Supplementary Methods

Mn₃O₄ nanoparticles maintain ROS homeostasis to modulate stomatal aperture to improve cotton drought tolerance

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Supplementary method 1: Measurement of $\cdot O_2^-$ and H_2O_2 content

The content of superoxide anion ($\cdot O_2^-$) and hydrogen peroxide (H_2O_2) were measured by following previous paper, with some modifications.[1] After 24 h of drought stress, 0.1 g cotton leaves from PMO or TES buffer treatment were mixed with 1 mL extract solution (50 mM PBS buffer, pH 7.5) and then mixture was ground at 4 °C. The supernatant was collected by centrifuging the mixture at 12,000 g for 20 min.

For $\cdot O_2^-$ assay, a mixture was prepared containing 0.2 mL of supernatant, 0.05 mL of hydroxylamine hydrochloride solution (10 mM), 0.4 mL of β-aminobenzenesulfonic acid solution (17 mM), and 0.04 mL of α-naphthylamine solution (7 mM). This mixture was incubated at room temperature for 20 minutes, after which the absorbance was measured at 530 nm using a UV–Vis spectrophotometer. The $\cdot O_2^-$ content was calculated using the following formula:

$$\cdot O_2^- \text{ content (mM/g)} = 2 \times (At - Ac) \times C / w$$

Where, At represents the absorbance of the treatment solution, Ac is the absorbance of the PBS buffer, C = 1.75, and w is the weight of the sample (g).

For the H_2O_2 assay, 0.5 mL of supernatant was mixed with 1 mL of $Ti(SO_4)_2$ solution (5% w/v). After shaking, the samples were centrifuged at 3,000 rpm for 5 minutes, and the precipitates were solubilized in 1 mL of H_2SO_4 (2 M). The absorbance of the final solutions was measured at 415 nm using a UV–Vis spectrophotometer. The H_2O_2 content was calculated according to the following formula:

$$H_2O_2 \text{ content (mM/g)} = (At - Ac) \times C / w$$

Where, At is the absorbance of the treatment solution, Ac is the absorbance of the PBS buffer, C = 1.625, and w is the weight of the sample (g).

Reference

1 J. Liu, J. Gu, J. Hu, H. Ma, Y. Tao, G. Li, L. Yue, Y. Li, L. Chen, F. Cao, H. Wu and Z. Li, *Plant Biotechnol. J.,* 2023, **21**, 1935–1937.

Supplementary method 2: Measurement of antioxidant enzyme activities

Enzyme supernatants preparation: 0.1 g cotton leaves from PMO or TES buffer treatment under drought stress was mixed with 1 mL extract solution (200 mM PBS buffer, pH 7.0) and then the mixture was ground at 4 °C. The enzyme supernatants were collected from mixture by centrifuging at 12,000 g for 20 min.

1. SOD activity: 0.05 mL enzyme supernatant was added into 3 mL SOD reaction solution (130 mM methionine, 0.75 mM nitinol blue tetrazolium, 0.1 mM EDTA-Na$_2$ and 0.02 mM riboflavin). The mixed solution was incubated for 20 min at light conditions (200 μmol m^{-2} s^{-1} PAR). The absorbance of the mixture was measured at 560 nm by UV–Vis spectrophotometer. One unit (U) of SOD activity was defined as a 50% inhibition of the photochemical reduction of NBT caused by enzymatic activity. The SOD activity was calculated according to the following formula:

SOD activity (U/mg prot) = At – Ac × V / (0.5 × Ac × Cpr)

Where, At is the absorbance of treatment solution, Ac is the absorbance of PBS buffer, V is the volume of sample (mL), and Cpr is the protein concentration of sample.

2. POD activity: 0.076 mL guaiacol and 0.112 mL H$_2$O$_2$ (30%) were added into 200 mL PBS buffer (200 mM, pH 7.0) to prepare a POD reaction solution. 0.05 mL enzyme supernatant and 3 mL of POD reaction solution were mixed and subsequently the absorbance of the mixture was measured at 30 and 90 seconds of reaction at 470 nm by UV–Vis spectrophotometer, respectively. The POD activity was calculated according to the following formula:

POD activity (U/mg prot) = (A90-A30) × V / (0.01 × Cpr)

Where, A90 is the absorbance of treatment solution measured at 90s, A30 is the absorbance of treatment solution measured at 30s, V is the volume of sample (mL), and Cpr is the protein concentration of sample.

3. CAT activity: 0.3092 mL H$_2$O$_2$ (30%) were added into 200 mL PBS buffer (200 mM, pH 7.0) to prepare a CAT reaction solution. 0.05 mL enzyme supernatant and 3 mL of CAT reaction solution were mixed and subsequently the absorbance of the

mixture was measured at 30 and 90 seconds of reaction at 240 nm by UV–Vis spectrophotometer, respectively. The CAT activity was calculated according to the following formula:

CAT activity (U/mg prot) = (A90-A30) × V / (0.01 × Cpr)

Where, A90 is the absorbance of treatment solution measured at 90s, A30 is the absorbance of treatment solution measured at 30s, V is the volume of sample (mL), and Cpr is the protein concentration of sample.

Supplementary method 3: Determination of glutathione content

Cotton leaves (0.1 g) from PMO or control treatments under drought stress were homogenized with 1 mL of a 0.6% sulfosalicylic acid–Triton X solution at 4 °C. The resulting mixture was centrifuged at 8,000 g for 10 minutes at 4 °C to collect the supernatant.

1. **Glutathione (GSH) Assay:** A volume of 20 μL of the supernatant was incubated with 120 μL of a mixture containing glutathione reductase (3 units/mL) and 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB, 0.68 mg/mL) for 30 seconds. Subsequently, 60 μL of β-NADPH (0.68 mg/mL) was added. The absorbance of the resulting solution was measured at 412 nm using a spectrophotometer.

2. **Glutathione oxidized (GSSG) Assay:** In a 1.5 mL Eppendorf tube, 100 μL of the supernatant was combined with 2 μL of 2-vinylpyridine to derivatize glutathione. The reaction was allowed to proceed for 1 h at room temperature in a fume hood. The derivatized samples were then analyzed using the methodology described for the GSH assay.

Supplementary method 4: Determination of ascorbic acid content

After 24 hours of drought stress, cotton leaves treated with PMO or TES buffer were dried at 75 °C for 96 h until a constant weight was achieved. One gram of the sample was accurately weighed and ground in an electric grinder with the addition of 2 mL of 4% oxalic acid. The resulting mixture was further ground and filtered through four layers of muslin cloth. The final volume of the extract was adjusted to 25 mL with 4% oxalic acid in a standard flask.

The ascorbic acid content of the samples was determined using the 2,6-dichlorophenol indophenol (DCPIP) titration method as described by published paper.[1] 5 mL of the ascorbic acid working standard (100 µg/mL) and 10 mL of 4% oxalic acid were pipetted into a 100 mL conical flask. The contents of the flask were titrated against the dye solution (V1) until a pale pink color persisted for several minutes. Similarly, 5 mL of the test sample was titrated against the dye solution (V2). The ascorbic acid content in the test samples was calculated using the following formula:

Amount of ascorbic acid (mg/g) = (500 × V2 × 25) / (V1 × 5 × 5)

Where, 500 represents the µg of standard ascorbic acid used for titration; V1 is the volume of dye consumed by 500 µg of standard ascorbic acid; V2 is the volume of dye consumed by 5 mL of the test sample; 25 corresponds to the total volume of the extract; and 5 represents both the weight of the sample taken for extraction and the volume of the test sample taken for titration.

Reference

1 B, Dinesh, B, Yadav, R. D. Reddy, A. S. Padma, M. K. Sukumaran, *Int. J. Curr. Microbiol. App. Sci*, 2015, **4**, 864-868.

Supplementary method 5: Field trials

A 3-year field experiment was conducted from 2021 to 2023 at the Experimental Station in Xiangyang (N31°75′, E111°83′), Hubei, China. This site has a typical subtropical monsoon humid climate, and the annual mean air temperature is 21 °C. The annual total number of sunshine hours is 1562 h, and the frost-free period lasts for 240 days. A completely randomized block design was employed in this study, with two treatment groups that control (H_2O) and PMO group (50 mg/L, foliar application). Each treatment has three replicates, and the plot size is 30 m² (10 m × 3 m) with a planting density of 43,000–45,000 plants per acre. The first foliar application of nanoparticles was performed at the second true leaf stage of cotton, early in the morning around 8 a.m. to avoid high temperatures and strong sunlight. The second application was done at the flowering bud stage, which is a critical period when the plant transitions from vegetative to reproductive growth. Throughout the trial, standard field management practices were followed, including timely irrigation, fertilization, and pest control. Manual weeding ensured weed-free plots, and protective measures were taken as necessary to minimize damage from pests or other environmental factors. All data were collected consistently at the same time to reduce the impact of environmental variability. The final cotton yield was determined at harvest. All plants in each plot were harvested to determine the seed cotton yield and lint cotton yield.