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

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Research Article

Keywords: Phoenix dactylifera, DNA methylation, Somaclonal variation, ISSR markers, Plant Growth Regulators, Regeneration

Posted Date: August 24th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-777517/v1

Version of Record: A version of this preprint was published at Plant Cell, Tissue and Organ Culture (PCTOC) on April 12th, 2022. See the published version at https://doi.org/10.1007/s11240-022-02259-8.

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 nmol g^{-1} 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 (H_2O_2) and malondialdehyde (MDA); 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 13.8–18.93%.

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.

Introduction

In vitro tissue cultures are known to be genetically and/or epigenetically unstable (Bednarek and Orłowska 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* sub culturing 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.E.F 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). 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 genome-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 objective of the present study was to evaluate physiological the effect of 2,4-D on date palm caulogenic *in vitro* cultures from ‘Barhee’ cultivar. In this aim, we investigated both genetic and epigenetic variability via ISSR markers and Global Methylation Rate (GMR) in *in vitro* cultivated material.

Materials And Methods

Plant material and growth condition

All experiments were implemented using *in vitro* multiple bud cultures of *P. dactylifera* cv. Barhee, which derived from juvenile leaf explants (Fki et al. 2011). Buds were cultured for 2 months on an MS medium. The media were 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.

In vitro shoot proliferation

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

After successive transfers on multiplication media, RITA® bioreactors were used for the proliferation of *in vitro* cultures using 200 mL of liquid MS medium. The pH was adjusted to 5.8 before autoclaving (120 °C/1 bar/ 20 min). 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. The immersion cycle was 10 min every 24 h.

Elongated regenerated shoots derived from the RITA® system were divided into single or few shoots and then transferred to an MS agar-solidified medium for further elongation and root development.

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:

GR = t_1(fresh weight after 2 months) / t_0 (fresh weight of starting bud).

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). 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% sulphosalicylic 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 (6M)) then heated for 1h 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. In this experiment, the degree of browning in relation to treatments was scored according to Abul-Soad (2012).

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). 500µL of Folin Ciocalteu reagent (10%) and 5 mL of Na_2CO_3 were added to 100µL 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 of extract).

Antioxidative enzyme assays

CAT activity was determined according to the method described by Aebi (1984). The assay buffer contained 250 µL phosphate buffer (10 mM, pH 7), 50 µL H_2O_2 and 100 µL of enzyme extract (pH 7). The reaction was based on the consumption of H_2O_2 which was spectrophotometrically monitored at 240 nm.

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 allowed to run for 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 content was measured according to Velikova et al. (2000) at 390 nm. The amount of H_2O_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). The absorbance was measured at 532 nm.

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 1M (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 8 arbitrary ISSR primers were evaluated for polymorphism in genomic DNA samples. Based on resolution and reproducibility of banding patterns, 4 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 10X 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 1X 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 $NaCH_3CO_2$, 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 14000 rpm for 15 min in order to precipitate proteins in the pellet. The supernatant was 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 KH_2PO_4, 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:

GMR =100 * (5mdC)/[(dC) +(5mdC)].

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 investigate the effect of 2,4-D. The auxin 2,4-D was found to reduce growth and affect the multiplication factor 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 mg L^{-1} to 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 treatment of cultures for 2 months with high 2,4-D levels increased the intensity of the browning in the medium, which ranged from 8.3%, 95.8% and negatively impacted the growth rate of buds (Table 3). The use of lower 2,4-D concentrations (1 mg L^{-1}) was found to be efficient for the induction of callus.

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

Most of the observed tissues emitted sizeable quantities of phenolic compounds in culture media. Fig. 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, which was high when compared with the low accumulation of phenolic compounds found in control cultures (1.99 mg GAE/g) for the same periods.

Effect on chlorophylls contents

The total chlorophylls contents varied significantly when 2,4-D concentrations in the media were changed. Leaves were very sensitive to high concentrations of 2,4-D which disturbed the synthesis of chlorophyll pigments while causing chlorosis at 20 and 40 mg L^{-1} concentrations (Fig. 1). Increased concentrations of 2.4-D had a negative impact chlorophyll a content and the lowest chlorophyll a content was found when 5 mg L^{-1} of 2,4-D were used. Chlorophyll b content of *in vitro* cultures diminished with increasing the auxin level. The maximum chlorophyll b level was recorded in the control. however, the lowest content in MS medium was obtained with 5 mg L^{-1} of 2,4-D (Table 3). Results showed a rapid decline in total chlorophyll content which reached a minimum value of 0.016 mg mg^{-1} FW in response to 5 mg L^{-1} 2,4-D. The application of high PGR doses of 10, 20 and 40 mg L^{-1} caused an inhibition in chlorophyll biosynthesis 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 mg g^{-1} FW 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 Tuckey'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 H2O2 and MDA content

Abiotic stress is associated with increased oxidative stress due to the accumulation of ROS, particularly H_2O_2. We found that H_2O_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_2O_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 decrease with increasing 2,4-D concentrations in then medium.

In vitro shoot regeneration

Treated and non-treated *in vitro* cultures were subcultured onto free PGR media in order to evaluate their regenerative potential. During the transfer, necrotic cultures showed an ability to regenerate in the absence of PGR, and they gave rise to new structures. Figure 6a shows the formation of calli: some of them were friable, mucilaginous and resembled microbial contaminations (Fig.6b). Callus grew and showed an interesting regenerative capacity characterized by the growth of greenish structures which gave rise to shoot buds and later to complete leaves (Fig. 6c). Indeed, regeneration rates were high as they were found to range from 70.83% to 95.3% (Table 3). Therefore, after tissue rejuvenation and establishment of culture, shoot buds were subcultured continuously in medium containing low amounts of the phytohormone BAP, in order to maintain the regeneration potential of cultures. The regenerated culture buds organized, grew and showed growth. A high morphogenetic capacity was observed in this material. In order to improve shoot bud multiplication, cultures were transferred into RITA® temporary immersion-based bioreactors. Clumps of shoot buds showed some interesting morphological capacity, 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. 6d).

ISSR analysis

ISSR amplification products were generated by PCR using four primers (Fig 7). Results obtained for each primer are presented in Table 6. ISSR fingerprints were found to reveal some level of genetic variation among studied samples. The four studied primers generated different banding patterns that were uniformly obtained for all samples. Figure 6 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 3 to 7 with an average of 5,5 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 and 40 mg L^{-1} of 2,4-D were used. When the ISSR4 primer was used, we noticed a 700 bp-band in plants regenerated at 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 significally regenerants from all 2,4-D treatments, when compared to the control.

Discussion

In our study, several changes could be evidenced after the application of 2,4-D in culture media. When high concentrations were used, some inhibitory effect of 2,4-D on the growth of *in vitro* material was clearly observed (Islam et al. 2017). The major question is whether the inhibition is due to the auxin itself or to the effect of auxin on the production or movement of other substances. In the present study, the concentrations of auxin significantly affected the survival of date palm *in vitro* cultures. Growth was fully inhibited at concentrations higher than 20 mg L^{-1} of 2,4-D with induce 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 application of the auxin 2,4-D lead 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). An increase of H_2O_2 contents was recorded in our plant material. 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 were reduced 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. Islam et al. (2017) reported a maximum decline in the chlorophyll contents after a 2,4-D treatment of rice cultures.

In our experiment, *in vitro* cultivated material was transferred onto MS media devoid of auxin and they showed the ability to regenerate. 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. 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 (Rival et al. 1998). Also, the origin of neoformed calli has widely been studied (Drira and Benbadis 1985). The use of RITA® temporary immersion-based bioreactors enhanced the yield of regenerated shootlets compared to conventional semi-solid medium (Fki et al. 2011; Nasri et al. 2019). The major advantage of treating date palm undifferentiated material with high doses of 2,4-D was the establishment of extremely vigorous callus lines which proved free from any apparent endophytic contamination. Indeed, these new structures showed a higher capacity to regenerate plants which were proved much more vigorous than their counterparts originating from primary shoots.

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).

Somaclonal variation could be detected through ISSR markers (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. In our research, we performed molecular analyses to assess the risk of induced somaclonal variation. 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. 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 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 (Bhatia 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). 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). Alteration in genomic DNA methylation rates was attributed to the development of the 'mantled' somaclonal variant in oil palm (Eeuwens et al. 2002; Jaligot et al. 2011; Rival et al. 2013). 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 LoSchiavo 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.

Conclusion

The present study is a precise investigation on the effect of 2,4-D on the date palm tissue cultures. Our ultimate aim was to pave the way for the regeneration of elite planting material bearing key agronomic characteristics. Our results confirmed the toxic effects of high doses of 2,4-D on the cultured plant material. Such doses of 2,4-D (10, 20 and 40 mg L^{-1}) were shown to be lethal since severe necrosis was recorded in all treated cultures. It should be noted that probably some undifferentiated cells were able to continue their multiplication even in the presence of high doses of PGRs. From these cells, it was possible to regenerate somaclonal variant plants. Our results showed that high concentrations affect the growth of *in vitro* cultures without hampering their morphogenetic capacities. At the biochemical level, the effect of 2,4-D was also studied: 2,4-D treatments decreased the levels of chlorophyll pigments and increased the concentration of proline in tissues exposed to high doses. Our ISSR analyses and the estimation of global DNA methylation rates evidenced the presence of genetic and epigenetic variations, respectively. Further research is now focusing on the phenotypic analysis of regenerated date palms, as we are looking for variations of putative agronomic interest for the generation of future elite planting material.

Abbreviations

HPLC: High performance liquid chromatography

GMRs: Global methylation rates

2,4-D: 2,4-Dichlorophenoxyacetic acid

ISSR: Inter simple sequence repeat

5mdC: 5-Methyldeoxycytidine

FW: Fresh weight

CTAB: Cetyltrimethyl ammonium bromide

PGR: Plant growth regulator

Declarations

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-DIADE 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 thank the staff of UMR-DIADE (Montpellier, France) under the supervision of Mr. Thierry Beulé and Mrs. Sylvie Doulbeau. We gratefully thank Mr. Kamel Maaloul for English corrections.

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

Conflicts of interest/Competing interests: The authors have no conflicts of interest to declare.

Availability of data and material: (Not applicable)

Code availability: (Not applicable)

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Tables

Table 1: ISSR primers used for our study of genetic variability and their amplification performance.

Primer's code	Sequence	T° C	Total number of bands	Number of polymorphic bands	Percent of polymorphic bands
ISSR1	$(CT)_{10}A$	57	7	0	0
ISSR2	$(AG)_{10}T$	57	7	0	0
ISSR3	$(GC)_6CC$	44	5	1	20
ISSR4	$(GACA)_4$	47	7	1	14.28

Table 2: Effect of 2,4-D concentration on growth rates of *in vitro* cultures and browning degree after 2 months of culture.

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 ± 0.5^a	3 ± 0.3^a	2 ± 0.2^b	1.5 ± 0.2^c	1 ± 0.1^d	1 ± 0.1^d
Regeneration rates (%)	95.3	91.7	87.5	75	83.3	70.83

Table 3: Effect of 2,4-D on photosynthetic pigments following the application of 2,4-D on *in vitro* cultures of 'Barhee' cultivar. Different letters denote statistically significant differences ($p \leq 0.05$).

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 (mg mg^{-1} FW)	0.023 ± 0.007^{b}	0.005 ± 0.003^{a}	0.008 ± 0.001^{a}	0	0	0
Chl b (mg mg^{-1} FW)	0.010 ± 0.002^{a}	0.018 ± 0.003^{b}	0.008 ± 0.001^{a}	0	0	0
Chl a+ Chl b (mg mg^{-1} FW)	0.033	0.023	0.016	0	0	0

Chl a: chlorophyll a; Chl b: chlorophyll b; Chl a+ Chl b: chlorophyll a + chlorophyll b

Table 4: Protein content of date palm *in vitro* cultures treated with various 2,4-D concentrations. Different letters denote statistically significant differences ($p \leq 0.05$).

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 ± 0.08^{a}	1.5 ± 0.045^{b}	1.56 ± 0.08^{b}	1.1 ± 0.015^{a}	0.62 ± 0.01^{b}	0.6 ± 0.008^{b}

Figures

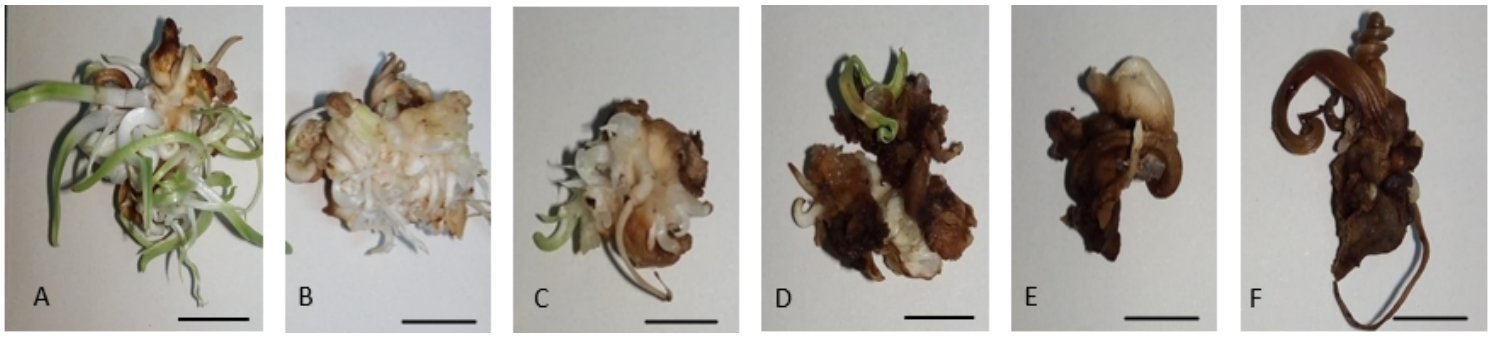

Figure 1

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. Scale bar: 1 cm.

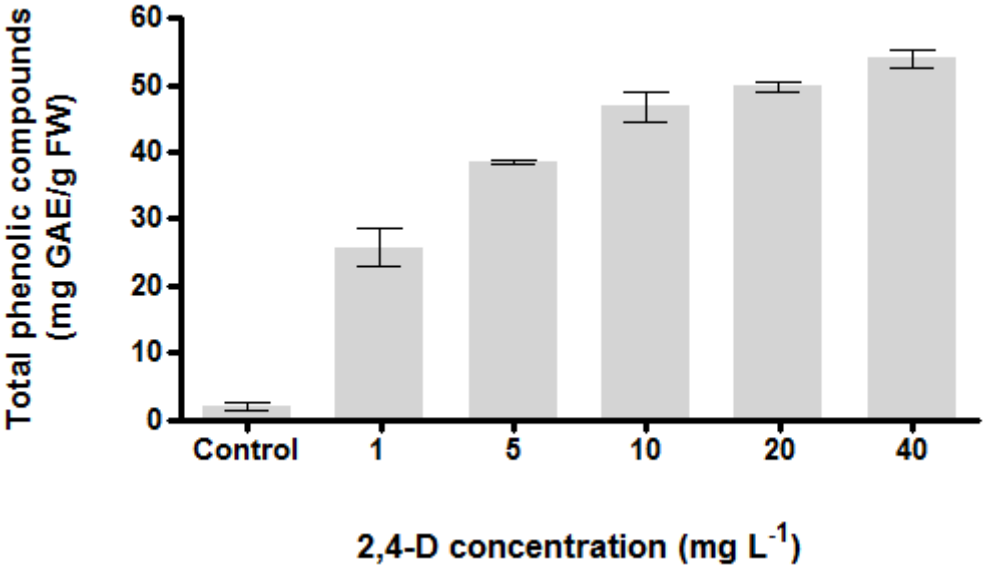

Figure 2

Total phenol content (mg GAE/g) of in vitro cultures of date palm cv. Barhee treated with different concentration of 2,4-D

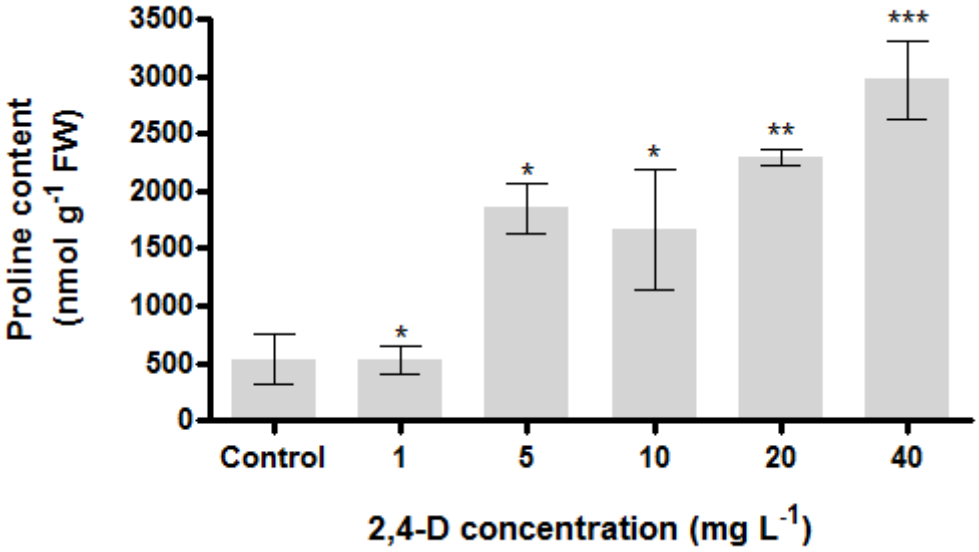

Figure 3

Proline content in in vitro cultures after 2-month cultivation on 2,4-D. *, **, *** indicates significant according to Tukey's test at $p < 0.05$, $p<0.01$ and $p < 0.001$, respectively.

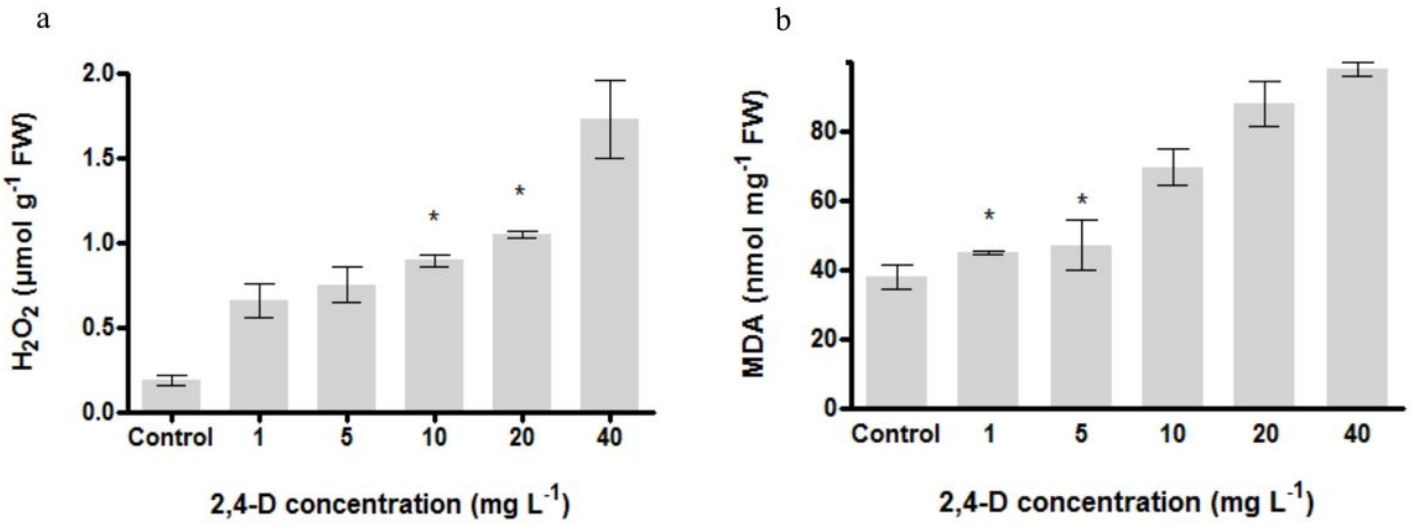

Figure 4

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 level of significant mean difference from control (Tuckey's test)

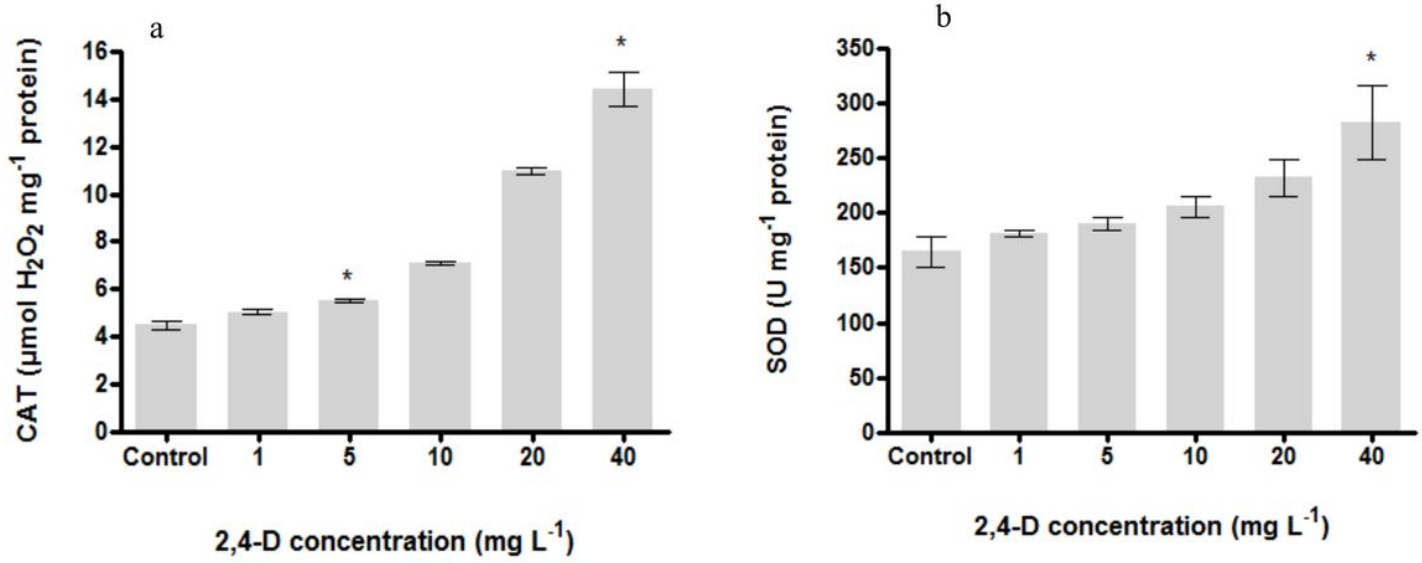

Figure 5

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)

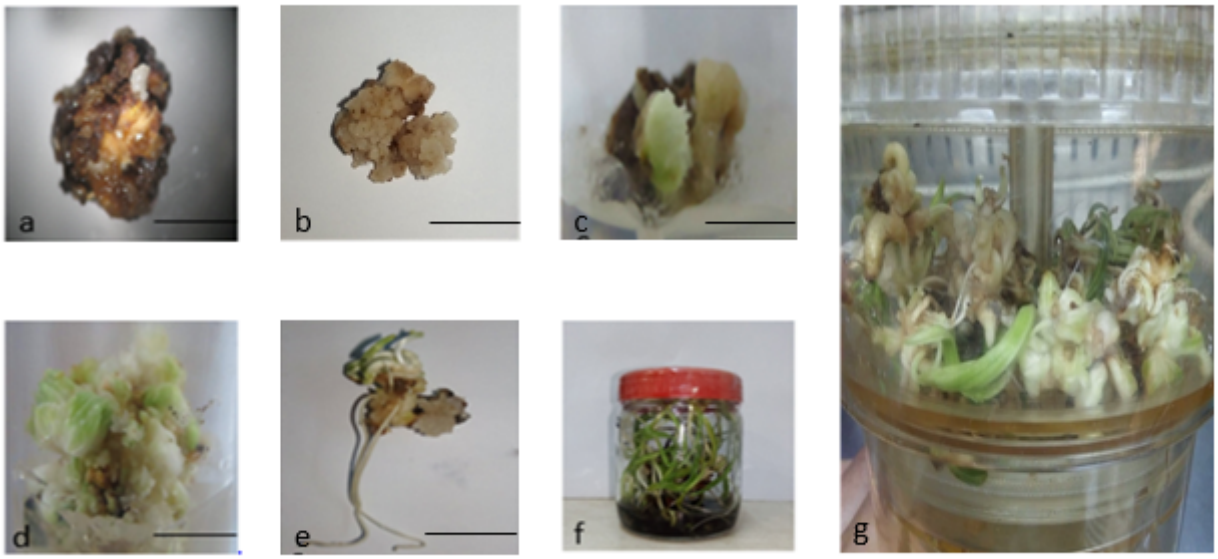

Figure 6

Morphology of in vitro cultures after transfer onto PGR-free media. a: neoformation of calli; b: calli differentiation; c: greenish structure; d: shoot multiplication; e and f: shoot elongation and subsequent rooting; g: in vitro shoot development in date palm cultures after transfer into the RITA system

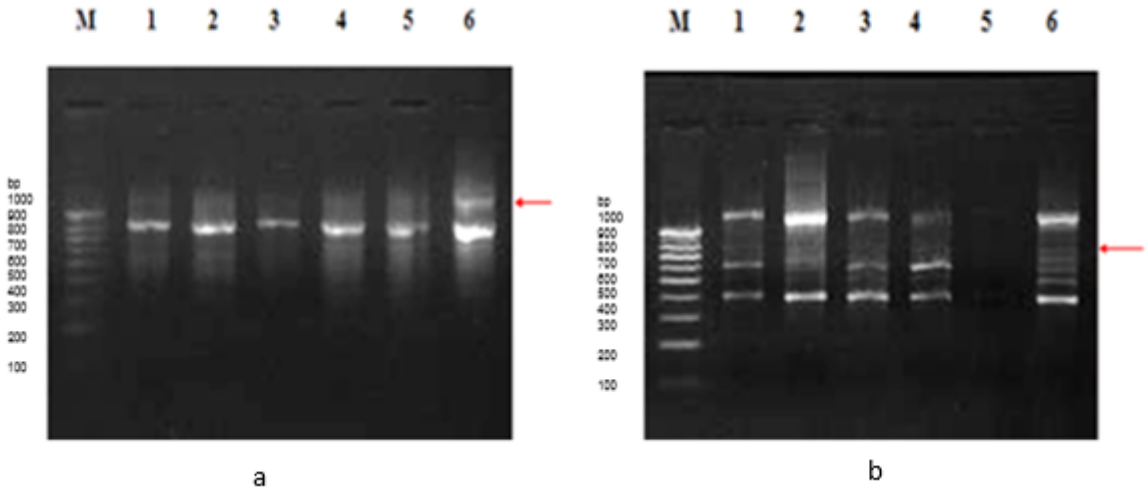

Figure 7

ISSR amplification patterns generated by primers ISSR3 (a) and ISSR4 (b) with Barhee cultivar. Lane M: molecular markers (from 100-bp to 1000-bp). Lane 1: control. Lane 2 to lane 6: 1, 5, 10, 20 and 40 mg L-1 2,4-D respectively.

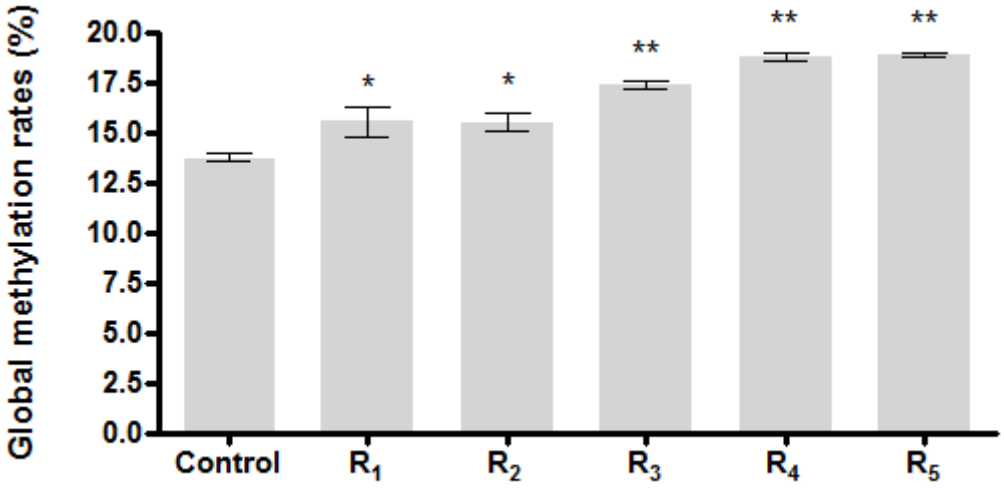

Figure 8

Global methylation rates in genomic DNA from different regenerants. Each value is the mean of three independent measurements. Control: 0 mg L-1 2,4-D; R1: 1 mg L-1; R2: 5 mg L-1; R3: 10 mg L-1; R4: 20 mg L-1; R5: 40 mg L-1. *, **indicates significant according to Tukey test at $p< 0.05$ and $p< 0.001$, respectively.