Supplementary material

Synergistic effects of exogenous melatonin and zinc oxide nanoparticles alleviating cobalt stress in *Brassica napus*: insights from stress-related markers and antioxidant machinery

Skhawat Ali[a], Basharat Ali[b], Imtiaz Ahmad Sajid[c], Shoaib Ahmad[d], Muhammad Arslan Yousaf[a], Zaid Ulhassan[a], Kangni Zhang[a], Shafaqat Ali[e,f], Weijun Zhou[a*], Bizeng Mao[c*]

[a] Institute of Crop Science, Ministry of Agriculture and Rural Affairs Key Laboratory of Spectroscopy Sensing, Zhejiang University, Hangzhou 310058, China.

[b] Department of Agricultural Engineering, Khwaja Fareed University of Engineering and Information Technology, Rahim Yar Khan 64200. Pakistan.

[c] Institute of Biotechnology, Ministry of Agriculture Key Lab of Molecular Biology of Crop Pathogens and Insects, Key Lab of Biology of Crop Pathogens and Insects of Zhejiang Province, Zhejiang University, Hangzhou 310058, China.

[d] State Key Laboratory of Pollution Control and Resource Reuse, School of the Environment, Nanjing University, Nanjing, Jiangsu 210023, China.

[e] Department of Environmental Sciences, Government College University, 38000, Faisalabad, Pakistan.

[f] Department of Biological Sciences and Technology, China Medical University, Taichung 40402, Taiwan.

[*]Corresponding author email: **wjzhou@zju.edu.cn** (WZ), **maobz@zju.edu.cn** (MB)

Text S1: Morphological attributes and root architecture

The healthy plants were collected following 15 days of treatment. Before harvesting, morphological traits were measured using scales and weighed and balanced. The plant shoot and roots samples were obtained and washed with 2% hydrochloric acid, afterwards and distilled water to remove the aerial deposition after the plant was harvested for its morphological characteristics. To evaluate the effect of Co stress on root morphological characteristics, root samples were manually separated using scissor, facilitating visual inspection of changes induced by Co exposure. Subsequent quantitative analysis of root architecture parameters, including, number of root tips, average diameter of roots and surface area of roots was performed via an automated root scanner apparatus (Model: MIN MAC, STD1600+) coupled with WinRHIZO software (Regent Instruments Co., Canada).[1] This integrated instrumental approach enabled precise measurements of the morphological alterations manifest in the root systems following Co treatment. The harvested plant was stored at 70°C for 48 hours to ascertain the dry weight samples.

Text S2: Quantifications of lipid peroxidations, reactive oxygen species (ROS) and histochemical analysis.

A mortar and pestle were used to crush 0.5 g of plant leaves and root samples with trichloroacetic acid (w/v); the sample solution was then centrifuged. 0.5 ml of plant samples extract enzyme was mixed with 0.5% TBA (w/v) in 20% trichloroacetic acid and incubated in a water bath at 95°C. Reaction was stopped by putting in a chilled ice bath. The supernatant was collected, and absorbance measured at 532 nm and 600 nm respectively, using UV-vis spectrophotometer. MDA content was calculated using the extinction coefficient of MDA. The method by,[2] was adopted for measuring MDA content. The method described by,[3] was adapted to measure H_2O_2 content. The plant leaf sample (0.5 g) was poured in mortar and crushed with pestle gently by adding 5 mL of 0.1% trichloroacetic acid (w/v) solution. After centrifugation at 12000 rpm for 20 minutes, the supernatant was collected. In a test tube, 0.5 mL of the supernatant was mixed with 0.5 mL of 50 mM phosphate buffer solution pH 7.0 and 1 mL of 1 M potassium iodide (KI) solution. The absorbance was measured at 390 nm, and the H_2O_2 content was estimated using a standard curve. Measurements for histochemical analysis were performed according to the methodology described by [4]. Leaves stained with nitroblue tetrazolium (NBT) and 3,3'-diaminobenzidine (DAB) were digitally imaged using a Canon DS126201 camera (Canon, Japan).

Text S3: Determination of Metal content and endogenous MT

Leaves and roots of each dry plant samples have been added in glassed flask after cut into small pieces by grinder. Plant dry samples digestion process have been conducted through di acid methods.[5] Atomic absorption spectrophotometer has been operated to determination the correct quantity of Co and Zn in leaves and shoots of the samples. MT content was quantified by enzyme-linked immunosorbent assay (ELISA), using the MT ELISA Kit (Product #MM-084802; MLBIO Tech.) according to the manufacturer's protocols. 0.1 g plant tissue was homogenized in phosphate-buffered saline (PBS) solution. The plant tissue was frozen and thawed 2 times. Then the tube was centrifuged at 10,000 × g for 5 min at cold temperature of 4°C. The liquid on top was taken out. The amount of light absorbed by the liquid was measured by spectrophotometer machine at the 450 nm wave length.[6]

Text S4: Topographical imaging of plant by using SEM and TEM

Text S4. 1: Scanning electron microscopy (SEM)

To prepared samples for scanning electron microscopy analysis, plant leaves have been cut into small pieces except the midrib part which have been then pre-treated with glutaraldehyde in 100 mM phosphate buffer having pH 7 for 1 day. In next step samples have been cleaned 3 times for 15 minutes by using phosphate buffer and then fixed in 1% osmium tetroxide again for 2 hours. samples have been cleaned again by using phosphate buffer for 15 minutes and then dehydrate with arranged series 30, 50, 70, 80, 90 and 95% ethanol for 15 minutes, absolute ethanol has been used for more dehydration of samples two times more for 20 minutes.[7] In last step dehydrated sample have been treated for 5 minutes against the gold palladium through ion sputter and carbon double sided adhesive tabs have been used to mount the samples on aluminum stub to carry out SEM analysis.

Text S4. 2: Transmission electron microscopy (TEM)

Leaf tissues excluding midrib of all samples have been preserved for 1 day in 2.5 % glutaraldehyde in phosphate buffer with 100 mM and pH 7 for TEM analysis. After 1-day samples have been cleaned by using phosphate buffer three times for 15 min. After cleaned the samples have been subsequently fixed for 2 h in 1% OsO_4 and then cleaned again using same buffer for 15 min. Afterward a series of ethanol with 30, 50, 70, 80,90, 95 and 100% for 15 min and absolute acetone for 20 min and two times are used for dehydration purpose. The final specimens were mounted on copper grids for transmission electron microscopy analysis, according the method detailed by [8].

Table 1S Treatments are shown in the completely randomized design.

Treatment No.	Description
T0	Control (No Cobalt + Melatonin + ZnO NPS applied)
T1	MT 50 µM (No Co applied)
T2	ZnO NPs 25 µM (No Co applied)
T3	MT 50 µM + ZnO NPs 25 µM (No Co applied)
T4	300 µM Co (alone)
T5	ZnO NPs 25 µM + Co 300 µM
T6	MT 50 µM + ZnO NPs 25 µM + Co 300 µM

Table S2. Primer sequences and data used for RT-PCR analysis.[9,10,11]

Gene ID	Gene Name	Forward	Reverse
SOD	Super oxide dismutase(SOD)	ACGGTGTGACCACTGTGACT	GCACCGTGTTGTTTACCATC
CAT	Catalase	TCGCCATGCTGAGAAGTATC	TCTCCAGGCTCCTTGAAGTT
APX	Ascorbate peroxidases	ATGAGGTTTGACGGTGAGC	CAGCATGGGAGATGGTAGG
PAL	phenylalanine ammonia-lyase	GGGTTGTCGTTGACGGAGTT	CATTATGGAGCACATCTTGG
PPO	Polyphenol Oxidase	GAATCTTGGGCTCTTTA	TTCCATTACGGTGACTT
CAD	Cinnamyl Alcohol Dehydrogenase	ATGATGTCTACACCGACGGA	ACGTGTGGAGCAAGAAACAC
PsbA	Photosystem II protein D1	GTATTTATTATCGCCTTCATCG	AGGACGCATACCCAAACG
PsbB	Photosystem II CP47 protein	TAGGCGTAACGGTGGA	AACATCTCGGAACAAGG
PsbC	Photosystem II CP43 protein	TAATACGGCTTATCCGAGTGAGTTT	TAATACGGCTTATCCGAGTGAGTTT
PsbD	Photosystem II D2 protein	GCCCTTGGTAGAGTTCCTAAAG	CGAAATAAGCGCAAGGAAAGAG
PsbO	oxygen-evolving enhancer protein 1	AGAGAGGCTCGGTGAAATAGA	CCAATCCCAGGGAACAGTAAAG

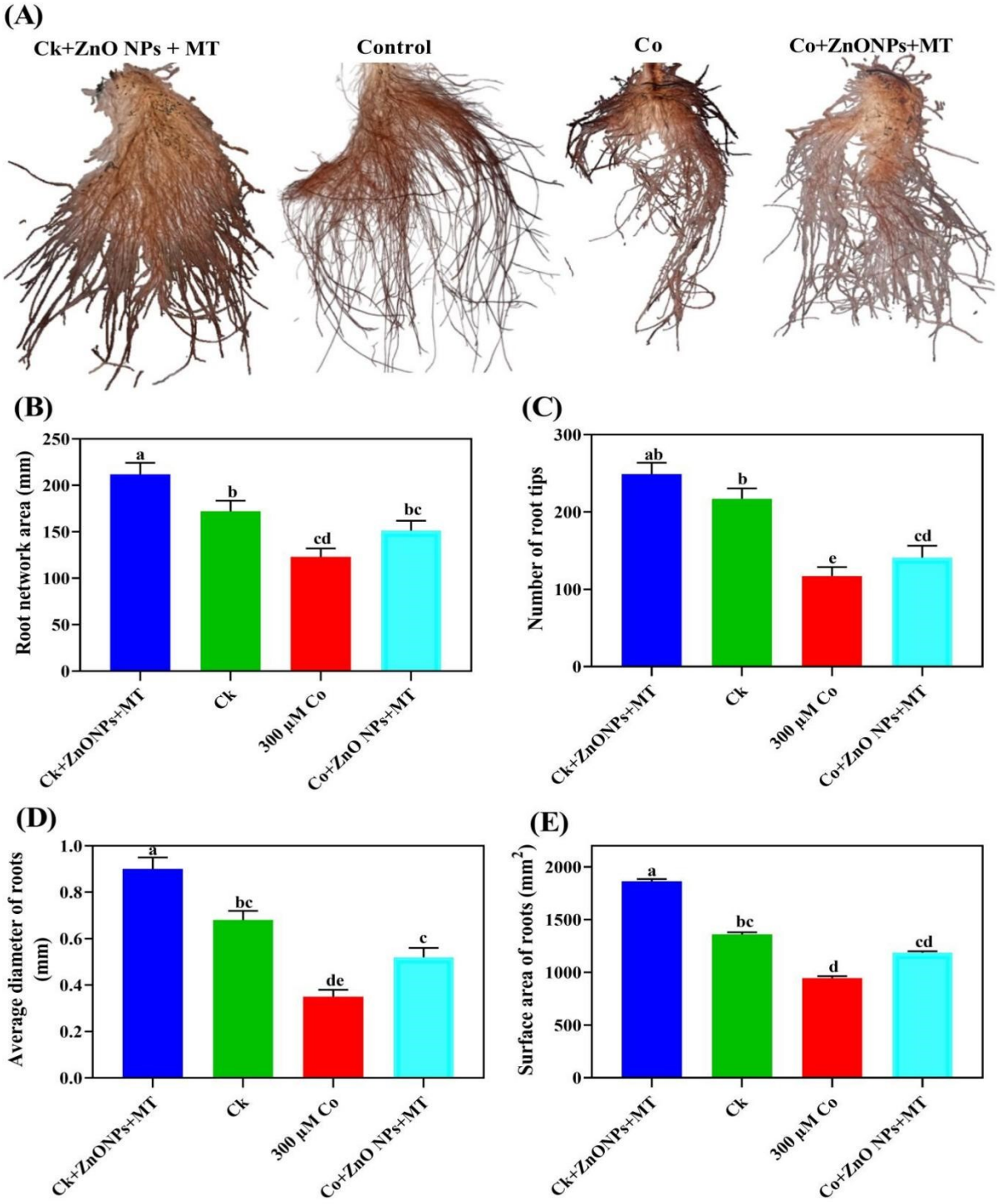

Fig. 1S Combined synergistic effects of exogenous melatonin (MT) and zinc oxide nanoparticles (ZnO NPs) in alleviating cobalt (Co) stress on root architecture. Effects of different treatments of exogenous MT (50 µM), ZnO NPs (25 µM) and Co (0, 300 µM) on **(A)** root appearance, **(B)** root network area, **(C)** number of root tips, **(D)** average diameter of roots, **(E)** surface area of roots in *Brassica napus* (ZS 758).

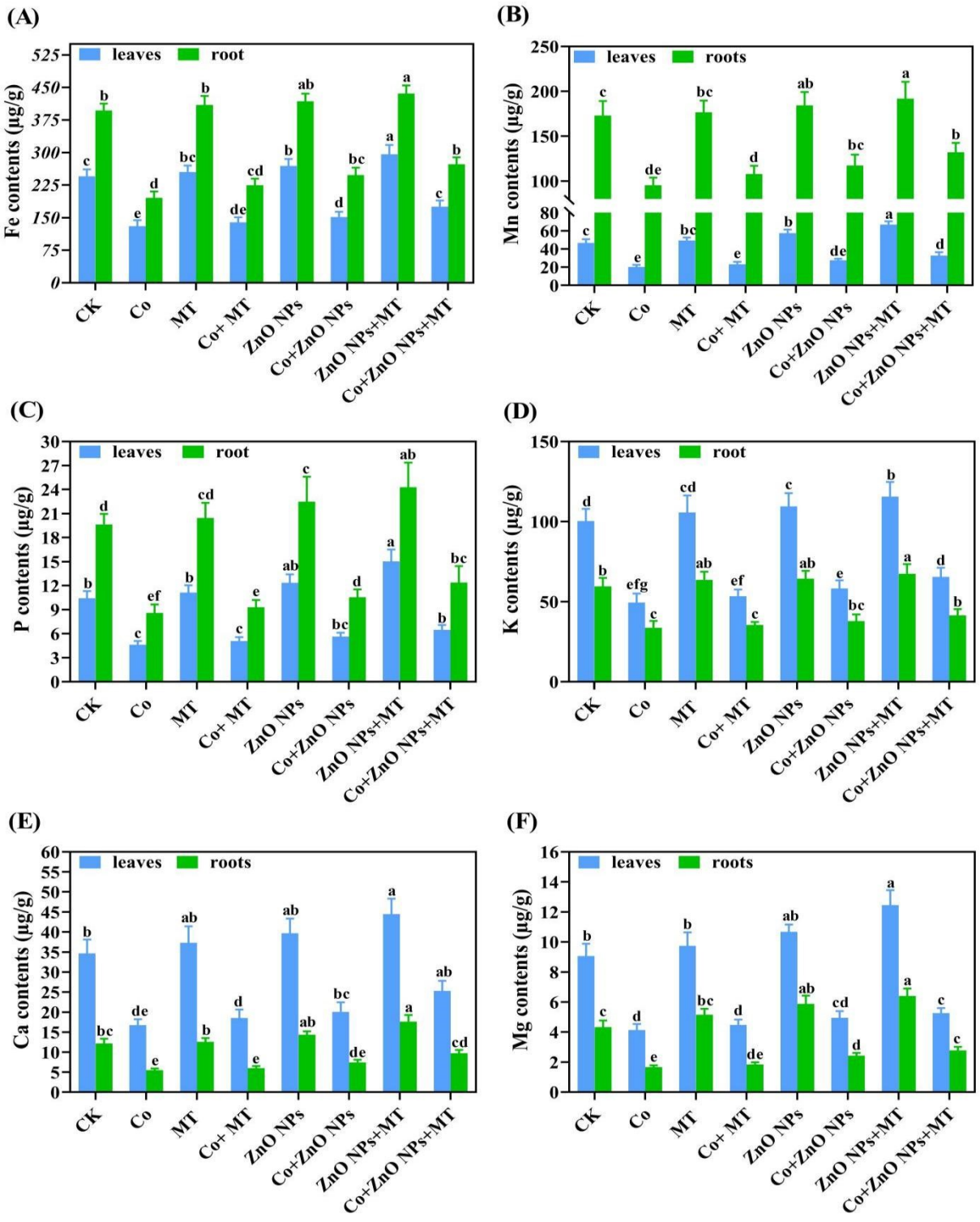

Fig. 2S Combined synergistic effects of exogenous melatonin (MT) and zinc oxide nanoparticles (ZnO NPs) in alleviating cobalt (Co) stress on elemental analysis. Effects of different treatments of exogenous MT (50 µM), ZnO NPs (25 µM) and Co (0, 300 µM) on the **(A)** Fe contents (µg/g), **(B)** Mn contents (µg/g), **(C)** P contents (µg/g), **(D)** K contents (µg/g), **(E)** Ca contents (µg/g), **(F)** Mg contents (µg/g) in the leaves and roots of *Brassica napus* (ZS 758).

References

1 Jin, X.F., Liu, D., Islam, E., et. al., 2009. Efects of zinc on root morphology and antioxidant adaptations of cadmium-treated *sedum alfredii* H. Journal of Plant Nutrition. 32:1642–1656. https://doi.org/10.1080/01904160903150909.

2 Hong, T.B., Rahumatullah, A., Yogarajah, T., Ahmad, M., & Yin, K.B., 2010. Potential effects of chrysin on MDA-MB-231 cells. International journal of molecular sciences. 11(3), 1057–1069. https://doi.org/10.3390/ijms11031057.

3 Chen, L., Xu, B., Liu, L., Luo, Y., Yin, J., Zhou, H., Chen, W., Shen, T., Han, X., Huang, S., 2010. Hydrogen peroxide inhibits mTOR signaling by activation of AMPK α leading to apoptosis of neuronal cells. Lab Invest. 90 (5), 762–773. https://doi.org/10.1038/labinvest.2010.36.

4 Romero-Puertas, M.C., Rodriguez-Serrano, M., Corpas, F.J., Gomez, M., del Rio, L.A., Sandalio, L.M., 2004. Cadmium-induced subcellular accumulation of $O_2^{\bullet-}$ and H_2O_2 in pea leaves. Plant, Cell & Environment. 27, 1122–1134. https://doi.org/10.1111/j.1365-3040.2004.01217.x.

5 Rehman, M.Z., Rizwan, M., Ghafoor, A., Naeem, A., Ali, S., Sabir, M., Qayyum, M.F., 2015. Effect of inorganic amendments for in situ stabilization of cadmium in contaminated soils and its phyto-availability to wheat and rice under rotation. Environmental Science and Pollution Research. 22, 16897–16906. https://doi.org/10.1007/s11356-015-4883-y.

6 Farooq, M.A., Islam, F., Ayyaz, A., Chen, W., Noor, Y., Hu, W., ... & Zhou, W., 2022. Mitigation effects of exogenous melatonin-selenium nanoparticles on arsenic-induced stress in *Brassica napus*. Environmental Pollution. 292, 118473. https://doi.org/10.1016/j.envpol.2021.118473.

7 Ali, S., Gill, R.A., Ulhassan, Z., Zhang, N., Hussain, S., Zhang, K., ... & Zhou, W. (2023). Exogenously applied melatonin enhanced the tolerance of *Brassica napus* against cobalt toxicity by modulating antioxidant defense, osmotic adjustment, and expression of stress response genes. Ecotoxicology and Environmental Safety. 252, 114624. https://doi.org/10.1016/j.ecoenv.2023.114624.

8 Azhar, W., Khan, A.R., Muhammad, N., Liu, B., Song, G., Hussain, A., Yasin, M.U., Khan, S., Munir, R., Gan, Y., 2020. Ethylene mediates CuO NP-induced ultrastructural changes and

oxidative stress in *Arabidopsis thaliana* leaves. Environmental Science Nano. 7(3), 938–953.https://doi.org/10.1039/C9EN01302D.

9 Mehar, F., Iqbal, N., Sehar, Z., Alyemeni, M.N., Kaushik, P., Khan, N.A., & Ahmad, P., 2021. Methyl jasmonate protects the PS II system by maintaining the stability of chloroplast D1 protein and accelerating enzymatic antioxidants in heat-stressed wheat plants. Antioxidants. 10(8), 1216. https://doi.org/10.3390/antiox10081216.

10 Alyammahi, O., & Gururani, M.A., 2020. Chlorophyll-a fluorescence analysis reveals differential response of photosynthetic machinery in melatonin-treated oat plants exposed to osmotic stress. Agronomy. 10(10), 1520. https://doi.org/10.3390/agronomy10101520.

11 Ali, S., Gill, R.A., Ulhassan, Z., Najeeb, U., Kanwar, M.K., Abid, M., ... & Zhou, W., 2018b. Insights on the responses of *Brassica napus* cultivars against the cobalt-stress as revealed by carbon assimilation, anatomical changes and secondary metabolites. Environmental and experimental botany. 156, 183–196. https://doi.org/10.1016/j.envexpbot.2018.07.004.