Combined impacts of tetracycline and multi-walled carbon nanotubes on the growth of *Chrysanthemum coronarium* L. and its root environment

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1. Materials and methods

1.1 Tetracycline extraction

Extraction of tetracycline (TC) in seedlings: Fresh leaves or roots of 0.20 g was exacted with 0.1 g of anhydrous sodium sulfate, 0.02 g of anhydrous sodium acetate, 0.01 g of disodium EDTA, 0.015 g of anhydrous calcium chloride and 3 mL of acetonitrile. The extraction was centrifugated in16,000 $r \cdot min^{-1}$ for 5 min, and the step was done for three times. These supernatants were mixed and extracted with 9 mL of hexane for two times. The extraction was evaporated on a rotary evaporator (N-1300S, Tokyo RIKEN, Japan) to near dryness. Subsequently, the residues were dissolved in 10 mL of EDTA-Mcllvaine buffer in an ultrasonic cleaner, and then the mixtures were enriched using the HLB column. Before using, the HLB column was first activated with 6 mL of methanol and then flowed through with 6 mL of distilled water, followed by 3 mL of EDTA-Mcllvaine buffer. The samples were passed through HLB column with a flow rate of 60-90 drops min^{-1}, and then rinsed with 6 mL of distilled water and left for 20 min to elute with 3 mL of methanol (flow rate of 40 drops min^{-1}). Eluates were blown to near dryness with nitrogen under a water bath at 40 °C. The residues were in

dissolved in 1 mL of methanol-water (3:2 V/V) complex solution, and the mixtures were filtered with a 0.22 μm membrane.

Extraction of TC in subcellular: Fresh leaves or roots of 0.5000 g were mixed with 20 mL of cellular component extract containing 0.25 mol L^{-1} sucrose and 50 mmol L^{-1} Tris-HCl buffer (pH=7), then ground and homogenized, and run for 5 min in a refrigerated centrifuge (D1524R, DLAB, Beijing) at 600 r min^{-1} to precipitate the cell wall fraction (F1). The supernatant was run at 2,000 r min^{-1} for 15 min, and the chloroplast (for leaves) or trophoplast (for roots) fraction (F2) were precipitated. The resulting supernatant underwent centrifugation at a speed of 10,000 r min^{-1} for 20 min, and the membrane and organelles fraction (F3) were precipitated. And the remaining supernatant was the soluble fractions, such as the cytoplasm and vacuoles (F4).

1.2 DNA extraction and 16S rRNA gene high-throughput sequencing

To investigate the changes in rhizosphere bacterial, the solutions from the lower and higher concentration groups were collected for microbial 16S rRNA gene sequencing. Genomic DNA extraction of samples by proteinase K lysis, DNA concentration and purity were monitored by agarose gel electrophoresis. The V3-V4 region of the bacterial 16S ribosomal RNA gene was amplified via polymerase chain reaction (PCR) using the primers 357 F (5'-ACTCCTACGGRAGGCAGCAG-3') and 806 R (5'-GGACTACHVGGGTWTCT AAT-3'). PCR conditions consisted of initial denaturation at 94 °C for 2 min, followed by 25 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s and extension at 72 °C for 30 s, with a final extension of 72 °C for 5 min. The barcoded PCR products were purified using a DNA gel extraction kit (Axygen, USA) and quantified using the FTC -3000TM real-time PCR (Funglyn, Shanghai). The library was constructed by adding the required connectors, sequencing primers and barcode to both ends of the target fragment on the Illumina platform, and the constructed library was sequenced by Novaseq 6000 SP 500 Cycle Reagent Kit (Illumina USA) at Suzhou Taihe Biotechnology Co., Ltd (Jiangsu, China). The 16S sequences were analyzed using a combination of software mothur (version 1.33.3), UPARSE (usearch version v8.1.1756, http://drive5.com/uparse/), and R (version 3.6.3). Alpha diversity of rhizosphere bacterial were calculated by QIIME2 (Version QIIME2-

202006).

Figure S

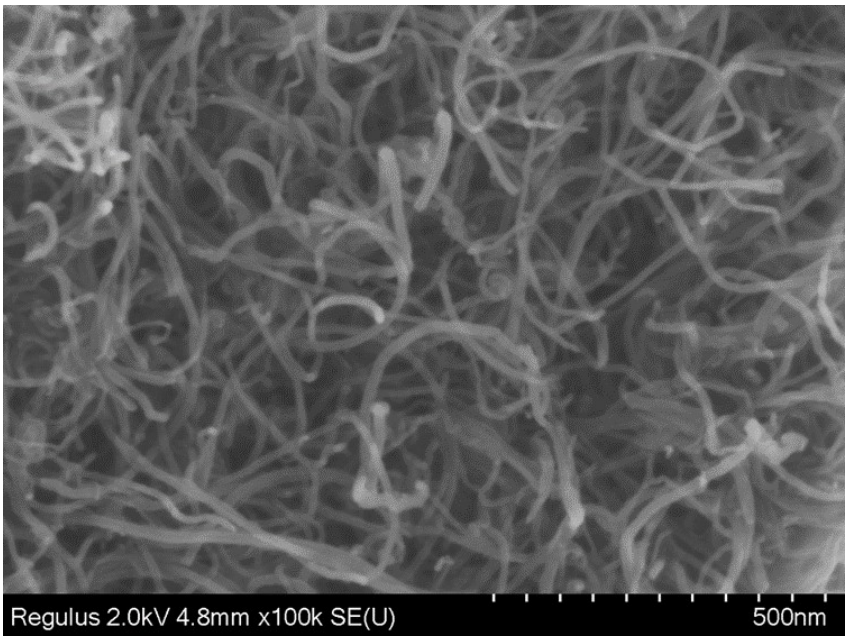

Fig. S1 Scanning electron microscope image of multi-walled carbon nanotube

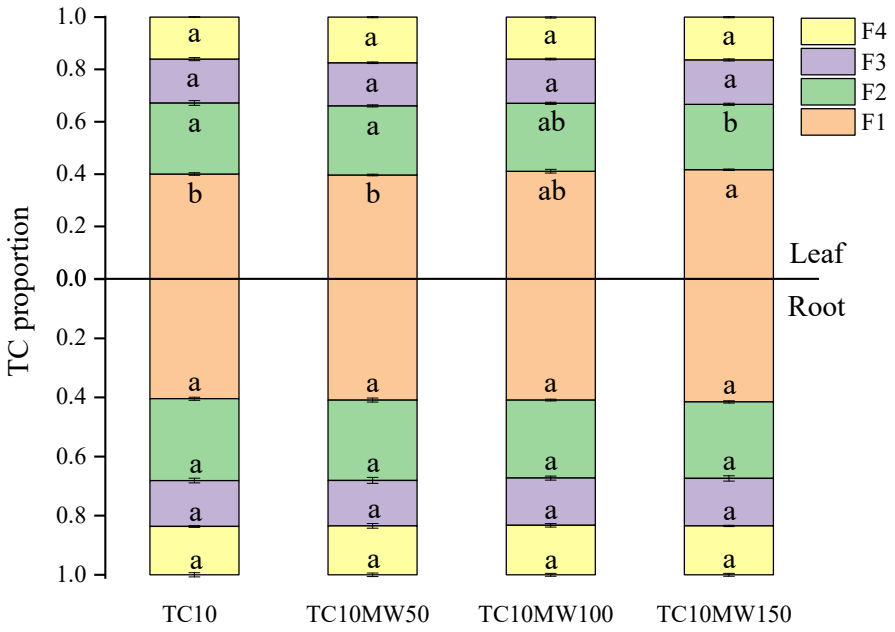

Fig. S2 Proportions of tetracycline (TC) in subcellular fractions of leaves and roots under different treatments. The numbers behind TC and multi-walled carbon nanotube (MW) represent their concentration (mg L^{-1}). F1, cell wall fraction; F2, chloroplast (for leaves) or trophoplast (for roots) fraction; F3, membrane and organelle fraction; F4, cytoplasm and vacuole fraction. Different lowercase letters represent significant differences in the TC proportion of the same subcellular fraction among different treatments.

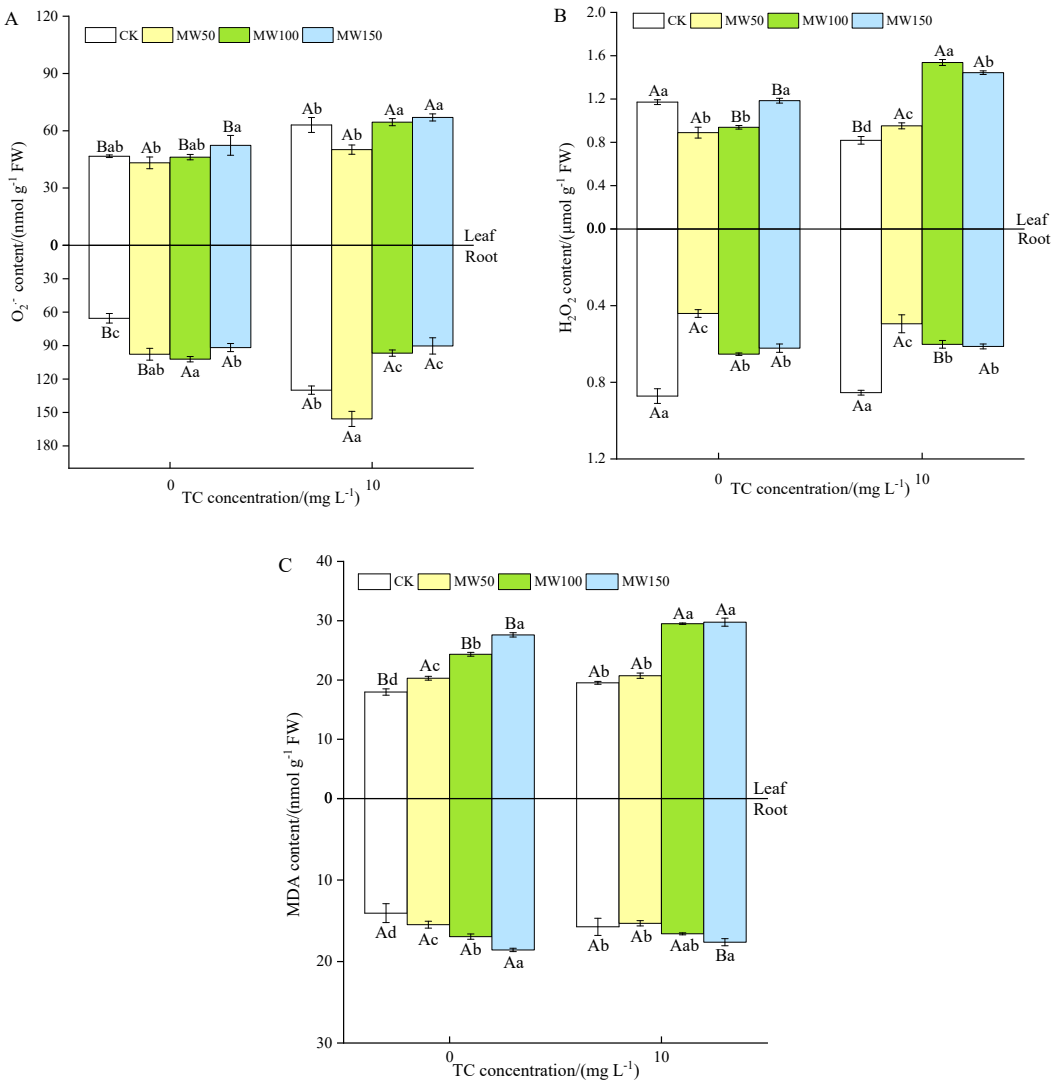

Fig. S3 Effect of multi-wall carbon nanotube (MW) and tetracycline (TC) on superoxide anion ($O_2^{\cdot-}$) (A), hydrogen peroxide (H_2O_2) (B) and malondialdehyde (MDA) content (C) in leaves and roots. The numbers 50, 100, and 150 behind MW in the legend represent MW concentrations (mg L^{-1}). Different lowercase and capital letters indicate significant differences in different groups at the same TC and MW concentration, respectively.

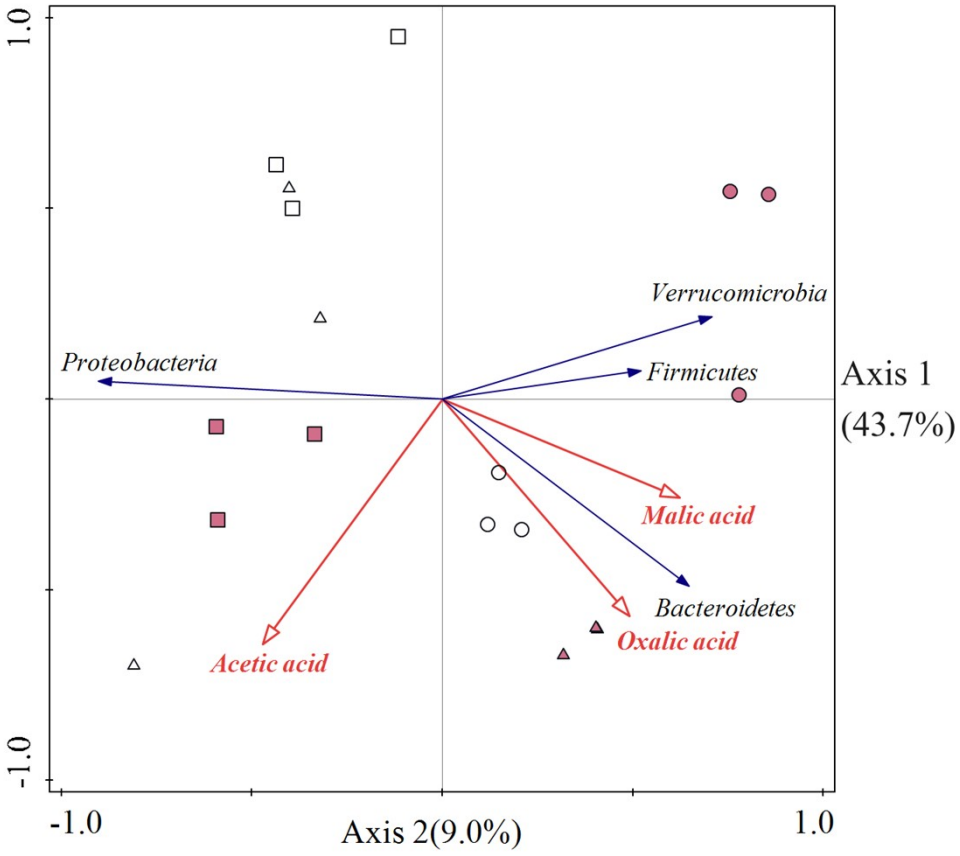

Fig. S4 Redundancy analysis of bacterial community and low molecular weight organic acids. The symbols used in the figure are as follows: circles, triangles and squares represent the MW concentrations of 0, 50 and 150 mg L^{-1}, respectively. The colors blanked and red represent samples treated with and without TC, respectively. The numbers behind tetracycline (TC) and multi-walled carbon nanotube (MW) represent their concentrations.

Table S

Table S1 Size distribution and zeta potentials of multi-walled carbon nanotube (MW) suspensions in different concentrations

MW concentration/ ($mg \cdot L^{-1}$)	Particles size /nm	Zeta potential /mV
50	705±42.0b	-14.1±1.10b
100	734±32.3b	-12.4±0.75b
150	1535±129a	-5.53±0.41a

Different letters indicate statistically significant differences ($P < 0.05$).

Table S2 Information of Hoagland nutrient solution formulation

Compounds	Concentration（$mg \cdot L^{-1}$）
$Ca(NO_3)_2$	945
KNO_3	607
$NH_4 \cdot H_2PO_4$	115
$MgSO_4$	493
$C_{10}H_{12}FeN_2NaO_8$	30
$FeSO_4$	15
H_3BO_3	2.86
$MnSO_4$	2.13
$CuSO_4$	0.05
$H_8MoN_2O_4$	0.02
$ZnSO_4$	0.22

Table S3 Two-way ANOVA of TC/MW for different physiological and biochemical parameters of *Chrysanthemum coronarium L.*

Treatments	Parameter	DHA	RuBisCO	H_2O_2		$O_2^{\cdot-}$		MDA		SOD		CAT	
		Root	Leaf	Leaf	Root	Leaf	Root	Leaf	Root	Leaf	Root	Leaf	Root
TC	*P*	＜0.001	0.009	＜0.001	0.403	＜0.001	＜0.001	＜0.001	0.863	＜0.001	＜0.001	＜0.001	＜0.001
MW	*P*	＜0.001	＜0.001	＜0.001	＜0.001	＜0.001	＜0.001	＜0.001	＜0.001	＜0.001	＜0.001	0.410	＜0.001
TC*MW	*P*	＜0.001	0.189	＜0.001	0.460	0.053	＜0.001	＜0.001	0.058	＜0.001	＜0.001	0.644	0.336

TC: tetracycline; MW: multi-walled carbon nanotube; DHA: dehydrogenase; MDA: malondialdehyde SOD: superoxide dismutase; CAT: catalase; H_2O_2: hydrogen peroxide; $O_2^{\cdot-}$: superoxide anion.

Table S4 Description of fluorescence spectrum parameters.

	Treatments		FI	BIX	HIX
	CK		0.729±0.009d*	0.437±0.009b*	0.813±0.020a
	MW50		1.603±0.017b	0.543±0.025a	0.630±0.029b
	MW100		1.310±0.016c	0.103±0.009c	0.467±0.004c
	MW150		2.567±0.069a	0.037±0.004d	0.243±0.017d
	TC10		0.573±0.012D	0.056±0.005D	0.893±0.012A
	TC10MW50		0.743±0.017C	0.190±0.016A	0.590±0.014B
	TC10MW100		1.323±0.019B	0.133±0.012B	0.617±0.021B
	TC10MW150		1.603±0.033A	0.100±0.008C	0.483±0.016C
Two-way ANOVA	TC	*P*	＜0.001	＜0.001	＜0.001
	MW	*P*	＜0.001	＜0.001	＜0.001
	TC*MW	*P*	＜0.001	＜0.001	＜0.001

Humification Index (HIX), Autochthonous Index (BIX), Fluorescence Index (FI). The numbers behind tetracycline (TC) and multi-walled carbon nanotube (MW) represent their concentration (mg L^{-1}). Different letters indicate significant differences in different groups at the same TC concentration (P<0.05), and "*" indicates significant differences among different groups at the same MW concentration (P<0.05).