Flavonoid content and isozyme analysis of tissue culture-derived date palm clones

(Accepted: 16.05.2002)

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ABSTRACT

Flavoniod content of tissue culture-derived date palm (Phoenix dactylifera L) clones indicated that all flavonoid derivatives detected in mother trees of the cultivars Zaghlool and Amhat were present in the clones, but differed in the case of cultivar Samany. Characteristic flavonoid profiles of the date palm cultivars (Zaghlool, Amhat and Samany) were also developed. Flavonoid profiles may be useful either for analysis of tissue culture-derived date palm plants for genetic stability or cultivar identification. Polyacrylamide gel electrophoresis of peroxidase (PER), polyphenol oxidase (POD), acid phosphatase and sodium dedocyle sulfate polyacrylamide gel electrophoresis of proteins (SDS-PAGE) showed mostly similar banding pattern of all tested plants. However, remarkable and reproducible variations in esterase (EST) and glutamate oxaloacetate transaminase (GOT) isozyme banding patterns were detected in some clones. In general, falvonoid profiles and isozyme banding patterns data can be used as an early test to screen tissue culture-derived date palm clones for genetic stability.

Key words: *Phoenix dactylifera L., tissue culture, isozymes, flavonoids, somaclonal variations.*

INTRODUCTION

Date palm (*Phoenix dactylifera L.*) is a member of the family palmace, inhabiting tropical and sub-tropical habitats and the majority of palms are found in the Old World (Moore, 1973). In Egypt, date palm is cultivated and grown everywhere (Taekholem, 1974). There is no doubt regarding the economic importance of date palm, because in addition to the nutritional value of its fruits (Toutain, 1967), the wood and leaves of palm tree are as important as the fruit itself. It is used to provide fiber, fuel, clothing, furniture, hats, baskets and bungalows. Nowadays, palm trees and leaves are very important component in decorations, accordingly it is called the tree of life (Martin, 1978).

This situation makes palm tree the major plantation crop in Arabic world. In Saudi Arabia, for example, date palm trees are grown on about 90% of the cultivated land (Shaheen, 1990). Improvement of date palm is a tedious endeavor due to its long life cycle, strongly heterozygous nature, insufficient and expansive offshoots (planting material) needed for new cultivation and impossibility to

determine sex at early stages of development. Recent advances in plant biotechnology may offer alternative solutions for these problems (Moursy and Saker, 1996). However, the majority of published data on date palm biotechnology are focussing on its rapid mass propagation using plant tissue culture techniques (Tisserat, 1982, Bhansali *et al.*, 1988, Dass *et al.*, 1989, Saker *et al.*, 1998, Bekheet and Saker, 1998; Bekheet *et al.*, 2001a,b).

Moreover, very limited data are available regarding molecular analysis of tissue culture-derived palm plants. In this context, Salman *et al.* (1988) found in *in vitro* propagated date palms similar isozyme patterns and chromosome polyploidy only in one case, suggesting high genetic uniformity of tissue culture derived plants. Recently, a molecular marker linked to somaclonal variations in oil palm has been identified by Rival *et al.* (1998). Similarly, Saker *et al.* (2000) found that genetic instability of tissue culture-derived date palm plantlets is age-dependent and was detected only in morphologically abnormal plantlets. Flavoniods are a major group of plant secondary metabolites and are detected in different members of family palmace. Searching of published data indicated that few cases employed falvonoid profiles in date palm classification. In this connection, Quafi *et al.* (1988) mentioned that flavonoids from acid hydrolysates of palm leaves could be used in taxonomy and classification of palm cultivars.

Nowadays, great debate regarding either genetic stability or instability of tissue culture-derived date palm plants appeared. This is because a study dealing with field evaluation of tissue-culture derived date palm plants indicated that high percentages of tissue culture derived -date palm plants cultured in Saudi Arabia during 1992 and 1993 were not able to set seed (Wasel, 2001). This background was the main reason for purposing the present study. In this study, isozyme banding patterns, quantitative and qualitative determination of flavonoids were employed to analyze tissue culture-derived date palm clones for genetic stability.

MATERIALS AND METHODS

Plant material

Date palm offshoots of the cultivars Zaghlool, Samany and Amhat, grown in the regions of Edco and El-Badrasheen (El-Behaira and Giza Governerates, respectively) were used. Offshoots of different cultivars were selected and carefully separated from certified cultivars during the flowering and fruiting season (September 1998). All offshoots were secured and separated from the mother tree during fruiting seasons, to eliminate any doubt in cultivar identification. The same offshoot used as source of explants for initiation of *in vitro* cultures was considered as mother tree (control) in biochemical analysis.

Tissue culture

Offshoots were dissected acropetally using serrated knife. Mature leaves were carefully separated. During dissection process, anti-oxidant solution (150 mg/l each of ascorbic and citric acid) was sprayed over hands and the offshoot. When all outer leaves are removed, the shoot tip was kept in an anti-oxidant solution and transferred to air laminar flow for surface sterilization. Explants were soaked in 50 % Clorox (5.25% NaOCl) for 20 min and thoroughly washed with sterile distilled water. The pale white leaves were trimmed till obtaining explants (1 cm^2). The explants were soaked in 30 % Clorox for 10 min, then washed with sterilized distilled water and soaked in sterilized anti-oxidant

solution. Leaves were removed acropetally and transferred to new sterilized petri dishes and additional leaves were removed till obtaining the smallest size of shoot tip explants. Shoot tip explants were cultured onto Murashige and Skoog (MS) medium containing 10 mg/l 2,4-D and 3 mg/l 2iP. The proliferated embryogenic callus developed shoots onto basal MS medium. Proliferated shoots having the same age, origin, proliferated and developed on the same medium were used for subsequent experiments.

Extraction and identification of flavonoids

Extraction of flavonoids from date palm leaves and tissue culture-derived clones was carried out using 70% ethanol, followed by 50% ethanol and extracts were dried under vacuum. Flavonoids were separated on polyamide column using water followed by increasing concentrations of ethanol. Fractions were further purified on Sephadex LH-20. Flavonoids of small samples (tissue culture-derived clones) were separated by paper chromatography. Flavonoids were identified using standard methods (Harborne; 1967; Mabry *et al.*, 1970; Makham, 1982). These included complete and mild acid hydrolysis, hydrogen peroxide oxidation, enzymatic hydrolysis, co-chromatography UV spectrophotometry and H-NMR. Solvents used for co-chromatography were n-butanol, acetic acid and water at a ratio 4:1:5, acetic acid and water at a ratio 15:85 and phenol.

Enzyme activities

The activity of acid phosphatase was determined according to the method described by Dinan *et al.* (1983). Peroxidase activity was determined according to Chance and Maehly (1955). Esterase activity was determined as described by Gottlieb (1974). Polyphenol oxidase activity was determined according to Wilson *et al.* (1975). Glutamate oxaloacetate transaminase (GOT) was determined according to Reitman and Frankel (1957).

Electrophoresis

Electrophoresis was performed using 7.5% (w/v) acrylamide slab gels, using Tris-glycine buffer, pH 8.3. Samples (20 µl of crude extract per well) were electrophoresed for 3 hrs at constant current 3 mA per well, using mini vertical electrophoresis unit of Bio-Rad. Esterase activity was detected on the gel according to Gottlieb (1974). Peroxidase activity was detected on the gel according Gottlieb (1973a). Acid phosphatase activity was localized on the gel using fast blue reaction according to Scandalios (1969). GOT activity was localized on the gel using fast blue reaction according to Gottlieb (1973b). For SDS-PAGE analysis, protein of one-gram fresh proleaves tissue, was extracted in 2 ml extraction buffer. Electrophoresis was performed using 10% acrylamide in the separating gel and 3% in the stacking gel.

RESULTS AND DISCUSSION

Tissue culture

Shoot tip cultures were established on (MS) medium supplemented with 100 mg/l myo-inositol, 50 mg/l adenine sulfate, 1.5 g/l activated charcoal, 10 mg/l 2,4-D and 3 mg/l 2ip and incubated under dark conditions at 27 ± 2 °C. Shoot tip explants were transferred to the same fresh medium at two-week intervals. Embryogenic callus was proliferated after two months cultivation. The resulting embryogenic callus was subcultured monthly onto callus proliferation medium and finally transferred to shoot development medium containing GA3 (unpublished data). The developed shoots were morphologically examined and all plants having any type of morphological

abnormalities including curled and twisted leaves, as well as rosy clusters of faint green plantlets were excluded. Figure (1) points to morphologically normal date palm clones of the cultivar Zaghlool grown onto basal MS medium devoid of GA3.

Fig. (1): Five tissue culture derived clones of data palm (CV. Zaghlool), regenerated from embryogenic callus onto basal MS medium. The embryogenic callus was proliferated from shoot tip explants onto MS medium containing 10 mg/l 2,4-D + 3 mg/l 2ip.

Because this comparative study aimed to biochemical evaluation of tissue culture-derived date palm clones, and because the biochemical status (gene expression) of the *in vitro* cultures is highly affected by environmental conditions, all plant material used here was subjected to the same media regime and conditions. Also, precautions were given to the morphology of the selected plants (Saker *et al.*, 2000). They stated that all morphologically abnormal plantlets showed genetic variations at the DNA and gene expression levels (isozyme). The most striking observation in this context was the observation of Wasel (2001), who recorded variations among tissue culture-derived date palm trees in Saudi Arabia. The recorded variations were only detected at the phenotypic level (morphological variations) which included delay in fruiting, fruit set failure and abnormal female spikelets numbers.

Flavonoid content of tissue culture-derived date palm clones

Falvonoids of both control mother tree and tissue cultured derived-date palm clones (Fig. 1) were determined as detailed in material and methods. Data of phytochemical analysis, including mild and complete acid hydrolysis, enzymatic hydrolysis, hydrogen peroxide oxidation, UV spectrophotometry and NMR are not shown here. The data of phytochemical analysis were used to identify different flavonoids and flavonoid derivatives, as well as their concentrations and distribution among tissue culture-derived date palm clones and their mother tree (control). Data summarised in Table (1) indicate clearly that the tree flavonoid derivatives (Ap-7-G, vitexin and iso-vitexin) were detected in both tissue culture- derived clones and the control tree. Although high concentrations of Ap-7-G were detected in control tree as well as clone no.1, the highest concentrations of vitexin and iso-vitexin were recorded in clones no. 2 and 3 (Table 1). Briefly, variations in different flavonoid concentrations, among the investigated clones, may be a first step for employing flavonoid profiles as biochemical markers for quality control test of tissue culture-derived date palm plants.

Table (1): Distribution of different flavonoid and flavonoid derivatives among tissue culture-derived date palm somaclones of the cultivar Zaghlool.

Date palm somaclones	Ap-7-G	Vitexin	Iso-Vitexin
Control	+ + +	+ +	+ +
1	+ + +	+ +	+ +
2	+	+ + +	+ + +
3	+ +	+ + +	+ + +
4	+ +	+ +	+ +
5	+ ++	+ +	+ +

Flavonoid profiles of other tissue culture-derived date palm clones, belonging to different cultivars were investigated. Data presented in Tables (2) and (3) summarise the flavonoid profiles of some tissue culture-derived date palm clones and their mother control trees belonging to the cultivars Amhat and Samany, respectively. As in the case of date palm clones of the cultivar Zaghlool, all falvonoid derivatives detected in mother plants of cultivar Amhat were also detected in tissue culture derived plants (Table 2). In the case of cultivar Samany, both the type and level of falvonoids of the mother plant and tissue culture derived plants were not the same (Table 3). For instance, some flavonoids, including Lut-5-G, Lut-7-G and Lut-7-Gal were detected in mother tree only and were completely absent in tissue culture-derived plants (Table 3). These results directly points to variations in falvonoid profiles between mother tree and date palm plantlets cloned from it.

Table (2): Flavonoid and flavoniod derivatives of date palm somaclone (CV. Amhat) and the donor date palm tree (mother tree).

Date palm Somaclones	Ap-5-G	Ap-7-G	Lut-5-G	Lut-7-G	Vitexin
Mother tree	+	+ +	+	+	+ + +
T.C. clone	+	+ +	+	+	+ + +

Table (3): Flavonoid and flavonoid derivatives of a date palm somaclone (CV. Samany) and the donor date palm tree (mother tree).

Date palm Somaclones	Ap-5-G	Ap-7-G	Ap-4'-G	Lut-5-G	Lut-7-G	Lut-7-Gal	Lut-7-rut	Vitexin	Iso-vitexin	Lut 4'/7 di G
Mother tree	+	+ + +	+	+	+ +	+ +	+ +	+ + +	+ + +	+
T.C. clone	+	+	+				+ +	+ + +	+ + +	+

+++ major, ++ high, + present
AP = Apigenin, Lut = Luteolin, G = Glucoside, Gal = Galactoside,
di = diglucoside, rut = rutinoside T.C. = Tissue culture

The most striking observation extracted from the analysis of flavonoid profiles of tissue culture-derived date palm clones, is the detection of some flavoniod derivatives characteristics for different date palm cultivars. Flavonoid profiles presented in Tables (1-3), indicate clearly that Lut-7-rut and Lut, 4,7-di G were peculiar for cultivar Samany. Meanwhile, iso-vitexin is detected in both Samany and Zaghlool but was completely absent in Amhat. The developed characteristic flavonoid profiles of different date palm cultivars (Zaghlool, Amhat and Samany) were age-independent, i.e., they were stable either in mother trees (*ex-vitro* level) or tissue culture-derived plantlets (*in vitro*). Accordingly, flavonoid profile may be useful either for analysis of tissue culture-derived date palm plants for genetic stability or cultiver identification, both *in vitro* and *ex-vitro* level. These results are in accordance with that of Quafi *et al.* (1998), who found that the presence or absence of individual flavonoids and their level allowed the identification of different date palm cultivars from each others. Moreover, flavonoid profiles are stable in spite

of the developmental stage (age) and environmental conditions. This stability may be an advantage for flavonoid profiles as biochemical markers, in comparison with other biochemical markers, such as isozymes, which depend on gene expression (Castiglione *et al.*, 1993).

Enzyme activity and isozyme analysis

The same plant material subjected to flavonoid analysis was also subjected to isozyme analysis. Activity levels (Table 4) and isozyme patterns (Table 5) of different enzymes were used as biochemical markers for biochemical characterization of tissue culture-derived date palm plantlets (CV. Zaghlool). Data presented in Table (4) and Fig. (2) show the activity levels as well as electrophoretic separation of esterase isozymes. The clone 5 had the highest esterase activity (89 units g^{-1} h^{-1}) and the esterase activity of the control. The clones from 1 to 5 were ranging from 26-89 units g^{-1} h^{-1}. The isozyme patterns of esterase show the similarity between the control and the clones no. 3, 4, 5 (3 bands with RF 0.13, 0.21, 0.38). The clones no. 1 and 2 have 5 similar bands (Rf 0.21, 0.38, 0.43, 0.5 and 0.58). On the other hand, the peroxidase activity of the control and the five clones ranged from 555-1267 Units g^{-1} h^{-1} (Table 4). Unlike esterase, the isozyme patterns of peroxidase were similar in the control and the five clones (Table 5 and Fig. 2).

Table (4): The activity levels of different enzymes in tissue culture-derived clones of date palm.

Clone No	Esterase	Peroxidase	Polyphenol oxidase	Acid phosphatase	GOT	Total protein
Control	60	5 5 5	9.7	34	700	1. 396
1	46	647	15. 05	46	602	1. 24
2	26	1267	16. 6	31. 3	640	1. 281
3	38	882	16. 5	34. 1	800	1. 54
4	43	976	22. 2	33	520	1. 045
5	89	1020	34. 68	66. 1	1000	3. 05

Table (5): Number of bands and relative mobilities (RF) of different enzymes in five clones of tissue culture-derived date palm.

Enzyme	1		2		3		4		5		Control	
	bands	RF	bands	RF	bands	RF	bands	RF	bands	RF	bands	RF
Esterase	5	0.13	6	0.13	5	0.21	6	0.21	6	0.21	6	0.21
		0.21		0.21		0.38		0.38		038		038
		0.38		0.38		0.43		0.43		0.43		0.43
		0.5		0.43		0.5		0.5		0.5		0.5
		0.58		0.50		0.58		0.58		0.58		0.58
				0.58				0.71		0.71		0.71
Peroxidase	1	0.076	1	0.076	1	2	2	0.079	1	0.076	1	0.076
								0.19				
Polyphenol oxidase	2	0.079	2	0.079	2	0.079	2	0.79	2	0.79	2	0.79
		0.19		0.19		0.19		0.19		0.19		0.19
Acid phosphatase	3	0.11	4	0.11	3	0.11	3	0.11	3	0.11	4	0.11
		0.2		0.22		0.2		0.2		0.22		0.2
		0.4		0.33		0.4		0.4		0.4		0.33
				0.4								0.44
GOT	1	0.22	2	0.22	1	0.22	1	0.22	1	0.22	1	0.22
				0.32								

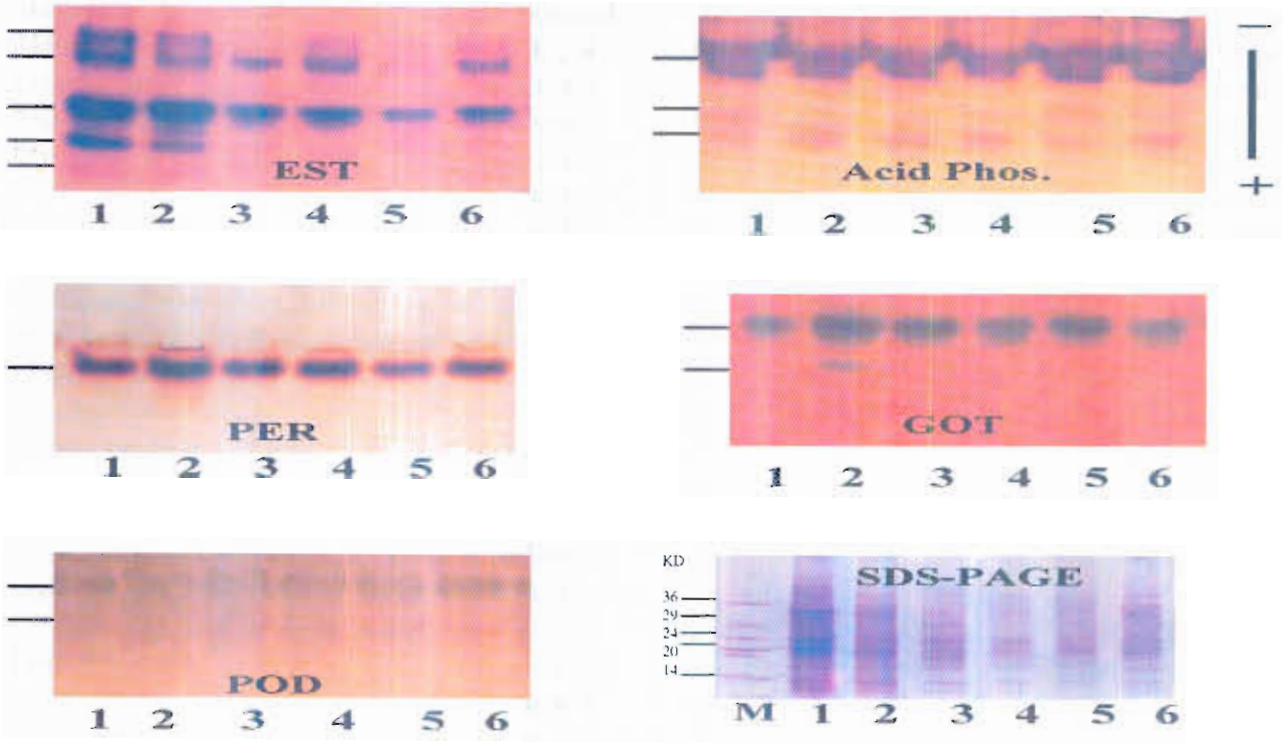

Fig. (2): Isozyme banding patterns and SDS-PAGE protein profiles of tissue culture- derived data palm clones (lanes 1-3) and control mother tree (lane 6). M = low molecular weight standard protein of Sigma.

In the case of polyphenol oxidase, the control date palm and the five different clones have polyphenol oxidase activities ranging from 9.7 to 34.68 Units g^{-1} h^{-1} (Table 4). The isozyme patterns of polyphenol oxidase (Table 5 and Fig. 2) showed similarity between the control and the five different clones (2 bands with RF 0.79 and 0.19). Data presented in Table (4) and Fig. (2) clearly point to variations in both activity levels and number of acid phosphatase isomers among tissue culture-derived date palm plantlets and the control mother tree. The control date palm and the five different clones of tissue culture date palm have acid phosphatase activities ranging from 31.3 to 66.1 Units g^{-1} h^{-1}). The isozyme patterns of acid phosphatase showed similarity between the control mother tree and the clones no. 1 and 5 (3 bands with RF 0.11, 0.2 and 0.4). Isozyme banding patterns presented in Fig. (2) and RF values tabulated in Table (5) also indicated that the clones no. 2, 3 and 4 have similar banding patterns (2 bands with RF 0.11, 0.3 and 0.4).

The glutamate oxaloacetate transaminase (GOT) activity levels (Table 4) of the control date palm tree and the five different tissue culture-derived clones ranged from to 520 to 1000 Units g^{-1} h^{-1}. The isozyme patterns of GOT showed similarity between the control

tree and the clone no. 1 (1 band with RF 0.22). Meanwhile, the clones no 2, 3, 4 and 5 showed different banding patterns (Table 5 and Fig. 2). The total protein of the control and the five clones ranged from 1.045 to 3.05 mg protein g^{-1} (Table 5). SDS-PAGE (Fig. 2) showed similarity between control tree and the five clones. Variations in expression levels were visible for some polypeptide bands but the results were not reproducible.

Based on the obtained data for enzyme activity levels and isozyme banding patterns, it is concluded that there is a variation in activity levels between the mother tree and the tissue culture clones, as well as among different tissue culture clones. There is no definite relationship between variations in activity levels recorded here and flavonoid profiles of the same clone. It is worth to mention that all clones showed variations in flavonoid profiles showed variations in isozyme banding patterns, with some exceptions. For instance, the flavonoid profile of the clone no. 2 differed from that of the mother tree (Table 1), the same clone had different EST and GOT pattern (Fig. 2).

Different authors have reported variations in enzyme activity levels and isozyme banding patterns in tissue culture-derived date palm somaclones (Saker *et al.*, 2000; Baaziz *et al.*, 1994) and in different tissue culture-derived plants (Torres and Al-Jibouri, 1989). These variations were explained on the basis that a possible genetic modification may have occurred during callus proliferation stage. Since izozymes are gene products, such variations may be responsible for triggering of specific genes to produce additional isomers or repressing specific genes, which may result in fewer numbers of isomers. In general falvonoid profiles and isozyme banding patterns data may be used as an early test to screen tissue culture-derived date palm clones for genetic stability.

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الملخص العربي

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٢ : قسم تصنيف النباتات و الفلورا المصرية-المركز القومي للبحوث-مصر
٤ : قسم البيولوجيا الجزيئية-المركز القومي للبحوث-مصر

أوضحت نتائج تقدير المركبات الفلافونويدية و مشتقاتها تشابها بين النباتات الناتجة من زراعة الأنسجة عند مقارنتها بالنخلة الأم وذلك في صنفي زغلول و أمهات، أما في الصنف سماني، فان المحتوى الفلافونويدى قد اختلف من حيث نوع المركبات وتركيزها. أوضحت الدراسة أيضا أن هناك أنماط فلافونويدية مميزة لكل صنف من أصناف النخيل تحت الدراسة، وبالتالى فان تقدير مركبات الفلافونويدات مفيدا في دراسة الثبات الوراثي و كذلك تمييز أصناف النخيل المختلفة. ومن ناحية أخرى فقد أظهرت دراسة أنماط التفريد الكهربي للمتشابهات الأنزيمية تشابها في عدد المتشابهات الأنزيمية لأنزيمات: البيروكسيديز (PER) ، البولي فينول أوكسيديز (POD) ، الاسد فوسفاتيز (Acid phosphatase) وأنماط التفريد الكهربي للبروتينات (SDS-PAGE) و ذلك بين النباتات الناتجة من زراعة الأنسجة و النبات الام. هذا و قد سجلت اختلافات واضحة و ثابتة في المتشابهات الأنزيمية لأنزيميي الايستريز (EST) و الجلوتوميت أوكسالواستيت (GOT)، و ذلك في بعض الحالات. وبصفة عامة فان دراسة أنماط توزيع الفلافونويدات و المتشابهات الأنزيمية يمكن أن يكون اختبارا مبكرا للكشف عن التغيرات الوراثية في نباتات النخيل الناتجة من زراعة الأنسجة.