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Impact of Carbon Nanotube Exposure to Seeds of Valuable Crops

Mohamed H. Lahiani,[†] Enkeleda Dervishi,[‡,#] Jihua Chen,[∥] Zeid Nima,[‡] Alain Gaume,[⊥] Alexandru S. Biris,[‡,§] and Mariya V. Khodakovskaya*[,†]

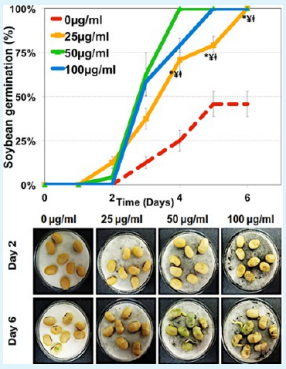
[†]Department of Applied Science, [‡]Center for Integrative Nanotechnology Sciences, and [§]Department of Systems Engineering, University of Arkansas at Little Rock, Little Rock, Arkansas 72204, United States

[∥]Center for Nanophase Materials Sciences, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37831, United States

[⊥]Syngenta Crop Protection AG, Basel, Switzerland

Supporting Information

ABSTRACT: Multiwalled carbon nanotubes (MWCNTs) affected seed germination, growth, and the development of three important crops (barley, soybean, corn). Early seed germination and activation of growth in exposed seedlings was observed when MWCNTs were added to sterile agar medium. Similarly, seed germination was activated for all tested crop species when MWCNTs were deposited on seed surfaces. The ability of MWCNTs to penetrate the seed coats of corn, barley, and soybean was proven by detection of nanotube agglomerates inside MWCNT-exposed seeds using Raman spectroscopy and transmission electron microscopy (TEM). Reverse transcription polymerase chain reaction (RT-PCR) analysis revealed that the expression of genes encoding several types of water channel proteins was increased in soybean, corn, and barley seeds coated with MWCNTs compared with uncoated control seeds. Our results indicate that the positive effect of MWCNTs on the germination and growth of seedlings is reproducible between crop species and can be observed for different methods of delivering carbon nanotubes. Such studies could prove the significant potential of carbon nanotubes as regulators of germination and plant growth.



KEYWORDS: *multiwalled carbon nanotubes, nanoparticles, germination, aerosol spray, aquaporins, Raman spectroscopy*

1. INTRODUCTION

Carbon nanomaterials are expected to find a range of applications from nanomedicine, optics, and electronics to materials science.[1] Eventually, these carbon nanomaterials will make their way into the environment (through recycling or waste), possibly triggering adverse effects in diverse representatives of the plant kingdom. Thus, it is important to understand the possible impact of carbon nanomaterials on the physiology and development of plant systems. Furthermore, a better understanding could open new frontiers in other fields such as agriculture where carbon nanomaterials could soon emerge as novel technology. However, reports related to understanding carbon nanomaterial−plant interactions are still limited and sometimes contradictory. The positive effect of carbon nanotubes on the root growth of onion and cucumber has been previously described.[2] Similarly, an increase in the root length of ryegrass in response to the application of carbon nanotubes was observed.[3] On the contrary, the application of multiwalled carbon nanotubes (MWCNTs) resulted in reduction of zucchini biomass,[4] and application of single-walled carbon nanotubes (SWCNTs) to rice resulted in the decreased yield of rice plants.[5] Recently, we demonstrated that application of SWCNTs or MWCNTs to tomato plants can lead to increased growth in exposed tomato seedlings and activate the expression of many stress-responsive tomato genes.[6] Also, a direct correlation between the activation of growth of tobacco cell culture exposed to MWCNTs and the up-regulation of marker genes for cell division, cell wall extension, and water transport was observed.[7] We have attempted to clarify links between specific properties of nanotubes and the response of plants exposed to CNTs. As a result, we found the level of agglomeration, the type and charge of functional groups on the surface, as well as specific attachments, can play critical roles in the physiological and genetic responses of tomato plants after application of carbon nanotubes.[8,9] Therefore, we believe that some of the contradictory results may be due to the processing and the specific characteristics of the nanostructural materials used in the various experiments. In addition to the actual size, number of concentric walls, length, and diameter, the surface chemistry of the nanotubes along with their ability to cluster or the presence of nanocrystalline carbon also play role in the reported physiologic effects in plants. Advanced methods for detecting nanoparticles, such as transmission electron microscopy,[10] Raman spectroscopy,[6−8] and photothermal/photoacoustic analysis,[6,11] were used for the detection of carbon nanomaterials inside plant organs and plant cells. All of these techniques confirmed the ability of carbon nanotubes to be uptaken by the root system of plants, to move between organs, and even to reach the reproductive organs of plants.[6,12] The ability of carbon nanotubes to interact with the walls of tobacco cells was also experimentally proved.[7,13]

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Table 1. Nucleotide Primers Used for RT-PCR Analysis of Aquaporin Genes from Corn, Barley, and Soybean

crop	gene	gene bank number	sequence of primers	size (bp)	cycle #
Glycine max	*PIP1-2-like*	NM_001254219.2	F-5′AGTGACGTTCGGTTTGTTTC3′ R-5′CACACCAGCACCACAGATAG3′	100	28
Hordeum vulgare	*PIP1;1*	AB286964.1	F-′CCAGACAGAGGACTTGTATGTAATC3′ R-5′GGGCACTACCTGAAACAACT3′	99	28
	TIP-like	X80266.1	F-5′CATGAGTCGTTTAAGTTTGCTTTG3′ R-5′GTCGATCGTCACAGGTTTCA3′	100	23
Zea mays	*PIP1.1*	NM_001254816.1	F-5′TGGATTCGTGGCTGTTTCA3′ R-3′TTACAAGTCTGGGTTCGGATAAA′3	100	28
	TIP1.11	AY243803.1	F-5′CTTCCTTGGACCAGCAGTC3′ R-5′ACTCAGGCAAATGCACAAATC3′	98	28
	SIP1.1	EU961206.1	F-5′CATGCTTGCTGGATGGATATTTAG3′ R-5′ACCAGCATCCATAAACGAGTAA3′	95	28
	NIP1.1	EU964298.1	F-5′CTCCGTCCCATTTATCCAGAAG3′ R-5′TTGCTCGCGTTGATCGT3′	96	23

To understand the biological mechanisms that take place in plants and plant cells after exposure to carbonaceous nanosized materials, many questions still need to be addressed. How universal are the positive effects of carbon nanotubes with the same properties on the germination and growth of various plant species? Can carbon nanomaterials penetrate the seed coats of different crops and affect the expression of key genes involved in the process of germination? Does any correlation exist between the physiological effects of CNTs and their method of delivery to the seeds/plants? In this report, we demonstrate that MWCNTs deposited on the seed surface by airspray techniques or added in growth medium can penetrate seed coats of all tested plant species (barley, corn, soybean) and activate germination of MWCNT-exposed seeds. Additionally, it was observed that the application of carbon nanotubes to the seeds of barley, corn, and soybean can stimulate expression of water channel genes (aquaporins) that play a critical role in the process of seed germination.

2. MATERIALS AND METHODS

2.1. Synthesis and Characterization of Carbon Nanotubes. MWCNTs were synthesized on a Fe:Co:$CaCO_3$ catalyst system with a stoichiometric composition of 3.35:1.65:95 wt %. The catalyst was prepared following the same steps as previously described.[14] Next, the catalyst system was placed on a graphite crucible and inserted inside a quartz tube. Carbon nanotubes were synthesized using a radio frequency catalytic chemical vapor deposition technique at 720 °C. First, the tube was flushed with nitrogen at 200 mL/min for 10 min after which the radio frequency generator was turned on. Once the temperature of the catalyst reached 720 °C, acetylene was introduced at 3.5 mL/min for 30 min. At the end of the reaction, the samples were cooled and purified in HCl diluted solution. Finally, the nanotubes were functionalized with carboxylic groups to ensure a homogeneous dispersion in water.[9] The purity of the samples was determined using a thermogravimetrical analysis (Mettler Toledo TGA/SDTA 851e.) technique. Transmission electron microscopy analyses were also performed using a JEOL field-emission transmission electron microscope, model JEM-2100F. The functionalized nanotubes were dispersed in iso-propanol using a bath sonicator, and a few drops of the solution were deposited on the TEM grid. Raman scattering spectra were recorded at room temperature using a Horiba Jobin Yvon LabRam HR800. The 785 nm wavelength laser was used as an excitation source, and the Raman shifts were calibrated with a silicon wafer at a peak of 521 cm^{-1}.

2.2. Plant Growth and Seed Germination. *2.2.1. Germination on Agar Medium.* Seeds, including soybean hybrid S42-T4, barley hybrid Robust, and corn hybrid N79Z 300GT, were obtained from Syngenta, Inc. (Greensboro, NC). Standard Murashige and Skoog medium (MS medium) was supplemented with different concentrations of CNTs (50, 100, and 200 μg/mL). The MS medium without CNTs was used as the control medium. All seeds were germinated under sterile conditions. Soybean seeds were sterilized with chlorine gas overnight as previously shown by Paz et al.[15] Barley seeds were treated with a solution of 50% H_2SO_4 for 15 min and washed thoroughly using deionized autoclaved water. Corn seeds were pretreated with soapy water and 80% EtOH, kept in 50% bleach for 15 min, and washed thoroughly in deionized autoclaved water. All seeds were kept in Magenta boxes and incubated at 25 °C. At day 10 and day 11, phenotypic measurements of corn and soybean seedlings were assessed, respectively. Root, shoot, and leaf lengths were evaluated. The fresh weight of plants was also assessed. Seedlings were dried in a hot oven at 75 °C overnight, and the dry weight was measured. All data were analyzed using ANOVA and posthoc analysis using the Tukey test for treatment differences.

2.2.2. Germination of Seeds Coated with MWCNTs by the Airspray Method. The functionalized nanotubes were dispersed in water at three different concentrations (100 μg/mL (heavy), 50 μg/mL (medium), and 25 μg/mL (light)). Next, the seeds were separately sprayed using an airbrush system with the "heavy", "medium", and "light" solutions. Each holder containing 10 seeds was sprayed with 10 mL of nanotube solution. For comparison, controls were also sprayed with just 10 mL of water. All seeds were monitored daily for the appearance of the emerging radical. Pictures were also taken daily to assess any phenotypical differences. The experiment was repeated three consecutive times.

2.3. Statistics for Tests of Germination and Plant Growth. All assays were performed in triplicate. All figures are represented as mean values ± SE (standard errors). All data were analyzed using SPSS software by performing repeated measure ANOVA for time-effect analysis and ANOVA and posthoc analysis using the Tukey test for treatment differences. Statistical significance was determined by $p < 0.05$.

2.4. Sequence Alignment. The sequences of aquaporin proteins were obtained from the FASTA Genbank using the PubMed database. ClustalX software was used to create the multiple alignments. Sequence similarity was determined after running protein alignment using the blastp program of protein−protein BLAST. The aligned sequences were analyzed using a Molecular Evolutionary Genetic Analysis package (MEGA 5.0).

2.5. Detection of Multiwalled Carbon Nanotubes Inside Seeds by Raman Spectroscopy. Raman-scattering spectra were obtained with a Horiba Jobin Yvon LabRam HR800 spectrometer equipped with 1800 and 600 lines mm^{-1} gratings and a Peltier-cooled CCD camera. The spectral resolution was below 1 cm^{-1}; in most experiments, the collected signal for each data point was the average of three measurements. Raman spectra were calibrated with a standard silicon wafer using the specific peak of 521 cm^{-1} of silicon. The scattered light was collected in the backscattering geometry by a confocal Raman microscope on an Olympus BX51 platform with 10, 50, and 100X micro-objectives. An automated stage provided 3D ($X-Y-Z$) spatial scanning with a variable step size. The laser excitation was 785 nm with a power at the sample surface of 1.02 mW.

2.6. TEM Analysis of Seeds Exposed to MWCNTs. Samples of soybean seeds exposed to MS medium containing MWCNTs and control seeds exposed to regular MS medium were dissected to isolate the endosperm and fixed in 3% GTA in 0.075 M Sörensen's buffer, pH 7.2 at 4 °C, for at least 48 h. Samples were washed for 3 × 30 min in

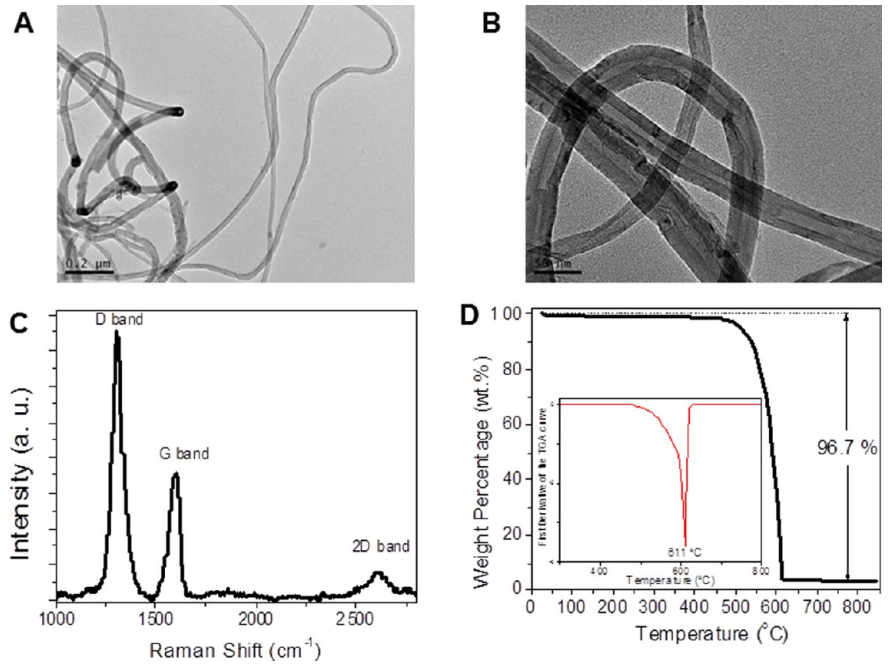

Figure 1. Characterization of MWCNTs used for biological experiments. (A) and (B): Low- and high-resolution TEM images of the purified MWCNTs, respectively. (C) Raman scattering spectrum of the MWCNTs obtained using a 785 nm wavelength laser. (D) The weight loss profile of the purified MWCNTs obtained from the TGA analysis. Inset of (D): First derivative of the TGA curve.

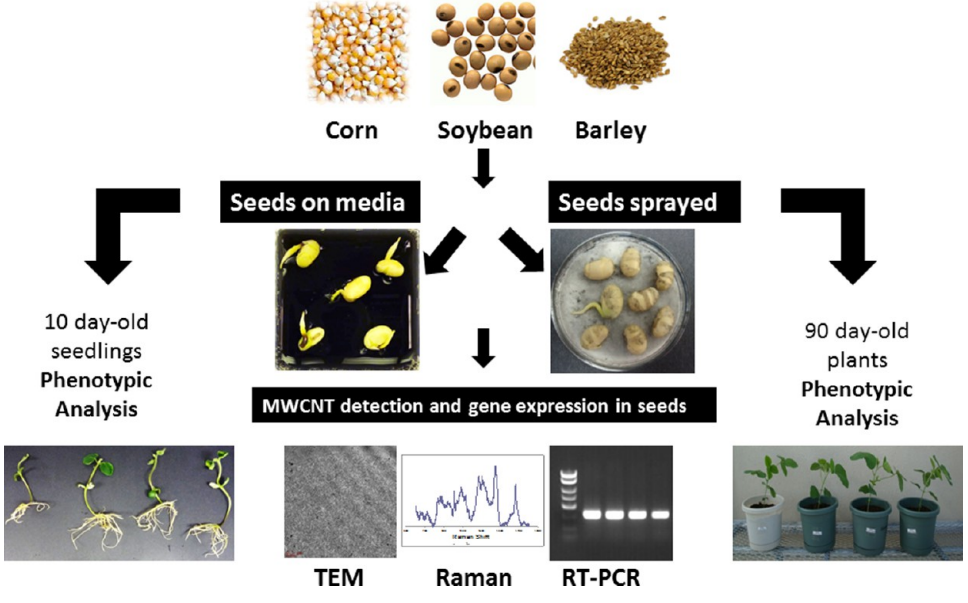

Figure 2. Schematic diagram illustrating the experimental design for this study. The seeds of corn, soybean, and barley were exposed to MWCNTs by addition to the medium and airspray. Germination rate and phenotypical differences between MWCNT-treated and control seeds/seedlings were recorded. Detection of MWCNTs inside seeds was performed by Raman spectroscopy and TEM imaging. Expression of aquaporin-encoded genes was analyzed by RT-PCR.

cold 0.075 M Sörensen's buffer, pH 7.2, then postfixed in 1% OsO4 in the same buffer for 2 h, on ice and in the dark, and finally washed for 3 × 30 min in the same buffer. Samples were dehydrated with a graded ethanol series (30, 50, 70, 95, and 3 × 100%) and then infiltrated with Spurr's resin. Blocks were sectioned using an LKB NOVA Ultramicrotome at 75−90 nm; sections were collected on 150-mesh Formvar/carbon grids (Ladd Research). Grids were stained with 4% uranyl acetate for 1 h at room temperature and 4 min in Reynolds' lead citrate. TEM samples were imaged with a Zeiss Libra 120 at 120 kV. A minimal electron dose condition was used, along with an emission current as low as 5 μA, to minimize electron-beam-induced sample damage.

2.7. RT-PCR Analysis of Expression of Aquaporin's Genes in Crop Seeds. Total RNA samples from control soybean seeds and soybean seeds exposed to MWCNTs by airspray and incubated for 1 day in a growth chamber were isolated using RNeasy Plant Mini Kit (Qiagen Inc. Valencia, CA). RNA from exposed and unexposed corn and barley seeds was extracted using the PureLink Plant RNA reagent protocol (Life Technologies, Grand Island, NY). Residual DNA was removed by on-column DNA digestion using the RNase-free DNase Kit (Qiagen Inc., Valencia, CA). Residual DNA from all RNA samples

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was removed by on-column DNA digestion using the RNase-free DNase Kit (Qiagen Inc., Valencia, CA). Synthesis of cDNA for all RNA samples was carried out using a SuperScript III First Strand Synthesis System Kit (Invitrogen, Carlsbad, CA) with dT16-oligonucleotide primers according to the manufacturer's protocol. Following synthesis, cDNA was used for the PCR reaction using gene-specific primers. The primers of all tested genes and the number of used PCR cycles are listed in Table 1. The 18S rRNA gene was used as an internal control. The following primers for 18S rRNA were used: F-5′AGGCCGCGGAAGTTTGAGGC3′; R-5′AGGCCGCG-GAAGTTTGAGGC3′. PCR products were separated on 1% agarose gels by electrophoresis for 30 min at 5 V cm^{-1}.

3. RESULTS AND DISCUSSION

3.1. Synthesis and Characterization of Multiwalled Carbon Nanotubes (MWCNTs). The carbon nanotubes synthesized via chemical vapor deposition were thoroughly characterized by several techniques. Figure 1A,B shows the high- and low-resolution TEM images of the MWCNTs used for this project. The functionalized nanotubes were found to be several micrometers long with outer diameters varying between 15 and 40 nm. Raman spectroscopy is a nondestructive technique often used to characterize carbon nanomaterials. There are three main features observed in the Raman spectra of MWCNTs: The D band, G band, and the 2D band. The D band usually arises due to defects present in the structure of nanotubes and is often observed at around 1300 cm^{-1}. The G band and 2D band (observed at ∼1600 and 2600 cm^{-1}, respectively) are often used to evaluate the crystallinity of the nanotubes. Figure 1C shows the Raman spectrum of the functionalized nanotubes. Often, during the functionalization process, defects are introduced on the surface of the nanotubes. Figure 1D shows the TGA curve (black curve) of the functionalized carbon nanotubes with a purity of ∼97%. The first derivative (red curve) of the TGA curve demonstrates that the decomposition temperature of the nanotubes is at 611 °C, indicating the presence of only one type of carbonaceous material.

To better understand the consequences of interactions between MWCNTs and commercially valuable crop species, we designed a complex experiment focused on observing the germination of seeds exposed to MWCNTs through two separate methods: airspray or by addition of MWCNTs to the growth medium, detailed phenotypical analysis of germinated seedlings/plants, detection of MWCNTs inside exposed seeds, and monitoring of the expression of selected genes in MWCNT-exposed seeds (Figure 2). Seeds of two monocot plants (barley, corn) and one dicot plant (soybean) were chosen for our study.

3.2. Involvement of Carbon Nanotubes in the Growth Medium Accelerates Germination of Barley, Corn, and Soybean Seeds. To test whether MWCNTs can affect germination, seeds of barley, corn, and soybean were sterilized and placed on Murashige and Skoog medium (MS) containing MWCNTs in concentrations of 50, 100, and 200 μg/mL. We monitored the seeds daily for the appearance of the first root as a sign of germination. As shown in Figure 3, the exposure of seeds of all tested crops to MWCNTs resulted in the acceleration of the process of seed germination. Barley, soybean, and corn seeds placed on MS medium supplemented with MWCNTs started to germinate after the first day of exposure. In the same time frame, no signs of germination on the first day were observed for seeds placed on the regular medium (control seeds). We continued to monitor the seed germination process for the next 5 days. Statistical analysis of the experiment was conducted using the Tukey Post-ANOVA test and Wilk's lambda test. On the first day post-treatment, MWCNTs increased the germination rate ($p < 0.05$) and decreased the germination time of barley seeds ($p < 0.05$). Significant acceleration of germination was observed when MWCNTs were used in doses of 100 and 200 μg/mL; however, no significant effect was observed for the lowest MWCNT concentration (50 μg/mL) (Figure 3A). Germination of corn was also significantly affected by treatment with MWCNTs (Figure 3B). Corn seeds treated with MWCNTs germinated earlier by comparison to the control seeds. A significant effect on germination was observed when MWCNTs were used in the higher concentrations of 100 and 200 μg/mL ($p < 0.05$); however, the application of MWCNTs in a lower concentration of 50 μg/mL did not lead to any significant effects. When compared with controls, the overall germination

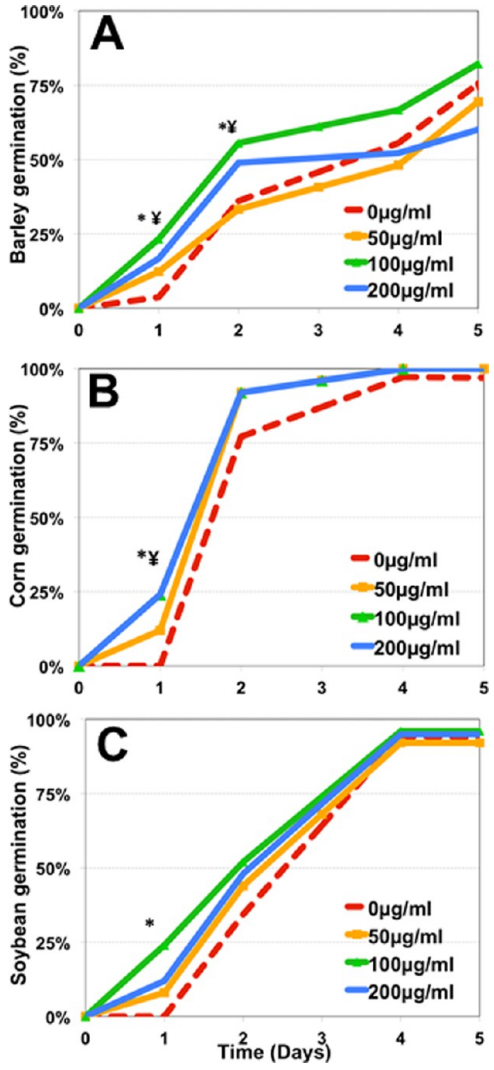

Figure 3. Germination of crop seeds (barley, corn, soybean) exposed to MWCNTs through introduction of nanotubes in agar medium. MWCNTs were added in MS medium in concentrations of 50, 100, and 200 μg/mL. MWCNTs affect the germination time of barley (A), corn (B), and soybean (C) seeds. Results are shown as an average of measurement of 25 seeds per each condition. * $p < 0.05$ using Tukey Post-ANOVA test, MWCNT (100 μg/mL) compared to control; ¥ $p < 0.05$ using Tukey post-ANOVA test, MWCNT (200 μg/mL) compared to control. Error bars represent standard error value.

Table 2. Growth Characteristics of 10-Day-Old Seedlings of Barley, Corn, and Soybean Germinated from Seeds Exposed to MWCNTs[a]

crop	treatment	root length	shoot length	leaf length	root fresh weight	shoot fresh weight	root dry weight	shoot dry weight
barley	control	3.23 ± 1.09	2.31 ± 0.83	9.79 ± 2.69	0.10 ± 0.05	0.10 ± 0.07	0.0100 ± 0.07	0.014 ± 0.05
	MWCNT_50	4.19 ± 0.84	3.72 ± 1.34	10.55 ± 3.1	0.12 ± 0.05	0.16 ± 0.05	0.0005 ± 0.05	0.007 ± 0.05
	MWCNT_100	4.88 ± 1.12	2.79 ± 0.96	9.97 ± 1.94	0.11 ± 0.03	0.12 ± 0.06	0.0105 ± 0.06	0.007 ± 0.03
	MWCNT_200	4.32 ± 1.10	3.02 ± 0.83	10.47 ± 2.70	0.17 ± 0.04	0.15 ± 0.08	0.0101 ± 0.08	0.009 ± 0.04
corn	control	14.98 ± 2.74	9.15 ± 3.18	2.36 ± 1.43	1.66 ± 0.03	0.46 ± 0.05	0.24 ± 0.03	0.05 ± 0.05
	MWCNT_50	15.75 ± 1.77	11.025 ± 2.23[b]	5.35 ± 2.25[b]	1.91 ± 0.04	0.68 ± 0.03[b]	0.23 ± 0.04	0.05 ± 0.03
	MWCNT_100	16.13 ± 1.89	12.63 ± 2.18[b]	8.02 ± 2.07[b]	2.07 ± 0.07	0.76 ± 0.05[b]	0.24 ± 0.07	0.06 ± 0.05
	MWCNT_200	16.15 ± 1.98	12.5 ± 2.50[b]	6.87 ± 1.43[b]	1.98 ± 0.08	0.76 ± 0.09[b]	0.23 ± 0.08	0.06 ± 0.09
soybean	control	6.67 ± 1.82	11.98 ± 2.66	0.93 ± 0.67	0.69 ± 0.17	0.99 ± 0.17	0.05 ± 0.31	0.15 ± 0.17
	MWCNT_50	8.44 ± 1.59[b]	13.44 ± 1.29	1.15 ± 0.88	0.74 ± 0.26	1.16 ± 0.26	0.05 ± 0.13	0.17 ± 0.26
	MWCNT_100	8 ± 1.17[b]	13.55 ± 1.35	1.05 ± 0.59	0.75 ± 0.15	1.04 ± 0.15	0.06 ± 0.16	0.15 ± 0.15
	MWCNT_200	7.95 ± 0.93[b]	12.78 ± 2.39	0.97 ± 0.59	0.61 ± 0.19[b]	0.97 ± 0.19	0.05 ± 0.17	0.14 ± 0.19

[a]MWCNTs were added to growth medium as supplement in concentrations (50, 100, 200 μg/mL). ± SE (Standard Error). [b]$p < 0.05$, compared to control.

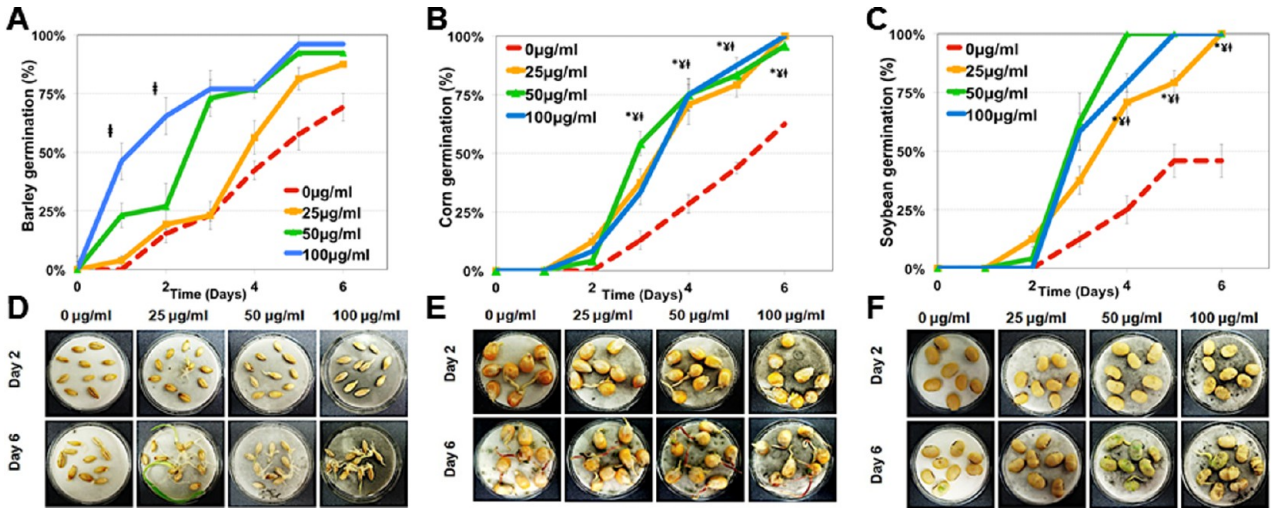

Figure 4. Germination of crop seeds (soybean, barley, corn) exposed to MWCNTs through the airspray technique. MWCNTs affect the germination time and rate of barley (A), corn (B), and soybean (C) seeds. Results are shown as an average of measurement of 24 seeds per each condition. Water solutions of MWCNTs in concentrations of 50, 100, and 200 μg/mL were used for airspray. *: $p < 0.05$, CNTs (25 μg/mL) compared to control. ¥: $p < 0.05$, MWCNT (50 μg/mL) compared to control. ‡: $p < 0.05$, MWCNT (100 μg/mL) compared to control. Phenotype of control and MWCNT-coated seeds of barley (D), corn (E), and soybean (F) are presented on the second and sixth day after MWCNT spray treatment.

rate remained higher for MWCNT-exposed seeds, but the difference was not statistically significant on the third, fourth, and fifth days after exposure (Figure 3B). The germination rate of soybeans in regular medium was found to reach 30% during the second day after exposure to MS medium (Figure 3C). The nanotube treatment altered the percentage of germination up to 50% during the second day and at a concentration of 100 μg/mL.

As the next step, we investigated the effect of MWCNTs on the growth and development of seedlings germinated from MWCNT-exposed seeds (Table 2). We noticed that corn seedlings growing on medium supplemented with MWCNTs had more developed leaves than control seedlings ($p < 0.05$). Corn seedlings growing on 100 μg/mL of MWCNTs revealed the highest mean value of leaf length (8 cm). MWCNTs in all tested concentrations increased the total fresh shoot weight compared to the shoot weight of unexposed corn seedlings. No significant effects of MWCNTs on the phenotype of barley seedlings were documented. MWCNT-treated soybean seedlings produced significantly longer root systems compared to control seedlings. On the basis of this experiment, we concluded that MWCNTs in the tested doses did not show any toxic effects for barley, corn, and soybean plants in early stages of development.

3.3. Deposition of Carbon Nanotubes on the Seed Surface Induce Germination of Barley, Corn, and Soybean Seeds. To determine if the method of delivering MWCNTs to seeds plays an important role in the documented acceleration of seed germination, we deposited MWCNTs on the seed surfaces through an airspray technique in three different concentrations (100 μg/mL (heavy spray), 50 μg/mL (medium spray), and 25 μg/mL (light spray)). The germination and development of control and MWCNT-coated seedlings were monitored daily. As shown in Figure 4 and Figure S1 (Supporting Information), the deposition of MWCNTs on seed surfaces affected germination of all tested plant species. At a low concentration of MWCNTs (25 μg/mL), no significant effect on time or rate of germination of

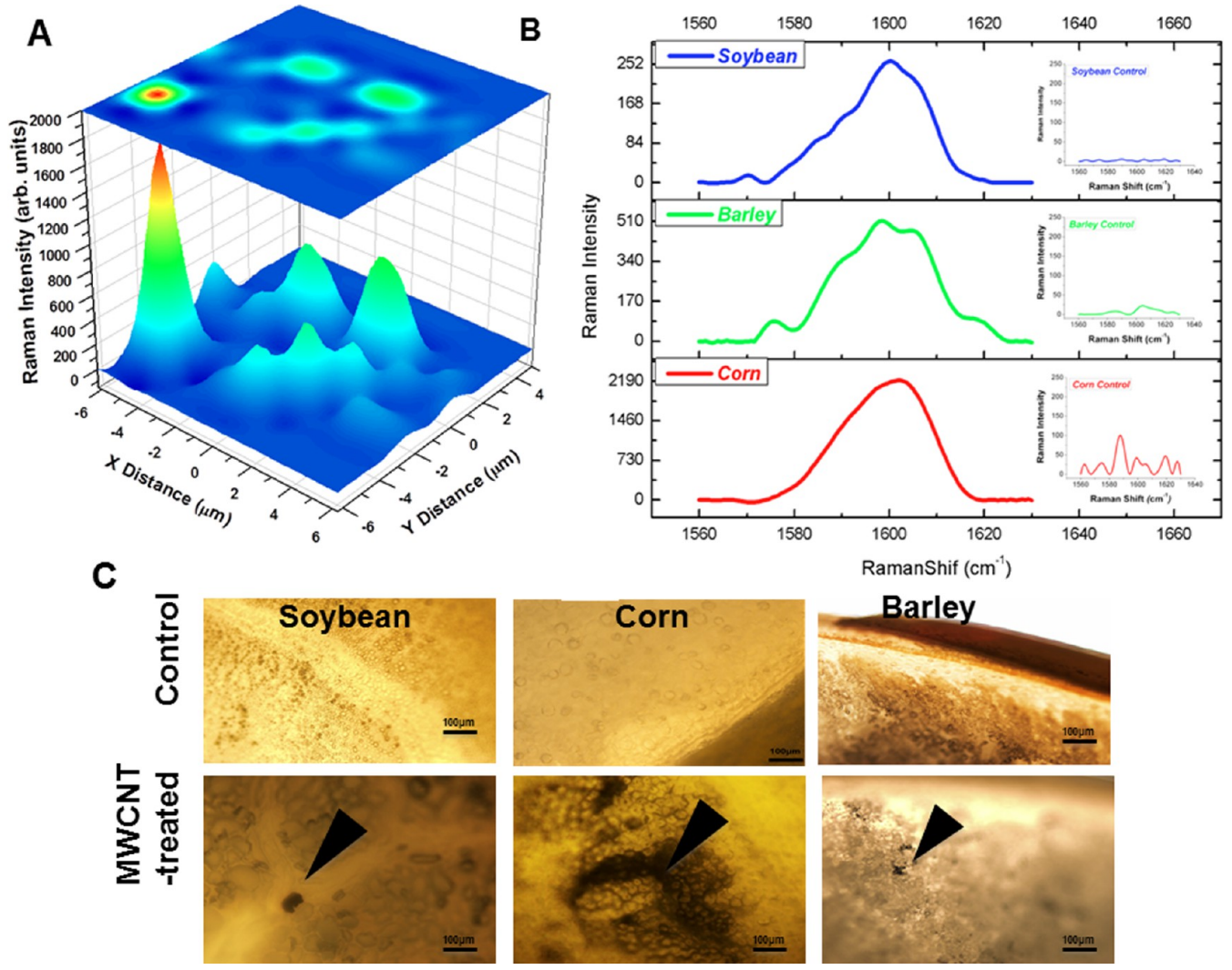

Figure 5. Detection of carbon nanotubes in seeds exposed to MWCNTs by air spray technique. (A) 3D (and corresponding 2D-inset) Raman mapping based on the G band intensity of a soybean seed indicating the presence of MWCNTs in small clusters. (B) Representative G band detection in the seeds of the three crops: barley, soybean, and corn. (C) Optical visualization of the MWCNT clusters inside the seeds of corn, barley, and soybean. (10X objective was used for these studies.) Arrows indicate location of large agglomerates of MWCNTs inside crop seeds.

barley seeds was observed. However, at a concentration of 100 μg/mL, the rate of seed germination reached a maximum and showed a significant increase compared to the control (Figure 4A; Figure S1, Supporting Information). MWCNT-treated barley seeds were able to germinate earlier compared to the controls (Figure 4D). While the control seeds germinated on the second day after planting, 46% of MWCNT-treated seeds were able to germinate during the first day post treatment. Seed germination of corn was also affected by the MWCNT deposition. After 6 days, all MWCNT-treated seeds reached a rate of 100% germination compared to the control seeds which only reached 63% (Figure 4B; E). An early germination of MWCNT-treated seeds was also observed. Treated corn seeds were able to germinate on the second day, while control seeds started to germinate on the third day (Figure 4B). A similar trend was documented for MWCNT-coated seeds of soybean. Soybean seeds treated with 25 μg/mL of MWCNTs reached 100% germination of seeds on the fourth day. However, the germination rate for unexposed seeds was only 25% (Figure 4C).

We monitored the development and growth of mature plants generated from MWCNT-coated seeds and did not detect any symptoms of toxicity or any negative effects on the development of plants (Table S1; Figure S2, Supporting Information). No significant differences in shoot length, number of leaves, and number of fruits were noticed between plants originated from untreated (control) and MWCNT-coated seeds of all tested crop species (Table S1, Supporting Information).

3.4. Carbon Nanotubes Were Found to Penetrate Seeds of Barley, Corn, And Soybean. Different plant species vary in seed coat texture and seed coat permeability. It was demonstrated early that seed coat permeability to heavy metals can vary among different plant species.[16] For example, only 28% of 25 tested plant species produced seeds with coats permeable to lead ions, and 39% had seeds permeable to barium ions.[16] Thus, it is possible that specific properties of seed coats may affect the uptake of carbon nanomaterials by seeds of different crop species. To understand if deposition of MWCNTs on the surface of crop seeds by airspray can lead to penetration of the seed coats of barley, corn, and soybean, we

analyzed the endosperm of control seeds and seeds exposed to MWCNTs for 24 h using Raman spectroscopy (Figure 5).

We used the 785 nm laser excitation for the detection of MWCNTs inside the seeds, based on the unique G band, which is a characteristic of these nanostructures. Figure 5A presents the 2D and 3D mapping of the G band intensity collected for a soybean seed. The measurement was made with an acquisition time of 55 s per point, and each collection of data was averaged 3 times. The presence of nanotube clusters of various sizes can clearly be seen inside the seed, which is in excellent correlation with the optical visualization of the presence of MWCNTs inside all of the crop seeds (Figure 5C). Figure 5B shows representative G band measurements inside the three types of seeds. The relative variation in the intensity of the bands does not represent a comparative quantification of the MWCNTs inside the various seeds but confirms the presence of MWCNT aggregates inside seeds that were exposed to MWCNTs. To demonstrate that MWCNTs can be also uptaken by soybean seeds placed on medium supplemented with MWCNTs, we performed TEM analysis of the endosperm of seeds exposed to MWCNTs (100 μg/mL) for 24 h. TEM imaging demonstrated the presence of black aggregates inside MWCNT-exposed seeds of soybean (Figure 6).

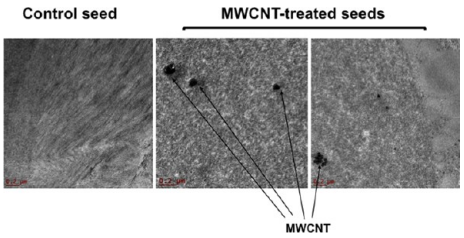

Figure 6. TEM detection of MWCNTs in soybean seeds exposed to nanomaterials by addition of MWCNTs in the growth medium. Soybean seeds were exposed to MWCNTs for 24 h, carefully washed, dried by filter paper, and opened by a longitude cut. The seed endosperm was prepared for TEM as described in the Materials and Methods section.

Interestingly, the Raman mapping analysis, as well as the TEM imaging, clearly indicate the presence of MWCNT clusters of various sizes inside the seeds. The property of the MWCNTs to easily move inside seeds and be present in large aggregates in the endosperm raises questions about the capacity of carbon nanomaterials to be transported into the plants germinated from MWCNT-treated seeds. Also, it is possible that carbon nanoparticles can move into the second generation of plants. For example, Lin et al.[5] were able to detect carbon nanomaterial C_{70} in the first generation exposed seeds and leaves and in second generation leaves of rice plants.[5] Taking into consideration safety issues and environmental protection, it is important to address such questions in future experiments.

The ability of MWCNTs to penetrate the thick seed coats of barley, corn, and soybean provides supporting evidence to suggest that the effect of MWCNTs on the complex process of seed germination can be associated with MWCNT-induced changes at the genomic level. In our earlier studies, we showed that MWCNTs can drastically affect the expression of genes involved in plant stress response.[6] In particular, we observed that the expression of water channel genes (aquaporin, *LeAqp2*) was activated in response to the application of MWCNTs to roots of tomato seedlings[6,9] and tobacco cells.[7] We suggested that carbon nanotubes can be sensed as a specific stress factor by plant cells and can affect the expression of the water channel proteins (aquaporins)[6] that are crucial for the process of seed germination and plant growth.[17]

3.5. MWCNTs Activate Expression of Water Channel Genes (Aquaporins) in Seeds of Barley, Corn, And Soybean. As the next step in our investigation, we decided to clarify if MWCNTs can regulate expression of different types of aquaporin genes in seeds and if such an effect can be observed in other than tomato plant species. For this task, we identified the number of aquaporins in barley, soybean, and corn using the NCBI database (Figure S3, Supporting Information). Aquaporins belong to the large family of major intrinsic proteins (MIPs) and five subfamilies:[18] plasma membrane intrinsic proteins (PIP),[19] tonoplast intrinsic proteins (TIP),[20] Nodulin-26 like proteins (NIP),[21] small basic intrinsic proteins (SIP),[22] and X-intrinsic proteins (XIP).[23] On the basis of sequence homology, aquaporins were found to be abundant in many plants. For example, *Arabidospsis* and corn have about 35 different aquaporins,[18,24] while rice contains 33 aquaporins.[25] The most abundant aquaporins (PIPs) are localized in the plasma membrane and comprise two groups: PIP1 and PIP2. The blast of tomato aquaporin protein (*LeAqp2*) sequences has shown 77% homology to soybean PIP1 protein, 73% to barley PIP1 protein, and 72% to corn PIP1 protein (Figure S3, Supporting Information). Sequences of aquaporins representing PIP, TIP, NIP, and SIP subfamilies from soybean, corn, and barley, as well as tomato aquaporin (*LeAqp2*), were grouped together using a UPGMA phylogenetic analysis (Figure S4, Supporting Information). This analysis revealed that PIP proteins from all three crop species are closest to tomato aquaporin protein. However, some level of homozygosity was found for TIP, NIP, and SIP aquaporins from the tested species as well (Figures S3,4, Supporting Information). The expression of aquaporins in seeds correlated with the germination process of many crops, such as *Mesembryanthemum crystallinum*,[26] *Arabidopsis thaliana*,[27] and rice.[28] Studies using gravimetric methods and nuclear magnetic resonance (NMR) spectroscopy have shown the association of water exchange with seed imbibition and embryo growth.[29,30]

Taking into account the importance of aquaporins for initiation of seed germination, we monitored the expression of genes encoding PIP1 proteins from soybean (*S_PIP1−2*), barley (*B_PIP1−1*), and corn (*ZM_PIP1−1*); TIP proteins from barley (*B_TIP1−1*) and corn (*ZM_TIP1−1*); and SIP/NIP proteins from corn (*ZM_SIP1−1*), (*ZM_NIP1−1*). As shown in Figure 7, the coating of crop seeds with MWCNTs resulted in significant activation of the expression of aquaporin genes from different gene subfamilies. Thus, the expression of soybean aquaporin *S_PIP1−2* was activated in soybean seeds coated with MWCNTs at all tested concentrations.

The highest level of *S_PIP1−2* gene expression was found in seeds coated with the highest concentration of MWCNTs (100 μg/mL) (Figure 7A). Similarly, gene expression in barley *PIP1* (*B_PIP1−1*) was up-regulated in a dose-dependent manner in seeds exposed to MWCNTs (Figure 7B). An increase in the expression of corn PIP1 gene (*ZM_PIP1−1*) was observed in MWCNT-exposed corn seeds, as well (Figure 7C). Vander Willigen et al.[17] reported that the level of expression of all 13 plasma membrane aquaporins (PIP) was low in dry and germinating seeds of *Arabidopsis*. Our data related to expression of PIP proteins in unexposed (control) seeds of all tested species (barley, corn, soybean) are in good correlation with this report. PIP gene expression in control seeds was very low

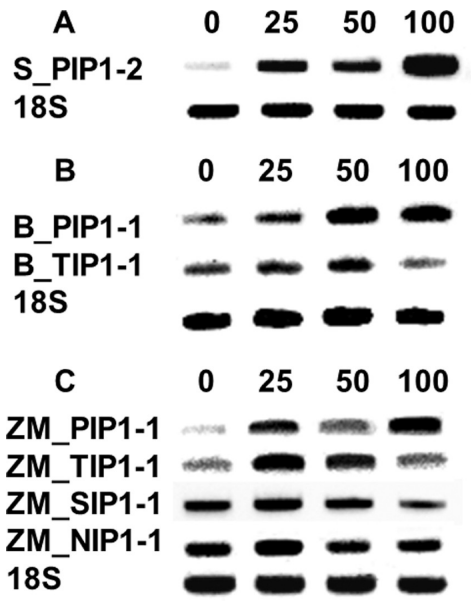

Figure 7. RT-PCR analysis of expression of major aquaporin genes in crop seeds coated with MWCNTs (25, 50, and 100 μg/mL) by airspray. The expression of aquaporins was detected in soybean (A), barley (B), and corn (C) seeds using specific to different aquaporin primers (Table 1). 18S rRNA was used as an internal control.

(Figure 7). The ability of MWCNTs to significantly up-regulate expression of naturally low-expressed PIP genes in seeds of all test species is intriguing. However, it was reported that aquaporins are stress-inducible genes and can be regulated by application of a variety of stress factors including heavy metals[31] and attachment/penetration caused by plant parasites.[32] Further genetic and biochemical experiments would be required to clarify the biological reason for such an effect. The trend toward up-regulation in response to the application of MWCNTs (25 μg/mL, 50 μg/mL) to seeds was noticed also for tonoplast intrinsic proteins (TIPs) in exposed barley and corn seeds (Figure 7B,C). However, expression of TIPs decreased significantly in both species at the highest used MWCNT concentration (100 μg/mL). It is interesting that a minute or no effect on activation of gene expression for small basic intrinsic protein (SIP) and Nodulin-26 like intrinsic protein (NIP) in corn seeds exposed to low doses of MWCNTs (25 μg/mL) was observed. Down-regulation of NIP and SIP was noticed at the highest used concentration of MWCNTs (Figure 7C).

4. CONCLUSION

The recent discoveries of nanotechnology can benefit a number of fields in plant biology, agriculture, or horticulture. In the present study, we demonstrated that the application of MWCNTs to seeds of three crop species (corn, barley, soybean) using two different methods of nanotube application (through agar growth medium and by seed coating) resulted in the acceleration of seed germination and had no negative effect on development of plants generated from exposed seeds. This study proves that the positive effect of carbon nanotubes on seed germination that we described earlier using a tomato model system[6,9] is reproducible for other valuable crop species. Our study also demonstrated that a positive effect on seed germination is achievable through different methods of MWCNT delivery to the seed surface. The ability of MWCNTs to penetrate seed coats of different crop species was proved by analytical techniques, such as Raman spectroscopy and transmission electron microscopy. For the first time, we demonstrated that MWCNTs can activate the expression of seed-located water channel genes (aquaporins) that belong to different gene families of aquaporins (PIP, TIP, SIP). This observation supported our hypothesis about the involvement of carbon nanotubes in regulating the activity of water channels in MWCNT-exposed plant organs or cells. Our research has proved that carbon nanotubes in low doses can be an attractive nanosized material for application in various areas of agriculture, including seed enhancement.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Details regarding germination of crop seeds (soybean, barley, corn) exposed to MWCNTs through airspray technique, phenotype of mature plants generated from MWCNT-coated and uncoated seeds, and sequences of aquaporin's proteins from different crops showing high similarity are presented. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: mvkhodakovsk@ualr.edu. Phone: 501-683-2656. Fax: 501-569-8020.

Present Address

[#]Center for Integrated Nanotechnologies, Materials Physics and Application Division, Los Alamos National Laboratory, Los Alamos, New Mexico 87544, USA.

Notes

The authors declare no competing financial interest.

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