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NITRIC OXIDE-MEDIATED MODULATION OF BIOCHEMICAL RESPONSES AND LEAF SPOT DISEASE PROGRESSION IN DATE PALM (*Phoenix dactylifera* L.) UNDER DROUGHT STRESS

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ABSTRACT

This study investigates the role of sodium nitroprusside (SNP) in enhancing drought tolerance and mitigating leaf spot disease in date palm (*Phoenix dactylifera* L.). Three SNP concentrations (0, 75, and 150 µM) were applied under progressive drought stress (1, 3, and 6 weeks). The results showed that drought stress significantly increased oxidative stress markers, including malondialdehyde (MDA) and hydrogen peroxide (H_2O_2), while SNP, particularly at 150 µM, reduced their levels. Additionally, SNP treatment enhanced antioxidant enzyme activities (catalase and peroxidase). SNP also reduced the severity of leaf spot symptoms caused by *Alternaria alternata*, limiting lesion size. These findings suggest that SNP modulates physiological and biochemical responses to drought stress, improving plant defense and disease resistance. Thus, SNP application could be a promising strategy to support the long-term viability of date palm cultivation, particularly in arid regions.

Keywords: Antioxidant enzymes, Oxidative stress, Plant disease resistance, Sodium nitroprusside

The date palm (*Phoenix dactylifera* L.) is one of the oldest cultivated crops, with a long history of agricultural and cultural significance in arid regions worldwide. Due to its economic value, high nutritional content, and exceptional resilience to harsh environmental conditions, date palm cultivation remains a cornerstone of agriculture in arid and semi-arid regions (Al-Khayri *et al.*, 2018). However, the sustainability of date palm production is increasingly threatened by the growing impacts of climate change, particularly the heightened frequency and intensity of drought events (Ali-Dinar *et al.*, 2023).

Drought stress represents a multifaceted challenge to the productivity of date palms and other plants, as it disrupts numerous physiological and biochemical processes. These include reductions in photosynthetic efficiency, alterations in water relations, and the induction of oxidative stress (Safronov *et al.*, 2017). Mitigating the adverse effects of drought on date palm productivity requires the exploration of innovative strategies. One promising avenue involves the role of nitric oxide (NO), a highly reactive and versatile signaling molecule that has garnered significant attention for its involvement in plant stress responses, including those triggered by drought (Graska *et al.*, 2023). Recent studies, such as that by Allagulova *et al.* (2023), have elucidated the role of NO in regulating various physiological processes, including hormonal signaling, antioxidant defense mechanisms, and stomatal regulation, all of which are critical for plant adaptation to environmental stressors.

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The potential of NO as a signaling molecule to enhance drought tolerance in plants has been the subject of increasing investigation in recent years (Nabi *et al.*, 2019). Exogenous application of NO, or modulation of its endogenous levels, has been shown to improve drought tolerance across a variety of plant species. This is achieved through mechanisms such as enhanced water-use efficiency, bolstered antioxidant defenses, and modulation of stress-related gene expression (Ullah *et al.*, 2021). However, despite promising results in other plant species, the role of NO in alleviating drought stress in date palms remains largely unexplored, highlighting a significant gap in the current literature. Given the unique physiological characteristics of date palms and their profound economic importance, understanding how NO influences drought tolerance in this species is of critical significance.

This study aims to address this gap by investigating the effects of NO on the physiological and biochemical responses of date palms subjected to drought stress. The objective is to evaluate the potential of NO as a means to enhance the drought tolerance of date palms, thereby contributing to the development of more sustainable agricultural practices for this vital crop.

MATERIALS AND METHODS

The study was conducted simultaneously in two distinct orchards within the Basra Governorate of southern Iraq. The first orchard is located in the Shatt al-Arab region (30°32’16”N, 47°51’01”E), while the second is situated in the Karmat Ali region (30°35’01”N,

47°45'27"E). Uniform six-year-old *Phoenix dactylifera* cv. Al-Sayer date palm trees were selected for this study. Three concentrations of sodium nitroprusside (SNP), a nitric oxide (NO) donor, were applied (0, 75, and 150 µM). Drought stress was induced by withholding irrigation for varying durations: one, three, and six weeks. A total of 27 trees per orchard were used, with three individual trees receiving each treatment. SNP was applied as a foliar spray one week prior to the initiation of the drought treatments. Distilled water was used to prepare SNP solutions at the concentrations of 75 µM and 150 µM, which were then evenly sprayed on the leaves of the date palm trees to ensure uniform coverage. The control group was treated with distilled water only. The experimental trees were maintained under typical orchard conditions throughout the duration of the study, with drought stress applied according to the designated treatment schedules. Leaf samples were collected from each treated and control tree after one, three, and six weeks of treatment. To minimize the effects of diurnal light variation, all sampling was performed in the morning. Approximately 5 grams of fresh leaf tissue was harvested from each tree for subsequent biochemical analysis.

Estimation of Malondialdehyde (MDA) content

The MDA content in the leaf tissue was determined according to the method outlined by Heath and Packer (1968). A 0.5 g sample of leaf tissue was homogenized in 5 mL of 0.1% trichloroacetic acid (TCA) solution (w/v). The resulting mixture was then centrifuged at 10,000 rpm for 5 minutes at 4°C. Following centrifugation, 4 mL of 0.5% thiobarbituric acid (TBA) solution, prepared by dissolving TBA in a 20% TCA solution (w/v), was added to 1 mL of the supernatant. The mixture was incubated in a water bath at 100°C for 30 minutes to allow the reaction to proceed. The reaction was then terminated by placing the samples on ice. After cooling, the mixture was centrifuged at 10,000 rpm for 15 minutes at 4°C. As a control, a solution of TBA without the sample extract was prepared. The absorbance of the supernatant was measured using a spectrophotometer at wavelengths of 532 nm and 600 nm to determine the MDA content. The absorbance at 532 nm corresponds to the MDA-TBA adduct, while the absorbance at 600 nm is used to correct for interfering substances.

The content of MDA was calculated using the following equation:

$$\text{MDA content} = \frac{1000\ (OD\ 532 - OD\ 600)}{155}$$

where 155 $mM^{-1}\ cm^{-1}$ is the extinction coefficient of MDA.

Estimation of hydrogen peroxide (H_2O_2) content

The hydrogen peroxide (H_2O_2) content in leaf tissues was measured using the method described by Sergiev *et al.* (1997). Briefly, 0.5 g of fresh leaf tissue was homogenized in 5 mL of 0.1% (w/v) trichloroacetic acid (TCA) solution. The homogenate was then centrifuged at 13,000 rpm for 15 minutes at 4°C. Following centrifugation, 1 mL of the supernatant was mixed with 0.5 mL of 10 mM potassium phosphate buffer (pH 7.0) and 1 mL of 1 M potassium iodide (KI) solution, prepared by dissolving 166 g of KI in 1 liter of distilled water. The absorbance of the resulting mixture was measured at 390 nm using a spectrophotometer. A control sample was prepared in the same manner, but without the addition of the leaf extract. The hydrogen peroxide concentration was calculated based on a standard curve constructed using known concentrations of hydrogen peroxide.

Estimation of catalase (CAT) activity

Catalase activity in leaf tissue was determined following the method outlined by Goth (1991). Briefly, 0.2 mL of leaf tissue extract was mixed with 1 mL of a reaction mixture containing 60 mM potassium phosphate buffer (pH 7.0) and 65 mM hydrogen peroxide (H_2O_2). The reaction mixture was incubated at 25°C for 4 minutes. To terminate the reaction, 1 mL of 32.4 mM ammonium molybdate was added. The absorbance of the resulting solution was then measured at 405 nm using a spectrophotometer. Catalase activity was calculated using the following formula:

$$\text{Catalase interest} = \frac{\text{Sample} - \text{Blank 1}}{\text{Blank 2} - \text{Blank 3}} \times 271$$

where:

Sample: contains 0.2 ml of extract, 1 ml of ammonium molybdate, and 1 ml of the hydrogen peroxide-buffer solution. Blank 1: contains 0.2 ml of extract, 1 ml of ammonium molybdate, and 1 ml of the buffer solution (without hydrogen peroxide). Blank 2: contains 0.2 ml of buffer, 1 ml of ammonium molybdate, and 1 ml of the hydrogen peroxide-buffer solution. Blank 3: contains 0.2 ml of buffer, 1 ml of ammonium molybdate, and 1 ml of buffer solution only.

Estimation of peroxidase (POD) activity

The activity of peroxidase (POD) was determined using the method described by Kim and Yoo (1996). The test solution consisted of hydrogen peroxide (0.02 M), guaiacol (0.05 M), and sodium acetate buffer (0.1 M, pH 5.5). The components were mixed with distilled water in a 1:1:1:7 (v/v/v/v) ratio. To initiate the reaction, 200 µL of enzyme extract was added to 2.8 mL of the prepared

reaction mixture. The mixture was gently agitated, and the increase in absorbance was measured at 470 nm using a spectrophotometer. Measurements were taken after incubating the reaction mixture for 3 minutes at room temperature. One unit of peroxidase activity was defined as the amount of enzyme required to cause an increase of 0.1 in absorbance per minute per gram of fresh weight (units·g^{-1}·min^{-1}). A reaction mixture without enzyme extract was used as the blank.

Monitoring of leaf spots disease development

The fungus *Alternaria alternata* (accession number PP330024) was used to inoculate date palm leaves, as it is one of the most prevalent pathogens affecting date palms (Alasadi, 2024a). Inoculation was performed to investigate the interaction between drought stress and fungal infection, given that drought conditions can increase the susceptibility of date palms to fungal diseases (Majeed *et al.*, 2025). A 5-mm disc of 10-day-old *A. alternata* mycelium, grown on potato dextrose agar (PDA), was placed on the leaf surface and sealed with parafilm to maintain humidity, as described by Alasadi (2024b). Control leaves were inoculated with a sterile PDA disc using the same procedure. Three replicates were used for each concentration. The development of leaf spot symptoms was monitored by measuring lesion diameter with a digital Vernier caliper at the end of the experimental period. Drought symptoms on the date palm trees were visually observed throughout the experiment.

Statistical analysis

The experimental data were analyzed using analysis of variance (ANOVA). Data analysis was performed with SPSS version 21, and the means were compared using the least significant difference (LSD) test at a significance level of 0.05.

RESULTS AND DISCUSSION

Malondialdehyde (MDA)

Malondialdehyde (MDA) content exhibited significant variation across different concentrations of sodium nitroprusside (SNP) (0, 75, and 150 µM) over time. In the first week, MDA levels were relatively low, with minor differences among treatments: 1.27, 1.14, and 1.01 nmol g^{-1} for 0, 75, and 150 µM SNP, respectively. A similar trend was observed at week three, where MDA contents were 1.42, 1.29, and 1.19 nmol g^{-1}, respectively. However, by week six, significant differences became apparent. The 0 µM SNP treatment, which served as the control, recorded the highest MDA content (4.57 nmol g^{-1}), which was significantly greater than the values observed for the 75 µM (2.97 nmol g^{-1}) and 150 µM (2.12 nmol g^{-1}) treatments (Fig. 1).

A noticeable decrease in MDA accumulation was observed with increasing SNP concentrations, highlighting the protective role of SNP against oxidative stress, particularly in the later stages of the experiment. Lipid peroxidation, a hallmark of oxidative stress, leads to MDA production, making it a reliable indicator of membrane damage under drought stress (Farooq *et al.*, 2010). The observed increase in MDA levels aligns with previous studies indicating that prolonged drought exacerbates oxidative damage (Møller *et al.*, 2007). The application of SNP alleviated this effect, suggesting that SNP plays a role in mitigating oxidative stress through nitric oxide (NO)-mediated regulation of reactive oxygen species (ROS) homeostasis (Ullah *et al.*, 2021; Suhim *et al.*, 2017).

Hydrogen Peroxide (H_2O_2)

The different concentrations of sodium nitroprusside (SNP) (0, 75, and 150 µM) and the progression of

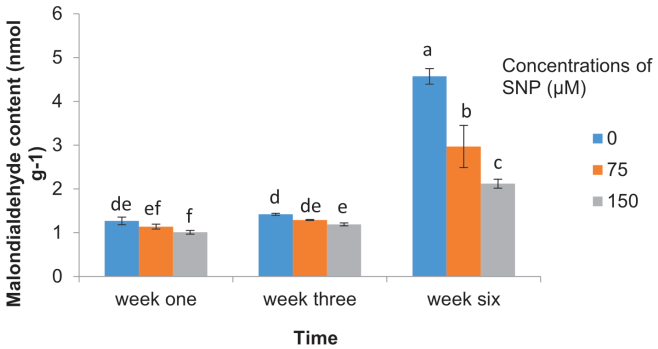

Fig. 1. Effect of drought duration (weeks) and application of different concentrations of sodium nitroprusside (SNP) on malondialdehyde (MDA) content in date palm leaves
Bars represent the standard error.
Values with different letters are significantly different at *p≤0.05*.

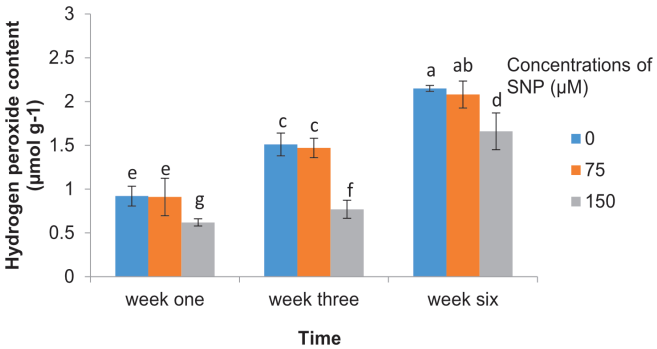

Fig. 2. Effect of drought duration (weeks) and foliar application of different concentrations of sodium nitroprusside (SNP) on hydrogen peroxide (H_2O_2) content in date palm leaves
Bars represent the standard error.
Values with different letters are significantly different at *p≤0.05*.

time significantly influenced hydrogen peroxide (H_2O_2) content. In the first week, there were no significant differences between the 0 and 75 µM treatments, with values of 0.92 and 0.91 µmol g^{-1}, respectively. In contrast, the 150 µM treatment exhibited a significantly lower H_2O_2 level (0.62 µmol g^{-1}). By week three, no significant differences were observed between the 0 and 75 µM treatments (1.51 and 1.47 µmol g^{-1}, respectively), while a significant reduction in H_2O_2 content was observed in the 150 µM treatment (0.77 µmol g^{-1}). In week six, the highest H_2O_2 content was recorded in the 0 µM treatment (2.15 µmol g^{-1}), followed closely by the 75 µM treatment (2.08 µmol g^{-1}), with no significant difference between them. However, the 150 µM treatment showed a significantly lower value (1.66 µmol g^{-1}) (Fig. 2).

H_2O_2 is a key reactive oxygen species (ROS) generated under abiotic stress, and if not properly regulated, it can lead to oxidative damage (Kocsy *et al*., 2005). The significant rise in H_2O_2 levels over time confirms the increase in oxidative stress under drought conditions. The SNP treatment effectively reduced H_2O_2 levels, suggesting its role in mitigating stress by enhancing antioxidant enzyme activity and maintaining ROS homeostasis (Shareef and Al-Khayri, 2021). These results indicate that SNP application, particularly at 150 µM, plays a protective role in reducing oxidative stress damage in a drought environment.

Catalase (CAT) activity

Catalase activity (units•g^{-1}•min^{-1}) was significantly influenced by both time and SNP concentration. In the first week, the highest catalase activity was observed in the 150 µM SNP treatment (24.74), which was significantly higher than the 0 µM treatment (21.47) and the 75 µM treatment (22.19). No significant difference was observed between the 0 µM and 75 µM treatments, but the difference between 0 µM and 150 µM indicated a statistically significant increase in catalase activity with SNP application. By week three, catalase activity reached its peak in the 150 µM SNP treatment (27.51), which was significantly higher than both the 75 µM (23.38) and 0 µM (22.41) treatments, confirming that the 150 µM SNP treatment significantly increased catalase activity. In week six, catalase activity remained high across all treatments, with values of 25.28 (0 µM), 25.47 (75 µM), and 26.18 (150 µM). At this stage, no significant differences were observed between treatments, suggesting that catalase activity had stabilized over time, regardless of SNP concentration (Fig. 3).

Overall, the application of 150 µM SNP consistently enhanced catalase activity during the first and third weeks, indicating a strong antioxidative response. However, by week six, catalase activity appeared to plateau, which could be attributed to adaptive stress responses or enzymatic saturation. Catalase (CAT) plays a critical role in scavenging H_2O_2 and protecting plant cells from oxidative stress (Ahmad *et al*., 2014). The increase in catalase activity observed in this study suggests a defensive response to prolonged drought stress, with SNP further enhancing this response, possibly through nitric oxide (NO) signaling, which activates antioxidant defenses (Wang *et al*., 2013).

Peroxidase (POD) activity

Peroxidase (POD) activity significantly increased over time under various SNP concentrations (0, 75, and 150 µM), with distinct differences observed across treatments and time points. In week one, the lowest peroxidase activity was recorded in the 0 µM treatment (15.52), which was significantly lower than both the 75 µM (17.58) and 150 µM (17.07) treatments. However, no significant difference was found between the 75 µM

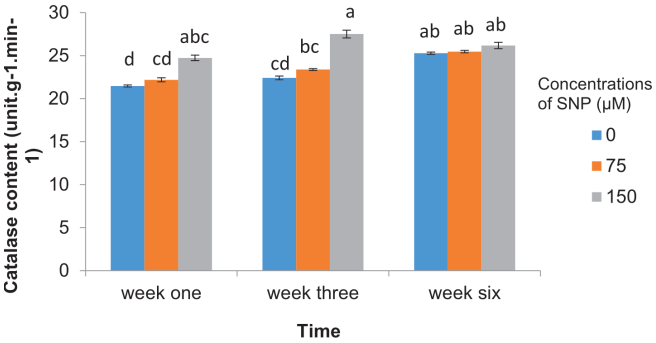

Fig. 3. Effect of drought duration (weeks) and foliar application of different concentrations of sodium nitroprusside (SNP) on catalase (CAT) content in date palm leaves
Bars represent the standard error.
Values with different letters are significantly different at *p≤0.05*.

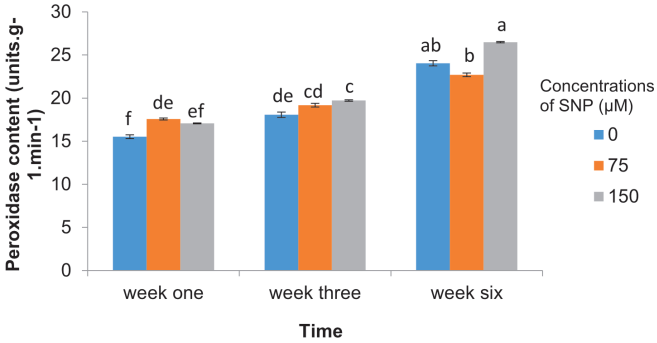

Fig. 4. Effect of drought duration (weeks) and foliar application of different concentrations of sodium nitroprusside (SNP) on peroxidase activity in date palm leaves
Bars represent the standard error.
Values with different letters are significantly different at *p≤0.05*.

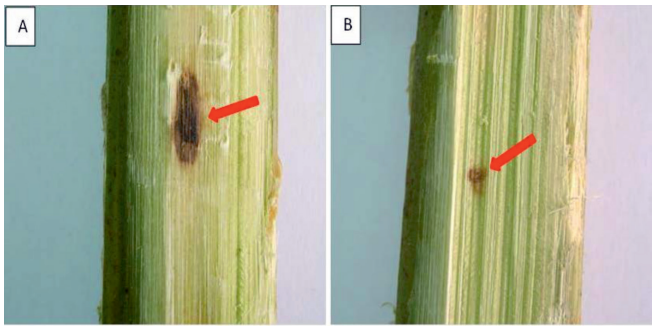

Fig. 5. Effect of sodium nitroprusside (SNP) on date palm leaves under drought stress inoculated with *Alternaria alternata*: (A) Leaf spot at 0 µM SNP concentration (red arrow). (B) Leaf spot at 150 µM SNP concentration (red arrow)

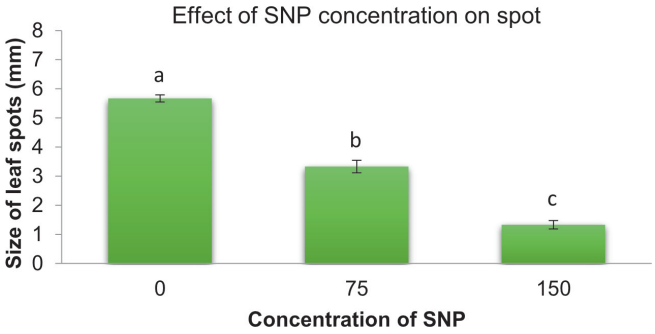

Fig. 6. Size of spots on date palm leaves treated with different concentrations of sodium nitroprusside (SNP)
Values are significantly different at *p≤0.05*. Bars represent the standard error (SE).

and 150 µM treatments at this stage. By week three, peroxidase activity continued to increase. The 0 µM treatment recorded 18.07 units•g^{-1}•min^{-1}, followed by 75 µM at 19.18 and 150 µM at 19.72. Significant differences emerged between the 0 µM treatment and the 150 µM treatment. However, the increase between the 75 µM and 150 µM treatments was not statistically significant. In week six, peroxidase activity reached its peak across all treatments. The highest value was observed in the 150 µM SNP treatment (26.49), which was significantly higher than the 75 µM (22.7) and 0 µM (24.04) treatments. This supports a statistically significant increase in POD activity at 150 µM compared to the 75 µM treatment. However, no significant difference was observed between the 0 µM and 150 µM treatments (Fig. 4).

These results indicate that drought stress induces POD activity, which is consistent with previous studies showing that reactive oxygen species (ROS)-stimulated antioxidant enzymes respond to drought stress (Mafakheri *et al.*, 2011). SNP application at 150 µM effectively enhanced POD activity, suggesting its role in mitigating oxidative stress. Overall, peroxidase activity progressively increased over time in all treatments, with the 150 µM SNP treatment producing the highest levels by week six. This demonstrates that under drought stress conditions, SNP application may strengthen the plant's antioxidant defenses.

Monitoring of leaf spot disease development

Drought-stressed leaves exhibited symptoms of chlorosis, necrosis, and the appearance of brown or dark spots, particularly in the middle and tip regions. The severity of leaf spot symptoms increased with the duration of drought stress, with significant differences observed among SNP treatments (*p<0.05*). The largest spot size was recorded in untreated leaves (5.6 mm), while treatments with SNP at 75 µM and 150 µM reduced spot sizes to 3.3 mm and 1.3 mm, respectively (Fig. 5 and 6).

Drought stress exacerbated the severity of leaf spot disease, possibly due to compromised plant defenses and an increased susceptibility to pathogen infection. SNP treatment effectively mitigated the severity of the symptoms, possibly by enhancing the plant's antioxidant defense system. Similar findings have been reported in other crops, where SNP increased resistance to fungal infections by stimulating defense-related enzymes and secondary metabolites (Zheng *et al.*, 2017; Khaliq *et al.*, 2021).

CONCLUSION

This study demonstrated that drought stress significantly impacts the biochemical status of date palm leaves, as evidenced by increased levels of hydrogen peroxide (H_2O_2) and malondialdehyde (MDA), both of which are indicative of heightened oxidative stress. However, foliar application of sodium nitroprusside (SNP) at 150 µM effectively mitigated these effects, reducing both H_2O_2 and MDA levels. Furthermore, SNP treatment enhanced the activity of key antioxidant enzymes, peroxidase (POD) and catalase (CAT), suggesting a strengthening of the plant's antioxidant defense system under stress conditions. In addition, the application of SNP reduced the severity of leaf spot disease, with a noticeable decrease in spot size as SNP concentration increased. These results highlight the potential of SNP as a valuable tool to alleviate drought-induced oxidative stress and improve disease resistance in date palms. Overall, the application of SNP appears to be an effective strategy for enhancing date palm resilience, promoting plant health, and improving their productivity under harsh environmental conditions.

Authors' contribution

Conceptualization of research work and designing of experiments (RMSA, ASM, FMA); Execution of field/

lab experiments and data collection (RMSA, ASM, FMA); Analysis of data and interpretation (RMSA, ASM, FMA); Preparation of manuscript (ASM, RMSA).

Conflicts of interest

Authors declare that they have no conflicts of interest.

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