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Electronic Supplementary Information

Phytotoxicity of halloysite nanotubes using wheat as a model: Seed germination and growth

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Supplemental experiment

Experiment S1. Cell suspension cultures

Seeds of tobacco (*Nicotiana tabacum* L.) were purchased from Guangzhou Baihui Biological Technology Co., Ltd, Guangzhou, China. The seeds were immersed in distilled water for 24 h, then immersed in 75% alcohol for 0.5 min and rinsed with sterile water 4 times, additionally immersed in 0.1% (w/v) mercuric chloride solution for 15.0 min followed by four rinses in sterile water. The surface-sterilized seeds were dried by sterile filter paper and implanted on Murashige and Skoog (MS) medium. Plants of tobacco were sub-cultured every 4 weeks. Leaves of tobacco were used as explants to induce callus. Leaves were cut into small pieces about 0.5 cm × 0.5 cm and cultured horizontally on MS solid medium with 1.0 mg L^{-1} α-naphthaleneacetic acid (NAA) and 3.0 mg L^{-1} 6-benzyladenine (6-BA). Callus appeared in 2 weeks. And then, callus was subsequently cut from the explant and further cultured in MS solid medium containing 1.0 mg L^{-1} NAA and 1.0 mg L^{-1} 6-BA. Suspension culture cells of tobacco were obtained by inoculating the callus in MS liquid medium with 1.0 mg L^{-1} NAA and 1.0 mg L^{-1} 6-BA. Unless otherwise stated, all media were contained 30 g L^{-1} sucrose，and 8 g L^{-1} agar for solid media, and adjusted to pH 5.8 before autoclaving at 121^{o}C for 20 min. All plant tissue cultures were maintained at 24 ± 1^{o}C. Plantlets were illuminated with 16/8 (day/night) photoperiod, while the cell were cultured in the dark. Suspension culture cells were cultured in dark with rotating shaking of 100 rpm.

Experiment S2. The cell viability

Suspension culture cells of tobacco were passaged three times before use, once

every 12 days. 20 mL tobacco suspension culture cells (approximately 6 g) were poured into 250 mL Erlenmeyer flask containing 80 mL MS liquid medium with 1.0 mg L^{-1} NAA and 1.0 mg L^{-1} 6-BA. After pre-cultured for 10 d, HNTs was added into the suspension culture cells, and further cultured for another 24, 48 and 72 h, respectively. Cell suspension samples (1 mL) were washed aseptically with 50 mM phosphate buffer (pH 7.5) twice. Then the samples were resuspended in 1 mL of the same buffer.

2,3,5-Triphenyl-2H-tetrazolium chloride (TTC) stock solution, which was freshly prepared by dissolving 0.2 g TTC in 10 mL sterile 50 mM phosphate buffer (pH 7.5), was added to a final concentration of 2.5 mM, and then the samples were incubated for 8 h in the dark at 25°C. Afterwards, formazan salts were solubilized with 50% methanol (containing 1% SDS) at 60°C for a period of 30 min. The sample was centrifuged at 1500 × g for 5 min and the supernatant was recovered. The supernatants were pooled and quantified absorbance at 485 nm using microplate reader.[1, 2]

Experiment S3. Effect of HNTs on the pH of the culture medium

pH value of the culture medium was determined at a serial time interval as 0, 1, 2, 3, 4, 5, 6, 12, 18, 24 h after the addition of HNTs.

Experiment S4. The PAL (phenylalanine ammonia lyase) of tobacco cells

The activity of PAL was determined by measuring trans-cinnamic acid, the catalytic product of PAL.[3] 5 mL suspension culture cells were centrifuged at 1500 × g for 5 min to remove the supernatant, and a small amount of polyvinylpyrrolidone (PVP) was then added. Next, pre-cooled enzyme extract (2 mL containing 5 mmol L^{-1} mercaptoethanol, 1% PVP, 0.1 mol L^{-1} boric acid buffer) and quartz sand were ground

on ice. The grinding solution was transferred to a 5 mL centrifuge tube and centrifuged at 10,000 × g for 15 min to take the supernatant as the crude enzyme extract. 0.02 mol L^{-1} phenylalanine (1 mL) was added to 1 mL of enzyme solution, and 1 mL of distilled water was used to replace enzyme solution for control. The absorbance of the reaction solution was measured at 290 nm before the reaction. Then the solution was kept in a constant temperature water bath at 30°C for 0.5 h, and the absorbance of the reaction solution was measured. The amount of enzyme required to change the absorbance value of 0.01 every 5 min before and after the reaction was defined as a unit (U). Cell enzyme activity was expressed in enzyme units per gram of wet cells (U g^{-1}).

Experiment S5. The total phenols

2 mL of tobacco suspension culture cells was mixed with 10 mL ethyl acetate, then sonicated and statically extracted for 2 h. The ethyl acetate extract was dried naturally in a flow hood, and the residue was dissolved in 3 mL of 75% ethanol. The absorbance was measured at 280 nm, with 3 mL of 75% ethanol as the control, salicylic acid as the standard, and the absorbance of 1 μg salicylic acid at 280 nm representing one unit of phenol accumulation.[4]

Experiment S6. Catalase (CAT) activity

Tobacco cells of 0.1 g was suspended in 3 mL of phosphate buffer (50 mM, pH 7.8), followed by the addition of 0.1 M H_2O_2 solution. And then, the time was immediately counted, and the absorbance was measured at 240 nm per minute. The measurement repeated 3 times, each as A_1, A_2, A_3. For the control A_0, cells were treated in boiling water for 10 min and the absorbance was measured at the same condition. ΔA_{240}

represented the change of CAT activity, and the calculation formula was:[5] $\Delta A_{240}=A_0-(A_1+A_2+A_3)/3$.

Experiment S7. The Glucose-6-phosphate dehydrogenase (G6PDH)

The activity of G6PDH was determined by measuring nicotinamide adenine dinucleotide phosphate (NADPH), the catalytic product of G6PDH. 3 mL of suspension cells were centrifuged to remove the supernatant, and 0.5 g PVP was added. Then 1 mL pre-cooled enzyme extract (containing 0.42 mol L^{-1} mannitol, 0.005 mol L^{-1} KCl, 0.005 mol L^{-1} $MgS0_4$, 0.05 mol L^{-1} Tris-HCl) and quartz sand were ground on ice. The grinding solution was transferred to a 5 mL centrifuge tube, and centrifuged at 10,000 × g for 15 min. The supernatant was taking as the crude enzyme extract. 0.1 mL enzyme solution was incubated with a mixed solution (0.09 mol L^{-1} G-6-P-2Na, 0.003 mol L^{-1} NADP, 12.5 mmol L^{-1} $MgCl_2$, 0.05 mol L^{-1} Tris-HCl) at 35°C in a water bath for 5 min. Afterwards, the absorbance at 340 nm before and after the reaction was measured using ultraviolet spectrophotometer. The amount of enzyme required to change the absorbance value by 0.01 every 5 min before and after the reaction was defined as a unit U. Cell enzyme activity was expressed in enzyme units per gram of wet cells (U g^{-1}).[6, 7]

Experiment S8. The biomass

Plant cell biomass was calculated based on dry cell weight (DW), and wet cells were air-dried to constant weight at 45°C.

Supplemental Discussion

Discussion S1. Effect of HNTs on tobacco cells

Cells are more sensitive to external substances and react faster. Some reaction signals of nanoparticles can be reflected at the cell level, but not necessarily at the plant level. Therefore, we carried out plant cell exposure experiments in the following section. Tobacco is a common research model to understand in different areas of plant biology such as tissue culture studies and genetic engineering. Tobacco cell and tissue cultures were generally employed for identifying the interactions of plant cells with numerous environmental pollutants including nanoparticles.[8, 9] The effect of HNTs on the viability of tobacco cells was investigated by the TTC method. As shown in Fig. S1A, HNTs had no obvious toxicity to tobacco cells under 80 μg mL^{-1}, and the cell survival rate was higher than 80%. But at high concentrations (greater than 160 μg mL^{-1}), HNTs showed significant toxicity to tobacco cells, and the toxicity was time- and dose-dependent. These results are similar to the effect of HNTs on wheat growth in a dose-dependent manner. Considering that when the concentration of HNTs was greater than 80 μg mL^{-1}, the viability of tobacco cells was significantly reduced with the extension of cultivation time. So, the HNT concentration (80 μg mL^{-1}) and its half-diluted HNT concentration (40 μg mL^{-1}) that affected the tobacco cells viability were used for the biochemical properties' studies. As shown in Fig. S1B, the biomass of tobacco cells increased significantly after the addition of low concentration HNTs, suggesting that HNTs in low concentration could stimulate the growth of tobacco cells.

Under normal conditions, the intracellular and extracellular pH of plant suspension cells should be kept basically constant. However, when stressed by external factors, such as elicitors, the cytoplasm will be acidified.[10] The alkalization of plant cell culture

media was considered to be the initial marker event of plant defense response. As a result of the ion exchange inside and outside the cell, the culture medium will be alkalized accordingly. As shown in the Fig. S1C, after giving low doses of HNTs for 1 h, the pH of the cell culture medium increased significantly, indicating that the tobacco cells culture medium alkalized by the induction of HNTs. The result indicates that HNTs activated the ion pump of the tobacco cell membrane. This is the first sign of the defense response of plants.

The phenylpropane metabolic pathway is an important biochemical pathway for the defense reaction of plants and the formation of secondary metabolites, such as the formation of phytoalexins. It is involved in the synthesis of phenol, benzoic acid, plant signal molecule salicylic acid and a series of compounds related to plant defense response. PAL is a key enzyme in the metabolic pathway of phenylpropane and also a symbolic enzyme in plant defense reactions.[11] The result in Fig. S1E showed that the PAL activity in plant cells increased first and then decreased after the addition of HNTs, reaching the maximum value at 24 h, which was 2.18 times higher than that of the control group. This suggested that HNTs could stimulate cell defense response, PAL activity, and promote phenolics accumulation. Phenolic compounds in plants are an important compound closely related to the defense response of plants. A proper concentration of phenol in the cell is beneficial to the accumulation of secondary metabolites. Some phenolic compounds form lignin through gathering, thicken cell walls, and improve plant disease resistance through lignin.[12] In addition, phenols are antioxidants, which can reduce the harmful effects of reactive oxygen molecules

produced in defense reactions on cells. The synthesis of phenol precursors is catalyzed by PAL enzyme, so the change of PAL enzyme activity ultimately affects the accumulation of phenol. Fig. S1D showed that the addition of HNTs could effectively increase the accumulation of phenolics.

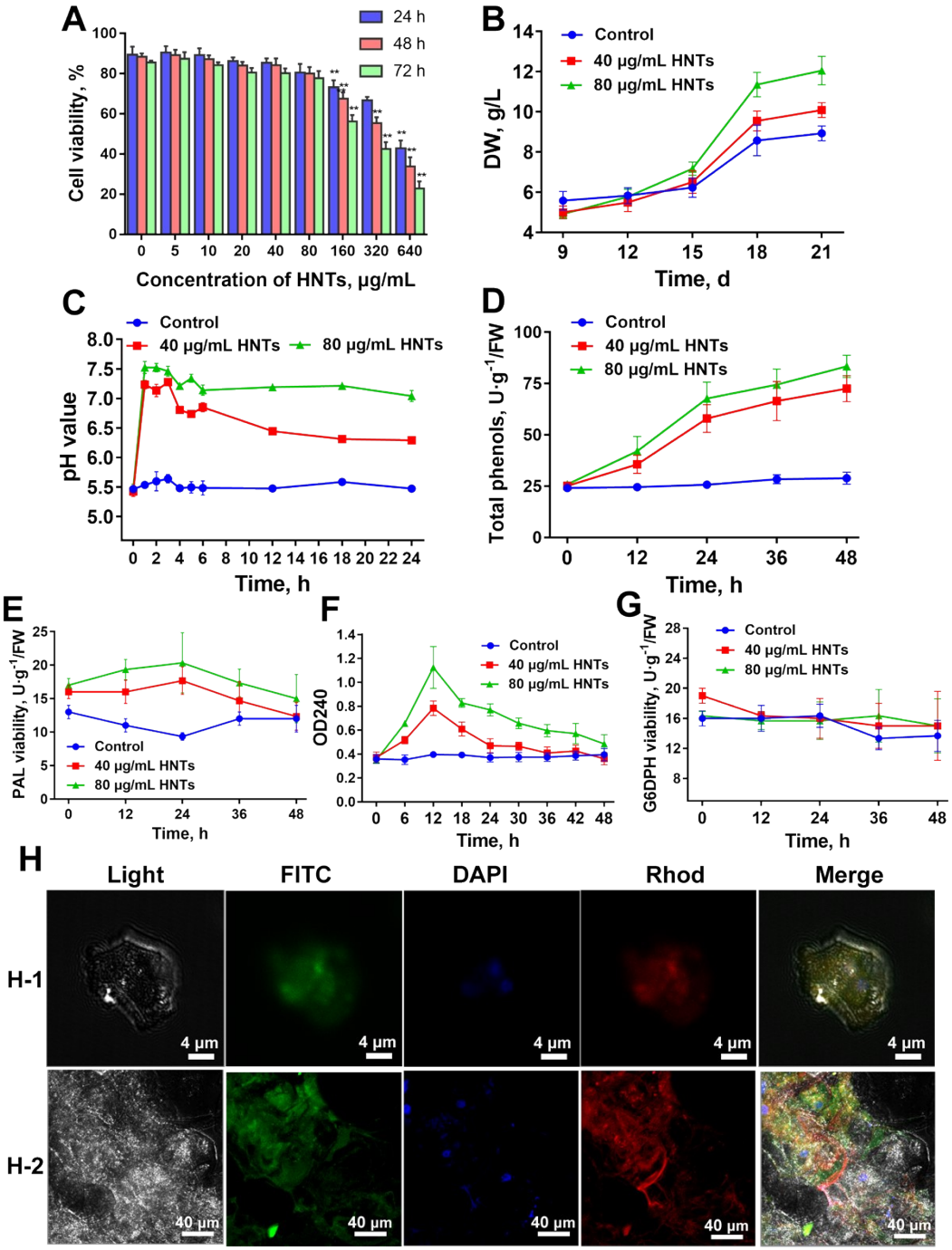

Fig. S1. Effect of HNTs on the growth and biochemical properties of tobacco cells. (A. Cell

viability at 24, 48 and 72 h, *P<0.05, **P<0.01. B. The biomass. C. pH of culture medium. D. Total phenols. E. PAL activity. F. CAT activity. G. G6PDH activity. H. Observation of HNTs uptake by tobacco cells with laser confocal microscope; H-1: laser confocal microscope map of single cell; H-2: laser confocal microscope map of multiple cells; Light: laser confocal microscope map of tobacco cells under natural light source; FITC: laser confocal microscope map of FITC-labeled HNTs co-cultured with tobacco cells; DAPI: laser confocal microscope map after DAPI staining the nucleus of tobacco cells; Rhod: laser confocal microscope map of tobacco cytoskeleton stained by Rhod; Merge: Overlapping the above four maps, it was found that HNTs existed in the nucleus, cytoskeleton and other parts of tobacco cells.

Catalase is widely distributed in plant cells and belongs to active oxygen scavengers. It can decompose the active oxygen generated in the body's metabolism, such as hydrogen peroxide and superoxide anion.[13] These substances have a toxic effect on the body, especially the plasma membrane. CAT activity is closely related to the physiological activities of plants such as stress resistance, cold resistance, disease resistance and regulation of cell apoptosis.[14] The results showed that after the addition of HNTs, with the extension of the treatment time, the CAT activity of cells showed a trend of first increasing and then decreasing. Fig. S1F showed that when low concentrations of HNTs were used to treat plant cells, they stimulated the plant cell's resistance to stress, and responded to environmental stresses through changes in cell CAT activity.

G6PDH (EC 1.1.1.49) is widely present in animals, plants, microorganisms and cultured cells. It is a key enzyme in the pentose phosphate pathway. It catalyzes the

oxidation of 6-phosphoglucose to 6-phosphogluconolactone and at the same time reduces NADP + to NADPH. It plays an important role in metabolite biosynthesis and maintaining the reducing capability of cells. Therefore, the activity of glucose 6-phosphate dehydrogenase can reflect to a certain extent in metabolite biosynthesis and the antioxidant capacity of the body. The activity of G6PDH was determined by measuring NADPH, the catalytic product of G6PDH.[15, 16] As shown in the Fig. S1G, after treatment with HNTs, the trend of G6PDH activity was not significantly different from the control. Therefore, HNTs might not play a role in G6PDH. Fig. S1H show the laser confocal microscope images of tobacco cells treated by HNTs. The FITC labeled HNTs was observed in the nucleus, cytoskeleton and other parts of tobacco cells, suggesting HNTs pass the cell membranes and entered the plant cells.

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