Carbon Nanotubes Are Able To Penetrate Plant Seed Coat and Dramatically Affect Seed Germination and Plant Growth

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ABSTRACT Carbon nanotubes (CNTs) were found to penetrate tomato seeds and affect their germination and growth rates. The germination was found to be dramatically higher for seeds that germinated on medium containing CNTs (10−40 μg/mL) compared to control. Analytical methods indicated that the CNTs are able to penetrate the thick seed coat and support water uptake inside seeds, a process which can affect seed germination and growth of tomato seedlings.

KEYWORDS: carbon nanotubes · tomato plants · enhanced germination · seed coat · water uptake

During the past decade, there has been a rapid growth of research in the areas of nanomaterials and nanoscience because of the realization that these small size materials can be used in a multitude of industrial and biomedical processes. The great potential of using nanoscale particles for different biological and medical applications including gene and drug delivery, biosensing, diagnostic, and tissue engineering was widely documented during the last several years.[1−6]

Most investigations were focused on studying the effects of different nanomaterials on the cellular morphology, behavior and functions, and selective killing in order to understand how such structures would affect animals and humans at various levels.[7−11] Moreover, thorough studies and reliable information regarding the effects of nanomaterials such as carbon nanotubes on plant physiology and plant development at the organism level are very limited. However, there is an extensive interest to investigate the ability of nanoparticles to penetrate plant cell walls and work as smart treatment-delivery systems in plants. Several research groups reported that different types of nanoparticles are able to penetrate plant cell walls. Thus, it was shown that gold-capped mesoporous silica nanoparticles (MSNs) were able penetrate cell wall and delivery DNA into plant cell by using a bombardment method.[12] Recently, Liu and co-authors[13] demonstrated the capability of single-walled carbon nanotubes (SWNTs) to penetrate the cell wall and cell membrane of tobacco cells. Additionally, methods of visualization of carbon-coated iron nanotubes in plant cells using pumpkin plants as the model were reported.[14] There is an extensive interest in applying nanoparticles to plants for agricultural and horticultural use.[15] To achieve the goals of "nano-agriculture", detailed studies on the effects of nanotubes on seed germination and development of seedlings of valuable agricultural plant species are needed. Penetration of plant seeds could be more complicated as compared to plant cell walls and mammalian cell membranes due to the significant thickness of seed coat covering the whole seed.[16] However, it was shown that seed coats of different plant species are selectively permeable to heavy metal ions such as Pb^{2+} and Ba^{2+}.[17] On the basis of this observation, it is logical to assume that some nanosize materials will be able to penetrate plant seed coats and affect seed germination. This study is the first report, in our best knowledge, that describes the effect of penetration of plant seed coats by carbon nanotubes. Here, we demonstrated that the exposure of carbon nanotubes to seeds of valuable crops, such as tomatoes, can increase the germination percentage and support and enhance the growth of seedlings. Furthering these findings could result in significant developments of improved plants for the area of

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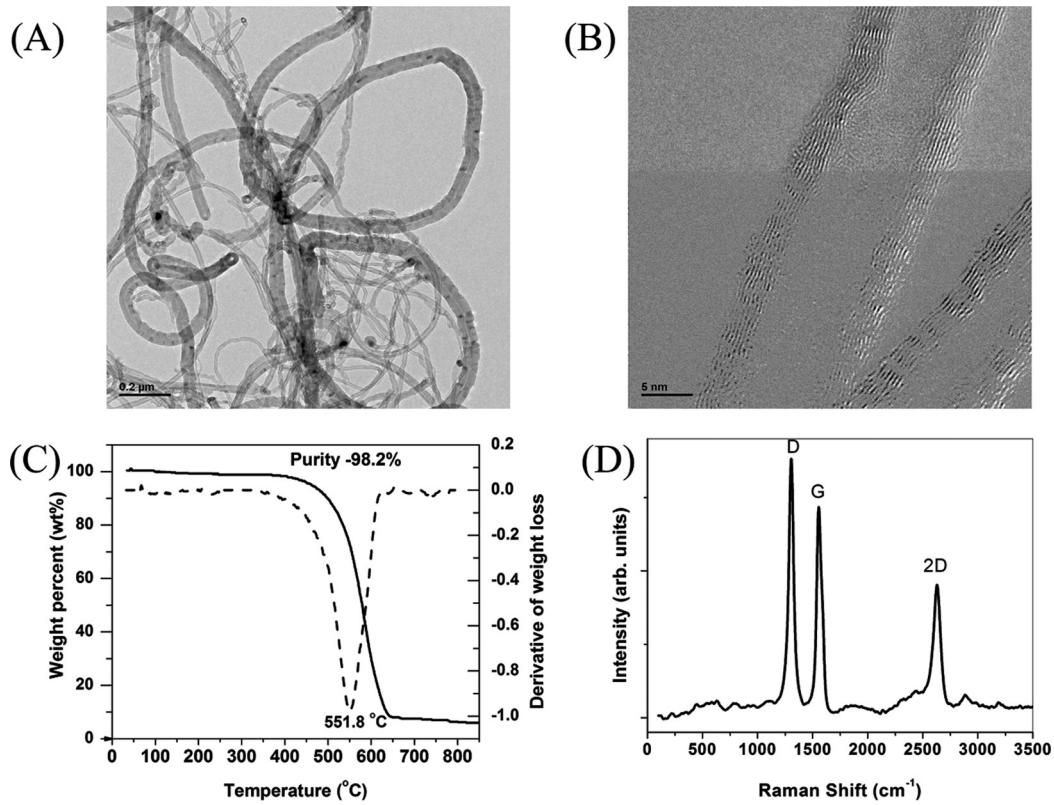

Figure 1. (A) Low- and (B) high-resolution TEM images of the CNTs obtained over Fe−Co/$CaCO_3$ catalyst, the weight loss profile and the oxidation rate of the CNTs (C), and their corresponding Raman scattering spectra (D).

energy, by taking advantage of the enhancement in the biomass of the plants when they are exposed to nanosized materials and fertilizers.

RESULTS AND DISCUSSION

Carbon Nanotube Analysis. The multiwall carbon nanotubes (CNTs) used in this study were produced on an Fe−Co/$CaCO_3$ catalyst with a Fe/Co/$CaCO_3$ weight ratio of 2.5:2.5:95 using acetylene as carbon source at 720 °C. The yield was found to be around 80%. The low- and high-magnification TEM images of CNTs are shown in panels A and B of Figure 1, respectively. Thermogravimetric analysis (TGA) was performed to characterize the purity of the purified CNTs in an airflow rate of 150 mL/min. The first derivative of the TGA curve determines the decomposition temperature of the sample. Figure 1C shows the weight loss profile of the purified nanotubes, which were heated from 25 to 850 °C at a rate of 5 °C/min. The normalized TGA curve and its first derivative indicate a significant mass drop at around 551 °C, which corresponds to the weight loss due to the combustion of the CNTs. The quantitative analysis revealed that, after the single-step purification in HCl, the purity of the CNT product was higher than 98%. Raman spectroscopy has been widely used to analyze the crystallinity and the diameter distribution of CNTs. The Raman scattering spectrum of the CNTs grown on Fe−Co/$CaCO_3$ is shown in Figure 1D. The characteristic bands for CNTs are the D band, G band, and the 2D band. The D band is present between 1305 and 1330 cm^{-1} and is related to the presence of defects and impurities in the carbon nanotube. The G band, present between 1500 and 1605 cm^{-1}, is also known as the tangential band and arises from the E_{2g} mode of the graphite plane. The G band position is relatively constant for CNT material excited at different energies.[18−20] The last important mode observed in the Raman spectrum of CNTs is the 2D band or the second-order harmonic of the D band, which is often present between 2450 and 2650 cm^{-1}. The 2D band is also highly dispersive and associated with the degree of CNT crystallinity. The relative intensities between the G and the D band (I_G/I_D) and between the 2D and G band (I_{2D}/I_G) are found to be 0.81 and 0.63, respectively. These values indicate an interplanar distance of 0.342 nm between the graphite layers, as shown by Yoshida *et al.*[21]

Carbon Nanotubes Affect the Germination Rate. To test whether the synthesized carbon nanotubes could affect germination and development of crop seedlings, we placed sterile tomato seeds (cv. Micro-Tom) on standard agar Murashige and Skoog medium (MS medium) supplemented with different concentrations of CNTs (10, 20, 40 μg/mL). The MS medium without CNTs was used for control experiments. As shown in Figure 2A,B, addition of carbon nanotubes to agar medium was found to accelerate the process of seed germination and significantly shortened the germination time. Tomato seeds placed on medium with CNTs (10, 20, 40

μg/mL) germinated on the third day, while the tomato seeds placed on regular MS did not germinate at that time (Figure 2B). The germination percentage rates during the next days were dramatically higher for seeds that were treated with nanoparticles. The germination percentage for seeds that were placed on regular medium averaged 32% in 12 days and 71% in 20 days, while germination percentage of the seeds placed on medium supplemented with CNTs averaged 74−82% in 12 days and 90% in 20 days (Figure 2A). Seedlings with developed cotyledons and root system were recognized as fully germinated in this experiment.

We further investigated effects of CNTs on the growth and development of seedlings germinated on medium supplemented with nanoparticles (Figure 3A−E). Tomato seedlings germinated and developed on the medium with different concentrations of CNTs (10, 20, 40 μg/L) exhibited a dramatic increase in vegetative biomass (Figure 3A). Fresh weight of total biomass (leaves, stems, and roots) increased 2.5-

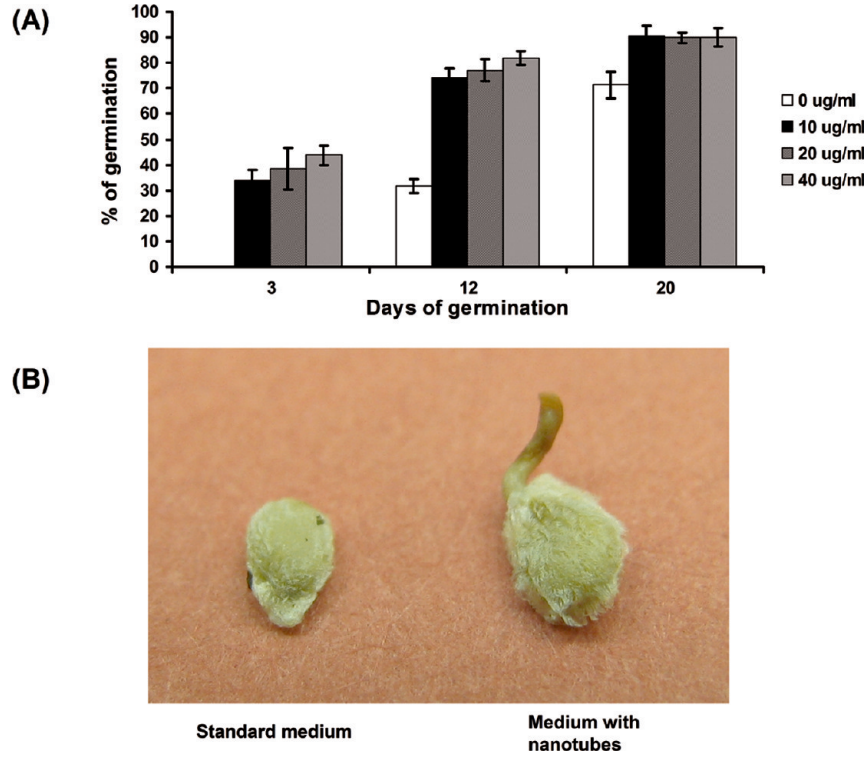

Figure 2. Effect of CNTs on tomato seed germination. (A) Time of germination and germination percentages of seeds incubated with and without CNTs during 20 days. Seedlings with developed cotyledons and root system were recognized as fully germinated in this experiment. (B) Phenotype of tomato seeds incubated during 3 days without (left) or with (right) CNTs on MS medium. Results are shown as average ± SE of three independent experiments.

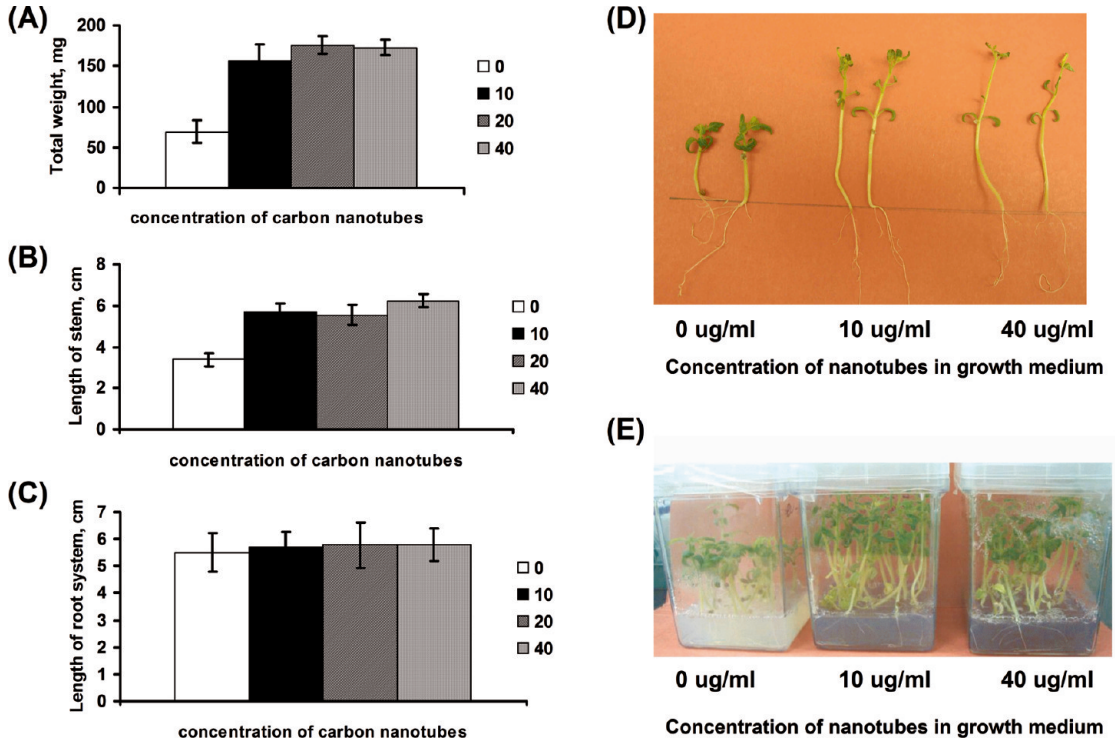

Figure 3. Effect of CNTs on growth and development of tomato seedlings. Results are shown as average ± SE of measurements of 10 plants per each condition. Twenty-seven-day-old seedlings were used for all measurements. (A) Weight of total fresh biomass of tomato seedlings growing on medium with and without CNTs. (B) Length of stem of tomato seedlings growing on medium with and without CNTs. (C) Length of root system of tomato seedlings growing on medium with and without CNTs. (D) Phenotypes of 27-day-old tomato seedlings growing on medium with and without CNTs. (E) Phenotypes of 25-day-old tomato seedlings growing on medium without and with CNTs (10 and 40 μg/mL).

fold for the seedlings germinated and grown on CNTs containing medium compared with seedlings developed on the standard medium. CNT-exposed tomato seedlings had longer stems and were more developed but presented similar lengths of root system compared with control (CNTs nontreated) seedlings (Figure 3B−E). Previously, limited reports indicated both positive and negative effects of different nanoparticles on plant physiology.[22] It was demonstrated that nano-TiO_2 treatment in proper concentration accelerated the germination of the aged spinach seeds and increases its vigor. The mechanism of observed effect was not investigated.[23] Recently, Lin and Xing made a comprehensive toxicity profile for five types of nanoparticles at a concentration of 2000 mg/L using six plant species.[24] They found that seed germination rates of all tested plant species were not affected by different types of nanoparticles except for the seeds of ryegrass and corn treated with nano-Zn particles. In this case, the inhibition effects of nano-Zn were documented. These experimental data indicate that the effects of nanomaterials on the plant growth and development are dependent upon the type of nanoparticles, concentration, plant species, and specific conditions of experiments including the method of nanoparticle uptake into the plant organisms. Additionally, some authors indicated that size and specific surface characteristics may play an important role in phytotoxicity of nanoparticles.[25] Cañas *et al.*[26] recently reported that application of carbon nanotubes resulted in inhibition of root elongation in tomato and enhancement of root elongation in onion and cucumber. In contrast with this observation, our results (Figure 3D) did not indicate any toxic effects of the CNTs on root development and root elongation of tomato seedlings, at least in the concentration range that was used. Water is a major required factor for plant seed germination. Mature seeds are relatively dry and need to uptake significant amounts of water before cellular metabolism and growth can resume. The rate of water imbibition is dependent on the permeability of the seed coat and available amount of water in the germination area.[27,28] We hypothesize that the observed activation of germination by CNTs is based on the role of CNTs in the process of water uptake inside the seed embryo.

Carbon Nanotubes Promote Water Uptake Inside the Seeds. To better understand the mechanism of activation of plant seed germination by application of carbon nanotubes, we performed experiments to measure the level of moisture of the tomato seeds by thermogravimetric analysis (TGA). Total level of moisture (%) present in the tomato seeds was determined by measuring the total mass loss of the seeds (Figure 4A,B) when heated from room temperature to 250 °C and maintained at this temperature for 120 min. First, we measured the level of moisture in dry tomato seeds before any treatments, and these data were used as reference. Then, dry seeds were placed on MS medium with and without CNTs and after 2 days of incubation, and the moisture levels for the seeds (both exposed and not exposed to CNTs) were measured. It was founded that seeds that were exposed to CNTs had a significantly higher level of moisture compared with the seeds that were not treated with CNTs. Thus, 18.4% of moisture level was detected in dry seeds before the experiment; seeds exposed to CNTs accumulated about 57.6% of moisture, and seeds unexposed to CNTs kept only 38.9% of moisture. This result suggested that carbon nanotubes could significantly enhance the water uptake inside tomato seeds.

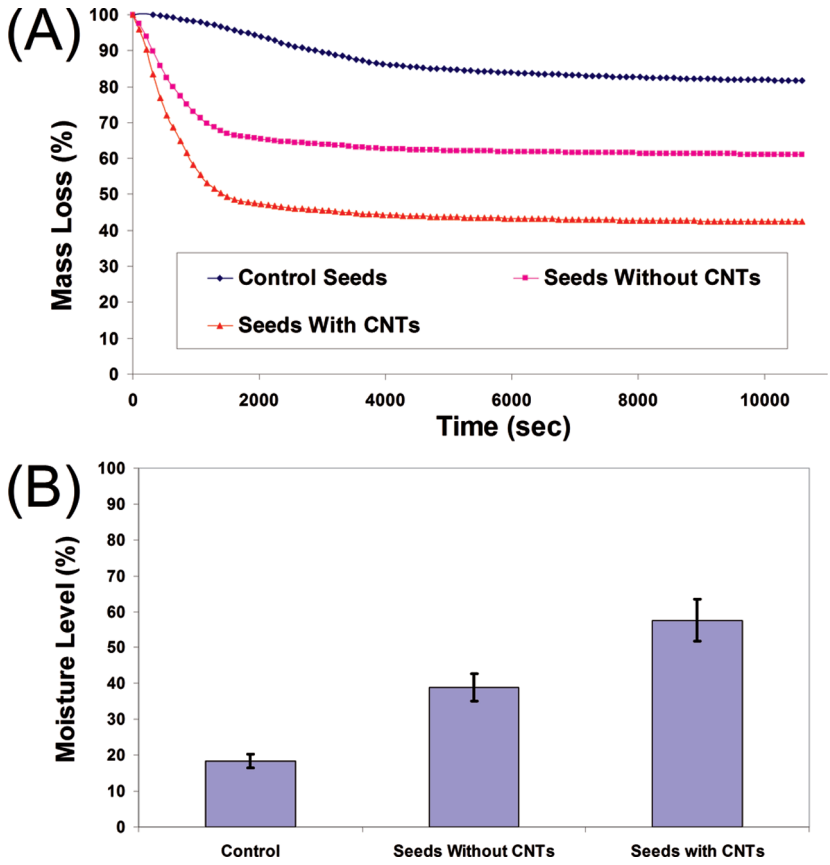

Figure 4. Mass loss (A) and moisture level (B) of seeds incubated with or without CNTs during 2 days.

One possible explanation of this observed effect could be based on the assumption that nanotubes are able to penetrate seed coat while supporting and allowing water uptake inside the seeds. To test such a possibility, Raman spectroscopy was used to detect the possible presence of the CNTs inside the seed embryos exposed and unexposed to CNTs. Raman spectroscopy is a technique that can give accurate information for the presence of

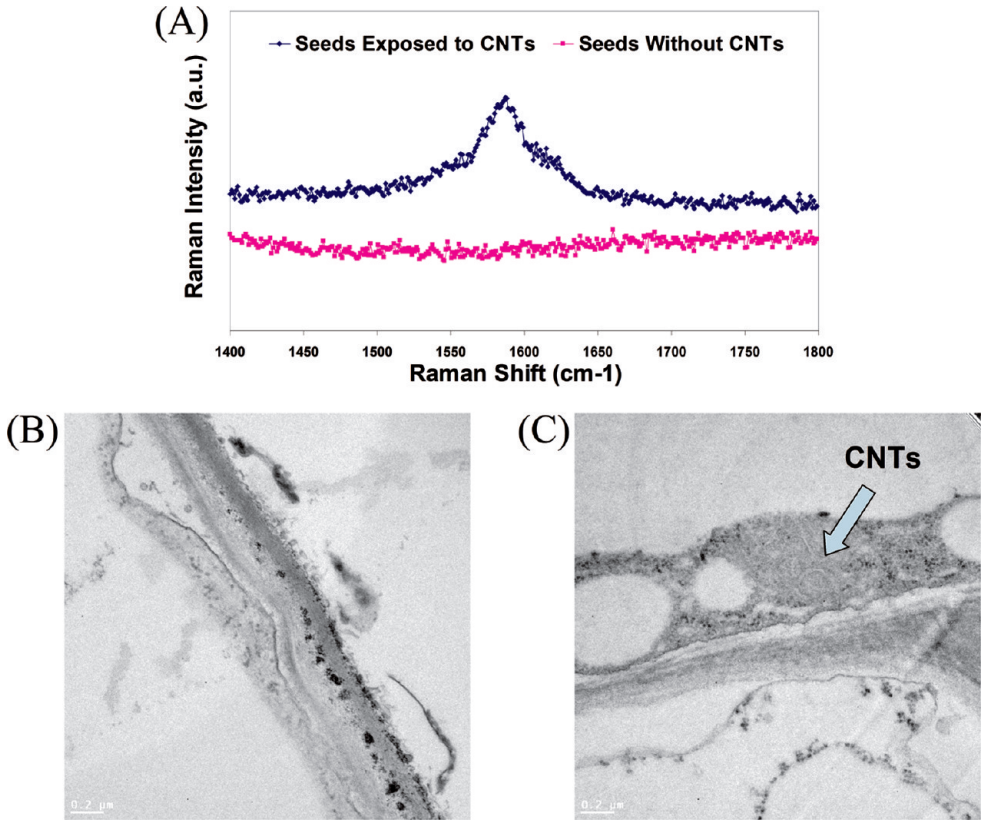

Figure 5. Detection of CNTs inside tomato seeds incubated with CNTs by Raman spectroscopy (A); TEM images of the root system of 25-day-old tomato seedlings growing on medium without CNTs (B) and with CNTs (C).

graphitic materials, such as CNTs, inside a biological systems, given the unique Raman spectrum of the CNTs and their strong scattering properties. For this experiment, tomato seeds were placed on regular agar MS medium (control) and MS medium supplemented with carbon nanotubes (40 μg/mL). Two days after the seeds were incubated under both conditions, they were removed from the medium, washed with water, opened by longitudinal cut, and dried, and the freshly exposed surfaces were analyzed by Raman spectroscopy. Raman spectroscopy has the ability to monitor and identify the CNTs during their transportation from the medium to the seeds. The strong and specific Raman scattering properties of individual CNTs and their clusters made it possible to use Raman spectroscopy for monitoring the CNTs among the biological tissues of the seeds. As shown in Figure 5A, a Raman signal of the CNT's G band (1569 cm^{-1}) was detected inside seeds exposed to CNTs, while no signal was detected in control seeds that were incubated on medium without nanoparticles. Even for relatively long acquisition times (over 80 s), the Raman spectra of the biological tissues did not show any peak at 1568 cm^{-1} (which is therefore specific only to CNTs). Therefore, this G band can be used as a marker for the presence of nanotubes and its intensity could reflect the amount of nanotubes present in the focal volume of the laser. The CNTs' corresponding G band was not observed when parts of the grown plants were further analyzed (roots, stems, leaves), which does not indicate that the CNTs were not present, but rather that possibly their amounts were below the detection level of the Raman spectrometer.

These results were further supported by high-magnification TEM imaging of the roots collected from plants with and without exposure to CNTs (Figure 5B,C). It can be seen in Figure 5C the clear morphology of several CNTs, which are completely missing in the images of the control samples. These studies indicate that the CNTs were able to penetrate both the seeds as well as the root systems of the more developed plants.

These results clearly indicate that the various nanomaterials can be uptaken by the tomato seeds and significantly affect their biological activity, most probably by enhancing the amount of water that penetrates inside the seeds during the germination period.

The mechanism by which nanoparticles can support water uptake inside seeds is not clear yet. It is possible that nanoparticles can create new pores for water permeation by penetration of seed coat. Another explanation could be based on the assumption that carbon nanotubes are able to regulate gating of existent water channels (aquaporins) in the coat of plant seeds. Systematic investigation of the mechanisms of the gating of water channels in plant cells is lacking. However, it was shown that activity of water channels could be regulated by different stresses such as high osmotic

pressure, anoxia, heavy metals, pH, salinity, and others.[29] Expression of several water channel genes including important PIP genes was characterized during rice seeds germination.[30] Possible effects of carbon nanotubes on aquaporins on genomic and protein level will be subject for our future investigations. Also, further genomic and proteomic studies are needed in order to fully understand the complex interaction between nanomaterials and various plant species. In particular, the use of nanomaterials for accelerated plant growth could open a new research direction for areas such as biofuels for which the total biomass is crucial for the ultimate production yield. On the downside, the possible unknown toxic effects, mostly related to the nanoparticle concentrations delivered to various plant lines, should be of interest for further advanced studies. We recognize that some of the positive results presented in this work could be completely different if the experimental conditions would change. Such factors would include the type of plants and nanomaterials, concentrations of the nanostructures, as well as their chemical and biological surface functionalizations.

CONCLUSIONS

Our results demonstrated, for the first time, that carbon nanotubes can penetrate thick seed coat and support water uptake inside seeds. The activated process of water uptake could be responsible for the significantly faster germination rates and higher biomass production for the plants that were exposed to carbon nanotubes. Molecular mechanisms of CNT-induced water uptake inside plants seeds are not clear and require further investigation. However, an observed positive effect of CNTs on the seed germination could have significant economic importance for agriculture, horticulture, and the energy sector, such as for production of biofuels.

METHODS

Synthesis of Carbon Nanotubes. The multiwall carbon nanotubes (CNTs) used in this study were produced on an Fe−Co/$CaCO_3$ catalyst with a Fe/Co/$CaCO_3$ weight ratio of 2.5:2.5:95 using acetylene as carbon source at 720 °C. First, the Fe/Co/$CaCO_3$ catalyst was prepared as follows: The distilled water solutions of the $Fe(NO_3)_3 \cdot 9H_2O$ and $Co(CH_3COO)_2 \cdot 4H_2O$ salts were poured over a $CaCO_3$ suspension in water under continuous stirring. The pH of the solution was maintained constant at 7−7.5 by adding ammonia solution (25%). The solvent was evaporated on a steam bath under continuous stirring, and the resulting solid matter was further dried overnight at 125 °C and powdered in a mortar.

For carbon nanotube growth, 150 mg of the Fe/Co/$CaCO_3$ catalyst was uniformly dispersed onto a graphite susceptor and introduced into the quartz reactor (2 cm diameter and 80 cm length) positioned in the middle of a water-cooled copper coil connected to a high-frequency generator (5 kW, 350 kHz). A nitrogen flow of 200 mL/min was introduced into the reactor for 15 min to remove the air, followed by inductive heating at 720 °C. This process was followed by the administration of acetylene (3 mL/min) for 30 min. The removal of the catalyst from the CNT final product was done by ultrasonication in HCl (1:1) for 30 min, washing with distilled water, and drying overnight at 120 °C. The efficiency of the reaction is defined as percent ratio between the mass of product obtained after purification and the initial mass of catalyst. The morphology of the nanotubes was studied by scanning electron microscopy (SEM-JEOL 7100 FE) and transmission electron microscopy (TEM- JEOL2100 FE). For this analysis, carbon nanotubes were dispersed in 2-propanol and sonicated for 10 min. A few drops of the suspension were deposited on the TEM grid then dried and evacuated before analysis. Raman scattering studies of the CNTs were performed at room temperature using Horiba Jobin Yvon LabRam HR800 equipped with a charge-coupled detector, a spectrometer with a grating of 600 lines/mm, and a He−Ne laser (633 nm) and Ar^+ (514 nm) as excitation sources. The laser beam intensity measured at the sample was kept at 20 mW. The microscope focused the incident beam to a spot size of <0.01 mm^2, and the backscattered light was collected 180° from the direction of incidence. Raman shifts were calibrated with a silicon wafer at a peak of 521 cm^{-1}. Thermogravimetrical analysis (TGA Mettler Toledo 815e) was done in airflow (150 mL/min) and a heating rate of 5 deg/min.

Germination of Tomato Seeds. Seeds of tomato (cv. Micro-Tom) were sterilized by 10 min treatment with 50% Chlorox solution and then rinsed five times with sterile water. Sterile tomato seeds were placed on Murashige and Skoog medium (MS) without or with carbon nanoparticles (10, 20, 40 μg/mL) for germination. Sterile Magenta boxes were used for all germination experiments.

Transmission Electron Microscopy. Tomato samples (roots) were pinned onto Silgard-coated plastic Petri dishes and overlaid with a fixing solution containing 2% paraformaldehyde, 2.5% glutaraldehyde, 1.5 mM calcium chloride ($CaCl_2$), and 1.5 mM $MgCl_2$ in 0.05 M PIPES buffer, pH 6.9. Small pieces were then cut with a razor blade from the apical leaf tips and pinned in place to keep them submerged. Dishes were covered, and fixation proceeded for 5.5 h at room temperature. Thereafter, leaf pieces were washed three times for 20 min each in 0.05 M PIPES buffer containing 1.5 mM $CaCl_2$ and 1.5 $MgCl_2$ and placed at 4 °C in the same solution overnight. Samples were washed one more time in the buffer rinse and then briefly postfixed at room temperature for 20 min in 1% osmium tetroxide, 0.8% potassium ferricyanide, 1.5 mM $CaCl_2$, and 1.5 mM $MgCl_2$ in 0.05 M PIPES buffer, pH 6.9, after which time Kodak Photo-flo was added (3.5% v/v) as a surfactant to reduce surface tension. After several minutes, pieces were unpinned from the Petri dishes and transferred to small shell vials containing fresh fixative without Photo-flo. Postfixation continued for an additional 2.25 h. After fixing, tissues were restored to 4 °C by rinsing in cold distilled water three times for 20 min each and dehydrated in an ascending ethanol series from 10 to 70% ethanol (EtOH), in 10% increments for 20 min each. Tissues were then stained in 1% uranyl acetate in 70% EtOH for 1.5 h at 4 °C, followed by two 5 min rinses in 70% EtOH, with the temperature brought back to room temperature during the second rinse. Dehydration was continued by washing tissues once in 85 and 95% EtOH and twice in 100% EtOH, 15−20 min per step. Finally, two washes in propylene oxide for 10 min each preceded the embedment of material into Spurr's resin. Thin sections were cut from the embedded samples using an ultramicrotome equipped with a diamond knife. Sections were mounted on copper grids. The sections were examined by transmission electron microscope (JEOL 2100 FE).

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