2,4-D induction of somaclonal variations in in vitro grown date palm (*Phoenix dactylifera* L. cv Barhee)

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Received: 15 November 2021 / Accepted: 17 February 2022 / Published online: 12 April 2022
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Abstract

The present study is a part of a program designed at improving the date palm, *Phoenix dactylifera* L. cv. Barhee, through induced somaclonal variation. In this work, caulogenic cultures were subcultured on MS media supplemented with 0, 1, 5, 10, 20 and 40 mg L\(^{-1}\) 2,4-D in order to induce genetic and epigenetic variations. The highest doses of 2,4-D were found to induce severe negative effects on in vitro cultures, although some tissues were able to survive and to produce calli with high morphogenetic capacities. Our analysis showed some significant effect of 2,4-D on several physiological parameters. Indeed, chlorophyll and growth rates were found to drastically decrease while proline content increased from 535 to 2973 nmol g\(^{-1}\) FW when 40 mg L\(^{-1}\) 2,4-D were used. In vitro cultures showed several signs of oxidative stress, such as high levels of hydrogen peroxide and malondialdehyde; likewise, the specific activity of several antioxidant enzyme was found to increase. Plant regeneration from in vitro cultures treated with 2,4-D was obtained after subculturing explants onto PGR-free media. The ISSR analysis of 2,4-D-treated material showed that this plant growth regulator (PGR) induced measurable genetic variations. The global DNA methylation rates (GMR) as estimated through the HPLC analysis of nucleosides also confirmed the presence of epigenetic changes caused by 2,4-D as GMRs increased from 13.8 to 18.93%.

Key message

Results demonstrate that the 2,4-D can affect physiological and molecular parameters of vitrocultures when used at high concentrations without hampering their morphogenetic capacities. It was found to be efficient at inducing genetic and epigenetic variations.

Keywords *Phoenix dactylifera* · DNA methylation · Somaclonal variation · ISSR markers · Plant growth regulators · Regeneration

Abbreviations

2,4-D  2,4-Dichlorophenoxyacetic acid  5mdC  5-Methyldeoxycytidine

Communicated by Bart Panis.

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Introduction

In vitro tissue cultures are known to be genetically and/or epigenetically unstable (Bednarek and Orlowska 2020) and may be used as a potential way to regenerate superior genotypes (Karim et al. 2016; Azizi et al. 2020). Much genomic variability can be induced by in vitro subculturing of the plant material. Several factors can be at the
origin of the instability of gene expression and somaclonal variation in higher plants (Etienne et al. 2016). The nutrient composition of the culture media is a source of variation (Hartke and Lorz 1989) and the exposure of tissues to growth regulators, like auxins and particularly 2,4-D, has the capacity to stimulate a disorganized growth through uncontrolled mitosis during the callus proliferation (George 1993). Several studies have specifically evidenced the role of plant growth regulators in inducing such variations (Ahmed et al. 2004; Sales and Butardo 2014). The nature, the concentration of growth regulators in the medium and the tissue source have been shown to influence the rate of somaclonal variation (Garcia et al. 2019).

Somaclonal variation arising from in vitro propagation is associated with a novel and heritable DNA-based phenotypic variation (Lukens and Zhan 2007). Larkin and Scowcroft (1981) coined a general term “somaclonal variation” for plant variants derived from any form of cell or tissue cultures. Somaclonal variation can advantageously be utilized as a source of new variation in crops (Karp 1995) and allow plants to tolerate the biotic or abiotic stress. Molecular markers are valuable tools in the characterization and evaluation of genetic diversity within and among species and populations, as different markers might reveal different classes of variation (Powell et al. 1996; Russell et al. 1997).

Epigenetic changes, linked to the alteration in DNA methylation rate and patterns, have been intensively studied in higher plants (Zhang et al. 2018; Omony et al. 2020). The addition of a methyl group to the C5 position of cytosine is the most common DNA modification in plants (Cervera et al. 2002). Variation in DNA methylation patterns in regenerants has been described in rice (Müller et al. 1990), maize (Kaeppler and Phillips 1993), potato (Harding 1994), tomato (Smulders et al. 1995) and more recently, in oil palm (Jaligot et al. 2000) and banana (Sales and Butardo 2014).

DNA methylation is of particular interest to biotechnologists who search for crop improvement and it opens doors to a better understanding of plant development monitoring. In this aim, the quantification of global DNA methylation rates enables the determination of wide-methylation changes (Johnston et al. 2005; Osorio-Montalvo et al. 2020).

The date palm, Phoenix dactylifera L., is a species with high ecological, economic and social interests. It is one of the most important fruit trees cultivated in Asia and North Africa, showing a wide adaptability to various agro-ecological conditions. Studies aiming at the improvement of the date palm are very few, as the genetics of the species is very poorly known (Gros-Balthazard et al. 2020). Traditional breeding techniques are long, laborious and uncertain. Recognizing the importance of this sector, our group is implementing innovative Research and Development in order to contribute to the development of the date palm sector.

Several studies have described the negative impact of somaclonal variation in date palm (Cohen 2020; Mirani et al. 2020) although there is a possibility for research on the exploitation of induced genetic/epigenetic variability for the improvement of this species.

The aim of this study was to explore the effect of the synthetic auxin 2,4-D on the various physiological and biochemical aspect of in vitro cultures of date palm cultivar Barhee and to assess the somaclonal variation in regenerated cultures.

Materials and methods

Plant material and growth condition

All experiments were conducted using in vitro multiple bud cultures (actively proliferating buds) of P. dactylifera cv. Barhee, that were established from juvenile leaves sampled from 2-year-old offshoots, as previously described by Fki et al. (2011). Explants were incubated for an average 2 years at 28 °C in the dark and maintained by sequential subculturing every 8 weeks. Bud cultures were then cultivated for 2 months on an MS medium supplemented with 2,4-D at different concentrations (0, 1, 5, 10, 20 and 40 mg L⁻¹), 50 g L⁻¹ sucrose and 8 g L⁻¹ agar. The media were adjusted to pH 5.8 before autoclaving at 120 °C for 15 min. All cultures were incubated at 25 °C with a photoperiod of 16 h under fluorescent light with intensity of 30 μmol m⁻² s⁻¹.

In vitro shoot proliferation

After 2 months of incubation on MS medium enriched with various concentrations of 2,4-D, cultures were individually transferred into jars containing PGR-free MS medium, then incubated in 25 °C in a culture room. Proliferating tissues were maintained by sequential subculturing every 2 months.

Tissues bearing buds or calli were transferred onto multiplication media supplemented with 0.5 g L⁻¹ BAP and 50 g L⁻¹ sucrose to regenerate multiple shoot buds.

In order to improve biomass production and proliferation, regenerated shoot buds cultured during 3 months on multiplication media were transferred into temporary immersion system (RITA® bioreactors) with 200 mL liquid MS PGR-free medium supplemented with 50 g L⁻¹ sucrose. The RITA® system is a simple bioreactor composed of two parts separated by a filter. In vitro cultures were placed in the upper compartment and the liquid medium in the basal compartment. Overpressure is applied by pumping sterile air into the lower compartment, which induces an upward flow of liquid. The immersion occurred during 10 min every 24 h.

After 8 weeks, regenerated shoots derived from the RITA® system were divided into either single or grouped...
shoots and then transferred onto MS medium for further elongation and root development.

**Shoot elongation and rooting**

Elongation and rooting of shoots were achieved using liquid MS media supplemented with 50 g L\(^{-1}\) sucrose and 1 g L\(^{-1}\) activated charcoal without any auxin and IBA at 1 mg L\(^{-1}\) respectively. All cultures were incubated at 25 ± 2 °C under a 14 h/24 h light/dark and a light intensity of 30 μmol m\(^{-2}\) s\(^{-1}\) using white-light lamps.

**Physiological and biochemical analysis**

**Growth rate estimation**

To determine the effect of 2,4-D concentration, growth rates were calculated according to the following formula based on changes in fresh mass of eighteen repeats per treatment

\[
GR = \frac{t_f}{t_0} \left( \frac{\text{fresh weight after 2 months}}{\text{fresh weight of starting bud}} \right)
\]

**Estimation of chlorophyll content**

For this experiment, 0.1 g of fresh leaves were homogenized in a mortar using 2.5 mL of 80% acetone (Scheer et al. 1989). The homogenate was transferred to a 1.5 mL tube, and centrifuged at 10,000 rpm for 10 min. The absorbance of the supernatant was measured at 663 nm for chlorophyll a (Chl a) and 645 nm for chlorophyll b (Chl b) in triplicates. Chlorophylls contents were determined using Arnon’s formula (1949).

**Total protein and free proline contents**

The quantitative measurement of total soluble protein was estimated by the Bradford method. Five microliters of the raw enzymatic extract were added to 795 μL of millipore water and 200 μL of Bradford reagent. The optical density was measured spectrophotometrically at 595 nm and the protein content was determined by reference to a range of calibration established with increasing amounts of bovine serum albumin from 0 to 15 μg.

Free proline was extracted and determined as described by Bates et al. (1973). Samples were ground in a mortar using liquid nitrogen then 1.5 mL of 3% sulphisalicylic acid were added to 150 mg of powder. After stirring the mixture, the homogenate was centrifuged at 13,000 rpm for 15 min at 4 °C. A 200 μL aliquot of the supernatant solution was added to 800 μL of reagent composed of ninhydrin (1.25 g of ninhydrin + 30 mL of glacial acetic acid + 20 mL of phosphoric acid (6 M)) then heated for 1 h in a water bath at 100 °C. After cooling, 1 mL of toluene was added to the mixture before stirring. After 4 h of settling, two phases appeared and the upper phase (toluene) contained proline. The proline content was determined spectrophotometrically at 520 nm.

**Browning percentage**

The browning of in vitro cultures of cv. Barhee treated with various 2,4-D concentration was investigated by visual observation. Browning percentage was calculated by subtracting number of necrotic clusters from that of the total repetitions multiplied by 100.

**Determination of total phenolic content**

The total phenolic content of plant material was determined using the Folin Ciocalteu method, as reported by Cicco et al. (2009). 500 µL of Folin Ciocalteu reagent (10%) and 5 mL of Na\(_2\)CO\(_3\) were added to 100 µL of the extract. The reaction mixture was left standing for 30 min, then absorbance was measured at 727 nm. Gallic acid was used as a standard. Results are expressed as mg of gallic acid equivalent, GAE (mg of GAE g\(^{-1}\) of extract).

**Antioxidative enzyme assays**

Crude enzymatic extract for SOD and CAT activity was prepared by grinding 0.1 g of leaves originating from 2,4-D-treated cultures with 1 mL of extraction buffer (50 mM phosphate buffer, pH 7, 1 mM EDTA, 5% PVP, 3 mM DTT and 0.1% Triton) using precooled mortar and pestle. Extracts were then incubated for one hour on ice then centrifuged at 10,000×g at 4 °C for 20 min and supernatant was used as enzyme.

Catalase activity (CAT) was determined according to the method described by Aebl (1984). The assay buffer contained 250 μL phosphate buffer (10 mM, pH 7), 50 μL H\(_2\)O\(_2\) and 100 μL of enzyme extract (pH 7). The reaction was based on the consumption of H\(_2\)O\(_2\) which was spectrophotometrically monitored at 240 nm.

Superoxide dismutase (SOD) activity was measured by monitoring the inhibition of nitro blue tetrazolium (NBT) reduction at 560 nm (Beauchamp and Fridovich 1971). The reaction mixture contained 50 mM phosphate buffer (pH 7.5), 0.1 mM EDTA, 2 μM riboflavin, 10 mM methionine 75 μM NBT and crude enzymatic extract. The reaction was initiated by illuminating the samples under a 15 W fluorescent tube during 10 min then stopped by turning off the light.
Blanks and controls were run in the same manner. Blanks were without irradiation and controls were run without enzymatic extract.

**Hydrogen peroxide level**

Hydrogen peroxide (H$_2$O$_2$) content was measured according to Velikova et al. (2000) at 390 nm. Leaves were homogenized in an ice bath with 0.1% TCA. The homogenate was then centrifuged at 12,000×g for 15 min and 0.5 mL of the supernatant was added to 10 mM potassium phosphate buffer (pH 7.0) and 1 M KI. The concentration of generated H$_2$O$_2$ was calculated using a standard curve and expressed as µmol g$^{-1}$ FW.

**Estimation of lipid peroxidation**

Lipid peroxidation was determined by estimating the total amount of malondialdehyde (MDA) contents, according to Hernández and Almansa (2002). Bud material (500 mg) was homogenized in 5 mL TCA (0.1%). The homogenate was centrifuged at 10,000×g for 20 min. the supernatant (0.5 mL) was added to 1 mL TBA (0.5%) in 20% TCA. The mixture was incubated in boiling water for 30 min, and the reaction stopped by placing the reaction tubes in an ice bath. Then the samples were centrifuged at 10,000×g for 5 min. The absorbance was measured at 532 nm. The level of lipid peroxidation is expressed as nmol of MDA formed using an extinction coefficient of 155 mM$^{-1}$ cm$^{-1}$.

**Molecular analysis**

**DNA extraction**

DNA was extracted from the regenerated callus of *Phoenix dactylifera* using CTAB protocol following the method of Rogers and Bendich (1985). After purification, DNA concentrations were determined using a Nanodrop® (Thermo scientific) spectrophotometer. DNA integrity was checked by electrophoresis in 1% agarose gel (Sigma, Ultra-pure) in TBE buffer 1 M (Tris; Borate; EDTA) at 70 V for 30 min to allow proper resolution. The gel was stained by 0.5 µg mL$^{-1}$ ethidium bromide (Sigma) and observed under UV.

**Primers and ISSR assay**

A total of eight arbitrary ISSR primers were evaluated for polymorphism in genomic DNA samples. Based on resolution and reproducibility of banding patterns, four primers were selected (Table 1).

For PCR amplifications, a 25 µL reaction mixture was used, which contained 50 ng of total DNA (1 µL), 10 µM of primer (1 µL), 2.5 µL of 10× Taq DNA polymerase reaction buffer, 0.5 unit of Taq DNA polymerase (Bio Basic) and 10 mM of dNTP mixture (DNA polymerization mix (Bio Basic)). Amplifications were performed on a DNA amplification thermocycler (Techne TC-312) which was programmed using the following conditions: a denaturation step of 5 min at 94 °C, followed by 35 cycles composed of 30 s at 94 °C, 90 s at the annealing temperature, and 90 s at 72 °C. A final extension of 72 °C for 5 min was included. A negative PCR control sample devoid of DNA was used to verify the purity of amplification reactions. Amplifications were performed at least twice and only reproducible products were considered for further data analysis. Amplification products were separated on 1.8% agarose gel in 1× TBE buffer and detected by staining with ethidium bromide (0.5 µg mL$^{-1}$) according to Reid (1991). The size of generated amplicons was estimated according to a 100-bp ladder (Bio basic INC).

**DNA methylation study**

**Enzymatic hydrolysis of DNA**

Enzymatic hydrolysis of total genomic DNA into nucleosides was performed using two enzymes: alkaline phosphatase and P1 nuclease (Jaligot et al. 2000). To this aim, DNA samples (5 µg) were added to 5 µL of a 0.5 U mL$^{-1}$ solution of nuclease P1 (Sigma N8630) and 17.5 µL of a 0.0168 U mL$^{-1}$ solution of alkaline phosphatase (Sigma P4252) and the reaction volume adjusted to 100 µL with the digestion buffer (30 mM NaCH$_3$CO$_2$, 0.1 mM ZnCl$_2$, pH 5.3). The mixture was incubated for 4 h at 37 °C with regular stirring every 30 min. The reaction was stopped by the addition of 245 µL of absolute ethanol to the hydrolyzed mixtures then centrifuged at 14,000 rpm for 15 min in order to precipitate proteins in the pellet. The supernatant was

| Primer’s code | Sequence | T °C | Total number of bands | Number of polymorphic bands | Percent of polymorphic bands |
|---------------|----------|------|-----------------------|-----------------------------|-----------------------------|
| ISSR1 | (CT)$_n$A | 57 | 7 | 0 | 0 |
| ISSR2 | (AG)$_n$T | 57 | 7 | 0 | 0 |
| ISSR3 | (GC)$_n$CC | 44 | 15 | 2 | 13.33 |
| ISSR4 | (GACA)$_n$ | 47 | 27 | 3 | 11.12 |
transferred to a new tube. Ethanol was removed by evaporation under a laminar flow cabinet. Nucleosides were dissolved in 500 µL of sterile water then filtered through nylon filters (0.2 µm) prior to HPLC analysis.

**HPLC analysis of nucleosides**

An isocratic elution method was followed according to Cock et al. (2010). 50 mM KH₂PO₄, 8% (V/V) methanol through a supelcosil LC-18S reverse-phase column (SUPELCO Inc; column length 25 cm; diameter 4.6 mm; particle diameter: 5 mm), with a flow rate of 0.8 mL min⁻¹ and a run time of 30 min for clear separation of the peaks. The effluent was monitored at the wavelength of 285 nm with a photodiode array detector (Beckman Coulter).

GMRs were calculated as percentages using the following formula:

\[
\text{GMR} = 100 \times \left( \frac{5\text{mdC}}{(dC) + (5\text{mdC})} \right)
\]

**Statistical analyses**

All assays were undertaken in triplicate and data were analyzed using the statistical package GraphPad Prism 4. HPLC analyses were evaluated via one-way ANOVA t-test using GraphPad Prism 4. 01 computer program. Standard error (S.E.) was used at p < 0.05 in Tuckey and Duncan’s test to establish significant differences between treatments in all experiments.

**Results**

**Effects of 2,4-D on the various morphological and physiological parameters**

**Effect on growth**

Shoot growth of treated and control in vitro cultures was evaluated after 2 months, in order to highlight the effect of 2,4-D. The auxin 2,4-D was found to reduce growth of cultures as a response to the PGR treatment. An inhibitory effect was evidenced when 2,4-D concentrations were used at highest concentrations (5–40 mg L⁻¹). Shoot buds did show some intensive browning then became completely necrotic after 2 months of culture in media containing 10, 20 and 40 mg L⁻¹ 2,4-D. Our results showed a decrease in growth rates which was significantly affected by 2,4-D treatment with p < 0.05 (Table 2). Some growth inhibition could be observed through changes in fresh biomass. The toxicity of the highest auxin concentration induced severe necroses in tissues (Fig. 1). In all treatment regime (except at 1 mg L⁻¹ of 2,4-D) in vitro cultures showed symptoms of toxicity—especially browning—as a result of tissue damage. The signs of toxicity were lower with 2,4-D at 5 mg L⁻¹ compared to the highest concentration of the auxin. The treatment of cultures for 2 months with high 2,4-D levels increased the intensity of the browning of tissues and released phenolic substances in the medium, which ranged from 8.3 to 95.8% and negatively impacted the growth rate of buds (Table 2). The use of lower 2,4-D concentrations (1 mg L⁻¹) was found to be efficient for the induction of callus and allowed the normal growth of buds (Fig. 1).

**Effect of various concentrations of 2,4-D on the production of phenolics**

Most of the observed tissues emitted phenolic substance in culture media. Phenolic compounds are usually produced as a defense in a stressful environment by plant tissues. Figure 2 shows that the incubation of in vitro cultures for 2 months under different concentrations of 2,4-D caused some accumulation of phenolic compounds. They were found to range between 25.74 and 53.95 mg GAE g⁻¹, which was high when compared with the low accumulation of phenolic compounds found in control cultures (1.99 mg GAE g⁻¹) for the same periods.

**Effect on chlorophylls contents**

The total chlorophylls contents varied significantly when 2,4-D concentrations were changed. As noted, the auxin 2,4-D caused severe necrosis of shoot buds which disturbed the synthesis of chlorophyll pigments. Results showed a rapid decline in total chlorophyll content which reached a minimum value of 16 µg mg⁻¹ FW in response to 5 mg L⁻¹ 2,4-D (Table 3). The application of high PGR doses of 10,

| 2,4-D concentration | Control | 1 mg L⁻¹ | 5 mg L⁻¹ | 10 mg L⁻¹ | 20 mg L⁻¹ | 40 mg L⁻¹ |
|---------------------|---------|----------|----------|-----------|-----------|-----------|
| Browning percentage (%) | 8.3     | 8.3      | 41.66    | 79.16     | 83.3      | 95.8      |
| Growth rates        | 3.00 ± 0.5a | 3.01 ± 0.3a | 2.07 ± 0.2b | 1.52 ± 0.2c | 1.08 ± 0.1d | 1.00 ± 0.1d |
| Regeneration rates (%) | 95.3    | 91.7     | 87.5     | 75        | 83.3      | 70.83     |

The regeneration rate was calculated after the transfer of cultures onto PGR-free medium. Different letters denote statistically significant differences (p ≤ 0.05) according to Duncan’s test.
Fig. 1 Effect of 2,4-D on in vitro cultures of ‘Barhee’ cultivar. A control: 0 mg L\(^{-1}\), B 1 mg L\(^{-1}\), C 5 mg L\(^{-1}\), D 10 mg L\(^{-1}\), E 20 mg L\(^{-1}\), F 40 mg L\(^{-1}\). a-f Corresponds to the start material of each treatment. Scale bar: 1 cm

Fig. 2 Total phenol content (mg GAE g\(^{-1}\)) of in vitro cultures of date palm cv. Barhee treated with different concentration of 2,4-D. *, **, *** Indicates significant according to Tukey’s test at \(p < 0.05\), \(p < 0.01\) and \(p < 0.001\), respectively

Table 3 Effect of 2,4-D on photosynthetic pigments following the application of 2,4-D on in vitro cultures of ‘Barhee’ cultivar

| Media composition | 0 (Control) | 1 mg L\(^{-1}\) | 5 mg L\(^{-1}\) | 10 mg L\(^{-1}\) | 20 mg L\(^{-1}\) | 40 mg L\(^{-1}\) |
|-------------------|-------------|----------------|----------------|----------------|----------------|---------------|
| Chl a (µg mg\(^{-1}\) FW) | 23.1 ± 7\(^b\) | 5.2 ± 3\(^a\) | 8.02 ± 1\(^a\) | 0 | 0 | 0 |
| Chl b (µg mg\(^{-1}\) FW) | 10.02 ± 2\(^a\) | 18.3 ± 3\(^b\) | 8.04 ± 1\(^a\) | 0 | 0 | 0 |
| Chl a + Chl b (µg mg\(^{-1}\) FW) | 33.12 | 23.5 | 16.06 | 0 | 0 | 0 |

Different letters denote statistically significant differences (\(p \leq 0.05\))

\(Chl\ a\) chlorophyll a; \(Chl\ b\) chlorophyll b; \(Chl\ a + Chl\ b\) chlorophyll a + chlorophyll b
20 and 40 mg L$^{-1}$ caused a severe necrosis thus an inhibition in chlorophyll biosynthesis was noticed which made those pigments undetectable.

**Protein and proline content**

The trend in protein content in analyzed samples was similar to that found for chlorophyll content (Table 4). Increasing concentrations had a significant negative impact on the soluble protein content ($p < 0.05$). In fact, total protein content rapidly reduced with 20 mg L$^{-1}$ and 40 mg L$^{-1}$ of auxin concentration and the lowest protein content was 0.6 mg g$^{-1}$ FW in cultures grown on 40 mg L$^{-1}$ 2,4-D. Total soluble protein contents varied from 1.8 to 0.6 mg g$^{-1}$ FW.

Figure 3 shows changes in proline contents recorded when 2,4-D-enriched media were used. Changes were found to be statistically significant according to Tukey’s test. The highest amount of proline (2973 nmol g$^{-1}$ FW) was obtained in severe stress conditions due to the application of 40 mg L$^{-1}$ of 2,4-D and the lowest amount (535 nmol g$^{-1}$ FW) was measured in non-treated tissues. The proline content of in vitro cultures was indeed found to increase with the auxin concentration.

**Effect of 2,4-D on H$_2$O$_2$ and MDA content**

Abiotic stress is associated with increased oxidative stress due to the accumulation of reactive oxygen species (ROS), particularly H$_2$O$_2$. We found that H$_2$O$_2$ in in vitro cultures tended to rise under stress provoked by 2,4-D treatment (Fig. 4a). The increased accumulation of MDA in treated cultures compared to control is used as an indicator of lipid peroxidation and enhanced ROS production (H$_2$O$_2$). Significant differences in MDA content were noted. MDA contents of shoots increased with increasing 2,4-D concentration (Fig. 4b). Maximum increases in MDA content were recorded in media containing 20 and 40 mg L$^{-1}$ 2,4-D.

**Antioxidant enzyme activities induced by 2,4-D**

In order to investigate how 2,4-D treatment impacts the antioxidative defense system, we measured antioxidant enzymatic activities namely SOD and CAT. Figure 5 show changes in CAT and SOD enzymes activity under different concentrations of 2,4-D. SOD activity was found to increase linearly with increasing the 2,4-D level from 0 to 40 mg L$^{-1}$. The highest level of SOD was recorded in the shoots cultivated in a medium supplied with 40 mg L$^{-1}$ 2,4-D, while the lowest level of activity was observed in the control treatment after 2 months of culture. CAT activity was found to increase with increasing 2,4-D concentrations in then medium.

**In vitro shoot regeneration**

Treated and non-treated in vitro cultures were subcultured onto PGR-free media in order to evaluate their regenerative potential. During the transfer, necrotic cultures showed an ability to regenerate after 2 weeks in the absence of PGR.

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### Table 4 Protein content of date palm in vitro cultures treated with various 2,4-D concentrations

| 2,4-D concentration | Control | 1 mg L$^{-1}$ | 5 mg L$^{-1}$ | 10 mg L$^{-1}$ | 20 mg L$^{-1}$ | 40 mg L$^{-1}$ |
|---------------------|---------|--------------|--------------|--------------|--------------|--------------|
| Protein content (mg g$^{-1}$ FW) | 1.8 ± 0.08a | 1.5 ± 0.045b | 1.56 ± 0.08b | 1.1 ± 0.015a | 0.62 ± 0.01b | 0.6 ± 0.008b |

Different letters denote statistically significant differences ($p \leq 0.05$)
and they gave rise to new structures. Figure 6a shows the formation of calli. Neoformed calli showed differences in texture and morphology and only regenerative ones were selected, some of them were friable, mucilaginous and resembled microbial contaminations. Callus grew and showed a regenerative capacity characterized by the growth of greenish structures which gave rise to shoot buds and later to complete leaves (Fig. 6b, c). PGR-free medium seemed to be good for growth and for the maintain of regeneration potential. Indeed, regeneration rates were high as they were found to range from 70.83 to 95.3% (Table 2). Therefore, after tissue rejuvenation and establishment of culture, shoot buds were subcultured continuously in medium containing low amounts of the phytohormone BAP and the number of shoots increased with each culture cycle. The regenerated culture buds organized and showed growth. A high morphogenetic capacity was observed in this material. In order to improve shoot bud multiplication and biomass production, cultures were transferred into RITA® temporary immersion-based bioreactors (Fig. 6d). High-frequency shoot production was determined and rooted shootlets could be produced (Fig. 6g). The rooted shootlets were sub-cultured and grown on MS medium supplemented with 1 g L⁻¹ active charcoal (Fig. 6f, g) then transferred to liquid MS medium to convert into plantlets (Fig. 6h). The addition of 1 mg L⁻¹ IBA improved the development of roots.

**ISSR analysis**

ISSR amplification products were generated by PCR using four primers (Fig. 7). Results obtained for each primer are presented in Table 1. ISSR fingerprints were found to reveal some level of genetic variation among studied samples. The

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**Fig. 4** Effect of different concentrations of 2,4-D the content of H₂O₂ (a) and MDA (b). Data presented are means ± standard error of three independent experiments. *, **: p < 0.05 and p < 0.01 respectively level of significant mean difference from control (Tuckey's test).

**Fig. 5** Effect of 2,4-D on the activities of CAT (a) and SOD (b) in shoots of date palm after 2 months of treatment. Data presented are means ± standard error of three independent experiments. *: p < 0.05 level of significant mean difference from control (Tuckey's t test).
four studied primers generated different banding patterns that were uniformly obtained for all samples. Figure 7 shows representative examples of banding patterns produced by primers ISSR3 and ISSR4 which gave rise to one polymorphic band. These two primers showed reproducible banding patterns and they generated polymorphic bands while other primers produced monomorphic bands (50%). The number of bands varied from 13 to 27 with an average of 11 bands per ISSR primer. The size of the scorable amplified fragments ranged from 200 to 1000 bp. A 900 bp—polymorphic band was obtained with ISSR3 primer and it was absent in regenerants obtained when 20 mg L\(^{-1}\) of 2,4-D were used and a 700 bp polymorphic band was obtained with the same probe in 40 mgL\(^{-1}\). When the ISSR4 primer was used, we noticed absence of a 800 bp-band in plants regenerated at 10, 20 and 40 mg L\(^{-1}\) of 2,4-D when compared to the control. The present study clearly demonstrates the efficiency of ISSR markers for revealing the genetic impact of 2,4-D treatments.

**Global DNA methylation rates**

The relative amounts of 5-methyldeoxycytidine in the DNA of date palm tissues were determined through HPLC.
analysis. DNA methylation levels were found to vary significantly among regenerants. The calculated GMR values ranged from 13.8 to 18.93% (Fig. 8) and they did not show any significant variation within regenerants. The highest GMR value (18.93%) was observed for R5 material (40 mg L\(^{-1}\) 2,4-D treatment) and the lowest was measured in the control (13.8%) sample. Global genomic DNA methylation rates were found to increase significantly in regenerants from all 2,4-D treatments, when compared to the control.

**Discussion**

In this study, buds of Barhee cultivar grown on media containing high concentrations of the auxin 2,4-D showed visible signs of toxicity. Growth was fully inhibited at concentrations higher than 20 mg L\(^{-1}\) of 2,4-D with induced necrosis of cultured tissues and subsequent browning of media. The browning degree was related to the emission of phenolic compounds. Several studies illustrated the increase of phenolics in cultures under stress conditions, in both potato (Daneshmand et al. 2010) and olive (Petridis et al. 2012). The photosynthetic pigments are considered as one of the most important parameters in evaluating stress. Present study revealed that the total chlorophyll content reduced after exposure to 2,4-D. Several studies have been reported reduction in total chlorophyll content in many plant species exposed to heavy metals (Piotrowska et al. 2009). Level of chlorophyll contents was affected indirectly with 2,4-D which led to the necrosis of buds. Our finding was in accordance with Islam et al. (2017) who reported a maximum decline in the chlorophyll contents after a 2,4-D treatment of rice cultures. Some reduction of chlorophyll content in the presence of 2,4-D was noted in cultures of pea, tomato and potato according to Hildebrandt et al. (1963).

The application of the auxin 2,4-D beleads to the disruption of the balance between ROS production and scavenging. Indeed, 2,4-D was able to induce lipid peroxidation resulting from the production of ROS which is in disagreement with Martín-Romero et al. (2008). MDA content increases in a concentration-dependent manner under 2,4-D stress which induces a higher level of ROS production (Hu et al. 2012). Increased lipid peroxidation has also been reported by Mondal et al. (2015) under Hg stress. An increase of H\(_2\)O\(_2\) contents was recorded in our plant material which might be due to the inactivation of H\(_2\)O\(_2\) scavenging enzymes. According to Grossman et al. (2001), the application of auxinic herbicides led to an overproduction of H\(_2\)O\(_2\) which was involved in the induction of tissue damage and cell death. Antioxidant enzyme activities are frequently related to mechanisms of herbicides toxicity and resistance (Kusvuran et al. 2016).

Therefore, we evaluated the effect of 2,4-D on the activity of several antioxidant enzymes, namely, SOD and CAT. The activities of CAT under 2,4-D treated cultures showed linear increase compared to the control, which might be the reason of enhanced ROS production and lipid peroxidation. On the other hand, SOD activity typically increased with increasing levels of 2,4-D. Several studies on 2,4-D application suggest an increase or decrease of antioxidant enzyme activities depending on the period of treatment and concentration (Pazmiño et al. 2011; Islam et al. 2016).

Our study also showed that 2,4-D treatment activated the accumulation of proline. High doses of PGRs are often described as a stress factor leading to the accumulation of this amino acid (Siddique et al. 2018). Proline is considered as a marker of stress and adaptation (Din et al. 2011) as it plays several roles: (i) it intermediates in the osmotic adjustment (Karimi et al. 2018) as an osmo protective agent (Kavi Kishor et al. 2005), (ii) it could act as an antioxidant (Sharma and Dietz 2006; Vendruscolo et al. 2007) and as a regulator of cytosolic acidity (Sivakumar et al. 2000), (iii) it can also be a source of carbon and nitrogen reserves after the disappearance of stress (Kala and Godara 2011). Indeed, several reports suggest a positive relationship between proline accumulation and plant stress (Al Mayahi and Fayadh 2015). Tahri et al. (1998) also showed the effect of high PGR.
doses on the levels of proline and chlorophyll pigments, as chlorophyll contents decreased when herbicide (Sunohara and Matsumoto 2004) were used.

The capacity of in vitro cultures to acquire competence and assume new developmental fate after modulation of the growth conditions is the basis for the establishment of plant regeneration systems and the ability to regenerate new tissues, organs, or the whole individual. In our experiment, in vitro cultivated material was transferred onto MS media devoid of auxin and they showed the ability to regenerate. In cultured tissue material, histological studies are able to reveal two possible origins for neoformed tissues; an internal origin relative to perivascular cells and a superficial origin involving epidermal cells which is the case in oil palm (Schwendiman et al. 1988; Rival et al. 1998; Kanchanapoom and Domyoas 1999; Sané et al. 2006; Gomes et al. 2017). Also, the origin of neoformed calli has widely been studied (Drira and Benbadis 1985). The emergence of calli from necrotic tissues indicates their higher morphogenetic capacity. High rates of shoot bud multiplication were obtained on PGR-free medium. This was consistent with the findings of Fki et al. (2003) on date palm. The same result was observed in pineapple plants (Ananas comosus L.) with high rooting in PGR-free media (Sripaoraya et al. 2003). Arrabal et al. (2002) also observed root formation in Cryptanthus sinuosus grown in auxin-free medium, showing that some species present higher rhizogenic potential than others. The use of RITA® temporary immersion-based bioreactors enhanced the yield of regenerated shootlets compared to conventional semi-solid medium (Fki et al. 2011; Gomes et al. 2016; Nasri et al. 2019). Indeed, these new structures showed a higher capacity to regenerate plants which were proved much more vigorous than their counterparts originating from primary shoots.

In in vitro plant cells, 2,4-D induces mitotic and meiotic irregularities (Khalkar and Bhargava 1982). Ozkul and Galderisi (2016) showed a negative cytogenetic effect of 2,4-D in plant tissue cultures that could result in undesirable variations affecting the genetic integrity of regenerated material. Regenerated plants are affected by artificial conditions through a high probability of both epigenomic and genomic changes (Neelakandan and Wang 2012). Ahmadi and Bakhshandeh (2009) studied the interaction between 2,4-D and DNA. Studies indicated that 2,4-D acts like groove binder of DNA.

In the present study, genetic variations were assessed by ISSR markers that revealed polymorphism among the control and the regenerants (Martínez-Estrada et al. 2017). The ISSR technique presented in this study provides a powerful tool to investigate DNA variation in different in vitro cultured tissues and treatments, and it showed very interesting results. Few studies have been published on the prevalence of variants among date palm plants regenerated by in vitro propagation. The small range of variation in DNA evidenced by ISSR analysis may be due to various factors such as the in vitro process, auxin concentration, and stress prone cultivation conditions. All of these are known to induce somaclonal variation. The incidence of somaclonal variation in plant regeneration process has been reported to be the result of point mutations (Gady et al. 2009), DNA amplification (Tiwari et al. 2010) and epigenetic variation (Smulders and de Klerk 2011). Thummar et al. (2015) failed to detect genetic variation between mother plants and two date palm clones using the ISSR approach, while Mohamed et al. (2018), in their study on date palm offshoots using five different ISSR markers, found that high lead (Pb) concentration induced changes in DNA patterns. The ISSR approach has been widely used to analyze the genetic stability of vitroplants as well as for varietal characterization in date palm (Zehdi et al. 2002). In other species, ISSR markers have been successfully used to detect genetic differences or similarities in micropropagated plants including gerbera (Bhatta et al. 2009), anthurium (Gantait and Sinniah 2011), and grape (Nookaraju and Agrawal 2012).

The present work also evaluated the effect of 2,4-D on global DNA methylation levels by HPLC. For many years, HPLC has been the favored technique for the quantitative determination of global DNA methylation rates (Kuo et al. 1980). HPLC is considered as a reliable and sensitive technique to simply assess DNA methylation (Wagner and Capesius 1981; Fraga and Esteller 2002) as it involves the digestion of DNA to nucleotides, nucleosides or bases, which are separated and quantified using UV detection. HPLC analysis of nucleosides can be used to characterize gross epigenetic changes during stress, growth and development (Johnston et al. 2005). Stressful conditions in in vitro culture may induce epigenetic changes to face new situation. Here, 2,4-D was found to led to incur changes in GMRs measured in date palm regenerants. In vitro plant tissue culture is also known to undergo high levels of oxidative stress due to the formation of reactive oxygen within the cells, which is known to cause DNA damages such as DNA methylation, histone modifications, and RNA interference (RNAi). The frequency of variation can be genotype-independent. Increased 2,4-D concentration was found to promote cytosine methylation levels which was in accordance with Neelakandan and Wang (2012) and Sales and Butardo (2014). It has been reported that 2,4-D resulted in enhanced DNA ploidy levels and methylation events in in vitro plant regeneration cultures (Da Silva et al. 2014). Several studies attribute the alteration in genomic DNA methylation rates to the development of the ‘mantled’ somaclonal variant in the case of oil palm (Eeuwens et al. 2002; Jaligot et al. 2011; Rival et al. 2013) which can be caused by hypomethylation of mantled locus (Ong-Abdullah et al. 2015). Increasing amounts of PGRs were found to enhance methylation levels in carrot cultures.
regenerants (Kaeppler et al. 2000). Changes in GMRs were also studied by LoSchiaivo et al. (1989) who showed that global methylation levels changed in response to hormone concentration in the media of carrot cultures; these authors also showed that DNA methylation levels increased with increasing amounts of 2,4-D, which was in accordance with our present results. Finally, to be of practical value, viable regenerants should be further screened under field conditions in order to assess their genetic/epigenetic stability and to select superior genotypes.

Conclusion

Besides of the toxic effect of 2,4-D, used in high levels, on in vitro cultures of date palm Barhee cultivar, we have successfully regenerated callus and shoot buds from necrotic buds. Our results showed the higher morphogenetic capacities of the in vitro cultures. The use of BAP and RITA® bioreactors was found to be efficient to enhance biomass production. Shoot buds were converted into plantlets using liquid MS medium. The assessment of somaclonal variation among regenerants via ISSR markers was established. To uncover epigenetic changes, HPLC was used to study global DNA methylation.

Acknowledgements Our research work was undertaken at the Faculty of Sciences of Sfax, Tunisia. The present research was partly implemented at the Institute of Research for Development (IRD) at UMR-DIADÉ in Montpellier, France. The authors would like to thank the COST office (FA1405 coordinator) for financial support. COST is an EU-funded program that enables researchers to set up their interdisciplinary research networks in Europe and beyond. The authors gratefully acknowledge the European Cooperation in Science and Technology (COST office (FA1405 coordinator)).

Funding This work was supported by the European Cooperation in Science and Technology (COST office (FA1405 coordinator)).

Data availability Not applicable.

Code availability Not applicable.

Conflict of interest The authors have no conflicts of interest to declare.

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