Biological Forum – An International Journal **15(5a): 362-367(2023)**

ISSN No. (Print): 0975-1130
ISSN No. (Online): 2249-3239

Melatonin Seed Priming Improves Chlorophyll content under High Temperature Stress by Modulating Heat Responsive Genes in Wheat (*Triticum aestivum* L.)

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(Received: 01 April 2023; Revised: 27 April 2023; Accepted: 04 May 2023; Published: 15 May 2023)
(Published by Research Trend)

ABSTRACT: Extreme temperature swings and a sharp decline in crop productivity brought on by global climate change contribute to global food insecurity. One of the biggest barriers to plant growth and development is high temperature, profoundly impact plant physiology, biochemistry, and molecular processes. Biostimulants melatonin (MT), which serves as a "defence molecule" to protect the body, have a multifunctional purpose. Melatonin (MT) is a pleiotropic signaling molecule which positively modulates the effects of different environmental stressors including heat stress (HS). Heat stress Here, to analyze the effect of melatonin in modulating the chlorophyll content we carried out transcript expression profiling of heat marker genes heat shock protein 70 (Hsp70) and antioxidant enzyme gene superoxide dismutase (SOD) under ambient as well as high temperature stress. Seeds of wheat cv HD2967 were primed with a series of melatonin at MT1 (15 mgl^{-1}), MT2 (30 mgl^{-1}), MT3 (50 mgl^{-1}), MT4 (60 mgl^{-1}), and MT5 (75 mgl^{-1}) and water alone as control. MT3 (50 mgl^{-1}), and MT4 (60 mgl^{-1}) remarkably improved the chlorophyll content. Further, seeds primed with (50 mgl^{-1}) were given heat stress at 37°C for 4hrs and analysed for heat effect on chlorophyll. Results show the effectiveness of MT in enhancing chlorophyll content and induced expression of heat related genes under ambient as well as after high temperature exposure.

Keywords: Melatonin, Seed priming, Wheat, Chlorophyll, germination, HSP70, SOD.

INTRODUCTION

The agricultural output, crop phenology, plant vulnerability, and livelihood of the world's eight billion residents are all seriously threatened by the effects of climate change (Bouabdelli *et al*., 2022). The rise in air temperature brought about by global warming is imposing an unprecedented degree of heat stress (HS), which has lead to significant losses in agricultural output around the globe (Ohama *et al*., 2017; Basavaraj *et al*., 2020**)**. HS upsets cellular equilibrium through increased production of reactive oxygen species (ROS) (Hasanuzzaman *et al.*, 2013). The analysis of heat stress tolerance during seed germination take into account changes in the germination indicators viz germination index (GI), germination potential (GP), and germination rate (GR) (Zhang *et al*., 2017). Thylakoids' structural organisation is impacted by heat stress, resulting in the loss of grana stacking and the enlargement of grana under heat stress (Rodríguez *et al*., 2005; Ashraf and Hafeez 2004). Activity of the (PSII) significantly decreases or even ceases under high temps (Morales *et al*., 2003).

The negative impact of heat on the photosynthetic machinery and chlorophyll content leads to the formation of harmful reactive oxygen species (ROS) (Camejo *et al*., 2006; Guo *et al*., 2007). Heat stress causes oxidative damage by raising the level of excessive reactive oxygen species (ROS) generation in the plant cell, which ultimately leads to cell death. Plant cell metabolism generates reactive oxygen species (ROS), which include hydrogen peroxide, superoxide anions, hydroxyl radicals, singlet oxygen, and triplet oxygen (Gu *et al*., 2022). But a sophisticated antioxidant defence mechanism effectively controls the oxidative profile by boosting the activities of antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), and ascorbate peroxidase (APX), and lessens the effects of stresses (Buttar *et al*., 2020). Sessile plants can't escape environmental stress as their defence depend on changing their metabolism to withstand challenging circumstances. These defence mechanisms include the synthesis of the chemical melatonin, which aids the cell in neutralising ROS by serving as a free oxygen radical scavenger and strengthening the other already-existing enzymatic and non-enzymatic antioxidant activities, as well as raising the tolerance to abiotic stress (Pardo *et al*., 2020). The overall production potential of the wheat crop is greatly impacted by abiotic restrictions such as a changing environment, increases in ambient temperature, and unexpected spikes in temperature (Dubey *et al*., 2020). High temperature stress often result in reduced water availability for seed or seeds that are germinating, which slows down metabolic processes and prevents or delays seed germination and hence, has an adverse effect on the seed's subsequent development phases (Bai *et al*., 2018). Melatonin, a naturally occurring substance found in both plants and mammals. Its application to crop plants may boost food output. According to a recent study, using melatonin enhances crop output under environmental stress by

20% and under drought stress alone by 18%. Additionally, under drought, salt, and cold stress conditions, melatonin-treated plants' photosynthetic rates rise by 44%, 42%, and 48%, respectively which ultimately increases yield (Yang *et al*., 2023). To boost the production of maize, mung beans and cucumbers in organic farming without fertiliser, seed priming with melatonin has been employed successfully (Kołodziejczyk and Posmyk 2016). When nitrogen application is reduced by 15% in rice, melatonin treatment may improve the genes involved in sucrose transporter and nitrogen absorption (Qin *et al*., 2023). The sustainability of soybean production is lowered during the seedling and seed-filling stages due to abiotic stress. Melatonin treatment successfully increases soybean production with better fatty acid levels under salt and drought stress (Zou *et al*., 2019; Wei *et al*., 2015). Similar to this, melatonin priming in maize seeds increases yield and improves salt tolerance (Hussain *et al*., 2022; Ahmad *et al*., 2021).

Towards analyzing the effect of MT, we designed this study to first optimize the effective concentration of MT in boosting germination and grow wheat seed germination indices, seedling growth, then further under applied heat stress and reduce the effects of heat stress. Chlorophyll content and transcript profiling of two heat stress inducible marker genes SOD and HSP were carried out predicting photosynthetic efficiency and the function of MT in modulating the antioxidant defence machinery for the purpose of comprehending systemic stress-induced injuries.

MATERIALS AND METHODS

A. Plant material, seed priming and growth conditions

The seeds of wheat *(Triticum aestivum* L.) cv HD 2967 used in this experiment and were obtained from Division of Genetics, ICAR-Indian Agriculture Research Institute (IARI), New Delhi, India during March 2023. Healthy seeds were separated and surface sterilised for 3 min in a solution of 1% sodium hypochlorite. After rinsing with sterilised water several times, the seeds were allowed to air dry on filter paper. The sterilised seeds were treated with the fungicide Bavistin 1% for another 2hours. After that seeds were air-dried and soaked in different concentrations of MT solutions for priming the seeds. Melatonin (molecular weight: 232.28) was purchased from Sigma Aldrich with >99% purity. To make a stock solution, 75mg MT was weighed and dissolved in an appropriate amount of anhydrous ethanol. Later, deionised water was added and made the final volume of the solution up to 1ml to get a stock solution. Further dilutions were made to prepare different concentrations of melatonin as MT1 ($15mgl^{-1}$), MT2 ($30mgl^{-1}$), MT3 ($50mgl^{-1}$), MT4 ($75mgl^{-1}$), NP (No Priming, only water), and seeds were dipped in the prepared solutions for 24 h at 22 °C ± 2 °C under dark conditions. After that seeds were air-dried thoroughly at room temperature and germinated in petri plates containing moistened blotting paper. After 1-2 days when radicle emergence started, seeds were transferred to pots containing soilrite for further growth in a growth chamber at 22 °C.

B. Chlorophyll measurements

The Witham *et al*. (1971) technique was used to estimate the total chlorophyll content. 20 ml of 80% acetone were added to 100 mg of a finely chopped, evenly mixed fresh leaf sample and pulverised in a mortar and pestle. After centrifuging the samples at 5000 rpm for 5 min, the supernatant was filtered using Whatman No. 1 filter paper and transferred to a 250 ml volumetric flask. Once more, residues were pulverised in 20 ml of 80% acetone, centrifuged, and the supernatant was added to the same volumetric flask. The process was repeated until the residue become colourless. In the volumetric flask, the final volume was made up of 100 ml with 80% acetone. With 80% acetone used as a blank, the extract's absorbance was measured using UV-Vis spectrophotometers at 645 nm and 663 nm wavelength filters to determine the amount of total chlorophyll present by using the following formulae:

Chlorophyll a mg /g tissue= 12.7 (A_{663})- 2.69 (A_{645})* V/1000*W; chlorophyll b-mg/g tissue= 22.9 (A_{645}) - 4.68 (A_{663})* V/1000*W; Total chlorophyll/g tissue= 20.2 (A_{645})+ 8.02 (A_{663})* V/1000*W , where, A= Absorbance at specific wavelengths, V= Final volume of chlorophyll extracts in 80% acetone and, W= Fresh weight of tissue extracted.

C. RNA Isolation, Semi quantitative reverse transcription PCR

Total RNA was prepared from 10 days old wheat seedlings primed with different MT concentration using one-step reagent (Bio-Basic, Canada). First strand cDNA was synthesized from 2mg of total RNA using a Prime Script II First-Strand cDNA synthesis Kit (TakaRa Dalian, China). For PCR amplification, the 18SrDNA gene sequence (Accession no. Y357916) from wheat was used as an internal standard for normalizing cDNA concentration, equal loading and relative quantification. Primers for the two heat marker genes and internal control gene were as follows in Table 1.

D. Heat stress treatment

After analysing the data, MT3 ($50mgl^{-1}$) was selected for further heat stress germination analysis. One set of seeds primed with melatonin MT3 ($50mgl^{-1}$) as described above and the pots were divided into two groups and both the sets of pots was exposed to heat stress in an incubator at 37°C for 4 hrs after 7 days of growth (Fig. 1). Samples were collected from one set and another set was kept for recovery. After 11 days all the pots were taken out and related parameters were measured. Respective control samples without heat stress treatment were also maintained for sample collection.

E. Statistical Analysis

The findings are presented as means and standard error (S.E.). Duncan's multiple range tests were used to identify the significant difference (at $p < 0.05$) between treatments for all morphological and physiological parameters, and analysis of variance (ANOVA) was used to assess the results. SPSS 10.0 (SPSS Inc.,

Chicago, IL, USA) and Microsoft Excel were used to calculate the ANOVA and critical difference value.

RESULT AND DISCUSSION

The above analysis of measurement of chlorophyll content and gene expression profiling of heat related genes under both control and heat stress conditions post melatonin seed priming, following observations were recorded.

A. Melatonin effect on chlorophyll content

The photosynthetic function of a plant depends on the quantity of chlorophyll per unit area. Chlorophyll is the green colour pigment which imparts a characteristic green colour to the plants**.** Priming with different concentration of melatonin improved the chlorophyll content differently. Our findings shows that seed pre-treatment with melatonin had a positive effect on chlorophyll content. Compared to the control plants the total chlorophyll content improved under different melatonin concentration.

In Fig. 2(a), MT1 (15mgl^{-1}), MT2 (30mgl^{-1}), MT3 (50mgl^{-1}), MT4 (60mgl^{-1}) and MT5 (75mgl^{-1}) significantly improved the chlorophyll concentration by 58.8%, 78.2%, 108%, 106% and 82% respectively as compared to NP or no priming control. A similar trend was observed in chlorophyll *a* and chlorophyll *b* content under different melatonin treatments. In case of chlorophyll a, MT1, MT2, MT3, MT4 and MT5 enhanced the chlorophyll concentration by 84%, 86%, 114%, 112% and 81% respectively (Fig. 2b). Similarly, in case of chlorophyll b there was an increase in concentration by 84%, 86.4%, 114%, 95.4% and 84.7% respectively (Fig. 2c). By analysing the data above, amongst all treated concentrations of melatonin, the 50mgl^{-1}(MT3), 60mgl^{-1} (MT4) showed significant impacts on chlorophyll concentration. Therefore, we selected these two concentrations of melatonin to carry out further analyses.

B. Effect of melatonin priming on expression of Hsp70 and SOD transcripts in wheat seedlings

Transcript expression profiling of Hsp70 and SOD gene was carried out in wheat seedlings under different melatonin priming. Differential expression of both the genes was observed when analyzed by semi-quantitative RTPCR with respect to control.

In the present study, in Fig. 3, different melatonin priming showed differential expression of SOD (Superoxide dismutase) and HSP70. Here, MT1 (15mgl^{-1}) MT2 (30mgl^{-1}) MT3 (50mgl^{-1}) shows high expression as compared to MT (60mgl^{-1}), MT (75mgl^{-1}) as compared to control.

C. Effect of MT seed priming under heat exposure

Transcript expression profiling of Hsp70 and SOD gene was carried out in wheat seedling in melatonin primed vs non- primed condition under heat stress. Differential expression of both the genes was observed when analyzed by semi-quantitative RTPCR with respect to control. In Fig. 4 (a) SOD expression was enhanced as compared to heat stress under primed condition. While, in case of Fig. 4 (b) Hsp70 the expression remains same under both heat stress and primed condition alone which may be attributed to general role of HSP70 as chaperone in various growth and development.

D. Melatonin seed priming has positive effects on chlorophyll content under heat stress

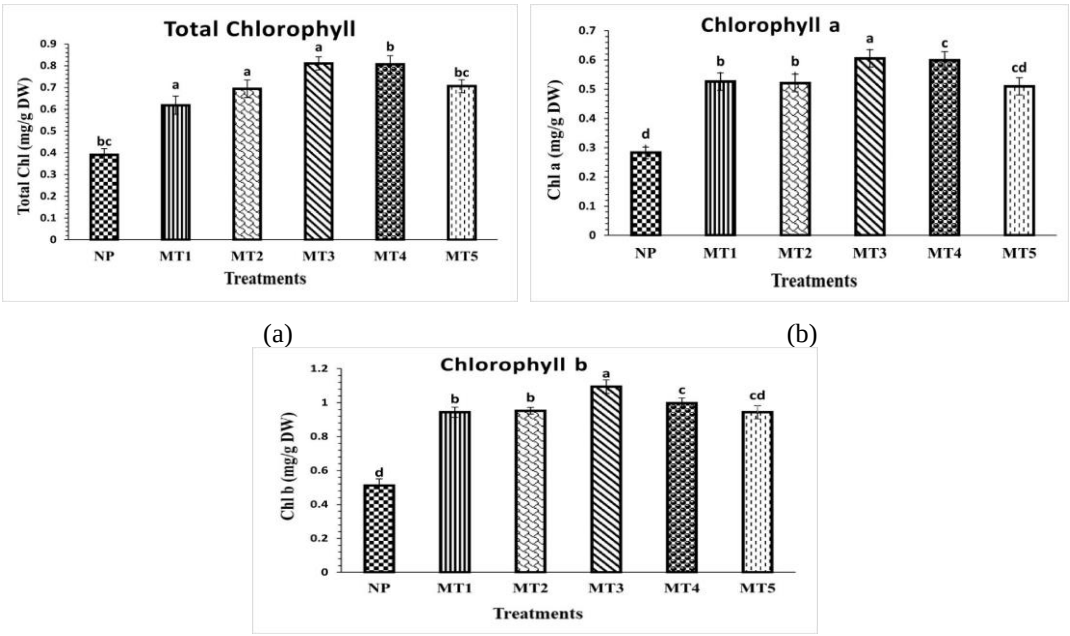
The chlorophyll content was recorded at seedling stage and the effect of priming over non- primed condition was compared. We found that under heat stress condition the average level of total chlorophyll decreased by 28.34% over non primed condition, while in case of primed condition, the average total chlorophyll increased by 64% over non- primed condition. A similar trend was observed in chlorophyll a and b content under heat stress. Heat stress reduced the chlorophyll a content by 28.34% and chlorophyll content by 11.6% respectively. The melatonin priming combated the stress by improving chlorophyll a by 52.9% and chlorophyll b by 34% respectively (Fig. 5).

The above findings of different concentrations melatonin seed priming under and chlorophyll measurement showed that MT3 and MT4 as better option as compared to other concentrations and MT3 showed improved results under heat stress condition over control condition. Awan *et al*. (2023); Barman *et al*. (2019) reported protective effect of melatonin in seed germination in soybean and at physiological maturity in rice respectively. Heat stress raises the activity of the enzyme chlorophyllase and reduces the quantity of photosynthetic pigments, which results in decreased plant photosynthesis and increased respiratory activity (Sharkey and Zhang 2010). Our findings on total chlorophyll, chlorophyll a and chlorophyll b shows a large drop caused by heat stress that might attributable to an increase in oxidative damage. Collectively, our finding shows that melatonin reduce chlorophyll damage under heat stress and maintain its activity under stress which maybe as a result of increased antioxidant ability needed further investigation. The heat responsive genes Hsp70 and SOD (superoxide dismutase) showed differential inducibility under heat stress exposure. Buttar *et al*. (2020) documented that under heat stress condition the heat responsive genes showed higher induction and upregulates the antioxidant machinery genes after melatonin priming.

Table 1: List of primers of two heat marker genes and internal control gene.

Hsp70F	5′ GAGCAAGGAGGAGATTGAGAAG 3′
Hsp70R	5′CAG ACAAATCGCTCCACCAA 3′
SOD F	5′ CTGCATCCTTTGTTGGGAATTG3′ 3′
SODR	5′GTTCACCACCTTCCAGATGTT 3′
18SrDNAF	5′TTTGACTCAACACGGGGAAT3′
18SrDNAR	5′CAG ACAAATCGCTCCACCAA3′

Fig. 1. Plants exposed to heat stress at 37°C for 4 hrs. to MT3 (50 mgl^{-1}) seeds.



(a) (b)

(c)

Fig. 2. Effect of different concentrations of melatonin treatment on (a) total chlorophyll content (b) chlorophyll a content, (c) chlorophyll b content in seedling stage. Here, NP (No Priming), MT1(15 mgl^{-1}), MT2 (30 mgl^{-1}), MT3 (50 mgl^{-1}), MT4 (60 mgl^{-1}), MT5 (75 mgl^{-1}). Values are the means ± SD (n=3). Different letters on the bars show a statistical significance level at $p<0.05$.

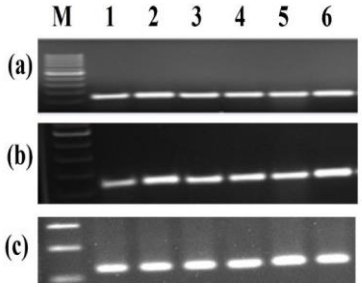


Fig. 3. Transcript expression profiling by semi quantitative RT-PCR of (a) SOD and (b) Hsp70 gene taking (c) 18S rDNA as housekeeping gene in wheat seedling under different melatonin priming concentration, where M is 100 bp plus ladder, Lane 1 (NP-No Priming), Lane 2 (MT1-15 mgl^{-1}), Lane 3(MT2-30 mgl^{-1}), Lane 4 (MT3-50 mgl-1), Lane 5 (MT4-60 mgl^{-1}), Lane 6 (MT5-75 mgl^{-1}).

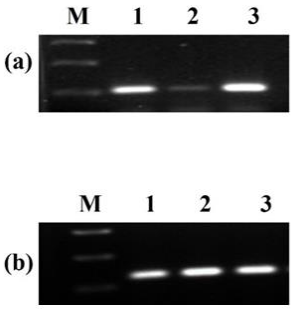


Fig. 4. Expression of (a) SOD and (b) Hsp70 under priming with MT3 (50mgl^{-1}), where M is 100 bp plus ladder, Lane1- NP (No Priming), Lane 2- Heat stress at 37°C for 4hrs (HS), Lane3- HS+MT3 (Heat stress+ MT- 50 mgl^{-1}).

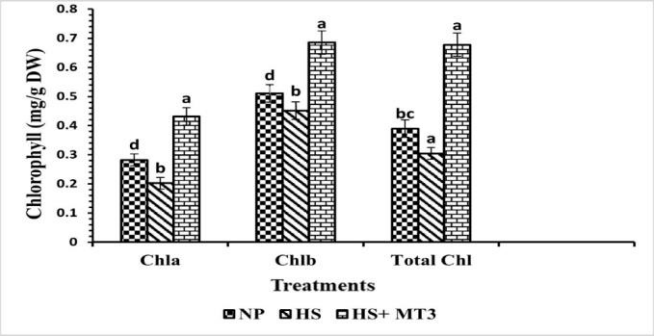


Fig. 5. Effect of priming with MT3 (50 mgl^{-1}) as well as heat stress (HS) (37°C for 4hrs) on chlorophyll.

CONCLUSIONS

Based on the experimental findings, we conclude that melatonin seed priming differentially regulated and positively improved the germination indices and other physiological processes. Pre-treatment of melatonin at 50 mgl^{-1} and 60mgl^{-1} was found significant in improving chlorophyl content. Our study concluded that melatonin has potential role to improve chlorophyll content under different concentration with 50mgl^{-1} being an optimum value to sustain photosynthetic efficiency. At the molecular level transcript expression of Hsp70 showed similar level under different melatonin concentration. At the specific concentrations of melatonin (50mgl^{-1} and 60mgl^{-1}) can positively improve chlorophyll concentration. The seeds primed with 50mgl^{-1} was given heat stress and further compared with control seedlings and positive effect of melatonin was recorded. The expression of SOD enzyme gene, an antioxidant enzyme was increased under melatonin priming as compared to no priming under heat stress condition. Chlorophyll content also improved in primed condition under heat stress, hence improving the photosynthetic capacity. This study provides a valuable base in showing the protective role melatonin in germination and seedling growth of wheat under normal and heat stress environment.

FUTURE SCOPE

Further, molecular and whole genome transcriptome and functional analysis would reveal the melatonin-mediated modulation of various metabolic pathways and identify important genes which can be utilised towards improved crop production and maintaining productivity under a changing climate scenario.

Acknowledgements. The work described here was carried out under the aegis of ICAR-NIPB in- house project, NRCPB-RPP 2012-2017-2021/02 of SB. SS acknowledges PG School, ICAR-Indian Agricultural Research Institute for a research fellowship.

Conflict of Interest. None.

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How to cite this article: Shaziya Sultana and Sharmistha Barthakur (2023). Melatonin Seed Priming Improves Chlorophyll content under High Temperature Stress by Modulating Heat Responsive Genes in Wheat (*Triticum aestivum* L.). *Biological Forum – An International Journal*, *15*(5a): 362-367.