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Studies on phytotoxicities of carbon nanomaterials in seedling stage

植物の実生段階における炭素ナノ材料の毒性評価に関する研究

A dissertation submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in the field of
Environmental Science

By

Refi Ikhtiari



Hokkaido University
2014

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ABSTRACT

Carbon nanomaterials (CNMs) which represented by graphene oxide (GO) and carbon nanotubes (CNTs) have been widely applied in nano science and nano industry. The use of CNMs was supposed contribute to negative consequences related to their environmental exposure to human, animal and plant. Therefore the biological effect of CNMs has been evaluated intensively and urgently suggested that the studies of their environmental fate would be very important in the field of nano-toxicology.

The effects of graphene oxide (GO) on root and shoot growth, biomass, shape, cell death, and reactive oxygen species (ROS) of cabbage, tomato, red spinach, and lettuce, were investigated using a concentration range from 500 to 2000 mg/L. The results of the combined morphological and physiological analyses indicate that after 20 days of exposure under our experimental conditions, GO significantly inhibited plant growth and biomass compared to a control. The number and size of leaves of the GO - treated plants were reduced in a dose-dependent manner. Significant effects also were detected showing a concentration dependent increase in ROS and cell death as well as visible symptoms of necrotic lesions, indicating GO -induced adverse effects on cabbage, tomato, and red spinach mediated by oxidative stress necrosis. Little or no significant toxic effect was observed with lettuce seedlings under the same conditions. The potential effect of GO largely depends on dose, exposure time, and plant.

Abundant experimental data have shown that multiwall carbon nanotubes (MWNTs) are toxic to plants, but the potential impacts of exposure remain unclear. The objective of the present study was to evaluate possible phytotoxicity of MWNTs at 0, 20, 200, 1000, and 2000 mg/L with red spinach, lettuce, rice, cucumber, chili, lady's finger,

and soybean based on root and shoot growth, cell death, and electrolyte leakage at the seedling stage. After 15 days of hydroponic culture, the root and shoot lengths of red spinach, lettuce, and cucumber were significantly reduced following exposure to 1000 mg/L and 2000 mg/L MWNTs. Similar toxic effects occurred regarding cell death and electrolyte leakage. Red spinach and lettuce were most sensitive to MWNTs, followed by rice and cucumber. Very little or no toxic effects were observed for chili, lady's finger, and soybean.

Further, we investigated the phytotoxicity of MWNTs on lettuce regarding inhibition effect on plant growth development as well as cell damage on root and leaves. We have cultured the plant seedling of lettuce using Hoagland's Media treated with MWNTs hydroponically. After two weeks, we observed the toxic symptoms in the presence of MWNTs in a dose-dependent manner as compared to carbon black (CB). Statistically, MWNTs could inhibit the seed germination, plant growth and plant biomass. Microscopic analysis showed the attachment of MWNTs on the root surface area, deterioration of root tip and an image of penetration of MWNTs into plant cell wall. We also showed that MWNTs caused cell death on root and leaves. In situ detection of hydrogen peroxide has proposed the Reactive oxygen species (ROS) for mechanism of toxic effect of MWNTs.

In general conclusion, CNMs may not negatively affect all crop species at low concentration; however in some cases apoptosis were found. But at high concentration, CNMs could induce the overproduction of ROS through oxidative stress mechanism which visibly detected as necrotic lesions.

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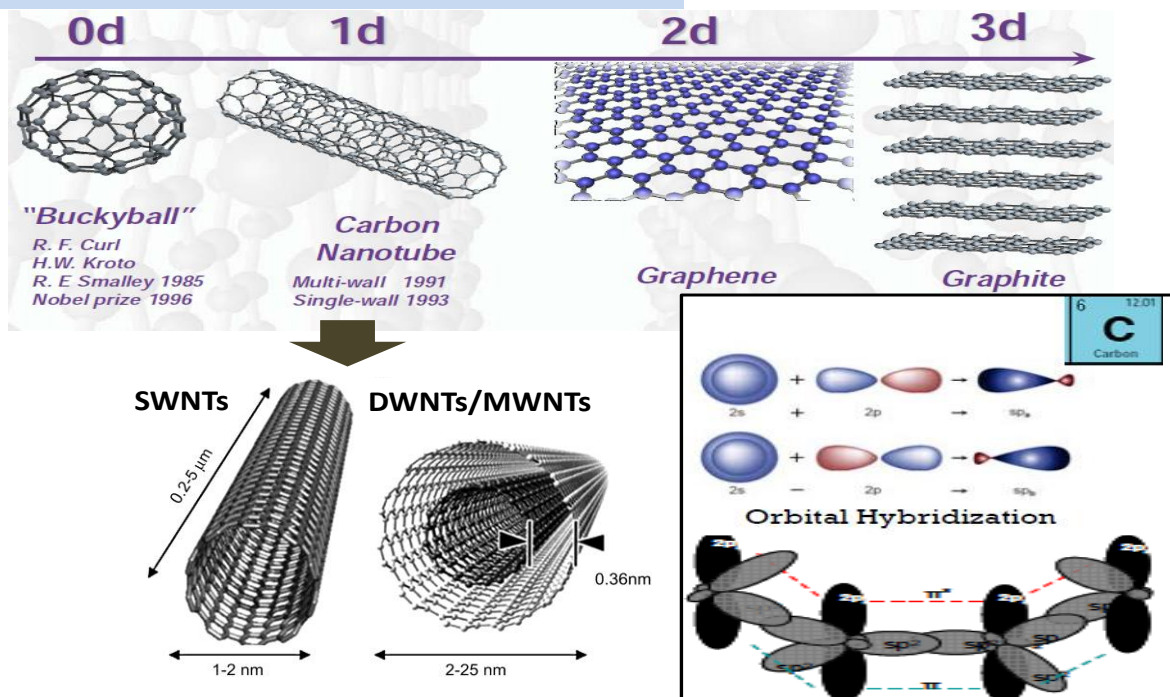
Chapter 1st

General Introduction

1.1 Basic theory of carbon nanomaterials (CNMs)

In the periodic table, carbon is the 6th element with six electrons which occupy $1s^2$, $2s^2$ and $2p^2$ atomic orbital. It can hybridize in sp (e.g. C_2H_2), sp^2 (e.g. graphite) or sp^3 (e.g. CH_4) forms. This property is unique to carbon in its particular group in periodic table. The discoveries of very stable nanometer size sp^2 carbon bonded materials such as fullerenes [1], carbon nanotubes [2] and graphene [3] had stimulated the research in the field of carbon nanomaterials (CNMs) [Figure 1].

Carbon nanomaterials (CNMs)



Credit image : <http://www.nobelprize.org/novoselov-lecture-slide>

Figure 1 Types of carbon nanomaterials (CNMs) including graphene and carbon nanotubes.

1.1.1 Graphene

It has been a decade since Nobel laureates A. Geim and K. Novoselov at the University of Manchester, UK, first reported graphene from lumps of graphite by using sticky tape to peel atomically thin layers [3]. Remarkably, graphene is the thinnest and strongest material reported so far, exhibiting outstanding properties such as excellent conductance of both heat and electricity, and being the strongest material ever measured (100 times stronger than steel). The remarkable properties of graphene arise from the atomically thin mesh of carbon atoms arranged in a honeycomb pattern. The huge number of symmetric carbon-carbon bonds forming the net produces an exceptionally high strength-to-weight ratio as well as the possibility of conducting electricity very efficiently. In this regard, electrons move through graphene as if they have no mass. Graphene which exhibits electron mobility 100 times that of silicon, could surpass in many aspects the properties of silicon for high-tech applications. Furthermore, the carbon atoms in graphene are so tightly packed that not even helium atoms can pass through it. Because of these appealing properties gathered in the same material, graphene has caught the attention of companies to transfer those encouraging features to market. As a consequence, graphene has been the subject of more research papers and patent applications reported in science in the last few years [4].

Because of the aforementioned features of graphene, efforts have been firstly oriented towards the development of more reliable methods for preparing these single-layer honeycomb carbon sheets. Among them, mechanical cleavage of graphite [3], epitaxial or chemical vapor deposition (CVD) growth [5], and the use of solvents for graphite exfoliation [6] have been the most successful procedures. A modification of the

last approach by using amphiphilic intercalators has also recently been reported, affording high quality single and few layer graphene in bulk production [7].

1.1.2 Graphene oxide (GO)

Graphene oxide (GO) was denoted as graphene contains a range of reactive oxygen functional groups [Figure 2], which renders it a good candidate for use in the applications through chemical functionalization. The most common source of graphite used for chemical reactions, including its oxidation, is flake graphite, which is a naturally occurring mineral that is purified to remove heteroatomic contamination. As such, it contains numerous, localized defects in its p-structure that may serve as seed points for the oxidation process [8].

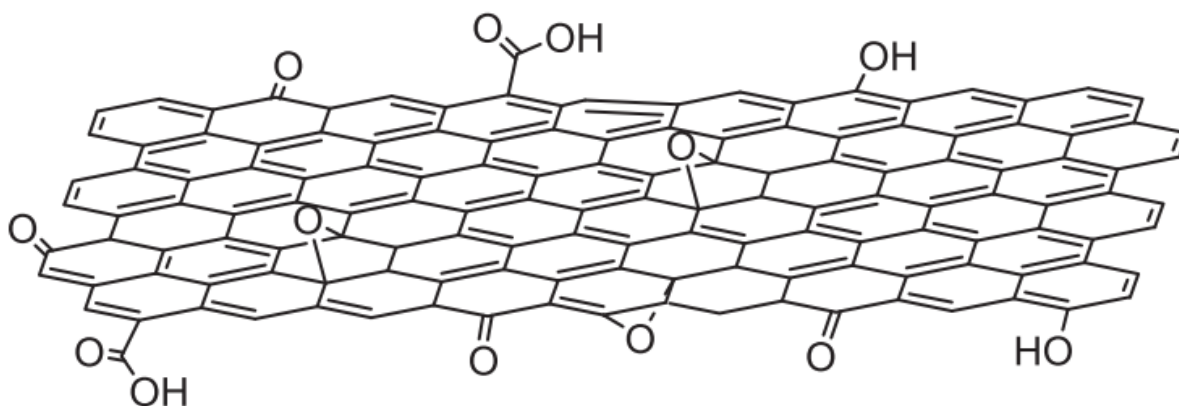


Figure 2 Schematic illustration of graphene oxide (GO) proposed by Singh [8].

Hummers and Offeman have developed an alternate oxidation method by reacting graphite with a mixture of potassium permanganate (KMnO_4) and concentrated sulfuric acid (H_2SO_4) [9]. A modified method for mass production of GO was also introduced by Fugetsu Lab members [10]. The typical intercalating agent (H_2SO_4) was capable of penetrating into the expanded graphite; this has further expanded internal distance

between the basal planes and as a result, a foam-like intermediate was obtained. The foam-like intermediate was more easily oxidized in reaction with the oxidant (KMnO_4) to form GO. Fully exfoliated GO was obtained with the expanded graphite having the median diameter $\sim 15\ \mu\text{m}$ as the precursors. This procedure was claimed as more safe and productive in scalable applications than the conventional Hummers methods [10].

In this experiment, we used the as prepared GO in our Lab which has good dispersibility in water. The maximum dispersibility of graphene oxide in solution, which is important for processing and further derivatization, depends both on the solvent and the extent of surface functionalization imparted during oxidation [Figure 3]; to date it has been found that the greater the polarity of the surface, the greater the dispersibility [11].

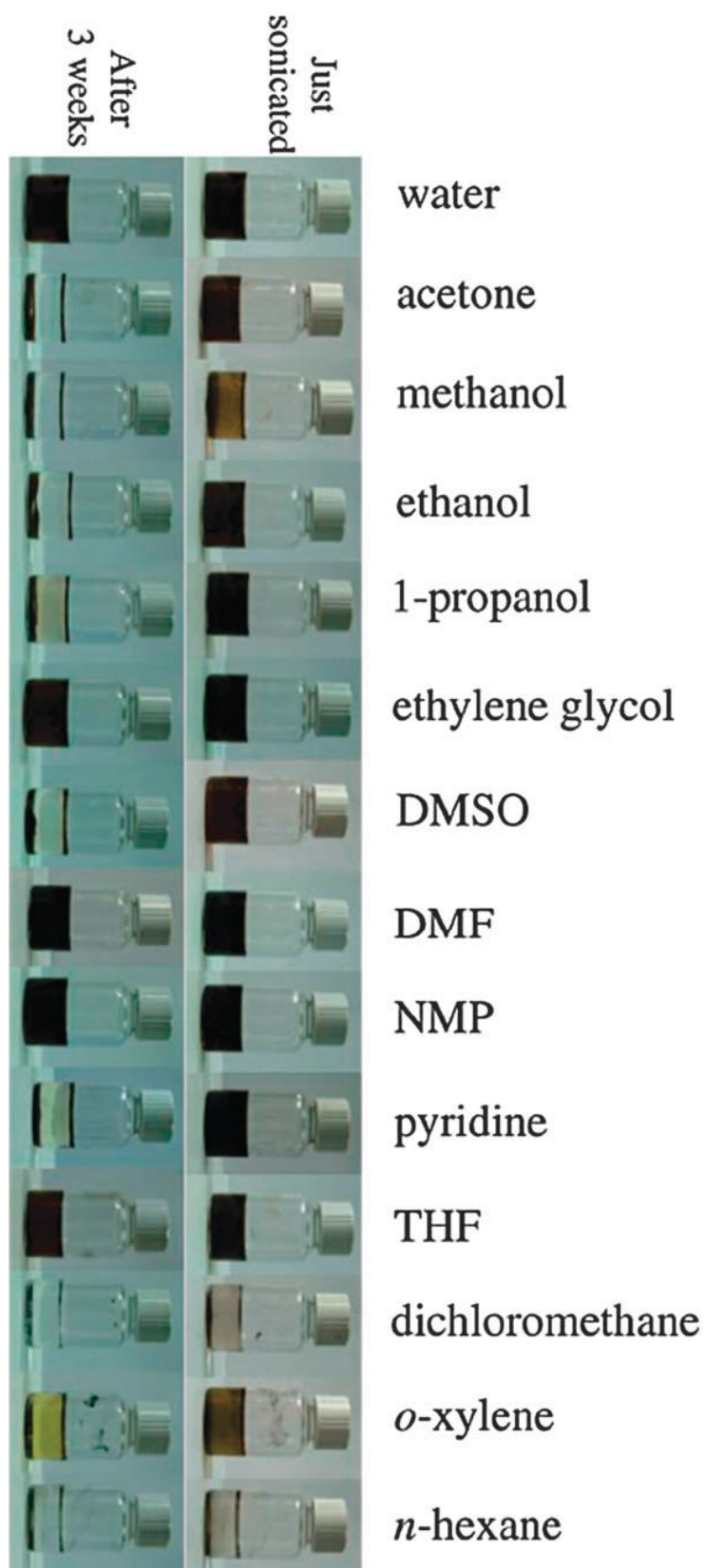


Figure 3 Digital pictures of as-prepared GO dispersed in water and 13 organic solvents through ultrasonication (1 h).

1.1.3 Carbon nanotubes (CNTs)

Carbon nanotubes (CNTs) are seamless cylinders of one or more layers of graphene (denoted single-wall as SWNT, or multiwall as MWNT), with open or closed ends [12]. However, rolling up graphene is not the actual way a nanotubes forms; its actual synthesis process will be explained in this section. Diameters of SWNTs and MWNTs are typically 0.8 to 2 nm and 2 to 25 nm respectively [Figure 1], although MWNTs diameters can exceed 100 nm. CNTs lengths range from less than 100 nm to several centimeters, thereby bridging molecular and macroscopic scales.

Chemical vapor deposition (CVD) is the dominant mode of high-volume CNT production and typically uses fluidized bed reactors that enable uniform gas diffusion and heat transfer to metal catalyst nanoparticles. CVD growth of CNTs start with the gas phase carbon feedstock. Several different carbon containing compounds have been used as precursors such as; carbon monoxide (CO), Methane (CH₄), Ethylene (C₂H₄), Acetylene (C₂H₂), benzene (C₆H₆), toluene (C₇H₈), Ethanol (C₂H₅OH), and Methanol (CH₃OH). Each gas has a particular decomposition temperature, thereby resulting in a different nanotubes growth temperature. Nanotubes are typically grown in a temperature range from 550°C to 1000°C. Temperature can also significantly affect the growth depending upon ramping rates. Ramping up the furnace temperature at different rates is shown to have profound effect on the CNT growth kinetics. Nanoparticles of Fe, Co, Mo, Ni, Cu, Au, etc. have been tried as catalyst either in pure metallic form or as alloys. These transition metal nanoparticles have common advantages of high carbon solubility, carbon diffusion rates and high melting temperatures [13].

The uses of low-cost feedstock, yield increases, and reduction of energy consumption and waste production have substantially decreased MWNTs prices. However, large-scale CVD methods yield contaminants that can influence CNT properties and often require costly thermal annealing and/or chemical treatment for their removal. These steps can introduce defects in CNT sidewalls and shorten CNT length. Currently, bulk purified MWNTs are sold for less than \$100 per kg, which is 1- to 10-fold greater than commercially available carbon fiber [14].

Currently, bulk CNT powders are incorporated in diverse commercial products ranging from rechargeable batteries, automotive parts, and sporting goods to boat hulls and water filters. Advances in CNT synthesis, purification, and chemical modification are enabling integration of CNTs in thin-film electronics and large-area coatings. CNT yarns and sheets already have promising performance for applications including super capacitors, actuators, and lightweight electromagnetic shields [15].

In this experiment, we used the purchased one of MWNTs from TMBaytubes. The MWNTs solution was treated with ultra-sonication and dispersed with non-toxic surfactant [Table 1].

Property	Value	Unit	Test Method
Carbon purity	> 96	wt%	Elementary analysis
Free amorphous carbon	Undetected	wt%	TEM
Outer mean diameter	13-16	nm	TEM
Inner mean diameter	4	nm	TEM
Length	1 to 10	μm	TEM
Bulk density	140-160	kg/m ³	EN ISO 60
Loose agglomerate size (>90 wt%)	0.1-1	mm	PSD

Table 1 Physical properties of MWNTs data from TMBaytubes.

1.2 *Environmental fate of CNMs*

Carbon nanomaterials (CNMs) are currently incorporated into various consumer products, and numerous new applications and products containing CNMs are expected in the future. The potential negative effects caused by CNMs release into the environment are a prominent concern and numerous research projects investigated possible environmental release pathways, fate, and toxicity [Figure 4].

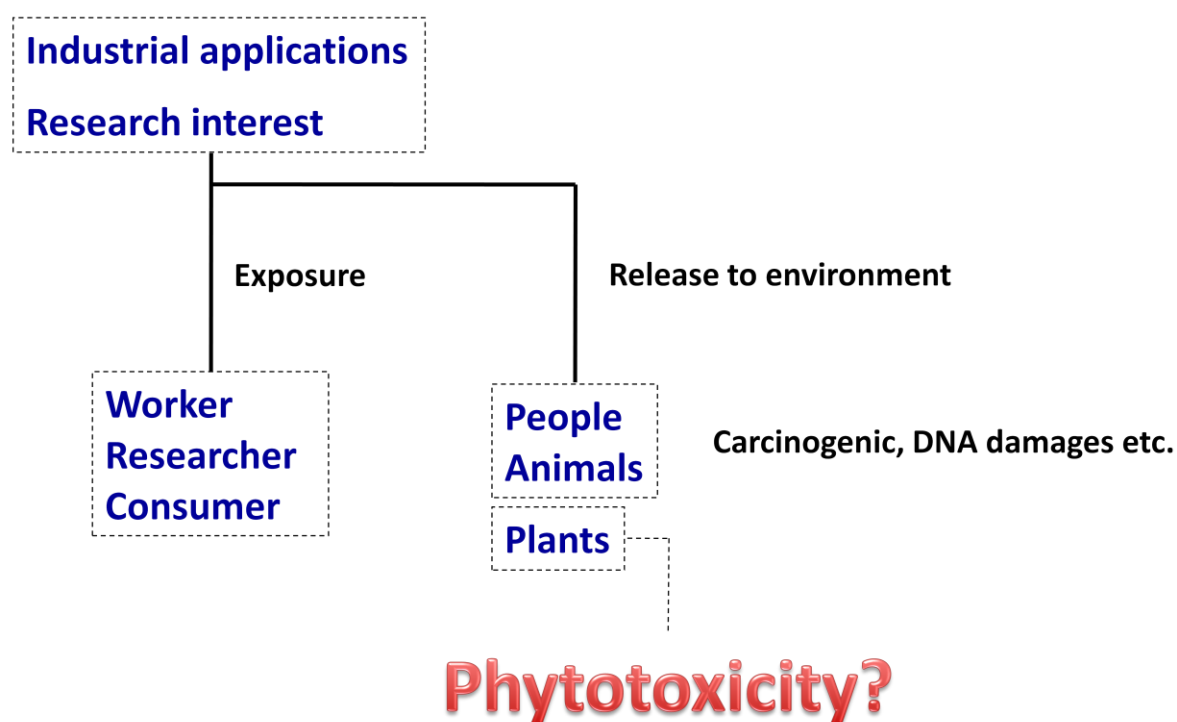


Figure 4 **Scheme of potential risks of CNMs in the environment.**

The possible toxic effect of CNMs on human has been reviewed by considering the exposure routes of inhalation, ingestion, and dermal penetration [16]. However, the major limitation of current literature is about standardized and valid assessment of phytotoxicity testing of CNMs on plant seedlings instead of plant cell lines at higher concentration [Table 2].

References	Materials	Organisms	Symptoms
Canas et al. 2008	SWNTs	Lettuce	Toxic
Lin et al.2009 and 2010	Fullerene C70	Rice cells	Toxic
Lin et al.2009	MWNTs	Arabidopsis T87	Toxic
Tan et al.2009	MWNTs	Rice cells	Toxic
Khodakovskaya et al.2011	MWNTs	Tomato	No Toxic
Khodakovskaya et al.2012	MWNTs	Tobacco cells	No Toxic
Parvin et al .2012	MWNTs	Red spinach	Toxic
Parvin et al.2013	Graphene	Arabidopsis T87	Toxic

Table 2 Recent reports on toxic effects of CNMs in plants.

Most of studies worked on *in vitro* experiment of suspension cell lines at high concentration of CNMs. Still, lack of knowledge on how CNMs affect the plant seedling at more broad range or lower concentration. Toxic effect of CNMs at high concentration might due to physical effect of nanomaterials which also could induce physical damage and systematic damages on plants. By considering lower concentration we could determine the environmental fate of CNMs which depend on dose, time exposure and plant species which deserve further attention.

1.3 Aim of Studies

We highlighted the aim of these studies as the following:

1. To understand on how GO and MWNTs affect the environment which represented by several crop species.
2. To propose the toxicity mechanism of CNMs in the seedling stage.

Chapter 2nd

Phytotoxicity of Graphene Oxide

2.1 Introduction

The use of nanostructures with unusual novel properties in agriculture [17] and for technological applications has been an active and exciting area of research in recent years. Graphene, the most recently discovered carbon allotrope, is a two dimensional building block of atomic thickness that can be stacked into three-dimensional graphite, rolled into one dimensional nanotubes, or wrapped into zero-dimensional fullerenes [18]. The unique electronic and transport properties of graphene [19], compatible with existing manufacturing processes [20], and the absence of the energy gap in the electronic spectra have opened up increasingly rich possibilities in the development of future electronic devices [19,21] and the graphene-based quantum electronics [22] that offer many benefits. If these trends in nanotechnology continue, graphene may ultimately be released into the aquatic, terrestrial, and atmospheric environments, where its fate and behavior are largely unknown. Exposure to nanoparticles in higher plants is expected to have an effect because these plants strongly interact with their atmospheric environments [23]. The nanoparticles, with their ultra-small size, specific shape, geometric structure, and unique properties, may have the potential for increased toxicity [24–27]. Nanoparticles can drastically modify their physicochemical properties compared to particles of bulk size [28]. Carbon nanoparticles can penetrate plant cells [29, 30] and induce phytotoxicity at high doses [31–33], leading some authors to conclude that certain carbon nanoparticles are not 100% safe. Therefore, great caution is suggested when considering the introduction of nanoparticles-based products to the market, and there is an urgent need for research related to the broad area of

nanotoxicology. A recent study, for example, has pointed to the possible adverse effects of graphene on human health [34] and in bacteria [35].

In response to these concerns, we explored whether GO can induce phytotoxicity at high doses in terrestrial plants grown hydroponically and exposed to varying concentrations (0–2000 mg/L) of graphene. According to the US EPA guidelines [36] the highest concentration of graphene used for this study was 2000 mg/L. In addition, Rico et al. [37] have described that nanoparticles showing a manifest toxic effect on the food crops commonly appears in the range of concentration of 1000–4000 mg/L of the nanoparticles. Toxicity studies on the food crops were commonly carried out at high concentrations (2000–5000 mg/L) of the nanoparticles [33, 38, and 39]. Khodakovskaya et al. [29] reported insignificant toxicities of graphene in the growth of tomato, although they used only one low concentration (50 mg/L) of chemically functionalized graphene with few layers and with a thickness of 2–5 nm. In a study by Sayes et al. [40], carbon nanoparticles functionalization led to a remarkable decline in toxic effects. The effects of nanoparticles on different plant species can vary greatly with plant growth stages and method and duration of exposure and depend also on nanoparticles size, concentration, chemical composition, surface structure, solubility, shape, and aggregation [28].

Thus, to our knowledge, the possible adverse effect of GO in terrestrial plants is almost totally unknown. Here, we use cabbage, tomato, red spinach, and lettuce as the selected crops to investigate the toxic effects of GO and to identify appropriate target plant species for further studies associated with GO. Potential targets include various terrestrial plants that are normally protected by specific barriers such as a cell wall. Targeting can be made more effective by prolonged exposure of different plant species

to carbon nanoparticles and application of a high concentration, which can lead to aggregation on the root surface [41], penetration within the cells [29], and a contribution to toxic effects [31,32,42]. This report is the first to describe the phytotoxicity effect of GO in terms of seedling growth, cell death, reactive oxygen species (ROS) generation, and morphology change. Our results showed the greatest toxic effect of GO on cabbage and tomato, followed by red spinach, with no clear toxic effect for lettuce.

2.2 Experimental Section

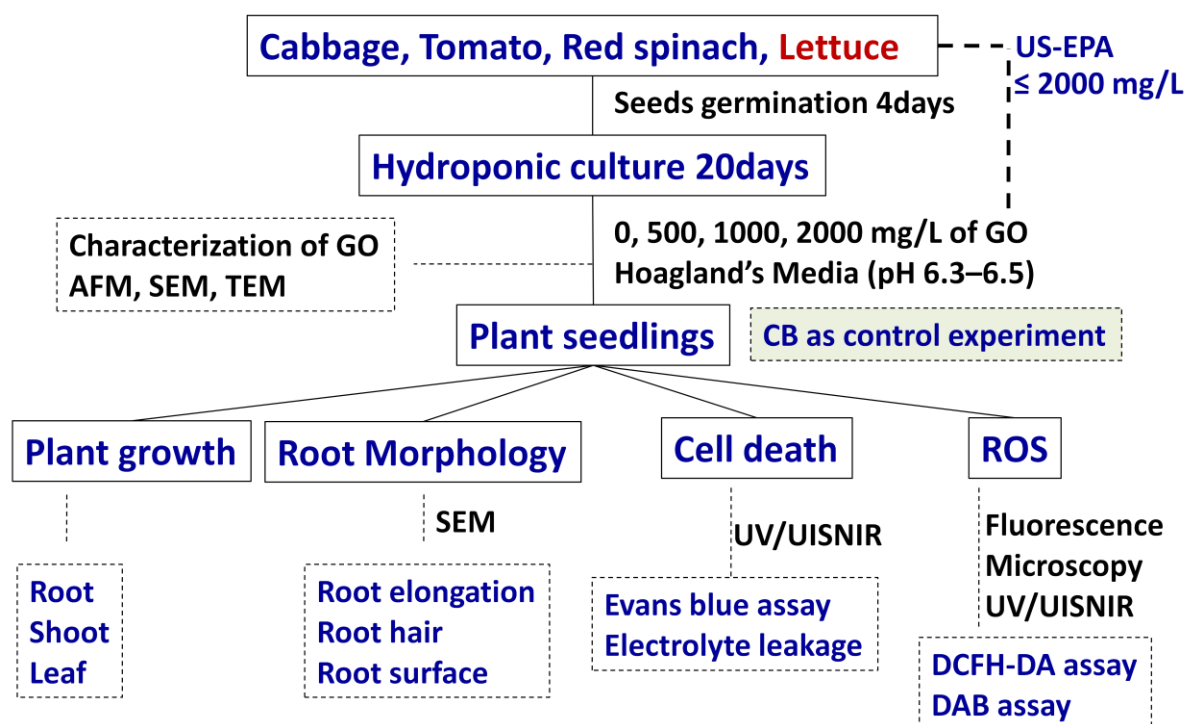


Figure 5 Scheme of phytotoxicity testing of GO in seedling stage

Chemicals used in this experiment were purchased from Kanto Chemical Co. Inc., Wako Pure Chemical Industries, Ltd., or Sigma–Aldrich Inc., Japan. Seeds of three plant species (cabbage, *Brassica oleracea* var. *capitata*; tomato, *Lycopersicon esculentum*; and lettuce, *Lactuca sativa*) used in this study were purchased from Homac, Sapporo, Japan. Red spinach (*Amaranthus tricolor* L. and also *Amaranthus lividus* L.) seeds were purchased from Dhaka, Bangladesh.

Water-soluble graphene or graphene oxide (GO) was obtained from natural graphite (SP-1, Bay Carbon) by using a modified Hummers and Offeman's method [9, 10]. In a typical treatment, 100 g of the graphite powders and 50 g of sodium nitrate and 2000 mL sulfuric acid were mixed in an ice bath. Next, 100 g of potassium permanganate was slowly added and well mixed. Once mixed, the suspension was placed in a water bath at 35 ± 3 °C and mixed for about 30 min. About 5 L of deionized water was added to the suspension, the temperature was increased to 90 ± 3 °C, and the suspension was mixed further for about 30 min. The suspension was finally diluted to about 10 L with warm deionized water and about 200 mL of 30 wt% of hydrogen peroxide (H_2O_2). The warm suspension was filtered, and a yellow– brown filtered cake was obtained. The filtered cake was carefully washed with a large amount of warm deionized water and then dispersed in deionized water by mechanical mixing to prepare a stock GO aqueous suspension containing approximately 2 wt% water-soluble graphene. An aqueous 0.1 mol/L sodium hydroxide solution was used to neutralize the graphene solution to a pH 6.32.

The sterile seeds of the selected plant species were soaked in solution with different concentrations (0, 500, 1000, and 2000 mg/L) of GO overnight at 25 °C in the dark. Seeds were placed on wet filter paper and then exposed to 3mL of the test solution

with and without GO and incubated at 25 °C until germination. After germination, the number of germinated seeds was counted. The seedlings were transferred to plastic pots containing 200 mL modified Hoagland medium [43]. Values of pH of the Hoagland medium remained almost unchanged during the 20 days of the GO exposure. They were restricted in 6.3–6.5. These pH values were desirable for plant growth [44]. Seedlings were treated with different concentrations of GO solution (0, 500, 1000, or 2000 mg/L). At 2000 mg/L GO was stable with very little settling.

After 20 days of exposure, roots and shoots were separated and washed with water to remove the growth medium and dried with wipes to remove the surface water. Their length and fresh weights were recorded. Leaf numbers were counted and leaf area measured using a RHIZO 2004b instrument.

For visualization and measurement of ROS by spectroscopy, oxidative sensitive probes 20,70-dichlorofluorescein diacetate (DCFH-DA) and 3,30-diaminobenzidine (DAB) were used. Fresh leaves of the treated and untreated plants were incubated in DCFH-DA for 2 h for the ROS measurements and the H₂O₂ detection. After three PBS (phosphate-buffered saline) rinses, the images for visualization were captured using fluorescence microscopy (Olympus IX70). We performed a measurement of DCFH fluorescence intensity in the leaves using a spectrofluorometer (Hitachi F-4500) by suspending the leaves in PBS buffer with an excitation wavelength at 485 nm and emission wavelength at 522 nm. The values were expressed as % of fluorescence intensity relative to control. This experiment was performed without exposure to light.

H₂O₂ was detected using DAB by the method previously described by Thordal-Christensen et al. [45]. The fresh leaves from 20-day-old treated and untreated plants were placed in 1 mg/mL DAB-HCl, pH 3.2–3.8 and incubated under vacuum for 8 h. DAB

deposits were revealed after washing leaves in boiled 100% (v/v) ethanol for 15 min to decolorize the leaves except for the deep brown polymerization product from the reaction of DAB with H_2O_2 . The images were observed and recorded using light microscopy (Transmit Light Microscope BX51, With Olympus DP72 Camera). The amount of formazan formation in leaves was measured spectrophotometrically at A700 (V-530 UV/UISNIR Spectrophotometer, Jasco, Japan) after leaves were ground in liquid nitrogen and solubilized in a mixture of 2 M KOH and DMSO at a ratio of 1:1.167 (v/v).

The cell death of the selected plant roots was evaluated by the method previously described by Baker and Mock [46] using Evans blue (0.025% v/v) for 2 h after 20 days of exposure to different concentrations (0, 500, 1000, and 2000 mg/L) of graphene. For quantitative assessment, after several washes with water, the Evans blue was extracted using 1% (w/v) SDS in 50% (v/v) methanol at 50 °C for 15 min, and the absorbance (optical density) was measured spectrophotometrically at 597 nm (V-530 UV/UISNIR Spectrophotometer, Jasco, Japan). Cell death was expressed as absorbance of treated roots in relation to untreated roots (control).

Cell death was also evaluated by measurement of ion leakage from leaf. The percent of injury of the membrane was measured from the electrolyte leakage of treated and untreated plants by a conductivity method based on the procedure of Lutts et al. [47] with some modifications. Leaf samples (100 mg) were placed in test tubes containing 10 mL of distilled water after three washes with distilled water to remove surface contamination. Test tubes were covered and incubated at room temperature (25 °C) on a shaker (100 rpm) for 24 h. Electrical conductivity of the solution (Lt) was determined. Samples were then autoclaved at 120 °C for 20 min, and a final conductivity reading (L0) was obtained upon equilibration at 25 °C. Electrolyte leakage was defined

as: electrolyte leakage (%) = $(L_t/L_0) \cdot 100$. The extent of membrane injury (leakage of electrolytes) from cells was determined with a portable conductivity meter (pH/Cond Meter, Horiba D-54).

For AFM, an Agilent Series 5500 AFM instrument was used. The samples were prepared by casting a diluted aqueous GO suspension on the surfaces of mica. The images were obtained using the tapping mode at a scanning rate of 1 Hz. For SEM, a few drops of the GO suspension were deposited on the aluminum stub, dried, sputter-coated, and observed using a Hitachi S-4000 SEM (Hitachi, Ibaraki, Japan) with an acceleration voltage of 10 kV. For SEM, the roots were fixed in 2.5% glutaraldehyde (GA) and 2% paraformaldehyde (PA) in 0.1 M phosphate buffer (PB), pH 7.4, post fixed in 1% osmium tetroxide, dehydrated, critical-point dried, sputter coated, and observed. For TEM, the GO were homogeneously dispersed in 2-propanol under ultrasonication for 30 min. A few drops of the suspension were deposited on the TEM grid covered with a Formvar membrane, dried, and evacuated before analysis. For root TEM, the roots were fixed in 2.5% GA and 2% PA in 0.1 M PB buffer, pH 7.4, post fixed in 1% osmium tetroxide, dehydrated, infiltrated with ethanol: Epon, embedded in pure Epon and polymerized at 60 °C for 2 days. Ultra-thin sections were stained with 2% uranyl acetate and lead citrate. The preparations were observed using a Hitachi H-800 TEM. The acceleration voltage was 75 kV.

Each treatment was conducted in triplicate. Phytotoxicity endpoints for all measurements were compared to those of the unexposed controls. Statistical analysis was performed using Student's t-test values of $P \leq 0.01$ were considered significant. Data are presented as mean $\pm$ SD (standard deviation).

2.3 Results and Discussions

A previous study uncovered evidence supporting that carbon based nanoparticles have adverse effects on terrestrial plants [33], opening the way to further investigation. Consequently, we assessed the potential impact of GO on the growth of tomato, cabbage, red spinach, and lettuce based on tests suggested and encoded by US EPA guidelines [36], which include consideration of studies on seed germination and seedling growth (root and shoot growth, leaf number), often accompanied by other evaluations of cell death, ROS production, and morphological studies using SEM, useful for obtaining evidence of in situ symptoms of possible toxicity. Altogether, the current work investigating the potential effect of GO demonstrates possible adverse effects on plants, underscoring the need for ecologically responsible disposal of GO and for more research on the potential effects of GO on agricultural and environmental systems.

2.3.1 Characterization of GO solution

Water-soluble graphene or GO with sodium ions as the counter ions (solution pH 6.32), was used throughout this study. AFM was used to evaluate the morphologies of the GO.

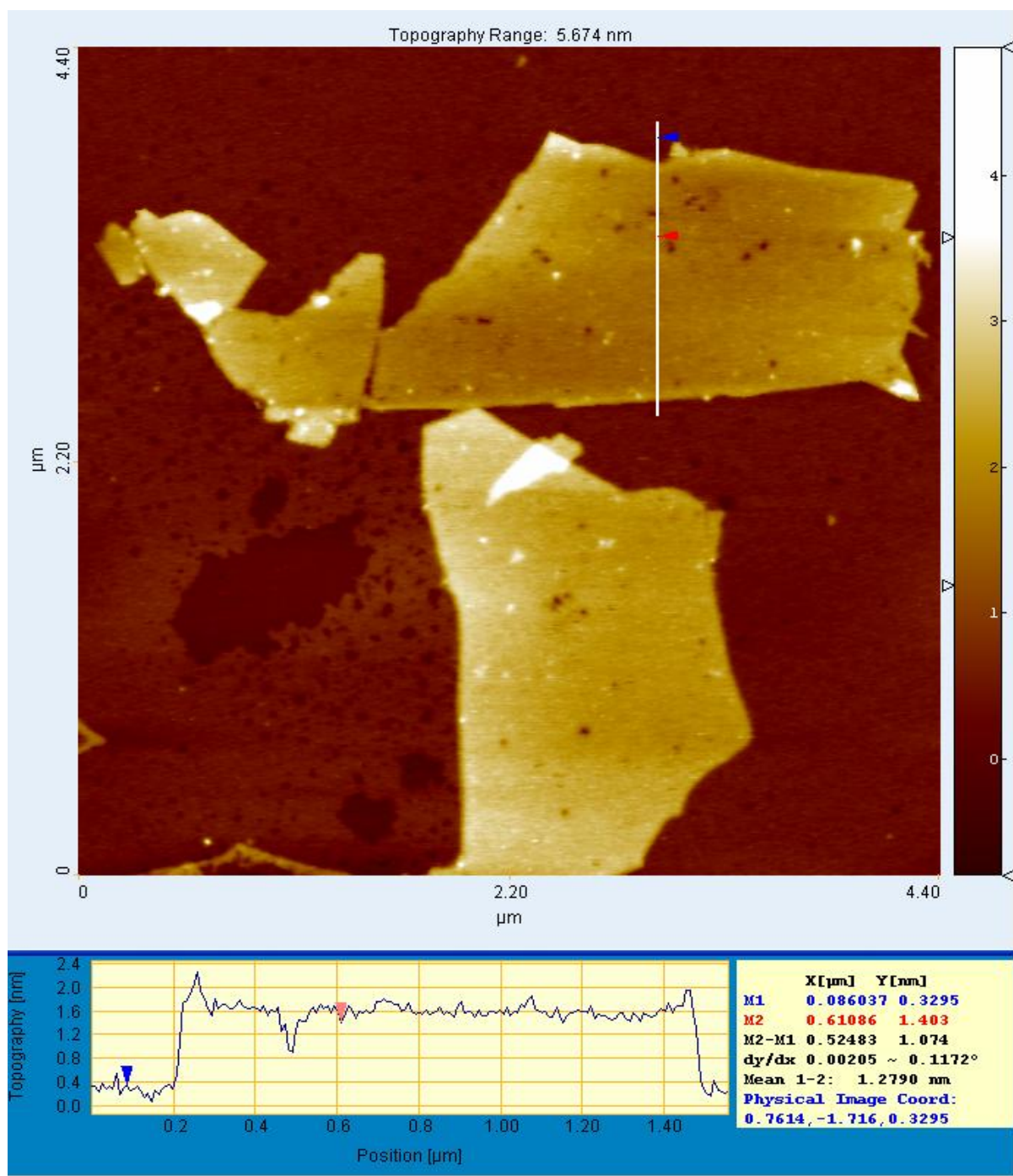
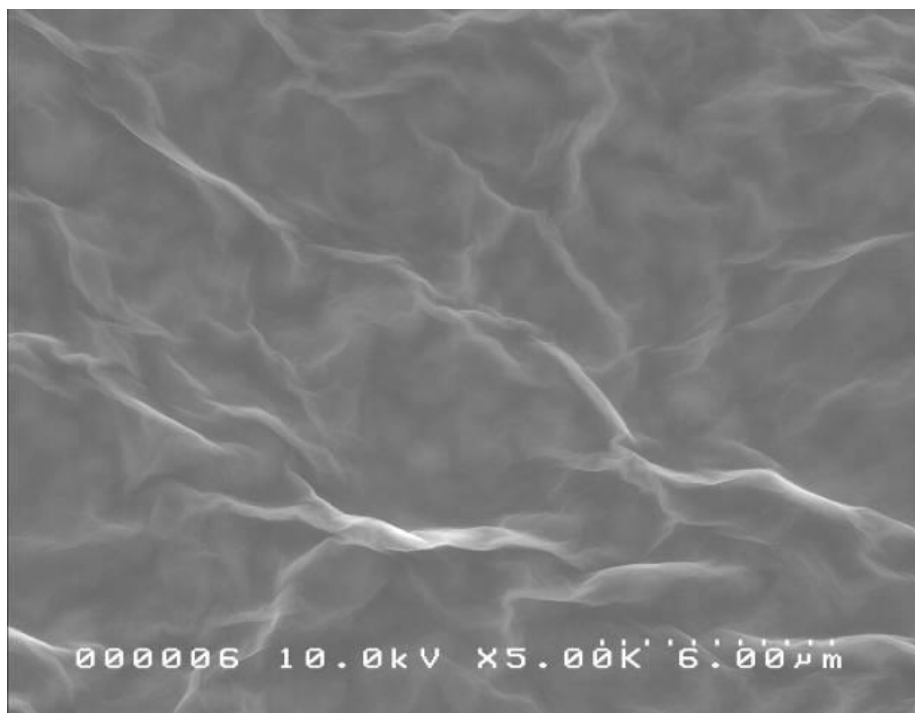


Figure 6 Typical AFM image of GO solution.

Figure 6 above shown the apparent heights of all the GO observed were found to be around 1 nm, indicating that GO was fully exfoliated into individual sheets with the size of length · breadth ranged from 0.5 · 0.6 to 1.5 · 6.5 μm for 30 pieces of the GO.

[Figure 7a] shows typical SEM image of the GO, revealing the morphology of the graphene sheets. [Figure 7b] shows a TEM image of the GO, which exhibits a typical wrinkled structure [48] with the corrugation and scrolling that are fundamental to graphene [49].

a



b

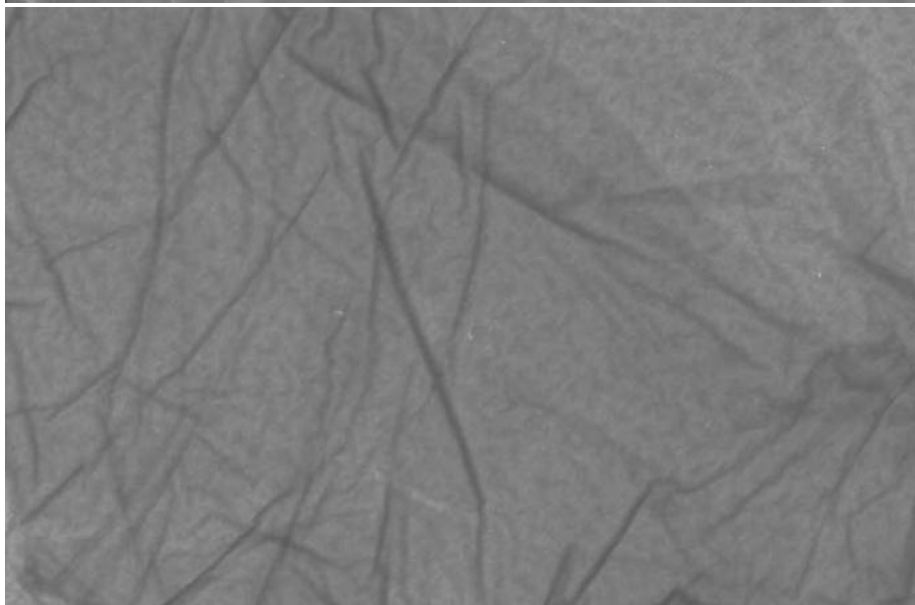


Figure 7 (a) Typical SEM image of GO, (b) Typical TEM image of GO.

2.3.2 Effect of GO on germination and plant seedlings

We conducted a series of tests of the potential effect of GO on the growth of tomato, cabbage, red spinach, and lettuce. Treated and untreated seeds were germinated after 4 days of incubation at 25 °C in the dark. Fully germinated seedlings with developed cotyledons and root system are shown in [Figure 8].



Figure 8 Effect of GO on germination seeds after 4 days incubation.

Cotyledons and root system were retarded with increasing GO concentration. Only in the case of lettuce was there no significant influence of GO on the cotyledons and root system after 4 days (data not shown). On the other hand, GO had a clear negative influence after 20 days on root and shoot length and biomass of tomato, cabbage and red spinach exposed to GO. The observed influence depended on the concentration of GO and the duration of the experiment. In hydroponics experiments, when plants were treated with different concentrations of GO (0, 500, 1000, and 2000 mg/L), the 20-day-old plants were characterized by inhibition of plant growth and leaf number and leaf size decreased with increasing GO concentration in comparison with control [Figure 9] and showed toxicity symptoms.



Figure 9 Effect of GO on plant seedlings after hydroponic 20 days.

As can be seen in Figs. 8 and 9, the primary roots were much shorter and the root hairs almost disappeared for plants treated with 2000 mg/L and as a result, the overall amount of GO aggregated on the surface of the roots was not in proportional to the GO concentrations. Similar results were also observed for *Brassica juncea* and *Phaseolus mungo* plants treated with other types of the carbon nanoparticles [31]. Root tips and root hairs produce large amounts of mucilage, a highly hydrated polysaccharide coating on the root surface [50], which are the key species responsible for absorbing the nanoparticles onto the root surface. No significant effect of GO on the parameters tested (seed germination and growth) was noted in our experiment in the case of lettuce. A certain species of plants responds in its very own manners to the nanoparticles. Difference in structures of the xylem would be the key physiological reason responsible for this fact. In the present studies, cabbage, tomato and red spinach contain one primary root and several smaller lateral roots, whereas lettuce has numerous small roots with a primary root. And as a result, no significant phytotoxicity of GO on lettuce was observed compared to cabbage, tomato and red spinach. Canas and co-workers [51] described in their studies that the species would respond differently to nanomaterials, and responses among species did, in fact, vary. Lee et al. [52] also reported that nanoparticles toxicity depends on probably due to differences in root anatomy because xylem structures determine the speed of water transport and different xylem structures may demonstrate different uptake kinetics of nanoparticles [53]. The following discussion is focused on the results obtained for tomato, cabbage and red spinach.

2.3.3 Effect of GO on plant growth

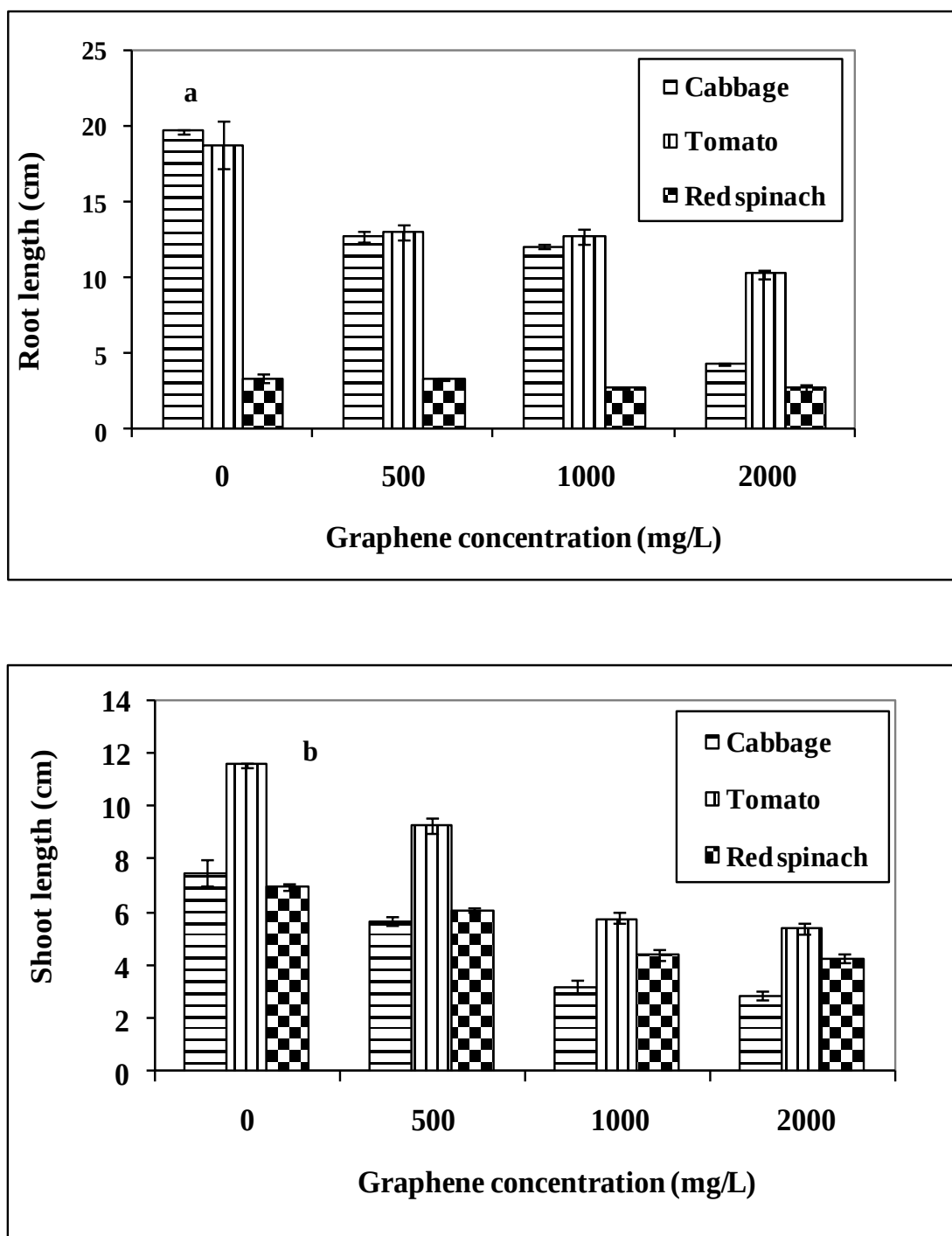


Figure 10 Inhibition effect of GO effect on root length (a) and shoot length (b).

The presence of GO has resulted in decreased root and shoot growth [Figures 10a and b]. However, GO at a lower concentration (500 mg/L) resulted in only a slight decrease in root and shoot length. A marked inhibition effect was observed with the highest concentration of GO (2000 mg/L). In the case of cabbage, there was a significant increase in root and shoot growth inhibition greater than 78% and 61%, respectively, compared to control. The 2000 mg/L concentration GO resulted in root and shoot growth inhibition of tomato of 46% and 53%, respectively, compared to control. Inhibition of shoot growth was noted in red spinach (13%) at 500 mg/L, and the highest concentration (2000 mg/L) resulted in further significant inhibition of shoot growth (39%). However, no significant effect of GO on the root growth of red spinach at 500 mg/L was observed while root growth of red spinach was inhibited by 18% at the higher GO concentrations (1000 and 2000 mg/L).

2.3.4 Effect of GO on plant biomass

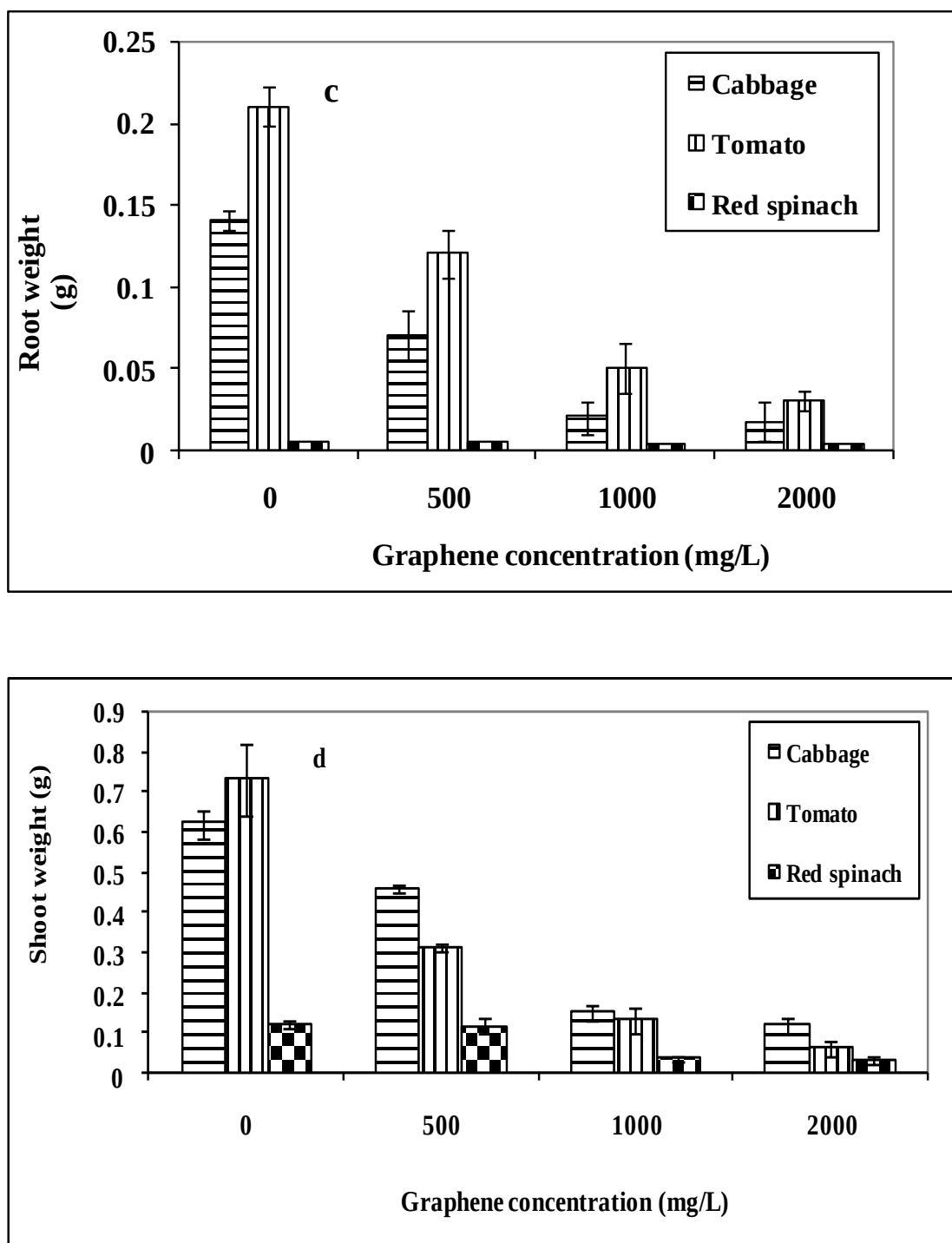


Figure 11 Inhibition effect of GO effect on root weight (c) and shoot weight (d).

The presence of GO resulted in decreased root and shoot weight [Figures 11c and d]. With cabbage seedlings grown in hydroponic culture, the root and shoot weight was sensitive to GO, and weight decreased by 88% and 81%, respectively, at the highest concentration (2000 mg/L) compared to control. The root and shoot weight of tomato was also sensitive to GO, and weight decreased by 86% and 92%, respectively, at the highest concentration (2000 mg/L) compared to control. With red spinach, the shoot weight appeared to be more sensitive to GO compared to root; the root and shoot weight of red spinach decreased by 39% and 75% at the highest concentration (2000 mg/L), respectively, compared to control. Tomato seedlings were somewhat more sensitive to GO than were cabbage and red spinach seedlings.

2.3.5 Effect of GO on leaves

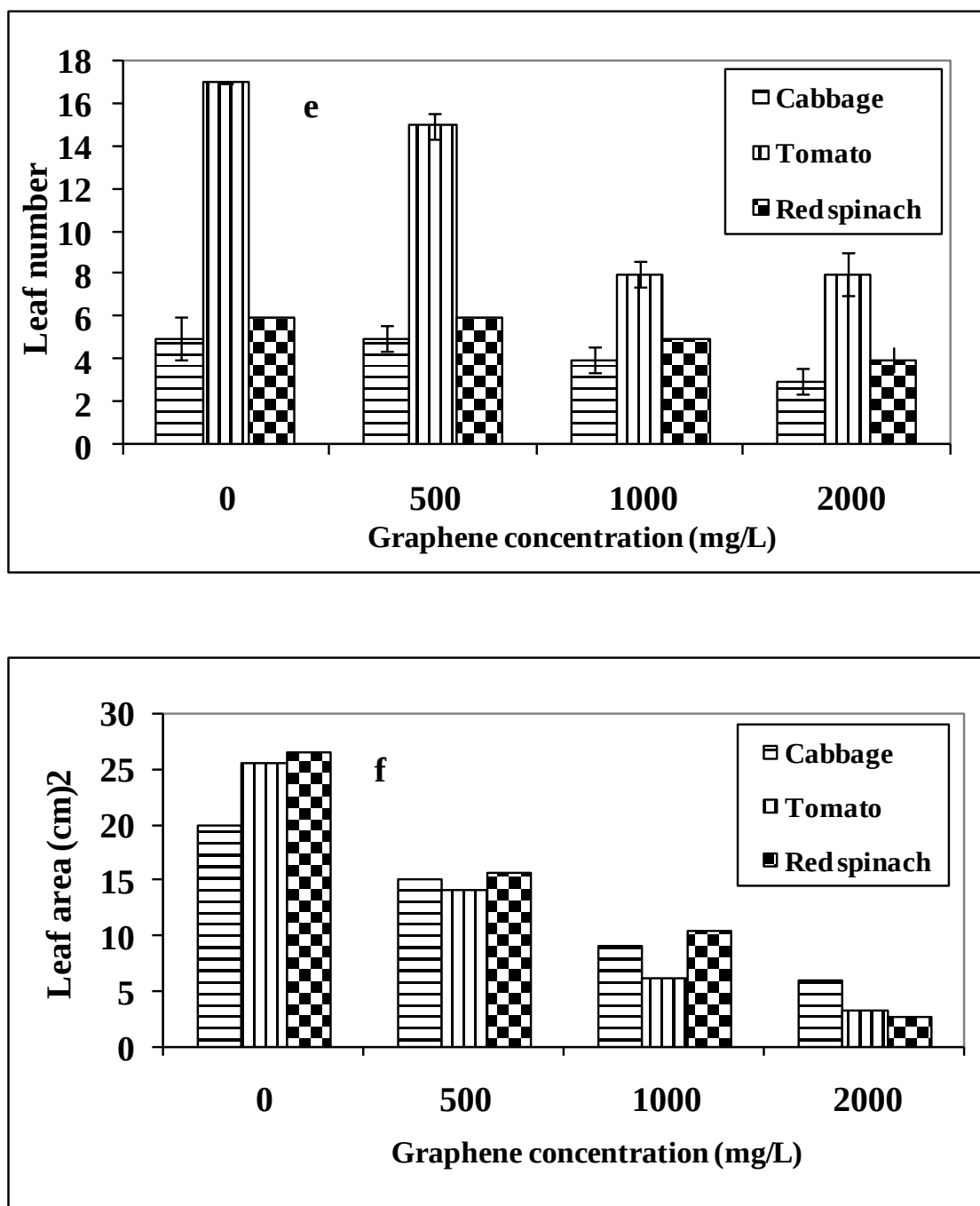


Figure 12 Inhibition effect of GO effect on leaf number (e) and leaf area (f).

Graphene oxide (GO) has influenced the leaves of all tested plants in a dose dependent manner [Figure 12e]. The number of leaves decreased with increasing GO concentration compared with control. For example, control tomato without GO developed an average of 17 leaves each, whereas the treated tomato developed only 8 leaves each at 1000 and 2000 mg/L [Figure 12e].

The leaf numbers of the treated plants were considerably decreased by 40%, 53%, and 33% at the highest GO concentration (2000 mg/L) in cabbage, tomato, and red spinach, respectively. The leaves exhibited reduced size and wilting symptoms, as evidenced by visual observation [Figure 9]. Of interest, the leaf area (visual observation, Figures 9 and 12f) of all treated plants was gradually reduced and continued to decline with increasing GO level. A significantly reduced leaf area of the treated plants was clearly observed at GO concentration (2000 mg/L, Figure 12f) compared to control. Furthermore, the leaf area of cabbage was reduced by 25% at 500 mg/L and by 71% at 2000 mg/L compared to control, indicating a dose-dependent reduction. Tomato had an 88% leaf area reduction at 2000 mg/L [Figure 12f] and 45% at 500 mg/L. The leaf area of red spinach was reduced by 91% at 2000 mg/L compared to control [Figure 12f].

We found that cotyledons and the root system of cabbage, tomato, and red spinach were inhibited after four days and observed further inhibition in seedling growth at different GO concentrations. Although 500 mg/L GO had a slight effect, 2000 mg/L resulted in a notable effect on the seedling stage of cabbage, tomato, and red spinach. By comparison, GO at 50 mg/L increased growth rates with no sign of significant toxicity for tomato in the seedling stage [29]. Khodakovskaya et al. [29] investigated the effect of four carbon materials (single-multiwall carbon nanotubes, few layer graphene, and activated carbon) on the seedling stage of tomato at 50 mg/L. As

noted, a high concentration of graphene was not employed in that study, and the authors used only one low concentration.

2.3.6 Effect of GO on reactive oxygen species (ROS)

Plants continuously produce ROS as byproducts of various metabolic pathways, but the excess accumulation of ROS leads to oxidative stress and cell death [54, 55]. ROS products, whether inside or outside the cell, can be key factors in the toxicological effects of nanostructure materials [28]. Carbon nanotubes (CNTs) are known to have phytotoxicity effects in plant cells because of aggregation [41], causing cell death and accumulated ROS in a dose-dependent manner [56].

GO may have the ability to generate ROS production, based on the similarities of some of the properties of GO sheets of CNTs. Based on the assumption that GO can be involved in ROS production in the leaf, we tested this possibility using 20-day-old leaves from test plants for the detection of ROS by means of the ROS-sensitive dye DCFH-DA.

We evaluated ROS accumulation (oxidative stress) by means of H₂O₂ detection after infiltration with DCFH-DA of treated and untreated leaves. The accumulation of H₂O₂ was visualized under a microscope; it can be imaged under fluorescence microscopy after removal of DCFH-DA from the leaves by washing with PBS buffer [Figure 13].

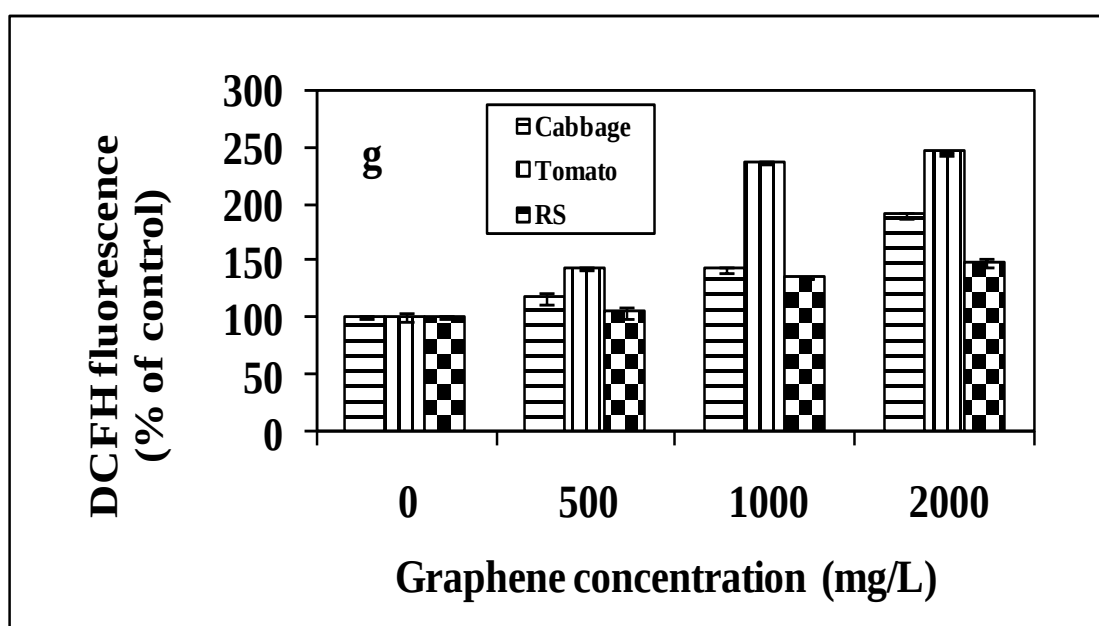
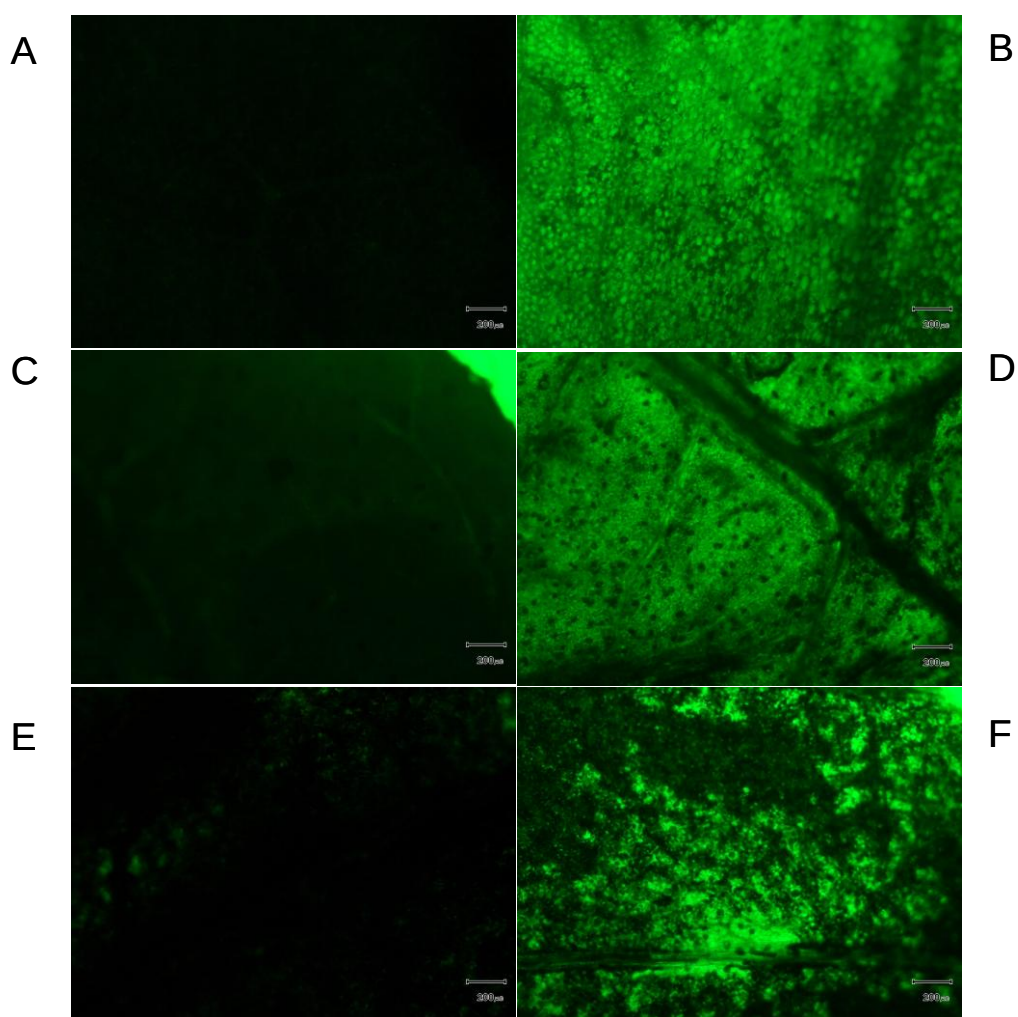


Figure 13 DCFH-DA Fluorescence assay of treated leaves with GO

In Figure 13 above, panel B, D and F illustrate that GO-treated leaves showed an increase in DCFH fluorescence compared to control leaves (panel A, C and E) of cabbage, tomato, and red spinach, respectively. Measurement of DCFH fluorescence by spectrofluorometer demonstrated the dose-dependent increase in ROS content in GO-treated leaves [Figure 13g] compared to control. The accumulation of ROS in the leaf was measured with the excitation wavelength at 485 nm and emission wavelength at 522 nm. As Figure 13g shows, as the concentration of graphene in medium increased, a progressively enhanced DCFH response was observed, strongly suggesting that graphene can cause an oxidative stress reaction in plant cells.

In the case of red spinach, graphene and the control without graphene shared similar fluorescence intensity specifically at the low GO concentration (500 mg/L). This finding supports that a low concentration of GO is not responsible for induction of ROS. In contrast, GO at a high concentration elicited a sharply increased ROS level compared to control [Figure 13g].

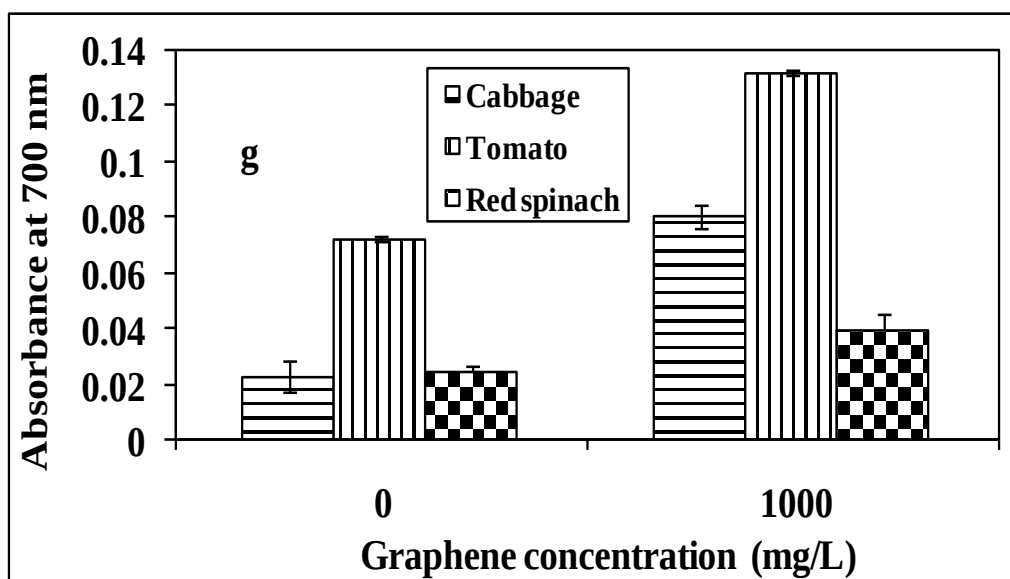
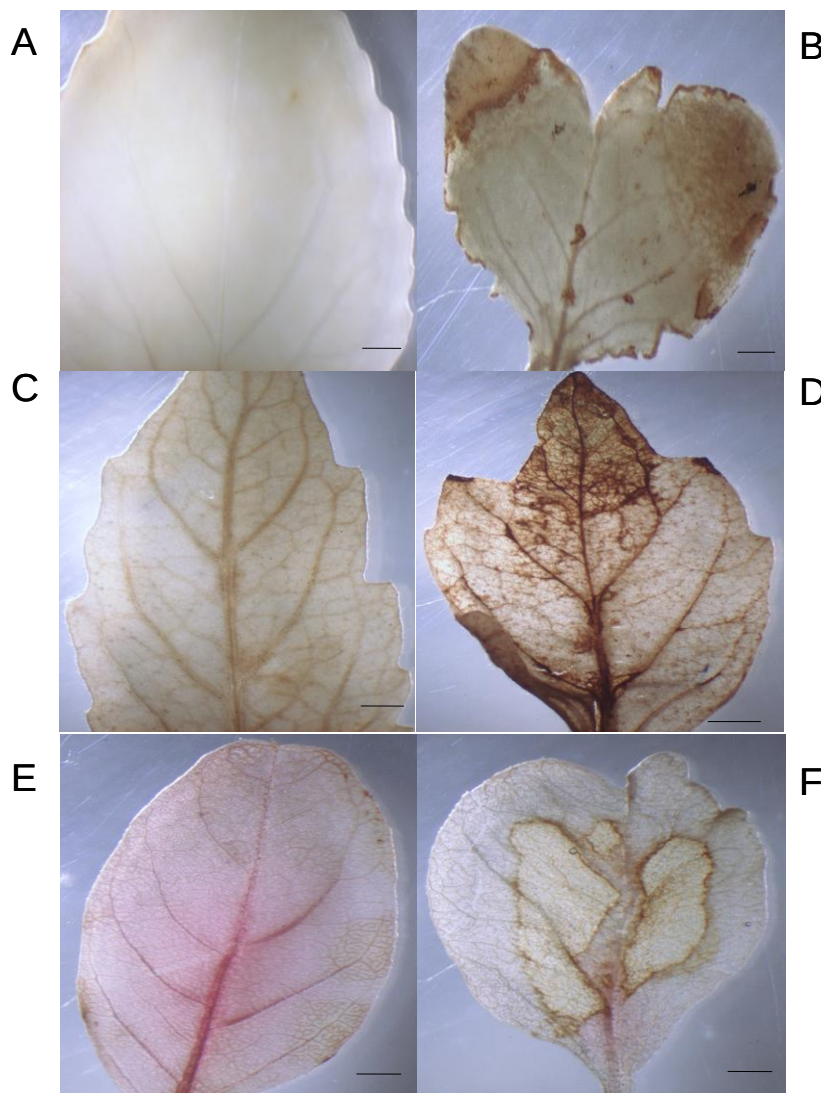


Figure 14 DAB assay of treated leaves with GO

We further tested the ROS accumulation induced by GO in 20-day-old leaves of cabbage, tomato, and red spinach by means of the ROS-sensitive dye DAB. Infiltration of leaves with DAB allowed the detection of H₂O₂. The location of insoluble deep radish brown polymerization product produced when DAB reacts with H₂O₂ was visualized under a light microscope; it can be imaged after removal of chlorophyll from the leaves by boiling for 15 min in ethanol.

Imaging of deep radish brown polymerization can be considered to indicate the accumulation of H₂O₂ in Figure 14 (panel B, D and F) in cabbage, tomato, and red spinach, respectively, and formation of H₂O₂ would be expected as a result of the ROS accumulation by GO induction. In control leaf (panel A, C and E) of cabbage, tomato, and red spinach, respectively, no distinctive deep radish brown polymerization of H₂O₂ was detectable. The plants exposed to concentrations of 1000 mg/L showed significant toxicity after 20 days, with the DAB assay indicating increasing ROS. The amount of formazan formation in leaves was determined at A₇₀₀. As shown in Figure 13g, higher levels of GO (1000 mg/L) triggered production of more ROS. The excess ROS production at 1000 mg/L may be indicative of concentration-dependent ROS generation.

ROS production estimated by DCFH-DA was correlated to an increase in ROS predictable by DAB assays. However, the results of both assays demonstrated the direct presence of ROS that are produced inside the leaf in those plants grown with GO. The ROS localization using DCFH-DA and DAB described above was also supported by the quantitative determinations of DCFH and deep brown polymerization at a higher concentration of GO (1000 mg/L) [Figures. 13g and 14g]. These findings are in agreement with those of Zhang et al. [34], who exposed neural pheochromocytoma-derived PC12 cells to graphene and demonstrated that graphene-induced ROS were

involved in the toxic mechanism. In fact, ROS are the key signaling molecules and could be induced by many exogenous stimuli [54].

Evidence for the accumulation of ROS induced by GO includes that the ability of GO to reduce plant growth was positively correlated with the generation of ROS with GO concentration in a dose-dependent manner, as was estimated by DCFH-DA. In previous studies, graphene was found to be also capable of generating ROS to A549 cells, a typically adeno carcinoma human alveolar basal epithelial cell line [57, 33]. The ROS induced oxidative stress is considered to be the common toxicological mechanism of the nanoparticles [58 - 61]. Graphene obtained using Hummer's method was seen to be electron paramagnetic resonance active (EPR-active) with $\sim 10^{16}$ electron spins/g [62]. Graphene oxide (GO) catalyzes the generation of ROS to plants (cabbage, tomato, and red spinach) once it has been covering on the roots of plants.

2.3.7 Effect of GO on cell death

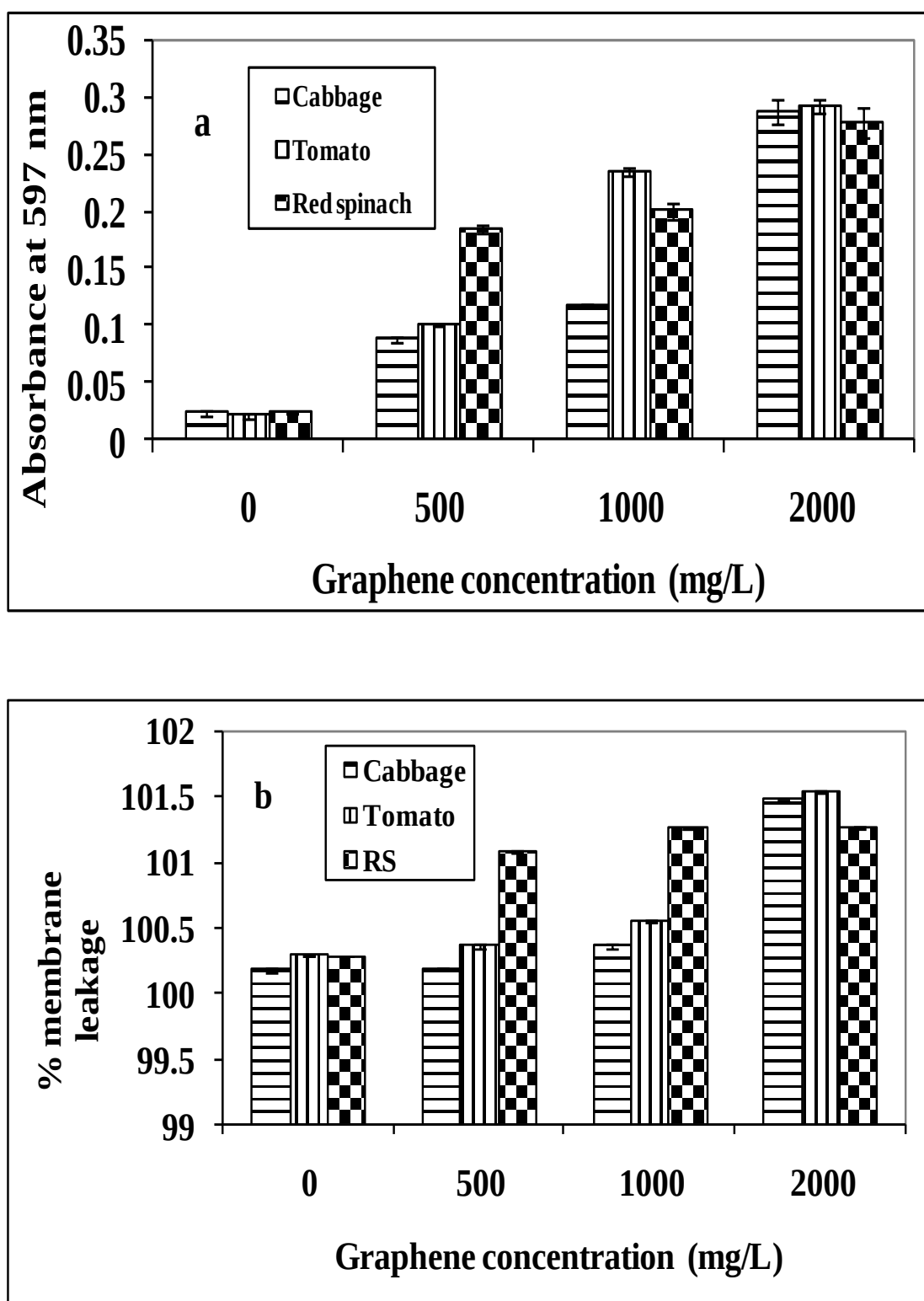


Figure 15 (a) Evans blue assay of treated roots,
(b) % Membrane leakage of treated leaf.

To evaluate the toxicity of GO through cell membrane damage *in vivo* in hydroponic culture conditions; we used a cell death assay. The roots of treated and untreated seedlings were examined by measuring cell death using Evans blue staining and with OD values at 597 nm after a 20-day exposure to different concentrations (0, 500, 1000, and 2000 mg/L) of GO. The measurement of the Evans blue extracted from roots showed a concentration-dependent induction [Figure 15a].

With the concentration of GO increasing to 1000 and 2000 mg/L in the case of cabbage, the amount of Evans blue uptake in root cells increased by about 5- and 12.3-fold, respectively, compared to that of control roots. A significant level of Evans blue uptake was observed after both 1000 and 2000 mg/L of GO treatment of tomato roots, representing 11.5- and 14.4-fold, respectively, of that in control roots. The higher concentrations of GO (1000 and 2000 mg/L) caused 8.5- and 11.8-fold higher uptake, respectively, of Evans blue in roots of red spinach compared to that in control roots. Thus, these results indicate that the loss of plasma membrane integrity occurred with exposure to GO, as it was greater than in the control plants. Higher (1000 and 2000 mg/L) GO concentrations caused severe stress on plant growth, inducing the excess generation of ROS. The effects of the ROS may be reflected in the rapid rise of Evans blue uptake. The toxic effect on plant roots appeared after exposure to the lower concentration of GO, and the differences between controls and GO -treated plants became even more prominent at higher concentrations of GO, as observed under a light microscope (data not shown).

We analyzed another cell death index to further demonstrate that GO causes cell membrane damage (electrolyte leakage/plasma membrane sensitivity) in the treated leaf. Electrolyte leakage experiments indicated that the leaves of the GO -treated plants

had decreased membrane integrity (Figure 15b). For these experiments, we used 20-day old seedlings with leaves, investigating the effect of increasing concentrations of GO on membrane integrity. Cell membrane damage was noted at 500 mg/L in cabbage, tomato, and red spinach, and 1000 and 2000 mg/L resulted in further damage, indicating dose-dependent membrane integrity and disruption of the plasma membrane. In addition, as shown in Figure 15b, the results of the electrolyte leakage experiments indicated that the tomato had significantly greater leakage at the highest concentration (2000 mg/L) compared to cabbage and red spinach. A comparison of [Figures 15 a and b] shows that enhancement of Evans blue uptake and loss of plasma membrane integrity gradually increased up to 2000 mg/L GO under the same conditions. Evans blue staining showed that extensive cell death/membrane injury was induced in plant roots by different concentrations of GO treatment. The electrolyte leakage assay confirmed the membrane injury resulting from GO treatment. This result indicates that the loss of plasma membrane integrity was induced by GO at the lower concentration and continued at higher concentrations. These results also suggest that intracellular ROS might have a crucial role in induction of cell death induced by graphene. It has been reported that the accumulation of ROS causes cell death, which is demonstrated by electrolyte leakage from cells [63].

The effect of GO on cell death was evaluated by Evans blue staining and a conductivity method (% of membrane leakage). These two assays have been considered as indexes of cell death in plants [64], although they give information only about plasma membrane integrity. The Evans blue assay is based on the accumulation of blue color inside dead cells (but not in living cells). The conductivity assay is based on electrolyte leakage from dying cells, with ion leakage used as a marker of cell death.

2.3.8 Effect of GO on root morphology

To provide clues of GO toxicity in the seedling stage of terrestrial plant roots, we next visualized the morphological alteration of cabbage, tomato, and red spinach using SEM of the treated roots and compared them to untreated controls [Figures. 16–18, respectively].

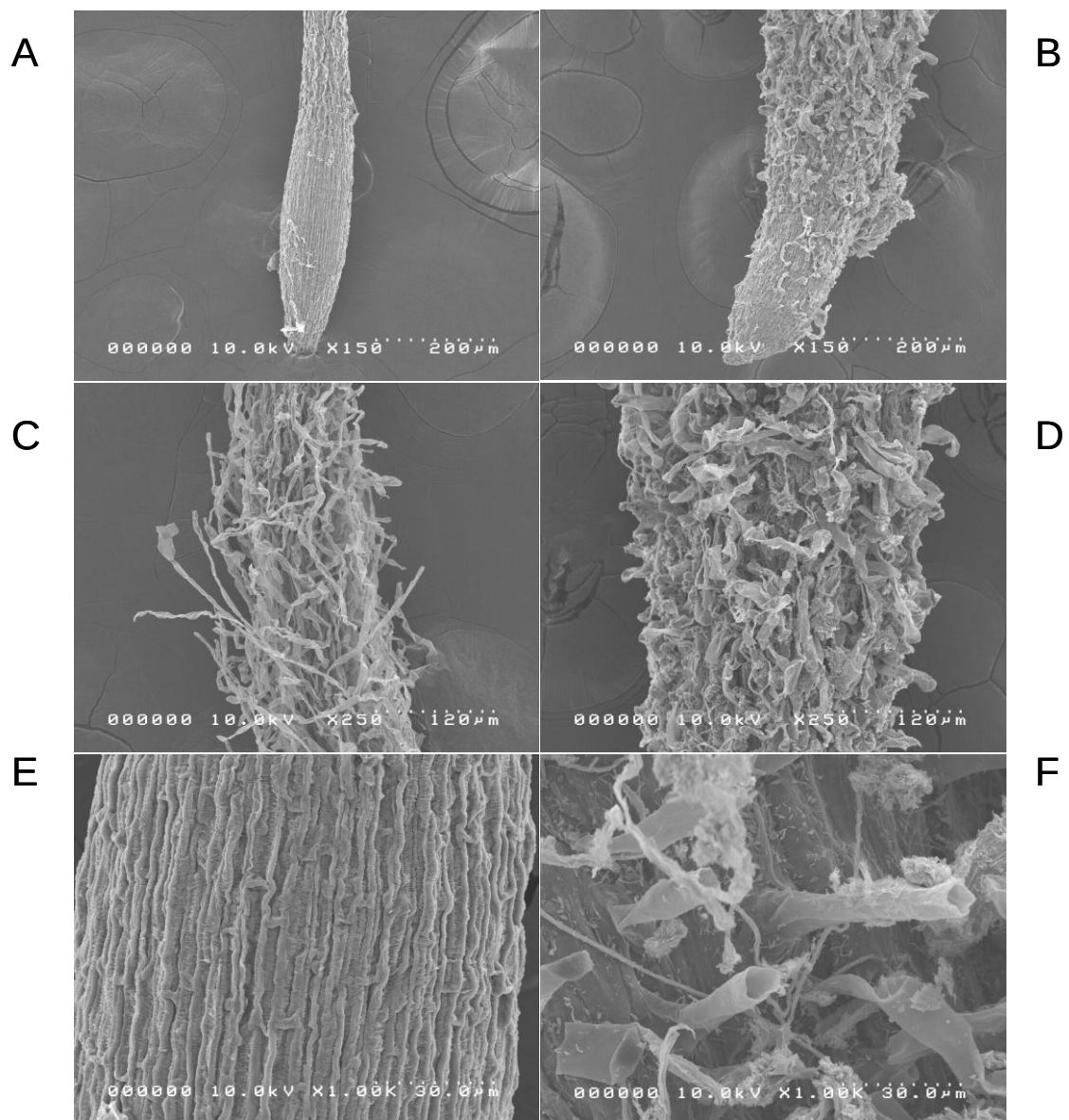


Figure 16 Root morphology of cabbage

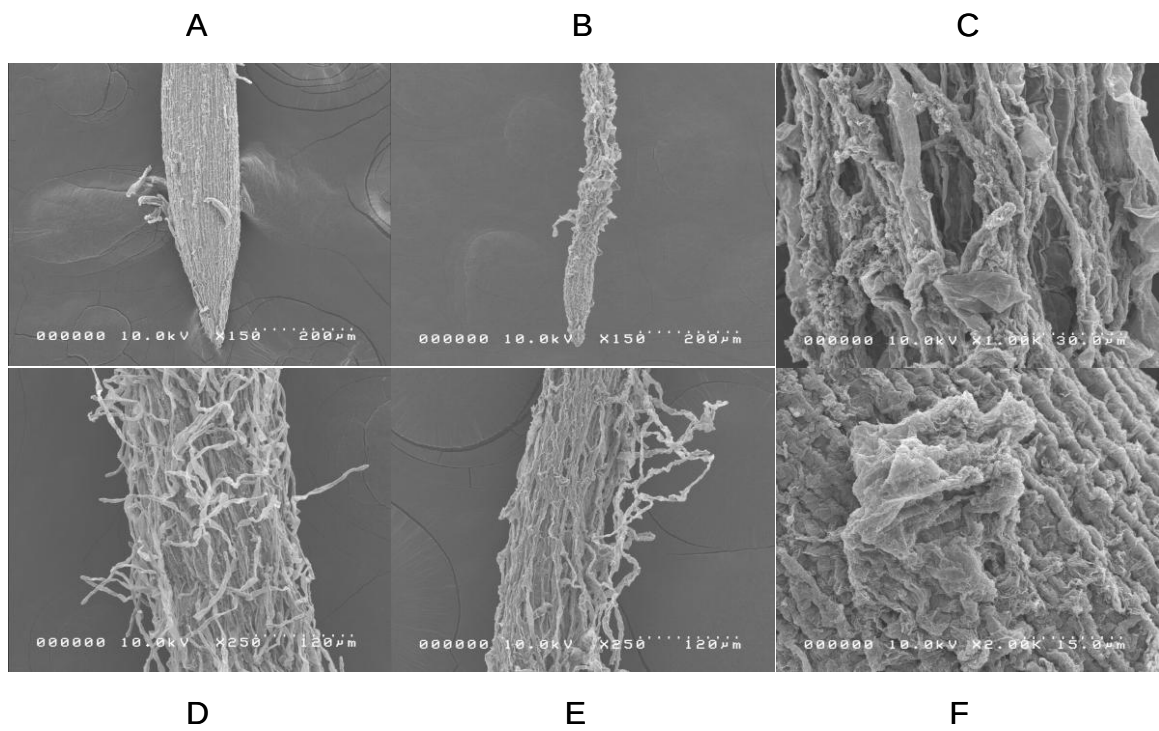


Figure 17 Root morphology of tomato

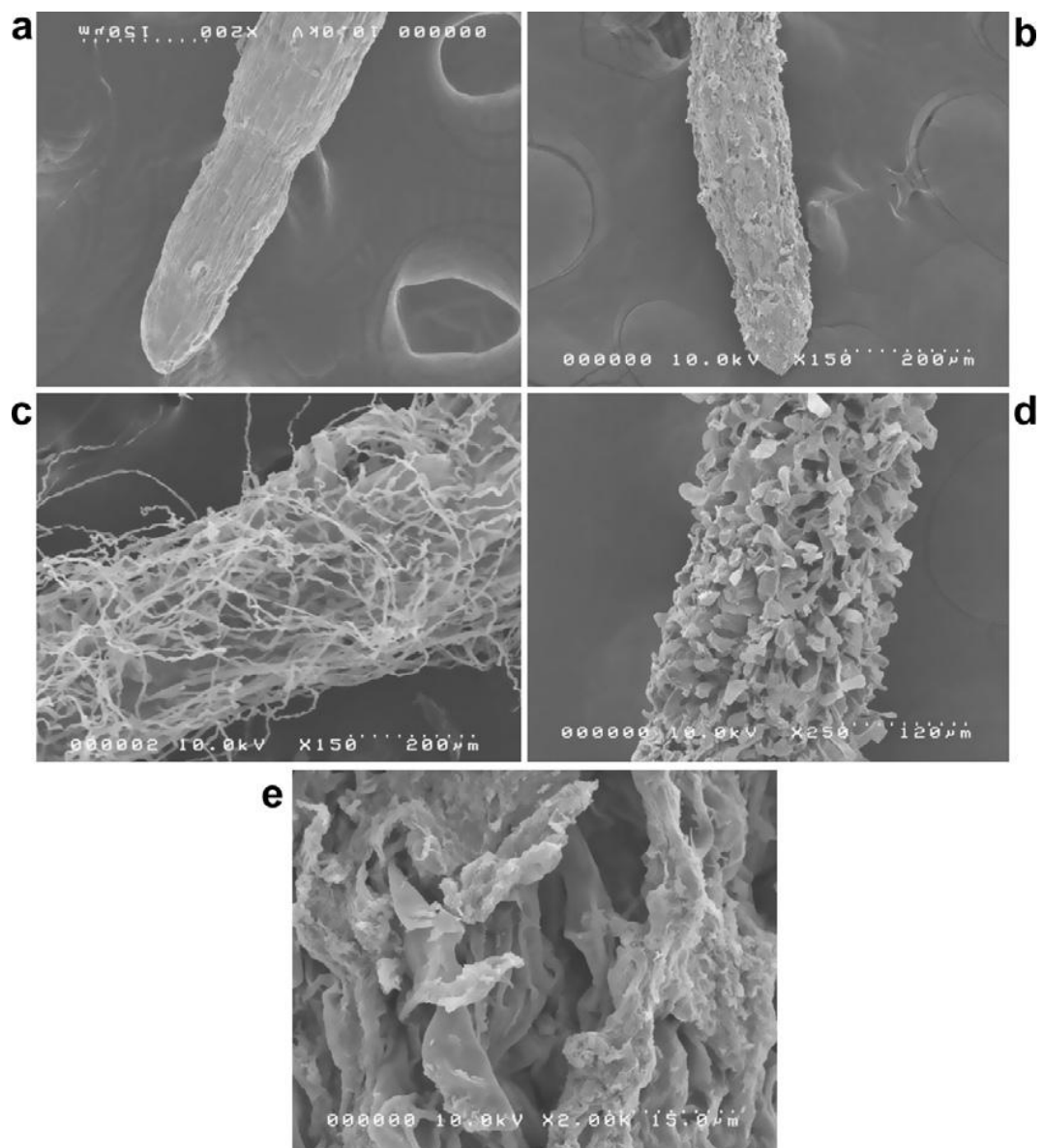


Figure 18 **Root morphology of red spinach**

SEM examination of the tested plants exposed to a higher concentration of graphene (1000 mg/L) revealed several morphological alterations compared to the control plants grown in Hoagland medium. At 20 days without exposure to GO, the majority of the root surface appeared healthy; little to no alteration in morphology was observed. Regarding morphology at 1000 mg/L of GO, root shape changed especially above the elongation area of cabbage and tomato [Figures 16 and 17, respectively] compared to control. Epidermis of the treated tomato and red spinach roots were loosely detached or even completely detached compared to control, which was characteristic for membrane damage as determined by Evans blue. We note that specific differences could be observed after Evans blue staining at the light microscopic level (data not shown) with respect to the cell morphology between roots exposed to different concentrations of GO. Thus, differences in cell death determined from Evans blue assays do reflect the sensitivity of cells to different concentrations of GO, although the morphological alterations observed from light microscopy indicate a common cell death pathway. These results imply that the root membrane with a higher GO concentration (1000 mg/L) had no tolerance to GO stress. An aggregation of GO was noted on the surface of the root of cabbage, tomato, and red spinach, indicating that aggregation of GO contributes at least in part to its toxic properties, as is the case for CNTs [37]. Uptake, translocation, and accumulation of CNTs in roots and leaves were reported for tomato [29]. In our studies with GO as the typical carbon nanoparticles, insights into the penetration, translocation, and/or accumulation of GO into the intracellular locations were not observed for all of the four species (cabbage, tomato, red spinach, and lettuce). This was confirmed by observing the plant specimens by TEM during the courses of the plant incubation.

The root surface area of the GO -treated cabbage was different from the untreated control [Figure 16]. The root surface area of cabbage was significantly increased by GO treatment, and it may be that an excess of GO resulted in swelling. This observation is similar to results previously reported for *Origanum vulgare* by Panou-Filotheou and Bosabalidis [65] and in cowpea by Kopittke et al. [66]. At a higher GO concentration (1000 mg/L), the root hair growth of cabbage and red spinach was significantly decreased compared to control.

2.3.9 Proposed mechanism of GO phytotoxicity

The two forms of carbon nanoparticles, CNTs and graphene, have similar chemical composition and crystalline structure. Based on this fact, a reasonable inference is that the two would be similar in many ways, such as biological activity. But they are different in shape; CNTs are tubular while graphene is flat atomic sheets [67]. As a result, atomically flat graphene nanostructures have been estimated to have an even stronger interaction with biological systems than the tubular shaped CNTs. There is evidence that CNTs could translocate to systemic sites (roots, leaves, and fruits) and engage in a strong interaction with the cells of tomato seedlings, resulting in significant changes in total gene expression in roots, leaves, and fruits [29] and exerting toxic effects [31,32,42]. CNTs are also known to have phytotoxic effects in plant cells because of aggregation [41], causing cell death and accumulated ROS in a dose-dependent manner [56]. Considering these aspects, it is not unexpected to find toxic effects of GO on terrestrial plant species, as we did in tomato, cabbage, and red spinach in the current study. Oxidative stress has been regarded as a suitable mechanism for explaining the toxicity of GO.

The phytotoxicity of GO is likely the result of its ability to generate ROS as represented predominantly by H₂O₂ using the ROS-sensitive dyes DCFH-DA and DAB. Reports regarding GO-induced oxidative stress are available [34]. Concerning the relationship between graphene-mediated ROS generation and cell death in plants, we investigated physiological and morphological endpoints, including visual symptoms, ROS production, SEM observation, and cell death-related responses after exposure to GO for 20 days. GO-treated leaves showed significant accumulation of H₂O₂ compared with control [Figure 13]. This observation was closely associated with visible signs of

necrotic damage lesions and cellular ROS accumulation monitored by DAB [Figure 14], and membrane damage [Figure. 15b]. The evidence of massive cell death (electrolyte leakage assay) as well as visual signs with the GO -treated leaves demonstrated that at 20 days following exposure to GO, necrosis was the predominant death response in treated leaf cells. In addition, the spatial pattern of H₂O₂ production was mainly located in the close proximity of visible symptomatic areas and the sites undergoing cell death.

Plant cell death occurs either by apoptosis or by necrosis. Necrosis is passive and characterized by a progressive loss of membrane integrity that results in cytoplasmic swelling and release of cellular constituents [68]. Furthermore, we also detected the effect of GO on the morphology of roots, finding that the epidermis of the treated tomato and red spinach roots was loosely detached or even completely detached. Further, the aggregation of GO was noted on the surface of the roots of all GO (1000 mg/L)-treated plants, indicating that aggregation of GO contributed at least in part to its toxic properties, as is the case for CNTs [41]. On the basis of these results, we infer that GO phytotoxicity involved the oxidative stress mechanism mediated through the necrotic pathway. In biological systems, ROS production and disturbances of cellular redox potentials have been found to be involved either directly or indirectly in the death of individual cells and/or the development of necrotic lesions [69, 70]. Enhanced generation of ROS (hydrogen peroxide) and plasma membrane rupture have been traditionally associated with pathogenic events including necrotic cell death, which is recognized by morphological signs and is usually considered to be uncontrolled [71].

2.4 Conclusion

In summary, we showed that GO exerted toxic effects on terrestrial plant species such as cabbage, tomato, and red spinach. The overproduction of ROS induced by GO could be responsible for significant plant growth inhibition and biomass reduction compared to control. Nel et al. [28] reported that the production of ROS can be a key factor in the toxicological effects of nanostructure materials. Observation of accumulation ROS production by means of H₂O₂ visualization along with visible signs of necrotic damage lesions and evidence of a massive electrolyte leakage all indicated an oxidative stress mechanism mediated through the necrotic pathway, which requires further study. We suggest an evaluation of graphene toxicity to targets on terrestrial plant species and applying a prolonged exposure period with different concentrations to measure any potential risk.

Chapter 3rd

Preliminary screening of Phytotoxicity of MWNTs on Lettuce (*Lactuca sativa*).

1.1 Introduction

Multi-walled carbon nanotubes (MWNTs) have attracted remarkable interest in terms of both fundamental research and technological development [72] due to their unique nanostructures and extraordinary properties [73]. Hundreds of nanotechnology based products are currently in the marketplace, with common applications in agriculture, food production, food packaging, and food protection [17]. These nanomaterials can end up in the environment [74]; biological or environmental systems may be exposed to the nanomaterials, may internalize the nanomaterials, and may experience a subsequent biological response and/or an ecological effect. Nanomaterials have been shown to generate harmful reactive oxygen species (ROS) due to their large surface areas, thus increasing cellular oxidative stress and possibly attacking DNA, proteins, and membranes, resulting in cellular injury [51]. We therefore suggest great caution before introducing nano products into the marketplace.

In terms of phytotoxicity, several studies have focused on terrestrial plant species exposed to MWNTs [31, 32, 51], but no continual, full, or multigenerational life-cycle studies appear in the literature, demonstrating the need for wide-ranging study of interactions between carbon nanoparticles and terrestrial plants, as well as the ultimate fates of exposed plants. To address this need, we investigated the interaction between MWNTs and the vegetable/commercial crops, red spinach, lettuce, rice, cucumber, chili, lady's finger, and soybean, focusing on root and shoot growth and cell death at the

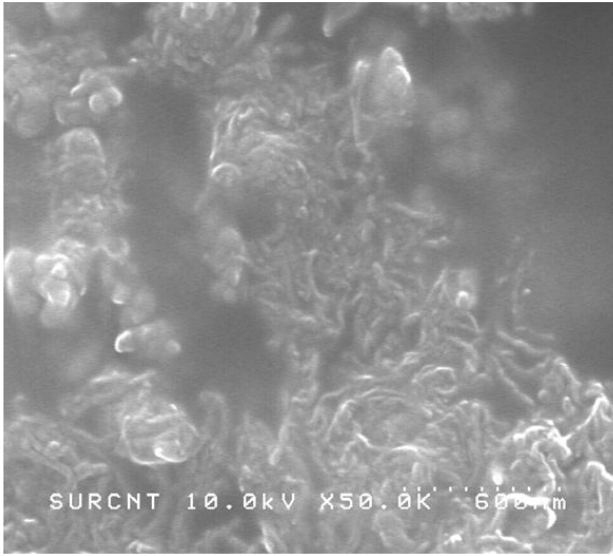
seedling stage. The most successful plant species were then used in phytotoxicity studies in association with MWNTs. Our experiment demonstrated that red spinach, lettuce, rice, and cucumber experience much higher toxicity from MWNTs at the seedling stage, indicating the need for further research including a wide range of terrestrial plant species associated with carbon nanoparticles.

3.2 *Experimental Section*

MWNTs were purchased (Bayer Material Science, Germany) as powders with a loose agglomerate size of 0.1–1 mm, outer mean diameter ~13 nm, inner mean diameter ~4 nm, and length >1 μ m. The powders were first wet with deionized water overnight at ~40 °C, then milled into smaller sizes with a continuously operating bead-mill system in the absence of dispersants (surfactants).

For scanning electron microscopy (SEM), a few drops of the MWNTs suspension were deposited on the aluminum stub, dried, sputter-coated, and observed using a Hitachi S-4000 scanning electron microscope (Ibaraki, Japan) with an acceleration voltage of 10 kV [Figure 19 a]. For transmission electron microscopy (TEM), the MWNTs were homogenously dispersed in 2-propanol under ultrasonication for 30 min. A few drops of the suspension were deposited on the TEM grid and covered with the formvar membrane, dried, and evacuated before analysis. The preparations were observed using a Hitachi H-800 transmission electron microscope at an acceleration voltage of 75 kV [Figure 19b].

a



b

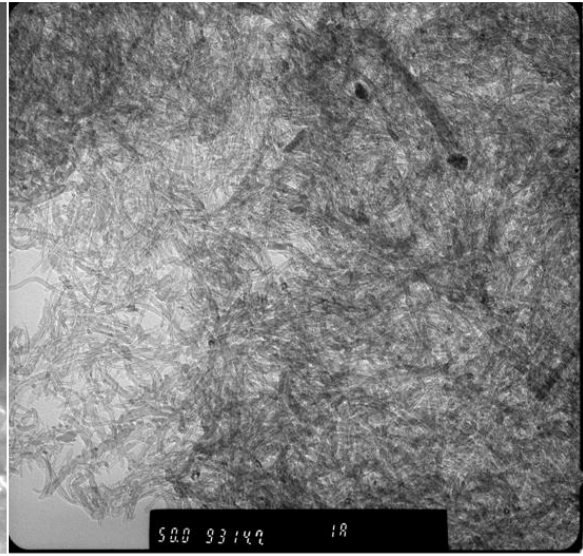


Figure 19 (a) SEM image of MWNTs , (b) TEM Image of MWNTs.

Chemicals were purchased from Kanto Chemical Co. Inc, Wako Pure Chemical Industries, Ltd. and Sigma–Aldrich Inc., Japan. Seeds of lettuce (*Lactuca sativa*), rice (*Oryza sativa* L.), and cucumber (*Cucumis sativus*) were purchased from Sapporo, Japan. Red spinach (*Amaranthus tricolor* L.), lady’s finger (*Abelmoschus esculentus*), and chili (*Capsicum annuum* L.) seeds were purchased from Dhaka, Bangladesh. Professor Toru Miura kindly provided soybean (*Glycine max*) seeds. Seeds were sterilized, soaked overnight, and incubated at 25 °C, and the seedlings were transferred to plastic pots containing 300 mL Modified Hoagland Media [43] with 0, 20, 200, 1000, or 2000 mg/L MWNTs. After 15 days of hydroponic culture, seedling roots and shoots were separated; the fresh weights were measured and the lengths of individual seedlings were determined. Cell death in untreated and MWNTs-treated roots was tested with Evans blue as previously described [46], and the percent of membrane injury was measured after 15 days of exposure from electrolyte leakage via the conductivity method based on [47]. Each treatment was conducted in triplicate. Statistical analysis was performed using Student’s t-test, with $P \leq 0.01$ considered significant. Data are presented as mean $\pm$ SD (standard deviation).

3.3 Results and Discussions

3.3.1 Effect of MWNTs on plant seedlings

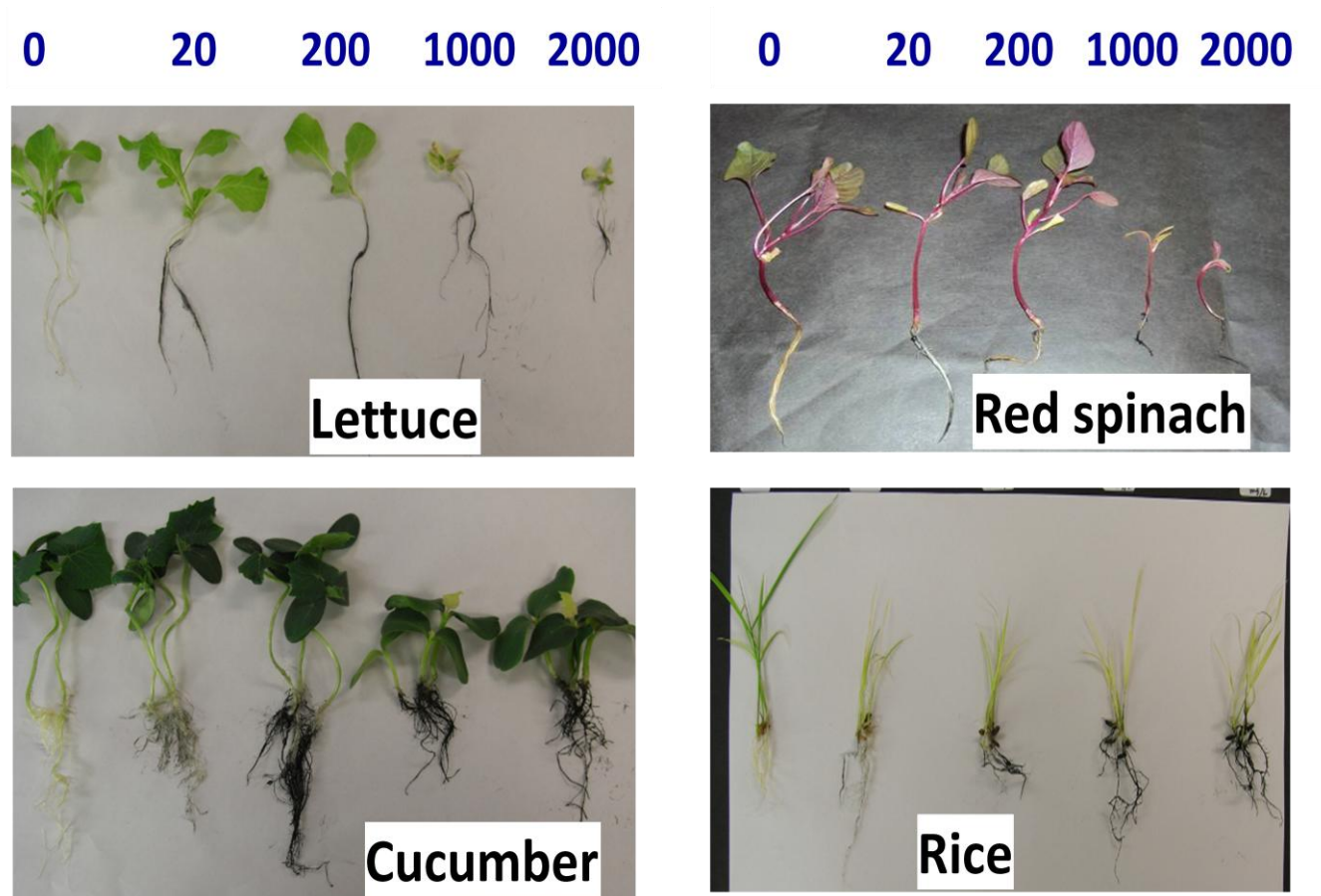


Figure 20 The effects of MWNTs exposure on plant seedlings.

The physiology and morphology of red spinach, lettuce, rice, cucumber, chili, soybean, and lady's finger were assessed following hydroponic culture with MWNTs [Figure 20]. Rice is a monocot, and the other tested species are dicot; the seeds of red spinach, lettuce, and cucumber are smaller than those of soybean and lady's finger.

MWNTs showed no toxic effects on chili, lady's finger, or soybean (data not shown), but the root and shoot growths of lettuce, red spinach, cucumber, and rice were reduced compared to control. The MWNTs-treated plants displayed a gradual change in leaf color to light green/yellowish with increasing MWNTs dose; untreated plants did not exhibit any change in color. Wilting of leaves of reduced size was evident by visual observation in MWNTs treated red spinach, lettuce, and rice. The attachment of MWNTs to plant roots was characterized by visual observation [Figure 20].

3.3.2 Effect of MWNTs on plant biomass

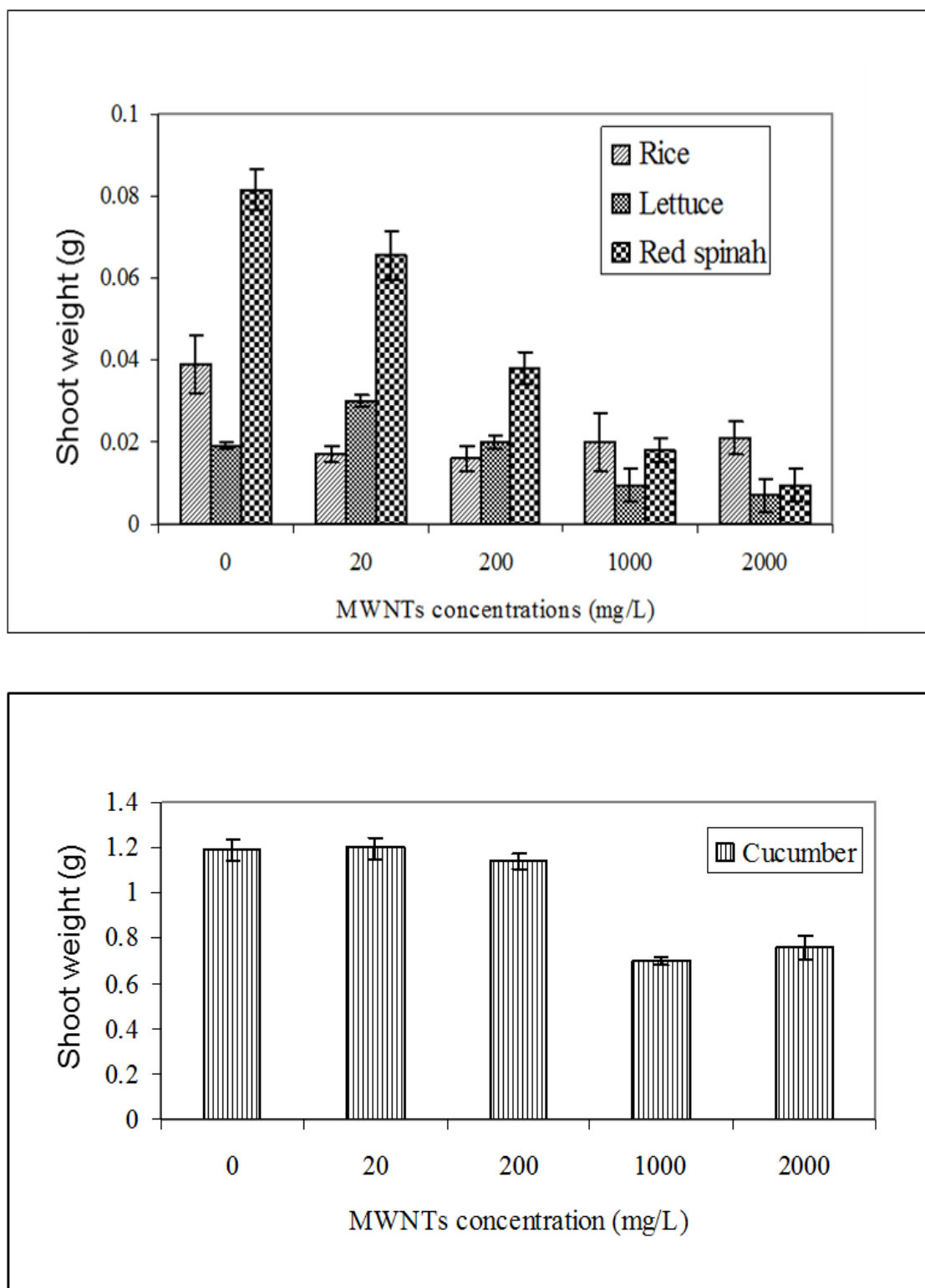


Figure 21 Effect of MWNTs on shoot weights (a) and (b).

The root and shoot growth of red spinach, lettuce, rice, and cucumber exposed to MWNTs at 2000 mg/L were significantly different from the control plants (19). The reductions in shoot fresh weight of red spinach, lettuce, rice, and cucumber plants at 2000 mg/L exposure were 88%, 63%, 46%, and 36%, respectively [Figures 21a and b]. Interestingly, the shoot fresh weight of rice was reduced 56% at 20 mg/L and 46% at 2000 mg/L compared to control [Figure 21a].

The root fresh weights of red spinach and lettuce at 2000 mg/L declined 81% and 79%, respectively [Figure 22c]. The average root fresh weight of untreated rice was 0.0173 g, while the weights of treated rice at 20, 200, 1000, and 2000 mg/L MWNTs were 0.0063, 0.004, 0.0037, and 0.0036 g, respectively [Figure 22c]. The average root fresh weight of control cucumber was 0.405 g, and the average root weight of treated cucumber decreased to 0.395, 0.36, 0.24, and 0.22 g following exposure to 20, 200, 1000, and 2000 mg/L MWNTs, respectively [Figure 22d].

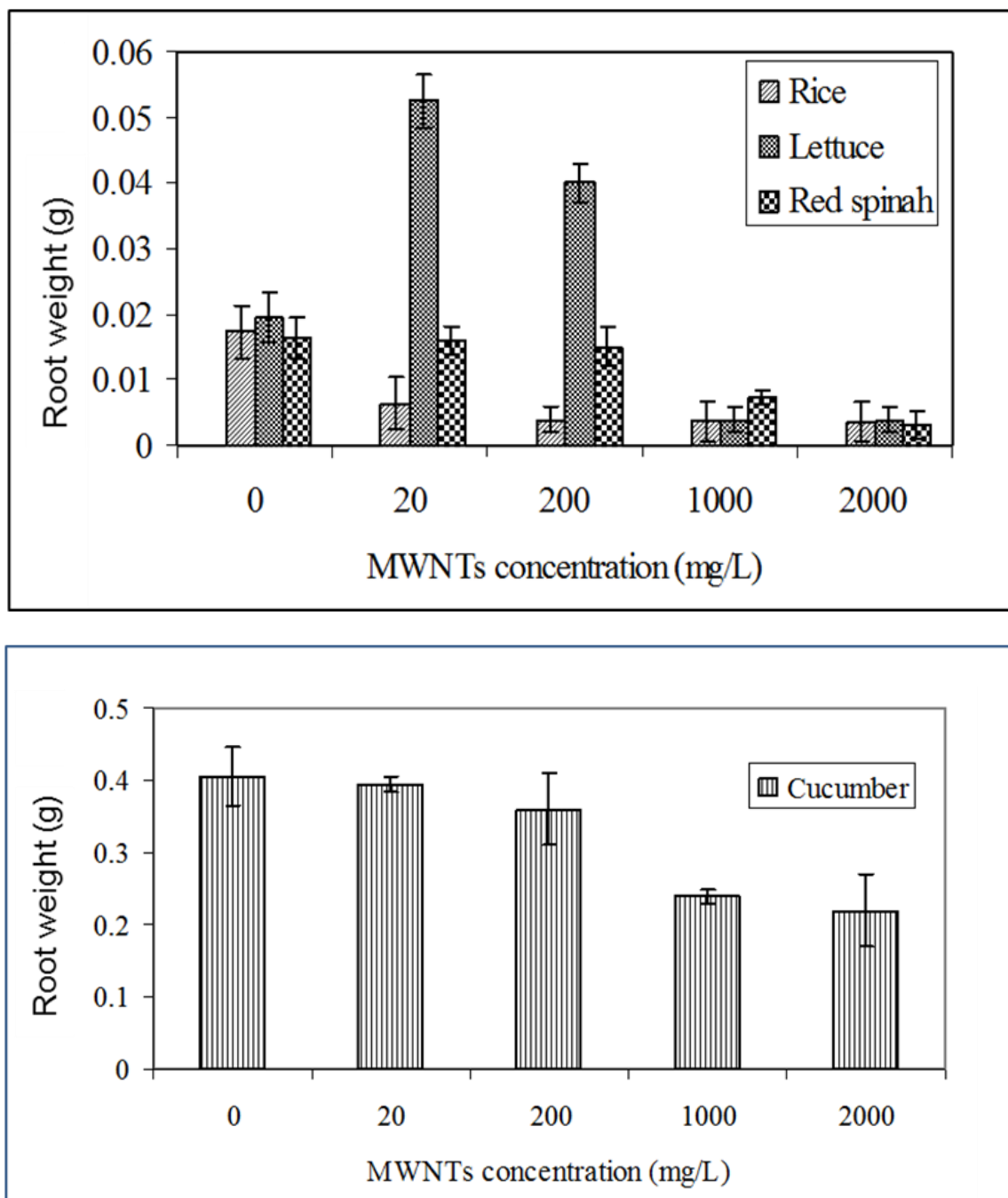


Figure 22 Effect of MWNTs on root weights (c) and (d).

3.3.3 Effect of MWNTs on plant growth

The reductions in the root lengths of red spinach and lettuce at 2000 mg/L exposure were 67% and 45%, respectively [Figure 23 a]. The average root length of untreated rice was 5.3 cm, and those of treated rice were 6, 4.7, 6.83, and 5.5 cm at 20, 200, 1000, and 2000 mg/L MWNTs, respectively. The reduction in shoot and root growth for rice was not in a concentration dependent manner. The average root length of untreated cucumber was 10 cm, a length that decreased to 8, 8.7, 6.3, and 9.5 cm following exposure to 20, 200, 1000, and 2000 mg/L MWNTs, respectively [Figure 23 a].

Red spinach, lettuce, and cucumber shoot lengths at 2000 mg/L exposure decreased by 80%, 50%, and 66%, respectively [Figure 23 b]. and the shoot length of rice was reduced by 48% at 20 mg/L and by 35% at 200 mg/L compared to [Figure 23 b].

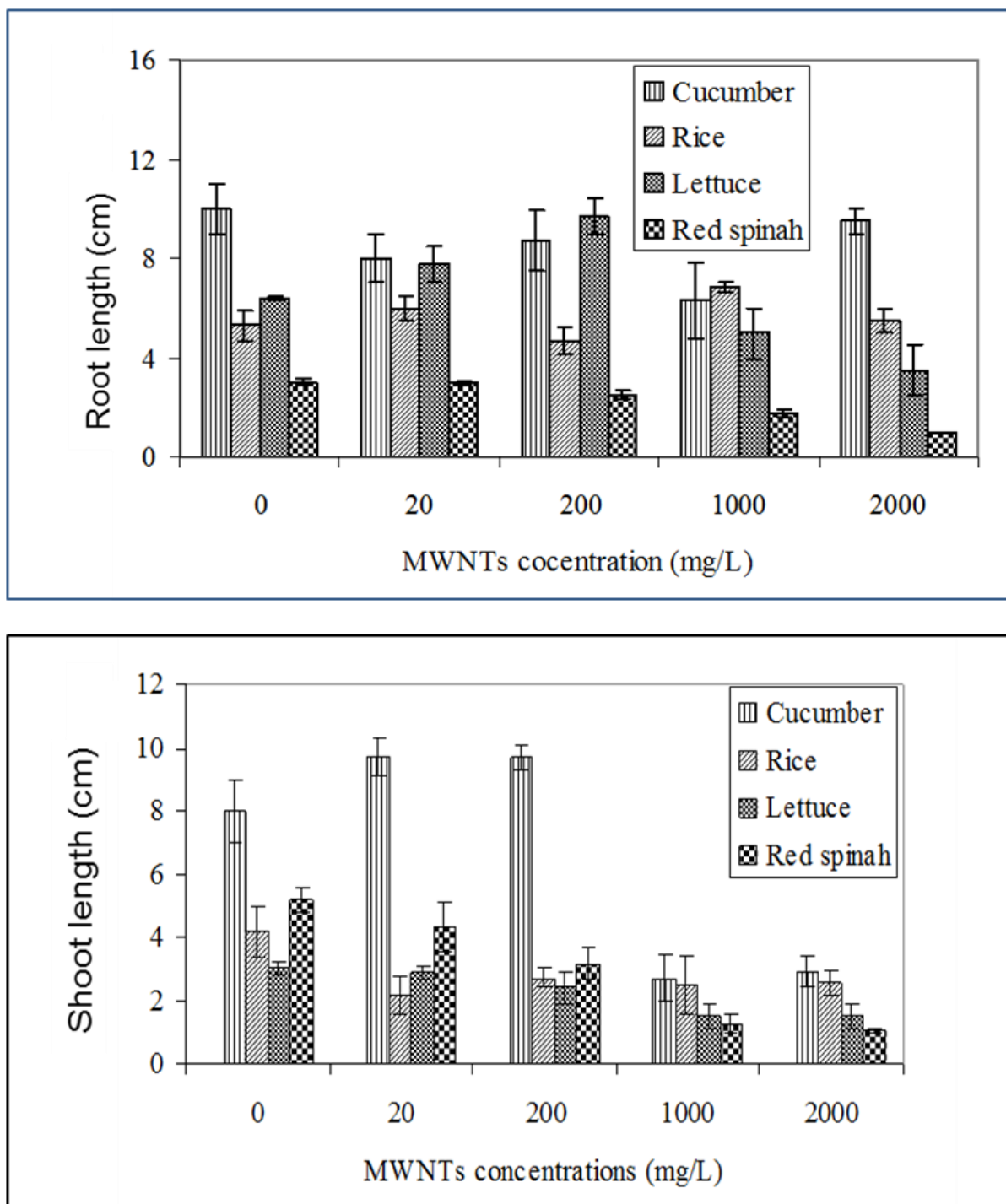


Figure 23 Effect of MWNTs on root length (a) and shoot length (b).

3.3.4 Effect of MWNTs on cell death

Cell death assay revealed that exposure of plants to MWNTs led to a higher capacity to fix the dye than lack of exposure; measurement of the Evans blue dye extracted from roots indicated that dye concentration was dependent on induction [Figure 24a]. At 20 mg/L and 200 mg/L MWNTs, the leaves displayed little increase in electrolyte leakage after 15 days of exposure, while 1000 mg/L and 2000 mg/L exposure led to a drastic increase in electrolyte leakage [Figure 24b], reflecting dose-dependent electrolyte leakage.

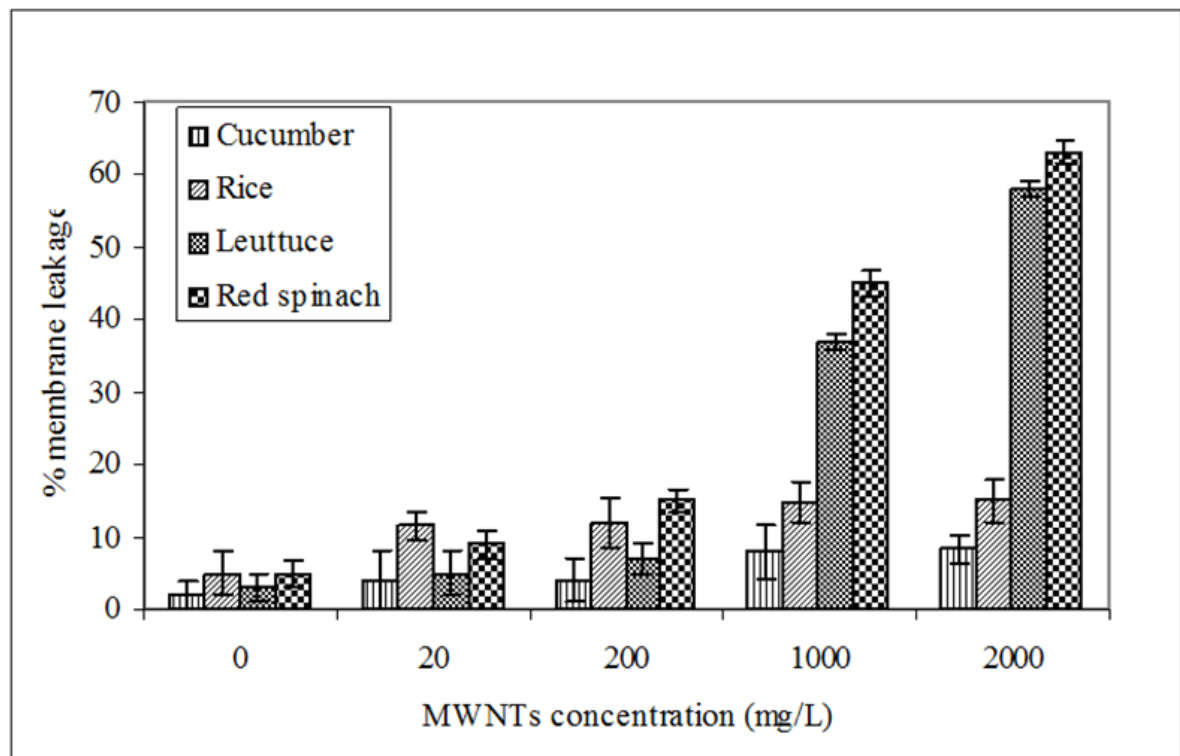
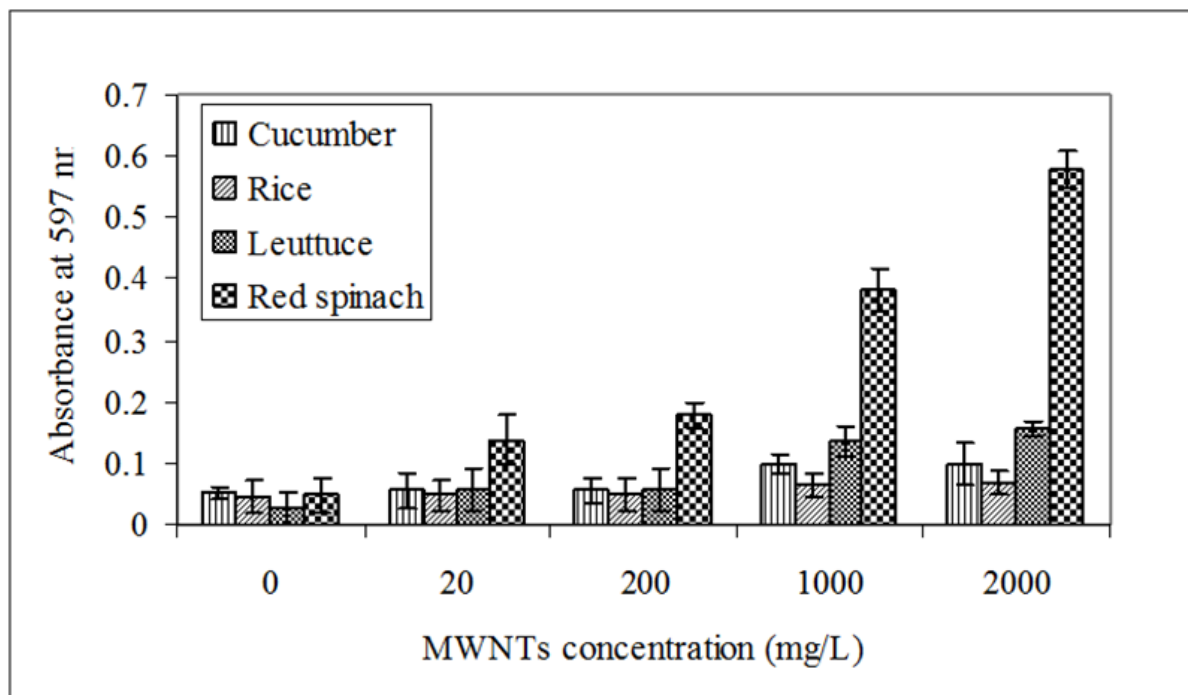


Figure 24 Cell death by Evans blue assay (a) and membrane leakage (b).

Despite different nanomaterials lengths and diameters [32], as well as the use of a variety of experimental techniques such as sonication [41] and functionalization [51], MWNTs have been consistently shown to aggregate and exert adverse effects in plants and plant cells. Our experimental data indicate that MWNTs are toxic to red spinach, lettuce, rice, and cucumber, but not to chili, lady's finger, and soybean. Plants of different species respond with their very own behaviors to the nanoparticles. Difference in structures of the xylem would be the key physiological reason responsible for this fact [78]. Canas et al., [51] described in their studies that the species would response differently to the nanomaterials, even under the identical experimental conditions. This differential toxicity tentatively suggests that agricultural use of MWNTs may not negatively affect all crop species; positive effects of MWNTs have also been reported [75].

Red spinach and lettuce have the smallest seed sizes and displayed the greatest phytotoxicity among the seven species tested here. Seed size can be an important factor in how a seed responds to MWNTs. For example, small-seeded species have a large surface area to volume ratio, which is conducive to higher sensitivity to toxicants [76] than large-seeded species such as lady's finger and soybean. Size may therefore cause seeds to be more sensitive to nanoparticles exposure, but it does not dictate whether phytotoxicity will be experienced.

Growth (root, shoot length, and biomass) retardation is the most general symptom of MWNTs toxicity in plants. Exposure to MWNTs at 1000 mg/L and 2000 mg/L resulted in significant decreases in red spinach and lettuce root and shoot growth compared to untreated plants. These observations are in agreement with those of Stampoulis et al. [32], who exposed zucchini to MWNTs at 1000 mg/L for two weeks.

Determination of leaf number is a non destructive method for assessing nanomaterials phytotoxicity, and our observation of a decrease in the number of leaves corresponds to inhibition of *Arabidopsis thaliana* growth [77]. Significantly fewer leaves were present on red spinach exposed to 1000 mg/L and 2000 mg/L MWNTs, corroborating the potential phytotoxicity of MWNTs. According to USEPA guidelines [36] the highest concentration of MWNTs used for this study was 2000 mg/L. Toxicity studies on the food crops were commonly carried out at high concentrations (2000–5000 mg/L) of the nanoparticles [33,39].

A number of investigations have indicated that ROS generation and oxidative stress are mechanisms of MWNTs-induced plant toxicity. MWNTs-induced stress at 1000 mg/L and 2000 mg/L caused cell death and membrane damage in red spinach, lettuce, rice, and cucumber after 15 days of exposure, suggesting that MWNTs may induce ROS formation, promoting cell death and electrolyte leakage in the root and leaf, respectively. Carbon nanoparticles were found to be capable of enhancing the generation of ROS in plants once they have been covering on the roots of plants [78], ROS accumulation occurred; this leading to the electrolyte leakage and the cell death [56,63]. In addition, MWNTs aggregation was noted on the root surfaces, contributing at least a portion of the toxic effects of MWNTs [41]; the attachment of carbon nanotubes on the root surface may alter essential biochemical processes necessary for plant growth and survival [51]. It is therefore possible that some nanoparticles may exert their toxic effects as aggregates or through the release of toxic materials.

3.4 Conclusion

This investigation underscores the importance of effective screening strategies for exposure of agricultural and environmental systems in which MWNTs can penetrate the root and be transported to leaf and fruits [29]. Little is currently known about plant uptake of carbon nanotubes, or about how toxicity is affected by plant type. In the future, long-term studies are necessary to identify target plants and to understand MWNTs-induced phytotoxicity; such studies will be relevant to use and disposal of engineered nanoparticles.

Chapter 4th

Phytotoxicity of MMWNTs on Lettuce (*Lactuca sativa* L)

4.1 Introduction

The multiwalled carbon nanotubes (MWNTs) are built by rolled graphene sheets as one type of nanoparticles composed by extensive sp^2 carbon atoms. The diameter up to tens of nanometer and length vary substantially up to tens of micrometer [79]. Rising interest among scientists and industrialists are related to their extraordinary properties in mechanical, electronic and biomedical applications [15]. Recently by the large production of MWNTs, it may have potential release, environmental fate and ecological risk which correlated with plant species, type of media, treatment system, dose, exposure time, and properties of carbon nanotubes [80, 81]. Consequently, it deserves further attention. Instead of preliminary screening of several plants species as previously reported [82], we further investigate lettuce (*Lactuca sativa*) regarding the potential effect of MWNTs on plants conducted under hydroponic conditions. Concentration of MWNTs and CB used in this study is varied by 0, 125, 250, 500 and 1000 mg/L. It is important to show the significant effect of MWNTs on the seedling stage of lettuce for the understanding of toxic symptoms on root and leaves. In this report we also identified the reactive oxygen species (ROS) which may responsible for the phytotoxicity of MWNTs.

4.2 Experimental Section

MWNTs were purchased from Bayer MaterialScience, Germany. The as-received, raw MWNTs were the powders with a loose agglomerate size of 0.1 – 1 mm, outer mean diameter (~ 13 nm), inner mean diameter (~ 4 nm) and length (> 1 μm), bulk density ($140\text{-}160$ kg/m^3), carbon purity (> 96 wt%) obtained from Bayer MaterialScience. We treated the hydroponic culture with MWNTs (3% bay-cho-40-25G-SUG-10) and Carbon Black (CB) as the control experiment. We have considered that MWNTs were ready to use without any pre-treatment due to its high carbon purity and free of amorphous carbon. Plant seeds were obtained from Homac Sapporo Japan. Modified Hoagland media was prepared based on protocol from Hoagland and Arnon, 1950 [43]. Other chemicals and reagents were purchased from Kanto Chemical Co. Inc, Wako Pure Chemical Industries, Ltd. and Sigma Aldrich Inc. Japan.

Seeds were sterilized, germinated and then seedlings grown in the hydroponic culture as described in our previous report [82]. To see the effect of MWNTs on germination, we immersed the lettuce seeds in MWNTs solution (0, 125, 250, 500, 1000 mg/L) at 4°C . After one night, 15 seeds were placed in Petri-dish containing 3 ml media (Hoagland media + MWNTs) with variation of concentration, then stored in incubator at 25°C . Germinated seeds were counted after 3-4 days. Then we transferred the seeds into 200 ml box for hydroponic culture containing Hoagland's media treated with MWNTs (0, 125, 250, 500, 1000 mg/L) and CB as control experiment at 224°C under continuous light. We harvested the plants after 17 days exposure. Statistical analysis, images of root and leaves morphology of treated plants were then examined. All treatments were done by triplicate ($n=3$). Statistical analysis was performed using

Student's *t*-test, with $P \leq 0.01$ considered significant. Data are presented as mean $\pm$ SD (standard deviation).

We have applied Evans blue assay to observe the cell death in root and leaves. We used 3,3'-diaminobenzidine (DAB staining assay) for *in situ* detection of hydrogen peroxide (H_2O_2) on leaves. The leaves were treated based on our previous study [78].

To observe the toxic symptoms on the root surface, we have performed Scanning Electron Microscopy (SEM) analysis using HITACHI S-4000. The preparation of specimen refers to previous method [78]. To observe the membrane damage of deformed cells in the cross sectioning tissue and penetration of MWNTs into plant cell wall, we have used Transmission Electron Microscopy (TEM) analysis using HITACHI H-800. The preparation of specimen refers to existing protocol [78].

4.3 Results and Discussions

4.3.1 Effect of MWNTs on germination

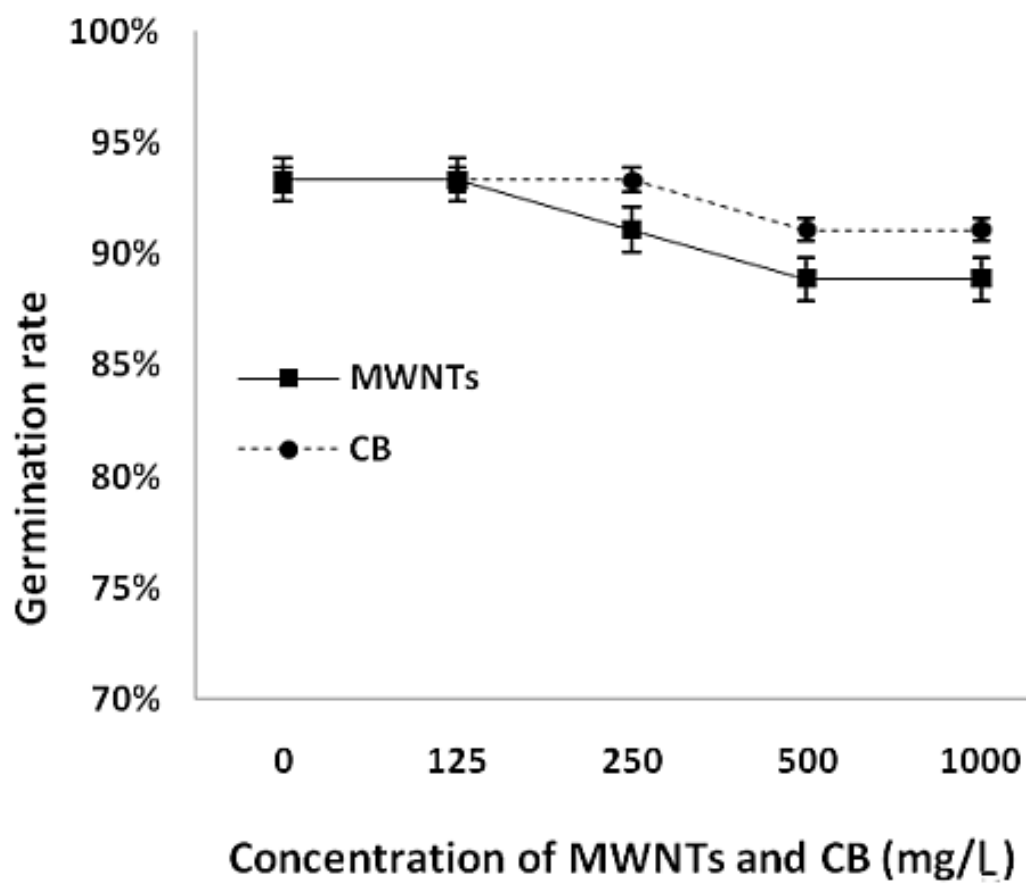


Figure 25 Effect of MWNTs on seeds germination of lettuce.

During 3-4 days of germination stage [Figure 25], the interference of germination rate was found in higher concentration, almost no significant effect of treatment by MWNTs and CB at lower concentration compared to control. However during hydroponic culture, images of plant seedlings were recorded. Based on our observation, the disparity on plant growth was started after two weeks of hydroponic culture. We recorded the data after 17 days in order to perceive the significance of plant growth inhibition [Figure 26].

Germination is a complex physiological process which triggered by imbibitions of water. In this study, we immersed the lettuce seeds in treated solutions containing MWNTs or CB with variation of concentration (0, 125, 250, 500, 1000 mg/L). This treatment will allow the water and media content to fully penetrate the plant seeds coat. Interestingly, our result showed no significant effect of MWNTs and CB at lower concentration (125 mg/L) [Figure 25]. In despite of single-walled carbon nanotubes (SWNTs) have been reported to penetrate plant seeds coat and dramatically affect seed germination and plant growth of tomato [75]. The existing contrast argument should be correlated with distinction of experimental condition, plant species, properties of nanomaterials in used etc.

4.3.2 Effect of MWNTs on plant root growth

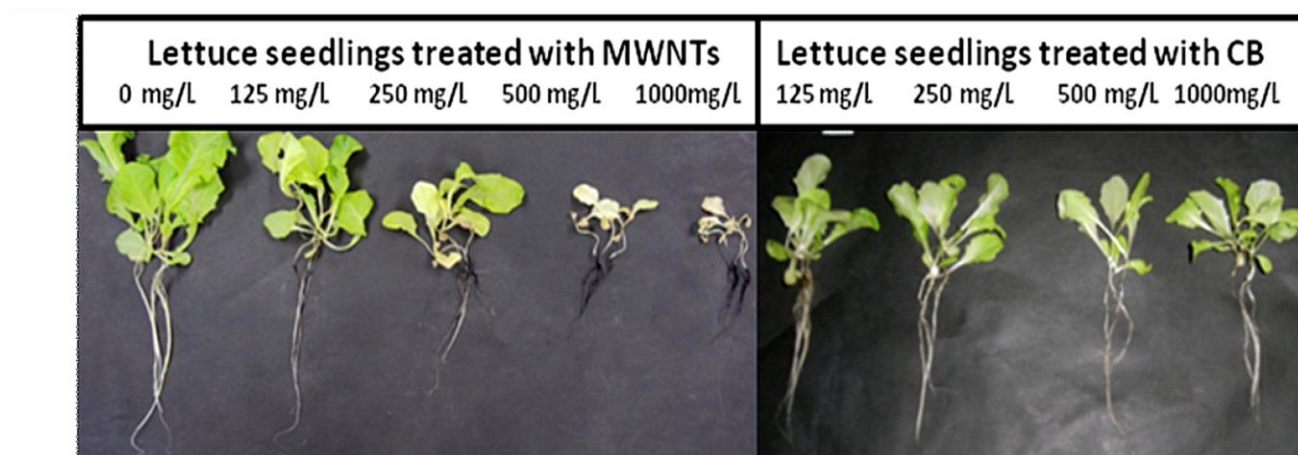


Figure 26 Effect of MWNTs on plant seedlings of lettuce.

Figures above have showed the significant effect of MWNTs on plant seedlings compared to CB. After harvesting, we have measured the averages of root lengths at different concentration of MWNTs (0, 125, 250, 500 and 1000 mg/L) which are 14.17, 11.50, 9.83, 5.60 and 5.53 cm respectively [Figure 27]. MWNTs also extremely decreased the root fresh weight up to 70% even though at lower concentration (125 mg/L). In the other hand, the averages of root fresh weight of MWNTs (0, 125, 250, 500 and 1000 mg/L) are 0.27, 0.07, 0.05, 0.05 and 0.04 g respectively [Figure 28].

Inhibition effect of SWNTs on root elongation has been reported previously [76]. They suggested that negative effect caused by lessening the available root surface area as SWNTs covered the surface of root. The data of plant growth inhibition presented here was strongly correlated to our previous study [78] where as lettuce plant growth was inhibited by the exposure of MWNTs 1000 mg/l and 2000 mg/l compared to other plant species relatively.

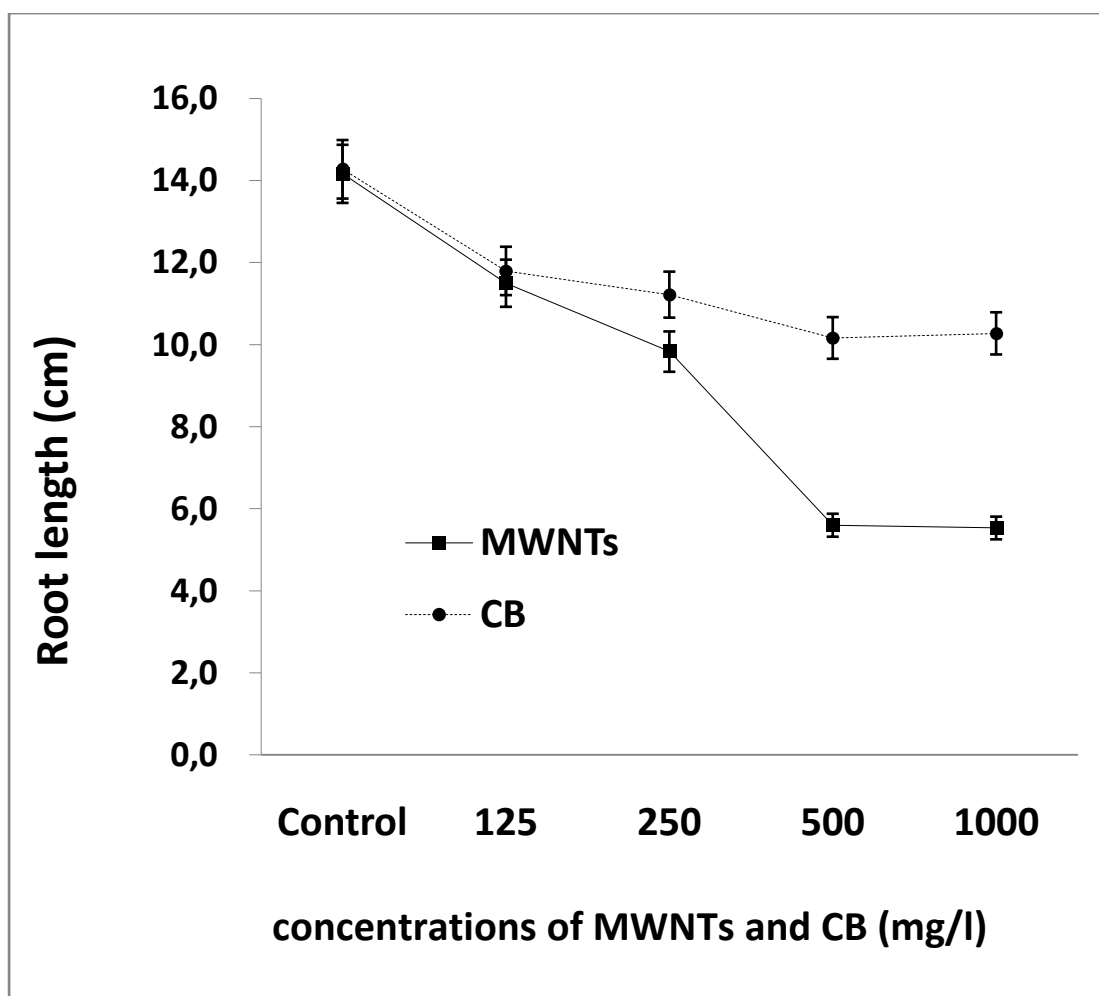


Figure 27 Effect of MWNTs on root length of lettuce.

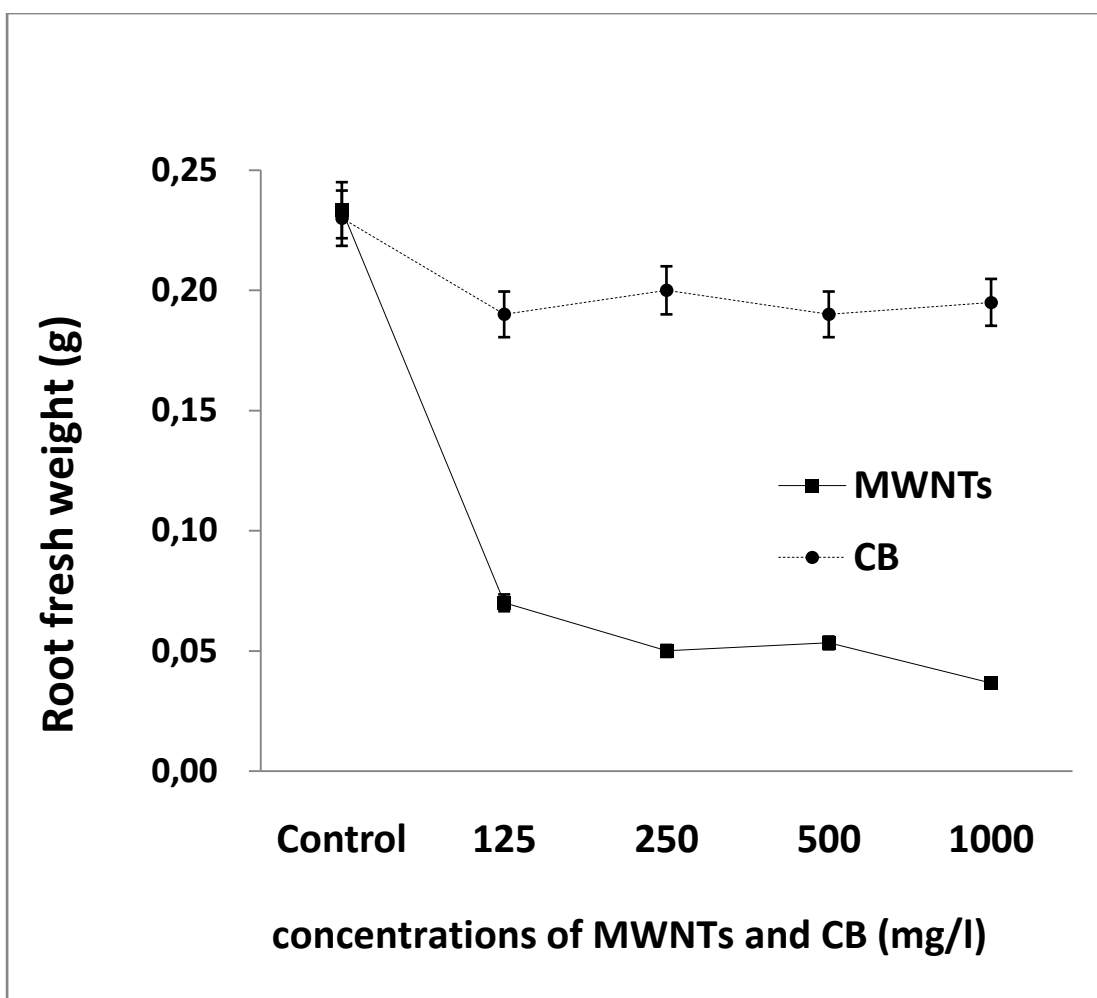


Figure 28 Effect of MWNTs on root weight of lettuce.

4.3.3 Effect of MWNTs on plant leaves

Leaf area and leaf fresh weight were also affected by the presence of MWNTs. We measured the average values of leaf area; 30.28, 21.57, 11.98, 6.80 and 5.03 cm² at different concentrations of MWNTs (0, 125, 250, 500 and 1000 mg/L) respectively [Figure 29]. The fresh weight of leaves was reduced; 2.66, 1.16, 0.49, 0.15 and 0.09 g with increasing of concentrations of MWNTs (0, 125, 250, 500 and 1000 mg/L) respectively [Figure 30].

We have used a non-toxic surfactant to stabilize the dispersion of hydroponic solution containing the Hoagland media treated by MWNTs and CB. Hence, no effect of surfactant against plant growth development (data is not shown). MWNTs could inhibit the plant growth at all concentration (125, 250, 500, 1000 mg/L) resulting the total decreased of root length up to 60.9% [Figure 27] and loss of root fresh weight up to 82.6% [Figure 28]. Furthermore, MWNTs also reduced leaf area [Figure 29] and leaf fresh weight [Figure 30] up to 99.8% and 96.6% respectively.

Even though root length [Figure 27] and leaf area [Figure 29] showed no different effect between MWNTs and CB at concentration of 125 mg/L. However, MWNTs tend to showed inhibition effect on root length, root fresh weight, leaf area and leaf fresh weight at all variation of concentration (125, 250, 500 and 1000 mg/L). In the other hand, CB tends to show the inconsistency of their inhibition effect due to physical properties of bulk material used in this study.

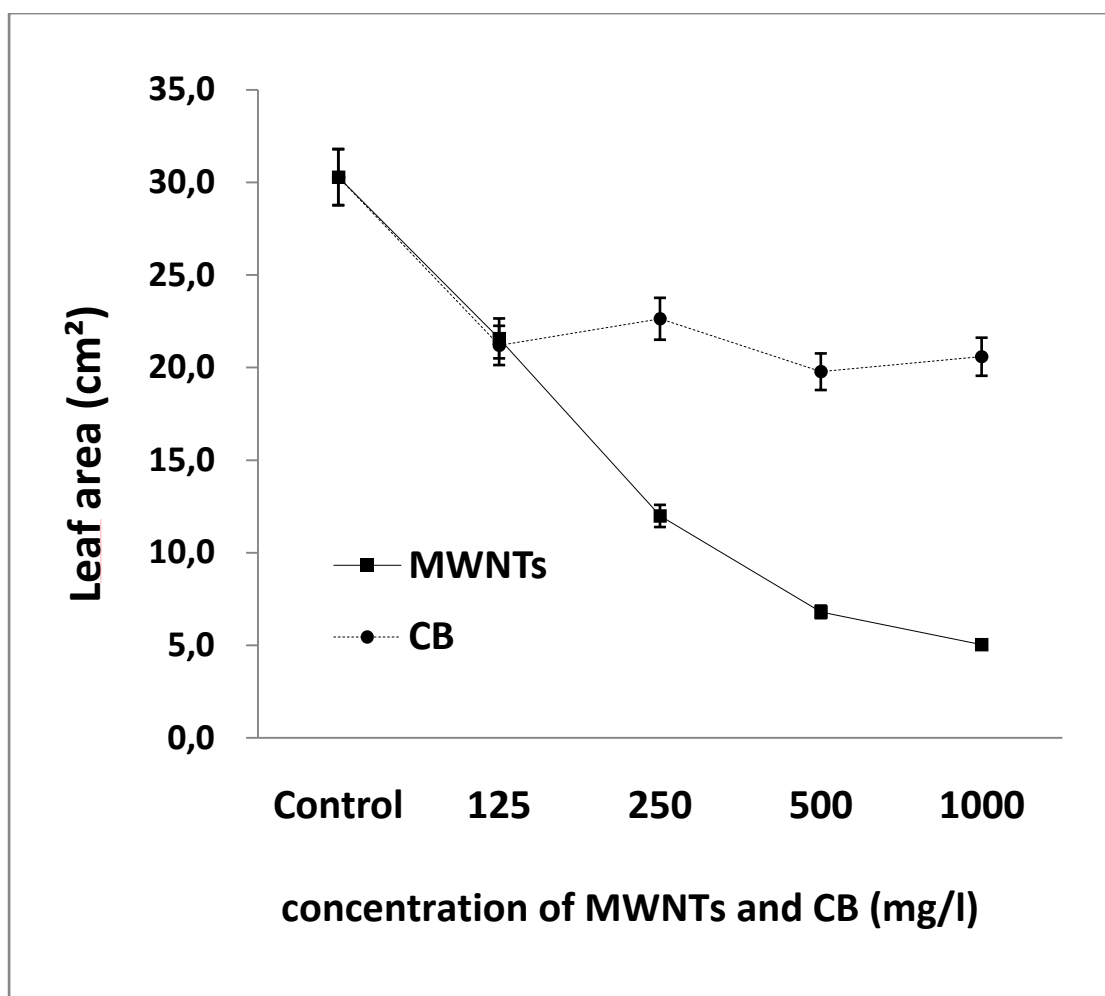


Figure 29 Effect of MWNTs on leaf area of lettuce.

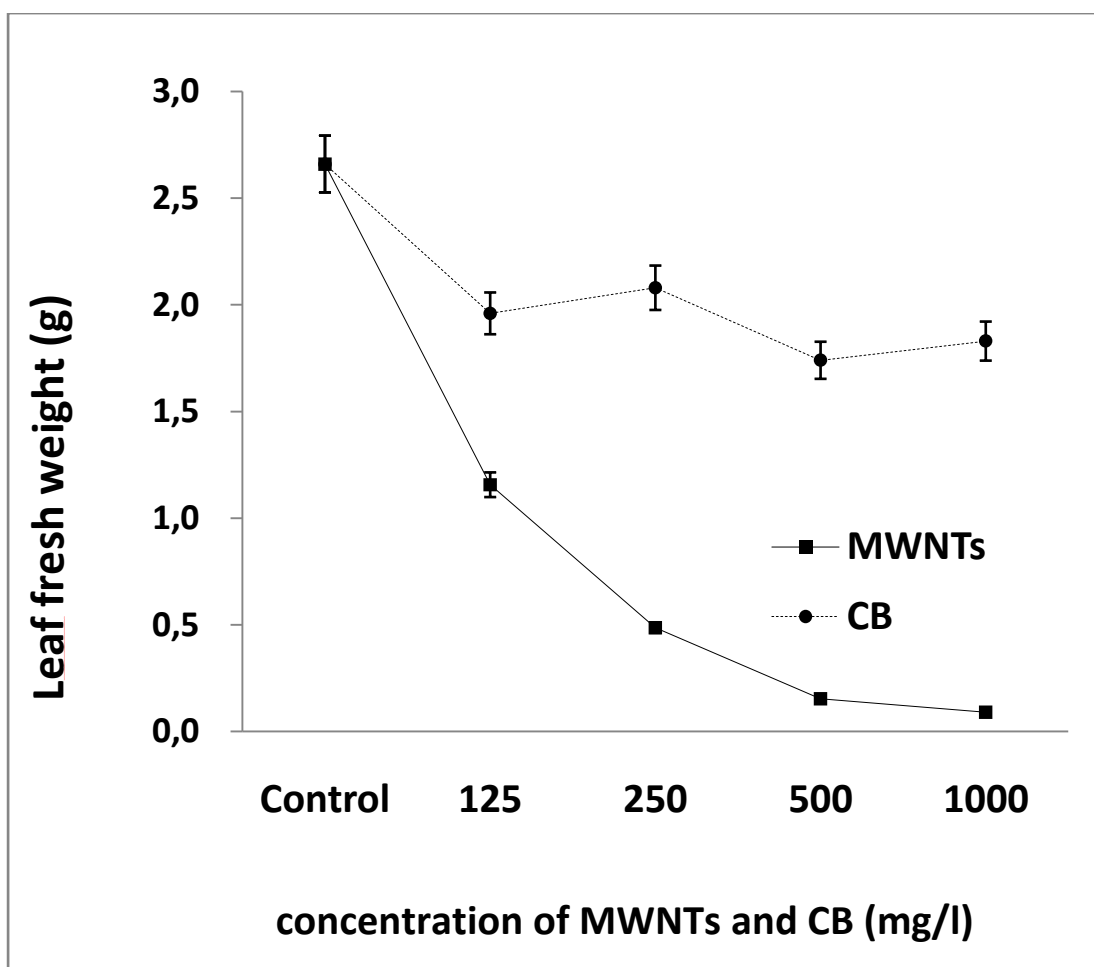


Figure 30 Effect of MWNTs on leaf fresh weight of lettuce.

4.3.4 Effect of MWNTs on cell death

After harvesting, root and leaves were treated by Evans blue assay to observe the cells death under light microscope [Figure 31]. Blue staining images showed on root and leaves indicated cell death.

Stampoulis *et.al*, [32] has demonstrated that seed germination and root elongation are not enough to evaluate the toxicity of terrestrial plants. Cell viability or cell death is an important variable to be measured. But in this paper we only show the identification of cell death along root tips and leaves qualitatively [Figure 31]. The quantitative measurements of viability and membrane leakage have been reported in the previous study [78]. Here we strongly emphasize that MWNTs caused cell death on vascular tissue and leave veins by a dose dependent manner. We could observe roughly that cell death on root and leaves indicated by the blue color of staining assay under light microscopy. Leaf senescence began at 125 mg/L followed by increasing intensity of blue color at higher concentration (250, 500 and 1000 mg/L) indicated that dye concentration was dependent on induction [Figure 31].

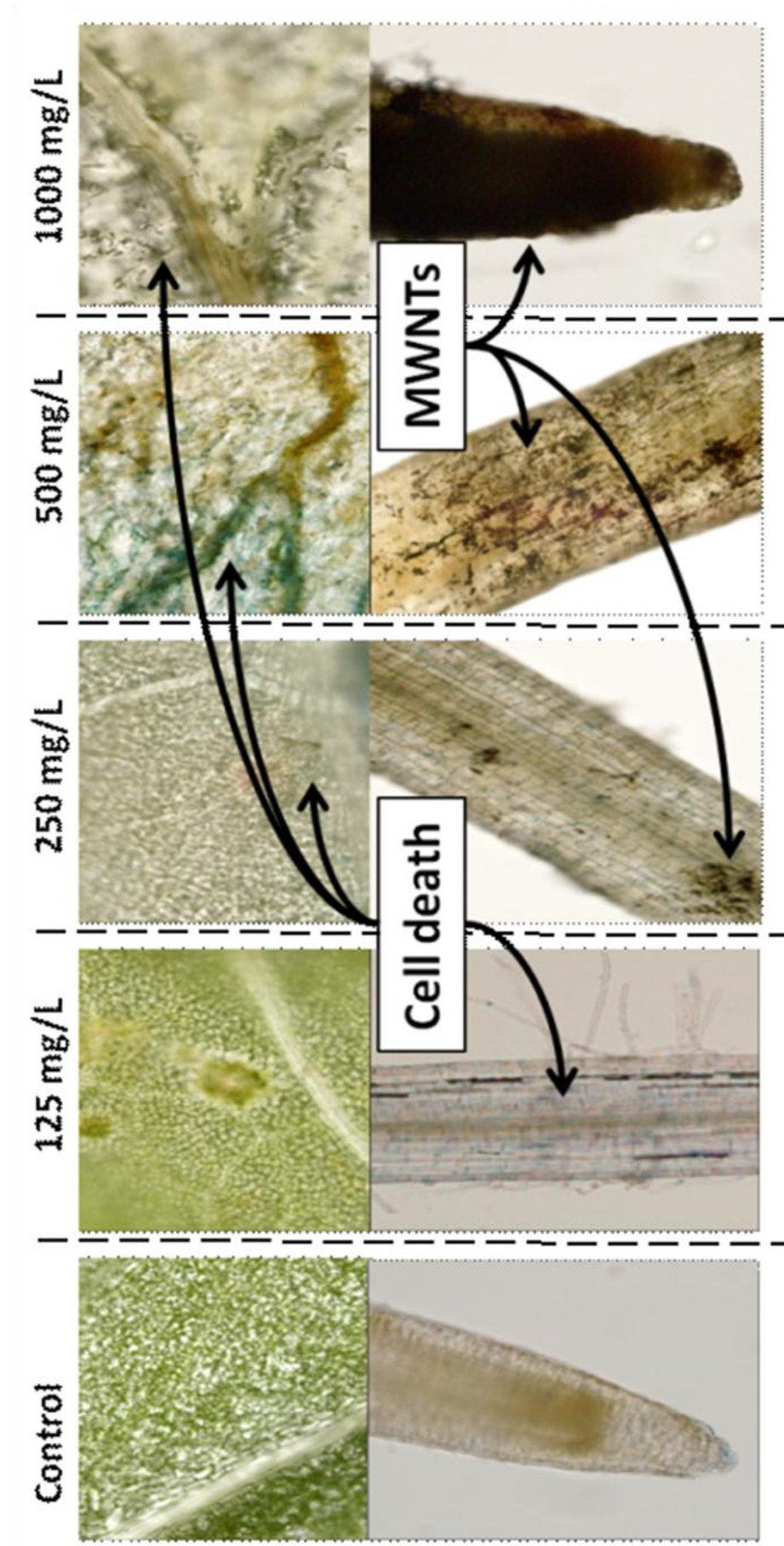


Figure 31 Cell death detection on leaf and root of lettuce.

4.3.5 Effect of MWNTs on reactive oxygen species (ROS)

To investigate the accumulation of H₂O₂ on leaves, we used DAB as labeling to generate a brown colored polymeric oxidation product. The DAB reaction product is discretely localized, providing high resolution images of sub-cellular antigen distribution on leaves [

Figure 32 ROS detection on leaf by DAB assay.].

Figure below showed that polymerization of DAB against H₂O₂ on leaves was gradually increased by dose dependent manner. Those oxidative burst has contributed to Programmed Cell Death (PCD) and rapid activation of defense in adjacent cells. Therefore we proposed the apoptosis as one of PCD categories in case of plant – MWNTs interactions.

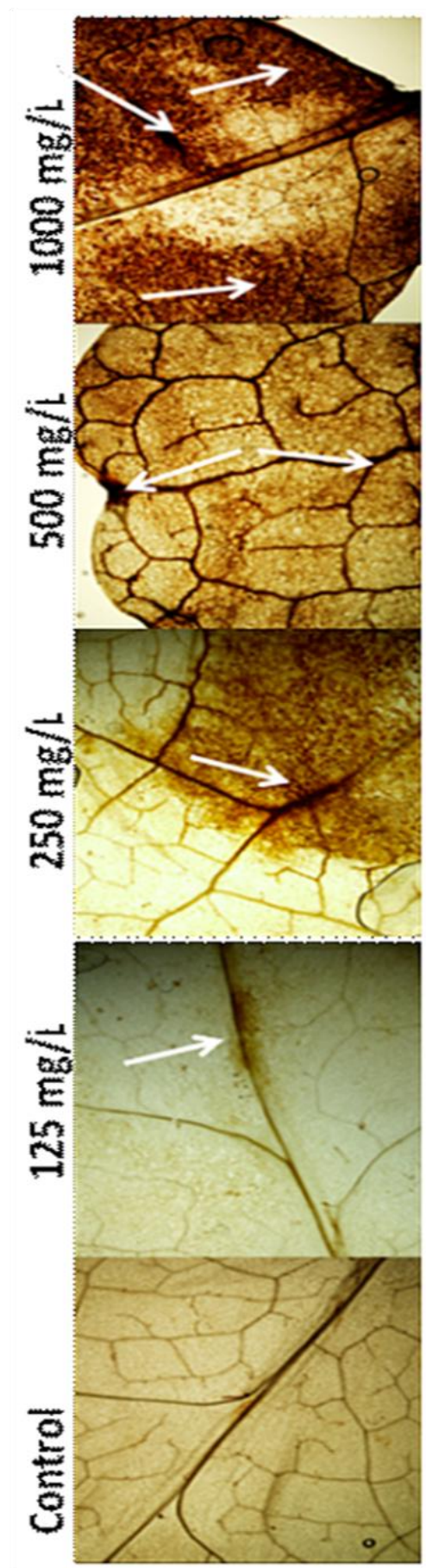


Figure 32 ROS detection on leaf by DAB assay.

4.3.6 Effect of MWNTs on root morphology

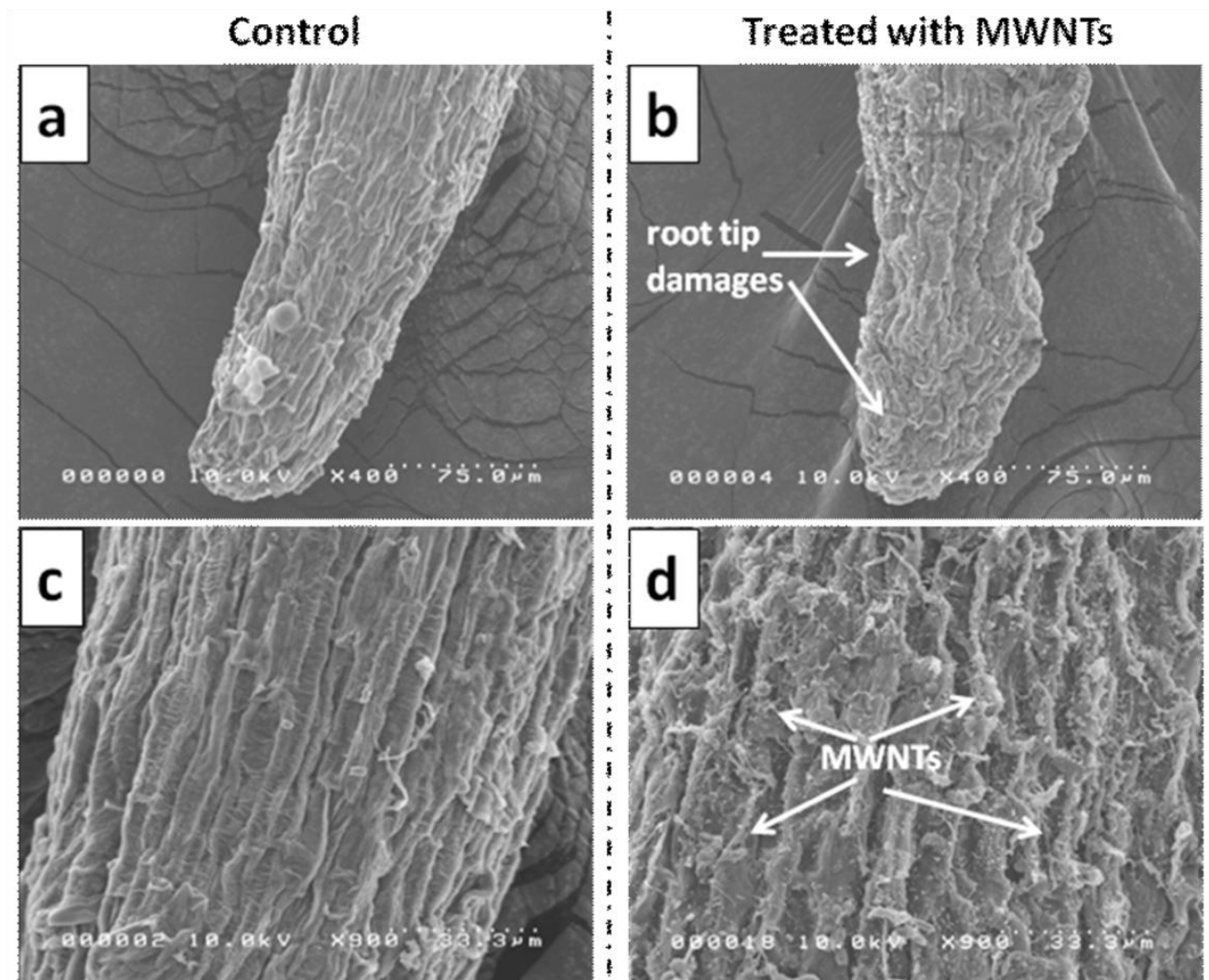


Figure 33 Effect of MWNTs on root morphology of lettuce.

There were morphological damages on the root tip and epidermis as consequence of exposure to MWNTs [Figure 33]. Easily we found the attachment and aggregation of MWNTs on root surface by the increasing concentration of MWNTs.

Deteriorations have been observed under SEM including epidermical injury, damages around root tip and cortex structure [Figure 33]. It has resulted similar symptoms with ZnO nanoparticles reported previously.

4.3.7 Root uptake and translocation of MWNTs

Root tip and root hair could secrete a large amount of mucilage, a highly hydrated polysaccharide, which might contribute to the attachment of MWNTs on the root surface [76]. We considered that MWNTs which covered the root surface could influence the water and nutrient uptake. Finally, it could induce whole process of plant growth development. However, MWNTs also found to be localized on the root surface, very little of MWNTs are found inside of vascular tissue by cross-sectioning analysis showed in figure below:

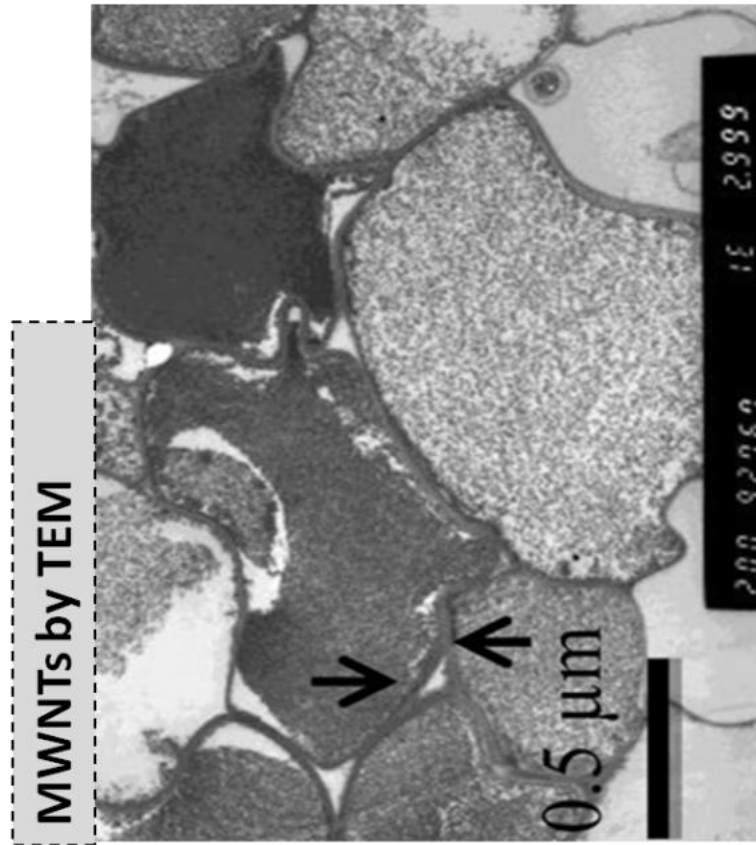
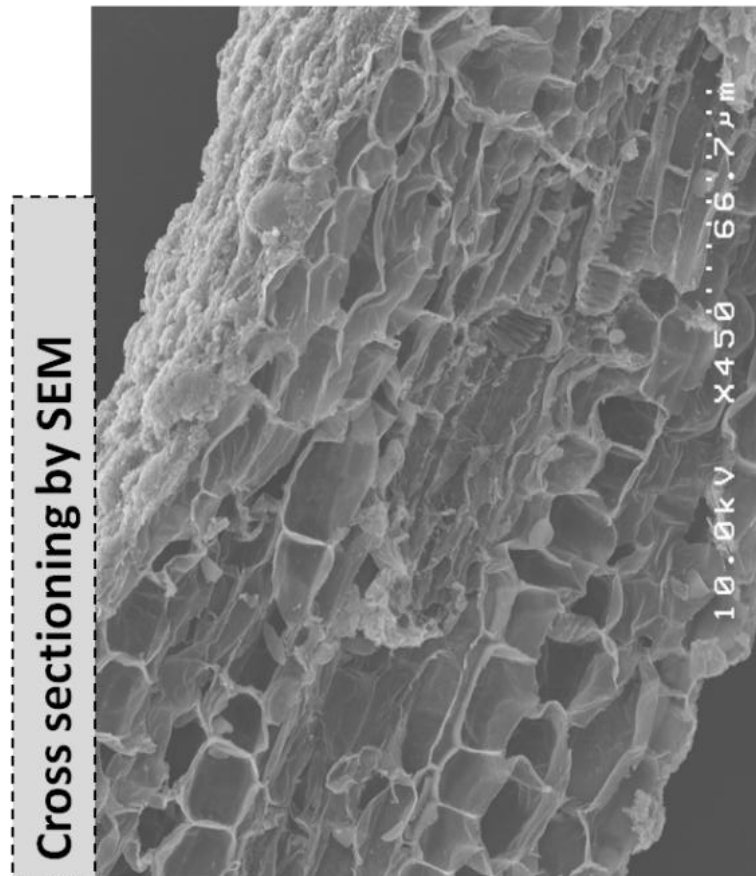


Figure 34 Cross sectioning analysis using SEM and TEM.

Based on figures above, MWNTs have caused the perturbation in epidermal cellular structure. We early assumed that MWNTs were translocated through vascular veins and triggered a systematic damage in entire plant tissue. Other reported the existences of MWNTs inside the root and leaves have been detected by photothermal and photoacoustic method [29].

Interestingly, here we provided the evidence of translocation of MWNTs from root to leaf toward water uptake along vascular vein detected by RAMAN spectroscopy. Localization of aggregated MWNTs on the root surface was strongly in agreement with the previous report [51]. Majority of MWNTs only attached on the surface, almost none of them could pass the rigid structure of plant cell wall (Figure 34). Still, we need further analysis to identify the uptake and translocation of MWNTs through plant tissue at higher magnification as well as another observation method available. Even though plant cell wall is harder than cell membrane but penetration ability of MWNTs through endocytosis mechanism was reported [83].

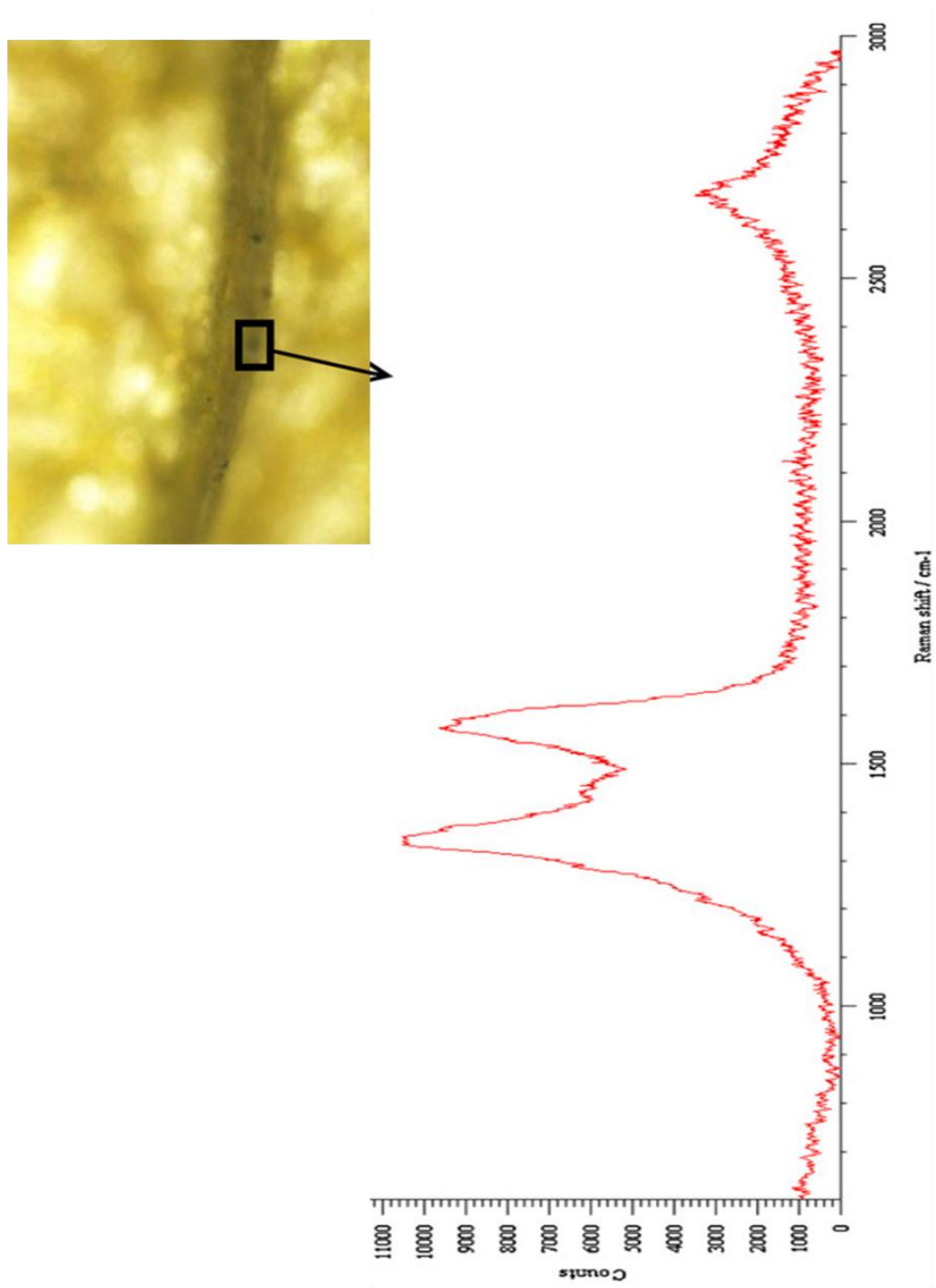


Figure 35 Detection of MWNTs translocated in leaf by RAMAN spectroscopy.

4.4 Conclusion

Morphological assessment of toxic effect of MWNTs compared to CB has been done. Significant results of inhibition effect on root length, root fresh weight, leaf area and leaf fresh weight have emphasized that MWNTs in all concentration range of 125 to 1000 mg/L could decrease the plant growth in a dose dependent manner. Plant cell death was examined by Evans blue assay on root and leaves. ROS was observed and considerably contributed to programmed cell death (PCD) on root and leaves. Attachment and aggregation of MWNTs on root surface might contribute to water or mineral uptake which affects the plant growth development. The uptake, localization and translocation of MWNTs on plant seedling deserve further attention. It may bring a depth understanding on how MWNTs influence the whole process of plant growth development.

Generally materials affect biological organism through the form of dissolved ions which is dominant in either nutrition or (acute) toxicity. This chemical effect is enhanced with downsizing, since specific surface area (surface area/volume) is increased reciprocally proportional to particle size. In the case of bioinert like carbon or non-dissolvable materials like titanium, cells do not react if their size is macro or larger than cell size. But if the size becomes smaller than cell size (about 10 micron), cells regard them automatically foreign objects and make reaction such as phagocytosis. During this bioreaction, cells emit superoxides, cytokines and induce inflammation in tissue in in vivo circumstances. Through this biological process the different function from macro and specific to nano appears like the toxicity of titanium abraded particles produced in the artificial joint although titanium is originally biocompatible.

Nanoparticles are bioreactive and biointeractive in the sense that their size induces the intrinsic function of cells and tissue [84].

Chapter 5th

General Conclusions

- 1. Graphene exerted toxic effects on terrestrial plant species such as cabbage, tomato, and red spinach. Less/no effect on lettuce.**
- 2. MWNTs are toxic to red spinach, lettuce, rice, and cucumber, but not to chili, lady's finger, and soybean.**
- 3. Toxicity of CNMs may depend on dose, time exposure, plant defense mechanism and root structure.**
- 4. Significance of study is the first reported on the phytotoxicity of GO in seedling stage of vegetative plants. The screening test revealed the diverse response of plant species to CNMs.**
- 5. Typical response of each plant corresponds with their physiological characteristic such as seed size, root structure, root numbers and plant defense mechanism.**
- 6. Carbon is inert. That is as material not toxic, but if their size becomes smaller than cell size, it induces cell reaction like superoxide emission.**
- 7. The proposed phytotoxicity mechanism is oxidative stress, more explanations would be described in several figures below;**

Proposed mechanism of phytotoxicity

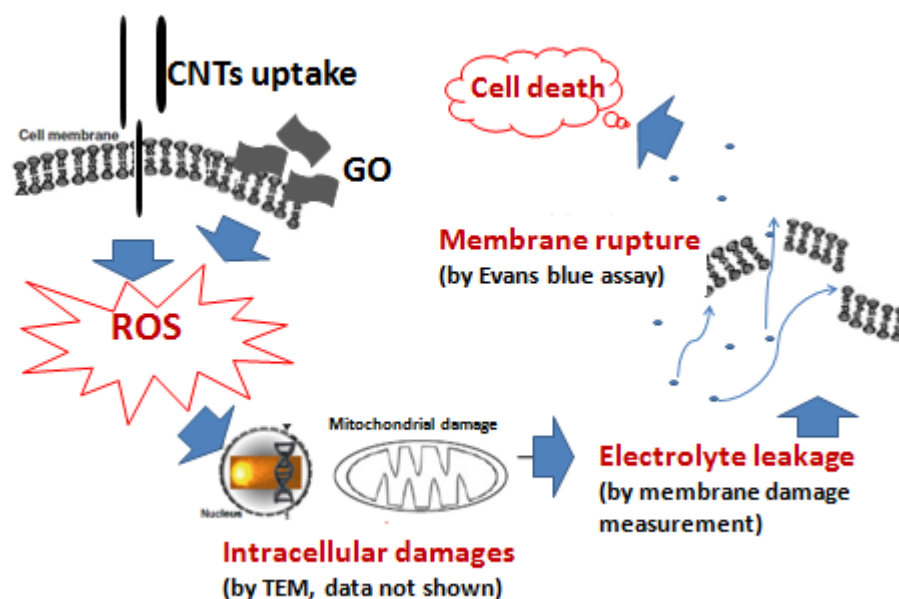
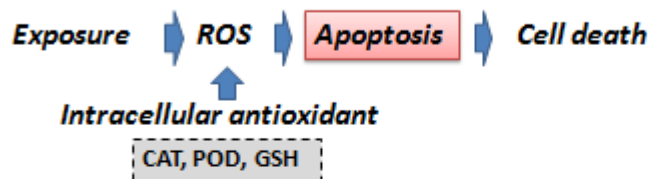


Figure 36 Mechanism of CNMs induce toxicity in plant cells.

Effect of concentration of CNMs

At low concentration (20 – 125 mg/L)



At medium - high concentration (200 – 2000 mg/L)

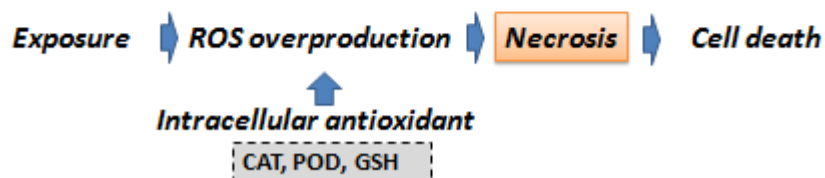


Figure 37 Effect of concentration on phytotoxicity mechanism.

Effect of physical characteristics of CNMs

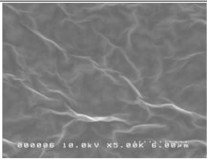
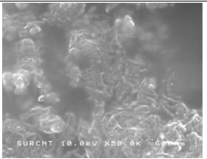
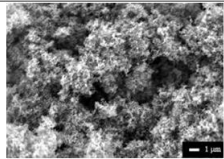
Physical Characteristics	GO	MWNTs	CB
			
Shape	Planar	Cylindrical	Amorphous
Dimension	Thickness about 1 nm Lateral size up to um	Diameter 13 nm Length up to um	Hundreds nm to um
Surface area	Up to 2675 m ² /g	100 - 500 m ² /g	under 100 m ² /g
Colloidal stability	Water soluble GO	Non toxic surfactant	Water soluble CB
Level of toxicity	(++)	(+++)	(+)

Figure 38 Effect of physical characteristics of CNMs on level of toxicity.

Findings of research

1. SPECIFIC CONDITION!

We have confirmed that species used in this experiment were in the first time tested under specific experimental condition. Instead of accumulation of scientific data, the output might be also useful for next study at real environmental condition.

2. EASY HANDLING!

The first report on toxic effect of GO on seedling stage in term of macro scale / in vivo experiment.

3. BROAD RANGE!

The first report on proposing the apoptosis at low level and necrosis at high level of CNMs.

List of Achievements

International publications

1. **Refi IKHTIARI**, Parvin BEGUM, Fumio WATARI, and Bunshi FUGETSU. Toxic Effect of Multiwalled Carbon Nanotubes on Lettuce (*Lactuca Sativa*). *Nano Biomedicine*;5(1):18-24 (Jun 30, 2013).
2. **Refi IKHTIARI**, Parvin BEGUM, Bunshi FUGETSU. Studies of phytotoxicity of carbon-based nanoparticles on edible plants model. *Toxicology Letters*; 211:S164, Supplément, (June17, 2012).
3. Parvin BEGUM, **Refi IKHTIARI**, Bunshi FUGETSU, Makoto MATSUOKA, Tsukasa AKASAKA, Fumio WATARI. Phytotoxicity of multi-walled carbon nanotubes assessed by selected plant species in the seedling stage. *Applied Surface Science*; 262:120– 124 (March 15, 2012).
4. Parvin BEGUM, **Refi IKHTIARI**, Bunshi FUGETSU. Graphene phytotoxicity in the seedling stage of cabbage, tomato, red spinach, and lettuce. *Carbon*; 49; 3907–3919 (May 18, 2011).

International Conference

The 3rd International Symposium on Surface and Interface of Biomaterials (SIB 2011), Sapporo July 12-15th 2011, Poster : 13P23, Toxicity studies of multi-walled carbon nanotubes on plants.

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End of Dissertation