Determination of uptake, accumulation, and stress effects in corn (*Zea mays L.*) grown in single-wall carbon nanotube contaminated soil

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Abstract

Single-wall carbon nanotubes (SWNTs) are projected to increase in usage across many industries. Two studies were conducted using *Zea Mays L.* (corn) seeds exposed to SWNT spiked soil for 40 d. In Study 1, corn was exposed to various SWNT concentrations (0, 10, and 100 mg/kg) with different functionalities (non-functionalized, OH-functionalized, or surfactant stabilized). A microwave induced heating method was used to determine SWNTs accumulated mostly in roots (0 to 24 $\mu\text{g/g}$), with minimal accumulation in stems and leaves (2 to 10 $\mu\text{g/g}$) with a limit of detection at 0.1 $\mu\text{g/g}$. Uptake was not functional group dependent. In Study 2, corn was exposed to 10 mg/kg SWNTs (non-functionalized or COOH-functionalized) under optimally grown or water deficit conditions. Plant physiological stress was determined by the measurement of photosynthetic rate throughout Study 2. No significant differences were seen between control and SWNT treatments. Considering the amount of SWNTs accumulated in corn roots, further studies are needed to address the potential for SWNTs to enter root crop species (i.e., carrots), which could present a significant pathway for human dietary exposure.

Key words: Microwave induced heating, photosynthetic rate, single-wall carbon nanotube, drought plant stress

1. Introduction

The use of carbon nanotubes (CNTs) is growing in applications across industries such as wastewater treatment, environmental remediation, and agricultural practices. Single-wall carbon nanotubes (SWNTs) and multi-wall carbon nanotubes (MWNTs) can be functionalized with groups such as surfactant (Irin et al., 2012; Kharissova et al., 2013), amine, and carboxylic acid (Tripathi et al., 2011; Villagarcia et al., 2012; Zhai et al., 2015), and polymer groups (Canas et al., 2008; Hong et al., 2013; Chen et al., 2015a). These chemical functional groups allow for reactivity and dispersibility specific to a range of applications. As a result, worldwide production of CNTs is estimated to increase significantly (Nowack et al., 2013; Mitrano et al., 2015). Uses for CNTs include nanosensor detectors for pesticides and other chemicals in food science and safety industries (Baruah and Dutta, 2009; Das et al., 2009; Husen and Siddiqi, 2014). In agriculture, CNTs can be used as smart delivery systems with nano-encapsulated agrochemicals that are relatively time controlled, self-regulated and specifically targeted compounds that are used for plant pathology (Nair et al., 2010), fertilizers (Liu and Lal, 2015) and pesticides (Hong et al., 2013; Chen et al., 2015b). Although beneficial, applications such as these involve direct contact with soil and vegetation which creates a risk for human and ecological exposure through dietary intake.

Investigating CNT uptake in plants is a critical issue considering its projected applications in the agricultural sector (Hong et al., 2013; Husen and Siddiqi, 2014), however limited studies have addressed uptake and stress effects in plants. The effects of CNTs on vegetation have been studied on plants grown in various media, such as agar and hydroponic systems (Khodakovskaya et al., 2011; Tripathi et al., 2011;

Miralles et al., 2012b; Smirnova et al., 2012; Schierz et al., 2014), with variable results regarding uptake into plants (Rico et al., 2011). While effects on growth have been seen in plants such as delayed flowering and yield in rice (Lin et al., 2009), no phytotoxic effects have been seen in cabbage, carrot, tomato, onion, and lettuce plants (Canas et al., 2008) when exposed to CNTs. However adsorption onto roots of cucumber plants after a 48 h exposure period was observed, suggesting CNT interaction with roots (Canas et al., 2008). Introduction of nanomaterials through soil media presents a more realistic approach and is the likely route of exposure to plants in the environment (Pan and Xing, 2012; Maurer-Jones et al., 2013). Past authors have shown root uptake of non-functionalized MWNTs by corn grown in spiked soil for 40 d. MWNT concentrations were detected at $< 10 \mu\text{g/g}$ in roots and translocation was observed into leaves and stems but at lower concentrations.

Environmental stressors such as drought can negatively affect overall plant activity, growth, and yield via a number of effects such as reduced radiation use efficiency for photosynthesis (Osborne et al., 2002; Earl and Davis, 2003), decreased biomass (Stone et al., 2001), increased physical deformations in xylem and root structure (de Silva et al., 2012), and increased oxidative stress (Ashraf et al., 2015). These factors combined with contaminants such as CNTs may cause additional stress in plant water and nutrient uptake especially in arid environments, where periods of drought are common. Additionally, CNTs have been suggested to move concurrently with other nutrients in soil (Nair et al., 2010; Rico et al., 2011) and may have an effect on plant stress response mechanisms under drought conditions. Thus, further studies addressing drought stress and CNTs should be investigated.

Well established methods have been used for CNT detection, including radioactive isotopes (Petersen et al., 2008), near-infrared (Cherukuri et al., 2004; Schierz et al., 2014), and Raman spectroscopy (Hartschuh et al., 2003; Dresselhaus et al., 2005) have been used in CNT detection. Though versatile, these methods include extensive sample preparation, can be costly, often require trained personnel to operate instrumentation, and present difficulty when detecting CNTs in complex media. Microwave-induced heating of CNTs is an alternative method which can quantitatively determine CNTs in biological samples such as plants and earthworms (Irin et al., 2012; Li et al., 2013). This analysis method is advantageous because of its low cost in equipment, safety, and simple design. Heat is emitted from the sample when CNTs absorb microwaves, and the change in temperature can be recorded. Microwave-induced heating is not only quantitative in contrast with other analytical methods, but also presents a low detection limit ($\sim 0.006 \mu\text{g CNT/mg sample}$).

This paper addresses uptake, accumulation, and stress effects of SWNTs in corn (*Zea mays L.*) grown in spiked soil under optimally watered and water deficit conditions. A microwave-induced heating method was used to determine concentrations of SWNTs taken up into corn following exposure to 10 mg/kg and 100 mg/kg after 40 d. These studies aim to expand on the current knowledge of potential for human nanotube dietary exposure.

2. Materials and methods

2.1 Materials and carbon nanotube characterization

Non-functionalized, covalently functionalized (hydroxyl (OH)), and carboxylic acid (COOH) single-wall nanotubes (1-4 nm OD, >90 wt% purity, <1.5 wt% ash content, 5-30 μm length), were purchased from Cheaptubes, Inc (Cambridgeport, VT, USA). Sodium dodecylbenzenesulfonate (SDBS) was added as a surfactant for purchased non-functionalized SWNTs to prepare surfactant-stabilized SWNTs. Characterization of CNT size distribution was done using transmission electron microscopy (TEM, Hitachi H-8100) and thermogravimetric analysis (TGA, Q50-TA Instrument). Non-functionalized SWNTs dispersed in SDBS were used for TEM as all types of SWNTs used in the study were these same SWNTs with different functional groups.

2.2 Preparation and characterization of SWNT dispersion

SWNT dispersion was prepared according to procedures described in previous studies (Irin et al., 2012; Li et al., 2013). Briefly, 10 mg/mL SDBS was dissolved in 20 mL DI water and then 0.2 mg/mL SWNT was added into the solution. The mixture was then tip sonicated for an hour at a power of 50 watts with a 6.35 mm diameter probe tip at an amplitude of 30-35 %. The sonicated dispersion was then centrifuged for 4 h at a speed of 3500 rpm. After centrifugation, the supernatant was collected and characterized by using transmission electron microscopy (TEM, Hitachi H-8100) to determine the average diameter of the tubes. The concentration of SWNT in the dispersion (0.85 mg/mL) was calculated from the UV-vis absorbance (A) data according to the Lambert-Beer Law ($A = \alpha Lc$); here, α = extinction co-efficient ($3389 \text{ mL mg}^{-1} \text{ m}^{-1}$), L = path length, and c = concentration of SWNT.

2.3 Preparation of soil and plants

A fine sandy loam soil (Amarillo field soil) from the USDA-ARS field site in Lubbock, TX was used. Soil was characterized by Agvise Laboratories (Northwood, ND, USA). Commercially available potting mix and sand were also used to create the soil mixture. For the soil mixture, a ratio of soil: potting mix: sand (2:1:1, wt/wt/wt) was used and mixed for 2 min in a cement mixer to ensure thorough mixing.

For accumulation experiments, which will be referred to henceforth as Study 1, SWNTs (non-functionalized, OH-functionalized, or surfactant-stabilized) were added to the soil mixture at concentrations of 10 and 100 mg/kg (wt/dry wt). Each soil treatment was contained in a five gallon bucket, covered, and placed in a cement mixer for 4 h. The soil mixture (1.5 kg) was then placed in 12.7 cm diameter pots (1 L). For drought experiments, referred to hereafter as Study 2, SWNTs (non-functionalized and COOH-functionalized) were added to the soil mixture at a concentration of 10 mg/kg (wt/dry wt) and mixed as stated above. Each soil treatment (4.5 kg) was then placed in 3 L pots.

2.4 Growing conditions and measurements for experiments

For all experiments, five Sweet Corn Southern Gentleman (*Zea mays L.*) seeds (Molalla, OR, USA) previously sanitized with soap and water were added to each pot and allowed to germinate. There were 5 replicate pots per type of SWNT. After a week seedlings were thinned to one plant per pot. Plants were grown for 40 d in a greenhouse with natural day and night conditions (approximately 14/12 h day/night cycle) and average day/night temperatures of 28/20 °C. Pots were irrigated to field capacity every 3 days and fertilized with a diluted Hoagland's solution weekly.

For Study 2, plants were prepared as described above, however irrigation was stopped 21 days after germination and drought was induced for 10 days. Leaf-level gas-exchange was used to monitor plant response to water deficit stress using a LI-6400XT Portable Photosynthesis System (LI-COR Inc, Lincoln, NE, USA). Chamber conditions were set approximate ambient growth conditions: reference [CO₂] was 400 ppm, chamber block temperature was 28 °C, and photon flux density was 1800 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

2.5 Sample preparation and microwave analysis

After 40 d, plants were carefully soaked in warm tap water to remove excess soil from roots. All plants were separated into roots, stems, and leaves and gently scrubbed to remove any SWNTs on outer surfaces. After washing, plant root length was measured. Plants were oven dried at 80 °C for 3 d to remove all moisture. Samples were then crushed using a mortar and pestle into a fine homogenous mixture and placed into a glass capillary tube (4 cm I.D., 6.5 cm long). Samples in capillary tubes were then analyzed using a microwave-induced heating method (Irin et al., 2012). A K-type thermocouple (Model SC-GG-K-30–36, ungrounded, 0.032” diameter, Omega Engineering Inc.) attached to a digital multi-meter (Model HHM209, Omega Engineering Inc.) was inserted into the capillary tube with the sample and placed into a microwave generator (Model MPG 4 RF, LAB-909, Opthos Instruments Inc). Microwaves were guided by an optimized copper rectangular waveguide (WR-284). The sample inside the generator was under a constant nitrogen flow to prevent ignition of nanotubes. The sample was exposed to 30 W microwave power for 10 s and the temperature rise was recorded. All sample measurements were fitted onto a plant calibration curve to verify

sample concentration (Irin et al., 2012). This curve was constructed using 9 points (0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, and 0.8 μg). To construct this curve, plant roots were injected with known concentrations of dispersion using a precision syringe and dried before microwave analysis. Each point consisted of 12 to 18 mg of spiked plant root and was measured using 30 W of microwave power for 10 s. To validate the calibration curve, each point was measured 10-15 times. There was a poor dependency on root type and temperature rise which allowed for the use of this curve to measure corn samples (Irin et al., 2012). A check standard from the calibration curve was used to detect fluctuations in the microwave generator.

2.6 Statistical analysis

All results were analyzed using R version 3.0.3 software (2014, The R foundation for Statistical Computing). Data was tested for homoscedasticity and normality using the Bartlett Test and Shapiro-Wilk test, respectively. For comparisons of SWNT concentrations between treatments two-way analysis of variance (ANOVA) $P \leq 0.05$ was used with Tukey Multiple Comparisons of Means. Data for drought stressed corn studies were transformed using the squared root of each data point.

3. Results and discussion

3.1 Nanomaterial characterization

The average outer diameter was determined to be 2.5 nm; this confirmed the size provided by the manufacturer (Figure 1). SWNT functionalization from the manufacturer was confirmed using thermogravimetric analysis (Figure 2).

3.2 Study 1: Uptake and accumulation of SWNTs in corn

Analysis of corn samples using the microwave-induced heating method (Irin et al., 2012), revealed that SWNTs were taken up into roots at concentrations between 0 and 24 $\mu\text{g/g}$ (Figure 3). There was uptake at all exposure concentrations except in one plant in the 10 mg/kg surfactant stabilized SWNT treatment and one plant in the 10 mg/kg OH-functionalized SWNT treatment. Translocation of SWNTs was observed in stems and leaves at concentrations between 2 and 10 $\mu\text{g/g}$. Previous studies have reported qualitative results such as the adsorption of CNTs onto root surfaces (Canas et al., 2008), into plant cell membranes (Tan et al., 2009), and have been qualitatively detected in edible plants (Lin et al., 2009; Tripathi et al., 2011; Miralles et al., 2012b; Yan et al., 2013). These results however, do not include actual concentrations of CNTs in plant samples. This study is unique because it quantitatively demonstrated accumulated concentrations of SWNTs in corn roots, stems and leaves. A limited number of studies have shown methods of quantification of CNTs. Schierz et al. used near-infrared fluorescence spectroscopy (NIRF) to quantitatively detect SWNTs in mesocosms at concentrations of $< 0.5 \mu\text{g/L}$, though no bioaccumulation was seen in aquatic plants (Schierz et al., 2014).

3.3 SWNT functionality and effect on plant uptake

In Study 1, the effects of OH-functionalized and surfactant stabilized SWNTs were compared to non-functionalized SWNTs. Significant differences in uptake between SWNT functional group treatments ($P < 0.05$) were detected using Tukey Multiple

230 Comparisons of Means. Significant differences were observed between treatments of
231 non-functionalized (100 mg/kg) and OH-functionalized SWNTs (10 mg/kg and 100
232 mg/kg). Root length was significantly higher in plant exposed to 100 mg/kg of non-
233 functionalized SWNTs (S3). This is contrary to what was seen with corn plants exposed
234 at higher concentrations of 2000 mg/L non-functionalized MWNTs, where roots were not
235 significantly different from the control (Lin and Xing, 2007). Generally, plant uptake of
236 SWNTs was not dose dependent, however there was dose dependency seen between
237 10 and 100 mg/kg non-functional group treatments. Correlations between an increase in
238 plant growth and negatively functionalized MWNTs have been demonstrated
239 (Villagarcia et al., 2012). However, taking this into account, SDBS was used as an
240 anionic surfactant stabilizer in treatments. It is possible that SWNTs were more
241 bioavailable to roots at lower concentrations than at higher concentrations. The lowest
242 concentration of SWNTs in roots was detected in the 10 mg/kg OH-functionalized
243 SWNT exposure group (Figure 3). Surfactant-stabilized SWNTs (uptake as high as 22
244 $\mu\text{g/g}$) and non-functionalized SWNTs (uptake as high as 26 $\mu\text{g/g}$) were taken up more
245 than the OH-functionalized SWNTs. At low exposure concentrations of 10 mg/kg, plant
246 root uptake followed the trend of surfactant stabilized > non-functionalized > OH-
247 functionalized. However, at higher exposure concentrations of 100 mg/kg, plant root
248 uptake followed a different trend of non-functionalized > surfactant stabilized > OH-
249 functionalized. Studies have observed differences in plant uptake and bioavailability
250 based on CNT surface functionalization and aggregation. Villagarcia et al. demonstrated
251 an increase in the growth of tomato plants exposed to well dispersed non-functionalized
252 and COOH-functionalized MWNTs (Villagarcia et al., 2012). Our results did not show

plant uptake to be dependent on functional group dispersibility (Figures S1 and S2), differing from what has been observed in other studies (Liu et al., 2009; Nair et al., 2010; Rico et al., 2011). In future work, shorter or oxidized types of SWNTs may create an easier dispersion in the soil and may be used to better understand the interaction between SWNTs and plant roots.

3.4 Study 2: Effect of CNTs on water deficit stress response

Corn was grown in a controlled greenhouse environment over a 40 d time period and exposed to water deficit conditions for 10 d. SWNTs were detected in roots at concentrations of 1.6 µg/g in plants exposed to non-functionalized SWNTs and 2.6 µg/g in plants exposed to COOH-functionalized SWNTs under drought conditions (Figure 4). Trace amounts of translocated SWNTs were observed in stems at concentrations of 2 µg/g in water deficit COOH-functionalized SWNT treatments. No SWNT concentrations were detected in optimally grown treatments in Study 2. Root length was measured and was not significant from the control (S4), indicating that there was no effect of SNWTs on root growth.

Similar to Study 1, uptake and translocation of SWNTs in Study 2 was observed but at lower concentrations. For plant exposures, seeds were allowed to germinate in SWNT-laden soil. As a result, emerging seedlings may have been directly exposed to SWNTs. However, Lin *et al* showed no alteration of growth and germination in corn seedlings when exposed to MWNTs at concentrations of 2000 mg/L (Lin and Xing, 2007). On contrary to plants in Study 1 which had accumulated SNWTs, no SWNTs were detected in optimally grown plants in Study 2. These observations could have

276 been due to the difference between soil volumes used in each study. A larger soil
277 volume of 3 L was used for each plant replicate in Study 2 to prevent stress due to
278 limitations of space needed for root growth with the addition of stress from simulated
279 drought conditions. Exposure media plays a role in availability of CNTs to plant roots.
280 Although plants have been exposed to CNTs through agar (Yan et al., 2013) and
281 hydroponic systems (Tripathi et al., 2011), exposing plants to CNTs through soil
282 simulates environmentally relevant conditions. Soil consists of complex components
283 such as natural organic matter (NOM), microorganisms, and variable texture. These
284 components can play a role in CNT availability to plant roots (Miralles et al., 2012a).
285 CNTs are known to form complexes with NOM such as fulvic and humic acids. When
286 common divalent ions Ca^{2+} and Mg^{2+} are present in soil, these can neutralize negative
287 forces exhibited from fulvic and humic acids, releasing CNTs and causing aggregation
288 (Saleh et al., 2010; Grillo et al., 2015). Despite soil being homogeneously mixed, using
289 3 L of soil tripled the amount of NOM compared to that used in Study 1. The increase in
290 NOM may have had an effect on the localization and aggregation SWNTs in the soil
291 mixture. Another reason for variability between studies in uptake may have been due to
292 differences in seasonality. Plants were grown in the summer for Study 1 while Study 2
293 plants were grown in the winter. Although both studies had plants which were prepared
294 under the same conditions and grown in a greenhouse with a consistent temperature,
295 the difference in the amount of sunlight may have contributed to differences in uptake.
296 Some samples showed to have no SWNTs in stem, leaves, and roots; however, this
297 may be due to the detection limit of this method (0.1 $\mu\text{g/g}$). Furthermore, both studies

showed SWNTs to reside in the root of plants, which should be considered in root tuber species risk assessment and dietary exposure.

3.5 Effects of SWNTs on plant photosynthetic rate

To the knowledge of the authors, no studies to date have addressed SWNT implications on plant photosynthetic rate. The measurement of photosynthetic rate has been used as an indication of stress in plant drought studies (Saglam et al., 2014; Ashraf et al., 2015; Aslam et al., 2015) and was used here to assess the impact of SWNTs on the water-deficit stress response and general physiology of the plants. Net photosynthetic rate (A_n) was measured throughout the water deficit study over a 10 d stress period (Figure 5). After day 3, there was a decrease in A_n in water deficit plants with a continued decline to day 10. Plant responses in photosynthetic rate which were optimally watered were significantly different from those which had experienced water deficit conditions ($P < 0.05$); however no treatments within a growing condition were significant ($P > 0.05$). Results from this study suggest no stress due to SWNT interactions with roots, similar to Larue *et al.* which exposed wheat and rapeseed to [^{14}C] MWNTs for 7 d. The study showed no negative physiological effects on photosynthetic pigments, root elongation, and growth due to MWNT exposure (Larue et al., 2012) despite low accumulation in roots and leaves (0.005 %). On the contrary, Yan *et al.* showed SWNT concentrations induced stress in exposed maize plants grown with commercially available SWNTs for 72 h. SWNTs were responsible for the up-regulation and modification of enzyme genes which can lead to global deacetylation of histone H3, an indication of a common stress signal in plants (Yan et al., 2013). In Study 2, plant

stress is measured using photosynthetic rate. It may be feasible to measure other parameters concurrently with oxidative stress to confirm total stress effects (Ashraf et al., 2015).

4. Conclusions

Using a microwave-induced heating technique, concentrations of accumulated and translocated SWNTs in exposed corn grown for 40 d in soil were determined. SWNTs were taken up into the roots of corn with minimal concentrations seen in stem and leaf parts. Both studies showed that uptake of SWNTs was independent of functionalization but dependent on volume and composition of soil. The water deficit study identified no compounding stress of exposure to CNTs in plants grown under water deficit conditions. Additional investigation addressing uptake of CNTs with different surface groups in root tuber species should be done to better predict the amount of CNTs available for potential human dietary exposure.

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Figure 1: Non-functionalized single-walled carbon nanotubes dispersed in SDBS as characterized by transmission electron microscopy (TEM). Outer SWNT diameter is 2.5 nm.

Figure 2: Thermal decomposition profiles of pristine and (-OH, -COOH) functionalized SWNTs during TGA.

Figure 3: Single-walled nanotube uptake in roots, stems, and leaves of corn exposed for 40 d. Treatments included treatment control (CRT), Non-functionalized (NF), OH-functionalized (F), Surfactant (SDBS) stabilized (S). Asterisks represent significant differences of $P < 0.05$. Limit of detection (LOD) is indicated as $\sim 0.006 \mu\text{g CNT/mg sample}$. Error bars represent standard error (SE) in the data.

Figure 4: Uptake of COOH-functionalized SWNTs by corn roots, grown in optimally watered (OP) and water deficit conditions (D). Plant treatments were control (CRT), non-functionalized (NF), and COOH-functionalized (F). Error bars represent standard error (SE) in the data.

Figure 5: Net photosynthetic rate of plants exposed to 10 mg/kg SWNTs over a 10 day period. Treatments included optimally watered (OP) and water deficit (D) growing conditions. SWNT plant treatments were control (CRT), non-functionalized (NF), and COOH-functionalized (F). Error bars represent standard error between replicates.

Figure 1

Figure 2

Figure 3.

Figure 4

Figure 5