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Effect of Conservation on Steroids Contents of Callus Explants of Date Palm cv. Sakkoti

El-Dawayati, M.M., Zaid, Z.E. and Elsharabasy S.F.

The Central Lab of Date palm Researches and Development, A. R. C., Cairo, Egypt

Abstract: In vitro plant cell cultures have potential for commercial production of secondary metabolites. Date palm tissues produced steroids which have important medicinal value. There are some studies about the precursors which can be used to enhance and increased the production of date palm plant cell of these important secondary metabolites. The focus of the present paper is to discuss the application of tissue culture technology by studying the effect of slow growth conditions storage at $15C^{o}$ with the addition of different type of sugar as osmotic agents (sucrose, sorbitol and mannitol) on the total steroids contents of conserved callus after (4, 8, and 12 months) also survival of conserved callus was demonstrated. Results showed that, the highest significant value of total steroid contents recorded after eight months from conservation period when callus cultured on conservation medium supplemented with sucrose, also gave the best results in the survival of the conserved callus explants during conservation periods .Mannitol sugar in conservation medium revealed a reduction in total steroids contents of conserved callus in all studied conservation period.

Key words: Date palm, *In vitro*, Callus, Secondary products, Steroids, Slow growth condition.

INTRODUCTION

Date palm (*Phonix dactylifera* L.) is considered one of the most important commercial crops in the Arab worlds. Biotechnological applications of plant cell cultures presents the most updated reviews on current techniques in plant culture in the field, rapid propagation of date palm through tissue culture is the most promising technique for production of sufficient planting materials (off shoots) and obtaining high quality (Watanabe, 1997). The evolving commercial importance of the secondary metabolites has in recent years a great interest, in secondary metabolism, and particularly in the possibility to alter the production of bioactive plant metabolites by means of cell culture technology. The principle advantage of this technology is that it may provide continuous, reliable source of plant pharmaceuticals and could be used for the large-scale culture of plant cells from which these metabolites can be extracted. Advances in the area of cell cultures for the production of medicinal compound have made possible the production of a wide variety of pharmaceuticals like alkaloids, terpenoids, steroids, saponins, phenolics, flavanoids, and amino acids. Successful attempts to produce some of these valuable pharmaceuticals in relatively large quantities by cell cultures are illustrated. (Mulabagal *et al*., 2004).

Steroid belong to a large group of compounds known as terpenoids or isopernoids Nothing may well be suggested concerning drugs which are utilized in the curing of sterility except date palm pollen grains, which have been recognized by the Egyptian and Arabs to be nutritive and used as antisterility agent. Cholesterol and coprostanol are the animal sterols, while, B-sitosterol, campestral, stigmasterol, ergosterol and brassicasterol are the principal plant sterols, (Bailey, 1964). Cholesterol, which is the main animal sterol, has lately been established to be to a certain extent widely dispersed among plants. Up to now, cholesterol has been recognized in the pollen of many plants as well as the date palm (Bennett *et al*., 1996) and in oil palm (Slover *et a*l., 1983). Some of the medicinal compounds localized in morphologically specialized tissues or organs of native plants have been produced in culture systems not only by inducing specific organized cultures, but also by undifferentiated cell cultures.

Elsharabasy, (2000) separated and identified the steroids cholesterol and ß-sitosterol from the tissues of two kinds of date palm Zaghloul and Sewi cultivars by Thin Layer Chromatography (TLC), besides the identification of stigma sterol which was detected in pollen grains. The spectrophotometric verification of total steroids in the tissues of Zaghloul and Sewi cultivars demonstrated higher values in pollen grain and shoot tip of in vivo tissues. Also in callus tissues leaf and roots of the in vitro tissues. (Abdel-Aal , 2011) studied the effect of MS salt strength of nutrient culture medium and some micro elements, some vitamins and some amino acids as a precursors of secondary metabolites (total steroids) production in callus tissues of date palm cv. Sakkoti. Callus cultures are usually maintained by subculture. However, serial transfer may induce polyploidy, genetic variation, loss of ability in morphogenesis and deterioration of biosynthesis of secondary metabolites. As the subculture of many clones is labor-intensive and costly, prolongation of the duration of subculture of the callus by decreasing the metabolism is a useful method for solving these problems (Moriguchi *et al*., 1988). To maintain regeneration potential of callus, many approaches have been employed, Slow growth in vitro may be

Corresponding Author: El-Dawayati, M.M., The Central Lab of Date palm Researches and Development, A. R. C., Cairo, Egypt
E-mail: zemmz2005@yahoo.com

obtained by low temperature, Osmotic stress (Withers, 1991), or a low concentration of nutrients (Engelmann, 1991). Minimal growth storage is a very simple technique that allows storage of plants *in vitro* for periods ranging from 6 months to 5 years, depending on species. These stored plants can be micropropagated rapidly when desired. The aim of this study is to discuss the effect of slow growth condition at 15C^{o} of conservation temperature during three duration (4, 8 and 12 months) of conservation period by using osmatic agents (sucrose, sorbitol and mannitol) which implicated in conservation medium on the callus contains of steroids as secondary products and the ability of conserved callus explants to survive and to resume their development after returning to normal growth conditions.

MATERIALS AND METHODS

The experimental work was performed at the laboratory of date palm researches and development during the period from 2009 to 2011. Callus explants which were obtained from sterilized shoot tip explants of date palm were used as explants material for conservation under minimal growth condition. Callus explants divided into pieces each piece was approximately about 1cm x1cm as mentioned by EL-Dawayati, 2000.These callus pieces were conserved on conservation media which consists of MS (Murashige and Skoog medium 1962) basal nutrient medium + 10.0 mg/l 2,4-D + 3.0mg/l 2ip +1.5 g/l activated charcoal with addition of different sugars (sucrose, sorbitol or Mannitol) at 0.3M concentration. The pH of conservation medium was adjusted to 5.7 ± 0.1 prior to addition of 8.0 mg/L agar. The medium of each treatment was distributed into culture jars (150 mL) where each one contained 40 mL. The culture jars were immediately capped with polypropylin closure and then the medium was sterilized by autoclaving at 121˚C and 15 Ibs/in2 for 20 min. The culture jars conserved under complete darkness at 15˚C for 4, 8 and 12 months. Each treatment = 3 replicates and each replicate = 3 culture jars. The data were calculated on the total steroids contents of conserved callus explants and survival of conserved callus explants after4, 8 and 12 months

Recovery:

The survival percentage and viability of callus explants were evaluated at the end of each conservation period (4,8and 12 months).The explants of each conservation treatment were transferred at the end of different conservation period and recultured for 4 weeks on normal growth medium for callus induction which consists of MS basal nutrient medium supplemented with 10.0mg/l 2,4-D+3.0 2ip mg/l and 30.0 g/l sucrose (Tisserat, 1981). Each jar contained one conserved callus explants incubated under normal growth condition (27 ± 2 C^{o} of incubation temperature under complete darkness for 24 hrs).

Total Steroids Determination:

Data were taken about the contents of total steroids of conserved callus explants which recultured on normal growth medium for callus induction for 4 weeks under normal condition of growth after each conservation duration (4,8 and 12 month) to record the changes in total steroids production during storage conditions.

Total steroids were calculated and determined by spectrophotometer according the methods described by (Pharco 1993, El-Sharabasy, 2000, Abdel-Aal, 2011).

Layout of the Experiments:

The randomized factorial design was used and data were subjected to analysis of variance. Separation of means among treatments was determined using L.S.D test at 5% according to Snedecor and Cochran (1972).

RESULTS AND DISSCUTION

Effect of Different Sugar Type on Survival of Conserved Callus at 15C˚During Conservation Period (4, 8 and 12 Months)

Our data in **Table (1)** showed that, the survival percentage of conserved callus explants of date palm cv. Sakkoti was significantly affected by the addition of different sugar type (sucrose, sorbitol and mannitol) at 0.3M to conservation medium at 15C˚ of conservation temperature under complete dark, conserved callus explants which cultured on conservation medium with mannitol exhibited death rate when they returned to culture on recovery for 4 weeks after conservation period to give the lowest significant value of survival percentage as (85.18%) while when sucrose or sorbitol were added to conservation medium during conservation period no death rate were observed for conserved callus explants when returned to culture on recovery for 4 weeks after conservation period the survival percentage was (100%)

In this concern El-Dawayati, 2008 found that Different sugar (sucrose ,sorbitol or mannitol) concentrations, (0.1, 0.3, 0.5 or 0.7) added to conservation media did not affect significantly the survival percentage of conserved callus explants of date palm Gundila cv. at 15 C^{o} of slow growth condition but showed that these

different sugar type added to conservation media gave significant effect on the survival percentage of the callus explants and all callus explants conserved on medium supplemented with sucrose or sorbitol were able to survive as the survival percentage was 100% while, 86.10% of callus explants conserved on medium supplemented with mannitol able to survive. In General Plants have adapted particularly to resource stress causing osmotic imbalance, Leading to metabolic changes and altered physiological states there by affecting developmental progression (Siobhan *et al*., 2003). Sucrose at high concentrations is known to have adverse effects on morphogenesis (Danso and Ford – LIoyd, 2004). Staikidou *et al*., (2005) mentioned that it is difficult in separating substrate and osmotic effects of sucrose. Sugar alcohols are not usually metabolized by plant tissues and generally cannot be used as carbon sources. For this reason mannitol and sorbitol are frequently employed as osmotica to modify the water potential of a culture medium. In these circumstances sufficient sucrose must also be present to supply the energy requirement of the tissue.

Osmotic regulators, such as sucrose and mannitol, act as growth retardants by causing osmotic stress to the material under conservation. When added to the culture medium, these carbohydrates reduce the hydric potential and restrict the water availability to the explants (Fortes & Scherwinski-Pereira 2001; Shibli *et al*., 2006).

Table 1: Effect of different sugar type supplemented in conservation medium during different conservation period (4, 8 and 12 months) on the survival percentage of callus of date palm cv Sakkoti at 15 C˚

Sugar type Mol	Conserved period			
	4	8	12	Mean
Sucrose	100	100	100	100
Sorbitol	100	100	100	100
Manitol	100	85.18	70.36	85.18
Mean	100	95.06	90.12	

We can indicate that the tested duration (4, 8 and 12 months) of conservation period effected significantly on the survival percentage of the conserved callus explants when they returned to culture on recovery for 4 weeks after conservation period ,there was no death rate was recorded on recovery for 4 weeks after 4 months duration of conservation period as the highest significant value of survival percentage followed by the survival percentage of conserved callus explants during 8 months duration of conservation period as (100% and 95.06 % respectively), where conserved callus explants with 12 months duration of conservation period gave the lowest significant results of survival percentage as (90.12%) when conserved callus explants returned to culture on recovery for 4 weeks after conservation period

It could be observed from data that our result is confirmed with (El-Dawayati, 2008) who demonstrated that conservation period (6 and 12 months) affected significantly the survival percentage of callus explants of date palm Gundila cv. and all callus explants conserved for 6 months able to survive (100%).While, 90.73% of callus explants conserved for 12 months able to survive.

Regarding to the effect of interaction between the different type of sugar (sucrose, sorbitol and mannitol) which supplemented in conservation medium and the conservation period duration (4, 8 and 12 months) the presence of sucrose or sorbitol in conservation medium during (4, 8 and 12 months) duration of conservation period showed 100% survival percentage for all conserved callus explants when they returned to culture on recovery for 4 weeks after each conservation period but with addition of mannitol sugar to conservation medium only conserved callus explants stored for 4 months at 15 C^o of conservation temperature did not record death rate when they returned to culture on recovery medium for 4 weeks, it is clearly that the death rate of conserved callus explants increased when mannitol sugar was used in conservation medium with the increased of the storage duration (8 and 12 months) the lowest significant survival percentage was obtained for conserved callus explants when they returned to culture on recovery for 4 weeks after 12 months duration of conservation period as (70.36%) followed by the survival percentage for conserved callus explants when they returned to culture on recovery for 4 weeks after 8 months duration of conservation period as (85.18%). El-Dawayati, 2008 found that when callus explants of date palm Gundila cv. conserved on mannitol conservation media at 5 or 15 C^o for at least 12 months showed the lowest survival percentage compared with those obtained from callus explants conserved on sucrose or sorbitol media. Bekheet *et al*., (2001) showed that healthy shoot bud cultures of date palm were obtained after 6 months of storage on medium containing 40g/L sorbitol. However, this period extended for 9 months in the case of callus cultures.

Effect of Different Sugar Type on the Total Striods Contents (Mg/G Dry Weight) of Conserved Callus Explants at 15C˚During Conservation Period (4, 8 and 12 Months):

From **Fig**. 1 we can conclude that sugar type (sucrose, sorbitol & mannitol) which used as osmotic agents supplemented in conservation medium had clear effects on the production of total steroids as secondary products of date palm callus tissue during the three tested conservation period (4, 8 and 12 months) under 15C˚ of conservation temperature. Results showed that the conserved callus explants gave the highest significant average value of total striodes contents as (0.812 mg/g d. w) when cultured on conservation medium

supplemented with sucrose sugar followed by average value of total steroids contents as (0.714 mg/g d. w) of conserved callus cultured on conservation medium supplemented with sorbitol sugar ,where the lowest significant average value of total steroids contents as (0.310 mg/g d. w) of conserved callus explants were obtained when cultured on conservation medium supplemented with mannitol sugar. From data we can conclude that conservation period had a great effect on the contents of steroids of conserved callus which recorded the highest average value of total steroids contents after 8 months of conservation period followed significantly by the steroids contents of conserved callus for 12 months of conservation period (0.786 and 0.622 mg/g d. w respectively) where the lowest average value of total steroids contents of conserved callus was recorded after 4 months of conservation period as (0.427 mg/g d. w)

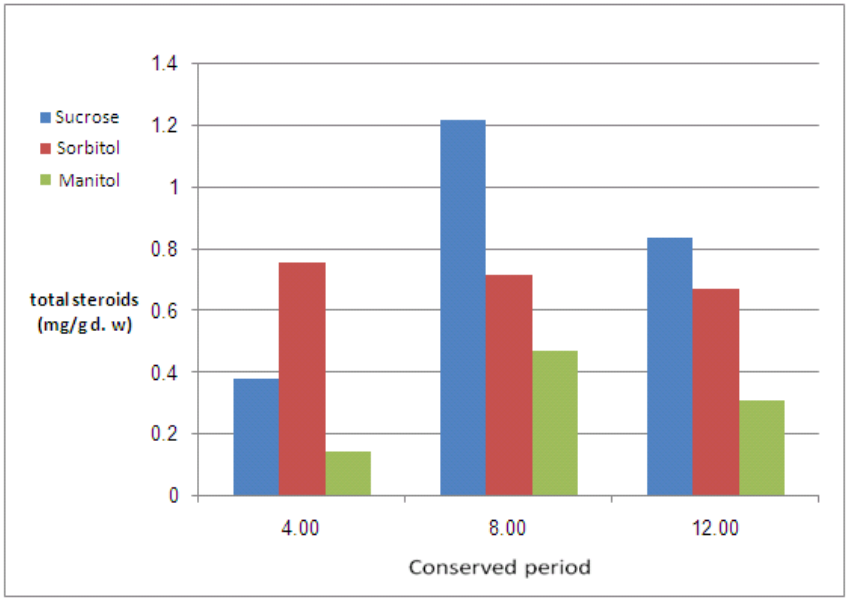

Fig. 1: Effect of different sugar type supplemented in conservation medium during different conservation period (4, 8 and 12 months) on the production of total steroids (mg/g d. w) in the conserved callus explants of date palm cv Sakkoti at 15 C˚

Regarding of the interaction effect between the implicated sugar type in conservation medium and the conservation period on the total steroids contents of conserved callus explants under 15 C^{o} we can observed from data that conservation medium supplemented with sorbitol was superior significantly during 4 months of conservation period in the yield of total steroids contents of conserved callus explants but when sucrose supplemented in conservation medium for 8 months of conservation period the highest average value of total steroids contents of conserved callus explants was obtained, whereas conservation medium supplemented with mannitol gave the lowest average value of total steroids contents of conserved callus explants after each tested conservation period (4, 8 and 12 months)

Our data revealed that the addition of different types of sugar to conservation medium at 15 C^{o} of low temperature plays a clear role with the contents of date palm callus cells of total steroids during conservation period which take our result in parallel with (Collin 2000) who reported that The formation of secondary products in plant tissue cultures is reviewed and the conditions for the enhanced production of secondary products, which include alkaloids, terpenoids, steroids and phenolics, can be regulated in a number of ways by varying the nutrient composition of the growth medium, light, temperature and pH. From our results we can confirmed that preservation of date palm Sakkoti cv. callus explants under slow growth condition for 8 months at 15^{o}C with the addition of sucrose to conservation medium can sustain the potential of plant cells of date palm production of total steroids at higher contents and this agreed with Loureiro and Everson(2011) reported that because of the economic potential of the species with secondary metabolites germplasm conservation is important strategies to guarantee not only the conservation of the species, but also their sustainable use. Slow growth is usually achieved by reducing the culture temperature, by modifying culture media with supplements of osmotic agents and growth inhibitors, or by removing growth promoters to reduce the cellular metabolism of the material, striving to maximize the time between subcultures (Gonçalves & Romano, 2007; Lata *et al*., 2010; Scherwinski-Pereira *et al*., 2010). Carbohydrates strongly affect growth and physiology of plants in all *in vitro*

culture phases, including conservation, as they serve both as carbon sources for cultured tissues and as osmotic regulators in the medium (Pruski *et al*., 2000).

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