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Full Length Article

An Efficient Direct Somatic Embryogenesis Protocol for Genetic Stability and Gene Transfer of Egyptian Date Palm Cultivars Sewi and Hayani

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Abstract

The agronomy of date palm (*Phoenix dactylifera* L.) has benefited significantly from biotechnology. Primary aim of current research was to develop a direct somatic embryogenesis (DSE) regeneration protocol of date palm shoot tip explant and study the genetic similarity of these directly regenerated plantlets compared to other indirectly regenerated plantlets via recommended indirect somatic embryogenesis (IDSE) regeneration protocol. Also, the DSE procedure was used for the first time to perform an *Agrobacterium*-mediated transformation. For the study of DSE protocol, data was collected for browning appearance degree, embryonic cell formation degree, globularization degree and number of direct somatic embryos produced. The results support that a nutrient culture medium of NAA at 0.25 mg/L for developing an efficient regeneration protocol by DSE for both date palm cultivars. Start codon targeted (SCoT) polymorphism was utilized to study the genetic stability between mother plants and regenerated plants via DSE and IDSE protocols. Results confirmed that direct plantlets regeneration had the highest genetic stability compared to indirect plantlets regeneration. Also, the genetic similarity of DSE regenerated plantlets of Sewi was higher (0.99%) than that of IDSE regenerated plantlets of Hayani (0.98%). Successful *Agrobacterium*-mediated genetic transformation of date palm via DSE was achieved with a transformation percentage of 4.8 for Sewi and 4.2 for Hayani. This work represents a vital step towards stable genetic transformation for this sustainable cash crop of monocot species.

Key words: *Agrobacterium tumefaciens;* Date palm; Direct somatic embryogenesis; *Gus* gene; Somaclonal variation
Abbreviations: DSE, Direct somatic embryogenesis; IDSE, Indirect somatic embryogenesis; SCoT, Start codon targeted; *A. tumefaciens*, *Agrobacterium tumefaciens*; NAA, Naphthaleneacetic acid; 2, 4-D, 2, 4-dichlorophenoxy acetic acid; NOA, 2-naphthoxyacetic acid; BA, 6-benzyladenine; 2iP, 6-g, g-dimethylallylaminopurine; dNTPs, deoxynucleoside triphosphates; TBE, Tris/Borate/Ethylenediaminetetraacetic acid

Introduction

Date palm is a monocotyledon fruitful tree belonging to Arecaceae family. It is considered an essential crop in the hot arid regions in the Middle East and North Africa (Jain 2012; Gantait *et al.* 2018). Date palm contributes to settlement and food security, being a staple food of countries concerned about its plantation, so therefore this crop has special attention from the Arab governments and scientists. Meanwhile, other countries also are interested in date palm cultivation. The countries that produce the most date palm on a global scale include Egypt (17%), Iran (16%), Saudi Arabia (8.8%) and Iraq (7.3%) (FAO 2020). In general, date palm trees provide integrated benefits for human life, represented in the delicious fruits of high nutrient values and abundant bioactive medicinal compounds in date palm tissues as anticancer and antibacterial, antifungal, antioxidants and anti-sterility (Eldawayati *et al.* 2022), besides various food dates industries, and traditional or advanced industries utilizing raw material of whole tree parts (Nasser *et al.* 2016; Awad *et al.* 2021).

Date palm tree is characterized by its resilience to various biotic and abiotic stresses of ecological conditions and high cultivars biodiversity, which permit a felicitous plantation under different ranges of environmental conditions (Patankar *et al.* 2019; Elarabi *et al.* 2021; Bouhouch *et al.* 2021). Date palm propagation using sexual propagation by seeds is not assured for coming progeny due to its higher genetic heterozygosity and its dioecious nature. Mainly, date palm traditional propagation is conducted by using the

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mother plant off shoots. However, higher offshoot costs and limited number per tree, especially for the economic and rare cultivars, obstruct the expansion of date palm plantation (Chao and Krueger 2007; Jain 2012). Furthermore, for date palm breeding programs, long tree life cycle and its large size are main challenges for date palm cultivars development (El Hadrami *et al.* 2011; Kordrostami *et al.* 2022).

Biotechnology has pronouncedly influenced date palm agronomy by addressing difficulties associated with date palm propagation (Johnson 2011; Ibrahim 2018; Kordrostami *et al.* 2022). Tissue culture enables a fast and high-mass propagation method for date palm cultivars as an alternative reliable propagation method (Mazri *et al.* 2018) depending on the totipotency potential of mature meristematic cell tissues of the mother plant such as shoot tips (Mazri *et al.* 2017; Zein El Din *et al.* 2021), axillary bud (Al-Khateeb 2006; Moghaieb *et al.* 2011) and inflorescences (Zayed 2011; Sidky and Eldawayati 2012; Abd Elaziem *et al.* 2022). A considerable number of studies have covered the date palm micropropagation protocols and discussed different remarkable factors to optimize production process protocols (Eldawayati 2000; Mazri and Meziani 2015; Zayed 2020). Several republic agencies besides private sectors are commercially working on date palm tissue culture. *In vitro* regeneration of date palm is achieved by three protocols. First protocol is an indirect somatic embryogenesis, where the callus phase interferes during initial differentiation, which is intensively discussed by numerous studies and is characterized by high production of regenerated plantlets (Fki *et al.* 2011; Al Mayahi 2015; Eldawayati *et al.* 2018). Second is direct shoot organogenesis (Bekheet 2013; Meziani *et al.* 2016). Third one is direct somatic embryogenesis (Sidky and Zaid 2011), where date palm plantlets proliferation through direct protocols are achieved without middle callus phase interference, which offers to confirm a low opportunity for the occurrence of somaclonal variation in the regenerated plantlets. However, there are limited studies on direct morphogenesis regeneration in date palm because of difficulties concerning different date palm genotypic responses (Baghdady *et al.* 2018). It is recognized that growth regulators have a significant role in inducing the morphogenesis process for cells in most plant species (Raspor *et al.* 2021; Asghar *et al.* 2023). Different auxins like naphthalene acetic acid (NAA), 2, 4-dichlorophenoxy acetic acid (2, 4-D), 2-naphthoxyacetic acid (NOA), and cytokinins like N6-benzyladenine (BA), kinetin, 6-g, g-dimethylallyl aminopurine (2iP) are implemented in date palm micropropagation protocols (Mazri *et al.*2017; Zayed 2020).

Complementary to the progression in date palm propagation by tissue culture techniques, biotechnology also highlights new technologies in molecular markers (Marsafari and Mehrabi 2013; Al-Khalifah and Shanavaskhan 2017), gene transformation (Mousavi and Fard 2019), germplasm conservation (Bekheet 2017; Eldawayati 2020) and genomics (Al-Dous *et al.* 2011; Asaf *et al.* 2018; Kordrostami *et al.* 2022). Worth to mention that improving the quality and quantity yield of date palm varieties is an essential requirement for the sustainable development of this significant cash crop. Gene transfer technology could be a key for a novel improved date palm breeding, skipping the selection programs time consumed, only target genetic changes are done without altering the whole remaining genetic makeup (Jain 2012; Sattar *et al.* 2017). Importantly, the genetic transformation of date palm is still in its first steps, where countable studies have been conducted so far, and several factors have been studied for complete protocol of gene transformation of date palm (Saker *et al.* 2007). These pioneering studies from Egypt (Saker *et al.* 2009; Badr-Elden *et al.* 2017), Iran (Mousavi *et al.* 2009; Mousavi *et al.* 2014a, b), Bangladesh and India (El-Rakashy *et al.* 2011; Aslam *et al.* 2015) conducted by using two methods. The first one employed T-DNA delivery mediated by strains of soil bacterium *A. tumefaciens* (Mousavi *et al.* 2014a; Aslam *et al.* 2015; Badr-Elden *et al.* 2017), or by direct gene transfer with microprojectile bombardment (Saker *et al.* 2009; Mousavi *et al.* 2009; Mousavi *et al.* 2014a, b). Transformation protocols have been studied for different varieties of tropical and subtropical fruit species of families of both dicots and monocots based on embryogenic and organogenic regeneration routes (Mousavi *et al.* 2019; Kong *et al.* 2020). In date palm, both of the two T-DNA delivery methods were conducted via embryonic callus (Saker *et al.* 2009; Mousavi *et al.* 2009; Mousavi *et al.* 2014a; Badr-Elden *et al.* 2017) or mature somatic embryo of indirect somatic embryogenesis protocol (Mousavi *et al.* 2014b; Aslam *et al.* 2015).

The most published trials of date palm transformation through *Agrobacterium*-mediated or microprojectile bombardment systems used *gus* (β-glucuronidase) as a gene reporter, which is easy to conduct genetic assay (Leclercq *et al.* 2010; Chaparro-Pulido *et al.* 2014). In the light of this quick overview of micropropagation and gene transformation studies in date palm tree and in our knowledge, there is no report on *Agrobacterium*-mediated genetic transformation of date palm via direct somatic embryogenesis (DSE) regeneration protocol. The present work mainly aims to conduct an efficient *Agrobacterium*-mediated genetic transformation of date palm for the first time via direct globular embryonic cells of two Egyptian date palm Sewi and Hayani cultivars, which is collected to study the influence of lowering the naphthalene acetic acid (NAA) concentration in regeneration protocol medium of direct shoots morphogenesis to develop a regeneration protocol of DSE of date palm shoot tip explant and studying genetic similarity of direct regenerated plantlets compared to indirect regenerated plantlets via IDSE protocol, for both date palm cultivars Sewi and Hayani.

Materials and Methods

Plant material

Healthy selected offshoots of Egyptian cultivars Sewi (semi-

dry) and Hayani (soft), about 15–25 kg weight and 60–100 cm were separated from certified donor mother trees of date palm. The verification of cultivars and plant's sex identification has been done during the fruiting season.

Establishment of shoot tip explants

The preparation and sterilization procedures of shoot tip explants were according to Eldawayati and Zayed (2017) protocol, which were started as usual by removing the outer leaf bases and hard fibrous of the offshoot with their surrounding young leaves. The obtained shoot tip explants (approximately 4.0 cm length × 3.0 cm width) were transferred to aseptic conditions. Shoot tip explants go through a double surface sterilization process using 0.1 mg/L mercuric chloride ($HgCl_2$) solution for 10 min, followed by 50 min and three rinses with sterilized distilled water. To establish the cultures of DSE and IDSE, disinfected and peeled shoot tip explants (consisting of 4–5 primordial leaves around the apical meristem) were longitudinally cut into four equal segments.

DSE protocol

To investigate the influence of lowering NAA concentration in the regeneration protocol medium of the direct shoots morphogenesis to develop a regeneration protocol of direct somatic embryogenesis of the date palm shoot tip explant, all the established longitudinal segments of the shoot tip (LSS) explants of date palm studied cultivars were cultured on basics culture medium composition, which consisted of nutrient salts of MS medium (Murashige and Skoog 1962) with the addition of 100 mg/L myo-inositol, 0.1 mg/L biotin, 0.5 mg/L nicotinic acid, 170 mg/L $NaH_2PO_4.2H_2O$, 200 mg/L glutamine, 40 mg/L adenine sulfate, 1.0 mg/L thiamine hydrochloride, 0.1 mg/L pyridoxine HCl, 3.0 mg/L activated charcoal, 40 mg/L sucrose and supplemented with a recommended growth regulators for direct organogenesis by Khierallah and Bader (2006) of 1.0 mg/L NAA, 1.0 mg/L NOA, 2.0 mg/L 2ip, 1.0 mg/L BA (M0) as a control treatment. The LSS explants were also cultured on the same previous basics culture medium composition plus the same growth regulators but with lowering of NAA concentration to (0.25, 0.5 and 0.75 mg/L) as treatments as follows:

M1 = 0.25 mg/L NAA, 1.0 mg/L NOA, 2.0 mg/L 2ip, 1.0 mg/L BA
M2 = 0.5 mg/L NAA, 1.0 mg/L NOA, 2.0 mg/L 2ip, 1.0 mg/L BA
M3 = 0.75 mg/L NAA, 1.0 mg/L NOA, 2.0 mg/L 2ip, and 1.0 mg/L BA

For all treatments, the pH was adjusted to 5.8 before adding agar and all prepared culture media were autoclaved at 1.5 kg/cm^2 pressure and 121°C for 20 min. All cultured explants on treatments and control treatment were subcultured every six weeks. Data were recorded visually in every subculture for the browning degree appearance of the cultures, embryonic cell appearance degree, globularization degree of the embedded embryonic tissue and number of produced direct somatic embryos. According to recommendations, the data indicating "degree of appearance" were rated numerically e.g., 0 = no change, 1 = below average, 2 = average, 3 = above average, 4 = high, and 5 = very high (Eldawayati *et al.* 2020).

IDSE protocol

This experiment was conducted according to the recommended protocol for callogenesis morphogenesis to obtain indirect plantlets regeneration (Eldawayati *et al.* 2018).

Somatic embryos germination, shoots multiplication and plantlets development

All normal matured somatic embryos received individually or in repetitive clusters from direct somatic embryogenesis treatments or from the indirect somatic embryogenesis protocol were transferred to the germination and shoot multiplication media consisted of all components of basics culture medium as mentioned above, without glutamine and supplemented with growth regulators of 0.05 mg/L BA, 0.1 mg/L NAA and 0.1 g/L activated charcoal. The macro-elements of MS nutrients were modified to avoid the hyperhydrisity as described by Eldawayati and Zayed (2017). Each culture was incubated for 16 h under cool-white, fluorescent light (25 $mol/m^2/s$) at a temperature of 27±2°C. All the proliferated shoots about 6–7 cm received from the DSE and IDSE were transferred to the rooting culture nutrient medium of 3/4 MS supplemented with of growth regulators 1.0 mg/L NAA, 1.0 mg/L indole-3-butyric acid (IBA), 0.4 mg/L paclobutrazol (Pbz), and at 27.2°C, all cultures were incubated for 16 h under cool-white fluorescent light (42 $mol/m^2/s$), the rooting protocol followed was of Abd Elzaher *et al.* (2019) to obtain the full plantlets. All experiments were repeated three times.

Determination of genetic similarity of regenerated plantlets from protocols of DSE, IDSE and mother plants

Using SCoT markers, the genetic similarity between the mother plants of the Sewi and Hayani cultivars and the regenerated date palm plantlets acquired from the DSE and the IDSE was studied. The DNA was extracted from the DSE and the IDSE regenerated plantlets (50 plantlets) and the mother plants utilizing Qiagen Dneasy plant mini kit (Qiagen, cat. no. 69104). Ten arbitrary SCoT primers were utilized (Table 1). The reaction was performed in 25 mL volume containing of 10 ng DNA, 200 M deoxynucleoside triphosphates (dNTPs), 1 M primer, 0.5 units of Red Hot *Taq* polymerase (AB-gene Housse, UK), and 10-X *Taq* polymerase buffer (AB-gene House, UK). For DNA amplification, a Perkin Elmer thermal cycler (2720) was created as follows: Denaturation: 5 min at 94°C. After that, there were 35 cycles at 94°C for 0.45 sec, annealing at 52°C

Table 1: SCoT primers name and sequences

No.	Primer name	Sequence (5′-3′)
1	SCoT 30	CAACAATGGCTACCACCT
2	SCoT 31	CAACAATGGCTACCACGA
3	SCoT 33	AAGCAATGGCTACCACCA
4	SCoT 34	ACGACATGGCGACCAACG
5	SCoT 35	AACCATGGCTACCACCAC
6	SCoT 36	CACCATGGCTACCACCAT
7	SCoT 39	CAACAATGGCTACCACGG
8	SCoT 40	CAACAATGGCTACCACGT
9	SCoT 41	CAACAATGGCTACCAGCA
10	SCoT 42	ACGACATGGCGACCATCG

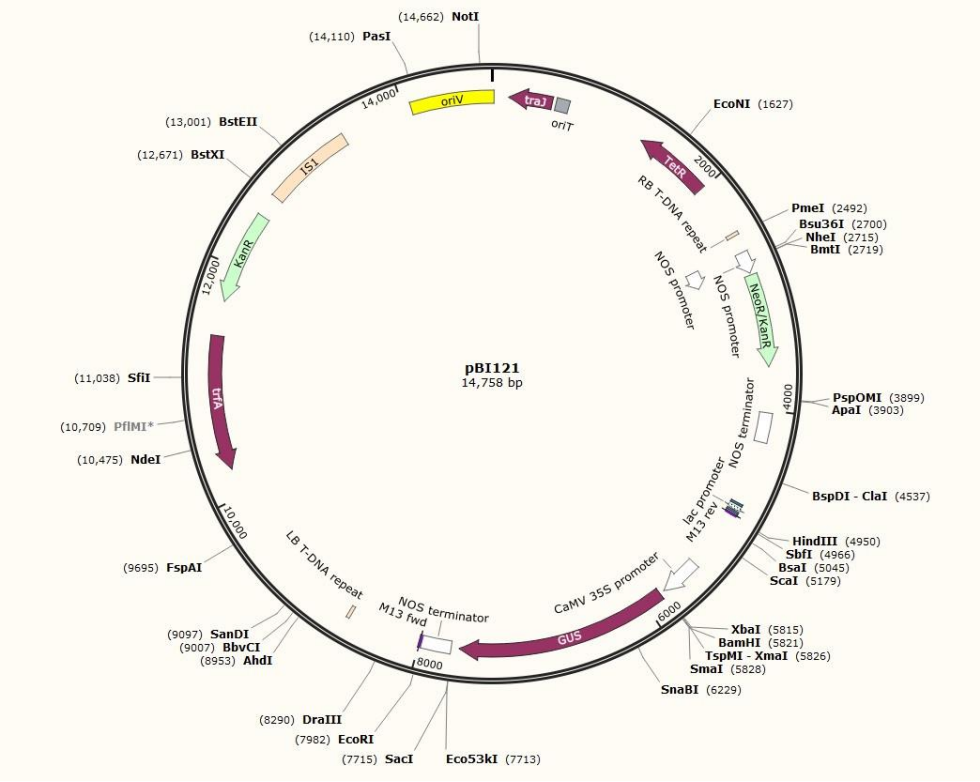

Fig. 1: Schematic illustration of pBI-121 plasmid; Abbreviations: RB: right border; LB: left border; *gus:*β-glucurodinase; *Kan^R:neomycinphosphotransferase-II* gene and CaMV35SP: Cauliflower Mosaic Virus promoter (Jefferson *et al.* 1987)

for 1 min, extension at 72°C for 7 min (Adawy *et al.* 2004). In a 1 X Tris/Borate/Ethylenediamine tetra acetic acid (TBE) buffer, the amplification products were separated on a 2% (w/v) agarose gel before being seen with ethidium bromide staining.

Agrobacterium-mediated gene transformation of date palm

Direct globular embryonic cells collected from the developed DSE protocol of date palm cv. Sewi and Hayani were used as explants at 1 cm size for the gene transformation study. Dr. Reda E.A. Moghaieb, Cairo University, Egypt, generously provided *A. tumefactions* strain LBA4404 carrying the binary vector pBI121, for use in the date palm transformation (Fig. 1) following Jefferson *et al.* (1987). In 30 mL of LB medium containing 100 mg/L kanamycin sulphate (Sigma-Aldrich, Japan) at 28°C, *A. tumefaciens* was cultivated overnight. The explants spent 5 min submerged in the bacterial suspension (Elarabi *et al.* 2021). The explants were then transferred onto a nutritional medium for shoot multiplication supplemented with (0.1 mg/L NAA + 0.05 BA mg/L), 1000 mg/L cefotaxime, and 100 mg/L kanamycin after being blotted on a sterilized Whatman No. 1 filter paper (Aslam *et al.* 2015). On shoot multiplication medium, the explants were subcultured monthly until all of the somatic embryos had developed into shoots.

Transformation conformation using PCR

The potential transgenic plantlets were examined by PCR to confirm permanent insertion of the T-DNA into date palm genome. Using a Qiagen DNeasy plant mini kit (Qiagen, Cat. No. 69104), total genomic DNA was extracted from fresh leaves of transgenic date palm. The *gus* specific primers, 5'AGTGTACGTATCACCGTTTGTGTGAAC-3'(forward) and 5'-ATCGCCGCTTTGGACATACCATCCGTA-3'(reverse) were utilized to amplify about 730 bp of the coding region for *gus* gene. A 10 µL volume of the amaR OnePCR™ (Cat. No. SM213) master mix, 5 µL of purified DNA (10 g/L), 0.5 µL of each primer (2 mM/L), and 3 µL of dH_2O were used in the reactions. To achieve PCR amplification, the reaction was first denatured at 94°C for 4 min, then 35 cycles of annealing at 58°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 4 min, with a hold given to prolong any premature DNA synthesis. On a 1% agarose gel, the PCR products were subsequently separated.

Histochemical GUS assay

The method developed by Moghaieb *et al*. (2010) was used to perform the histochemical assay of GUS activity. Proliferated transgenic shootlets produced from DSE were treated at 37°C for overnight with a leaf immersed in a substrate solution containing 100 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 0.3% (m/v) X-Gluc, and 0.1% (v/v) and Triton X-100. The expression of *gus* gene was seen after destaining the tissues by soaking and washing in 70% ethanol numerous times.

Statistical analysis

Using four replications per treatment and five samples per replication, the experiment was repeated twice. One-way ANOVA was performed on the received data. Using the SPSS program (Version 20, SPSS Inc. Chicago, USA), the significant variation in the treatment data (mean±SE) was examined using Duncan's Multiple Range Test (Duncan 1955) at $P \leq 0.05$. For SCoT analysis, the fraction of common bands amplified by each pair of primers was compared pair-by-pair to assess differences between date palm genotypes (Nei 1978). Statistical package SPSS was used to calculate the similarity coefficient (Stat Soft Inc., Tulsa OK, USA).

Results

DSE protocol

Results indicated that LSS explants of Sewi and Hayani cultivars under the applied treatment (M0, M1, M2 and M3) of culture nutrient media started to swell after about first four weeks of the cultures period and stained by browning appearance after about first eight weeks. The browning degree of all cultured explants of both cultivars on applied treatments was not significantly affected by lowering NAA concentration in the culture media composition (Fig. 2). However, the browning degree was significantly higher in the cultured explants of the Sewi cultivar than what appeared on the cultured explants of Hayani cultivar (Fig. 2a).

Formation of early embryonic cells

After second subculture (12 weeks), a glossy, spongy structure of smooth tissues, embryonic with light yellow to light brown colorist was developed on the edges of longitudinal segments of shoot tip explants, particularly near upper edges of explant bases (Fig. 3a). During the third subculture (18 weeks interval), these tissues became thicker, increased in volume and started to granulate, looking like embryogenic cells. Results revealed that explants of Sewi and Hayani cultivars cultured on M1 treatment of NAA at 0.25 mg/L were superior in the formation degree of early embryonic cells, followed by degree values of early embryonic cells obtained from cultured explants of both the cultivars under M2 and M3 treatments (Fig. 2b). LSS explants of Sewi and Hayani cultured on M0 treatment gave the lowest degree values for early embryonic cell formation degree. It was also observed that LSS explants of Hayani were superior in forming an early embryonic cell than LSS explants of Sewi, which was significantly exhibited lower result (Table 2; Figs. 3b–c).

Globular structure formation (globulrization degree)

After the third subculture (18 weeks), all of these early embryonic cells of all cultured explants of both cultivars on different treatments began to form globular structures varying from small cells to clear spherical embryos structure (Fig. 3d), attached to cultured explants or spread on the culture medium surface (Fig. 4). Results revealed that the cultured explants on M0 treatment of NAA at 1.0 mg/L recorded the lowest degree value of globular structure from early embryonic cells. However, a highly significant value of globularization degree for the produced embryonic cells was observed with cultured explants on M1 NAA treatment of Sewi and Hayani, followed by globule formation degree values of the produced embryonic cells from shoot tip explants of both cultivars, cultured on M2 NAA treatment (Fig. 2c). The globulrization degree of embryonic cells from shoot tip explants, cultured on M3 NAA treatment at 0.75 mg/L, without significant difference in between M2 and M3. It was also indicated that there was no significant difference between both genotypes of Sewi and Hayani explants in globulrization degree for the produced embryonic tissues.

Table 2: PCR amplicons obtained from SCoT markers between Hayani DSE and IDSE regenerated plants and the mother plant

No.	DSE protocol				IDSE protocol			
	Primer name	Total bands	Polymorphic band	Polymorphism (%)	Primer name	Total bands	Polymorphic band	Polymorphism (%)
1	30	12	2	16.6	30	11	3	27.3
2	31	9	3	33.3	31	8	4	50
3	33	10	3	30	33	7	3	42.8
4	34	11	2	18.1	34	10	2	20
5	35	11	3	27.2	35	11	6	54.5
6	36	8	1	12.5	36	7	0	0
7	39	7	4	57.1	39	6	4	66.6
8	40	5	3	60	40	5	3	60
9	41	8	2	25	41	8	3	37.5
10	42	7	1	14.2	42	11	7	63.6
	Total	88	24	27.3	Total	84	35	41.6

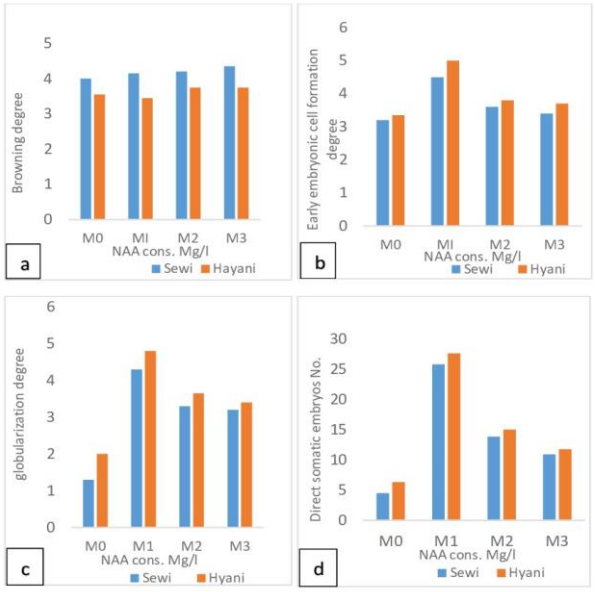

Fig. 2: Influence of reducing NAA on (a) browning (degree at 6-point scale), (b) early embryonic cell formation (degree at 6-point scale) (c) globular embryos formation (degree at 6-point scale) and (d) direct somatic embryo numbers of date palm Sewi and Hayani). Statistically significant difference at P\0.05 based on one-way analysis of variance followed by Duncan's multiple range test (Duncan 1955)

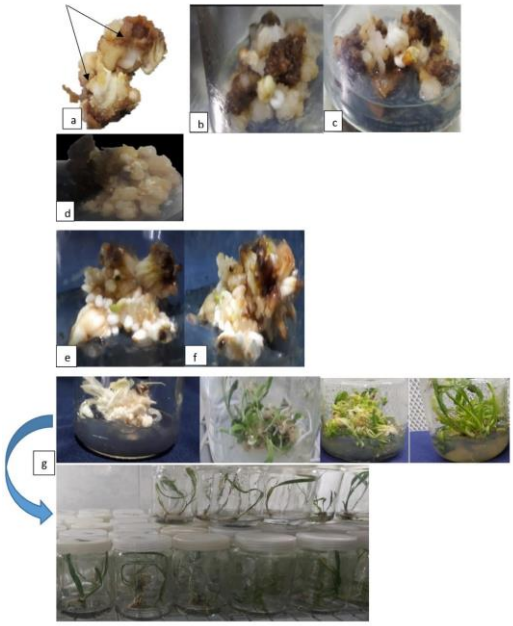

Fig. 3: The developed direct somatic embryogenesis protocol. Initial appearance of the developed embryonic cells on the edges of LSS explants after 12 weeks on culture treatments (a). Cultured LSS explants of Hayani (b) were superior in forming an early embryonic cell than the cultured LSS explants of Sewi (c) on the best treatment of NAA at 25 mg/L. Appearance of direct globular embryo after 18 weeks on the best treatment of NAA at 25 mg/L which is selected to be an explant material for *Agrobacterium*-mediated genetic transformation of date palm cultivars Sewi and Hayani (d). LSS explants of Hayani (e) exhibited a highly significant difference in the number of direct somatic embryos produced than the cultured LSS explants of Sewi (f), after 24 weeks of culture period. Subsequent growth development stages of the collected direct somatic embryos through stages of germination, shoots multiplication and full plantlets production by the developed DSE regeneration protocol (g)

At the end of fourth subculture (24 weeks), the bipolar cotyledonary somatic embryos developed and attached to or surrounded the cultured segments of shoot tip explants of Sewi and Hayani cultivars under different treatments. Results showed that LSS explants of Sewi and Hayani cultured on M0 NAA treatment recorded the lowest significant result for the number of direct somatic production embryos (Fig. 2d). On the other hand, LSS explants of Sewi and Hayani cultivars cultured on M1 NAA treatment recorded the highest number of direct somatic embryos. Moreover, the cultured LSS explants of Hayani were significantly higher in number of direct somatic embryos produced than cultured LSS explants of Sewi after 24 weeks of culture period (Figs. 3e–f). All direct somatic embryos from all treatments of both cultivars were transferred to continue their growth development of multiplication and rooting stages, till full plantlets formation, which could be successfully transfer to acclimatization stage.

Determination of genetic similarity of regenerated plantlets from protocols of DSE, IDSE and mother plants

SCoT analysis for Hayani cultivar: Among Hayani regenerated plantlets, a total of 88 alleles were detected from DSE protocol; only 24 of them (27.3%) were polymorphic markers. The primer SCoT–30 produced a maximum number of bands (12 bands), whilst SCoT–4 produced a minimum number of bands (5 bands). The IDSE protocol showed 35 polymorphic alleles out of 84 (41.6%) alleles produced using SCoT marker (Table 3; Fig. 4).

SCoT analysis for Sewi cultivar: Total number of amplicons amplified by the SCoT markers for Sewi date palm cultivar resulted from DSE protocol was 90 amplicons, with 16 polymorphic amplicons (21.1%). A highest number of amplicons was 13 SCoT–35, while a lowest number was 5 amplicons amplified by both SCoT–39 and SCoT–40 primers. Out of a total number of 88 amplicons, 31 were polymorphic fragments resulting from IDSE protocol. The polymorphic amplicons ranged from 0 to 4 amplified by SCoT–6 and SCoT–1, respectively. The percentage of polymorphism ranged from 0% by SCoT–6 to 37.5% by SCoT–42 primer (Table 3; Fig. 4).

Genetic similarity among mother plant and regenerated plants by SCoT markers

The generated SCoT markers were utilized to determine the genetic similarity among mother plant and regenerated plantlets for both the date palm cultivars. For Hayani, the genetic similarity ranged from 86–98% between mother plant and DSE plantlets, while similarity ranged from 77–93% between mother plant and indirect regenerated plantlets (Table 4). For Sewi, the genetic similarity ranged from 88–99% between mother plant and regenerated plantlets by DSE protocol, while the similarity ranged from 80–96% between mother plant and regenerated plantlets by IDSE protocol (Table 4). Regenerated plantlets by DSE protocol gave higher genetic similarity than those regenerated by IDSE protocol. Meanwhile, the genetic similarity of DSE regenerated plantlets of Sewi was higher (0.99%) than that found in DSE regenerated plantlets of Hayani (0.98%).

Cluster analysis

The SCoT direct results divided the Hayani regenerated plants and the mother plant into two main clusters. One included the mother plants and regenerated plant number 2 and the other cluster included the other regenerated plants (Fig. 5A). Indirect SCoT analysis results for Hayani regenerated plants divided the regenerated plants also into two clusters. One cluster included mother plants and regenerated plants number 9 and 7 (Fig. 5B). In addition, the relationships between mother and regenerated plants for Sewi cultivar are represented as a dendrogram. In SCoT analysis, the dendrogram divided results of direct regenerated protocol into two main clusters: the first one included three regenerated plants with mother plant, and the other cluster only included one regenerated plant (Fig. 5C). Results from indirect regenerated protocol divided regenerated plants into two clusters. The first one included two regenerated plants with mother plant and the other cluster included two regenerated plants (Fig. 5D).

***Agrobacterium*-mediated genetic transformation of date palm**

Direct globular embryonic cells derived during DSE protocol of date palm Hayani and Sewi were co-cultivated with *A. tumefaciens* LBA-4404 harboring the binary vector pBI-121 for three days. After that, infected globular embryonic cells were placed on the nutrient medium with low selection pressure. During selection, the untransformed globular embryonic cells failed to develop as shoots clusters and eventually bleached and became necrotic within three weeks. However, successfully transformed globular embryonic cells continued to grow vigorously and differentiated as germinated complete direct dipolar somatic embryos, which was subsequently converted to produce shoot clusters. Putative transgenic shoot clusters were regenerated on the nutrient medium components of somatic embryo germination and shoots multiplication, which supplemented with 0.05 mg/L BA, 0.1 mg/L NAA as growth regulators. During cultures selection, the explants were sub-cultured on fresh nutrient media with the addition of 100 mg/L kanamycin, which greatly reduced the number of escapes. The transformation for the two tested date palm cultivars after *Agrobacterium* infection and the selection were 4.8 and 4.2 for cultivars Sewi and Hayani, respectively (Table 5).

Conformation of GUS transformation using PCR

To confirm the presence of T-DNA in regenerated date palm shoot clusters. All T0 shoots were subjected to PCR analysis with specific primers for *gus* gene. The integration of *gus* gene in the genome of date palm Sewi and Hayani was confirmed by the presence of an amplified fragment of 730 bp. Amplification of this fragment was not observed in non-transformed shoots (Fig. 6).

Conformation of *GUS* expression

The histochemical GUS assay confirmed the presence and stable expression of integrated *gus* gene into the genome of date palm transgenic regenerated shoots of both cultivars. In this work, non-transgenic plants did not exhibit any GUS activity while kanamycin-resistant plants expressed GUS (Fig. 7).

Table 3: PCR amplicons obtained from SCoT markers between Sewi mother plant and DSE and ISDE regenerated plantlets

No.	Primer name	DSE protocol			IDSE		
		Total bands	Polymorphic band	Polymorphism (%)	Total bands	Polymorphic band	Polymorphism (%)
1	30	12	4	33.3	11	2	18.1
2	31	8	2	25	9	3	33.3
3	33	10	3	30	9	4	44.4
4	34	12	2	16.6	11	5	45.4
5	35	13	2	15.3	11	2	18.1
6	36	9	0	0	9	0	0
7	39	5	1	20	8	7	87.5
8	40	5	1	20	5	1	20
9	41	8	1	12.5	8	3	37.5
10	42	8	3	37.5	7	4	57.1
	Total	90	16	21.1	88	31	35.2

Table 4: Genetic similarity matrix of date palm Hayani and Sewi based on SCoT analysis

No.	Hayani DSE regenerated plantlets					No.	Hayani IDSE regenerated plantlets				
	1	2	3	4	5		1	6	7	8	9
1	1.00					1	1.00				
2	0.96	1.00				6	0.84	1.00			
3	**0.86**	0.90	1.00			7	0.90	**0.77**	1.00		
4	0.91	0.93	0.93	1.00		8	0.87	**0.93**	0.82	1.00	
5	0.91	0.92	0.92	**0.98**	1.00	9	0.91	0.84	0.89	0.82	1.00
	Sewi DSE regenerated plantlets						Sewi IDSE regenerated plantlets				
	10	11	12	13	14		10	15	16	17	18
10	1.00					10	1.00				
11	0.96	1.00				15	0.82	1.00			
12	0.93	0.94	1.00			16	0.85	0.95	1.00		
13	0.95	0.93	**0.88**	1.00		17	0.95	**0.80**	0.85	1.00	
14	0.96	0.95	0.90	**0.99**	1.00	18	0.95	0.79	0.84	**0.96**	1.00

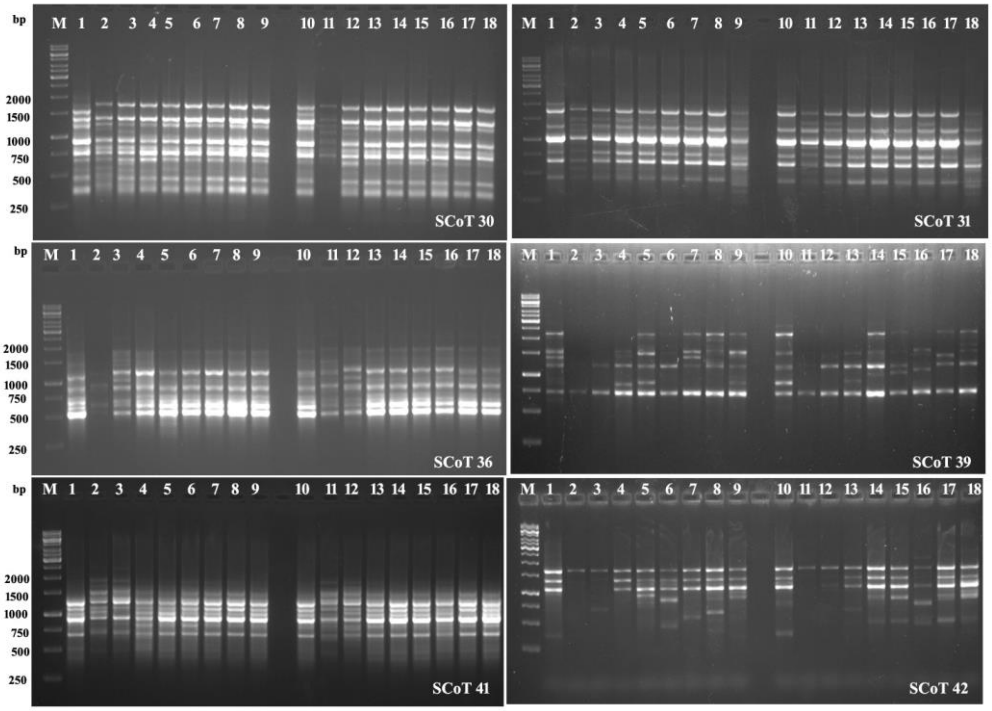

Fig. 4: SCoT profile demonstrating polymorphism among the two date palm cultivars. M refers to 1 Kb DNA ladder marker of 1Kb ladder. Lane 1 represented Hayani mother plant and lane 2–5 represents the regenerated plantlets resulted from the DSE protocol while lane 6–9 represents the regenerated plantlets resulted from IDSE protocol. Lane 10 represents Sewi mother plant and lane 11–14 represents the regenerated plantlets resulted from DSE protocol while lane 15–18 represents the regenerated plantlets resulted from IDSE protocol

Table 5: Transformation percentage of date palm cultivars infection with *Agrobacterium*

Cultivars	No. shoot	Gus positive	PCR positive	Transformation %
Sewi	251	12	12	4.8
Hayani	216	9	9	4.2

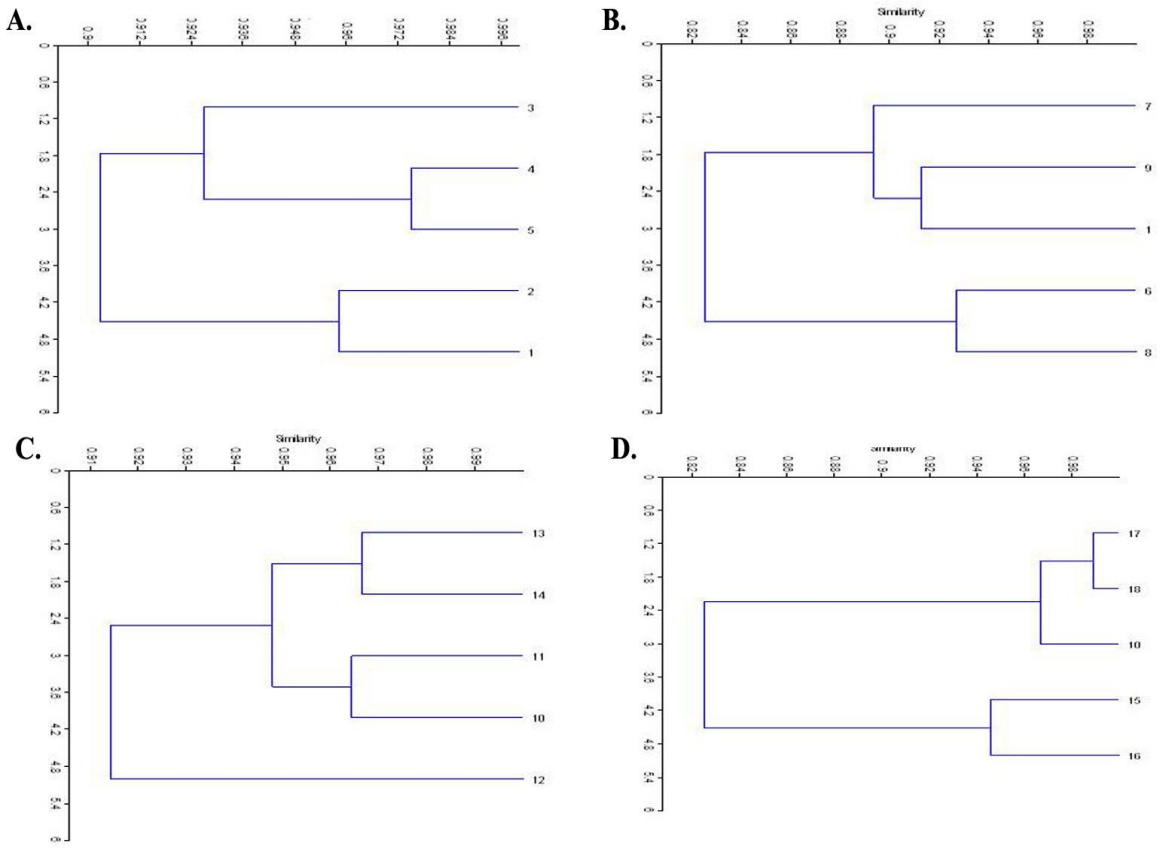

Fig. 5: Cluster analysis of two date palm cultivars. (A) Cluster analysis of Hayani mother plant and regenerated plantlets by DSE protocol, (B) Hayani mother plant and the regenerated plantlets by IDSE protocol, (C) Cluster analysis of Sewi mother plant the regenerated plantlets by DSE protocol and (D) Sewi mother plant and the regenerated plantlets by IDSE protocol

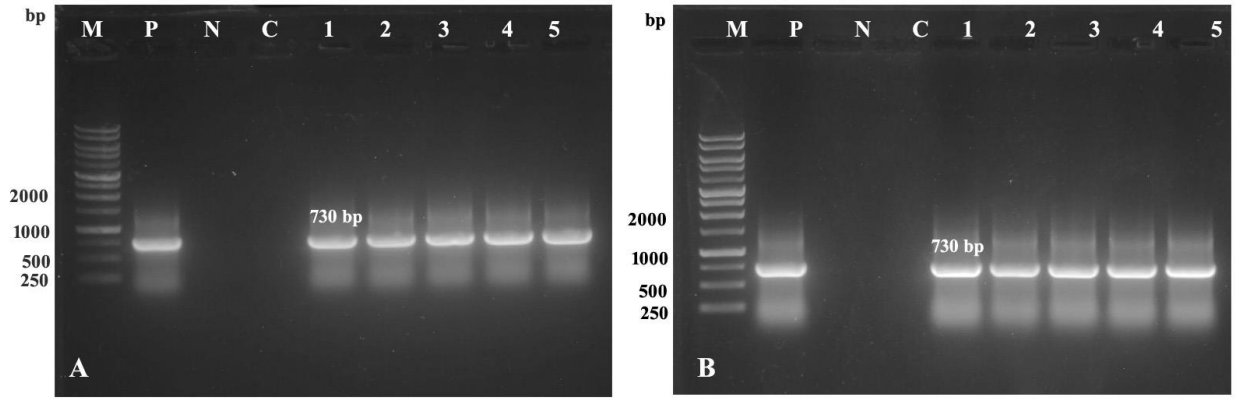

Fig. 6: Detection of the *gus* gene into the transformed regenerated plantlets of two date palm cultivars. (A) Hayani cultivar and (B) Sewi cultivar. M: 1 Kb DNA ladder, P: plasmid as positive control, N: dH_2O as negative control, N: non transformed date palm regenerated shoots and lines (1-5) the transformed regenerated date palm plants

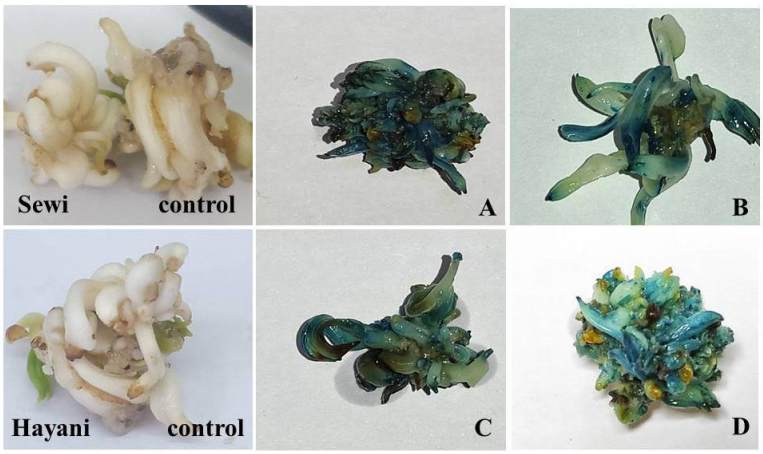

Fig. 7: Histochemical GUS assay showing gene expression in transgenic date palm Sewi and Hayani. A and B GUS expression for Sewi cultivar while C and D GUS expression for Hayani cultivar

Discussion

Given that tissue browning was a physiological problem in many plant species during *In vitro* propagation, the occurrence of tissue browning in cultured explants of both of date palm cultivars under study was examined. It has also been claimed that some explants release harmful secondary metabolites or phenolic compounds from cut surfaces, which oxidize later and turn the medium brown (El-Shafey *et al.* 1999; Helaly *et al.* 2008). Our findings showed that there was no observable difference in the degree of browning between examined treatments. It was concluded that normal tissues with normal separation of polyphenol oxidase and phenolic substances by membrane cell structure did not brown (Ju *et al.* 1988; Poudyal *et al.* 2008). Therefore, membrane disruption resulted in browning (Poudyal *et al.* 2008; Khan and Bibi 2012). In general, date palm tissues were abundant with phenolic compounds, the problem of browning was clearly evident during date palm micropropagation, while several studies have discussed this phenomenon (Abohatem *et al.* 2011; Mazri 2015; Eldawayati *et al.* 2020). According to reports, the primary factor contributing to the browning of explants was high concentration of caffeoylshikimic acids in date palm tissues (Loutfi and El Hadrami 2005). As a result, incubating tissues in complete darkness for three months in particular and incorporating activated charcoal into the culture medium may help lessen this phenomenon while also improving the responsiveness of tissues (Eldawayati *et al.* 2020). In addition, culturing explants during winter and spring seasons, pretreatment of explant soaking in an antioxidant solution. Small, cultured explants size and frequent reculturing to fresh culture medium are also found to reduce the incidence of tissue browning (Al-Khateeb 2008; Zaid *et al.* 2011). So, no sever browning was observed in the applied treatments because appropriate steps of decreasing browning degree were followed in this study. However, the browning degree was significantly higher in cultured shoot tip explants of Sewi cultivar than what appeared on cultured shoot tip explants of Hayani cultivar. This corroborates with the findings of Kriaa *et al.* (2012) who stated that the date palm genotype factor imposes one of the greatest limitations in the process of date palm regeneration by tissue culture technique. This is largely related to the level of richness of tissues in phenolic compounds, which inhibits cell proliferation process and hinder strain establishment because of toxicity in the culture media.

Somatic embryogenesis declares a complete model of the cells' totipotency and involves the action of a complex signaling network and reprogramming for gene expression patterns, which are regulated in a specific way. Where that gene regulation occurs as a result of response to exogenous stimuli of using plant growth regulators (PGR) or exposure to certain conditions of stress (Nic-Can Loyola-Vargas 2016). For the differentiation changes of shoot tip explants of Sewi and Hayani cultivars cultured on different treatments of nutrient culture media of direct morphogenesis, all indicated observations about the appearance of early embryonic cell formation, then the globular embryos formation till the production of direct somatic embryos production (Hassan *et al.* 2021). In this context, it was recognized that in direct somatic embryogenesis system, pre-embryogenic determined cells have the responsibility to initiate embryos directly from explant tissue committed to embryonic development and need only to be released. Many studies are interested in the role of auxin(s) in plant embryogenesis process. Each auxin may interfere through a different route due to the involvement of different signal transduction and gene programming in the plant cell (Méndez-Hernández *et al.* 2019). The present study confirmed that reducing NAA to

0.25 mg/L with other growth regulators of 1 mg/L NOA + 2 mg/L 2ip+ 1 mg/L BA was significantly superior in direct somatic embryos production from shoot tip explants of both studied Egyptian cultivars of date palm, during only six months compared to the control medium, of NAA at 1 mg/L with 1 mg/L NOA + 2 mg/L 2ip + 1 mg/L BA, gave the lowest significant results in producing direct somatic embryos. In date palm studies of direct shoot organogenesis protocol NAA at 1 mg/L combined with different cytokinins concentrations such as BA, 2ip, and kinetin was applied mainly in the culture media (Bekheet 2013; Jazinizadeh *et al.* 2015; Sidky 2017). Regarding our obtained result, it could be supposed that the interference of exogenously added auxin at a defined concentration could alter the endogenous hormone levels in the explant and may develop conditions that could encourage the embryogenic transition in somatic cells, which is suggested by different studies in this concern (Nic-Can and Loyola-Vargas 2016). It was stated that developing growth *in vitro* is highly concentrated on the balance between naturally endogenous growth substances of hormones in the plant cells and added concentrations of analogous synthetic substances to culture nutrient medium (George *et al.* 1993; Kahia *et al.* 2016). The presence of proper auxin concentration with the presence of proper cytokinin concentration appears to be more important to induce direct somatic embryogenesis in most micropropagated plants (Moradi *et al.* 2017). In this study, no direct shoot organogenesis was observed in the shoot tip explants of Egyptian cultivars Siwe and Hayani under control condition, although its composition was recommended for direct organogenesis from the shoot tip of Arabian cultivar Maktoom (Khierallah and Bader 2006). Our explanation is that many factors that affect the process of somatic embryogenesis, the origin of the explant and composition of culture medium, particularly the type and concentration of growth regulators, play a crucial role in success of micropropagation protocols, especially for direct morphogenesis protocols (Abohatem *et al.* 2011; Zaid *et al.* 2011). Most of the reports on direct morphogenesis protocols of direct shoot organogenesis or direct somatic embryogenesis of date palm are confined to specific genotypes, e.g., there is genotype specificity, and even closely related cultivars have different growth behavior under the same stimulants (Al-Khayri and Al-Bahrany 2004; Al-Khateeb 2008).

The SCoT markers were employed to examine the genetic stability and development of somaclonal variants between the mother plant and the regenerated plants produced using the DSE and IDSE protocols. For breeding strategies, the production of precise DNA fingerprints to define plants and detect genetic variety is very valuable. Despite being economically significant, there are extremely few breeding programs that choose desirable features. The advancement of agricultural, horticultural, and nutritional value greatly benefits from the development of precise DNA fingerprints to classify date palm cultivars and the detection of genetic diversity and somaclonal variations (Tanya *et al.* 2011; Xie *et al.* 2011). Previously attempts to create molecular markers in date palms were well documented and relied on ISSR, RAPD, AFLP, and SSR approaches (Adawy *et al.* 2004; Adawy *et al.* 2005; Al-Hadidi *et al.* 2010; Rhouma-Chatti *et al.* 2011; Xie *et al.* 2011). The identification of cultivars and investigation of genetic diversity and evolution benefit greatly from these markers. Vegetative reproduction by tissue culture or offshoots should produce offspring that are genetically identical to the mother tree and each other. Yet, it is generally recognized that plants propagated through tissue culture do experience somaclonal variation, resulting in occasionally different plants (Kaeppler *et al.* 2000). In this study ten primers SCoT markers were used to evaluate the somaclonal variations between mother plant and regenerated plants results from direct and indirect protocols in the two date palm cultivars.

In plant biotechnology, *Agrobacterium*-mediated transformation is a common approach. *A. tumefaciens* is a well-known method for facilitating effective gene transfer in both monocots and dicots. Genetic engineering and *Agrobacterium*-mediated transformation have some benefits over conventional gene breeding techniques (Kharte *et al.* 2016). Monocot development by genetic engineering is typically hampered by the absence of adequate improvement systems, low cell competence, and necessary wound responses. Apart from these challenges, several transgenic monocots have been produced using *Agrobacterium* transformation approach with great efficiency by modifying such factors that control efficient transport and integration of transgene(s) into the plant genome (Sood *et al.* 2011). In this research, *Agrobacterium* was utilized to transfer the reporter *gus* gene into date palm Sewi and Hayani cultivars for the first time using direct globular embryonic cells of regeneration protocol of direct somatic embryogenesis. The PCR and GUS assay confirmed the integration of reporter *gus* gene into date palm genome. The results showed that the percentage of transformation was higher in Sewi cultivar (4.8%) than in Hayani cultivar (4.2%). According to certain reports, pre-induction of *Agrobacterium* and/or the addition of acetosyringone to the incubation medium can both increase transformation efficiency (Sunikumar *et al.* 1999; Yao 2001). Moreover, El-Rakashy *et al.* (2011) demonstrated that transgenic date palm expressing an endotoxin Cry3Aa gene had its red palm weevil resistance, yield and fruit quality assessed in the field. The use of super-binary vectors with a hypervirulent strain has led to better transformation efficiency (Singh and Prasad 2016).

Conclusion

Lowering NAA concentration induced direct somatic embryos in date palm cultivars. Moreover, high genetic similarities were observed as assessed by SCoT analysis. GUS transgenic date palm plants were produced using DSE.

To our knowledge, there is no earlier report on *Agrobacterium*-mediated transformation of date palm via DSE. This approach may help maintain a commercial tolerant date palm submitted to biotic and abiotic stress.

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Author Contributions

NIE, MME and DSA designed the study; NIE, MME, MH, AAM and DSA performed field work and collected data; NIE, MME, MH, AAM and DSA analyzed data and drafted manuscript. All the authors read and approved the final manuscript.

Conflicts of Interest

The authors declare that they have no conflict of interest.

Data Availability

Data presented in this study will be available on a fair request to the corresponding author.

Ethics Approval

Not applicable to this paper.

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