



BIOCHEMICAL AND MOLECULAR BASIS OF SECONDARY

METABOLITE BIOSYNTHESIS IN *Silybum marianum* L. UNDER STRESS CONDITIONS

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Abstract: *This study provides a comprehensive investigation of the biochemical and molecular mechanisms underlying secondary metabolite biosynthesis in *Silybum marianum* L. (milk thistle), a medicinal plant widely recognized for its hepatoprotective properties. Special attention is given to the phenylpropanoid pathway, which plays a central role in the formation of flavonoids and flavonolignans, collectively known as the silymarin complex. Experimental analyses were conducted under controlled and salinity-induced stress conditions to evaluate variations in total phenolic content, flavonoid accumulation, silymarin concentration, and antioxidant activity. The results demonstrate that salinity stress significantly enhances the biosynthesis of secondary metabolites through the activation of key enzymatic systems and gene expression pathways. These findings highlight the adaptive significance of secondary metabolism in stress tolerance and provide a scientific basis for the pharmacological and biotechnological utilization of *Silybum marianum*.*

Keywords: *Silybum marianum, silymarin, phenylpropanoid pathway, flavonoids, secondary metabolites, oxidative stress, antioxidant system, gene expression*

1. Introduction: The study of secondary metabolites in medicinal plants has gained increasing importance due to their extensive applications in pharmacology, nutraceuticals, and biotechnology. Among

such plants, *Silybum marianum* L., commonly known as milk thistle, occupies a prominent position due to its rich content of biologically active compounds, particularly the silymarin complex. Silymarin consists primarily of flavonolignans such as silybin, silydianin, and silychristin, which exhibit strong antioxidant, anti-inflammatory, and hepatoprotective activities. Unlike primary metabolites, which are directly involved in growth and development, secondary metabolites serve ecological and protective functions. They play a crucial role in plant adaptation to environmental stress factors such as salinity, drought, temperature extremes, and ultraviolet radiation. These compounds act as chemical defenses, signaling molecules, and antioxidants, enabling plants to survive under adverse conditions. The biosynthesis of secondary metabolites in *Silybum marianum* is primarily governed by the phenylpropanoid pathway, a highly regulated metabolic network that converts phenylalanine into a wide range of phenolic compounds. This pathway integrates multiple enzymatic steps and is tightly controlled at both biochemical and genetic levels. Understanding these mechanisms is essential for optimizing the production of valuable phytochemicals and enhancing plant stress tolerance.

2. Materials and Methods.2.1 Plant Material and Growth

Conditions: Seeds of *Silybum marianum* were collected and cultivated under controlled greenhouse conditions. The plants were grown in sterilized soil with a uniform nutrient composition to ensure consistency across experimental treatments. Environmental parameters such as temperature ($25 \pm 2^{\circ}\text{C}$), light intensity (16-hour photoperiod), and humidity were carefully maintained throughout the growth period.

2.2 Experimental Design: The experiment was conducted using three treatment groups: a control group grown under normal conditions, a moderate salinity group treated with 50 mM NaCl, and a high salinity group exposed to 100 mM NaCl. Each treatment was replicated three times to ensure statistical reliability. Salinity stress was applied gradually to avoid osmotic shock, and plants were monitored over a 30-day period.

2.3 Biochemical Analysis: Leaf and seed samples were collected at regular intervals and subjected to biochemical analysis. Total phenolic content was determined using the Folin–Ciocalteu method, which measures the reducing capacity of phenolic compounds. Flavonoid content was quantified using the aluminum chloride colorimetric assay, with absorbance measured at 415 nm. Silymarin components were analyzed using high-performance liquid chromatography (HPLC), allowing precise identification and quantification of individual flavonolignans. Antioxidant activity was evaluated using the DPPH radical scavenging assay, which provides insight into the free radical neutralization capacity of plant extracts.

2.4 Enzyme Activity and Gene Expression Analysis

The activity of key enzymes involved in the phenylpropanoid pathway, including phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS), and flavonol synthase (FLS), was measured using spectrophotometric methods. For molecular analysis, total RNA was extracted from plant tissues, and quantitative PCR (qPCR) was performed to assess the expression levels of genes encoding these enzymes.

2.5 Statistical Analysis: All experimental data were analyzed using analysis of variance (ANOVA), and differences between treatments were considered statistically significant at $p < 0.05$.

3. Results: The results of this study clearly indicate that salinity stress significantly influences the biosynthesis of secondary metabolites in *Silybum marianum*. Under moderate salinity conditions (50 mM NaCl), total phenolic content increased by approximately 35–40% compared to the control group. Under high salinity conditions (100 mM NaCl), this increase reached up to 70%, demonstrating a strong correlation between stress intensity and phenolic accumulation. Flavonoid content showed a similar trend, with a 1.5-fold increase under moderate stress and nearly a twofold increase under severe stress conditions. These findings suggest that flavonoid biosynthesis is highly responsive to environmental stress and plays a critical role in plant defense mechanisms. The concentration of silymarin, particularly its major component silybin, increased significantly under salinity stress. HPLC analysis revealed that the relative proportion of individual flavonolignans also shifted, indicating a possible reprogramming of metabolic flux within the phenylpropanoid pathway. Enzyme activity assays demonstrated a marked increase in PAL activity, which nearly doubled under high salinity conditions. Similarly, CHS and FLS activities showed significant enhancement, reflecting the activation of the flavonoid biosynthetic pathway. Gene expression analysis confirmed these findings, with upregulation of PAL, CHS, and FLS genes observed in stressed plants. Antioxidant activity, as measured by DPPH assay, increased proportionally with metabolite accumulation, confirming the functional role of these compounds in mitigating oxidative stress.

4. Discussion: The observed increase in secondary metabolite production under salinity stress can be attributed to the activation of complex biochemical and molecular mechanisms. Salinity stress disrupts cellular homeostasis, leading to the accumulation of reactive oxygen species (ROS), which can cause oxidative damage to proteins, lipids, and nucleic acids. In response to this challenge, plants activate their antioxidant defense systems, including both enzymatic and non-enzymatic components. The phenylpropanoid pathway plays a central role in this response by generating phenolic compounds that can scavenge ROS and stabilize cellular structures. The enzyme phenylalanine ammonia-lyase (PAL) acts as the entry point to this pathway and is often considered a key regulatory step. Its increased activity under stress conditions indicates a metabolic shift toward the production of protective compounds. Downstream enzymes such as CHS and FLS further channel metabolic intermediates into the synthesis of flavonoids and flavonolignans. At the molecular level, stress signals are transmitted through signaling pathways involving calcium ions (Ca^{2+}), mitogen-activated protein kinases (MAPKs), and transcription factors that regulate gene expression. These pathways coordinate the activation of biosynthetic genes, leading to enhanced metabolite production. Silymarin, as a major secondary metabolite in *Silybum marianum*, plays a dual role as both an antioxidant and a membrane stabilizer. It inhibits lipid peroxidation and protects cellular integrity, thereby enhancing the plant's resilience to environmental stress.

5. Conclusion: This study demonstrates that secondary metabolite biosynthesis in *Silybum marianum* is a highly dynamic and regulated process that is significantly enhanced under salinity stress conditions.

The coordinated increase in phenolic compounds, flavonoids, and silymarin is mediated by the activation of key enzymes and genes within the phenylpropanoid pathway. These findings provide valuable insights into the adaptive mechanisms of medicinal plants and highlight the potential of *Silybum marianum* as a source of bioactive compounds for pharmaceutical applications. Furthermore, understanding these processes opens new opportunities for metabolic engineering and sustainable production of high-value phytochemicals.

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