A Scanning electron microscopy and X-ray microanalysis studies during the induction of embryogenesis in date palm (*Phoenix dectylifera* L.) cv. Gundila

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Abstract: A scanning electron microscopy (SEM) study during the induction of embryogenesis from cell suspension culture of (*Phoenix dectylifera* L.) cv. Gundila showed that somatic embryogenesis had different developmental stages such as embryo with globular shape as the first stage of somatic embryogenesis followed by torpedo shape as the second stage and finally matured embryo as the third stage in comparison with zygotic embryo. Results from Energy dispersive x-ray microanalysis (EDX) during induction of embryogenesis showed no differences between mature somatic and zygotic embryos in C, O, N, K, Na, Cl, and Zn elements accumulation while there was difference in Al, Ca and Cu accumulation obtained for both mature somatic and zygotic embryo. So we suggest that Energy dispersive x-ray microanalysis (EDX) can be used as signal marker to distinguish between somatic embryo stages.

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1. Introduction

Date palm (***Phoenix dectylifera L.*),** a monocotyledonous species belonging to the palmaceae family, is widely cultivated in arid regions of the middle east and north Africa **(Al-Khayri *et al.*, 2001)** and is one of the most important fruit crops in Egypt. It can be propagated by seeds but rooted offshoots are performed for conventional propagation because they produce true-to type trees with fruit quality identical to that of the mother tree **(Bajaj, 1992; Al-Khayri *et al.*, 2001).** However, there are many problems associated with this system **(Popenoe, 1973);** the availability of offshoots is limited because the few number of offshoots produced by each tree and the difficulty in controlling their growing **(Veramendi *et al*., 1996; Al-Khayri *et al*., 2001).** Also, the offshoots must remain attached to its parent for at least 2-3 years until they have their own roots **(Bajaj, 1992)**.

Furthermore, the way of excision is complicated and time consuming and the percentage of offshoots successfully established in soil is highly variable (30-80 %) **(Veramendi *et al*., 1996; Al-Khayri *et al*; 2001)**. For these above mentioned reasons, micro-propagation gives an attractive alternativesystem for large-scale propagation and commercial production of date palm trees **(Al-Khayri *et al.*,2001).** This is especially true with somatic embryogenesis **(Eshraghi *et al.*, 2005)** which leads to the production of bipolar structures nominally capable of germination without separated shoot and root induction phases. Somatic embryogenesis is the process by which somatic cells, under induction conditions, generate embryogenic cells, which go through a series of morphological and biochemical changes that result in the formation of a somatic embryo. It is also expressed as a model system for studying the morphological, physiological, molecular and biochemical events occurring during the onset and development of embryogenesis in higher plants (**Francisco *et al*., 2006)**, and considered the most efficient regeneration process for date palm micro-propagation **(Fki *et al.*, 2003).** The microscopic study of ultra-structural and elemental changes during the induction of embryogenesis appeared to be an important step to understand the transition process from embryogenic calli to somatic embryos. The information about the accumulation of elements during developmental stages in vitro could be useful for formulating a media for induction of high frequency of embryogenesis in plants **(Desai *et al.*, 2006)**. X-ray microanalysis is the most versatile technique for the quantitative estimation of special distribution of chemical elements in a biological samples **(Zglinicki, 1992)**. This technique can be a useful tool for studying the concentration and distribution changes of certain elements, such as calcium in cells and tissues undergoing morphogenesis **(Fortes and Pais, 2001)**. The aim of

this work was to (i) compare between the external morphology of somatic embryo stages and dry zygotic embryo taken from date palm seeds using scanning electron microscopy (SEM), (ii) study elemental type, and elemental changes occurring during the induction of somatic and zygotic embryogenesis in date palm (Phoenix *dactilifera* L.) cv. Gundila, (iii) determine if elemental compositions accumulated in mature somatic embryos similar to those found in mature zygotic ones using energy dispersive x-ray microanalysis (EDX) as a good tools to achieve this goal. We believe that the obtained information may be useful for those who are developing artificial seeds. Also analysis of elemental composition would offer valuable information on specificity of the elements correlated with a stage of development and to provide data that can be used in formulating suitable nutrient medium to favor a developmental pattern.

2. Material and Methods

This study has been carried out at The Central Laboratory of Date Palm Researches and Development, Agriculture Research Center (ARC), Giza, Egypt. Scanning electron microscopy and energy dispersive X-ray micro analysis studies were done at the Regional center of Mycology and Biotechnology, Al-azhar university, Cairo, Egypt during the period from 2011-2013.

2.1 Plant material and culturing medium.

Friable callus has been donated from the Central Laboratory of Date Palm Researches and Development, Agriculture Research Center (ARC), Giza, Egypt and dissected using sterile scalpel and chopped to small pieces (fine parts) as possible and transferred aseptically into 50 ml of half strength MS liquid medium supplemented with 32 mg l^{-1} Fe–EDTA, 30 g l^{-1} sucrose, 100 mg l^{-1} myo-inositol, 2 mg l^{-1} glycine, 100 mg l^{-1} glutamine, 120 mg l^{-1} KH_2PO_4, 30 mg l^{-1} adenine, 300 mg l^{-1} activated charcoal and 0.5mg/l^{-1} 2,4-D. Cultures were maintained on a rotary shaker with continuous shaking at 120 rpm at 28 ± 2^{o} C under dark conditions. The suspension cultures were passed through a 500 µm mesh filter. Cell suspension cultures and pro embryos were subculture every 15 days. The proliferation and maturation of somatic embryos occurred simultaneously in the same culture vessel.

2.2 Preparation of somatic and zygotic embryos for scanning electron microscopy.

Undifferentiated calli, embryogenic calli and three different stages of somatic embryos obtained from suspension culture of date palm cv. gundila (globular, torpedo and mature stages) were prepared for scanning electron microscopy (SEM). The zygotic embryos used in this experiment were isolated from mature fruit pits of date palm cv. Gundila. Samples were prepared for SEM examination as follows.

2.2.1. Preparation method: chemical fixation, critical point drying.

Samples were fixed by glutaraldhyde 2.5 % for 24 h at 4 ^{o} C and dehydrated by serial dilution of ethanol using automatic tissue processor (Leica EM TP). The samples were dried using CO_2 critical point drier (tousimis audosadri-815). The samples coated by gold sputter coater (spi-module). Finally the samples examined and photographed by scanning electron microscopy (JEOLJSM-5500 LV) using high vacuum mode at the Regional center of Mycology and Biotechnology, Al-azher university, Cairo, Egypt.

2.2.2. Tissue preparation for EDX analysis.

The samples were examined under X-ray micro-analyzer (Module Oxford 6587 INCA x-sight) attached to JOEL JSM-5500 LV scanning electron microscopy at 20 kV after gold coating using SPI-Module sputter coater. The elemental changes of (C, O, N, Na, Mg, Si, Al, P, S, Cl, K, Ca, Fe, Cu and Zn) occurring during somatic embryogenesis of date palm (***Phoenix dactylifera* L.)** cv. gundila were recorded using energy dispersive x-ray microanalysis (EDX)at 20 kV. Samples were prepared according to protocol of SEM lab at the Regional center of Mycology and Biotechnology, Al-azher university, Cairo, Egypt.

3. Results and Discussion

In the present study, cell suspension culture was initiated from callus tissue of date palm cv. Gundila on half strength MS liquid medium containing 0.5 mg l^{-1} 2,4-D, 32 mg l^{-1} Fe–EDTA, 30 g l^{-1} sucrose, 100 mg l^{-1} myo-inositol, 2 mg l^{-1} glycine, 100 mg l^{-1} glutamine, 120 mg l^{-1} KH_2PO_4, 30 mg l^{-1} adenine, 300 mg l^{-1} activated charcoal. In the initial stage, the de-differentiated calli developed into embryogenic type.

3.1. Scanning electron microscopy study:

Scanning electron microscopy was used to compare the external morphology between the somatic embryo of date palm cv. Gundilam at different developmental stages and those of zygotic one. Images presented in Fig. 1. illustrated the morphology of both somatic embryos at different developmental stages and that of zygotic one. Image of A showed the somatic embryo with globular shape while that of B showed the somatic embryo at torpedo shape. Image of C showed somatic embryo at mature stage compared to that of mature zygotic one (D). The altered morphology of somatic embryos is probably due to the tissue culture environment where access to growth hormones and nutrients would not be regulated as they are in the developing seed (Fowke *et al.,*1994). The different developmental stages are depicted in Fig.1.

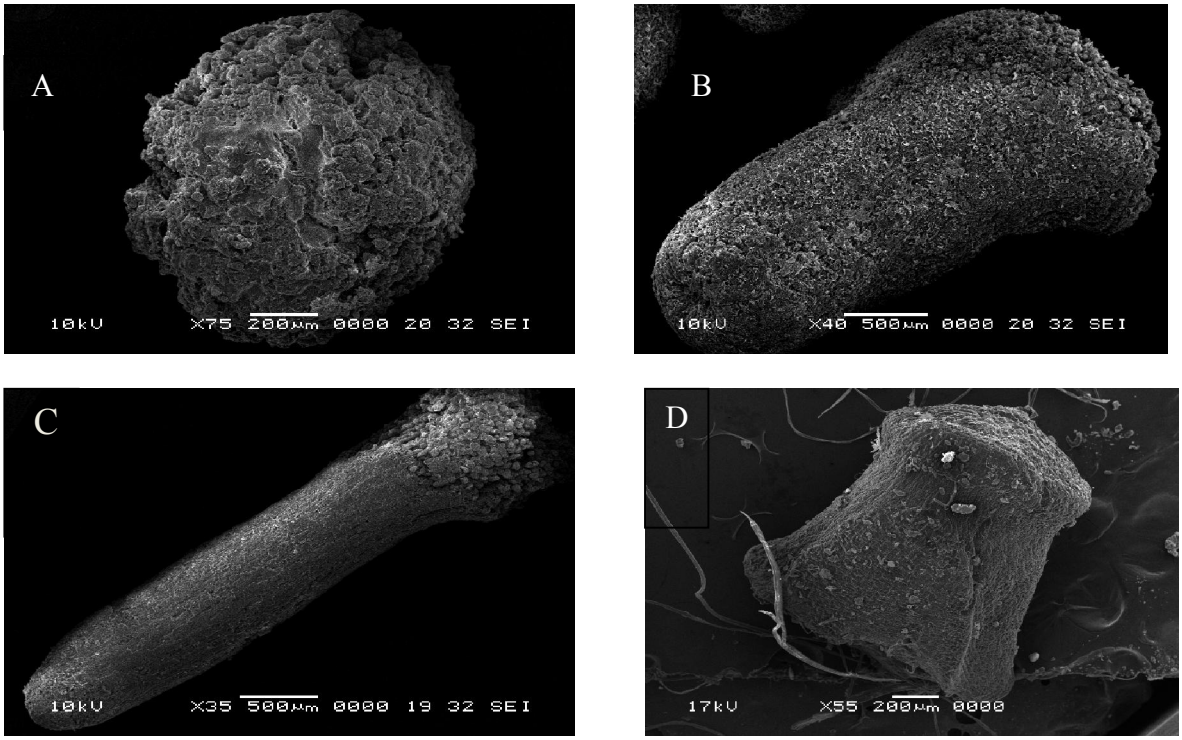


Fig.1. Scanning electron microscopy of date palm cv. Gundila somatic embryos at different developmental stages compared to that of zygotic embryo.
A: Somatic embryo at globular stage, B: Somatic embryo at torpedo stage, C: Somatic embryo at mature stage and D: zygotic embryo.

3.2. X-ray microanalysis study.

X-ray spectra were collected from different developmental stages such as de- differentiated calli (S1), embryogenic calli (S2), embryo with globular shape (S3), embryo with torpedo shape (S4), cotyledonary embryos (S5) and zygotic embryos (S6). The elemental changes (Table 1) and Fig. 2 of different developmental stages showed much variation in (C, O, N, Na, Si, P, Cl, K, Ca, Fe, Zn, Al, Cu, and S) accumulations. Higher (44.02 %) accumulation of C in the torpedo stage (S4) was observed followed by those of zygotic embryo (S6) which gained (41.5 %) compared to de- differentiated calli (S1) which possessed the lowest values of C (29.847) indicating the important role of C accumulation during somatic embryogenesis and embryogenic callus development. Regarding O accumulation, data presented in Table 1 and Fig. 2 showed that higher accumulation of O (56.7 %) was observed in zygotic embryo (S6) followed by that of mature somatic embryo (S5) (55.5 %) compared to embryogenic callus with globular stage (S3) which accumulated 90.4 % of (S6) stage indicating the important role of O accumulation during embryogenic callus development. N was detected in de- differentiated calli (S1), embryogenic calli (S2) only whereas no N was detected either in the other somatic embryogenesis stages or even in zygotic embryo. The higher (3.51 %) Na accumulation was detected in globular stage (S3) followed by embryo with torpedo stage (S4) compared to de-differentiated calli (S1) and embryogenic calli (S2), which possessed (19.94 %) of (S3) indicating the important role of Na accumulation during somatic embryogenesis and embryogenic callus development. This result agree with **Cristena Pedroso and Salome Pais (1992)** who reported that elements other than calcium particularly Na plays an important role in the induction of morphogenesis. Our results in Table 1 and Fig. 2 indicated that light element such as Mg is not detected at any stage of either de- differentiated calli (S1) and embryogenic calli (S2) at any developmental stage or ever in the samples of zygotic embryos. **Cristena Pedroso and Salome Pais,(1994)** also reported that the lower levels of Mg can be due to the greater absorption of low energy X-rays, and/or to Mg^{2+} loss by diffusion. The presence and absence of detectable Mg^{2+} can eventually be related with Mg^{2+} binding to DNA in the absence of synthesis of competing polyamines and to its removal, when they

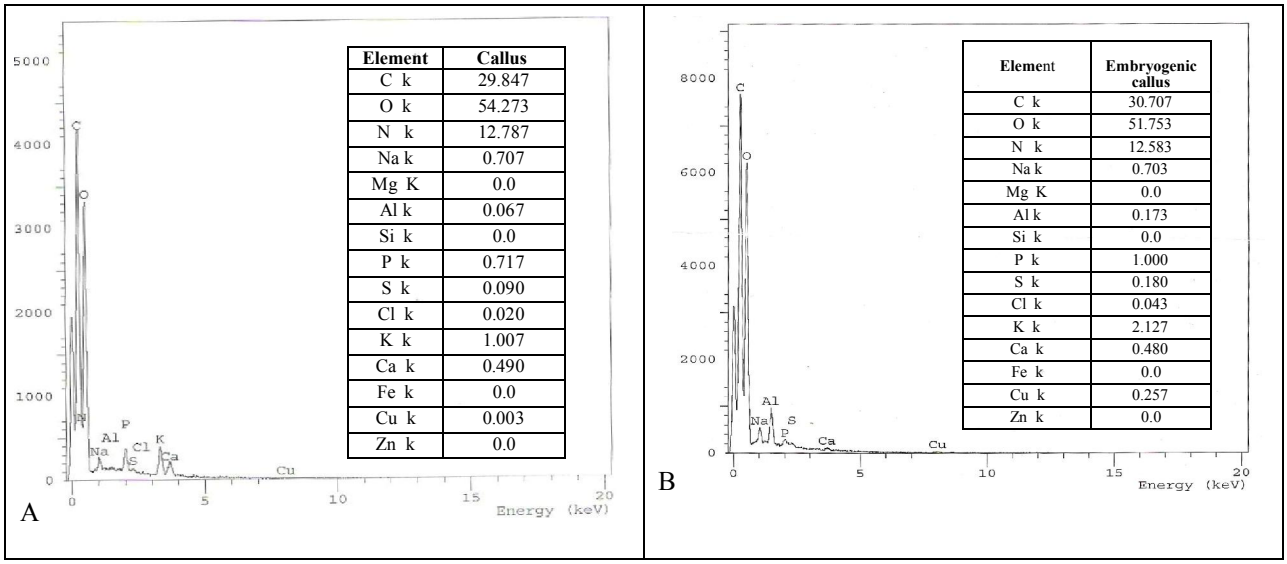
are present,. In the 1st case, Mg^{2+} may hold DNA in one conformation while, in the 2nd case, DNA is switched both in structure and function **(Frausto da silva and Williams,1991)**. The higher Al accumulation (0.463 %) was recorded in globular embryo stage (S3) followed by that of zygotic embryo (0.447) and other stages. Data indicated that there is no big difference between the Al –ratio in zygotic embryo stage (S6)(0.447 %) and torpedo somatic embryo stage (S4) which gained (0.463 %). Regarding phosphorus (P) accumulation, data presented in Table 1 and Fig. 2 showed that higher accumulation of P (1.0 %) in embryogenic calli was observed followed by that of mature embryo (S5) (0.90) and other stages. High P levels were detected during embryogenic calli (S2) and mature somatic embryos (S5) suggests an interaction between Phosphate and metabolism and the activation of particular pathway of differentiation **(Fortes and Pais, 2001)**. Phosphate is involved in metabolism and bioenergetics particularly in DNA, RNA, proteins, polysaccharide and lipid synthesis, metabolic energy is constantly required to maintain gradients for biosynthesis prior to cell division for cell division and cell wall stabilization. Many important enzymes have their synthesis controlled at the gene level by proteins, the binding conformation of which is affected by phosphorylation, then much of proteins synthesis is under control of phosphate metabolism **(Frausto da silva and Williams,1991).** Data presented in Table 1 and Fig.2 also indicated that fluctuation in S accumulation was noticed in regard to embryogenic calli stage where it accumulated the highest values at mature embryo stage (S5) (0.573 %) compared to embryogenic callus stage (S2) and other stages. In spite of certain fluctuation, S increased throughout the induction periods (S1,S2) and not detected at (S3,S4) while it attained the maximum value at mature embryo (S5). This increase in S is not surprising since S play an important role in protein synthesis and as constituent of several coenzymes and prosthetic groups has an important function in various redox reactions **(Frausto da silva and Williams,1991).** Bio-chemical studies on ***Camellia japonica*** **L.** produced by **Garces, 1998** during somatic embryogenesis induction showed the presence of metallo-thionins during this morphogenic process. Regarding Cl data presented in Table 1and Fig.2 showed a fluctuation in Cl accumulation during somatic embryogenesis were it recorded lowest value at de-differentiated callus (S1) and gradually increased to reach the maximum value at globular embryo stage(S3) where it gained (0.560 %) compared to other stages no Cl was detected at either mature somatic embryo or zygotic embryo. Potassium accumulation was higher in embryogenic calli (S2) stage where it recorded (2.12 %) followed by callus stage (S1) (1.007 %) and other stages. On the other hand no K was detected at both mature somatic embryo (S5) or zygotic embryo (S6). Our results are in agreement **Sandra *et al.*, 2002**, who cleared that cell elongation, a common feafure of callus cells was found to be associated with increased K^{+}. Also, **Pullman *et al.* (2003)** observed stage specific elemental accumulation in pinus zygotic embryo with increased potassium, calcium, magnesium, manganese and zinc during the embryo development zygotic embryos, when the medium was enriched with these elements. Data presented in Table 1, Fig.1 also reveled that Ca^{2+} accumulation was fluctuated from stage to other where maximum values was recorded at globular embryo stage (S3) (1.060 %) followed by mature somatic embryo (S5) (0.863 %) and other stages, suggesting that availability of Ca^{2+} is crusade for successful embryogenesis. Apparently the success of morphogenesis induction depends on the availability of Ca^{2+}. EPMA of Zea Mays roots indicates that high calcium levels are associated with cells under differentiation **(Jounin *et al.*,1991).** Increases in Ca^{2+} concentration of the basal medium enhanced the frequency of carrot somatic embryogenesis **(Jansen *et al.*,1993)**. Generally, calcium plays a central role in cell signaling and in mediating plant response to osmotic stress **(Sanders *et al.*,2002)**. The changes in external concentration of calcium in tissue culture can enhance embryogenesis in sandalwood (**Veena and Rao, 2000).**

Also, Suprasanna *et al.* (2004) cleared that identifying normal and abnormal gene activity may provide stage specific markers to improve somatic embryogenesis. They also reported that the expiration of calmodulin gene during different stages of somatic embryogenesis is showed significant CaM expression signifying calcium mediated CaM role in the pathway of somatic embryogenesis in sugarcane. Regarding Fe, Cu and Zn, data presented in Table 1 and Fig.2 showed that somatic embryo with globular stage (S3) possessed the higher level of Fe (2.177 %) followed by somatic embryo with torpedo stage (S4) which possessed (0.460 %). No Fe accumulation was detected at other stages. Cu accumulation was higher (1.043) at torpedo stage (S4) compared to (S2) and other stages. Data reveled that Zn accumulation reached the highest value (0.567 %) at torpedo stage (S4) flowed by globular stage (S3) suggesting that availability of Fe, Cu and Zn are playing an important role in successful embryogenesis. Our results are in agreement with **Foyer *et al.* (1994)** who reported that superoxide dismutase (SOD) depends on the availability of Zn, Cu and Fe and hence the increased accumulation of these elements can allow activation

of superoxide dismutase (SOD). Iron is a vital micronutrients however, the insolubility of iron restricts its uptake. In the recent studies, an iron transport protein (ITP) of Arabidopsis was shown to have strong similarity to late embryogenesis abundant (LEA) proteins from a number of specific **(Kruger *et al.*, 2002)**. In this regard, it is interesting that one of the postulated roles of plant LEA proteins in embryogenesis and drought tolerant is the sequestration of ions **(Imai *et al.*, 1996).**

Table 1. X-ray microanalysis of the elemental changes during different developmental stages of date palm cv. gundila somatic embryos.

ELEMENT (%)	Undifferentiated Callus	Embryogenic calli	Somatic embryo stages			Mature zygotic embryo
	(S1)	(S2)	Globular (S3)	Torpedo (S4)	Mature (S5)	Zygotic (S6)
C k	29.847	30.707	39.813	44.023	40.953	41.500
O k	54.273	51.753	51.310	51.460	55.5	56.737
N k	12.787	12.583	0.0	0.0	0.0	0.0
Na k	0.707	0.703	3.513	1.483	0.930	0.953
Mg K	0.0	0.0	0.0	0.0	0.0	0.0
Al k	0.067	0.173	0.0	0.463	0.0	0.447
Si k	0.0	0.0	0.373	0.0	0.203	0.0
P k	0.717	1.000	0.730	0.750	0.900	0.160
S k	0.090	0.180	0.0	0.0	0.573	0.077
Cl k	0.020	0.043	0.560	0.270	0.0	0.0
K k	1.007	2.127	0.400	0.153	0.0	0.0
Ca k	0.490	0.480	1.060	0.823	0.863	0.063
Fe k	0.0	0.0	2.177	0.460	0.0	0.0
Cu k	0.003	0.257	0.0	1.043	0.0	0.063
Zn k	0.0	0.0	0.063	0.567	0.0	0.0



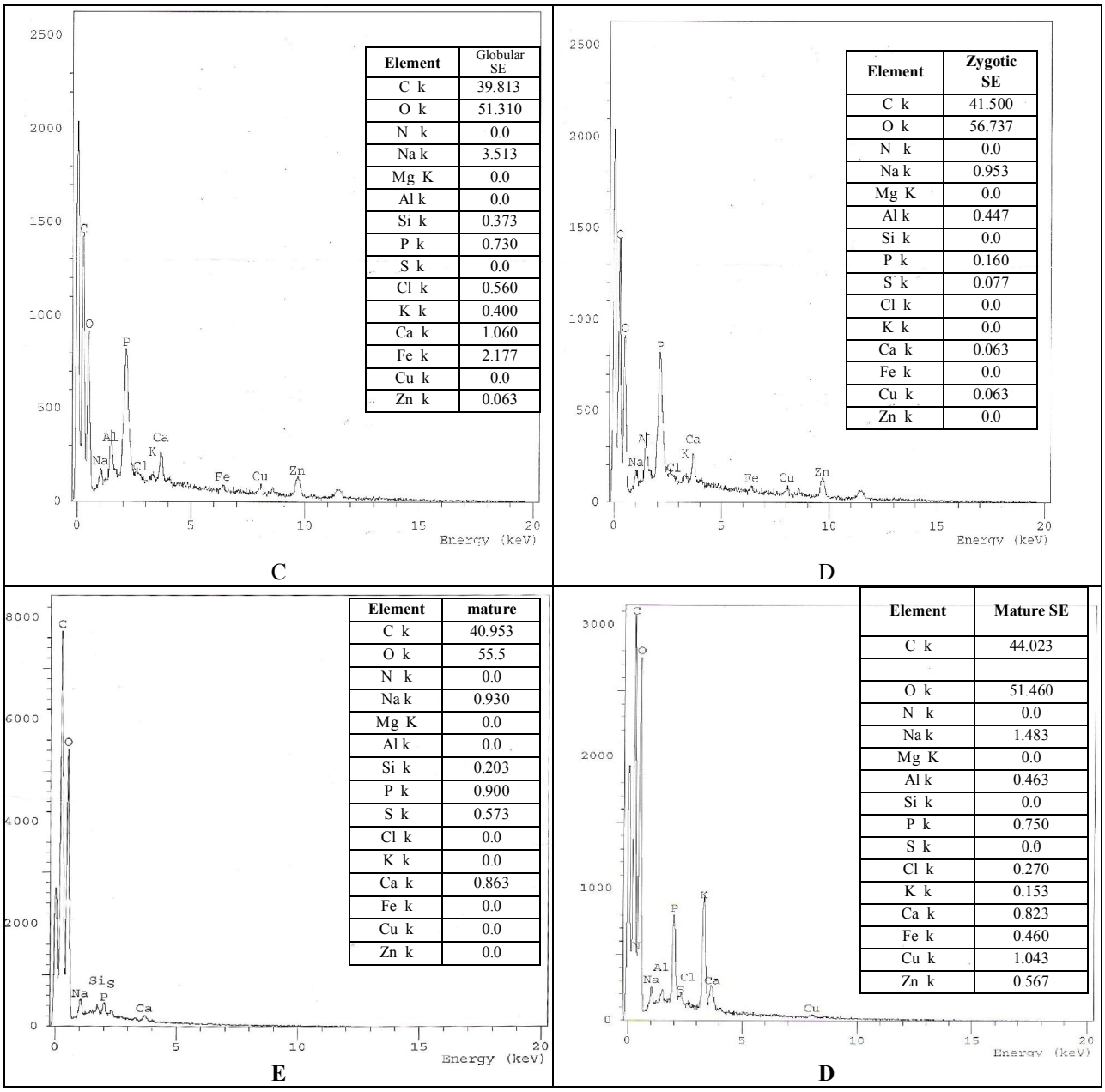


Fig. 2. Energy dispersive X-ray spectra collected from callus tissue of Date palm (*Phoenix dactilifera* L.) cv. Gundila in (A) Callus tissues. (B) Embryogenic callus tissues. (C) somatic embryo at Globular stage. (D) somatic embryo at Torpedo stage. (E) Mature somatic embryo. (F) Mature Zygotic embryo.

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