Effect of gamma rays and colchicine on silymarin production in cell suspension cultures of *Silybum marianum*: a transcriptomic study of key genes involved in the biosynthetic pathway

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Abstract

The aim of this study was to investigate secondary metabolite production in *Silybum marianum* L. cell suspension cultures obtained from seeds treated with gamma rays (200 and 600Gy) and 0.05% colchicine. The effects of these treatments on callus induction, growth, viability, and silymarin production were studied, along with the changes in the transcriptome and DNA sequence of chalcone synthase (*CHS*) genes. The effect of gamma radiation (200 and 600 Gy) on silymarin production in *S. marianum* dry seeds was also studied using HPLC-UV. All three treatments induced high callus biomass production from leaf segments. The viability of the cell suspension cultures was over 90%. The flavonolignan content measured in the extracellular culture medium of the *S. marianum* cell suspension was highest after treatment with 600 Gy, followed by 0.05% colchicine, and finally, 200 Gy, after a growth period of 12 days. In general, an increased expression of *CHS1*, *CHS2*, and *CHS3* genes, accompanied by an increase of silymarin content, was observed in response to all the studied treatments, although the effect was greatest on *CHS2* expression. Bioinformatics analysis confirmed that the three *CHS2* clones exhibited the highest genetic variation, both in relation to each other and to the *CHS1* and *CHS3* clones. Based on the results, *S. marianum* plants obtained from seeds previously exposed to 600 and 200Gy as well as colchicine constitute a renewable resource with the potential to obtain large amounts of silymarin.

Keywords: *Silybum marianum*; gamma rays; Colchicine; Cell suspension; HPLC; Flavonolignans; *Chalcone synthase* (*CHSs*) genes, Bioinformatics.

Introduction

Silymarin, a secondary metabolite produced in *S. marianum* L. plants, has gained the attention of the scientific community as a safe medicinal compound (KÖksal et al., 2009; Kandemir et al., 2017). Silymarin is composed of an isomeric mixture of the flavonolignans silybin A, silybin B, isosilybin A, isosilybin B, silydianin, silychristin, isosilychristin, and the flavonoid taxifolin (Lee and Liu, 2003; Lv et al., 2017a; Lv et al., 2017b). It has several pharmacological applications due to anti-fibrotic (El-Lakkany et al., 2012), anticancer (Cheung et al., 2010), antiviral, antibacterial, anti-inflammatory, and antiallergic properties (KÖksal et al., 2009; Kandemir et al., 2017). Furthermore, silymarin is clinically used for its hepatoprotective, cardioprotective, neuroprotective, UV-protective, and hypo-cholesterolemic effects (Roubalová et al., 2017).

Silymarin is released to the extracellular medium of *S. marianum* cultures, and its production can be stimulated by different elicitors (Madrid and Corchete, 2010; Hidalgo et al., 2017). Elicitation is one of the

most effective techniques currently in use for improving the biotechnological production of metabolites via *in vitro* plant cell cultures (Singh and Dwivedi, 2018). Elicitors are biotic or abiotic molecules that stimulate a group of defense or stress responses and activate specific genes encoding the enzymes involved in the biosynthesis of secondary metabolites (El-Garhy et al., 2016; Giri and Zaheer, 2016). This has opened up a novel area of research that could have important economic benefits for the pharmaceutical industry. The effect of elicitors on the production of secondary metabolites via *in vitro* culture depends on several factors, such as elicitor dosage and duration of exposure, among others (Singh and Dwivedi, 2018). The traditional cultivation of *S. marianum* L. plants has several limitations that cause a reduction in the total yield of silymarin. It is difficult to manually manipulate *S. marianum* L. plants, particularly during harvesting, due to the spiny margins of leaves and flowers (Alikaridis et al., 2000; Rady et al., 2018). Therefore, plant cell tissue and organ cultures are used as a promising alternative approach for the large-scale production of silymarin, the yields depending on the components of the culture medium (Akula et al., 2011). Furthermore, these cultures are also a source of flavonolignans, although the yields of these compounds without elicitation are poor (Hasanloo et al., 2008).

Chalcone synthase (CHS, EC 2.3.1.74) is an allosteric enzyme that catalyzes the formation of naringenin chalcone, thus playing a key role in the biosynthesis of flavonolignans (Sanjari et al., 2015). Several genes encoding CHS have been cloned, sequenced, and characterized in *S. marianum* L. and many other plant species (Pitakdantham et al., 2010). CHS provides the starting material for a diverse set of metabolites such as flavonoids, constituents of the flavonolignans (Feng et al., 2015). Therefore, the identification of CHS-encoding genes in *S. marianum* can be of great importance (Sanjari et al., 2015).

Genes encoding CHS are elicitor-responsive (El-Garhy et al., 2016). In addition, *SmCHS1*, *SmCHS2*, and *SmCHS3* genes from *S. marianum* L. are involved in the silymarin biosynthetic pathway (Sanjari et al., 2015). Torres and Corchete (Torres and Corchete, 2016) observed a relationship between the expression of silymarin pathway genes (*SmCHS*) and the production of these metabolites in *S. marianum*. Molecular pathways involved in radiation-induced stress response were first discovered using traditional biochemical approaches that monitored the activation of a single gene. Different physiological mechanisms are activated in living cells after exposure to radiation through gene modifications, which can be determined by qRT-PCR (Roy et al., 2009). Gamma radiation, as a physical mutagen, plays an effective role in improving silymarin yield and constitutes a promising tool to enhance genetic variation for the selection of stable mutated genotypes with a high content of silymarin

(Soliman et al., 2018). The use of different elicitors and determining their effects on the expression and regulation of biosynthetic pathways is an important step toward the commercial production of silymarin. We used cell suspension cultures to investigate the enhancing effect of elicitation on secondary metabolite production. Specifically, the aims of this work were:

1) To study the effect of gamma radiation (200 and 600 Gy) on the silymarin content in *S. marianum* dry seeds using HPLC-UV.
2) To establish cell suspension cultures obtained from *S. marianum* L. plantlets germinated from the seeds treated with gamma radiation (200 and 600 Gy) as well as 0.05% colchicine and determine the silymarin content in the cell suspension culture medium.
3) To evaluate the transcript levels of *CHS1*, *CHS2*, and *CHS3* genes in response to gamma radiation (200 and 600 Gy) and 0.05% colchicine treatments using quantitative real-time PCR (qRT-PCR).
4) To characterize and analyze the genetic fidelity of the sequence of *CHS1*, *CHS2*, and *CHS3* genes in the cell suspension cultures obtained from the treated seeds of *S. marianum* L. using different bioinformatics tools.

Materials and Methods

Plant material and seed germination

Seeds of *Silybum marianum* L. (annual variety) were obtained from the Genetics and Genetic Engineering Dept., Faculty of Agriculture, Benha University, Egypt. Dry seeds were distributed into four groups: the first group was the control (non-treated), the second and third groups were elicited by exposure to 200 and 600 Gy gamma-irradiation doses, respectively, while the fourth group was treated by soaking in 0.5 mg/mL colchicine for 30 min. Exposure to Gamma rays was carried out at the Egyptian Atomic Energy Authority using Mega Gamma-1 type J 6600 cobalt-60 irradiation at the Cyclotron Dept., Nuclear Research Center. For germination, seeds of different groups were surface sterilized by washing with tap water for 30 min, then soaking in hydrogen peroxide (H_2O_2) for 20 min, followed by washing three times with sterile distilled water under a laminar air-flow hood. Sterilized seeds were cultured in jars (40 ml capacity) containing 10 ml Murashige & Skoog (MS) medium, 2.2 g/L (Murashige and Skoog, 1962), containing half-strength basic salts and vitamins. This medium was supplemented with 3 % (w/v) sucrose, solidified with 3 gL^{-1} phytagel and its pH was adjusted to 5.7 before autoclaving for 20 min at 121 °C and 1.2–1.3 $kgcm^{-2}$ pressure; the sterilized seeds were cultured with only one seed/jar. The cultures were incubated under complete darkness until seedling emergence, and then in a growth

chamber at 22 ±2ºC with a 16-h light/8-h dark photoperiod (illumination of about 40 $mmolm^{-2}s^{-1}$ provided by cool white fluorescent lamps) to complete their growth. These seedlings were used for callus induction**.**

Callus induction

Leaf segments (about 0.25 cm in length) of all treatments (200, 600 Gy and 0.05% colchicine) as well as the control were excised from 30-day-old seedlings of *S. marianum* and cultured on MS medium supplemented with 3 % (w/v) sucrose, 0.25 mgL^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 0.05 mgL^{-1} 6-benzylaminopurine (BAP), 50 mgL^{-1} asparagine, and 50 mgL^{-1} myo-inositol. The pH of the medium was adjusted to 5.7. The medium was solidified and autoclaved as for the seed germination. Also, the incubation conditions used for the cultures were the same as for seed germination. For further experiments, the callus cultures were sub-cultured monthly for mass production (Rady et al., 2014).

Establishment of cell suspension cultures

Cell suspension cultures were established using 1g (fresh weight) of 2-month-old friable calli in 25 mL liquid MS medium, with the same composition as the callus induction medium, in 100 mL Erlenmeyer flasks. The cultures were incubated at 25ºC in darkness with agitation at 120 rpm on an orbital shaker (model ISF–4-V, Adolf Kühner AG. Schweiz).

Growth

To establish the growth curve of a culture, we chose 5 points (days) that covered the entire curve (lag, growth and stationary phase). Thus, samples were taken and weighed at days 3, 6, 10, 12, and 15 to determine the growth (fresh weight). The dry weight was obtained after drying the samples in a hot air oven at 40 °C until a constant weight (Tůmová et al., 2006)**.** The growth index was calculated using the following formula:

Growth index = (final fresh weight – initial fresh weight) / initial fresh weight

Cell viability

The cell viability was determined in the cell suspension cultures as the percentage of living cells out of the total number of cells using fluorescein diacetate (FDA) and propidium iodide (PI), both at 0.01% (w/v) (Walker and Pollard, 1990).

Extraction and determination of flavonolignans

Extraction

Flavonolignans (silychristin, silydianin, silybin A and B, iso silybin A and B, and taxifolin) were extracted from 1 g ground dry seeds (control, 200 and 600 Gy) or 1 ml cell suspension medium (control, colchicine 0.05%, 200 and 600 Gy) using 80% methanol at 60 ºC for 4 h under vacuum. The extracts were filtered, dried at below

40 ºC and resuspended in 1 mL methanol (Wianowska and Wiśniewski, 2015). The cell suspension medium was separated from the biomass culture by filtration. For the flavonolignan extraction from the cell suspension medium, samples were taken at day 3 (from the control only) to detect and confirm the production of flavonolignans during the first 3 days, and at days 10 and 12, samples were taken from all the studied treatments plus the control, with three replicates each.

All the standards of flavonolignans (silychristin, silydianin, silybin A and B, iso silybin A and B, and taxifolin) were purchased from Sigma-Aldrich, U.S.A. HPLC-grade methanol and acetic acid were used for the chromatographic assay.

Chromatographic conditions

The determination of flavonolignans was performed using high-performance liquid chromatography-UV (HPLC-UV) on an HPLC system from Agilent Technologies, Santa Clara, California, USA. The column used was Spherisorb ODS–2 (5 µm) reversed-phase column (4.6 × 250 mm). The HPLC conditions were: temperature 35 °C; mobile phase, methanol:acidic water (34:66 v/v), the acidic water containing H_2O:acetic acid (5:55, v/v); flow rate: 1 $mLmin^{-1}$. Determination and quantification were done using a UV detector (280 nm) (Sánchez-Sampedro et al., 2005a).

Molecular analysis

Primer design

The primers used in this study (Table 1) were designed according to the sequence of the genes encoding CHS1 (JN182805.1), CHS2 (JN182806.1), CHS3 (JN182807.1), *18S rRNA*, (AJ831537.1), and dehydrogenase (*NADH*) (KC589999.1) from *S. marianum*. The primers were designed using the online PCR primer design tool Primer3plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/) (El-Garhy et al., 2016). The primers were tested using an *in-silico* PCR tool (http://insilico.ehu.eus/PCR/). In this study, *18S rRNA* and *NADH* genes were used as reference genes for normalization of the qRT-PCR data. Primers were ordered from Invitrogen™, Germany.

Table 1. Oligonucleotide name and sequence of qRT-PCR and sequencing primers.

Gene	Oligonucleotide name and sequence of qRT-PCR primers		Reference
Chalcone	CHS1F 5-TCTTGATTCCCTCGTTGGTC-3	101	**(El-Garhy et al., 2016)**
synthase1 (CHS1)	CHS1R 5- TCTCAAACAACGGCCTCTCT-3		**GenBank: JN182805.1**
Chalcone	CHS2F 5- AGGACATTGCGGAAAACAAC-3	184	**(El-Garhy et al., 2016)**
synthase2 (CHS2)	CHS2R 5- AACGGCCTCTCTGTCTTCAA-3		**GenBank: JN182806.1**

Chalcone synthase3 (CHS3)	CHS3F 5- ACCCACCTCATCTTTTGCAC-3 CHS3R 5- CATCATGAGGCGTTTGATTG-3	105	**(El-Garhy et al., 2016)** **GenBank: JN182807.1**
NADH (reference gene)	ndhchs_L 5-TTCCGCATTTTGGAAATACC-3 ndhchs_R 5-CCCGTCTTGATTGAAAGGAA-3	134	**(El-Garhy et al., 2016)** **GenBank: KC589999.1**
18S rRNA gene (reference gene)	18sRNA_L1 5-CCGTGAACCATCGAGTTTTT-3 18sRNA _R1 5- GAAGGCCTTAACAACCACCA-3	221	**(El-Garhy et al., 2016)** **GenBank: AJ831537.1**
Oligonucleotide name and sequence of sequencing primers			
CHS1	CHS1-F 5-TCTTGATTCCCTCGTTGGTC-3 qCHS2-R2 5-ACCGTCTCCACTGTCAAACC-3	622	**(El-Garhy et al., 2016)** **GenBank: JN182805.1**
CHS2	CHS2-F 5- AGGACATTGCGGAAAACAAC-3 qCHS2-R2 5-ACCGTCTCCACTGTCAAACC-3	622	**(El-Garhy et al., 2016)** **GenBank: JN182805.1, JN182806.1**
CHS3	CHS3-F 5- ACCCACCTCATCTTTTGCAC-3 CHS3_R1 5- GCCCTCAATTTTCCCTTCTC-3	605	**(El-Garhy et al., 2016)** **GenBank: JN182807.1**

Total RNA extraction and complementary deoxyribonucleic acid (cDNA) synthesis

Total RNA was extracted from *S. marianum* cell suspension samples for all the studied treatments as well as the control. The samples were collected at day 12 of culture and kept at -80 ºC. Total RNA was extracted using the SV Total RNA Isolation System Kit (Cat. No. Z3100) according to the manufacturer's protocol. The residual genomic DNA was eliminated by treating RNA with gDNA Wipeout Buffer, which was included in the QuantiTect® Reverse Transcription Kit, according to the manufacturer's instructions. Reverse transcription (RT) of the treated RNA was carried out using the QuantiTect® Reverse Transcription Kit (Qiagen, Cat. No. 205311). The cDNA samples were stored at -20 °C until use.

Differential expression analysis by quantitative real-time PCR

The quantitative real-time PCR (qRT-PCR) for each analyzed sample as well as the non-template control (NTC) was performed in triplicate. Each PCR reaction mixture consisted of 500 ng of cDNA (except for NTC and cDNA control), 12.5 µL SYBR Green PCR MasterMix (Maxima SYBR Green qPCR, Thermo Fisher Scientific, Cat. No. k0251), 0.3 µM of each forward and reverse primers (Table 1), 10 nM/100 nM ROX Solution, and nuclease-free water to a final volume of 25 µL. The reactions were performed on an AriaMx Real-Time PCR System (Agilent Technologies) using a two-step cycling protocol, under the following conditions: 95 °C for 10 min and 40 cycles of 95 °C for 15 s followed by 60 °C for 60 s. The expression levels of *CHS1*, *CHS2*, and *CHS3* genes were normalized to that of the housekeeping NADH gene, which was more stable than the *18S rRNA* gene. All the experimentally induced changes in the expression of the studied genes were presented as n-fold changes relative to the corresponding controls. The relative gene expression ratios (RQ) between treated and control groups were calculated using the formula: $RQ=2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001).

DNA extraction and PCR amplification for sequencing

Total genomic DNA from cell suspensions of all the studied groups (control, 200 Gy, 600 Gy and 0.05% colchicine) was extracted using the QIAGEN DNeasy Plant DNA extraction Mini Kit (Cat. No. 69104) according to the manufacturer's instructions. The sizes of the expected PCR amplicons for the *CHS1*, *CHS2*, and *CHS3* genes were 622 bp, 622 bp, and 605 bp, respectively. The DNA fragments were amplified using the primers listed in Table 1 (El-Garhy et al., 2016). The PCR reaction was performed in a 50 µL mixture containing 0.4 µM of each primer with a concentration of 10 pmol, 400 µM of each dNTP mix, 5 µL of 10x PCR reaction buffer, 2 µM $MgCl_2$, 2.5 U of Taq DNA polymerase (TAKARA, Cat. No. R001AM), 1 µL of template DNA, and the final volume was adjusted using sterilized doubled distilled water (d.dH_2O). The PCR reactions were performed using a thermocycler (BioRad) under the following conditions: initial denaturation at 95 °C for 3 min, followed by 40 cycles at 95 °C for 50 sec, annealing at 58.9 °C for 1 min, and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. The amplified PCR products were purified and stored at –20 °C for further downstream applications. About 5 µL of each PCR-amplified product was loaded on a 1.2% agarose gel, stained with ethidium bromide, and electrophoresed. GeneRuler™ 1 kb DNA ladder (Cat. No.: SM0313) was used as the molecular weight reference. The gel was then visualized under UV Transilluminator (BioRad).

Cloning and Sequencing

Positive PCR amplicons were obtained using a DNA template extracted from control, and colchicine- and 200 Gy-treated cells. No amplicon was obtained using the DNA template extracted from 600 Gy cells, although this experiment was repeated many times to confirm the absence of handling error. Therefore, in a further study we will try to find the reason for this negative result and try to solve it via designing new primers with different target regions in the sequence of the studied genes isolated from the plantlets treated with 600 Gy. The resulting PCR amplicons of approximately 622 and 605 bp were eluted from the agarose gel and purified using QIAquick Gel Extraction Kit (Cat. No. 28704). The purified DNA fragments were ligated into pGEMR-T Easy Vector Systems (Cat. No. A1360) according to the manufacturer's instructions. The competent cells of *E. coli* top 10 strains were prepared and transformed as previously described (Inoue et al., 1990). From LB/Amp/Xgal plates, white-colored colonies were selected and inoculated on LB/Amp broth media. They were then incubated overnight at 33 °C with shaking to stabilize the plasmid inside the transformed cells. The alkaline method of Bimboim and Doly (1979) was used to isolate the plasmid. To confirm the recombinant plasmids, the purified plasmids were examined by electrophoresis on 1.5% agarose gel using a GeneRuler™ 1 kb DNA Ladder as the molecular size marker (Cat. No. SM0313). The sequencing of the obtained recombinant plasmids was carried out by the

Macrogen Company (South Korea). The obtained sequences of *CHS1*, *CHS2*, and *CHS3* genes from all treatments were examined for vector contamination using the VecScreen tool (http://www.ncbi.nlm.nih.gov/tools/vecscreen). Single nucleotide polymorphisms (SNPs) were viewed using the Jalview software (http://www.jalview.org) and a consensus was obtained from the alignment of our obtained sequences with the closest strains in NCBI database (Waterhouse et al., 2009). The phylogenetic tree was constructed using the Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) and MEGA7 software (Kumar et al., 2016). The obtained sequences were deposited in the NCBI database (http://www.ncbi.nlm.nih.gov) under accession numbers MG751175.1, MG751176.1 and MG751177.1 for *CHS1* clone1 (control samples), *CHS1* clone2 (control samples treated with 0.05% colchicine) and *CHS1* clone3 (samples from 200Gy), respectively; MG751178.1, MG751179.1, and MG751180.1 for *CHS2* clone1 (control), *CHS2* clone2 (200Gy) and *CHS2* clone3 (colchicine), respectively; and MG751181.1, MG751182.1, and MG751183.1 for *CHS3* clone1 (control), *CHS3* clone2 (colchicine) and *CHS3* clone3 (200Gy), respectively.

Multi-sequence alignment (MSA) and phylogenetic analysis

The sequence-alignment tool DELTA-BLAST was used to identify similarities in sequences with homologous DNA and proteins from other organisms, on the obtained *CHS1*, 2, and 3 DNA, and the deduced amino acid sequences of *S. marianum*. The software Clustal Omega (http://www.ebi.ac.uk/tools/msa/clustalo/) was used to generate multiple sequence alignment for the obtained DNA sequence. The maximum likelihood method was used to build the phylogenetic tree (Saitou and Nei, 1987). A bootstrap value of the compared algorithms was attached to each branch to indicate the confidence level.

Statistical analysis

All data were presented as the mean ±Standard Error (SE). The data were evaluated by one-way ANOVA using the statistical software SPSS (ver.16). The means were compared through the least significant difference (LSD). The differences were considered statistically significant at $p < 0.05$. Percentage changes from the control were calculated according to the formula [(Treatment–Control)/ (Control)] × 100.

Results and discussion

Due to the morphological features of *S. marianum* plants, their manual manipulation in traditional agriculture during different stages of growth is difficult, which causes a reduction of the total yield of silymarin (Alikaridis et al., 2000; Rady et al., 2018). Therefore, in this study, we investigated the enhancing effect of elicitation on

secondary metabolite production in cell suspension cultures as a promising alternative approach to cultivation in the field.

Seed germination

Seed germination is one of the most critical periods for plant development and productivity, as seeds are sensitive to different types of abiotic stress. Seeds of *S. marianum* were cultured on half-strength MS medium supplemented with 3% (w/v) sucrose and solidified with 3 g/L phytagel. The use of half-strength MS medium for seed germination gave comparable results with those of Elsharnouby and Hassan (2018), who reported that it was better than the full-strength medium, due to the osmotic pressure of the latter. Also, the germination process was enhanced by incubating the seeds in complete darkness until seedling formation. These results are corroborated by Elsharnouby and Hassan (2018), who stated that light was unnecessary in the germination period of *S. marianum* seeds due to its inhibitory effect **(**Khan et al., 2013**)**.

Effect of gamma radiation and colchicine elicitation on callus induction and growth.

Leaf segments from seedlings obtained from 200 Gy-, 600 Gy- and 0.05% colchicine-treated and control seeds were used for callus induction. In general, all the treatments resulted in a high biomass production, and the calli appeared after 30 days of culture (with two subcultures) on MS medium solidified with 3 g/L phytagel and supplemented with 0.25 mg/L 2,4-D + 0.25 mg/L BAP (incubated in darkness). Moreover, the obtained calli were friable, which facilitated the establishment of cell suspension cultures (**Fig. 1A)**.

These results agree with those of Rady et al. (2014), who initiated callus formation from leaf explants of different *S. marianum* genotypes and reported that MS medium supplemented with 0.25 mg L^{-1} 2,4-D + 0.25 mg L^{-1} Kinetin (Kin) was the ideal medium for the induction and conservation of friable calli. Meanwhile, Eari et al. (2017) found that 1 mg/L or 1.5 mg/L 2,4-D and Kin were the most effective concentrations for callus induction from root explants of *S. marianum*. Furthermore, the culture of different explants of *S. marianum* seedlings on MS medium supplemented with 1 mg/L 2,4-D, in combination with 0.5 mg/L BA, produced large undifferentiated friable calli (Elsharnouby and Hassan, 2018). Supplementing MS medium with 2,4-D + Kin caused a significant increase in the callus induction rate. Auxin is known to facilitate cell elongation, cell division, and callus formation in the medium culture, while cytokinin promotes cell division and differentiation (Elhaak et al., 2016).

In previous studies, callus tissues have been produced from cotyledons (Elhaak et al., 2016), leaf explants **(**Rady et al., 2014**;** Rady et al., 2018**)**, hypocotyl segments (Elsharnouby and Hassan, 2018), and a

combination of hypocotyl and root segments (Riasat et al., 2015). MS medium appeared to promote cell growth faster than other media. It is generally supplemented with 30 g/L sucrose and/or 100 mg/L myo-inositol, vitamins, different plant growth regulators, auxins (2,4-D, IBA, or IAA), and cytokinins (Kin, BA, or Zeatin) for the induction of callus cultures from different explants of *S. marianum* (Rady et al., 2018). 2,4-D, at levels ranging from 0.25 to 4.0 mg/L, was the most effective auxin for callus induction, used alone (John and Koperuncholan, 2012) or in combination with BA (Elsharnouby and Hassan, 2018). Moreover, BA at levels ranging from 0.05 to 5 mg/L was the most effective cytokinin used alone (Abbasi et al., 2010), or in combination with 2,4-D (Elsharnouby and Hassan, 2018) or NAA**.**

Cell Viability

Cell viability was monitored using a staining technique with fluorescein di-acetate. Viable cells appeared as fluorescein green while dead cells appeared as fluorescein red (Figs. 1B and 1C, respectively). Cell viability was over 90% in the cell suspension cultures. The results showed that control, 200 Gy-, 600 Gy-, and 0.05% colchicine-treated cells exhibited a normal shape (Figs. 1B and 1C**)**, whereas treatment with 600 Gy also produced abnormal sickle-shaped cells (Fig. 1D**)**.

Figure 1. Callus induction and viability of cell suspensions in the different treatments. (A) Callus tissues from *Silybum marianum* explants grown on MS medium supplemented with 0.25 mg/l 2,4-D + 0.25 mg/l BAP; (B and C) viability of the cell suspensions of 200 and 600 Gy as well as 0.05% colchicine treatments; (D) abnormal cell shape of cell suspensions treated with 600Gy.

Effect of gamma radiation and colchicine elicitation on fresh and dry weights as well as the growth rate of *S. marianum* cell suspension cultures

Cell suspension cultures were established by transferring 1 g (fresh weight) of callus from 2-month-old friable calli originated from control, 200 Gy-, 600 Gy-, and 0.05% colchicine-treated seedlings to the same MS liquid medium used for callus induction, without phytagel. The cell suspensions were incubated in growth chambers at 25 °C with agitation at 120 rpm in darkness for 15 days, and the growth was measured every three days. Figs. 2A and 2B illustrate the growth curve as fresh (FW) and dry weights (DW), respectively, of *S. marianum* cell suspension cultures after 3, 6, 10, and 12 days of culture in response to the studied treatments. The FW and DW increased most with the highest radiation dose, followed by 0.05% colchicine. The highest FW and DW were recorded at day 12 in all the treatments (Fig. 2A, 2B). These findings indicated that gamma rays and colchicine

had no inhibitory effect on the growth of *S. marianum* callus cultures. All treatments increased the growth index compared to the control; the highest (3.7 g) was obtained in cultures elicited with 600 Gy for 12 days, followed by 200 Gy (3.2 g) and 0.05% colchicine (2.8 g) (Fig. 2C). The overall data revealed that calli originating from 600 Gy explants possessed the highest FW, DW, and growth index, after 12 days of culture. Improvement in the growth index in response to radiation and colchicine elicitation is logical due to the enhancement of callus FW. Similar results have been reported in a previous study, which observed an improvement in the *S. marianum* callus growth index due to an increase in FW as a result of using $CuSO_4$ elicitation in the culture media (Elsharnouby and Hassan, 2018).

Figure 2. Effect of gamma radiation and colchicine treatments on *S. marianum* cell suspension cultures. (A) Fresh weight (B) dry weight and (C) growth index, after 12 days of culture, of *S. marianum* cell suspension cultures in response to radiation (200 and 600 Gy) and colchicine (0.05%) treatments.

Flavonolignan profile in response to gamma radiation and colchicine treatments in *S. marianum* dry seeds.

Silymarin flavonolignans in *S. marianum* dry seed powder were quantified using the HPLC-UV method. *S. marianum* seeds were treated with 200 Gy and 600 Gy. The flavonolignan profile obtained (Table 2) confirmed that treatement with 600 Gy resulted in a significantly higher content of silymarin compared to 200 Gy and the control.

The quantitative profile of silymarin was compared with standards of taxifolin, silychristin, silydianin, silybin A, silybin B, and isosylibin A+B silymarin. Six principal peaks were observed, with each peak identified as one of the flavonolignan constituents of the silymarin samples. Quantitative results (Table 2) showed that total silymarin levels increased significantly ($p < 0.05$) in 600 Gy dry seeds (47% more than the control), and increased slightly in 200 Gy dry seeds (7% more than control).

S. marianum and other plants accumulate flavonoids predominantly in their seeds or the skin of their fruit (Lv et al., 2017b). The only reasonable explanation for the accumulation of silybin in the seed is that taxifolin is synthesized in the flower and transported to the seed coat, where it couples with equimolar amounts of radicalized coniferyl alcohol, resulting in the synthesis and accumulation of silybin (Lv et al., 2017a; Lv et al., 2017b). Also, gamma radiation might be a potential tool to obtain promising mutated genotypes of *S. marianum*, with enhanced expression of the genes involved in the silymarin biosynthetic pathway (El-Garhy et al., 2016).

The results of the current study are in accordance with those of El-Garhy et al. (2016), who used LC-MS/MS analysis for the quantification and separation of silybin A+B in *S. marianum* dry fruit extracts from plants exposed to six doses of gamma radiation (100, 200, 400, 600, 800, and 1000 Gy). Their findings indicated that the plants irradiated with 200 Gy had the highest silybin content; the silybin A+B content was higher when the irradiation dose was increased from 100 to 600 Gy but decreased at higher doses (800 and 1000 Gy). Abdel Halim (2011) reported that the use of different doses of gamma radiation caused a significant increase in the silymarin content of *S. marianum* seeds. It has also been demonstrated that different doses of gamma radiation, as an abiotic elicitor, change the active components of *S. marianum* responsible for its medicinal properties (El Sherif et al., 2013). Finally, increasing the genetic variation of *S. marianum* was found to be an effective approach for obtaining genotypes with high silymarin yields.

Table 2. Silymarin production (mg/g DW) in *Silybum marianum* dry seeds, control and treated with gamma rays (200 and 600 Gy).

Groups	Differential silymarin profile mg/g dried powder						
	Taxifolin	Silychristin	Silydianin	Silybin A	Silybin B	Iso Sb A+B	total Silymarin
Control	2.87 ± 0.08^{b}	2.5 ± 0.07^{b}	1 ± 0.03^{ab}	1.7 ± 0.05^{b}	2.56 ± 0.07^{b}	0.84 ± 0.02^{b}	12.25 ± 0.32^{b}
200 Gy	3.27 ± 0.09^{b}	2.65 ± 0.07^{b}	0.95 ± 0.03^{b}	1.9 ± 0.05^{ab}	2.74 ± 0.08^{ab}	0.84 ± 0.02^{b}	13.11 ± 0.36^{b}
600 Gy	5.14 ± 0.15^{a}	3.31 ± 0.09^{a}	1.3 ± 0.04^{a}	2.39 ± 0.06^{a}	3.61 ± 0.1^{a}	1.15 ± 0.03^{a}	18.01 ± 0.5^{a}

Within each column, means followed by same letter/s indicate no significant difference among treatments at P<0.05.

Extracellular production of flavonolignans in cell suspension cultures of *S. marianum* from seedlings treated with 200 Gy, 600 Gy or 0.05% colchicine.

As previously reported, *S. marianum* cultures accumulate silymarin in the extracellular medium upon elicitation with biotic and abiotic elicitors (Torres and Corchete, 2016). In the current study, HPLC-UV was used to quantify silymarin components in the control medium at day 3 of growth as well as at days 10 and 12 of growth in the medium of cell suspension cultures of calli derived from *S. marianum* seedlings germinated from control, 200 Gy-, 600 Gy-, or 0.05% colchicine-treated seeds. The chromatographic separation of different flavonolignans resulted in the identification of silydianin, silychristin, isosilybin B, silybin B, and silybin A, obtained in high purity comparable to the standards, and giving a standard curve at different concentrations. Chromatographic separation yielded a high resolution with optimum retention time separating each peak from the others, high linearity, and accuracy, with r^2 ranging from 0.9997 to 0.9999.

In the control cell suspension samples at day 3 the presence of flavonolignans was detected in the extracellular medium in correspondence with the culture growth rate. Elicitation with gamma radiation at 600 Gy and 0.05%

colchicine significantly enhanced the extracellular accumulation of silydianin, silychristin, isosilybin B, silybin A, and silybin B after either 10 or 12 days of incubation compared to the control, with 200 Gy observed to have a lower effect (Table 3). The highest production of flavonolignans was observed in cell suspensions treated with 600 Gy, followed by 0.05% colchicine and 200 Gy, after 12 days of culture. The data showed that the percentage of silybin B in the flavonolignan profile was highest at both 10 and 12 days of culture.

Silymarin is a mixture of flavonolignans extracted from *S. marianum*, the major components being silybin, silydianin, silychristin, and taxifolin (Singh and Dwivedi, 2018). Silymarin is synthesized through 4-coumaroyl-CoA, which enters the flavonoid as well as the monolignol pathway, resulting in two immediate precursors, taxifolin (Tx) and coniferyl alcohol (CA). The formation of silymarin occurs via the oxidative radicalization of Tx and CA and it accumulates at high levels during the final stages of maturation of *S. marianum* fruits. In contrast, the production of silymarin was significantly reduced in *S. marianum* cell cultures, although the suspensions were able to excrete silymarin compounds into the medium upon treatment with different elicitors (Torres and Corchete, 2016).

The elicitors methyl B cyclodextrin and methyl jasmonate (MeJA) increased the levels of bioactive compounds of *S. marianum* in cell suspension cultures (Corchete and Bru, 2013). In addition, chitosan, MeJA, and salicylic acid (SA), used as elicitors of metabolite production, enhanced the accumulation of silybin A+B in the callus cultures of *S. marianum* (Gabr et al., 2016). Higher antioxidant activity was observed in the callus cultures of *S. marianum* compared to regenerated plantlets or wild-grown plants (Abbasi et al., 2010). Also, HPLC analysis of total silymarin revealed that treatment with chitosan slightly increased the silymarin content (5.60 µg/g DW) in *S. marianum* callus culture (Gabr et al., 2016). A remarkable improvement in silymarin accumulation in the *in vitro* cultures of *S. marianum* due to biotic and abiotic elicitors has been documented in previous studies (Elsharnouby and Hassan, 2018).

Table 3. Flavonolignan profile in the medium of *Silybum marianum* cell suspensions derived from control explants and those treated with gamma rays (200 and 600 Gy) and 0.05% colchicine.

		Differential silymarin profile/Sample concentration µg/ml				
Cultivation period	**Groups**	**Silydianin**	**Silychristin**	**Iso Sb B**	**Silybin A**	**Silybin B**
Day 3	Control	13.4± 0.35	25.5 ± 0.71	11.3 ± 0.32	23.0 ± 0.63	29.6 ± 0.82
Day 10	Control	15.3± 0.41^{c}	31.2 ± 0.84^{c}	14.6± 0.39^{b}	29.7± 0.82^{c}	39.0± 1.03^{d}

	200 Gy	17.7± 0.47^{c}	35.6± 0.97^{bc}	16.6± 0.45^{b}	36.7± 0.96^{b}	46.0± 1.25^{c}
	600 Gy	31.8± 0.85^{a}	63.8± 1.81^{a}	29.8± 0.80^{a}	65.9± 1.80^{a}	79.7± 2.20^{a}
	Colchicine 0.05%	22.7± 0.59^{b}	40.2± 1.08^{b}	18.1± 0.47^{b}	39.2± 1.07^{b}	55.4± 1.46^{b}
Day 12	Control	15.6± 0.44^{c}	31.4± 0.85^{d}	15.5± 0.43^{c}	32.2± 0.84^{c}	44.4± 1.23^{c}
	200 Gy	19.9± 0.55^{c}	40.1± 1.13^{c}	18.6± 0.51^{bc}	41.3± 1.16^{b}	49.9± 1.34^{c}
	600 Gy	36.3± 0.96^{a}	72.8± 1.99^{a}	34.0± 0.89^{a}	75.2± 2.00^{a}	90.9± 2.43^{a}
	Colchicine 0.05%	26.1± 0.70^{b}	49.0± 1.36^{b}	20.7± 0.56^{b}	39.4± 1.04^{b}	59.5± 1.65^{b}

Within each column, means followed by same letter/s indicate no significant difference among treatments at P<0.05.

Differential expression profiling of *CHS1*, *2*, and *3* genes in response to different abiotic elicitors

Knowledge of gene expression is important to understand the silymarin biosynthetic pathway and develop approaches to increase production in cell cultures (Torres and Corchete, 2016). Therefore, to explore the relationship between the expression of silymarin pathway genes and the metabolite production, the expression levels of three genes (*CHS1, CHS2*, and *CHS3*) involved in flavonolignan biosynthesis were quantified in the elicited cell suspensions using qRT-PCR. Fig. 3 presents the expression profiles of the three studied genes in *S. marianum* cell suspension cultures obtained from explants of seedlings treated with gamma rays (200 and 600 Gy) or 0.05% colchicine.

In general, the expression of *CHS1*, *CHS2*, and *CHS3* genes was upregulated in response to all the studied treatments, which corresponded with an increase in silymarin content. However, the results showed that *CHS2* was elicited the most (40.8, 10.4, and 5.7-fold increase with 600 Gy, 200 Gy, and 0.05% colchicine treatments, respectively). The mRNA transcripts of *CHS1*, *CHS2*, and *CHS3* were the most abundant (13.7, 40.8, 7.1-fold increase, respectively) under the effect of 600 Gy. This enhancement of the expression of *CHS* genes under the effect of 600 Gy was accompanied by an increase in levels of silymarin metabolites in both dry seeds and cell suspension cultures. Studies of the molecular pathways affected by different elicitors have revealed that elicitation is an effective method to activate multiple signaling pathways of intracellular defense mechanisms and regulate the expression of related genes, which could explain the accumulation of important metabolites such as silymarin in the *S. marianum* plant (Lv et al., 2017b). It has been suggested that after plant cells are treated with radiation, MeJA, or cyclodextrins (CD), different mechanisms are stimulated through the enhancement of gene expression, measured by qRT-PCR (El-Garhy et al., 2016). Therefore, gamma radiation could be a promising tool to trigger desirable and beneficial genetic variations for the generation of genetically modified genotypes with a high content of silymarin. Moreover, several studies report how irradiation can alter the expression of genes involved in the

394 silymarin biosynthetic pathway. The results of the current work agree with the findings of El-Garhy et al. (2016),
395 who observed that exposure of *S. marianum* seeds to six doses of gamma radiation (100, 200, 400, 600, 800, and
396 1000 Gy) increased the expression of the *CHS1, CHS2*, and *CHS3* genes in the plant leaves. The stimulation of
397 the expression of silymarin pathway genes is positively correlated with an increase in the levels of this metabolite
398 (El-Garhy et al., 2016). In addition, the use of colchicine can lead to chromosomal duplication and thus enhance
399 the content of silymarin. Using qRT-PCR, Sanjari et al. (2015) showed that *SmCHS1*, *SmCHS2*, and *SmCHS3*
400 genes from *S. marianum* were involved in the silymarin biosynthetic pathway. Our results also agree with those
401 of Sánchez-Sampedro et al. (2005b), who observed that MeJA enhanced *CHS* activity and increased silymarin
402 levels in cell cultures of *S. marianum*. In addition, Torres and Corchete (2016) observed a relationship between
403 the expression of silymarin pathway genes (*SmCHS*) and the metabolite production in *S. marianum*. Their results
404 indicate that the observed increase in silymarin accumulation may reflect the differential expression of the studied
405 genes in response to different elicitors. The *S. marianum APX1* (ascorbate peroxidase 1) gene was reported to play
406 a role in the biosynthesis of secondary metabolites in plants, as the concentration profiles of silybin and its
407 precursors, as well as RNA-Seq analysis of the expression of this gene, revealed that the amount of taxifolin and
408 the activity of peroxidase served as limiting factors in silybin biosynthesis (Lv et al., 2017b).
409
410 **Figure 3. Differential expression profiling of chalcone synthase genes, *CHS1, CHS2,* and *CHS3*, in cell**
411 **suspension cultures of *Silybum marianum* from 200 Gy-, 600 Gy-, and colchicine (0.05%)-treated**
412 **explants at day 12 of the culture. Data were normalized using *NADH* as an internal reference gene.**
413 **Sequencing and bioinformatics analysis of *CHS1, CHS2, and CHS3* genes**
414 To confirm the specificity of the primers designed for the three *CHS* genes and the results of qRT-PCR, the
415 genes were amplified using *S. marianum* DNA as a template for PCR reactions. The PCR products of *CHS1,*
416 *CHS2* (622 bp), and *CHS3* (605 bp) genes were partially sequenced and registered in the NCBI database under
417 the accession numbers MG751175.1, MG751178.1, and MG751181.1, respectively.
418 The obtained DNA sequences were aligned with other sequences of *S. marianum* and other plant species
419 available in the NCBI database using the BLAST alignment algorithm (http://www.ncbi.nlm.nih.gov/BLAST).
420 BLAST results indicated that *CHS1* (MG751175.1), *CHS2* (MG751178.1), and *CHS3* (MG751181.1), from the
421 current study, were similar to *CHS1* (JN182805.1), *CHS2* (JN182806.1), and *CHS3* (JN182807.1) in the NCBI
422 database for *S. marianum*, with an identity ratio of 99%.

The effect of gamma rays (200Gy) and colchicine (0.05%) on the genetic fidelity of *S. marianum CHS1*, *CHS2*, and *CHS3* genes

In vitro culture and genetic fidelity as biotechnological tools can be effective for the production of secondary metabolites from *S. marianum* cultures (Rady et al., 2018). Determination of genetic diversity within and between the populations of medicinal plants is highly important for the improvement of these plants (Rady et al., 2018).

In this part of the study, the genetic fidelity and variation of *CHS1*, *CHS2*, and *CHS3* genes within *S. marianum* genomic DNA isolated from the cell suspensions derived from explants from 200 Gy-, 600 Gy- and 0.05% colchicine-treated seedlings were investigated. The PCR products of these three genes and their clones are represented in Fig. 1S. The data show positive PCR amplicons for the three genes from control, and 200 Gy- and 0.05% colchicine-treated tissues. The bioinformatics analysis of the obtained sequences of *CHS* gene clones and their genetic fidelity under the effect of the studied abiotic elicitors (200 Gy and 0.05% colchicine) as well as the control are discussed in the following sections.

Bioinformatics analysis and genetic fidelity of CHS1 gene and its clones

The BLAST results indicated that *CHS1* clone1 (control), clone 2 (200 Gy), and clone 3 (0.05% colchicine) (MG751175.1, MG751176.1, MG751177.1, respectively) in the current study were similar to *S. marianum CHS1* (JN182805.1) in the NCBI database, with an identity ratio of 99% for all the clones. Pairwise alignment analysis of the partial sequences of *S. marianum* revealed the presence of two SNPs and one gap between *CHS1* clone 1 and JN182805.1 in the NCBI database (Fig 4A), while *CHS1* clone 2 and clone 3 exhibited no SNPs or gaps (Fig 4B and 4C, respectively).

Phylogenetic analysis showed that all the studied clones (clones 1, 2, and 3) for the *CHS1* gene were related to each other with variable distances. In addition, clones 2 and 3 were observed to be closer to the gene JN182805.1 than clone 1, which is in line with the previous results for the SNPs. It exhibited highly similar identity ratios on the roots of clades and reflected their similarity with the closest sequence in the NCBI database (JN182805.1) (99% identity ratio, Fig 4D). A comparison between the DNA sequences of *CHS1* clones showed that clone 1 and 3 were closer to each other than clone 2, which again confirmed the previous conclusion (Fig 4E).

Fig. 4. Bioinformatics analysis of *CHS1* gene clones revealed; pairwise alignment analysis and SNPs among the partial sequences of A: *Silybum marianum CHS1* clone1, control, (MG751175.1), B: *CHS1* clone2, 200Gy, (MG751176.1), C: *CHS1* clone3, colchicine 0.05%, (MG751177.1) and the nearest deposited

sequence in NCBI database *CHS1* (JN182805.1), D: Phylogenetic tree showing the relation between *CHS1* clones1, 2, 3 and the nearest deposited chalcone synthase *CHS1* (JN182805.1) gene from the NCBI, and E: comparative analysis among the partial sequences of *S. marianum CHS1* clones 1, 2, 3, (MG751175.1, MG751176.1, MG751177.1, respectively).

Bioinformatics analysis and genetic fidelity of the *CHS2* gene and its clones

For this gene, the order of the clones submitted to NCBI was changed as follows: *CHS2* clone 1 (control) (MG751178.1), *CHS2* clone 2 (0.05% colchicine) (MG751179.1), and *CHS2* clone 3 (200 Gy) (MG751180.1). The results of the bioinformatics analysis confirmed that the sequence of the *CHS2* clone 3 exhibited a higher variation than *CHS2* clone 1 and 2. Furthermore, *CHS2* clones 1, 2, and 3 in this study were similar to *CHS2* (JN182806.1) in the NCBI database, with identity ratios of 99%, 99%, and 96%, respectively. The data exhibited the presence of two SNPs between *CHS2* clone 1 gene sequence and the NCBI sequence JN182806.1 (Fig 5A), four SNPs and two gaps between *CHS2* clone 2 and JN182806.1 (Fig 5B), and 18 SNPs and no gaps between *CHS2* clone 3 and JN182806.1 (Fig. 5C). An increase in the number of SNPs in the last case reflects the relatively low identity ratio (96%), which could be attributed to the mutagenic effect of gamma radiation on the treated seeds that resulted in the genetically modified explants, the source of the studied DNA sequence. These results suggested that *CHS2* clones 1 and 2 were closer to each other than *CHS2* clone 3. The phylogenetic tree also confirmed the finding and showed the same identity ratios on the roots of clades. The phylogenetic tree (Fig. 5D) indicated that *CHS2* clones 1 and 2 were closely related to each other, while *CHS2* clone 3 was more distant from the two other clones, thus confirming the lower identity ratio (96%) due to the radiation effect. The comparison between the DNA sequences of *CHS2* clones also confirmed these findings (Fig. 5E).

Fig 5. Bioinformatics analysis of *CHS2* gene clones revealed; pairwise alignment analysis and SNPs among the partial sequences of A: *Silybum marianum CHS2* clone1, control, (MG751178.1), B: *CHS2* clone2, colchicine 0.05%, (MG751179.1), C: *CHS2* clone3, 200Gy, (MG751180.1) and the nearest deposited sequence in NCBI database *CHS2* (JN182806.1), D: Phylogenetic tree showing the relation between *CHS2* clones1, 2, 3 and the nearest deposited chalcone synthase *CHS2* (JN182806.1) gene from the NCBI, E: comparison analysis among the partial sequences of *S. marianum CHS2* clones 1, 2, 3, (MG751178.1, MG751179.1, MG751180.1, respectively).

Bioinformatics analysis and genetic fidelity of the *CHS3* gene and its clones

Clone 1 (control), clone 2 (200 Gy) and clone 3 (0.05% colchicine) of the *CHS3* gene (MG751181.1, MG751182.1, MG751183.1, respectively) were observed to be similar to *CHS3* from *S. marianum* (JN182807.1) in the NCBI database, with respective identity ratios of 99%, 98%, and 99%. Regarding pairwise alignment, four SNPs were observed between the *CHS3* clone 1 gene sequence and JN182807.1, six SNPs, and two gaps between *CHS3* clone 2 and JN182807.1, and four SNPs and two gaps between *CHS3* clone 3 and JN182807.1 (Figs. 6A, B, C, respectively). The presence of SNPs and gaps between the clones of the *CHS3* gene confirmed the mutagenic effect of 200 Gy and its transfer to the explants, which is in accordance with the previous results for the clones of *CHS1* and *CHS2*. The phylogenetic tree showed similar identity ratios on the roots of clades, indicating the close similarity between *CHS3* clone 1 and clone 3. On the other hand, *CHS3* clone 2 was distant from the other clones, indicating less similarity between them (Fig. 6D). The comparison between the DNA sequences of the *CHS3* clones confirmed these findings, wherein clone 1 and clone 3 were observed to be closer to each other than to clone 2 (Fig. 6E).

In conclusion, the bioinformatics analysis confirmed that the clones of the *CHS2* gene exhibited the highest genetic variation, both in relation to each other and to the other studied gene clones, *CHS1* and *CHS3*. These results were accompanied by a high expression of the *CHS2* gene, which could play a significant role in stimulating and enhancing silymarin production.

Fig. 6. Bioinformatics analysis of *CHS3* gene clones revealed; pairwise alignment analysis and SNPs among the partial sequences of A: *Silybum marianum CHS3* clone1, control, (MG751181.1), B: CHS3 clone2, 200Gy, (MG751182.1), C: *CHS3* clone3, colchicine 0.05%, (MG751183.1) and the nearest deposited sequence in NCBI database *CHS3* (JN182807.1), D: Phylogenetic tree showing the relation between *CHS3* clones1, 2, 3 and the nearest deposited chalcone synthase *CHS3* (JN182807.1) gene from the NCBI, E: comparitive analysis among the partial sequences of *S. marianum CHS3* clones 1, 2, 3, (MG751181.1, MG751182.1, MG751183.1, respectively).

Conclusions

Gamma rays (200 and 600 Gy) and colchicine (0.05%) did not inhibit the formation of *S. marianum* calli and promoted cell culture growth. Thus, callus induction from leaf segments of seedlings obtained from seeds treated with gamma rays (200 and 600 Gy), 0.05% colchicine or control conditions, showed high biomass production. The viability of the cell suspension cultures was over 90%. The flavonolignan profile of the radiated seeds confirmed the promising impact of the two studied treatments, given that 200 and 600 Gy positively influenced

the total silymarin production, and the effect was transferred to the fruits. Elicitation with both doses of gamma rays (200 and 600 Gy) and 0.05% colchicine significantly enhanced the extracellular accumulation of silydianin, silychristin, isosilybin B, silybin A, and silybin B in cell suspension cultures at day 10 and 12 of incubation. The highest production of flavonolignans was observed in the *S. marianum* cell suspension medium corresponding to the 600 Gy treatment, followed by 200 Gy, and 0.05% colchicine, after 12 days of culture. All three treatments upregulated the *CHS1*, *CHS2*, and *CHS3* genes, involved in the flavonolignan biosynthetic pathway, in the *S. marianum* cell suspensions, which was accompanied by an increase in silymarin content. The bioinformatics analysis confirmed that the three clones of the *CHS2* gene exhibited the highest genetic variation, both in relation to each other and to the clones of the other studied genes, *CHS1* and *CHS3*. The high expression of the *CHS*s genes could play a significant role in stimulating and enhancing silymarin production.

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