

## Enhancing drought tolerance in okra through melatonin application: A comprehensive study of physiological, biochemical and metabolic responses

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### Abstract

As climate change intensifies, drought stress presents a critical challenge for horticultural crops like okra (*Abelmoschus esculentus*). The effectiveness of melatonin in reducing drought stress is investigated in this study. The treatments include: Absolute control (fully irrigated), control (drought), drought and seed treatment with 100  $\mu$ M melatonin, drought and foliar spray of 100  $\mu$ M melatonin, and drought stress with combined effect of seed treatment and foliar spray of 100  $\mu$ M melatonin. Physiological parameters such as photosynthetic rate, stomatal conductance, transpiration rate, Fv/Fm ratio, and chlorophyll index were evaluated, alongside biochemical parameters including malondialdehyde, proline content, membrane stability index and antioxidant enzyme activities such as catalase and ascorbate peroxidase were quantified. Melatonin supplemented as seed treatment and foliar spray enhanced both physiological and biochemical parameters including antioxidant activity compared to drought control. Metabolite profiling identified bioactive compounds (mainly carbohydrates and amino acids) contributing to drought tolerance in okra. The results highlight that application of 100  $\mu$ M melatonin via seed treatment and foliar spray enhances drought tolerance in okra, suggesting its potential to enhance crop resilience under water-deficit conditions.

**Keywords:** drought; okra; melatonin; metabolites

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## Introduction

Climate change and the rising frequency of extreme weather events present substantial risks to global crop yield and their stability (Reyes *et al.*, 2021). It is predicted that sustained global warming will worsen the variability of the global water cycle, the global monsoon's precipitation, extreme wet and dry weather, and climate events and seasons (Lee *et al.*, 2023). As climate change advances, understanding its effects on various vegetable crops is vital. Certain crops may exhibit greater resilience to specific climatic conditions, necessitating strategic adaptation measures. Additionally, alterations in precipitation and temperature can influence pest and disease life cycles, impacting crop yield and quality. Drought is a complex stressor that primarily arises from reduced rainfall and prolonged dry spells (Gogoi and Tripathi, 2019).

Each vegetable crop responds uniquely to drought, depending on its severity and the plant's growth stages. Okra (*Abelmoschus esculentus* [L.] Moench), a significant crop grown for its fruits, vegetables, and seed oil, flourishes in tropical, subtropical, arid, and semi-arid climates. Vegetable crops are pivotal for human nutrition, providing essential nutrients in daily diets. Consumption of okra has been linked to health advantages, such as reducing sugar levels in blood, lowering lipid levels and mitigating constipation (Gemede *et al.*, 2015). In India, okra cultivation spans an area of 554 thousand hectares, yielding an annual production of 7252 thousand metric tons for the 2023–2024 period (Indiastat, 2023). Nonetheless, okra cultivation is notably vulnerable to the effects of climate change, directly impacting its yield. Abiotic stress, particularly drought, causes okra yield losses ranging from 30% to 100%, notably during flowering and pod-filling stages (Mbagwu and Adesipe, 1987). Yield reductions depend on cultivar variation and the phenological stage of drought occurrence. Drought stress caused reduction in stomatal conductance (gs), transpiration rate (T), net photosynthetic rate (A), maximum photochemical efficiency (Fv/Fm), actual photochemical efficiency of PSII ( $\phi$ PSII), photochemical quenching (qP) and electron transport rate (ETR) in various okra varieties (Mkhabela *et al.*, 2023). Drought stress resulted in reduced leaf water status, chlorophyll content, carotenoid levels, and total protein ( $\mu$ g/g) at a drought level of 25% field capacity (FC). Conversely, levels of malondialdehyde (MDA), proline content along with the activities of catalase (CAT) and peroxidase (POD) increased (Ayub *et al.*, 2021). Enhancing the tolerance of vegetables to drought necessitates a multifaceted approach, integrating novel cultivation techniques and external regulatory technologies to enhance the growth and development of vegetables globally (Dhankher and Foyer, 2018; Razi and Muneer, 2021).

Melatonin (N-acetyl-5-methoxytryptamine) was identified as a notable bioactive compound in vascular plants in 1995 (Dubbels *et al.*, 1995). Initially noted for its potent antioxidant properties, melatonin has since been recognized for its diverse roles in plant development (Sheshadri *et al.*, 2018). It contributes to various physiological processes, including seed germination (Zhang *et al.*, 2017) root elongation (Arnao and Hernández-Ruiz, 2019), photosynthesis enhancement (Li *et al.*, 2017), and leaf senescence regulation (Wang *et al.*, 2022). As a plant hormone, melatonin plays a crucial role in growth regulation and developmental pathways (Arnao and Hernández-Ruiz, 2019). It is present in multiple plant tissues, including seeds, roots, leaves, and fruits (Zhang *et al.*, 2017). Research has extensively examined melatonin's potential to improve plant growth and regulatory processes (Sun *et al.*, 2020).

Melatonin is an effective antioxidant compound known for its reactive oxygen species (ROS) detoxification properties. It enhances plant resilience to various stresses by neutralizing free radical species through single-electron and hydrogen transfer mechanisms. It effectively scavenges ROS such as superoxide anions ( $O_2^{\bullet-}$ ) and hydroxyl radicals (OH $\cdot$ ), reactive nitrogen species (RNS) including nitric oxide, nitrogen dioxide radicals, azide radicals, and peroxy nitrite radicals, as well as other oxidative agents. Melatonin reduces oxidative damage induced by nickel in both tomato leaves and roots by inhibiting the production of superoxide anion ( $O_2^{\bullet-}$ ) and hydrogen peroxide ( $H_2O_2$ ) (Jahan *et al.*, 2020). It also enhances Arabidopsis' tolerance to high light levels by scavenging both  $H_2O_2$  and  $O_2^{\bullet-}$  (Yang *et al.*, 2021). Moreover, exposure to various environmental stresses triggers the biosynthesis of endogenous melatonin upon the application of exogenous melatonin. This

process effectively suppresses the accumulation of  $H_2O_2$ ,  $O_2^{\bullet-}$  and MDA, thereby mitigating cell membrane disruption and enhancing photosynthetic efficiency (Chen *et al.*, 2021; Imran *et al.*, 2021). Hence, Melatonin and its metabolites directly scavenge ROS and RNS in stressed plants. Melatonin contributes to the removal of ROS and enhances the activity of antioxidant enzymes, mitigating the impact of water stress (Zhang *et al.*, 2019).

Exogenous melatonin significantly boosted the performance of antioxidant enzymes and improved the yield of soybean plants (Oliveira-Spolaor *et al.*, 2022). Recent findings suggests that external application of melatonin enhances salt tolerance in okra by increasing proline accumulation and the  $K^+/Na^+$  ratio. This regulation aids in maintaining water equilibrium and ionic stability, preserves membrane stability, photosynthetic process and activates the reactive oxygen species detoxification system (Wang *et al.*, 2024). Melatonin can be applied exogenously either by seed priming (Bai *et al.*, 2020), root or rhizosphere application (Wang *et al.*, 2022) (Imran *et al.*, 2021) or foliar spray (Cherono *et al.*, 2021). However, the effect of melatonin on plant growth and health depends on the method of application. Comprehensive studies are required to evaluate the efficacy of melatonin supplemented through seed treatment, foliar spray and their combination on plants. Hence, further in-depth studies are necessary to explore stress signaling pathways, homeostasis maintenance mechanisms and the most effective mitigation strategies for melatonin application under abiotic stress conditions. Limited research exists on the stress resilience of important but underutilized vegetable crop like okra. The present study aims at unravelling the potential of melatonin in counteracting the detrimental effect of drought stress in okra by utilizing various application methods (seed treatment, foliar spray and their combination) and modulating the physiological and biochemical attributes including antioxidant systems.

## Materials and Methods

### *Planting material and source of melatonin*

Okra hybrid seeds CO 4, Arka Anamika, Arka Abhay and Arka Nikita sourced from Tamil Nadu Agricultural University, Coimbatore, Kerala Agricultural University, Thrissur, and Indian Institute of Horticultural Research, Bangalore respectively. The soil mixture consisted of red soil, clay, and farmyard manure in 2:1:1 ratio. The physio-chemical properties of soil are represented in Table 1. Melatonin was sourced from Sigma-Aldrich Pvt. Ltd.

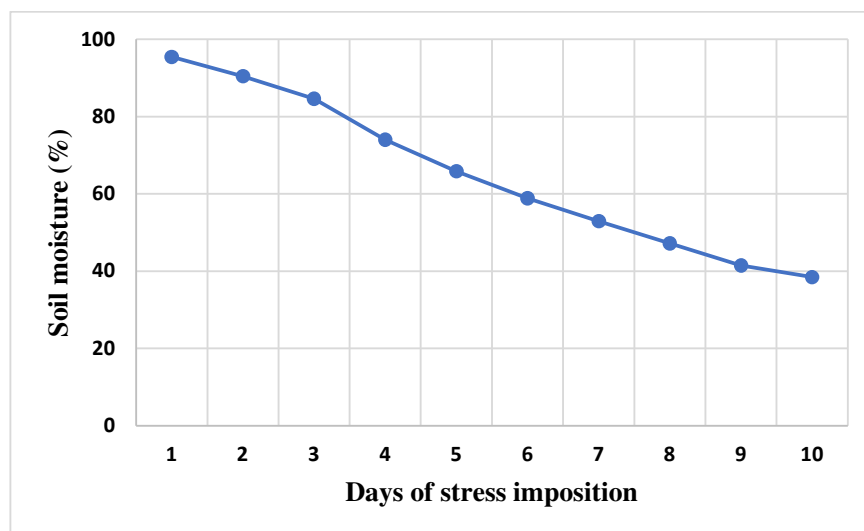
**Table 1.** Physio-chemical properties of soil

Soil properties	Range
Texture	Red loam
pH	6.42
Electrical conductivity ( $ds\ m^{-1}$ )	0.62
Organic carbon (%)	1.32
Available Nitrogen ( $kg\ ha^{-1}$ )	247.52
Available phosphorous ( $kg\ ha^{-1}$ )	20.74
Available potassium ( $kg\ ha^{-1}$ )	541.6

### *Plant growth condition and treatment imposition*

Preliminary screening of okra hybrids (CO 4, Arka Anamika, Arka Abhay and Arka Nikita) were carried out for standardization of optimum concentration of melatonin for seed treatment and foliar spray. Based on laboratory screening, among the melatonin concentrations (50, 100, 150, 200 and 250  $\mu M$ ), 100  $\mu M$  melatonin (MT) was identified as the most effective concentration of melatonin for enhancing drought tolerance in okra. This concentration underwent further evaluation in pot culture experiment on the four okra hybrids (CO 4, Arka Anamika, Arka Abhay and Arka Nikita). The formulated pot study was conducted during July to

September 2023 at Department of Crop Physiology, Tamil Nadu Agricultural University, Coimbatore, India by following five treatments and four replications. Treatments include, absolute control (T1: fully irrigated AC), control (T2: Drought control C), Drought and seed treatment (ST) with 100  $\mu\text{M}$  melatonin (T3: MT - ST), Drought and foliar spray (FS) of 100  $\mu\text{M}$  melatonin (T4: MT - FS), and drought stress with combined effect of seed treatment and foliar spray of 100  $\mu\text{M}$  melatonin (T5: MT - ST + FS). For seed treatment, seeds from each hybrid were surface sterilization with 75% ethanol followed by treatment with 100  $\mu\text{M}$  melatonin by soaking for 6 hours at 25 °C and shade dried. Then, seeds were dibbled in pot at a depth of approximately half an inch and the pots were maintained under ambient condition. After seed germination, each pot with two plants were regularly irrigated and managed according to standard crop management practices until flowering. Upon the onset of flower initiation, the pots were relocated to a controlled greenhouse setting at Department of Crop Physiology, Tamil Nadu Agricultural University, Coimbatore. Drought stress was induced naturally by ceasing irrigation, with daily monitoring of moisture levels by HD2 mobile moisture meter (Figure 1), while the remaining pots (AC) continued to receive irrigation. The stress period spanned for ten days (from the 31<sup>st</sup> to the 42<sup>th</sup> day) during the flowering stage. Five days after the initiation of stress, plants were treated with a spray of 100  $\mu\text{M}$  melatonin (requisite amount of melatonin were dissolved in 99.9% ethanol and the final volume was adjusted using distilled water, absorption efficiency was enhanced by addition of tween 20). At the termination of the stress period, leaf samples from all the treatments were taken to analyze biochemical parameters.



**Figure 1.** Soil moisture content under drought stress for a time period of ten days

#### *Photosynthetic measurements*

Gas exchange parameters were measured on fully expanded uppermost leaves during a bright sunny day from 9:00 to 11:30 AM using the CI-340 handheld photosynthesis system (CID BioSciences Inc). Recorded parameters included photosynthetic rate ( $A$ ,  $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ ), transpiration rate ( $E$ ,  $\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$ ), and stomatal conductance ( $C$ ,  $\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$ ). Chlorophyll fluorescence was measured using a chlorophyll fluorometer (Heinz Walz, Germany), with leaves dark-adapted for 30 min prior to measurement.

$$\text{Maximum quantum yield of PS II (Fv/Fm) was computed as } Fv/Fm = (Fm - Fo)/Fm. \quad (1)$$

Chlorophyll index was determined using a SPAD meter (Minolta), based on the ratio of light transmitted at wavelengths of 650 nm and 940 nm.

*Relative water content (RWC)*

Fresh samples of most juvenile and fully extended leaves were collected, cut into 1 cm pieces, and weighed (0.5 g). These samples were then immersed in water for 4 hours, after which their turgid weight was recorded. Following hydration, the samples were dried in an oven at 80 °C for 24 h to determine their dry weight.

Relative Water Content (RWC) was computed using the formula proposed by González and González-Vilar (2001).

$$\text{RWC (\%)} = \frac{(\text{FW} - \text{TW})}{(\text{TW} - \text{DW})} \times 100 \quad (2)$$

FW = Fresh weight, TW = Turgid weight, DW = Dry weight

*Membrane stability index (MSI)*

MSI was determined following the method by (Premachandra *et al.*, 1991), modified by (Sairam, 1994). Fresh leaf material (1 g) was cut into discs, cleaned, and put in glass test tubes along with a blank. To immerse the discs, 10 mL of deionized water were poured to each tube. For 30 min, the tubes were incubated in a water bath at 45 °C. Electrical conductivity ( $C_1$ ) was measured after cooling. After adding water once more, the leaf discs were incubated for 10 minutes at 100 °C in a water bath before their final electrical conductivity ( $C_2$ ) was determined.

The membrane stability index was determined using percent conductivity, as given by the formula:

$$\text{MSI (\%)} = 1 - C_1 / C_2 \times 100 \quad (3)$$

Where  $C_1$  = Initial electrical conductivity  $C_2$  = Final electrical conductivity

*Proline content*

Proline levels were quantified according to the method described by (Bates *et al.*, 1973). Fresh leaf samples (0.1 g) were homogenized in 3% aqueous sulfosalicylic acid and centrifuged at 5000 rpm for 10 min. The supernatant was mixed with acid ninhydrin, glacial acetic acid 2 ml of enzyme extract, and the mixture was heated in a hot water bath for 1 h, then cooled on an ice bath. Proline was extracted with 4 ml of toluene and vigorously mixed, vortexed for 10 s. The absorbance of the resultant solution was measured at 520 nm, with toluene serving as the blank reference.

*Malondialdehyde content*

MDA levels were determined as per the protocol outlined by (Hodges *et al.*, 1999). Frozen leaf samples weighing 0.1 g each were pulverized in 1 ml of 0.1% trichloroacetic acid (TCA). The resulting homogenate underwent centrifugation at 4 °C for 30 min at 15000 rpm. To the supernatant, 4 ml of 20% trichloroacetic acid containing 0.5% thiobarbituric acid was added and heated at 95 °C for 15 min, then immediate cooling on ice. The MDA concentration ( $\text{nmol mL}^{-1}$ ) of the filtrate was measured at 532 nm and expressed as  $\text{nmol MDA g}^{-1}$  FW.

*Antioxidant enzyme assays*

Fresh leaf samples (100 mg) were homogenized using 50 mM sodium phosphate buffer along with 0.05% Triton X-100, 2% polyvinylpyrrolidone (PVP), and 1 mM EDTA for enzyme extraction in APX and CAT assays. The extract was subjected to centrifugation at 18,000 rpm at 4 °C for 20 min, and the resulting extraction buffer was used for enzyme analysis.

For the determination of APX activity, 0.3 g of tissue was homogenized in 3 mL of extraction buffer followed by addition of 1 mL of 5 mM ascorbate. After centrifugation at 10,000 rpm for 20 min at 4 °C, the resulting aliquot was mixed with reaction buffer containing 2 mL of  $\text{KH}_2\text{PO}_4$  and  $\text{K}_2\text{HPO}_4$  (pH 7.3). Absorbance readings were taken at 290 nm with 30 s intervals for 3 min (extinction coefficient =  $2.8 \text{ mM}^{-1} \text{ cm}^{-1}$ ) (Nakano *et al.*, 1981).

CAT activity was assessed using a 50 mM phosphate buffer containing 100  $\mu\text{L}$  of enzyme extract and 15 mM  $\text{H}_2\text{O}_2$ . Plant tissue samples (approximately 0.2 g) were homogenized in an extraction buffer. After centrifugation at 10,000 rpm for 20 min, the supernatant was combined with a reaction buffer ( $\text{K}_2\text{HPO}_4$ ,  $\text{KH}_2\text{PO}_4$ ) and  $\text{H}_2\text{O}_2$  as the enzyme substrate. Absorbance was monitored at 240 nm for 3 min at 30 s intervals, using an extinction coefficient of  $39.4 \text{ mM}^{-1} \text{ cm}^{-1}$ , for the determination of  $\text{H}_2\text{O}_2$  scavenging activity (Cakmak *et al.*, 1992).

#### *Identification of bioactive compounds through GC-MS/MS*

GC MS analysis was conducted at Centre for Plant Molecular Biology (CPMB), Tamil Nadu Agricultural University, Coimbatore. Two Samples (in triplicates) were frozen and ground using liquid nitrogen. Samples of about 0.3 g each were taken in 2 ml microcentrifuge tubes followed by addition of 1.4 ml of 100% methanol and vortexed. The tubes were subjected to incubation at 70 °C with continuous shaking for 15 min. The samples were centrifuged at 12000 rpm for 20 min at 4 °C. Supernatant was transferred through a 0.2  $\mu\text{m}$  syringe filter followed by addition of 1.4 ml water and 750  $\mu\text{l}$  of chloroform. The tubes were again subjected to centrifugation at 12000 rpm for 10 min. The upper polar phase (1 ml) was transferred to new tubes. The upper phase was concentrated using concentrator at 45 °C for 3 h. Derivatization was carried out using 50  $\mu\text{l}$  of methoxamine hydrochloride and 80  $\mu\text{l}$  of MSTFA followed by incubation and centrifugation at 10000 rpm for 3 min.

GC-MS/MS analysis of okra leaf extract in methanol was done using SHIMADZU NEXIS-GC-2030 GCMS - TQ8040NX spectrometer. The SH-Rxi-5Sil MS column (30 m length, 0.25 mm diameter, and 0.25  $\mu\text{m}$  film thickness) was used in the GC-MS system. The carrier gas used was Helium, with a flow rate of 1ml  $\text{min}^{-1}$ . Initial temperature was 70 °C (hold 1 min), ramped to 225 °C (wait 3 min) then to 300 °C (hold 5 min) with a total experiment run length of 55 min. A sample of about 2  $\mu\text{L}$  was injected. The injector and detector were both kept at 230 °C. Electron ionisation at 70 eV was used to acquire mass spectra with a spectral range of  $m/z$  50-700 amu. The GC-MS spectra were analysed using NIST library. The bioactive compounds with highest peak area were selected for analysis. A heat map was generated for the selected compounds using MetaboAnalyst 5.0.

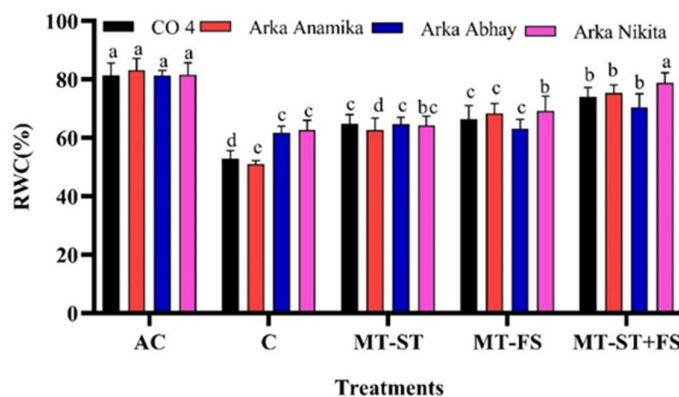
#### *Statistical analysis*

The study utilized a Factorial Completely Randomized Design (FCRD) to analyse the efficacy of different methods of application of melatonin on stress tolerance of four okra hybrids. Data collected for various traits were statistically analyzed using SPSS software (version 16.0) via a two-way Analysis of Variance (ANOVA). To assess differences among group means, the Least Significant Difference (LSD) test was applied, and the Critical Difference (CD) was calculated at a significance level of 0.05 ( $p \leq 0.05$ ). Graphs were produced using GraphPad Prism (version 8.2.0). Principal component analysis and correlation were carried out using R software (version 4.3.1).

## Results

### *Changes in RWC of drought stressed okra hybrids under varying melatonin treatments*

The study represents a significant variation in the relative water content (RWC) of okra hybrids, CO 4, Arka Anamika, Arka Abhay, and Arka Nikita under different treatments. The highest RWC, 74.65%, was achieved with a combined treatment of 100  $\mu$ M melatonin as seed treatment and foliar spray (Figure 2). Although the differences among the hybrids were not significant ( $p < 0.05$ ). The interaction between the hybrids and the treatments showed a significant effect ( $p < 0.05$ ).

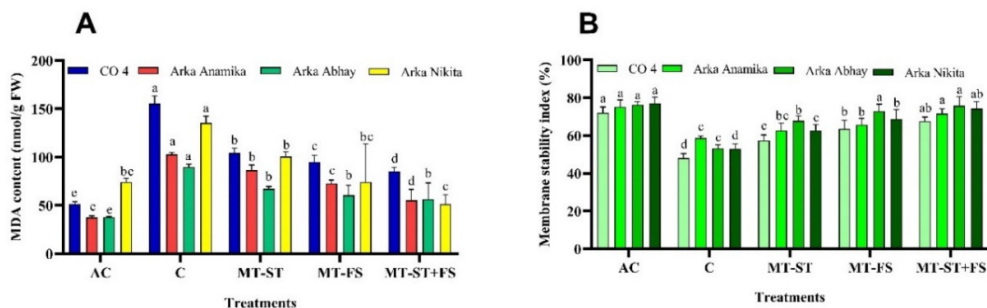


**Figure 2.** Effect of melatonin on relative water content (RWC) under drought stress in okra  
AC: Absolute Control, C: Control (Drought), MT-ST: Seed treatment of 100  $\mu$ M melatonin, MT-FS: Foliar spray of 100  $\mu$ M melatonin, MT- ST+FS: Combined effect of seed treatment and foliar spray of 100  $\mu$ M melatonin. Data in the figure are expressed as mean  $\pm$  SE. Different letters between the treatments and hybrids differ significantly at  $P \leq 0.05$  by LSD

### *MDA and MSI under different melatonin treatment combinations*

The ability of plant to withstand drought stress can be assessed through quantification of MDA and MSI. The inverse relation between MDA and MSI is evident among okra hybrids. The results reveal that plants experiencing water deficit showed increase in malondialdehyde content (120.77  $\text{nmol g}^{-1}$  FW) compared to absolute control (AC). Application of 100  $\mu$ M of melatonin (MT- ST+ FS) reduced the MDA content by 46.22% in case of Arka Anamika compared to seed treatment and foliar spray. Among hybrids, CO 4 accumulated more MDA (98.27  $\text{nmol g}^{-1}$  FW) and Arka Abhay the least (62.19  $\text{nmol g}^{-1}$  FW) (Figure 3).

Among hybrids, Arka Abhay exhibited higher membrane stability index (69.22%) and CO 4 recorded the least (61.71%). Membrane stability index reduced significantly in case of drought stressed treatment (C) (53.20%). Application of 100  $\mu$ M of melatonin as seed treatment and foliar spray enhanced the membrane stability index by 40.16% in case of CO 4 (Figure 3) compared to other treatments.

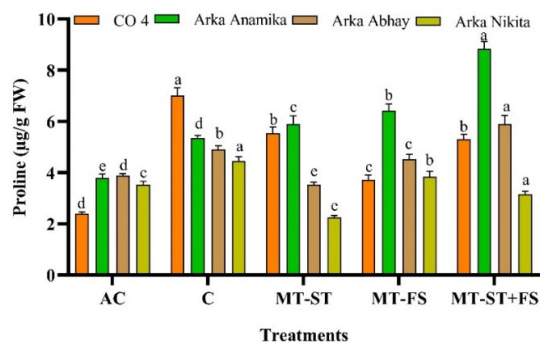


**Figure 3.** Effect of melatonin on A) malondialdehyde content (MDA) and B) membrane stability index (MSI) under drought stress in okra

AC: Absolute Control, C: Control (Drought), MT-ST: Seed treatment of 100  $\mu\text{M}$  melatonin, MT-FS: Foliar spray of 100  $\mu\text{M}$  melatonin, MT- ST+FS: Combined effect of seed treatment and foliar spray of 100  $\mu\text{M}$  melatonin. Data in the figure are expressed as mean  $\pm$  SE. Different letters between the treatments and hybrids differ significantly at  $P \leq 0.05$  by LSD

#### *Changes in proline content across different melatonin treatments*

When plants face drought stress, the accumulation of proline is a key indicator of their response. In comparison to absolute control, the plants that received a treatment combination (MT- ST+FS) of 100  $\mu\text{M}$  melatonin (5.80  $\mu\text{g g}^{-1}$  FW) and the drought stressed plants (5.42  $\mu\text{g g}^{-1}$  FW) accumulated more proline. Among hybrids, Arka Anamika recorded higher proline content (6.05  $\mu\text{g g}^{-1}$  FW) and Arka Nikita showed the least value (3.44  $\mu\text{g g}^{-1}$  FW). Application of 100  $\mu\text{M}$  of melatonin as seed treatment and foliar spray enhanced the proline levels in Arka Anamika (Figure 4). The interaction effect between treatment and hybrids are significant.



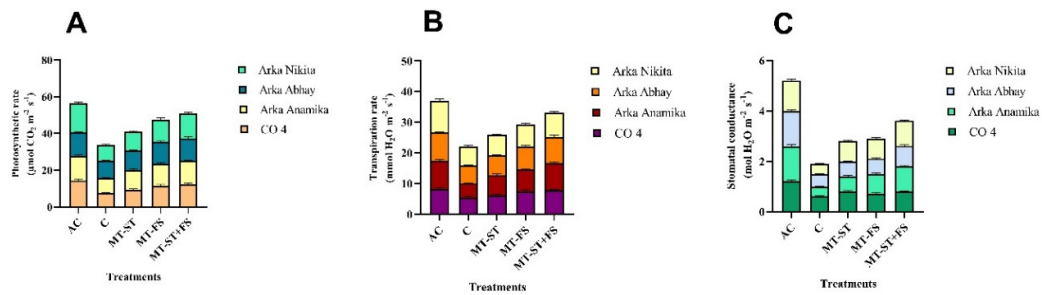
**Figure 4.** Effect of melatonin on proline content under drought stress in okra

AC: Absolute Control, C: Control (Drought), MT-ST: Seed treatment of 100  $\mu\text{M}$  melatonin, MT-FS: Foliar spray of 100  $\mu\text{M}$  melatonin, MT- ST+FS: Combined effect of seed treatment and foliar spray of 100  $\mu\text{M}$  melatonin. Data in the figure are expressed as mean  $\pm$  SE. Different letters between the treatments and hybrids differ significantly at  $P \leq 0.05$  by LSD

#### *Gas exchange indicators*

Gas exchange parameters such as photosynthetic rate, stomatal conductance and transpiration rate were significantly influenced by treatments and their interaction with hybrids. Among the treatments, drought stressed plants recorded a decrease in photosynthetic rate (8.41  $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ ), transpiration rate (5.52  $\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$ ) and stomatal conductance (0.47  $\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$ ) whereas the combined application of 100  $\mu\text{M}$  of melatonin as seed treatment and foliar spray showed an increment in photosynthetic rate (12.72  $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ ), transpiration rate (8.27  $\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$ ) and stomatal conductance (0.90  $\text{mmol H}_2\text{O}$

$m^{-2} s^{-1}$ ). Among hybrids, Arka Nikita outperformed other hybrids in photosynthetic rate, transpiration rate and stomatal conductance (Figure 5). Interaction effect between hybrids and treatments are significant ( $p < 0.05$ ).

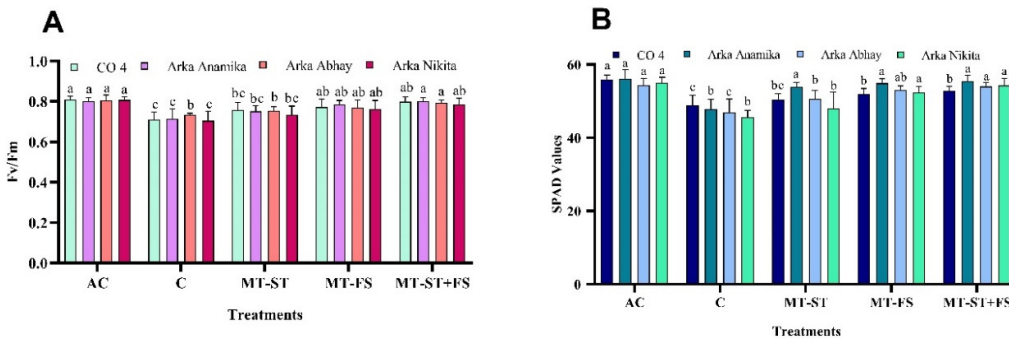


**Figure 5.** Effect of melatonin on A) Photosynthetic rate, B) Transpiration rate and C) Stomatal conductance under drought stress in okra

AC: Absolute Control, C: Control (Drought), MT-ST: Seed treatment of 100  $\mu$ M melatonin, MT-FS: Foliar spray of 100  $\mu$ M melatonin, MT- ST+FS: Combined effect of seed treatment and foliar spray of 100  $\mu$ M melatonin. Data in the figure are expressed as mean  $\pm$  SE. Different letters between the treatments and hybrids differ significantly at  $P \leq 0.05$  by LSD

*Fv/Fm and SPAD*

Plants exposed to drought stress showed reduced  $F_v/F_m$  ratio (0.716) over absolute control (0.807). Melatonin treatment significantly increased  $F_v/F_m$  ratio. Treatment effects are significant. Among the treatments, application of 100  $\mu$ M of melatonin as seed treatment and foliar spray showed higher  $F_v/F_m$  ratio (0.796) in comparison to other treatments. Among hybrids, Arka Anamika (0.80) exhibited higher  $F_v/F_m$  compared to other hybrids. The chlorophyll index (SPAD values) declined significantly in case of drought stressed plants (47.31) compared to absolute control (55.35). Application of melatonin as seed treatment and foliar spray improved chlorophyll index (54.13). All hybrids performed better when supplemented with 100  $\mu$ M of melatonin as seed treatment and foliar spray, with Arka Anamika exhibiting higher chlorophyll index (55.40) (Figure 6).

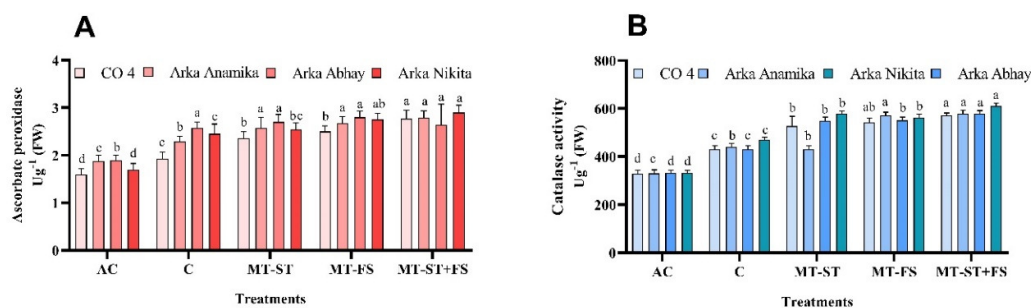


**Figure 6.** Effect of melatonin on A) Chlorophyll fluorescence and B) SPAD values under drought stress in okra

AC: Absolute Control, C: Control (Drought), MT-ST: Seed treatment of 100  $\mu$ M melatonin, MT-FS: Foliar spray of 100  $\mu$ M melatonin, MT- ST+FS: Combined effect of seed treatment and foliar spray of 100  $\mu$ M melatonin. Data in the figure are expressed as mean  $\pm$  SE. Different letters between the treatments and hybrids differ significantly at  $P \leq 0.05$  by LSD

*Ascorbate peroxidase (APX) and catalase (CAT) activity*

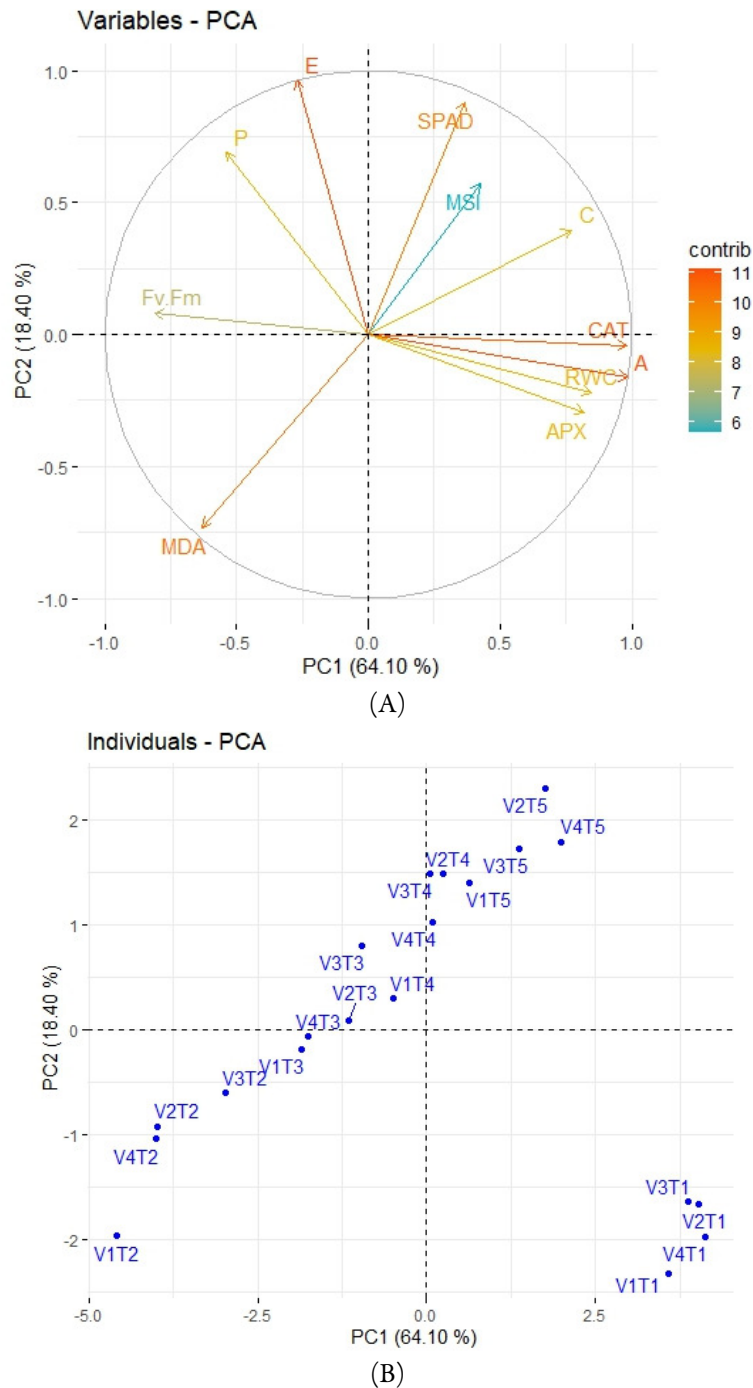
Enzymatic antioxidant activity such as APX and CAT increased significantly under drought stress compared to absolute control by 23.47% and 25.31% respectively. The enzyme activity was further enhanced on application of 100  $\mu\text{M}$  of melatonin (MT- ST+ FS) by 36.46% and 43.42% in case of ascorbate peroxidase and catalase. Among the hybrids, Arka Abhay (2.52  $\text{Ug}^{-1}\text{FW}$ ), Arka Nikita (2.47  $\text{Ug}^{-1}\text{FW}$ ) and Arka Anamika (2.44  $\text{Ug}^{-1}\text{FW}$ ) exhibited almost similar ascorbate peroxidase activity, whereas Arka Nikita (510.70  $\text{Ug}^{-1}\text{FW}$ ) recorded higher catalase activity (Figure 7). The interaction effects between treatment and hybrids are significant.



**Figure 7.** Effect of melatonin on A) Ascorbate peroxidase and B) Catalase under drought stress in okra AC: Absolute Control, C: Control (Drought), MT-ST: Seed treatment of 100  $\mu\text{M}$  melatonin, MT-FS: Foliar spray of 100  $\mu\text{M}$  melatonin, MT- ST+FS: Combined effect of seed treatment and foliar spray of 100  $\mu\text{M}$  melatonin. Data in the figure are expressed as mean  $\pm$  SE. Different letters between the treatments and hybrids differ significantly at  $P \leq 0.05$  by LSD

*Principal component analysis (PCA)*

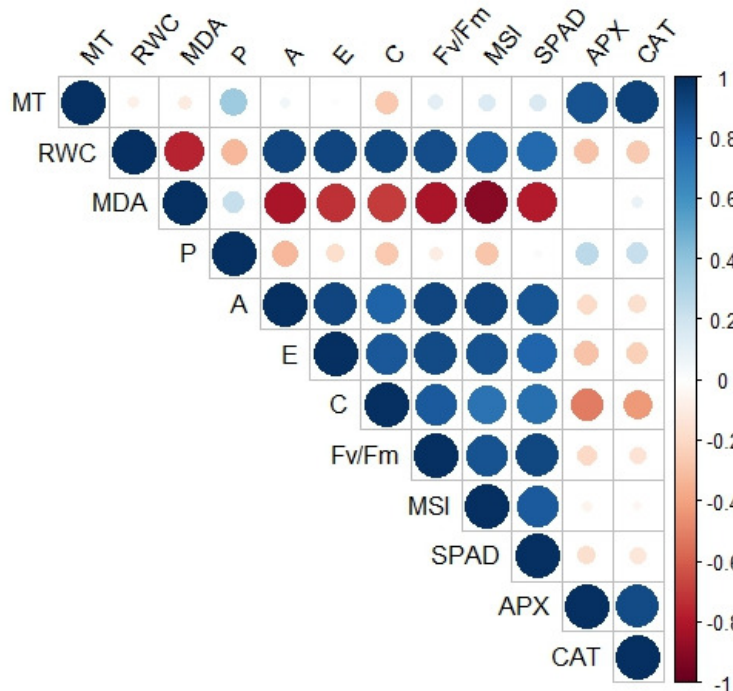
Principal component analysis (PCA) were used to indicate the relationships among okra hybrids for the parameters (MSI, SPAD, Fv/Fm, E, A, RWC, C, APX, CAT, P and MDA) under melatonin treatment (Figure 8). First two principal components exhibited eigenvalues  $> 1$  and accounted for 82.56% of total phenotypic variation. The parameters SPAD, MSI, A, RWC, CAT, APX and C were positively correlated with PC1, which accounted for 64.1% of the total variation, whereas PC2 was positively correlated with P, E and Fv/Fm, while MDA negatively correlated with PC2, which accounted for 18.4% of the total variation. Traits presented by parallel vectors or those close to each other revealed a strong positive association (MSI, SPAD, CAT, APX, A and RWC) whereas those located nearly opposite (at  $180^\circ$ ) showed a highly negative association (MSI and MDA). Treatment combinations namely, V1T5, V2T5, V3T5, V4T5, V2T4, V3T4, V4T4 are clustered together towards right side based on close correlation with MSI, SPAD, CAT, APX, A and C. V1T2, V2T2, V3T2 and V4T2 are grouped based on close association with MDA. V1T3, V2T3, V3T3 and V4T3 are grouped based on Fv/Fm, E and P. This is a clear indicative that hybrids namely CO 4 (V1), Arka Anamika (V2), Arka Abhay (V3) and Arka Nikita (V4) treated with 100  $\mu\text{M}$  of melatonin as seed treatment and foliar spray exhibited positive correlation with all variables except for MDA, suggesting the ameliorative role of melatonin in combating drought stress by enhancing physiological and biochemical parameters.



**Figure 8.** Principal component analysis of okra subjected to melatonin treatment under drought condition. A) Loading plot of variables and B) Scoring plot of treatments. Variables are Proline (P), photosynthetic rate (A), chlorophyll fluorescence (Fv/Fm), membrane stability index (MSI), chlorophyll content (SPAD), ascorbate peroxidase activity (APX), catalase activity (CAT), relative water content (RWC), malondialdehyde content (MDA), transpiration rate (E) and stomatal conductance (C). Treatments include T1 (Absolute control), T2 (Control), T3 (MT-ST), T4 (MT-FS) and T5 (MT-ST+FS). V1 (CO 4), V2 (Arka Anamika), V3 (Arka Abhay), V4 (Arka Nikita)

### Correlation analysis

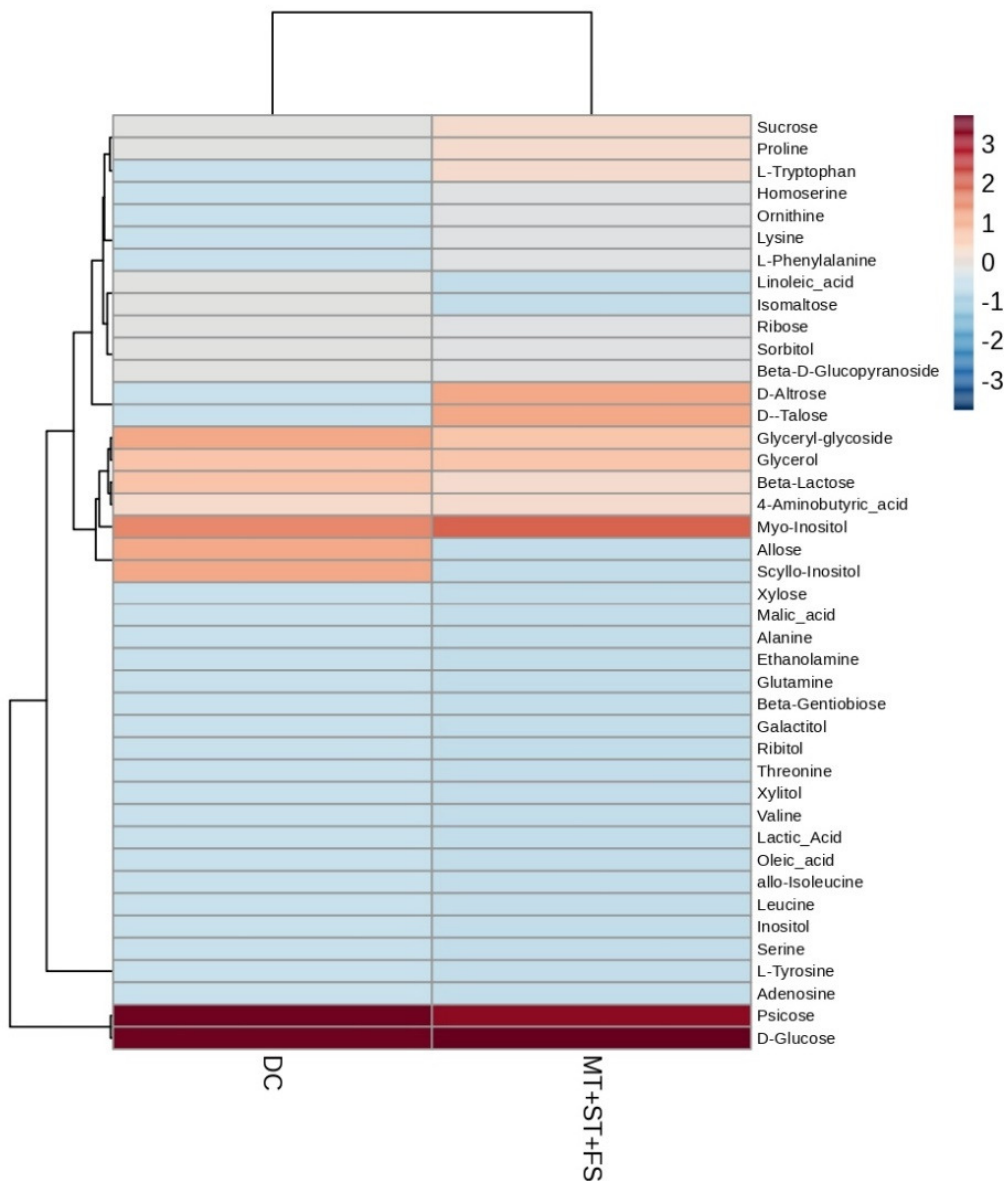
Pearson correlation analysis was carried out to check the effect of melatonin treatments on physiological and biochemical parameters in okra which include proline (P,  $r^2 = 0.36$ ), photosynthetic rate (A,  $r^2 = 0.051$ ), chlorophyll fluorescence (Fv/Fm,  $r^2 = 0.112$ ), membrane stability index (MSI,  $r^2 = 0.149$ ), chlorophyll content (SPAD,  $r^2 = 0.15$ ), ascorbate peroxidase activity (APX,  $r^2 = 0.867$ ) and catalase activity (CAT,  $r^2 = 0.92$ ). However, the relative water content (RWC,  $r^2 = -0.073$ ), malondialdehyde content (MDA,  $r^2 = -0.101$ ), transpiration rate (E,  $r^2 = -0.018$ ) and stomatal conductance (C,  $r^2 = -0.267$ ) were negatively correlated with the melatonin concentration (Figure 9). A strong positive correlation (dark green circle) was observed between A and E, indicating that higher photosynthetic rates are associated with higher transpiration rates. This is expected as both processes are regulated by stomatal opening. C showed a strong positive correlation with both A and E, underscoring the role of stomata in facilitating both gas exchange for photosynthesis and water vapour loss through transpiration. Moderate positive correlations were found between SPAD and A, E, and C, suggesting that higher chlorophyll content, indicative of healthy and productive plants, is associated with increased photosynthetic and transpiration activities. A strong negative correlation between RWC and MDA suggests that higher water content within the plant tissues is linked to reduced oxidative stress. This relationship is crucial for maintaining cellular integrity and function under stress conditions. A strong positive correlation was noted between Fv/Fm and MSI, indicating that efficient functioning of photosystem II is associated with enhanced membrane stability. This relationship highlights the interdependence of photosynthetic efficiency and cellular integrity. MT displayed a high positive correlation with CAT, suggesting that higher melatonin levels are associated with increased catalase activity. Melatonin is known for its role in mitigating oxidative stress, and this correlation underscores its protective effects in plants. Proline exhibited low correlations with other variables, indicating it might be an independent stress indicator.



**Figure 9.** Pearson correlation analysis between different parameters and okra plant subjected to melatonin treatments under drought stress. Variables include MT- Melatonin, RWC- Relative water content, MDA- Malondialdehyde content, P- Proline, A- Photosynthetic rate, E- Transpiration rate, C- Stomatal conductance, Fv/Fm- Chlorophyll fluorescence, MSI- Membrane stability index, SPAD- Chlorophyll content, APX- Ascorbate peroxidase, CAT- Catalase

*Metabolite profiling of bioactive compounds detected in water stressed and melatonin treated plants*

The effect of application of melatonin to drought stressed (MT+ST+FS) okra plants with that of drought control (C) plants were analysed through metabolite profiling (GC-MS/MS). Based on the percentage of peak area both treatments accumulated metabolites in varying degrees. The drought control (DC) treatment showed higher probability of the compounds mainly psicose (10.86%), D- Glucose (10.64%), glyceryl-glycoside (4.47%), Allose (4.25%), Scyllo-inositol (4.16%) whereas melatonin treated sample exhibited the presence of the following compounds, sucrose (2.01%), proline (2.33%), L- Tryptophan (2.56%), D- Altrose (4.12%), D- Talose (4.02%), Myo-inositol (6.07%), psicose (9.03%) and D- Glucose (10.73%). Other bioactive compounds were almost comparable in both the treatment combinations (Figure 10).



**Figure 10.** Heat map analysis of different bioactive compounds subjected to drought stress (Drought control, DC) and melatonin application as seed treatment and foliar spray (MT-ST+FS)

## Discussion

Abiotic stress, which encompasses environmental factors such as dry spell, salt stress, thermal extremes, free radical stress and metal-induced toxicity, poses a significant challenge to crop productivity worldwide (Bulgari *et al.*, 2019; Raza *et al.*, 2023). In vegetable crops, there are critical growth stages where irrigation is crucial to prevent substantial yield loss and quality deterioration. For okra, a vegetable prized for its nutritional quality, the flowering and pod formation stages are particularly sensitive to water availability (Velavan *et al.*, 2023). Insufficient irrigation during these periods can markedly reduce yield and hinder fiber development.

Relative Water Content is used as a metric to assess the hydration status of plants, and its regulation is linked to the plant's adaptation to drought stress (Liang *et al.*, 2020). In the present study, a combination of seed treatment and foliar spray of 100  $\mu$ M melatonin resulted in enhanced RWC. The reduction in Relative Water Content after 14 days of drought stress was alleviated by melatonin application. Drought stress significantly lowers leaf relative water content in tomato plants, resulting in reduced water transport from roots to stems, mesophyll flaccidity, reduced leaf water status and reduced soil moisture (Altaf *et al.*, 2022). Similar findings by (Huang *et al.*, 2019) and (Dai *et al.*, 2020) suggest that melatonin enhances photosynthesis. The decline in RWC may be linked to decreased plant growth factors. Melatonin pre-treatment significantly improved water status in rice under salinity and drought stress (Khan *et al.*, 2024). These results align with (Turk *et al.*, 2014), indicating that melatonin likely modulates stomatal behaviour, thus regulating stomatal opening and closure to reduce water loss. Exogenous melatonin application increased the total leaf cuticular wax load and the expression of certain wax biosynthetic genes in tomato plants under water deficit conditions, thereby effectively reducing water loss in plants (Ding *et al.*, 2018).

The study reveals that okra plants subjected to water stress exhibit oxidative damage, indicated by elevated malondialdehyde (MDA) levels and a decreased membrane stability index (MSI), in alignment with the findings of Nalina *et al.* (2021) and Talaat (2015, 2023). Interestingly, our experiment showed a significant reduction in MDA accumulation in drought stressed plants treated with melatonin. The higher MSI and lower electrolyte leakage (EL) values observed in these treated plants can be attributed to reduced MDA and hydrogen peroxide ( $H_2O_2$ ) production. Multiple studies have reported that MT-treated plants under stress conditions show lower MDA and EL values and higher MSI levels. The external application of MT appears to alleviate water stress-induced cellular damage and preserve membrane integrity by reducing  $H_2O_2$  production and lipid peroxidation. Additionally, MT is suggested to stabilize biological membranes under stress by maintaining optimal fluidity (Arnao and Hernández-Ruiz, 2019) and regulating the expression of lipid peroxidase genes (Gao *et al.*, 2019).

Photosynthesis, the fundamental process for harnessing light energy to produce carbohydrates, is integral to plant growth. Nevertheless, photosynthesis is particularly vulnerable to drought stress, with water deficiency markedly suppressing photosynthetic activity in numerous plant species (Velikova *et al.*, 2018; Zhou *et al.*, 2019; Sharma *et al.*, 2020). In general terms, the decrease in photosynthetic activity is limited by reduced  $CO_2$  diffusion to the chloroplasts, resulting from the closure of stomata (Liu *et al.*, 2013; Ye *et al.*, 2016). In response to drought stress, plants typically exhibit stomatal closure, resulting in reduced stomatal conductance and consequent limitations on photosynthesis (Meloni *et al.*, 2003; Liu *et al.*, 2013). Melatonin mitigates chlorophyll degradation under drought stress conditions, thereby enhancing photosynthesis, transpiration, and stomatal conductance (Liang *et al.*, 2018; Karaca *et al.*, 2019). Melatonin helps maintain cellular turgor, enhancing stomatal opening and conductance (Meng *et al.*, 2014). This increased conductance improves water and  $CO_2$  movement, ultimately favouring photosynthesis in melatonin-treated plants (Cui *et al.*, 2017).

Chlorophyll fluorescence serves as a crucial indicator for assessing the photosynthetic capacity and energy conversion efficiency of Photosystem II (PSII) in plants (Mathur *et al.*, 2019). Numerous studies suggest that prolonged water deficit induces photoinhibition in the reaction center of Photosystem II (PSII)

in plants (Huang *et al.*, 2019; Zhou *et al.*, 2019). In line with these findings, a significant reduction in Fv/Fm was observed in drought-stressed maize seedlings, indicating that drought stress caused substantial damage to the PSII complexes (Zhao *et al.*, 2021). This was attributed to the restricted diffusion of ambient CO<sub>2</sub> to the carboxylation sites, resulting in a relative surplus of light energy and electron sinks, thereby causing photoinhibition or photooxidation (Atkin and Macherel, 2009; Zhong *et al.*, 2018). Exogenous application of melatonin enhanced photosynthetic efficiency and protected the maize plant from photoinhibition (Zhao *et al.*, 2021). The application of melatonin exerts a protective effect on chlorophyll, thereby reducing damage to the photosynthetic apparatus (Campos *et al.*, 2019; Li *et al.*, 2021).

Application of exogenous melatonin enhances the activity of key antioxidant enzymes, such as catalase and ascorbate peroxidase, which play a critical role in shielding plants from oxidative stress caused by reactive oxygen intermediates. This melatonin-induced enhancement facilitates plant survival under drought stress conditions. Numerous studies have demonstrated that melatonin effectively promotes plant survival and growth by strengthening ROS scavenging mechanisms under various abiotic stress conditions (Sharma *et al.*, 2020; Gao *et al.*, 2018). Foliar application of melatonin mitigates oxidative impairment in corn seedlings (Ahmad *et al.*, 2019). Under water stress, plants develop adaptation strategy, such as ROS detoxifying enzymes to prevent free radical bursts and maintain ROS balance (Abid *et al.*, 2018; Imran *et al.*, 2021; Li *et al.*, 2018a). Melatonin enhances the transcript levels and activities of antioxidant enzymes (Arnao and Hernández-Ruiz, 2019). The activity of these antioxidant enzymes is linked to the regulation of key genes encoding them, ensuring cellular redox balance under stressful conditions (Sharma *et al.*, 2020).

Metabolomic assessment have revealed the retention of various osmoregulatory solutes, including carbohydrates such as glucose and sucrose, polyols like sorbitol, and amino acids such as proline, lysine, L-tryptophan, leucine, and tyrosine. These osmolytes are crucial for reducing osmotic gradient and maintaining cellular pressure by promoting water uptake. They also play a significant role in stabilizing cellular membranes, enzymes, and proteins (Jorge and António, 2018; Sharma *et al.*, 2019). Additionally, the osmolytes buildup aids in regulating ROS levels, supplies energy to manage stress, facilitates repair mechanism, and supports continued growth (Silva *et al.*, 2018; Fàbregas and Fernie, 2019). Present study reported the presence of certain carbohydrates, including sucrose, psicose, talose and myo- inositol in case of drought stressed okra plants subjected to melatonin treatments. Similar findings were reported by (Sharma *et al.*, 2019) wherein the following compounds (sucrose, tagatose, psicose, glucoheptose, allose, talose, cellobiose and sugar alcohol inositol) were shown to exhibit a sharp increase in response to salinity. D-psicose affects plants by increasing sugar content while preserving leaf water content. One potential mechanism for this effect involves osmotic adjustment, where the leaf enhances its water absorption capacity by elevating the soluble content within its cells (Yamada *et al.*, 2014). L-tryptophan (Try) is a well-recognized amino acid essential for plant growth, functioning effectively under both normal environmental conditions and various abiotic stresses. In the present metabolite profiling, try-tryptophan levels were higher in case of plants treated with melatonin which is in accordance to the findings of (Chen *et al.*, 2009) that in higher plants, L-tryptophan functions as a physiological precursor to melatonin. As an osmolyte, try-tryptophan plays a crucial role in facilitating the transport of nutrient ions, modulating stomatal aperture, and mitigating the adverse effects of heavy metals (Rai, 2002). Additionally, tryptophan enhances various biochemical attributes by regulating plant growth and divergence and improving nutrient and water uptake efficiency (Talaat *et al.*, 2005; Dawood and Sadak, 2007).

Moreover, the selection of appropriate method of application is important to achieve the fullest drought mitigation potential of melatonin in okra. In the present study, both seed priming and foliar application of melatonin enhanced plant MSI, APX and CAT enzyme activity and SPAD value except MDA compared to drought. However, foliar application of melatonin performed better with respect to measured parameters under drought compared to seed biotized with melatonin. This improved defense mechanism in foliar spray of melatonin was due to immediate action of melatonin during the critical growth stage. Moreover, seed treatment

promotes overall plant vigour by promoting plant metabolic events, whereas foliar application offers targeted protection at critical growth stages (Marthandan *et al.*, 2020; Kuppusamy *et al.*, 2023).

### **Conclusions**

Melatonin acts as growth regulator and stress protector, playing key role in enhancing both physiological and biochemical responses in plants, making it a promising tool for improving drought tolerance. Seed priming with melatonin improves plant metabolic processes at early stage of seed germination therefore the long term effect is not pronounced at later stages of the plant, whereas foliar application of melatonin imparts immediate defense response at critical growth stage of okra. The combined effect of melatonin seed treatment and foliar spray proved to be an effective strategy in mitigating drought stress in okra as plants are better equipped to handle stress, as they benefit from early-stage systemic resilience (from seed treatment) and ongoing targeted protection (from foliar spray), thus showing enhanced stress tolerance compared to the more limited protection from seed priming and foliar spray alone. This integrated approach significantly enhanced photosynthetic parameters and antioxidant activity while reducing lipid peroxidation and maintaining membrane stability index. These improvements highlight the potential of melatonin applications to bolster drought tolerance in okra, thereby promoting better growth and productivity under water-limited conditions. Future research should explore the underlying mechanisms of melatonin's protective effects and investigate its efficacy across different okra varieties and environmental conditions. Additionally, studies should aim to optimize application methods and dosages to maximize the benefits of melatonin treatments in agricultural practices.

### **Authors' Contributions**

Conceptualization, A.G, R.V, V.D, A.L, R.S, and J.R; methodology, A.G and R.V; experiments carried out, A.G; statistical analysis, A.G; validation, A.G, R.V, V.D, A.S., A.L, R.S, M.P., and J.R; writing original draft preparation, A.G. and R.V; writing—review and editing, A.G, V.R., V.D, A.L, R.S, P.M. and J.R.

All authors read and approved the final manuscript.

### **Ethical approval** (for researches involving animals or humans)

Not applicable.

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## Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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