Journal of Genetic Engineering and Biotechnology

RESEARCH Open Access

In vitro propagation and assessment of genetic stability in date palm as affected by chitosan and thidiazuron combinations

Ahmed Madi Waheed Al-Mayahi*

Abstract

Background: Mass propagation of date palm has attracted the interest of commercial producers. However, this technique still faces many obstacles that hinder production. This study investigated the effect of chitosan (CHT) at various concentrations for the possibility to apply it in combination with *thidiazuron* (TDZ) on the growth and development of tissue cultures of Barhee cultivar.

Results: The results showed that CHT and TDZ on in vitro proliferation of Barhee date palm cultivar were significant. The highest response rate and the number of shoots per jar were found in MS media supplemented with 15 mgL^{-1} CHT and 0.5 mgL^{-1} TDZ combination. Furthermore, we found that the combined application between 20 mg L^{-1} CHT+ 1.0 mg L^{-1} TDZ resulted in the highest shoots content of endogenous IAA, compared with other treatments. At the same time, the data revealed that the maximum cytokinins (CKs) content of shoots occurred in a medium supplemented with 15 mg L^{-1} CHT and 0.5 mg L^{-1} TDZ. The genetic stability of the discussed micropropagation protocol was confirmed in this study by DNA-based technique RAPD (random amplified polymorphic DNA). The results may indicate that the micropropagation protocol developed in this research paper was appropriate and applicable for producing genetically stable date palm cv Barhee plants.

Conclusion: Applying the strategy of culture treatment with (CHT) and (TDZ) can be valuable for improving the propagation of date palm cv Barhee in vitro.

Keywords: Micropropagation, Culture media, Multiple shoots, RAPD-PCR marker

Background

Date palm is a monocotyledon plant from the Arecaceae family. The exact origin of the date palm (*Phoenix dactylifera* L.) is not known precisely. However, according to Mesopotamian antiquities, this plant likely originated in southern Iraq at least 6000 years ago [1]. The high nutritional composition, profitability, and environmental advantages make the date palm a major role in food security and job creation in various countries [2, 3].

The vegetative propagation of the date palm is traditionally achieved by offshoots. This type of offshoots multiplication has limitations such as a slow propagation rate making them less effective in establishing new plantations, the transmission of disease-causing pathogens and insects, and the production of a limited number of offshoots for a certain period through the juvenile phase [4–6]. In vitro culture methods have attracted great interest in overcoming these limitations and producing pathogen-free plants [7].

The positive effect of CHT on in vitro propagation has been proved in some plant species [8–10]. CHT appears as a potential agent used to enhance the plant's defence mechanism, as a growth stimulator, as an antimicrobial agent; besides this, CHT is used as a carrier to improve nutrient delivery [11]. Cytokinin (CKs) promotes cell division, and stimulates lateral shoot growth [12]. Some

*Correspondence: hng_1988@yahoo.com
Date Palm Research Centre, University of Basrah, Basrah, Iraq

commonly used CKs in plants micropropagation include Thidiazuron (TDZ) 1-phenyl-3 (1,2,3-thiadiazol-5-yl) urea. TDZ is the cytokinin-like substance that promotes micropropagation of a wide array of woody species, including date palm, because of its tremendous ability to stimulate shoots proliferation [13, 14]. TDZ is also a powerful regulator of plant propagation in vitro and subsequent growth [15]. Many reports show that the use of TDZ leads to a better shoot proliferation capacity than other CKs [16, 17]. If date palm micropropagation is successful, an evaluation to determine the genetic stability of the cultures would need to be established before a large-scale tissue culture method could be developed for commercial use. Although it may present a severe problem for commercial micropropagation, molecular markers can efficiently verify clonal variations. These variations during tissue culture depend largely on many factors, among them the media composition. Plant tissue culture is particularly suitable for evaluating the compounds and plant growth regulator's effects on plants that multiply and differentiate slowly, such as the date palm. However, there is no study examining the effect of CHT and TDZ on the in vitro propagation of date palms and related gene expression. Hence, this study investigated the effect of CHT at various concentrations for the possibility to apply it in combination with TDZ on the growth and multiplication in vitro of date palm buds, some physicochemical parameters, and genetic stability; RAPD indicators were used to determine the genetic stability of in vitro multiplied materials to determine the protocol effectiveness.

Methods

Experiments were conducted in the Department of Date Palm Propagation, Date Palm Research Center, Basrah University, Basrah-Iraq.

Young offshoots (3 years old) of date palm cv. Barhee was detached from the mother palm (Fig. 1a). Outer leaves and fibrous tissues at their bases were removed gradually until exposure to the shoot tip zone (Fig. 1b, c). Sheathing leaf base enclosing the very young leaves of the heart of the offshoot was left in place to protect it from disinfection solutions. Four apical buds were used in the experiment; each apical bud was sectioned longitudinally into four sections. To callus induction, quarters of the apical buds were transferred to MS basal medium [18], supplemented with 3 mg L^{-1} 6-(dimethylallyl amino) purine (2iP), 30 mg L^{-1} naphthalene acetic acid (NAA), 2.0 g L^{-1} activated charcoal and solidified with Agar-Agar at 7.0 g L^{-1}. The cultures were transferred to fresh media, with the same composition every 6 weeks until the callus had initiated (Fig. 1d, e). All cultures were incubated in a culture room under darkness at 27 ± 2 °C for 180 days to initiate callus. The percentage of callus formation reached 62.5%. For callus propagation; it was transferred and grown in jars containing 25 ml of the MS medium supplemented as mentioned above, except NAA at 6 mg L^{-1} and 2iP at 2 mg L^{-1}, with reducing the activated charcoal (AC) concentration to 0.5 mg L^{-1} for use in subsequent experimental techniques experiments, where it lasted for 10 weeks. For differentiation and multiplication, the yellow compact calluses on growth media was divided and subcultured on differentiation, and multiplication media. To study the effects of CHT and TDZ, supplementation of these compounds at various concentrations in the culture media was assessed. MS medium was modified at five concentrations of CHT (0.0, 5, 10, 15, and 20 mg L^{-1}) alone or in combination with TDZ at three concentrations (0.0, 0.5, and 1.0 mg L^{-1}). In order to obtain the best nutritional composition capable of stimulating shoot organogenesis and changes in phytochemicals. Treatments were consisted of 15 media, as shown in Table 1. All cultures were kept in the incubator at a temperature of 25 ± 2 °C. A photoperiod (2000 lux) of 16 h using cool white fluorescent tubes were also provided. The medium pH was adjusted to 5.7 before autoclaving. Subculturing was carried out in a fresh medium every 5 weeks for up to 3 months. The results of the experiments regarding the effect of MS medium on the percentage of shoot regeneration

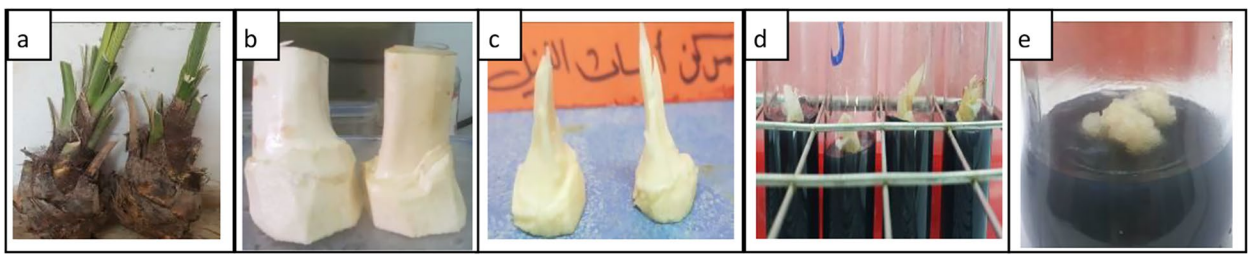

Fig. 1 Induction of callus of date palm (*P. dactylifera* L. cv. Barhee) from apical buds of an offshoot: **a** offshoots, **b** trimmed offshoots, **c** apical buds, **d** quarters of apical buds on MS medium, **e** callus formation (× 0.8)

Table 1 Treatments that applied in this study

No.	Treatments (mgL^{-1})	No.	Treatments (mgL^{-1})
1	0.0 CHT + 0.0 TDZ	**9**	10 CHT + 1.0 TDZ
2	0.0 CHT + 0.5 TDZ	**10**	15 CHT + 0.0 TDZ
3	0.0 CHT + 1.0 TDZ	**11**	15 CHT + 0.5 TDZ
4	5 CHT + 0.0 TDZ	**12**	15 CHT + 1.0 TDZ
5	5 CHT + 0.5 TDZ	**13**	20 CHT + 0.0 TDZ
6	5 CHT + 1.0TDZ	**14**	20 CHT + 0.5 TDZ
7	10 CHT + 0.0 TDZ	**15**	20 CHT + 1.0 TDZ
8	10 CHT + 0.5 TDZ		

and shoot number per jar were evaluated 12 weeks after the inoculation of callus on the media. There were twelve replicates (jars) of each treatment.

Extraction and measurement of auxin (IAA)

Auxins were extracted and quantified according to [19]. Five grams of leaves after various treatments with CHT and TDZ were homogenized using 80% methanol. The extract has been filtered through the Whatman filter paper (no. 1) and evaporated at 4 °C in dark conditions under a vacuum. The supernatant was dried in a vacuum, withdrawn by 0.1 M phosphate potassium (pH 8.1). Eluate was obtained using 1 N hydrochloric acid (HCI) and by partitioning (4×) with diethyl ether, in dryness, in water with a pH set to 2.5. The injection in reversed HPLC, C18 column, in the isocratic elution mode by the concentrate, determined phytohormones using a portable acetone step (26:74) with 30 mM of phosphoric acids. A UV detector (2996PDA detector) with 280 nm was passed through the column eluants, and auxins were detected and quantified. Standard auxins were used as the source (IAA).

Determination of cytokinins (CKs)-like substances

The CKs-like substances in the shoot tissue were extracted and quantified [20]. Spectrophotometer UV-Visible Shimadzu determined the shoot samples' CK-like substances at the wavelength of 265 nm. The values content of the endogenous CK was calculated using the linearized standard curve in which benzyl adenine was used, and the results were expressed in micrograms. The values were corrected, and the endogenous CKs content was calculated using the linearized standard curve in which benzyl adenine was used, and the results were expressed in micrograms.

Genetic stability among regenerated date palm plantlets

In order to study the genetic similarities, several regenerated plantlets were analyzed at the molecular levels using RAPD analysis.

Table 2 The RAPD primers and their sequences used for the genetic fidelity evaluation

Primers	Sequences
OPA-02	**TGCCGAGCTG**
OPB-05	**TGCGCCCTTC**
OPE-15	**ACGCACAACC**
OpO-07	**CAGCACTGAC**

RAPD analysis

Total genomic deoxyribonucleic acid (DNA) was isolated from regenerated date palm plantlets using the CTAB method described in [21]. Polymerase chain reaction (PCR) reactions were conducted using a set of four arbitrary 4-mer primers (Operon Technology, Inc., Alameda, CA, USA). These primers and their sequences are presented in Table 2.

The PCR mixture

The reaction mixture (20 µl) contained 10 ng DNA, 200 µM deoxynucleotide triphosphates (dNTPs), 1 µM primer, 0.5 units of Red Hot Taq polymerase (AB-gene Housse, UK) and 10-X Taq polymerase buffer (AB-gene Housse, UK). For DNA amplification, a Perkin Elmer thermal cycler (2720) programmed as follows: Denaturing: 95 °C for 5 min 94 °C for 0.45 min. Then, annealing (35 cycles) 35 °C for 1 min. This is followed by 72 °C for 1 min and 30 s and finally Extension: at 72 °C for 7 min [22]. The amplification products were separated in 1% (w/v) agarose gel in 1× Tris/Borate/Ethylenediaminetetraacetic_acid (TBE) buffer and visualized by staining with ethidum bromide. The reproducibility of DNA profiles was determined by replicating all RAPD reactions at least three times using DNA markers. The primers were evaluated from wise pair comparison for the proportion of shared bands amplified [23]. The similarity coefficient was calculated by using the statistical software package STATISTICA-SPSS (Stat Soft Inc.).

Statistical data analysis

In all experiments, each treatment consisted of 12 replicates. Data were statistically analyzed by analysis of variance (ANOVA). The least significant difference (LSD) method was used to test the difference between treatments, and $p \leq 0.05$ was considered statistically significant. Statistical analyses were performed with SPSS packet software.

Results

Effect of CHT and TDZ on shoot induction

According to the results obtained, the combination between CHT and TDZ application had the highest

response percentage and number of shoot compared with either individual application or control treatment (Fig. 2e, f, h, i, k, l, n, and o) after 12 weeks from culture. The response rate and the number of developing shoots varied among the different treatments (Table 2). The highest response rate and the number of developing shoots, 75% and 16.11 respectively, were obtained on the medium supplemented with 15 mg L^{-1} CHT and 0.5 mg L^{-1} TDZ (Fig. 2k).

Endogenous hormones levels

Endogenous IAA levels

Figure 3 shows the effect of CHT and TDZ on endogenous IAA content under in vitro conditions. Among all treatments, CHT and TDZ were applied. The highest IAA content (3.757 µg kg^{-1}) in the shoots was obtained in the MS medium supplemented with 20 mg L^{-1} CHT + 1.0 mg L^{-1} TDZ. On the other hand, the lowest contents were recorded in shoots grown in the control medium.

Endogenous cytokinin (CK) levels

Figure 4 shows the effect of CHT and TDZ on endogenous CKs concentrations. Thus, increasing the CHT concentration of the medium from 0.0 to 20 mg L^{-1} resulted in a proportionally increasing content of endogenous CK of tissues, as observed when TDZ concentration was increased from 0.0 to 1.0 mg L^{-1}. Furthermore, in regeneration media with 15 mg L^{-1} CHT + 0.5 mg L^{-1} TDZ , the highest CKs content was recorded (2.327 µg kg^{-1},) which was significantly different than what was reported at the shoots grown in the other media ($p < 0.05$). The lowest contents were recorded in shoots grown in the control medium (with no additives).

RAPD-based genetic relationships

In the present study, we regenerated plants from callus tissues under the influence of different CHT and TDZ. Since morphological evaluation is unreliable in characterizing variability among the tissue culture-derived plantlets with the mother plant, it becomes necessary to check

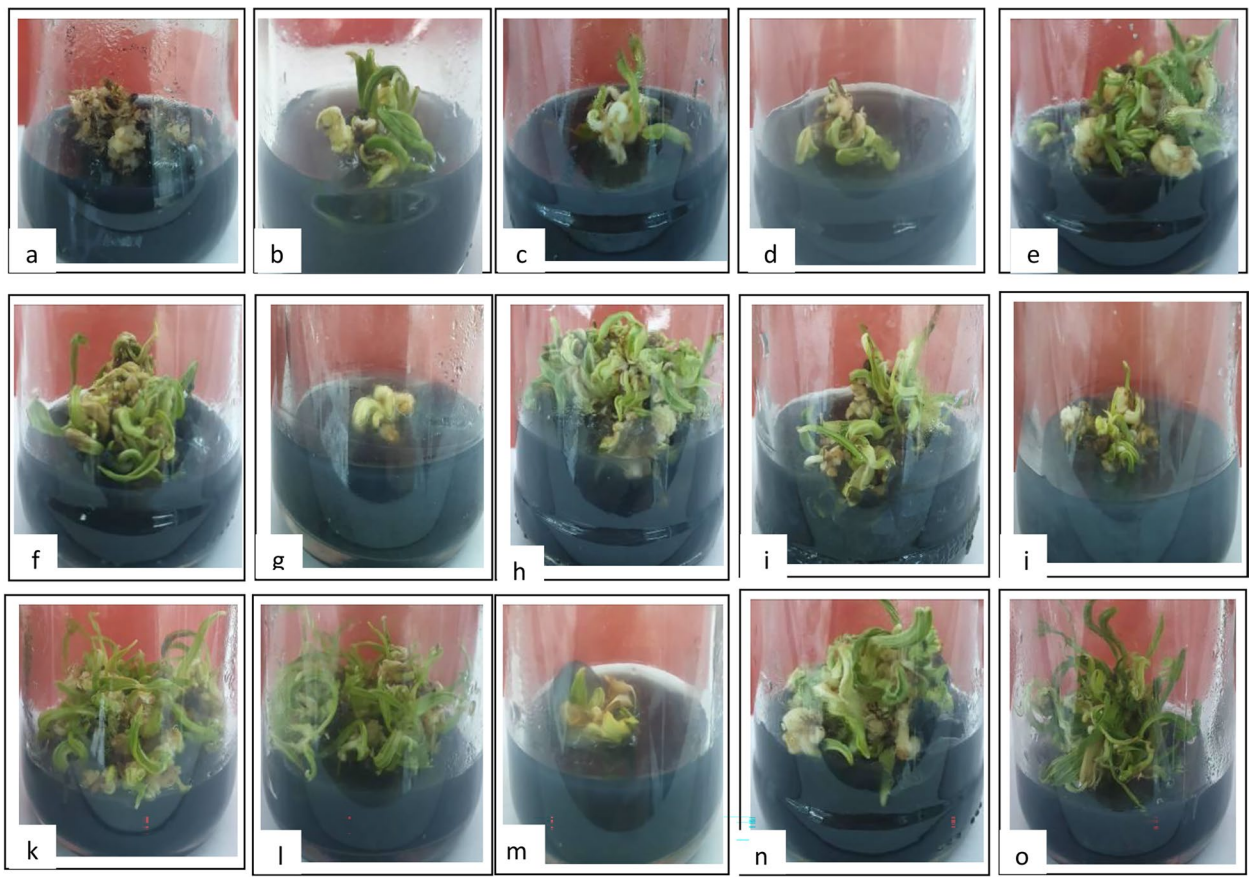

Fig. 2 Initiation of multiple shoot formation after 12 weeks of culture on MS media supplemented with chitosan (CHT) alone or in combination with thidiazuron (TDZ): mgL^{-1}: **a** control, **b** 0 CHT + 0.5 TDZ, **c** 0 CHT + 1.0 TDZ, **d** 5 CHT + 0.0 TDZ, **e** 5 CHT + 0.5 BA, **f** 5 CHT + 1.0 TDZ, **g** 10 CHT + 0.0 TDZ, **h** 10 CHT + 0.5 TDZ, **i** 10 CHT + 1.0 TDZ, **j** 15 CHT + 0.0 TDZ, **k** 15 CHT + 0.5 TDZ, **l** 15 CHT + 1.0 TDZ, **m** 20 CHT + 0.0 TDZ, **n** 20 CHT + 0.5 TDZ, **o** 20 CHT+ 1.0 TDZ. (× 0.8)

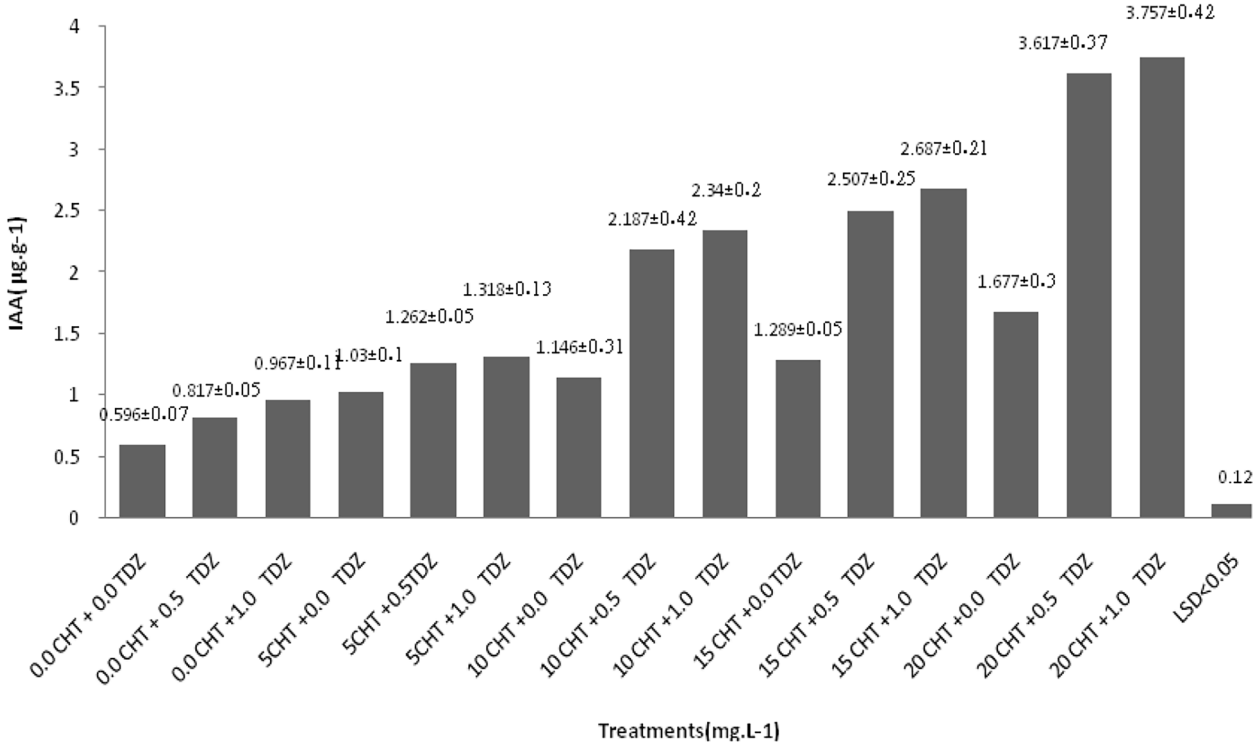

Fig. 3 Effect of chitosan (CHT) and thidiazuron (TDZ) on endogenous indole acetic acid (IAA) content. ± Standard error (n15)

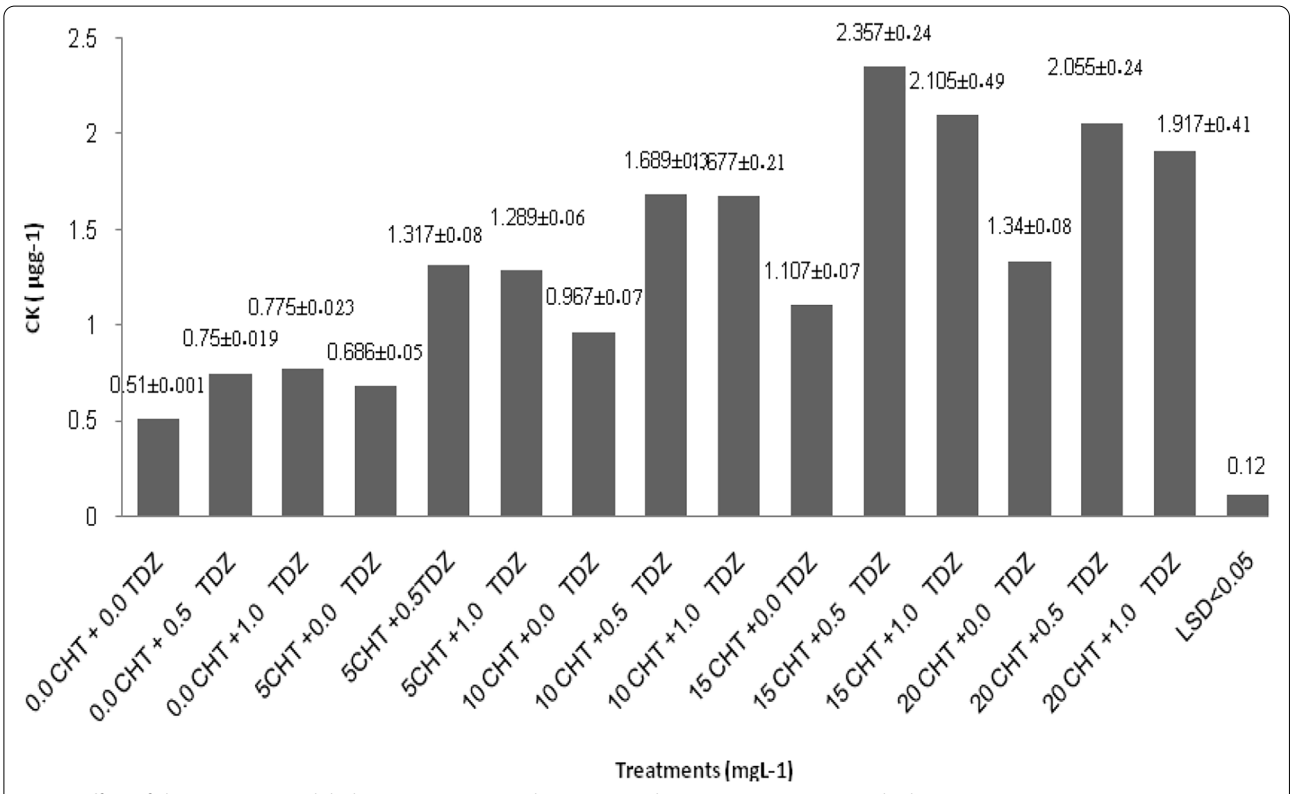

Fig. 4 Effect of chitosan (CHT) and thidiazuron (TDZ) on endogenous cytokinin (CK) content. ± Standard error (n15)

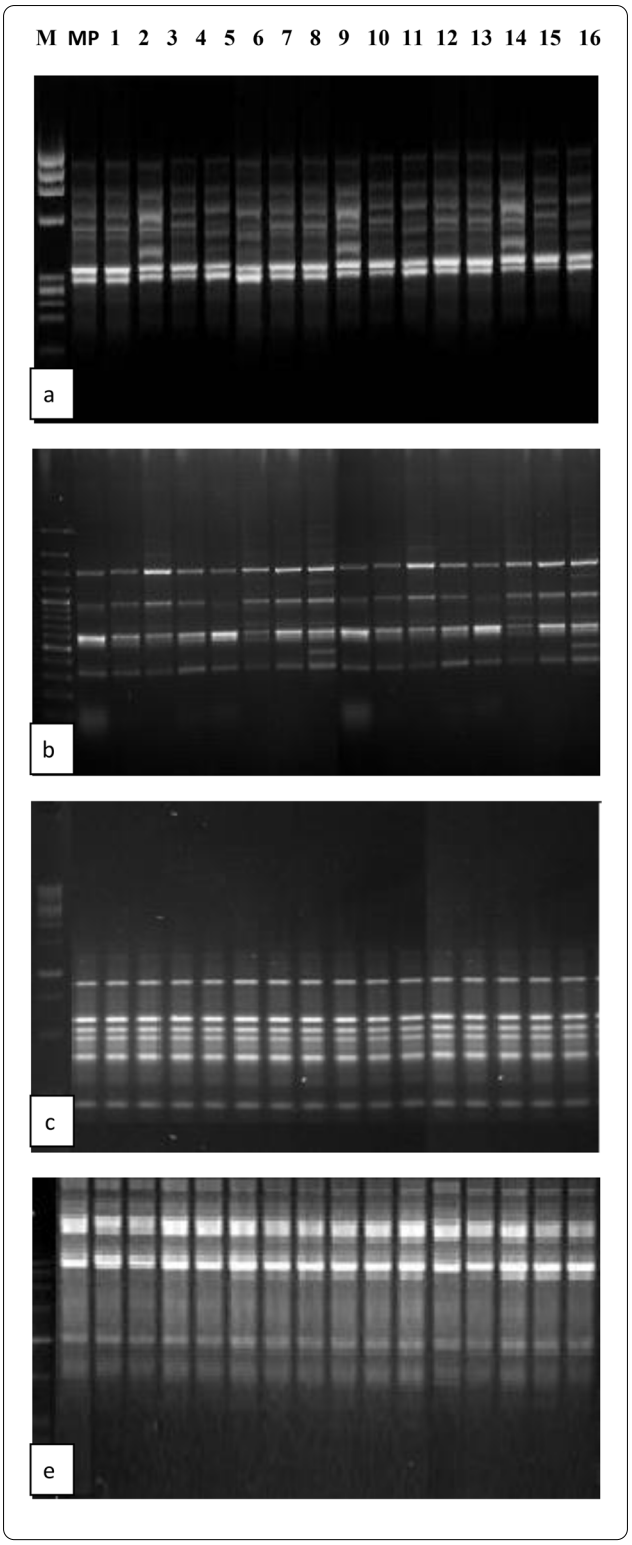

Fig. 5 **a** OPB-05, **b** OPE-08, **c** OPE-15, and **d** OpO-07). RAPD pattern of regenerated plants of *Phoenix dactylifera* L. on MS medium supplemented with chitosan (CHT) alone or in combination with thidiazuron (TDZ): mgL^{-1}:(M) size marker (1) (1) MP: mother plant, (2) Control (3) 0 CHT + 0.5 TDZ (4) 0 CHT + 1.0 TDZ (5) 5 CHT + 0.0 TDZ (6) 5 CHT + 0.5 BA (7) 5 CHT + 1.0 TDZ (8) 10 CHT + 0.0 TDZ (9) 10 CHT + 0.5 TDZ (10) 10 CHT + 1.0 TDZ (11) 15 CHT + 0.0 TDZ (12) 15 CHT + 0.5 TDZ (13) 15 CHT + 1.0 TDZ (14) 20 CHT +0.0 TDZ (15) 20 CHT + 0.5 TDZ(16) 20 CHT + 1.0 TDZ

the genetic stability of these regenerated plants. Four operon primers were used (OPB-05, OPE-08, OpO-07, and OPE-15). All of them have given good amplification with scorable DNA bands. The results also revealed that the regenerated plants did not have any polymorphism with the mother plant (Fig. 5a-d). DNA bands produced by amplification with RAPD markers were monomorphic across the mother plant and micropropagated plantlets (Fig. 5). The genetic profiles obtained in RAPD analysis suggest no genetic changes between the tissue culture-derived plantlets treated with CHT and TDZ with the mother plant. This suggests that the in vitro conditions in this study provide relatively genetic stability of date palm cv Barhee on regeneration in vitro, which confirms the true-to-type and genetically stable nature of those plants. The fidelity of the micropropagation protocol to produce true–to–type date palm plants indicate that the use of CHT and TDZ during in vitro propagation phases showed a similar banding pattern in vitro regenerated and mother plant in RAPD profiles of this date palm cv. Barhee.

Discussion

In vitro propagation of date palm depends on the appropriate selection of explants and hormones [24, 25]. Due to the preferred properties of CHT, it can be used as a growth stimulator in vitro of date palm. To determine a suitable medium for an efficient in vitro propagation of date palm cv. Barhee, callus tissues were cultured on MS media supplemented with different CHT and TDZ combinations. MS media supplemented with CHT in combination with TDZ gave the best response on most growth criteria studied as compared with no additives (control treatment) or with their individual application. The highest response rate and the number of shoots per jar were achieved in cultures incubated on MS medium supplemented with 15 mg L^{-1} CHT + 0.5 mgL^{-1} TDZ (Table 1). The interesting property of CHT suggests its potential as a growth regulator for the micropropagation of date palm. Using MS media with different chitosan concentrations may enhance the response rate and the number of shoots produced per jar. CHT can be used as a plant growth enhancer. Perhaps CHT stimulates a signal for the synthesis of plant hormones such as gibberellins. In addition, CHT may promote growth and development

through some signaling pathways related to auxin biosynthesis by the tryptophan-independent pathway [26, 27] who indicated that the use of CHT stimulated the accumulation of auxin. CHT, a natural alternative to commonly used synthetic plant growth regulators, in vitro effects of chitosans have been mainly investigated based on morphometric parameters such as the number of shoots, roots, and leaves [28, 29]. Chitosan treatment has been shown to improve the growth of in vitro-cultured plantlets of bananas and enhance immunity against various plant pathogens [30]. This study confirmed that CHT could act as a plant growth stimulator for date palm cv Barhee micropropagation. These results agree with those of [31], who reported that CHT was a stimulator for enhancing the growth of safflower and sunflower plants, and with [32] in *Petunia atkinsiana* D. don.). Thus, suggested that CHT was able to enhance the growth of date palm 'Barhee' was reported in this paper was in accordance to many many other researches in many plants [8, 9, 30, 33]. On the other hand, TDZ is necessary for date palm propagation in in vitro. The use of TDZ in culture media enhanced the growth traits. This is probably due to the difference in endogenous levels of growth regulators, where TDZ plays a vital role in stimulating endogenous cytokinin biosynthesis or altering cytokinin metabolism. An examination of the different media and plant growth regulators combinations that have been utilized for tissue culture of date palm plants reveals that shoot regeneration requires the presence of both auxins (Aux) and cytokinins (CKs) [34]. This was consistent with findings on applications of TDZ in shoot regeneration and the synergistic effect of TDZ with auxins on the proliferation higher number of shoots [16]. Auxins play a vital role in the differentiation of cell aggregates which is a prerequisite for regeneration. Additionally, auxin is one of the plant hormones necessary for the growth and development of in vitro organs [35]. Aux (e.g., IAA) is produced in response to physiological or metabolic changes [36]. We found that increasing CHT leads to the accumulation of IAA and CKs related to plant growth. In addition, there is evidence that CHT can be used as an alternative to commonly used growth factors such as Aux or CKs [37, 38] due to its stimulating effects [39]. In our study, tissue exposure to increased chitosan levels leads to the accumulation of IAA in buds. This hormone is known to be increased by CHT [27]. The results also showed that the presence of TDZ had a significant effect on the IAA levels of the tissues compared to those grown on a TDZ-free medium. Application of TDZ elicited effects typically associated with CKs. Studies have indicated that TDZ may act by modulating endogenous plant growth regulators [40]. In this regard, [41] reported that the application of TDZ in tissue culture medium increases internal CKs levels. There are also indicates the induction of accumulation or synthesis of endogenous CKs by TDZ [42]. Also, TDZ is involved in auxin synthesis by increasing levels of IAA and its precursor tryptophan and modification of cell membranes, energy levels, nutrient uptake, or nutrient assimilation [43]. There has been some evidence that TDZ may influence the endogenous content of the IAA. Peanut plants grown on a culture medium containing TDZ showed increased IAA, indicating that TDZ may enhance Aux synthesis [44]. A combination of CKs with auxins showed superior results for the shoot regeneration in date palm [34]. It has also been shown that the relative amounts of Auxs and CKs are essential for many physiological processes. The decrease in IAA levels at 15 mg L^{-1} CHT + 0.5 mg L^{-1} TDZ and increase in tissues treated with 20 mg L^{-1} CHT + 1.0 mg L^{-1} TDZ is consistent with the previous results putative mechanisms of shoot regeneration. The buds are regenerated when there is a high ratio of CKs to Auxs. Differences in regeneration abilities under different treatments may be due to differences in endogenous levels of growth hormones in these tissues. Several researchers have well documented the utility of molecular analysis of in vitro regenerated plantlets [35, 45, 46]. Somaclonal variation is often induced by the culture media and subculture cycles [47]. Therefore, testing of genetic stability of in vitro raised plants is necessary to date palm plantlets production. The applicability of RAPD technology has captured the interest of many researchers. Perhaps the main reason for the success of RAPD analysis is the acquisition of many genetic markers that require small amounts of DNA without cloning, sequencing, or any other form of molecular genome characterization. RAPD analysis micropropagated plants date palm cv Barhee indicated a profile similar to that of the mother plant that clearly showed the genetic stability of those plants (Fig. 5a–d). This confirmed that the in vitro regenerated P. *dactylifera* L. cv Barhee was genetically stable and free of clonal variations. For induction of defense responses and stimulation of cell division, CHT may directly affect gene expression interacting with chromatin [48]. A successful in vitro propagation method should give true-to-type plantlets without the occurrence of genetic mutations or morphological alteration [49–51]. Similarly, no variation appeared in genetic variation using RAPD has been reported in many cases of in vitro propagated of chestnut rootstock and almond plantlets hybrids [52, 53]. Terminalia arjuna [54], and medicinal herb–Coleus aromaticus Benth L. [55] *P. dactylifera* L. cv Ashgar [35]. On the other hand, cytokinins play a major role in DNA synthesis, cell division, and plant regeneration and regulate protein synthesis responsible for forming the mitotic spindle [56]. The RAPD technique showed genetic conformity of micro propagated plants of

Table 3 Effect of chitosan (CHT) and thidiazuron (TDZ) on a response percentage (%) of callus for bud formation, and a number of buds/100 mg callus for date palm, cv. Barhee after 12 weeks of culturing

Treatments (mgL^{-1})	frequency [%]	Shoot number	Treatments(mgL^{-1})	frequency [%]	Shoot number
0.0 CHT + 0.0 TDZ	0.0 ± 0.0	0.0 ± 0.0	**10 CHT + 1.0 TDZ**	41.67 ± 3.89	6.60 ± 0.58
0.0 CHT + 0.5 TDZ	25.0 ± 4.81	3.0 ± 0.50	**15 CHT + 0.0 TDZ**	25.0 ± 4.81	3.33 ± 0.4
0.0 CHT + 1.0 TDZ	16.67 ± 1.53	2.5 ± 0.22	**15 CHT + 0.5 TDZ**	75.00 ± 4.60	16.11 ± 0.99
5 CHT + 0.0 TDZ	8.34 ± 1.21	2.0 ± 0.19	**15 CHT + 1.0 TDZ**	58.34 ± 4.81	13.2 ± 0.59
5 CHT + 0.5 TDZ	41.67 ± 3.89	7.4 ± 0.58	**20 CHT + 0.0 TDZ**	25.0 ± 4.81	3.66 ± 0.42
5 CHT + 1.0 TDZ	33.34 ± 3.05	6.25 ± 0.4	**20 CHT + 0.5 TDZ**	58.34 ± 4.81	11.14 ± 0.17
10 CHT + 0.0 TDZ	16.67 ± 1.53	2.5 ± 0.22	**20 CHT + 1.0 TDZ**	41.67 ± 3.89	9.2 ± 0.21
10 CHT + 0.5 TDZ	58.34 ± 4.81	7.85±0.1			
LSD < 0.05	**13.9**	**0.7**		**13.9**	**0.7**

± Standard error (n12)

H. procumbent, pretreated or non-pretreated with TDZ [57] (Table 3).

Conclusions

To the best of our knowledge, this is the first study on the efficacy of CHT and TDZ together on genetically stable multiple shoot developments of date palm. This report provides an efficient protocol for a higher frequency of genetically stable multiple shoot development and a complete plant proliferation system through callus tissues. The combination of CHT and TDZ showed a positive effect on multiple shoot induction compared with either individual application or control treatment. Adding 15 mgL^{-1} CHT to the regeneration medium supplemented with 0.5 mgL^{-1} TDZ was the best combination to promote the growth of date palm cv Barhee as it resulted in the highest response rate and the number of shoots. CKs content increased in in vitro shoots regenerated in the same combination mentioned above. Random amplified polymorphic DNA of treatments with CHT and TDZ showed genetic similarity to mother plants. We conclude that applying the strategy of cultures treatment with CHT and TDZ may be valuable for improving date palm cv Barhee in vitro propagation. However, further studies are required to evaluate the effects of CHT.

Abbreviations

NAA: Naphthaleneacetic acid; 2iP: N6-(2-Isopentenyl) adenine; IAA: Indole acetic acid introduction; Aux: Auxins; CKs: Cytokines; CHT: Chitosan; TDZ: Thidiazuron; MS: Murashige and Skoog Basal Medium; RAPD profiles: Random amplified polymorphic DNA.

Acknowledgements

The author thanks and appreciates all the staff at the Date Palm Research Center, especially in the Date Palm propagation Lab.

Author's contributions

Al AMW preparing the culture media and the conduct of plant tissue culture of the date palm, and the follow of the growth and development of cultures. The author also analyzed the physiological characteristics of the tissues and wrote the manuscript. The authors read and approved the final manuscript.

Funding

The work was no funding was received.

Availability of data and materials

All data generated or analyzed during this study are included in this article.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The author declares that they have no competing interests.

Received: 10 August 2022 Accepted: 4 December 2022
Published online: 14 December 2022

References

1. Chao CT, Krueger RR (2007) The date palm (*Phoenix dactylifera*L.): an overview of biology, uses, and cultivation. Hortic Sci 42:1077–1082
2. Arias E, Hodder AL, Oihabi L (2016) FAO support to date palm development around the world: 70 years of activity. Emir J Food Agric 28(1):1–11
3. Jasim AM, Al-Mayahi AMW, Attaha AHM (2009) Propagation of four rare cultivars of date palm (*Phoenix dactylifera* L.) by tissue culture techniques. Bas J Date Palm Res 8(1):72–99
4. Gueye B, Ahmed HS, Morcillo F, Borgel A, Sane D, Hilbert JL, Verdeil JL, Blervacq AS (2009) Callogenesis and rhizogenesis in date palm leaf segments: are there similarities between the two auxin-induced pathways? Plant Cell Tissue Organ Cult 98:47–58
5. Al-Mayahi AMW, Ali AH, Shareef HJ (2018) Influence of cold pretreatment on shoot regeneration from callus in date palm (*Phoenix dactylifera* L.) cv. 'Barhee'. J Genet Eng Biotechnol 16:607–612
6. Al-Mayahi AMW (2021) The effect of humic acid (HA) and zinc oxide nanoparticles (ZnO-NPS) on *in vitro* regeneration of date palm (*Phoenix dactylifera* L.) cv. Quntar. Plant Cell Tiss Organ Cult 145:445–456
7. Al-Mayahi AMW (2021) I*n vitro* plant regeneration system for date palm (*Phoenix dactylifera* L.): effect of chelated iron sources. J Genet Eng Biotechnol 19:83
8. Nge KL, New N, Chandrkrachang S, Stevens WF (2006) Chitosan as a growth stimulator in orchid tissue culture. Plant Sci 170:1185–1190

9. Mastuti R, Batoro J, Waluyo B (2020) Elicitor effect of chitosan on in vitro culture of different explants of physalis accessions from east java. Adv Biol Sci Res 14:382–386 Proceedings of the 3rd KOBI Congress, International and National Conferences
10. Tang C, Lamasudin DU, Asrul WM, Abdullah NW, Moo C, Chiew M, Chai Q, Abdullah J, Lai K (2021) Enhanced *in vitro* shoot regeneration and biochemical properties of *Stevia rebaudiana* using chitosan. Sains Malaysiana 50(3):667–676
11. Pandey P, Verma MK, De N (2018) Chitosan in agricultural context- a review. Bull Env Pharmacol Life Sci 7(4):87–96
12. Al-Mayahi AMW (2019) Effect of calcium and boron on growth and development of callus and shoot regeneration of date palm cv. Barhee. Can J Plant Sci 100(4):357–364
13. Al-Mayahi AMW (2014) Thidiazuron induced *in vitro* bud organogenesis of the date palm (*Phoenix dactylifera* L.) cv. Hillawi. Afr J Biotechnol 13(35):3581–3590
14. Al-Asadi AZ, Abdulwahid AH, Al-Mayahi AMW (2019) The effect of thidiazuron on callus and *in vitro* shoots development of date palm (*Phoenix dactylifera* L.) cv. Barhee. Basrah J Agric Sci 32(Spec Issue):258–264
15. Cappelletti R, Sabbadini S, Mezzetti B (2016) The use of TDZ for the efficient *in vitro* regeneration and organogenesis of strawberry and blueberry cultivars. Sci Hortic 207:117–124
16. Yildirim AB, Turker AU (2009) *In vitro* adventitious shoot regeneration of the medicinal plant meadowsweet (*Filipendula ulmaria* (L.) maxim). In Vitro Cell Devel Bio 45(2):135–144
17. Guo B, Abbasi BH, Zeb A, Xu LL, Wei YH (2011) Thidiazuron a multi-dimensional plant growth regulator. Afr J Biotechnol 10(45):8984–9000
18. Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15(3):473–497
19. Niedz RP, Evens TJ (2006) A solution to the problem of ion confounding in experimental biology. Nat Methods 3:417–417
20. Abbas MF, Fandi BS (2001) Endogenous hormone level during fruit development in jujube (*Ziziphusmauritiana* lam). Basrah J Agric Sci 14(1):15–22
21. Rogers SO, Bendich AJ (1985) Extraction of DNA from milligram amounts of fresh, herbarium and mummified plant tissues. Plant Mol Biol 5:69–76
22. Adawy SS, Hussein Ebtissam HA, Saker MM, El-Itriby Hanaiya A (2004) Intra- and inter-varietal variation of upper Egypt date palm cultivars (*Phoenix dactylifera* L.): I. as revealed by RAPD and inter simple sequence repeat markers. Proc Int Conf Genet Eng Appl 8–11:165–179 (Sharm El-Sheikh, South Sinai, Egypt)
23. Nei M (1978) Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetic 89:583–590
24. Al-Mayahi AMW (2019) Effect of aluminium on the growth of the *in vitro* culture tissues of the date palm (*Phoenix dactylifera* L.) cv. Um-Adelhin. Folia Oecol 46(2):164–169
25. Al-Mayahi AMW, Jafar ON, Mohsen KA (2020) Effect of glutathione (GSH) on date palm (*Phoenix dactylifera* L.) micropropagation. Folia Oecol 47(1):64–69
26. Uthairatanakij A, Teixeira da Silva JA, Obsuwan K (2007) Chitosan for improving orchid production and quality. Orchid Sci Biotech. 1(1):1–5
27. Lopez-Moya F, Escudero N, Zavala-Gonzalez EA, Esteve-Bruna D, Blázquez MA, Alabadí D (2017) Induction of auxin biosynthesis and WOX5 repression mediate changes in root development in Arabidopsis exposed to chitosan. Sci Rep 7:16813
28. Salachna P, Zawadzinska A (2014) Effect of chitosan on plant growth, flowering and corms yield of potted freesia. J Ecol Eng 15(3):97–102
29. Acemi A, Bayrak B, Çakır M, Demiryürek E, Gün E, El Gueddari NE, Özen F (2018) Comparative analysis of the effects of chitosan and common plant growth regulators on *in vitro* propagation of *Ipomoea purpurea* (L.) Roth from nodal explants. In Vitro Cell Dev Biol Plant 54:537–544
30. Kandha L, Kumar R, Sethi SK, Bindhani BK (2021) Chitosan enhances growth and survival rate of in vitro-cultured plantlets of banana cultivar"Grand Naine." J Crop Improv 35(6):848–865
31. Jabeen N, Ahmad R (2013) The activity of antioxidant enzymes in response to salt stress in safflower (*Carthamus tinctorius* L.) and sunflower (*Helianthus annuus* L.) seedlings raised from seed treted with chitosan. J Sci Food Agric 93:1699–1705
32. Krupa-Małkiewicz M, Fornal N (2018) Application of chitosan *in vitro* to minimize the adverse. Effects of salinity in *petunia* × *atkinsiana* D. don. J Ecol Eng 19(1):143–149
33. Obsuwan K, Sawangsri K, Thongpukdee A, Thepsithar C (2013) The response of growth and development from in vitro seed propagation of *dendrobium* orchid to chitosan. In: Proc. of the Int. Conf. On quality Management in Supply Chains of ornamentals – QMSCO. Acta Hort 970, ISHS
34. Ibrahim MA, Waheed AM, Al-Taha H, Al-Taha H (2013) Plantlet regeneration from root segments of date palm tree (*Phoenix dactylifera* L. cv Barhee) producing by *in vitro* culture. Bioflux 5(1):45–50
35. Al-Mayahi, A.M.W. The Effect of Phenyl Acetic Acid (PAA) on Micropropagation of Date Palm Followed by Genetic Stability Assessment. J Plant Growth Regul 41:3127–3137. https://doi.org/10.1007/s00344-021-10500-5
36. Asami T, Nakagawa Y (2018) Preface to the special issue: brief review of plant hormones and their utilization in agriculture. Pestic Sci 43:154–158
37. Ahmad Z, Shahzad A, Sharma S (2019) Chitosan versus yeast extract driven elicitation for enhanced production of fragrant compound 2-hydroxy-4-methoxy benzaldehyde (2H4MB) in root tuber derived callus of *Decalepis salicifolia* (Bedd. Ex Hook.F.) venter. Plant Cell Tissue Organ Cult 136:29–40
38. Acemi A (2020) Chitosan versus plant growth regulators: a comparative analysis of their effects on *in vitro* development of *Serapias vomeracea* (Burm.F.) Briq. Plant Cell Tissue Organ Cult 141:327–338
39. Malerba M, Cerana R (2016) Chitosan effects on plant systems. Int J Mol Sci 17:996
40. Taiz L, Zeiger E, Møller IM, Murphy A (2014) Plant physiology and development, Sixth edn. Sinauer Associates, Inc., Publishers, Sunderland, p 761
41. Hare PD, Staden JV (1994) Inhibitory effect of Thidiazuron on the activity of cytokinin oxidase isolated from soybean callus. Plant Cell Physiol 35:1121–1125
42. Ferreiraa WM, Kerbauyb GB, Krausb JE, Pescadorc R, Suzuki RM (2006) Thidiazuron influences the endogenous levels of cytokinins and IAA during the flowering of isolated shoots of Dendrobium. J Plant Physiol 163:1126–1134
43. Murthy BNS, Murch SJ, Saxena PK (1998) Thidiazuron: a potent regulator of in vitro plant morphogenesis. In Vitro Cell Dev Bio 34(4):267–275
44. Murthy BNS, Murch SJ, Saxena PK (1995) TDZ-induced somatic embryogenesis in intact seedlings of peanut (*Arachis hypogaea*): endogenous growth regulator levels and significance of cotyledons. Physiol Plant 94:268–276
45. Piatcza E, Kuzma L, Sitarek P, Wysokinska H (2015) Shoot organogenesis, molecular analysis and secondary metabolite production of micropropagated Rehmannia glutinosa Libosch. Plant Cell Tissue Organ Cult 120:539–549
46. Bhalang D, Prabhuling G, Hipparagi K, Raghavendra S, Prakash DP, Babu AG (2018) Analysis of the genetic stability of banana tissue culture propagated plantlets cv. Ney Poovan (AB) using morphological and molecular markers. Int J Curr Microbiol App Sci 7(1):1007–1018
47. Bidabadi SS, Meon S, Wahab Z, Mahmood M (2010) Study of genetic and phenotypic variability among somaclones induced by BAP and TDZ in micropropagated shoot tips of banana (*Musa spp.*) using RAPD markers. J Agric Sci 2:49–60
48. Hadwiger LA (2015) Anatomy of a nonhost disease resistance response of pea to Fusarium solani: PR gene elicitation via DNase, chitosan and chromatin alterations. Front Plant Sci 6:373
49. Prakash L, Middha SK, Mohanty SK, Swamy MK (2016) Micropropagation and validation of genetic and biochemical fidelity among regenerants of Nothapodytes nimmoniana (Graham). Mabb employing ISSR markers and HPLC. 3 Biotech 6:1–9
50. Safarpour M, Sinniah UR, Subramaniam S, Swamy MK (2017) A novel technique for *Musa acuminata* Colla 'grand Naine'(AAA) micropropagation through transverse sectioning of the shoot apex. In Vitro Cell Develop Biol-Plant 53(3):226–238
51. Khatab IA, Youssef MS (2018) Micropropagation and assessment of genetic stability of *Musa* sp. cv. Williams using RAPD and SRAP markers. Egypt J Bot 58(3):371–380
52. Carvalho LC, Goulao L, Oliveira C, Goncalves JC, Amancio S (2004) RAPD assessment for identification of clonal identity and genetic stability of *in vitro* propagated chestnut hybrids. Plant Cell Tissue Organ Cult 77:23–27
53. Martins M, Sarmento D, Oliveira MM (2004) Genetic stability of micropropagated almond plantlets as assessed by RAPD and ISSR markers. Plant Cell Rep 23:492–496
54. Gupta AK, Harish Rai MK, Phulwaria M, Agarwal T, Shekhawat NS (2014) *In vitro* propagation, encapsulation, and genetic fidelity analysis of Terminalia arjuna: a cardioprotective medicinal tree. Appl Biochem Biotechnol 173(6):1481–1494

55. Govindaraju S, Indra Arulselvi P (2018) Effect of cytokinin combined elicitors (L-phenylalanine, salicylic acid and chitosan) on *in vitro* propagation, secondary metabolites and molecular characterization of medicinal herb– Coleus *aromaticus Benth* (L). J Saudi Soc Agric Sci 17:435–444
56. Karunadasa S, Kurepa J, Timothy E, Shull Smalle JA (2020) Cytokinin-induced protein synthesis suppresses growth and osmotic stress tolerance. New Phytol 227:50–64
57. Grabkowska R, Sitarek P, Wysokińska H (2014) Influence of thidiazuron (TDZ) pretreatment of shoot tips on shoot multiplication and *ex vitro* acclimatization of Harpagophytum procumbens. Acta Physiol Plant 36:1661–1672

Publisher's Note