zinc sulfate concentrations, the highest significant value (0.40 mg/g fresh weight) was for Sakkoty cultivar

embryogenic callus grown on medium contained(17.2 mg/l) zinc sulfate. The lowest value (0.076 mg/g fresh weight) was for Bartamuda cultivar embryogenic callus grown on medium contained (8.6 mg/l) zinc sulfate.

Effect of zinc sulfate on the total phenols content (mg/g fresh weight)

Table 6: Effect of zinc sulfate on the total phenols content (mg/g fresh weight).

Cultivar (A)	zinc sulfate mg/ l (B)			
	8.6	17.2	25.8	Mean (A)
Bartamuda	0.81	0.31	1.20	0.78
Sakkoty	0.66	0.44	1.24	0.78
Mean (B)	0.73	0.38	1.22	
L.S.D 0.05: A=N.S, B=0.22, AB=0.31				

Data in Table (6) showed that no significant differences were formed between the two cultivars under investigation (0.78, 0.78 mg/g fresh weight respectively), zinc sulfate concentration 25.8mg/l was the most effective as it induced, the highest significant value was (1.22 mg/g fresh weight), concerning the interaction between cultivars and zinc sulfate concentrations, the highest significant value (1.24 mg/g fresh weight) was for Sakkoty cultivar embryogenic callus grown on medium contained 25.8 mg/l zinc sulfate. The lowest value (0.31 mg/g fresh weight) was for Bartamuda cultivar embryogenic callus grown on medium contained 17.2 mg/l zinc sulfate.

Effect of Cupric Sulfate (CuSO₄.5H₂O) concentration on some chemical component (total of amino acids , Indoles and Phenols) in embryogenic callus stage of in vitro date palm (Sakkoty and Bartamuda cultivar)

Effect of cupric sulfate on total amino acids content (mg/g fresh weight)

Table 7: Effect of cupric sulfate on total amino acids content (mg/g fresh weight).

Cultivar (A)	cupric sulfate mg/l (B)			
	0.025	0.050	0.075	Mean (A)
Bartamuda	1.36	0.61	0.30	0.75
Sakkoty	1.14	0.84	0.31	0.76
Mean (B)	1.25	0.72	0.31	
L.S.D 0.05: A=N.S, B=0.11, AB=0.16				

Data in Table (7) showed that no significant differences were found between the two cultivars under investigation (0.75, 0.76 mg/g fresh weight respectively),cupric sulfate concentration 0.025mg/l was the most effective as it induced, the highest significant value (1.25 mg/g fresh weight), concerning the interaction between cultivars and cupric sulfate concentrations, the highest significant value (1.36 mg/g fresh weight) was for Bartamuda cultivar embryogenic callus grown on medium contained 0.025 mg/l cupric sulfate. The lowest value (0.30 mg/g fresh weight) was for Bartamuda cultivar embryogenic callus grown on medium contained 0.075 mg/l cupric sulfate.

Effect of cupric sulfate on total indoles content (mg/g fresh weight)

Table 8: Effect of cupric sulfate on total indoles content (mg/g fresh weight).

Cultivar (A)	cupric sulfate mg/l (B)			
	0.025	0.050	0.075	Mean (A)
Bartamuda	0.40	0.16	0.09	0.22
Sakkoty	0.25	0.11	0.07	0.14
Mean (B)	0.32	0.14	0.08	
L.S.D 0.05: A=0.033, B=0.040, AB=0.057				

Data in Table (8) showed that, significant differences were found between the two cultivars under investigation (0.22, 0.14 mg/g fresh weight respectively), cupric sulfate concentration 0.025mg/l was the most effective as it produced the highest significant value (0.32 mg/g fresh weight), concerning the interaction between cultivars and cupric sulfate concentrations, the highest significant value (0.40 mg/g fresh weight) was for Bartamuda cultivar embryogenic callus grown on medium contained 0.025 mg/l cupric sulfate. The lowest value (0.07 mg/g fresh weight) was for Sakkoty cultivar embryogenic callus grown on medium contained 0.075 mg/l cupric sulfate.

Effect of cupric sulfate on total phenols content (mg/g fresh weight)

Table 9: Effect of cupric sulfate on total phenols content (mg/g fresh weight).

Cultivar (A)	cupric sulfate (CuSO ₄ .5H ₂ O)mg/l (B)			
	0.025	0.050	0.075	Mean (A)
Bartamuda	0.34	1.28	1.53	1.05
Sakkoty	0.47	1.56	1.83	1.28
Mean (B)	0.41	1.04	1.68	
L.S.D 0.05: A=0.18, B=0.23, AB=0.32				

Data in Table (9) clearly showed that, significant differences were found between the two cultivars under investigation (1.05, 1.28 mg/g fresh weight respectively), cupric sulfate concentration 0.075mg/l was the most effective as it produced the highest significant value was (1.68 mg/g fresh weight), concerning the interaction between cultivars and cupric sulfate concentrations, the highest significant value (1.83 mg/g fresh weight) was for Sakkoty cultivar embryogenic callus grown on medium contained 0.075 mg/l cupric sulfate. The lowest value (0.34 mg/g fresh weight) was recorded by Bartamuda cultivar embryogenic callus grown on medium contained 0.025 mg/l cupric sulfate.

The present results regarding the beneficial effect of manganese sulfate (MnSO₄.4H₂O) zinc sulfate (ZnSO₄.7H₂O) and cupric sulfate (CuSO₄.5H₂O) as microelements in congeniality with the findings of several investigators on some species. Plant-produced secondary compounds have been contributed into a wide range of commercial and manufacturing applications. Obviously, in many cases, rigorously controlled plant *in vitro* cultures can generate the same valuable natural

products [7]. There are many studies made on the method that could be used for the enhancement of the production of valuable secondary metabolites. Microelements are required in trace amounts (Manganese, iodine, copper, cobalt, boron, molybdenum, iron, and zinc) usually comprise the microelements for plant growth and development, and have many diverse roles. The effects of the medium employed in various processes have been reported [8]. It has been reported that proper concentration microelements have been considered as nutrient factors or as abiotic elicitors, which trigger the formation of secondary metabolites [9]. Where, Metal ions cause stress at elevated concentrations and stress has been implicated in secondary metabolite production. Many studies were undertaken to assess the role of metal stress on growth and differentiation as well as on secondary metabolite production in plants [17]. Copper, for example, is essential for the function of many oxidases and oxygenases with a key role in secondary metabolism. Also, Copper deficiency strongly inhibits the activities of diamine oxidase, which is essential for the metabolism of the diamines putrescine and cadaverine of polyphenoloxidase, and of superoxide dismutase (Cu/ZnSOD). Micronutrients without redox functions are also directly or indirectly involved in plant secondary metabolism. Zinc is required for the activity of thousands of plant proteins. Manganese is essential for Mn-SOD activity. In the shikimate pathway, Mn stimulates the pre-chorismate step catalyzed by the metalloenzyme 3-deoxy-D-arabinoheptulosonate 7-phosphate synthase [9]. It could be suggested that the production of preformed phytochemicals can also be significantly enhanced by those, trace elements which are being widely used to stimulate the production of active principles from callus cultures.

Summary

Studies in this area could lead to the successful manipulation of secondary metabolism and could significantly increase the amounts of the compounds. It should be possible to achieve the synthesis of a wide range of compounds in date palm callus cultures.

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