Summary of MSc Thesis

"Biotechnological production of Silymarin using cell suspension culture of Silybum marianum"

This study was funded by the EU Commission, joint master's degree, tempus project no.543865. It is a scientific cooperation between Genetics and Genetic engineering dept., Faculty of Agriculture, Benha University, Egypt, and Plant Physiology dept., Faculty of Pharmacy, Barcelona University, Spain.

In this study, the enhancing effect of gamma irradiation (200, 600 GY) and colchicine (0.05%) on silymarin (secondary metabolites) production using cell suspension culture of *Silybum marianum* L. was investigated. Also, chalcone synthase *(CHS)* genes expression was evaluated in all treatments as well as control using qRT-PCR, quantitative real time PCR.

To achieve these goals, the M3 stable mutated seeds of *Silybum marianum* L. which were treated with different doses of gamma irradiation (200GY, 600GY) in previous study for **El-Garhy et al., 2016** were used. In addition, we used the M1 seeds soaked in colchicine (0.5 mg/L) for 30 min comparing with control seeds. Hence, calli were obtained from irradiated and colchicine treated seedlings using MS medium. Moreover, the cell viability of the obtained callus was determined where viable cells appeared as fluorescein green while dead cells appeared as fluorescein red. After confirming the callus cells viability, cell suspensions were established from 3-month-old undifferentiated callus.

In addition, the fresh weight, dry weight and growth rate were evaluated after 12 days of incubation periods under the effect of the

studied different treatments. Also, the flavonoid in both dry seeds and cell suspension culture were determined by HPLC.

The total RNA was extracted from all the studied treatments as well as the control of *S. marianum* callus samples for evaluating the differential expression of *CHS1, 2, 3* genes in response to gamma radiation and colchicine. On the other hand, conventional PCR using cDNA as a template was performed to confirm the amplicon lengths (101, 184, 105 and 134bp) and primer specificity of *CHS1, CHS2, CHS3* genes and *NADH*, internal reference gene for qRT-PCR.

Total genomic DNA from callus of all the studied groups (control, colchicine, M4200 and 600Gy explants) was extracted. For sequencing , the purified PCR fragments 622bp for *CHS1,CHS2* as well as 605bp for *CHS3* were cloned , sequenced and registered in the NCBI database under accession numbers (MG751175.1,*CHS1*clone1; MG751176.1,*CHS1*clone2;MG751177.1,*CHS1*clone3; MG751178.1,*CHS2*clone1;MG751179.1,*CHS2*clone2; MG751180,*CHS2*clone3;MG751181.1,*CHS3*clone1; MG751182.1,*CHS*3 clone2 and MG751183.1,*CHS3*clone3). Bioinformatics analysis using VecScreen tool, blast, Jalview software, Clustal Omega and MEGA7 software were performed on the obtained sequences.

The obtained results can be summarized:

Tissue culture results showed that all treatments exhibited high growth index compared to control, where callus originated from 600Gy of M4 explants possessed the highest growth Rate (1.3 gm) and dry weight (0.3gm). Also, the flavonolignans profile of the studied M3 mutated dry seeds showed that the amount of total Silymarin were 18.01, 13.1 and 12.3 mg/g d.w for 600GY, 200GY and control samples, respectively

On the other hand ,silymarin components were quantified in the extracellular medium using HPLC assay of cell suspension culture originated from *S. marianum* M4 (200, 600 Gy seedlings) and 0.05% colchicine treated seedlings at 10 and 12 days incubation and the result showed that 600GY treatment recorded the highest content of silymarin comparing with 200GY treatment, colchicine and control. Moreover the obtained results of gene expression showed that *CHS2* expression was more elicited (40.8, 10.4 and 5.7-fold) increase under the effect of 600GY, 200GY and colchicine (0.05%) treatments, respectively, than *CHS1* and *CHS3* genes. From results, the mRNA transcripts of *CHS1, CHS2* and *CHS3* had the highest abundance, 13.7, 40.8 and 7.1-fold increase, respectively, under the effect of 600 GY. Further, the specificity of the primers of the three *CHSs* genes were checked by normal PCR.The PCR products of *CHS1, CHS2* and *CHS3* were 622 bp and 605 bp respectively. On the other hand, Blast results indicated that *CHS1* (MG751175.1), *CHS2* (MG751178.1) and *CHS3* (MG751181.1) from the current study are similar to *CHS1* (JN182805.1), *CHS2* (JN182806.1) and *CHS3* (JN182807.1) in the NCBI from *S. marianum* with 99% max identity. The phylogenetic analysis also showed that all the studied *CHS* genes *CHS1, 2* and *3* were related to each other with variable distances which confirmed the same identity ratios on the roots of clades and reflects the close similarity in accordance with the relatively high identity 99%.

Blast, SNPs, Gaps, phylogenetic tree, protein SNPs and DNA comparison analysis were used for studying the genetic fidelity of *CHS1, 2, 3* genes and their clones. All the obtained results confirmed the stability of the mutagenic effect of gamma irradiation (200 GY) on the treated M3 seeds and their resulted M4 explants, the source of the studied DNA, and the inheritance of these genetic changes (mutations) through

generations where these changes were detected in the DNA sequence from the M4 explants.