Journal of Pineal Research

WILEY

Journal of Pineal Research
Molecular, Biological, Physiological and Clinical Aspects of Melatonin

ORIGINAL ARTICLE OPEN ACCESS

Microbial Melatonin Production Improves Plant Metabolic Function in Short-Term Climate-Induced Stresses

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Received: 29 March 2025 | **Revised:** 29 March 2025 | **Accepted:** 28 April 2025

Keywords: abiotic stresses | *Bacillus velezensis* | heat stress | ICP-MS | melatonin | melatonin producing microbes | metabolites | metabolomics | PGPB | salt stress

ABSTRACT

Climate change, specifically high temperatures, can reduce soil moisture and cause hypersaline conditions, which creates an unsustainable agro-production system. Microbial symbionts associated with plants relinquish stressful conditions by producing stress-protecting substances. Melatonin is a signaling and stress-protecting molecule for plants, but is least known for microbial symbionts and their function in stress protection. Here, our study shows that the melatonin-synthesizing *Bacillus velezensis* EH151 (27.9 ng/mL at 96 h) significantly improved host plant (*Glycine max* L.) growth, biomass, photosynthesis, and reduced oxidative stress during heat and salinity stress conditions than the non-inculcated control. The EH151 symbiosis enhanced the macronutrient (P, Ca, and K) and reduced Na uptake in shoots during stress conditions. The microbial inoculation significantly expressed the *high-affinity* K^+ *transporter*, *MYB transcription factor*, *Salt Overly Sensitive 1*, Na^+/H^+ *antiporter 2*, and *heat shock transcription factors* in spatio-temporal orders during heat and salinity stress (H&S 1, 3, 10, and 14 h). We observed that microbial strain significantly increased the plant's endogenous abscisic acid (49.5% in H&S 10 h), jasmonic acid (71% in H&S 10 h), and melatonin biosynthesis (418% in H&S 14 h). Metabolome map of plant defense response showed that EH151 enhanced activation of amino acid metabolism pathways (e.g., glutamate (34%) L-aspartate (82%), glycine (18.5%), and serine (58%) under H&S 14 h compared to non-inoculation). Conversely, the free sugars and organic acids within the central carbon metabolism were significantly activated in non-inoculated combined heat and salinity stress compared to inoculated plants—suggesting lesser defense energy activated for stress tolerance. In conclusion, the current results show promising effects of the microbial abilities of melatonin that can regulate host growth and defense responses. Utilization of beneficial strains like *B. velezensis* EH151 could be the ideal strategy to improve stress tolerance and overcome the adverse impact of climate-induced abrupt changes.

1 | Introduction

A growing world population necessitates that agricultural production double by 2050. However, over 15% of global land is expected to experience heat stress, affecting food production and human health [1]. The major staple crops such as rice, wheat, maize, and soybean [2] meet two-thirds of human energy needs and provide 33% of human dietary protein [1]. Global warming has heightened the risk of heat and salinity stress influencing crop yields, specifically short episodes of high temperature at early seedling stages. Heat and its resulting subsidiary salinity stress in agricultural lands affects 20%–33% of cultivated and irrigated land worldwide, with adverse effects projected to reach 50% by 2050 [3, 4]. Similarly, it has been observed that the increasing soil sodicity, inducing salinity stress in crops, has threatened the sustainable agri-production. Hence, it is crucial to understand the mechanism of crops enduring multiple stresses during agricultural production and their mitigation measures to stimulate real-world conditions [5, 6].

Every abiotic stress induces a certain physiological pattern in crops, which may potentially lead to plant death. In the case of heat stress, it affects respiration by altering mitochondrial activity, which can increase or decrease depending on the crop [7, 8]. Increasing temperature exponentially raises the respiration rates of the plants; however, once a critical threshold is exceeded, respiration rate declines due to damage to the respiratory mechanisms [9]. Plasma membranes, the primary site of injury in plant cells, are considered the most heat-sensitive components [10]. Heat injury transforms the membranes of sensitive plants from a solid gel structure to a flexible liquid crystal structure [11]. When temperatures exceed the optimal range, proteins deactivate, altering enzyme activity and increasing reactive oxygen species (ROS) levels [11, 12]. An increase in temperature is coupled with a loss of soil moisture, which results in salinity stress [13]. With salt stress, the initial disruption in metabolism begins with ionic imbalance, resulting in osmotic stress [14]. This becomes a double-edged sword for plants to deal with and maintain a high flux of defense responses. However, such mitigating measures can be quickly exhaustive for plants and rely on more symbiotic microbes to help [15].

The interaction of plant–microbe–soil interplay plays a crucial role in regulating metabolites to confer stress tolerance. Metabolites are fundamental components in biochemical and ecological processes and serve as direct precursors of phenotypes [16]. Under abiotic stress, plants can adapt without exhibiting phenotypic changes until reaching a "point of no return", beyond which mortality becomes inevitable [17]. In contrast, molecular and metabolic responses typically occur rapidly, almost instantaneously, after the onset of stress, well before physiological stress symptoms are observable [18]. Consequently, these responses offer insights into the underlying mechanisms of stress, which can inform the development of more effective management strategies and targeted conservation efforts [17, 19].

Microbes, including bacteria, fungi, algae, and protozoa, inhabit most soils, with bacteria being the most prevalent [20]. Plant roots release sugars, amino acids, and organic acids, leading to higher bacterial concentrations in the rhizosphere than in the bulk soil [21]. Bacteria living in the rhizosphere, phyllosphere, or endosphere that enhance plant growth are termed "plant probiotic bacteria" or "PGPB" [22]. Microorganisms introduced to stressed plants can improve growth, yield, and overall health [23]. Consequently, many commercially available bacterial inoculants are now used as biofertilizers and biopesticides [24]. Therefore, using beneficial bacteria as bioinoculants is a potentially cost-effective and environmentally friendly strategy to enhance crop productivity on marginalized agricultural lands [25]. Inoculating plants with phytohormone-producing plant growth- promoting rhizobacteria (PGPR) can enhance crop physiology, biomass, and yield by increasing plant biomass and leaf area, indicating better metabolic activity at different growth stages [24, 26, 27]. Similar benefits have been observed with exogenous application of various growth regulators such as auxins (indole acetic acid—IAA), gibberellin, proline, ascorbic acid, and organic acids, which work as crucial plant growth regulators under normal and stressful conditions [28–32]. Likewise, secondary metabolites, such as melatonin, synthesized by the plant but recently reported to be produced by microbes, have been proposed as key signaling molecules [33, 34].

Numerous studies have shown that melatonin regulates plant growth, promotes rhizogenesis, acts as an antioxidant, delays leaf senescence, and enhances stress tolerance and redox signaling when applied exogenously [35, 36]. The term "phytomelatonin" was introduced in 2004 [37], and ongoing studies have quantified melatonin levels in various plants [38]. Recent research has also highlighted melatonin synthesis in microorganisms, revealing that their production capacity often surpasses that of plants [39, 40]. However, PGP microorganisms that produce melatonin have yet to be extensively studied, unlike those producing other phytohormones and secondary metabolites, and the synthetic pathway of melatonin in bacteria remains unclear.

To our knowledge, melatonin-producing plant growth-promoting microorganisms (PGPMs) hold significant potential to enhance plant growth and stress resistance, similar to the effects of other exogenous phytohormones [34, 39]. Still, only a few comprehensive studies have investigated the influences of melatonin-producing PGPB on crops under abiotic stresses, and especially, there has been no related research conducted on combined heat and salt stress. Thus, we hypothesized that application of melatonin-producing EH151 would ameliorate the toxicity of the combined heat and salinity stress and confer stress tolerance in soybean. Here, this study has three main objectives: (1) to isolate melatonin-producing PGPB, establish the melatonin production pathway of the bacteria, and determine the optimal conditions for melatonin production; (2) to further investigate its physiological parameters related to antioxidant activation, phytohormones modulation, and ion interplay with its associated transcriptomes in soybean plants on exposure to the combined heat and salinity stress; and (3) to examine the process through which melatonin-producing microorganisms alleviate high-temperature and high-salt stress in soybeans, focusing on the central carbon

metabolism (CCM) of plant leaves by analyzing glycolysis, the tricarboxylic acid (TCA) cycle, and respiratory chain pathways. This study is novel and innovative not only in its discovery of new melatonin-producing microorganisms with PGP capabilities but also in its exploration of whether these microorganisms can be utilized as an environmentally friendly agricultural tools to alleviate combined salinity and heat stresses; this approach may also contribute to developing heat-tolerant soybean varieties in response to dramatic climate change and it can share potential alternatives to the urgent issue of food shortages resulting from climate change faced by humanity.

2 | Materials and Methods

2.1 | Selection of Melatonin-Producing *Bacillus velezensis* EH151

For this study, 751 bacterial strains were isolated from the rhizosphere soils of high-salinity greenhouse facilities cultivating strawberries, oriental melons, and tomatoes located in Gunwi, South Korea (36°06′38.2″ N 128°38′38.4″ E). Then, a series of assays were conducted to evaluate the plant growth-promoting (PGP) properties of these isolates. The isolates were inoculated on CAS-Blue media, phosphate solubilization media, and EPS production media to assess siderophore production, phosphate solubilization ability, and extracellular polymeric substance (EPS) production, respectively. The auxin production was quantified with Salkowski reagent under dark conditions, followed by absorbance measurement at 530 nm using a spectrophotometer.

Based on the results of these assays, 100 bacterial strains with superior PGP capabilities were selected. These selected strains were further evaluated for their melatonin production. For this, the strains were cultured for 2 days and then, each of 100 different culture broths was centrifuged. The supernatant was then mixed in a 1:1 ratio with ethyl acetate and vortexed, then the upper layer was concentrated under reduced pressure using a rotary evaporator. Finally, the melatonin content in the culture supernatant was determined using a melatonin ELISA kit (Enzo Life Sciences, USA) with spectrophotometric analysis.

Then, the highest melatonin-producing 10 microbes were selected and tested again for melatonin synthesis in 150 mM NaCl and at 42°C. The PGPB that produced the highest melatonin quantity under these conditions was selected for identification through 16S rRNA sequencing. The results identified the isolate as *Bacillus valenzensis*, and it was submitted to the NCBI database (GenBank accession number: PP967898).

2.2 | Quantification of Melatonin Produced by EH151 Under Various Conditions at Time Intervals

For further validation, the strain EH151 was grown in LB broth cultures spiked with tryptophan and serotonin at 28°C for 1, 3, 6, 12, 24, 48, 72, and 96 h to check the melatonin biosynthesis precursors of EH151. The cultures were centrifuged at 1000 *g* for 10 min at 4°C. Subsequently, 5 mL of the resulting supernatant was separated, vortexed, and partitioned with an equal volume of ethyl acetate. Furthermore, the EH151 were grown in LB broth spiked with 150 mM NaCl at 42°C for 1, 3, 6, 12, 24, 48, 72, and 96 h, and the cultures were analyzed with melatonin quantification.

2.3 | Plant Materials and Experiments

2.3.1 | NaCl, Heat, and EH151 Treatment for Soybean Plants

Soybean seeds (var. Pungsan) were germinated, and equal-sized seedlings at the VC stage were transplanted into pots (Inner diameter: 11.4 cm Height: 10.6 cm) (one seedling/pot) containing autoclaved sandy soil. Figure 1A describes how these plants were maintained under greenhouse conditions at 70% RH and a temperature of 25 ± 4°C and irrigated with EH151 cultures. After completing the EH151 treatments, the bacteria could acclimate and associate with plant roots for 15 days. The seedlings were then subjected to 150 mM NaCl and 42°C heat stress in a growth chamber (light intensity 1000 E $m^{-2} s^{-1}$ and relative humidity 60%–70%) according to the volumes and time duration detailed in Figure 1A. Following the salt and heat stress treatments, the plants were irrigated for 3 days, and relevant data were recorded. Plant samples were then collected in liquid nitrogen and stored at −80°C for further analysis.

2.3.2 | Hyper-Spectral Imaging

The effect of EH151-mediated mitigation on plant growth under heat and salinity stress was evaluated by measuring growth indices using hyperspectral imaging. Vegetative indices, including the normalized difference vegetation index (NDVI), photochemical reflectance index [41], and anthocyanin reflectance index (ARI), were calculated with a Specim IQ hyperspectral camera (Specim, Finland). These indices are commonly used in remote sensing and plant sciences to assess plant health, stress levels, and overall productivity [42].

2.3.3 | Melatonin Quantification in Soybean

Melatonin levels in the leaves of control and treated soybean plants were quantified using the Ultra-sensitive Melatonin ELISA kit (Enzo Life Sciences, USA) following the manufacturer's instructions. Leaf samples weighing 0.05 g were homogenized in 125 μL of 1× stabilizer. After adding 750 μL of ethyl acetate, the mixture was vigorously vortexed. The samples were then incubated on ice for 5 min and centrifuged at 1000 *g* for 10 min. The organic layers were transferred to fresh tubes and dried using a SpeedVac ([SPD2030, Thomas Scientific, USA] vacuum centrifuge with a vapor trap). The dried samples were resuspended in 400 μL of 1× stabilizer and quantified following the protocol of the kit. Optical density was measured at 450 nm using a spectrophotometer (Multiskan GO, Thermo Fischer Scientific, USA) as previously described [43].

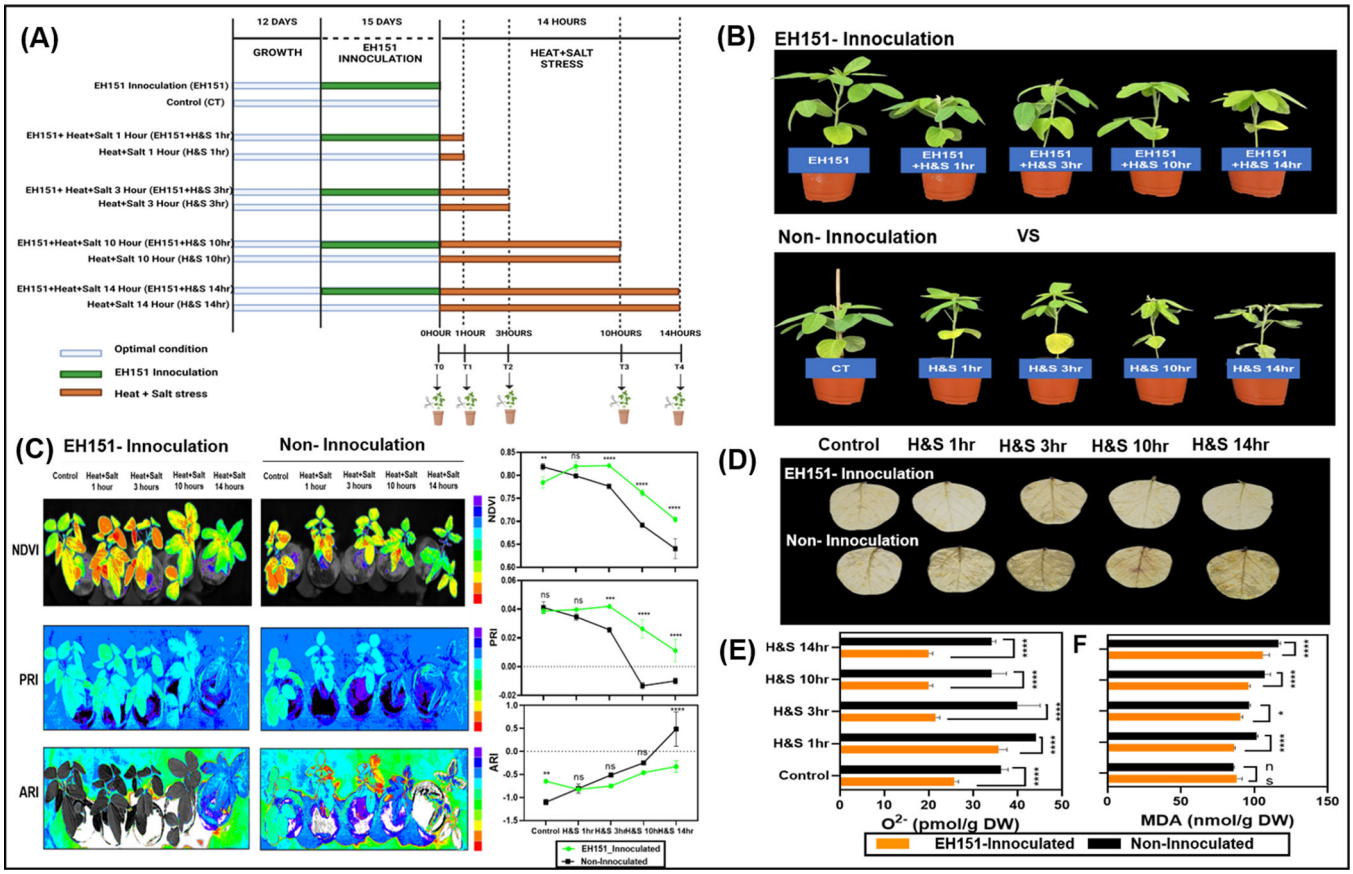

FIGURE 1 | *B. velezensis* EH151 improves plant growth and development and mitigates oxidative stress induced by the combined heat and salinity stress. (A) Effect of EH151 soil application on the growth and development of soybean plants subjected to H&S stress. (B) Close-up images of soybean plants showing symptoms of H&S-induced toxicity and the alleviation of these symptoms by EH151. (C) Hyperspectral imaging analysis showing mean values of NDVI, PRI, and ARI. (D) In situ detection of ROS in soybean leaves subjected to H&S stress following EH151 inoculation. (E and F) O_2^- and MDA in soybean leaves. Data represents the means of at least three independent biological replicates. Error bars represent standard deviations. Error bars represent standard deviations. Means were compared using Tukey's post hoc test. * indicates $p < 0.05$, ** indicates $p < 0.01$, and *** indicates $p < 0.001$.

2.3.4 | ICP Ion Analysis for K^+, Na^+, Ca^{2+}, P, and Mg^{2+}

K^+, Na^+, Ca^{2+}, P, and Mg^{2+} levels in plant samples were measured using inductively coupled plasma mass spectrometry (ICP–MS) with an Optima 7900DV system (PerkinElmer, USA). Freeze-dried, ground samples weighing 0.05 g were resuspended in 1.5 mL of 70% HNO_3 on a heating block for 1.5 h at 110°C. The samples were then diluted with deionized water to a final volume of 15 mL, filtered using 0.22 μm syringe filters (StarLab, Germany), and analyzed directly with the ICP–MS.

2.3.5 | ABA, JA, and SA Analysis in Soybean

ABA was quantified using the method described by [44]. During the sample extraction process, an ABA standard [(±) −3,5,5,7,7,7-d6] was added to detect and compare peak values. Quantification was performed using GC-MS with a 6890 N Gas Chromatograph (Agilent, USA). The ion responses were analyzed with ThermoQuest software (Manchester, UK), with m/e of 162 and 190 for Me-ABA and 166 and 194 for Me-(2H6)-ABA.

SA measurements were conducted as previously described by [45]. The extraction was performed using 100% methanol, followed by filtration, drying, concentration, and partitioning with a solvent mixture of ethyl acetate, cyclopentane, and isopropanol (100:99:1, v/v). The resulting solution was analyzed using HPLC with a C18 reverse-phase column (HP hypersil ODS, particle size 5 μm, pore size 120 Å, Waters; dimensions 3.9 × 300 mm) at a flow rate of 1.0 mL/min. The standard protocol was followed to measure JA content in the plant shoots [46, 47]. Before adding double-distilled H_2O and the JA standard, 0.1 g of freeze-dried samples was extracted with acetone. After evaporating the acetone, 0.1 M phosphate buffer was added, and the pH was adjusted to 2–3. To remove chlorophyll, DEAE cellulose was used, followed by chloroform for screening. The solution was then filtered using an NH_2 cartridge and quantified using GC-SIM.

2.3.6 | Analysis of Antioxidant Enzymes

The O^{2-} contents were measured according to [48]. Briefly, 0.2 g of fresh leaves were homogenized in 2 mL of 50 Mm

phosphate buffer (pH 7.8), followed by centrifugation at 10 000 *g* at 4°C for 15 min. Subsequently, 0.5 mL of 50 mM phosphate buffer (pH 7.8) and 0.1 mL of 10 mM hydroxylamine hydrochloride were mixed with 0.5 mL of the supernatant and incubated at room temperature for 25 min. After incubation, 17 mM sulfanilamide and 1 mL of 7 mM naphthylamine were added and incubated at room temperature for 30 min. The absorbance was measured at 530 nm, and O^{2-} production was calculated using a $NaNO_2$ standard curve.

Hydrogen peroxide (H_2O_2) contents were determined using the method described by Velikova et al. [49]. Furthermore, an extinction coefficient (ε) of 0.28 mM cm^{-1} was applied to calculate the H_2O_2 content in three biological replicates, expressed as μM g^{-1} FW. Lipid peroxidation was quantified by measuring malondialdehyde (MDA) levels according to the protocol by Imran et al. [50]. The soybean leaves were homogenized in 5% TCA and centrifuged at 6000 *g* for 10 min at 4°C. The supernatant was mixed with 4 mL of TBA and heated at 80°C for 25 min. The optical absorbance was measured at 532 and 600 nm using a UV spectrophotometer (Multiskan GO, Thermo Fischer Scientific, USA), and MDA levels were calculated as μmol g^{-1} FW for three biological replicates. In situ ROS accumulation was assessed using diaminobenzidine [51] staining [52].

2.3.7 | Quantitative Real-Time PCR

Total RNA was extracted using the RNA Prep Kit (BioFact, Republic of Korea) following the instruction of the manufacturer. Subsequently, cDNA was synthesized from 1 μg of RNA with the BioFactTM RT kit (BioFact, Korea). Real-time PCR was performed in replicates using the CFX Duet real-time PCR system (Biorad, USA). β-tublin served as the housekeeping gene, and a no-template control was included. The resulting data were normalized, and gene expression was calculated using the Delta-Delta Ct method (Supporting Information S1: Table 1).

2.3.8 | Metabolites Analysis

GC-MS was used to analyze primary water-soluble and lipid-soluble soybean metabolites, with the methodology developed based on a prior study [53]. Free amino acid (FAA) contents were measured using an L-8800 high-speed amino acid analyzer (Hitachi, Japan) according to the referenced methodology [54]. A 3% trichloroacetic acid solution diluted 1.0 g of freeze-dried buckwheat sprouts. The sample was left at room temperature for 1 h and then centrifuged at 10 000 *g* for 15 min. The supernatant was collected and filtered through Millipore 0.45 μm syringe filters (Milford, USA). The filtrate was analyzed using an amino acid analyzer. The standard amino acid solutions, type AN-II and type B, were obtained from Wako (Wako-shi, Japan). The quantified mass spectra of primary metabolites were then cross-referenced with reference spectra from the National Institute of Standards and Technology (NIST) library [55].

2.3.9 | Statistical Analysis

Analyses were conducted in triplicate, and means and standard deviations were calculated. Analysis of variance (ANOVA) was performed to identify significant differences among treatments at $p < 0.05$, $p < 0.01$, and $p < 0.001$, using Tukey's multiple range test. All statistical analyses were conducted using GraphPad Prism (v8.01, GraphPad Software Inc., San Diego, California, USA). Metabolite data were analyzed using MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/). Different treatment groups were compared using principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA).

3 | Results

3.1 | Biochemical Analysis of EH151

3.1.1 | Characterization of *Bacillus velezensis* EH151 and Its Melatonin-Producing Trait

Soil samples were collected and processed to isolate microbes. The aim was to isolate bacteria capable of growing under NaCl-induced salt and heat stresses. Bacteria that thrived at 150 mM NaCl and 42°C were successfully isolated and purified. Following isolating halotolerant and heat-tolerant bacteria, they were identified through 16S rRNA sequencing. The bacterium *Bacillus valezensis* (strain EH151) was identified, showing 100% sequence identity to the *B. valezensis* strain CBMB205 (GeneBank accession CP014838) (Figure 2A). Furthermore, a BLAST search of the 16S rRNA sequence on NCBI revealed over 90 hits with various strains of *B. valezensis*, all showing 100% sequence identity and coverage. *B. valezensis* exhibited exponential growth since the beginning 6 h postinoculation and entered the stationary phase after 3 days (Figure 2B). In plants and animals, tryptophan undergoes a series of conversions to produce melatonin, progressing through the intermediates tryptamine and serotonin (tryptophan → tryptamine → serotonin → melatonin). When strain EH151 was inoculated into LB media supplemented with tryptophan (1 mg/mL) or serotonin (0.1 mg/mL), it produced the highest amount of melatonin—27.9 ng/mL—96 h after inoculation in the serotonin-supplemented media and 17.6 ng/mL—96 h after inoculation in the tryptophan-supplemented media. These were significantly higher than the 1.97 ng/mL melatonin produced in basal media after 96 h. Given that EH151 exhibits an innate ability to resist high salinity and temperature-induced stress while producing melatonin, changes in melatonin production were investigated in NaCl-supplemented media at 42°C over 96 h. In basal media, the highest melatonin concentration of 2.9 ng/mL was observed in NaCl-spiked media at 42°C after 48 h of EH151 inoculation, which was significantly higher than the melatonin produced in normal media (Figure 2D).

3.2 | Biochemical Analysis of Soybean Plants

3.2.1 | Effect of *Bacillus velezensis* EH151 on Plant Growth and Development

Combinational stress assays were conducted to investigate the responses of the soybean seedlings under the combined heat

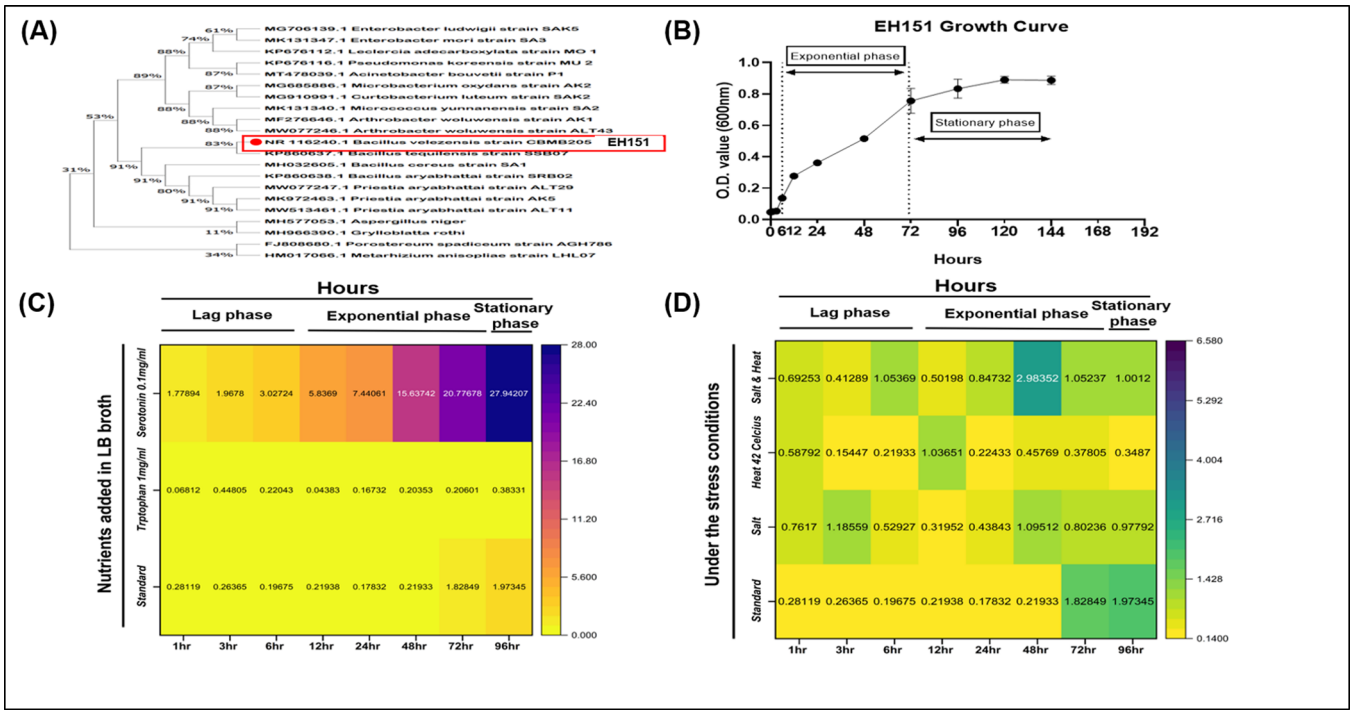

FIGURE 2 | Identification of *B. velezensis* EH151 and its melatonin production capability. (A) Phylogenetic classification of EH151 and 16S rRNA sequence alignment with other *B. Velezensis* strains. (B) Growth rates of EH151 were measured using OD readings at 600 nm. (C) Heatmap showing melatonin production (unit: ng/mL) by EH151 during different growth phases in tryptophan- and serotonin-supplemented LB broth. (D) Heatmap illustrating melatonin production by EH151 during different growth phases in LB broth with sole and combined heat (42°C) and salinity (150 mM) stress. Data represents the means of at least three independent biological replicates. indicates $p < 0.05$, ** indicates $p < 0.01$, and *** indicates $p < 0.001$.

and salinity (H&S) stress after EH151 inoculation. The soybean seedlings at the Vegetative Cotyledon (VC) stage were inoculated with EH151 and exposed to combined heat (42°C) and NaCl (150 mM) stress after 15 days. The seedlings were measured for photosynthetic parameters at 0, 1, 3, 10, and 14 h. Subsequently, the seedlings were collected, frozen in liquid nitrogen, and stored at −80°C for further analysis (Figure 1A). The EH151-inoculated plants exhibited recovery under H&S at 10 and 14 h, while the non-inoculated plants showed incomplete recovery at 10 h and died under H&S at 14 h (Figure 1B and Supporting Information S1: Figure 3). To further evaluate the effectiveness of EH151 on enhancing stress tolerance in soybean plants, the focus was shifted to investigating plant responses, specifically at 10 and 14 h of the combined H&S stress.

To evaluate the mitigation of H&S stress through the EH151 application, the vegetative indices of soybean plants, such as the Normalized Difference Vegetation Index (NDVI), Photochemical Reflectance Index [41], and Anthocyanin Reflectance Index (ARI), were assessed using hyperspectral imaging (Figure 1C). H&S stress results in a significant reduction in NDVI and PRI values. As the duration of the combined stress increased, these values substantially decreased, indicating poor plant health. Conversely, the EH151 application significantly increased the NDVI by 5.7%, 10%, and 9.8% and significantly enhanced the PRI values compared to the plants subjected to combined stressed plants for 3, 10, and 14 h without EH151 inoculation. The ARI value of "H&S 14 h" was higher than that of "EH151 + H&S 14 h", indicating that EH151 inoculation mitigates the effects of H&S (Figure 1C). This finding demonstrates that EH151 inoculation enhances photosynthetic performance and protects against chlorophyll degradation caused by oxidative stress, alleviating the combined H&S stress.

ROS levels were higher in the non-inoculated group than in the EH151-inoculated group. The superoxide anion (O_2^-) levels in the non-inoculated group increased by 40%, 23%, 85%, 71%, and 71%, respectively, relative to those in the EH151-inoculated group (0, 1, 3, 10, and 14 h) (Figure 1E). A similar pattern was observed for malondialdehyde (MDA) levels (Figure 1F), which is further corroborated by the DAB staining analysis. DAB staining of soybean leaves revealed a higher intensity of dark staining in non-inoculated plants under prolonged H&S stress, indicating increased accumulation of H_2O_2 and O_2^- (Figure 1D).

3.2.2 | *Bacillus velezensis* EH151 Mitigates Combined Heat and Salinity Stress by Modulating Photosynthetic Parameters

To gain insight into the effect of EH151 inoculation on soybean plants under combined H&S stress, several physiological properties were investigated. Plants subjected to continuous exposure to combined heat and salt stress exhibited significantly reduced net photosynthesis and electron transport rate (ETR). Plants inoculated with EH151 exhibited higher net photosynthesis and ETR values than non-inoculated plants during 1, 3, 10, and 14 h of combined stress (Figure 3A,B). To further

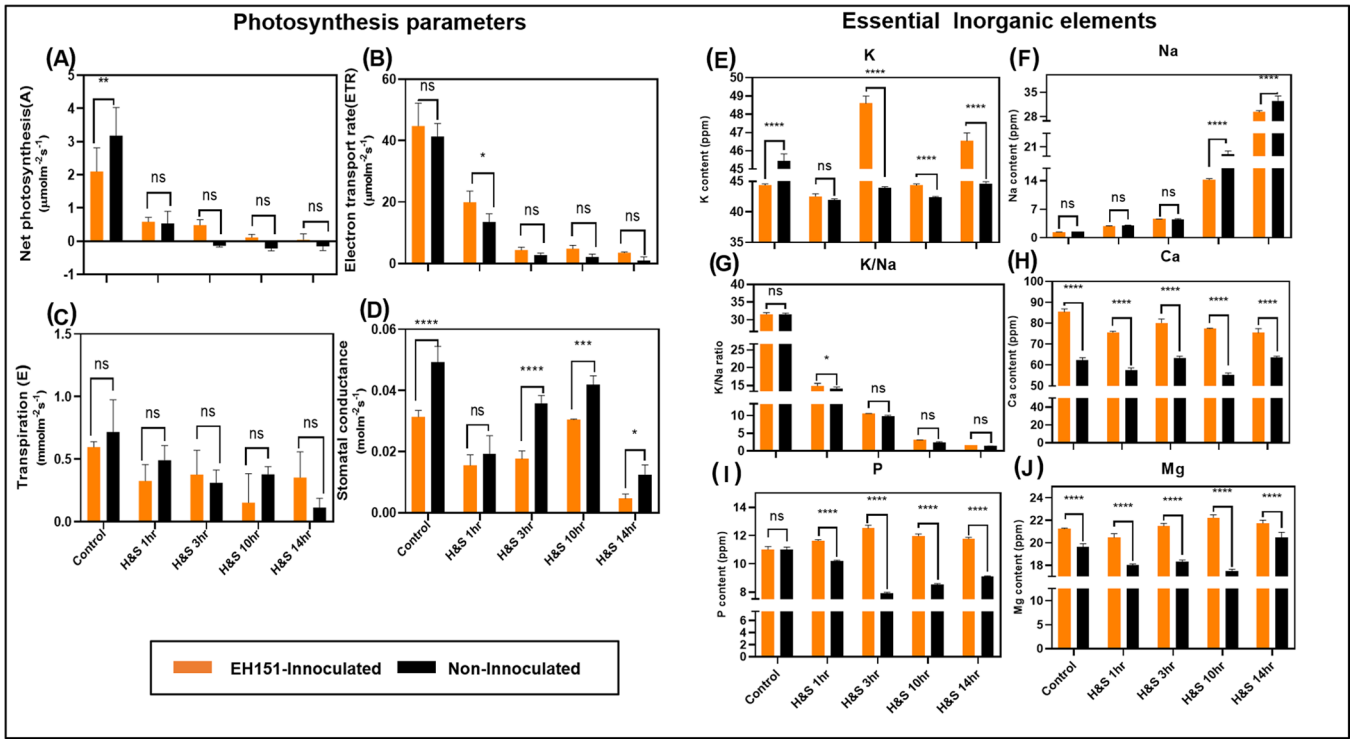

FIGURE 3 | *B. velezensis* EH151 enhances the stress resistance via regulating phytohormone biosynthesis and essential inorganic elements in soybean. (A–D) Photosynthetic parameters: (A) Net photosynthesis rate, (B) Electron transport rate, (C) Transpiration, and (D) Stomatal conductance [31]. Inorganic nutrient in soybean leaves: (E) Quantification of K content, (F) Na content, (G) K/Na ratio, (H) Ca content, (I) P content, and (J) Mg content in leaves of H&S-stressed soybean plants pretreated with EH151. Data represent the means of at least three independent biological replicates. Error bars represent standard deviations. Means were compared using Tukey's post hoc test. * indicates $p < 0.05$, ** indicates $p < 0.01$, and *** indicates $p < 0.001$.

characterize the photosynthetic apparatus, stomatal conductance was analyzed. Stomatal conductance decreased at 1 h compared to that of the Control group, but after 1 h, it increased until 10 h before declining again at 14 h, indicating a similar trend in EH151-inoculated and non-inoculated treatments. However, EH151 inoculation decreased stomatal conductance by 19.4%, 50.6%, 27.4%, and 62.6% compared to that of non-inoculated stressed plants at 1, 3, 10, and 14 h, respectively (Figure 3D).

3.2.3 | *Bacillus velezensis* EH151 Regulates Essential Inorganic Elements in Soybean Plants

Potassium (K) is an essential macronutrient required for plant growth and development. ICP–MS analysis demonstrated that EH151 differentially modulates Na^+ and K^+ ion contents, affecting the K^+/Na^+ ratio in the leaf tissues (Figure 3E–G). Overall, EH151 increased K^+ content in stressed plants by 1.4%, 10.6%, 4.6%, and 4.2% at 1, 3, 10, and 14 h, respectively, compared to that of non-inoculated plants under stress conditions (Figure 3E). In contrast, EH143 decreased Na^+ ion concentrations in stressed plants compared to that of non-inoculated plants (Figure 3F). Salinity stress is characterized by high Na^+ ion concentrations at the tissue level, which disrupts ionic balance, impairs K^+ uptake, and ultimately affects the water balance [56]. Under salt stress, plants maintain ion homeostasis by adjusting the acquisition and distribution of K^+ and Na^+ [57]. To mitigate Na^+ toxicity, plants limit Na^+ absorption, enhance Na^+ exclusion, alter the cellular ionic balance (especially K^+/Na^+ ratio), and redistribute Na^+ to the leaves [58]. In this study, a higher K^+/Na^+ ratio in EH151-inoculated plants by 6.1%, 8.5%, 29.9%, and 15.7% compared to that of non-inoculated plants after 1, 3, 10, and 14 h, respectively, was observed (Figure 3G).

Calcium and phosphorus are essential plant macronutrients, playing a crucial role in stabilizing cell membranes and walls under stress conditions. EH51 significantly increased Ca^{2+} content under normal and salt stress conditions (Figure 3H). EH151 inoculation significantly increased the phosphorus uptake in soybeans under normal and combined H&S stress across all time durations (Figure 3I). Deficiencies in mineral nutrients, particularly magnesium (Mg), increase ROS production and chloroplast damage [59, 60]. The ICP analysis revealed that Mg levels in non-inoculated treatments (0, 1, 3, 10, and 14 h) decreased by 8%, 13.5%, 17.4%, 27%, and 6%, respectively, compared to EH151-inoculated treatments at the same time points (0, 1, 3, 10 h, and 14 h) (Figure 3J).

3.2.4 | *Bacillus velezensis* EH151 Mitigates Combined Heat and Salinity Stress by Modulating Stress-Related Gene Expression

Real-time PCR analysis indicated significant changes in the expression of heat shock protein 90, alpha 2 (HSP90A2), salt tolerance 1 (ST1), nitrate reductase [61], and S-nitrosoglutathione

reductase (GSNOR) (Figure 4A–D). In this study, a decrease in HSP90A2 expression levels was observed at 1, 3, 10, and 14 h of H&S stress compared to the expression in unstressed control plants. Despite the continuous decrease in HSP90A2 expression levels under the combined H&S stress, a slight increase in HSP90A2 expression levels was observed with EH151 application after 1 h of H&S stress. Additionally, the EH151 application reduced HSP90A2 expression levels by 75%, 63.6%, 38%, 42%, and 5.6% at 0, 3, 10, and 14 h under stress conditions, respectively (Figure 4A). ST1, known for its strong tolerance to salt stress, exhibited increased sensitivity to ABA and decreased production of ROS under salt stress. In this study, the transcription pattern of ST1 was assessed in soybean plants. A decrease in ST1 expression levels was observed in plants subjected to combined H&S stress (1, 3, 10, and 14 h) compared to those of unstressed plants. While ST1 expression levels decreased over time, EH151 application increased ST1 expression by 120%, 135%, 17%, 37.9%, and 27.5% at 0, 1, 3, 10, and 14 h under stress conditions, respectively (Figure 4B).

Nitric oxide (NO), a chemical messenger, is essential for plant growth, development, and responses to biotic and abiotic stresses. Additionally, NO interacts with antioxidants, melatonin, hydrogen sulphide, and ROS. NR can be used as a cofactor to help other enzymes in producing NO. Furthermore, the enzyme GSNOR deconstructs intracellular SNO levels, which are derived from NO. Upon examining EH151-inoculated and non-inoculated plants, the gene expression of GSNOR was observed to decrease continuously as the combined stress persisted. However, the non-inoculated plants exhibited increased GSNOR expression levels by 112.7%, 101.6%, 714.6%, 253.6%, and 95.5% at 0, 1, 3, 10, and 14 h, respectively, under stress conditions compared to those of the EH151-inoculated plants (Figure 4C). Furthermore, for NR, which synthesizes NO, the non-inoculated plants exhibited increases of 263%, 59%, and 92.8% at 3, 10, and 14 h, respectively, under stress conditions compared to EH151-inoculated plants (Figure 4D). Therefore, despite the decrease in GSNOR expression in EH151-inoculated and non-inoculated plants under stress, NR was significantly upregulated in the non-inoculated plants compared to those of the EH151-inoculated plants after 3 h of combined stress. This suggests that NO levels were higher in the non-inoculated groups, where NO potentially scavenged ROS generated through the combined H&S stress.

3.2.5 | *Bacillus velezensis* EH151 Enhances K/Na Balance by Regulating K and Na Inorganic Element Transport-Related Genes

In this study, a higher K^+/Na^+ ratio in EH151-inoculated plants by 6.1%, 8.5%, 29.9%, and 15.7% compared to that of non-inoculated plants after 1, 3, 10, and 14 h, respectively, was observed (Figure 3G). This suggests that EH151 enhances K^+ absorption, transport, and accumulation in plants while restricting the transport of Na^+ ions. The higher expression of the ion regulator genes HKT1 and MYB76 in EH151-inoculated plants under all

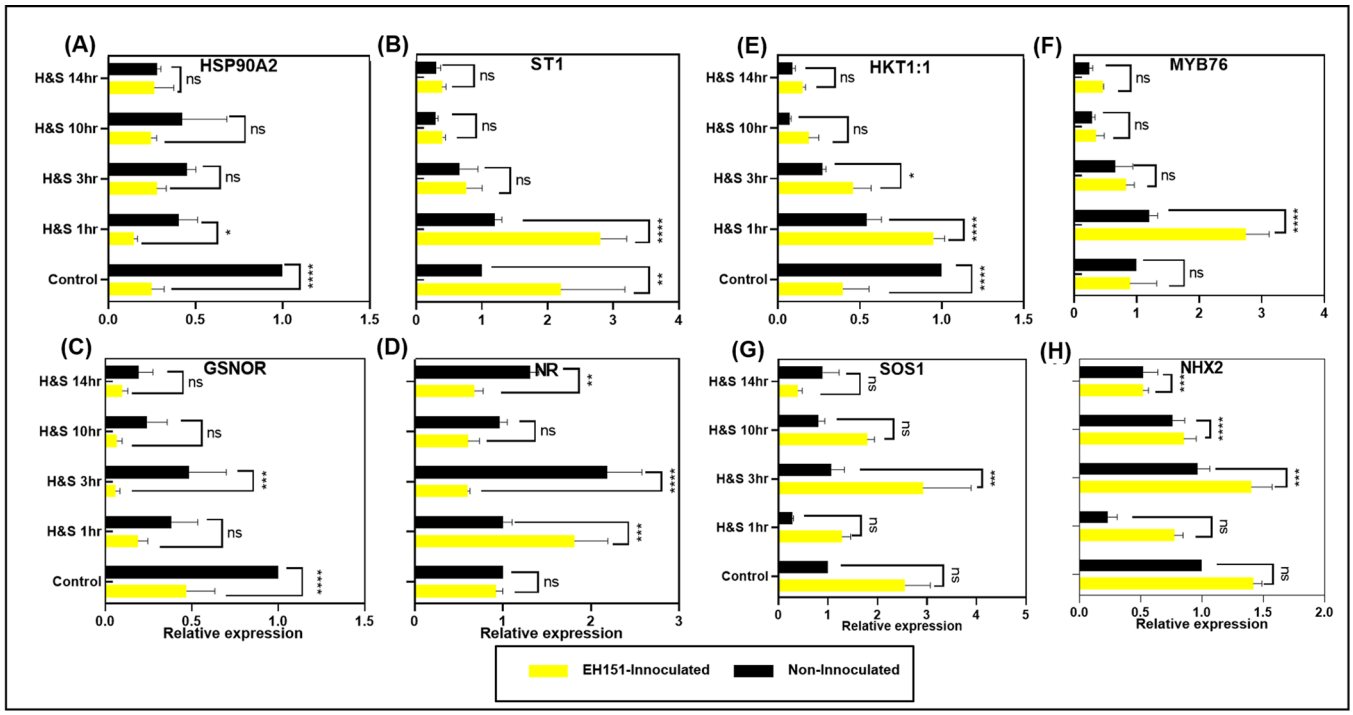

FIGURE 4 | *B. velezensis* EH151 mitigates H&S stress by modulating stress-related genes and Na and K transport-related genes. (A–D) Real-time PCR analysis showing changes in the expression of genes involved in resistance to H&S stress: (F) Heat shock protein A2 (HSP90A2), (G) Salt tolerance1 (ST1), (H) S-nitrosoglutathione reductase (GSNOR), and (I) Nitrate reductase [61]. (E–H) Real-time PCR analysis showing changes in the gene expression of transporters involved in Na^+ and K^+ transport: (E) High-affinity K^+ transporter 1;1 (HKT1;1), (F) MYB transcription factor (MYB76), (G) Salt Overly Sensitive 1; (SOS1), and (H) Na^+/H^+ antiporter 2 (NHX2). Data represent the means of at least three independent biological replicates. Error bars represent standard deviations. Means were compared using Tukey's post hoc test. * indicates $p < 0.05$, ** indicates $p < 0.01$, and *** indicates $p < 0.001$.

conditions further validated these findings (Figure 4E,F). To understand the regulation of salt- and heat-responsive genes in response to combined salt and heat stress, the relative expression level of selected genes was investigated after 0, 1, 3, 10, and 14 h of stress exposure (Figure 4E–H). The HKT1;1 expression increased by 75.5%, 69.9%, 160%, and 70% in EH151-inoculated plants compared to expression in non-inoculated plants after 1, 3, 10, and 14 h, respectively (Figure 4E). Furthermore, MYB76 expression was higher in EH151-inoculated plants than in untreated stressed plants, with increases of 127.8% after 1 h, 26.7% after 3 h, 20% after 10 h, and 91.5% after 14 h under H&S stress (Figure 4F). Salt Overly Sensitive 1 (SOS1) is an extensively characterized Na^+ efflux transporter in multiple plants. The expression level of SOS1 increased by 156%, 352%, 173%, and 124% in EH151-inoculated plants compared to non-inoculated plants after 0, 1, 3, and 10 h of H&S stress, respectively (Figure 4G). Intracellular Na^+/H^+ antiporter (NHX2), playing a crucial role in maintaining cellular pH and Na^+, K^+ homeostasis, exhibited similar expression trends to the SOS1 gene (Figure 4H).

3.2.6 | *Bacillus velezensis* EH151 Enhances Stress Resistance by Regulating Phytohormone Biosynthesis

Phytohormones regulate every aspect of plant growth and development, from germination to maturity and senescence (Figure 5A–D). Additionally, phytohormones are crucial in enabling plants to adapt to stressful conditions [62]. The capacity of crop varieties to effectively maintain phytohormone homeostasis determines their stress tolerance or susceptibility. This study demonstrates that the soil application of EH151 led to a consistent increase in jasmonic acid (JA) content in plants from 1 h up to 10 h under combined stress conditions (Figure 5A). In contrast, JA content continuously decreased in non-inoculation plants during the same period. The accumulation of salicylic acid (SA) significantly increased with the duration of stress (0 h, 1, 3, 10, and 14 h) in EH151-treated and nontreated groups (Figure 5B). However, EH151 application increased endogenous SA compared to non-inoculated plants by 55.8% and 57.6% after 3 and 10 h of stress, respectively, but decreased SA content by up to 47.7% after 14 h.

Under water-deficient conditions, the plant hormone abscisic acid (ABA) regulates stomatal movements. ABA reduces transpirational water loss by closing stomata, decreasing stomatal conductance. ABA levels increased with prolonged exposure to H&S stress until 10 h, while stomatal conductance decreased, as previously discussed (Figures 3D and 5C). Moreover, EH151-inoculated plants accumulated significantly higher levels of endogenous ABA at 0, 1, 3, 10, and 14 h compared to those of the non-inoculated plants, indicating an opposite trend in stomatal conductance (Figure 5C).

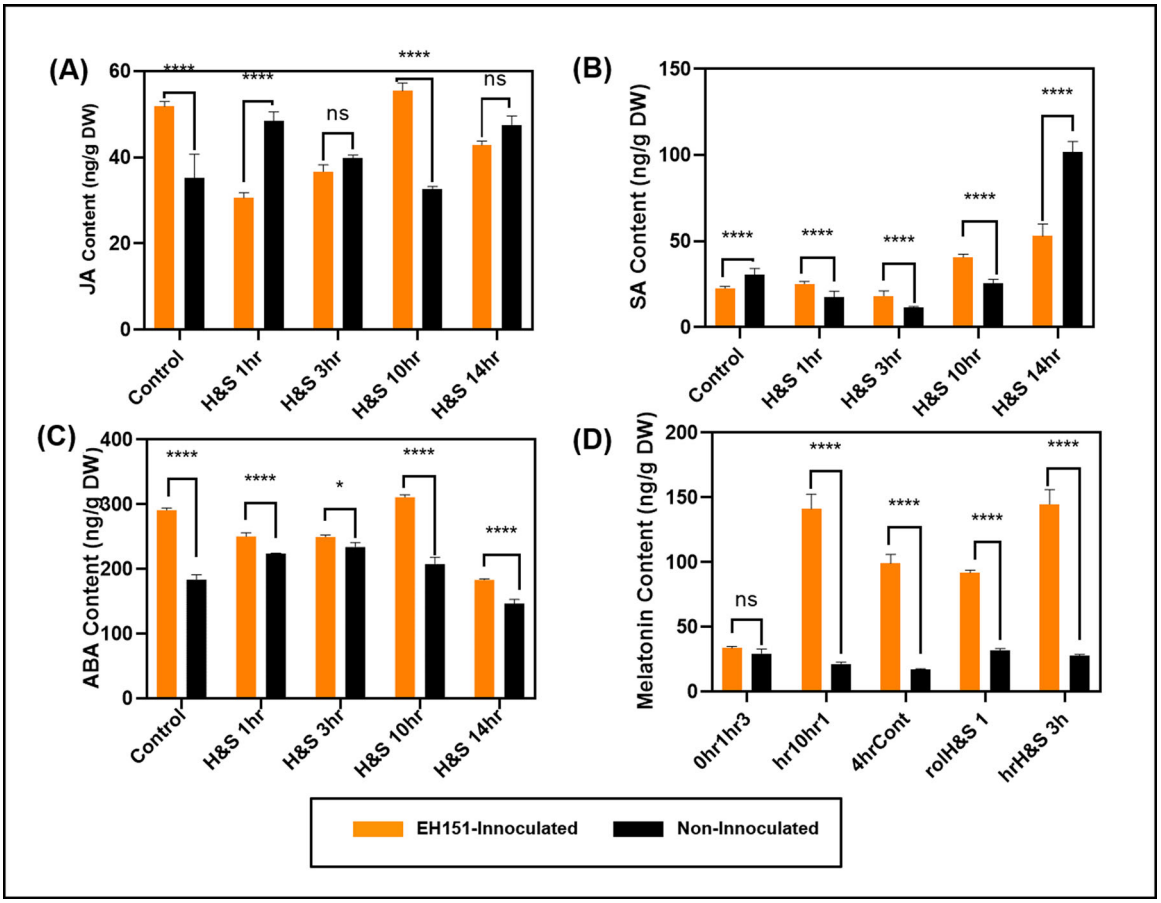

FIGURE 5 | *B. velezensis* EH151 enhances the stress resistance via regulating phytohormone biosynthesis. (A–D) Quantification of stress-related phytohormone: (A) JA concentration, (B) SA concentration, (C) ABA concentration, and (D) Melatonin concentration in soybean plants. Data represent the means of at least three independent biological replicates. Error bars indicate standard deviations. Means were compared using Tukey's post hoc test. *indicates $p < 0.05$, **indicates $p < 0.01$, ***indicates $p < 0.001$ and ****indicates $p < 0.0001$.

Melatonin content in plants displayed different patterns under stress conditions, depending on whether the plants were inoculated or non-inoculated. Under EH151 inoculation, endogenous melatonin significantly increased after just 1 h of combined H&S stress and remained high until the end of the experiment. However, non-inoculated plants showed no significant change in melatonin levels during the stress duration compared to the control group (Figure 5D).

3.3 | Metabolite Analysis of Soybean Plants

3.3.1 | *Bacillus velezensis* EH151 Regulates Central Carbon Metabolism Pathway Metabolites in Soybean Under Combined Heat and Salinity Stress

Soybean leaves were analyzed using GC-MS and an amino acid auto-analyzer. Consequently, 27 free amino acids and their derivatives, 18 organic acids and their derivatives, four lipids and their derivatives, and five free sugars and four sugar alcohols were detected. Principal components analysis (PCA) of the 58 metabolites (Figure 6A) revealed that the primary factors 1 and 2 accounted for 57.4% and 27.6% of the variance among the groups: "Control", "H&S 10 h", "H&S 14 h", "EH151", "EH151 + H&S 10 h", and "EH151 + H&S 14 h", respectively. In the loading plot, the content of most of the free sugars, sugar alcohols, and organic acids was relatively high in the "Control" group. In contrast, L-alanine, sucrose, and L-proline were more prevalent in the "H&S 14 h" group. Saturated fatty acids and free amino acids, such as L-serine, arginine, L-histidine, and L-threonine, were found in higher concentrations in the "EH151 + H&S 14 h" group (Figure 6A).

A heatmap was developed based on these findings to visually illustrate the metabolites of the central carbon metabolism (CCM) pathway in soybeans. The heatmap highlights the varying levels of free amino acids, free sugars and sugar alcohols, organic acids, and saturated fatty acids in the "Control", "EH151 + H&S 14 h", and "H&S 14 h" groups. Among the examined compounds, the "EH151 + H&S 14 h" group exhibited significant levels of free amino acids, including ammonia, and displayed a similar trend for saturated fatty acids such as methyl linoleate, palmitic acid methyl ester, and behenic acid compared to those of the "H&S 14 h" group. In contrast, the "H&S 14 h" showed relatively higher concentrations of free sugars and sugar alcohols, including sucrose, myo-inositol, and D-glucose, than the "EH151 + H&S 14 h" group. Additionally, the "EH151 + H&S 14 h" group demonstrated abundant levels of organic acids such as citric acid, fumaric acid, and oxalic acid, compared to those of the "H&S 14 h" (Figure 6B). These

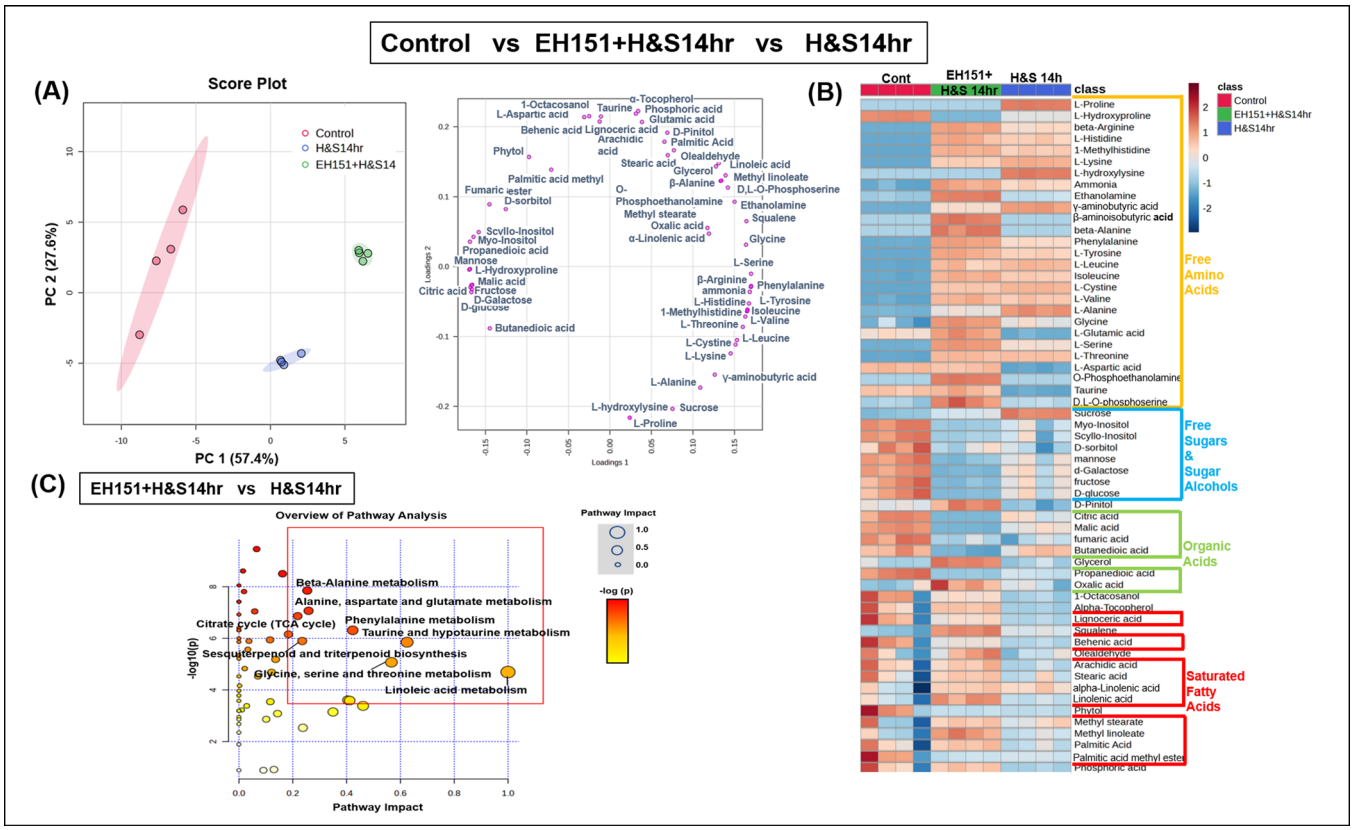

FIGURE 6 | *B. velezensis* EH151 regulates the metabolites of CCM pathway in soybean under the stress. (A) Principal component analysis (PCA) of metabolites in three treatment groups: "Control," "EH151 + H&S 14 h", and "H&S 14 h". Results are displayed as score plots (left column) and loading plots [63] to visually distinguish between the groups. Certain metabolites are marked using their compound names in the loading plots based on Tukey's HSD test ($p < 0.05$). (B) Heatmap showing the top 59 significant primary metabolites in the CCM pathway, including 27 free amino acids, 6 organic acids, 8 free sugars and their derivatives, and 10 saturated fatty acids, across the "Control", "EH151 + H&S 14 h", and "H&S 14 h" treatments. (C) Pathway analysis of soybean leaves comparing two treatments, "EH151 + H&S 14 h" and "H&S 14 h". Data represent the mean ± SD ($n = 4$).

changes are more distinctly observable when comparing the "EH151 + H&S 14 h" and "H&S 14 h" groups only, excluding the "Control" group (Supporting Information S1: Figure 5).

To gain further insights into the metabolites between the "EH151 + H&S 14 h" and "H&S 14 h" groups, the Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway was used to analyze the metabolites of the groups. This analysis was aimed at characterizing the effect of EH151 under continuous H&S stress in soybean seedlings over 14 h. Fold Change (FC) and *t*-tests were used for Volcano plot analysis. Three metabolic pathways with the highest *p* values and one with the greatest pathway effect were identified. Beta-alanine metabolism, alanine, aspartate, glutamate metabolism, and the TCA cycle had the highest *p* values, while linoleic acid metabolism exhibited the most significant pathway effect. Under extreme H&S stress, plants prioritize respiration and the TCA cycle to generate energy for survival. In contrast, inoculation with EH151 caused the plants under the H&S stress to focus on producing free amino acids, such as alanine, aspartate, and glutamate, along with synthesizing saturated fatty acids, such as those involved in linoleic acid metabolism. This adjustment helps plants to cope with heat stress and enhances their stress resistance (Figure 6C).

Based on the results shown in Figure 6A–C, "EH151 + H&S 14 h", "H&S 14 h", and "Control" groups were compared, demonstrating clear differences in the phenotypic and physiological traits of the leaves (Figures 1 and 3–5). This comparison highlights the effect of EH151 on soybean plants subjected to combined H&S stress.

3.3.2 | Overview of Metabolites in the Central Carbon Metabolism Pathway in Soybean Leaves Treated With *Bacillus velezensis* Eh151 Under Combined Heat and Salinity Stress

The CCM pathways in soybean leaves differed significantly among the "Control", "H&S 14 h", and "EH151 + H&S 14 h" treatments. These differences arise from the activation of distinct CCM pathways depending on whether the plants were exposed to abiotic stress or stress-mitigating nutrients during abiotic stress. This highlights how metabolomics can be used to uncover the response mechanisms of combined H&S stress, particularly with the inoculation of melatonin-producing *Bacillus velezensis* EH151. To enhance understanding, the scores were transformed into *Z*-scores (Figure 7A), expressed as standardized deviations from their means. These *Z*-scores have a mean of 0 and a standard deviation of 1. *Z*-score of 0 indicates that the score of the data point is identical to the mean score. *Z*-scores can be positive or negative, indicating whether the data point is above or below the mean. These changes were displayed in relation to the respiratory pathway. The most significant change in metabolites under "H&S 14 h" was the increase in most TCA cycle intermediates (citrate, succinate, malate) compared to those of "EH151 + H&S 14 h" while these metabolites were still lower than in the "Control" group. However, in the case of oxaloacetate (OAA), "EH151 + H&S 14 h" showed a significant upregulation, with a *Z*-score reaching 1.5, the highest among "Control", "EH151 + H&S 14 h", and "H&S 14 h". Carbohydrates at the beginning of the glycolysis pathway (glucose) and free sugars (sucrose and fructose) were significantly upregulated in "H&S 14 h" compared to those in "EH151 + H&S 14 h". However, fructose and glucose exhibited the highest values in the "Control". This indicates that most metabolites in the glycolysis and TCA cycle under "H&S 14 h" increased compared to those under "EH151 + H&S 14 h" but were lower than in the "Control" (Figure 7A).

In the free amino acid metabolism pathway, "EH151 + H&S 14 h" significantly enhanced free amino acid metabolism compared to that of the "Control" and "H&S 14 h". In glutamate metabolism, "EH151 + H&S 14 h" demonstrated that glutamate, 4-aminobutanoate (GABA), and ammonia (nitrogen sources for amino acids synthesis) had *Z*-scores greater than 1, while the "Control" and "H&S 14 h" were down-regulated. Furthermore, glutamate is interchangeable with α-ketoglutarate (α-keto) in the TCA cycle. Subsequently, glutamic acid was converted to GABA and later to succinic acid. In the "EH151 + H&S 14 h" treatment, succinate and citrate (the precursor metabolite before α-keto) were depleted to a *Z*-score of −1.5, unlike in "H&S 14 h" and "Control". The data suggest that "EH151 + H&S 14 h" prioritized free amino acid synthesis over respiration and the TCA cycle compared to "Control" and "H&S 14 h". This conclusion is supported by the fact that many intermediates in the TCA cycle and glycolysis interact bidirectionally with free amino acids in CCM. Similarly, phenylalanine metabolism exhibited a comparable trend, with tyrosine and L-phenylalanine showing the highest levels of upregulation compared to those of the "Control" and "EH151 + H&S 14 h". These metabolites originate from shikimate and phosphoenolpyruvate (PEP), which are key precursors of aromatic amino acids. In alanine and aspartate metabolism, aspartate levels were enhanced in "EH151 + H&S 14 h" compared to that in "H&S 14 h". Aspartate plays a crucial role in the biosynthesis of phenylalanine and glutamic acid [64], which were significantly upregulated in "EH151 + H&S 14 h". The metabolism of glycine, serine, and threonine, all metabolites (*o*-phospho-serine, L-serine, glycine, and L-threonine) were significantly upregulated, demonstrating a similar trend to other amino acids metabolic pathways. However, alanine and proline in "EH151 + H&S 14 h" were downregulated compared to those in "H&S 14 h", unlike other amino acids. Several studies have reported that proline and alanine act as a stress marker in severely stressed plants [65, 66] whereas the reduced stress conditions leads to lower proline accumulation in plants [67]. Thus, the less accumulated proline in "EH151 + H&S 14 h" might reflect that the inoculated plants got less exposed to H&S stress and did not need the excessive proline synthesis for the stress mitigation as much as "H&S 14 h" needed (Figure 7A).

To gain insight into CCM and its biological processes in response to combined H&S stress, the relative gene expressions of enzymes involved in the respiratory chain, glycolysis, the TCA cycle, and free amino acid metabolism pathways were analyzed. Among the enzymes in the glycolysis pathway, the relative gene expression of phosphofructokinase (PFK) and Fructose-1,6-bisphosphate aldolases (FBA) was observed to decrease as the duration of combined stress increased under EH151 inoculation, while non-inoculated treatments exhibited the opposite trend (Figure 7B). The gene expression of pyruvate

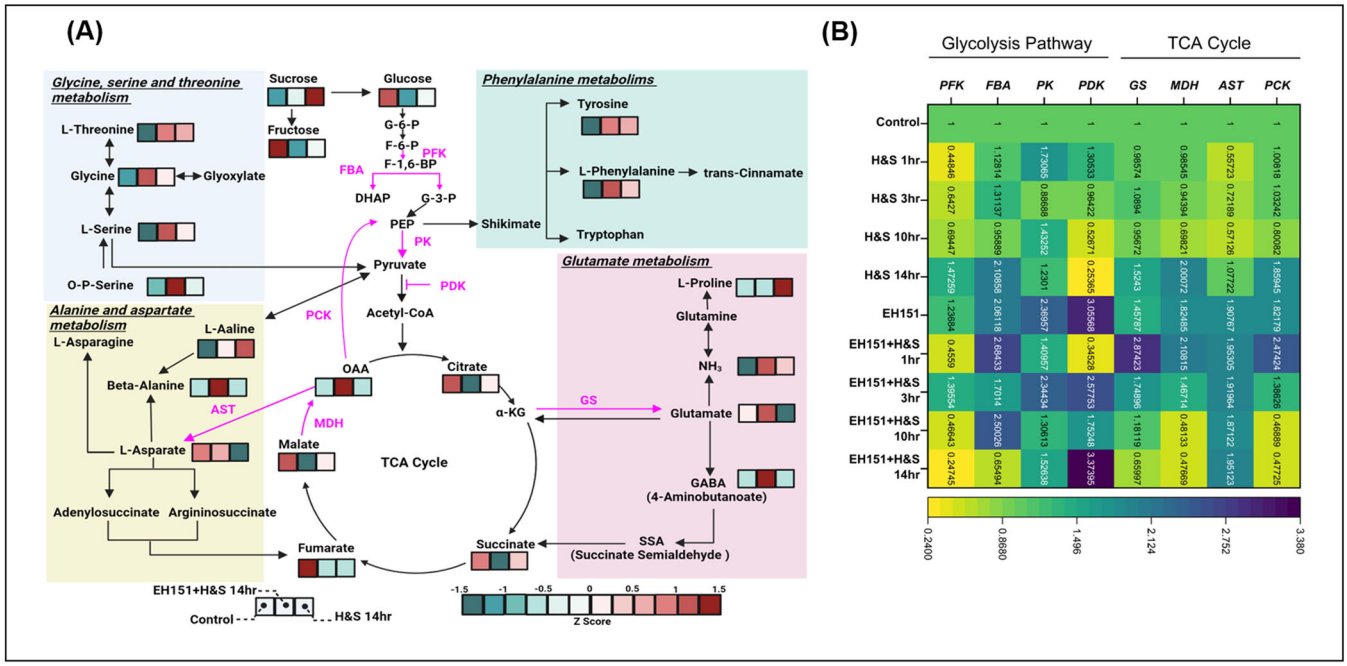

FIGURE 7 | Pathway overview of metabolites of central carbon metabolism (CCM) with *B. velezensis* Eh151 Application Effects in soybean leaves under the combined heat and salinity stress. (A) Changes in metabolite levels in soybean plants under "Control", "EH151 + H&S 14 h", and "H&S 14 h" conditions. The levels of 3 sugars, 13 free amino acids, and 5 organic acids expressed as *Z*-scores, calculated using the formula: $Z = (x - \mu)/\sigma$, where "x" is the relative abundance of the metabolite, "μ" is the mean relative abundance of the metabolite across 17 rice brans, and "σ" is the standard deviation of the relative abundance of the same metabolite across 17 cultivars. (B) Gene expression profiling of enzymes in the CCM pathway using RT-qPCR. Quantitative data represent the means ± SD of three independent experiments, each with at least three technical replicates. Abbreviations: AST, aspartate aminotransferase; FBA, fructose-1,6-bisphosphate aldolase; GS, glutamate synthase; MDH, malate dehydrogenase; PCK, phosphoenolpyruvate carboxykinase; PFK, phosphofructokinase; PDK, pyruvate dehydrogenase; PK, pyruvate kinase.

dehydrogenase (PDK), which deactivates the PDK enzyme to prevent the conversion of pyruvate into acetyl-CoA, was downregulated under EH151 inoculation during combined stress but showed an opposite trend in non-inoculated treatments. The expression of the gene encoding Glutamate synthase (GS) was upregulated in EH151-inoculated plants until 10 h into the combined H&S stress, compared to that of non-inoculated plants. This explains the higher accumulation of glutamate in "EH151 + H&S 14 h" than the "Control" and "H&S 14 h". A similar trend was observed in the expression of the gene encoding aspartate aminotransferase (AST); EH151-inoculated treatments exhibited higher AST gene expression than non-inoculated treatments throughout the entire period of H&S stress (Figure 7B). These results support the suggestion that EH151 inoculation plays a critical role in acclimatizing soybean to H&S stress by shifting its focus towards free amino acid metabolism rather than respiration through glycolysis and the TCA cycle.

4 | Discussion

Rising temperatures and salinity are among the most impending environmental challenges affecting agricultural production globally. The individual roles of PGPB and melatonin in alleviating the detrimental effects of heat or salinity stress have been extensively researched, focusing on the underlying physiological and molecular mechanisms [68, 69]. However, research focusing on how melatonin and PGPB mitigate heat and salinity stress based on plant metabolomic mechanisms has not been conducted well yet. Also, unlike numerous studies on the role of melatonin in plants and animals, studies on melatonin production by microbes are limited [70, 71]. Only a few strains, including certain yeasts and pseudomonads, are known to synthesize melatonin independently, and the mechanism of melatonin production in the microorganisms still remains unclear [70, 71]. Therefore, this study is the first to speculate the melatonin synthesis pathway of *B. velezensis* strains and investigate the synergistic effects of melatonin-producing *B. velezensis* EH151 on plants under the H&S stresses in its various metabolomic and genetic perspectives.

4.1 | Elucidation of the Melatonin Biosynthetic Pathway in *B. velezensis* EH151

Here, we found that the innate ability of EH151 to synthesise melatonin was significantly enhanced when each of serotonin and tryptophan was added to the LB medium (Figure 2C). This suggests that the fundamental steps involved in EH151's melatonin synthesis are aligned with those observed in plant and animal systems where serotonin works as the precursor of melatonin [72]. Furthermore, the observed increase in melatonin levels under heat and salinity stress conditions confirmed that *B. velezensis* EH151

possesses resistance to high-salinity and high-temperature stress. Therefore, it has been confirmed that *B. valezensis* EH151 can serve as a stress mitigation microorganism in the present soy experiments involving combined high-salinity and high-temperature stress conditions.

4.2 | Photosynthetic Attributes, Ionic Fluxes, and Phytohormonal Response of Soybean Plants

Combined H&S exposure significantly decreased plant vegetative growth and also showed the increased anthocyanin biosynthesis with the onset of chlorophyll degradation, which eventually downregulated the net photosynthesis (A) and ETR indices (Figure 3A,B). On the contrary, the EH151 interaction with soybean improved the vegetative indices and photosynthetic parameters, as demonstrated in Figure 1C and Figure 3A–D. Furthermore, the regulation of ion balance is crucial for maintaining cellular homeostasis under abiotic stress [34]. The EH151 treatment enhanced K^+ uptake but restricted Na^+ absorption, eventually increasing the K/Na ratio. These results are corroborated by EH151's regulation of the SOS, NHX, and HKT signalling genes (Figure 4E–H). The positive regulation of *GmSOS1*, *GmNHX2*, and *GmHKT1;1* facilitates the exclusion of Na^+ from the cytoplasm and the uptake of K^+ in plants [73], which could mitigate the adverse effects of H&S stress. Also, previous studies show that salt- and heat-induced stress typically inhibits the regulation and uptake of Ca, which reduces the efflux of Na^+ to the apoplast through Ca^{2+}-dependent SOS signalling pathways and leads to the over-accumulation of Na^+. The excess Na^+ competes with K^+ for HKT and AKT transporters, causing the overproduction of ROS through the Ca^{2+}-dependent SOS signalling pathway [74]. In our studies, Ca accumulation was observed under EH151 inoculation (Figure 3H), which could help the plants to lessen Na+ and ROS accumulation. Thus, these findings demonstrate that the application of EH151 efficiently controls nutrient uptake and assimilation in soybeans, enabling them to cope with the combined H&S-induced oxidative stress.

The assimilation of mineral ions is followed by the modulation of phytohormones such as ABA, JA and SA to confer stress tolerance against both H&S. EH151 increased the accumulation of ABA in soybean plants and enhanced the expression of ABA-related gene ST1, increasing the sensitivity of ABA to plants and strengthening plants resistance under combined stress [75] (Figures 4B and 5C). The HSP90A2 gene was downregulated in EH151-inoculated plants under stress conditions (Figure 4A). Given that HSPs are one of the most recognized mechanisms for responding to potential damage caused by high temperature and salinity stresses [76], this result suggests that EH151 inoculation may mitigate and prevent damage from combined extreme H&S stresses in soybean plants. Also, the study showed that EH151 increased the accumulation of JA and MEL in soybean plants under H&S stress (Figures 5A,D). Similar results were reported that JA and melatonin play a crucial role in improving tolerance to H&S and act as a signal transducer in a cascade of intracellular signals triggered by these stresses [77].

4.3 | Influence on Metabolic Pathway of Soybean Plants With EH151 Inoculation Under Heat and Salinity Stress

CCM includes a series of biochemical processes such as glycolysis, the pentose phosphate pathway, the tricarboxylic acid cycle, and the respiratory chain that regulate the photosynthetic components. Here, our studies found that a leaf CCM pathway was significantly activated under non-inoculated plants compared to EH151 inoculated plants under H&S 14 h stress (Figure 6A–C and Supporting Information S1: Figure 4). Additionally, our study focused on substrate–product relationships across the respiratory network to identify key metabolites correlating strongly with H&S stress and EH151 inoculation (Figure 7A). It was observed that several soluble carbohydrates (fructose, galactose, mannose, sucrose) including sugar alcohols, such as myo-inositol and D-sorbitol were significantly higher in "H&S 14 h" than in "EH151 + H&S 14 h" (Supporting Information S1: Figure 1). Myo-inositol, in particular, has been shown to respond to salt and heat stress in plants and it can function as a precursor for protein-stabilizing osmolytes, which typically accumulate under heat stress [61]. Additionally, the upregulation of sugar alcohols such as ribitol, sorbitol, and mannitol has been associated with salt stress in terrestrial plants [78]. Overall, it can be inferred that the nontreated plants are more sensitive and vulnerable to H&S stress when compared to EH151-treated plants under H&S stress conditions.

TCA cycle intermediates can respond to a higher respiratory rate in photosynthesizing tissues with an elevation of temperature. These are further triggered by the amino acids metabolism as demonstrated by [79], where glutamic acid was metabolized to 2-oxoglutarate, GABA, succinate, fumarate, malate, and some amino acids. We observed the higher accumulation of citrate, succinate, and malate in "H&S 14 h" than in "EH151 + H&S 14 h", suggesting that these metabolites increased the formation of TCA cycle intermediates under H&S without EH151 treatment (Figure 7A) for making more energy to resist the combined stress (Figure 7A). However, this increase in TCA cycle intermediates was not observed in the "EH151 + H&S 14 h" condition, where TCA cycle intermediates were downregulated (Figure 7A). We can interpret these findings as EH151 inoculated plants experienced less stress and did not require the highly activated TCA cycle to make the energy against H&S stress compared to "H&S 14 h".

Under H&S stress, the amino acid synthesis pathway and metabolism play a crucial role in conferring plant stress tolerance [80]. Here we observed a significant rise in accumulation of several amino acids in "EH151 + H&S 14 h" group compared to those in the "H&S 14 h" and "Control" (Figures 6A–C and 7A). In plants, aspartate plays a crucial role as a metabolic hub, contributing to energy production, nutrition, reproduction, development, growth, stress response, and defense [64]. Our findings showed that the aspartate pathway in EH151-inoculated plants was significantly more activated than in the "H&S 14 h" treatment (Figure 7A). Furthermore, aspartate is involved in the biosynthesis of phenylalanine and glutamic acid [64]. The glutamate is used as a precursor for several essential amino acids, such as arginine, ornithine, and lysine, thereby indirectly regulating many metabolic activities [81] and works

as an important signalling molecule under different conditions in many plants [82]. Also, under biotic and abiotic stresses, glutamate serves as the precursor for the important biomolecule γ-aminobutyrate (GABA). GABA plays a crucial role in carbon metabolism and the biosynthesis of proline, acting as an antioxidant and osmolyte in stressed plants [83], showing a significant increase in "EH151 + H&S 14 h" compared to those of the "Control" and "H&S 14 h" treatments. Moreover, glutamate contributes to carbon and N metabolism by converting to α-ketoglutarate through the action of the enzyme glutamate dehydrogenase enzyme (GDH) [84] and it is involved in ammonia assimilation [83], which aligned with the rise in the "EH151 + H&S 14 h" group, serving as a primary source of inorganic N used for amino acid synthesis [85] (Figures 6B and 7A).

In response to salt stress, the levels of several amino acids, including alanine, serine, and glycine, varied in different plant organs. For example, in maize, wheat, and barley, the leaves showed a significant increase in these amino acids, indicating an increase in photorespiration, as these amino acids are actively involved in this process [41, 86, 87]. This finding aligns with that of this study, where the levels of glycine, serine, and alanine increased in the "EH143 + H&S 14 h" and "H&S 14 h" after 14 h of combined H&S stress compared to those in the "Control" (Figures 6B and 7A). In particular, serine and glycine are crucial for photosynthesis and growth, playing key roles in stomatal conductance and biomass accumulation in *Arabidopsis thaliana* [88]. Our studies show that glycine and serine are more enhanced in "EH143 + H&S 14 h" than in "H&S 14 h" (Figure 7A). In case of alanine, an expanded glycolysis pathway for energy production and the accumulation of pyruvate as a storage form of pyruvate are associated with elevated levels of alanine [89]. Also, alanine plays a role in protecting the photosynthetic apparatus from photoinhibition induced by salt stress [66]. Unlike glycine and serine, which are more enhanced in "EH143 + H&S 14 h" than in "H&S 14 h", alanine accumulates to a greater extent in "H&S 14 h" than in "EH143 + H&S 14 h" (Figure 7A). This suggests that respiration in soybeans is more activated in "H&S 14 h" than in "EH143 + H&S 14 h", leading to the increased alanine levels in "H&S 14 h" to enhance the glycolysis pathway for energy production. These findings confirm that "H&S 14 h" prioritizes respiration and energy production; most metabolites in the TCA and glycolysis cycles are relatively more accumulated in "H&S 14 h" than "EH143 + H&S 14 h".

Under heat stress, severe cellular damage and even death may occur within minutes due to a catastrophic collapse of cellular organization [90]. Fatty acid desaturation and alterations in lipid composition are strongly associated with acclimation to cold and hot temperatures. At high temperatures, unsaturated fatty acids are less stable, as cis double bonds can become saturated, isomerized into trans configurations, or undergo other oxidative processes [91]. The loss of membrane integrity results in the activation of lipid-based signalling cascades, increased Ca^{2+} influx, and cytoskeletal reorganization [92]. Saturated fatty acids (methyl stearate, stearic acid, arachidic acid, behenic acid, and lignoceric acid) are upregulated in "EH151 + H&S 14 h" compared to those in "H&S 14 h". This indicates that EH151 inoculation effectively protects plants from cellular damage caused by heat stress to prevent the desaturation of fatty acids (Supporting Information S1: Figure 2).

This study identified a significant role of TCA-dependent metabolites, including sugars, organic acids, and amino acids, in the acclimation of respiration under H&S stress. Intermediates of glycolysis (glucose) and the TCA cycle (citrate, succinate, fumarate, and malate), along with their derivatives (alanine and proline), were strongly correlated with the enhanced respiration to adjust to H&S stress. In contrast, EH151 inoculation decreased the intermediates of glycolysis and the TCA cycle while enhancing several free amino acids (glutamate, L-aspartate, L-serine, *o*-phospho-serine, glycine, L-threonine) under combined H&S stress, compared to soybean seedlings subjected to only H&S stress. These findings offer insights into soybean's the potential metabolic determinants for respiratory acclimation under the combined H&S stress. Specifically, this study demonstrates the effectiveness of melatonin-producing EH151 on adjustments of soybean's metabolites under extreme combined stress.

5 | Conclusion

Overall, our findings demonstrated that the melatonin-secreting potential of EH151 includes the innate ability to produce melatonin along with several plant growth-promoting (PGP) metabolites. Upon inoculation, it enhanced K^+ and Ca^{2+} influx, improved nutrient uptake of P and Mg, and strengthened key biochemical processes such as JA and melatonin synthesis, glycolysis, the TCA cycle, central carbon metabolism (CCM), and the amino acid pathway. These mechanisms potentially contributed to reducing oxidative stress and detoxifying harmful radicals. Currently, the significance of EH151 application could be recommended under controlled conditions. A key challenge lies in the application and sustenance of these microbes under field conditions. However, a major advantage of this study is the novel discovery of melatonin synthesis through biological means. EH151 can be regarded as an important source of melatonin synthesis and a safe biological tool for enhancing crop tolerance against periodic high temperature and salinity stress. Also, considering that melatonin is marketed as both a pharmaceutical and a health supplement product, EH151 could be utilized in the development of food or medicinal products. Building on these possibilities, ongoing research aims to clarify the molecular and genomic mechanisms underlying microbial melatonin biosynthesis. In parallel, efforts are underway to identify melatonin-producing microbial consortia and evaluate their role in alleviating crop stress. Additionally, forthcoming studies will explore the practical applications of engineered melatonin-producing microbes as bio-tools in agriculture, as well as in the pharmaceutical and food industries.

Author Contributions

Eunhae Kwon: design of research, investigation, and writing – original draft. **Abdul Latif Khan, Sang-Mo Kang:** review and editing. **Eunsu Do, Nusrat Jahan Methelaa, Chung-Yeol Lee:** data preparation. **Arjun Adhikari:** data preparation and writing – original draft.

Kang-Mo Ku, Byung-Wook Yun: data preparation and writing – original draft. **In-Jung Lee:** supervision and funding acquisition.

Acknowledgments

This study was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT) (RS-2025-00516246).

Ethics Statement

The authors have nothing to report.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.