Supplementary Information

Fe_3O_4 nanoenzymes as a novel plant growth promoter for enhancing salt tolerance in cucumber seedlings: integrated analysis of growth physiology and transcriptional metabolism

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Text S1. Detection of POD activity of Fe_3O_4 NPs.

Fe_3O_4 NPs exhibit inherent peroxidase-like enzyme activity under acidic pH conditions, and this enzymatic activity constitutes the fundamental mechanism driving the oxidation of substrates (e.g., TMB). During this process, Fe_3O_4 NPs can catalyze the decomposition of H_2O_2, leading to the oxidation of TMB from a colorless state to a blue product. This oxidized form exhibits strong ultraviolet absorption at 650 nm.

Buffer solution preparation: Mix 0.3 mL of glacial acetic acid and 525 mL of deionized water by vortex, then add 750 μL of 1 M NaOH. Finally, obtain 10 μM of buffer solution with pH=4.

There are a total of 6 treatments as follows:

1. Buffer solution (3 mL) + TMB (100 μL) + H_2O_2 (100 μL) + Fe_3O_4 NPs (100 μL);
2. Buffer solution (3 mL) + TMB (100 μL) + H_2O_2 (100 μL) + deionized water (100 μL);
3. Buffer solution (3 mL) + TMB (100 μL) + Fe_3O_4 NPs (100 μL) + deionized water (100 μL);
4. Buffer solution (3 mL) + H_2O_2 (100 μL) + Fe_3O_4 NPs (100 μL) + deionized water (100 μL);
5. Buffer solution (3 mL) + TMB (100 μL) + deionized water (100 μL);
6. Buffer solution (3 mL) + Fe_3O_4 NPs (100 μL) + deionized water (100 μL).

Prepare six treatment groups using 5-mL centrifuge tubes. Observe the color change in Treatment 1, and after 15 minutes, transfer a droplet of the mixed solution onto a UV-Vis spectrophotometer to measure the absorbance change.

Text S2. Transcriptome analysis method

RNA was extracted by ethanol precipitation method, and total RNA was identified and quantified using Qubit fluorescence quantifier and Qsep400 high-throughput biological fragment analyzer. mRNA with polyA tails was enriched through Oligo (dT) magnetic beads. fragmentation buffer was added to use the short fragments interrupted by RNA as templates. The first strand of cDNA was synthesized using six-base random primers. The second strand of cDNA was synthesized by adding buffer solution, dNTPs and DNA polymerase. After purifying the double-stranded cDNA using DNA purification magnetic beads, the end was repaired and the sequencing connector was connected. The inserted fragments of 250-350bp were selected and PCR enrichment was performed to obtain the final cDNA library. After the library construction was completed, the quality of the library was detected. The Qubit dye method was used for preliminary quantification, and the fragment size was detected using a fragment analyzer. After the library inspection was qualified, different libraries were pooled according to the requirements of effective concentration and target offline data volume for Illumina sequencing, and 150bp paired end readings were generated. Quality control was carried out on the sequencing data to remove low-quality sequences and connector sequences. After alalignment the high-quality sequences to the reference genome and obtaining the differentially expressed genes, GO functional significance and KEGG significance analyses were conducted.

Text S3. Metabolome analysis method

The biological samples were placed in a freeze dryer (Scientz-100F) for vacuum freeze-

drying for 63 hours and ground into powder using a grinder (MM400, Retsch). Weigh 50 mg of the sample and add 1.2 mL of 70% methanol water pre-cooled at -20 ℃ as the internal standard extract. After centrifugation, the supernatant was aspirated. The sample was filtered through a 0.22 μm filter membrane and stored in an injection bottle for liquid chromatography-mass spectrometry (UPLC-MS) analysis. Based on OPLS-DA (Orthogonal Partial least squares discriminant Analysis), differential metabolites are selected according to the multiple relationship of metabolite content of samples with different treatments being greater than 1. Subsequently, a significance analysis of KEGG pathways was performed on the identified differential metabolites.

Supplemental Figures

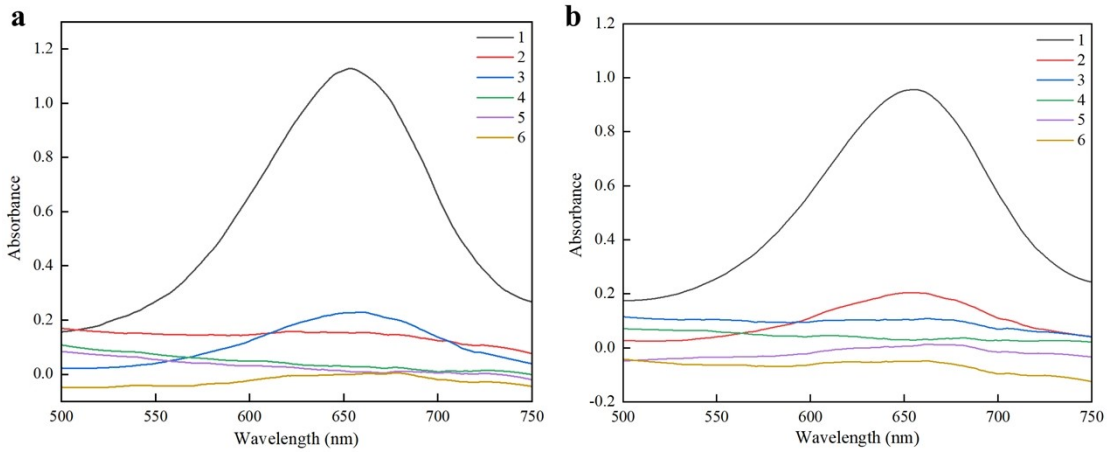

Fig. S1 Changes of POD activity absorbance in FNPs and FNPs-G.

Note: 1, 2, 3, 4, 5, and 6 in the figure represent different treatment groups respectively (The detection method of POD activity of Fe_3O_4 NPs in Text S1)

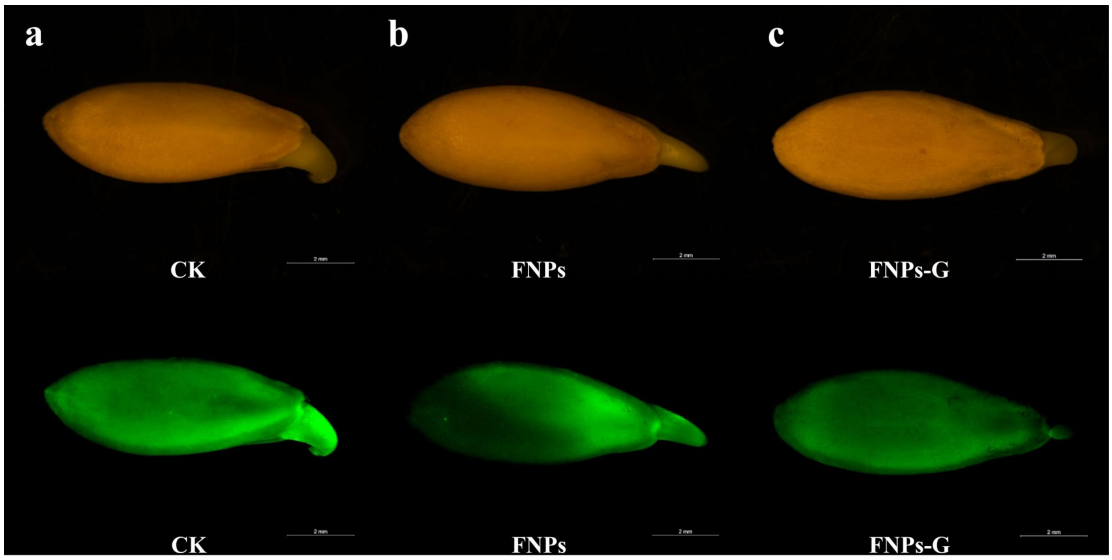

Fig. S2 Visualization of reactive oxygen species in cucumber seeds. (a-c) They respectively represent the images of bright field and dark field imaging under a fluorescence microscope after seed soaking treatments of CK, FNPs and FNPS-G.

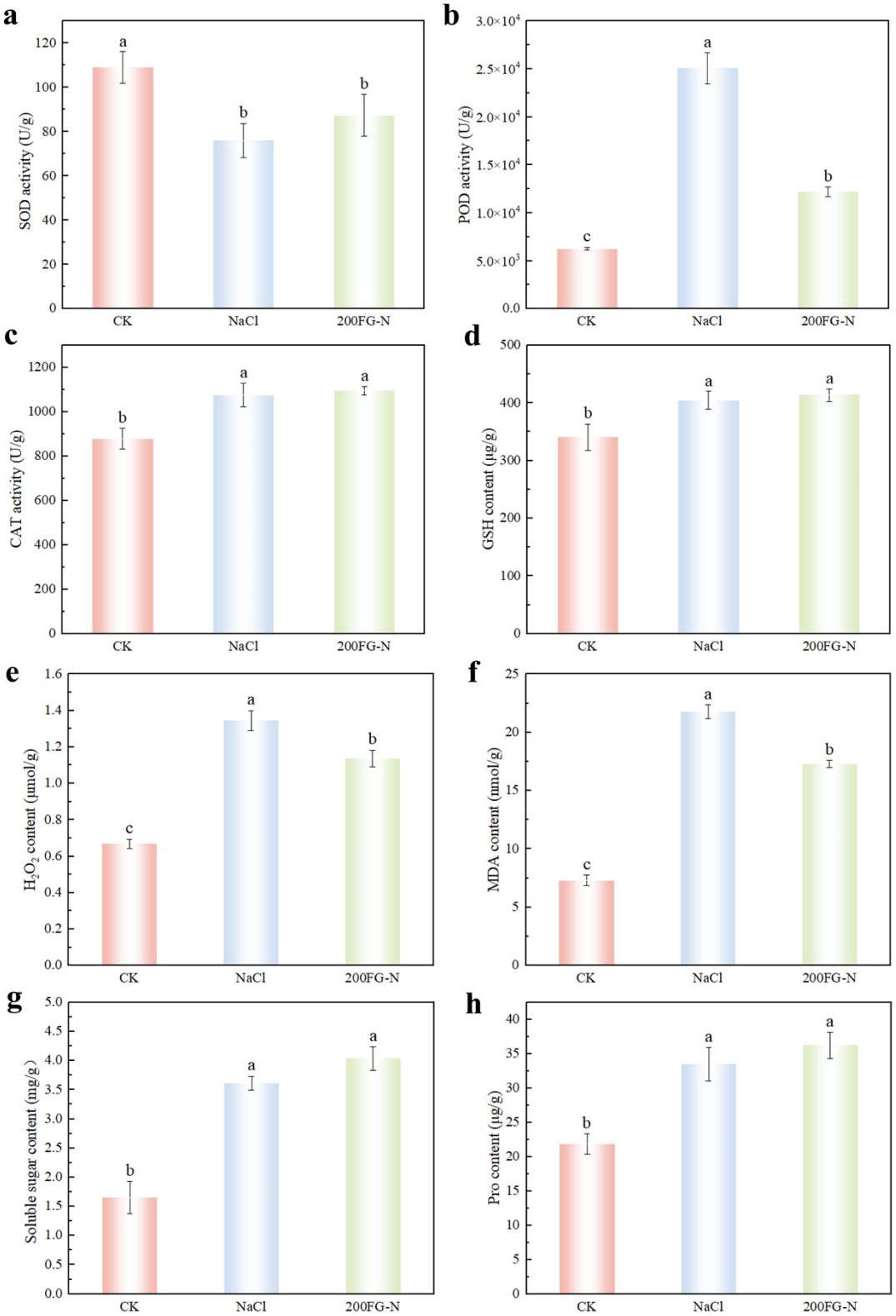

Fig. S3 The effects of 200 mg L^{-1} FNPs-G seed soaking treatment on physiological parameters of cucumber seedlings under salt stress, including (a) SOD activity, (b) POD activity, (c) CAT activity, (d) GSH content, (e) H_2O_2 content, (f) MDA content, (g) soluble sugar content and (h) Pro content. The images and charts shown represent at least three independent biological experiments. Statistical comparisons were conducted using univariate ANOVA. The letters denote statistical significance with respect to untreated seeds ($P \leqslant 0.05$).

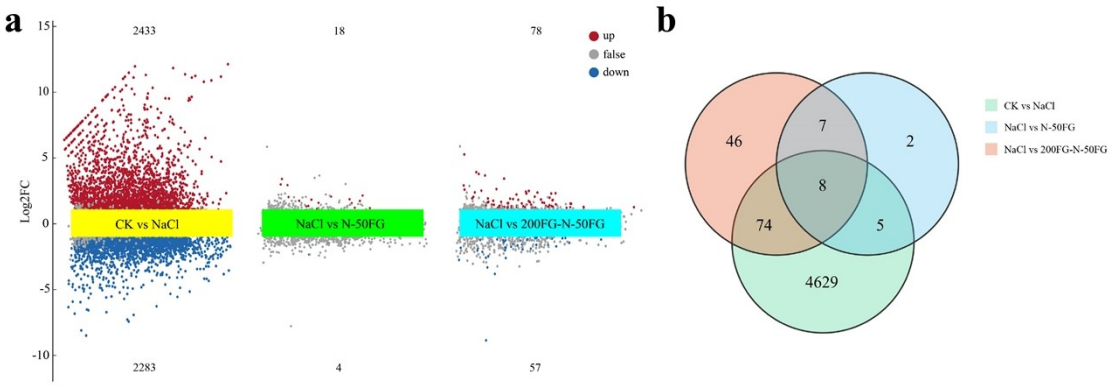

Fig. S4 (a) Quantity of genes exhibiting upregulation and downregulation in the CK vs NaCl, NaCl vs N-50FG and NaCl vs 200FG-N-50FG groups. (b) Venn diagram.

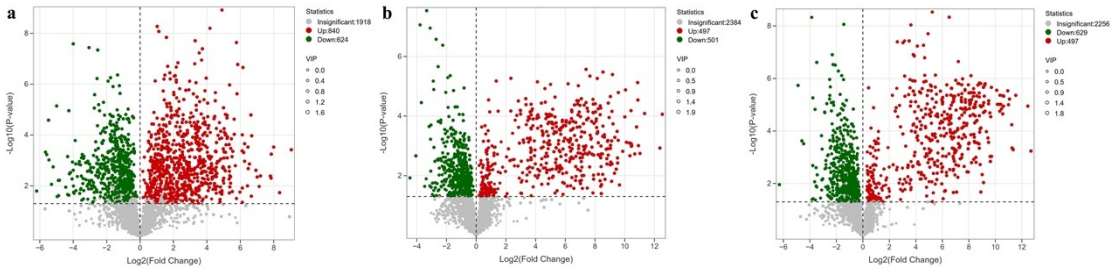

Fig. S5 Volcano plots of differential metabolites of (a) CK vs NaCl, (b) NaCl vs N-50FG, and (c) NaCl vs 200FG-N-50FG.

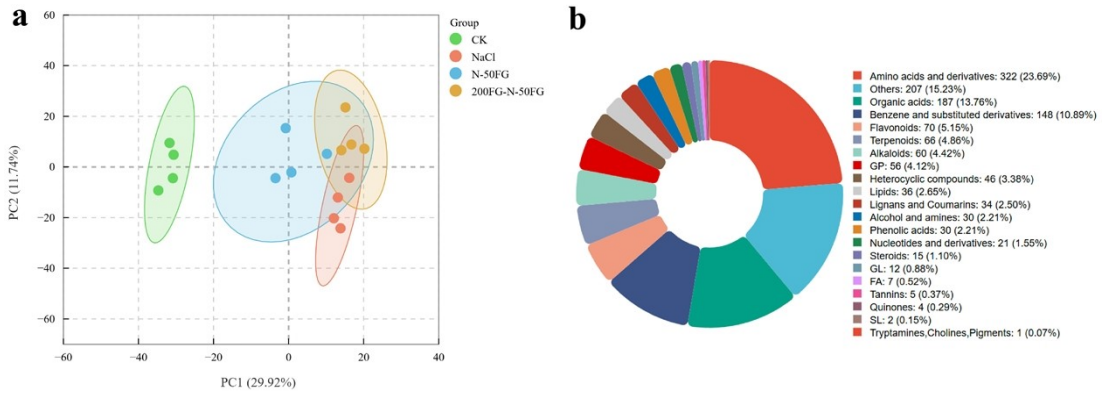

Fig. S6 (a) PCA scatter plots comparing different treatments. (b) Circular diagram illustrating metabolite classes.

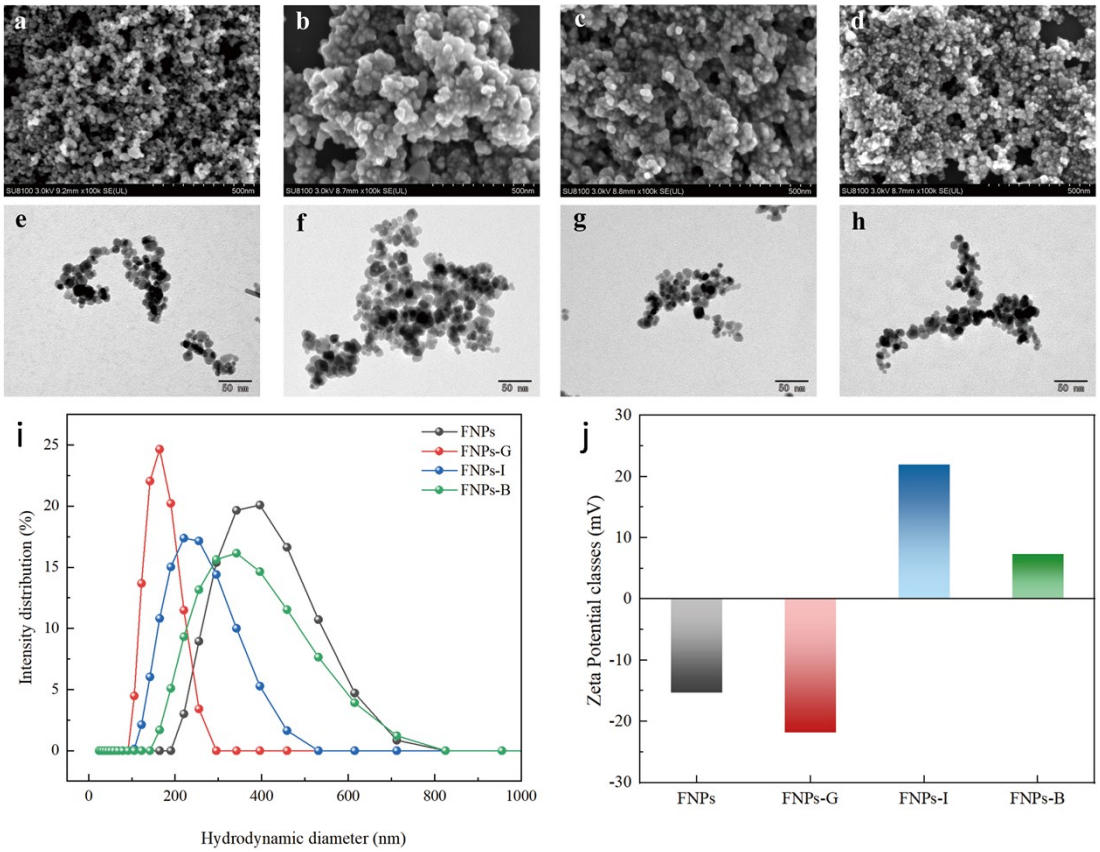

Fig. S7 SEM (a-d), TEM (e-h), Hydrodynamic diameter (i) and Zeta potential (j) characterization of Fe_3O_4 NPs

Note: Fe_3O_4 NPs includes FNPs, FNPs-G, FNPs-I, and FNPs-B, the same below.

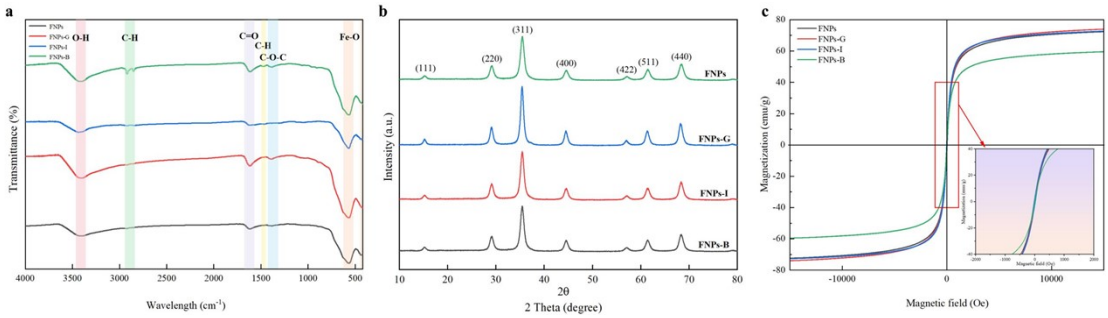

Fig. S8 FTIR (a), XRD (b) and magnetization curve (c) characterization of Fe_3O_4 NPs

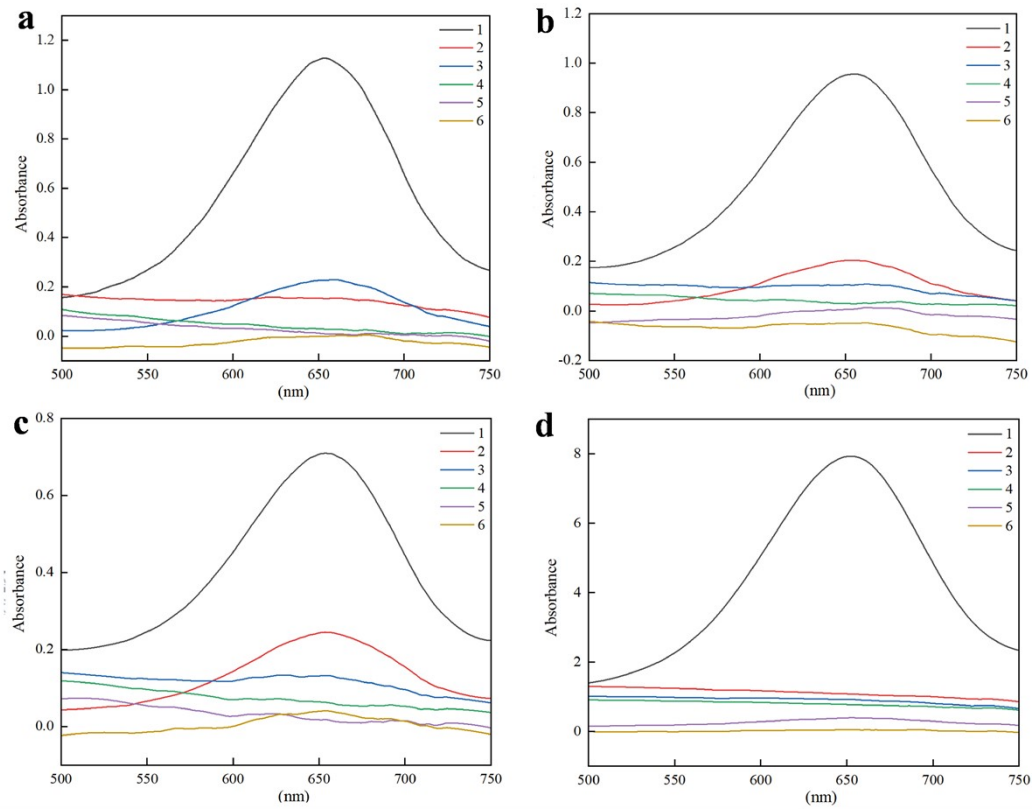

Fig. S9 Changes of POD activity absorbance in Fe_3O_4 NPs

Note: 1, 2, 3, 4, 5, and 6 in the figure represent different treatment groups respectively (POD activity detection of Fe3O4 nanoenzymes in the test method). a, b, c, and d stand for FNPs, FNPs-G, FNPs-I, and FNPs-B respectively.

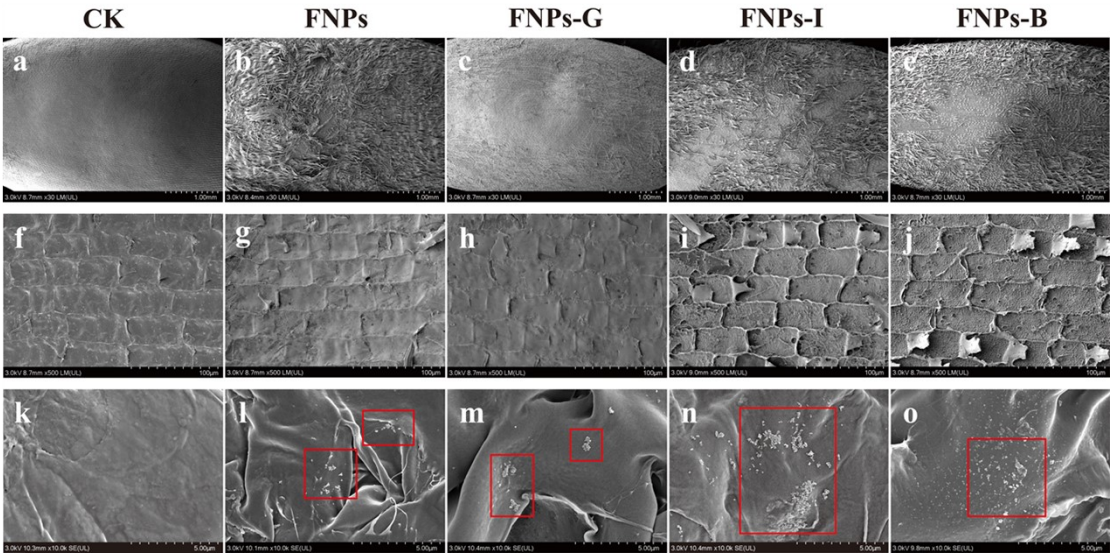

Fig. S10 SEM images of Fe_3O_4 NPs inside the epidermis and seed coat of cucumber seeds

Note: a-e represents the effect of different treatments on cucumber seed skin, the scale is 1 mm; f-j represents the effect of different treatments on the cell wall of cucumber seeds with a scale of 100 μm. k-o represents the presence of Fe_3O_4 NPs observed in the interior of cucumber seed coat on a scale of 5 μm.

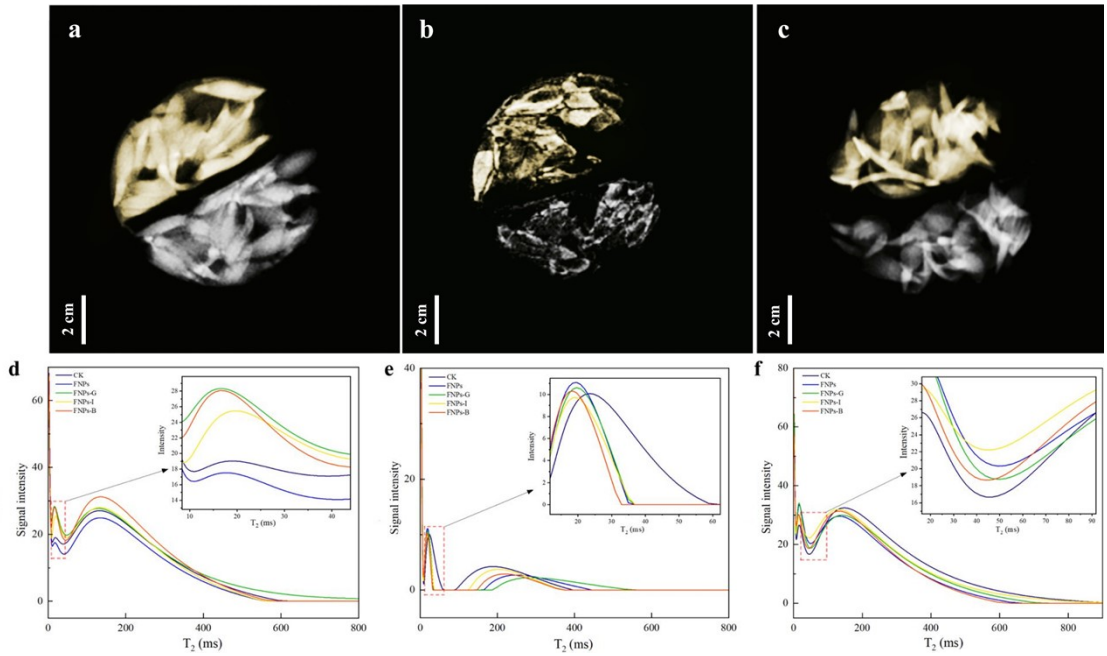

Fig. S11 Seed (a), seed coat (b), and endosperm (c) represent 2D central axial sections; the signal intensity of seed (d), seed coat (e) and endosperm (f) after soaking with 100 mg/L Fe_3O_4 NPs as a function of T2 relaxation time (ms)

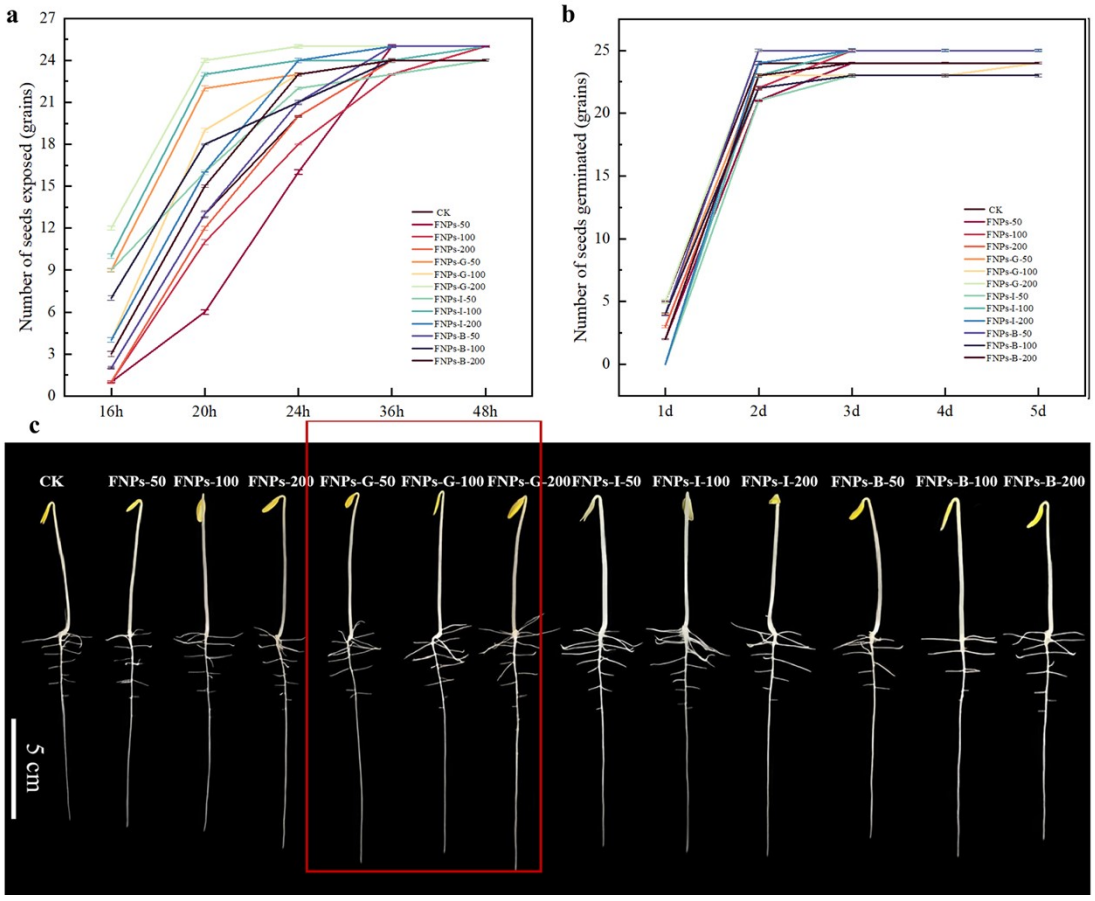

Fig. S12 Trends in the number of cucumber seed dews and germination over time (a,b); Images of

cucumber seedlings on day 5 after different Fe_3O_4 NPs and different concentrations of seed immersion treatments (c)

Note: a stand for the variation trend of cucumber seed whitening quantity during 16 h-48 h after soaking with different concentrations of FNPs, FNPs-G, FNPs-I and FNPs-B; b represents the trend chart of germination number of cucumber seeds after soaking with different concentrations of FNPs, FNPs-G, FNPs-I and FNPs-B for 1 d to 5 days.

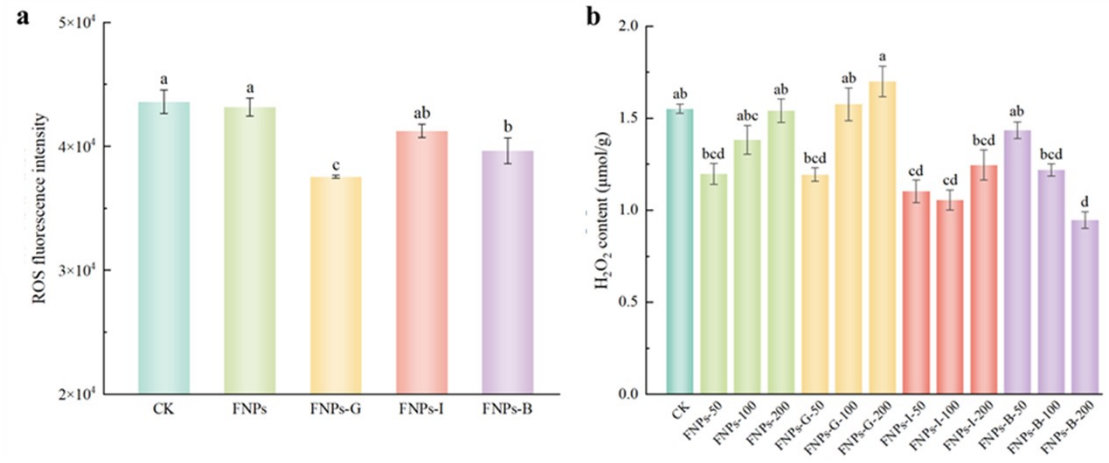

Fig. S13 ROS fluorescence intensity (a) and Effects of different concentrations on H2O2 content in seedlings soaked with Fe3O4 NPs (b)

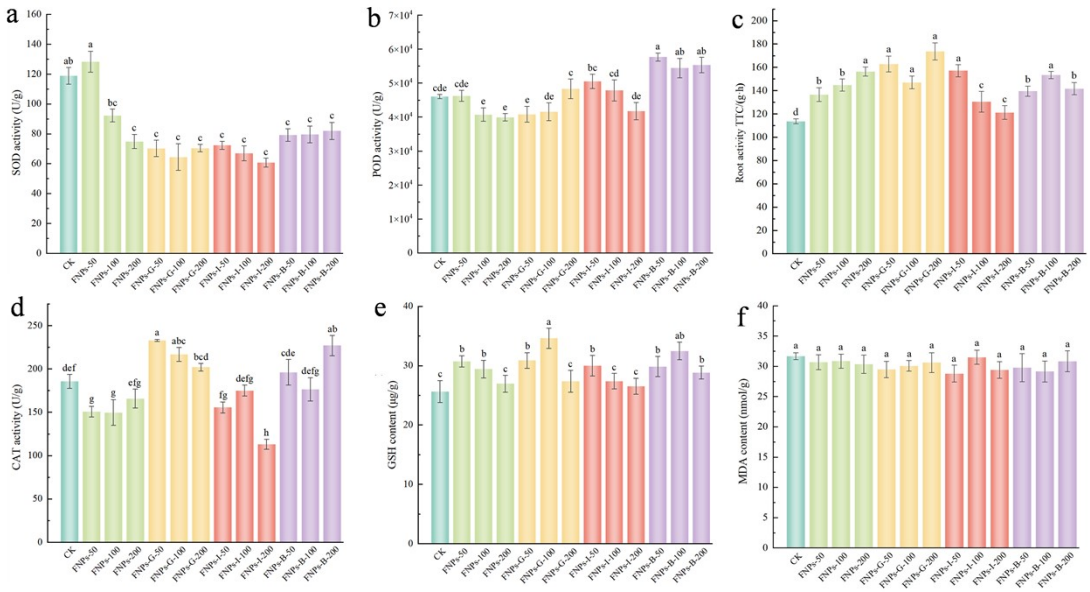

Fig. S14 Effects of different concentrations of Fe_3O_4 NPs on antioxidant system of seedlings (a,b,d,e); Effects of different concentrations of Fe_3O_4 NPs on root activity of cucumber (c); Effect of seed dipping with different concentrations of Fe3O4 NPs on MDA content of cucumber seedlings(f)

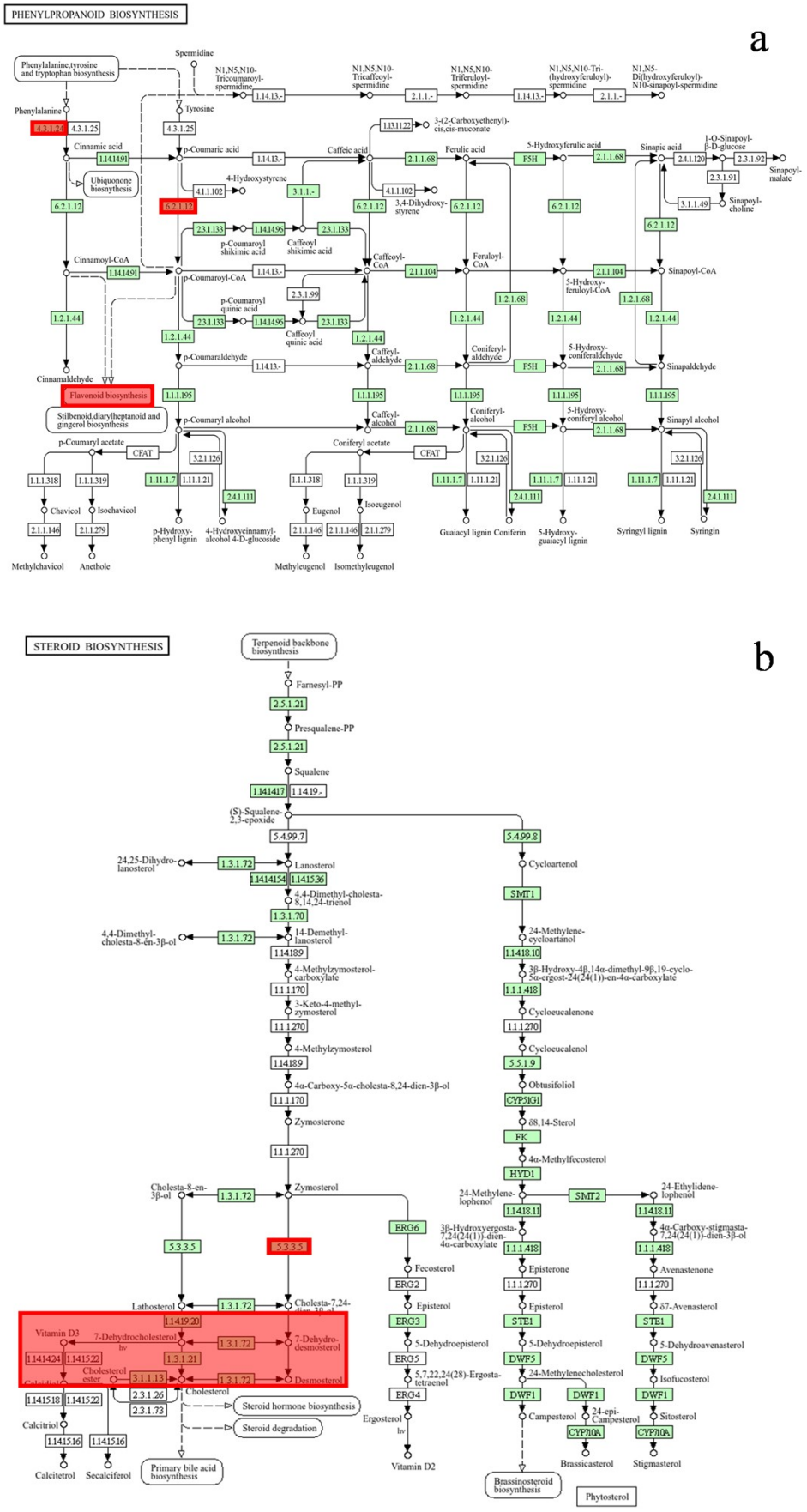

Fig. S15 KEGG pathway map of phenylpropanoid biosynthesis (a) and steroid biosynthesis (b).

Note: (a) Phenylpropanoid biosynthesis pathway (ko00940). Red circles indicate upregulated enzymes under FNPs-G treatment: PAL (phenylalanine ammonia-lyase, EC 4.3.1.24), 4CL (4-coumarate-CoA ligase, EC 6.2.1.12), and CHS (chalcone synthase, EC 2.3.1.74, corresponding to module M00137). (b) Steroid biosynthesis pathway (ko00100). The red shaded area indicates the downstream region containing key upregulated enzymes, including SMT (sterol 24-C-methyltransferase, EC 2.1.1.41) and EBP (sterol 8,7-isomerase, EC 5.3.3.5). Original KEGG maps are available at https://www.kegg.jp/pathway/ko00940 and https://www.kegg.jp/pathway/ko00100.

Supplemental Tables

Table S1. Sample sequencing data quality summary

Sample	Raw Reads	Clean Reads	Q20(%)	Q30(%)	Reads mapped
CK-1	60896734	55756586	97.53	93.7	53816279(96.52%)
CK-2	47958288	43499668	97.52	93.6	41946257(96.43%)
CK-3	48985092	44539948	97.52	93.57	42967803(96.47%)
CK-4	48696464	44077092	97.49	93.51	42519882(96.47%)
NaCl-1	50769922	45240444	97.42	93.39	43567797(96.30%)
NaCl-2	69965202	63576750	97.47	93.49	61316872(96.45%)
NaCl-3	59374136	54247578	97.45	93.48	52277533(96.37%)
NaCl-4	60959198	55290400	97.49	93.61	53274062(96.35%)
N-50FG-1	54305878	49724410	97.43	93.48	47841090(96.21%)
N-50FG-2	49433464	43573324	97.47	93.52	42026717(96.45%)
N-50FG-3	48643870	43905088	97.54	93.61	42402428(96.58%)
N-50FG-4	51597498	47707824	97.61	93.75	46187739(96.81%)
200FG-N-50FG-1	52889218	47009474	97.5	93.58	45296256(96.36%)
200FG-N-50FG-2	63365828	58102972	97.45	93.48	55981641(96.35%)
200FG-N-50FG-3	58987724	52635918	97.49	93.55	50754187(96.43%)
200FG-N-50FG-4	55653772	49884456	97.52	93.56	48107060(96.44%)

Table S2. Effects of different concentrations of NaCl on the growth of cucumber seedlings

NaCl (mmol/L)	Fresh weight (g)	Dry weight (g)	Plant height (cm)	Root length (cm)	Chlorophyll content (SPAD)	Nitrogen content (mg/kg)
0	7.532	0.241	2.92	27.64	41.29	15.71
50	8.843	0.317	2.9	30.79	44.52	16.74
100	6.578	0.24	3.56	28.34	44.06	16.58
150	4.687	0.182	3.7	28.81	33.44	13.22
200	2.897	0.115	2.74	23.06	31.37	12.58

Table S3. Correlation analysis of physiological parameters in cucumber seedlings under FNPs-G treatment

Parameter	CK	NaCl	N-50FG	200FG-N	200FG-N-50FG	Correlation coefficient with H_2O_2	Correlation coefficient with MDA	Correlation coefficient with T-AOC
SOD (U/g)	109.09	76.02	42.15	87.35	67.08	r = 0.13	r = 0.04	r = -0.10
POD (U/g)	6236.07	25078.2	34613.6	12207.53	16101.4	r = -0.93*	r = -0.93*	r = -0.43
CAT (U/g)	878.58	1075.79	1263.35	1095.85	1152.47	r = -0.86	r = -0.89*	r = -0.66
GSH	340.58	404.49	442.17	413.77	425.51	r = -0.61	r = -0.67	r = -0.71
H_2O_2	0.67	1.34	1.01	1.14	1.07	1	r = 0.98**	r = 0.36
MDA	7.29	21.75	14.4	17.29	14.78	r = 0.98**	1	r = 0.26
Proline	21.86	33.46	51.74	36.24	48.91	r = -0.60	r = -0.60	r = -0.62
Soluble sugar	1.65	3.61	5.16	4.04	4.51	r = -0.55	r = -0.58	r = -0.73
T-AOC	2.28	2.55	2.22	1.82	2.3	r = 0.36	r = 0.26	1

Note: Data are presented as mean ± SD (n=3). Different lowercase letters indicate significant differences among treatments at $p < 0.05$. Correlation coefficients (r) were calculated based on treatment means (n=5). *$p < 0.05$, **$p < 0.01$ (two-tailed test). Unmarked correlations are not significant ($p \geq 0.05$).