

SCREENING FOR SUITABLE MELATONIN APPLICATION SCHEME TO MODULATE DROUGHT STRESS IN BULGARIAN WHEAT

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Abstract

Drought occupies a dominant share among the environmental factors that directly cause significant economic losses. Wheat is one of the most important crops for humankind, and although it tolerates drought to a certain extent, prolonged water deficit provokes a number of unfavourable alterations in plants, which lead to a significant decrease in productivity. Because of the broadening climate changes, the search for ecological biostimulants as an innovative approach to reduce the harmful effects of drought is gaining more popularity in modern agriculture. Melatonin is a natural product of plant metabolism with growth-regulating properties – it is non-toxic and is safe for the environment. We aimed to investigate the possibilities to modulate the negative effects of drought through application of melatonin using a comparative analysis of the physiological reactions of young wheat seedlings. We studied the effects of melatonin applied before drought on the physiological responses of two Bulgarian wheat cultivars grown as a soil culture. Different concentrations of melatonin ranging from 5 to 500 μM were tested and 75 μM was selected as a promising one. Additionally, we tested the method of application of melatonin – leaf spray or root supplement. Based on the content of selected stress markers we could make a conclusion that leaf spraying of plants before stress had some attenuating effect on the negative consequences of drought, but one dose of root application appeared to be more effective.

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Abbreviations: MDA – malondialdehyde, Mel – melatonin, ROS – reactive oxygen species

Introduction. Drought is a frequent stress factor in wheat growing farmlands, and may induce distress or even plants' death [1]. It may happen during each growth stage and cause negative consequences on plant physiology and biochemistry, which may substantially reduce wheat growth and yield [1]. Among these consequences, the accumulation of reactive oxygen species (ROS): hydrogen peroxide, superoxide anions, hydroxyl radical, singlet oxygen, may amplify as an avalanche process and damage macromolecules and biological membranes [2].

Melatonin (N-acetyl-5-methoxytryptamine, Mel) is a natural metabolite. In plants it plays various regulatory functions on plant metabolism throughout the ontogenesis, and recently is extensively studied as plant growth regulator or biostimulant [2, 3]. Moreover, Mel is reported as an effective endogenous ROS scavenger under diverse stress conditions like exceptionally high or low temperatures, salinity, drought, heavy metals, UV-radiation, pathogens, etc., and as a modulator of the stress protection mechanisms in plants [2, 3]. There are few reports targeting the effect of melatonin pretreatment on soil-grown wheat [4, 5], and there is no information about melatonin application on Bulgarian wheat varieties. This is a first attempt to modulate drought stress by melatonin application in Bulgarian wheat cultivars. Sadovo-1 cultivar is a Bulgarian wheat standard for bread wheat, which possesses high drought resistance. The cv. Fermer also has a good drought tolerance, which is relatively lesser than that of cv. Sadovo-1 [6].

This study aimed to determine a suitable scheme of melatonin supply (concentration, method and number of doses of melatonin application) intended to modulate the negative drought effects on soil grown Bulgarian wheat via monitoring the alterations of the content of typical stress-related indices – malondialdehyde (MDA) and free proline.

Material and methods. Plant material and treatments. Bulgarian wheat cultivars (*Triticum aestivum* L., cv. Sadovo-1 and cv. Fermer) were soil-grown in growth chamber with the following parameters: 22 °C/19 °C (day/night) temperatures, 14/10 h (day/night) photoperiod, providing 200 $\mu\text{mol}/\text{m}^2/\text{s}$ photon flux density and 60% relative air humidity. Plants were grown in plastic pots containing 560 g of soil. The soil moisture was maintained to 75% of the field soil capacity by daily watering. Twenty seedlings were planted in each pot. Every variant of treatment consisted of 6 pots. In order to screen the appropriate concentration and method of melatonin application, young seedlings with fully developed second leaf were foliar sprayed or root supplemented with 10 ml of melatonin water solution before drought stress. Drought stress was applied to 17-days old plants by

withholding water supply. The plant samples were collected from above-ground parts and used for analyses of the selected stress marker contents.

Stress markers content. Approximately 250 mg of fresh leaf material was ground with quartz sand in 4 ml of 1% (w/v) cold trichloroacetic acid and then centrifuged for 30 min ($15000 \times g$, 4°C). Appropriate aliquots of the resultant supernatants were used for measurement of the stress markers free proline and malondialdehyde (MDA) content. Free proline and MDA were determined according to the method of BATES et al. [7] and KRAMER et al. [8], respectively, with some modifications. The proline reaction mixture consisted of 0.5 ml supernatant, 0.5 ml 1% trichloroacetic acid, 1 ml conc. acetic acid, and 1 ml ninhydrin reagent. It was incubated on boiling water bath for 1 h. The reaction was terminated on ice. The absorbance was read at 520 nm and proline concentration was calculated by standard curve. The level of lipid peroxidation was estimated by the concentration of MDA in the plant tissues [8]. Half milliliter of supernatant was used to determine the concentration of MDA by incubation with 1 ml 0.5% thiobarbituric acid in 20% trichloroacetic acid for 45 min at 100°C . After termination of the reaction on ice and centrifugation (5 min at $3000 \times g$), the absorbance was read at 532 nm and 600 nm. Molar extinction coefficient of $155 \text{ mM}^{-1} \text{ cm}^{-1}$ was used to calculate the MDA content.

Statistical analysis. The results are from two independent experiments each performed in six replicates. The data presented in figures are mean values $\pm$ standard error (SE). One-way ANOVA with Duncan's post-hoc multiple range test was used to evaluate the significant differences at a level $p \leq 0.05$.

Results and discussion. Screening of the most effective concentration of melatonin to alleviate drought stress. Since melatonin has not been studied on Bulgarian wheat cultivars, we tested its application in different concentrations and monitored its effect on the Bulgarian wheat standard Sadovo-1. Melatonin was leaf applied in a wide range of concentrations (5 to 500 μM), including those reported to be the most effective and frequently used according to the literature survey, and 24 hours later drought stress was imposed for 4 days. We found that drought doubled MDA content and increased proline more than 26-fold as compared to the control levels (Fig. 1). The preliminary applications of 75 μM , 100 μM , and 500 μM melatonin were found to decrease MDA content by 18%, by 16%, and by 7%, respectively, as compared to drought stress only. The preliminary treatment with the tested concentrations additionally augmented the proline content, which reached highest level after 200 μM melatonin.

A number of researchers studied the most effective concentrations of melatonin application that lessen the negative outcomes of drought stress in different crops [5, 9, 10]. Accumulation of ROS usually follows drought stress, which damages biomembranes. MDA is a typical stress marker indicating biomembrane injuries, which is substantially increased due to stress [2]. Proline is rapidly accumulated under drought conditions, but its accumulation might be also a cellular

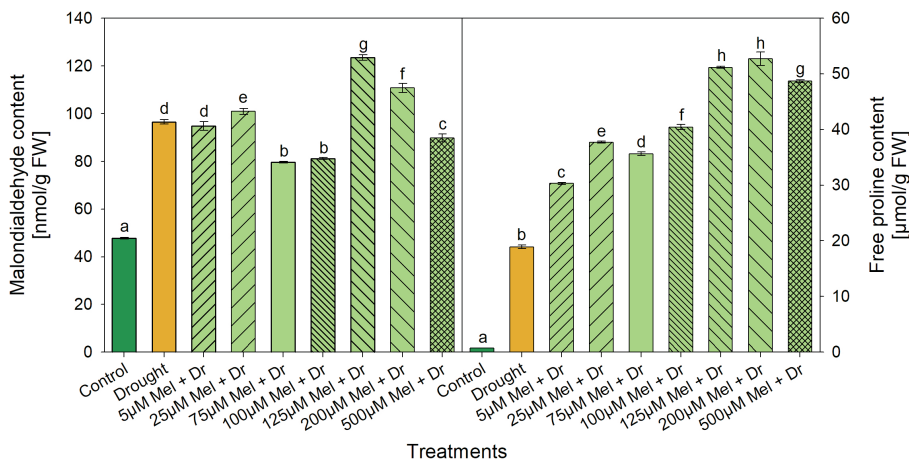


Fig. 1. Malondialdehyde and free proline content in the leaves of wheat seedlings (cv. Sadovo-1) foliar pretreated with different concentration of melatonin (Mel) and 24 h later subjected to drought (Dr) for 4 days. Data are presented as mean values with standard error. Different small letters designate statistically significant differences between the treatments at $p \leq 0.05$

protector against stress [2]. In general, while screening wide range of melatonin concentrations, the authors chose the one which decreases the content of stress markers to the greatest degree as compared to drought stress only. The decrease of MDA after melatonin application was reported to indicate an effective diminution of drought stress injuries [5,9,10]. The fact that Mel was described to possess ROS scavenger properties [2,3] could explain these observations. Moreover, exogenous melatonin usually provoked an increase of the cellular compatible protectants like soluble sugars and other osmolytes including free proline, aiming preservation of cell integrity by balancing the osmotic pressure under various abiotic stress conditions, including drought [2,11]. However, the proline severe buildup could be toxic for cells [12]. Therefore, based on our results we selected 75 μM as the most promising concentration of melatonin, which could diminish the negative drought stress consequences in the Bulgarian wheat standard. This concentration combined at the same time a greater decrease of MDA, and mid-range increase of free proline.

Screening of an effective method of melatonin application to alleviate drought stress. The selected 75 μM concentration of melatonin was tested further on another Bulgarian wheat cultivar Fermer. We observed that the levels of stress markers of cv. Farmer (Fig. 2) also reached these of cv. Sadovo-1 (Fig. 1) but a day earlier which could be expected because these cultivars are characterized to differ in their drought tolerance [6]. It should be noted that under our experimental conditions cv. Fermer showed a strong tendency of lodging, which

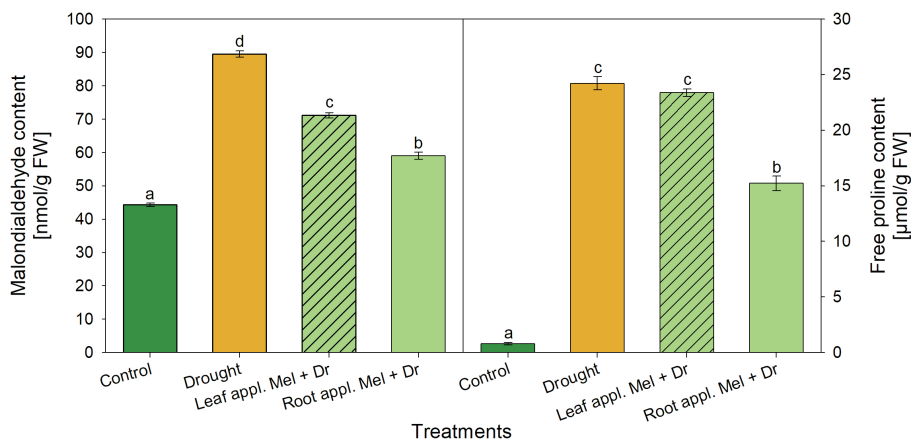


Fig. 2. Malondialdehyde and free proline content in the leaves of wheat seedlings (cv. Fermer) foliar or root pretreated with 75 μ M melatonin (Mel) and 24 h later subjected to drought (Dr) for 3 days. Data are presented as mean values with standard error. Different small letters designate statistically significant differences between the treatments at $p \leq 0.05$

to a considerable extent compromised the application of melatonin by leaf spraying. Therefore, we searched for a reliable method of application of melatonin by comparing the effectiveness of leaf spray and root supplement. We found that after 3 days of drought the levels of MDA and proline (Fig. 2) were significantly increased (two-fold and 30-fold, respectively). The preliminary treatment with leaf applied melatonin decreased MDA as compared to drought stress alone but its content was still 60% above the control level. No significant alteration was detected in proline content after leaf application and its level was comparable with that after drought stress alone. The data obtained showed that the root supply of melatonin reduced MDA and proline much closer to the control values than the leaf application, which suggests that melatonin application facilitated maintenance of physiological processes in drought treated plants. Based on these observations we can suppose that the root application of melatonin is more effective than leaf spraying in order to attenuate the destructive drought stress effects. Other authors also conclude that in general, the root delivery method demonstrated a better protective effect than foliar spraying, probably because the signal of melatonin applied via root could be transported effectively across the entire plant [9, 13, 14].

Screening for delivery doses of melatonin application to alleviate drought stress. Other authors [15, 16] indicated that more doses of melatonin application were better in preventing different stress consequences. Therefore, we tested the appropriate number of delivery doses for further investigation of

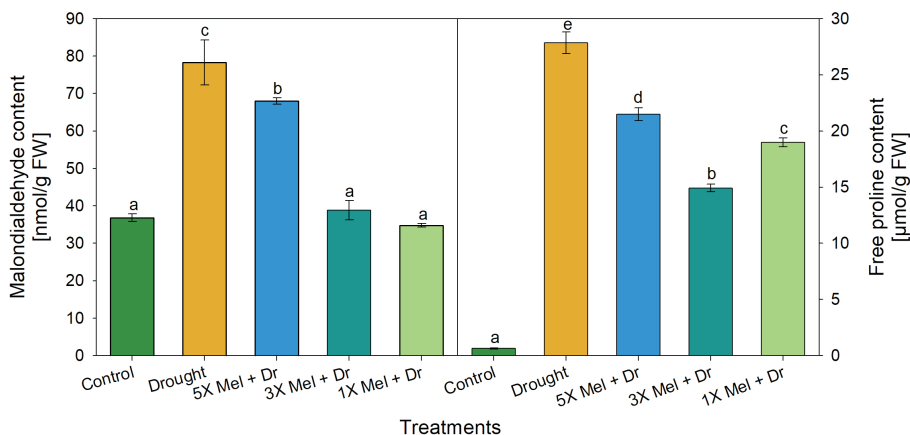


Fig. 3. Malondialdehyde and free proline content in the leaves of wheat seedlings (cv. Fermer) root pretreated with 75 μ M melatonin (Mel) and subjected to drought (Dr) for 3 days. Data are presented as mean values with standard error. Different small letters designate statistically significant differences between the treatments at $p \leq 0.05$

the alleviating effect of root applied melatonin to prevent drought stress injuries. For this purpose the seedlings of cv. Fermer were irrigated with 75 μ M Mel by the following 3 schemes. The first one (5X Mel + Dr) included irrigation with water solution of 75 μ M melatonin once per day for 5 consecutive days just before the subjection of plants to drought stress. The second scheme (3X Mel + Dr) included 3 times delivery of the same solution on each odd day. The third scheme (1X Mel + Dr) comprised of only one delivery of the solution 24 h before drought stress. The data obtained revealed that similarly to previous two experiments, drought provoked substantial increase in MDA and proline content (Fig. 3). All tested melatonin delivery doses led to decreased content of the stress indicators measured, and 5X Mel was less effective in alleviating the negative drought stress outcomes. HUANG et al. [9] showed that irrigation with Mel was more effective in preventing drought stress than multiple leaf spraying of maize. Similarly, ZHAO et al. [16] showed that two applications of 100 μ M melatonin resulted in the highest inhibitory effects on the local infection in *N. glutinosa* and systemic infection in *S. lycopersicum* inoculated with TMV as compared to one application. CORTÉS-MONTAÑA et al. [15] also found that 3X melatonin pre-harvest application is more effective than 2X pre-harvest application in preserving shelf-life cold storage and post-harvest quality of plum fruits.

Although we expected that 5X melatonin application should be most effective, probably it provokes a supersaturation equal to a single high-concentration dose, which as we have previously found (Fig. 1) does not result in a stress mitigation

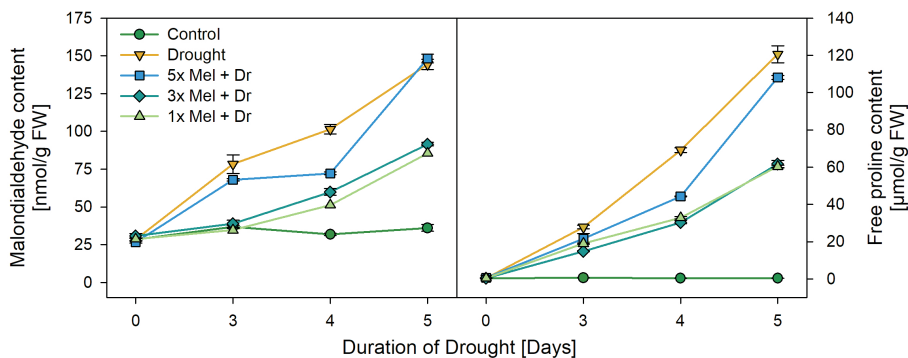


Fig. 4. Malondialdehyde and free proline content in the leaves of wheat seedlings (cv. Fermer) root pretreated with 75 μ M melatonin (Mel) and subjected to drought (Dr). Data are presented as mean values with standard error

effect. HERNÁNDEZ-RUIZ et al. [17] showed that melatonin had positive effect on the growth of wheat when applied at low concentrations, but had negative effect at high concentrations.

Effects of melatonin application in the time-course of drought stress duration. We tested also to what extent melatonin is effective in the time-course of drought duration, which is related to the stress severity. For this purpose, the same 5X, 3X, and 1X application delivery doses were used, but the stress duration was prolonged for 5 consecutive days. The drought negative outcomes deepened with stress advancement and the amounts of MDA and proline were progressively increased. We found that 5X melatonin application was not able to prevent stress injuries and under severe drought (5 days) there were no statistically significant differences in MDA content of drought-treated and 5X Mel pretreated plants.

GUO et al. [18] showed that the beneficial effects of melatonin in mitigation of drought decreased with stress severity, although melatonin alleviated drought stress-induced ROS accumulation and the corresponding oxidative damage in maize plants. However, we found that during the experiment 3X and 1X melatonin application mitigated the stress consequences and the level of stress markers was approximately two-fold lower than that in drought-treated wheat seedlings. There were no considerable differences between 3X and 1X melatonin application on parameters studied and we could assume that the effects of both treatments were comparable. However, the 1X application is economically reasonable.

Conclusion. Based on the results of this study, it is obvious that the potential of melatonin to alleviate drought stress is closely related to its appropriate concentration and depends on the application method and number of doses applied. We can conclude that 1X delivery dose of 75 μ M melatonin applied to

roots is capable to mitigate the effects of drought stress in Bulgarian wheat cultivar Fermer. However, the beneficial outcome depends also on the severity and duration of the drought. Further investigation on the antioxidant defense mechanisms, molecular biological analyses of the expression of selected genes encoding antioxidant and proline biosynthetic enzymes, and other important physiological traits such as photosynthesis in cv. Fermer and other Bulgarian wheat varieties are necessary to confirm our findings.

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