PHYTOTOXICITY OF CARBON NANOTUBES IS ASSOCIATED WITH DISTURBANCES OF ZINC HOMEOSTASIS

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Keywords: soybean, common bean, maize, seedling development, nutrient availability, multi-walled carbon nanotubes, nanomaterials.

Effects of short-term seed treatments with multi-walled carbon nanotubes (MWCNTs) on seedling development in soil culture and of root-exposure in hydroponics were studied on soybean, considered as model plant system. At 8 days after sowing and in later stages of seedling development, stunted growth and poor fine root production were detected. More detailed investigations revealed zinc (Zn) deficiency as a major growth-limiting factor. The growth of affected plants was recovered by foliar application of $ZnSO_4$ or by cultivation in nutrient solution supplied with soluble $ZnSO_4$. Since Zn is an important co-factor of enzymes involved in detoxification of reactive oxygen species (ROS), such as copper-zinc superoxide dismutases, stunted plant growth in response to MWCNTs treatments may be related to oxidative damage associated with lipid peroxidation and excessive oxidative degradation of auxin as growth hormone important for lateral root formation and leaf expansion.

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Introduction

Carbon nanotubes (CNTs) are currently among the most important additives for composite materials with a predicted market increase up to USD 5.91 billion by 2018.[1] During manufacturing, utilization and disposal, these materials are intentionally or by chance released into the environment.[2-6] Due to a very high volume to surface ratio, nanomaterials can exhibit novel properties leading to yet unknown environmental interactions,[7-9] complicating predictions on the fate of engineered nanoparticles released into environmental compartments, such as soil, air, and water. There are still controversial discussions whether nanoparticles exert beneficial or adverse effects on living organisms, on their ability to penetrate living tissues/barriers, and incorporation into food chains.[10–12] Accordingly, in the recent past, various studies addressed the impact of metal-based[13,14] as well as carbon-based nanomaterials[15–21] also on germination and early growth of higher plants. The resulting findings are frequently controversial, with positive as well as negative effects of CNTs, depending on plant species, source of CNTs, their physico-chemical properties, applied concentrations of nanotubes and the culture systems.

The effects of MWCNTs on seed germination and seedling development were studied in various plant species, such as tomato (*Solanum lycopersicum* L.),[15,16] radish (*Raphanus sativus* L.), rapeseed (*Brassica napus* L.), ryegrass (*Lolium perenne* L.), lettuce (*Lactuca sativa* L.), maize (*Zea mays* L.), cucumber (*Cucumis sativus* L.),[17] zucchini (*Cucurbita pepo* L.)[18] and others. In some cases MWCNTs did not affect germination rates but in different ways influenced further seedling development. Ghodake et al.[19] reported no effect of MWCNTs at 10−40 mg L^{-1} on germination of mustard (*Brassica juncea* L.) and gram (*Vigna mungo* (L.) Hepper) but root elongation of mustard seedlings was doubled as compared to the control at 20 mg L^{-1}, while higher concentrations had inhibitory effects on root hair formation. In the majority of studies, which focused on the influence of nanoparticles on seedling development, artificial growth media or hydroponic culture systems where employed but experiments with soil-grown plants are rare. Begum et al.,[20] Begum and Fugetsu [21] and Stampoulis et al.[18] reported negative effects of MWCNTs added to a Hoagland nutrient solution in concentrations up to 2000 mg L^{-1} on the development of various plant species, namely red spinach (*Amaranthus tricolor* L.), lettuce, cucumber and zucchini, while chili (*Capsicum annuum* L.), okra (*Abelmoschus esculentus* (L.) Moench) and soybean (*Glycine max* (L.) Merr) remained unaffected.

From the eco-toxicological point of view it is very important to uncover the mechanisms of interactions between nanomaterials and plants, since plants are an important component of ecosystems, exhibiting close interactions with other living organisms as well as with inorganic components such as air, soil and water. Moreover, numerous applications are under development using nanomaterials for the development of novel plant growth stimulators, fertilisation and plant protection.[22] Therefore, investigation of genotypic differences and identification of the most MWCNT-sensitive plant species and cultivars, as well as determination of toxic thresholds under different environmental conditions is urgently needed. However, the high variability of reported results and a wide range of different types of CNTs, makes comparisons difficult.

In a previous pilot study[23], we have investigated the responses of three crop species (soybean; common bean, *Phaseolus vulgaris* (L.); and maize) to short-term seed exposure (36 h) of a defined industrial MWCNT batch applied at a low (50 µg $seed^{-1}$) and high (500 µg $seed^{-1}$) dosage in a standardised germination test under controlled environmental conditions according to the rules of the International Seed Testing Association (ISTA).[24] MWCNT treatments increased germination percentage and reduced the proportion of abnormal seedlings[24] particularly in soybean associated with a reduction in the speed of water uptake during imbibition. However, early development of seedling was affected particularly by inhibition of root growth (fine root production) in all plant species first detectable at 8 days after sowing (DAS).

In the present study the consequences of these treatment effects on early growth were monitored in different culture systems (hydroponics, soil culture) with contrasting availability of water and nutrients.

Experimental

MWCNTs and preparation of MWCNT suspensions

Industrial multi-walled carbon nanotubes, MWCNTs (NanoTechCenter Ltd., Tambov, Russia) were used for the experiments. The MWCNTs have a minimum length of 2 µm, an external diameter of 20−70 nm and an internal diameter of 5−10 nm. The material was produced by chemical vapor deposition with purity above 98% (Appendix A). The selected concentrations of the MWCNT working suspensions used in the experiments (50, 100, 500 and 1000 mg L^{-1}) were in the range previously employed for various other studies on plant effects of MWCNTs.[15,19-21] For preparation of working suspensions, MWCNTs were mixed directly with deionized (DI) water and dispersed by ultrasonification (SONOREX SUPER RK 510 H; 35 KHz, Bandelin Electronic, Berlin, Germany) for 30 min.

Test plants

For the experiments three plant species were used: soybean (*Glycine max*. (L.) Merr cv. BR16 Conquista, Embrapa, Brazil), common bean (*Phaseolus vulgaris* L. cv. Bohnen maxi, Baywa AG, Germany) and maize (*Zea mays* L. cv. Surprise, Saaten Union GmbH, Rastatt, Germany).

Seed treatments

Suspension of MWCNTs in DI water was used in a concentration of 1000 mg L^{-1} corresponding to a dose of 500 µg $seed^{-1}$. Deionized (DI) water was used as a control. Five mL of MWCNTs suspensions or DI water were added to plastic Petri dishes (diameter 96 mm, Greiner, Nürtingen, Germany) with 3 layers of filter paper (Blue ribbon MN 640d, Macherey und Nagel, Düren, Germany) at the bottom, and ten soybean seeds were evenly distributed per Petri dish and homogenously moistened with the treatment solutions. The Petri dishes were covered with lids and placed into an incubator (BD 115, Binder, Tuttlingen, Germany) at 25 °C for 36 h in the dark before emergence of the radicles, and subsequently transferred to different growth mediums without MWCNTs addition: (a) in filter paper rolls wetted with DI water for 10 days, (b) in filter paper rolls wetted with DI water for 3 days, then into rhizoboxes with silty loam soil for 10 days, (c) in filter paper rolls wetted with DI water for 6 days, then into hydroponic culture with fill nutrient solution for 9 days, (d) into pots with loess subsoil for 38 days and (e) into pots with loess subsoil and foliar Zn application for 33 days (Figure 1, experimental set-up 1). The rhizobox experiment was performed additionally with maize and common beans.

Seed vitality staining

Seed vitality staining was performed after 36 h of MWCNTs treatments according to ISTA rules.[24] Seeds were stained with 1% (w/v) 2, 3, 5-triphenyltetrazolium chloride (TTC) (pH 6.5−7.5) for 18 h. After rinsing the seeds with DI water, they were cut lengthwise with a razor blade and staining intensity of seed organs was evaluated under a binocular microscope (Stemi 2000-C, Zeiss, Germany). In metabolically active cells, TTC is reduced by dehydrogenases, forming red formazan and therefore, color intensity reflects the degree of metabolic activity in the stained tissues. Finally, embryos were excised, and formazan was quantitatively extracted with 2 M KOH/DMSO (1:1.16 v/v) using mortar and pestle. After removal of solid material by centrifugation, absorption of the supernatant was measured spectrophotometrically at 485 nm.

Seedling growth in filter rolls

As a pre-culture for hydroponic and rhizobox experiments seedlings were germinated in filter paper rolls: one sheet of filter paper (58×58 cm, MN710, Macherey und Nagel, Düren, Germany) was folded lengthwise four times and was wetted with 60 ml of DI water. Ten treated with MWCNTs seeds were placed along the edge of the paper which was subsequently folded, forming a paper roll with the seeds inside. The paper rolls were placed in upright position into a plastic germination box (30×20×10 cm), the lids of the box was opened and it was placed for 3−6 d (until rootlets reach 2.0−2.5 cm) into a climate chamber with a 14 h light period and an average temperature of 23 °C with regular additions of 25 ml DI water per filter roll to compensate for evaporation.

Hydroponic culture

Hydroponic culture was employed to investigate the impact of the MWCNTs on seedling development of soybean under full, freely available nutrient supply. Seed treatments with MWCNTs (1000 mg L^{-1}) and pre-culture in filter rolls were performed as described above. Seedlings with a root length of 2.0−2.5 cm were transferred from filter rolls to pots with 2.5 L nutrient solution, aerated with an aquarium pump and containing 2 mM $Ca(NO_3)_2$, 100 µM KH_2PO_4, 0.7 mM K_2SO_4, 0.1 mM KCl, 0.5 mM $MgSO_4$, 10 µM H_3BO_3, 0.5 µM $MnSO_4$, 0.5 µM $ZnSO_4$, 0.2 µM $CuSO_4$, 0.01 µM $(NH_4)_6Mo_7O_{24}$, and 20 µM Fe(III)-EDTA, which was replaced in 3 day-intervals. In each pot, 8 seedlings were fixed with foam strips in perforated lids with 4 replicates per treatment. Cultivation was performed in a climate chamber with a 14 h light period (200 µmol $m^{-2}s^{-1}$) at 23 °C. After a culture period of 16 d, seedlings were harvested for biomass and root length determination. Alternatively, non-treated seeds were pre-cultured in moist filter rolls, and thereafter exposed for 6 days to nutrient solution amended with MWCNTs (50, 100, 500 mg L^{-1}) and subsequently cultivated in nutrient solution without MWCNTs supply for 7 days (Figure 1, experimental set-up 2).

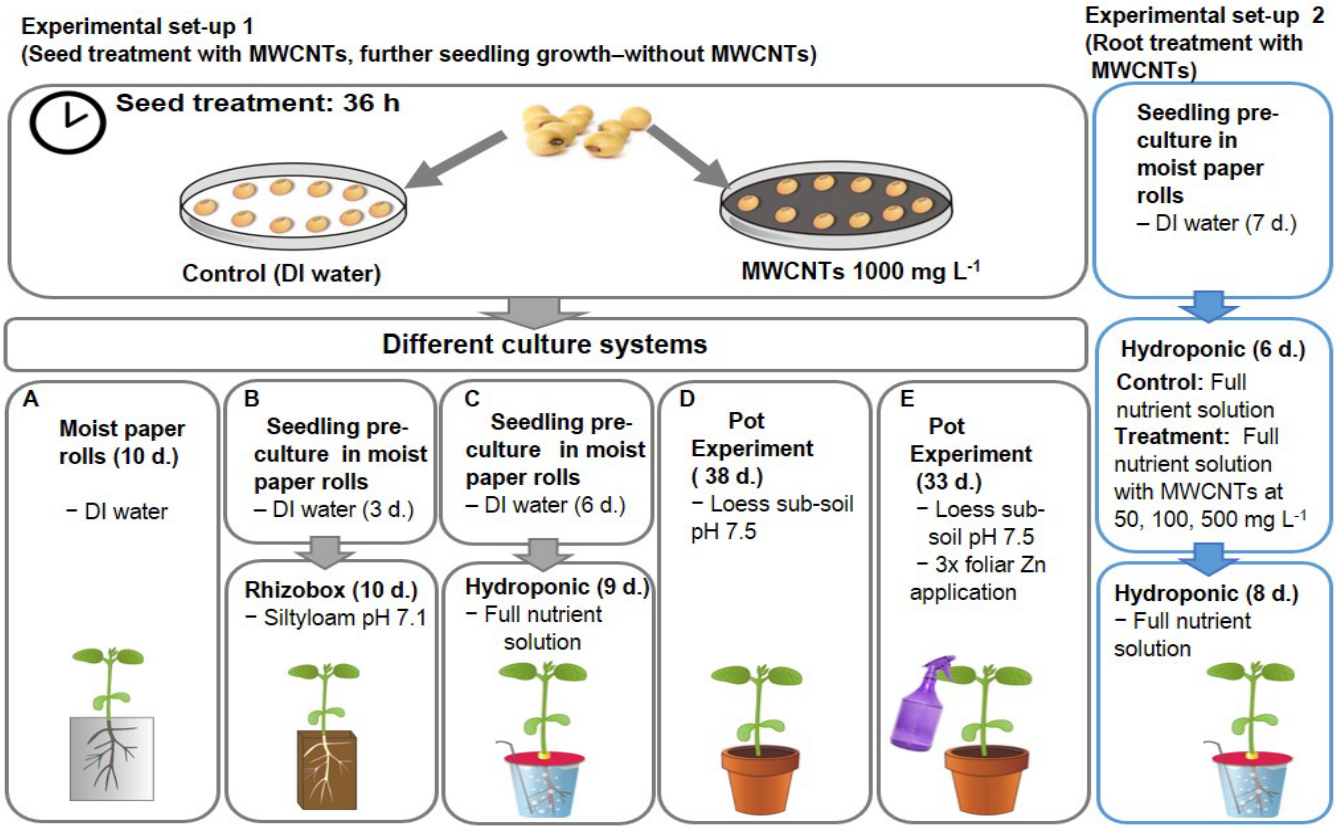

Figure 1. Schematic representation of the performed experiments: experimental set-up 1 and 2.

Soil culture - seedling growth in rhizoboxes

A rhizobox experiment was performed to monitor root development in soil culture. Seed treatments in Petri dishes, and pre-culture in filter rolls were performed as described above. Each of two seedlings with a root length of 2.0−2.5 cm were transferred into rhizoboxes equipped with transparent root observation windows[25] filled with 0.5 kg of a clay loam field soil (pH 7.1) taken from the Heidfeldhof experimental station in Hohenheim (Stuttgart, Germany). During the culture period, soil moisture was adjusted gravimetrically to 20% (w/w) by supplying DI water via holes on the backside of the boxes. Cultivation was performed in a climate chamber with a 14 h light period (200 µmol m^{-2} s^{-1}) at 23 °C. After a culture period of 15 days, the seedlings were harvested for biomass and root length determination.

Soil culture - pot experiments

Pot experiments were conducted to investigate the effects of short-term MWCNTs seed treatment and foliar Zn application on early growth of soybean on a soil substrate with limited nutrient solubility. A calcareous loess subsoil low in available P (P CAL 5 mg kg^{-1}), total N (0.02%), calcium chloride-diethylenetriaminepentaacetic acid (CAT) extractable micronutrient concentrations (mg kg^{-1} soil): Mn, 15; Fe, 7.8; Zn, 0.6; B, 0.2; organic matter (0.1 %), pH 7.6 was used for the experiments. Each pot was filled with 1 kg of a mixture of loess subsoil and quartz sand (50 % w/w). Basal fertilization for the substrate comprisedof N (100 mg kg^{-1}) as $Ca(NO_3)_2$; K (150 mg kg^{-1}) as K_2SO_4, Mg (50 mg kg^{-1}) as $MgSO_4$, P (80 mg kg^{-1}) as $Ca(H_2PO_4)_2$. Seed treatment with MWCNTs (1000 mg L^{-1}; 36 h) was performed as described above. Thereafter, 4 seeds per pot were sown at depth of 1 cm and the pots were cultivated in a climate chamber with a 14 h light period (200 µmol m^{-2} s^{-1}) at 23 °C. During the culture period the moisture content of the substrate was adjusted gravimetrically in to 20% (w/w) by regular supply of DI water. At 10 DAS thinning was performed to final number of two seedlings per pot with 10 replicates per treatment. Harvests were performed at 26 DAS and 38 DAS for each 5 replicates for the determinations of biomass, root length and nutritional status.

Plant analysis in the first experiment revealed a critical Zn-nutritional status associated with growth depressions for soybean plants, developed from MWCNT-treated seeds. Therefore, in a second experiment, after unfolding of the 1st trifoliate leaves (15 DAS) foliar Zn applications were applied once a week with 0.5 mM or 5 mM $ZnSO_4$ or DI water as negative control (8 replicates per treatment). Final harvest was performed at 33 DAS for the determination of leaf area (young fully developed leaf) plant height, biomass and root length.

Mineral analysis of plant tissues

For mineral nutrient analysis, dried shoots of soybean plants were ground to a fine powder and each 250 mg of dry plant material were ashed in a muffle furnace at 500 °C for 4 h. After cooling, the samples were extracted twice with 2.5 mL of 3.4 M HNO_3 and evaporated to dryness to precipitate SiO_2. The ash was dissolved in 2.5 mL of 4 M HCl, subsequently diluted ten times with hot deionized water, and boiled for 2 min. After cooling, the solutions

were adjusted to 25 ml with DI water and passed through blue ribbon filters (Macchery & Nagel, Düren, Germany). Zinc concentrations in the extracts were determined by atomic absorption spectrometry (iCE 300 Series, Thermo Fisher Scientific, United Kingdom).

Analysis of leaf area and root morphology

Fresh root samples, stored in 30% (v/v) ethanol were carefully separated on transparent Perspex trays and subsequently digitalised with an Epson Expression 10000Xl scanner (Epson, USA) which was also used for scanning of leaves. Analysis was performed using the WinRHIZO software (Regent Insruments, Quebec, Canada).

Statistical analysis

All experiments were performed in a completely randomized design. Statistical analysis was conducted with the SigmaPlot 11.0 software package using the Student t-test for comparison of two treatments and one-way ANOVA for comparison of multiple treatments. The level of significance was determined at a P value ≤ 0.05. All results in tables and graphs are presented as mean values ± SE (standard error of a mean).

Results and Discussion

It has been already reported that short-term seed exposure (36 h) medium (50 µg seed^{-1}) and high (500 µg seed^{-1}) dosages of MWCNTs even prior to radicle emergence can induce significant effects on germination and early seedling development.[23] In a pilot study with three crop species (soybean, *Glycine max*; common bean, *Phaseolus vulgaris*; and maize, *Zea mays*), germination rate was increased, associated with reduced formation of abnormal seedlings according to the ISTA classification[24] particularly in soybean. This effect could be attributed to a reduction in the speed of water uptake, detectable already during the first 12 h of seed imbibition. However, during later seedling development, inhibition of root growth (mainly fine root production) was recorded in seedlings of all plant species, first detectable at 8 DAS, and associated with a reduced metabolic activity of the root tissue. A minimum time period of 36 h MWNCT seed-exposure was required for induction of root damage.[23]

The results of the present study confirmed penetration of MWCNTs and its localization in the embryonic axis (Figure 2 E) even in non-germinated seeds, as has been described for other plant species also.[15,26]This was associated with a reduced metabolic activity of the embryonic tissue detected by 2,3,5-triphenyltetrazoliumchoride staining (Figure 2 B, C) at 36 h of seed imbibition, and most probably cauding the inhibition of root growth during later seedling development.[23] In this context, a closer look to the effective dosage of MWCNTs shows that only an extremely small proportion of the applied MWCNT dose (e.g. 500 µg seed^{-1} supplied in a treatment suspension with a concentration of 1000 mg L^{-1}) was really in contact with the seed surface, since the majority of the applied MWCNTs was sticking to the germination paper (Figure 2 A). A quantitative evaluation of the MWCNT proportion finally taken up into the seeds and interacting with the plant metabolism would require incubation experiments with radioactively-labeled MWCNT tracers as previously described by Larue et al.[27] However, even without the availability of exact quantitative data on MWCNT uptake it is obvious that even trace amounts MWCNTs entering the seeds exhibit a high metabolic activity with the ability to induce both, positive and negative effects on plant development.

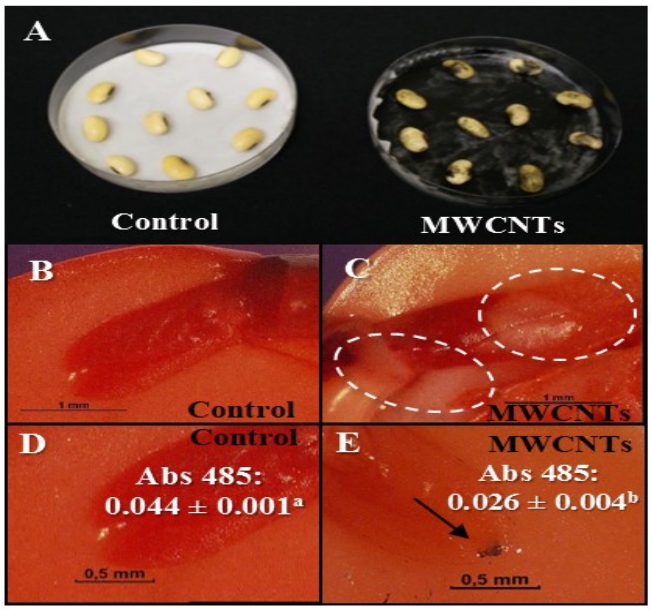

Figure 2. (A) Soybean seed treatment in Petri dishes: control (DI water) and MWCNTs (1000 mg L-1) variants; (B−E) Embryos of MWCNT-treated and non-treated soybean seeds (2 DAS), stained with 1% 2,3,5-triphenyl tetrazolium chloride for 18 h. The weakly-stained regions highlighted in (C) indicate embryo tissues with a low metabolic activity. The numeric values in (D) and (E) represent absorption intensity of tetrazolium extracted from the embryos after photometric determination (means ± SEM). Different letters (a, b) indicate significant differences between the treatments (Student t-test, P ≤ 0.05). The black arrow in (E) indicates MWCNTs accumulation next to the embryo. DI−deionised water, MWCNTs−multi-walled carbon nanotubes, DAS−days after sowing.

Interestingly, the further development of plants grown from MWCNT-treated seeds was strongly dependent on the culture conditions. Cultivation of soybean seeds, identified as most sensitive to MWCNT treatments[23], in a hydroponic culture system with all essential plant nutrients provided in sufficient and freely available amounts, induced a complete recovery of root growth in plants grown from MWCNT-treated seeds within three weeks of the culture period, without any growth differences to control plants (Table 1, Figure 3 A). This finding suggests that nutrient limitation of the seedlings was a major problem induced by the MWCNT treatments, which could be supplemented by freely available nutrient supply in the hydroponic culture system. However, root growth inhibition was maintained even in nutrient solution with unlimited nutrient supply when the presence of MWCNTs was not restricted to the imbibition stage and MWCNTs were applied during plant growth in nutrient solution exerting inhibitory effects already at low concentrations of 50 mg L^{-1} (Table 1). By contrast, shoot growth remained unaffected (Table 1).

Table 1. Growth characteristics of soybean plants: (I) developed from seeds treated for 36 h with MWCNTs (1000 mg L^{-1}) and DI water (Control), pre-germinated in filter paper rolls without MWCNTs for 6 d and subsequently grown in full nutrient solution without MWCNTs for 9 d (see Figure 1, experimental set-up 1 C); (II) developed from non-treated seeds, pre-germinated in filter paper rolls for 7 d, grown in nutrient solution amended with MWCNTs (50, 100, 500 mg L^{-1}) for 6 d and finally grown in nutrient solution without MWCNTs for 8 d (see Figure 1, experimental set-up 2).

Treatment	Plant height, cm	Shoot dry matter, g	Rroot dry matter, g	Root length, cm	Root diameter, mm
(I) Seed treatment (36 h)					
Control	24.6 ± 0.3 a	0.17 ± 0.01 a	0.03 ± 0.00 a	268.9 ± 23.3 a	0.50 ± 0.01 a
MWCNTs 1000 mg L^{-1}	25.0 ± 0.5 a	0.17 ± 0.00 a	0.02 ± 0.00 a	244.8 ± 4.0 a	0.51 ± 0.01 a
(II) Root treatment in hydroponics (6 days)					
Control	39.3 ± 1.5 a	0.29 ± 0.03 a	0.04 ± 0.00 a	609.3 ± 33.2 a	0.35 ± 0.01 a
MWCNTs 50 mg L^{-1}	41.2 ± 0.9 a	0.32 ± 0.01 a	0.03 ± 0.00 a	493.3 ± 29.5 b	0.40 ± 0.02 a
MWCNTs 100 mg L^{-1}	40.6 ± 0.2 a	0.31 ± 0.01 a	0.03 ± 0.00 a	457.6 ± 31.2 b	0.42 ± 0.02 b
MWCNTs 500 mg L^{-1}	40.3 ± 0.7 a	0.31 ± 0.01 a	0.03 ± 0.00 a	497.3 ± 24.6 b	0.42 ± 0.00 b

Note: Results represent mean values ± SEM of four replicates. Different letters (a, b) indicate significant difference between treatments (Student t-test for the (I) seed treatment experiment, $P \leq 0.05$; one-way ANOVA, Tukey test for the (II) root treatment in hydroponics experiment, $P \leq 0.05$). MWCNTs−multi-walled carbon nanotubes.

Table 2. Growth characteristics of soybean, common bean and maize seedlings, developed from seeds treated for 36 h with MWCNTs (1000 mg L^{-1}) and subsequently grown in soil culture in rhizoboxes.

Treatment	**Plant height, cm**	**Shoot dry matter, g**	**Root dry matter, g**	**Root length, cm**	**Root diameter, mm**
Soybean *(Glycine max* (L.) Merr*), 15 DAS					
Control	15.4 ± 0.6 a	0.11 ± 0.00 a	0.03 ± 0.00 a	350.7 ± 25.3 a	0.85 ± 0.01 b
MWCNTs 1000 mg L^{-1}	14.3 ± 1.1 a	0.11 ± 0.01 a	0.02 ± 0.00 a	264.1 ± 17.5 b	0.89 ± 0.02 a
Common bean (*Phaseolus vulgaris* L.), 17 DAS					
Control	8.6 ± 0.5 a	0.14 ± 0.01 a	0.06 ± 0.00 a	922.9 ± 92.0 a	0.32 ± 0.01 a
MWCNTs 1000 mg L^{-1}	8.7 ± 0.7 a	0.14 ± 0.01 a	0.04 ± 0.01 a	575.9 ± 98.4 b	0.36 ± 0.02 a
Maize (*Zea maize* L.), 14 DAS					
Control	24.9 ± 1.0 a	0.08 ± 0.01 a	0.09 ± 0.01 a	618.1 ± 46.5 a	0.81 ± 0.01 b
MWCNTs 1000 mg L^{-1}	21.5 ± 2.4 a	0.06 ± 0.01 a	0.08 ± 0.01 b	452.6 ± 48.7 b	0.86 ± 0.01 a

Note: Results represent mean values ± SEM of five replicates. Different letters (a, b) indicate significant difference between treatments (t-student test, $P \leq 0.05$). DAS−days after sowing, MWCNTs−multi-walled carbon nanotubes.

Apparently, the freely available nutrient supply in hydroponics was sufficient to maintain normal shoot growth even with a smaller root system affected by the MWCNTs treatments. Root growth effects of MWCNTs have been documented also in previous studies with different plant species, and responses ranged from growth inhibition, no effects and even growth stimulation particularly at lower MWCNT concentrations.[18,20,21]

In an additional experiment, further development of soybean seedlings was monitored in soil culture on a clay loam field soil (pH 7) and a calcareous Loess subsoil pH 7.6 with full macronutrient (N, P, K, Mg) fertilizers, carried out in rhizoboxes, equipped with root observation windows for monitoring of root growth effects and in pot experiments. In contrast to the experiment in hydroponic culture, inhibitory effects of short-term MWCNTs treatments during seed imbibition on root growth, persisted in soil culture up to 6 weeks after sowing even in absence of further root contact with MWCNTs. The effects were not only detectable in soybean (Figure 3 B), but similarly also in common bean and maize (Table 2), affecting mainly root length development. Typical symptoms comprised of reduction of average root length, associated with increased average root diameter in the plants developed from MWCNT-treated seeds (Table 2), which could be attributed to a reduction in lateral and fine root production (Figure 4). This was associated with stunted shoot growth, inhibited leaf expansion, and chlorosis of young leaves (Figure 5 A, B) as a typical indicator for zinc deficiency (little leaf syndrome[28]). In accordance with the visual symptoms, shoot nutrient analysis revealed critically low zinc levels at final harvest of plants exposed to MWCNT-seed treatments, reaching less than 20 % of the Zn concentrations of untreated control (Figure 5 C). No comparable effect could be observed for other nutrients such as phosphate (P) or potassium (K) (data not shown). A critical role particularly of Zn as limiting nutrient in MWCNT-treated soybeans was further confirmed by the observation that depressions of root and shoot growth could be restored by repeated foliar Zn applications throughout the culture period (Figure 4). Zinc limitation can induce oxidative stress since certain superoxide dismutases involved in detoxification of free radicals require Zn as a co-factor.[29,30] Apart from lipid peroxidation of membranes[31] Zn limitation may also promote oxidative degradation of indole acetic acid (IAA). The resulting low IAA levels have been discussed as a cause for limited leaf expansion and may be similarly responsible also for limited lateral fine root production observed in MWCNT-treated plants.[32,33] Exposure of plants to MWCNTs can induce increased formation of reactive oxygen species (ROS)[21] and the related oxidative stress may be responsible for the inhibition of root growth already in young seedlings (10 DAS).

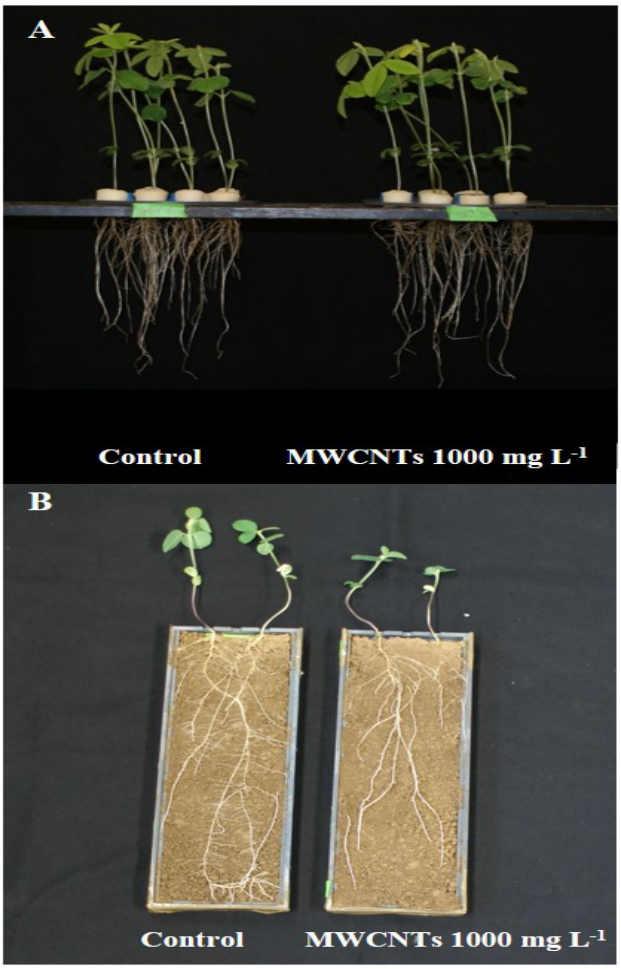

Figure 3. (A) Absence of growth effects after short-term MWCNTs seed treatment (36 h; 1000 mg L^{-1}) of soybean, subsequently grown in hydroponic culture with full nutrient supply (16 DAS); (B) negative influence of short-term MWCNTs seed treatment (36 h; 1000 mg L^{-1}) on root and shoot development of soybean seedlings in soil culture (15 DAS). DAS−days after sowing, MWCNTs−multi-walled carbon nanotubes.

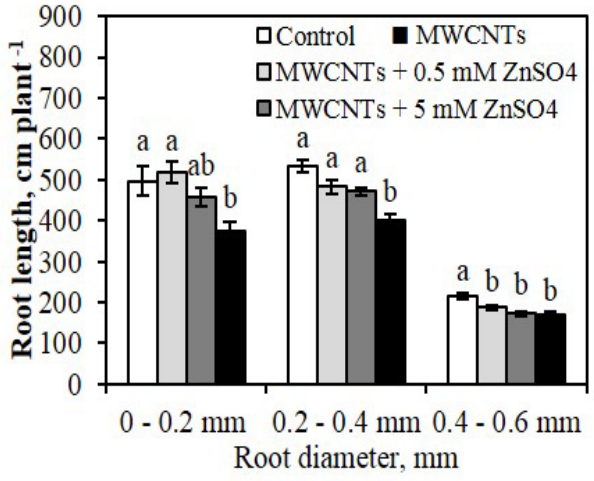

Figure 4. Effects of short-term MWCNTs seed treatments (36 h; 1000 mg L^{-1}) and 3x-foliar Zn application (0.5 and 5 mM) on development of fine roots (different diameter classes) of soybean plants in soil culture (33 DAS). Results represent mean values ± SEM of eight replicates. Different letters (a, b) indicate significant difference between treatments (one-way ANOVA, Tukey test, $P \leq 0.05$). DAS−days after sowing, MWCNTs−multi-walled carbon nanotubes.

Whether this effect is already linked with zinc deficiency, remains to be established. When these seedlings are supplied with high amounts of freely available nutrients e.g. in hydroponic culture, sufficient nutrients are taken up even by the smaller root systems of MWCNT-treated plants finally leading to a compensation of the inhibitory effects on plant growth. In soil culture, sparingly soluble nutrients and particularly Zn are obviously not acquired in sufficient amounts by the stunted root systems and the Zn demand can only be covered by additional foliar Zn application.

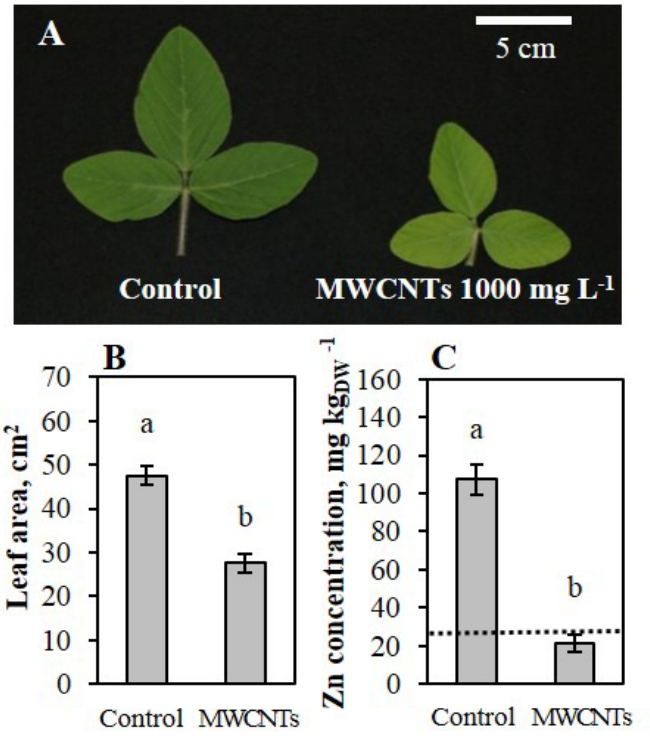

Figure 5. (A) First fully developed leaves of soybean plants with and without short-term MWCNTs seed treatment (38 DAS). Leaf chlorosis (pale-green color) and inhibited leaf expansion (little-leaf syndrome) in the MWCNTs variant as a typical symptom of Zn deficiency. (B) Effect of short-term seed treatments with MWCNTs on surface area of the first fully developed leaf of soybean plants (38 DAS). (C) Zinc concentration of the soybean shoots (38 DAS). The dotted line indicates the level of zinc deficiency.[34] Results represent mean values ± SEM of five replicates. Different letters (a, b) indicate significant difference between treatments (Student t-test, $P \leq 0.05$). DAS−days after sowing, MWCNTs−multi-walled carbon nanotubes.

Conclusions

Our findings suggest that short-term exposure to small amounts of MWCNTs during germination can induce long lasting inhibitory effects during the development of soybean plants. Moreover, the expression of effects strongly depends on the culture conditions and application time. This may at least partially explain the high variability of responses reported in the literature, comprising both stimulatory and inhibitory effects. Different concentrations and types of applied MWCNTs have been discussed as additional factors. The close link of MWCNTs applications with production of ROS[21] may provide an explanation for the expression of both, positive and negative effects. The formation of ROS

has been discussed as a physiological base for hormesis effects at low concentrations by stimulation of seed germination[35] and plant defence mechanisms and for negative effects induced by oxidative stress at higher concentrations. In this context also the sensitivity of plants may vary during their ontogenesis and in response to the environmental conditions. However the physiological base of MWCNTs effects on plant metabolism and potential relationships with oxidative stress responses still require a more detailed characterisation. This is of particular importance in face of agricultural applications of MWCNTs currently under development or already on the market.[36,37]

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The authors declare that they have no conflict of interest.

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