Materials Research Proceedings **11** (2019) 213-218 doi: https://doi.org/10.21741/9781644900178-16

Effect of some Micro-Elements on Steroids Production from Embryogenic Callus of *in vitro* Date Palm Sakkoty and Bartamuda Cultivars

Sherif F. El sharabasy[1,a*], Abdel-Aal W. B.[1], Hussein A. Bosila[2], Bayome M. Mansour[2] and Abdel-Monem A. Bana[1]

[1]The Central Lab of Date Palm Researches and Development, ARC, Egypt.

[2]Floriculture, Medicinal & Aromatic Plants. Dept., Fac. of Agric., Al-Azhar University, Egypt.

[a]sharabasydates@yahoo.com

Keywords: micro-elements, steroids, embryogenic callus, manganese sulfate, zinc sulfate, cupric sulfate, date palm cultivars

Abstract. The ability of plant cell, tissue, and organ cultures to produce and accumulate many of the same valuable chemical compounds as the parent plant in nature has been known almost since the inception of *in vitro* technology. Date palm has been recognized as an important crop containing high valuable secondary metabolism. Some microelements such as, manganese sulfate ($MnSO_4 2H_2O$), zinc sulfate ($ZnSO_4 7H_2O$) and copper sulfate ($CuSO_4 5H_2O$) were used as precursor to produce steroids from embryonic callus two date palm dry cvs. In this study, embryogenic callus explants were cultured on MS nutrient medium supplemented with different concentrations of ($MnSO_4 2H_2O$), (22.3, 44.6 and 66.9 mg/l), $ZnSO_4 7H_2O$ (8.6, 17.2 and 25.8 mg/l) and $CuSO_4 5H_2O$ (0.025, 0.050, 0.075 mg/l). The highest significant value of total steroids (0.94 mg/g dry weight) was recorded when embryogenic callus of Sakkoty cv. was cultured on medium contained (22.3mg/l) $MnSO_4 2H_2O$. Where embryogenic callus of Bartamuda cv. cultured on nutrient medium supplemented at (17.2 mg/l) $ZnSO_4 7H_2O$ gave the highest significant value of total steroid (0.92 mg/g dry weight).

Introduction

Date palm (*Phoenix dactylifera* L.) is a member of (Arecaceae) family it is a heterozygous and dioecious tree it was known in ancient Egypt since 4000 years ago and this fact can be simply indicated from date palm inscriptions appearing on the walls of ancient Egyptian temples. In general, the importance of this tree all over in its cultivation region in North Africa and the Middle East was referred to the numerous advantages from its fruits and from the tree as a whole [1]**.** Biotechnology approach has a great deal for the production of chemicals and pharmaceuticals from *in vitro* plant cell culture [2].

Steroids are a set of cholesterol derivative lipophilic that are low molecular weight and may found in synthetic sources. They include sterols, hormones gonadal and adrenal ones, hydrocarbons and bile acids. Steroids family plays an important role in the biochemistry and composition of organisms [3]. Steroids are used as anti-cancer agents, antibiotics, and anti-inflammatory, and anti-hormones drugs [4]. First study for the steroids production in date palm tissues was documented by El Sharbasy *et al*., [5] who found that total steroids in tow Egyptian cultivars tissues demonstrated higher values in pollen grain and shoot tip of in vivo tissues, and also in leaf and roots of the *in vitro* tissues. Also**,** the separation and identification of cholesterol and ß-sitosterol from callus cultures by Thin Layer Chromatography (TLC), was detected. El-Sharabasy [6] indicated also, that the precursors have great effect in the biosynthesis of steroids

Materials Research Proceedings **11** (2019) 213-218 doi: https://doi.org/10.21741/9781644900178-16

in date palm callus and embryogenic callus cells. Enhancement in secondary metabolite production can be obtained by selection of high-producing and medium optimizations [7,8]. Microelements are required in trace amounts for plant growth and development and have many diverse roles [9]. Manganese, iodine, copper, cobalt, boron, molybdenum, iron, and zinc usually comprise the microelements, although other elements, such as nickel and aluminum, are frequently found in some formulations. Iron is usually added as iron sulphate, although iron citrate can also be used. The aim of this work is to study the effect of Microelements on steroids production content (mg/ g dry weight) in embryonic callus stage of *in vitro* date palm Bartamuda, Sakkoty cultivars in order to optimize strategy for enhancing steroids production from *in vitro* date palm tissues by targeting manipulation of culture media composition.

Materials And Methods

Preparation of plant material

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 Na_2HPO_4,0.1 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). Pyruvic acid was added at 0.01 mg/l to induce steroids compounds production [13]

Micro elements compounds, manganese sulfate ($MnSO_42H_2O$), zinc sulfate heptahydrate ($ZnSO_47H_2O$) and copper sulfate ($CuSO_4.5H_2O$) 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 ($MnSO_42H_2O$) were added at (22.3, 44.6 and 66.9 mg/l)

2- Zinc sulfate Heptahydrate ($ZnSO_47H_2O$) were added at (8.6, 17.2 and 25.8 mg/l)

3- Copper sulfate ($CuSO_4.5H_2O$) 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/cm^2 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 ($MnSO_42H_2O$), Zinc sulfate Heptahydrate ($ZnSO_47H_2O$) and copper sulfate ($CuSO_4.5H_2O$) for both Bartamuda and Sakkoty cv. for total steroids assay

Determination of total steroids (mg/g dry weight)

Total steroids were calculated as β-sitosterol and determined by spectrophotometer according to the methods described by [11] as follows.

Test solution preparation: - 0.5 g weight of embryogenic callus sample is dried in an oven at 75 °C for 48 h. dried embryogenic callus sample is placed in a clean flask, with addition of 100 ml of 5% potassium hydroxide solution in alcohol (90% v/v) and are heated on a water bath at 50°C to smooth reflux for 2 hours, then are cooled for 5 min, then the flask contents are transferred to a separator funnel. The residual contents of flask were washed for two times, firstly with 100 ml water followed by 100 ml diethyl ether then the washings were transferred

Materials Research Proceedings **11** (2019) 213-218 doi: https://doi.org/10.21741/9781644900178-16

into the same separator funnel and they are shacked altogether slowly by hand for 3 min. To separate the formed layer the aqueous phase was removed from separator funnel. This layer was washed in a separator funnel four times with 100 ml diethyl ether then, is placed in a clean flask. The received ethereal extracts are washed with three successive portions of 40 ml water (shaking was gently to avoid emulsions), 40 ml 5% w/v hydrochloric acid, and 40 ml 3% w/v potassium hydroxide aqueous solution. Successive portions of 40 ml water (each wash) are edited until the washings become neutral to phenolphthalein solution (2 drops 1% phenolphthalein in 70% ethanol and 2 N NaOH until rose color is stable). One drop of 0.1 N HCl is added to sample and rapidly mix until the rose color disappears. Hundred mg anhydrous sodium sulfate powder is added to the sample with well shacking, then the mixture is filtered through folded Whatman filter paper. The resulted solution is evaporated in water bath at 50°C until fully dry. 100 ml glacial acetic acid is added to the residue with stirring for 30 min in small glass bowl.

Test solution: - 2ml of previous resulted solution is transferred to a 20 ml volumetric flask and dilute to 20 ml with glacial acetic acid.

The reference solution preparation:- 40 mg β-sitosterol is dissolved in 100 ml glacial acetic acid then 5 ml of this solution is taken then diluted to 50 mL with glacial acetic acid.

The deniges reagent preparation: - This reagent is consisting of mixing of two solutions (solution A) is prepared by adding 100 ml sulfuric acid to 50 ml glacial acetic acid. (solution B) is prepared by dissolving 5g mercury oxide (HgO_2) and 20 ml sulfuric acid into 100 ml water. 100 ml of solution (A) is added to 1 ml of solution (B), then are mixed and filtered through a sintered glass filter (grade G4) before use.

Finally, 5 ml of Deniges reagent mixture solutions is added to test tube filled with 1 ml (Test solution) and 1 ml (Reference solution) for evaluation of β-sitosterol amount.

The blank is carried out by 1 ml glacial acetic acid instead of the sample in a test tube. Both tubes are lifted on the stand under the dark for 15 min. The absorbance is read using a spectrophotometer at 510 nm against the blank reading. The amount of steroids is calculated as β-sitosterol from a standard curve prepared by dissolving 40 mg of β-sitosterol in 10 ml glacial acetic acid. Series of standards are prepared as 5, 10, 20, and 40 mg/100 ml, respectively; 1 ml of each is mixed with 5 ml deniges reagent and read at 510 nm against the blank. The absorbance of each concentration is plotted against the absorbance obtained from the standard curve.

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 analyses according to the randomized complete block design with three replicates [14].

Results and Discussion

1. Effect of manganese sulfate on total steroids content (mg/g dry weight).

Data in Table 1 clearly showed that no significant differences were found between the two cultivars under investigation (0.53, 0.61 mg/g dry weight) was for Bartamuda and Sakkoty respectively). The manganese sulfate concentration 22.3 mg/l was the most effective forming the highest significant value (0.83 mg/g dry weight), concerning the interaction between cultivars and manganese sulfate concentrations, the results illustrated that the highest significant value(0.94mg/g dry weight)of total steroids was for Sakkoty cultivar embryogenic callus grown on medium contained 22.3mg/l manganese sulfate. The lowest value (0.25mg/g dry weight) was for Bartamuda cultivar embryogenic callus grown on medium contained 66.9 mg/l.

Materials Research Proceedings **11** (2019) 213-218 doi: https://doi.org/10.21741/9781644900178-16

Table 1: Effect of manganese sulfate on total steroids content (mg/g dry weight).

Cultivar (A)	Manganese sulfate mg/ l (B)			
	22.3	**44.6**	**66.9**	**Mean (A)**
Bartamuda	0.72	0.61	0.25	0.53
Sakkoty	0.94	0.59	0.31	0.61
Mean (B)	0.83	0.60	0.28	
L.S.D 0.05: A=0.057, B=0.070, AB=0.099				

2. Effect of zinc sulfate on the total steroids content (mg/g dry weight).

Table 2: Effect of zinc sulfate on total steroids content (mg/g dry weight).

Cultivar (A)	zinc sulfate mg/ l (B)			
	8.6	**17.2**	**25.8**	**Mean (A)**
Bartamuda	0.70	0.92	0.52	0.71
Sakkoty	0.59	0.83	0.44	0.62
Mean (B)	0.64	0.88	0.48	
L.S.D 0.05: A=0.033, B=0.040, AB=0.057				

Data in Table 2 showed that significant differences were found between the two cultivars under investigation (0.71, 0.62 mg/g dry weight respectively), the zinc sulfate concentration(17.2mg/l) was the most effective, forming the highest significant value (0.88 mg/g dry weight), concerning the interaction between cultivars and zinc sulfate concentrations, the highest significant value(0.92 mg/g dry weight)was for Bartamuda cultivar embryogenic callus grown on medium contained 17.2 mg/l zinc sulfate. The lowest value (0.44 mg/g dry weight) was for Sakkoty cultivar embryogenic callus grown on medium contained 25.8 mg/l zinc sulfate.

3. Effect of copper sulfate on total steroids content (mg/g dry weight).

Table 3: Effect of copper sulfate on total steroids content (mg/g dry weight).

Cultivar (A)	cupric sulfate mg/l (B)			
	0.025	**0.050**	**0.075**	**Mean (A)**
Bartamuda	0.92	0.61	0.32	0.62
Sakkoty	0.85	0.63	0.43	0.63
Mean (B)	0.88	0.62	0.38	
L.S.D 0.05: A=N.S, B=0.040, AB=0.057				

Data in Table 3 showed that no significant differences were found between the two cultivars under investigation (0.62, 0.63 mg/g dry weight respectively), cupric sulfate concentration 0.025mg/l was the most effective as it produced the highest significant value (0.88 mg/g dry weight), There are some reports showing effects of some trace minerals on the production of secondary metabolites, i.e. Cu^{2+} had a positive effect on ginseng saopnin and polysaccharide production in cell cultures of *Panax notoginseng*. [15]. concerning the interaction between cultivars and cupric sulfate concentrations, the highest significant value (0.92 mg/g dry weight) was for Bartamuda cultivar embryogenic callus grown on medium contained 0.025 mg/l copper sulfate. The lowest value (0.32 mg/g dry weight) was for Bartamuda cultivar when the embryogenic callus grown on medium contained 0.075 mg/l cupric sulfate. 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. 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 [8,9,16]. Andrijany *et al*. [17] found in callus cultures of *Agave amaniensis* the relatively high concentration of magnesium, cobalt and copper ions simultaneously inhibited the sapogenin steroid formation while the absence of calcium ions in media induced the increasing in the sapogenin steroid content. It seems to be that the productions of specific useful secondary metabolites by plant cell cultures have intensive researches for economic implications, but until now only a few studies addressing the possibility of date palm steroids production.

Summary

Concentration of microelements have significant role in enhancing steroids accumulation in date palm callus. It could be suggested that medium components at certain concentrations have been considered as nutrient factors or precursors, which trigger the in vitro formation of steroids in date palm metabolites. More intensive studies are needed in this approach.

References

[1] S. Gantait, M.M. El-Dawayati, J. Panigrahi, C. Labrooy, S.K. Verma, The retrospect and prospect of the applications of biotechnology in (Phoenix dactylifera L.), App. Microbial. Biotech. 102 (2018) 8229–8259. https://doi.org/10.1007/s00253-018-9232-x

[2] K. Jamwal, S. Bhattacharya, S. Puri, Plant growth regulator mediated consequences of secondary metabolites in medicinal plants, Journal of applied research on medicinal and aromatic plants (2018). https://doi.org/10.1016/j.jarmap.2017.12.003

[3] A. Sultan, A.R. Raza, Steroids: a diverse class of secondary metabolites Med Chem, 5-7 (2015), 310–317.

[4] S.S. Jovanovi Santa, E.T. Petri, O.R. Klisuric, M. Szecsi, R. Kovacevic, Antihormonal potential of selected D-homo and Dsecoestratriene derivatives Steroids, 97 (2015) 45–53. https://doi.org/10.1016/j.steroids.2014.08.026

[5] S.F. El-Sharabasy, H. Bosila, S.Mohamed, I. Ibrahim, K. Refay, Production of some secondary products from date palm (Phoenix dactylifera) tissue cultures, Sewi cultivar using

some precursors during Embryogenesis stage, Second Emirates conference of date palm. Abu Dhabi (2001) 540-545.

[6] S.F. El-Sharabasy, Effects of some precursors on development of secondary products in tissues and media of embryogenic callus of date palm cv. Sewi. Arab J. Biotech. 7(2004) 83-90.

[7] M.I. Dias, M.J. Sousa, R.C. Alves, I.C. Ferreira, Exploring plant tissue culture to improve the production of phenolic compounds: A review, Industrial Crops and Products 1-82(2016) 9-22. https://doi.org/10.1016/j.indcrop.2015.12.016

[8] I. Smetanska, Production of secondary metabolites using plant cell cultures, In Food biotech Springer, Berlin, Heidelberg (2008) 187-228.

[9] W. Yue, Q.L. Ming, B. Lin, K. Rahman, C.J. Zheng, T. Han, L.P. Qin, Medicinal plant cell suspension cultures: pharmaceutical applications and high-yielding strategies for the desired secondary metabolites, Critical reviews in biotechnology 36-2 (2016) 215-32. https://doi.org/10.1016/j.indcrop.2015.12.016

[10] Z.E. Zayed, Enhanced Indirect Somatic Embryogenesis from Shoot-Tip Explants of Date Palm by Gradual Reductions of 2, 4-D Concentration. In Date Palm Biotechnology Protocols, Humana Press, New York 1 (2017) 77-88. https://doi.org/10.1007/978-1-4939-7156-5_7

[11] M.M. El-Dawayati, H.S. Ghazzawy, M. Munir, Somatic embryogenesis enhancement of date palm cultivar Sewi using different types of polyamines and glutamine amino acid concentration under in-vitro solid and liquid media conditions, Int J Biosci, 12 (2018) 149-159. https://doi.org/10.12692/ijb/12.1.149-159

[12] T. Murashige, F.A. Skoog, revised medium for rapid growth and bioassays with tobacco tissue cultures, Physiol Plant 15 (1962) 473-497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x

[13] El-Sharabasy, M.M. El-Dawayati, Bioreactor steroid production and analysis of date palm embryogenic callus. in: J.M. Al-Khayri, S.M. Jain, D. Johnson (Eds), Date palm biotechnology protocols, Tissue culture and applications, Humana Press, New York 1 (2017) 309–318. https://doi.org/10.1007/978-1-4939-7156-5_25

[14] G.W. Snedecor, W.G. Cochran, "Statisical Methods". Oxford and J.B.H. Publishing Co., 6th edition, 1980, pp. 507.

[15] X.W. Pan, Y.Y. Shi, X. Liu, X. Gao, Y.T Lu, Influence of inorganic microelements on the production of camptothecin with suspension cultures of Camptotheca acuminate, Plant growth regulation 44-1 (2004) 59-63. https://doi.org/10.1007/s10725-004-1654-z

[16] D. Pavokovic, M. Krsnik-Rasol, Complex biochemistry and biotechnological production of -betalains, Food Tech. Biotech 49-2 (2011) 145.

[17] V.S. Andrijany, G. Indrayanto, L.A. Soehono, Simultaneous effect of calcium, magnesium, copper and cobalt ions on sapogenin steroids content in callus cultures of Agave amaniensis, Plant cell, tissue and organ culture 55-2 (1998) 103-108. https://doi.org/10.1023/a:1006119600153