

COMBINED APPLICATION OF SODIUM NITROPRUSSIDE AND MELATONIN ENHANCED PHOTOSYNTHETIC AND ANTIOXIDANT CAPACITY OF WHEAT UNDER DROUGHT STRESS

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Abstract

Exogenous application of sodium nitroprusside (SNP) and Melatonin improved drought Wheat's ability to withstand stress (*Triticum aestivum* L.) plants. Therefore, the current study investigated how melatonin and nitric oxide (NO) could improve the tolerance of wheat under drought stress. Treatments used for wheat plants included control, drought stress, SNP, melatonin treatment, combination SNP and melatonin treatment, abiotic stress with SNP, abiotic stress with melatonin, and abiotic stress with combined SNP and melatonin. The concentration of both SNP and melatonin used in the treatments for wheat plants was 100 μ M, while the level of drought stress applied was at a standard level. In this study, the relationships between shoot length and a range of physiological parameters are investigated quantitatively. The results showed that there are significant positive correlations with tillers per plant ($r = 0.918$), SPAD value ($r=0.977$), chlorophyll-a ($r=0.974$), chlorophyll-b ($r=0.718$), and protein content ($r=0.864$). Still, there were negative correlations between enzyme activities and oxidative stress markers. Significant variations ($p<0.05$) were found between the treatments for shoot length, tillers per plant, SPAD value, chlorophyll-a, and chlorophyll-b, indicating a strong influence of SNP, melatonin and their combinations on plant growth, development, and photosynthetic activity under drought stress. Moreover, using SNP, melatonin, and their combination treatment restored these enzymes' activity, demonstrating their potential to strengthen antioxidant defence systems. This study concludes that NO and melatonin application significantly affected wheat development and reduced the adversative consequences of drought stress. Besides, the potential of these chemicals to be used as useful approaches to increase wheat tolerance to drought stress, hence advancing the development of sustainable agricultural methods.

Key words: Sustainable agriculture; Drought stress; Melatonin; Sodium nitroprusside; Antioxidant defence systems.

Introduction

One of the most important abiotic stresses limiting wheat productivity worldwide is drought. This is due to its detrimental effect on the growth of wheat, leading to an inhibition in plants' different physiological and biochemical processes. As such, stomatal closure, decreased transpiration and photosynthesis, growth inhibition, the generation of antioxidants, and alterations in hormone composition can all result from drought (Nabi *et al.*, 2019). Moreover, drought stimulates the generation of reactive oxygen species (ROS), counting singlet oxygen (1O_2), hydrogen peroxide (H_2O_2), and superoxide (O_2^-), which could cause loss to proteins, plant DNA, lipids, nucleic acids and carbohydrates (Boyarshinov & Asafova, 2011). Plants have evolved an efficient defence mechanism against this damage over their antioxidant system, which produces catalase (CAT) (R. Ahmad *et al.*, 2019), ascorbate peroxidase (APX), and peroxidase (POD) (Bahador & Tadayon, 2020; Rezaei-Chiyaneh *et al.*, 2020), and superoxide dismutase (SOD) (Stephenie, Chang, Gnanasekaran, Esa, & Gnanaraj, 2020). Plants release ROS in response to stress; however, higher accumulations of ROS in plants are poisonous and have both negative and positive impacts on plants' different cells and organelles. To control the higher accumulation of ROS under stress conditions, NO interacts with various signalling components (Gechev & Petrov, 2020). Scientists have been interested in NO, a gaseous free radical that has a vital function in plant signalling, and it participates in several developmental, environmental,

and physiological reactions (Oz, Eyidogan, Yucel, & Öktem, 2015). For example, NO guards against oxidative damage to plant cells via inducing the movement of antioxidant enzymes, reducing the harmful concentrations of lipid-free radicals, H_2O_2 , and superoxide anions (Dwivedy, Singh, Prakash, & Dubey, 2018). Furthermore, NO reduces the detrimental impact of ROS by boosting the MAPK pathway's generation of antioxidant enzymes, encouraging photosynthesis, lipid peroxidation, and triggering other signalling pathways (Ifthikhar and Perveen 2024; Wani *et al.*, 2021). Furthermore, enhanced APX, SOD, CAT, and dehydroascorbate reductase accumulation by S-nitrosylation reduced the harmful effects of ROS (Sharma *et al.*, 2019).

Plants experiencing drought circumstances encounter a disruption in their cellular redox balance, leading to alterations in oxidative stress levels and subsequent cell damage (Uddin, Hossain, & Burritt, 2016; Trabelsi *et al.*, 2024). Therefore, plants must adapt their morphology, biochemistry, molecular, and physiological processes in response to the stress of drought (Kumar, Berwal, & Saroj, 2019). One of the critical redox-related signalling molecules in this stress adaptation response cascade is the synergistic effect of NO in crops. It is a gaseous signalling molecule rapidly formed in response to several hormonal and environmental stressors. It may be resolved in lipids and water (Yang *et al.*, 2021). NO could scavenge the excessive levels of ROS by regulating transcriptional alterations involved in various translocation processes such as signal transduction, as well as cell death, plant

defense, and ROS metabolism (Sachdev, Ansari, Ansari, Fujita, & Hasanuzzaman, 2021). Crop yield has been greatly affected by recent changes in the climate, which have induced drought stress, impacting the development and productivity of the most strategic crops (Raza *et al.*, 2019). Therefore, several agronomic techniques, such as phytohormones or bio-stimulators in crop production, must increase plant tolerance in response to abiotic stressors like drought. These phytohormones enhance various plant biochemical, physiological, and molecular mechanisms that support resilience development and growth control (Wahab *et al.*, 2022). Investigating the role and mechanism of these phytohormones is thus crucial for enhancing plant development and productivity in drought-stressed environments.

With its natural origin, multifunctionality, safety, universality, and regulatory qualities, Melatonin has diverse impacts on plant species because of its low molecular weight (Nawaz *et al.*, 2020). Thus far, melatonin has been shown to strengthen defenses against different abiotic stressors, including disease-causing agents, extreme heat or cold, salt, heavy metals, chemicals, and pathogens (Gull, Lone, & Wani, 2019). Melatonin improved fruit ripening, root growth (rhizogenesis), antioxidant activity, root structure, seed sprouting, photosynthesis, and seedling growth at the cellular and tissue levels (Altaf *et al.*, 2022). In particular, greater melatonin content was linked to improved ROS homeostasis and antioxidant capability in *Actinidia deliciosa* (Liaquat *et al.*, 2020) *Althaea rosea* (Rahim *et al.*, 2022), *Eriobotrya japonica* (Bohra, Sanadhya, & Chauhan, 2015), and *Carthamus tinctorius* (Yamazaki, Ueda, Mukai, Ochiai, & Matoh, 2018) under drought stress.

Preceding studies have shown how melatonin enhanced the root architecture system in kiwifruit (Liang *et al.*, 2019), balance of protected minerals in *C. cathayensis* (Eun, Ali, Jung, Kim, & Kim, 2019), improved photosynthetic efficiency in *E. japonica* (Yamazaki *et al.*, 2018), reduced ROS accumulation and lipid peroxidation in *Moringa oleifera* (Qi *et al.*, 2019). Melatonin enhances antioxidant enzyme systems in fenugreek (Gulcin, 2020), increases ascorbic acid and glutathione levels in kiwifruit seedlings (Liang *et al.*, 2019), boosts osmoregulatory substances in tomato (Khan *et al.*, 2020), protects chloroplast structures in wheat (Zhao *et al.*, 2023) and Brassica napus, and regulates genes related to antioxidants and antioxidant enzymes in *Solanum lycopersicum* (Khan *et al.*, 2019). Most of the material in the research has used melatonin externally but not in the plant's rhizosphere. This research aims to determine whether melatonin and SNP might stimulate wheat growth in drought-stressed environments. This work aimed to regulate the impact of melatonin and SNP treatments on the development, growth and stress tolerance of wheat plants subjected to drought

stress. The research aimed to achieve the following objectives: I) To determine how treatments with SNP and melatonin influence shoot length, the number of tillers per plant, and SPAD value in wheat plants under drought stress. II) determine the effects of drought-induced oxidative stress on cell damage of wheat plants and determine if melatonin and SNP treatment synergistically mitigate these effects. III) To investigate the potential pathways by which melatonin and SNP treatments influence the growth and development of wheat plants under drought stress.

Material and Methods

Plant type, setup, and growth environment: A pot experiment in a shed house was conducted at Lanzhou University (Yuzhong campus), Gansu Province, China. The seeds of hexaploid spring wheat (*Triticum aestivum* L.) Longchun 28 were used in this experiment. After sterilization and germination, the seeds were planted as per the given instructions (Liu, Deng, Zheng, Wang, & Ni, 2022). The seeds were cultivated in late March based on crop phenology observed in the area. As shown in (Table 1) the pots were split into eight treatments, each consisting of three duplicates. After four weeks post-transplanting, the plants were given 100 μ M SNP treatment and melatonin (Rahim *et al.*, 2022).

Measurements of plant parameters and biomass: The leaf area of every plant was determined using a leaf area meter (LI-3000; Li-Cor, Lincoln, NE, USA). A micrometer was used to measure the thickness of the leaves. The height of the plant was determined using its base to the tip of its tallest leaf. By chopping each plant off at the base and measuring the fresh weight, the biomass of each plant was determined. To calculate their dry weight, the samples were then dried for 24 hours at 60°C in an oven.

Quantification of chlorophyll contents: Three plants for each treatment were used for net photosynthetic rate measures made using a portable photosynthetic system, Li-6400 (Li-Cor, Lincoln, NE, USA). 85% propanol was used to extract chlorophyll from leaves (Cui *et al.*, 2017). The quantity of chlorophyll present was determined using a spectrophotometer by measuring the absorption values of light at 663 nm and 645 nm of the chlorophyll pigment extract; propanol was used as the control solution. Leaf samples were obtained twice during the development period to determine the physiological characteristics of the leaves: first in the middle of June, which is the reasonable development stage, and again in the middle of August, which is the compensatory growth stage. It was observed that wheat was in blossom around the middle of June. Mid-August was the time for harvesting wheat.

Table 1. Experimental design and treatment conditions.

Group	Treatment	Concentration
Control (C)	Untreated	-
Drought Stress (DS)	Water stress	40% reduction in water amount
SNP	SNP	100 μ M
Melatonin	Melatonin	100 μ M
SNP + Melatonin	SNP + Melatonin	100 μ M + 100 μ M
Drought Stress + SNP	Drought stress + SNP	100 μ M + 40% reduction in water amount
Drought Stress + Melatonin	Drought stress + Melatonin	100 μ M + 40% reduction in water amount

Quantification of oxidative stress: The measurement protocol for relative electrolyte leakage. After preparing three ultrapure water washes for five fresh leaves, 50 mL test tubes were set up (Cui *et al.*, 2017). The electrical conductivity of the ultrapure water was determined using an electrical conductivity analyzer (Thermo Fisher, USA) as EC0, EC1, and EC2 before to submerging the portion of leaf, following two hours of submersion at 32°C, and after 20 minutes of boiling. The formula used to compute the comparative electrolyte leakage (REL) was $REL = \frac{EC1 - EC0}{EC2 - EC0}$. Foliage samples (0.3 g) were crushed in 5 mL 100 mM pre-cooling phosphate buffer (pH 7.0) for MDA, H₂O₂, and superoxide anion analysis. For 15 minutes, the homogenate was centrifuged at 12,000 g and 4°C. For the next examination, the supernatant was used. MDA was prepared by mixing 1 mL of supernatant with 2 mL of thiobarbituric acid (0.6%, w/v). After 30 minutes of heating and cooling, the mixture was centrifuged for 15 minutes at 3000~g. The absorbance at 450, 532, and 600 nm was determined using the supernatant. To measure H₂O₂ (Cui *et al.*, 2017). The combination, which included 1 mL of supernatant and 1 mL of KI (1 M), was incubated for 1 hour at 25°C in the dark. The absorbance at 390 nm was measured using the standard curve, and the H₂O₂ concentration was computed. The methodology quantified superoxide anion with minor adjustments (Cui *et al.*, 2017). The mixture was left to incubate for 20 minutes at 25 °C. It included 0.25 mL hydroxylamine hydrochloride (10 mM) and 1 mL supernatant. After adding 1 mL of sulfanilic acid (17 mM) and 1 mL of α -naphthylamine (7 mM) to the mixture, the reaction happened at 30 °C for 30 minutes. The absorbance at 530 nm was measured using a reference curve, and the superoxide anion concentration was determined.

Lipid peroxidation quantification: The quantity of MDA, a consequence of membrane bilayer oxidation, was calculated to determine the degree of lipid peroxidation (M. A. Khan *et al.*, 2020).

Antioxidant activity estimation: After harvesting, FW was immediately measured and dried in an oven for 15 minutes at 105 °C. The material was then dried at 80 °C until its weight remained constant (DW). The leaf water content was calculated using the following formula: $WC = \frac{FW - DW}{FW} \times 100\%$, FW (Rahim *et al.*, 2022) declare that the using plasma's ferric-reducing activity, antioxidant capacity was calculated (FRAP) approach. A 0.1 g leaf sample was crushed in 2 mL deionized water and centrifuged at 15,000 g, 4°C for 10 minutes. The supernatant's capacity as an antioxidant was assessed. The components of the FRAP reagent were 10 mM tripyridyltriazine (TPTZ, in 40 mM HCl), 20 mM FeCl₃ (10/1/1, v/v/v), and 0.3 M NaAc-HAc buffer (pH 3.6). A reaction solution containing 100 mL of supernatant and 2.4 mL of FRAP reagent was combined and incubated for 10 minutes at 37°C. The absorbance at 593 nm was measured, and the outcome was normalized using the absorbance of 1.0 mM FeSO₄.

Evaluation of statistics: In this study, we used a fully randomized design (CRD) to conduct independent experimental studies in triplicate. All data were statistically evaluated using R-studio version 5.1 to determine the

statistical implication between the control and SNP-supplied plants and between the control and melatonin-supplied plants. The R-studio was used to visualize the graphs and display the data visually.

PCA and data correlation analysis were performed using RStudio and relevant R packages. The dataset was preprocessed for the PCA analysis, which included managing missing values and standardizing variables. The PCA analysis was made more accessible by the "FactoMineR" software, which used covariance and correlation matrices. Variable loadings were analyzed to determine the contribution of each variable to the main components, and scores and coordinates were evaluated to find groupings and patterns in the dataset. Biplots were produced because the principal components were determined during the PCA analysis. R Studio was utilized to compute Pearson correlation coefficients (r) and determine p-values, which indicate the significance of correlations, for data correlation analysis. R tools were used to create a correlation heat map, showing the variable's correlations. The statistical assessments and important insights obtained from these RStudio studies of the dataset aided in discovering underlying patterns and relationships between the observed parameters.

Results

Physiological response of SNP and melatonin-treated under drought stress: The current findings demonstrated that SNP treatments substantially improved photosynthetic activity, as shown by greater photosynthetic rate and photosystem II peak efficiency (Fv/Fm) compared to the control group, melatonin. Moreover, SNP treatments reduced oxidative damage in drought-stressed wheat seedlings as reflected by lower levels of ROS and oxidative damage markers such as superoxide anion, hydrogen peroxide, MDA, and relative conductivity. The treatments notably enhanced the structural integrity of photosynthetic membranes and organelles in drought-stressed wheat, resulting in thicker laminae, enhanced leaf structure and more intact chloroplast grana lamella. The treatments included the administration of 100 μ M SNP and melatonin four weeks after transplantation. These findings suggest that in drought-stressed plants, melatonin and SNP synergistically contribute to increased photosynthetic efficiency, guard against oxidative damage, and maintain the structural integrity of photosynthetic membranes and organelles. It could be concluded that SNP and melatonin treatments could be used as a promising approach to improve wheat crop growth under drought stress conditions by increasing photosynthetic activity, decreasing oxidative damage, and bolstering the mechanical integrity of photosynthetic membranes and organelles.

Growth and development: After six weeks of growth, the control group exhibited better growth and development than other treatments, with an average shoot length of 10 cm, three tillers per plant, and a SPAD value of 30. This suggests that sufficient water is necessary for higher seedling expansion and productivity of wheat plants. Drought stress reduced the growth and development of wheat seedlings as associated to the control group. The plant exhibited an average shoot length of 5 cm, with two tillers per plant and a SPAD value 20, as illustrated in Figure 1. This implies that

wheat plant growth and development were severely affected by drought stress (Fig. 1). The SNP treatment showed considerably improved growth and development compared to the drought stress group, with an average shoot length of 8.5 cm and three tillers per plant. This suggests that SNP has the potential to mitigate the detrimental impact of drought stress on wheat crops.

The melatonin treatment (d) exhibited superior growth and development compared to the drought stress treatment group, with an average shoot length of 9.00 cm and three tillers per plant. The detrimental effects of drought stress on wheat plants can also be mitigated by melatonin. Applying SNP and melatonin recorded higher plant growth and development than the individual treatments. These results suggest that wheat plants responded significantly more to combination than individual treatments.

Transpiration rate, photosynthetic rate, and stomatal conductance under drought stress:

In the absence of drought stress, plants exhibited higher transpiration and photosynthetic rates and stomatal conductance than those grown under drought stress conditions. The findings indicate that drought stress significantly reduces wheat plants' photosynthetic capacity and water transport efficiency. The melatonin therapy notably increased the drought resistance of wheat sprouts by enhancing membrane integrity, maintaining intact grana lamella, and preserving leaf structure. Melatonin has a good impact on wheat plants' photosynthetic and water transport capacity in drought-stress conditions. Additionally, the rate of transpiration and photosynthetic rate, and under drought stress, the stomatal conductance of the plants treated with melatonin was considerably greater than that of the control group. The combination treatment of both melatonin and SNP exhibited the highest transpiration rate, stomatal conductance, and photosynthetic rate compared to the individual treatments.

Chlorophyll fluorescence as impacted by SNP and melatonin under drought stress:

The chlorophyll fluorescence characteristics were considerably lower under drought stress treatment than under control treatment. The SNP treatment considerably improved chlorophyll fluorescence in contrast to the control during the drought. The group experiencing drought stress did not display the same levels of fluorescence characteristics (Fv/Fm, PSII) in chlorophyll as the group receiving melatonin therapy (d). This suggests that the detrimental consequences of drought stress on wheat plants' chlorophyll fluorescence were mitigated by melatonin. The combination of melatonin and SNP recorded the highest metrics values (Fv/Fm, Δ PSII) (Fig. 2).

Oxidative stress markers and photosynthetic activity:

The findings demonstrated that MDA, H₂O₂, and superoxide anion concentrations were significantly higher in wheat plants exposed to drought stress. The MDA level was 3.8 nmol/g in the drought-stressed group and 1.2 nmol/g in the control group. The combined treatment resulted in the lowest average of MDA level (1.9 nmol/g). The results showed that drought stress decreased transpiration rates, stomatal conductance, and photosynthetic activity in wheat plants. The photosynthetic rate of the plants treated with melatonin was 11.2 μ mol/m²s,

while the average rate for the plants treated with SNP was 10.8 μ mol/m²s. The combined treatment group had the most significant average photosynthetic rate (13.1 μ mol/m²s) (Fig. 3).

SNP and melatonin regulate enzymatic antioxidant activities in response to drought stress:

PPO activity affectedly increased, with values in the drought-stressed group being 3.50 U/g and in the control, group being 1.20 U/g. PPO activity varied in the melatonin and SNP-treated seedlings, ranging from 2.20 U/g to 2 U/g. Melatonin and SNP combined produced the maximum PPO activity (3 U/g). POD activity significantly increased under drought stress in comparison to plants under management. While the presence of the combination of SNP and melatonin treatments significantly increased the POD activity under drought stress. The drought-stressed group displayed a CAT activity of 4.0 U/g, surpassing the control group with a CAT activity of 1.50 U/g. The SNP and melatonin treatments showed increased CAT activities of 2.0 U/g and 2.50 U/g, respectively. The combination of melatonin and SNP recorded higher activity of CAT (4.20 U/g). The SOD activity level measured was 2.0 U/g, and it was shown that the group under drought stress had much higher SOD activity levels (1.0 U/g) than the control group. Similarly, the combination treatment (SNP and melatonin) increased levels of SOD activity as compared to the individual treatment and the control. Furthermore, heightened APX activity was seen at 2.0 U/g and 2.50 U/g in reaction to the SNP and melatonin treatments. The maximum quantity of APX activity was produced at 3.20 U/g by melatonin and SNP combined.

SNP and melatonin improving plant water relations and photosynthesis:

The drought stress group showed a important decrease in biomass, plant height, leaf area, and leaf thickness in contrast to the group under authority. These metrics also indicated dropping values for the SNP and melatonin treatments, but not as sharply as those in the drought stress group. The drought stress group had a considerable decrease in stomatal conductance, photosynthetic rate, and transpiration rate, as well as a huge increase in water consumption efficiency compared to the control group. The SNP and melatonin treatments also showed lower values for transpiration rate, photosynthetic rate, and stomatal conductance and greater values for water use efficiency. However, the effects were less pronounced than those in the drought stress group. Photosynthetic rate and stomatal conductance increased somewhat when SNP and melatonin were combined, although they remained lower than in the control group. The drought stress group exhibited a large rise in Fv/F0 and a significant drop in Fv/Fm and PSII compared to the control group. Reduced Fv/Fm and PSII and elevated values for Fv/F0 were also seen in the SNP and melatonin treatments but less dramatically than in the drought stress group. Fv/Fm and PSII increased somewhat due to the SNP and melatonin combination, but they were still lower than those in the control group.

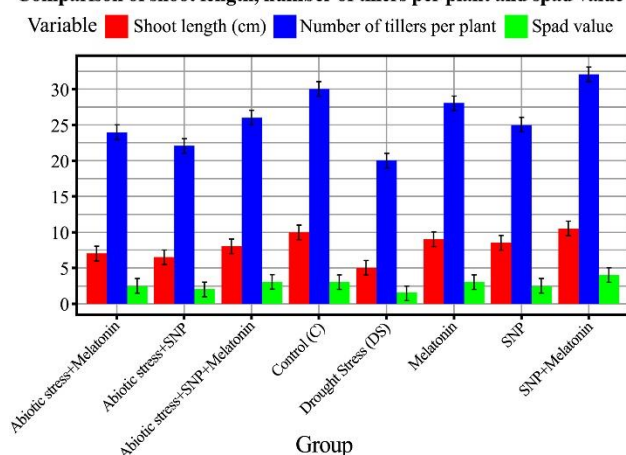
Comparison of shoot length, number of tillers per plant and spad value

Fig. 1. Effects of drought stress, SNP, and melatonin on shoot length, number of tillers per plant, and SPAD value in wheat plants.

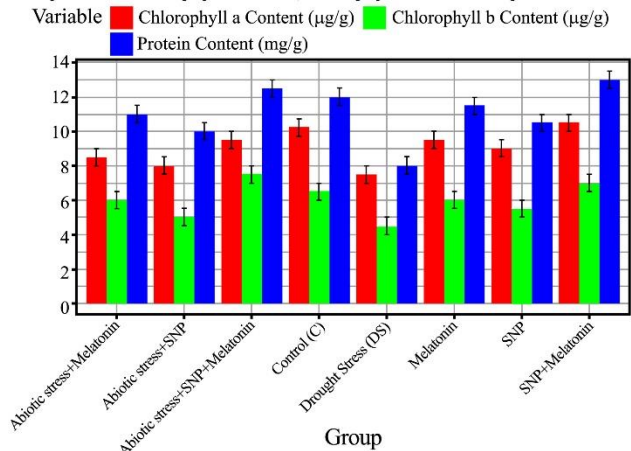
Comparison of chlorophyll a content, chlorophyll b content and protein content

Fig. 2. Effects of drought stress, SNP, and melatonin on chlorophyll a and b contents, and protein content in wheat plants.

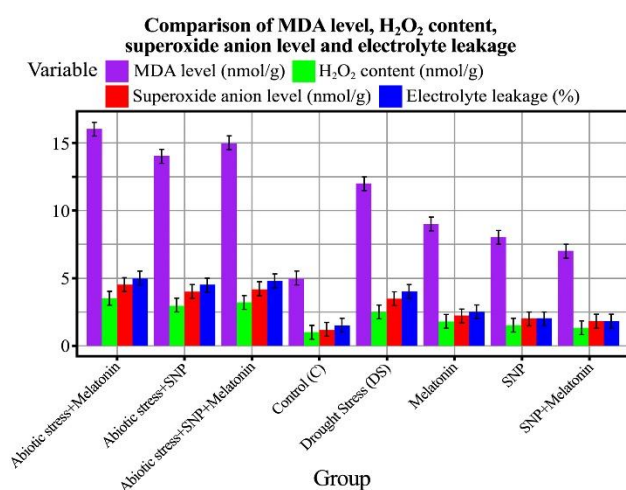


Fig. 3. Effects of drought stress, SNP, and melatonin on oxidative stress parameters in wheat plants.

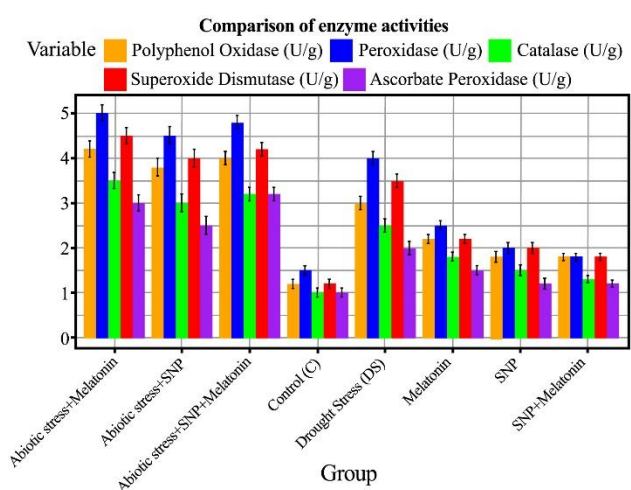


Fig. 4. Effects of drought stress, SNP, and melatonin on antioxidant enzymes in wheat plants.

PCA analysis: Two main components, PC1 and PC2, were identified by principal component analysis (PCA) of the observed data. These components account for PC1 23.1% and PC2 75.6% of the data variance, respectively. Three clusters were formed from the variables according to how well they loaded on the main components. Chlorophyll a and b content, shoot length, tillers per plant, SPAD value, and other growth and development-related variables made up Cluster 1. The positive correlation between these factors and PC1 and PC2 suggests that they are associated with the general health and productivity of the plant, as well as its capacity to photosynthesize and generate biomass. Cluster 2 included components of the antioxidant defense system, including ascorbate peroxidase, polyphenol oxidase, catalase, superoxide dismutase, and peroxidase. The positive correlation between these factors and PC2 suggests they are associated with the plant's capacity to scavenge ROS and defend against oxidative stress. The cluster included stress response variables such as MDA concentration, H₂O₂ content, superoxide anion concentration, and electrolyte leakage. The fact that these factors had a negative correlation with PC1 and PC2 suggests that they have something to do with how the plant reacts to oxidative damage and stress. The PCA analysis

indicates that the plant's growth and development, antioxidant defense system, and stress response are represented by two primary components that account for the shoot data (Fig. 5).

Correlation: The correlation analysis's findings showed that there were important connections between the physiological measures that were examined. There was a notable positive correlation between shoot length and the number of tillers per plant ($r = 0.918$) and SPAD value ($r = 0.977$). Likewise, favourable correlations were observed between shoot length and chlorophyll-a ($r = 0.974$) and chlorophyll-b ($r = 0.718$). A positive connection ($r = 0.864$) was found between shoot length and protein content. In contrast, there were negative correlations found between shoot length and the following: MDA level ($r = -0.758$), H₂O₂ content ($r = -0.720$), superoxide anion level ($r = -0.766$), electrolyte leakage ($r = -0.724$), polyphenol oxidase activity ($r = -0.758$), peroxidase activity ($r = -0.720$), catalase activity ($r = -0.766$), superoxide dismutase activity ($r = -0.597$), and ascorbate peroxidase activity ($r = -0.690$). The findings imply that several physiological parameters affect shoot length, including protein content, oxidative stress indicators, and chlorophyll concentration (Fig. 6).

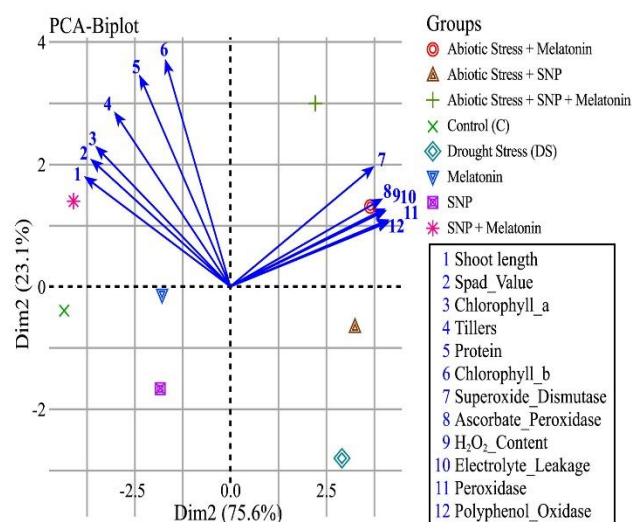


Fig. 5. PCA Analysis Results Visualized in a Biplot.

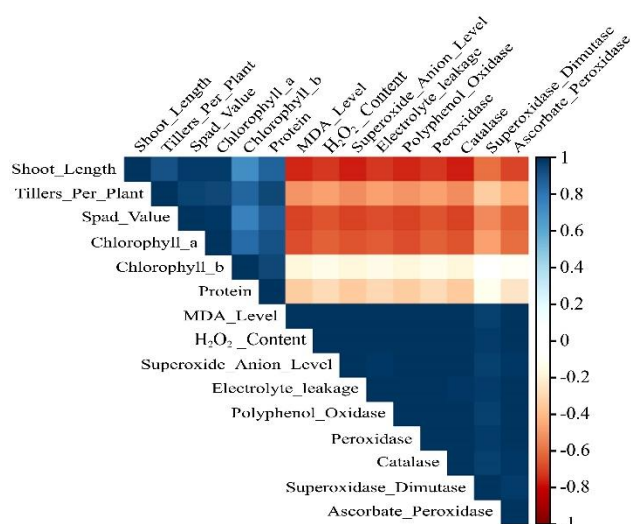


Fig. 6. Shows the correlation analysis of Shoot Length, Number of Tillers per Plant, and SPAD Value under different stress conditions. It illustrates the relationships between these variables and highlights their interdependencies in response to various stressors.

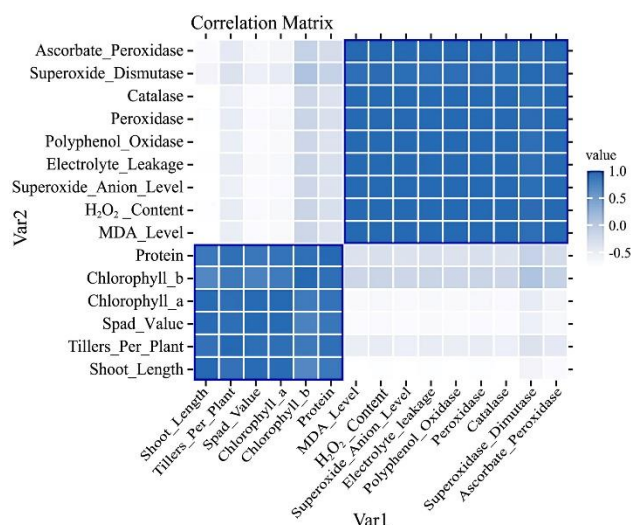


Fig. 7. Correlation Matrix of Physiological and Biochemical Parameters under Different Stress Conditions.

Significant and non-significant findings in physiological and biochemical responses to different treatments: Our study examined how different treatments affected plants' biochemical and physiologic characteristics. After data analysis, we determined both significant and non-significant treatment differences. Shoot Length ($p = 0.001297055$), Tillers per Plant ($p = 2.91E-05$), SPAD Value ($p = 4.15E-05$), Chlorophyll-a ($p = 0.044932065$), and Chlorophyll-b ($p = 0.005627108$) were among the significant findings. These findings showed significant effects on photosynthetic activity and plant development. However, there were no discernible variations between the treatments for measures including protein content, MDA level, H_2O_2 content, superoxide anion level, electrolyte leakage, Polyphenol Oxidase, Peroxidase, Catalase, Superoxide Dismutase, and Ascorbate Peroxidase. These results draw attention to the distinct physiological and biochemical reactions impacted by the treatments, highlighting the importance of some parameters while pointing out that there are no appreciable variations in others (Fig. 7).

Discussion

The current research explains the mechanism underlying melatonin and SNP-improved wheat plant development under the pressures of drought. Our results indicated that NO and melatonin provide additional protection against drought stress and that both substances positively impact plant growth and development. In earlier research, nitric oxide and melatonin have also been shown to have significant functions in plant stress responses. NO is a signalling molecule in plant defensive responses, controlling activities including photosynthesis, antioxidant defence, and stomatal conductance (P. Ahmad *et al.*, 2020). It has been shown that melatonin promotes plant growth and development by improving antioxidants and hormones in drought-stressed plants (El-Zohri *et al.*, 2024 P. Ahmad *et al.*, 2020). Although the exact processes by which melatonin and nitric oxide combine to mitigate drought stress in wheat plants are unknown, several potential explanations exist. While melatonin has antioxidant and hormone-like properties that encourage the expansion and advancement of plants, NO functions as a signalling molecule to activate genes linked to stress. The advantageous benefits of one molecule are enhanced when the two are combined, a phenomenon known as a synergistic effect.

Numerous studies have shown the critical functions that NO and melatonin are essential in mitigating stress effects on plants. For instance, (Altaf *et al.*, 2022) exposed that melatonin and NO had a joint role in controlling stomatal mobility and photosynthesis in wheat under the pressures of drought. As a response to drought stress in wheat, (Cui *et al.*, 2017) showed that melatonin and NO impacted antioxidant defense regulation and oxidative damage reduction. SNP and melatonin have a complicated and diversified function in plant growth and response to stress. Research demonstrates that NO regulates the growth and development of plants and how plants react to stress via several different pathways (Camejo, Guzmán-Cedeño, & Moreno, 2016). Melatonin has been linked to plant stress responses, particularly drought stress, and has been shown to have antioxidant and hormone-like properties (Wei *et al.*, 2022).

The current work contributes to the increasing research showing that melatonin and NO significantly affect plant stress responses. Our results imply that NO and melatonin provide wheat plants with more defense against drought stress and that these substances work in concert to mitigate the effects of drought. Several potential explanations exist for how NO and melatonin combine to mitigate drought stress in wheat plants. However, the underlying processes and signalling pathways remain unclear (Ertuğrul *et al.*, 2024; Hussain *et al.*, 2024; Lau *et al.*, 2021; Moustafa-Farag *et al.*, 2020). While melatonin has antioxidant and hormone-like properties that promote the growth and development of plants, NO functions as a signalling molecule to activate genes linked to stress (Kaur *et al.*, 2021). The current work offers compelling evidence that these substances are crucial to plant stress responses and help increase crop resistance to abiotic stress. The current work has important ramifications for crop enhancement and sustainable agriculture. Natural stress-relieving substances like melatonin and SNP provide an alternative to conventional stress-reduction techniques, which often depend on environmentally hazardous chemical fertilizers and pesticides. Farmers can decrease the usage of chemical stress management measures, increase crop resistance to drought stress, and advance sustainable agriculture by implementing SNP and melatonin into agricultural methods.

Furthermore, the current work is significant in creating novel crop enhancement methods. Using melatonin and SNP as naturally occurring stress-relieving agents stimulates the creation of novel crop types that are more resilient to stress and better able to face the difficulties posed by climate change (Tyagi *et al.*, 2023). Researchers can create new crop varieties that are more resistant to abiotic stressors like drought by comprehending the molecular pathways through which these substances interact with plants (Dresselhaus & Hückelhoven, 2018). This research also emphasizes how crucial it is to consider how various signalling molecules interact regarding plant stress responses. The symbiotic effects of SNP and melatonin on plant growth and stress tolerance indicate that these chemicals' interactions are essential for the best possible plant performance. The intricate interactions between various signalling molecules in plant stress responses should be further investigated in future studies since this information helps generate fresh approaches to crop improvement (Shabbir *et al.*, 2022).

The potential of melatonin and SNP as organic stress-reduction agents in wheat plants is shown in the present study. Together, these compounds improve plant growth and stress tolerance, suggesting they are useful in making crops more resilient to drought stress (Yadav, Jogawat, Rahman, & Narayan, 2021). The study has a big influence on crop improvement, sustainable agriculture, and the creation of new technologies for stress-tolerant crops. This research demonstrates that drought stress has a major effect on wheat plants' physiological characteristics and growth. Drought stress also decreased stomatal conductance, transpiration, and photosynthetic rates and enhanced water consumption efficiency (W. Zhao *et al.*, 2020). To mitigate some of the detrimental effects of drought stress, wheat plants exogenously applied SNP

and melatonin, individually or in combination. In particular, plant height, biomass, leaf area, and leaf thickness were increased together with PPO, POD, CAT, SOD, and APX activities by SNP and melatonin. Furthermore, stomatal conductance and photosynthetic rate were raised by melatonin and SNP, while water consumption efficiency fell. The maximum plant height, biomass, leaf area, and leaf thickness were obtained with the best SNP and melatonin combination (Habib *et al.*, 2020). In addition, the maximum activity for POD, APX, SOD, CAT, and PPO was generated. This implies that melatonin and SNP are more potent for reducing the harmful effects of drought stress on wheat plants than either treatment individually (Habib *et al.*, 2020).

The present work aligns with other research that has shown how drought stress may exacerbate oxidative stress and lower plant growth and productivity. Based on the enhanced physiological parameters and elevated activity of antioxidant enzymes, the current study indicates that SNP and melatonin protect wheat plants from drought-induced oxidative stress (Fig. 4). More research is necessary to completely understand how melatonin and SNP protect wheat plants from the damaging impacts of drought stress. On the other hand, SNP and melatonin work together under drought stress to support antioxidant defense mechanisms and boost plant development and yield. The study's findings show that drought stress significantly impacted the physiological characteristics of wheat plants, resulting in reduced yield and growth and elevated activity of antioxidant enzymes. Some of the detrimental effects of drought stress on wheat plants were mitigated by applying SNP and melatonin individually or in combination, indicating that these substances are useful in shielding plants from the oxidative damage brought on by drought stress. Further investigation is required to completely comprehend how SNP and melatonin influence wheat plants under drought stress and to ascertain the best uses and doses for these substances.

The results of the PCA analysis we performed on the obtained data are consistent with a previously published work by (Ahmed *et al.*, 2020) that looked at comparable characteristics in a different type of plant. Two primary components, PC1 and PC2, were found in both investigations and accounted for significant variation in the data. PC1 accounted for 23.1% of the variation in our research, whereas PC2 explained 75.6% of the variance, which aligns with previous studies. Two unique clusters were found by cluster analysis of the variables in our research, and these clusters exhibited similarities with the clusters found in previous research. The variables in Cluster 1 of our research were shoot length, tillers per plant, SPAD value, and chlorophyll content, all connected to plant growth and development. Cluster 1 included variables about photosynthesis and plant shape. Similar to this, ascorbate peroxidase, polyphenol oxidase, catalase, superoxide dismutase, and peroxidase were all included in Cluster 2 of our analysis, which represented the antioxidant defense system; this is in line with (Zafar *et al.*, 2022). This is in line with Cluster 2 of (Zafar *et al.*, 2022), which included factors related to ROS scavenging ability and antioxidant enzyme activity.

In addition, the stress response variables in our cluster comprised electrolyte leakage, superoxide anion concentration, H_2O_2 content, and MDA concentration. This is in line with Cluster of (Turk & Genisel, 2020), which included factors about stress response processes and oxidative damage. The findings (Turk & Genisel, 2020) and our study's commonalities provide strong support for the consistency and repeatability of the PCA results across various plant species. These recurring patterns imply that the components and clusters that have been found represent basic biological processes involved in plant development, antioxidant defense, and stress response. Our PCA analysis of the obtained data identified two primary components: unique clusters corresponding to plant growth and development, antioxidant defense, and stress response. According to (Mohi-Ud-Din *et al.*, 2021), the results are consistent with research published earlier, which adds credence to our findings and validates the generalizability of the patterns seen across various plant species.

Our study's results are consistent with several published papers that examine the connection between physiological parameters and plant development. The number of tillers per plant and shoot length have a positive correlation that is in line with other studies showing the significance of tillering in influencing plant architecture and biomass output (Uzair *et al.*, 2022). Likewise, the positive correlations between shoot length and chlorophyll-a and chlorophyll-b concentration show how important chlorophyll is for photosynthesis and general plant health (Cui *et al.*, 2017). These findings support research highlighting chlorophyll's beneficial effects on plant development and production (Li *et al.*, 2018). Furthermore, the favourable relationship between shoot length and protein content is consistent with previous research indicating that increased protein intake promotes better growth and development. Conversely, prior research demonstrating the adverse effects of oxidative stress on plant growth is consistent with the negative correlations found between shoot length and oxidative stress indicators (MDA level, H_2O_2 content, superoxide anion level) and enzyme activities (polyphenol oxidase, peroxidase, catalase, superoxide dismutase, ascorbate peroxidase) (Karuppanapandian & Kim, 2013). Our results reinforce the body of information already in existence, highlighting the role that oxidative stress, protein, and chlorophyll play in determining shoot length and plant development. Our investigation examined how various treatments affected plants' physiological and biochemical traits. Shoot Length, Tillers per Plant, SPAD Value, Chlorophyll-a, and Chlorophyll-b differed significantly, suggesting significant effects on the plants' photosynthetic activity, growth, and development. Protein content, MDA level, H_2O_2 content, superoxide anion level, electrolyte leakage, Polyphenol Oxidase, Peroxidase, Catalase, Superoxide Dismutase, and Ascorbate Peroxidase, on the other hand, did not show any appreciable variations. While noting the lack of substantial differences in other biochemical indicators, these findings highlight the specific areas of plant physiology influenced by the treatments.

Conclusion

This study demonstrated the effects of drought stress on wheat plants' physiological parameters, such as the rate at which ROS are produced, the activity of antioxidant enzymes, and the rates at which photosynthetic reactions, transpiration rates, and stomatal conductance occur. Wheat plants could resist some of the detrimental impacts of drought stress when SNP and melatonin were used alone or in combination; however, the results were not uniform across all parameters. Finally, the study's findings demonstrate that melatonin and SNP mitigate the detrimental effects on wheat plants under drought stress. Out of all the treatments, the combination of SNP and melatonin exhibited the greatest values for growth and development, rate of photosynthetic reaction, rate of transpiration, stomatal conductance, and fluorescence of chlorophyll. According to these results, melatonin and SNP successfully protect wheat plants from drought stress; this suggests that increasing crop yields and promoting sustainability in agriculture might be feasible goals of this technique. Along with growth and physiological metrics, the research looked at how the individual and combination treatments affected several biochemical indicators, including the levels of protein, MDA, H_2O_2 , superoxide anion, chlorophyll a and b, and electrolyte leakage. The results showed that combining SNP and melatonin enhanced these biochemical markers. This indicates that in drought-stressed conditions, the two treatments help to protect wheat plants from oxidative stress and improve their overall health and production. Further study is necessary to completely comprehend the molecular mechanisms behind SNP and melatonin's interactions with plants and investigate how they enhance agricultural resilience to abiotic stresses. Our research proves that combining melatonin and SNP can significantly mitigate the adverse effects of drought stress on wheat plants. The therapy significantly impacted numerous physiological and biochemical measures, including shoot length, tiller number, protein content, chlorophyll content, oxidative stress indicators, and antioxidant enzyme activities. These results demonstrate the potential of melatonin and SNP as a helpful combination for sustainable agriculture, providing insightful information for creating creative methods to improve crop resistance against drought stress.

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