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# The Synergistic role of serotonin and melatonin during temperature stress in promoting cell division, ethylene and isoflavones biosynthesis in *Glycine max*

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ABSTRACT

Serotonin (SER) and melatonin (MEL) are signalling molecules involved in signalling in various stress response mechanisms in plants. However, their role and molecular mechanism in the accumulation of isoflavones and biomass under temperature stress are not clearly defined. To explicate their function, SER and MEL at different ratios were applied to the *in vitro* cultures of soybean at 16, 24, and 32 °C and were analyzed for the accumulation of bioactive compounds, isoflavones content, and culture biomass followed by transcript levels of cell division, isoflavones, and ethylene biosynthesis genes. An increase in biomass, total phenolics, total flavonoids, and different forms of isoflavones content was evident in the treatments. The expression level of critical genes of isoflavone (*chalcone synthase, chalcone reductase, chalcone isomerase, and isoflavone synthase*), ethylene (*s-adenosyl methionine* and *1-aminocyclopropane-1-carboxylic acid oxidase*), cell division (*cyclin and cyclin-dependent kinase*), and MEL biosynthesis genes (*acetyl serotonin O-methyltransferase* and *serotonin N-acetyltransferase*) and transcription factors (*melatonin 2 hydroxylase, myeloblastosis like protein*) were co-expressed during temperature stress in the presence of SER and MEL. At 32 °C, the SER and MEL application showed maximum biomass and isoflavones content and was substantiated with the corresponding gene expression. We hypothesize that the SER and MEL ratio may play a role in increasing the biomass and isoflavones content under temperature stress in soybean cell culture.

## 1. Introduction

The plants are confronted with various biotic and abiotic environmental stress during their life-cycle. Among abiotic stress, the temperature is one of the vital ecological factors that affect plant growth and productivity [1]. Their survival during thermal stress depends on the activation of efficient protective responses [2]. The most common reaction of plants during environmental stress is the generation of reactive oxygen species, the primary source of signal transduction during biotic and abiotic stress [3,4]. The temperature stress disrupts the cellular redox homeostasis, leading to decreased biomass production and cell death [5]. The disruption in the equilibrium leads to an increase in intracellular reactive oxygen species levels. Soybean is one of the economically important crops, and it contributes significantly to edible oil and soy protein all over the world [6]. Various environmental factors, including temperature, play an important role in soybean production

*Abbreviations:* SER, serotonin; MEL, melatonin; 2,4-D, 2,4-dichloro phenoxy acetic acid; Kin, kinetin; °C, degrees Celsius; FW, fresh weight; %, percentage; MS, Murashige and Skoog; qPCR, quantitative real-time polymerase chain reaction; H, Hours; S, seconds; µL, microliter; µm, micrometer; µM, micromolar; mg, milligram; g, gram; rpm, rotation per minute; RNA, ribonucleic acid; $r^2$, coefficient of determination; cDNA, complementary deoxyribonucleic acid; GAEq, gallic acid equivalent; QEq, quercetin equivalent; UV, Ultraviolet; HPLC, High performance liquid chromatography; 18 s, *18 sribosomal ribonucleic acid*; 60 s, *60sribosomal RNA*; ACT, *actin*; ELF, *elongation factor*; GPD, *glucose-6-phosphate dehydrogenase*; UBQ-F, *ubiquitin family*; UBQ-E, *ubiquitin-conjugating enzyme-E2*; PAL, *phenylalanine ammonia-lyase*; CHS, *chalcone synthase*; CHR, *chalcone reductase*; CHI, *Chalcone isomerase*; IFS, *synthase*; ACCO, *s-adenosyl methionine and 1-aminocyclopropane-1-carboxylic acid oxidase*; SAMS, *s-adenosylmethionine synthase*; ASMT, *acetyl serotonin o-methyltransferase*; SNAT, *serotonin n-acetyltransferase*; M2H, *melatonin 2 hydroxylase*; NPR, *nonexpressor of pathogenesis-related genes*; MYBJ, *myeloblastosis like protein J*.

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and studies are documented temperature effects on soybean seed yield and seed quality [6]. As plants are indulged in coping with the excessive reactive oxygen species level to maintain cellular redox homeostasis for its survival [7], finding an efficient growth-regulator application to increase plant's thermal tolerance plays an important role in increasing soybean crop production. Recently, MEL application has proven to be an efficient method to increase tolerance against cold and heat stress in plants [8].

The SER and MEL are tryptophan-based indole molecules found ubiquitously across all the living forms on Earth from unicellular to the multicellular organism [9]. The pleiotropy of MEL and SER exhibits various physiological responses in plants, including morphogenesis, growth, development, stress tolerance, and defense mechanism [10,11]. SER and MEL act as a signaling molecule that plays a vital role in regulating various abiotic and biotic stress, by modulating abscisic acid, gibberellic acid, and salicylic acid pathways [12]. According to different environmental cues, the plants enhance MEL production for ecological adaptation to a hostile environment [13]. MEL treatment efficiently reduced oxidative stress by regulating lipid peroxidation, accumulation of hydrogen peroxide ($H_2O_2$), and biosynthesis of proline in *Triticum aestivum* seedlings [14]. However, the endogenous MEL deficiency under higher temperature aggravated oxidative stress in *Solanum lycopersicum* [15]. In high light/UV intensities, elevated MEL levels may play a role in direct antioxidant and photo protectant properties [16]. The exogenous MEL induced growth and stress tolerance in *Hevea brasiliensis* seedling under salt stress [17] and metal stress tolerance in *Citrullus lanatus* seedling [18].

Isoflavones occur primarily in leguminous plants like *Glycine max*, *Trifolium pretense,* and *Trifolium repens* [19]. Isoflavones occur in two forms, aglycones and glycoside. In soybean, mainly aglycones like daidzein, genistein, and their conjugates are present in larger quantities [20]. The isoflavones content dramatically depends on the plant tissues, growth stage, cultivar, and growing conditions [19]. Isoflavones were reported to show various health benefits, mainly by their estrogenic activity, hormonal therapy, breast and prostate cancer, and cardiovascular diseases [19].

Various stress molecules like methyl jasmonate, salicylic acid, and abscisic acid are reported to increase the isoflavone biosynthesis genes expression and isoflavones content in soybean [21]. The RNAseq analysis of legume plants under fungal pathogen and aphid infection demonstrated ethylene's role in the accumulation of isoflavones content [22,23]. Further, MEL enhanced the ethylene content by increasing the ethylene biosynthesis genes during carotenoid biosynthesis, ripening, salt stress, and drought [24].

Several studies elucidated on individual SER and MEL's function in many plant species [25]. But the exogenous application of different concentrations of SER and MEL is not explained concerning to cell division, isoflavones, and ethylene biosynthesis under different temperature stress.

Since the *in vitro* cultures offer the best method for producing valuable secondary metabolites in aseptic conditions, the soybean *in vitro* cultures provide the most controlled, convenient and simplified biological system for studying the influence of abiotic and biotic elicitors [21,26,27]. However, SER and MEL's role in the isoflavones content in soybean is not demonstrated. To know its function, in the present day, different ratios of SER and MEL were used to treat soybean cell culture, and analyzed the biomass production and isoflavones content (genistein, genistin, daidzein, daidzin, and glycitin) by High-Performance Liquid Chromatography. This is further substantiated by transcript profiles of critical genes involved in isoflavones (*phenylalanine ammonia-lyase, chalcone synthase, chalcone reductase, Chalcone isomerase,* and *isoflavone synthase*), ethylene (*S-adenosylmethionine synthase and s-adenosyl methionine* and *1-aminocyclopropane-1-carboxylic acid oxidase*), cell division (*cyclin and cyclin-dependent kinase*), MEL biosynthesis genes (*acetyl serotonin O-methyltransferase and serotonin n-acetyltransferase*), and transcription factors (*nonexpressor of pathogenesis-related genes, melatonin 2 hydroxylase, myeloblastosis like protein*) through qPCR analysis.

## 2. Materials and methods

### 2.1. Chemicals

HPLC standards: Serotonin (SER), melatonin (MEL), genistein, genistin, daidzein, daidzin, and glycitin were procured from Sigma-Aldrich, Bengaluru, India. HPLC grade solvents and plant growth regulators were purchased from Hi-media, Mumbai, India.

### 2.2. Establishment of soybean cell cultures

The soybean seed [*Glycine max* (L.) Merr.] variety KHSB2 was obtained from the University of Agricultural Sciences, Bangalore, India. The seeds were surface sterilized and inoculated for *in vitro* germination. The cotyledonary leaves were obtained from soybean seedling, grown on MS basal medium (Murashige and Skoog 1962) to initiate callus culture. The *in vitro* cotyledonary leaf was used as an explant, and it was inoculated on an MS medium comprising of 1.0 mg $L^{-1}$ 2,4-dichloro phenoxy acetic acid (2, 4-D) and 0.1 mg $L^{-1}$ Kinetin (Kin) and supplemented with 3% (w/v) sucrose along with 0.3 % (w/v) phytagel (Sigma-Aldrich). The cultures were incubated at 25 °C in 16/8 h of photoperiod. The induced calli were subcultured in the above-mentioned medium every three weeks to obtain friable callus. The obtained green friable callus was selected to initiate cell culture by inoculating 5 g $L^{-1}$ of inoculum into a 150 mL Erlenmeyer flask containing 40 mL liquid MS medium supplemented with 2, 4-D (1.0 mg $L^{-1}$), and Kin (0.1 mg $L^{-1}$). The cell cultures were incubated on an orbital shaker (ORBITEK ® India) set at 90 rpm in a culture room at 24 °C under a photoperiod of 16/8 h. The pH of the medium was adjusted to 5.8 before autoclaving and subcultured every 18 days.

### 2.3. Application of SER and MEL ratios to the soybean cell culture

After performing the growth curve experiment, the culture showed an exponential growth phase from 12 to 15 days. The different ratio of SER and MEL concentrations; 50.5: 50.5, 1.0: 100, 1.0: 50.5, 1.0: 1.0, 50.5: 1.0, 100: 100, and 100: 1.0 μM (based on response surface methodology) was added to the cell culture after the day 12 with 5 g $L^{-1}$ of inoculum in 150 mL flask containing 40 mL medium. The treated culture was maintained in the dark at 90 rpm with 16, 24, and 32 °C ± 1 in the shaker for 72 h. The duration and concentration of SER and MEL were selected based on the previously published work [28], whereas the low and high-temperature stress chosen for the study is based on the work carried by Alsajri et al., 2019 [6] and also based on the preliminary work carried out in the lab.

### 2.4. Estimation of fresh weight biomass

The cell cultures were treated with SER and MEL on the 12th day and harvested after the exponential phase (15 days). The cultures were harvested and filtered in a vacuum filter to separate media from the callus. The callus washed with distilled water, was weighed, and the fresh weight (FW) was recorded and expressed in g/L FW.

### 2.5. Extraction, identification, and quantification of Isoflavones by HPLC

The cell culture of soybean was taken for the extraction of isoflavones as per the earlier reported method (Devi et al., 2020), and the filtrate was filtered through a 0.45 μm filter unit (Cameo 13 N syringe-filter, nylon). The HPLC (Shimadzu model LC-10A) coupled with a UV detector (SPD-10AD model) was used to identify, separate, and quantify isoflavones from the samples. The gradient consisted of mobile phase A (0.1 % Trifluoroacetic acid and water) and B (0.1 % Trifluoroacetic acid and acetonitrile). Phenomenex C18 reversed-phase column (5 μm

diameter and 150 mm × 4.6 mm) was used for the sample separation, and 20 μL of sample volume was injected for the detection of isoflavones (genistein, genistin, daidzein, daidzin, and glycitin) at 254 nm wavelength. The gradient method was applied, where solvent B was introduced 10–100 % B (0–45 min) and then returned to 10 % for (45–53 min) at 1 mL/min flow rate. By calculating the standard area obtained from HPLC analyses, calibration curves for standards were obtained with linearity ($r > 0.979$–$0.991$). The standard of isoflavones was injected in triplicates to find the linearity. The chromatogram of standards and the samples are given in Fig. S1

### 2.6. Estimation of total phenolics content

The total phenolics content of the cell cultures was determined by the Folin-Ciocalteu reagent method with gallic acid as standard [29]. The extract (0.1 mL) was pipetted out into a test tube and used for further analysis. Absorbance values were measured at 650 nm. The amount of total phenolic was determined in the samples using an equation obtained from a gallic acid standard curve. The total phenolics content was expressed as mg 100 $g^{-1}$ fresh cell weight in gallic acid equivalent (GAEq.).

### 2.7. Estimation of total flavonoids content

The total flavonoids contents of the soybean cell culture were determined as described [29]. In brief, 1.0 mL of a diluted extract in absolute ethanol was mixed with 1 mL of 2 % (w/v) methanolic solution of $AlCl_3$. The solution was incubated for 15 min at room temperature (24 °C), and the absorbance was recorded at 430 nm. The results were expressed as quercetin equivalent (QEq.) in mg 100 $g^{-1}$ of fresh weight.

### 2.8. RNA isolation and qPCR analysis

Total RNA was isolated from cell cultures treated with SER and MEL using Tri-Reagent (Sigma-Aldrich) according to the manufacturer's protocol. The quality and quantity of RNA were determined by 1.2 % agarose gel electrophoresis and Nanodrop (ND1000, Thermo Scientific™, USA), respectively. One microgram of total RNA was reverse transcribed into cDNA with Verso cDNA Synthesis Kit (Thermo Scientific™), and the cDNA was diluted to 1:5 times before qPCR analysis. All the primers (Table S1) were designed using an oligo-analyzer from Integrated DNA Technologies and synthesized by Shrimpex Bangalore, India.

The qPCR performed in Applied Biosystems QuantStudioTM-5 using 10 μL reactions contained 5 μL of SYBR green master mix (SYBR Premix Ex Taq, TAKARA), 0.5 μL forward primer (2.5 μM), 0.5 μL reverse primer (2.5 μM), 1.0 μL of diluted cDNA and 3.0 μL of nuclease-free water. The PCR program was as follows: pre-denaturation at 95 °C for 5 min followed by 40 cycles of denaturation at 95 °C for 5 s, annealing at 55–57 °C for 10 s, and followed by melting curve program. The amplification efficiency of each primer pair was evaluated by the standard curve method using serial dilutions of pooled cDNA, 'qPCR Efficiency Calculator' from Thermo Fisher Scientific for efficiency calculations. The primers showed an efficiency between 85–110 % and the $r^2$ value of 0.98–0.99.

### 2.9. Selection of reference genes for normalisation of qPCR

The various reference genes include 18 s-rRNA (18 s), 60 s-rRNA (60 s), actin (ACT), elongation factor (ELF), glucose-6-phosphate dehydrogenase (GPD), ubiquitin family (UBQ-F), and ubiquitin-conjugating enzyme-E2 (UBQ-E) were quantified in soybean cell culture to identify the reference genes for normalization of qPCR data in response SER and MEL ratio.

The effect of treatments (SER and MEL) on isoflavone biosynthesis genes *phenylalanine ammonia-lyase* (*PAL*), *chalcone synthase* (*CHS*), *chalcone reductase* (*CHR*), and *isoflavone synthase* (*IFS*) was studied through qPCR analysis. The R2R3-type myeloblastosis-like protein J (*MYBJ)* transcription factor involved *CHS* and *IFS* activation [30], were selected for the study. The conversion of MEL from SER starts with *Acetyl serotonin O-methyltransferase* (*ASMT*) and *serotonin N-acetyltransferase (SNAT)*. The putative genes for *ASMT* and *SNAT* in *Glycine max* (soybean) were selected based on the protein domain (Conserved Data Domain) and also by analysing curated transcriptome data from *Arabidopsis thaliana* and *Oryza sativa* during the developmental stage with *Glycine max* from the GENEVESTIGATOR (https://genevestigator.com/ ). Based on the screening, the putative genes were selected and quantified. The enzymatic MEL degradation is achieved by *Melatonin 2-hydroxylase* (*M2H*); therefore, putative *M2H* is considered based on the homology and domain analysis.

To quantify the influence of SER and MEL treatment on ethylene biosynthesis, the expression of genes involved in ethylene biosynthesis was analysed. The genes selected from the soybean database, Soybase (https://soybase.org/), putative *S-adenosyl methionine* (*SAM*), and *1-aminocyclopropane-1-carboxylic acid oxidase* (*ACCO*) genes were quantified in the present study. The *Nonexpressor of the pathogenesis-related gene (NPR)* is a transcription factor involved in abiotic and biotic stress. It is known to influence a broad range of systems like salicylic acid (SA) biosynthesis and circadian rhythm [31]. Therefore, *NPR* was quantified to understand its role in SER and MEL treatments.

### 2.10. Statistical analysis

The variability of metabolites across the treated sample and control harvested after 72 h was measured by two-way ANOVA and Duncan's post-hoc test in R. The Pearson correlation among them was measured by the corrplot package [32] in R-studio [33]. The statistical analysis for the reference gene (*18 s, 60 s, ELF, CYC, CDK, UBQ-E,* and *UBQ-F*) was performed using the R packages, ReadqPCR and NormqPCR [34]. The most stable gene expression will have the least M values and provides the two best genes for normalization (Fig. S2). The relative gene expression levels were calculated with the $2^{-\Delta\Delta CT}$ method [35], and the significance of each gene under the treatments was calculated using a two-tailed *t*-test with a false discovery rate (FDR) used in the Enhanced Volcano plots [36]. The differential gene expression was represented as log-fold-change and was used for Complex Heatmap [37] and Enhanced Volcano plots.

## 3. Results

### 3.1. Isoflavones profile under SER and MEL ratios at 16 °C

The influence of SER and MEL at different ratio (50.5: 50.5 μM, 1.0: 1.0 μM, 100.0: 100.0 μM, and 100.0: 1.0 μM) on the various forms of isoflavones *viz.*, genistein, genistin, glycitin daidzein, and daidzin at 16 °C was compared to control (without SER and MEL) and is shown in Fig. 1. The aglycones (genistein and daidzein) content increased in all the treatments of SER and MEL compared to control, whereas glycosides of isoflavones content did not show any significant change in any ratio of SER and MEL compared to control (Fig. 1A and D). Under the lower temperature, at 16 °C, the equal ratio of SER and MEL concentration enhanced aglycones content compared to control and other combinations of SER and MEL. Among the treatments, SER and MEL ratio of 50.5: 50.5 μM showed the highest genistein and daidzein of 4.0 and 7.5-fold, respectively, compared to control. The glycosides: genistin, daidzin, and glycitin responded differently to the SER and MEL ratio. However, none of the ratios showed a significant increase or decrease in the respective isoflavones content than control at 16 °C (Fig. 1B, C, and E).

### 3.2. Isoflavones profile under SER and MEL ratios at 24 °C

The different ratio of SER and MEL (50.5: 1.0, 100.0: 50.5, 1: 50.5,

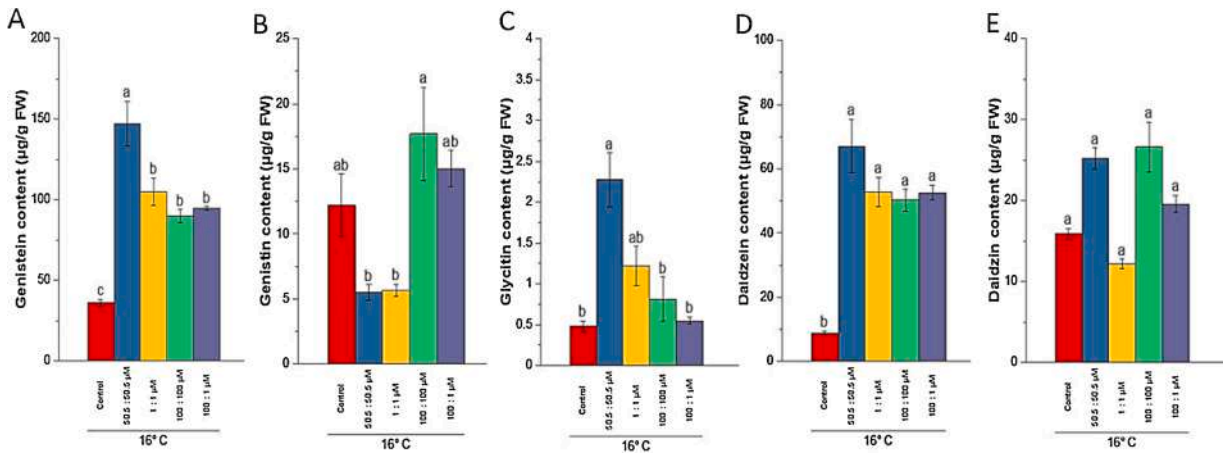


**Fig. 1.** Effect of SER and MEL ratio on isoflavones content at 16 °C A) Genistein (B) Genistin (C) Glycitin (D) Daidzein and (E) Daidzin from cell cultures of soybean. Data are represented as the mean ± standard deviation from four replicates. The Letters, a, b, c above the bars indicate statistical significance; different letters indicate significant differences between two samples and similar letters indicate no significant difference between two samples ($P < 0.05$).

and 50.5: 50.5 µM) showed considerable effects on genistein, genistin, daidzein, daidzin, and glycitin content at 24 °C compared to control (without SER and MEL) as shown in Fig. 2. Genistein and daidzein content significantly increased in all the given concentrations. The maximum content of 11.1 and 12.5-fold, respectively, was observed in 50.5: 1.0 µM compared to control (Fig. 2A and D). No significant change was observed in the glucosides (genistin and daidzin) compared to control in all the treatments (Fig. 2B and E). Whereas glycitin content was enhanced significantly by 9.7 and 12.0-fold in the presence of 50.5: 1.0 and 50.5: 50.5 µM concentration compared to control (Fig. 2E).

### 3.3. Isoflavones profile under SER and MEL ratios at 32 °C

The effect of different ratio of SER and MEL (1.0: 1.0, 100.0: 100.0, 100.0: 1.0, 1.0: 100.0, and 50.5: 50.5 µM) on various forms of isoflavones content at 32 °C is shown in Fig. 3. A 22-fold increase in the genistein content was achieved at 100.0: 100.0 µM ratio of SER and MEL compared to control at 32 °C (Fig. 3A). The genistin content was increased significantly up to 16.4-fold only in SER and MEL ratio of 100.0: 1.0 µM (Fig. 3B). Whereas glycitin showed a 13.3-fold increase in the lowest concentration (1.0: 1.0 µM) of SER and MEL ratio (Fig. 3C). The daidzein content was increased in all the given SER and MEL ratios, wherein the 9.4-fold increase was observed at the higher ratio (100.0: 100.0 µM) of SER and MEL (Fig. 3D). The daidzin content was not significantly affected by the different ratios of SER and MEL (Fig. 3E). Among the glycoside form of isoflavones, only the glycitin content was increased significantly by 11.2-fold at the lowest concentration of SER and MEL ratios (1.0: 1.0 µM) compared to control (Fig. 3C).

### 3.4. Effect of biomass of callus under SER and MEL ratios at different temperatures

The SER and MEL ratio of 50.5: 50.5 µM showed a significant increase in the biomass at 16 and 24 °C, respectively (Fig. 4A and B). However, at 32 °C, all the SER and MEL ratios showed a significant elevation in the biomass except in a lower ratio of (1.0: 1.0 µM) SER and MEL (Fig. 4C).

### 3.5. Effect of SER and MEL ratios on total phenolics and total flavonoids content at temperature stress

The total phenolics and total flavonoids were quantified using a spectrophotometer at the different SER and MEL ratios at 16, 24, and 32 °C. The significant increase in total phenolics content under SER and MEL treatment was recorded at all the temperatures compared to control (Fig. 5). The total phenolics content was found to be maximum (2.5-fold)

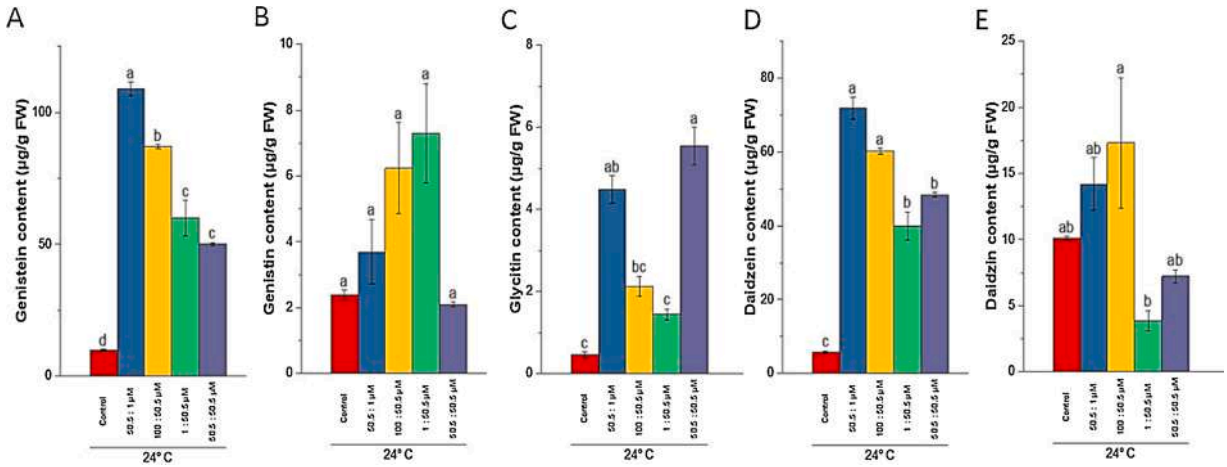


**Fig. 2.** Effect of SER and MEL ratio on isoflavones content at 24 °C A) Genistein (B) Genistin (C) Glycitin (D) Daidzein and (E) Daidzin from cell cultures of soybean. Data are represented as the mean ± standard deviation from four replicates. The Letters, a, b, c above the bars indicate statistical significance; different letters indicate significant differences between two samples and similar letters indicate no significant difference between two samples ($P < 0.05$).

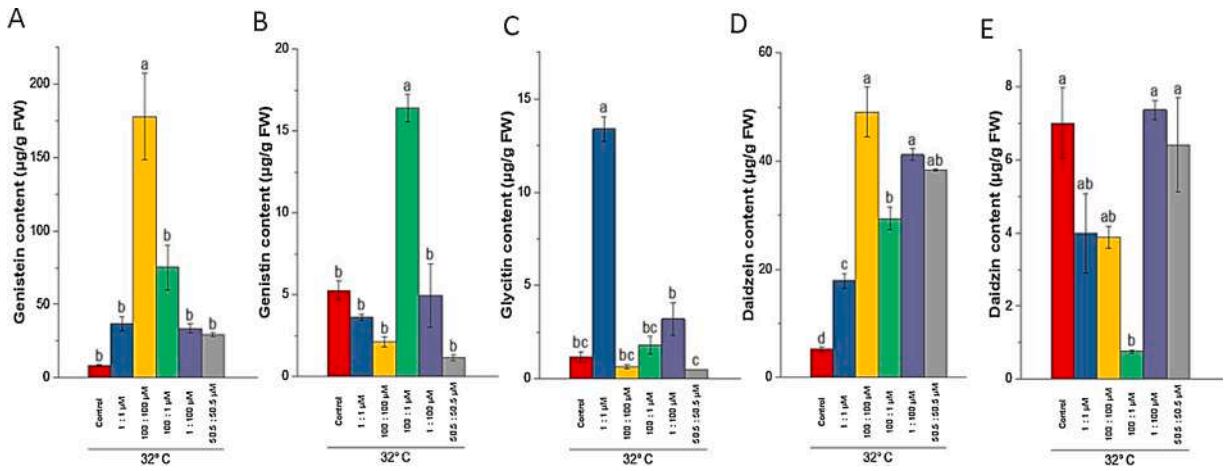


**Fig. 3.** Effect of SER and MEL ratio on isoflavones content at 32 °C A) Genistein (B) Genistin (C) Glycitin (D) Daidzein and (E) Daidzin from cell cultures of soybean. Data are represented as the mean ± standard deviation from four replicates. The Letters, a, b, c above the bars indicate statistical significance; different letters indicate significant differences between two samples and similar letters indicate no significant difference between two samples ($P < 0.05$).

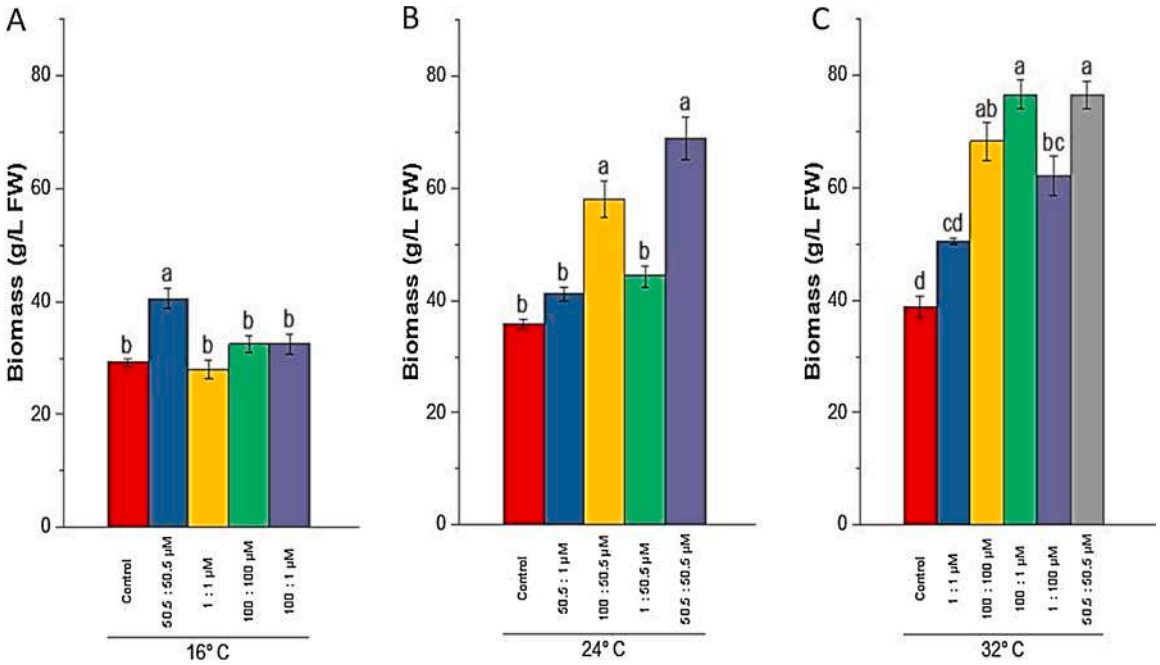


**Fig. 4.** Effect of SER and MEL ratio on biomass at 16 °C (A), 24 °C (B), and 32 °C (C) in cell cultures of soybean. Data are represented as the mean ± standard deviation from three replicates. The Letters, a, b, c above the bars indicate statistical significance; different letters indicate significant differences between two samples and similar letters indicate no significant difference between two samples ($P < 0.05$).

at 16 °C, in 100.0: 100.0 µM of SER and MEL ratio compared to control (Fig. 5A). At 24 °C, all the ratios except for SER and MEL ratio of 1.0: 50.5 µM showed a significant increase in total phenolics content compared to control. A maximum 2.0-fold increase was observed in the 100.0: 50.5 µM ratio of SER and MEL compared to control (Fig. 5B). At 32 °C, a 3.2-fold increase in total phenolics content at SER and MEL ratio of 100.0: 100.0 µM (Fig. 5C).

Varied response for the total flavonoids content was observed for SER and MEL ratios at 16, 24, and 32 °C. At 16 °C, most of the treatments did not show any significant increase in the total flavonoids contents compared to the control (Fig. 5D). At 24 °C, the only higher concentration of SER (100.0 µM) and a moderate level of MEL (50.5 µM) showed a 5.6-fold increase in the total flavonoids content compared to the control (Fig. 5E). At 32 °C, a maximum of 10.2-fold increase was observed in 1.0: 1.0 µM concentration of SER and MEL ratio (Fig. 5F).

### *3.6. Correlation analysis among biomass, total flavonoids, total phenolics, and isoflavone forms*

Pearson's correlation test was performed between the experimental parameters (biomass, total phenolics, total flavonoids, and isoflavones content shown in Fig. 6). A significant positive correlation was observed between the metabolites across different temperatures. At 16 °C, addition of SER and MEL ratio led to positive correlation between total phenolics against total flavonoids and daidzein ($r = 0.82^{***}$ and $0.72^{***}$), daidzein showed strong correlation against genistein ($r = 0.96^{***}$) (Fig. 6A). Similarly, at 24 °C, a significantly strong correlation was observed between total phenolics against total flavonoids and daidzein ($r = 0.82^{***}$ and $0.72^{***}$) and between daidzein and genistein ($0.96^{***}$) (Fig. 6B). Likewise, a salient correlation was detected between biomass and daidzein ($0.74^{***}$) and flavonoids and glycitin ($r =$

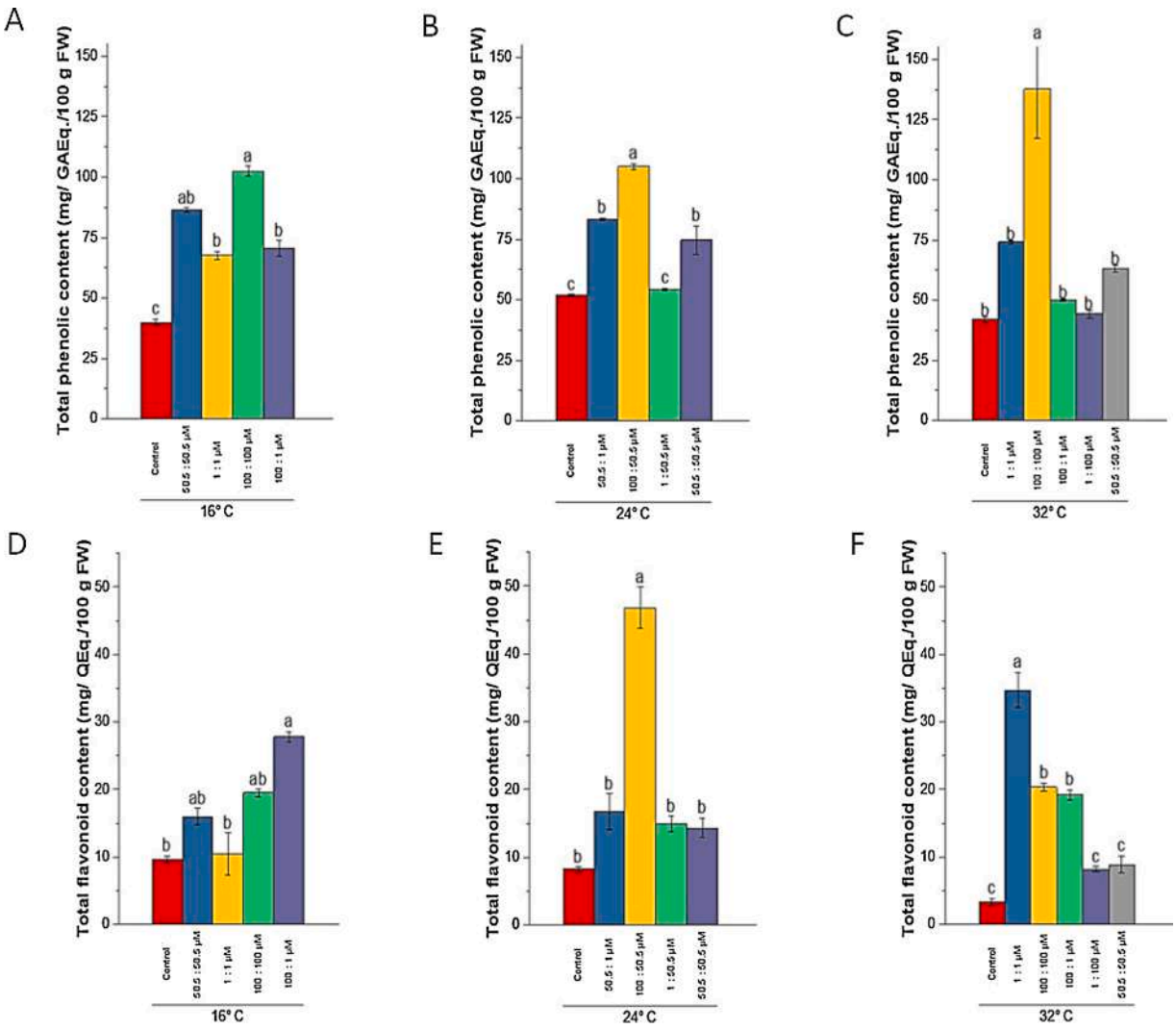


**Fig. 5.** Effect of SER and MEL ratio on accumulation of total phenolics content at 16 °C (A), 24 °C (B), and 32 °C (C). Effect of SER and MEL ratio on accumulation of total flavonoids content at 16 °C (D), 24 °C (E), and 32 °C (F) in cell cultures of soybean. Data are represented as the mean ± standard deviation from three replicates. The Letters, a, b, c above the bars indicate statistical significance; different letters indicate significant differences between two samples and similar letters indicate no significant difference between two samples ($P < 0.05$).

0.76***) at 32 °C (Fig. 6C).

### 3.7. Expression levels of reference genes across SER and MEL ratios

The reference genes (*ACT, ELF, GPD, UBQ-F, UBQ-E, 18 s,* and *60 s)* Cq/Ct values under the different ratios of SER and MEL concentration at 16, 24, and 32 °C are given in Fig. 7. Where lower Cq values correspond to higher expression, and higher Cq values correspond to lower gene expression. The expression of candidate reference genes showed variation across all the treatments and temperatures. Among these, *ACT, UBQ-F, UBQ-E,* and *GPD* had a lower expression, while the *60 s* and *ELF* showed average expression, and only *18 s* showed a higher expression across the treatments and temperatures.

### 3.8. Normalization of reference genes for qPCR analysis

To determine the stability of reference genes (*ACT, ELF, GPD, UBQ-F, UBQ-E, 18 s,* and *60 s)* transcripts under SER and MEL ratios at 16, 24, and 32 °C were analysed. The Ct/Cq values of reference genes are entered into geNorm to calculate the stability and designated M value for all the candidate genes. In geNorm analysis, low average expression stability (M) of candidate genes values corresponds to the stability of gene expression. Based on temperatures and SER and MEL ratios, the reference gene pairs are selected from the geNorm package. The ranking of all the genes based on different SER and MEL ratios at 16, 24, and 32 °C are given in Tables S2, S3, and S4, respectively.

At 16 °C, under control condition (without SER and MEL), *18 s* and *60 s* estimated to have the lowest M value of 0.0047; at SER and MEL (50.5: 50.5 µM) ratio, *UBQ.E,* and *UBQ.F* showed the least M values of 0.0012; at SER and MEL (1.0: 1.0 µM) ratio, *GPD,* and *UBQ.F* showed the least M values of 0.0019; at SER and MEL (100.0: 100.0 µM) ratio, *ACT,* and *UBQ.E* showed the least M values of 0.0044; at SER and MEL (100: 1.0 µM) ratio, *GPD,* and *UBQ.F* showed the least M values of 0.0050. The ranking of candidate reference genes at 16 °C is given in Table S2.

At 24 °C, under control condition (without SER and MEL), the *60 s* and *UBQ.E* are estimated to have the lowest M value of 0.0032; at SER and MEL (50.5: 1.0 µM) ratio, *18 s* and *ELF* showed the least M values of 0.0132; at SER and MEL (100.0: 50.5 µM) ratio, *18 s* and *GPD* showed the least M values of 0.0035; at SER and MEL (1.0: 50.5 µM) ratio, *ACT* and *UBQ.F* showed the least M values of 0.0026; at SER and MEL (50.5: 50.5 µM) *ELF* and *UBQ.F* showed the least M values of 0.0029. The ranking of candidate reference genes at 24 °C is given in Table S3.

At 32 °C, under control condition (without SER and MEL), the *18 s* and *UBQ.F* are estimated to have the lowest M value of 0.0041; at SER

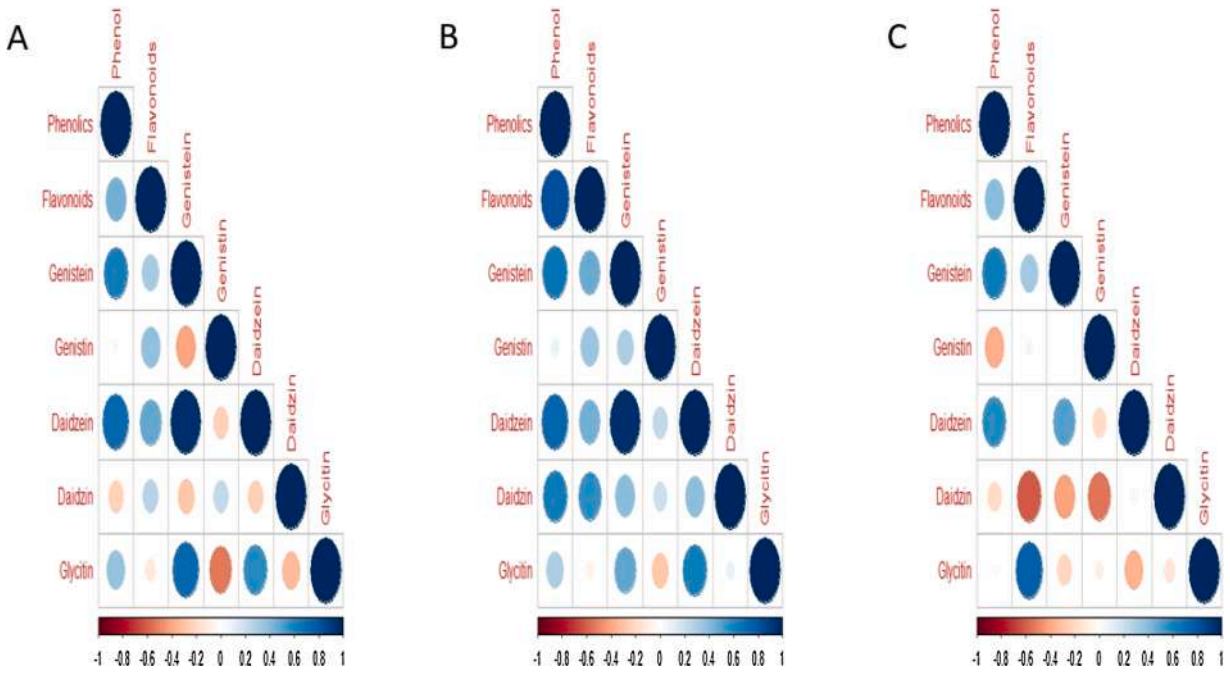


**Fig. 6.** Correlation matrix of biomass, total phenolics, total flavonoids and isoflavones under SER and MEL ratio at (A) 16 °C, (B) 24 °C and (C) 32°. The color and the shape of each dot in the triangular matrix show the strength of Pearson correlation (positive or negative) between metabolites. Positive strong correlations are in dark blue. Lighter colors indicate the weaker relations between the metabolites. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

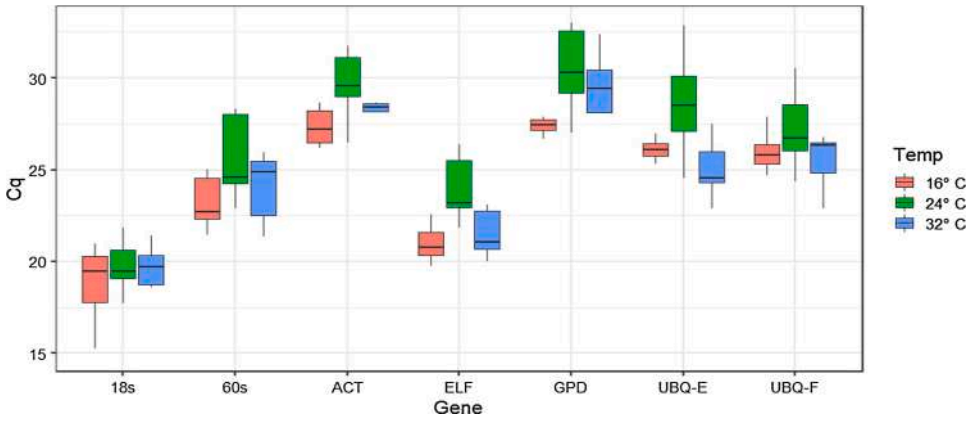


**Fig. 7.** Expression levels of reference genes across SER and MEL ratio in cell culture of soybean. The line across the box-plot indicates the median. Lower and upper boxes represent 25th and 75th percentiles. Whiskers represent the lowest and highest Cq values respectively.

and MEL (1.0: 1.0 μM) ratio, *18 s,* and *ELF* showed the least M values of 0.0037; at SER and MEL (100.0: 100.0 μM) ratio, *18 s* and *UBQ.F* showed the least M values of 0.0018; at SER and MEL (100.0: 1.0 μM) ratio, the *60 s* and *UBQ.F* showed the least M values of 0.0015; at SER and MEL (1.0: 100.0 μM) *GPD* and *UBQ.E* showed the least M values of 0.0025; at SER and MEL (50.5: 50.5 μM) *ACT* and *UBQ.F* showed the least M value 0.0207. The ranking of candidate reference genes at 32 °C is given in Table S4.

### 3.9. Differential expression of genes under SER and MEL ratios

To quantify the expression of genes under SER and MEL ratio at a different concentration, the soybean cell culture treated with different SER and MEL ratios were evaluated with the expression of biosynthesis pathways: isoflavone (*PAL*, *CHS*, *CHR*, and *IFS*), ethylene (*ACCO* and *SAM*), transcription factor (*MYB* and *NPR*). All the selected genes for the experiment showed different expressions across SER and MEL treatments. The R package, 'ComplexHeatmap' [37], and 'Enhanced-Volcano' [36] were followed to visualize the differential expression of genes.

### 3.10. Expression of isoflavone biosynthetic pathway genes under SER and MEL ratios

The isoflavone biosynthesis genes (*PAL, CHS, CHR, CHI, and IFS*) were selected to study the influence of the addition of SER and MEL ratio in the soybean cell culture. As shown in Fig. 8, the expression of *PAL, CHS, CHR, CHI, and IFS* increased considerably in all the given ratios compared to the control.

At 16 °C, *PAL* showed a slight difference in expression pattern among all the ratios of SER and MEL among all the isoflavone biosynthesis genes. *PAL* showed an 8.5-fold increase in SER and MEL ratio of 100.0: 1.0 μM compared to control (without SER and MEL). The *CHS* expression did not vary considerably among all the given ratios of SER and MEL,

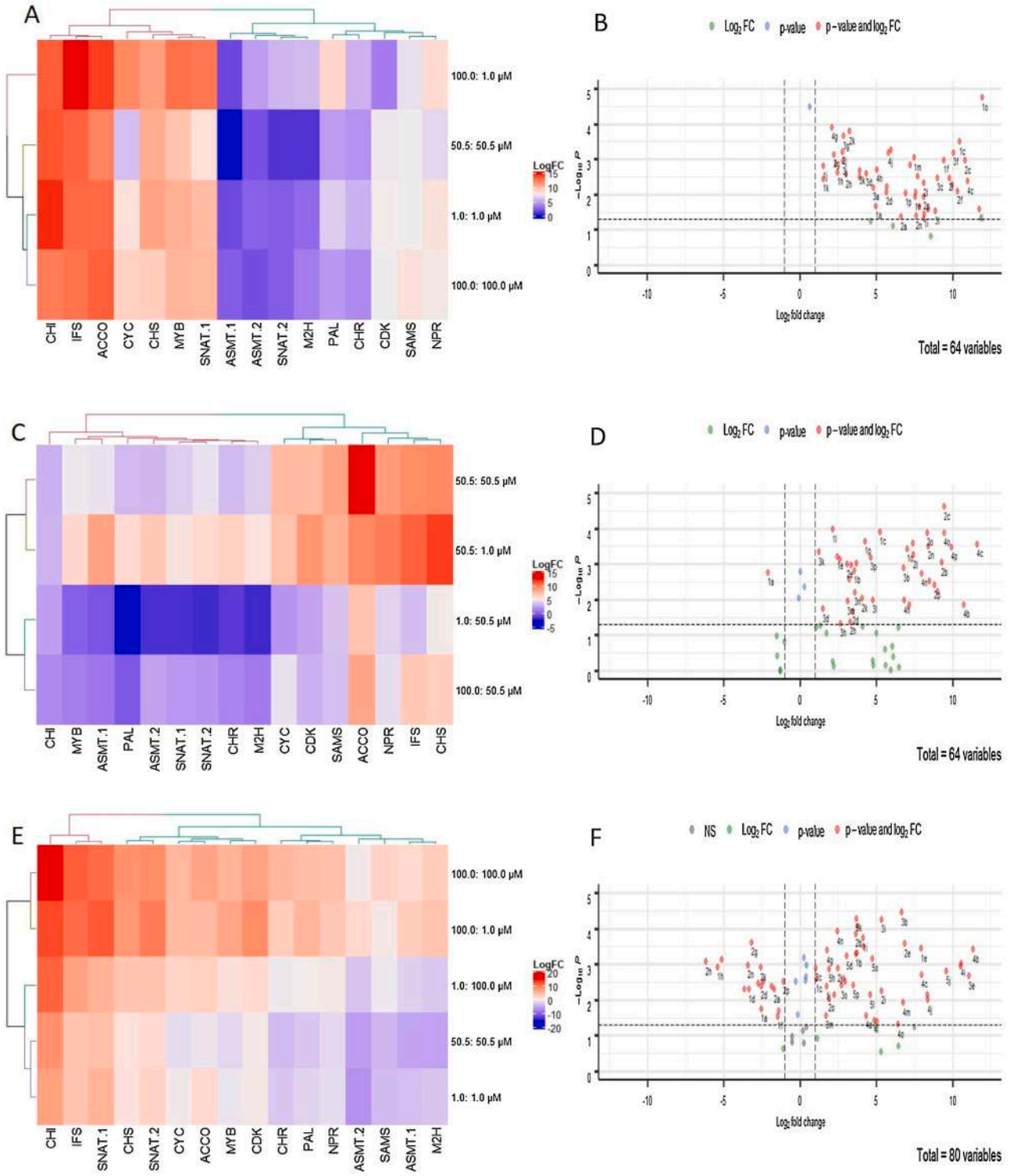


**Fig. 8.** The gene expression analysis of listed genes under SER and MEL ratio. The differential expression at 16 °C (A), 24 °C (C), and 32 °C (E) are shown in the clustered Heatmap for the dataset. The heatmap was generated in the complex Heatmap R package tab using the hierarchical clustering method and Euclidean distance measurements. Column (gene) clustering was enabled. Clustered heatmaps are useful for displaying expression changes across different concentration and treatments groups. Volcano plot of 16 °C (B), 24 °C (D), and 32 °C (E) highlighting genes that meet both log fold change and Padj cut-off of the datasets. The volcano plot was generated using enhanced volcano package in R with log fold change cut-off set to ± 1.5 and the Padj cut-off set to < 0.05. The volcano plot also illustrates the statistical significance of genes (the y-axis) and each significant fold changes are represented in red dot and the number and alphabet represent gene and ratio of SER and MEL concentration (Supplementary file. 1, 2, and 3). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

with a maximum expression of 10.9-fold was observed at 100.0: 1.0 μM (SER and MEL) ratio. The *CHR* expression was up-regulated by 5.6-fold both in 100.0: 1.0 and 1.0: 1.0 μM (SER and MEL) ratios showed similar expression, and 4.6-fold is observed in 50.5: 50.5 μM SER and MEL ratio. The *CHI* showed the maximum response of 14.1, 13.3, and 13.1-fold higher with SER and MEL ratio of 1.0: 1.0, 50.5: 50.5, and 100.0: 1.0 μM, respectively. The *IFS* showed maximum up-regulation of 14.5 and 13.02-fold in SER and MEL ratios of 100.0: 1.0 and 50.5: 50.5 μM compared to control. The *MYB* transcription factor involved in the regulation of *IFS* and *CHS* also showed up-regulation under all the SER and MEL ratios compared to the control condition. The *MYB* showed a maximum up-regulation of 12.5- fold in 100.0: 1.0 μM SER and MEL ratio (Fig. 8A and B).

At 24 °C, the expression of isoflavone biosynthesis genes varied drastically between the various ratios of SER and MEL. The expression of *PAL, CHS, CHR, CHI,* and *IFS* showed maximum fold changes in 50.5: 1.0 μM of SER and MEL ratios compared to other SER and MEL ratios and control. The *PAL, CHS, CHR, CHI,* and *IFS* showed maximum expression of 5.9, 11.6, 6.4, 3.2, and 10.7-fold, respectively, at 50.5: 1.0 μM SER and MEL ratio. The *MYB* transcription factor also showed a maximum up-regulation of 6.1-fold at 50.5: 1.0 μM of SER and MEL ratio (Fig. 8C and D).

At 32 °C, the expression of *PAL, CHS, CHR, CHI,* and *IFS* showed maximum up-regulation in both 100.0: 100.0 and 100.0: 1.0 μM (SER and MEL) ratios respectively. The maximum *PAL* expression was up-regulated by a 5.0-fold increase in the 100.0: 1.0 μM (SER and MEL) ratio. Whereas *CHS, CHR CHI,* and *IFS* showed maximum up-regulation of 7.9, 4.9, 14.4, and 11.3-fold respectively at 1: 100 μM (SER and MEL) ratios. The *MYB* transcription factor also showed maximum up-regulation of 6.4 and 1.1 5.2-fold at 100.0: 1.0 and 1: 100 μM (SER and MEL) ratios (Fig. 8E and F).

### 3.11. Expression of putative MEL biosynthesis genes under SER and MEL ratios

The MEL is synthesised from SER through the enzymatic activity of *ASMT* and *SNAT*. Two putative *ASMT* and *SNAT* enzymes showed varying effects at different ratios of SER and MEL at different temperatures. At 16 °C, *ASMT-1* showed a maximum up-regulation of 2.8-fold in SER and MEL ratio of 100.0: 100.0 μM. Whereas, *ASMT-2*, *SNAT-1,* and *SNAT-2* showed significant up-regulation of 5.0, 12.3, and 5.7-fold change respectively in 100.0: 1.0 μM SER and MEL ratio compared to the control culture (Fig. 8A and B). The higher SER ratio with respect to MEL has increased the expression of putative MEL biosynthesis genes at a lower temperature. The putative *M2H* involved in MEL degradation showed maximum up-regulation of 5.9-fold change in 100.0: 1.0 μM SER and MEL ratio, where the maximum up-regulation of *ASMT* and *SNAT* was observed.

At 24 °C, the maximum up-regulation of 8.4, 6.8, 5.5, and 6.0-fold change was observed in putative *ASMT-1*, *ASMT-2*, *SNAT-1,* and *SNAT-2*, under 50.5: 1.0 μM SER and MEL ratio compared to control. The expression of putative MEL synthesis genes showed down-regulation in the presence of a higher ratio of MEL (Fig. 8C and D). The putative M2H also showed a higher up-regulation of 5.6-fold in a similar concentration of 50.5: 1.0 μM SER and MEL ratio, where MEL biosynthesis genes were up-regulated.

At 32 °C, the maximum down-regulation of -4.9 and -6.1-fold was observed in *ASMT-1* and *ASMT-2* in 50.5: 50.5 and 1.0: 1.0 μM SER and MEL ratios. The maximum up-regulation of 2.8, 1.8, 11.6, and 9.5-fold in *ASMT-1, ASMT-2, SNAT-1,* and *SNAT-2* was observed in 100.0: 1.0 μM of SER and MEL ratio. The putative *M2H* also showed a maximum up-regulation of 3.6-fold in 100.0: 1.0 μM SER and MEL ratio (Fig. 8E and F). At 32 °C, the higher concentration leads to a higher expression of MEL biosynthesis and degradation genes.

### 3.12. Expression of cell division genes under SER and MEL ratios

*CYC* and *CDK* expressions are both up-regulated in all the given concentrations of SER and MEL ratio at 16 and 24 °C compared to control. At 16 °C, *CYC* and *CDK* expression showed maximum up-regulation of 11.7 and 7.6- fold with 100.0: 1.0 and 1.0: 1.0 μM SER and MEL ratios compared to control (Fig. 8A and B). The higher concentration of SER compared to the MEL ratio promoted a higher expression of *CYC* and *CDK*. At 24 °C, the *CYC* showed maximum up-regulation of 7.4 and 7.1-fold with 50.5: 50.5 and 50.5: 1.0 μM. The *CDK* showed maximum up-regulation of 8.9 and 7.3-fold 50.5: 1.0 and 50.5: 50.5 μM SER and MEL ratio compared to control (Fig. 8C and D). At 32 °C, the higher concentration of SER compared to the MEL ratio increased the expression of *CYC* and *CDK* genes compared to the control. The maximum up-regulation of 4.8 and 8.3-fold was observed in *CYC* and *CDK* at 1.0: 100.0 and 100.0: 1.0 μM of SER and MEL ratios (Fig. 8E and F).

### 3.13. Expression of ethylene biosynthesis genes under SER and MEL ratios

The expression of *SAMS* and *ACCO* involved in ethylene biosynthesis was up-regulated in all the given SER and MEL ratios at 16 and 24 °C. At 16 °C, the *SAMS* and *ACCO* showed similar pattern expression in all the treatments compared to the control. The *SAMS* and *ACCO* showed maximum up-regulation of 8.1 and 13.8 -fold with 100.0: 100.0 and 100.0: 1.0 μM SER and MEL ratios (Fig. 8A and B). At 24 °C, the equal ratio of SER and MEL (50.5: 50.5 μM) showed the maximum up-regulation of 8.4 and 12.3-fold in *SAMS* and *ACCO* gene expression compared to control (Fig. 8C and D). At 32 °C, the treatment showed both up-regulation and down-regulation of the ethylene biosynthesis genes. The lower concentration of SER in the SER and MEL ratio resulted in the down-regulation of *SAMS* and *ACCO* gene expression. The maximum down-regulation of -3.0 and -0.5-fold was observed in 50.5: 50.5 μM SER and MEL ratio (Fig. 8E and F). The *SAMS* and *ACCO* showed maximum up-regulation of 2.4 and 6.4-fold change in 1.0: 100.0 μM SER and MEL ratio.

### 3.14. Expression of NPR transcription factor under SER and MEL ratios

The *NPR* involved in stress response in plants was shown up-regulation in all the treatments of SER and MEL ratio at 16 and 24 °C (Fig. 8). Whereas, at 32 °C, the expression was down-regulated by -1.4 and -1.0-fold 50.5: 50.5 and 1.0: 1.0 μM SER and MEL ratios compared to control. At 16 °C, *NPR* showed maximum up-regulation of 8.3-fold at 100.0: 1.0 μM (Fig. 8A and 8B), at 24 °C, 9.9-fold expression was observed in 50.5: 1.0 μM (Fig. 8C and 8D), at 32 °C, 4.1-fold expression was observed in 1.0: 100.0 μM SER and MEL ratio (Fig. 8E and F).

## 4. Discussion

The SER and MEL ratios significantly influenced isoflavones content and cell biomass in soybean cell cultures in all temperatures. The role of MEL in increasing secondary metabolite was reported in *Leucojum aestivum in vitro* cultures [38]. The lower temperature limits the growth, development, and biomass of many plant species [39]. However, the exogenous SER and MEL application increased the growth, biomass, and isoflavones (genistein and daidzein) content at 16 °C in soybean culture compared to control. Application of MEL is reported to give resistance by scavenging reactive oxygen species, modulating redox balance, and polyphenols in the regulation of cold stress. In the ambient temperature of 24 °C, the SER and MEL ratio significantly increased genistein, daidzein, and glycitin content in a higher MEL concentration and a lower SER concentration. The higher temperature (37 °C) has been shown to decrease the isoflavones content in soybean seeds and pods [40], as observed in the present study in the control group at 32 °C. However, SER and MEL's application resulted in a significant increase in

isoflavones content (genistein, daidzein, genistin, and glycitin) compared to the control at 32 °C. Previous reports also indicate that the MEL application increased the heat tolerance in plants and increased the germination percentage in *Arabidopsis thaliana* [8].

The soybean cell culture biomass was increased significantly at higher temperatures (32 °C), in a higher ratio of SER and MEL compared to the control. Similarly, the SER and MEL treatment induced the growth and biomass under abiotic stress by regulating gene expression and metabolism associated with auxin-responsive pathways [13,41]. The exogenous application of all SER and MEL ratios in the present study resulted in a 1.0–3.2-fold increase in total phenolics content compared to their corresponding temperature control by increasing the expression of *ASMT* and *SNAT* genes (Fig. 5A–C). Also, the MEL treatment of Santa rose fruit showed increased total phenolics content and bioactive compounds during cold storage [42]. The SER and MEL ratio significantly improved total flavonoids in all the temperatures. Still, a maximum 10-fold increase was observed at 32 °C, compared to control, demonstrating its importance in elevating flavonoids content against temperature stress as a defensive strategy. Similarly, MEL treatment in *Vitis vinifera* cell suspension cultures enhanced total phenolics and flavonoids under temperature stress [43].

The expression of the selected reference genes varied considerably under SER and MEL's different ratios at different temperatures. Accurate gene expression studies are needed to validate the reference gene for the precise gene expression analysis, and it is essential to validate reference genes in other plant species and experimental conditions [44]. The selected reference genes ranking under SER and MEL ratio under different temperatures are given in Table. S2, S3, and S4. MEL and SER treatment significantly upregulated the expression of isoflavone biosynthesis genes at 16 °C (Fig. 8B). The *MYB* transcription factor involved in the regulation of isoflavone biosynthesis was also co-expressed with the *CHS* and *IFS* expression demonstrating the involvement in isoflavone biosynthesis genes up-regulation [30]. It shows SER and MEL's probable participation in influencing isoflavone and ethylene biosynthesis genes at different temperatures in soybean cell cultures. Exogenous MEL improved the endogenous MEL pool *in Arabidopsis thaliana* and *Solanum lycopersicum* by activating the MEL biosynthesis genes under temperature stress [45,46]. Similarly, in the present research, SER and MEL application increased the *ASMT* and *SNAT* expression involved in MEL biosynthesis. Exogenous application of SER and MEL increased *SNAT* expression compared to *ASMT* at 16 and 32 °C. Similar studies have revealed that overexpression of human *SNAT* plays a crucial role in cold resistance transgenic rice seedlings [47]. Whereas at 24 °C, the *ASMT* and *SNAT* expression recorded similar expressions in MEL biosynthesis. Similarly, in *Solanum lycopersicum*, the exogenous MEL triggered *ASMT* and *SNAT* expression during cold stress. The *ASMT* and *SNAT* deficient *Oryza sativa* lines showed decreased tolerance to salt and cold stress [15]. Also, the higher expression of *SNAT* has positively regulated the growth and salt tolerance in *Cucumis sativus* [48]. The MEL is enzymatically metabolized into 2-hydroxymelatonin by the plants *M2H* genes [43]. In our study, the higher expression of *ASMT/SNAT* correlated with the *M2H* gene expression in the SER and MEL ratio at different temperatures. Moreover, the expression of *ASMT* was transcriptionally induced by *M2H* genes in *Oryza sativa* [49].

*CDK* controls the cell division in plants and animals; its activity is controlled through the association of regulatory cyclin subunit, determining the timing of *CDK* activation and promoting progression through the cell cycle [50]. The SER and MEL application promoted cell proliferation and biomass by increasing *CYC* and *CDK* activity in cell cultures under temperature stress. Similarly, the MEL exhibited growth, development, and yield during abiotic stress in soybean plants [51].

## 5. Conclusion

The biotic and abiotic stress may significantly increase secondary metabolites content over the unfavorable condition, reducing the plant's growth and development. However, the present research suggests that SER and MEL can increase secondary metabolites without adverse effects on cell division and biomass production in soybean cell culture. The application resulted in regulating temperature stress and promoting cell growth and isoflavones accumulation in lower and higher temperature stress in soybean culture. The SER and MEL lead to the co-expression of isoflavone and ethylene biosynthesis genes suggesting the probable involvement of ethylene in increasing the isoflavone biosynthesis genes and, in turn, increasing isoflavones content under temperature stress. Further studies on exploring the interplay of SER and MEL synthesis and metabolism will confer a broader understanding of the mechanism by which isoflavone biosynthesis responds at different temperature stress in the agriculture fields. The outcome of the present study provides an insight into SER and MEL's role on plant growth and secondary metabolite production during adverse temperature thus by broadening SER and MEL biological function in plants.

## Author's contributions

The experiment was designed and supervised by PG, NPS, BP and MP who also assisted in writing and editing the manuscript. GK, KRS, and MA designed, performed the experiments and wrote the manuscript.

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## Intellectual property

Yes, we confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property.

## Authorship

Yes, all listed authors meet the ICMJE criteria. We attest that all authors contributed significantly to the creation of this manuscript, each having fulfilled criteria as established by the ICMJE.

Yes, we confirm that the manuscript has been read and approved by all named authors.

Yes, we confirm that the order of authors listed in the manuscript has been approved by all named authors.

## Declaration of Competing Interest

The authors report no declarations of interest.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.cpb.2021.100206.

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