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Novel impacts of functionalized multi-walled carbon nanotubes in plants: promotion of nodulation and nitrogenase activity in the rhizobium-legume system†

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The rhizobium-legume symbiosis system is critical for nitrogen-cycle balance in agriculture. However, the potential effects of carbon nanomaterials (CNMs) on this system remain largely unknown. Herein, we studied the effects of four carbon-based materials (activated carbon (AC), single-walled carbon nanotubes (SWCNTs), multi-walled carbon nanotubes (MWCNTs) and graphene oxide (GO)) on the rhizobium-legume symbiosis system consisting of *Lotus japonicus* and *Mesorhizobium loti* MAFF303099. Under non-symbiotic conditions, the bacterial growth and root development of plants were both clearly inhibited by SWCNTs and GO, while the elongation of plant stems was enhanced by MWCNTs to a certain degree. More importantly, only MWCNTs could increase the number of nodules and enhance the activity of nitrogenase in the rhizobium-plant interaction. Further analyses showed that the average number of nodules in plants treated with 100 μg mL^{-1} MWCNTs was significantly increased by 39% at 14 days post inoculation (dpi) and by 41% at 28 dpi. Meanwhile, the biological nitrogen fixation of the nodules was promoted by more than 10% under 100 μg mL^{-1} MWCNT treatment, which enhanced the above- and below-ground fresh biomass by 14% and 25% respectively at 28 dpi. Transmission electron microscopy images further indicated that MWCNTs penetrated the cell wall, and pierced through the cell membrane to be transmitted into the cytoplasm. In addition, gene expression analysis showed that the promotion of nodulation by MWCNTs was correlated with the up-regulation of certain genes involved in this signaling pathway. In particular, the expression of *NIN*, a crucial gene regulating the development of nodules, was significantly elevated 2-fold by MWCNTs at an early stage of nodulation. These findings are expected to facilitate the understanding and future utilization of MWCNTs in agriculture.

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1. Introduction

With the rapid development of nanotechnology, nanomaterials are being increasingly used for agricultural purposes, resulting in the emergence of nano-agriculture.[1,2] It has been revealed that the effects of nanomaterials on plants and the environment may determine their directions of application and their potential in agriculture to a large extent.[3–5] Currently, legumes and their symbiotic nitrogen fixation system, as an important part of the nitrogen cycle balance in agriculture,[6,7] are also affected by different nanomaterials in the environment.[8–10] However, little is known about the effects of different nanoparticles on legumes, and only a limited number of studies have shown that metal nanoparticles are destructive to this symbiotic nitrogen fixation system.[11–13] On the other hand, the emergence of Haber–Bosch nitrogen fixation has partly relieved the lack of nitrogen in agriculture and enhanced the yield of crops.[14] However, to meet the large demand for fertilizers, large amounts of energy are consumed in the chemical reduction of nitrogen, which causes the increase of greenhouse gas emission and triggers widespread eutrophication of the aquatic ecosystems.[15] Thus, to solve the global problem of nitrogen deficiency, multifarious techniques have been

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employed and the symbiotic nitrogen fixation system of legumes has been extensively studied during the past decade,[16–18] because compared with other commercial crops, most legumes can receive nitrogen from this system through the formation of nodules with their corresponding rhizobia.[19–21] Meanwhile, we have made great efforts to study the mechanism of this system, which has led to the identification of several genes related to the development of nodulation, such as *SIP2*, *ROP6* and *CHC*.[22–24] Therefore, the development of nanomaterials with various properties arouses researchers' interest in their effects on the rhizobium-legume symbiosis system, which may help to simply and effectively improve the function of this system.[25,26]

As important and common types of nanomaterials, carbon nanomaterials (CNMs), mainly including single-walled carbon nanotubes (SWCNTs), multi-walled carbon nanotubes (MWCNTs), graphene oxide (GO) and fullerene (C_{60}), are characterized by their special structures with unique electrical, mechanical and surface properties.[27,28] As ideal candidates for bio-imaging, bio-engineering and biomedical applications, CNMs and their derivatives have aroused the interest of many scientists for their particular effects and functions in regulating plant growth, especially MWCNTs.[29–33] Recently, there have been several reports about plant growth enhancement upon MWCNT exposure. Khodakovskaya *et al.* found that the germination percentage of tomato seeds germinated on Murashige and Skoog (MS) growth medium containing MWCNTs reached 82% in 12 days, which was significantly higher than that of the control (32% in 12 days); besides, MWCNT exposure increased the total fresh biomass of tomato seedling 2.5-fold compared with seedlings developed on the standard medium.[34] In a follow-up study, these researchers demonstrated that the growth of tobacco cells was 55–64% higher on MWCNT exposure (5–500 mg mL^{-1}) compared with that of the untreated controls, and further investigations revealed an upregulation of several genes related to the effect of MWCNT exposure, including that of an aquaporin (*NtPIP1*) involved in water transport and two genes (*CycB* and *NtLRX*) involved in cell wall formation and cell division.[35] Also, the effects of MWCNTs on the development of some valuable agricultural plant species have been reported, showing a positive effect of MWCNT exposure on the germination and growth of soybean, corn (*Zea mays*), and barley (*Hordeum vulgare*) in agar medium.[36] Similarly, our previous study demonstrated 50% and 32% increases in root elongation of wheat seedlings after 3 and 7 days of exposure to 40–160 mg L^{-1} MWCNTs, respectively.[37] Nevertheless, it is still unclear whether MWCNTs have the same promoting effects on the growth and development of legumes as found in other plants. Moreover, it is of great significance that the valuable characteristics of CNMs can be effectively utilized in the symbiotic nitrogen fixation system. So far, there have been no reports that any nanomaterial can promote the nodulation and biochemical nitrogen fixation in legumes.

For the above-mentioned reasons, this study evaluated the effects of three types of CNMs (SWCNTs, MWCNTs and GO) and activated carbon (AC) on the rhizobium-legume symbiosis system of a model legume (*Lotus japonicus*) inoculated with its corresponding rhizobium (*Mesorhizobium loti* MAFF303099), and further revealed the functional mechanism of MWCNTs in the nodulation and nitrogen fixation of this symbiosis system to a certain degree. In general, these findings not only deepen our understanding about the impact of CNMs on the interaction between plants and bacteria, but also indicate the application potential of carbon nanotubes in agriculture. Through clarifying the molecular mechanism of MWCNTs in the rhizobium-legume symbiosis system, a new method could be developed to efficiently elevate the availability of nitrogen in the soil, which could partly meet the nitrogen demand of agricultural cultivation.

2. Experimental section

2.1 Preparation of carbon-based materials

SWCNTs (purity >95 wt%, OD × length: 1–2 nm × 5–30 μm, –COOH content: 2.73 wt%, ash <1.5 wt%, SSA >380 m^2 g^{-1}, EC >10^2 s cm^{-1}), MWCNTs (purity >95%, OD × length: 8–15 nm × 5–50 μm, –COOH content: 2.56 wt%, ash <1.5 wt%, SSA >233 m^2 g^{-1}, EC >10^2 s cm^{-1}) and GO (purity >98 wt%, thickness: 0.55–3.74 nm, size: 0.5–3 μm, layers <10, SSA: 500–1000 m^2 g^{-1}) were purchased from Chengdu Organic Chemicals Co., Ltd (China). Activated carbon (vapor pressure <0.1 mmHg (20 °C), autoignition temp. 842 F, iodine-adsorption (0.05 mol I_2/I) > 70 mL g^{-1}, methylene blue-adsorption >12 mL per 0.1 g, 0.15% sol., ign. residue ≤2%, anion traces: chloride (Cl^-) ≤5000 mg kg^{-1} and sulfate (SO_4^{2-}) ≤5000 mg kg^{-1}, Fe ≤300 mg kg^{-1}, Zn ≤10 mg kg^{-1}, Pb ≤10 mg kg^{-1}) was purchased from Sigma-Aldrich.

Through accurate weighing, different carbon-based materials (including AC, MWCNTs, SWCNTs and GO) were prepared at a concentration of 1000 μg mL^{-1} in ultrapure water. As for the mother liquor, these suspensions of different carbon-based materials were all obtained by 30 min sonication using a sonicator bath (Elamsonic, S60H) at 37 kHz less than 550 W without adding any dispersant, which could produce a stable dispersion to the suspensions for two weeks at room temperature. Before being used for the bacterial or plant experiments, all suspensions of carbon-based materials were sterilized by ultraviolet radiation for more than 30 min. According to experimental requirements, the suspensions of different carbon-based materials were adjusted to desirable concentrations (50, 100, 150, 200, 500 μg mL^{-1}). Then, TEM images, Raman spectra, zeta potential and XPS were used for a general characterization of the carbon-based materials. Related experimental results are shown in Fig. S1.†

2.2 Bacterial cell growth and viability

Starter cultures of *Mesorhizobium loti* strain MAFF303099 were initiated by inoculation of a single colony in 20 mL YMA medium in a 50 mL flask, and grown for 48 h at 30 °C in a shaker (200 rpm). The cells were collected by centrifugation at

6000 rpm for 5 min and washed twice with sterile distilled water. Then, the cells were resuspended in water.

To obtain the growth curve of the bacterial cells, 250 μL cell suspensions were mixed with different carbon material suspensions (AC, MWCNTs, SWCNTs and GO) in the tubes to obtain a final concentration of 50 or 500 μg mL^{-1} and then incubated at 20 rpm shaking for 2 h at room temperature. Control samples contained 250 μL of the cell suspensions mixed with DI water equal to the amount of carbon material suspensions. After 2 h of treatment, these mixtures were transferred to 5 mL tubes, each containing 2 mL YMA medium, and the tubes were inoculated on a rotary shaker at 200 rpm and 30 °C. The value of the optical density (OD) at the wavelength of 600 nm was measured on a Nicolet Evolution 300 UV-VIS spectrometer every 8 h. Bacterial growth curves were obtained by plotting OD values *versus* time. All treatments were performed three times.

For the antibacterial activity test of bacterial cells, cell suspensions were adjusted to a concentration of 10^7–10^8 cells per mL. 200 μL cell suspensions of serial 10^6-fold dilutions with sterile distilled water were spread onto the medium plates, and the basic YMA medium was used as control. For the experimental conditions, the prepared YMA solid medium was heated to thaw out and cooled down to 45–55 °C. Different carbon-based material suspensions (AC, MWCNTs, SWCNTs and GO) were quickly added into this medium to obtain a final concentration of 50 or 500 μg mL^{-1}. Then, the medium and suspensions were mixed rapidly and spread onto the plates, which was intended to make the carbon-based materials highly disperse and effectively reduce aggregation in the medium. Colonies were counted and compared among different treated plates after growing for 48 h at 30 °C.

2.3 Plant growth and nodulation experiments

Plants of *Lotus japonicus* 'Miyakojima MG-20' were used in this experiment. Seeds were surface sterilized in concentrated sulfuric acid for less than 2 min with agitation, rinsed 5 times with sterile Milli-Q water, immersed in 10% sodium hypochlorite for 5 min with agitation, and then rinsed 10 times with sterile water. After that, the seeds were vernalized at 4 °C overnight before use.

To evaluate the effects of different carbon materials on nodulation, *Lotus japonicus* seedlings were planted into the "soil". This soil was composed of perlite and vermiculite at a 1 : 1 volume ratio without any nutrition (especially free of nitrogen), and was adequately blended by a soil mixer. After sterilization and vernalization, *Lotus japonicus* seeds were placed in a 50 mL flask with different 5 mL carbon material suspensions (50 or 500 μg mL^{-1} AC, MWCNTs, SWCNTs and GO, respectively) and 5 mL deionized water as control. Then, the seeds were cultured at 22 ± 1 °C in darkness for two days and in light (16 h-light/8 h-dark cycle at a light intensity of 150 μmol m^{-2} s^{-2}) for five days. After that, with the development of the radicle, the seedlings were gently removed to be grown in a pot filled with mixed soil watered with a one-half-strength Broughton and Dilworth (B&D) nitrogen-free medium, and were cultured at 22 ± 1 °C in growth cabinets with a 16 h-light/8 h-dark cycle at a light intensity of 150 μmol m^{-2} s^{-2}. Two days later, 1 mL carbon material suspension (50 or 500 μg mL^{-1} AC, MWCNTs, SWCNTs and GO, respectively) for each plant was added into the mixed soil and 1 mL deionized water was added for each plant as control. This step was repeated every seven days to ensure treatment efficacy.

Two days later, all these seedlings were inoculated with *Mesorhizobium loti* strain MAFF303099 for nodulation assays (or without rhizobia and using equal deionized water instead). Rhizobia were grown in YMA liquid medium for 48 h at 30 °C in a shaker (200 rpm). Then, the cells were collected by centrifugation at 6000 rpm for 5 min and washed twice with sterile distilled water. Subsequently, the cells were resuspended in water and adjusted to a concentration of 10^7–10^8 cells per mL. 1 mL cell suspension for each plant was added into the mixed soil. After 14 dpi, the plants were gently removed from the soil for washing and the nodule primordia and nodules were counted. At the same time, the length of the stems and roots of the seedlings were also measured carefully.

2.4 Experiments on MWCNTs

The following experiments were designed and conducted to further investigate the effect of MWCNTs on the rhizobium-legume symbiosis system.

2.4.1 Plant growth and nodulation experiments. To study the effect of MWCNTs on plants, sterile *Lotus japonicus* seeds were placed on a one-half-strength Broughton and Dilworth (B&D) nitrogen-free medium with/without MWCNTs (50, 100, 150, 200, 500 μg mL^{-1}). The seeds were cultured at 22 ± 1 °C in darkness and germination was observed every 12 h. After 48 h, these seedlings were allowed to continue to grow on the medium at 22 ± 1 °C with a 16 h-light/8 h-dark cycle at a light intensity of 150 μmol m^{-2} s^{-2}, and then the length of the stem and root of 7, 10, 14-d-old plants was measured. On the other hand, to evaluate the effect of different concentrations of MWCNTs on nodulation, similar experiments were carried out as mentioned above (section 2.3) with some minor adjustments. Instead, 1 mL MWCNT suspension at different concentrations (50, 100, 150, 200, 500 μg mL^{-1}) was added for each plant into the mixed soil medium and 1 mL deionized water was used for each plant as control. This step was repeated every seven days to ensure the treatment efficacy. Two days later, all these seedlings were inoculated with *Mesorhizobium loti* strain MAFF303099 for nodulation assays, which was resuspended in water to adjust to a concentration of 10^7–10^8 cells per mL. 1 mL cell suspension for each plant was added into the mixed soil. After 7, 10, 14, 21, 28 dpi, the seedlings were gently removed from the "soil" for washing and the nodule primordia and nodules were counted. At the same time, the length of the stem and root of the seedlings was measured and the vegetative biomass of plants at 21 and 28 dpi was quantified by electronic balance.

2.4.2 Nitrogenase activity measurement. Nitrogenase activity was examined as acetylene reduction activity (ARA).[38–40] To quantify the effects of MWCNT exposure on

nitrogenase activity in *Lotus japonicus* nodules, the reduction of acetylene (C_2H_2) to ethylene (C_2H_4) in the collected nodules of 28 dpi was measured as described below.

The amount of ethylene was measured using an East & West Analytical Instrument GC 4000A gas chromatograph. The gas chromatograph was configured as follows (injection: splitless, injector temperature: 60 °C, column: GS-Q 30 m × 530 μm × 20 μm, carrier: ultrahigh purity N_2, column flow: 3.9 mL min^{-1}, oven temperature: 120 °C, detector: flame ionization, detector temperature: 200 °C, H_2: 30 mL min^{-1}, air: 400 mL min^{-1}, makeup (N_2): 30 mL min^{-1}). The detection limit of the flame ionization detector was less than 3×10^{-11} g s^{-1} gas and the linear range of detection is 10^6. Before starting the test, the range of baseline fluctuation should be no more than 0.2 mV within 30 min.

C_2H_2 was generated by combining several grams of solid calcium carbide (CaC_2) with deionized water in a 1 L plastic flask. One end of a two sided needle was inserted into the flexible septum of the covered flask, and the other end of the needle was inserted into a collection bag, and the bag was filled with acetylene. The needle was removed from the bag and flask when the reaction was finished. Root nodules collected from individual plants were gently washed and the exact weight was recorded, and then the samples were placed into sterile 10 mL plastic jar. The lip was greased and the jar was sealed. 1 ml of air was removed from the jar with a gas-tight syringe and 1 ml C_2H_2 was injected into it, yielding a 10 mL 10% C_2H_2 mixture.

After the addition of C_2H_2, the sample jars were placed in low fluorescent light at 28 °C for 120 min, then 1 mL of gas from the jar was manually injected into the gas chromatograph equipped with a silica gel column. The oven temperature was held constant at 120 °C. Two distinct peaks were observed on the analytical software of gas chromatograph in the computer, with C_2H_4 having a shorter retention time compared with C_2H_2. Peak areas were estimated using an integration tool, and were converted into C_2H_2 and C_2H_4 concentrations using data from standard curves. The C_2H_4 production rate (μmol min^{-1}) was normalized to the total fresh mass of nodules in each sample (μmol min^{-1} g^{-1}).

2.4.3 Raman spectroscopy. The plants treated with/without 100 μg mL^{-1} MWCNTs at 14 dpi were gently removed from the soil for washing. Their clean root samples were cut open and washed with 0.1 mol L^{-1} pH 7.0 phosphate buffer to prepare them for the Raman-scattering analysis. Raman-scattering analysis was performed at room temperature using an inVia Raman spectrometer (Renishaw, UK) equipped with a confocal microscope (Leica, DM LM/P/11888500, Germany). Laser excitation of 633 nm (1.96 eV) was used for these studies and the laser beam intensity at the sample surface was 20 mW. Similarly, nodules from 14 dpi plants treated with/without 100 μg mL^{-1} MWCNTs were gently removed from the soil for washing and were treated as mentioned above.

2.4.4 Transmission electron microscopy. The roots and mature nodule tissues were observed by TEM to examine whether MWCNTs entered into the plant cells. 14 dpi plants treated with/without MWCNTs (100 μg mL^{-1}) were gently removed from the soil for washing, and their clean root samples were cut open, prefixed in 3.5% glutaraldehyde, washed with 0.1 mol L^{-1} pH 7.0 phosphate buffers, and post fixed in 1.0% osmium tetroxide. After fixation, the tissues were dehydrated in an ascending ethanol series, and embedded in Spurr's resin. Finally, thin sections were excised from the embedded samples using an ultramicrotome equipped with a diamond knife, and the ultrathin sections were mounted on copper grids for TEM examination. Similarly, nodules from 28 dpi plants treated with/without MWCNTs (100 μg mL^{-1}) were gently removed from the soil for washing and treated as above mentioned.

2.4.5 Real-time PCR analysis. Total RNAs were isolated from *Lotus japonicus* roots using Trizol reagent (Invitrogen). Primescript RT Reagent Kit (Takara) was used to eliminate genomic contamination of total RNAs and synthesize first strand cDNAs. qRTPCR was performed using the SYBR Select Master Mix (ABI) reagent. All PCR reactions were performed using an ABI ViiA 7 Real-Time PCR System under the standard cycling mode: 2 min at 50 °C for uracil-DNA glycosylase activation and 2 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. All of the expression levels were analyzed and normalized using the ubiquitin (*Ubi*) gene, which is constitutively expressed in *L. japonicus*. To compare the expression of nodulation genes treated with control, AC and MWCNTs, roots inoculated with/without rhizobia for 1, 3, 5, 7, 10, 14 d were harvested respectively for total RNA isolation and qRT-PCR analysis. Similarly, to compare the expression of nitrogenase genes treated with control, AC and MWCNTs, roots inoculated with/without rhizobia for 7, 10, 14, 21, 28 d were harvested respectively for total RNA isolation and qRT-PCR analysis. Primer sequences used in the expression analysis are shown in Tables S3 and S4.†

2.4.6 Rhizobial infection assay. For the rhizobial infection assay, the plants treated as mentioned above were inoculated with *M. loti* strain MAFF303099 constitutively expressing GFP. Five days after inoculation, the plants were gently removed from the soil for washing. The fluorescence of infection threads was observed using a Zeiss LSM510 laser scanning microscope.

2.5 Statistical analysis

Each experiment was performed with more than three replicates and arranged in a completely random design. The data were represented as mean values ± SE (standard errors). The statistical analysis of the experimental data was conducted using IBM SPSS Statistics software, and the statistical significance of the differences among the treatments was calculated using one-way analysis of variance (ANOVA) and covariance according to the Tukey–Kramer all-pair comparisons at a significance level of 0.05 ($p < 0.05$).

3. Results and discussion

3.1 Effect of CNMs on the growth of rhizobia and plants

To study the effect of carbon nanomaterials on the rhizobium-legume symbiosis system, we first investigated whether carbon CNMs could affect the legume plant and rhizobium separately (*Lotus japonicus* and *Mesorhizobium loti* MAFF303099 were used in this experiment).

Fig. 1A and B present the OD growth curves of *M. loti* incubated with aqueous dispersions of CNMs at high (500 μg mL^{-1}) and low (50 μg mL^{-1}) concentrations in YMA solution, respectively. It could be seen that the bacterial growth was clearly inhibited by 50 μg mL^{-1} GO or 500 μg mL^{-1} SWCNTs, suggesting that GO or SWCNTs at certain concentrations possess antibacterial activity against *M. loti*. However, MWCNTs and AC (as positive control, which is composed of carbon elements without nano structure) showed less toxicity to the bacteria, because whether at high or low concentrations, only slight or no significant difference was observed in the cell growth after treatment with these two materials. Furthermore, we also examined the survival rate of *M. loti* on YMA medium supplemented with different carbon materials at high and low concentrations. As shown in Fig. 1C and Fig. S2,† these carbon materials had no significant impact on the surface morphology of rhizobia after 48 h of growth. But the number of colonies was significantly decreased after treatment with 50 μg mL^{-1} GO or 500 μg mL^{-1} SWCNTs, which is in agreement with the results in the OD growth curves (Fig. 1D). Similarly, our

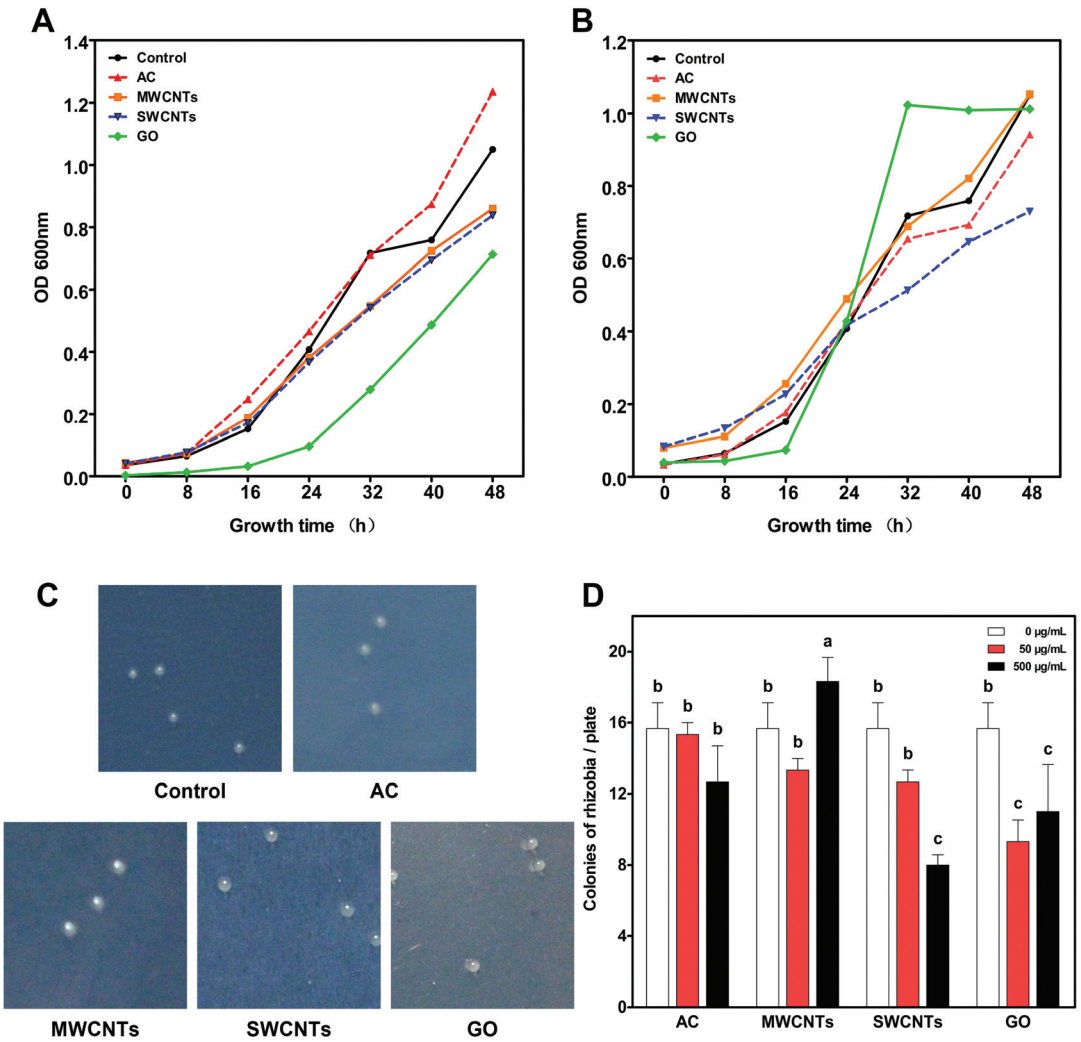

Fig. 1 Effects of different CNMs on the growth of rhizobia. OD growth curves of *M. loti* MAFF303099 in YMA solution at 30 °C after the cells were treated with AC, MWCNTs, SWCNTs and GO at (A) low (50 μg mL^{-1}) and (B) high (500 μg mL^{-1}) concentration for 2 h, respectively. (C) Phenotypes of rhizobia cultured for 48 h on standard YMA medium (control) or on YMA medium supplemented with 50 μg mL^{-1} AC, MWCNTs, SWCNTs and GO, respectively. 200 μL of serial 10^6-fold dilutions with sterile distilled water was spread onto YMA plates. (D) Colony-forming units of rhizobia cultured for 48 h on standard YMA medium (control) or on YMA medium supplemented with AC, MWCNTs, SWCNTs and GO at low (50 μg mL^{-1}) or high (500 μg mL^{-1}) concentration, respectively. Results are shown as average ± SE of two repeated experiments. Bars with different letters represent significant differences ($p < 0.05$).

previous study also showed that SWCNTs and GO have clear antibacterial effects on copper-resistant *Ralstonia solanacearum* to various degrees.[41,42] Because the survival of rhizobia is an important precondition for nodulation and nitrogen fixation, special attention should be paid to those CNMs that showed no or little effect on the growth of *M. loti* in this experiment, such as MWCNTs.

Compared with studying the effect of CNMs on rhizobia, the research about the effect of CNMs on legume plants is more complicated: on the one hand, the formation of nodules is usually more efficient in the soil than in the medium; on the other hand, we hoped to uncover the effect of CNMs on the rhizobium-legume symbiosis system under real planting conditions as far as possible. Therefore, *Lotus japonicus* seedlings were planted into the "soil" (a mixed soil medium containing perlite and vermiculite without nitrogen) supplemented with water soluble CNMs, AC and water (as control). Then, the growth of plants was monitored by measuring the stem and root length at the 14th day. Fig. 2A shows the growth of *Lotus japonicus* treated with different carbon materials at high (500 μg mL^{-1}) and low (50 μg mL^{-1}) concentrations. The results showed that whether at high or low concentrations, plant seedlings treated with MWCNTs exhibited a significantly higher stem elongation, while other carbon materials showed little effect on the stem (Fig. 2B). In contrast, the growth of roots was not promoted by MWCNTs, but was obviously inhibited by SWCNTs and GO, particularly in the plants treated with 500 μg mL^{-1} SWCNTs, whose root length was reduced by almost 30% compared with the control (Fig. 2C). Previously, some studies reported the positive or negative effects of different CNMs and their derivatives on plant physiology: both carbon nanotubes and their surface chemical functional groups can enhance the development of tomato plants, while water-soluble fullerenes were found to inhibit the growth of *Arabidopsis thaliana*.[34,43,44] Hence, based on these results and our experimental data, it can be inferred that the effects of CNMs on plants are mainly dependent on the type of carbon materials, plant species, and specific conditions of experiments, including the dose and duration of the treatment in the cultivation and the uptake mode of CNMs by the plant organisms.

3.2 Effect of CNMs on nodulation

To investigate whether CNMs could affect the nodulation in rhizobium-legume symbiosis system, seedlings of *Lotus japonicus*, which were planted in the soil and treated with water

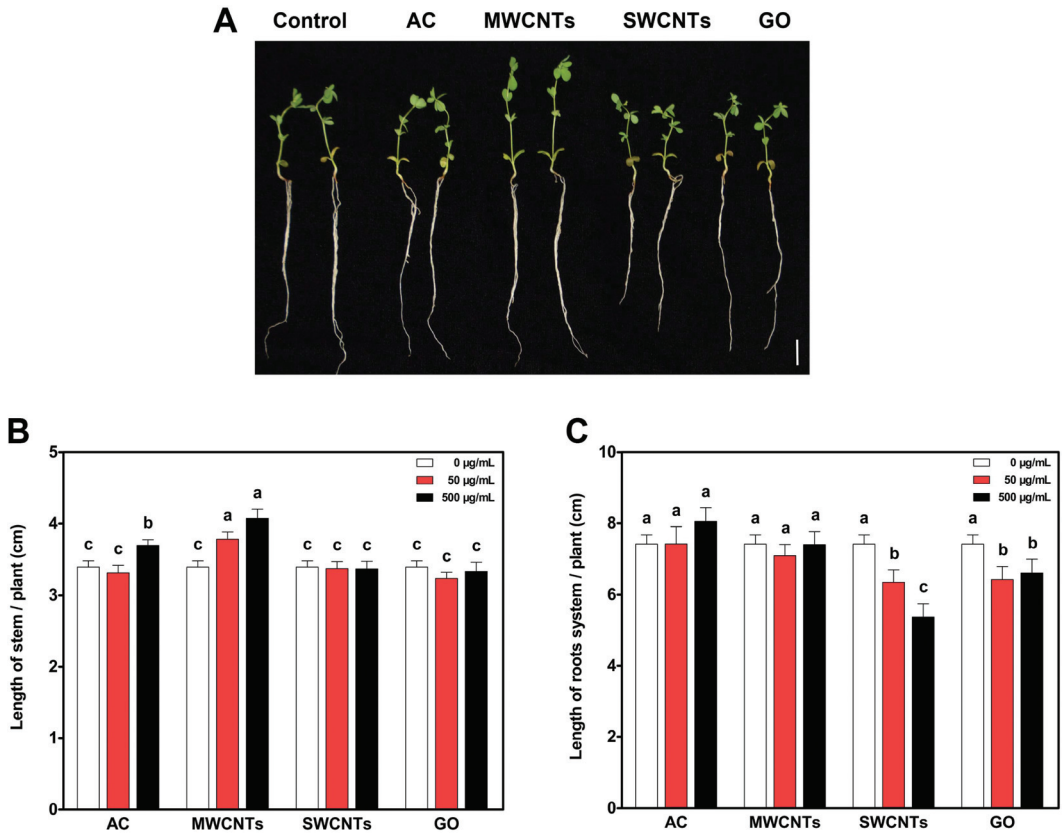

Fig. 2 Effects of different CNMs on the growth of legume plants. (A) Phenotypes of *Lotus japonicus* grown in soil treated with/without CNMs for 14 d. Plants from the left to the right were treated without any carbon material (control), or with AC, MWCNTs, SWCNTs and GO, respectively. Scale bar = 10 mm. (B) Length of stem and (C) root system of plants grown in soil treated without any carbon material (control), or with AC, MWCNTs, SWCNTs and GO at low (50 μg mL^{-1}) or high (500 μg mL^{-1}) concentration for 14 d, respectively. Results are shown as average ± SE of three repeated experiments, and $n \geq 25$ individual plants for each condition. Bars with different letters represent significant differences ($p < 0.05$).

soluble CNMs, AC and water, were inoculated with rhizobia *M. loti* MAFF303099. The subsequent nodule development was evaluated at 14 days post inoculation (dpi). Fig. 3A shows the growth of *Lotus japonicus* treated with different carbon materials at high (500 μg mL^{-1}) or low (50 μg mL^{-1}) concentration at 14 dpi, and Fig. 3B displays the nodulation on the plant roots under corresponding treatments. It can be roughly observed that compared with other treatments, MWCNT treatment resulted in more nodules in the plant (Fig. 3B). Statistical analysis of the nodule numbers demonstrated that

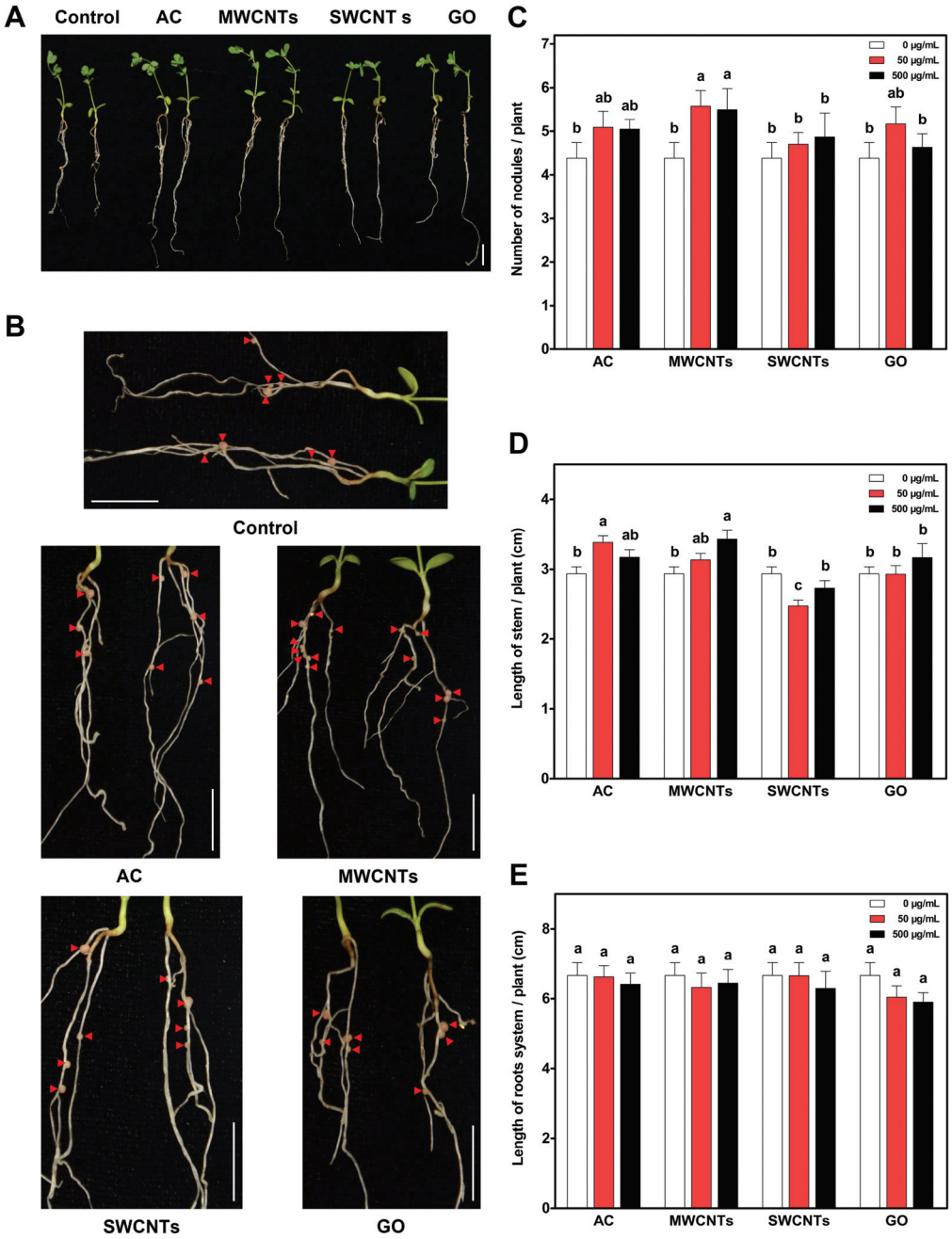

Fig. 3 Effects of different CNMs on the nodulation of legume plants. (A) Phenotypes of *Lotus japonicus* grown in soil treated with/without CNMs for 14 dpi. Plants from the left to the right were treated without any carbon material (control), or with AC, MWCNTs, SWCNTs and GO, respectively. Scale bar = 10 mm. (B) Phenotypes of nodules of plants grown in soil treated without any carbon material (control), or with AC, MWCNTs, SWCNTs and GO at low (50 μg mL^{-1}) or high (500 μg mL^{-1}) concentration for 14 dpi, respectively. Scale bar = 10 mm. Red triangles indicate nodules on the roots. (C) Number of nodules, (D) length of stem and (E) length of root system of plants grown in soil treated without any carbon material (control), or with AC, MWCNTs, SWCNTs and GO at low (50 μg mL^{-1}) or high (500 μg mL^{-1}) concentration for 14 dpi, respectively. Results are shown as average ± SE of three repeated experiments, and $n \geq 25$ individual plants for each condition. Bars with different letters represent significant differences ($p < 0.05$).

the amount of nodules on the roots treated with MWCNTs (about 5.5 nodules per plant) was increased by 35% compared with the control (about 4 nodules per plant) (Fig. 3C). On the other hand, the growth of the stem and root of plants inoculated with rhizobia under these treatments was also measured. Similar to the above results about the plants uninoculated with rhizobia, MWCNTs also resulted in increased stem length, particularly at high concentrations (Fig. 3D). It is interesting that SWCNTs and GO treatments did not show inhibitory effects on the growth of roots (Fig. 3E). We speculated that these results might be related to the formation of nodules, which might compensate for the growth inhibition of roots caused by these CNMs through biological nitrogen fixation.

So far, only a limited number of studies, which were mainly focused on metal nanoparticles, have reported the effects of nanomaterials on legume nodulation, and little was known about the impact of CNMs on the rhizobium-legume symbiosis system. It was found that when added to the soil in the form of powder, nano-ZnO did not affect the morphology of root nodules in soybean, and the number of nodules was similar to that of the control.[11] But recently, it was demonstrated that around the rhizosphere, the presence of homogeneous nano-ZnO suspension in the water would decrease root nodulation and cause early senescence of nodules in garden pea and its compatible bacterial partner, and similar results were also found in nano-TiO_2 treatment.[12,13] As an important organ that provides nitrogen to legumes, healthy and highly efficient nodules are critical for plant growth and productivity.[26] The current findings are almost all about the negative effects of nanomaterials on either the legumes or the rhizobia. However, different from previous studies, this study for the first time evaluated the effects of common carbon nanomaterials, including MWCNTs, SWCNTs and GO, on the rhizobium-legume symbiosis system. More importantly, we discovered that MWCNTs can promote nodulation in this system, which may facilitate the new application of CNMs in agriculture.[5]

3.3 MWCNTs enhance nodulation and promote nitrogenase activity

To further investigate the effect of MWCNTs on the rhizobium-legume symbiosis system, a series of gradient concentrations of MWCNTs were set up (50, 100, 150, 200, 500 μg mL^{-1}), and the variations of nodule development in different time periods were studied. Based on the observation of nodule phenotypes, MWCNTs did not affect nodule formation and development in different time periods, as no differences in phenotype or time of nodulation were observed between the control and MWCNT-treated nodules (Fig. S3†). However, compared with the control, plants treated with MWCNTs showed more nodules at 14 and 28 dpi, which is consistent with the results obtained in CNMs treatments (Fig. S4†). Furthermore, statistical analyses were performed on the numbers of nodules in plants treated by different concentrations of MWCNTs at different stages, including the early stages (7, 10, 14 dpi) and late stages (21, 28 dpi) (Fig. 4A). It was observed that 10 dpi later, plants treated with MWCNTs started to possess more nodules than the control, and this trend was continued and became more clear gradually with time, particularly at 14 dpi and 28 dpi, which well explains the above-mentioned observations. For example, under 100 μg mL^{-1} MWCNT treatment, there were 7.7 nodules per plant at 14 dpi and 9.5 nodules per plant at 28 dpi, both of which were about 40% higher than that of the control (5.5 nodules per plant at 14 dpi and 6.8 nodules per plant at 28 dpi) (Fig. 4A).

Additionally, we investigated the nitrogenase activity in the nodules, as the variation of nitrogen fixation could indicate the effects of MWCNTs on the N_2 fixation apparatus. Interestingly, it was observed that at 28 dpi, MWCNT-treated plants showed a more vigorous development than the control, suggesting that MWCNTs might positively affect the nitrogen fixation of nodules (Fig. S4†). To test whether MWCNTs could affect the nitrogen fixation in mature nodules, the nitrogenase activity of root nodules at 28 dpi was measured by the reduction of acetylene to ethylene. As presented in Fig. 4B, nitrogen fixation was enhanced in the root nodules exposed to MWCNTs at 28 dpi, and the nitrogen fixation ability was significantly elevated by 100, 150 and 200 μg mL^{-1} MWCNTs. On the other hand, the plant biomass under MWCNT-treatments was also measured, which, as an indirect indicator, may reflect the changes of nitrogen fixation to a certain extent. Fig. 4C and D shows the vegetative biomass of plants treated by MWCNTs at 21 and 28 dpi. The results showed that almost all concentrations of MWCNTs resulted in a dramatic increase in vegetative biomass. The fresh weight of the below-ground (roots and nodules) and the above-ground (stems and leaves) body of the plants germinated and grown with MWCNTs was about 20% higher than that of the control (Fig. 4C and D). We also attempted to measure the dry weight of plants at 21 and 28 dpi. But when the plants were dried, it was hard to determine the weight because of the too small mass of the plants, which would produce inaccurate results (data not shown). On the other hand, it was found that the stem length of plants was increased by MWCNTs, but the root length was unchanged (Fig. S5†). Both results are consistent with the results observed in the plants treated with different CNMs. It is noteworthy that in the plants uninoculated with rhizobia, MWCNTs still could stimulate the development of the plant stem in both the soil and the YMA medium (Tables S1 and S2†). However, all these effects are not dependent on the concentration of MWCNTs, and 100 μg mL^{-1} MWCNTs was regarded as the most favorable concentration for the rhizobium-legume symbiosis system considering both the dosage and effect in this experiment.

Previously, some studies indicated that MWCNTs possess positive effects on plant physiology: MWCNTs are able to promote the seed germination and plant growth in tomato, and can also enhance the growth of tobacco cells by activating water channels (aquaporins) and the major gene regulators of cell division and extension.[34,35] Recently, we have also found that the root elongation of wheat plants can be enhanced by MWCNTs at different concentrations.[37] On the other hand, nanomaterials may have the potential to improve the biological nitrogen fixation in plants, because it has been reported

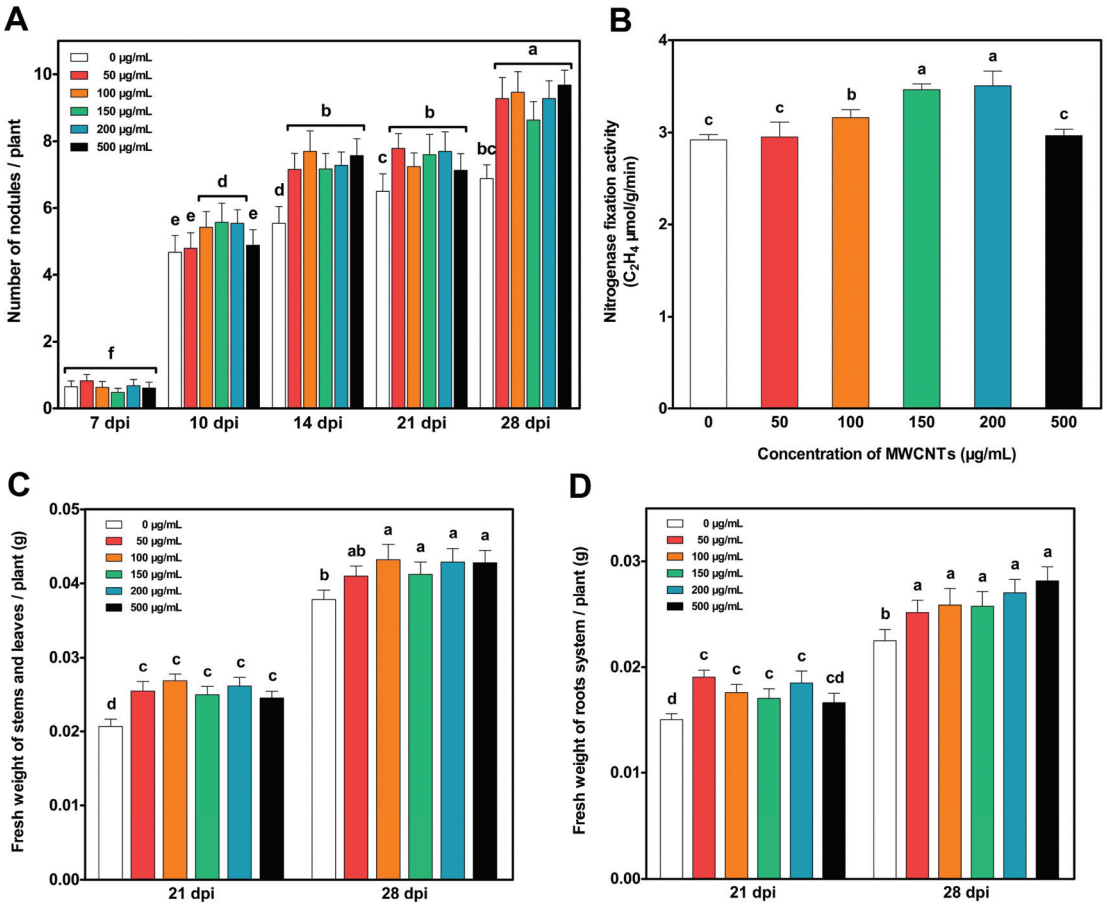

Fig. 4 Effects of MWCNTs on the nodulation and nitrogenase activity of legume plants. (A) Number of nodules per plant grown in soil treated with/without MWCNTs for 7, 10, 14, 21 and 28 dpi, respectively. (B) Nitrogenase fixation activity of nodules grown in soil treated with/without MWCNTs for 28 dpi. (C) Fresh weight of root system and (D) stems and leaves of plants grown in soil treated with/without MWCNTs for 21 and 28 dpi, respectively. Results are shown as average ± SE of three repeated experiments, and $n \geq 25$ individual plants for each condition. Bars with different letters represent significant differences ($p < 0.05$).

that nano-anatase TiO_2 exposed to sunlight could chemisorb N_2 directly or reduce N_2 to NH_3 in spinach leaves, which is transformed into organic nitrogen to improve the growth of spinach.[45] The results of this study also confirm these two findings, as we found that MWCNTs have positive effects on both the nodulation and nitrogen fixation in rhizobium-legume symbiosis system. Most symbiotic legumes can absorb nitrogen from the nodules, in which gaseous nitrogen is converted into ammonia by nitrogenase. In our experiments, besides nodulation and nitrogenase activity, MWCNTs also greatly affected the growth of legumes. Thus, the accelerated growth of plants demonstrates that MWCNTs can facilitate the production of more nodules and higher nitrogenase activity. More importantly, compared with most metal nanomaterials which would accumulate or form by-products in the cells, MWCNTs, a nanomaterial composed of carbon elements, did not change the composition of carbon and nitrogen in the plants (Fig. S6†). Meanwhile, we speculated that the observed induction of nitrogenase activity is due to the uptake of MWCNTs into root cells and nodule cells.

3.4 MWCNTs penetrate the cell wall into the cytoplasm

The interaction between MWCNTs and the plants (mainly roots and nodules) was investigated with Raman spectroscopy and additionally by transmission electron microscopy (TEM), which are two important approaches to demonstrate the functional mechanism of MWCNTs in rhizobium-legume symbiosis system. Due to the specific D band and G band intensity of carbon nanotubes, the detection of Raman spectroscopy could indicate the existence of MWCNTs in root and nodule cells.[46–48] As shown in Fig. 5A, it is obvious that the presence and relative intensity of the D band (1330 cm^{-1}) and G band (1581 cm^{-1}) were associated with the existence of MWCNTs in the root cells (blue line). Meanwhile, similar results could be obtained in nodule cells through Raman spectroscopy (red line), which is an interesting finding in cells composed of plant and microbial cells together (Fig. 5B). Raman analysis did not show any similar peak in the spectra of the control roots (black line). Additionally, to study the uptake of MWCNTs by plant cells in this symbiosis system, TEM analysis

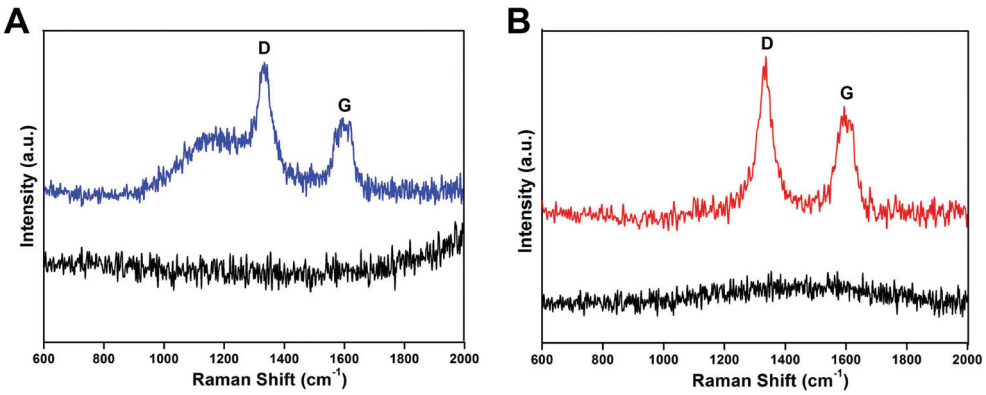

Fig. 5 Detection of MWCNTs inside root cells (A) and nodule cells (B) incubated with MWCNTs by Raman spectroscopy. Blue line indicates Raman analysis of the root cells exposed to MWCNTs, red line indicates Raman analysis of the nodule cells exposed to MWCNTs and black line indicates Raman analysis of control root or nodule cells exposed to MWCNTs (carbon nanotube-specific 1350 cm^{-1}-D band and 1580 cm^{-1}-G band).

was performed to reveal the ultrastructure of root and nodule cells grown with/without MWCNTs. Compared with the control (Fig. 6A), the presence of clustered carbon nanotubes was clearly observed in the root cells of MWCNT-treated plants (Fig. 6B–D). Under High-magnification view of the root cells, MWCNTs could be detected in various parts of root cells, including the cell wall (Fig. 6F), cell wall interval (Fig. 6G) and cytoplasm (Fig. 6H), indicating that MWCNTs can penetrate the cell wall and get into the cytoplasm. On the other hand, the microscopic structure of nodules was also detected by TEM (Fig. 7). The results showed that a certain number of bacteroids were surrounded by peribacteroid membrane in both the control and MWCNT-treated nodules (Fig. 7A and B). Whether the plants were treated with or without MWCNTs, the morphology of nodule cells and bacteroids was complete to ensure the normal nitrogen fixation capability (Fig. 7C and D). It is interesting that part of MWCNTs entered into the nodules and existed in the interval between the cell wall of two nodule cells (Fig. 7G), but whether MWCNTs could penetrate the peribacteroid and get into bacteroids is still not clear. In general, these findings through TEM are in line with the results of the Raman analysis, and suggest that MWCNTs do not change or destroy the basic structure of cells, demonstrating relatively weaker effects of MWCNTs on the physiological structure of roots and nodules from a different perspective. Actually, it has been reported that compared with other nanomaterials which also can penetrate into the cells, MWCNTs present less toxicity and do not destroy the structure or change the morphology of cells, and even bring some positive effects on the treated subjects in some studies.[35,49]

Previous studies have reported that CNMs are able to penetrate into plant and animal cells as well as into microorganisms.[33,50,51] Their results indicated that MWCNT internalization can be visualized by both Raman spectroscopy and transmission electron microscopy in the cells of tomato, tobacco, soybean and so on.[35,36,52] Also these findings are in agreement with what we previously found in wheat plant cells.[37] Some researchers proposed that water soluble MWCNTs may also be absorbed into root cells through the water channel when the roots assimilate nutrients and water from outside.[53] On the other hand, through investigating the capability of carbon nanotubes to penetrate the cell membrane of plant protoplasts, some researchers proposed that MWCNTs could be a tracer to target specific cellular substructures in plant cell biology.[54] Through our TEM imaging, it can be speculated that MWCNTs are first adsorbed on the root surface and a part of them penetrate the cell wall, then pierce through the cell membrane, and are finally transmitted into the cytoplasm. Different from the single cell sensitive to nanomaterials,[55] plant roots have more sophisticated protection mechanisms to select exogenous substance so that only part of MWCNTs can penetrate into part of root cells, which could well explain why there were no differences in the effect of MWCNT treatments at different concentrations in some studies as well. On the other hand, there were no differences in ultrastructure between the control and MWCNT-treated nodules. But it is unclear whether MWCNTs are absorbed by the root cells, or brought by rhizobia into nodules or directly absorbed by nodules. Besides, a previous study has demonstrated the uptake, accumulation and generational transmission of carbon nanoparticles in rice plants, which finally affect plant reproduction.[55] On the basis of these findings, it could be speculated that the uptake of MWCNTs and the interactions between them and root or nodule cells have the potential to induce significant responses at the genetic level.

3.5 MWCNTs regulate gene expression of nodulation and nitrogen fixation

The promotion of nodulation and nitrogen fixation together with the invasion into root and nodule cells suggest that MWCNTs could lead to the variations of symbiotic nitrogen fixation at the genetic level. Therefore, the expression of relevant genes was analyzed subsequently to examine the possible impact of MWCNTs on the molecular mechanism of symbiotic nitrogen fixation. At present, many studies have reported that the nodulation in legumes is mainly regulated by particular

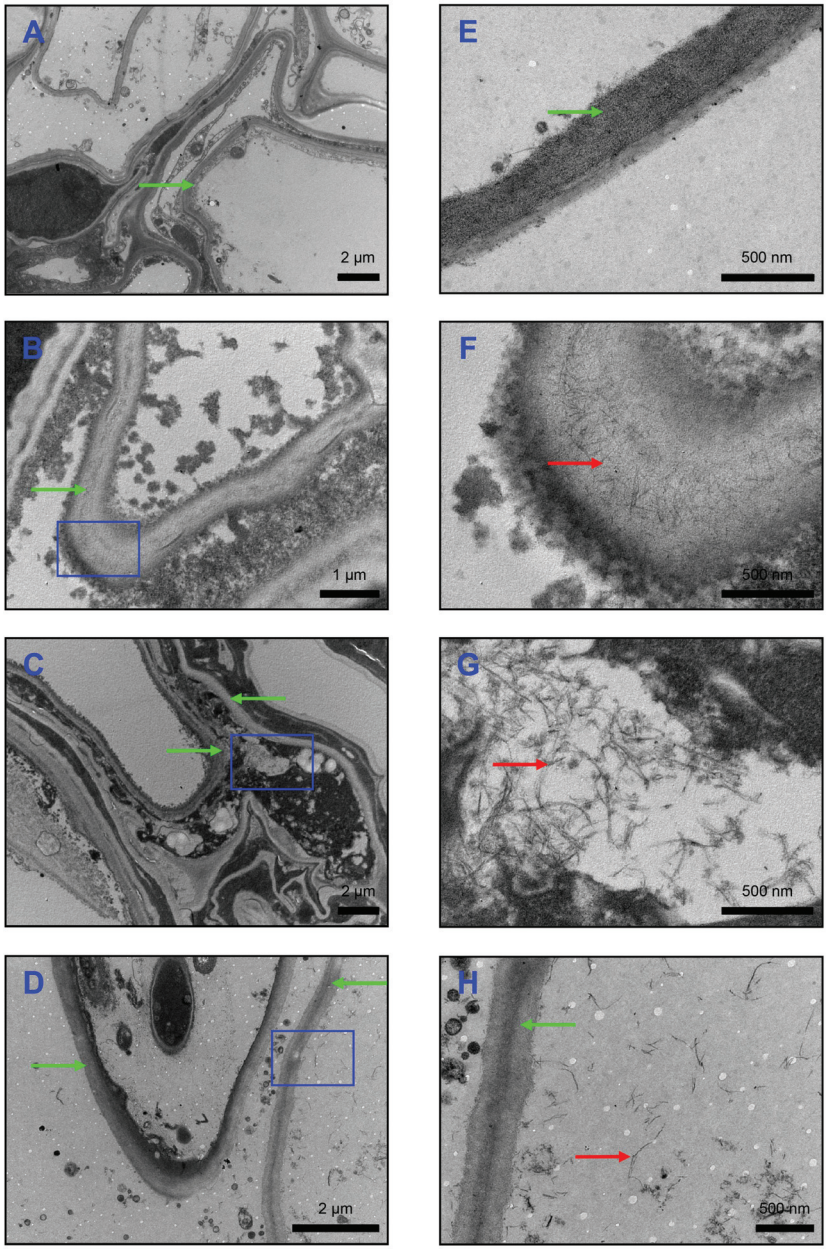

Fig. 6 Transmission electron microscopy images of root cells treated with/without MWCNTs. (A) Low-magnification view of root cells treated without MWCNTs (control) and (B)–(D) with MWCNTs. (E) High-magnification view of root cells treated without MWCNTs (control) and (F)–(H) with MWCNTs, which shows that MWCNTs existed in the root cells and were distributed in the cell wall, cell wall interval and cytoplasm, respectively. Red arrows indicate MWCNTs, green arrows indicate cell wall of root cells, and blue boxes indicate the enlarged region.

genes *via* a series of signaling pathways,[56–59] mainly including *NFR1* and *NFR5*,[60,61] *SYMRK*,[62] *NENA*, *NUP85* and *NUP133*,[63] *CASTOR* and *POLLUX*,[64] *CCaMK*,[65,66] *CYCLOPS*,[67] *NSP1* and *NSP2*,[68,69] and *NIN*,[70,71] which are essential for the formation of infection threads and the morphological development of nodules.[72] With the development of nodules, the nitrogen fixation genes (*nif* and *fix*) synthesize nitrogenase, which is responsible for atmospheric nitrogen fixation.[25]

Based on the model signaling pathway of nodulation in *Lotus japonicus*, the expression of twelve genes essential for nodulation mentioned above (except for *NENA*) was detected by the quantitative real-time PCR method in the roots treated with 100 μg mL^{-1} MWCNTs or AC when the plants were inoculated with/without rhizobia in different periods. As a result, two changing patterns in the expression of these genes under MWCNT- or AC-treatment could be observed. With/without the inoculation of rhizobia, the expression of some genes (such as *NFR1* and *NFR5*) showed insignificant fluctuations (0.5-fold to 1.5-fold) after MWCNT- (Fig. 8A and B) or AC- (Fig. S7A and B†) treatment compared with that of the control in corres-

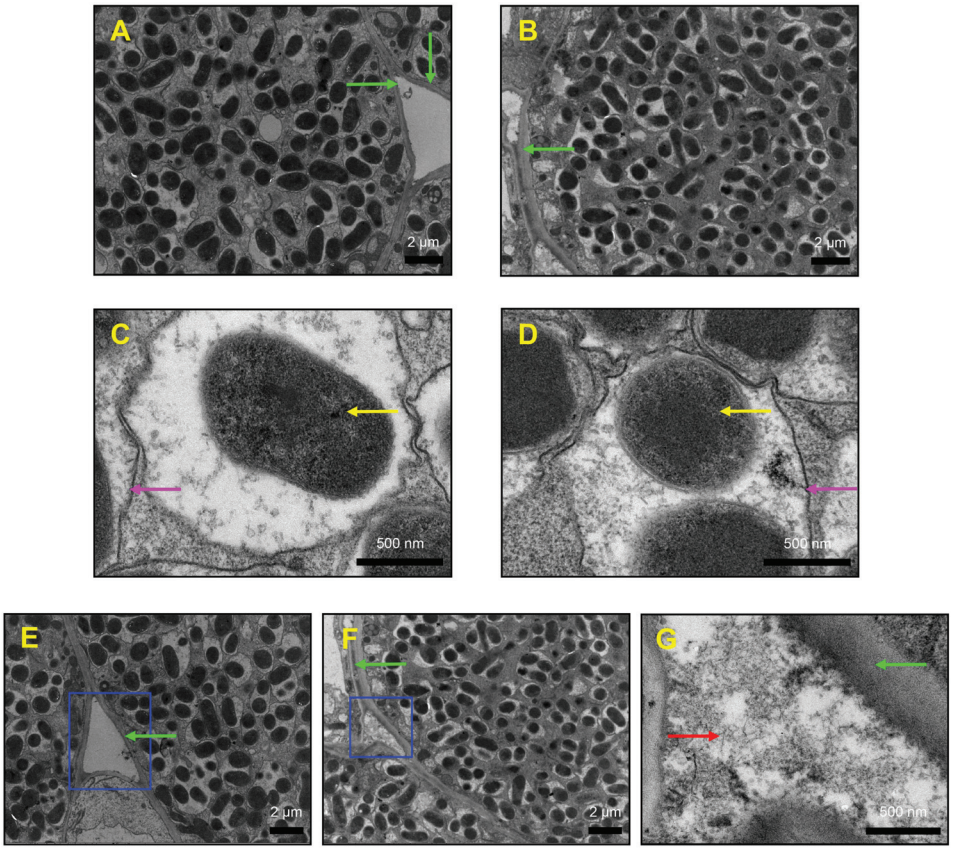

Fig. 7 Transmission electron microscopy images of nodule cells treated with/without MWCNTs. (A) Low-magnification view of nodule cells treated without MWCNTs (control), (B) with MWCNTs. (C) High-magnification view of nodule cells treated without MWCNTs (control), (D) with MWCNTs. (E) Low-magnification view of the interval between the cell wall of two nodule cells (blue boxes) treated without MWCNTs (control), (F) with MWCNTs. (G) High-magnification view of the interval between the cell wall of two nodule cells treated with MWCNTs, which shows that MWCNTs existed in the interval. Red arrows indicate MWCNTs, green arrows indicate cell wall of nodule cells, yellow arrows indicate bacteroids, and pink arrows indicate peribacteroid membrane.

ponding stages. On the other hand, in both uninoculated and inoculated roots, the expression of some other genes was up-regulated after MWCNT treatment, such as *NUP85*, *NUP133* and *NIN*, whose expression was elevated to more than 1.5-fold in most time periods (Fig. 8A and B). Similar results were also found in the roots treated with AC (Fig. S7A and B†), but by comparison, the up-regulation of such genes was not as dramatic as that under MWCNT-treatment. Among these up-regulated genes, *NIN* is an essential gene to directly regulate the number of nodules. As shown in Fig. 8C, the expression of *NIN* began to increase at 5 dpi in MWCNT-treated roots, and this increase lasted until 14 dpi. Compared with that of the control at 7 or 10 dpi, the expression of *NIN* was significantly elevated to nearly 2-fold, which is consistent with the increase of nodules in MWCNT-treated roots inoculated with rhizobia. Interestingly, the expression of the *NIN* gene was also promoted by MWCNTs in the roots without inoculation of rhizobia (Fig. 8C). However, these results could not be observed in AC treatments: whether with/without inoculation of rhizobia, the expression of the *NIN* gene was similar to that of the control (Fig. S7C†). These findings clearly demonstrate the specific promotion of MWCNTs on the nodulation in legume. Additionally, a similar analysis was also made of the genes that synthesize nitrogenase in nodules. Compared with that of the control, the expression of four *nif* genes in MWCNT-treated roots was elevated in early periods (7, 10 dpi), but was subsequently reduced in late periods (14, 21 and 28 dpi) (Fig. 8D). For example, the expression of *nif*H was enhanced in MWCNT-treated roots at 7 dpi; but at 14 dpi, it was significantly reduced by more than 2-fold, which lasted until 21 dpi (Fig. 8E). This phenomenon may be related to the enhancement of nitrogenase activity caused by MWCNTs, because more nitrogen oxides are catalyzed into ammonia in nodules, resulting in a feedback inhibition of the expression of *nif* genes.[73–75] The overall effect of AC treatment on *nif* genes was much less significant compared with that of MWCNT treatment (Fig. S7D†), though the expression of *nif*H was also found to be reduced in AC-treated roots at 14 dpi (Fig. S7E†).

Some studies have been focused on the interactions between nanomaterials and all kinds of living organisms at the molecular level in the past few years. Khodakovskaya *et al.* noted that many genes activated or inhibited by MWCNTs in

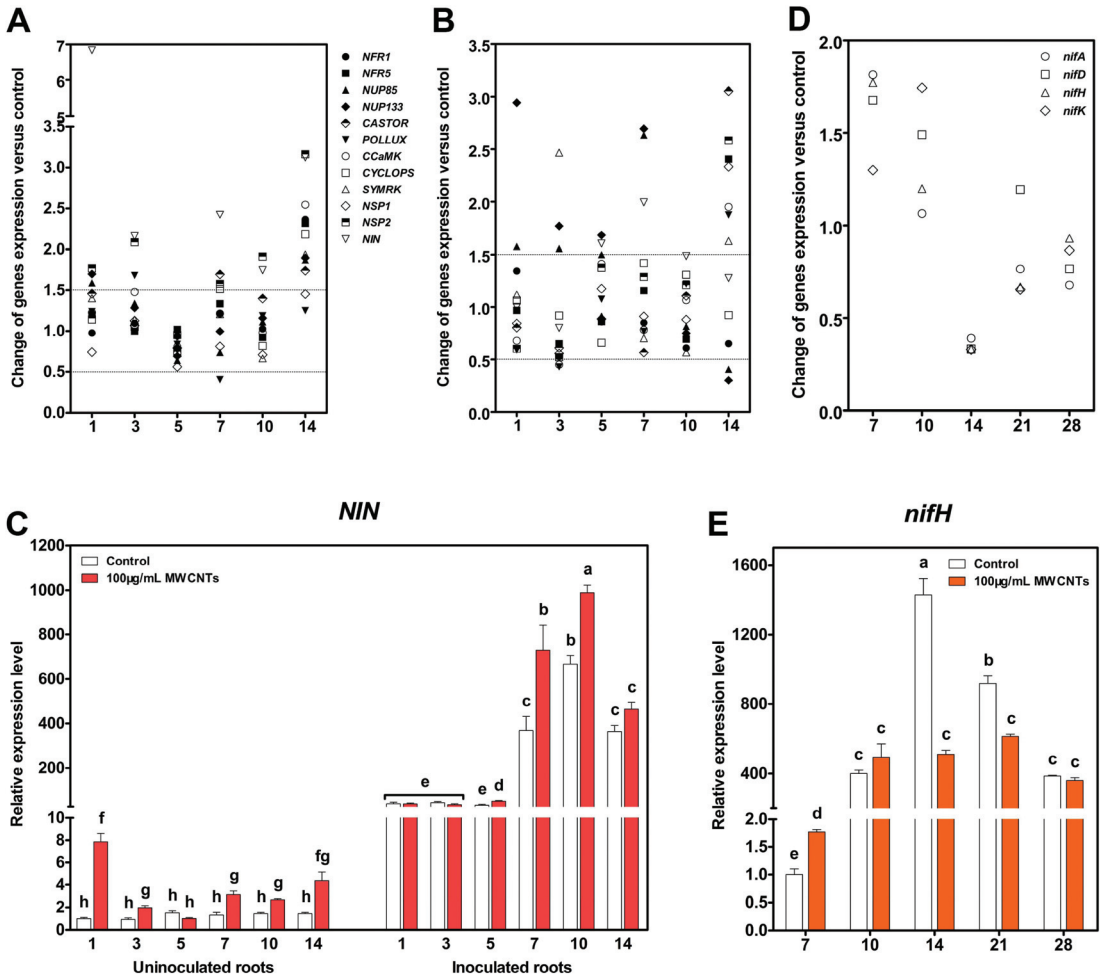

Fig. 8 Effect of MWCNTs on gene expression of nodulation and nitrogenase. Scatterplots of expression levels of early nodulation genes in (A) uninoculated and (B) inoculated roots treated with 100 μg mL^{-1} MWCNTs in different periods *versus* corresponding blank control. The dotted line indicates 0.5 and 1.5 of gene expression threshold *versus* blank control in the corresponding periods. (C) Relative expression levels of *NIN* in the root of plants uninoculated or inoculated with rhizobia and grown in soil treated with/without 100 μg mL^{-1} MWCNTs in different time periods. The ubiquitin gene was used as an internal control and the expression level was compared with that of the uninoculated control roots of 1 d. (D) Scatterplots of expression levels of nitrogenase genes in inoculated roots treated with 100 μg mL^{-1} MWCNTs in different time periods *versus* corresponding blank control. (E) Relative expression levels of *nifH* in root of plants inoculated with rhizobia and grown in soil treated with/without 100 μg mL^{-1} MWCNTs in different time periods. The ubiquitin gene was used as an internal control and the expression level was compared with that of the inoculated control roots of 7 d. Relevant measurement was implemented on quantitative PCR machine using the PCR array kit. Relevant results are shown as average ± SE of three repeated experiments. Bars with different letters represent significant differences ($p < 0.05$).

tomato plants are involved in plant stress-signal transduction and can be regulated by specific environmental stress.[52] They also found that in tobacco cells, both *NtLRX1* and *Cyc*B genes, which are involved in the regulation of cell cycle progression, exhibited the highest expression when the cells were treated with MWCNTs.[30] Consistently, our results also confirm that MWCNTs can affect the molecular mechanism of the interactions between microorganisms and plants, which could up-regulate specific genes in the signaling pathway of nodulation and eventually lead to increased root nodule number in *Lotus japonicas*. It is noteworthy that the up-regulation of these genes by MWCNTs is more dependent on the spatial-temporal expression of genes in this process. When the plants were inoculated with rhizobia, genes in the upstream of signaling pathway, such as *NUP85* and *NUP133*, were first up-regulated by MWCNTs in the earlier periods; afterwards, with the signal transduction over time, the expression of the downstream genes, such as *NSP1*, *NSP2* and *NIN*, was gradually increased in the later periods. Similar results were also obtained in the study of ZnO NPs in animal cells, which activate ER stress-responsive pathway, and the ER stress response might be used as an earlier and sensitive end point for nanotoxicological study.[76] Therefore, the gradual signal amplification brought by MWCNTs to the genes in the nodulation signaling pathway might be an important reason for the increase of nodules in legumes. This speculation was also well demonstrated by the study of MWCNT-treated infection threads of *Lotus japonicus*, an important phenotype of nodulation in early periods. As

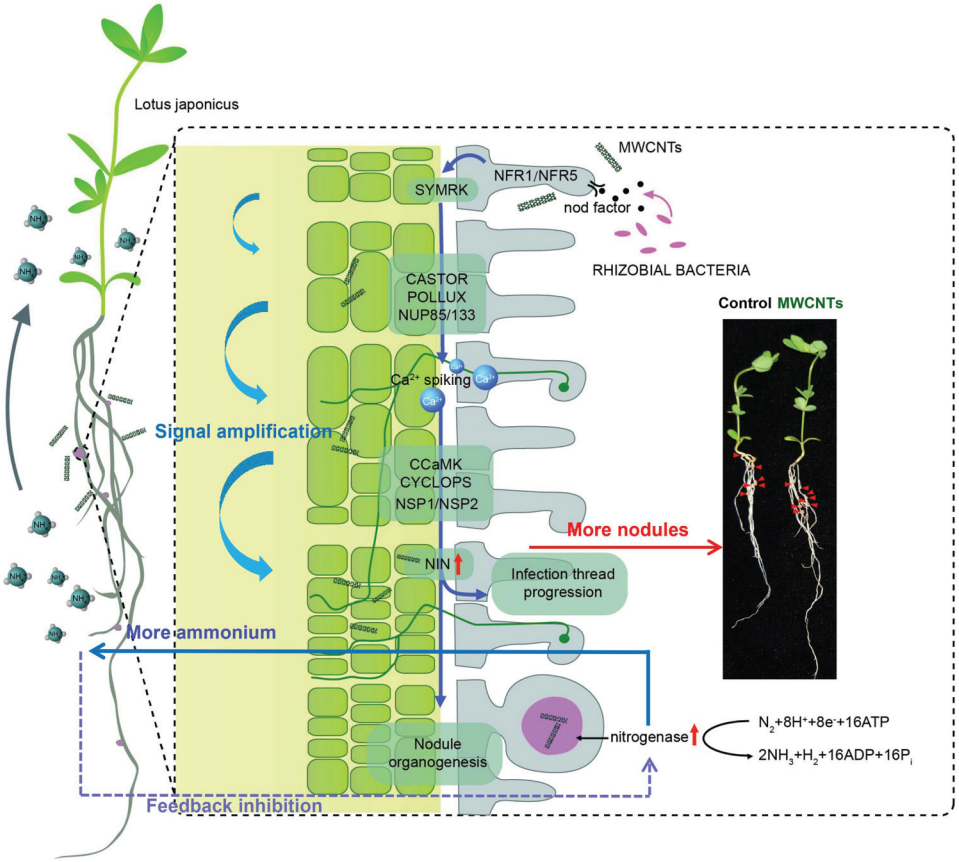

Fig. 9 Mechanisms of the positive effect of MWCNTs on the rhizobium-legume system. Schematic representation of the proposed mechanism how MWCNT treatment influences the nodulation signaling pathway and the biological nitrogen fixation in *Lotus japonicus*.

shown in Fig. S8A,† whether the plants were treated with control, AC or MWCNTs, infection threads could be normally formed on the roots of 5 dpi plants. However, the average density of infection threads on the MWCNT-treated roots was higher than that on the control or AC-treated roots, suggesting that MWCNTs are conducive to the invasion of rhizobia and initiate the expression of relevant genes in early nodulation (Fig. S8B†). Different from the effect of MWCNTs on directly regulating the genes in the nodulation signaling pathway, MWCNTs showed a more indirect effect on the structural genes of nitrogenase. Through the feedback inhibition caused by high-concentration ammonia, MWCNTs finally decrease the gene expression of nitrogenase while the activity of this enzyme is enhanced.

In conclusion, we hypothesized a model that can well illustrate the promotion effect of MWCNTs on rhizobium-legume symbiosis system in *Lotus japonicus* (Fig. 9). Water soluble MWCNTs are adsorbed on the root surface, and part of them penetrate into the cell wall and are further transmitted into the cytoplasm. When plant roots are inoculated with rhizobia to start nodulation, the expression of relevant genes is induced in the root cells. The existence of MWCNTs will effectively promote the expression of some genes in the signaling pathway of nodulation and gradually improve the signaling transduction of nodulation. Along with the downstream signal transmission in this process, the expression of *NIN* is finally up-regulated to increase the number of nodules. On the other hand, nitrogenase is synthesized to convert gaseous nitrogen into ammonia with the maturation of nodules. Because of the enhancement of nitrogenase activity by MWCNTs, more nitrogen oxides are catalyzed into ammonia in the nodules. Ultimately, the positive effect of MWCNTs on nodulation and nitrogen fixation promotes the growth of plants and increases the biomass.

4. Conclusions

In this study, we investigated the effect of four carbon-based materials on the rhizobium-legume symbiosis system of *Lotus japonicus*. Our results show that only MWCNTs have positive effects on this system as compared with AC, SWCNTs and GO. MWCNTs can effectively increase the number of nodules and enhance nitrogenase activity to promote nitrogen reduction in the nodules, which can be confirmed by the better growth and development of plants indicated by the increase in plant biomass. Further studies indicate that after penetrating into the root and nodule cells, MWCNTs could up-regulate the

expression of certain genes in the nodulation signaling pathway and lead to the gradual signal amplification in this physiological process, particularly the up-regulation of the *NIN* gene, which well explains the increase of nodules in this experiment. These findings facilitate a further and better understanding of the function of carbon nanomaterials in a complicated physiological process involving the interaction between plants and microorganisms. It is more important that with these simple methods and effective treatments, the positive effect of MWCNTs on legume plants provide an opportunity to solve the problems of ineffective invasion of rhizobia and low nitrogenase activity caused by the lack of microelements (*i.e.* molybdenum), which could increase the yield of legumes in practical production. On the other hand, biological nitrogen fixation capacity can be promoted by MWCNTs under normal circumstances, which can enhance the availability of nitrogen in the soil, and thus largely reduce the consumption of fertilizer to protect terrestrial resources. However, this hypothesis has been confirmed only in our limited experiments, and environmental safety must be taken into account when carbon nanotubes are used in the cultivation of food crops. Moreover, it should be noted that once CNTs are applied in legumes such as soybean, these nanomaterials will accumulate in the plants and gradually enter into the life activities of animals and human through the food chain. Thus, the influence of consumption of these contaminated plants on animals or humans must be clarified. Finally, whether MWCNTs could be used as an inorganic additive in agriculture needs further investigation from various perspectives, and we expect that the data presented in this study would further stimulate research interest on the practical application of various nanomaterials in agriculture.

Conflict of interest

The authors declare no competing financial interest.

Acknowledgements

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