

Full Length Article

Hormonal Basis of 'Shees' Fruit Abnormality in Tissue Culture Derived Plants of Date Palm

Sarfraz Hadi^{1*}, Nasser Swaleh Al-Khalifah² and Mohammed Abdo Moslem¹

¹Department of Botany and Microbiology, College of Science, P. Box 2455, King Saud University, Riyadh 11451, Saudi Arabia

²National Center for Agricultural Technology, King Abdulaziz City for Science and Technology (KACST), P. Box 6086, Riyadh 11442, Saudi Arabia

*For correspondence: shadi@ksu.edu.sa

Abstract

'Shees' fruit abnormality manifested as cluster of small fruit-like structures on a single peduncle occurs at a high frequency in tissue culture derived plants of date palm. The abnormality is akin to parthenocarpy, which is known to be caused by altered hormone profile of flowers/fruits in many species. This study was conducted to elucidate the hormonal profile of date palm flowers and early fruits and to identify the hormones associated with 'shees' fruit formation. Hormone levels in young flowers/fruits of normal and 'shees'-bearing plants of cultivars 'Barhy' and 'Nabtet-Saif' were studied with HPLC at the time of pollination and subsequently after 10 and 20 days. In all these samples, out of seven hormones detected by HPLC, levels of indole-3-acetic acid (IAA) and gibberellic acid (GA₃) were significantly higher, while that of a 'kinetin-like' compound and an unknown compound were significantly lower in 'shees' flowers/fruits as compared to the normal counterparts. Other three unknown compounds did not show significant variations between normal and 'shees' fruits. Kinetin-like compound showed the same elution properties as kinetin standard during HPLC procedures. The compound was purified and an attempt was made to characterize the molecule with gas chromatography-mass spectrometry (GC-MS). On the basis of Wiley-229 library hit (entry 101383) the compound was identified as Benzaldehyde, 2-hydroxy-[(2-hydroxyphenyl) methylene] hydrazone with formula: C₁₄H₁₂N₂O₂ and Mol weight: 240. We have tentatively designated the compound as BHH and are reporting the occurrence of this compound for the first time in date palm. © 2015 Friends Science Publishers

Keywords: Date palm; Fruit abnormality; (*Phoenix dactylifera* L.); Plant growth regulators; 'Shees' fruit

Introduction

Female flowers in date palm (*Phoenix dactylifera* L.) possess tricarpeal apocarpous pistil consisting of three independent carpels, while rudimentary androecium is represented by two whorls of three staminodes each alternately arranged in antepetalous and antesealous position (DeMason *et al.*, 1982). During normal development of the fruit, only one carpel grows after pollination and successful fertilization to form a single fruit while the other two degenerate into papery structures (Fig. 1a), which shed away as the normal fruit emerges out of the parianth whorl (Zaid, 1999). When pollination is not performed manually or is not affected due to absence of a male palm in the vicinity, all the three unfertilized carpels tend to grow together forming three parthenocarpic fruitlets of small size (Fig. 1b, c) on the same peduncle (Reuveni, 1986). These fruitlets are without seed and generally shed away within 3-4 weeks after formation. Even when

remaining attached to the rachis they do not mature into edible material. This condition is referred to as 'shees' in Arabic.

In tissue culture-derived populations of date palm it has been frequently observed that despite standard practice of pollination, besides female carpels, staminodes also grow to form a cluster of 3-9 tiny seedless fruit-like structures (Fig. 1b, d) within a single parianth whorl (Al-Wasel, 2000; Djerbi, 2000). This is an abnormal and complex development which results in frustratingly low yields in tissue culture-derived date palm plantations. The condition represents a special kind of parthenocarpy where in addition to the ovaries; staminodes which are rudiments of androecium in the unisexual female flowers also grow to form multiple fruit-like structures.

The process of fruit formation in higher plants like *Arabidopsis* and tomato is known to be mediated by hormones produced within the ovule as a result of fertilization (Vivian-Smith and Koltunow, 1999; Gorguet *et*

al., 2005; Serrani *et al.*, 2007). Acting as a hormone, ethylene has been found to be involved in fruit ripening processes in date palm as well (Serrano *et al.*, 2001). Generally, in the absence of fertilization fruit development either fails to take place at all or is severely restricted (O'Neill and Nadeau, 1997; Vivian-Smith *et al.*, 2001) probably due to inadequate level of endogenous hormones (Pharis and King, 1985). It has been shown in pea (Vercher and Carbonell, 1991; Rodrigo *et al.*, 1997) and citrus (Talon *et al.*, 1990a; Ben-Cheikh *et al.*, 1997) developing seeds also produce hormones which contribute to changes in ovary wall consequently leading to normal fruit formation. In some species, however, failure of fertilization leads to formation of parthenocarpic fruits in tomato (Gorguet *et al.*, 2005), mandarins (Talon *et al.*, 1992), and citrus (Talon *et al.*, 1990a). This suggests that in these fruits some pathways exist, which regulates ovary wall growth and senescence, which may operate without occurrence of fertilization or seed development.

It has been observed that naturally occurring parthenocarpic fruits have higher levels of endogenous gibberellins and auxins (Talon *et al.*, 1990b; 1992) and parthenocarpic fruits can also be produced by exogenous application of plant growth regulators including gibberellins, auxins and cytokinins, which apparently make up for inadequacy of the endogenous hormones (Schwabe and Mills, 1981). Conversely, in an experiment we could reduce the level of 'shees' fruit formation in tissue culture-derived date palm plants by exogenous application of kinetin on the stigmas before pollination (Al-Khalifah *et al.*, 2007a). These reports provide sufficient clue to the involvement of hormones in 'shees' formation in date palm. However, hormone profiles of developing fruits of date palm have not been worked out so far and cause of 'shees' remains unclear, though preliminary anatomical information is available about the genesis of this abnormality (Al-Khalifah *et al.*, 2007a). This study is an attempt to track hormonal activity during the course of development of date palm fruit so as to assess the role of hormones in 'shees' fruit formation in tissue culture derived plants.

Materials and Methods

Collection of Samples

Flower and fruit samples were collected from date palm orchards planted with crop geometry of 10 x 10 m. Eight to ten years old tissue culture-derived plants of cultivars 'Barhy' and 'Nabtet Saif' known for their normal fruiting behaviour and heavy 'shees' production were identified for collection of samples on the basis of their fruiting record during the two preceding seasons. Spathes selected for the experiment were covered with paper bags before opening to prevent chance out-crossing and to precisely control the pollination. After natural opening of the spathe three rachillae, each bearing 30-40 flowers were collected from

middle of the inflorescence just before pollination in the two types of plants. These were marked as zero days after pollination (0 DAP). Remaining flowers in the inflorescence were pollinated by sprinkling plenty of pollen on the flowers and the inflorescences were covered again with paper bags. After 10 days, normal and 'shees' forming fruits could be distinguished clearly and rachillae were collected from the two types of plants on the basis of the appearance of fruits. Similarly, rachillae were collected on 20th and 30th day after pollination from the same rachises. For the sake of replication, rachillae were collected from three normal and three 'shees' bearing plants in each cultivar. Samples were brought to lab and were stored at -20°C for 10-30 days before extraction of hormones.

Extraction of Phytohormones

Ten flowers/fruits were collected from the middle 1/3rd portion of the rachillae in each treatment. Extraction was performed by a procedure adapted from Mwange *et al.* (2003), Baydar and Ulger (1998) and Xu *et al.* (1998). Precisely weighed samples were homogenized in 10 mL of 80% methanol and 50 mg ascorbic acid; following which, volume of the homogenate was raised to 50 mL and it was stirred overnight with 500 mg polyvinylpyrrolidone (PVP) at 4°C. Subsequently, this suspension was centrifuged at 13,900 g for 30 min under refrigeration (4°C). The supernatant was collected and aqueous methanol was evaporated under low pressure at 25°C. The resulting residue was alkalized by adding 25 mL of phosphate buffer (pH 8.5) and was partitioned with equal volume of ethyl acetate three times. Aqueous phase was collected and was acidified (2.5 pH) by adding 20 mL of 1 N HCl. The solution was partitioned with equal volume of diethyl ether 3 times; following, which the diethyl ether phase was collected and was passed through dry sodium sulphate. Diethyl ether was evaporated under vacuum and residue containing hormones was dissolved in 500 µL of methanol.

HPLC Analysis

HPLC analysis was performed on Shimadzu Class VP-100 system using a C18 – ODS (5 µm): 250 x 4.6 mm column (Supelco Inc., USA) and a UV detector. Injection volume of 20 µL, column temperature of 40°C, flow rate of 1 mL min⁻¹, and run time of 25 min was maintained for all analyses. The system was calibrated with external standards of indole-3-acetic acid (IAA), gibberellic acid (GA₃), abscisic acid (ABA), zeatin, zeatin-riboside (ZR), N⁶-2-isopentyladenine (2iP), N⁶-benzyladenine (BA), and N⁶-furfuryl-adenine (kinetin). According to the procedure of Baydar and Ulger (1998) for detecting GA₃, methanol-water (30:70) adjusted to 3.0 pH with 0.1 M H₃PO₄ as mobile phase and the elutant was scanned at 208 nm; for IAA, 35% methanol prepared in 0.1 M acetic acid was used as mobile phase and reading wave length was 265 nm; and for ABA, 55% methanol in 0.1 M acetic acid and 280 nm were used as

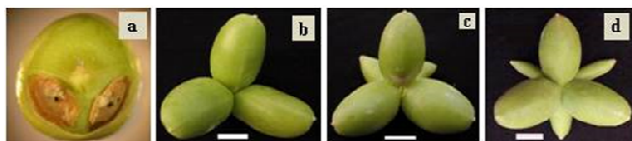


Fig. 1: Early stages of fruit development in date palm: (a) Young normal fruit - with rudiments of degenerated ovules; (b) 'Shees' - three ovules growing simultaneously to form tiny fruit-lets; (c) 'Shees' - three ovules and two staminodes growing simultaneously; (d). 'shees': three ovules and three staminodes growing simultaneously. Bar = 1 cm. (Adopted from Al-Khalifah *et al.*, 2007a)

mobile phase and reading wavelength respectively. Standards of Zeatin, ZR, 2iP, BA and kinetin showed satisfactory separation with the mobile phase and the reading wavelength used for IAA, therefore same conditions were applied for probing these compounds in all the samples. Molecules with peaks not coinciding with any of the used standards were designated as unknowns and numbered in their order of elution. Values for concentration in the extract were transformed into ng/g fresh weight (FW) on the basis of initial weight of the samples. The analysis was conducted with three replicates in each treatment and the means for content of different hormones in normal and 'shees' flowers/fruits were compared on the basis of SE ($P \leq 0.05$) worked out by F test.

GC-MS Analysis

Attempt was made to characterize the compound that showed the same elution properties as kinetin with the help of GC-MS; for kinetin is a synthetic cytokinin and is not known to occur in natural conditions. The fraction was purified by using 28:72 methanol-water mobile phase through the same column as mentioned previously and GC-MS analysis was conducted on Shimadzu QP-5050A system equipped with fused silica capillary column (RTX-5MS: 30 m, 0.25 mm, 0.25 μ m - Restec). The sample (2 μ L) was injected at an oven temperature of 70°C, which was raised to 250°C where it was retained for 9 min. Mass spectrum was acquired after 12 min and was referred to Wiley-229 library for tentative identification.

Statistical Analysis

Experiments yielding quantified results were conducted in three replicates; ensuing data were analyzed by F test and means were separated by SE at $P \leq 0.05$.

Results

Phytohormones Detected with HPLC

With the help of HPLC procedures seven hormones were detected in flower/fruit samples of the two cultivars at

different stages of development (Table 1). Hormone profile of cv. 'Barhy' at 10 DAP stage was elucidated as a representative case in Fig. 2a-d. With the method using 35% methanol in 0.1 M H_3PO_4 as mobile phase and reading wavelength of 280 nm, a group of six hormones including IAA and five unknown compounds were detected (Fig. 2a). First peak appeared at 6.201 min. This did not match with any of the standards used; therefore it was labeled as unknown-1. Then another unknown compound (unknown-2) appeared at 7.138 min. This compound showed exactly the same elution properties as kinetin standard (Fig. 2b); therefore, it was labeled 'kinetin-like' since kinetin is not known to occur in plants under natural conditions. After this, three more unknown compounds (unknown-3, 4, 5) peaked at 9.367, 10.077 and 12.397 min, respectively. IAA eluted in the last at 20.745 min. GA_3 was detected in separate runs with the method using methanol-water (30:70) as mobile phase and 208 nm as reading wavelength. It eluted at 11.970 min (Fig. 2c). ABA was not detected in any of the samples at any stage of development.

Level of Phytohormones

Level of different hormones in developing flowers/fruits of cultivars 'Barhy' and 'Nabtet-Saif' at different stages after pollination have been presented in table 1. In general, level of IAA, 'kinetin-like' compound (unknown-2) and unknown-1 increased between 0 and 10 DAP but dropped sharply by 20 DAP, while that of GA_3 and three remaining unknown compounds declined gradually through 0, 10 and 20 DAP. At 30 DAP stage, some of the hormones had disappeared while others had declined to very low levels (Fig. 2d). In flowers/fruits from the known 'shees'-bearing plants, level of IAA was significantly higher on 0 DAP and 10 DAP as compared to the samples from plants with normal fruiting behaviour; but on 20 DAP the level was similar in both types of fruits (Table 1). Similarly, GA_3 was also found to be significantly higher at the time of pollination (0 DAP) in 'shees'-forming flowers.

Characterization of an Unknown Compound

Appearance of a 'kinetin-like' compound (unknown-2) in normal as well as 'shees' bearing flowers/fruits in both cultivars was an unexpected observation of this study as kinetin is a synthetic cytokinin and is not expected to exist in plant systems; therefore this compound was purified by HPLC and was subjected to GC-MS analysis for verification (Fig. 3a). On the basis of Wiley-229 library hit (Fig. 3b, c) the compound had a formula: $C_{14}H_{12}N_2O_2$ with a mass of 240. Its name was provided in the library as Benzaldehyde, 2-hydroxy-[(2-hydroxyphenyl)-methylene]-hydrazone (entry: 101383). This is for the first time that occurrence and role of this compound as a hormone has come to light in developing flowers/fruits of date palm. We have tentatively designated this hormone as BHH in our study. Level of BHH was significantly lower in 'shees'-

Table 1: Phytohormone level (ng g⁻¹ fresh weight) in developing fruits of cvs. 'Barhy' and 'Nabtet'

Cultivar	Stage of flowers/fruits	IAA		GA ₃		Kinetin-like compound (BHH)		Unknown-1		Unknown-2		Unknown-3		Unknown-4	
		N	S	N	S	N	S	N	S	N	S	N	S	N	S
'Barhy'	0 DAP	2.3	8.6*	2.8	7.2*	37.4	4.7*	8.6	8.6	10.2	—	16.6	17.3	14.6	17.7
	10 DAP	4.2	10.3*	1.4	2.4	76.2	16.5*	151.3	118.9*	2.9	2.4	12.3	13.5	8.2	5.3
	20 DAP	0.8	1.0	0.8	1.1	4.3	2.1*	20.9	25.4	1.2	0.2	2.1	1.8	3.2	1.6
'Nabtet Saif'	0 DAP	1.8	9.5*	2.4	8.4*	29.6	3.9*	9.4	4.2*	8.8	11.8	20.4	24.4	18.9	18.2
	10 DAP	4.3	12.7*	1.8	3.5	70.7	15.3*	148.6	110.9	3.6	3.2	17.2	15.6	10.6	8.7
	20 DAP	1.2	2.1	1.2	2.3	5.6	2.2	12.8	16.5	1.9	1.2	1.3	2.5	2.4	1.8

DAP = days after pollination, N = normal fruit, s = flowers/fruits exhibiting indications of 'shees', BHH = Benzaldehyde, 2-hydroxy-(2-hydroxyphenyl) methylene-hydrazone, * = significantly different from the corresponding normal flower/fruit at $P \leq 0.05$

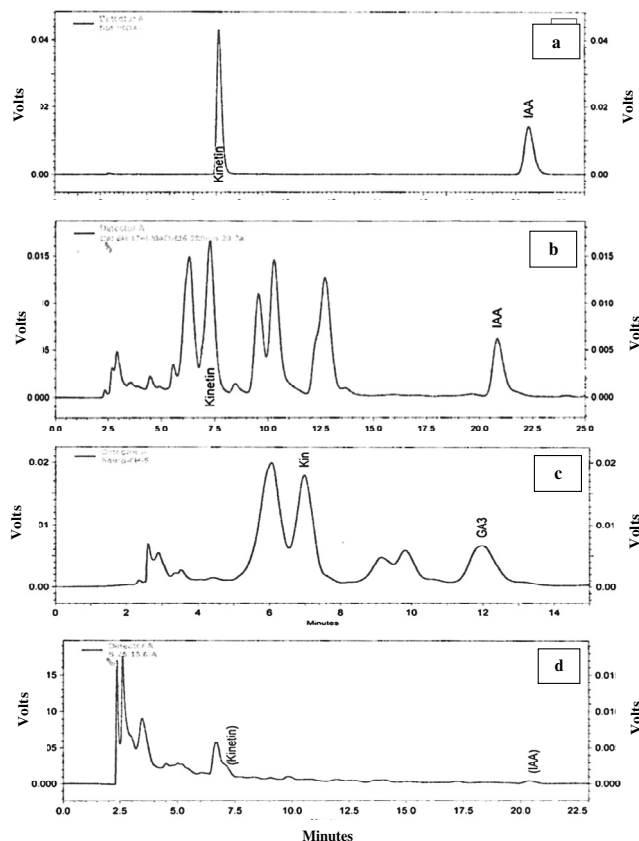


Fig. 2: HPLC chromatograms showing elution properties of some external standards and indigenous hormones of developing flowers/fruits of cv. 'Barhy'. (a) Kinetin and IAA standards. (b) IAA and five unknown compounds in flowers of cv. 'Barhy' at 0 DAP (A compound eluted at the same time as kinetin standard). (c) GA₃ in fruits of cv. 'Barhy' at 10 DAP stage. (d) Highly reduced hormone levels in fruits of cv. 'Barhy' at 30 DAP stage

forming flowers/fruits at all stage of development (Table 1). Another compound (unknown-1) that eluted immediately before BHH also showed significantly lower level in 'shees'-forming flowers at 0 DAP stage in both the cultivars. Level of this compound increased enormously in normal fruits by 10 DAP; but remained significantly lower in 'shees' fruits. The levels dropped sharply by 20 DAP in

both types of fruits. The compound is yet to be identified. Three more unknown compounds, all eluting after BHH in HPLC analyses, were detected with similar levels in normal and 'shees' flowers/fruits of both the cultivars (Table 1). Generally, their levels declined from 0 to 20 DAP. These three compounds are also being characterized.

Discussion

Observations of this study suggest that several hormones, including IAA and GA₃ are involved in development of fruit in date palm. Role of coordinated hormonal activity in fruit formation in various plant species is well documented (Liu *et al.*, 2008; Dorsey *et al.*, 2009; Wang *et al.*, 2009). ABA as an agent of abscission was expected in developing fruits of date palm since two out of three carpels in the flower degenerate and fall down during the course of normal fruit formation in this species. ABA is generally active in association with IAA and GA₃ in developing fruits (Buta and Spaulding, 1994; Setha *et al.*, 2004). Absence of ABA in our samples suggests that in date palm, the two non-fruit forming carpels just degenerate and shed away without the formation of an abscission layer. Among the expected cytokinins, Zeatin, ZR, and 2iP were also not detected in our procedures; while Zeatin and its derivatives are known to be present in coconut, a related palm (Ge *et al.*, 2004). Possibly, cytokinins other than these may be involved in date palm fruit formation peaking as unknowns in our samples.

Hormone constituents of normal and 'shees' flowers/fruits were similar to each other and were also similar in the two cultivars as well. Concurrent occurrence of hormones in two types of flowers/fruits indicates that abnormal development of 'shees' fruit may be due to the difference in the level of hormones or their ratio rather than due to absence or addition of some compounds. Higher than normal levels of IAA and GA₃ have been noted in many natural parthenocarpic fruits as well (George *et al.*, 1984; Talon *et al.*, 1990b; 1992). It has also been demonstrated that parthenocarpic fruit development can be induced by exogenous application of GA₃ and IAA (Schwabe and Mills, 1981). Possibly, elevated levels of these hormones in flowers of tissue culture derived plants of date palm may be among the factors responsible for formation of 'shees'

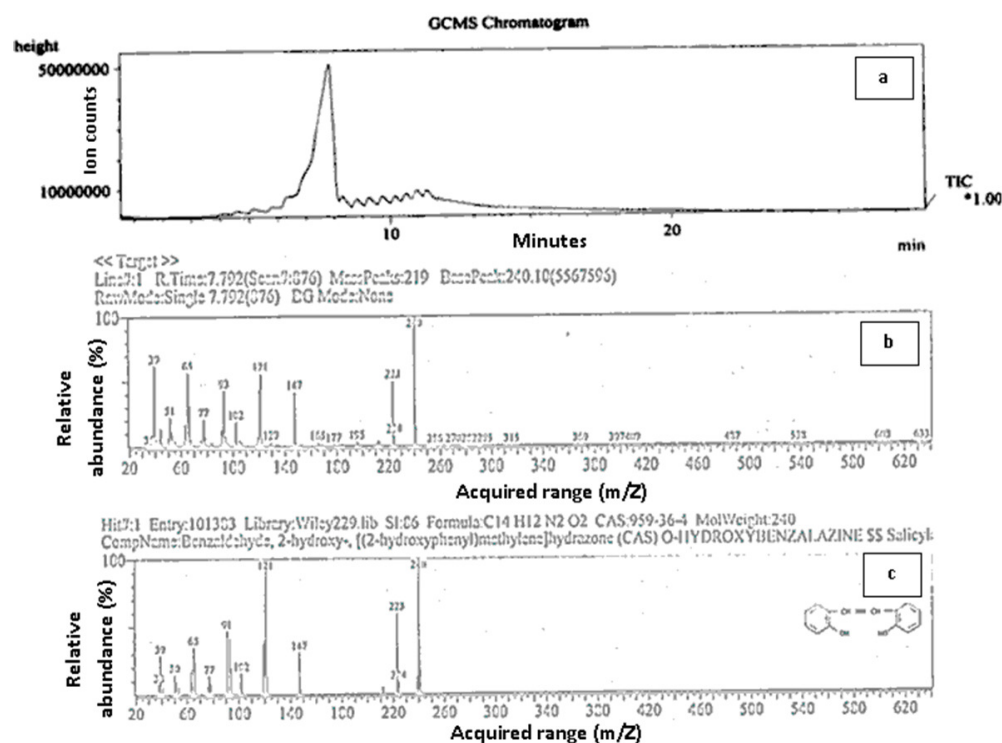


Fig. 3: GC-MS chromatogram of purified 'kinetin-like' compound (a), mass spectrum of purified 'kinetin-like' compound (b) and Hit-1 of Wiley-229 library (c)

fruits. Why these hormones are in greater abundance in tissue culture derived plants is not clear.

It has been suggested that failure of fertilization leads to parthenocarpy in some cases (Vivian-Smith and Koltunow, 1999) including date palm (Reuveni, 1986). However, present observations indicate that elevated levels of IAA and GA₃ in 'shees' flowers/fruits of tissue culture derived plants of date palm exist from pre-pollination/fertilization (0 DAP) stage; therefore, failure of fertilization may not be the cause of 'shees' in these plants. On the other hand, 'shees' appearing in normal offshoot-derived date palm plants in the absence of pollination/fertilization (Reuveni, 1986) may be the result of post-fertilization events, which may or may not be operating with the involvement of these two hormones.

It has been suggested that inadequate level of endogenous hormones may cause parthenocarpic fruit development (Vivian-Smith and Koltunow, 1999). Li *et al.* (2003) have shown that pollination and application of exogenous cytokinins enhanced the level of CycD3, a cyclic protein associated with cell division in white flower gourd. We have also found in a previous study (Al-Khalifah *et al.*, 2007a) that application of kinetin on stigmas before pollination in known 'shees' bearing plants greatly suppresses 'shees' formation. Assuming that exogenous cytokinins make up for insufficiency of the endogenous hormones, BHH and unknown-1 should have a cytokinin-like activity. Hence, lower levels of these two compounds

may be part of the causes associated with 'shees' formation.

Our attempts to elicit genetic markers associated with this fruit abnormality of tissue culture derived plants of date palm could not produce consistent results with the help of RAPD procedures (Al-Khalifah *et al.*, 2007b). Gurevich *et al.* (2005) also detected low level of genetic variation in culture derived plants of date palm which formed abnormal fruits ('shees') with the help of RAPD and AFLP techniques and could not establish authentic genetic markers for this abnormality. Apparently, the expression level of genes regulating synthesis of different hormones has been altered in this case without gross genomic changes, which could be detected by DNA profiling procedures. This may hold well for many other somaclonal variations (Karp, 1995; Kaeppler *et al.*, 2000) frequently appearing in tissue culture derived populations of different plant species. Present findings suggest that 'shees' fruit abnormality in tissue culture derived plants of date palm is caused due to disruption of hormone profiles of flowers/fruits as evidenced by elevated levels of IAA and GA₃ and lower levels of BHH and another unknown compound.

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