# DIFFERENTIAL IMPACT OF MULTI-WALLED CARBON NANOTUBES ON GERMINATION AND SEEDLING DEVELOPMENT OF *GLYCINE MAX*, *PHASEOLUS VULGARIS* AND *ZEA MAYS*

**Olga Zaytseva[a]* and Günter Neumann[a]**

**Keywords** germination, seedling growth, carbon nanotubes, soybean, common bean, maize.

This study is designed to investigate the effects of carbon nanomaterials (multi-walled carbon nanotubes, MWCNTs) under controlled conditions on three different plant species. The study covers the effects of MWCNT dosage, treatment duration, and the plant-developmental stage, including imbibition, germination and seedling development. Germination experiments are conducted under standardized laboratory conditions based on the protocols of the International Seed Testing Association with aqueous MWCNT suspensions at a dosage of 0, 100 and 1000 mg $L^{-1}$ applied as seed treatments during 36 h after sowing prior to radicle emergence, using soybean (*Glycine max* (L.) Merr. cv. BR-16 Conquista), common bean (*Phaseolus vulgaris* L. cv. Bohnen maxi) and maize (*Zea mays* L. cv. Surprise) as test plants. The seed treatment with MWCNTs reduced the speed of water uptake particularly by soybean seeds. This is associated with an increased germination percentage and reduced development of abnormal seedlings, while mean germination time is unchanged. However, during later seedling development, negative effects on root growth, particularly affecting fine root development are recorded for all investigated plant species. In soybean, this effect is first detected at 8 days after sowing and requires a minimum MWCNT seed exposure of 36 h. Inhibition of root growth is associated with reduced metabolic activity of the root tissue as indicated by tetrazolium vitality staining. The nitrate uptake was lower in MWCNT-treated plants, which is mainly attributed to the smaller root system. The results demonstrate that even under standardized experimental conditions, excluding environmental factors and effects induced by carbon nanomaterials, plant responses to MWCNT exposure exhibit differences, depending on plant species but also on the physiological status and the developmental stage of individual plants.

* Corresponding Authors
E-Mail: olga.zaytseva@uni-hohenheim.de
[a] Institute of Crop Science (340h), Faculty of Agriculture, University of Hohenheim, Stuttgart, 70593, Germany

## Introduction

Carbon nanotubes (CNTs) are nanostructured carbon allotropes of cylindrical shape, possessing outstanding physical and chemical properties. Multi-walled carbon nanotubes (MWCNTs) are the most important class of carbon nanomaterials with the highest production volumes and numerous technical applications. In the recent past, potential applications have been extended to agriculture with first patents as germination stimulants, plant growth promoters, fertilizers and fertilizer synergists, as well as delivery systems for agrochemicals, antifungal and antimicrobial agents. However, the published literature reflects a high heterogeneity of plant responses to MWCNT treatments, including both, positive and negative effects in some plant species and the complete absence of responses in others.[1-10] The subject matter is reviewed by O. Zaytseva and G. Neumann.[11]

Positive effects of MWCNTs added to agar media at concentrations of 10−40 mg $L^{-1}$ on seed germination and seedling growth of tomato (*Solanum lycopersicum* L.) are reported by Khodakovskaya et al.[1] and Morla et al.[2] Later, Lahiani et al.[3] reported accelerated germination of barley (*Hordeum vulgare* L.), maize (*Zea mays* L.) and soybean (*Glycine max* (L.) Merr.) seeds in a Murashige and Skoog medium amended with 50−200 mg $L^{-1}$ MWCNTs. Srivastava and Rao[4] reported that germination of wheat (*Triticum aestivum* L.), maize and peanut (*Arachis hypogaea* L.) was enhanced by application of 50 mg $L^{-1}$ MWCNTs. The reports listed above suggested that MWCNT application increased the seed water content by perforation of the seed coat. Additionally, Lahiani et al.[3] reported increased expression of aquaporin-related genes, playing a role in water uptake, related with germination, root elongation and plant growth. Positive effects of MWCNTs on seed germination have been also related to the presence of metal catalyst impurities in the applied MWCNT materials.[12]

Similarly, during later seedling development and early growth, positive effects of MWCNTs on root and shoot elongation have been reported for a range of plant species, such as tomato, wheat, soybean, maize, mustard (*Brassica juncea* L.), and black lentil (*Vigna mungo* (L.) Hepper), even in cases when the plant roots had direct contact with the MWCNTs. The expression of effects was concentration-dependent with beneficial effects at lower levels of MWCNT application and inhibition at higher concentrations. Induction of oxidative stress associated with formation of reactive oxygen species (ROS), membrane damage, electrolyte leakage, mitochondrial dysfunctions and DNA aberrations are characterized as determinants of MWCNT toxicity during seedling development and early growth of red spinach (*Amaranthus tricolor* L.), rice (*Oryza sativa* L.), lettuce (*Lactuca sativa* L.) and cucumber (*Cucumis sativus* L.), and small seeds being more sensitive than large seeds. By contrast, Lin and Xing[5] did not find any effect of MWCNTs

when applied in concentrations of 1000−2000 mg $L^{-1}$ on seed germination and root elongation of five plant species (radish (*Raphanus sativus* L.), rape (*Brassica napus* L.), ryegrass (*Lolium perenne* L.), lettuce, maize and cucumber. Similarly, Stampoulis et al.[6] reported no effects on germination of zucchini (*Cucurbita pepo* L.), and Miralles et. al.[12] observed no effects of MWCNTs on alfalfa (*Medicago sativa* L.) and wheat.

Various reasons such as genotypic differences, plant developmental stage, experimental setups, type, dosage, formulation and agglomeration have been discussed to explain the heterogeneity of plant responses to MWCNT treatments. In view of the highly variable effects of MWCNT on plant performance, reported in the literature, a systematic analysis of factors determining the effects of MWCNTs such as MWCNT dosage, duration of exposure to MWCNT treatments, plant-developmental stage including imbibition, germination and seedling development on selected plant species are studied. Soybean, common bean (*Phaseolus vulgaris* L.) and maize are selected as representative crops. Apart from plant growth responses, MWCNT effects are evaluated using assays for physiological activity of the test plants (nutrient uptake, vitality staining).

## Experimental

### MWCNTs and preparation of MWCNT suspensions

Multi-walled carbon nanotubes (MWCNTs) (NanoTechCenter Ltd., Tambov, Russia), produced by chemical vapor deposition (purity > 98%) with a minimum length of 2 µm, external diameter of 20−70 nm and internal diameter of 5−10 nm were used for the experiments. The selected concentrations for MWCNT application (100 and 1000 mg $L^{-1}$) were in the range reported in various other studies.[1-9] Working suspensions of MWCNTs were prepared directly in deionized (DI) water and dispersed by ultrasonification (SONOREX SUPER RK 510 H; 35 KHz, Bandelin Electronic, Berlin, Germany) for 30 min.

### Test plants

Three plant species: soybean (*Glycine max* (L.) Merr. cv. BR-16 Conquista), common bean (*Phaseolus vulgaris* L. cv. Bohnen maxi) and maize (*Zea mays* L. cv. Surprise) were selected. Seeds were stored in darkness at 4 °C and brought to room temperature one day before use.

### Impact of MWCNTs on seed water uptake

Hydration of seeds by imbibition is an important factor triggering the start of seed germination and seed hydration has been influenced by MWCNT treatments in various earlier reports.[1,3] Therefore, the effects of MWCNTs on seed water uptake were investigated during the first twelve hours of imbibition. A germination test was performed in Petri dishes (control: DI water; treatments: 100 and 1000 mg $L^{-1}$ MWCNTs) for studying the kinetics of water uptake by seeds. An additional control without MWCNT was included with seeds imbibing more slowly between four layers of moist filter paper: one sheet of filter paper (58×58 cm, MN710, Macherey und Nagel, Düren, Germany) was folded lengthwise two times to obtain a 4-layer paper strip, which was soaked with 60 ml of DI water according to its maximum water holding capacity. Ten seeds were placed along the upper edge of the paper strip, which is 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) and kept under the same growth conditions as the seeds in Petri dishes. Water uptake was recorded by determining weights of the seeds from the Petri dishes and from the filter paper rolls at 1 h intervals during 12 h.

### Impact of MWCNTs on the seed germination

The influence of MWCNTs on germination of the three plant species was estimated in standardized filter paper germination tests according to the ISTA rules[13]. For treatments, suspensions of MWCNTs in DI water were applied in concentrations 100 mg $L^{-1}$ and 1000 mg $L^{-1}$; deionized (DI) water was used as a control. Five mL of un-precipitated MWCNT suspensions or DI water were evenly distributed in plastic Petri dishes (diameter 96 mm, Greiner, Nürtingen, Germany) with 3 layers of filter paper (Blue ribbon MN 640d, Macherey und Nagel, Düren, Germany) on the bottom. Thereafter, ten seeds per Petri dish were distributed equidistantly into the MWCNT suspensions or DI water. Thus, the concentrations of MWCNTs in working suspensions (100 mg $L^{-1}$ and 1000 mg $L^{-1}$) translated into an actual MWCNT dosage of 50 and 500 µg $seed^{-1}$. Petri dishes were covered with lids and placed into an incubator (BD 115, Binder, Tuttlingen, Germany). Depending on the plant species, temperature during the germination test as well as test duration were maintained according to the ISTA rules[13] and presented in Table 1. During the germination tests, the number of germinated seeds was counted every 12 h and final germination percentage was determined on the day specified in Table 1. Seeds are considered to be germinated when the radicle length reaches 2 mm.[14] Two indices were calculated to describe the results of the germination tests: germination percentage (*GP*)[15] and mean germination time (*MGT*). [15]

**Table 1.** Conditions maintained during the germination test.

| Plant species | Temp., °C | First count, day | Final count, day |
|---|---|---|---|
| Maize | 25 | 4 | 7 |
| Soybean | 25 | 5 | 8 |
| Common bean | 25 | 5 | 9 |

### Impact of MWCNT seed treatment on seedling development

The germination test showed that radicles started to emerge after 36 h of seed imbibition. Therefore, the seed treatment with MWCNTs in the succeeding experiments was limited to 36 h to avoid direct interactions of MWCNTs with the emerging radicles. After 36 h of seed treatment in Petri dishes, seeds were transferred to filter paper rolls in germination boxes as described above.

The lids of the germination boxes were opened and the boxes placed into a climate chamber with a 14 h light period at an average temperature of 23 °C with regular additions of 25 ml DI water per filter roll to compensate for evaporation. No nutrients were supplied to the seedlings because cotyledons can provide organic and mineral nutrients to young seedlings for up to ten days after emergence.[16,17] At 10 DAS, the number of abnormal seedlings were counted as defined in ISTA rules.[13] In brief, seedlings are classified as abnormal if there are significant damages or deformations of essential structures (cotyledons, hypocotyl, primary leaves and primary roots), which can prevent normal plant development. Subsequently, shoot length of normal seedlings was recorded and the seedlings were harvested for biomass and root length determination. For root morphology analysis, 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). Root analysis was performed using the WinRHIZO software (Regent Instruments, Quebec, Canada).

#### Nitrate uptake by seedlings

A short (24 h) hydroponic experiment was performed to investigate the effect of short-term (36 h) MWCNT seed treatment on nutrient uptake by seedlings developed from the treated seeds. Soybean and common bean seeds were treated for 36 h in Petri dishes with 1000 mg $L^{-1}$ MWCNTs or DI water (control). Thereafter, seeds were transferred to filter paper rolls were moistened with DI water and were grown until 10 DAS as described above. Thereafter, three representative seedlings per replicate were transferred for 24 h into beakers containing 100 mL of nutrient solution (chemical composition of the nutrient solution is in Appendix A). To estimate the amount of nitrate ($NO_3^-$) absorbed by the seedlings, the volume of the nutrient solutions as well as the nitrate ($NO_3^-$) concentration in the beakers were measured before and after the incubation pweriod. The $NO_3^-$ concentration was determined by using nitrate-sensitive test strips (Merck KGaA, Darmstadt, Germany) and the color intensity on the strips was quantified colorimetrically (Hermann Wolf GmbH&Co.KG, Wuppertal, Germany).

After 24 h of seedlings exposure to nutrient solution, two of three seedlings were harvested for biomass and root length determination and one seedling per replicate was stained with 2, 3, 5-triphenyltetrazolium chloride (TTC) as a measure of the metabolic activity of the root tissue.

#### TTC reduction assay

Vitality staining of root tissues was performed with roots of soybean and common bean seedlings, developed from seeds treated for 36 h with 0 or 1000 mg $L^{-1}$ MWCNTs, which were grown in filter paper rolls until 10 DAS and subsequently incubated for 24 h in nutrient solution as described above. Prior to TTC staining the roots were rinsed with DI water and then placed into 50 mL of TTC solution (0.08% TTC in 0.05 M sodium phosphate buffer, pH 7.4) for 24 h in the dark.[18] In metabolically active cells, TTC is reduced by dehydrogenases, forming red-colored insoluble triphenylformazan (TF). The color intensity reflects the degree of metabolic activity in the stained tissues. After an incubation time of 24 h, the TTC solution was discarded, roots were rinsed three times with DI water, cut into segments of 1 cm and immersed into 10 mL of 95% (v/v) ethanol for 24 h at 4 °C for extraction of TF. Thereafter, the ethanol extract was filtered (Blue ribbon MN 640d, Macherey und Nagel, Düren, Germany) and the absorbance of the filtrate was measured spectrophotometrically at a wavelength of 485 nm (U-3300, Hitachi Ltd. Tokyo, Japan). Reduction of TTC is calculated as absorption of TF produced per root [Abs485 $seedling^{-1}$] and per unit root dry weight [Abs485 $gDW^{-1}$].

#### Statistical analysis

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

## Results

#### Seed water uptake

The speed of water uptake is calculated according to the formula:

$$v_{\text{water uptake}} = \frac{m_{\text{imbided seed}} - m_{\text{dry seed}}}{t}$$

where

$v_{\text{water uptake}}$: speed of water uptake [mg $seed^{-1}$ $h^{-1}$],

$m_{\text{imbibed seed}}$: average weight of imbibed seed [mg $seed^{-1}$]

$m_{\text{dry seed}}$: average initial seed weight [mg $seed^{-1}$] before the experiment,

$t$: time duration of imbibition [h].

The highest speed of water uptake is found in soybean seeds, followed by common bean and maize (Fig. 1). In soybean, most rapid imbibition is detected in the control variant (Fig. 1), while seeds imbibed between layers of moist paper show the slowest rate of water uptake. Similarly for the variant imbibing between layers of moist paper, the application of 100 and 1000 mg $L^{-1}$ MWCNTs significantly slowed down the seed water uptake by 12 % as compared to the control. Also in maize, seed imbibition between layers of moist filter paper and MWCNTs treatments reduced the speed of the water uptake. However, for MWCNT treatments, this reduction is not significant. A similar but not significant trend is observed for common bean.

#### Germination test

Germination percentage ($GP_i$) for each observation is calculated according to the formula[15]:

$$GP_i = 100\frac{n_i}{N}$$

where

$GP_i$: germination percentage at the $i^{th}$ observation [%],

$n_i$: number of seeds, germinated from the beginning of the experiment to the $i^{th}$ observation and

$N$: total number of seeds.

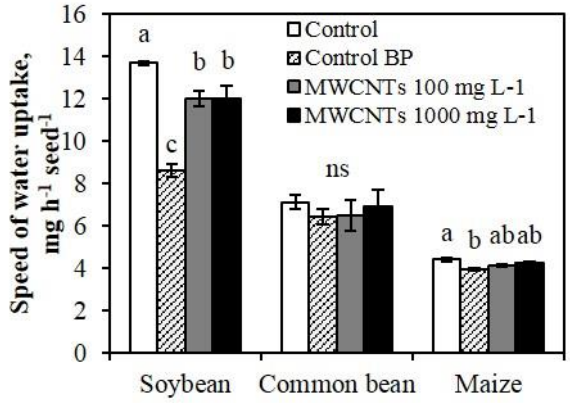


**Figure 1.** Speed of water uptake by soybean, common bean and maize seeds during 12 h of imbibition in Petri dishes (Control (DI water), MWCNTs 100 and 1000 mg $L^{-1}$) and in between filter paper (Control BP). Values represent mean values ± SEM of four replicates. Different letters (a, b, c) indicate significant difference between treatments (one-way ANOVA, Tukey test, $P \leq 0.05$). ns–not significant, MWCNTs–multi-walled carbon nanotubes, DI water–deionized water.

The mean germination time (*MGT*), days for germination of 50 % of all germinated seeds, is calculated as described by formula[15]:

$$MGT = \frac{\sum_{i=1}^{k} n_i t_i}{\sum_{i=1}^{k} n_i}$$

where

$t_i$: time from the beginning of the experiment to the $i^{th}$ observation [days],

$n_i$: number of seeds germinated on the $i^{th}$ day and

$k$: last day of germination.

Stimulation of germination percentage (*GP*, %) by MWCNT application is detectable only in soybean but not in common bean and maize (Fig. 2). Enhanced *GP* of soybean is first detectable on the second day of the experiment and remains elevated till the day of the final count (Fig. 2A). The application of MWCNTs with a concentration of 1000 mg $L^{-1}$ significantly increased the final *GP* of soybean seeds by 28% as compared to the control experiment. The treatment with a concentration of 100 mg $L^{-1}$ of MWCNT the *GP* increased by 25%, although this effect is not significant.

In common bean (Fig. 2B) and maize (Fig. 2C) the *GP* does not significantly differ. The mean germination time (*MGT*) of all the studied plant species is not affected by MWCNT application (data not shown).

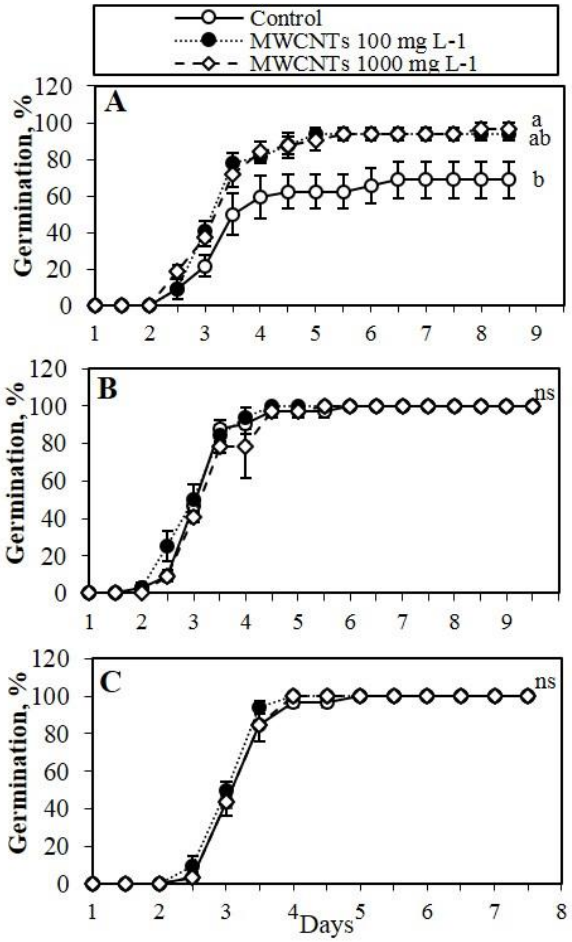


**Figure 2.** Germination percentage (*GP*, %) of (A) soybean, (B) common bean and (C) maize seeds in DI water (Control) and in MWCNTs (100 and 1000 mg $L^{-1}$) suspensions. Values represent mean values ± SEM of four replicates. Different letters (a, b) indicate significant difference between treatments (one-way ANOVA, Tukey test, $P \leq 0.005$), ns–not significant. MWCNTs–multi-walled carbon nanotubes, DI water–deionized water.

In all cases, the percentage of abnormal seedlings, according to the ISTA rules,[13] positively correlates with the speed of seed water uptake by soybean ($R^2$=0.92, $P$=0.08), common bean ($R^2$=0.99, $P$=0.01) and maize seeds ($R^2$=0.73, $P$=0.27) measured during the first 12 h of the seed imbibition (Fig. 3). For all tested plant species, the highest percentage of abnormal seedlings was observed in the control variants imbibed in Petri dishes in a film of free water, while the seeds imbibing between filter paper with the slowest rate of water uptake developed the smallest number of abnormal seedlings.

**Seedling development**

Abnormal seedlings were discarded at final harvest (10 DAS) and the measured growth characteristics refer to normal developed seedlings according to the ISTA rules.[13]

In soybean, the short-term (36 h) seed treatment with 100 and 1000 mg $L^{-1}$ of MWCNTs did not affect shoot length, shoot and root dry weight of seedlings. However, the total root length was significantly reduced, while average root diameter increased in both MWCNT treatments (Table 2). In the control between layers of moist paper the dry root weight of soybean seedlings decreased as compared to theother treatments and the total root length was shorter than in the control, but longer as compared to MWCNTs treatments.

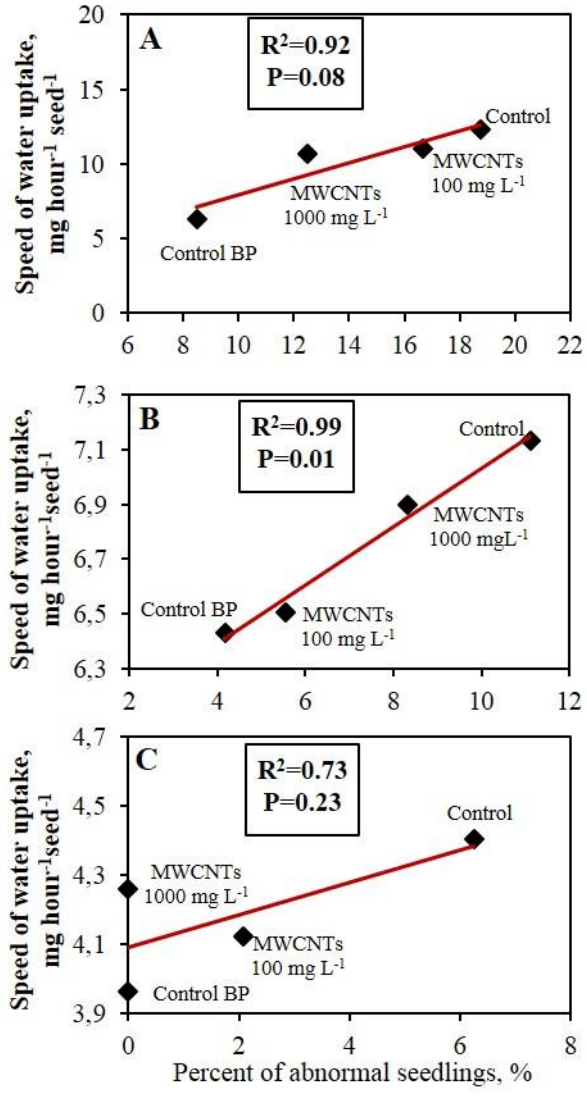


**Figure 3.** Correlation between speed of water uptake by seeds during the first 12 h of imbibition and formation of abnormal seedlings (%) at 10 days after sowing (DAS). Soybean (A), common bean (B) and maize (C) seeds were treated in Petri dishes for 36 h by 100 and 1000 mg $L^{-1}$ MWCNTs or DI water (Control) and by DI water between moist filter paper (control BP) and subsequently grown in filter paper rolls moistened with DI water. MWCNTs−multi-walled carbon nanotubes, DI water−deionized water.

The application of 1000 mg $L^{-1}$ MWCNTs reduced the root dry weight of common bean and increased the shoot dry weight of maize seedlings as compared to the untreated control. There is also a trend for declining total root length of common bean and maize seedlings induced by 1000 mg $L^{-1}$ MWCNTs as compared to the control, although not significant (Table 2).

The analysis of root diameter distribution reveals a reduction of fine root length (diameter: 0.0−0.2 and 0.2−0.4 mm) of soybean in both applied MWCNT concentrations, while the length of roots with a diameter of 0.4−0.6 mm decreased only by the application of 1000 mg $L^{-1}$ MWCNTs (Table 3). Similarly, in maize the length of fine roots (0.0−0.2 mm) decreased in the 1000 mg $L^{-1}$ MWCNT variant (Table 3).

**Assessment of root activity**

As indicators for root activity, nitrate uptake of the seedlings was measured by nitrate depletion of a nutrient solution. Additionally, vitality staining of the root tissue with 2, 3, 5-triphenyl tetrazolium chloride (TTC) was performed, followed by extraction and photometric quantification of the red triphenylformazan formed by the metabolic activity of the roots.

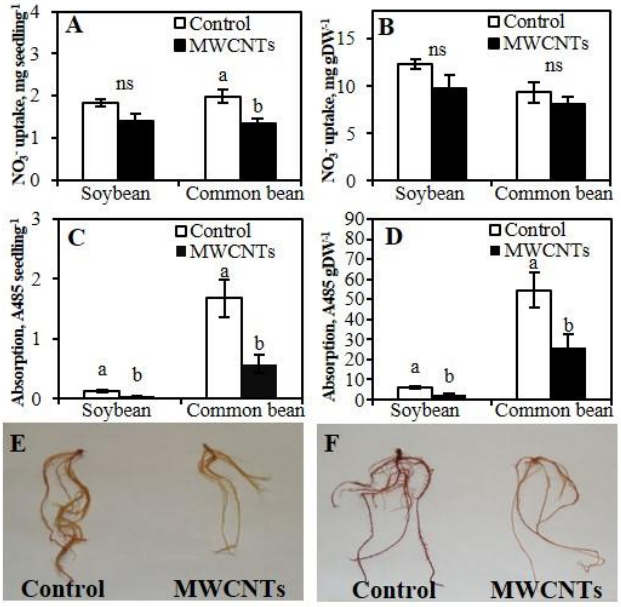


**Figure 4.** Assessment of root activity of soybean and common bean seedlings. (A) Nitrate uptake per plant [mg $seedling^{-1}$] and (B) per unit root dry weight [mg $g_{DW}^{-1}$] by 10-days old soybean and common bean seedlings cultivated in nutrient solution during 24 h. (C) Absorption of a triphenylformazan (TF) at 485 nm per seedling [A485 $seedling^{-1}$] and (D) per unit root dry weight [A485 $g_{DW}^{-1}$] as a product of 2, 3, 5-triphenyl tetrazolium chloride (TTC) reduction by the roots of 10-days old soybean and common bean seedlings immersed for 24 h in 0.08% TTC solution followed by ethanol extraction. (E) Roots of soybean and (F) common bean seedlings after staining with 0.08% TTC. The seedlings (A−F) were developed from seeds treated in Petri dishes for 36 h with 1000 mg $L^{-1}$ MWCNTs or DI water (Control) and subsequently grown in filter paper rolls moistened with DI water. Values represent mean values ± SEM of five replicates. Different letters (a, b) indicate significant differences between treatments (*t*-Student test, $P \leq 0.005$), ns−not significant. MWCNTs−multi-walled carbon nanotubes, DI water−deionised water.

**Table 2.** Growth characteristics of 10-days old soybean, common bean and maize seedlings, developed from seeds exposed to MWCNTs for 36 h and subsequently grown in filter paper rolls. The seeds were treated in Petri dishes either with DI water (Control) or MWCNTs suspensions (100 and 1000 mg $L^{-1}$) or imbibed slowly between filter paper with DI water (control BP).

| Treatment | Shoot length, cm $plant^{-1}$ | Shoot dry matter, mg $plant^{-1}$ | Root dry matter, mg $plant^{-1}$ | Total root length,cm $plant^{-1}$ | Average root diameter, mm |
|---|---|---|---|---|---|
| **Soybean (*Glycine max* (L.) Merr)** | | | | | |
| Control | 17.6 ± 0.7 a | 95.56 ± 3.69 a | 16.70 ± 1.26 a | 981.1 ± 23.0 a | 0.25 ± 0.00 b |
| Control BP | 12.6 ± 0.4 b | 94.35 ± 6.07 a | 15.50 ± 1.18 b | 820.2 ± 14.5 b | 0.26 ± 0.00 b |
| MWCNTs 100 mg $L^{-1}$ | 18.8 ± 0.5 a | 101.05 ± 4.21 a | 21.17 ± 1.38 a | 523.2 ± 18.4 c | 0.33 ± 0.01 a |
| MWCNTs 1000 mg $L^{-1}$ | 18.8 ± 0.7 a | 93.51 ± 2.85 a | 17.90 ± 0.75a | 458.5 ± 5.8 d | 0.33 ± 0.00 a |
| **Common bean (*Phaseolus vulgaris* L.)** | | | | | |
| Control | 17.2 ± 0.3 a | 161.20 ± 5.04 a | 37.26 ± 0.96 a | 137.7 ± 22.3 a | 0.39 ± 0.02 a |
| Control BP | 17.2 ± 0.3 a | 154.61 ± 6.19 a | 34.35 ± 1.53 a | 86.3 ± 9.2 a | 0.33 ± 0.02 a |
| MWCNTs 100 mg $L^{-1}$ | 16.7 ± 0.2 a | 143.45 ± 12.92 a | 31.81 ± 2.69 a | 91.7 ± 16.7 a | 0.30 ± 0.03 a |
| MWCNTs 1000 mg $L^{-1}$ | 16.5 ± 0.4 a | 143.42 ± 13.16 a | 28.41 ± 3.12 b | 91.2 ± 8.6 a | 0.31 ± 0.02 a |
| **Maize (*Zea maize* L.)** | | | | | |
| Control | 10.9 ± 0.5 a | 28.10 ± 0.76 b | 42.23 ± 1.93 a | 93.5 ± 5.5 a | 0.77 ± 0.02 a |
| Control BP | 11.0 ± 0.4 a | 25.88 ± 1.49 b | 41.00 ± 2.27 a | 92.1 ± 5.1 a | 0.76 ± 0.02 a |
| MWCNTs 100 mg $L^{-1}$ | 11.3 ± 0.4 a | 29.29 ± 2.25 b | 44.06 ± 2.04 a | 91.3 ± 6.8 a | 0.76 ± 0.01 a |
| MWCNTs 1000 mg $L^{-1}$ | 10.7 ± 0.4 a | 38.13 ± 2.09 a | 39.27 ± 1.86 a | 77.1 ± 7.1 a | 0.75 ± 0.03 a |

**Note:** Results represent mean values ± SEM of 6 replicates. Different letters (a, b, c, d) indicate significant differences between treatments (one-way ANOVA, Tukey test, $P \leq 0.05$). Control BP−control between paper, MWCNTs−multi walled carbon nanotubes, DI water−deionized water.

**Table 3.** Length of fine root fractions of 10-days old soybean, common bean and maize seedlings, developed from seeds exposed to MWCNTs for 36 h and subsequently grown in filter paper rolls. The seeds were treated in Petri dishes either with DI water (Control) or MWCNT suspensions (100 and 1000 mg $L^{-1}$) or imbibed slowly between filter paper with DI water (control BP).

| Treatment | Fine root length (0 ≤ 0.2 mm), cm $plant^{-1}$ | Fine root length (0.2 ≤ 0.4 mm), cm $plant^{-1}$ | Fine root length (0.4 ≤ 0.6 mm), cm $plant^{-1}$ |
|---|---|---|---|
| **Soybean (*Glycine max* (L.) Merr)** | | | |
| Control | 679.2 ± 42.7 a | 72.2 ± 6.3 a | 51.7 ± 3.6 a |
| Control BP | 586.2 ± 14.4 b | 65.2 ± 1.7 a | 53.9 ± 4.6 a |
| MWCNTs 100 mg $L^{-1}$ | 327.1 ± 18.3 c | 33.7 ± 2.5 b | 43.3 ± 3.5 ab |
| MWCNTs 1000 mg $L^{-1}$ | 287.4 ± 10.2 c | 26.8 ± 1.7 b | 36.4 ± 2.7 b |
| **Common bean (*Phaseolus vulgaris* L.)** | | | |
| Control | 0.5 ± 0.1 a | 14.5 ± 1.3 a | 79.8 ± 7.8 a |
| Control BP | 0.4 ± 0.0 a | 10.8 ± 1.8 a | 79.2 ± 3.5 a |
| MWCNTs 100 mg $L^{-1}$ | 0.5 ± 0.0 a | 14.4 ± 2.3 a | 69.9 ± 6.8 a |
| MWCNTs 1000 mg $L^{-1}$ | 0.5 ± 0.1 a | 13.3 ± 2.2 a | 93.0 ± 10.4 a |
| **Maize (*Zea maize* L.)** | | | |
| Control | 24.2 ± 2.6 a | 14.1 ± 2.7 a | 3.4 ± 0.3 a |
| Control BP | 24.6 ± 0.8 a | 10.5 ± 1.4 a | 4.6 ± 0.8 a |
| MWCNTs 100 mg $L^{-1}$ | 20.6 ± 1.1 ab | 16.0 ± 1.9 a | 3.7 ± 0.2 a |
| MWCNTs 1000 mg $L^{-1}$ | 15.7 ± 2.0 b | 17.3 ± 2.6 a | 4.1 ± 0.4 a |

**Note:** Results represent mean values ± SEM of 6 replicates. Different letters (a, b, c) indicate significant differences between treatments (one-way ANOVA, Tukey test, $P \leq 0.05$). Control BP−control between paper, MWCNTs−multi-walled carbon nanotubes, DI water−deionized water.

Nitrate uptake of common bean seedlings developed from seeds treated with MWCNTs (36 h, 1000 mg $L^{-1}$) was significantly reduced as compared to the untreated control (Fig. 4A) and a similar but not significant trend was also recorded for soybean. This was associated with a significant reduction in root length in the MWCNT variants of soybean and common bean (Appendix B). However, analysis of specific nitrate uptake per unit of dry root weight revealed no inhibition related with the application of MWCNTs (Fig. 4B).

The TTC reduction assay revealed significantly inhibited dehydrogenase activity in the seedlings roots exposed to MWCNT treatments. The visual evaluation of stained root samples showed less intensive red coloration of the soybean and common bean roots developed from the MWCNT-treated seeds (Fig. 4E, F). The amount of produced TF was approximately 50% less in MWCNT treatments compared to the corresponding controls (Fig 4C, D).

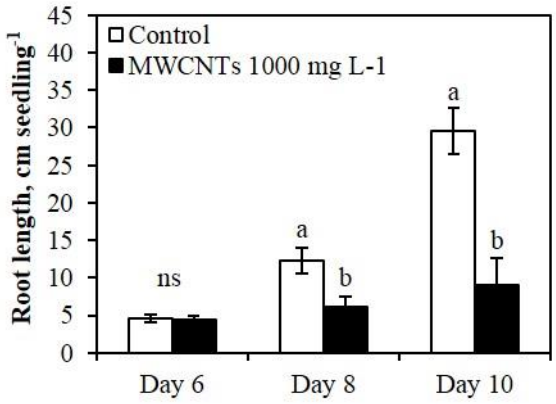


**Figure 5.** Total root length of 6, 8 and 10-days old soybean seedlings, developed from seeds treated in Petri dishes with and without MWCNTs (1000 mg $L^{-1}$ for 36 h) and grown in filter paper rolls moistened with DI water. Different letters (a, b) indicate significant differences between treatments (Student t-test, $P \leq 0.05$), ns−not significant. MWCNTs−multi-walled carbon nanotubes, DI water−deionised water.

**Table 4.** Total root length [cm $plant^{-1}$] of 10-days old soybean seedlings, developed from seeds treated in Petri dishes for 6, 12, 24, 30 and 36 h with MWCNTs (1000 mg $L^{-1}$) or DI water (Control) and subsequently grown in filter paper rolls.

| Seed treatment duration, h | Total root length, cm $plant^{-1}$ | |
|---|---|---|
| | Control | MWCNTs 1000 mg $L^{-1}$ |
| 6 | 91.2 ± 10.3 a | 87.7 ± 3.5 a |
| 12 | 89.3 ± 6.8 a | 82.2 ± 8.7 a |
| 24 | 77.2 ± 5.4 a | 72.7 ± 3.6 a |
| 30 | 73.4 ± 5.8 a | 65.9 ± 4.4 a |
| 36 | 70.8 ± 4.6 a | 60.6 ± 1.6 b |

**Note:** Results represent mean values ± SEM of 4 replicates. Different letters (a, b) in the same line indicate significant difference between treatments (Student t-test, P ≤ 0.05). MWCNTs−multi-walled carbon nanotubes, DI water−deionised water.

**MWCNT exposure time**

To determine the minimum time period for seed exposure required for induction of plant damage induced by MWCNTs in soybean seedlings developed from seeds treated with MWCNTs (1000 mg $L^{-1}$) were investigated after exposure times of 6, 12, 24 and 36 h. Significantly reduced total root length as compared to the control variant were first recorded after 36 h of seed exposure to MWCNTs (Table 4) and is detectable earliest at 8 DAS (Fig. 5).

## Discussion

The present study reveals differences in responses to short-term MWCNT seed exposure in three different plant species (*Glycine max* (L.) Merr), *Phaseolus vulgaris* L., *Zea mays* L.) in a standard germination test according to the ISTA rules,[13] showing both positive and negative effects on plant development. MWCNT suspensions were applied for 36 h prior to radicle emergence in Petri dishes on filter paper in two concentrations (100 and 1000 mg $L^{-1}$), as used in previous studies investigating CNT effects on plant growth[1-9] (reviewed by O. Zaytseva and G. Neumann[11]). The selected concentrations translated into a CNT dosage of 50 and 500 µg MWCNTs $seed^{-1}$. However, a closer look at the culture system shows that only traces of the applied MWCNTs had direct seed contact, while by far the majority of the MWCNTs remained sticking to the germination paper.

The most striking positive MWCNT effect on plant development is a 30% stimulation in germination, reflected in germination percentage (Fig. 2) and development of abnormal seedlings according to the ISTA classification (Fig. 3) recorded in soybean with a germination rate of approximately 65% in the untreated control. This effect is not detectable in common bean or maize, with untreated control variants, reaching almost 100 % germination. The variability in MWCNT-induced stimulation of germination may reflect interspecific differences. On the other hand, seed lot effects related with differences in seed quality and seed aging[19] indicated by different germination rates of the untreated controls, may offer an alternative explanation. A clear distinction would require a comparison of seed lots with a comparable vitality for all tested plant species.

Similar to this study, positive effects of MWCNTs exposure at concentrations between 10 and 200 mg $L^{-1}$ on seed germination and seedling growth have been reported for barley, wheat, maize, peanut (*Arachis hypogaea* L.), soybean and tomato.[1-4] Increased germination has been related with improved seed water uptake during imbibition as a putative consequence of a MWCNT-induced seed coat and cell wall perforation and increased expression of water channel proteins (aquaporins)[3] involved in water uptake, germination, root elongation and also in many stress responses.[20] Additionally, the beneficial impact on plant growth, frequently observed at lower doses of MWCNT has been attributed to hormesis effects[9,21] (reviewed by O. Zaytseva and G. Neumann[11]). However in the current study, the treatment with increasing MWCNT concentration, promoted the positive effects on seed germination in soybean (Fig. 2A), associated with a reduction in the speed of seed water uptake during imbibition (Fig. 1). Moreover, the speed of water uptake in all investigated plant species during seed imbibition is positively correlated with the formation of abnormal seedlings according to the ISTA classification[13] and the lowest rate is recorded for seeds imbibed very slowly between layers of moist filter paper (Fig. 3). Imbibition damages, such as disturbed reconstitution of cell membranes, resulting in reduced germination[22,23] often occur as a result of a rapid seed water uptake in large-seeded leguminous plants, exposed e.g. to excessive soil moisture levels. A large amount of

hydrophobic proteins on the seed coat surface of soybean[24] may allow preferential adsorption of the hydrophobic MWCNTs similar to seed dressing agents. Seed dressings can slow down the speed of water uptake, thereby reducing the risk of imbibition damage,[25] and obviously the MWCNT treatments had a similar function in our experiments. Moreover, seeds with impaired seed vitality (induced e.g. by seed aging) are particularly sensitive to additional stress factors, such as imbibition damage. The low germination rate recorded for the untreated soybean seed lot (65%) used in this study (Fig. 2) may indicate a similar seed vitality problem, associated with a high responsiveness to protective seed dressing treatments and therefore, also to MWCNT application.

However, despite the beneficial effects of short-term MWCNT seed treatments on germination, a negative impact on further seedling development is detected in all tested plant species. The development of root growth and fine root production was inhibited at 10 DAS (Tables 2, 3 and 4) which is particularly important for spatial nutrient acquisition. Accordingly, nitrate uptake measured as nitrate depletion in a hydroponic growth medium is significantly reduced in common bean seedlings with a similar trend also in soybean (10 DAS) exposed to 36 h of MWCNT seed treatments (Fig 4). However, only nitrate uptake per plant is reduced by the MWCNT treatments, while the specific uptake rate per unit root dry weight remains unaffected. This finding suggests that the reduction in nitrate uptake is mainly a consequence of inhibited root growth and not of a limitation in the specific uptake activity. By contrast, vitality staining of the root tissue with TTC (2, 3, 5-triphenyl tetrazolium choride) revealed reduced triphenylformazan (TF) formation per unit root dry biomass of the MWCNT-treated common bean and soybean seedlings. This findings indicate lower metabolic activity and lower vitality of the root tissue, which is potentially responsible for the limitation of root growth.

Apart from plant growth stimulation,[1,3,4] negative growth effects have been similarly reported in the literature for a range of plant species including red spinach, rice, lettuce and cucumber particularly at higher dosages of MWCNT application. Growth restrictions have been related with MWCNT-induced indication of oxidative stress, membrane damage, electrolyte leakage, mitochondrial dysfunctions and DNA aberrations.[7,8,26,27]

## Conclusion

The present study demonstrates that MWCNT effects on plant growth are highly variable depending on plant species, but also on the physiological status and the developmental stage of individual plants. The different plant responses to MWCNT treatments are observed under strictly controlled experimental conditions, largely excluding environmental factors and effects induced by carbon nanomaterials of different origin or agglomeration status.[28,29] Apart from the well-documented stimulation of germination by increased water uptake during imbibition associated with seed coat perforation and upregulation of aquaporin genes,[1,3,29,30] the results demonstrates that MWCNT seed treatments can also exert protective effects by reducing the speed of water uptake thereby minimizing the detrimental effects of imbibition damage,[31] particularly in seeds with limited vitality (Figs. 2 and 3). In soybean, the protective effect seems to be restricted mainly to the first 24 h of seedling development as approximate time period required for complete seed imbibition. A significant reduction in water uptake in MWCNT-treated seeds is already detectable after 12 h of imbibition (Fig. 1), although the promoting effect on germination rate started to appear at 3 DAS (Fig. 2).

By contrast, inhibitory effects on plant growth induced by MWCNT treatments are first detectable at 8 DAS (Fig. 5) and in all investigated plant species root growth is primarily affected (Table 2). In soybean, MWCNT exposure for at least 36 h prior to radicle emergence is required (Table 4) to induce root growth inhibition at 8 DAS (Fig. 5), associated with reduced metabolic activity of the roots (Fig. 4). The molecular and physiological events determining the inhibition of root growth during later seedling development already within 36 h after sowing, remain to be elucidated. A reduced establishment of a functional root system can act as a cause of inhibitory effects on further plant development with pleiotropic patterns, particularly under conditions of limited nutrient and water availability, requiring adaptive responses in root growth for adequate nutrient acquisition. This holds true for soil culture in general with additional impact of abiotic and biotic stress factors. This situation may further contribute to the reported variability of plant responses due to exposure to carbon nanomaterials.

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The authors declare that they have no conflict of interest.

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