Dose-dependent effects of multi-walled carbon nanotube on seed germination, growth, photosythetic pigments and lipid peroxidation of six plant species

Rayhaneh Amooaghaie
Biology Department, Science Faculty, Shahrekord University, Iran

Maryam Norouzi
Biology Department, Science Faculty, Shahrekord University, Iran

Abstract
The multi- walled carbon nanotubes (MWCNTs) are one of the most widely used engineered nanoparticles and are expected to enter natural ecosystems. More studies on plants, as a part of food chain, are needed to understand profoundly the toxicity and health risks of CNMs as ecotoxicological stressors. In this study, The effects of multi- walled carbon nanotubes (MWCNTs) with a length ۳۰ nm on seed germination and growth of six plant species () were evaluated in petri dish (direct exposure) and in soil in a greenhouse experiment (soil exposure). Data demonstrate that under both culture conditions, low or mild concentrations MWCNT either stimulated or had no effect on seed germination, root growth and vegetative biomass while high concentrations had an inhibitory effect. However, results showed that the impacts of MWCNT on plant growth in soil were partially consistent with those observed in pure culture. Based on data among above six species, lettuce and canola were the most susceptible and the most tolerant species to MWCNT toxicity. In all species root growth was more susceptible to MWCNT toxicity than seed germination or shoot growth. The high concentrations of MWCNT caused significant reductions in fresh and dry weight of arial parts and root and chlorophyll and carotenoids contents of all species which also coincided with further accumulation of malondealdehyde (MDA). These findings suggest that decreasing growth might be the result of an MWCNT-induced oxidative stress and disturbtion of photosynthesis systems.

Keywords: Chlorophyll, Growth, Malondealdehyde, MWCNT, Seed germination

Introduction
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. But in plant field, studies on the effect of nanomaterials are still emerging and basically evidence several negative effects on growth and development of plantlets. With the rapid growth of nanotechnology, there are growing concerns over the potential adverse impacts of engineered nanoparticles (ENPs) in the environment. However, our understanding of how

ENPs may affect organisms within natural ecosystems, lags far behind our rapidly increasing ability to engineer novel nanomaterials [١].

Carbon-based materials such as carbon nanotubes (CNTs), fullerene, and graphene are one of the most promising nanomaterials in nanotechnology due to their unique physico-chemical, electronic, and mechanical properties, which have led to their exploitation in diverse applications such as sensors, flat panel displays, energy and gas storage. The potential use of carbon-based materials has been actively explored in some important fields like biomedicine, biosensors, and tissue engineering (Baby et al. ٢٠١١; Tan et al. ٢٠٠٩), but the exploration of their extensive application in agriculture and plant science has just been initiated (Nair et al. ٢٠١٠; Kurepa et al. ٢٠١٠). Recently, it has been found that CNTs at relatively low doses can penetrate even thick seed coats, stimulate germination, and activate enhanced growth of tomato plants (Khodakovskaya et al. ٢٠٠٩). Moreover, CNTs can also work as an effective nanocargo to deliver DNA and small molecules into tobacco cells (Liu et al. ٢٠٠٩a, b). There are also reports about multiple nano-technological applications of CNTs in agriculture, from agrochemical treatments to genetic transformation for improved plant disease resistance, efficient nutrient utilization, and enhanced plant growth (Liu et al. ٢٠١٠; Nair et al. ٢٠١٠).

On the other hand, several studies have reported the toxicity of CNT exposure in animal and human cell lines, bacteria, and organisms (Zhu *et al*., ٢٠٠٦; Warheit *et al*., ٢٠٠٤; Porter *et al*., ٢٠٠٧; Manna *et al*., ٢٠٠٥; Kang *et al*., ٢٠٠٧; Poland *et al*., ٢٠٠٨). Tan and Fugetsu (٢٠٠٧) also found that MWCNTs interact directly with rice cells and may have a detrimental effect on rice growth. In sum, there are contradictory results on CNT toxicity in plants. Therefore, before the development of their application in agriculture such as the ''smart delivery system'', the effects and behavior of carbon-based materials on plant growth and development should be properly identified. To better understand the effects of CNTs on plant physiology and plant development, its biocompatible role in biological systems should be addressed first before any agricultural application is investigated. Therefore, it is still very necessary to continue the exploration and identification of the effects of CNTs on plant growth and physiology.

To date, research on the biological impacts of ENPs has primarily consisted of controlled lab studies of model organisms with single species in culture media [١،٢]. These types of highly controlled experiments are essential for elucidating the mechanisms of ENPs toxicity; however, pure culture research is rarely sufficient to predict the impacts of a potential contaminant in the complex soils, sediments, or waters found in natural environments. In the current study, we exposed ٦ species of plants to MWCNTs under pure culture conditions and after planting in natural soils. We asked: (١) Are the effects of MWCNTs on the germination and growth of each plant species is dose dependent manner? (٢) How do plant species differ in their response to MWCNTs exposure? and (٣) Are the effects observed in pure culture consistent in direction or magnitude with the effects observed in natural soils?.

Method and materials

Chemicals

Six plant species: wheat, soybean, tomato, rape, cucumber and lettuce were used in our study. The effects of multi- walled carbon nanotube (MWCNT) with a length ٣٠ nm, Surface area ٢٠٠ (m٢/g), spiral shape and purity ٩٩,٥٪ were evaluated on seed germination, the growth and physiology of six plant species. MWCNT were suspended in deionized water (DI-water) and vibrated ultrasonically (١٠٠ W, ٤٠ kHz) for ٣٠ min. To avoid aggregation of the particles suspensions were continuously mixed using a magnetic stirrer before use.

Response to MWCNT exposure in petri dishes

MWCNT suspensions, at ٠, ٥٠, ١٠٠, ٢٠٠, ٤٠٠, ٨٠٠, ١٦٠٠ mg L-١ concentrations, were used to investigate NPs toxicity on seed germination indices. The seeds of six plant species were immersed in ١٠٪ solution of sodium hypochlorite for ٣ min to ensure surface sterility. Seeds were then washed three times with distilled water and placed in large Petri dishes on filter paper, ١ cm apart. ١٠ ml of each concentration of NP suspensions were added to each dish (three replicates for each treatment dose) and kept at ٢٥°C. When radicle became visible (>٢ mm) the seed was noted as having germinated. Seed germination was monitored daily, over ١٠ days, and germination percentages were calculated. Germination tolerance index (GTI), relative seed germination (RSG), and relative root elongation (RRE) were calculated as described by Barrena et al. (٢٠٠٩) using following equations:

$\text{germination percentage} = \dfrac{\text{number of germinated seeds}}{\text{number of total seeds}} \times ١٠٠$	Equation ١
$\text{Relative root elongation} = \dfrac{\text{Mean root length with NPs}}{\text{Mean root length with control}} \times ١٠٠$	Equation ٢
$\text{Relative seed germination} = \dfrac{\text{seed germinated with NPs}}{\text{seed germinated with control}} \times ١٠٠$	Equation ٣
Germination Tolerance Index (GTI) = (RSG × RRE)/١٠٠	Equation ٤

Response to MWCNT exposure in Soil

The seeds of six plant species germinated in glass Petri dishes (١٠ cm) containing two sheets of autoclaved blotting paper moistened with ١٠ ml of distilled water at ٢٥°C in the dark. After ٣ days growth in Petri dishes, seedlings of uniform size were transferred to soil culture in plastic pots. Each pot contains four plants and irrigated with ٤٠ ml of half strength Hoagland's nutrient solution having pH ٦,٤. Pots were transferred to greenhouse and maintained at ٢٥ ± ٢°C and ٥٠ % relative humidity with photosynthetic photon flux density (PPFD) at ٥٠٠ µmol $m^{-٢}$ $s^{-١}$ by a combination of fluorescent tubes and tungsten lamps for ١٤/١٠ h day/night daily.

The ١٥ days old acclimatized seedlings were used for further experimental work. The suitable amounts of MWCNT solution were added to the nutrient solution until final concentration reached to ٥٠, ١٠٠, ٢٠٠, ٤٠٠ mg $L^{-١}$ A control with half strength Hoagland's nutrient solution without nanoparticles was used. The plants were grown in a growth chamber with above-mentioned growth conditions. After two week seedlings were harvested and used for further experiments.

Fresh and dry (٧٥ºC oven for ٤٨ h) weights of shoots and roots were recorded. Levels of chlorophyll a, b and carotenoids were determined by collecting fresh leaf samples from randomly selected plants per replicate as previously described by Lichtenthaler (١٩٨٧).

Lipid peroxidation measurment

Heath and Packer's method (١٩٦٨) for measurement of malondialdehyde (MDA) was followed to determine lipid peroxidation levels. Leave samples (٠,٥ g) were homogenized in ١٠ ml of ٠,١٪ trichloroacetic acid (TCA) and centrifuged at ١٥٠٠٠ ×g for ١٠ min. Supernatant was mixed with ٤,٠ ml of ٠,٥٪ thiobarbituric acid, and heated at ٩٥°C for ٣٠ min. Then, quickly was cooled in an ice bath. After centrifugation at ١٠٠٠٠ ×g for ١٠ min, the absorbance was recorded at ٥٣٢ nm and nonspecific absorbance at ٦٠٠ nm was subtracted. The MDA content was calculated using its absorbance coefficient of ١٥٥ $mmol^{-١}$ $cm^{-١}$ and expressed as µmol (MDA) per gram fresh weight.

Statistical analysis

Both experiments carried out as factorial with complete randomized design in ٣ replications. Data were subjected to analysis of variance using computer software of SPSS, version ١٣. Multiple Duncan test was applied to compare the treatment means at P < ٠,٠٥. In all figures; error bars are representing standard errors of the means.

Results

Response to MWCNT exposure in petri dishes

Exposure to MWCNT significantly affected seed germination and seedling growth. Germination responses differed between MWCNT concentrations and species.

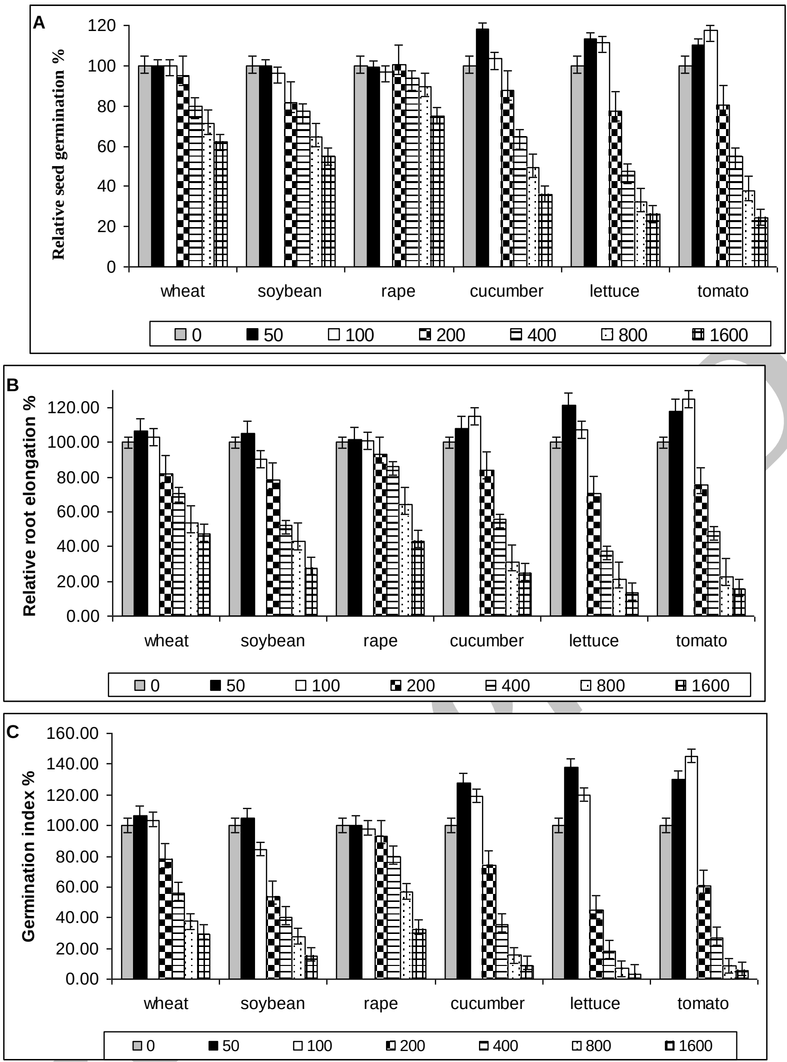


Fig ١. Effect of various concentrations of MWCNT on Relative seed germination, Relative root elongation and Germination index of six plant species

As shown in Fig. ١A exposure to low concentration of MWCNT (<١٠٠ mg/L) promoted germination percentage for ٣ of the ٦ species (cucumber, lettuce and tomato), while had no significant effect on final seed germination of ٣ other species (wheat, soybean and canola) (Fig. ١). However, at MWCNT concentrations further than ٢٠٠ mg/L (especially at ٨٠٠ to ١٦٠٠ mg/L), seed germination was reduced in all species.

We documented significant root growth responses to all MWCNT exposure concentrations which root growth occurred. In general, root growth was much more sensitive to MWCNT exposure than seed germination. The magnitude and direction of the effect differed by concentrations and species. Root growth of the wheat, soybean did not respond to MWCNT until ١٠٠ mg/L but did affected negatively at higher concentrations. Root growth of the rape did not change until ٢٠٠ mg/L.At ٥٠ and ١٠٠ mg/L cucumber, lettuce and tomato, responded to MWCNT treatments by growing significantly longer and more curved roots compared to root growth in control. However at concentrations higher than ٤٠٠ mg/L and especially at ٨٠٠ to ١٦٠٠ mg $L^{-١}$ root all species had significantly shorter roots (Fig ١B) than their contols. The magnitude of inhibition was greater for cucumber, lettuce and tomato than wheat, soybean and especially canola. Increasing concentrations of NPs lead to more sever reductions in germination index (Fig. ۱C) in all plant species. These reductions were more pronounced in cucumber, lettuce and tomato than wheat, soybean and especially canola.

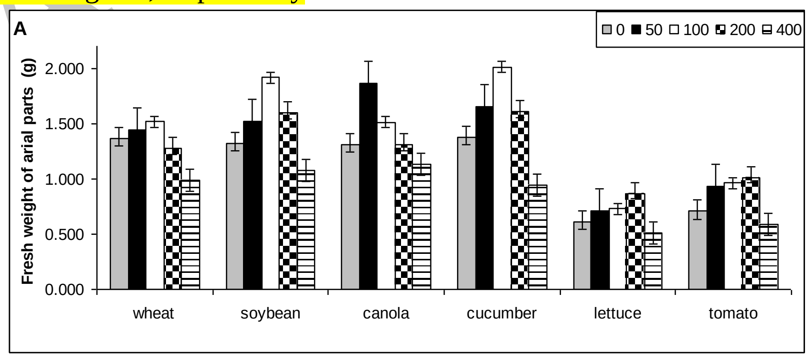
IC۵۰ (fifty percent inhibitory concentrations) for seed germination, root and shoot length of seedlings shown in Table ۱. Fifty percents inhibitory concentrations (IC۵۰) of MWCNT were more for seed germination and shoot length than root elongation in all species (Table ۱), indicating more susceptibility of root growth to NP toxicity than seed germination and shoot growth.

Table ۱. The IC۵۰ (fifty percent inhibitory concentrations) values of MWCNT for seed germination, root and shoot length of six plant species.

species	Seed germination	Root length	Shoot length
wheat	IC۵۰ =۱٦۰۰	IC۵۰ =۱٦۰۰	۸۰۰<IC۵۰<۱۶۰۰
soybean	۸۰۰<IC۵۰<۱۶۰۰	IC۵۰ =٤۰۰	۴۰۰<IC۵۰<۸۰۰
canola	IC۵۰>۱۶۰۰	۸۰۰<IC۵۰<۱۶۰۰	۸۰۰<IC۵۰<۱۶۰۰
cucumber	۴۰۰<IC۵۰<۸۰۰	IC۵۰ =٤۰۰	۴۰۰<IC۵۰<۸۰۰
lettuce	۲۰۰<IC۵۰<۴۰۰	۲۰۰<IC۵۰<۴۰۰	۲۰۰<IC۵۰<۴۰۰
tomato	۲۰۰<IC۵۰<۴۰۰	۲۰۰<IC۵۰<۴۰۰	۲۰۰<IC۵۰<۴۰۰

Response to MWCNT exposure in Soil

The only significant effect of MWCNT on germination in the soil exposure experiment was inhibition of germination of Phytolacca americana by MWCNT (Figure ۱). Though the effects on germination were minimal, MWCNT had significant impacts on aboveground biomass. The magnitude of plant biomass change was greater for GA-AgNPs than for PVP-AgNPs or AgNO۳. Exposure to MWCNT affected seedling growth for all tested plant species while exposure to PVP-AgNPs and AgNO۳ affected seedling growth for only one species each, L. multiflorum and Phytolacca americana, respectively (Figure ۲). Seedling growth responses differed between species. The grass L. multiflorum responded positively to MWCNT treatments, and its aboveground biomass increased by ٥٥٪ and ٤٥٪, respectively, as compared to control plants. In general, aboveground biomass of the other species demonstrated a negative response to the MWCNT treatments. Both Carex spp. and E. fistulosum had significantly reduced aboveground growth when exposed to MWCNT. Phytolacca americana aboveground growth was reduced ٦٢٪ and ٦٥٪ when exposed to PVP-AgNPs and GA-AgNPs, respectively



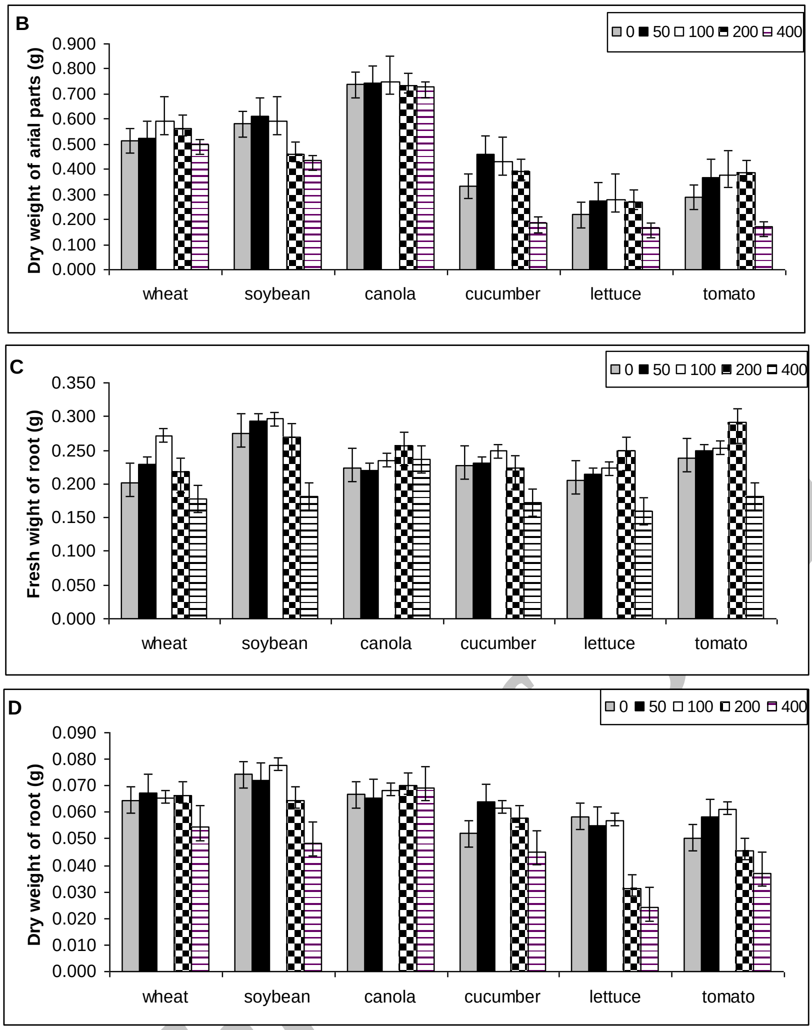


Fig ٢. Effect of various concentrations of MWCNT on fresh and dry weight of arial parts and roots of six plant species

Increasing NP concentrations in soil caused a significant reduction in fresh and dry weight of shoot and root of all species (Fig. ٢). In hydroponic system at ٥٠ mg L-١ MWCNT fresh and dry weights of shoots of tomato were significantly did not affect but fresh and dry weights of roots decreased. However, at ١٠٠-٤٠٠ mg L-١ MWCNT fresh and dry weight of shoot and root of tomato declined sharply. Application of ٥٠ mg L-١ nZn and nZnO increased significantly fresh and dry weight of shoot and fresh weight of root but did not influence dry weight of root. However, with increasing MWCNT to ٢٠٠ and ٤٠٠ mg L-١, fresh and dry weights of roots/shoots of wheat also were reduced significantly.

These growth reductions were more pronounced in the root in all species. In ٤٠٠ mg L-١ MWCNT, shoot dry mass decreased ٧٧,٧٧ and ٧١,١١٪ in tomato, and ٢٢,٧٢ and ٣٦,٣٦ % in wheat, as compared to their control (٠ NPs). While, root dry mass reduced ٩٠,١٦, ٨١,٩٦٪ in tomato and ٤٠,٤٨, ٤٨,٨٨٪ in wheat in respond to ٤٠٠ mg L-١ nZn and nZnO respectively (Fig. ٢).

Photosynthetic pigments decreased significantly and progressively with increasing concentrations of MWCNT (Fig. ٣), which resulted markedly yellowing of the leaves at ٢٠٠ mg L-١ MWCNT in tomato, whereas in wheat slight yellowing of the leaves observed at ٤٠٠ mg L-١ MWCNT.

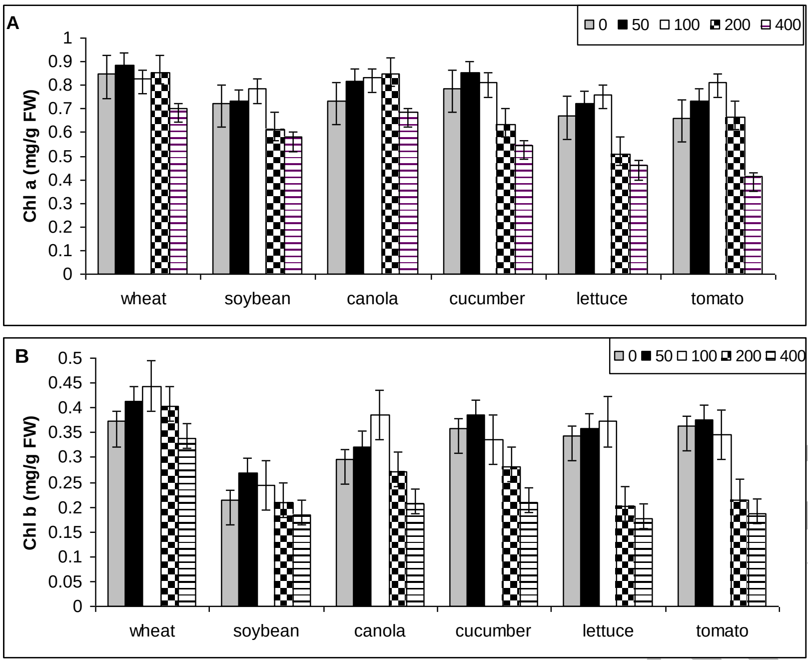
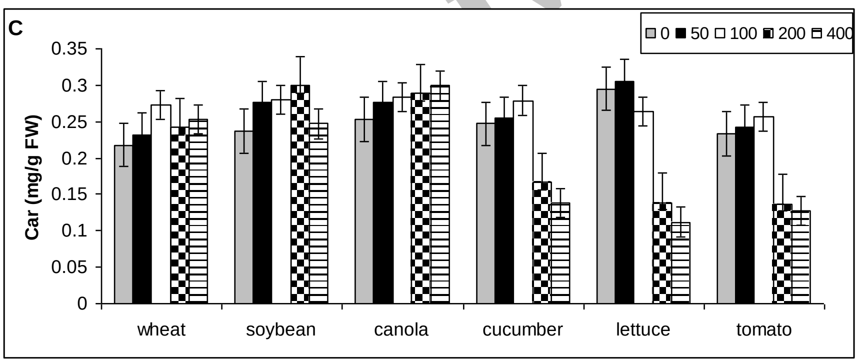


Fig ١. Effect of various concentrations of MWCNT on chlorophyll a, chlorophyll b and carotenoids of six plant species

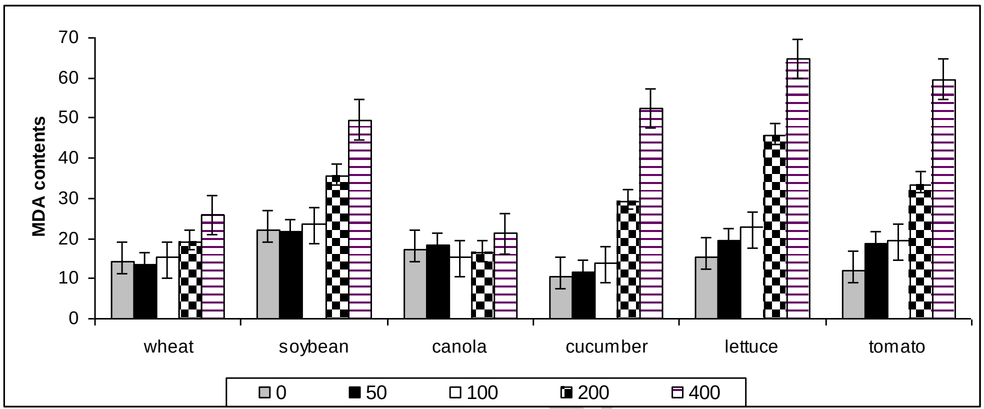


Lipid peroxidation and H_2O_2 content

To investigate nanoparticle-driven oxidative damage in species, lipid peroxidation was detected by evaluating MDA content of the leaves.

In tomato exposure to ١٠٠-٢٠٠ mg L-١ MWCNT increased MDA content significantly (Fig. ٤a, b), whereas significant change was not observed in level of MDA in wheat leaves subjected to ١٠٠ mg L-١ nanoparticles. However, at ٢٠٠ mg L-١ MWCNT concentrations a significant increase in MDA content was recorded in wheat. In ٢٠٠ mg L-١ MWCNT, the accumulation of H_2O_2 was in tomato ٢,٣٦, ٢,٦٦ times and in wheat ١,٤٢, ١,٥٣ times higher than ones in leaves of their control plants (Fig. ٤a, b).

All three forms of Ag affected leaf growth for some species although the direction and magnitude of the effects differed by treatment and species. Only C. lurida leaf growth responded positively to Ag exposure. All three Ag treatments elicited this response, however PVP-AgNPs had a significant effect only at the highest dose (٤٠ mg L٢١), AgNO٣ had a significant effect only at the intermediate dose, and GA-AgNPs had a stimulatory effect at the lowest dose and an inhibitory effect at the highest dose (Tables ١ and S٣). The grass L. multiflorum responded negatively to all three forms of Ag with significant negative effects of PVP- AgNPs and AgNO٣ were observed at the highest dose, and significantly less leaf growth from GA-AgNP exposure at both the intermediate and highest dosing scenario (Tables ١ and S٣). High dose (٤٠ mg Ag L٢١) exposures led to ٥٥٪, ٤٥٪ and ٢٥٪ reductions in leaf growth on average in the GA-AgNP, AgNO٣ and PVP-AgNP treatments respectively. Two species, C. scoparia and S. cyperinus had significantly reduced leaf growth when exposed to ٤٠ mg Ag/L GA-AgNPs but were unaffected by exposure to PVP-AgNPs or AgNO٣ (Table ١). Both C. crinita and C. vulpinoidea had similar reductions in leaf growth in response to high doses of either GA-AgNPs or AgNO٣ (Table ١).



Discussion

Carbon-based nanomaterials (CNMs), owing to their numerous potential applications, have been increasingly used for phytotoxic tests since their ability to enter the cell wall and plasma membrane was ascertained [٢٩].

Seed germination and root elongation is a rapid and widely used acute phytotoxicity test with several advantages: sensitivity, simplicity, low cost and suitability for unstable chemicals or samples (Wang et al., ٢٠٠١). Our results here demonstrate that under direct exposure in petri dishes, Low or intermediate concentrations MWCNT either stimulated or had no effect on seed germination, while high concentrations had an inhibitory effect.

Other studies also reported advantageous, disadvantageous, or no evident effects of nanotubes on plants both at phenothypic and genotypic levels. A stimulative effect, prevalently of MWCNTs, on seedling root elongation and/or seed germination was observed in onion and cucumber [٥٢], wheat [٩٢], mustard [٩٣], and tomatoes [٥٣،٥٦،٩٤،٩٥]. In tomatoes the MWCNTs penetrated the seed coat and seedling roots through new wall pores and enhanced the capacity of the plant to uptake water, with positive effect on germination and seedling growth. Assuming CNTs are able to induce new water entry points on the seed coat, one would expect a similar capacity on the root rhyzoderm instead of a suppression of water uptake. This point, repeatedly debated by several authors, is still waiting to be cleared up. However, Khodakovskaya et al. [٥٣], by means of advanced physical methods (Raman, Phototherma, and Photoacoustic methods), described a nanotube-dependent gene activation, both in roots and leaves, of either stress-related genes, among which were those switched on by pathogens, or the gene controlling the water channels on the plasma membranes. Seeds and seedlings responded to the gene upregulation by increasing germination and growth. Growth induced by the upregulation of genes controlling the cell division, cell wall formation, and the water flow through the membrane was also observed in tobacco cells cultured with MWCNTs [١٠١.[

Plant cell walls function as natural sieves, and NPs may have to penetrate cell walls. NPs may not affect seed germination if they cannot pass through seed coats, although they have obviously inhibitory effect on root elongation. It can be assumed that The different effects of MWCNTs between the six species might be partly attributed to the pore size of plant cell walls as well as concentrations. (Ma *et al*., ۲۰۱۰; Lin and Xing, ۲۰۰۷).
The effects of MWCNTs on root growth of the six species revealed similar tendency with their seed germination (Fig). The stimulation of root emergence and root growth was recently observed in in vitro cultures of *Rubus adenotrichos* treated with functionalized SWCNTs [۹٦ . Accumulation of MWCNTs and SWCNTs was observed on the root surface of several crop plants including tomato, wheat, cabbage, lettuce, carrot, cucumber, onion, and rice [٥٢–٥٥]. Of course Canas et al. [٥٢] also observed in several plants that CNTs did not penetrate the roots but produced effects on the root growth—adverse in tomato and advantageous in onion and cucumber— whose mechanisms of action were not fully clarified. Authors reported toxicity of nanoparticles and uptake into roots may be attributed to nature and the chemical composition, concentration, size, aggregation, functionalization, and experimental conditions including temperature and time, and method of exposure (seeds/seedlings/cell suspensions) and plant species (Ma *et al*., ۲۰۱۰, Nowack and Bucheli ۲۰۰۷).
Fifty percents inhibitory concentrations (IC٥٠) of MWCNT were more for seed germination than root elongation in all species (Table ۱), indicating more susceptibility of root growth to NP toxicity than seed germination. Seed coat plays a very important role in protecting the embryo from harmful external factors. Seed coats can have selective permeability (Lin and Xing ۲۰۰۷). Thus it is possible that pollutants which having obviously inhibitory effect on root growth, not affect germination if they cannot pass through seed coats. This is consistent with previous studies that report NPs had less of an effect on seed germination than seedling growth [۲۹،۳۰]. This may be explained by the protective effect of the seed coat [۳۱].

Based on seed germination and seedling growth among above six species, lettuce and canola were the most susceptible and the most tolerant species to MWCNT stress. Our data confirmed the effects of MWCNT on seed germination and seedling growth was not clearly linked to taxonomy of species. We tested ٦ species that among them, only wheat is a monocot, our representation of the full diversity of plants is limited, thus limiting our ability to tie observed responses to plant classification. Nonetheless, it is interesting to note that effects of MWCNT on seed germination partly appear to be predicted by seed size. Large seeds (e.g. wheat) would have a lower ratio of surface to volume than a small seed (Ma *et al*., ۲۰۱۰). Thus, more effects were expected in smaller-seeded species as were observed for tomato or cucumber in this study (Fig. ۱, ۳-٥). Size may render a seed more sensitive to nanoparticle exposure, but it does not dictate whether phytotoxicity will be exhibited.
However, consistent with our expectations, high concentrations, MWCNT were toxic and both seed germination and root elongation were inhibited in all species. These reductions were more pronounced in GTI (Fig. ۱). The germination tolerance index has been extensively used as an indicator of phytotoxicity in soils (Tiquia and Tam, ۱۹۹۸). It is worth to note that the germination tolerance index combines germination and root growth and consequently reflects the toxicity more completely.
Since roots are the first target tissue to confront with excess concentrations of pollutants, toxic symptoms seem to appear more in roots rather than in shoots (Lin and Xing ۲۰۰۷). This could explain why, shoots displayed higher IC٥٠ than roots and could grow to a certain degree even though root elongation was halted in the presence of MWCNT.

Of all species studied, only C. lurida responded to MWCNT exposures with significant increases in both leaf and root biomass, and this positive response was only observed in the PVP-AgNP exposure. Both Phytolacca americana and Panicum virgatum, also grew significantly longer roots in response to MWCNT treatments, in this case in response to MWCNT and without any measurable impact on leaf length.
While three species showed an increase in growth in response to MWCNT exposure, the majority of plants had significantly shorter roots upon exposure to MWCNT. We hypothesize that plants may

cope with the MWCNT toxicity either by an ''escape strategy'' involving rapid root elongation and curving or by a ''quiescence strategy'' involving persistence under high MWCNT concentration with minimal activity, as reported for plants when they suffer other stresses such as flooding [۳۵].

From petri dish culture experiments, we clearly observed that plant species differed in their susceptibility to MWCNT exposure. In the soil experiments, seeds were germinated in soil to examine whether the effects observed in petri dish on germination and shoot growth could be documented when plants are grown in the chemically complex environment of soil over a longer time period. We found that plant species differ in their susceptibility to MWCNT and that MWCNT' toxicity to plants under realistic growth conditions is only partially consistent with results from petri dish experiments. MWCNT significantly inhibited Phytolacca americana seed germination and Carex spp. aboveground growth both in soil and petri dish culture. MWCNT had relatively low toxicity to plant seed germination and seedling growth both in soil and petri dish culture. These results suggest that there is promising consistency between MWCNT toxicity observed in petri dish culture systems and potential MWCNT toxicity in the natural environment. However, for some species and MWCNT treatments, the effects of MWCNT on plants in soil were either attenuated when compared to petri dish culture, or in some cases the direction of the effect changed (i.e., stimulation instead of inhibition of growth). For example, MWCNT significantly promoted seed germination of E. fistulosum in petri dish culture, but had no effect on E. fistulosum grown in soil. In contrast, L. multiflorum responded negatively to MWCNT in petri dish culture, but positively to MWCNT in soil. There are several possible explanations for these changes in germination and growth. First, it could be that they represent a decrease in effective concentration due to dosing MWCNT in a larger volume of soil as opposed to on a much smaller volume in a petri dish with filter paper. Second, it could be that the seeds and seedlings experienced a decreased impact of MWCNT due to surface modification and interaction of MWCNT with the organic and mineral phases of soil. MWCNT may aggregate or be complexed by ligands which can cause a decreased in toxicity [۱،٤،۵]. Both dilution in soil and complexation/aggregation of MWCNT would lead to lower exposure to seeds and seedlings. This could both explain the lack of impact on germination in soil when compared to petri dish culture, as well as the increased growth of L. multiflorum, which could be subtoxic stimulation of growth–also observed in other species in petri dish culture. Therefore, the effects of CNTs on the plant growth and development are dependent upon plant species, the applied concentrations, the specific conditions of experiments, and the impact of surface properties (Khodakovskaya et al. ۲۰۰۹; Castiglione et al. ۲۰۱۰).

Data of soil culture showed that low concentrations of MWCNT was stimulative but high concentrations significantly reduced shoot and root dry weights (Fig. ۲) and caused yellowing of the leaves which was accompanied by a significant declining in chlorophyll a, b and carotenoids contents especially in tomato plants (Fig. ۳). Carbon nanotubes (CNTs) may either accumulate on the ryzoderm surface or penetrate its cell walls and, if they are compatible with the size, they may be translocated in aerial plant tissues or get trapped during translocation. Evidence for the absorption of carbon-based nanomaterials (CNMs) and translocation to aerial parts, including fruits, were provided by Smirnova et al. [۵۷] in *Onobrychis arenaria* seedlings with electron microscopy (TEM), and by Khodakovskaya et al. [۵۳] in tomato seedlings with TEM, Raman photothermal, and photoacoustic methods.

Shweta et al. (۲۰۱۱) reported that MWCNT-treated gram increased the growth rate in every part of the plant. They found that SWCNT, after being aligned along and attached to the root surface or an inner portion of root, can enhance the capillary action of water absorption, which leads to the faster growth in gram plants . It is known that CNTs can act as molecular channels for water (Liu et al. ۲۰۰۹a, b), supporting water uptake and enhancing plant growth. Wang et al. (۲۰۱۲) suggested that o-MWCNTs can significantly enhance root dehydrogenase activity, which in turn enhances the ability of water uptake and growth of the seedlings. One possible explanation is that o-MWCNTs may promote the dehydrogenase electron-transfer reaction. It is also found that the surface-modified MWCNTs can induce the increase of electrical conductivities (Lee et al. ۲۰۱۱), and that CNTs exhibit a strong and stable electrocatalytic response toward glucose and NADH due to their high surface area and good electron-transfer rate (Baby et al. ۲۰۱۱; Musameh et al. ۲۰۰۲; Ye et al . ۲۰۱۱.(

In contrast Ghosh et al. [۱۰۲] in roots of *Allium cepa*, observed cytological errors and damages to DNA associated with an apoptosis process, as a probable consequence of internalization of the nanotubes. Most recently Yan et al. [۱۰۳], by means of PCR analysis, immunostaining technique, and electron microscopy, provided evidence that in *Zea mays* the penetration and accumulation of SWCNTs inside roots may change the expression of genes controlling the seminal root and the hairs' root growth, of benefit to the first and disadvantage to the second.
In the present study, we observed that high doses of MWCNTs impose precocious oxidative stress and associated membrane damage (shown by lipid peroxidation) that can inhibit plant growth and development in all species (Fig. ٤ a,b). Lipid peroxidation is one of the major effects of oxidative stress, leading to the deterioration of cell membranes, as has been reported in many plant species following exposure to stresses heavy metals (Gomes et al. ۲۰۱۳; Li et al. ۲۰۱۳, Sytar et al. ۲۰۱۳). Hence, nanotoxicity, like other abiotic stresses could induce the accumulation of MDA, which is an indicator of NPs-driven oxidative damage to the membranes. Likewise, tomato showed higher lipid peroxidation rates than wheat in the presence of MWCNTs which also coincided with further decreasing growth parameters. Therefore we suggest that lipid peroxidation can consider as biomarker of MWCNT toxicity.
Our data also showed that the chlorophyll contents of all plants decreased significantly at the presence of high concentration of MWCNT that was correlated to generation of ROS in presence of MWCNT. Carotenoids are plant pigments that serve as Light Harvesting Complexes (with proteins) in photosynthesis and protect thylakoid membrane as well as chlorophylls. Carotenoids are important in antioxidative stresses as antioxidant agents. In our study, similar to chlorophyll, these pigment contents reduced. It is possible due to their oxidation by ROS. Studies on leaf cell cultures in *Arabidopsis* [۳٦] and *Oryza sativa* [۳۷] showed that treatments with multi-walled carbon nanotubes (MWCNTs) produced in the first plant a decrease of superoxide dismutase (SOD) activity associated with a decay of chlorophyll production, and in the second plant an increase of reactive oxygen species (ROS) and apoptotic processes. It is notable that symptoms of oxidative stress (ROS accumulation) and a dose-dependent programmed cell death were found in Arabidopsis and rice by Shen et al. [۲۸] after treating both protoplasts and integral leaves with SWNTs; these authors also provided some evidence in favor of the internalization of nanotubes through endocytosis-like processes. Recently, Giraldo et al. [۳۰] found that SWCNTs, once having penetrated the membranes of spinach chloroplasts, increased the flow of electrons and photosynthetic activity, most likely by a stimulating action on the uptake of light with wavelengths of the near-infrared. Otherwise, Santos et al. [۳۸] showed a toxic action of Fullerene C٦۰ on the aquatic plant *Lemna gibba*, which manifested itself in a decrease of photosynthetic activity and plant growth. The leaves were also shown to be sensitive to graphene oxides (GO). Cabbage, spinach, and tomato leaves decreased in size when the seedlings were treated with GO at ٥۰۰–۲۰۰۰ mg/L, and the leaves of tomato and cabbage also decreased in number at concentrations of ۱۰۰۰–۲۰۰۰ mg/L [۳۹]. Begum and Fugetsu [۹۹] treated spinach plants with MWCNTs and these were observed within the plant tissues to produce an increase of ROS and mechanisms of cell death. The toxicity was ascribed to oxidative stress induced by MWCNTs, as the toxic effects were reversed by adding antioxidants to the MWCNTs treatments.
In conclusion, it could be concluded that the phytotoxicity of MWCNT particles are possibly the result of induction of ROS generation that led to reduction of chlorophyll and carotenoids and the damage of photosynthesis systems and finally inhibition of plant growth. However confirm of this hypothesis needs further investigations.

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