Electronic supplementary Information (ESI)

Silver nanoparticles mitigated cadmium toxicity in tobacco by modulating biochemical, cellular and genetic responses

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Section 1

Soil spiking

Soil was collected from agricultural fields located in Hanzhong, Shaanxi province, China (33.0676° N, 107.0238° E) at a depth ranging from 0-15 cm. The collected soil was subsequently air dried and sifted through a 2mm sieve for establishment of the pot experiment.[1] For soil contamination; firstly, cadmium chloride was dissolved in deionized water, then saturated each pot containing 3.5 kg of soil, and next soil was air-dried to ensure the stable state for moisture content. Afterwards, the soil was uniformly mixed, and this wetting-drying cycle was repeated for 45 days under natural light conditions with the relative humidity of 30-40% and the temperatures ranged from 25-40°C during day while 10–11°C at night. Regular additions of deionized water were made to sustain a 60% moisture level in the soil, ensuring the stable and even distribution of metals.[2-4]

Section 2

Seed planting and seedling transplantation

The tobacco seeds were soaked in distilled water for 30 min, followed by surface sterilization using 75% alcohol for approximately 2 min. Afterwards, seeds were submerged in 1% NaClO for 5 min. Subsequently, the seeds were disinfected using distilled water and placed on filter paper to dry. The entire seed sterilization steps were performed under Laminar flow bench.[5,6] Following the completion of sterilization process, seeds were uniformly dispersed in seed propagation trays containing a moist commercial mix composed of 45% vermiculite, 35% peat moss and 20% organic matter in 128-cell seed propagation trays (~22 cm^3 per cell).[7,8] These trays were then placed in a controlled environment within a light incubator sustaining to a day/night cycle of 16 hours light and 8 hours dark, at a constant temperature of 28°C during the day and 23°C at night.[9] Meanwhile, propagation trays were enclosed with translucent covers to penetrate proper light until germination occurred, typically within 3-4 days. After emergence, seedlings were raised up in seed propagation trays up to 7^{th} leaf stage.[10] On the other hand, Upon measuring the dimensions of the pots (diameter 23 cm and a depth of 17 cm), each pot was filled up to 10 cm from the top with 3.5 kg of dry soil to maintain uniform bulk density. Ultimately, equally sized seedlings were precisely selected and transplanted into pots holding soil in a glasshouse setting for further experimentation in controlled environment of 28°C during day, 15°C for night, and 50% relative humidity. To ensure the adequate moisture level, 75% of the water holding capacity (WHC) was maintained for irrigating all pots—aside from the Ag-NP group—every two days with deionized water (dH_2O).[1,5] While, the Ag-NPs treatment group was irrigated with ultrapure water-based.

Section 3

Characterization of nanoparticles

Silver nanoparticles (Ag-NPs) (size<20nm, 99.99% purity) were purchased from Macklin Biochemical Co., Ltd (Shanghai, China). Structural evaluation was performed through field emission scanning electron microscope (SEM; Nano SEM 450, FEI Nova, USA) and transmission electron microscope (TEM; HT7800, Hitachi High-Technologies Corporation, Japan).[11] Briefly, the thin film of Ag-NPs was prepared on a carbon coated copper grid by drying Ag-NPs suspension on silicon wafer and then fixing it on a carbon tape in SEM at an accelerating voltage of 5 kV. For TEM analysis, a drop from the Ag-NPs suspension was mounted on copper grids followed by staining with a drop of 2% uranyl acetate. After that, samples were incubated for 3 min at RT and finally observed the images of the dried sample using TEM.[12] The elemental analysis was conducted using energy-dispersive x-ray spectroscopy (EDAX), equipped with SEM at 10 kV. The EDAX spectra of Ag-NPs was measured in parallel with SEM and percentage of various elements existing in the same field was quantified. Hydrodynamic size with dynamic light scattering technique (DLS) and zeta potential of particle was determined by using water as solvent in liquid zeta sizer/potentiometer (Litesizer500, Anton Paar GmbH, Austria).[13]

Section 4

Ag-NPs suspension preparation

The Ag-NPs suspension was prepared with the same procedures as described by Wan et al.[14] Briefly, 50 uM concentration of Ag-NPs solution was prepared using deionized water followed by stirring for 30 min at 40 kHz with ultrasonic homogenizers KQ-500DE (Kunshan Ultrasound Instrument Co., Ltd. China). The ultrasonication step was performed to avoid any aggregate and to get uniform suspension by ultrasonication.

Section 5

Photosynthetic activity

The net photosynthetic activity (Pn), stomatal conductance (Gs), intercellular CO_2 concentration (Ci) and transpiration rate (Tr) were assessed by portable photosynthesis measurement system LI-6400XT (LI-COR Corporation, USA). The readings were quantified around 9 – 10 AM. The leaves were positioned inside the chamber and seal was tightened enough to minimize air leakage for around five minutes to get stable readings.[15] Measurements were taken under following adjustments; light-saturating photosynthetic photon flux density (PPFD) of 800 μmol m^{-2} s^{-1}, CO_2 concentration of 400 μmol mol^{-1} to assess its impact on photosynthesis, 65% relative humidity and 25°C temperature inside the leaf chamber.[16] To evaluate leaf photochemical efficiency (Fv/Fm), plants were wrapped by black plastic bags to sustain darkness for 30 min prior to taking data. Values were taken by using plant chlorophyll fluorescence imaging system (IMAGING-PAM; WALZ, Germany).[17,18]

Section 6

Antioxidants level and Lipid peroxidation

Enzymatic and non-enzymatic antioxidants including superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), ascorbate peroxidase (APX), glutathione (GSH), and ascorbic acid (AsA) in leaves were investigated using 0.1 g of leaf sample by guaiacol method and ultraviolet absorption spectrometry as explained by Chen et al. (2023). MDA-a biomarker of lipid peroxidation was assessed by using glucosinolates barbituric acid colorimetric method for oxidative damage analysis.[10]

Section 7

Scanning electron microscopy (SEM)

Leaf samples were cut and placed in 4% glutaraldehyde solution and stored overnight at 4°C. Then, the sample were washed, dehydrated by ethanol series, passed through CO_2 drying step and final images were observed by scanning electron microscope (Nano SEM 450, USA).[19]

Section 8

Transmission electron microscopy (TEM)

Leaf sample were cut and dipped in glutaraldehyde solution. Then sample were washed three times with phosphate buffer (pH 7.2) and fixed using osmic acid, followed by three time washing with PBS. Afterwards, sample were dehydrated by series of ethanol and then washed with LR White. Final plant sample were fixed in Epon-Araldite resin. The ultrathin section was cut by ultramicrotome and then stained by using uranyl acetate and lead citrate. Then samples were observed using TEM (HT7800, Hitachi High-Technologies Corporation, Japan).[17]

Section 9

Primer list

NtActin F	GTATGTCGCCATTCAAGCCGTTCT	NtCSD1 F	CCACAATCCATCATTGGAAGAGC
NtActin R	ACGGAGGATAGCATGTGGCAAAGCAT	NtCSD1 R	CCGATGATACCACAAGCAAC
NtChl F	ATCAAATATGGGTGCTTCTTCTTGGAGG	NtAPX F	TGTTTTCTACAGAATGGGC
NtChl R	ATTATGTCAGGTGTAAGGGTGCCGAACA	NtAPX R	GTTGAGTATTTTG CTGCCAC
NtPsy2 F	TCAGAGATGTTGGAGAAGATGC	NtCAT4-F	AATCCTTGTTGGAAGATGAGGC
NtPsy2 R	GCTTCAATCTCGTCCAATATCTTG	NtCAT4-R	TAGTCACATCAAGCGGATCGA
NtSPL4a F	AGCCATAATAGGGACAGACACT	NtFSD1 F	CACACCGCTCCTCACCATAGAC
NtSPL4a R	GAGAGCAAGCATATCGTATCCA	NtFSD1 R	AGCCTAGAACTGACTGCTTCC
NtSPL15a F	TGAAGCTAGCAGCAGTTTGCA	Nt6b-Q-F	CAGCAACCTGGGTTATTGGCACATTA
NtSPL15a R	CGTCCAGTGCACATTCTGAACA	Nt6b-Q-R	CACAAGGTGCCTAATATTCCACAAAG

Section 10

Growth attributes of tobacco under control (CK), cadmium (Cd; 20 mg kg^{-1}), silver nanoparticles (Ag-NPs; 50 µM) and Cd+Ag-NPs (20 mg kg^{-1} + 50 µM).

Treatments	RFW (g/plant)	RDW (g/plant)	SFW (g/plant)	SDW (g/plant)	SL (cm)	RL (cm)
CK	12.05±0.70b	6.33±0.32b	85.93±4.03b	22.61±1.52b	74±3.31b	26.00±1.37a
Cd	4.31±0.36d	2.06±0.15d	23.04±1.64d	12.95±1.17d	35±2.68d	18.23±1.01c
Ag-NPs	16.4±0.68a	8.21±0.15a	114.50±3.65a	31.61±1.27a	97±3.58a	28.82±1.25a
Cd+Ag-NPs	9.28±0.22c	4.68±0.23c	68.63±3.55c	19.43±0.68c	52±2.08c	21.86±1.02b
p-value	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001

RFW, root fresh weight; RDW, root dry weight; SFW, shoot fresh weight; SDW, shoot dry weight; SL, shoot length; SL, root length.

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New Project

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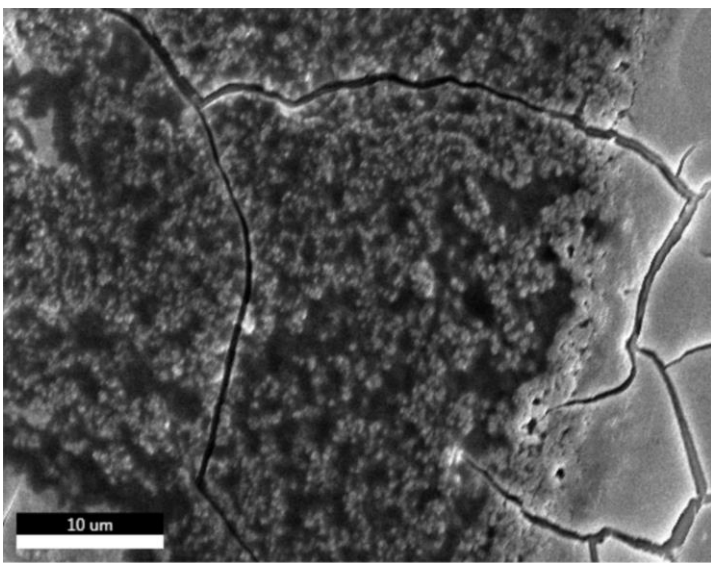
Sample Name: Silver nanoparticle (AgNP)

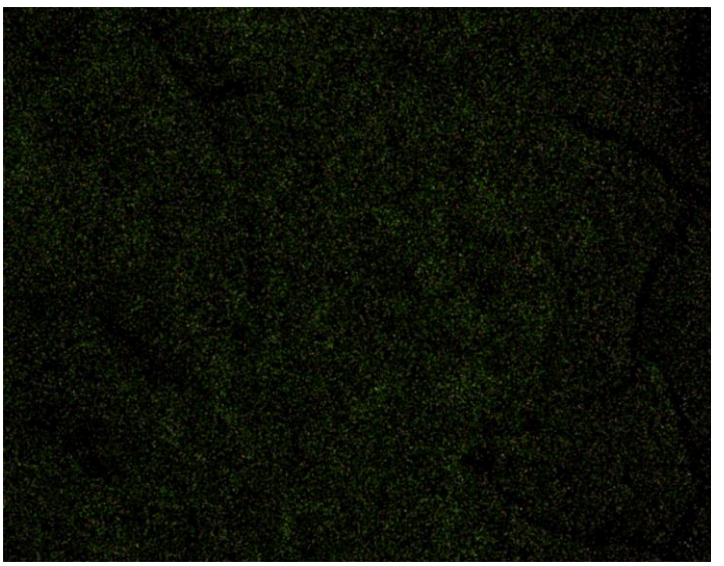
Area 9

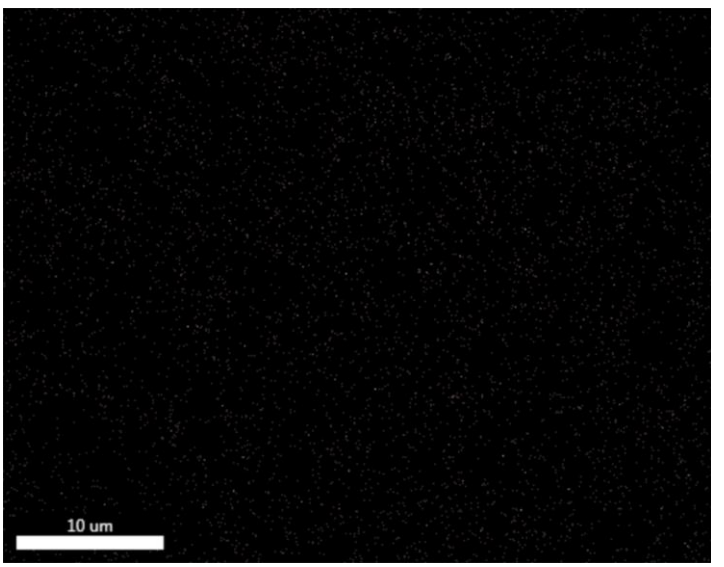
Live Map 1



Image

ElementOverlay





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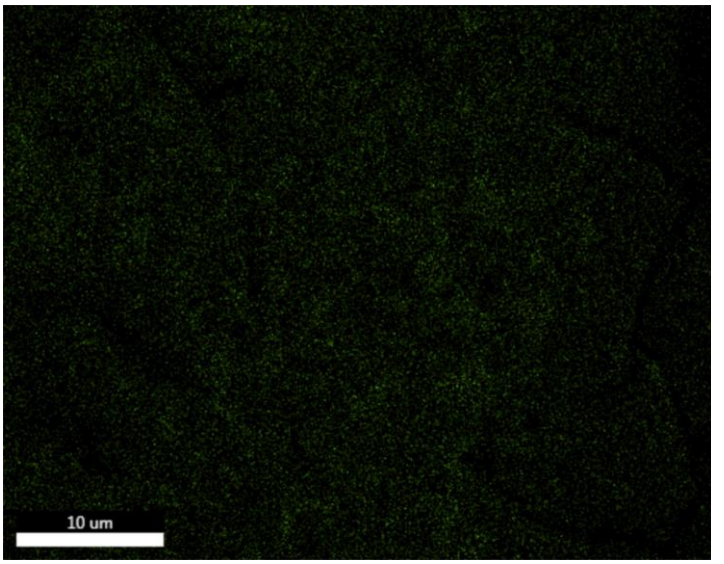
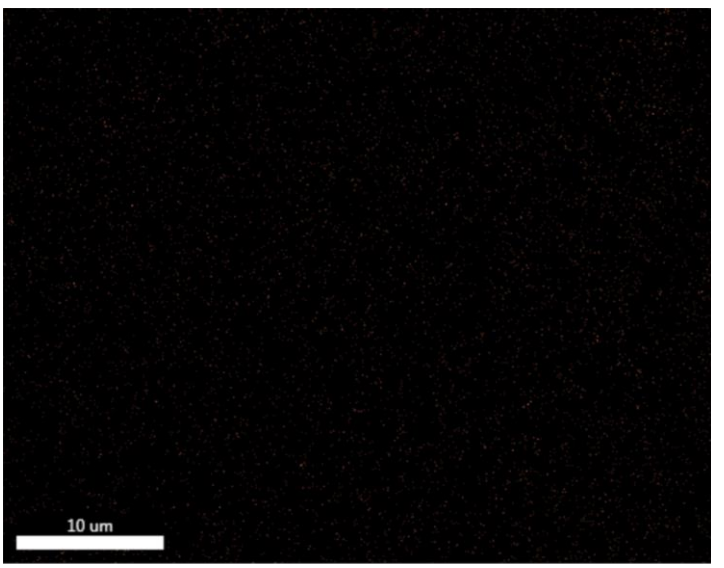


O K_ROI (6)



AgL_ROI (4)

Section 12

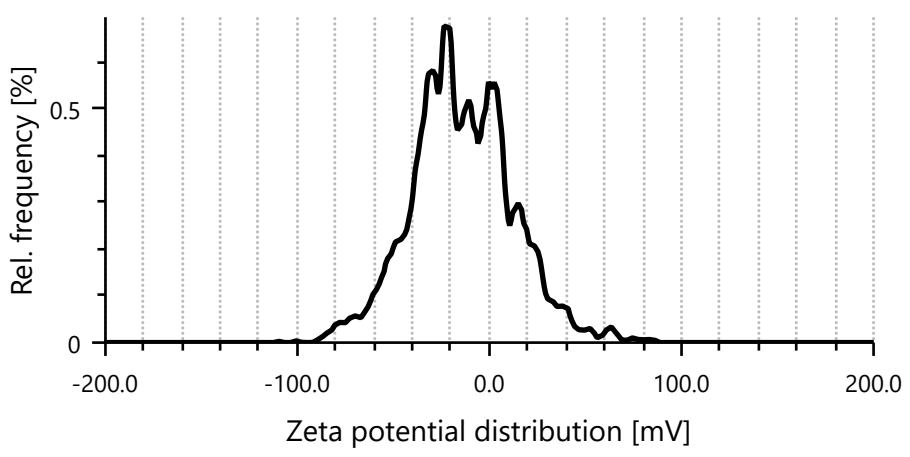
Standard Report - Zeta Potential

Anton Paar

Measurement information

Measurement name	Ag Zeta	**User**	lenovo
Method	-	**Time**	2024/5/29 17:21:14
Status	Succeeded	**Instrument type**	Litesizer 500
Measurement mode	Zeta potential		
Sample ID	-		
Batch number	-		
		Filter optical density	1.0506
Measurement cell	Omega cuvette Mat.No. 225288	**Solvent**	Water
Target temperature	25.0 °C	**Solvent refractive index**	1.3303
Equilibration time	0h 01m 00s	**Solvent viscosity**	0.0008903 Pa.s
Henry factor	1.5 (Smoluchowski)	**Solvent relative permittivity**	78.37
Adjusted voltage	200.0 V (Automatic Mode)		
Processed runs	200 (Manual)		

Zeta potential distribution



Result

Mean zeta potential	-26.0 mV	**Mean intensity**	726.6 kcounts/s
Standard deviation	1.8 mV	**Filter optical density**	1.0506
Distribution peak	-22.4 mV	**Conductivity**	0.034 mS/cm
Electrophoretic Mobility	-2.0300 µm*cm/Vs	**Transmittance**	11.7 %

Section 13

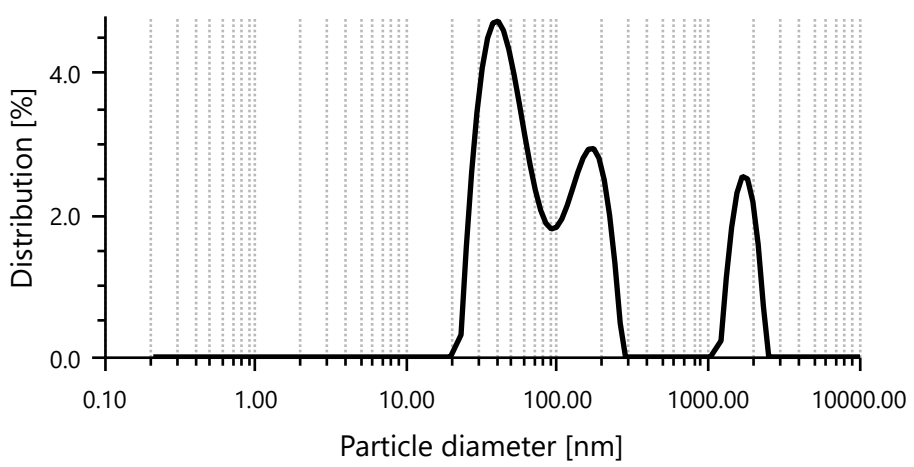
Standard Report - Particle Size

Anton Paar

Measurement information

Measurement name	Ag	**User**	lenovo
Method	-	**Time**	2024/5/29 17:04:39
Status	Succeeded	**Instrument type**	Litesizer 500
Measurement mode	Particle size		
Sample ID	-		
Batch number	-		
		Filter optical density	0.500 (Automatic)
Measurement cell	Disposable	**Focus position**	0.1 mm (Automatic)
Measurement angle	Side scatter (Automatic)	**Material**	Silver
Target temperature	25.0 °C	**Material refractive index**	0.1590
Equilibration time	0h 01m 00s	**Material absorption index**	4.3000
Analysis model	General	**Solvent**	Water
Cumulant model	Advanced	**Solvent refractive index**	1.3303
Processed runs	9 (Automatic)	**Solvent viscosity**	0.0008903 Pa.s
Time for each run	0h 00m 10s (Automatic)		

Particle size distribution (intensity)



Result

Hydrodynamic diameter	120.93 nm	**Mean intensity**	91.9 kcounts/s
Polydispersity index	30.1 %	**Absolute intensity**	290.9 kcounts/s
Diffusion coefficient	4.1 µm²/s	**Intercept g1²**	0.8144
Transmittance	66.0 %	**Baseline**	1.182

Particle size distribution peaks (intensity)

Peak name	Size [nm]	Area [%]	Standard deviation [nm]
Peak 1	46.53	54.55	13.43
Peak 2	156.89	30.43	49.96
Peak 3	1742.4	15.02	271.0