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Analysis of phytotoxicity and plant growth stimulation by multi-walled carbon nanotubes

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"There's Plenty of Room at the Bottom"

Richard P. Feynman,

a Nobel Prize Laureate in Physics

List of abbreviations

AC	activated carbon
AsA	ascorbic acid
ANOVA	analysis of variance
C60, C70	fullerenes
CAL	calcium acetate lactate method of phosphorous content determination
CNMs	carbon nanomaterials
CNPs	carbon nanoparticles
CNTs	carbon nanotubes
cv.	cultivar
CVD	chemical vapor deposition
d	days
DAS	days after sowing
DDE	dichlorodiphenyldichloroethylene
DDT	dichlorodiphenyltrichloroethane
DI water	deionized water
DNA	deoxyribonucleic acid
DMSO	dimethyl sulfoxide
DWCNTs	double-walled carbon nanotubes
EDTA	ethylenediaminetetraacetic acid
FMAD	fullerene-malonic acid derivative
fSWCNTs	functionalized single-walled carbon nanotubes
GO	graphene oxide
GSH	glutathione
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	high performance liquid chromatography

IAA	indole acetic acid
ISTA	International Seed Testing Association
IR spectroscopy	infra-red spectroscopy
MS medium	Murashige and Skoog medium
MWCNTs	multi-walled carbon nanotubes
NBT	nitro blue tetrazolium
NOM	natural organic matter
oMWCNTs	oxidized multi-walled carbon nanotubes
Pro	proline
ROS	reactive oxygen species
RT-qPCR	quantitative real-time polymerase chain reaction.
SA	salicylic acid
SEM	scanning electron microscopy
SOD	superoxide dismutase
SWCNTs	single-walled carbon nanotubes
TEM	transmission electron microscopy
TTC	2,3,5-triphenyl tetrazolium chloride
TF	triphenylformazan,
wsMWCNTs	water soluble multi-walled carbon nanotubes

Publications and conference contributions

Publications

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4**. "Oxidative stress in soybean (*Glycine max.*) seeds due to MWCNTs treatments and its impact on seedling development"** (Zaytseva O., Neumann G.). *Nanocarbon annual conference 2016*. Würzburg (Germany), 23−24 February **2016**.

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1 Summary-Zusammenfassung

1.1 Summary

Nanotechnology is a rapidly expanding area of science and technology, which has gained a great interest due extraordinary properties of nanomaterials with numerous potential fields for practical application. Meanwhile, carbon nanotubes (CNTs) are among the ten most-produced engineered nanomaterials worldwide with applications in automotive industry, building and construction, electronics, and many other industrial sectors, showing also a great potential for integration into environmental and agricultural applications. However, during the last decade it has been demonstrated that nanomaterials can exert significant and extremely variable effects also on living organisms. In higher plants, both, positive and negative responses on growth and development have been reported but the related mechanisms are still not entirely understood. This study presents a systematic assessment of CNT effects on representative crops under standardized conditions with special emphasis on interactions with plant nutrition.

After the introductory background (**Chapter 1**), presenting a comprehensive literature review on carbon nanomaterials with special emphasis on plant responses, environmental and agricultural applications, **Chapter 2** describes the impact of selected multi-walled carbon nanotubes (MWCNTs) on seed germination and early seedling development of different crops (soybean−*Glycine max*, maize−*Zea mays*, and common bean−*Phaseolus vulgaris*). In face of highly variable plant responses to CNT treatments reported in the literature, the study was designed as a systematic analysis under standardized growth conditions, dissecting the effects of one single type of MWCNTs, depending on plant species, MWCNT dosage, duration of exposure to MWCNT treatments, and plant-developmental stage, including imbibition, germination and seedling development. Short-term seed treatments (36 h) with MWCNTs reduced the speed of water uptake particularly by soybean seeds, associated with an increased germination percentage and reduced formation of abnormal seedlings. However, during later seedling development, negative effects on fine root production were recorded for

all investigated plant species. Inhibition of root growth was associated with reduced metabolic activity of the root tissue and a reduction of nitrate uptake, which could be mainly attributed to the smaller root system. The results demonstrated that even under standardized growth conditions largely excluding external factors, plant responses to MWCNT exposure exhibit differences, depending on plant species but also on the physiological status and the developmental stage of individual plants. Soybean was selected as a model plant for further studies since both, positive and negative effects of the same dose of MWCNTs (1000 mg L^{-1}) could be observed even in the same individual plants.

Chapter 3 investigates effects of short-term soybean seed exposure (36 h) to MWCNTs on seedling development, depending on the nutrient availability of the substrate. At 8 DAS stunted growth and poor fine root production were first detectable in seedlings germinating on moist filter paper without additional nutrient supply. This effect was preceded by reduced metabolic activity of the seedling tissues detectable by vital staining already at 2 DAS. Root growth inhibition was a long-lasting effect, detectable in soil culture up to 38 DAS. More detailed investigations revealed zinc (Zn) deficiency as a major growth-limiting factor. The growth of affected soil-grown plants was recovered by foliar application of $ZnSO_4$ or by cultivation in nutrient solution supplied with soluble $ZnSO_4$.

A more detailed investigation of the physiological mechanisms related with the inhibitory effects of MWCNTs on plant growth is presented in **Chapter 4.** Oxidative stress was identified as a major factor determining MWCNT-induced root growth inhibition in soybean, demonstrated by recovery of root development after external supplementation with antioxidants. Induction of oxidative stress by MWCNT application was detectable already after the 36 h imbibition period particularly in the tips of the radicle as indicated by accumulation of superoxide anions, reduced triphenyltetrazolium chloride vital staining, and induction of superoxide dismutase activity. The expression pattern of the oxidative stress indicators coincided with preferential accumulation of MWCNTs in the cells of the root tip and was reverted by external application of proline as antioxidant. MWCNT-induced plant damage could be

reverted by external supplementation of micronutrients (Zn, Cu, Mn) as important cofactors for various enzymes involved in oxidative stress defense (SOD, biosynthesis of antioxidative phenolics). Accordingly, SOD activity increased in seedling roots after Zn supplementation. During germination, the CNT treatments inhibited particularly the Zn translocation from the cotyledons to the growing seedling, and CNTs exhibited a selective adsorption potential for Zn and Cu, which may be involved in internal immobilization of micronutrients. Therefore, this study demonstrated for the first time that phytotoxicity of CNTs is linked with disturbances of micronutrient homeostasis during seedling development. Implications for environmental phytotoxicity assessment of MWCNTs and their potential applications in agriculture are discussed in a final overview presented in **Chapter 5**.

1.2 Zusammenfassung

Nanotechnologien repräsentieren ein extrem schnell expandierendes, wissenschaftliches und technologisches Arbeitsfeld, das aufgrund herausragender Eigenschaften und zahlreicher potenzieller, praktischer Anwendungsmöglichkeiten von Nanomaterialien derzeit große Aufmerksamkeit erfährt. Sogenannte „Carbon Nanotubes (CNTs)“ gehören mittlerweile weltweit zu den zehn meistproduzierten Nanomaterialen mit Anwendungen für Verkehrs,- und Transporttechnologien, im Bau-, und Ingenieurswesen, in der Elektrotechnik und vielen anderen Anwendungsbereichen, mit vielversprechenden Möglichkeiten auch in der Umwelttechnik und der Landwirtschaft. Während der vergangen zehn Jahre wurde allerdings deutlich, dass Nanomaterialien signifikante und oft extrem variable Wirkungen auch auf lebende Organismen haben können. Bei höheren Pflanzen wurden sowohl positive als auch negative Effekte auf das Wachstum und die pflanzliche Entwicklung berichtet, und die zugrundeliegenden Mechanismen sind nach wie vor unklar. In der vorliegenden Untersuchung wird eine systematische Bewertung von CNT Wirkungen auf repräsentative Kulturpflanzenarten unter standardisierten Bedingungen vorgestellt, mit besonderem Augenmerk auf mögliche Wechselwirkungen mit der pflanzlichen Ernährung.

Nach einem einleitenden Überblick (**Kapitel 1**), der eine umfassenden Literaturstudie zu Carbon-Nanomaterialien mit Schwerpunkt auf pflanzlichen Wechselwirkungen und Anwendungsperspektiven in der Umwelttechnologie und in der Landwirtschaft vorstellt, beschreibt **Kapitel 2** die Wirkungen ausgewählter, so genannter „Multiwalled Carbon Nanotubes (MWCNTs)“ auf die Keimung, und die Keimlingsentwicklung verschiedener Kulturpflanzenarten (Soja – *Glycine max*, Mais – *Zea mays*, und Phaseolus-Bohne – *Phaseolus vulgaris*). Vor dem Hintergrund der extrem heterogenen Wirkungen, die in der Literatur beschrieben werden, wurde eine systematische Analyse unter standardisierten Wachstumsbedingungen in Abhängigkeit der Pflanzenart, der MWCNT-Dosierung, der Dauer der MWCNT-Behandlung, und des pflanzlichen Entwicklungsstadiums durchgeführt. Kurzzeitige Saatgutbehandlungen mit MWCNTs (36 Std.) verminderten die Geschwindigkeit der Wasseraufnahme während der Einquellungsphase besonders bei Soja-Saatgut, was mit einer verbesserten Keimrate

und einem verminderten Anteil abnorm entwickelter Keimlinge verbunden war. Während der weiteren Keimlings-entwicklung ergaben sich jedoch negative Wirkungen auf die Feinwurzelbildung bei allen untersuchten Pflanzenarten. Die Wurzelwachstumshemmung ging mit verminderter Stoffwechselaktivität im Wurzelgewebe und verminderter Nitrataufnahme einher, die hauptsächlich auf das schwächer entwickelte Wurzelsystem zurückzuführen war. Die Ergebnisse zeigen, dass selbst unter standardisierten Anzuchtbedingungen, die externe Einflussfaktoren weitestgehend ausschließen, unterschiedliche Wirkungen von MWCNTs in Abhängigkeit von der Pflanzenart, doch auch vom physiologischen Status und dem pflanzlichen Entwicklungsstadium, selbst bei individuellen Pflanzen auftreten können. Soja wurde in diesem Zusammenhang als Modellpflanze für weiterführende Studien ausgewählt, da hier sowohl positive als auch negative Wirkungen bei identischer MWCNT-Dosierung (1000 mg L^{-1}) individuell an derselben Versuchspflanze auftraten.

Kapitel 3 untersucht die Wirkungen kurzzeitiger MWCNT-Saatgutexposition (36 Std.) auf die Keimlingsentwicklung von Soja in Abhängigkeit der Nährstoffverfügbarkeit im Anzuchtsubstrat. Bereits acht Tage nach der Aussaat wurde erstmals gehemmtes Pflanzenwachstum und verminderte Feinwurzelentwicklung bei Keimlingen nachweisbar, die auf Keimpapier ohne weiteres Nährstoffangebot wuchsen. Dieser Hemmung ging eine, durch Vitalfärbung nachgewiesene Verminderung der Stoffwechselaktivität, bereits zwei Tage nach Aussaat voraus. Die resultierende Wurzelwachstumshemmung war jedoch ein langfristiger Effekt, der auch noch 38 Tage nach Aussaat bei Pflanzen in Bodenkultur nachweisbar war. Genauere Untersuchungen ergaben, dass Zink-(Zn)-Mangel einer der wichtigsten Wachstumslimitierenden Faktoren war. Das Wachstum von Pflanzen in Bodenkultur konnte durch Blattdüngung mit Zinksulfat regeneriert werden, während Zinksulfat-haltige Nährlösung in hydroponischer Kultur wirksam war.

Eine detailiertere Analyse physiologischer Grundlagen der Hemmwirkung von MWCNTs auf das Pflanzenwachstum wird in **Kapitel 4** vorgestellt:

Oxidativer Stress wurde als Hauptfaktor für die MWCNT-induzierte Wurzelwachstums-hemmung bei Soja identifiziert, was durch die revertierende Wirkung der

Supplementierung mit Antioxidantien auf das Wurzelwachstum bestätigt wurde. Die durch MWCNTs ausgelöste oxidative Stressreaktion war bereits innerhalb der 36-stündigen Saatgutquellungsperiode anhand der Akkumulation von Superoxid-Anionen, verminderter Vitalfärbung mit Triphenyltetrazoliumchlorid und erhöhter Aktivität der Superoxiddismutase (SOD) nachweisbar und erfasste besonders die Keimwurzelspitzen. Das Expressionsmuster dieser oxidativen Stressindikatoren spiegelte die präferentielle Akkumulation von MWCNTs in den Zellen der Wurzelspitze wider und konnte durch externe Applikation von Prolin als Antioxidant revertiert werden. Die MWCNT-induzierten Pflanzenschäden waren ebenso durch die Supplementierung mit Mikronährstoffen (Zn, Cu, Mn) revertierbar, die als wichtige Co-Faktoren für Enzymsysteme der oxidativen Stressabwehr fungieren (SOD, Synthese antioxidativer Phenole). Entsprechend konnte ein Anstieg der SOD Aktivität im Wurzelgewebe MWCNT-behandelter Keimlinge nach Zn Applikation nachgewiesen werden. Die MWCNT-Behandlung hemmte während der Keimung besonders die Zn-Translokation aus den Kotyledonen in die jungen, wachsenden Gewebe des Keimlings und MWCNTs zeigten ein selektives Adsorptionspotenzial besonders für Zn und Cu, das so an einer intrazellulären Immobilisierung dieser Mikronährstoffe beteiligt sein könnte.

Damit zeigt die vorliegende Arbeit erstmals, dass die Phytotoxizität von CNTs mit Störungen der Mikronährstoffhomöostase während der Keimlingsentwicklung in Verbindung steht. Die Bedeutung für die Phytotoxizitätsbewertung von MWCNTs und ihrer potenziellen Anwendungen in der landwirtschaftlichen Praxis werden in einer Abschlussbetrachtung diskutiert (**Kapitel 5**).

2 Chapter I. General introduction. Carbon nanomaterials: production, impact on plant development, agricultural and environmental applications

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2.1 Abstract

During the relatively short time since the discovery of fullerenes in 1985, carbon nanotubes in 1991, and graphene in 2004, the unique properties of carbon-based nanomaterials have attracted great interest, which has promoted the development of methods for large-scale industrial production. The continuously increasing commercial use of engineered carbon-based nanomaterials includes technical, medical, environmental and agricultural applications. Regardless of the application field, this is also associated with an increasing trend of intentional or unintended release of carbon nanomaterials into the environment, where the effect on living organisms is still difficult to predict. This review describes the different types of carbon-based nanomaterials, major production techniques and important trends for agricultural and environmental applications. The current status of research regarding the impact of carbon nanomaterials on plant growth and development is summarized, also addressing the currently most relevant knowledge gaps.

Keywords: carbon-based nanomaterials, carbon nanotubes, fullerenes, graphene, germination, plant development, agricultural application.

2.2 Introduction

Some chemical elements are able to compose a range of different molecular structures from the same type of atoms–a unique feature known as "allotropy". Different chemical and physical properties of those materials are determined by the structural geometry of the atoms and the type of chemical bounds within the molecules. In this context, carbon is one of the most interesting elements, with the ability to form a wide range of structures, frequently with fundamentally different properties. Classical examples of carbon allotropes comprise "hard" diamond and "soft" graphite used in science and technology and in a wide range of products, including consumer goods in various areas of human activity [1].

The list of known carbon allotropes has expanded during the last decades of the 20th century after the discovery of several new low-dimensional carbon forms. The novel materials comprised carbon nanotubes (CNTs), fullerenes and graphene and attracted high interest from science and industry, since these materials exhibited a wide range of outstanding and novel features as promising materials for numerous application fields. Based on these properties, they were repeatedly termed as "wonder materials" in the scientific literature [2–5].

Natural carbon-based nanoparticles exist only in negligible quantities, and the overwhelming majority are engineered, or artificially synthesized. Therefore, their availability does not depend on natural reserves (such as diamonds), and theoretically production can be performed in unlimited quantities as long as raw materials for synthesis are available. According to latest forecasts, a constant increase of production volumes is expected during the next decade [6, 7]. However, despite the fact that carbon-based nanomaterials promote industrial progress there are concerns about a potential release into the environment and interactions of released nanomaterials with living organisms and incorporation into food chains with yet unknown consequences.

In the face of the increasing importance of practical applications, this review will focus on two major aspects associated with the handling of carbon-based nanomaterials considering:

(i) production and potential applications, with special focus on the environmental and agricultural sectors, and the significance for the improvement and development of novel, efficient products and technologies;

(ii) potential impact on living organisms with a special focus on plants, as a fundamental component of food chains in natural and agricultural ecosystems, where increased input of carbon nanomaterials can be expected as a consequence of intentional use in agricultural and environmental applications or by accidental release as unintended contamination.

2.3 Classification of carbon-based nanomaterials

Carbon is one of the few chemical elements (including also silicone) with the ability to polymerize at the atomic level, thus forming very long carbon chains. Due to the four electrons in the outer electron layer (Figure 2.1 A, B), carbon atoms have a valence of four and can be linked via single, double or triple covalent bonds, or also with other elements. These properties of carbon atoms can be attributed to their special electron structure and the smaller size compared with other elements of group IV.

For the reasons specified above, carbon can exist in a range of different molecular forms, composed by the same type of atoms but due to different structures, possessing different properties. These forms are termed as “allotropes” or “allotropic modifications” of a certain chemical element. Until recently, only two natural carbon allotropes were known: diamond and graphite. Meanwhile, various new allotropic forms have been described, including carbon nanomaterials. In general, nanomaterials are defined as materials containing particles with at least one dimension between 1 and 100 nm in size [8]. All nanomaterials composed of carbon atoms are termed as carbon-based or carbon nanomaterials. Classification of carbon-based nanomaterials is most commonly performed according to their geometrical structure. Carbon nanostructures include particles which can be tube-shaped, horn-shaped, spherical or ellipsoidal. Nanoparticles having the shape of tubes are termed as carbon nanotubes; horn-shaped particles are nanohorns and spheres or ellipsoids belong to the group of fullerenes. In the meantime, carbon nanomaterials have numerous technical applications including micro- and

nanoelectronics, gas storage, production of conductive plastics, composites, displays, antifouling paints, textiles, batteries with improved durability, gas biosensors and others [9–11].

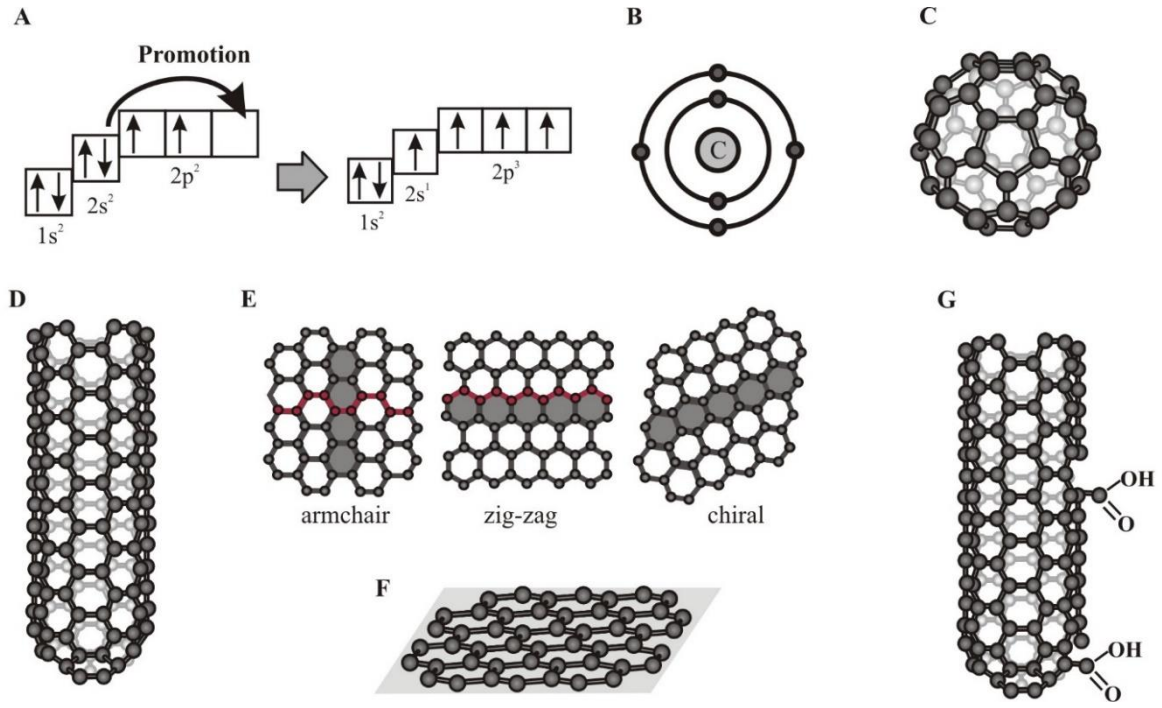

Figure 2.1. *Structure of a carbon atom and of carbon-based nanoparticles. (A) Electrone configuration of a carbon atom before and after promotion of one s-electrone; (B) schematic representation of a carbon atom structure with two electron orbtales arownd the nucleus and six electrones distributed on them; (C) structure of a fullerene C60; (D) structure of a single-walled nanotube; (E) different types of single-walled nanotubes: armchair, zig-zag and chiral; (F) structure of a graphene sheet; (G) structure of an oxidazed single-walled nanotube.*

2.3.1 Fullerenes

Fullerenes are an allotropic modification of carbon, often termed as a molecular form of carbon, or carbon molecules. Fullerenes were discovered in 1985 by H.W. Kroto, R.F. Curl and R.E. Smalley [12] who were later awarded with the Nobel Prize for Chemistry in 1996. The fullerene family includes a number of atomic C_n clusters (n > 20), composed of carbon atoms on a spherical surface. Carbon atoms are usually located on the surface of the sphere at the vertices of pentagons and hexagons. In fullerenes, carbon atoms are usually present in the sp^2-hybrid form and linked together by covalent bonds. Fullerene C60 is the most common and best-investigated fullerene. The spherical molecule is highly symmetric and consists of 60 carbon atoms, located at the vertices of twenty hexagons and twelve pentagons (Figure 2.1 C). The diameter of fullerene C60 is 0.7 nm [13].

2.3.2 Carbon nanotubes (CNTs)

Among other carbon-based nanomaterials, CNTs are one of the carbon allotropes with exceptional properties suitable for technical applications. They were discovered in 1991 by the Japanese researcher S. Iijima. Carbon nanotubes are characterized by cylindrical structures with a diameter of several nanometers, consisting of rolled graphene sheets (Figure 2.1 D). Carbon nanotubes may vary in length, diameter, chirality (symmetry of the rolled graphite sheet) and the number of layers. According to their structure, CNTs may be classified into two main groups: single-walled nanotubes (SWCNTs) and multi-walled nanotubes (MWCNTs). Some researchers additionally isolate double-walled carbon nanotubes (DWCNTs) as a separate class of CNTs. Generally SWCNTs have a diameter around 1−3 nm and a length of a few micrometers. Multi-walled CNTs have a diameter of 5−40 nm and a length around 10 μm. However, recently synthesis of CNTs with a length of even 550 mm has been reported [14]. The structure of CNTs leads to exceptional properties with a unique combination of rigidity, strength and elasticity compared with other fibrous materials. For instance, CNTs exhibit considerably higher aspect ratios (length to diameter ratios) than other materials, and larger aspect ratios for SWCNTs as compared with MWCNTs due to their smaller diameter. Additionally,

CNTs show high thermal and electrical conductivity compared to other conductive materials. Electrical properties of SWCNTs depend on their chirality or hexagon orientation with respect to the tube axis. Accordingly, SWCNTs are classified into three sub-classes: (i) armchair (electrical conductivity > copper), (ii) zigzag (semi-conductive properties) and (iii) chiral (semi-conductive properties) (Figure 2.1 E). By contrast, MWCNTs consisting of multiple carbon layers, frequently with variable chirality, can exhibit extraordinary mechanical properties instead of outstanding electrical characteristics.

2.3.3 Graphene

Graphene is a two-dimensional allotropic form of carbon, formed by single layers of carbon atoms (Figure 2.1 F). In graphene, carbon atoms exhibit sp^2-hybridization connected by σ- and π-bonds in a two-dimensional hexagonal crystal lattice with a distance of 0.142 nm between neighboring atoms of carbon hexagons. Graphene also represents a structural element of some other carbon allotropes, such as graphite, carbon nanotubes and fullerenes.

Theoretical studies on graphene began a long time before the real material samples were obtained. The Canadian theoretical physicist P. R. Wallace first explored the theory of graphene in 1947, while the first graphene samples were described 57 years later (in 2004) by A. Geim (Dutch-British physicist) and K. Novoselov (Russian-British physicist), awarded with a Nobel prize in 2010.

Despite the long history of theoretical investigation, the fact that the real material has been obtained only recently, implies that comprehensive studies on the properties of graphene are still ongoing. Graphene has many unique physical properties, such as extremely high mechanical rigidity and a high thermal stability. Also the electric properties of this carbon allotrope are fundamentally different from the properties of three-dimensional materials.

2.4 Synthesis of carbon-based nanomaterials

2.4.1 Industrial synthesis of carbon-based nanomaterials

2.4.1.1 Fullerenes

Since the discovery of carbon-based nanomaterials, their outstanding properties have been intensively studied and different methods for synthesis have been developed. The basic components for carbon nanomaterial production are carbon vapors. Fullerenes were produced for the first time by W. Krätschmer and D.R. Huffman in 1990 by evaporation of graphite electrodes in a helium atmosphere [15, 16]. Later, a reactor was modified by establishing an electric arc between two graphite electrodes. The resulting soot condenses on the cold surface of the reactor, and is collected and processed in boiling toluene, benzene, xylene, or other organic solvents. After evaporation of the solvents a black condensate is formed, containing about 10−15% of C60 and C70 fullerenes, as well as small amounts of higher fullerenes. Depending on the synthesis parameters, the ratio between the C60 and C70 fullerenes varies, but typically C60 represents the dominant fraction. The described arc-discharge method belongs to the large family of plasma methods which are most popular and commonly used compared to other techniques [17]. However, the practical use of fullerenes is limited due to high costs and the low productivity of the methods currently available for their synthesis.

2.4.1.2 Carbon nanotubes

Arc discharge, laser ablation and chemical vapor deposition (CVD) are basic methods for CNT synthesis [18]. Currently, one of the most investigated and commonly used techniques for CNT production is chemical vapor deposition (CVD) [19]. In contrast to two other methods (arc discharge and laser ablation), CVD synthesis requires simpler equipment and milder conditions in terms of temperature and pressure, making it more suitable for the large-scale production of CNTs [20]. CVD synthesis is based on decomposition of hydrocarbons to carbon, and subsequent synthesis of carbon nanostructures on various substrates containing catalysts on which the nanotubes are growing. Metal-based nanoparticles are frequently used as catalysts and their size

strongly correlates with the diameter of nanotubes synthesized on it (0.5−5 nm—for SWCNTs, 8−100 nm—for MWCNTs synthesis). Nickel, cobalt or iron nanoparticles are usually acting as catalysts for the synthesis of SWCNTs and MWCNTs.

Reactors for CVD synthesis generally consist of a reaction chamber and tubes filled with inert gas and hydrocarbon (Figure 2.2 A). Methane is frequently used for SWCNT production, while ethylene or acetylene for MWCNTs. As a simplified process description, the substrate is heated up to 850−1000 °C in case of SWCNT and up to 550−700 °C for MWCNT production. Carbon is formed by thermal decomposition of hydrocarbons and dissolves in the metal nanoparticle catalyst. After reaching a certain threshold concentration of carbon, it forms a semi-fullerene cap, as a starting structure for the growth of a cylindrical shell nanotube, formed by increased flow of carbon from the hydro-carbon source to the catalyst particle (Figure 2.2 B, C). Final removal of the catalysts from the tips of the nanotubes and further purification are still under development and optimization in order to yield CNTs of a higher quality [21, 22].

Industrial applications of CNTs and especially of SWCNTs require homogeneous materials with specific properties. However, shifting from well-controlled laboratory conditions to large-scale production frequently results in heterogeneous products, containing impurities of amorphous carbon, carbon fiber, catalyst residuals and other nanoparticles. Therefore, CNT production frequently requires further purification and separation steps, substantially increasing the production costs. One of the major challenges regarding controlled synthesis of SWCNTs is related to difficulties in obtaining small metal-catalyst particles, their equal dispersion on the substrate and prevention of aggregation. For instance, sintering of fine catalysts into larger particles, leads to an increased diameter of SWCNTs or to formation of DWCNTs and MWCNTs.

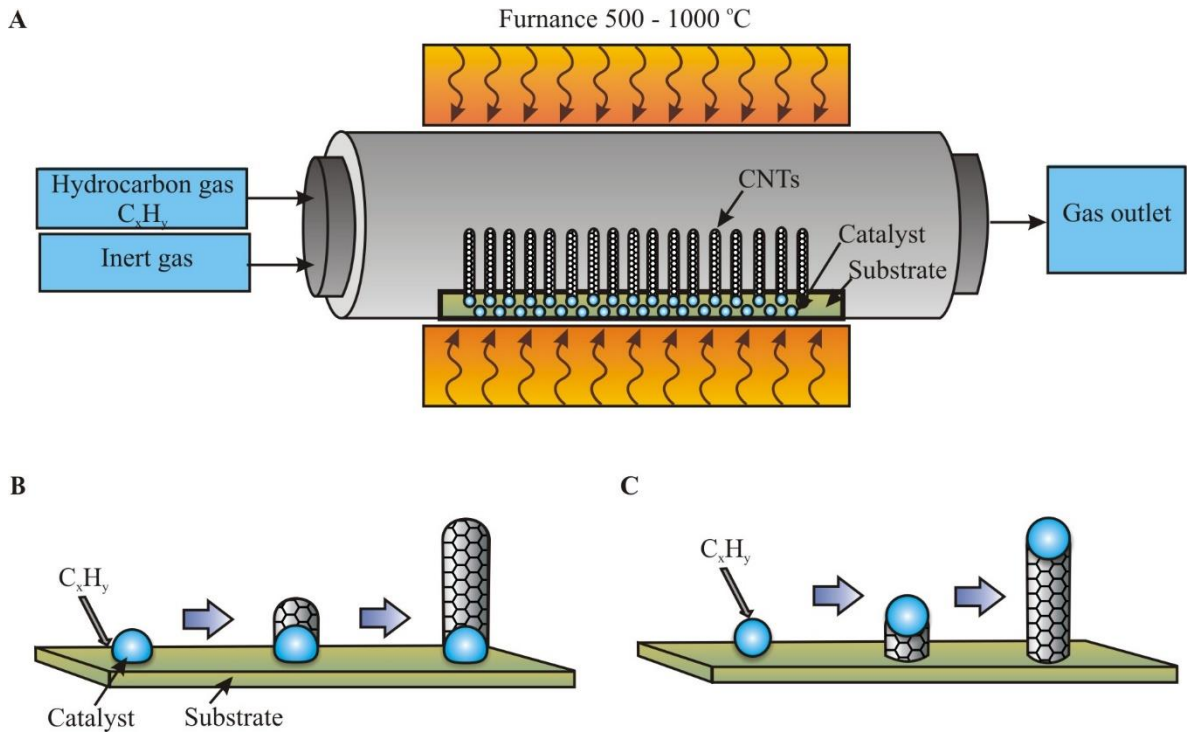

Figure 2.2. *Schematic representation of chemical vapor deposition (CVD) process. (A) Simplified scheme of a CVD reactor for CNTs synthesys; (B) base-growth model of CNT growth mechanism; (C) tip-growth model of CNT growth mechanism.*

Chirality, as another structural feature of SWCNTs, also depends on synthesis conditions and the growth mechanism of the nanotubes. Very often, the yielded products represent a mixture of conductive and semi-conductive SWCNTs, requiring further extraction steps to obtain SWCNTs with defined chirality for specific applications. Establishing convenient methods for sorting nanotubes according to their characteristics (diameter, chirality) and eliminating various undesired impurities will significantly contribute to the developmental progress towards improved applications. Another important area of research is synthesis of vertically or horizontally aligned CNTs, which have numerous structural advantages as compared to bundles of agglomerated CNTs. Production of MWCNTs seems to be less complicated and expensive. However, the controlled formation of inner and outer diameters or defined numbers of walls are still major challenges.

2.4.1.3 Graphene

Since graphene sheets were first obtained by A. Gheim and K. Novoselov, using mechanical splitting of graphite with adhesive tape [23], great progress in graphene research has been made, and meanwhile various methods for graphene production are available [24]. These techniques are based on obtaining nanoscale graphene sheets by splitting or cutting materials, such as graphite or nanotubes [25], using a range of physical or chemical methods. Production of graphene sheets by CVD synthesis or laser ablation methods is also possible. The different methods are able to provide graphene or reduced graphene oxide sheets of different qualities, depending on the requirements of the corresponding applications. Graphene of moderate quality for structural applications can be obtained in large quantities with relatively low production costs. High quality graphene for electronic devices produced in smaller quantities, are usually more expensive. Liquid phase and thermal exfoliation of graphite, CVD synthesis (potentially most cost effective) and synthesis on silicon carbide—are the major methods suitable for mass production of graphene [24].

2.4.2 Chemical functionalization of carbon-based nanoparticles

A wide variety of carbon-based nanomaterials can be further expanded by so-called chemical functionalization. Very often, nanoparticles are functionalized by linking certain molecules to the nanoparticle surface, in order to modify the physical and chemical properties of the particles [26], which in turn greatly expands the field of applications [26, 27]. One example for functionalization of carbon-based nanoparticles is an oxidation of CNTs. This process comprises an ultrasonic treatment of nanotubes in a mixture of acids, leading to attachment of carboxylic functional groups (–COOH) on the sidewalls of the nanotubes (Figure 2.1 G). Oxidized CNTs acquire solubility in aqueous solutions, but retain their mechanical and electrical properties. Moreover, carboxylic groups attached to the nanotube surface can serve as sites for further functionalization. There are also examples of functionalized fullerenes and graphene.

2.4.3 Naturally occurring carbon nanomaterials

Apart from engineered nanomaterials, naturally occurring carbon-based nanoparticles have also been identified [28, 29]. Velasco-Santos et al. [28] reported the presence of carbon nanotubes in a coal-petroleum mix. For SWCNT synthesis using the CVD method, Su and Chen [30] and Mracek et al. [31] used volcanic lava as a substrate and catalyst, containing particles of metal oxides. The authors speculated that this process may provide evidence for a possible formation of nanotubes under natural conditions when the temperature rises extremely high e.g. during volcano eruptions. Beside carbon nanotubes, there is also evidence for the occurrence of fullerenes in geological materials. Fullerenes have been detected in the natural mineral shungit from Karelia in low concentrations (2% w/w) [32–35] and also in meteorite samples of cosmic origin [36].

Interestingly, the spherical structure of fullerenes seems to be not only restricted to carbon nanomaterials. Recently, fullerene-like structures have been described in pollen grains of Chinese hibiscus (*Hibiscus rosa-sinensis*), with putative functions for mechanical stability and adaptive properties important for the pollination process [37].

2.5 Potential applications of carbon-based nanomaterials

The unique physical and chemical properties of carbon-based nanomaterials determine a wide range of options for practical applications, which in turn trigger the increase of their production. The most widespread field of applications has been reported for CNTs. In 2013, the industrial production of CNTs already exceeded several thousand tons [9]. Due to their mechanical properties, namely the high tensile strength, they are incorporated into polymers and other materials in order to create structural and composite materials with advanced properties [38]. For instance, a material obtained by directly growing CNTs in a cement matrix had double the compressive strength of the original [39]. Carbon nanotubes allow to producing not only very strong, but at the same time extraordinarily light materials with application fields in the production of wind-turbine blade materials [40, 41] and marine turbines [42], in the automotive industry [43], aviation [44] or in sport equipment [9]. Other areas where carbon nanotubes can be used include various electronic applications [9, 45]. Fullerenes and their derivatives

can be used in medicine [46], including drug and gene delivery [47] or in cosmetics [48–50]. Graphene has numerous applications as well: it can be used in electronics, various biochemical sensors, in solar cells and others summarized in a recently published review by Choi et al. [51]. Beside the examples listed above, carbon-based nanomaterials also have numerous potential applications in the environmental and agricultural sectors, summarized in Figure 2.3.

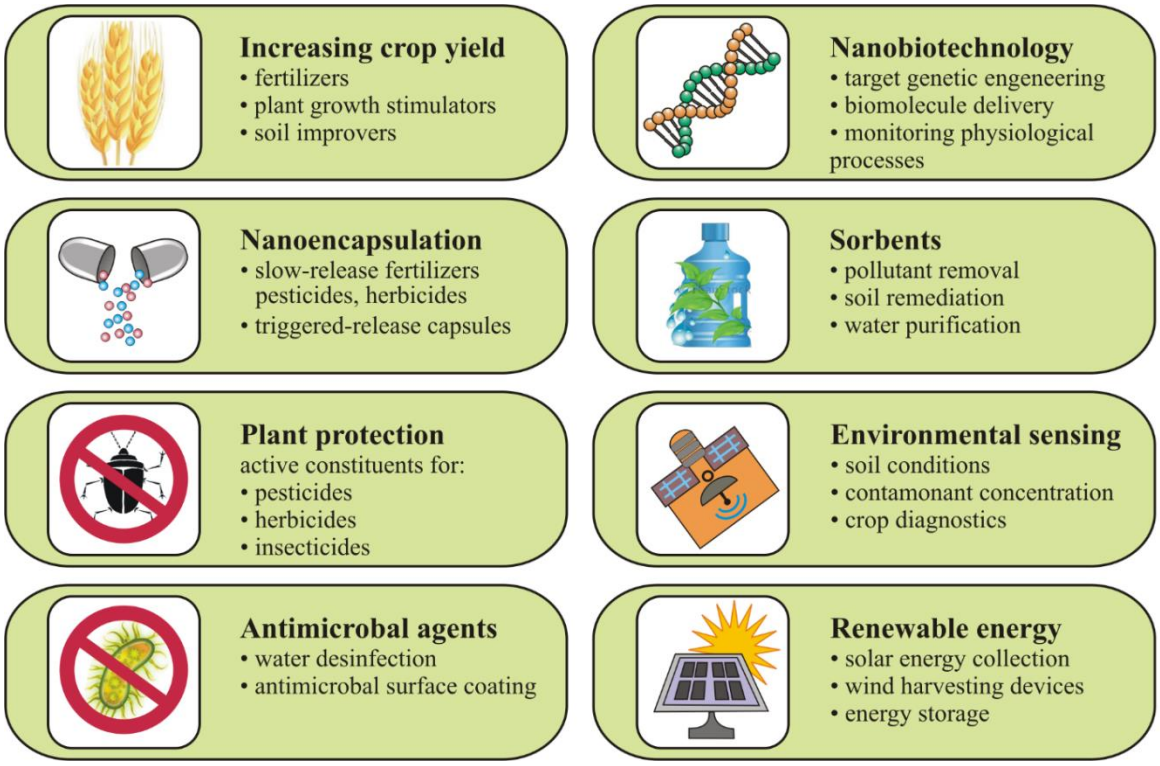

Figure 2.3. *Potential applications of carbon-based nanomaterials in environmetal and agricultural sectors. See text for details.*

2.5.1 Environmental applications of carbon-based nanomaterials

Environmental pollution is one of the major global challenges since pollutants of different nature contaminate urban and agricultural areas. In order to improve pollutant remediation strategies for environmental sustainability, there is a need to increase the efficiency of conventional methods or to introduce innovative approaches. In this context, nanotechnology, and especially carbon-based nanomaterials can greatly

contribute because they possess an enormous absorption potential due to their high surface area.

Activated carbon (AC) has been widely used as a sorbent for conventional wastewater treatment due to its large surface area and ability to adsorb a broad spectrum of organic and inorganic contaminants. However, AC has slow adsorption kinetics; it is a nonspecific adsorbent and its effectiveness against microorganisms is low. The presence of solids, oil and grease in wastewater often causes pore blockage in AC. Moreover, AC is frequently removed together with the adsorbed pollutants and therefore, needs to be replaced in regular intervals. In this context, utilization of carbon-based nanomaterials has a promising potential to improve wastewater filtration systems with numerous examples in the available literature [52–55]. It has been reported that the adsorption capacity of CNTs towards microcystins (cyanobacterial toxins) [56], lead [57] and copper (III) [58] was even stronger than that of AC. Multi-walled nanotubes have been also used for sorption of antibiotics [59], herbicides [60] or nitrogen and phosphorus in wastewater [61]. On the other hand, fullerenes as well as CNTs exhibit a mobilization potential for various organic pollutants [62], such as lindane (agricultural insecticide) [63] and persistent polychlorinated biphenyls [64]. The significant advantages of CNMs comprise their enormous surface area, mechanical and thermal stability, high chemical affinity for aromatic compounds [65], and potential antibacterial properties (described below). Moreover, contaminants can be desorbed from CNMs, and therefore, filters based on CNMs can be recycled [66, 67]. However, challenges related with high costs of CNM production, difficulties in obtaining of CNTs with uniform size and diameter distribution, uncertainties regarding the leaching potential of CNTs, as well as ecological safety and human health issues are still limitations for commercial applications in wastewater clean-up technologies.

Carbon-based nanomaterials also possess antimicrobial properties, although the mode of action is still under investigation. Some studies suggest possible applications of CNTs for disinfection purposes or antimicrobial surface coatings. Particularly silver-(Ag)-coated CNTs hybrid nanoparticles have shown antimicrobial activity [68] and the authors suggest that such materials could find their applications in biomedical devices

and antibacterial control systems. Al-Hakami et al. [69] described a method of water disinfection based on interactions of functionalized CNTs with microwaves. According to the authors, CNTs, functionalized with the aliphatic alcohol 1-octadecanol ($C_{18}H_{38}O$) had outstanding antimicrobial properties since the long carbon chains contributed to a better absorption of the microwaves by CNTs. Water purification technologies employing CNTs, including water disinfection have been described in the comprehensive reviews of Upadhyayula et al. [70] and Das et al. [71]. Studies on antibacterial and antifungal properties of graphene, CNTs and fullerenes, and their applications in agriculture are summarized in subsection 3.2.3 below.

Additionally, not only removal of pollutants from different environmental compartments, but also monitoring of contamination levels has considerable importance. Carbon-based nanomaterials can be employed for the development of novel, very efficient biochemical sensors for detecting of very low concentrations of chemical compounds in different environments. These sensors also exhibit application potential in agriculture and are discussed in more detail in section 2.5.2.3.

2.5.2 Agricultural applications of carbon-based nanomaterials

Increasing and optimizing agricultural production on limited areas of arable land sustainably with a minimum negative impact on the environment are major challenges in a future with a continuously increasing world population. Potential contributions of modern nanotechnology in this context comprise [72–74]:

(i) increase of crop productivity by use of plant growth promoters and new fertilizers based on nanomaterials;

(ii) application of nanomaterial-based plant protection products [75] including pesticides [76, 77] and herbicides [78];

(iii) a general reduction of applied agrochemicals using nanoencapsulated plant protection products and slow-release fertilizers;

(iv) nanotechnologies for optimization of agricultural practices by introducing precision farming [79].

According to Gogos et al. [75], 40% of all contributions of nanotechnology to agriculture will be provided by carbon-based nanomaterials acting as additives as well as active components. The majority of such applications are still in the developmental stage and a selection of promising approaches will be presented in this section.

2.5.2.1 Plant growth stimulators and fertilizers

A range of studies have reported a positive impact of carbon-based nanomaterials on plant growth (see section 2.6), stimulating research on nano-carbon containing fertilizers. Selected patents of various nano-fertilizers and soil improvers are listed in Supplemental Table S2.1. The majority of these fertilizers are based on amendments of mineral and organic fertilizers with nano-carbon, which in most cases acts as a fertilizer synergist with the aim of improving plant nutrient availability, reducing nutrient losses and stimulating plant growth.

2.5.2.2 Nanoencapsulation and smart delivery systems

Development of smart delivery systems—a promising technique for target delivery of agrochemicals—has numerous potential advantages. Encapsulated agrochemicals exhibit improved stability, and protection from degradation as a perspective to reduce the amount of applied agrochemicals and increase their use efficiency [80]. In this context Sarlak et al. [76] demonstrated that fungicides, encapsulated in MWCNTs functionalized with citric acid, had a higher toxicity against *Alternaria alternata* fungi compared to the not encapsulated bulk pesticide.

A still common practice of fertilizer application in conventional agriculture is broadcasting or foliar application by spraying. These techniques are often associated with considerable losses of nutrients by leaching or evaporation. So-called slow-release or controlled release fertilizers are employed for adaptation of the nutrient supply to the current demand of the plant, avoiding temporal overdoses, extending the time of function, and counteracting losses by leaching. Slow-release fertilizers can be encapsulated by graphene oxide films [81]. Even for very soil-mobile nutrients, such as potassium nitrate, encapsulation by graphene oxide considerably prolongs the process

of fertilizer release and large-scale production of encapsulates seems to be possible at a relatively low cost [81].

Smart delivery systems of agrochemicals and organic molecules including transport of DNA molecules or oligonucleotides into plant cells are potential applications of nano-biotechnology, based on the ability of carbon nanomaterials to penetrate through cell walls and membranes of plant cells [82]. A recently published study reported a possibility to deliver SWCNTs and ceria nanoparticles into isolated chloroplasts. These nanoparticles, passively penetrating through the chloroplast membrane via diffusion and were able to influence photosynthetic activity by supplying electrons into the photosynthetic electron transport chain [83]. Apart from agricultural applications, CNTs are also being investigated as molecular transporters also in animal cells for medical purpose [84–90]. In parallel, much attention is paid to research and development of techniques for directed modifications of CNTs to prevent cytotoxicity. Some of these approaches are summarized in the review by Jain et al. [91].

2.5.2.3 Antifungal and antibacterial agents

Due to antifungal properties, carbon-based nanomaterials are promising materials for the development of novel fungicides [92]. Among the various carbon nanomaterials including nanotubes, fullerenes and graphene oxide, tested against two plant pathogenic fungi *Fusarium graminearum* and *Fusarium poae*, SWCNTs showed the strongest antifungal activity. By contrast, fullerenes and activated carbon used in the assay were largely ineffective. According to Wang et al. [92], an important prerequisite determining the antifungal activity seems to be a tight contact of the nanoparticles with the fungal spores, which induces plasmolysis, associated with reduced water content and growth arrest.

In other studies, anti-microbial activity of graphene oxide (GO) has been attributed to induction of microbial membrane damage, disturbance of the membrane potential [93] and electron transport [94], as well as oxidative stress by increased production of reactive oxygen species (ROS) [95, 96]. Antibacterial properties of graphene oxide were also found to be dependent on the size of the GO sheets: larger GO sheets, wrapping

bacterial cells, can effectively isolate bacteria from their environment, and show stronger antibacterial activity as compared to small GO sheets [97]. Also the basal plane of GO sheets seems to play a key role in this mechanism [98] since masking of the basal plane by non-covalent protein adsorption with bovine serum albumin resulted in loss of antibacterial activity [98]. Accordingly, sticking the layers of GO sheets closely together [99] to avoid the interactions of bacteria with the sharp edges of single GO sheets, while keeping contact of the bacteria with the basal plane, retained the antimicrobial activity. These findings underline the importance of the basal plane as a central structural element mediating antibacterial properties of GO.

The CNMs with antifungal and antimicrobial properties described above received considerable attention due to potential applications as novel fungicides and disinfection agents suitable for agricultural purposes (e.g. in plant protection). However, due to the largely unknown behavior of CNMs in complex environmental matrices, it is quite difficult to predict to what extent the described *in vitro* properties will be manifested when the CNMs or CNM-containing products are released into the environment. This requires much more detailed investigations of the antifungal and antibacterial properties of CNMs, considering a wider range of crops and pathogens to be investigated under real field conditions [92]. On the other hand, for environmental risk assessment, the potential impact of CNMs on non-target organisms also requires comprehensive evaluation before commercialization of novel agrochemicals.

2.5.2.4 Sensing systems and precision agriculture

Carbon-based nanomaterials with novel chemical, physical and mechanical properties are employed to develop highly sensitive sensors and diagnostic devices for numerous agricultural and environmental applications. Nanosensors exhibit numerous operating principles [100] but a common mode of action is conversion of physicochemical properties into signals. The high sensitivity of these devices is determined by the nanoscale size of the sensing elements, such as carbon nanotubes. Therefore, a few molecules are frequently sufficient to influence the electrical properties (chemical to electrical transduction) of the nanoparticles. Moreover, the great surface area of carbon-based nanomaterials provides large spaces for interactions with the sensed molecules.

Development of novel technologies based on nanomaterials which can be successfully used for easy, rapid and highly sensitive chemical analysis, is documented in numerous studies. A graphene-based sensor for the detection of cadmium contaminations in water, which works effectively at concentration levels of 0.25 μg L^{-1} has been described by Wu et al. [101]. A highly sensitive sensor for the determination of nickel, not only in environmental samples but also in food, is based on modified nanotubes and has a detection limit of 4.9 ng L^{-1} [102]. CNT-based gas sensors for ammonia detection can be used for trace gas measurements [103]. Sensors to detect pesticides, herbicides and their metabolites in environmental samples have been developed based on modified MWCNTs [104, 105] or on GO [106, 106]. Moreover, nanosensors have been employed for monitoring soil moisture [107].

Nanosensor-based monitoring systems for crop health have also been described. In this context, CNT sensors were successfully used for *in vivo* monitoring of ROS formation in plant tissues as stress indicators [108]. An electronic device for real-time sensing of toxic gases based on CNTs, which could be placed onto insects or plants [109] can be applied in remote sensing systems for plant diseases. Covering a field site with multiple sensors can provide a full picture of the spatial distribution of disease severity at a field scale and allows the detection of hot spots, requiring a special treatment, as a significant contribution to reducing the input of agrochemicals.

However, currently the majority of nanosensors are still in the product development stage and large-scale production has not yet been established due to a number of restrictions. First of all, on the way from the lab to the market scale, long-term testing is required for calibration and validation of the developed devices [110]. Currently, only a limited number of studies reported measurements of real samples under natural conditions [100]. Challenges related with up-scaling production techniques and the high costs of the materials and equipment required for the production of nanosensors have been claimed by various authors [111, 112]. Major challenges still remain around the issues of safety, health aspects and risk assessment, requiring an entire life cycle analysis of a product [110]. However, despite the listed issues, several authors are expecting

intensive commercialization of nanosensor technologies [113] in the near future, also confirmed by recently issued market forecasts [114, 115].

2.6 Impact of carbon-based nanomaterials on plants

Due to the rapid expansion of nanotechnology and the production of engineered nanomaterials, it is essential to understand how nanoparticles interact with living organisms for bio-safety reasons. In the case of nanomaterial-plant interactions, knowledge gained in this direction is not only important in terms of an ecological risk assessment but may also contribute to the development of nanotechnological applications in agriculture towards improved crop yields and reduced input of agrochemicals. Phytotoxicology of engineered nanomaterials is a comparatively new field of research, which according to Nowack and Bucheli [116] did not exist before 2007. Accordingly, the potential toxicity of nanoparticles on plants has not yet been widely investigated, and the reported results are frequently descriptive and contradictory with very limited information on the underlying modes of action [117]. Physiological processes potentially affected by interactions with nanomaterials comprise alterations of gene expression [118, 119], DNA damage [120, 121] and increased formation of ROS [122, 123]. A high variability of responses can be observed between different plant species [124, 125], various stages of plant ontogenesis [126] and different varieties [127, 128]. The impacts of carbon-based nanomaterials on seed germination and plant development of different plant species are compiled in Tables 2.1 and 2.2, while penetration of carbon nanoparticles into plant tissues is summarized in Figure 2.4.

The high variability of nanomaterials in terms of chemical composition, size and shape, surface structure, solubility, aggregation and application modes are also factors most likely contributing to the heterogeneity of plant responses reported in the literature [129, 130]. Knowledge of the special structural features of nanomaterials determining the adverse effects on living organisms is a pre-requisite for the so called “safe-by-design” approach for a directed design of nanomaterials without negative environmental side effects [131–133].

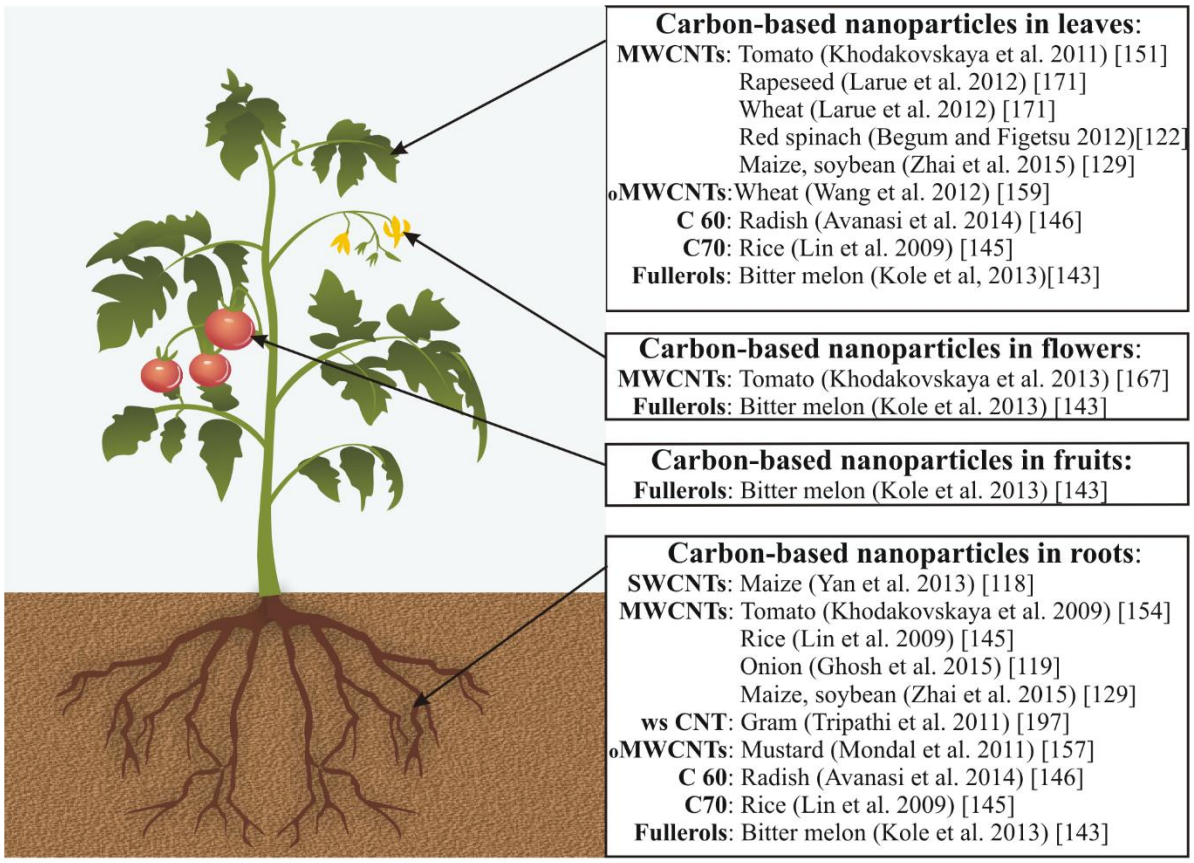

Figure 2.4. *List of plant species in which carbon-based nanoparticles were detected in different plant organs.*

Table 2.1. *Influence of carbon-based nanomaterials on seed germination*

Plant specie	Type of NPs	Size of NPs	Concentration	Germination medium	Exposure duration	Effects	Ref
Maize	SWCNTs	Ø 1−2 nm L 30 µm	20 mg L^{-1}	MS medium	72 hours	No effect on seed germination	[118]
Radish, rape, ryegrass, lettuce, maize, cucumber	MWCNTs	Ø 10−20 nm L 1−2 µm	2000 mg L^{-1}	DI water	5 days	No effect on seed germination	[158]
Alfalfa, wheat	MWCNTs	Ø 3 ± 4 nm	40−2560 mg L^{-1}	Agar medium	4 days	No effect on seed germination	[190]
Zucchini	MWCNTs	Ø 13 - 16 nm L 1 - 10 µm	1000 mg L^{-1}	Hoagland medium	12 days	No effect on seed germination	[126]
Mustard, black lentil	MWCNTs	Ø 110−170 nm L 5−9 µm	10, 20, 40 mg L^{-1}	DI water	7 days	No effect on seed germination	[160]
Wheat	oMWCNTs	Ø 6−13 nm L 2.5−20 µm	10, 20, 40, 80, 160 mg L^{-1}	DI water	7 days	No effect on seed germination	[159]
Wheat, maize peanut, garlic bulb	wsMWCNTs	Ø 10−20 nm, L 10−30 µm	20, 50 mg L^{-1}	DI water	5−10 days	Enhanced germination	[191]
Barley, maize, soybean	MWCNTs	na	50, 100, 200 mg L^{-1}	MS medium	10 days	Accelerated germination	[128]
Barley, maize, soybean,	MWCNTs	na	25, 50, 100 mg L^{-1} (spray)	Water	10 days	At 25 mg L^{-1} no effect, at 100 mg L^{-1} increased germination rate	[128]
Tomato	MWCNTs	na	10, 20, 40 mg L^{-1}	MS medium	20 days	Increased seed moisture content, accelerated germination, improved germination rate	[154]
Rice	CNTs	Ø 8 nm, L 30 µm	50, 100, 150 mg L^{-1}	MS medium	6 days	Enhanced germination speed and rate	[192]
Rice	CNTs	na	na	Basal growth medium	na	Increased seed water content, germination rate	[156]

Plant specie	Type of NPs	Size of NPs	Concentration	Germination medium	Exposure duration	Effects	Ref
Tomato, onion, turnip, radish	CNTs	Ø 8−15 nm L > 10 μm	10, 20 and 40 mg L^{-1}	Ultra-pure water	12 days	Improved germination of tomato and onion	[193]
Tomato	Graphene	na	40 mg L^{-1}	DI water	11 days	Accelerated germination	[172]
Rice	Graphene	na	5, 50, 100, 200 mg L^{-1}	na	16 days	Delayed germination with the increasing of graphene concentration	[173]
Wheat	Graphene, GO Graphene ribbon		200 mg L^{-1}	Water	5 days	Graphene and GO: inhibited germination, graphene ribbon: enhanced germination	[174]

Ø—diameter, L—length, DI water—deionized water, wsMWCNTs—water soluble MWCNTs, oMWCNTs—oxidized MWCNTs, MS medium—Murashige and Skoog medium, GO—graphene oxide.

Table 2.2. *Influence of carbon-based nanomaterials on plant growth and development*

Plant specie	Type of nanomaterial	Size of NPs	Concentration	Growth medium/substrate	Exposure duration	Effect	Ref
Cabbage, carrot,	SWCNT fSWCNT	Ø 3 nm	28, 160, 900, 5000 mg L^{-1}	DI water	2−3 days	No effect	[125]
Cucumber, onion,	SWCNT fSWCNT	Ø 3 nm	28, 160, 900, 5000 mg L^{-1}	DI water	2−3 days	Enhanced root elongation	[125]
Arabidopsis, rice	SWCNTs	Ø 1-2 nm L 5-30 µm	5−250 mg L^{-1}	Cell culture	6−72 hours	Cell aggregation, chromatin condensation, plasma membrane deposition, H_2O_2 accumulation.	[149]
Tomato lettuce,	SWCNT fSWCNT	Ø 3 nm	28, 160, 900, 5000 mg L^{-1}	DI water	2−3 days	Inhibited root elongation	[125]
Tomato	SWCNTs	Ø 0.86−2.22 nm	50 mg L^{-1}	MS medium	10 days	Increased fresh and dry plant biomass	[151]
Maize	MWCNTs	Ø 6−9 nm L 5 µm	5, 10, 20, 40, 60 mg L^{-1}	Nutrient agar medium	7 days	At 20 mg L^{-1} increased dry weight and Ca, Fe concentration. At high dose: depressed growth	[127]
Barley, maize, soybean	MWCNTs	na	50, 100, 200 mg L^{-1}	MS medium	10 days	Increased leave length and biomass in maize, increased root length in soybean	[128]
Tomato	MWCNTs	na	10, 20, 40 mg L^{-1}	MS medium	20 days	Increased stem length, biomass	[154]
Wheat, rapeseed	MWCNTs	Ø 42.2 nm	1000 mg L^{-1}	Hydroponic	7 days	No impact on development; uptake by roots and translocation to leaves	[171]
Wheat, rape seed	MWCNTs	Ø 10−150 nm	1000 mg L^{-1}	Ultrapure water	7 days	No effect on development	[194]
Arabidopsis	MWCNTs	Ø 9.5 nm L 1.5 µm	10, 60, 100, 600 mg L^{-1}	Cell culture	7 days	Decreased cell dry weight, cell viabilities, cell chlorophyll content, and superoxide dismutase activities	[163]

Plant specie	Type of nanomaterial	Size of NPs	Concentration	Growth medium/substrate	Exposure duration	Effect	Ref
Satureja	MWCNTs	Ø < 50 nm	25, 50, 100, 250 500 mg L^{-1}	Cell culture	3 weeks	Increased callus growth	[195]
Wheat, maize peanut, garlic	ws MWCNTs	Ø 10−20 nm, L 10−30 µm	20, 50 mg L^{-1}	DI water	5−10 days	Root and shoot elongation	[191]
Tomato	MWCNTs	Ø 25 nm		MS medium, later soil	9 weeks	Enhanced growth, flower and fruit number formation	[167]
Tomato	MWCNTs	Ø 10−35 nm L 6 µm	50, 100, 200 mg L^{-1}	MS medium	10 days	Increased total biomass, up-regulation of stress-related genes; penetration into roots, leaves, and fruits	[151]
Red spinach	MWCNTs	Ø 11 nm L > 1 µm	125−1000 mg L^{-1}	Modified Hoagland medium	15 days	Inhibited root and shoot elongation, reduced weight, leaf area, enhanced electrolyte damage and ROS	[122]
Onion	MWCNTs	Ø 7−15 nm, L 0.5−200 µm	5, 20, 50 mg L^{-1}	Filter sterile water		DNA damage	[120]
Onion	MWCNTs	na	5 and 10 mg L^{-1}	Filter sterile water		DNA damage	[119]
Cucumber, rice, lettuce, red spinach	MWCNTs	Ø 13 nm L > 1 µm	20, 200, 1000, 2000 mg L^{-1}	Modified Hoagland medium (hydroponic)	15 days	Decreased shoot length, biomass, increased membrane leakage	[124]
Zucchini	MWCNTs	Ø 13−16 nm L 1−10 µm	1000 mg L^{-1}	Hoagland medium	12 days	Reduced biomass	[126]
Rice	MWCNTs		na	Cell culture		Decreased cell density	[196]
Rice	MWCNTs	Ø <10 nm L 5−15 µm	20 mg L^{-1}	Cell culture MS medium	6 days	Decreased cell viability, increased ROS	[164]

Plant specie	Type of nanomaterial	Size of NPs	Concentration	Growth medium/substrate	Exposure duration	Effect	Ref
Maize, soybean	MWCNTs		10−50 mg L^{-1}	Hoagland solution	18 days	Stimulated growth of maize, inhibited growth of soybean; uptake and translocation	[129]
Wheat	oMWCNTs	Ø 6−13 nm L 2.5−20 µm	10, 20, 40, 80, 160 mg L^{-1}	DI water	7 days	Increased cell elongation in roots, root length, biomass	[159]
Common gram	ws CNTs	Ø 10−50 nm	6.0 mg L^{-1}	Water	10 days	Increased growth rate	[197]
Tobacco	CNPs	na	25,75, 125 mg pot^{-1}	na	na	Increased plant height, leaf area, dry matter accumulation, chlorophyll, soluble protein, N and K contents	[198]
Arabidopsis	CNTs	na	na	Basal salt medium	na	Reduced photosynthesis, transpiration, carbon gain, chlorophyll fluorescence	[199]
Rice	MWCNTs Fullerene C70	Ø 5−40 nm L 0.5−2 µm	2.5−800 mg L^{-1}	NOM suspension	2 weeks	MWCNTs: insignificant uptake C_{70}: uptake, accumulation and translocation within the plant	[145]
Radish	Fullerene C60	-	-	Sand substrate and hydroponic	2 weeks	Uptake by plants and transportation	[146]
Pumpkin	Fullerene C60	-	1670 mg kg^{-1} soil	Sandy loam soil with DDT residues	21 days	Increased shoot mass and decreased root mass	[139]
Green algae, arabidopsis	Fullerols	-	0.001−20 mg L^{-1} 1-200 mg L^{-1}	Cell culture	96 hours	Increased the algal culture density; enhanced growth in Arabidopsis	[142]
Bitter melon	Fullerols	-	0.943, 4.72, 9.43, 10.88, 47.2 nM	Milli-Q water	48 hours	Increased fruit length, number, weight and phytomedicines content	[143]
Tobacco	Nano-carbon sol	na	5−20 mg L^{-1}	Water culture	na	Increased biomass, nitrogen, potassium, calcium and magnesium content	[200]

Plant specie	Type of nanomaterial	Size of NPs	Concentration	Growth medium/substrate	Exposure duration	Effect	Ref
Tobacco	Nano-carbon sol	na	5−10, 10−20 mg L^{-1}	Water culture	na	Promoted root growth, increased shoot and root biomass and improved potassium content	[201]
Faba bean	GO	0.5–5 μm	400, 800 mg L^{-1}	DI water	na	Decreased levels of H_2O_2, and lipid and protein oxidation, and enhanced H_2O_2 decomposing enzymes; increased proline and seed-relative water content	[177]
Faba bean	GO	0.5−5 μm	400, 800 mg L^{-1}	DI water	na	Increased ratio of the reduced glutathione (GSH)-to oxidized GSH, reduced GSH pool and GSH reductase activity, decreased activities of GSH-metabolizing enzymes	[176]
Faba bean	GO	0.5–5 μm	100, 200, 1600 mg L^{-1}	DI water	na	Reduced growth, reduced activity of H_2O_2-decomposing enzymes; increased levels of electrolyte leakage, H_2O_2, and lipid and protein oxidation	[177]
Faba bean	GO	0.5–5 μm	100, 200, 1600 mg L^{-1}	DI water	na	Decreased (GSH) redox ratio, reduced GSH pool, elevated activities of GSH-regenerating and GSH-metabolizing enzymes	[176]
Wheat	GO	na	0.1–10 mg L^{-1}	Water	na	GO amplifies phytotoxicity of arsenic (As)	[179]
Cabbage, tomato, red spinach, lettuce	Graphene	na	500, 1000, 2000 mg L^{-1}	Modified Hoagland medium	20 days	Inhibited plant growth and biomass	[175]
Tomato	Graphene	na	40 mg L^{-1}	DI water	11 days	Longer roots and shoots, but reduced biomass	[172]

Plant specie	Type of nanomaterial	Size of NPs	Concentration	Growth medium/substrate	Exposure duration	Effect	Ref
Rice	Graphene	na	5, 50, 100, 200 mg L^{-1}	na	16 days	At 5 mg L^{-1}: improved seedling growth, at higher concentration: inhibited growth	[173]

SWCNTs—single-walled carbon nanotubes, fSWCNTs—functionalized single-walled carbon nanotubes, DI water—deionized water, MWCNTs—multi-walled carbon nanotubes, wsMWCNTs—water soluble multi-walled carbon nanotubes, Ø— diameter, L—length, MS medium—Murashige and Skoog medium, CNPs—carbon nanoparticles, NOM—natural organic matter, GSH—glutathione, GO—graphene oxide, DDT—dichlorodiphenyltrichloroethane.

2.6.1 Impact of fullerenes on plants

Germination. Only a limited number of studies report effects of fullerenes or their derivatives on seed germination. For instance, fullerenes applied in aqueous suspensions (10−500 mg L^{-1}) or by soil incorporation (1000 mg kg^{-1}) did not affect seed germination of wheat (*Triticum aestivum*), rice (*Oryza sativa*), cucumber (*Cucumis sativus*) and green gram (*Vigna radiata*) [134]. The authors explained the absence of fullerene effects on seed germination as a consequence of selective seed coat permeability. Similarly, Liu et al. [135] reported that fullerene malonic acid derivative (FMAD, $C_{70}(C(COOH)_2)_{4-8}$) did not affected the germination of Arabidopsis (*Arabidopsis thaliana*) seeds most likely due to protective effects of seed coat.

Plant growth and development. The majority of studies focusing on the effects of fullerenes on terrestrial and aquatic plants, report negative or no effects of fullerene C60 on plant growth and development. Tao et al. [136] found inhibition of photosynthesis and Mg uptake of phytoplankton exposed to fullerenes C60. Similarly, fullerenes inhibited growth and chlorophyll accumulation in duckweed (*Lemna gibba*) [137].

In a study of terrestrial plants with fullerene soil amendments used for immobilization of pesticide residues, reduced biomass accumulation was reported at higher fullerene concentrations (500-5000 mg kg^{-1} soil): up to 40% reduction for soybean (*Glycine max*), 44 % for maize (*Zea mays*) and 10% for tomato (*Solanum lycopersicum*) [138], while inhibition of root growth was detected in pumpkin (*Cucurbita pepo*) [139].

Several studies have demonstrated a potential of fullerenes to increase accumulation of organic contaminants in plants. Accordingly, in cottonwood (*Populus deltoides*) cuttings cultivated in hydroponics, accumulation of industrial solvent trichloroethylene added to the growth medium was increased by 80% in the presence of fullerene C60 (15 mg L^{-1}) [140]. The authors speculated that a fullerene-trichloroethylene complex, formed in the nutrient solution was taken up by the plants. In another study [141] carried out in a vermiculite substrate, fullerene C60 (40 mg pot^{-1}) increased the total plant content of the DDT metabolite dichlorodiphenyldichloroethylene (DDE) in zucchini (*Cucurbita pepo*), soybean (*Glycine max*) and tomato (*Solanum lycopersicum*) by

approximately 30, 45 and 62%, respectively. Although fullerenes C60 were detected mainly in the root tissue and at the root surface, a conjugated uptake of C60 together with the contaminant was postulated by the authors. Analyses of plant tissues revealed no membrane disruptions, suggesting that the contaminants did not enter the plant simply via damaged tissues. Genotypic differences in the uptake rates of DTT metabolites in the presence of fullerene C60 have been reported in plants grown on a vermiculite-soil mixture [138] with inhibitory effects recorded for maize (*Zea mays)* and tomato (*Solanum lycopersicum*) but stimulation in soybean (*Glycine max*) and zucchini (*Cucurbita pepo).* Interestingly, in a study conducted on a loamy field soil, containing naturally aged DDE residues, no impact of fullerene C60 amendments on contaminant uptake by pumpkin (*Cucurbita pepo*) was detectable [139]. These findings suggest that plant availability of fullerenes and/or organic contaminants depends not only on substrate properties, but also on differences between plant species.

By contrast, fullerols, as OH-functionalized fullerenes, frequently exerted positive effects on plant growth, such as stimulation of cell divisions in green algae cultures of *Pseudokirchneriella subcapitata* and of hypocotyl growth in Arabidopsis (*Arabidopsis thaliana)* [142]. Fullerol seed dressings even increased fruit number, fruit size and final yield by up to 128% in bitter melon (*Momordica charantia*), and are also associated with a higher content of bioactive compounds in fruits, such as cucurbitacin-B, lycopene, charantin and inulin [143]. These findings demonstrate perspectives of hydroxyfullerenes to improve crop yields and product quality. However, further investigations on potential food chain contaminations are still necessary, since fullerol residues have been detected in various plant organs including fruits. The exact mechanism behind plant-growth promotion induced by hydroxyfullerenes is not yet clear, but may be at least partially explained by antioxidant properties [144] connected with the ability of hydroxyfullerenes to accept up to six electrons and distribute them among the aromatic rings, thereby acting as “radical sponges”.

However, not all functionalized fullerenes exhibit stimulatory effects on plant growth. As an example Liu et al. [135] reported that a water-soluble fullerene-malonic acid derivative (FMAD), $C_{70}(C(COOH)_2)_{4\text{-}8}$ added to the growth medium, induced dose-

dependent inhibition of root elongation by up to 60% and deformation of root tips in Arabidopsis (*Arabidopsis thaliana)*, associated with a disruption of auxin transport in the root tips, aberrations of cell divisions in the root meristematic zone and reduction of intracellular ROS. Growth-inhibitory effects of carboxyfullerenes ($C_{70}(C(COOH)_2)_{2–4}$) have been similarly reported in tobacco (*Nicotiana tobacum*) cell cultures, connected with cell wall deformations and co-induction of oxidative stress [123]. These findings demonstrate that the type of functionalization is an important determinant for the effects of nanomaterials on plants.

Uptake of fullerenes and fullerols. Lin at al. [145] reported that rice seedlings (*Oryza sativa*) grown in hydroponic culture exhibit root uptake of fullerene C70 and translocation to shoots and leaves. The accumulation of C70 was observed in vascular tissues, in surrounding cells and intercellular spaces. Similar to hydroxyfullerenes described above, uptake of fullerenes added to a vermiculite growth substrate was found in root systems of soybean (*Glycine max*), tomato (*Solanum lycopersicum*) and in both the roots and shoots of zucchini (*Cucurbita pepo*) [146, 141]. Fullerene C60 accumulated mainly in the root tissue, but smaller quantities were detected also in leaves and stems [146]. Uptake of fullerene $^{14}C60$ was also reported for radish (*Raphanus sativus*), grown for two weeks in a sand substrate and hydroponic culture, while a rock wool substrate limited the availability of C60 for plant uptake [146]. Although the principal capacity of plant roots to take up fullerenes has been repeatedly demonstrated, unfortunately more detailed studies on plant availability and root uptake under real soil conditions are still lacking [138, 139].

2.6.2 Impact of carbon nanotubes on plants

Compared with fullerenes, many more studies on plant interactions with the various types of carbon nanotubes are available, describing effects on seed germination, early plant growth, cell culture, gene expression and various physiological processes. Concerning toxicity aspects, due to smaller size, SWCNTs seem to be more toxic than MWCNTs, and toxicity is further increased by functionalization of nanotubes [125].

Since CNTs exhibit great tensile strength, mechanical damage of tissues may be induced by piercing effects.

2.6.2.1 Impact of SWCNTs on plants

Germination. A few studies report effects of SWCNTs on germination rate. Stimulation of seed germination in response to SWCNTs treatments (10−40 mg L^{-1}), potentially induced by perforation of the seed coat, has been reported for salvia (*Salvia macrosiphon*), pepper (*Capsicum annuum*), and tall fescue (*Festuca arundinacea*) [147]. Among the tested treatments, the highest germination rates were obtained by applying moderate SWCNT concentrations: e.g.10 mg L^{-1} SWCNTs for pepper (*Capsicum annuum*) and 30 mg L^{-1} of for salvia (*Salvia macrosiphon*) and tall fescue (*Festuca arundinacea*). However, a similar concentration of SWCNTs (20 mg L^{-1}) did not affect the germination of maize (*Zea mays*) seeds [118].

Plant cell culture. For SWCNTs added to the growth media of plant cell cultures, positive as well as negative effects have been observed, depending on the applied dosage. In Arabidopsis (*Arabidopsis thaliana*) mesophyll cells, low concentrations of SWCNTs (10−50 mg L^{-1}) exerted stimulatory effects on cell growth, while higher concentrations (100 mg L^{-1}) induced the generation of ROS and toxic effects, associated with necrosis and apoptosis [148]. Similar dose-dependent effects including ROS accumulation and programmed cell death (at 25 mg L^{-1}) have been reported for cell cultures of Arabidopsis (*Arabidopsis thaliana*) and rice (*Oryza sativa*), potentially linked with the small particle size of SWCNTs, since particles of activated carbon did not cause similar damage. [149]. It has been speculated that SWCNTs might penetrate cell walls and cell membranes.

Plant growth and development. Effects of SWCNTs have been reported mainly for young seedlings grown in aqueous suspensions or various culture media amended with SWCNTs. Stimulatory effects on early seedling growth have been detected for a range of plant species including fig plants (*Ficus carica*) [150] maize (*Zea mays*) [118] and tomato (*Solanum lycopersicum*) seedlings [151]. A dose-dependent effect of SWCNTs was reported for salvia (*Salvia macrosiphon*), pepper (*Capsicum annuum*) and tall

fescue (*Festuca arundinacea*) [147], where 10−30 mg L^{-1} of SWCNTs increased the formation of seedling biomass, while at 40 mg L^{-1} SWCNTs exerted negative effects on seedling development. A similar response has been reported for blackberry (*Rubus adenotrichos*) grown *in vitro* in a culture medium supplemented with functionalized carboxy-SWCNTs (SWCNTs-COOH) [152].

However, contradicting effects were observed for short-term applications (24 and 48 h) of SWCNTs functionalized with poly-3-aminobenzenesulfonic acid and non-functionalized SWCNTs in six important crops (cabbage (*Brassica oleracea*), carrot (*Daucus carota*), cucumber (*Cucumis sativus*), lettuce (*Lactuca sativa*), onion (*Allium cepa*), and tomato (*Solanum lycopersicum*)). Non-functionalized nanotubes inhibited root elongation in tomato (*Solanum lycopersicum*) but exerted stimulatory effects on cucumber (*Cucumis sativus*) and onion (*Allium cepa*). Root elongation in lettuce (*Lactuca sativa*) was inhibited by functionalized nanotubes, while cabbage (*Brassica oleracea*) and carrot (*Daucus carota*) were not affected at all [125]. The authors speculated that the variability of the responses may be related (i) with genotypic differences of the test plants and (ii) with differences in seed size, since small-seeded species, such as lettuce (*Lactuca sativa*), onion (*Allium cepa*), and tomato (*Solanum lycopersicum*) appeared to be more sensitive compared with large-seeded species with a lower surface to volume ratio providing a lower surface area for interactions with SWCNTs.

A limited number of studies linked the effects of plant exposure to SWCNTs on morphological traits with modifications at the molecular level. In maize (*Zea mays*) grown on Murashige and Skoog medium amended 20 mg L^{-1} SWCNTs, increased formation of seminal roots was associated with increased expression of the respective genes (*SLR1, RTCS*), while inhibition of root hair growth was reflected by down regulation of root hair-related genes (*RTH1, RTH3*). Moreover, similar to plants exposed to stress conditions, SWCNTs could increase histone deacetylation [118], most likely as a response towards SWCNT accumulation in the root cortex.

Uptake of SWCNTs. The process of SWCNT penetration into plant cells has been first described in plant cell cultures. Liu et al. [82] reported intracellular penetration of water-

soluble SWCNTs with a length < 500 nm in cell cultures of tobacco (*Nicotiana tobacum*). The nanotubes showed the ability to penetrate both the hard cell wall, and the cell membrane, mediated most probably by means of fluidic-phase endocytosis, since nanotube penetration was minimal in presence of endocytosis inhibitors. Temperature did not affect SWCNT internalization into *Arabidopsis thaliana* mesophyll cells and therefore, the authors proposed a non-energy dependent endocytosis pathway [148]. Later, Shen et al. [149] reported the formation of endocytosis-like structures in the membranes of *Arabidopsis thaliana* leaf cells in response to SWCNT (5−30 μm) treatments. Successful intracellular penetration was shown for functionalized magnetic SWCNTs into canola (*Brassica napus*) and carrot (*Daucus carota*) cells driven by external magnetic forces. This behavior may reflect the potential of SWCNTs for development delivery carriers for biomolecules [153].

However, less evidence is available for SWCNT uptake by intact plants. In a study by Cañas et al. [125], none of the functionalized and non-functionalized SWCNTs supplied with the growth medium were found in the root tissues of young seedlings (2−3 days) of six plant species, and SWCNTs were mainly sticking to the external root surface. Accordingly, also in maize *(Zea mays*) the occurrence of SWCNTs was restricted to the root surface and the intercellular spaces of the root cortex [118]. In studies with cell cultures, it has been demonstrated that events of nanotube penetration occur more frequently with high concentrations of SWCNTs rarely present in experiments with whole plants.

2.6.2.2 Impact of MWCNTs on plants

Germination. In contrast to SWCNTs, stimulatory effects of MWCNTs have been reported for a wider range of different crops. Improved germination rates were described for tomato (*Solanum lycopersicum*) (already registered as a patent) [154, 155] and rice (*Oryza sativa*) [156], while germination speed was accelerated in barley (*Hordeum vulgare*), soybean (*Glycine max*), maize (*Zea mays*) [128, 157] and mustard seeds (*Brassica juncea*) [157]. One of the most frequently encountered theories to explain beneficial effects on germination is associated with improved water uptake, demonstrated for tomato (*Solanum lycopersicum*) [154], rice (*Oryza sativa*) [156], and

mustard (*Brassica juncea*) [157]. Accelerated water flow into the seeds has been related with the ability of CNTs to perforate the seed coat [154]. Later, a concentration-dependent effect of MWCNTs on the expression of aquaporin genes has been reported for germinating seeds of soybean (*Glycine max*), barley (*Hordeum vulgare*) and maize (*Zea mays*) [128]. Due to a central role of aquaporins in germination, it has been speculated that the beneficial effects of MWCNTs on seed water uptake and germination may be mediated by the described aquaporin effect. However, this assumption is still speculative since additionally to water uptake, aquaporins are involved in many physiological processes including stress responses also induced by CNTs and therefore, more detailed investigation of the involved aquaporin genes is necessary.

Apart from positive effects of MWCNTs on germination, there are also numerous reports claiming the absence of any MWCNT effect in a wide range of different plant species, including radish (*Raphanus sativus*), rape (*Brassica napus*), ryegrass (*Lolium perenne*), lettuce (*Lactuca sativa*), maize (*Zea mays*), cucumber (*Cucumis sativus*) [158], wheat (*Triticum aestivum*) [159], mustard (*Brassica juncea*), black lentil (*Phaseolus mungo*), and zucchini (*Cucurbita pepo*) [160]. On the one hand, this discrepancy may be attributed to genotypic differences or variability in seed lot quality of the tested seed material but it may also be the test conditions. Interestingly, it has been shown that different techniques of MWCNT application, such as dispersal in agar medium or spraying onto the seed surface did not change the beneficial effects on the germination of barley (*Hordeum vulgare*), soybean (*Glycine max*) and maize (*Zea mays*) [128]. However, the chemical composition of the growth media used for the germination tests may play an important role. These media can contain simply distilled water [158, 159], but also agar with and without supplementation of mineral nutrients [127] or even complete media used for plant tissue cultures, such as Murashige-Skoog medium [154, 128], supplemented with minerals, amino acids, vitamins and hormones as bioactive compounds [161]. Moreover, even contaminations of CNTs with catalytic impurities, such as Fe and Al_2O_3 can exert a stimulatory effects on seed germination [162].

<u>*Plant cell culture.*</u> Investigations of the influence of MWCNTs on suspension cells of *Arabidopsis thaliana* showed toxic effects of MWCNTs (10−600 mg L^{-1}) [163],

reflected by inhibited cell growth and cell viability, decreased chlorophyll content and superoxide dismutase (SOD) activity. Comparing two types of MWCNTs, forming larger and smaller agglomerates, revealed higher toxicity of the smaller particles, confirming again that the size of nanoparticles is an important factor determining their toxicity (see 2.6.2.1). Investigations of MWCNT effects on rice suspension (*Oryza sativa*) cultures revealed increased ROS formation, associated with reduced cell viability, mitigated by the application of ascorbic acid as an antioxidant [164], demonstrating once more that oxidative stress is another important determinant of CNT toxicity. Due to the similar size of CNTs and many pathogens, it has been speculated that CNTs are able to induce a pathogen-like hypersensitive response, associated with an oxidative burst, as a defense reaction of plants in response to various kinds of pathogen attacks.

However, positive effects of MWCNTs on plant cell cultures have also been reported. In tobacco (*Nicotiana tabacum*) cells [165] MWCNTs at a dosage of 100 mg L^{-1} stimulated biomass accumulation, associated with an upregulated expression of cell cycle genes (after 6 h), cell growth (after 4 days) and water transport (from 24 h to 4 days), while during the rest of the exposure time the gene expression did not differ from cells grown in control (MS medium).

Independent of positive or negative plant responses to MWCNT treatments, the direct cell contact of individual or agglomerated CNTs seems to be a prerequisite for the induction of any effects, with differences in plant sensitivity or different physical parameters of CNTs (diameter, length, degree of aggregation) as determinants for the variable expression of plant responses.

Plant growth and development. During seedling development and early growth, positive effects of MWCNTs on root and shoot elongation have been reported for a range of plant species, such as tomato (*Solanum lycopersicum*) [154, 151, 166], wheat (*Triticum aestivum*) [159], soybean (*Glycine max*), maize (*Zea mays*) [128], mustard (*Brassica juncea*), and black lentil (*Phaseolus mungo*) [160]. A high degree of MWCNT dispersion in the growth medium resulted in more intense stimulation of plant growth as compared to variants containing larger agglomerates of the same MWCNTs [166],

suggesting that a uniform and widely distributed contact of smaller MWCNTs with the plant tissues may be a prerequisite for stimulatory effects on plant growth. In many studies the expression of effects was concentration-dependent (see 2.6.2.1), with beneficial effects at lower levels of MWCNT application and inhibition at higher concentrations [127, 160]. As already described for cell culture experiments (see above and 2.6.2.1), induction of oxidative stress, associated with ROS formation, membrane damage, electrolyte leakage, mitochondrial dysfunctions, DNA aberration and cell death, has been characterized as determinant for MWCNT toxicity. This was also noted during seedling development and early growth of red spinach (*Amaranthus tricolor*), rice (*Oryza sativa*), lettuce (*Lactuca sativa*) and cucumber (*Cucumis sativus*) [122, 124, 119] and again small seeds were most sensitive (see also 2.6.2.1).

The majority of studies focused on MWCNTs effects on plant growth and development have used hydroponics or agar media as growth substrates, while soil culture has been employed very rarely. Apart from studies on germination and early growth, MWCNT effects have also been investigated in the reproductive stage of plant development. Khodakovskaya et al. [167] reported a doubling of flower setting and yield in tomato (*Solanum lycopersicum*), grown in soil with MWCNT amendments, which was not detectable in control soils simply treated with activated charcoal. On the other hand, De La Torre-Roche et al. [138] found no indications for effects of MWCNT soil amendments in zucchini (*Cucurbita pepo*) and tomato (*Solanum lycopersicum*). This is most likely due to limited mobility of MWCNTs in soil [168] related with a low probability of CNT contact with plant tissues.

Alteration of morphological traits in plants treated with MWCNTs is often associated with changes in gene expression and also with damage of DNA and chromatin structures. Ghosh et al. [119] reported DNA damage, micronucleus formation and chromosome aberration in onion roots (*Allium cepa*) in response to MWCNT treatments. In a study with tomato roots (*Solanum lycopersicum*) [151], application of MWCNTs to the growth medium triggered overexpression of various biotic stress-related genes, such as subtilisin-like endoprotease, meloidogyne-induced giant cell protein, threonine deaminase, and this was also observed after SWCNT treatments in maize (*Zea mays*)

seedlings [118]. Similarly, the increased expression of aquaporins (water channel proteins) reported for seedlings of tomato (*Solanum lycopersicum*) [151, 166], soybean (*Glycine max.*), maize (*Zea mays*), and barley (*Hordeum vulgare*) with MWCNTs seed treatments may reflect a common stress response [128]. In tomato seedlings (*Solanum lycopersicum*), up-regulation of aquaporin gene expression was triggered by highly dispersed MWCNTs with different functional groups attached to the surface, while MWCNTs in the form of large aggregates remained ineffective [166]. Taken together the findings suggest that CNTs are acting as stress factors with the ability to induce plant defense responses and hormesis effects, or toxicities depending on the intensity of the stimulus.

Uptake of MWCNTs. Despite the fact, that the diameter and length of MWCNTs are frequently greater than the size of fullerenes and SWCNTs, plant uptake and internal translocation has been reported also for MWCNTs. The majority of such studies have been carried out in hydroponics or agar-like growth media. Multiwalled nanotubes can penetrate not only cells of developing seedlings, but even rigid seed coats by perforation and creation of new pores [154]. For instance, MWCNTs with a diameter range of 15−40 nm were detected in germinating seeds of barley (*Hordeum vulgare*), soybean (*Glycine max*) and maize (*Zea mays*) [128]. Once nanotubes have passed the seed coat, contact with the radicle and other seedling organs is possible. Accordingly, small diameter MWCNTs (< 13 nm), present in the germination medium, could penetrate cell walls and were later detected in the roots of wheat (*Triticum aestivum*) [159] and red spinach (*Amaranthus tricolor*) seedlings [122]. Wild and Jones [169] demonstrated that MWCNTs with a diameter of 110−170 nm could pierce the epidermal cell wall and thus penetrate up to 4 μm into the cytoplasm of wheat (*Triticum aestvcum*) root hairs. MWCNTs taken up by plant roots were even detected in the xylem and in phloem cells [129]. A root to shoot translocation of MWCNTs is most probably driven by transpiration [170] as demonstrated for wheat (*Triticum aestivum*) and rapeseed (*Brassica napus*) [171]. In soil-grown tomato plants MWCNTs have been detected in vegetative shoot organs and even in the flowers [167]. Figure 2.4 summarizes the evidences of plant uptake and internal translocation of carbon-based nanomaterials.

Considering the above-described effects of CNTs on plants, several tentative conclusions can be formulated: the cases of positive effects on seed germination seem to be related to seed coat perforation by nanotubes and improved seed water uptake. The effects of CNTs on cell cultures are negative as well as positive, but in both cases a contact of CNTs and cells was observed. Many studies show plant responses to CNT treatments comparable with reactions induced by various biotic and abiotic stress factors. Generally, the interaction of CNTs (and other CNMs) with entire plants appears to be a highly complex process, in which three components (plant, CNMs and growth medium) are closely interlinked. Therefore, a variation in one of these components can completely change the expression profile of responses to CNT-plant interactions (Figure 2.5), as a main source of contradictions and variability in different studies.

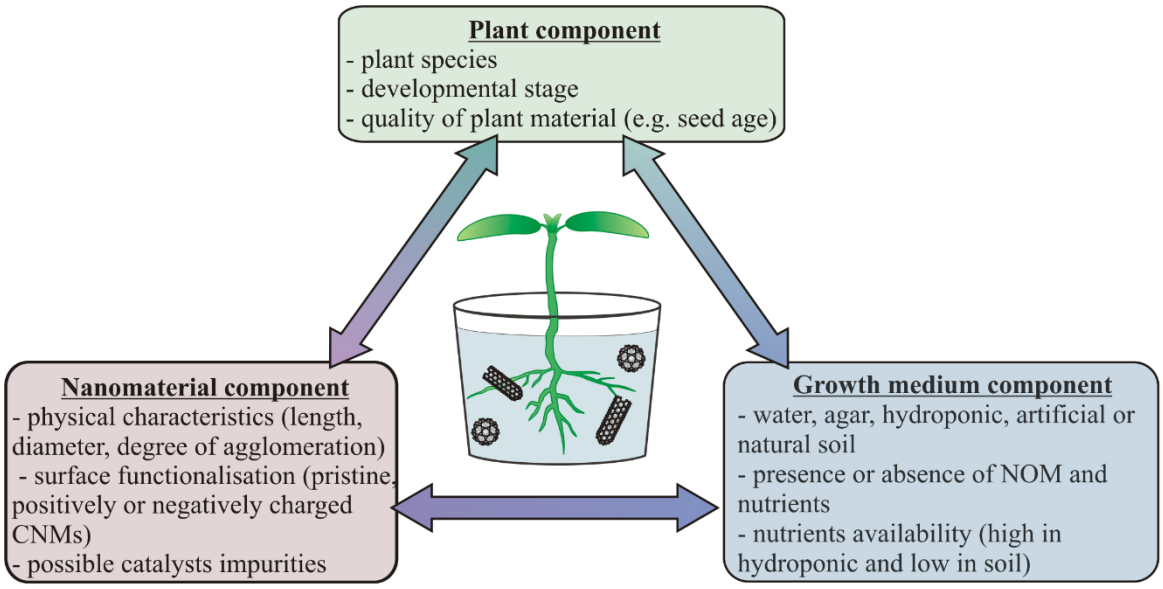

Figure 2.5. *Relationships between three components (plant, carbon nanomaterial and growth medium) as a complex system determining effects of nanomaterial influence of plant development.*

2.6.3 Impact of graphene on plants

Similar to other classes of nanomaterials, uptake into seeds and seedlings [172], plant growth stimulation [173, 174] at low concentrations (e.g. 5 mg L^{-1}) and growth inhibition in plants exposed to higher doses ($\geq$ 50 mg L^{-1}) as a response to oxidative stress [175] has been also demonstrated for graphene.

A very detailed investigation of oxidative stress, induced by different concentrations of graphene oxide in faba bean (*Vicia faba*) seedlings was performed by Anjum et al. [176, 177]. They showed that low and high concentrations of GO applications (100, 200 and 1600 mg L^{-1}) impaired the antioxidative glutathione metabolism [176] and increased the amount of ROS [177]. Interestingly, graphene oxide applied in moderate concentrations (400 and 800 mg L^{-1}) increased the glutathione pool [176] and reduced the formation of ROS [177], which was explained by an improved seed water content. Amendments of graphene oxide to native soils also reduced the activity of soil enzymes (xylosidase, 1,4-β-N-acetyl glucosaminidase, and phosphatase), which is probably related to its antimicrobial properties, but the microbial biomass was not affected [178]. Additionally, indirect toxicity of GO has been reported in wheat (*Triticum aestivum*) acting via increased phytotoxicity of arsenic (As) [179]. Mechanical damage of the cell wall and plasma membrane caused by the graphene oxide sheets, contributed to increased arsenic (As) uptake, which led to toxicity and further changes in metabolism. Similarly, mechanical damages of cell wall and other organelles (chloroplasts) due to GO treatments as well as enhanced formation of ROS have been detected in algal cells [180, 181]. Thus, the published data suggest that the main mechanisms of graphene toxicity are based on (i) mechanical damage of cells and tissues caused by the sharp edges of graphene sheets and (ii) formation of ROS which in small doses can also induce hormetic effects.

2.6.4 Methodological considerations

Many authors emphasize that in studies on nanomaterial toxicity, specific methodological considerations should be taken into account. Very often these studies have been criticized for the use of unrealistically high concentrations of the applied carbon-based nanomaterials. By analyzing the available literature on carbon nanomaterial-plant interactions, it can be concluded that the concentration of applied fullerenes, nanotubes, graphene and their derivatives is highly variable, ranging from the lowest applied concentration of 0.001 mg fullerenes L^{-1} [142] to the highest of 5000 mg carbon nanotubes L^{-1} [125]. Considering each group of nanomaterials separately, it turns out that fullerenes were tested in a concentration range between

0.001 mg L^{-1} and 200 mg L^{-1} [142], followed by graphene concentrations from 5 [173] to 2000 mg L^{-1} [175], and carbon nanotubes applied in a range from 5 mg L^{-1} [158] up to 5000 mg L^{-1} [125] (Figure 2.6). The real concentrations of carbon-based nanomaterials in different environmental compartments are yet unknown but modeled release rates of carbon-based nanomaterials into soils (for EU) are higher for CNTs (1.51 ng $kg^{-1}year^{-1}$), compared to fullerenes (0.058 ng $kg^{-1}year^{-1}$). However, certain management strategies such as sludge soil applications can dramatically increase these values (CNTs: 73.6 ng $kg^{-1}year^{-1}$, fullerenes: 2.2 ng $kg^{-1}year^{-1}$) [182]. These estimates provide important basic information concerning the range of potentially expected inputs, although the long-term behavior and persistence of CNMs in various environmental compartments still remains to be determined. At least some studies demonstrate the environmental transformations of CNMs including soil sorption and microbial degradation [146, 183–186] which can finally reduce the real CNM bioavailability.

For ecotoxicological studies, it is recommended to use an appropriate range of CNM concentrations with respect to the study objectives. However, the experimental concentrations of CNMs used to investigate potential consequences of CNM release as environmental contaminations (ppb range), in many cases largely exceed the levels arising from model calculations (ppt range). On the other hand, in studies aiming at the development of agricultural or biotechnological CNM applications, substantially higher concentrations frequently need to be investigated according to the envisaged product design. Therefore, a clear definition of study objectives is essential to obtain valuable results.

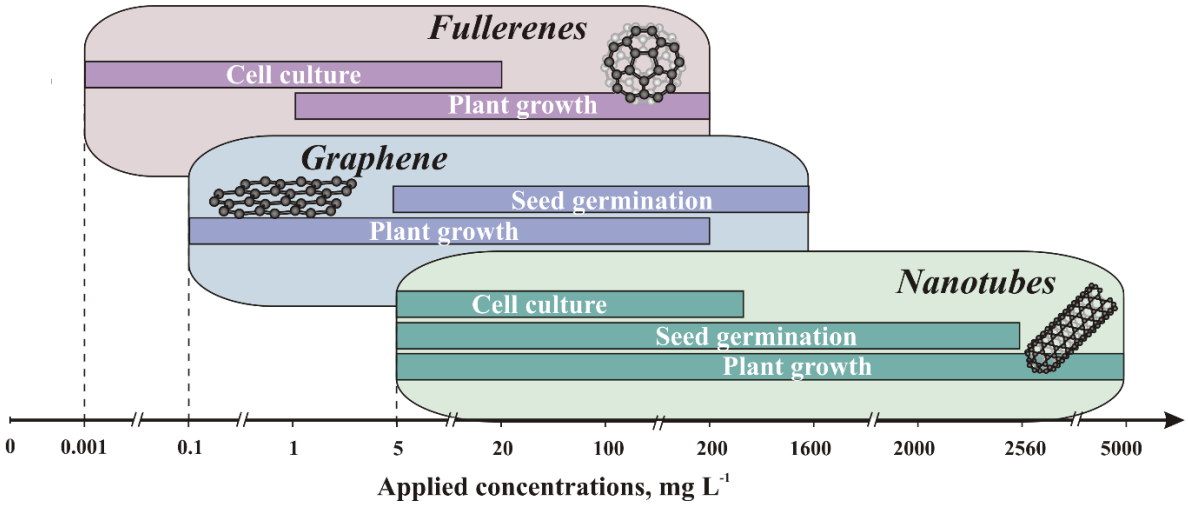

Figure 2.6. *Range of carbon-based nanomaterial concentrations, used in plant toxicity studies.*

Another important methodological issue is the availability of reliable techniques for CNMs detection in plant tissues. Qualitative methods of CNM detection in plant samples comprise light microscopy, transmission and scanning electron microscopy (TEM and SEM) able to detect carbon nanotubes, fullerenes and graphene particles in plant samples. Light microscopy was used as an easily available technique for visualization of MWCNT aggregates in contact with Arabidopsis (*Arabidopsis thaliana*) cell cultures or for the detection of MWCNTs at the root surface of red spinach (*Amaranthus tricolor*) seedlings, grown in CNT-amended nutrient solution [122]. However, light microscopy only allows detection of large CNMs aggregates but not individual CNM particles.

A significantly higher resolution can be obtained using TEM, which has been used for detection of graphene oxide in the tissues of wheat (*Triticum aestivum*) seedlings [179], the presence of graphene inside the husk of tomato (*Solanum lycopersicum*) seeds [172], localization of C70 in rice (*Oryza sativa*) leaves [145], MWCNTs in root and leaves of maize (*Zea mays*) and soybean (*Glycine max.*) seedlings [129]. Challenges related to TEM comprise low contrast between CNMs and plant tissue structures [130, 151], a complicated sample preparation and the need for analysis of large numbers of samples. Use of fluorescent labels attached to CNTs can significantly improve the feasibility of microscopic techniques [187]. However, this type of chemical CNT functionalization

can also lead to alterations of their physicochemical properties associated with altered effects on plants [166].

Scanning electron microscopy is another technique to visualize CNMs: it has been used to detect graphene sheets at the root surface of red spinach (*Amaranthus tricolor*), cabbage (*Brassica oleracea*) and tomato (*Solanum lycopersicum*) seedlings [175] and of MWCNTs associated with red spinach (*Amaranthus tricolor*) roots [122]. However, the resolution of SEM techniques is often smaller than in TEM. Visualization of CNMs at the surface or inside plant tissues can provide important information on interactions of CNMs with plant cells and cell structures, internalization mechanisms of CNMs, and transport and distribution of CNMs within the plant, with particular importance for the development of vehicle systems.

Alternative technique to identify the presence of carbon nanotubes in plant samples is Raman spectroscopy. In contrast to TEM, this method does not produce false negative results, but it cannot provide detailed information on intracellular CNM location. Therefore, both techniques are frequently used for complementary analyses. A combination of Raman spectroscopy and TEM was employed for detection of CNTs in tomato (*Solanum lycopersicum*) [154], wheat (*Triticum aestivum*) [171] and red spinach (*Amaranthus tricolor*) [122] seedlings and in tobacco (*Nicotiana tabacum*) cell cultures [165].

A promising novel approach for detection of MWCNTs in plant tissues is based on a combination of photothermal and photoacoustic mapping developed by Khodakovskaya et. al [151]. The method has demonstrated high sensitivity, and the obtained results were confirmed by optical imaging. In another study, infra-red (IR) spectroscopy was used for detection of fullerols in bitter melon [143], since fullerols exhibit specific infra-red absorption features.

Despite numerous examples of evidence for the uptake of carbon nanomaterials into plant organs, only limited information exists concerning the quantities of CNMs taken up by plants. However, in the recent past significant progress has been made in the development of techniques for CNMs quantification; fullerene C60 has been quantified

in zucchini (*Cucurbita pepo*) stems using high-performance liquid chromatography (HPLC) with UV-vis spectroscopic detection [141]. Another method for CNT quantification inside plant samples, based on microwave-induced heating [188] has demonstrated extraordinarily high accuracy associated with low detection limits (< 0.1 μg) and is now registered as a patent [189]. Also, radio-labeled CNMs have been employed to quantify CNMs in plant tissues. Application of ^{14}C-labeled C60 to radish (*Raphanus sativus*) grown in sand and hydroponic culture, revealed plant uptake of 7% of the applied fullerene dosage. In a study with wheat (*Triticum aestivum*) and rapeseed (*Brassica napus*) exposed to ^{14}C-radiolabeled MWCNTs (10−100 mg L^{-1}) in a hydroponic culture medium [171] it was demonstrated that less than 0.005‰ (≈200 ng g^{-1} plant dry matter) of the total applied nanomaterial, was taken up and translocated within the plants without beneficial or detrimental effects. The high discrepancy between the externally applied amount of CNTs and the fraction really taken up by the plants, as well as typical features of carbon nanomaterials, such as agglomeration or sedimentation in suspensions and surface adsorption to solid substrates, which can vary considerably depending on the composition of the incubation media [152], demonstrate that the simple indication of application concentrations is easy to use but only of little informative value with respect to the real effective dosage.

Moreover, frequently pristine nanomaterials are used in test systems to investigate their interactions with living organisms. This scenario hardly reflects realistic natural conditions, since depending on the composition of the incubation medium, carbon nanomaterials can undergo significant conformational changes (e.g. agglomeration) with significant impact on their properties in terms of bioavailability or toxicity [118, 152]. To define realistic application conditions, much more information is required concerning the behavior of the various types of nanomaterials in soils and planting substrates. For comparative analyses, the development of standardized test systems would be urgently needed. It has been also recommended to pay more attention to selected controls [130] by also including positive controls in addition to the commonly used negative controls, as well as other carbon (activated carbon) and non-carbon (contaminants in CNTs) controls that will eliminate any possible artifacts.

2.7 Conclusion

Nanotechnology develops rapidly and promises innovations in many fields of science and technology. Nanomaterials, including carbon-based nanomaterials, are ready to be produced on a large, industrial scale for a wide range of application fields including the environmental and agricultural sectors. However, a surprisingly limited body of information exists concerning the real concentrations and behavior of these materials in natural environments and their interactions with living organisms as a prerequisite for safety evaluations. This is further complicated by the wide range of nanomaterials with different properties and by conformational changes of carbon nanomaterials during interactions with the various constituents of different incubation media (e.g. agglomeration) with potential impact on bioavailability and toxicity [122, 171]. Apart from these limitations however, at least some principal properties of carbon nanomaterials, relevant for their interactions with plants have been identified:

(i) most carbon nanomaterials can be taken up by plants. This is frequently associated also with internal translocation;
(ii) small amounts of absorbed nanomaterials can induce physiological responses;
(iii) at higher external concentrations, frequently detrimental effects on plant growth are observed, while lower levels of carbon nanomaterials (<100 mg L^{-1}) exert beneficial or no effects;
(iv) induction of oxidative stress by formation of ROS seems to be a major common mechanism of phytotoxicity induced by carbon nanomaterials, while beneficial effects are probably based on hormesis, which is frequently observed during exposure to toxic agents at sub-toxic levels (e.g. glyphosate, Al^{3+}, pathogens), and often based on induction and strengthening of stress defense systems [176, 177]; in the case of improved seed germination there is also a stimulation of water uptake;
(v) chemical (functionalization) or conformational (agglomeration) modifications of carbon nanomaterials can significantly influence their toxicity potential;
(vi) in many studies of CNM phytotoxicity tested dosages significantly exceed the expected environmental concentrations.

As a major challenge for the future, a more comprehensive and systematic survey of the key factors important for interactions of the various carbon nanomaterials with living organisms and the environment will be important for both risk evaluation and the characterization of potential applications.

2.8 Appendix

2.8.1 Authors' contributions

OZ wrote the manuscript and prepared figures and tables. The manuscript was edited and improved by both authors.

2.8.2 Competing interests

The authors declare that they have no competing interests.

2.8.3 Acknowledgments

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2.8.4 Supplemental materials

The following additional data are available with the online version of this paper. Supplemental Table S2.1 listing patents of various nano-fertilizers and soil improvers.

Supplemental Table S2.1. *List of patents for nano-carbon containing agricultural products.*

Patent number	Patent name	Product type	Nano component	Size and amount of nanocomponent	Claims	Inventors
WO 2013110202 A1	Carbon nanotube production method to stimulate soil microorganisms and plant growth produced from the emissions of internal combustion	Soil improver	SWCNTs, DWCNTs, MWCNTs	na	Can improve plant growth characteristics	(Lewis G)
CN103772055 (A)	Preparation method of liquid state fertilizer synergist containing trace rare earth and nano carbon	Fertilizer synergist	Nano carbon	na	Low in production cost and capable of effectively utilizing the ammonium chloride waste water	(Yang J, Wu H)
CN103772044 (A)	Nano carbon organic compound fertilizer	Fertilizer	Nano carbon	na	Can enhance the cold, drought, disease and insect pest-resisting functions.	(Li L)
CN103772043 (A)	Nano carbon fertilizer	Fertilizer	Nano carbon	na	Can improve the cold, drought, disease and insect resistance of the crops.	(Li L)
CN103772007 (A)	Preparation method of nano cow dung fertilizer	Fertilizer	Nano carbon	na	For effectively increasment the yield of crops.	(Yang H)
CN103755479 (A)	Nanocarbon organic nitrogen fertilizer	Fertilizer	Nano carbon	na	Cold, drought and pest resistance of the crops can be strengthened.	(Li L)
CN103739396 (A)	Nano-carbon fertilizer special for paddy rice	Fertilizer	Nano carbon	3−10 %	Yield of paddy rice can be increased by 30-45 % and using amount of the fertilizer is saved by 30-50%.	(Zhang J)
CN103724066 (A)	Potato slow release fertilizer	Slow release fertilizer	Nano carbon	Unclear	High in yield and suitable for large-scale cultivation; the yield per ha is up to above 60000 kilograms.	(Wu A)

Patent number	Patent name	Product type	Nano component	Size and amount of nanocomponent	Claims	Inventors
CN103641606 (A)	Special fertilizer for greenhouse watermelons and preparation method thereof	Fertilizer	Nano carbon	na	The yield and the nutritive value of the greenhouse watermelons cultivated by the special fertilizer are high.	(Yang J, Liao H)
CN103613472 (A)	Method for preparing potato sustained-release fertilizer	Fertilizer	Nano carbon	Unclear	The yield per ha reaches over 60000kg, the fertilizer is applicable to large-scale cultivation and the method cost is low.	(Wu A)
CN103539585 (A)	Synergist fertilizer as well as preparation method thereof	Fertilizer synergist	Nano carbon	Unclear	Can adsorb free nitrogen in air and soil and can improve fertilizer efficiency, has a slow release effect and reduces a topdressing link in a growth process of the crops.	(Cao J)
CN103539559 (A)	Fertilizer specially used for cedars	Fertilizer	Nano carbon	Unclear	Can improve the quality of the cedars, improve the fertility of lands, enhance granular structure of the soil, improve the water and nutrient retention performance and increase the number of soil OM.	(Cao J)
CN103524237 (A)	Special nano-carbon fertilizer for sugarcane and preparation method thereof	Fertilizer	Nano carbon	1−10%	Sufficient potash fertilizer is provided in the growth period of sugarcane, the soil is improved, the cadmium pollution is prevented, and the production cost of the special fertilizer for sugarcane is reduced.	(Zhang J)
CN103497037 (A)	Special fertilizer for potato and its preparation method	Fertilizer	Nano carbon	Unclear	Has a slow release effect, can satisfy nutrient needs of the potato on vegetative growth and reproductive growth by one-time application.	(Liu X)
CN103408382 (A)	Special fertilizer for mountain tea tree and preparation method thereof	Fertilizer	Nano carbon	Unclear	The quality of the special fertilizer for a tea tree can be improved, and the cost can be lowered.	(Zhou J)

Patent number	Patent name	Product type	Nano component	Size and amount of nanocomponent	Claims	Inventors
CN103333003 (A)	Special fertilizer for nano carbon tea and preparation method of special fertilizer	Fertilizer	Nano carbon	Unclear	The fertilizer effect is prolonged, the special fertilizer is reasonable in preparation of nutrient elements, and has a controlled release effect.	(Zhang K)
CN103274806 (A)	Bio-organic fertilizer with weathered coal as main material, and preparation method thereof	Fertilizer	Nano carbon	Unclear	Can improve soil quality and structure, contains macroelements and microelements required by crops and a large amount of active substances produced during a microbial fermentation process.	(Lu Y)
CN103172442 (A)	Functional nutrient complex granular fertilizer and preparation method thereof	Fertilizer	Nano carbon	Unclear	Controllable in slow release (30-150 days), strong in pertinency (special fertilizer for wheat, rice and other crops) of fertilizer application, relaxed in limitation on the storage and transportation conditions and increased in service radius.	(Wu X, Liu F)
CN103011950 (A)	Lost control nano-composite fertilizer and preparation method thereof	Fertilizer	Nano carbon	Unclear	High in nutrition utilization rate, high in production increasing extent, convenient to apply, low in cost, high in input-output ratio, water-maintaining, soil-loosening, environment-friendly.	(Zhang Z, Liu Q)
CN102951970 (A)	Rabbit dung-Chinese herbal medicine organic fertilizer and preparation method thereof	Fertilizer	Nano carbon	Unclear	The physical and chemical properties of soil can be improved, the nutrient absorption of crops and the yield can be improved and the occurrence of diseases and insect pests of the crops can be further reduced.	(Wei C)
CN102826907 (A)	Nanometer concentrated enzyme organic fertilizer and preparation method thereof	Fertilizer	Nano carbon	2−4%	The utilization rate of the fertilizer is increased, by means of combining biologic enzyme with the nanometer materials, not	(Wu P, Song X)

Patent number	Patent name	Product type	Nano component	Size and amount of nanocomponent	Claims	Inventors
					only absorption for enzyme molecules and nanometer materials is guaranteed, but effective time of biologic enzyme in soil is increased, and use efficiency of organic fertilizer is raised.	
CN102816003 (A)	Nano carbon sulfate radical organic fertilizer and preparation method thereof	Fertilizer	Nano carbon	na	Can be widely applied to treating plant diseases and has a good effect on clearing cadmium pollution in soil.	(Zhang Z, Chen J)
CN102718584 (A)	Nano-carbon synergistic compound fertilizer specially used for tobacco and production method thereof	Fertilizer	Nano carbon	0.1− 0.5 wt%	Specially used for tobacco improves fertilizer utilization efficiency, a tobacco leaf yield and tobacco leaf quality.	(Xie J, Liu J)
CN102674936 (A)	Nano-carbon type special silicon fertilizer for rice and production process of fertilizer	Fertilizer	Nano carbon	Unclear	The special fertilizer is complete in nutrient, balanced, high in use ratio and low in environment pollution.	(Gao J, He S)
CN102617214 (A)	Preparation method for nano compound fertilizer	Fertilizer	Nano carbon	na	Increaces the seeding ratio to 92 percent, promotes plants to absorb potassium and the ratios of disease infection and insect damage infection are reduced.	(Zheng J)
CN102491815 (A)	High-concentration nano-carbon cotton special fertilizer and preparation method thereof	Fertilizer	Nano carbon	na	Has comprehensive nutrition, balanced fertilizer components and a high fertilizer utilization rate, and can improve a cotton yield and cotton quality.	(Gao J, He S)
CN 101633590 A	Nano-carbon rare-earth synergistic fertilizer and preparation method thereof	Fertilizer	Unclear	5−70 nm 0.01−0.9%	The nitrogen use efficiency is enhanced to 45 percent to 60 percent, and has obvious effects for increasing production and saving fertilizer.	(Zhang Z, Liu J)

Patent number	Patent name	Product type	Nano component	Size and amount of nanocomponent	Claims	Inventors
CN1701665 (A)	Highly effective nanometer seed coating agent with low toxicity and preparation method thereof	Seed coating agent	nano white carbon black	0.8−0.95%	Efficient and low toxicity of nano seed coating.	(Ding Y, Wu Q)
US2015007496 (A1)	Carbon Nanotube Production Method to Stimulate Soil Microorganisms and Plant Growth Produced from the Emissions of Internal Combustion	CNTs production system	CNTs		Carbon nanotubes applied to the plant growing medium, for example by using the agricultural implement to incorporate the conditioned exhaust into the soil.	(Lewis G)
CN103155846 (A)	Method of improving germination rates of isatis root seeds using multi-wall carboxylating carbon nano tubes	Method of improving germination rates	Multi-wall carboxylating CNTs	na	The germination rates of the isatis root seeds can be improved by about 20%.	(Yu J, Ren Q)
US2012233725 (A1)	Method of Using Carbon Nanotubes to Affect Seed Germination and Plant Growth	Method of increasing the probability and rate of seed germination	Carbon nanomaterial	10−200 μg/mL	Increases the probability and rate of seed germination, vegetative biomass, and water uptake in seeds.	(Biris A, Khodakovskaya M)
CN103155745 (A)	Method for enhancing germination rate of safflower seed by utilizing multi-wall carboxylic carbon nanotube	Method for enhancing germination rate	Multi-wall carboxylic CNTs	na	Germination rate of the safflower seeds is enhanced by 10%.	(Yu J, Ren Q.)
CN104030825 (A)	Mixed fertilizer for grapes and preparation method thereof	Mixed fertilizer	Nano carbon	Unclear	Promotes growth of the grapes, enhances the yield and sweetness, improves the structure of the soil, enhances the fertility of the soil.	(Jiang C)

Patent number	Patent name	Product type	Nano component	Size and amount of nanocomponent	Claims	Inventors
CN103210957 (A)	Solvent for improving antioxidation metabolic capacity of flue-cured tobacco	Solvent for improving the antioxidation metabolic capacity	Nano carbon sol	0.01−0.05%	The activities of superoxide dismutase, peroxidase and catalase of the tobacco leaf can be improved, the content of proline is increased, and the content of membranous maleicdialdehyde in the tobacco leaf is reduced.	(Liang T, Yin Q)
CN103125395 (A)	Culture medium for promoting adventive root of woody plant to root and grow and application of culture medium	Culture medium	Water-soluble CNTs	50−150 mg/L	The culture more capable of promoting the growth of the adventive root.	(Chen J, Xu Z)

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3 Objectives and research questions

This work was driven by the importance of understanding the fundamental processes underlying the interactions of higher plants and novel synthetic nanomaterials in face of increased passive release of these compounds into the environment or even direct agricultural applications. Selected industrial Multi-Walled Carbon Nanotubes (MWCNTs) were employed as model compounds. In the existing literature there are evidences of both simulative and inhibitory effects of MWCNTs on plant growth and development, but knowledge on the specific mechanisms of these impacts is limited. This thesis focused on characterization of the mechanisms of MWCNT impacts on various growth stages of higher plants including seed germination, seedling development and early growth of representative crops. The main objectives of the thesis comprised:

Objective 1.

To identify a potential impact of MWCNTs on germination and early growth of representative crops; to characterize the underlying mechanisms; and to select the most responsive plant species as a model plant system for further investigations.

Research questions:

1. Can MWCNTs promote or inhibit germination of various crops?

2. Is there any difference in responsiveness between various plant species?

3. Which MWCNTs concentrations and treatment durations can cause detectable effects?

4. Is it possible to identify the underlying mechanisms of potential MWCNT effects on germination and seedling development?

Objective 2.

To investigate the role of MWCNT treatments on further plant development and to characterize the underlying mechanisms.

Research questions:

1. Is it possible to identify persistent morphological and physiological changes after plant exposure to MWCNTs?

2. Can nutrient availability in growth substrates impact on MWCNT-plant interactions?

3. What is the physiological background of potential MWCNT-plant interactions?

4 Chapter II. Differential impact of multi-walled carbon nanotubes on germination and seedling development of *Glycine max*, *Phaseolus vulgaris* and *Zea maize*

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4.1 Abstract

This study is designed to investigate the effects of carbon nanomaterials (multi-walled carbon nanotubes, MWCNTs) under controlled conditions on three different plant species. The study covers the effects of MWCNT dosage, treatment duration, and the plant developmental stage, including imbibition, germination and seedling development. Germination experiments are conducted under standardized laboratory conditions based on the protocols of the International Seed Testing Association with aqueous MWCNT suspensions at a dosage of 0, 100 and 1000 mg L^{-1} applied as seed treatments during 36 h after sowing prior to radicle emergence, using soybean (*Glycine max* (L.) Merr. cv. BR-16 Conquista), common bean (*Phaseolus vulgaris* L. cv. Bohnen maxi) and maize (*Zea mays* L. cv. Surprise) as test plants. The seed treatment with MWCNTs reduced the speed of water uptake particularly by soybean seeds. This is associated with an increased germination percentage and reduced development of abnormal seedlings, while mean germination time is unchanged. However, during later

seedling development, negative effects on root growth, particularly affecting fine root development are recorded for all investigated plant species. In soybean, this effect is first detected at 8 days after sowing and requires a minimum MWCNT seed exposure of 36 h. Inhibition of root growth is associated with reduced metabolic activity of the root tissue as indicated by tetrazolium vitality staining. The nitrate uptake was lower in MWCNT-treated plants, which is mainly attributed to the smaller root system. The results demonstrate that even under standardized experimental conditions, excluding environmental factors and effects induced by carbon nanomaterials, plant responses to MWCNT exposure exhibit differences, depending on plant species but also on the physiological status and the developmental stage of individual plants.

Key words: germination, seedling growth, carbon nanotubes, soybean, common bean, maize.

4.2 Introduction

Carbon Nanotubes (CNTs) are nanostructured carbon allotropes of cylindrical shape, possessing outstanding physical and chemical properties. Multi-walled carbon nanotubes (MWCNTs) are the most important class of carbon nanomaterials with the highest production volumes and numerous technical applications. In the recent past, potential applications have been extended to agriculture with first patents as germination stimulants, plant growth promoters, fertilizers and fertilizer synergists, as well as delivery systems for agrochemicals, antifungal and antimicrobial agents. However, the published literature reflects a high heterogeneity of plant responses to MWCNT treatments, including both, positive and negative effects in some plant species and the complete absence of responses in others [1–10]. The subject matter is reviewed by O. Zaytseva and G. Neumann [11].

Positive effects of MWCNTs added to agar media at concentrations of 10 to 40 mg L^{-1} on seed germination and seedling growth of tomato (*Solanum lycopersicum* L.) are reported by Khodakovskaya et al. [1] and Morla et al. [2]. Later, Lahiani et al. [3] reported accelerated germination of barley (*Hordeum vulgare* L.), maize (*Zea mays* L.) and soybean (*Glycine max* (L.) Merr.) seeds in a Murashige and Skoog medium

amended with 50−200 mg L^{-1} MWCNTs. Srivastava and Rao [4] reported that germination of wheat (*Triticum aestivum* L.), maize and peanut (*Arachis hypogaea* L.) was enhanced by application of 50 mg L^{-1} MWCNTs. The reports listed above suggested that MWCNT application increased the seed water content by perforation of the seed coat. Additionally, Lahiani et al. [3] reported increased expression of aquaporin-related genes, playing a role in water uptake, related with germination, root elongation and plant growth. Positive effects of MWCNTs on seed germination have been also related to the presence of metal catalyst impurities in the applied MWCNT materials [12].

Similarly, during later seedling development and early growth, positive effects of MWCNTs on root and shoot elongation have been reported for a range of plant species, such as tomato, wheat, soybean, maize, mustard (*Brassica juncea* L.), and black lentil (*Vigna mungo* (L.) Hepper), even in cases when the plant roots had direct contact with the MWCNTs. The expression of effects was concentration-dependent with beneficial effects at lower levels of MWCNT application and inhibition at higher concentrations. Induction of oxidative stress associated with formation of reactive oxygen species (ROS), membrane damage, electrolyte leakage, mitochondrial dysfunctions and DNA aberrations are characterized as determinants of MWCNT toxicity during seedling development and early growth of red spinach (*Amaranthus tricolor* L.), rice (*Oryza sativa* L.), lettuce (*Lactuca sativa* L.) and cucumber (*Cucumis sativus* L.), and small seeds being more sensitive than large seeds. By contrast, Lin and Xing [5] did not find any effect of MWCNTs applied in concentrations of 1000−2000 mg L^{-1} on seed germination and root elongation of five plant species (radish (*Raphanus sativus* L.), rape (*Brassica napus* L.), ryegrass (*Lolium perenne* L.), lettuce, maize and cucumber). Similarly, Stampoulis et al. [6] reported no effects on germination of zucchini (*Cucurbita pepo* L.) and Miralles et. al. [12] observed no effects of MWCNTs on alfalfa (*Medicago sativa* L.) and wheat.

Various reasons such as genotypic differences, plant developmental stage, experimental setups, type, dosage, formulation and agglomeration have been discussed to explain the heterogeneity of plant responses to MWCNT treatments. In view of the highly variable

effects of MWCNT on plant performance, reported in the literature, a systematic analysis of factors determining the effects of MWCNTs such as MWCNT dosage, duration of exposure to MWCNT treatments, plant-developmental stage including imbibition, germination and seedling development on selected plant species are studied. Soybean, common bean (*Phaseolus vulgaris* L.) and maize are selected as representative crops. Apart from plant growth responses, MWCNT effects were evaluated using assays for physiological activity of the test plants (nutrient uptake, vitality staining).

4.3 Experimental

4.3.1 MWCNTs and preparation of MWCNT suspensions

Multi-walled carbon nanotubes, MWCNTs (NanoTechCenter Ltd., Tambov, Russia), produced by chemical vapor deposition (purity > 98%) with a minimum length of 2 µm, an external diameter of 20−70 nm and an internal diameter of 5−10 nm were used for the experiments. The selected concentrations for MWCNT application (100 and 1000 mg L^{-1}) were in the range reported for various other studies [1–9]. Working suspensions of MWCNTs were prepared directly in deionized (DI) water and dispersed by ultrasonification (SONOREX SUPER RK 510 H; 35 KHz, Bandelin Electronic, Berlin, Germany) for 30 min.

4.3.2 Test plants

Three plant species: soybean (*Glycine max* (L.) Merr. cv. BR-16 Conquista), common bean (*Phaseolus vulgaris* L. cv. Bohnen maxi) and maize (*Zea mays* L. cv. Surprise) were selected. Seeds were stored in darkness at 4 °C and kept at room temperature one day before use.

4.3.3 Impact of MWCNTs on seed water uptake

Hydration of seeds by imbibition is an important factor triggering the start of seed germination and seed has been influenced by MWCNT treatments in various earlier reports [1, 3]. Therefore, the effects of MWCNTs on seed water uptake were

investigated during first twelve hours of imbibition. A germination test was performed in Petri dishes (control: DI water; treatments: 100 and 1000 mg L^{-1} MWCNTs) for studying the kinetics of water uptake by seeds. An additional control without MWCNT was included with seeds imbibing more slowly between four layers of moist filter paper: one sheet of filter paper (58×58 cm, MN710, Macherey und Nagel, Düren, Germany) was folded lengthwise two times to obtain a 4-layer paper strip which was soaked with 60 ml of DI water according to its maximum water holding capacity. Ten seeds were placed along the upper edge of the paper strip, which was subsequently folded, forming a paper roll with the seeds inside. The paper rolls were placed in upright position into a plastic germination box (30×20×10 cm) and kept under the same growth conditions as the seeds in Petri dishes. Water uptake was recorded by determining weights of the seeds from the Petri dishes and from the filter paper rolls in 1 h intervals during 12 h.

4.3.4 Impact of MWCNTs on the seed germination

The influence of MWCNTs on germination of the three plant species was estimated in standardized filter paper germination tests according to the ISTA rules [13]. For treatments, suspensions of MWCNTs in DI water were applied in concentrations 100 mg L^{-1} and 1000 mg L^{-1}; deionized (DI) water was used as a control. Five mL of un-precipitated MWCNT suspensions or DI water were evenly distributed in plastic Petri dishes (diameter 96 mm, Greiner, Nürtingen, Germany) with 3 layers of filter paper (Blue ribbon MN 640d, Macherey und Nagel, Düren, Germany) on the bottom. Thereafter, ten seeds per Petri dish were distributed equidistantly into the MWCNT suspensions or DI water. Thus, the concentrations of MWCNTs in working suspensions (100 mg L^{-1} and 1000 mg L^{-1}) translated into an actual MWCNT dosage of 50 and 500 µg $seed^{-1}$. Petri dishes were covered with lids and placed into an incubator (BD 115, Binder, Tuttlingen, Germany). Depending on the plant species, temperature during the germination test as well as test duration were maintained according to the ISTA rules [13] and indicated in Table 4.1. During the germination tests, the number of germinated seeds was counted every 12 h and final germination percentage was determined on the day specified in Table 4.1. Seeds were considered to be germinated when the radicle length reaches 2 mm [14]. Two indices were calculated to describe the results of the

germination tests: germination percentage (GP_i) [15] and mean germination time (MGT) [15].

Table 4.1. *Conditions maintained during the germination test.*

Plant species	Temp., °C	First count, day	Final count, day
Maize	25	4	7
Soybean	25	5	8
Common bean	25	5	9

4.3.5 Impact of MWCNT seed treatment on seedling development

The germination test showed that radicles started to emerge after 36 h of seed imbibition. Therefore, the seed treatment with MWCNTs in the succeeding experiments was limited to 36 h to avoid direct interactions of MWCNTs with the emerging radicles. After 36 h of seed treatment in Petri dishes, seeds were transferred to filter paper rolls in germination boxes as described above.

The lids of the germination boxes were opened and the boxes placed into a climate chamber with a 14 h light period at an average temperature of 23 °C with regular additions of 25 ml DI water per filter roll to compensate for evaporation. No nutrients were supplied to the seedlings because cotyledons can provide organic and mineral nutrients to young seedlings for up to ten days after emergence [16, 17]. At 10 DAS, the number of abnormal seedlings were counted as defined in ISTA rules [13]. In brief, seedlings are classified as abnormal if there are significant damages or deformations of essential structures (cotyledons, hypocotyl, primary leaves and primary roots), which can prevent normal plant development. Subsequently, shoot length of normal seedlings was recorded and the seedlings were harvested for biomass and root length determination. For root morphology analysis fresh root samples, stored in 30 % (v/v) ethanol, were carefully separated on transparent Perspex trays and subsequently digitalised with an Epson Expression 10000Xl scanner (Epson, USA). Analysis was performed using the WinRHIZO software (Regent Instruments, Quebec, Canada).

4.3.6 Nitrate uptake by seedlings

A short (24 h) hydroponic experiment was performed to investigate the effect of short-term (36 h) MWCNT seed treatment on nutrient uptake by seedlings developed from the treated seeds. Soybean and common bean seeds were treated for 36 h in Petri dishes with 1000 mg L^{-1} MWCNTs or DI water (control). Thereafter, seeds were transferred to filter paper rolls were moistened with DI water and were grown until 10 DAS as described above. Thereafter, three representative seedlings per replicate were transferred for 24 h into beakers containing 100 mL of nutrient solution (chemical composition of nutrient solution in Appendix in Supplemental Table S4.1). To estimate the amount of nitrate (NO_3^-) absorbed by the seedlings, the volume of the nutrient solutions as well as the nitrate (NO_3^-) concentration in the beakers were measured before and after the incubation period. The NO_3^- concentration was measured by using nitrate-sensitive test strips (Merck KGaA, Darmstadt, Germany) and the color intensity on the strips was quantified colorimetrically (Hermann Wolf GmbH&Co.KG, Wuppertal, Germany).

After 24 h of seedlings exposure to nutrient solution, two of three seedlings were harvested for biomass and root length determination and one seedling per replicate was stained with 2, 3, 5-triphenyl tetrazolium chloride (TTC) as a measure of the metabolic activity of the root tissue.

4.3.7 TTC reduction assay

Vitality staining of root tissues was performed with roots of soybean and common bean seedlings, developed from seeds treated for 36 h with 0 or 1000 mg L^{-1} MWCNTs, which were grown in filter paper rolls until 10 DAS and subsequently incubated for 24 h in nutrient solution as described above. Prior to TTC staining the roots were rinsed with DI water and then placed into 50 mL of TTC solution (0.08% TTC in 0.05 M sodium phosphate buffer, pH 7.4) for 24 h in the dark [18]. In metabolically active cells, TTC is reduced by dehydrogenases, forming red-colored insoluble triphenylformazan (TF). The color intensity reflects the degree of metabolic activity in the stained tissues. After an incubation time of 24 h, the TTC solution was discarded, roots were rinsed three

times with DI water, cut into segments of 1 cm and immersed into 10 mL of 95% ethanol for 24 h at 4 °C for extraction of TF. Thereafter, the ethanol extract was filtered (Blue ribbon MN 640d, Macherey und Nagel, Düren, Germany) and the absorbance of the filtrate was measured spectrophotometrically at a wavelength of 485 nm (U-3300, Hitachi Ltd. Tokyo, Japan). Reduction of TTC was calculated as absorption of TF produced per root [Abs_{485} seedling^{-1}] and per unit root dry weight [Abs_{485} g_{DW}^{-1}].

4.3.8 Statistical analysis

All experiments were performed in a completely randomized design. Statistical analysis was conducted with SigmaPlot 11.0 software using the Student's t-test for comparison of two treatments and one-way ANOVA for comparison of multiple treatments. The level of significance was determined at a P value ≤ 0.05. All results in tables and graphs are presented as mean values ± SE (standard error of a mean).

4.4 Results

4.4.1 Seed water uptake

The speed of water uptake was calculated according to the formula:

$$v_{\text{water uptake}} = \frac{m_{\text{imbibed seed}} - m_{\text{dry seed}}}{t}$$

where $v_{\text{water uptake}}$: speed of water uptake [mg seed^{-1} h^{-1}], $m_{\text{imbibed seed}}$: average weight of imbibed seed [mg seed^{-1}] and $m_{\text{dry seed}}$: average initial seed weight [mg seed^{-1}] before the experiment, t: time duration of imbibition [h].

The highest speed of water uptake is found in soybean seeds, followed by common bean and maize (Figure 4.1). In soybean, most rapid imbibition is detected in the control variant (Figure 4.1), while seeds imbibed between layers of moist paper showed the slowest rate of water uptake. Similarly to the variant imbibing between layers of moist paper, the application of 100 and 1000 mg L^{-1} MWCNTs significantly slowed down the seed water uptake by 12% as compared to the control. Also in maize seed imbibition between layers of moist filter paper and MWCNTs treatments reduced the speed of the

water uptake. However, for MWCNT treatments this reduction was not significant. A similar but not significant trend is observed for common bean.

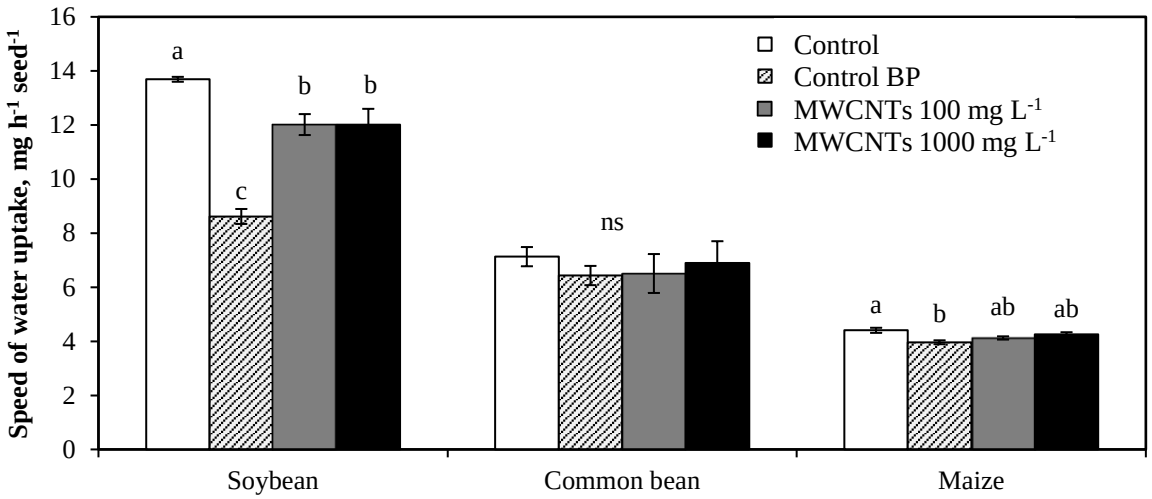

Figure 4.1. *Speed of water uptake by soybean, common bean and maize seeds during 12 h of imbibition in Petri dishes (Control (DI water), MWCNTs 100 and 1000 mg L^{-1}) and in between filter paper (Control BP). Values represent mean values ± SEM of four replicates. Different letters (a, b, c) indicate significant difference between treatments (one-way ANOVA, Tukey test, P ≤ 0.05). ns−not significant, MWCNTs−multi-walled carbon nanotubes, DI water−deionized water.*

4.4.2 Germination test

Germination percentage (GP_i) for each observation is calculated according to the formula [15]:

$$GP_{\mathrm{i}} = 100\,\frac{n_{\mathrm{i}}}{N}$$

where GP_{i}: germination percentage at the i$^{\mathrm{th}}$ observation [%], n_{i}: number of seeds, germinated from the beginning of the experiment to the i^{th} observation and N: total number of seeds.

The mean germination time (*MGT*), days to germination of 50 % of all germinated seeds, is calculated as described by formula [15]:

$$MGT = \frac{\Sigma_{i=1}^{k} n_i t_i}{\Sigma_{i=1}^{k} n_i}$$

where t_i: time from the beginning of the experiment to the i^{th} observation [days], n_i: number of seeds germinated on the i^{th} day and k: last day of germination.

Stimulation of germination percentage (*GP*, %) by MWCNT application is detectable only in soybean but not in common bean and maize (Figure 4.2). Enhanced *GP* of soybean in MWCNT treatments is first detectable on the second day of the experiment and remained elevated till the day of the final count (Figure 4.2 A). The application of MWCNTs with a concentration of 1000 mg L^{-1} significantly increased the final *GP* of soybean seeds by 28% as compared to the control experiment. The treatment with a concentration of 100 mg L^{-1} of MWCNT the *GP* was increased by 25%, although this effect was not significant.

In common bean (Figure 4.2 B) and maize (Figure 4.2 C) the *GP* does not significantly differ. The mean germination time (*MGT*) of all the studied plant species is not affected by MWCNT application (data not shown).

In all cases, the percentage of abnormal seedlings according to the ISTA rules [13], positively correlates with the speed of seed water uptake by soybean (R^2=0.92, P=0.08), common bean (R^2=0.99, P=0.01) and maize seeds (R^2=0.73, P=0.27) measured during the first 12 h of the seed imbibition (Figure 4.3).

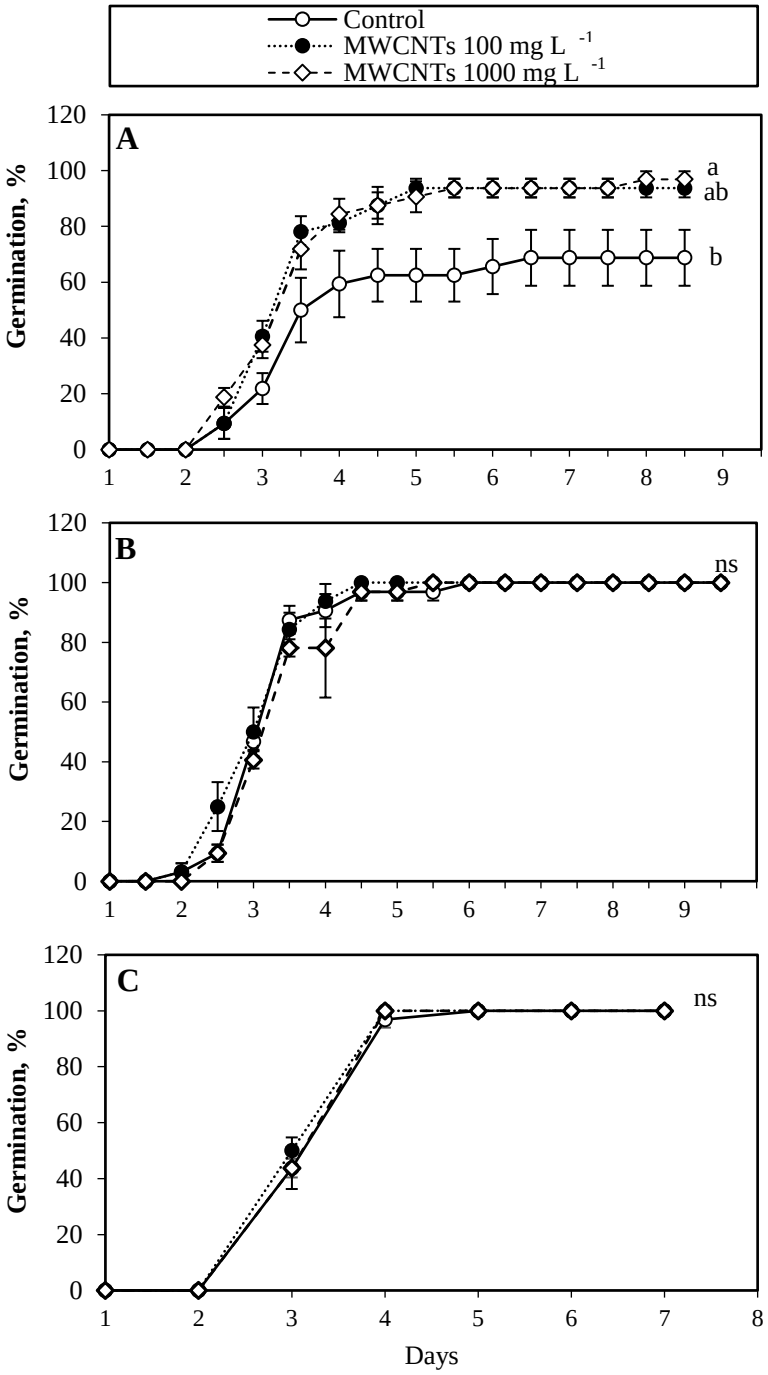

Figure 4.2. *Germination percentage (GP, %) of (A) soybean, (B) common bean and (C) maize seeds in DI water (Control) and in MWCNTs (100 and 1000 mg L^{-1}) suspensions. Values represent mean values ± SEM of four replicates. Different letters (a, b) indicate significant difference between treatments (one-way ANOVA, Tukey test, $P \leq 0.05$). ns−not significant, MWCNTs−multi-walled carbon nanotubes, DI water−deionized water.*

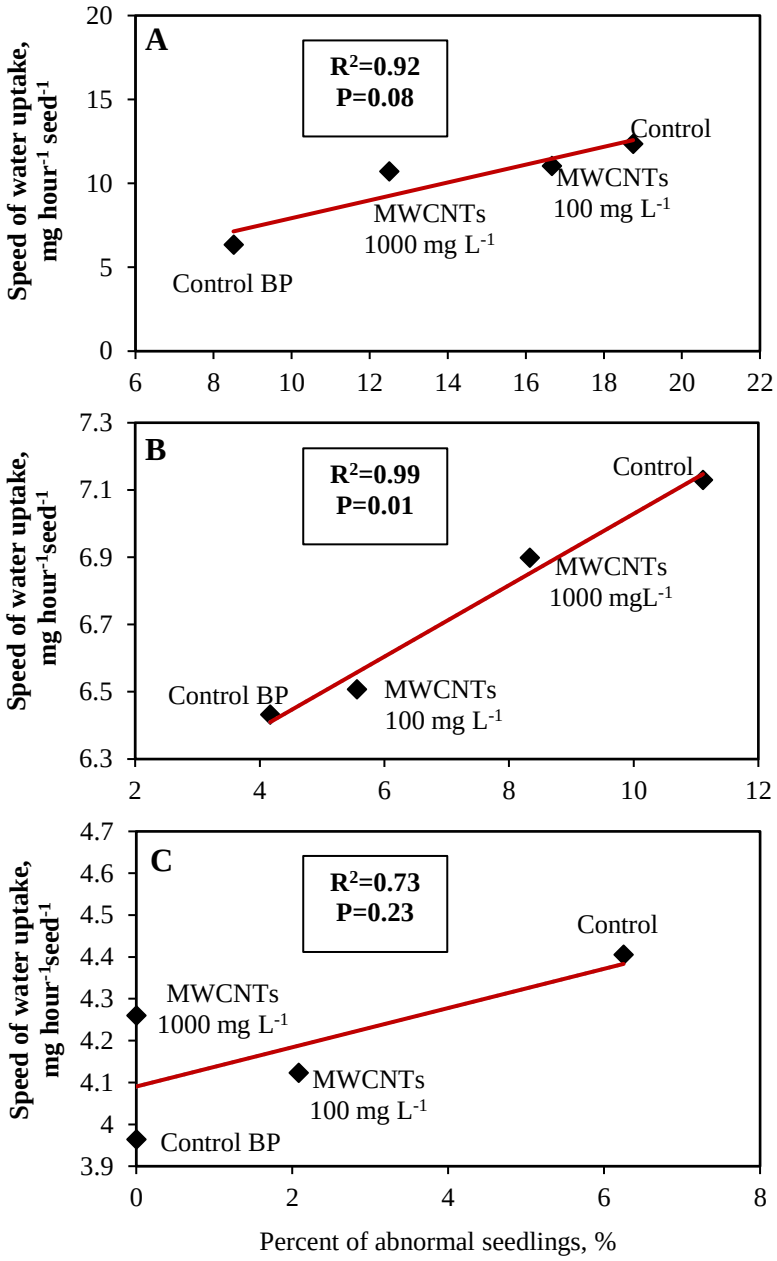

Figure 4.3. *Correlation between speed of water uptake by seeds during first 12 h of imbibition and formation of abnormal seedlings (%) at 10 days after sowing (DAS). The soybean (A), common bean (B) and maize (C) seeds were treated in Petri dishes for 36 h by 100 and 1000 mg L^{-1} MWCNTs or DI water (Control) and by DI water between moist filter paper (control BP) and subsequently grown in filter paper rolls moistened with DI water. MWCNTs−multi-walled carbon nanotubes, DI water−deionized water.*

For all tested plant species the highest percentage of abnormal seedlings was observed in the control variants imbibed in Petri dishes in a film of free water, while the seeds imbibing between filter paper with the slowest rate of water uptake developed the smallest number of abnormal seedlings.

4.4.3 Seedling development

Abnormal seedlings were discarded at final harvest (10 DAS) and the measured growth characteristics refer to normal developed seedlings according to the ISTA rules [13].

In soybean, the short-term (36 h) seed treatment with 100 and 1000 mg L^{-1} MWCNTs did not affect shoot length, shoot and root dry weight of seedlings. However, the total root length was significantly reduced, while average root diameter was increased in both MWCNT treatments (Table 4.2). In the control between layers of moist paper the root dry weight of soybean seedlings was decreased as compared to other treatments and the total root length was shorter than in the control, but longer as compared to MWCNTs treatments.

The application of 1000 mg L^{-1} MWCNTs reduced the root dry weight of common bean and increased the shoot dry weight of maize seedlings as compared to the untreated control. There is also a trend for declining total root length of common bean and maize seedlings induced by 1000 mg L^{-1} MWCNTs as compared to the control, although not significant (Table 4.2).

The analysis of root diameter distribution reveales a reduction of fine root length (diameter: 0.0−0.2 and 0.2−0.4 mm) of soybean in both applied MWCNTs concentrations, while the length of roots with a diameter of 0.4−0.6 mm decreased only by the application of 1000 mg L^{-1} MWCNTs (Table 4.3). Similarly, in maize the length of fine roots (0.0−0.2 mm) decreased in the 1000 mgL^{-1} MWCNT variant (Table 4.3).

Table 4.2. *Growth characteristics of 10-days old soybean, common bean and maize seedlings, developed from seeds exposed to MWCNTs for 36 h and subsequently grown in filter paper rolls. The seeds were treated in Petri dishes either with DI water (Control) or MWCNTs suspensions (100 and 1000 mg L^{-1}) or imbibed slowly between filter paper with DI water (control BP).*

Treatment	Shoot length, cm plant^{-1}	Shoot dry matter, mg plant^{-1}	Root dry matter, mg plant^{-1}	Total root length, cm plant^{-1}	Average root diameter, mm
			Soybean *(Glycine max)*		
Control	17.6 ± 0.7 a	95.56 ± 3.69 a	16.70 ± 1.26 a	981.1 ± 23.0 a	0.25 ± 0.00 b
Control BP	12.6 ± 0.4 b	94.35 ± 6.07 a	15.50 ± 1.18 b	820.2 ± 14.5 b	0.26 ± 0.00 b
MWCNTs 100 mg L^{-1}	18.8 ± 0.5 a	101.05 ± 4.21 a	21.17 ± 1.38 a	523.2 ± 18.4 c	0.33 ± 0.01 a
MWCNTs 1000 mg L^{-1}	18.8 ± 0.7 a	93.51 ± 2.85 a	17.90 ± 0.75a	458.5 ± 5.8 d	0.33 ± 0.00 a
			Common bean *(Phaseolus vulgaris)*		
Control	17.2 ± 0.3 a	161.20 ± 5.04 a	37.26 ± 0.96 a	137.7 ± 22.3 a	0.39 ± 0.02 a
Control BP	17.2 ± 0.3 a	154.61 ± 6.19 a	34.35 ± 1.53 a	86.3 ± 9.2 a	0.33 ± 0.02 a
MWCNTs 100 mg L^{-1}	16.7 ± 0.2 a	143.45 ± 12.92 a	31.81 ± 2.69 a	91.7 ± 16.7 a	0.30 ± 0.03 a
MWCNTs 1000 mg L^{-1}	16.5 ± 0.4 a	143.42 ± 13.16 a	28.41 ± 3.12 b	91.2 ± 8.6 a	0.31 ± 0.02 a
			Maize *(Zea maize)*		
Control	10.9 ± 0.5 a	28.10 ± 0.76 b	42.23 ± 1.93 a	93.5 ± 5.5 a	0.77 ± 0.02 a
Control BP	11.0 ± 0.4 a	25.88 ± 1.49 b	41.00 ± 2.27 a	92.1 ± 5.1 a	0.76 ± 0.02 a
MWCNTs 100 mg L^{-1}	11.3 ± 0.4 a	29.29 ± 2.25 b	44.06 ± 2.04 a	91.3 ± 6.8 a	0.76 ± 0.01 a
MWCNTs 1000 mg L^{-1}	10.7 ± 0.4 a	38.13 ± 2.09 a	39.27 ± 1.86 a	77.1 ± 7.1 a	0.75 ± 0.03 a

Note: *Results represent mean values ± SEM of 6 replicates. Different letters (a, b) indicate significant difference between treatments (one-way ANOVA, Tukey test, $P \leq 0.05$). Control BP−control between paper, MWCNTs−multi walled carbon nanotubes, DI water−deionized water.*

Table 4.3. *Length of fine root fractions of 10-days old soybean, common bean and maize seedlings, developed from seeds exposed to MWCNTs for 36 h and subsequently grown in filter paper rolls. The seeds were treated in Petri dishes either with DI water (Control) or MWCNTs suspensions (100 and 1000 mg L^{-1}) or imbibed slowly between filter paper with DI water (control BP).*

Treatment	Fine root length (0 ≤ 0.2 mm), cm $plant^{-1}$	Fine root length (0.2 ≤ 0.4 mm), cm $plant^{-1}$	Fine root length (0.4 ≤ 0.6 mm), cm $plant^{-1}$
	Soybean *(Glycine max)*		
Control	679.2 ± 42.7 a	72.2 ± 6.3 a	51.7 ± 3.6 a
Control BP	586.2 ± 14.4 b	65.2 ± 1.7 a	53.9 ± 4.6 a
MWCNTs 100 mg L^{-1}	327.1 ± 18.3 c	33.7 ± 2.5 b	43.3 ± 3.5 ab
MWCNTs 1000 mg L^{-1}	287.4 ± 10.2 c	26.8 ± 1.7 b	36.4 ± 2.7 b
	Common bean *(Phaseolus vulgaris)*		
Control	0.5 ± 0.1 a	14.5 ± 1.3 a	79.8 ± 7.8 a
Control BP	0.4 ± 0.0 a	10.8 ± 1.8 a	79.2 ± 3.5 a
MWCNTs 100 mg L^{-1}	0.5 ± 0.0 a	14.4 ± 2.3 a	69.9 ± 6.8 a
MWCNTs 1000 mg L^{-1}	0.5 ± 0.1 a	13.3 ± 2.2 a	93.0 ± 10.4 a
	Maize *(Zea maize)*		
Control	24.2 ± 2.6 a	14.1 ± 2.7 a	3.4 ± 0.3 a
Control BP	24.6 ± 0.8 a	10.5 ± 1.4 a	4.6 ± 0.8 a
MWCNTs 100 mg L^{-1}	20.6 ± 1.1 ab	16.0 ± 1.9 a	3.7 ± 0.2 a
MWCNTs 1000 mg L^{-1}	15.7 ± 2.0 b	17.3 ± 2.6 a	4.1 ± 0.4 a

Note: *Results represent mean values ± SEM of 6 replicates. Different letters (a, b, c) indicate significant difference between treatments (one-way ANOVA, Tukey test, P ≤ 0.05). Control BP−control between paper, MWCNTs−multi-walled carbon nanotubes, DI water−deionized water*

4.4.4 Assessment of root activity

As indicators for root activity, nitrate uptake of the seedlings was measured by nitrate depletion of a nutrient solution. Additionally vitality staining of the root tissue with 2, 3, 5-triphenyl tetrazolium chloride (TTC) was performed, followed by extraction and photometric quantification of the red triphenylformazan formed by the metabolic activity of the roots.

Nitrate uptake of common bean seedlings developed from seeds treated with MWCNTs (36 h, 1000 mg L^{-1}) was significantly reduced as compared to the untreated control (Figure 4.4 A) and a similar but not significant trend was also recorded for soybean. This was associated with a significant reduction in root length in the MWCNT variants of soybean and common bean (Supplemental Table S4.2. in Appendix). However, analysis of specific nitrate uptake per unit of root dry weight revealed no inhibition related with the application of MWCNTs (Figure 4.4 B).

The TTC reduction assay revealed significantly inhibited dehydrogenase activity in the seedlings roots exposed to MWCNT treatments. The visual evaluation of stained root samples showed less intensive red coloration of the soybean and common bean roots developed from the MWCNT-treated seeds (Figure 4.4 E, F). The amount of produced TF was approximately 50% less in MWCNT treatments compared to the corresponding controls (Figure 4.4 C, D).

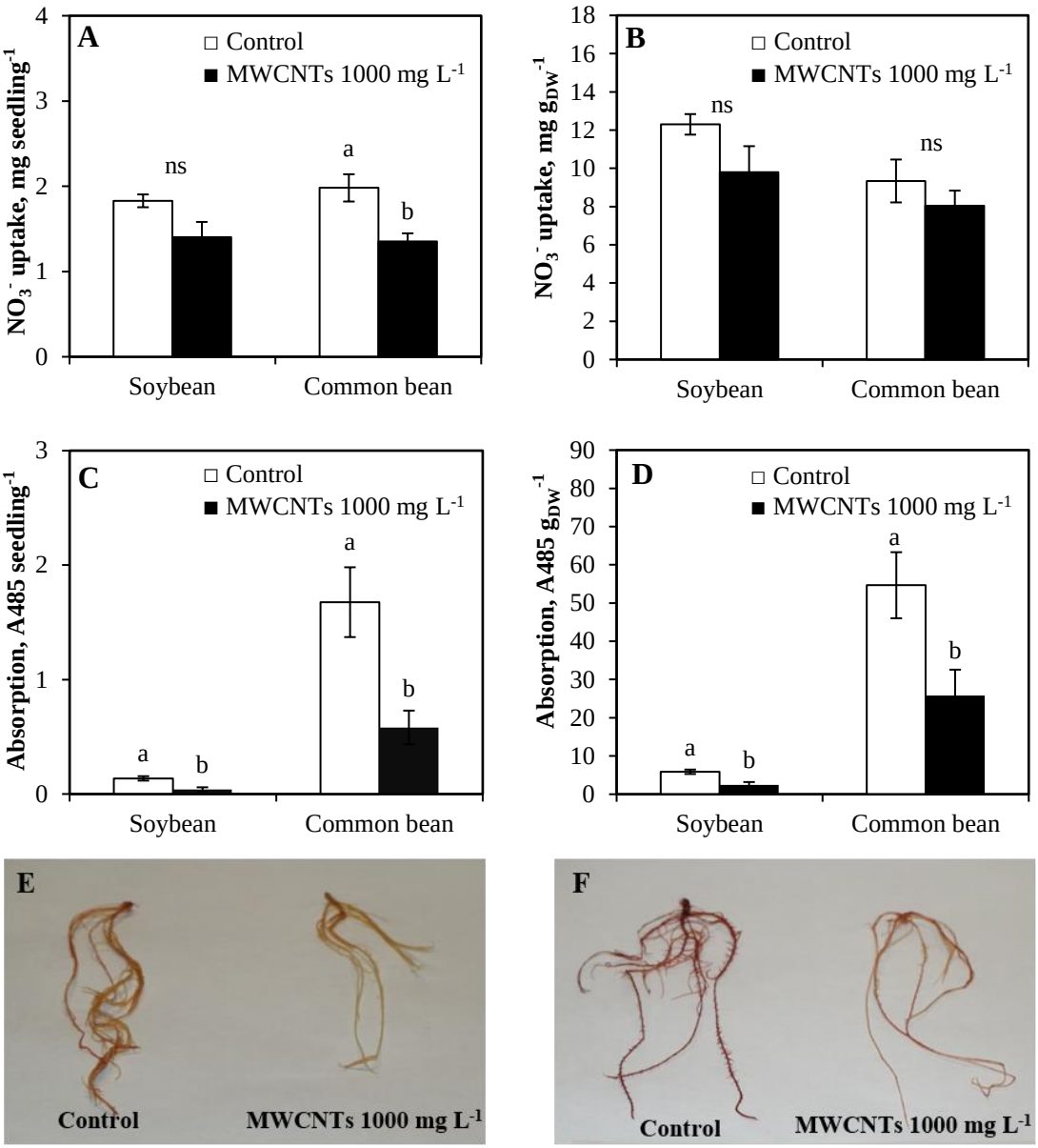

Figure 4.4. *Assessment of root activity of soybean and common bean seedlings. (A) Nitrate (NO_3^-) uptake per plant [mg seedling^{-1}] and (B) per unit root dry weight [mg g_{DW}^{-1}] by 10-days old soybean and common bean seedlings cultivated in nutrient solution during 24 h. (C) Absorption of a triphenylformazan (TF) at 485 nm per seedling [A485 seedling^{-1}] and (D) per unit root dry weight [A485 g_{DW}^{-1}] as a product of 2, 3, 5-triphenyl tetrazolium chloride (TTC) reduction by the roots of 10-days old soybean and common bean seedlings immersed for 24 h in 0.08% TTC solution followed ethanol extraction. (E) Roots of soybean and (F) common bean seedlings after staining with 0.08% TTC. The seedlings (A−F) were developed from seeds treated in Petri dishes for 36 h by 1000 mg L^{-1} MWCNTs or DI water (Control) and subsequently grown in filter paper rolls moistened with DI water. Values represent mean values ± SEM of five replicates. Different letters (a, b) indicate significant difference between treatments (Student's t-test, $P \leq 0.005$). ns−not significant, MWCNTs−multi-walled carbon nanotubes, DI water−deionized water.*

4.4.5 MWCNT exposure time

To determine the minimum time period of seed exposure required for induction of plant damage induced by MWCNTs soybean seedlings developed from seeds treated with MWCNTs (1000 mg L^{-1}) were investigated after exposure times of 6, 12, 24 and 36 h. Significantly reduced total root length as compared to the control variant were first recorded after 36 h of seed exposure to MWCNTs (Table 4.4) and was detectable earliest at 8 DAS (Figure 4.5).

Table 4.4. *Total root length [cm plant^{-1}] of 10-days old soybean seedlings, developed from seeds treated in Petri dishes for 6, 12, 24, 30 and 36 h with MWCNTs (1000 mg L^{-1}) or DI water (Control) and subsequently grown in filter paper rolls.*

Seed treatment duration, h	Total root length, cm plant^{-1}	
	Control	MWCNTs 1000 mg L^{-1}
6	91.2 ± 10.3 a	87.7 ± 3.5 a
12	89.3 ± 6.8 a	82.2 ± 8.7 a
24	77.2 ± 5.4 a	72.7 ± 3.6 a
30	73.4 ± 5.8 a	65.9 ± 4.4 a
36	70.8 ± 4.6 a	60.6 ± 1.6 b

Note: *Results represent mean values ± SEM of 4 replicates. Different letters (a, b) within a line indicate significant difference between treatments (Student's t-test, $P \leq 0.05$). MWCNTs−multi-walled carbon nanotubes, DI water−deionized water.*

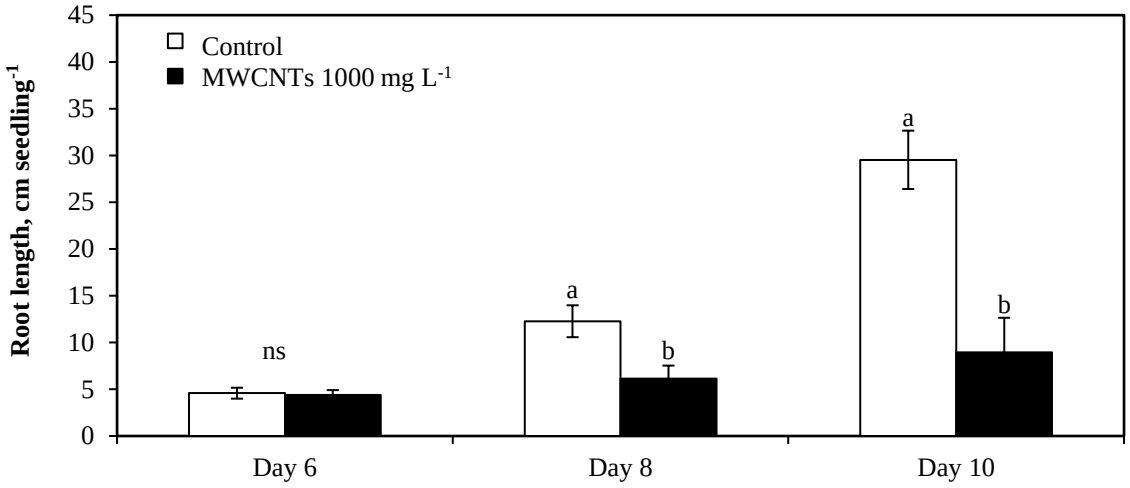

Figure 4.5. *Total root length of 6, 8 and 10-days old soybean seedlings, developed from seeds treated in Petri dishes with and without MWCNTs (1000 mg L^{-1} for 36 h) and grown in filter paper rolls moistened with DI water. Different letters (a, b) indicate significant difference between treatments (Student's t-test, $P \leq 0.05$). ns−not significant. MWCNTs−multi-walled carbon nanotubes, DI water−deionized water.*

4.5 Discussion

The present study revealed differences in responsiveness to short-term MWCNT seed exposure in three different plant species (*Glycine max* (L.) Merr, *Phaseolus vulgaris* L., *Zea mays* L.) in a standard germination test according to the ISTA rules [13], showing both, positive and negative effects on plant development. MWCNT suspensions were applied for 36 h prior to radicle emergence in Petri dishes on filter paper in two concentrations (100 and 1000 mg L^{-1}), as used in previous studies investigating CNT effects on plant growth [1–9] (reviewed by O. Zaytseva and G. Neumann [11]). The selected concentrations translated into a CNT dosage of 50 and 500 µg MWCNTs $seed^{-1}$. However, a closer look at the culture system shows that only traces of the applied MWCNTs had direct seed contact, while by far the majority of the MWCNTs were sticking to the germination paper.

The most striking positive MWCNT effect on plant development is a 30% stimulation in germination, reflected in germination percentage (Figure 4.2) and development of abnormal seedlings according to the ISTA classification (Figure 4.3) recorded in

soybean with a germination rate of approximately 65% in the untreated control. This effect is not detectable in common bean or maize, with untreated control variants, reaching almost 100% germination. The variability in MWCNT-induced stimulation of germination may reflect interspecific differences. On the other hand, seed lot effects related with differences in seed quality and seed aging [19] indicated by different germination rates of the untreated controls, may offer an alternative explanation. A clear distinction would require a comparison of seed lots with a comparable vitality for all tested plant species.

Similar to this study, positive effects of MWCNTs exposure at concentrations between 10 and 200 mg L^{-1} on seed germination and seedling growth have been reported for barley, wheat, maize, peanut (*Arachis hypogaea* L.), soybean and tomato [1–4]. Increased germination has been related with improved seed water uptake during imbibition as a putative consequence of a MWCNT-induced seed coat and cell wall perforation and increased expression of water channel proteins (aquaporins) [3] involved in water uptake, germination, root elongation and also in many stress responses [20]. Additionally, the beneficial impact on plant growth, frequently observed at lower doses of MWCNT application, has been attributed to hormesis effects [9, 21] (reviewed by O. Zaytseva and G. Neumann [11]). However in the current study, the treatment with increasing MWCNT concentration, promoted the positive effects on seed germination in soybean (Figure 4.2 A), associated with a reduction in the speed of seed water uptake during imbibition (Figure 4.1). Moreover, the speed of water uptake in all investigated plant species during seed imbibition was positively correlated with the formation of abnormal seedlings according to the ISTA classification [13] and the lowest rate was recorded for seeds imbibed very slowly between layers of moist filter paper (Figure 4.3). Imbibition damages, such as disturbed reconstitution of cell membranes, resulting in reduced germination [22, 23] often occur as a result of a rapid seed water uptake in large-seeded leguminous plants, exposed e.g. to excessive soil moisture levels. A large amount of hydrophobic proteins on the seed coat surface of soybean [24] may allow preferential adsorption of the hydrophobic MWCNTs similar to seed dressing agents. Seed dressings can slow down the speed of water uptake, thereby reducing the risk of imbibition damage [25], and obviously the MWCNT treatments had a similar function

in our experiments. Moreover, seeds with impaired seed vitality (induced e.g. by seed aging) are particularly sensitive to additional stress factors, such as imbibition damage. The low germination rate recorded for the untreated soybean seed lot (65%) used in this study (Figure 4.2) may indicate a similar seed vitality problem associated with a high responsiveness to seed dressing treatments and therefore, also to MWCNT application.

However, despite the beneficial effects of short-term MWCNT seed treatments on germination, a negative impact on further seedling development is detected in all tested plant species. The development of root growth and fine root production was inhibited at 10 DAS (Tables 4.2, 4.3 and 4.4) with is particularly important for spatial nutrient acquisition. Accordingly, nitrate uptake measured as nitrate depletion in a hydroponic growth medium is significantly reduced in common bean seedlings with a similar trend also in soybean (10 DAS) exposed to 36 h MWCNT seed treatments (Figure 4.4). However, only nitrate uptake per plant is reduced by the MWCNT treatments, while the specific uptake rate per unit root dry weight remained unaffected. This finding suggests that the reduction in nitrate uptake is mainly a consequence of inhibited root growth and not of a limitation in the specific uptake activity. By contrast, vitality staining of the root tissue with TTC (2, 3, 5-triphenyl tetrazolium choride) revealed reduced triphenylformazan (TF) formation per unit root dry biomass of the MWCNT-treated common bean and soybean seedlings. This findings indicate lower metabolic activity and lower vitality of the root tissue, which was potentially responsible for the limitation of root growth.

Apart from plant growth stimulation [1, 3, 4], negative growth effects have been similarly reported in the literature for a range of plant species including red spinach, rice, lettuce and cucumber particularly at higher dosages of MWCNT application. Growth restrictions have been related with MWCNT-induced indication of oxidative stress, membrane damage, electrolyte leakage, mitochondrial dysfunctions and DNA aberrations [7, 8, 26, 27].

4.6 Conclusion

The present study demonstrates that MWCNTS effects on plant growth are highly variable depending on plant species, but also on the physiological status and the developmental stage of individual plants. The different plant responses to MWCNTs treatments are observed under strictly controlled experimental conditions, largely excluding environmental factors and effects induced by carbon nanomaterials of different origin or agglomeration status [28, 29]. Apart from the well-documented stimulation of germination by increased water uptake during imbibition associated with seed coat perforation and upregulation of aquaporin genes [1, 3, 29, 30], the results demonstrates that MWCNT seed treatments can also exert protective effects by reducing the speed of water uptake thereby minimizing the detrimental effects of imbibition damage [31], particularly in seeds with limited vitality (Figures 4.2 and 4.3). In soybean, the protective effect seems to be restricted mainly to the first 24 h of seedling development as approximate time period required for complete seed imbibition. A significant reduction in water uptake in MWCNT-treated seeds is already detectable after 12 h of imbibition (Figure 4.1), although the promoting effect on germination rate started to appear at 3 DAS (Figure 4.2).

By contrast, inhibitory effects on plant growth induced by MWCNT treatments are first detectable at 8 DAS (Figure 4.5) and in all investigated plant species root growth was primarily affected (Table 4.2). In soybean, MWCNT exposure for at least 36 h prior to radicle emergence is required (Table 4.4) to induce root growth inhibition at 8 DAS (Figure 4.5), associated with reduced metabolic activity of the roots (Figure 4.4). The molecular and physiological events determining the inhibition of root growth during later seedling development within 36 h after sowing, remain to be established. A reduced establishment of a functional root system can act as a cause of inhibitory effects on further plant development with pleiotropic patterns, particularly under conditions of limited nutrient and water availability, requiring adaptive responses in root growth for adequate nutrient acquisition. This holds true for soil culture in general with additional impact of abiotic and biotic stress factors. This situation may further contribute to the reported variability of plant responses due to exposure to carbon nanomaterials.

4.7 Appendix

4.7.1 Authors' contributions

OZ participated in designing experiments, conducted experiments and laboratory analysis, performed statistical analyses and results interpretation, drafted and participated in reviewing the manuscript. GN designed experiments, was involved in proof reading and provided final editing of the manuscript.

4.7.2 Acknowledgments

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The authors declare that they have no competing interests.

4.7.3 Supplemental materials

Supplemental Table S4.1. *Chemical composition of the nutrient solution, utilized for the hydroponic experiment.*

Nutrient	Form	Concentration in the solution
K	K_2SO_4	0.7 mM
Cl	KCl	0.1 mM
Mg	$MgSO_4$	0.5 mM
P	KH_2PO_4	0.1 mM
B	H_3BO_3	10 µM
Mn	$MnSO_4 \times H_2O$	0.5 µM
Zn	$ZnSO_4 \times 7\ H_2O$	0.5 µM
Cu	$CuSO_4 \times 5\ H_2O$	0.2 µM
N and Mo	$(NH_4)Mo_7O_{24} \times 4\ H_2O$	0.01 µM
N and Ca	$Ca(NO_3)_2 \times 4\ H_2O$	2 mM
Fe	Fe-EDTA	20 µM

Supplemental Table S4.2. *Total root length and average root diameter of 10-days old soybean and common bean seedlings, developed from seeds treated in Petri dishes with and without MWCNTs (1000 mg L^{-1} for 36 h), grown in filter paper rolls, wetted with DI water and exposed to full nutrient solution for 24 h. Different letters (a, b) indicate significant difference between treatments (Student's t-test, $P \leq 0.05$). ns−not significant, MWCNTs−multi-walled carbon nanotubes.*

Treatment	Total root length, cm plant^{-1}	Average root diameter, mm	Fine root length (0≤0.2 mm), cm plant^{-1}	Fine root length (0.2≤0.4 mm), cm plant^{-1}	Fine root length (0.4≤0.6 mm), cm plant^{-1}
Soybean (*Glycine max*)					
Control	186.6 ± 12.2 a	0.5 ± 0.0 a	28.5 ± 3.9 a	60.6 ± 4.5 a	54.3 ± 7.4 a
MWCNTs 1000 mg L^{-1}	99.0 ± 18.4 b	0.5 ± 0.0 a	16.8 ± 3.2 b	25.8 ± 6.3 b	28.6 ± 5.0 b
Common bean (*Phaseolus vulgaris*)					
Control	216.4 ± 8.8 a	0.5 ± 0.0 a	40.3 ± 2.5 a	57.0 ± 3.0 a	52.1 ± 1.4 a
MWCNTs 1000 mg L^{-1}	169.4 ± 6.7 b	0.5 ± 0.0 a	32.5 ± 2.3 a	47.7 ± 2.2 b	40.7 ± 2.8 b

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5 Chapter III. Phytotoxicity of carbon nanotubes is associated with disturbances of zinc homeostasis

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5.1 Abstract

Effects of short-term seed treatments with multi-walled carbon nanotubes (MWCNTs) on seedling development in soil culture and of root-exposure in hydroponics were studied on soybean, considered as model plant system. At 8 days after sowing and in later stages of seedling development, stunted growth and poor fine root production were detected. More detailed investigations revealed zinc (Zn) deficiency as a major growth-limiting factor. The growth of affected plants was recovered by foliar application of $ZnSO_4$ or by cultivation in nutrient solution supplied with soluble $ZnSO_4$. Since Zn is an important co-factor of enzymes involved in detoxification of reactive oxygen species (ROS), such as copper-zinc superoxide dismutases, stunted plant growth in response to MWCNTs treatments may be related to oxidative damage associated with lipid peroxidation and excessive oxidative degradation of auxin as growth hormone important for lateral root formation and leaf expansion.

Keywords: soybean, common bean, maize, seedling development, nutrient availability, multi-walled carbon nanotubes, nanomaterials.

5.2 Introduction

Carbon nanotubes (CNTs) are currently among the most important additives for composite materials with a predicted market increase up to USD 5.91 billion by 2018 [1]. During manufacturing, utilization and disposal, these materials are intentionally or by chance released into the environment [2–6]. Due to a very high volume to surface ratio, nanomaterials can exhibit novel properties leading to yet unknown environmental interactions [7–9], complicating predictions on the fate of engineered nanoparticles released into environmental compartments, such as soil, air, and water. There are still controversial discussions whether nanoparticles exert beneficial or adverse effects on living organisms, on their ability to penetrate living tissues/barriers, and incorporation into food chains [10−12]. Accordingly, in the recent past, various studies addressed the impact of metal-based [13, 14] as well as carbon-based nanomaterials [15−21] also on germination and early growth of higher plants. The resulting findings are frequently controversial, with positive as well as negative effects of CNTs, depending on plant species, source of CNTs, their physico-chemical properties, applied concentrations of nanotubes, and the culture systems.

The effects of MWCNTs on seed germination and seedling development were studied in various plant species, such as tomato (*Solanum lycopersicum* L.) [15−16], radish (*Raphanus sativus* L.), rapeseed (*Brassica napus* L.), ryegrass (*Lolium perenne* L.), lettuce (*Lactuca sativa* L.), maize (*Zea mays* L.), cucumber (*Cucumis sativus* L.) [17], zucchini (*Cucurbita pepo* L.) [18] and others. In some cases MWCNTs did not affect germination rates but in different ways influenced further seedling development. Ghodake et al. [19] reported no effect of MWCNTs at 10−40 mg L^{-1} on germination of mustard (*Brassica juncea* L.) and gram (*Vigna mungo* (L.) Hepper) but root elongation of mustard seedlings was doubled as compared to the control at 20 mg L^{-1}, while higher concentrations had inhibitory effects on root hair formation. In the majority of studies, which focused on the influence of nanoparticles on seedling development, artificial growth media or hydroponic culture systems where employed but experiments with soil-grown plants are rare. Begum et al. [20], Begum and Fugetsu [21] and Stampoulis et al. [18] reported negative effects of MWCNTs added to a Hoagland nutrient solution in

concentrations up to 2000 mg L^{-1} on the development of various plant species, namely red spinach (*Amaranthus tricolor* L.), lettuce, cucumber and zucchini, while chili (*Capsicum annuum* L.), okra (*Abelmoschus esculentus* (L.) Moench) and soybean (*Glycine max* (L.) Merr) remained unaffected.

From the eco-toxicological point of view it is very important to uncover the mechanisms of interactions between nanomaterials and plants, since plants are an important component of ecosystems, exhibiting close interactions with other living organisms as well as with inorganic components such as air, soil and water. Moreover, numerous applications are under development using nanomaterials for the development of novel plant growth stimulators, fertilisation and plant protection [22]. Therefore, investigation of genotypic differences and identification of the most MWCNT-sensitive plant species and cultivars, as well as determination of toxic thresholds under different environmental conditions is urgently needed. However, the high variability of reported results and a wide range of different types of CNTs, makes comparisons difficult.

In a previous pilot study [23], we investigated the responses of three crop species (soybean; common bean, *Phaseolus vulgaris* L.; and maize) to short-term seed exposure (36 h) of a defined industrial MWCNT batch applied at a low (50 µg $seed^{-1}$) and high (500 µg $seed^{-1}$) dosage in a standardised germination test under controlled environmental conditions according to the rules of the International Seed Testing Association (ISTA) [24]. MWCNT treatments increased germination percentage and reduced the proportion of abnormal seedlings (ISTA) [24] particularly in soybean associated with a reduction in the speed of water uptake during imbibition. However, early development of seedling was affected particularly by inhibition of root growth (fine root production) in all plant species first detectable at 8 days after sowing (DAS).

In the present study the consequences of these treatment effects on early growth were monitored in different culture systems (hydroponics, soil culture) with contrasting availability of water and nutrients.

5.3 Experimental

5.3.1 MWCNTs and preparation of MWCNT suspensions

Industrial multi-walled carbon nanotubes, MWCNTs (NanoTechCenter Ltd., Tambov, Russia) were used for the experiments. The MWCNTs have a minimum length of 2 µm, an external diameter of 20−70 nm and an internal diameter of 5−10 nm. The material was produced by chemical vapor deposition with purity above 98% (Supplemental Table S5.1 in Appendix). The selected concentrations of the MWCNT working suspensions used in the experiments (50, 100, 500 and 1000 mg L^{-1}) were in the range previously employed for various other studies on plant effects of MWCNTs [15, 19–21]. For preparation of working suspensions, MWCNTs were mixed directly with deionized (DI) water and dispersed by ultrasonification (SONOREX SUPER RK 510 H; 35 KHz, Bandelin Electronic, Berlin, Germany) for 30 min.

5.3.2 Test plants

For the experiments three plant species were used: soybean (*Glycine max*. L. Merr cv. BR16 Conquista, Embrapa, Brazil), common bean (*Phaseolus vulgaris* L. cv. Bohnen maxi, Baywa AG, Germany) and maize (*Zea mays* L. cv. Surprise, Saaten Union GmbH, Rastatt, Germany).

5.3.3 Seed treatments

Suspensions of MWCNTs in DI water was used in a concentration of 1000 mg L^{-1} corresponding to a dose of 500 µg $seed^{-1}$. Deionized (DI) water was used as a control. Five mL of MWCNTs suspensions or DI water were added to plastic Petri dishes (diameter 96 mm, Greiner, Nürtingen, Germany) with 3 layers of filter paper (Blue ribbon MN 640d, Macherey und Nagel, Düren, Germany) on the bottom, and ten seeds were evenly distributed per Petri dish and homogenously moistened with the treatment solutions. The Petri dishes were covered with lids and placed into an incubator (BD 115, Binder, Tuttlingen, Germany) at 25 °C for 36 h in the dark before emergence of the radicles, and subsequently transferred to different growth mediums without MWCNTs

addition: (a) in a filter paper rolls wetted with DI water for 10 days, (b) in filter paper rolls wetted with DI water for 3 days, then into rhizoboxes with silty loam soil for 10 days, (c) in filter paper rolls wetted with DI water for 6 days, then into hydroponic culture with fill nutrient solution for 9 days, (d) into pots with loess subsoil for 38 days and (e) in pots with loess subsoil and foliar Zn application for 33 days (Figure 5.1, experimental set-up 1).

5.3.4 Seed vitality staining

Seed vitality staining was performed after 36 h of MWCNTs treatments according to ISTA rules [24]: seeds were stained with 1% (w/v) 2, 3, 5-triphenyl tetrazolium chloride (TTC) (pH 6.5−7.5) for 18 h. After rinsing the seeds with DI water, they were cut lengthwise with a razor blade and staining intensity of seed organs was evaluated under a binocular microscope (Stemi 2000-C, Zeiss, Germany). In metabolically active cells, TTC is reduced by dehydrogenases, forming red formazan and therefore, color intensity reflects the degree of metabolic activity in the stained tissues. Finally, embryos were excised, and formazan was quantitatively extracted with 2 M KOH/DMSO (1:1.16 v/v) using mortar and pestle. After removal of solid material by centrifugation, absorption of the supernatant was measured spectrophotometrically at 485 nm.

5.3.5 Seedling growth in filter rolls

As a pre-culture for hydroponic and rhizobox experiments seedlings were germinated in filter paper rolls: one sheet of filter paper (58×58 cm, MN710, Macherey und Nagel, Düren, Germany) was folded lengthwise four times and was wetted with 60 ml of DI water. Ten treated with MWCNTs seeds were placed along the edge of the paper which was subsequently folded, forming a paper roll with the seeds inside. The paper rolls were placed in upright position into a plastic germination box (30×20×10 cm), the lids of the box was opened and it was placed for 3−6 d (until rootlets reach 2.0−2.5 cm) into a climate chamber with a 14 h light period and an average temperature of 23 °C with regular additions of 25 ml DI water per filter roll to compensate for evaporation.

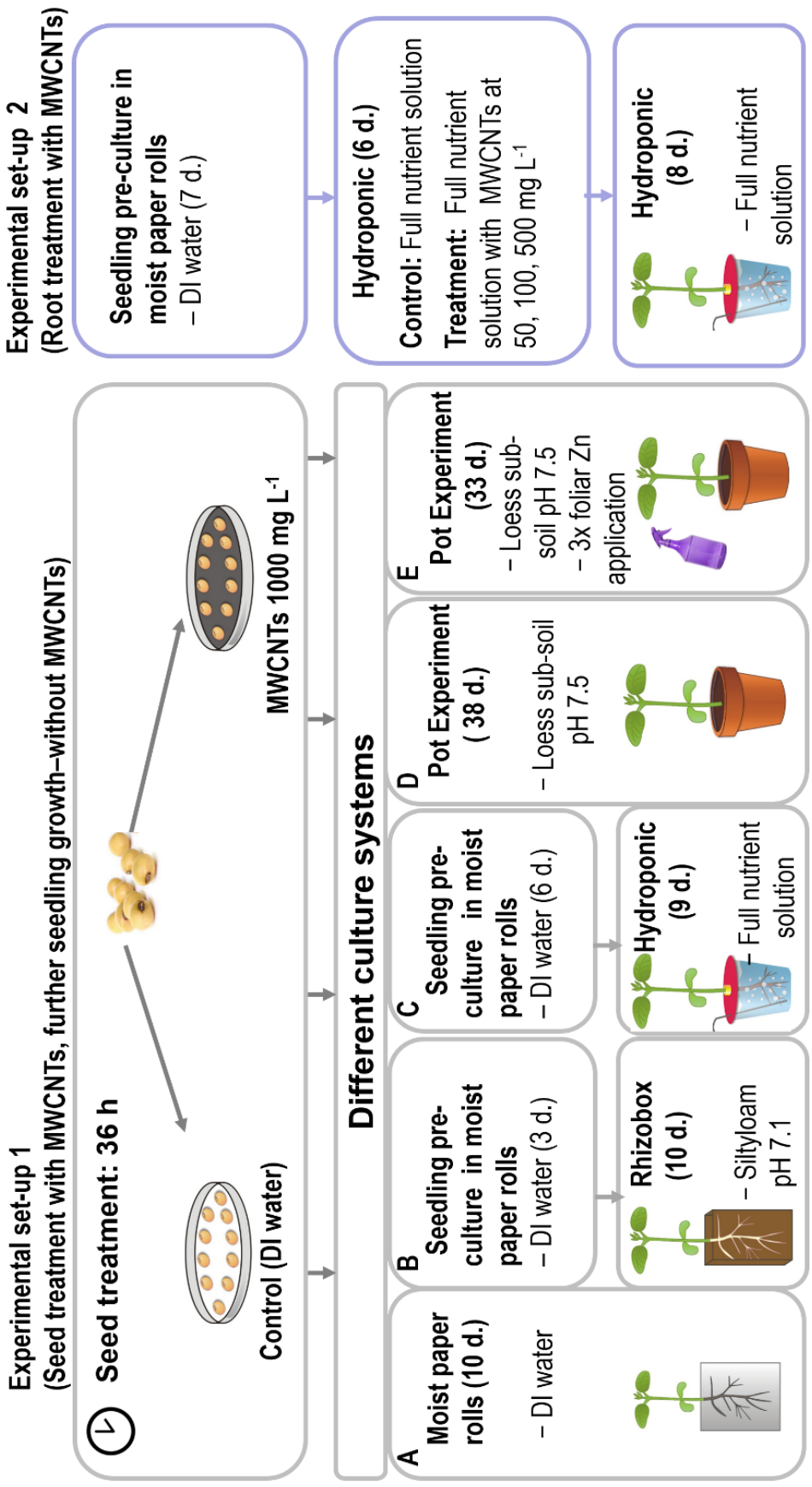

Figure 5.1. *Schematic representation of the performed experiments. experimental set-up 1 and 2.*

5.3.6 Hydroponic culture

Hydroponic culture was employed to investigate the impact of the MWCNTs on seedling development of soybean under full, freely available nutrient supply. Seed treatments with MWCNTs (1000 mg L^{-1}) and pre-culture in filter rolls were performed as described above. Seedlings with a root length of 2.0−2.5 cm were transferred from filter rolls to pots with 2.5 L nutrient solution, aerated with an aquarium pump and containing 2 mM $Ca(NO_3)_2$, 100 µM KH_2PO_4, 0.7 mM K_2SO_4, 0.1 mM KCl, 0.5 mM $MgSO_4$, 10 µM H_3BO_3, 0.5 µM $MnSO_4$, 0.5 µM $ZnSO_4$, 0.2 µM $CuSO_4$, 0.01 µM $(NH_4)_6Mo_7O_{24}$, and 20 µM Fe(III)-EDTA, which was replaced in 3 day-intervals. In each pot, 8 seedlings were fixed with foam strips in perforated lids with 4 replicates per treatment. Cultivation was performed in a climate chamber with a 14 h light period (200 µmol m^{-2} s^{-1}) at 23 °C. After a culture period of 16 d, seedlings were harvested for biomass and root length determination. Alternatively, non-treated seeds were pre-cultured in moist filter rolls, and thereafter exposed for 6 d to nutrient solution amended with MWCNTs (50, 100, 500 mg L^{-1}) and subsequently cultivated in nutrient solution without MWCNTs supply for 7 d (Figure 5.1, experimental set-up 2).

5.3.7 Soil culture – seedling growth in rhizoboxes

A rhizobox experiment was performed to monitor root development in soil culture. Seed treatments in Petri dishes, and pre-culture in filter rolls were performed as described above. Each of two seedlings with a root length of 2.0−2.5 cm were transferred into rhizoboxes equipped with transparent root observation windows [25] filled with 0.5 kg of a clay loam field soil (pH 7.1) taken from the Heidfeldhof experimental station in Hohenheim (Stuttgart, Germany). During the culture period, soil moisture was adjusted gravimetrically to 20% (w/w) by supplying DI water via holes on the backside of the boxes. Cultivation was performed in a climate chamber with a 14 h light period (200 µmol m^{-2} s^{-1}) at 23 °C. After a culture period of 15 d, the seedlings were harvested for biomass and root length determination.

5.3.8 Soil culture – pot experiments

Pot experiments were conducted to investigate the effects of short-term MWCNTs seed treatment and foliar Zn application on early growth of soybean on a soil substrate with limited nutrient solubility. A calcareous loess subsoil low in available P (P CAL 5 mg kg^{-1}), total N (0.02%), Calcium chloride-diethylenetriaminepentaacetic acid (CAT) extractable micronutrient concentrations (mg kg^{-1} soil): Mn, 15; Fe,−7.8; Zn, 0.6; B,0.2; organic matter (0.1%), pH 7.6 was used for the experiments. Each pot was filled with 1 kg of a mixture of loess subsoil and quartz sand (50% w/w). Basal fertilization for the substrate comprised of N (100 mg kg^{-1}) as $Ca(NO_3)_2$; K (150 mg kg^{-1}) as K_2SO_4, Mg (50 mg kg^{-1}) as $MgSO_4$, P (80 mg kg^{-1}) as $Ca(H_2PO_4)_2$. Seed treatment with MWCNTs (1000 mgL^{-1}; 36 h) was performed as described above. Thereafter, 4 seeds per pot were sown at depth of 1 cm and the pots were cultivated in a climate chamber with a 14 h light period (200 µmol m^{-2} s^{-1}) at 23 °C. During the culture period the moisture content of the substrate was adjusted gravimetrically in to 20% (w/w) by regular supply of DI water. At 10 DAS thinning was performed to final number of two seedlings per pot with 10 replicates per treatment. Harvests were performed at 26 DAS and 38 DAS for each 5 replicates for determinations of biomass, root length and nutritional status.

Plant analysis in the first experiment revealed a critical Zn-nutritional status associated with growth depressions for soybean plants, developed from MWCNT-treated seeds. Therefore, in a second experiment, after unfolding of the 1st trifoliate leaves (15 DAS) foliar Zn applications were applied once a week with 0.5 mM or 5 mM $ZnSO_4$ or DI water as negative control (8 replicates per treatment). Final harvest was performed at 33 DAS for determination of leaf area (young fully developed leaf) plant height, biomass and root length.

5.3.9 Mineral analysis of plant tissues

For mineral nutrient analysis, dried shoots of soybean plants were ground to a fine powder and each 250 mg of dry plant material were ashed in a muffle furnace at 500°C for 4 h. After cooling, the samples were extracted twice with 2.5 mL of 3.4 M HNO_3

and evaporated to dryness to precipitate SiO_2. The ash was dissolved in 2.5 mL of 4 M HCl, subsequently diluted ten times with hot deionized water, and boiled for 2 min. After cooling, the solutions were adjusted to 25 ml with DI water and passed through blue ribbon filters (Macchery & Nagel, Düren, Germany). Zinc concentrations in the extracts were determined by atomic absorption spectrometry (iCE 300 Series, Thermo Fisher Scientific, United Kingdom).

5.3.10 Analysis of leaf area and root morphology

Fresh root samples, stored in 30% (v/v) ethanol were carefully separated on transparent Perspex trays and subsequently digitalised with an Epson Expression 10000Xl scanner (Epson, USA) which was also used for scanning of leaves. Analysis was performed using the WinRHIZO software (Regent Insruments, Quebec, Canada).

5.3.11 Statistical analysis

All experiments were performed in a completely randomized design. Statistical analysis was conducted with the SigmaPlot 11.0 software package using the Student t-test for comparison of two treatments and one-way ANOVA for comparison of multiple treatments. The level of significance was determined at a P value ≤ 0.05. All results in tables and graphs are presented as mean values ± SE (standard error of a mean).

5.4 Results and discussion

It has been reported that short-term seed exposure (36 h) to medium (50 µg $seed^{-1}$) and high (500 µg $seed^{-1}$) dosages of MWCNTs even prior to radicle emergence can induce significant effects on germination and early seedling development [23]. In a pilot study with three crop species (soybean, *Glycine max*; common bean, *Phaseolus vulgaris*; and maize, *Zea mays*), germination rate was increased, associated with reduced formation of abnormal seedlings according to the ISTA classification [24] particularly in soybean. This effect could be attributed to a reduction in the speed of water uptake, detectable already during the first 12 h of seed imbibition. However, during later seedling development, inhibition of root growth (mainly fine root production) was recorded in

seedlings of all plant species, first detectable at 8 DAS, and associated with a reduced metabolic activity of the root tissue. A minimum time period of 36 h MWNCT seed-exposure was required for induction of root damage [23].

The results of the present study confirmed penetration of MWCNTs and localization in the embryonic axis (Figure 5.2 E) even in non-germinated seeds, as has been described for other plant species also [15, 26]. This was associated with a reduced metabolic activity of the embryonic tissue detected by 2,3,5-triphenyl-tetrazoliumchoride staining (Figure 5.2 B, C) at 36 h of seed imbibition, and most probably causing the inhibition of root growth during later seedling development [23]. In this context, a closer look to the effective dosage of MWCNTs shows that only an extremely small proportion of the applied MWCNT dose (e.g. 500 µg $seed^{-1}$ supplied in a treatment suspension with a concentration of 1000 mg L^{-1}) was really in contact with the seed surface, since the majority of the applied MWCNTs was sticking to the germination paper (Figure 5.2 A). A quantitative evaluation of the MWCNT proportion finally taken up into the seeds and interacting with the plant metabolism would require incubation experiments with radioactively-labeled MWCNT tracers as previously described by Larue et al. [27]. However, even without the availability of exact quantitative data on MWCNT uptake it is obvious that even trace amounts MWCNTs entering the seeds exhibit a high metabolic activity with the ability to induce both, positive and negative effects on plant development.

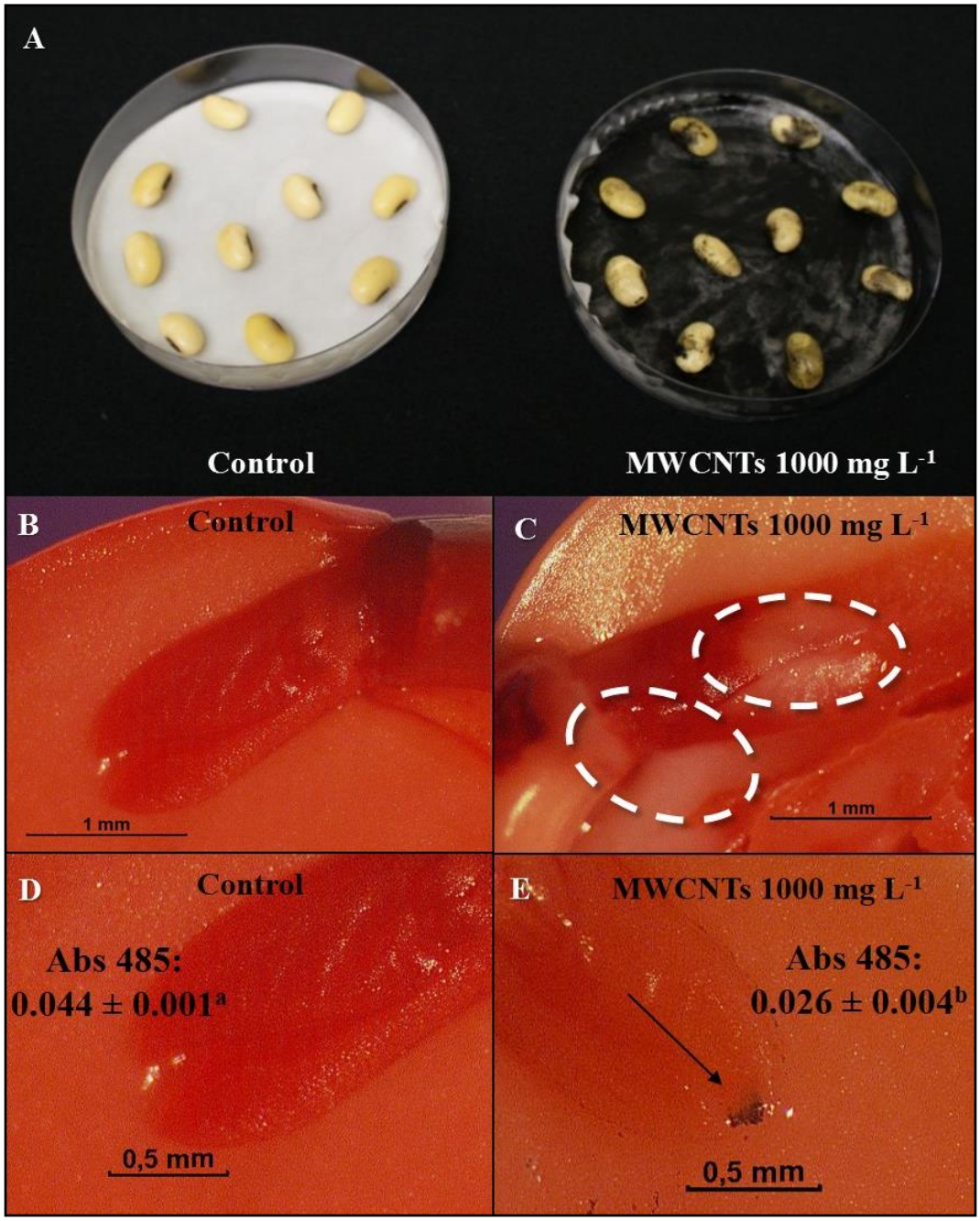

Figure 5.2. *(A) Soybean seed treatment in Petri dishes: control (DI water) and MWCNTs (1000 mg L^{-1}) variants; (B−E) Embryos of MWCNT-treated and non-treated soybean seeds (2 DAS), stained with 1% 2,3,5-triphenyl tetrazolium chloride for 18 h. The weakly-stained regions highlighted in (C) indicate embryo tissues with a low metabolic activity. The numeric values in (D) and (E) represent absorption intensity of tetrazolium extracted from the embryos after photometric determination (means ± SEM). Different letters (a, b) indicate significant differences between the treatments (Student's t-test, $P \leq 0.05$). The black arrow in (E) indicates MWCNTs accumulation next to the embryo. DI−deionized water, MWCNTs−multi-walled carbon nanotubes, DAS−days after sowing.*

Interestingly, the further development of plants grown from MWCNT-treated seeds was strongly dependent on the culture conditions. Cultivation of soybean seeds, identified as most sensitive to MWCNT treatments [23] in a hydroponic culture system with all essential plant nutrients provided in sufficient and freely available amounts, induced a complete recovery of root growth in plants grown from MWCNT-treated seeds within three weeks of the culture period, without any growth differences to control plants (Table 5.1, Figure 5.3 A). This finding suggests that nutrient limitation of the seedlings was a major problem induced by the MWCNT treatments, which could be supplemented by freely available nutrient supply in the hydroponic culture system. However, root growth inhibition was maintained even in nutrient solution with unlimited nutrient supply when the presence of MWCNTs was not restricted to the imbibition stage and MWCNTs were applied during plant growth in nutrient solution exerting inhibitory effects already at low concentrations of 50 mg L^{-1} (Table 5.1). By contrast, shoot growth remained unaffected (Table 5.1).

Apparently, the freely available nutrient supply in hydroponics was sufficient to maintain normal shoot growth even with a smaller root system affected by the MWCNTs treatments. Root growth effects of MWCNTs have been documented also in previous studies with different plant species, and responses ranged from growth inhibition, no effects and even growth stimulation particularly at lower MWCNT concentrations [18, 20, 21].

Table 5.1. *Growth characteristics of soybean plants, (I) developed from seeds treated for 36 h with MWCNTs (1000 mg L^{-1}) and DI water (Control), pre-germinated in filter paper rolls without MWCNTs for 6 d and subsequently grown in full nutrient solution without MWCNTs for 9 d (see Figure 5.1, experimental set-up 1 C); (II) developed from non-treated seeds, pre-germinated in filter paper rolls for 7 d, grown in nutrient solution amended with MWCNTs (50, 100, 500 mg L^{-1}) for 6 d and finally grown in nutrient solution without MWCNTs for 8 d (see Figure 5.1, experimental set-up 2).*

Treatment	Plant height, cm	Shoot dry matter, g	Root dry matter, g	Root length, cm	Root diameter, mm
(I) Seed treatment (36 h)					
Control	24.6 ± 0.3 a	0.17 ± 0.01 a	0.03 ± 0.00 a	268.9 ± 23.3 a	0.50 ± 0.01a
MWCNTs 1000 mg L^{-1}	25.0 ± 0.5 a	0.17 ± 0.00 a	0.02 ± 0.00 a	244.8 ± 5.0 a	0.51 ± 0.01a
(II) Root treatment in hydroponics (6 days)					
Control	39.3 ± 1.5 a	0.29 ± 0.03 a	0.04 ± 0.00 a	609.3 ± 33.2 a	0.35 ± 0.01 b
MWCNTs 50 mg L^{-1}	41.2 ± 0.9 a	0.32 ± 0.01 a	0.03 ± 0.00 a	493.3 ± 29.5 b	0.40 ± 0.02 b
MWCNTs 100 mg L^{-1}	40.6 ± 0.2 a	0.31 ± 0.01 a	0.03 ± 0.00 a	457.6 ± 31.2 b	0.42 ± 0.02 a
MWCNTs 500 mg L^{-1}	40.3 ± 0.7 a	0.31 ± 0.01 a	0.03 ± 0.00 a	497.3 ± 24.6 b	0.42 ± 0.00 a

Note: *Results represent mean values ± SEM of four replicates. Different letters (a, b) indicate significant difference between treatments (Student t-test for the (I) seed treatment experiment, $P \leq 0.05$; one-way ANOVA, Tukey test for the (II) root treatment in hydroponics experiment, $P \leq 0.05$). MWCNTs−multi-walled carbon nanotubes.*

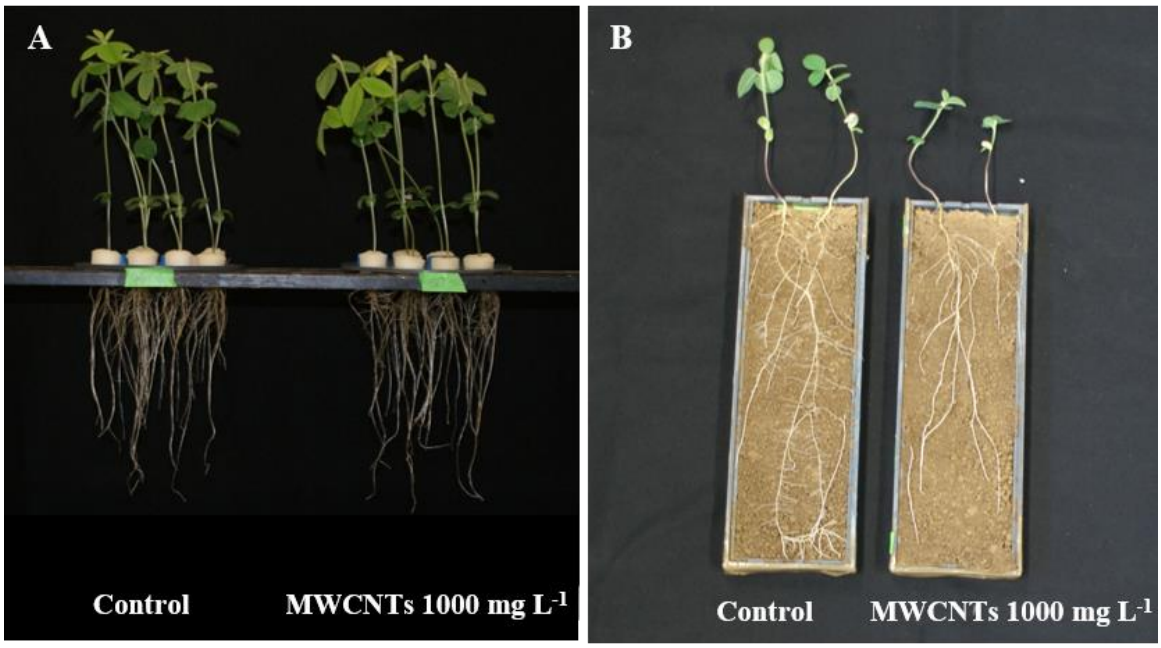

Figure 5.3. *(A) Absence of growth effects after short-term MWCNTs seed treatment (36 h; 1000 mg L^{-1}) of soybean, subsequently grown in hydroponic culture with full nutrient supply (16 DAS); (B) negative influence of short-term MWCNTs seed treatment (36 h; 1000 mg L^{-1}) on root and shoot development of soybean seedlings in soil culture (15 DAS). DAS−days after sowing, MWCNTs−multi-walled carbon nanotubes.*

In an additional experiment, further development of soybean seedlings was monitored in soil culture on a clay loam field soil (pH 7) and a calcareous Loess subsoil pH 7.6 with full macronutrient (N, P, K, Mg) fertilizers, carried out in rhizoboxes, equipped with root observation windows for monitoring of root growth effects and in pot experiments. In contrast to the experiment in hydroponic culture, inhibitory effects of short-term MWCNTs treatments during seed imbibition on root growth, persisted in soil culture up to 6 weeks after sowing even in absence of further root contact with MWCNTs. The effects were not only detectable in soybean (Figure 5.3 B), but similarly also in common bean and maize (Table 5.2), affecting mainly root length development. Typical symptoms comprised of reduction of average root length, associated with increased average root diameter in the plants developed from MWCNT-treated seeds (Table 5.2), which could be attributed to a reduction in lateral and fine root production (Figure 5.4). This was associated with stunted shoot growth, inhibited leaf expansion, and chlorosis of young leaves (Figure 5.5 A, B) as a typical indicator for zinc deficiency (little leaf syndrome [28]).

Table 5.2. *Growth characteristics of soybean, common bean and maize seedlings, developed from seeds treated for 36 h with MWCNTs (1000 mg L^{-1}) and subsequently grown in soil culture in rhizoboxes.*

Treatment	Plant height, cm	Shoot dry matter, g	Root dry matter, g	Root length, cm	Root diameter, mm
Soybean *(Glycine max* (L.) Merr*)*, 15 DAS					
Control	15.4 ± 0.6 a	0.11 ± 0.00 a	0.03 ± 0.00 a	350.7 ± 25.3 a	0.85 ± 0.01 b
MWCNTs	14.3 ± 1.1 a	0.11 ± 0.01 a	0.02 ± 0.00 a	264.1 ± 17.5 b	0.89 ± 0.02 a
Common bean *(Phaseolus vulgaris* L.*)*, 17 DAS					
Control	8.6 ± 0.5 a	0.14 ± 0.01 a	0.06 ± 0.00 a	922.9 ± 92.0 a	0.32 ± 0.01 a
MWCNTs	8.7 ± 0.7 a	0.14 ± 0.01 a	0.04 ± 0.01 a	575.9 ± 98.4 b	0.36 ± 0.02 a
Maize *(Zea maize* L.*)*, 14 DAS					
Control	24.9 ± 1.0 a	0.08 ± 0.01 a	0.09 ± 0.01 a	618.1 ± 46.5 a	0.81 ± 0.01 b
MWCNTs	21.5 ± 2.4 a	0.06 ± 0.01 a	0.08 ± 0.01 b	452.6 ± 48.7 b	0.86 ± 0.01 a

Note: *Results represent mean values ± SEM of five replicates. Different letters (a, b) indicate significant difference between treatments (Student's t-test, $P \leq 0.05$). DAS−days after sowing, MWCNTs−multi-walled carbon nanotubes.*

In accordance with the visual symptoms, shoot nutrient analysis revealed critical zinc (Zn) levels at final harvest of plants exposed to MWCNT-seed treatments, reaching less than 20% of the Zn concentrations of untreated controls (Figure 5.5 C). No comparable effect could be observed for other nutrients such as phosphate (P) or potassium (K) (data not shown). A critical role particularly of Zn as limiting nutrient in MWCNT-treated soybeans was further confirmed by the observation that depressions of root and shoot growth could be restored by repeated foliar Zn applications throughout the culture period (Figure 5.4). Zinc limitation can induce oxidative stress since certain superoxide dismutases involved in detoxification of free radicals require Zn as a co-factor [29, 30]. Apart from lipid peroxidation of membranes [31] Zn limitation may also promote oxidative degradation of indole acetic acid (IAA). The resulting low IAA levels have been discussed as a cause for limited leaf expansion and may be similarly responsible also for limited lateral fine root production observed in MWCNT-treated plants [32, 33].

Exposure of plants to MWCNTs can induce increased formation of reactive oxygen species (ROS) [21] and the related oxidative stress may be responsible for the inhibition of root growth already in young seedlings (10 DAS). Whether this effect is already linked with zinc deficiency, remains to be established. When these seedlings are supplied with high amounts of freely available nutrients e.g. in hydroponic culture, sufficient nutrients are taken up even by the smaller root systems of MWCNT-treated plants finally leading to a compensation of the inhibitory effects on plant growth. In soil culture, sparingly soluble nutrients and particularly Zn are obviously not acquired in sufficient amounts by the stunted root systems and the Zn demand can only be covered by additional foliar Zn application.

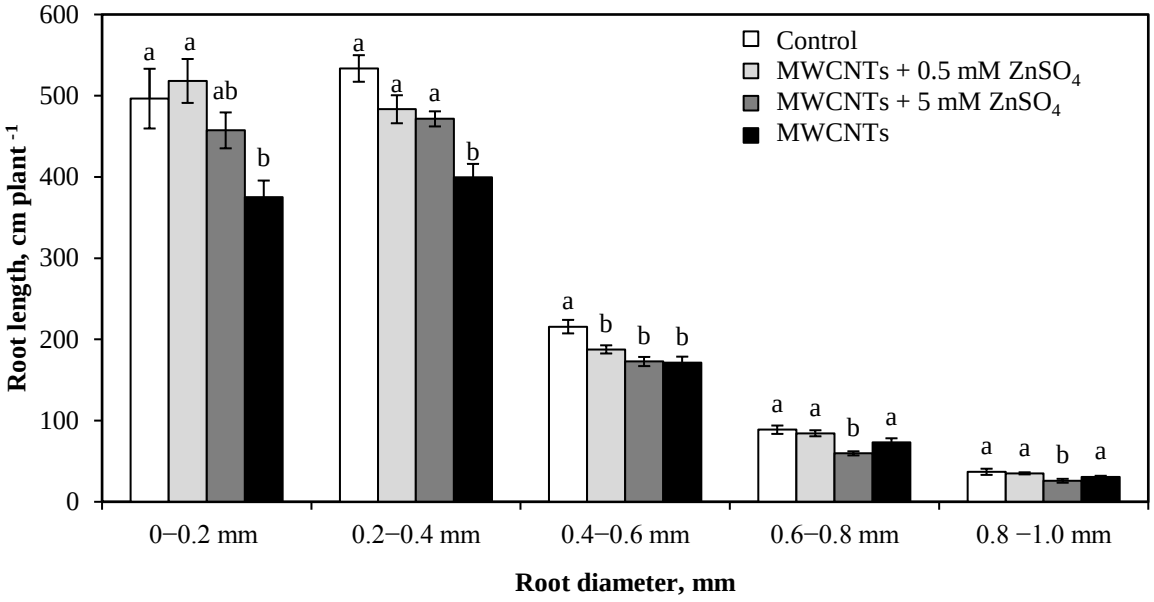

Figure 5.4. *Effects of short-term MWCNTs seed treatments (36 h; 1000 mg L^{-1}) and 3x-foliar Zn application (0.5 and 5 mM) on development of fine roots (different diameter classes) of soybean plants in soil culture (33 DAS). Results represent mean values ± SEM of eight replicates. Different letters (a, b) indicate significant difference between treatments (one-way ANOVA, Tukey test, $P \leq 0.05$). DAS−days after sowing, MWCNTs−multi-walled carbon nanotubes.*

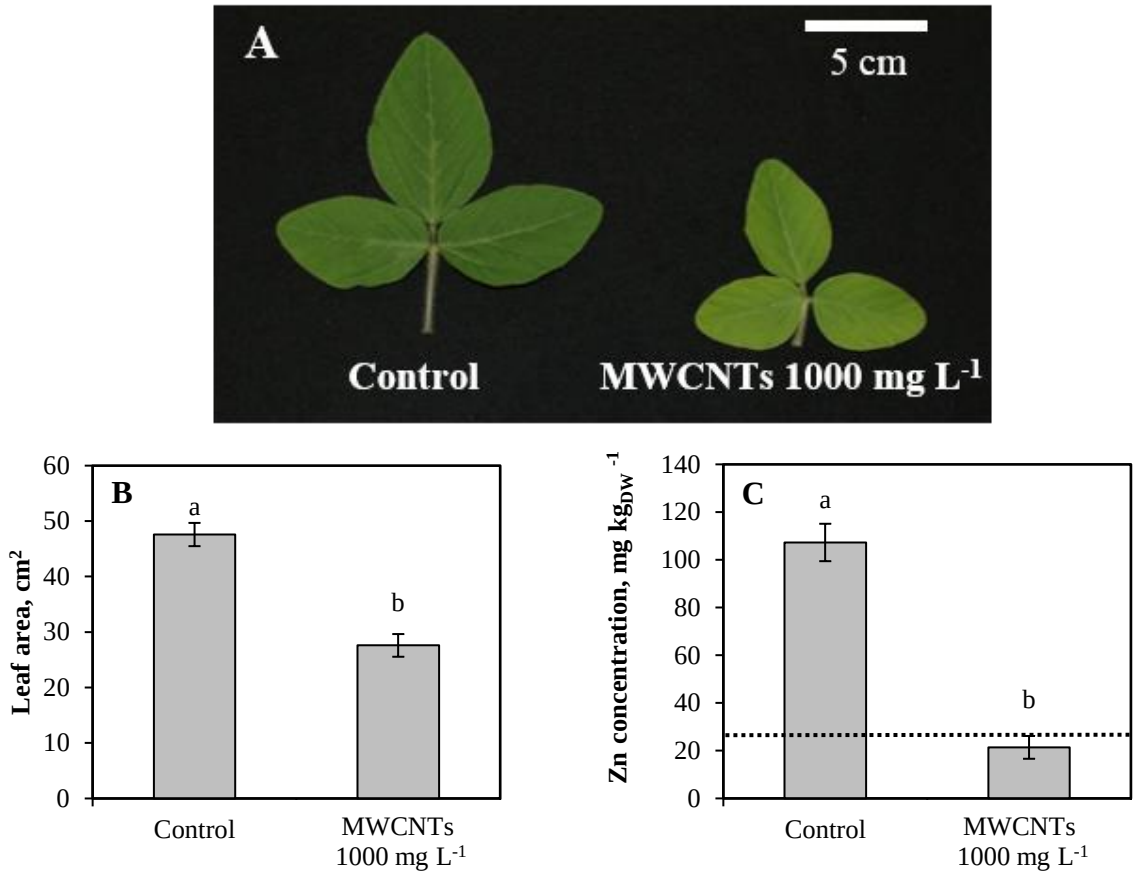

Figure 5.5. *(A) First fully developed leaves of soybean plants with and without short-term MWCNTs seed treatment (38 DAS). Leaf chlorosis (pale-green color) and inhibited leaf expansion (little-leaf syndrome) in the MWCNTs variant as a typical symptom of Zn deficiency. (B) Effect of short-term seed treatments with MWCNTs on surface area of the first fully developed leaf of soybean plants (38 DAS). (C) Zinc concentration of the soybean shoots (38 DAS). The dotted line indicates the level of zinc deficiency [34]. Results represent mean values ± SEM of five replicates. Different letters (a, b) indicate significant difference between treatments (Student t-test, P ≤ 0.05). DAS−days after sowing, MWCNTs−multi-walled carbon nanotubes.*

5.5 Conclusions

Our findings suggest that short-term exposure to small amounts of MWCNTs during germination can induce long lasting inhibitory effects during the development of soybean plants. Moreover, the expression of effects strongly depends on the culture conditions and application time. This may at least partially explain the high variability of responses reported in the literature, comprising both stimulatory and inhibitory effects. Different concentrations and types of applied MWCNTs have been discussed as additional factors. The close link of MWCNTs applications with production of ROS [21] may provide an explanation for the expression of both, positive and negative effects. The formation of ROS has been discussed as a physiological base for hormesis effects at low concentrations by stimulation of seed germination [35] and plant defence mechanisms and for negative effects induced by oxidative stress at higher concentrations. In this context also the sensitivity of plants may vary during their ontogenesis and in response to the environmental conditions. However, the physiological base of MWCNTs effects on plant metabolism and potential relationships with oxidative stress responses still require a more detailed characterisation. This is of particular importance in face of agricultural applications of MWCNTs currently under development or already on the market [36, 37].

5.6 Appendix

5.6.1 Authors' contributions

OZ participated in designing experiments, conducted experiments and laboratory analysis, performed statistical analyses and results interpretation, drafted and participated in reviewing the manuscript. GN designed experiments, was involved in proof reading and provided final editing of the manuscript.

5.6.2 Acknowledgments

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The authors declare that they have no competing interests.

5.6.3 Supplemental materials

Supplemental Table S5.1. *Amount of impurities (% mass) in the MWCNTs used for the experiments. (Data provided by NanoTechCenter Ltd., Tambov, Russia).*

Chemical formula	Amount, % mass	Confidence interval, % mass
Al_2O_3	0.237	± 0.04
Fe_2O_3	1.980	± 0.12
TiO_2	0.0018	± 3.0e-04
MgO	0.140	± 0.031
SiO_2	0.09	± 0.018
P_2O_5	0.002	± 3.4e-04
CaO	0.02	± 0.003
Na_2O	0.0033	± 3.8e-04
Sum of all metal oxides	**2.471**	**± 0.213**
Mn	0.0018	± 2.5e-04
Cu	0.0004	± 3.8e-05
Co	0.0003	± 2.9e-05
V	0.0148	± 0.0014
Zn	0.067	± 0.004
Cr	0.0077	± 4.2e-04
Sum of all metals	**0.025**	**± 0.006**

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6 Chapter IV. Phytotoxicity of carbon nanotubes in soybean as determined by interactions with micronutrients

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6.1 Abstract

Carbon nanomaterials released into the environment exert extremely variable effects on living organisms. In this study, we used soybean (*Glycine max*) to investigate early responses to seed exposure to multi-walled carbon nanotubes (MWCNTs, outer diameter 20–70 nm, inner diameter 5–10 nm, length of > 2 μm). Soybean seeds were imbibed with deionised water (control) or MWCNT suspension (1000 mg L-1) and were analysed for MWCNT contamination using light microscopy. The seedlings vitality status was evaluated by staining with triphenyltetrazolium chloride and measurement of oxidative stress indicators in the root tissue. Micronutrient (Zn, Mn, Cu) availability in different seedling organs was assessed and the effects of antioxidants and micronutrients supplementation was investigated. Oxidative stress induction by MWCNTs was detectable in radicle tips, coincided with MWCNTs accumulation and was reverted by external application of proline as antioxidant and micronutrients (Zn, Cu, Mn) as

cofactors for various enzymes involved in oxidative stress defence. Accordingly, SOD activity increased after Zn supplementation. During germination, the MWCNT treatments reduced Zn translocation from the cotyledons to the seedling and MWCNTs exhibited adsorption potential for Zn and Cu, which may be involved in internal micronutrients immobilisation. This study demonstrates for the first time that MWCNT phytotoxicity is linked with oxidative stress-related disturbances of micronutrient homeostasis.

Keywords

Carbon nanotubes, micronutrients, soybean, superoxide dismutase, superoxide radicals, tissue vitality.

6.2 Introduction

Multi-walled carbon nanotubes (MWCNTs) represent the most important class of carbon-based nanomaterials, structurally characterised by multiple cylindrical layers of graphene sheets with outer diameter less than 100 nm and length in the micrometre range [1]. Due to their exceptional physicochemical properties, MWCNTs have attracted increased interest with numerous industrial, environmental and even agricultural applications [2–6] reviewed by [7]. In recent past, significant improvements of production methods enabled large-scale production, and meanwhile, MWCNTs belong to the ten most widely distributed engineered nanomaterials [8]. As a consequence of the intensified production and widespread use of CNTs it is likely that the environment and living organisms will be increasingly exposed to nanomaterials either by intentional application or by unintended contamination. Therefore, it is of particular interest to investigate the potential effects of CNTs on living organisms and the underlying mechanisms.

Plant responses to MWCNT exposure reported in the literature are highly variable ranging from stimulatory growth effects up to severe toxicity symptoms associated with growth depressions [9–15]. Apart from potential genotypic differences in sensitivity of

the target plants and variable culture conditions, this discrepancy may be explained also by the wide range of available nanomaterials with different properties and the potential for conformational changes during interactions with the constituents of different incubation media, influencing bioavailability and toxicity [16, 17]. Nevertheless, at least some principal properties of carbon nanomaterials, relevant for plant interactions have been identified so far: (i) many carbon nanomaterials can be taken up by plants and (ii) internalisation already of small amounts can induce distinct physiological responses. (iii) Higher external concentrations frequently induce detrimental effects on plant growth while lower levels of carbon nanomaterials (< 100 mg L^{-1}) exert beneficial or no effects. (iv) Induction of oxidative stress by formation of ROS seems to be a major common mechanism of phytotoxicity induced by various carbon nanomaterials [7].

Based on earlier investigations, this study was focused on MWCNT interactions with soybean (*Glycine max* (L) Merr.) as a model plant since in this case, both positive and negative effects, induced by the same concentrations and type of MWCNTs have been detected, depending on the plant developmental stage and the culture conditions [18, 19]. The aim of the investigation was the characterisation of early events, determining the physiological plant responses after exposure to the MWCNT treatments.

6.3 Material and methods

6.3.1 Nanomaterials and exposure media

Industrial multi-walled carbon nanotubes (MWCNTs) (NanoTechCenter Ltd., Tambov, Russia) produced by chemical vapor deposition (purity above 98%) with an outer diameter of 20–70 nm, inner diameter of 5–10 nm, and length of > 2 µm were used for the experiments. Media for plant exposure to MWCNTs (1000 mg L^{-1}) were prepared directly in deionized (DI) water or in DI water-based solutions of ascorbic acid (1 mM, 5 mM or 10 mM), salicylic acid (0.2 mM, 0.5 mM or 1.0 mM) or proline (5 mM or 10 mM) and dispersed by ultrasonication (SONOREX SUPER RK 510 H; 35 KHz, Bandelin Electronic, Berlin, Germany) for 30 min. The freshly prepared un-precipitated suspensions were immediately used for seed treatments.

6.3.2 Seeds

Soybean seeds (*Glycine max* L. Merr. cv. BR-16 Conquista, Embrapa, Brazil) were used for the experiments. Seeds were stored in darkness at 4 °C and kept at room temperature one day before use.

6.3.3 Combined seed application of CNTs with antioxidants

Soybean seeds (10 per replicate) were equidistantly distributed in plastic Petri dishes (diameter 90 mm, Greiner, Nürtingen, Germany) containing three discs of filter paper (Blue ribbon MN 640d, Macherey und Nagel, Düren, Germany) and wetted with 5 mL of exposure media. The applied treatments and composition of corresponding exposure media are listed in Table 6.1. Petri dishes containing soybean seeds exposed to the incubation solutions were covered with lids and incubated in the dark for 36 h at 25 °C. Thereafter, seeds were transferred to paper rolls made of folded (four layers) filter paper sheets (58 × 58 cm, MN710, Macherey und Nagel, Düren, Germany) and moistened with DI water (60 mL per roll). The filter rolls were transferred in upright position into germination boxes, and the seeds were germinated in a climate chamber at 23°C with a 14-h light period (200 µmol m^{-2} s^{-1}). At 10 days after sowing (DAS), the seedlings were harvested; roots and shoots were separated; and roots, stored in 30% ethanol (v/v) were digitalised with an Epson Expression 10000Xl scanner (Epson, USA). Root morphology analysis was performed using the WinRHIZO software (Regent Instruments, Quebec, Canada).

6.3.4 Oxidative stress status of seed tissues

For assessing the oxidative stress status, soybean seeds were exposed to MWCNT suspensions (1000 mg L^{-1}), DI water (control) or 10 mM proline in a MWCNT suspension (1000 mg L^{-1}) for 36 h in Petri dishes as described above. Physiological indicators for oxidative stress were determined immediately after 36 h imbibition period by (i) detection and quantification of superoxide ($O_2^{\bullet-}$) anions [20], (ii) estimation of superoxide dismutase (SOD) activity [21–23], and (iii) assessment of tissue vitality by staining with triphenyltetrazolium chloride [24].

Table 6.1. *Seed treatments and composition of incubation media used for combined application of MWCNTs with antioxidants (MWCNTs multi-walled carbon nanotubes, DI water deionized water).*

Treatment abbreviation	Composition of exposure media		
	Concentration of MWCNTs, mg L^{-1}	Antioxidants and their concentration	Solvent
Control	-	-	DI water
MWCNTs	1000	-	DI water
MWCNTs + 1 mM AsA	1000	1 mM ascorbic acid	DI water
MWCNTs + 5 mM AsA	1000	5 mM ascorbic acid	DI water
MWCNTs + 10 mM AsA	1000	10 mM ascorbic acid	DI water
MWCNTs + 0.2 mM Sa	1000	0.2 mM salicylic acid	DI water
MWCNTs + 0.5 mM Sa	1000	0.5 mM salicylic acid	DI water
MWCNTs + 1 mM Sa	1000	1 mM salicylic acid	DI water
MWCNTs + 5 mM Pro	1000	5 mM proline	DI water
CNTs + 10 mM Pro	1000	10 mM proline	DI water

6.3.4.1 In situ detection and quantification of superoxide anions

Accumulation sites of superoxide anions in tissues of treated soybean seeds were detected by mean of a histochemical staining assay with nitro blue tetrazolium (NBT) modified after Ivanchenko et al. [20]. After the 36 h of treatment period, coat-free intact soybean seeds were carefully rinsed with DI water to remove residues of the exposure media and subsequently immersed in 20 mM MES buffer containing 0.2 mM NBT (pH 6.1) for 30 min at room temperature. During the staining, NBT penetrates into plant tissues and reacts with superoxide anions producing a blue-coloured formazan, indicating accumulation sites of superoxide anions, while staining intensity reflects the amount. Stained seeds were examined under a binocular macroscope (Stemi 200-C, Zeiss, Oberkochen, Germany), equipped with a digital camera (DXC-390P, Sony, Japan), and images were obtained at 1.6 x magnification. The colour reaction was

stopped by immerging the seeds into 20 mM MES buffer. Extraction of the blue-coloured formazan for spectrophotometric quantification was performed according to Ramel et al. [25]. Stained tissues were excised from seeds, ground in liquid nitrogen and homogenised in a mixture of 2 M KOH and DMSO (1:1.16, v/v). After centrifugation at 10000 x g for 5 min (Hettich 1003, Tuttlingen, Germany) the absorption of the supernatant was determined spectrophotometrically at 630 nm (Genesys 10 S, Thermo Electron Corporation, Madison, USA).

6.3.4.2 SOD activity assay

For measurement of SOD (EC 1.15.1.1) activity in germinating soybean seeds, the method of Beauchamp and Fridovich [21] considering modifications of Hajiboland and Hasani [22] and Semen et al. [23] has been optimised for soybean tissues. Frozen plant material was ground using a pre-chilled mortar and pestle in an ice bath and homogenised in extraction buffer containing 0.25 M HEPES and 1 mM EDTA. The ratio between plant material and extraction buffer maintained as 1:50 (e. g. 0.05 g in 2.5 mL). Subsequently, the homogenate was centrifuged at 10000 x g at 4°C for 3 min and the supernatant was used for further processing. A reaction mixture, containing 0.1 mL 62.5 mM HEPES, 0.05 mL 1.0 mM EDTA, 0.05 mL 500 mM Na_2CO_3, 0.1 mL 120 mM L-Methionine, 0.3 mL 750 µM NBT, 0.1 mL plant extract and 0.3 mL 10 µM riboflavin was prepared in 1 mL plastic cuvettes (Lab Logistics Group GmbH, Meckenheim, Germany). The cuvettes were exposed to light with 8000 Lux for 30 min at 23 °C, and the reaction was stopped by switching off the light and covering the cuvettes with an aluminium foil. Reaction tubes, containing the extraction buffer instead of plant extract and exposed to light, were used as a control, while those exposed to dark were used as blanks. Subsequently, the absorption of the reaction mixture was measured spectrophotometrically at 650 nm against the blank. One unit (U) of SOD activity was defined as the amount that inhibits the NBT photo-reduction by 50% [21] and the results were expressed as units of SOD activity per unit fresh weight of plant material [U g FW^{-1}].

6.3.4.3 Vitality staining of soybean seeds

For vitality testing of MWCNT-treated soybean seeds, the widely used tetrazolium staining assay was performed according to ISTA rules [24]. Seed coats from MWCNT-treated and MWCNT-untreated control seeds were removed; seeds were rinsed with DI water and incubated in 0.07 M phosphate (KH_2PO_4 and Na_2HPO_4) buffer (pH 7) containing 0.5% (w/v) 2, 3, 5-triphenyl tetrazolium chloride (TTC) in the dark at 25 °C for 18 h. Subsequently, the seeds were rinsed again with DI water, dissected with a razor blade, and staining of different organs was investigated under a binocular macroscope (Stemi 200-C, Zeiss, Oberkochen, Germany) with photographic documentation at 3.2x magnification using a digital camera (DXC-390P, Sony, Japan). The red-coloured triphenylformazan (TF), produced by enzymatic reduction in metabolically active tissues, was extracted by grinding tissue samples with mortar and pestle in a mixture of 2 M KOH and DMSO (1:1.16, v/v). After centrifugation at 10,000x g for 5 min, the absorption of the supernatant was measured spectrophotometrically at 485 nm for quantitative evaluation.

6.3.5 Light microscopy

Soybean seeds were incubated in Petri dishes with and without MWCNTs (36 h, 1000 mg L^{-1}) as described above. Subsequently, the seed coats were removed and whole seeds were fixed in 2.5% (w/w) glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 24 h at 4 °C. Thereafter, the seeds were washed three times in 0.1 M phosphate buffer (pH 7.2), dehydrated by incubation in solutions with increasing ethanol concentrations (30, 50, 70, 90, 95, 95, 100, 100%, 1.5 h each step), infiltrated in a 0.1% safranin-O solution in *tert*-butanol and, thereafter, slowly infiltrated with paraffin (60 h). Sections (8−10 μm) were prepared using sliding microtome (JUNG AG, Heidelberg, Germany), and the cuttings were mounted onto glass slides. The prepared samples were observed under a light microscope (Axioscop 2, Carl Zeiss Jena GmbH, Jena, Germany) equipped with a digital camera (Axiocam color, Carl Zeiss Jena GmbH, Jena, Germany).

6.3.6 Distribution of micronutrient seed reserves in germinating soybean seeds

In order to study micronutrient (Zn, Mn, Cu) seed reserve mobilization during germination, micronutrient contents in cotyledons and in the remaining seedlings were determined separately at 4 and 10 DAS. Soybean seeds were treated with MWCNTs (1000 mg L^{-1}) or DI water (control) for 36 h and subsequently germinated in filter rolls moistened with DI water. At 4 DAS and 10 DAS, half of the seedlings were harvested and cotyledons were separated from the remaining seedlings. Plant tissues were dried in paper bags for 2 days at 60 °C and used for a mineral analysis by atomic absorption spectroscopy. In brief, finely grinded dry plant material (100 mg) was ashed in a muffle furnace (500°C, 4 h), cooled, extracted twice with 1.0 mL of 3.4 M HNO_3 and evaporated to dryness on a heating plate. Thereafter, the ash was dissolved in 1.0 mL of 4 M HCl, diluted tenfold with hot DI water and boiled for 2 min. After cooling, the solutions were adjusted to 10 mL with DI water, filtered through blue ribbon paper (Macchery & Nagel, Düren, Germany) and Zn, Cu and Mn concentrations in the extracts were measured by atomic absorption spectrometry (iCE 300 Series, Thermo Fisher Scientific, United Kingdom).

6.3.7 External micronutrient supplementation to germinating soybean seeds

Soybean seeds were treated with MWCNT suspension (1000 mg L^{-1}) and DI water (control) in Petri dishes for 36 h as described above and pre-germinated in filter rolls moistened with DI water. At 5 DAS, when the length of rootlets had reached 2–3 cm, the seedlings from MWCNT treatments were divided into four groups and transferred into new filter rolls moistened with one of the following solutions: DI water, 0.25 µM $ZnSO_4$, 0.25 µM $MnSO_4$ or 0.1 µM $CuSO_4$, while the untreated control seedlings were transferred to paper rolls moistened with DI water only (Table 6.2). Analysis of root morphology was performed as described above. To test the effect of Zn supplementation on SOD activity in the seedlings, the experiment was repeated with 0.25 µM $ZnSO_4$ application only, and SOD activity was measured in roots at 4 DAS (just prior to Zn application) and at 10 DAS as described above.

6.3.8 Nutrient adsorption by CNTs

To assess the nutrient adsorption potential of MWCNTs a concentrated modified Hoagland solution for plant culture (40 mM $Ca(NO_3)_2$, 200 µM KH_2PO_4, 14 mM K_2SO_4, 2 mM KCl, 10 mM $MgSO_4$, 200 µM H_3BO_3, 10 µM $MnSO_4$, 10 µM $ZnSO_4$, 4 µM $CuSO_4$, 0.2 µM $(NH_4)_6Mo_7O_{24}$, and 400 µM Fe(III)-EDTA) was mixed with MWCNTs in 100-mL glass bottles at concentrations 2500 mg L^{-1}. The suspension was sonicated in an ultrasonic bath for 30 min (SONOREX SUPER RK 510 H; 35 KHz, Bandelin Electronic, Berlin, Germany) and incubated for 36 h on a shaker. Subsequently, MWCNTs were removed from the suspension by filtration (Blue ribbon MN 640d, Macherey und Nagel, Düren, Germany), followed by centrifugarion at 15000x g. Nutrient concentrations in supernatants were determined by atomic absorption spectrometry (iCE 300 Series, Thermo Fisher Scientific, United Kingdom).

Table 6.2. *Description of treatments in the experiment with micronutrients applications during seedling growth (DAS days after sowing, DI water deionized water, MWCNTsmulti-walled carbon nanotubes)*

Treatment	Seed treatment during 36 h (Petri dishes)	Seedling growth up to 5th DAS (moist paper rolls)	Seedling growth from 5th to 10th DAS (moist paper rolls)
Control	DI water	DI water	DI water
MWCNTs	1000 mg L^{-1} MWCNTs	DI water	DI water
MWCNTs + 0.25 µM Zn	1000 mg L^{-1} MWCNTs	DI water	0.25 µM $ZnSO_4$
MWCNTs + 0.25 µM Mn	1000 mg L^{-1} MWCNTs	DI water	0.25 µM $MnSO_4$
MWCNTs + 0.1 µM Cu	1000 mg L^{-1} MWCNTs	DI water	0.1 µM $CuSO_4$

6.3.9 Relative expression of auxin-, and ethylene-related genes

Total RNA was isolated from rootlets frozen in liquid nitrogen of 6-day-old soybean seedlings with or without 36 h MWCNT treatments using the innuPREP Plant RNA kit (Analytik Jena AG, Germany) and contaminating genomic DNA was removed with the RNase-Free DNase Set (Qiagen, Hilden, Germany) according to manufacturer's instructions. The complementary DNA (cDNA) was synthesised using the QuantiTect

Reverse Transcription Kit (Qiagen, Hilden, Germany). Gene-specific primers were designed using Primer3Plus. Quantitative RT-PCR was performed as described elsewhere [26]. The reference genes were used as recommended in [27–29] and comprised: *ACT11*, *SKIP16*, *UKN1*, *UKN2*, *ELF1B* and *CYP2*. The selected auxin-, and ethylene-related genes were: *PIN1*, *YUC2*, *LAX3*, and *ACO*. Primer sequences of reference genes and selected auxin-, and ethylene-related genes as well as their functions are listed in Table 6.3.

6.3.10 Statistical analysis

All experiments were performed in a completely randomised design. Statistical analysis was conducted using the SigmaPlot 11.0 software package. Treatment differences were evaluated using the Student t-test, with $P \leq 0.05$ considered significant. Data are presented as mean values ± SE (standard error of the mean).

6.4 Results

6.4.1 Combined application of MWCNTs with antioxidants

As similarly observed in earlier experiments [18], the total root length of seedlings developed from seeds exposed to a MWCNT suspension (1000 mg L^{-1}) during 36 h of imbibition was reduced by 29% as compared to the untreated control (Fig. 6.1; Table 6.4) and formation of lateral roots as well as shoot growth was inhibited (Fig. 6.1). However, simultaneous application of antioxidants, particularly proline, in different concentrations (5−10 mM) during the imbibition period was able to alleviate MWCNT-induced growth inhibition and the treated seedlings showed no significant differences as compared with the control variant. Gradually, 10 mM and 5 mM proline and 1 mM ascorbic acid were most efficient in restoration of plant growth (Fig. 6.1; Table 6.4).

Table 6.3. *Description of the reference genes and selected auxin-, and etylene-related genes used for RT-qPCR.*

Gene symbol	Primer sequences		Gene function
	Forward primer sequence [5'-3']	Reverse primer sequence [5'-3']	
	Reference genes		
ACT11	ATCTTGACTGAGCGTGGTTATTCC	GCTGGTCCTGGCTGTCTCC	Actin
SKIP16	GAGCCCAAGACATTGCGAGAG	CGGAAGCGGAAGAACTGAACC	Protein binding
UKN1	TGGTGCTGCCGCTATTTACTG	GGTGGAAGGAACTGCTAACAATC	Unkown
UKN2	GCCTCTGGATACCTGCTCAAG	ACCTCCTCCTCAAACTCCTCTG	Unkown
ELF1B	GTTGAAAAGCCAGGGGACA	TCTTACCCCTTGAGCGTGG	Eukaryotic elongation factor 1 beta
CYP2	CGGGACCAGTGTGCTTCTTCA	CCCCTCCACTACAAAGGCTCG	Protein folding
	Selected auxin- and ethylene-related genes		
PIN1	TGGGCCATAACCTTGTTCTC	ACTGGAGGACCACAATTTGC	Auxin efflux carrier
ACO	GAAGCTATGAAGGCCAATGC	TCCAAGGACACCAAACACTG	Aminocyclopropanecar-boxylate (ACC) oxidase
YUC2	TGAAGGGATGGGAGAGTTTG	AGGACGAGCATTATGGTTGC	Auxin biosynthesis
LAX3	GACTTTTGGGCAAACACTGG	AGGGATGAAGACCGTTGTTG	Auxin influx carrier

Figure 6.1. *Habitus of 10-days old soybean seedlings exposed to DI water (control), 1000 mg L^{-1} MWCNTs and combinations of MWCNTs (1000 mg L^{-1}) with various concentrations of ascorbic acid (AsA), salicylic acid (SA) or proline (Pro) during the first 36 h of seed imbibition. DI water deionized water, MWCNTs multi-walled carbon nanotubes*

Table 6.4. *Total root length of 10-days old soybean seedlings exposed to DI water (control), 1000 mg L^{-1} MWCNTs and combinations of MWCNTs (1000 mg L^{-1}) with various concentrations of ascorbic acid (AsA), salicylic acid (SA) or proline (Pro) during the first 36 h of seed imbibition.*

Treatment	Total root length, cm	Treatment	Total root length, cm	Treatment	Total root length, cm
Control	96.96 ± 8.19 a	Control	96.96 ± 8.19 a	Control	96.96 ± 8.19 a
MWCNTs	68.91 ± 7.56 a	MWCNTs	68.91 ± 7.56 a	MWCNTs	68.91 ± 7.56 b
MWCNTs + 1 mM AsA	90.88 ± 11.61 a	MWCNTs + 0.2 mM Sa	88.86 ± 10.03 a	MWCNTs + 5 mM Pro	94.13 ± 3.75 a
MWCNTs + 5 mM AsA	75.21 ± 10.28 a	MWCNTs + 0.5 mM Sa	74.36 ± 7.38 a	MWCNTs + 10 mM Pro	98.59 ± 4.03 a
MWCNTs + 10 mM AsA	82.80 ± 9.21 a	MWCNTs + 1 mM Sa	89.39 ± 8.44 a		
P value	0.279		0.148		0.009

Data represent mean values ± SEM of six replicates. Different letters (a, b) indicate significant differences between treatments (one-way ANOVA, Tukey test, $P \leq 0.05$).

6.4.2 Oxidative stress status of seed tissues

In face of the beneficial effects in mitigation of MWCNT-induced growth inhibition in soybean seedlings induced by seed imbibition with antioxidants, physiological indicators of oxidative stress were determined in the soybean seeds directly after termination of the 36 h imbibition period with or without MWCNTs. Vital staining with triphenyltetrazoliumchloride revealed distinct decolorations of embryonic tissues (Fig. 6.2 e) and reduced colour development in CNT-treated seeds, indicating reduced metabolic activity (Fig. 6.2 i). This effect was reverted completely by simultaneous application of 10 mM proline as antioxidant (Fig. 2 i). Accordingly, the damaged tissue in CNT-treated seeds showed a stronger accumulation of superoxide anions detected by nitroblue-tetrazolium (NBT) staining with a particularly intense expression in the apical zones of the radicles (Fig. 6.2 b, g), which was reverted by application of the proline as antoxidant (Fig. 6.2 c, g). A moderatetly increased activity of the free-radical detoxifying enzyme superoxide dismutase (SOD) further confirmed the induction of oxidative stress in MWCNT-treated seeds (Fig. 6.2 h).

6.4.3 Uptake and localization of CNTs in plant tissues

Microscopic examination of microtome cuttings revealed uptake of MWCNTs into the embryonic tissues of the imbibing soybean seeds (Fig. 6.3), although the seed coats did not show any visible signs of mechanical damage. In accordance with the preferential localisation of superoxide anions as indicators for oxidative stress, MWCNTs accumulated particularly in the calyptrogen and calyptra cells of the radicle tip (Fig. 6.3 b, d). Hot spots of MWCNT accumulation were also found close to the meristem and in plerome cells forming the central cylinder as origin of lateral root formation during later development (Fig. 6.3 b, f). The MWCNT-contaminated cells were smaller particularly in the longitudinal direction than the corresponding cells in control plants (Fig. 6.3 e, f) and MWCNTs were visible as multiple granulated agglomerations filling the whole cell content but not sticking to the cell walls in higher amounts (Fig. 6.3 g).

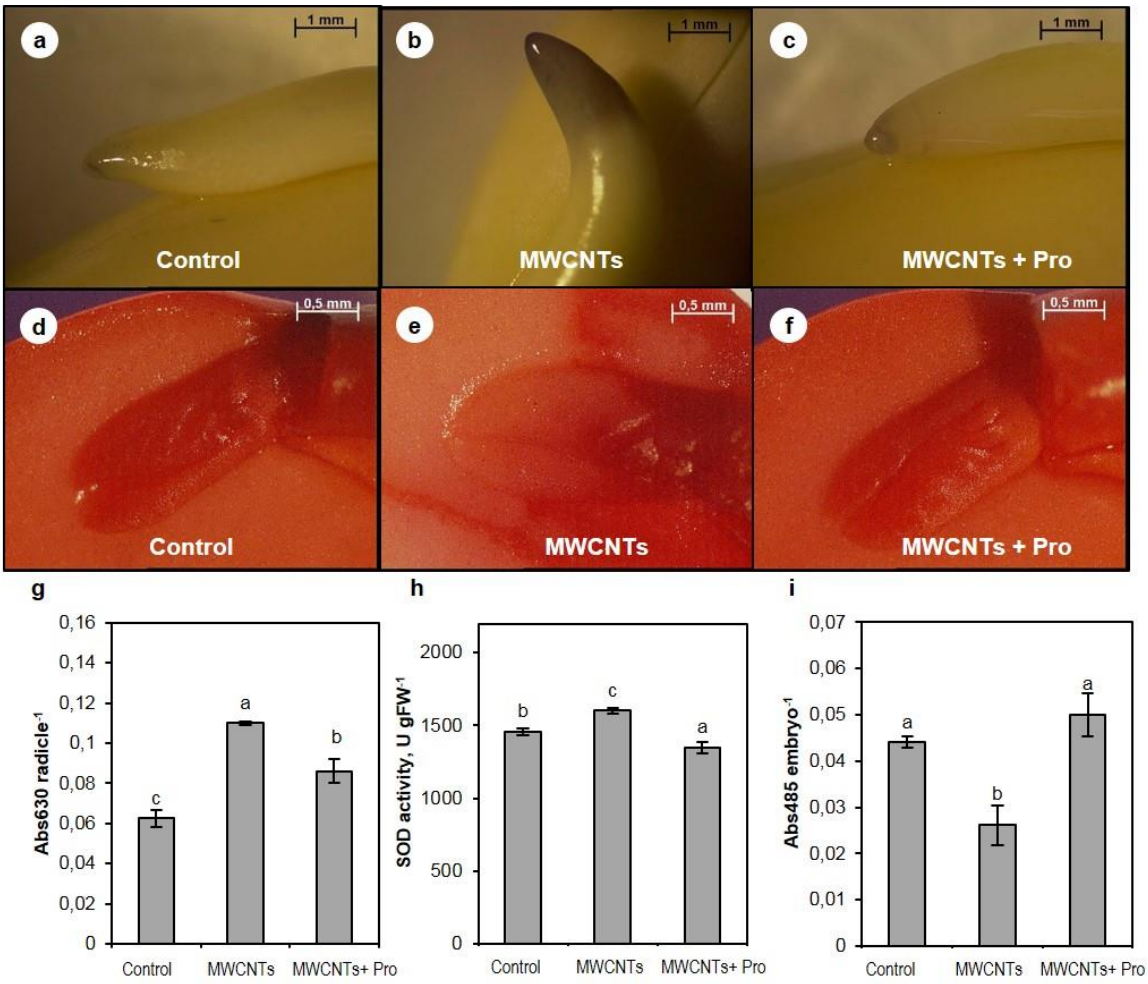

Figure 6.2. *Detection of oxidative stress indicators in soybean seeds imbibed for 36 h with 1000 mg L^{-1} MWCNTs, combined application of 1000 mg L^{-1} MWCNTs with 10 mM proline (MWCNTs + Pro) or DI water (control). (a-c): Radicles stained with nitroblue tetrazolium (NBT): localisation and intensity of dark staining indicates accumulation sites and amount of superoxide anions; (d-f): Soybean seeds, stained with 2, 3, 5-triphenyl tetrazolium chloride (TTC): darker staining induced by dehydrogenase activities as indicator for metabolic activity; (g): Spectrophotometric quantification of blue formazan, produced per radicle in soybean seeds stained with NBT, which indirectly indicates the amount of superoxide anions; (h): Activity of superoxide dismutase (SOD) in radicles of soybean seedlings; (i): Spectrophotometric quantification of red formazan, produced per embryo in soybean seeds stained with TTC, reflecting the vitality status of the tissues. Results (g-i) represent mean values ± SEM of six replicates; different letters (a, b) indicate significant treatment differences (one-way ANOVA, Tukey Test, $P \leq 0.05$). DI water deionized water, MWCNTs multi-walled carbon nanotubes.*

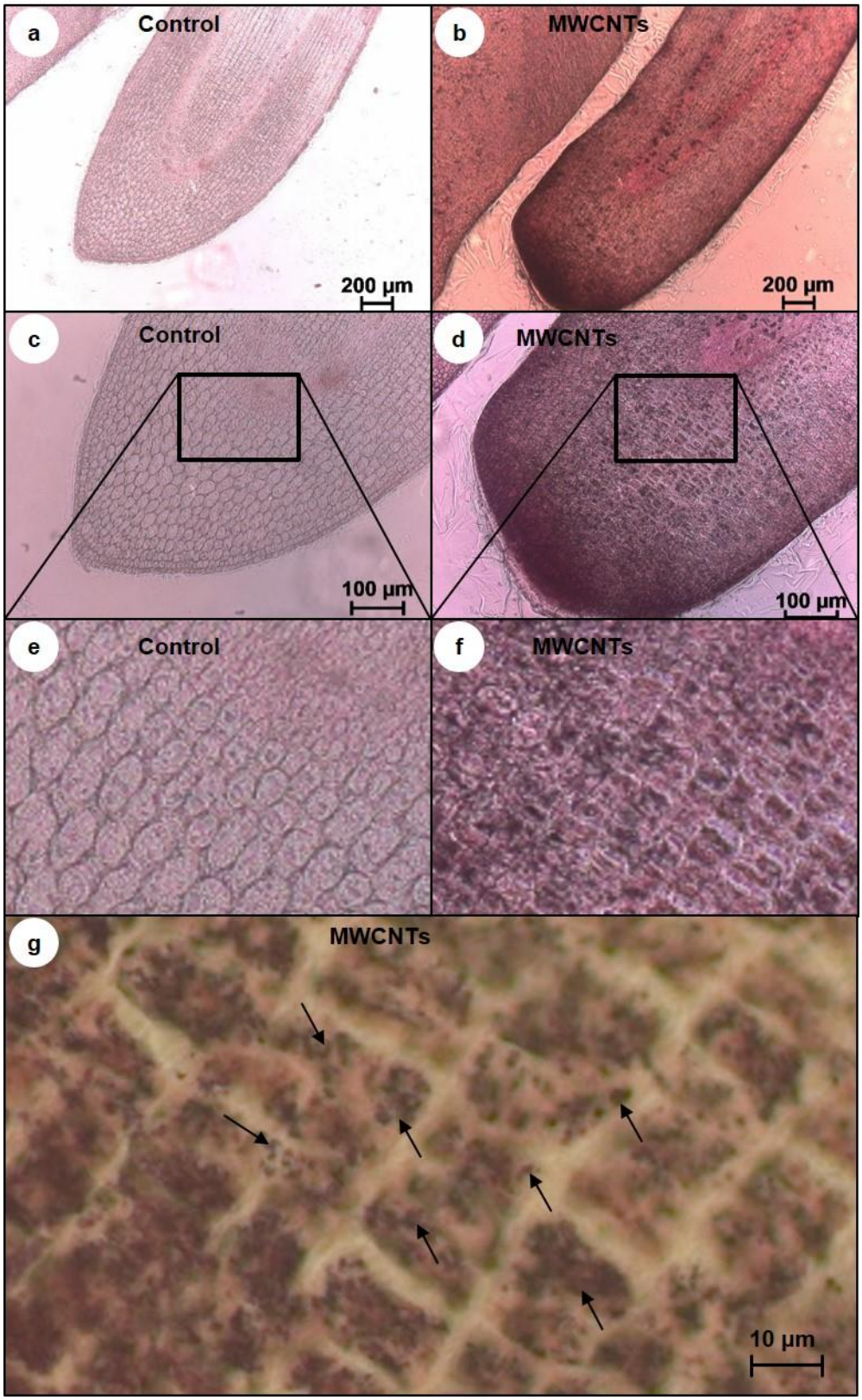

Figure 6.3. *Light-microscopic examination of microtome cuttings obtained from radicles of soybean seeds imbibed for 36 h in DI-water (control, a, c, e) or in MWCNT suspension (1000 mg L^{-1}; b, d, f, g). (a, b): overview; (c, d): tip region; (e, f, g): calyptrogen. Arrows indicate MWCNT agglomerates. DI water deionized water, MWCNTs multi-walled carbon nanotubes.*

6.4.4 External micronutrient supplementation

In face of the function of micronutrients, such as Zn, Mn and Cu as co-factors for enzymes involved in antioxidative defence reactions [30], a supplementation experiment was conducted. Soybean seeds exposed to 36 h imbibition in MWCNT suspension (1000 mg L^{-1}) or in DI water were subsequently germinated during 5−10 DAS between filter papers soaked with water or with solutions of the respective micronutrients. As similarly observed in the previous experiment (Fig. 6.1), the MWCNT treatment affected seedling development, reflected by stunted shoot growth (Fig. 6.4 a), reduction in root elongation (Fig. 6.4 a, b) and reduced formation of lateral roots (Fig. 6.4 a). The C MWCNT-induced root growth inhibition was mitigated by all micronutrient treatments (Fig. 6.4 a) with restoration of root elongation particularly expressed for Zn and Mn supplementation (Fig. 6.4 b-d). Also, external application of $ZnSO_4$ has restored SOD activity in 10-day-old soybean seedlings, while in sole MWCNT treatment, the enzyme activity was significantly decreased. (Table 6.5).

Table 6.5. *Superoxide dismutase (SOD) activity in roots of 4- and 10-days-old soybean seedlings developed from seeds treated for 36 h with MWCNT suspension (1000 mg L^{-1}) or DI water (control) and subsequently germinated in filter rolls moistened with deionized water or with a 0.25 µM Zn solution.*

Treatment	SOD activity in roots, U gFW^{-1}	
	4 DAS	10 DAS
Control	812.6 ± 13.4 b	510.1 ± 3.6 a
CNTs	851.1 ± 8.5 a	470.4 ± 5.8 b
CNTs + 0.25 *µM* Zn	-	495.4 ± 7.7 a
P value	0.026	0.003

Results represent means ± SEM of five replicates. Different letters in one column (a, b) indicate significant difference between treatments (Student's t-test for SOD activity at 4 DAS and one-way ANOVA, Tukey test for SOD activity at 10 DAS, $P \leq 0.05$).
DI water deionized water, MWCNTs multi-walled carbon nanotubes, DAS days after sowing.

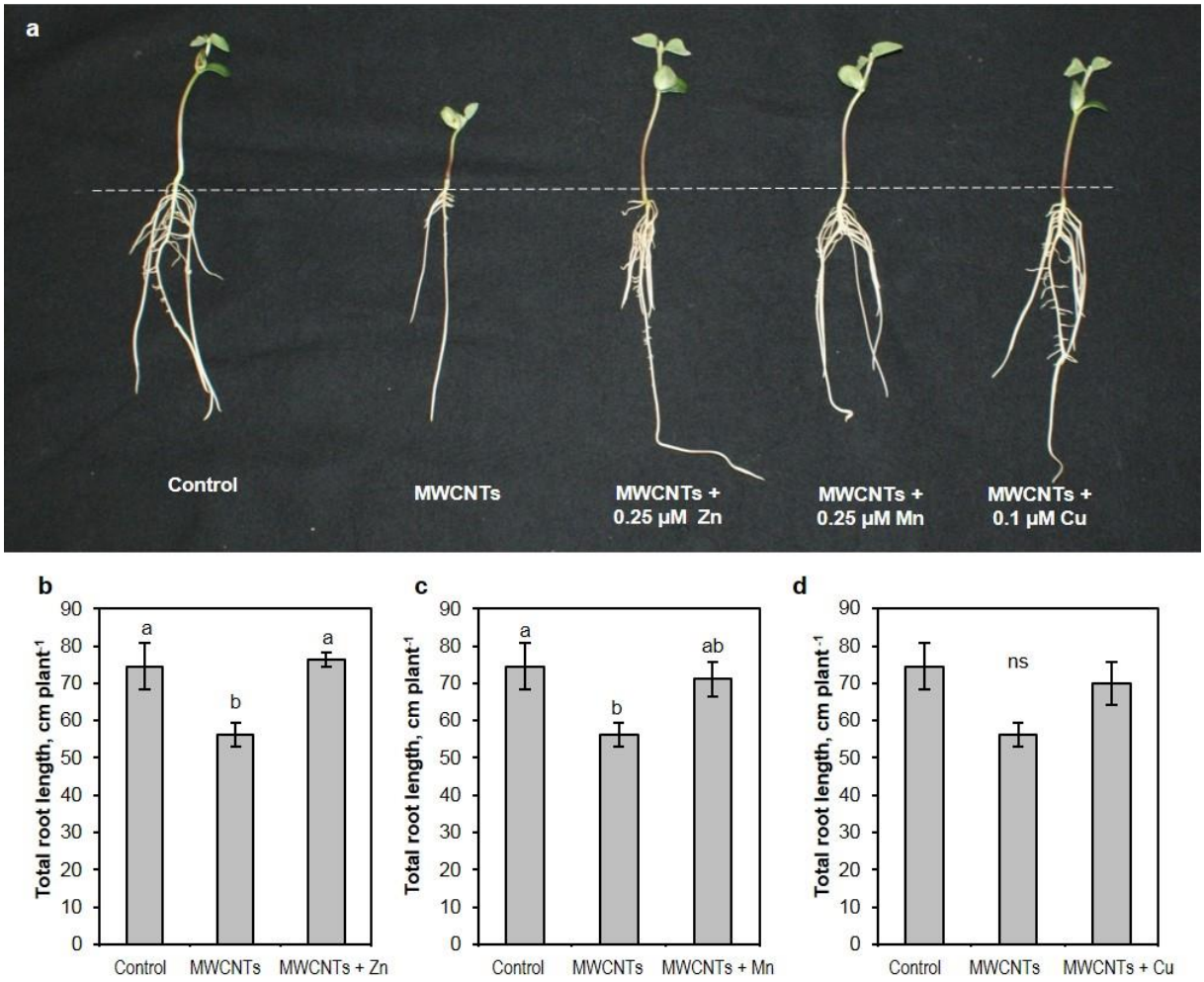

Figure 6.4. *Habitus (a) and total root length (b-d) of 10-days old soybean seedlings, developed from seeds treated for 36 h with 1000 mg L^{-1} MWCNTs or deionized water (control) and germinated in rolls of filter paper between 5 and 10 DAS moistened with DI water (control) or solutions of micronutrients in different concentrations (0.25 µM Zn, 0.25 µM Mn or 0.1 µM Cu). Results (b-d) represent mean values ± SEM of five replicates, different letters (a, b) indicate significant treatment differences (one-way ANOVA, Tukey Test, $P \leq 0.05$). DI water−deionized water, CNTs–multi-walled carbon nanotubes, DAS−days after sowing.*

6.4.5 Organ-specific distribution of micronutrients in germinating soybean seeds

Since symptoms of MWCNT toxicity in germinating soybean seeds without external nutrient supply could be specifically mitigated by supplementation of micronutrients, internal disturbance of the micronutrient homeostasis by MWCNT treatments is a likely cause for the detrimental effects observed during seedling development (Figs. 6.1, 6.4). One possible explanation could be a disturbed remobilisation of micronutrient seed

reserves and/or re-translocation of micronutrients from the cotyledons as storage organs to the developing seedling. Therefore we investigated alterations of micronutrient contents (Zn, Mn, Cu) in the cotyledons and the remaining seedling during 4 and 10 days of germination between filter paper supplied with distilled water lacking additional nutrients, with and without exposure to MWCNT suspension (1000 mg L^{-1}) within the first 36 h of seed imbibition. During the 6 days observation period, the Zn content of untreated control seedlings (excluding the cotyledons) increased by 1.73 µg while this increase reached only 1.35 µg in the CNT pre-treated seedlings, resulting in significantly lower Zn contents (Table 6.6). A similar but not significant trend was observed also for Mn and Cu. From the difference in total content of micronutrients in the seedling organs between 4 and 10 DAS (cotyledons + remaining seedling), also losses induced by micronutrient leaching were calculated without significant differences between the treatments (Table 6.6).

6.4.6 Nutrient adsorption by MWCNTs

Since high accumulation of MWCNTs was detected particularly in young growing tissues of soybean roots (Fig. 6.3), we investigated also the possibility of nutrient immobilisation by adsorption effects of MWCNTs. To simulate a balanced nutrient supply providing both, macronutrients and micronutrients for higher plants, a modified Hoagland nutrient solution was supplemented with 2500 mg L^{-1} MWCNTs. After 36 h incubation time on a shaker, MWCNTs were removed from the suspension by filtration and centrifugation and remaining nutrient concentrations in the supernatant were determined. The results demonstrated significant adsorption of Zn and Cu particularly by MWCNTs (Table 6.7). The concentration of Mn even increased and no significant effects were detected for Fe and selected macronutrients (K, Mg, Ca) (Table 6.7).

Table 6.6. *Average zinc (Zn), copper (Cu) and manganese (Mn) contents in cotyledons [µg pair of cotyledons^{-1}] and remaining seedlings [µg seedling^{-1}] of 4- and 10-days-old soybean seedlings, developed from seeds, treated for 36 h with MWCNT suspension (1000 mg L^{-1}) or DI water (control) and subsequently germinated in filter paper rolls moistened with deionized water.*

Treatment	Micronutrient content, µg				Amount of nutrient mobilized between 4th and 10th DAS, µg seedling^{-1}	Amount of leached nutrient between 4th and 10th DAS, µg seedling^{-1}
	4-days old seedlings		10-days old seedlings			
	Pair of cotyledons	Remaining seedling	Pair of cotyledons	Remaining seedling		
	Zinc (Zn) content, µg					
Control	5.51 ± 0.19	0.95 ±0.05	2.86 ± 0.21	2.68 ± 0.19	1.73 ± 0.24	0.91 ± 0.25
MWCNTs	4.97 ± 0.21	0.71 ± 0.09 *	3.04 ± 0.18	2.04 ± 0.19 *	1.35 ± 0.24	0.60 ± 0.26
	Manganese (Mn) content, µg					
Control	2.21 ± 0.23	0.16 ± 0.01	1.44 ± 0.17	0.64 ± 0.07	0.49 ± 0.26	0.22 ± 0.38
MWCNTs	2.45 ± 0.34	0.13 ± 0.02	1.86 ± 0.25	0.47 ± 0.06	0.35 ± 0.27	0.24 ± 0.38
	Copper (Cu) content, µg					
Control	2.41 ± 0.18	0.31 ± 0.05	0.97 ± 0.07	1.01 ± 0.09	0.70 ± 0.26	0.78 ± 0.37
MWCNTs	2.60 ± 0.61	0.24 ± 0.04	1.13 ± 0.16	0.79 ± 0.09	0.55 ± 0.25	0.91 ± 0.38

Results represent mean values ± SEM of six replicates. DI water deionized water, MWCNTs multi-walled carbon nanotubes, DAS days after sowing
** Significant difference as compared to a corresponding control (Student's t-test, $P \leq 0.05$)..*

Table 6.7. *Concentrations of zinc (Zn), copper (Cu), manganese (Mn), iron (Fe), potassium (K), magnesium (Mg) and calcium (Ca) in a nutrient solution incubated for 36 h with MWCNTs (2500 mg L^{-1}).*

Nutrient	Nutrient concentration, mg L^{-1}	
	Nutrient solution	Nutrient solution + 2500 mg L^{-1} MWCNTs
Zn	0.605 ± 0.009 a	0.574 ± 0.010 b
Cu	0.297 ± 0.004 a	0.252 ± 0.013 b
Mn	0.349 ± 0.011 b	0.418 ± 0.010 a
Fe	19.263 ± 0.086 ns	19.119 ± 0.030 ns
Ca	1624.8 ± 2.131 ns	1671.75 ± 3.038 ns
Mg	258.265 ± 3.464 ns	256.393 ± 2.989 ns
K	1448.4 ± 3.356 ns	1447.8 ± 2.956 ns

Results represent mean values ± SEM of five replicates, different letters (a, b) indicate significant treatment differences (Student's t-test, $P \leq 0.05$). ns not significant. MWCNTs multi-walled carbon nanotubes.

6.4.7 Relative expression of auxin-, and ethylene-related genes

In face of the strong inhibitory effects of MWCNT treatments on root growth of soybean seedlings (Figs.6.1 and 6.4) and the well-documented role of hormonal factors, such as auxin and ethylene in root development [31], the expression of auxin-, and ethylene-related genes was investigated in the root tissues of soybean seedlings with and without seed exposure to MWCNT suspensions. The RT-qPCR analysis revealed a trend for increased relative expression of all investigated genes (*ACO*, *PIN1*, *YUC2*, *LAX*) in the roots of 6-day-old soybean seedlings, developed from seeds treated for 36 h with 1000 mg L^{-1} MWCNTs (Table 6.8), although differences were not significantly different as compared with the untreated control variant.

Table 6.8. *Real-time qPCR analysis of transcripts involved in auxin and ethylene metabolism in roots of 6-day-old soybean seedlings developed from seeds treated for 36 h with MWCNT suspension (1000 mg L^{-1}) or DI water (control) and subsequently grown in filter paper rolls moistened with deionized water.*

Treatment	Relative gene expression			
	ACO	***PIN1***	***YUC2***	***LAX3***
Control	0.57 ± 0.14	1.08 ± 0.16	0.77 ± 0.13	1.98 ± 0.51
CNTs	0.79 ± 0.17 ns	1.14 ± 0.09 ns	1.14 ± 0.23 ns	3.34 ± 2.0 ns

The expression level is indicated relative to the reference genes specified in Table 6.3. Results represent mean values ± SEM of two (control) and three (CNTs) replicates, ns not significant from the control (Student's t-test, $P \leq 0.05$).

6.5 Discussion

Investigations on plant responses to MWCNT exposure frequently show variable results. Positive effects e.g. on germination and seedling development have been reported particularly at lower doses of MWCNT treatments [32, 33]. Hormesis effects, often based on induction and strengthening of defence systems in response to moderate stress levels [10, 34] as well as improved water uptake during germination as a consequence of MWCNT-induced seed coat perforation and induction of aquaporins were discussed as possible mechanisms [9, 17, 35]. At higher levels of MWCNT exposure, induction of oxidative stress by formation of reactive oxygen species (ROS) has been identified as a major cause for inhibitory effects of MWCNT treatments on plant growth [36, 11]. In soybean, both, positive and negative effects were reported to be induced by application of moderately high MWCNT concentrations (100 – 1000 mg L^{-1} equivalent to 50 and 500 µg $seed^{-1}$) during the first 36 h of seed imbibition [18]. In this case, beneficial effects on germination could be attributed to a reduction in the speed of water uptake by a seed dressing effect of the MWCNTs, tightly adhering to the seed coat [18], thereby reducing imbibition damage due to rapid water uptake as a well-documented stress factor for germination of large-seeded leguminous plants [37, 38]. However, despite initially improved germination, MWCNT-treated soybean seedlings exhibited stunted shoot and root growth during further seedling development, first detectable already at 8 days after sowing (DAS). The inhibitory effects of short-term

MWCNT exposure during seed inhibition could be reverted by hydroponic cultivation of the seedlings with freely available supply of all macro- and micro-nutrients but long-lasting inhibition of shoot and root growth was observed in soil culture. In this context zinc was identified as a critical nutrient [19].

6.5.1 Oxidative stress in MWCNT-treated soybean seedlings

In the present study, a major focus was placed on the identification of stress factors involved in the early events of MWCNT-induced inhibition of seedling growth in soybean. To test the hypothesis that the well documented role of MWCNTs in triggering oxidative stress in plant tissues [36, 11] might be involved also in the induction of plant damage in soybean, various antioxidants, such as ascorbic and salicylic acids as well as proline [39–45], were applied during the 36 h imbibition period in presence of MWCNTs. As expected for an oxidative stress syndrome, the application of the antioxidants exerted a protective effect on the development of MWCNT-treated soybean seedlings (Fig. 6.1) as previously reported also for other plant species [36, 11]. Among the tested antioxidants, proline was most effective in recovering root growth (Table 6.4). This may be explained by multiple functions of proline in biotic and abiotic stress resistance, including not only ROS quenching, but also activation of oxidative stress defence systems system by mean of increasing of enzymes (SOD, catalase, ascorbate peroxidase) activity and protection of membranes and protein structure [46].

The oxidative stress hypothesis was further confirmed by measuring superoxide anions in the seedling tissues as one of the most toxic radical species [56] and precursor for formation of other ROS [30]. MWCNT-induced superoxide anion accumulation was detected as an early event already at 36 h of the imbibition period, preferentially localised in the tip region of the radicle (Fig. 6.2 b), and was almost completely suppressed by simultaneous application of 10 mM proline as antioxidant (Fig. 6.2 c). Microscopic examination of microtome cuttings prepared from germinating soybean seeds, revealed that the accumulation of superoxide anions coincided with preferential accumulation of MWCNTs in the tip of the radicle (Fig. 6.4 d). As reported in earlier studies [9, 35, 14], obviously, MWCNTs were able to penetrate the seed coat and the

cell walls of embryonic tissues leading to surprisingly intense granular agglomerations, particularly in the cells of the calyptrogen and the calyptra but also in the plerome of the radicle (Fig. 6.3 d-g). The highly localized MWCNT accumulation in the young growing parts of the radicle, even detectable by light microscopy (Fig. 6.3 g), suggests the presence of a preferential uptake pathway apart from general seed coat penetration. In this context, the micropyle may act as a preferential entry point, since it promotes water uptake during seed imbibition, which may mediate also uptake of MWCNTs suspended in the imbibition water. Moreover, as the site of radicle emergence, the micropyle is located close to the tip of the radicle (Fig. 6.5 a) and this localisation would explain a preferential exposure of the radicle to MWCNTs entering the seed during imbibition and intense local expression of defence reactions including oxidative burst, comparable with responses to pathogen attack [57].

Radicle cells contaminated with MWCNTs were smaller as compared with those from untreated control plants (Fig. 6.4 e, f), reflecting problems with cell expansion already prior to radicle emergence. This effect is most probably related with the inhibition of primary root growth observed during later seedling development (Fig. 6.1). Inhibition of lateral root growth (Fig. 6.1) may be explained by MWCNT accumulation also in the plerome (Fig. 6.4d), later forming the central cylinder as origin of lateral root formation [58].

The ability of carbon nanotubes to penetrate cell walls and cell membranes has been similarly demonstrated for other plant species [9, 35] and membrane permeability has also been reported for animal cells [58]. Accordingly, carbon nanotubes were considered as potentially suitable carrier materials for development of smart delivery systems for agrochemicals (reviewed in [7]). The same penetration properties may be employed also in medicine and pharmacology for development of targeted delivery carriers for drugs, biomolecules, genes and biosensors for therapeutic use and diagnostic purposes [48]. However, the results of safety assessments reporting potential toxicity of MWCNTs for living organisms indicate a clear requirement for more detailed investigations on the conditions and mechanisms determining CNT toxicity, as a pre-requisite for the development of CNT-based medical, pharmacological or agricultural applications.

6.5.2 Role of superoxide dismutase

Superoxide anions are acting as a substrate during ROS detoxification driven by superoxide dismutase (SOD) as a key enzyme [30]. Therefore, SOD activity was determined in germinating soybean seedlings with or without short-term exposure to MWCNTs during the first 36 h of seed imbibition. A moderate but significant increase of SOD activity was triggered by MWCNT treatments in the radicle tissue both at 36 h and 96 h after sowing (Fig. 6.2 h, Table 6.5), indicating the induction of ROS detoxification. However, obviously the expression of the detoxification response was not sufficient to prevent ROS-induced damage of the embryonic tissues as indicated by a reduction in vital staining with triphenyltetrazolium chloride (Fig. 6.2 e, i) as indicator for metabolic activity [59]. Accordingly, vital staining of MWCNT-treated seeds was completely recovered by application proline as protective antioxidant (Fig. 6.2 f, i), associated with a decline of SOD activity to the background levels characteristic for untreated control seeds (Fig. 6.2 h).

Generation of ROS in response to MWCNT exposure has been observed not only in plants but also in animal tissues [49, 50]. Similar to the results of the present study, MWCNT-induced oxidative stress in animal tissues was associated with enhanced formation of ROS, declining levels of antioxidants (glutathione) and reduced activities of enzymes involved ROS detoxification SOD [51-53]. Accordingly, deficiency of antioxidants, such as vitamin E [54] increases the toxicity of CNTs in animal tissues. The results of in vivo studies suggest that in general, pristine hydrophobic CNTs are more toxic than CNTs chemically modified by functionalization [48]. Moreover, pristine CNTs are able to cause asbestos-like lung inflammation [50, 55], raising concerns regarding occupational lung diseases among workers in CNT production industry.

6.5.3 Role of micronutrient homeostasis

Since micronutrients, such as Zn, Mn and Cu play an important role as co factors for enzymes involved in antioxidative stress responses including SOD (Zn, Mn, Cu, Fe) [30, 60] and for various enzymes mediating biosynthesis of phenolic compounds (Mn,

Cu) [61, 62], which frequently exhibit antioxidative properties, a micronutrient supplementation experiment was conducted with soybean seeds after short-term exposure (36 h) to MWCNT treatments. Similar to the results of the experiment investigating the supplementation of antioxidants (Fig. 6.1, Table 6.4) the application of micronutrients completely restored MWCNT-induced growth retardation of the soybean seedlings (Fig. 6.4) with Zn and Mn applications as the most efficient treatments. This is the first report demonstrating that external micronutrient supplementation can alleviate adverse effects of MWCNTs and suggests a MWCNT-induced micronutrient limitation of the antioxidative defence systems in germinating soybean seeds, further confirmed by a significant reduction of root SOD activity in 10 day old seedlings exposed to MWCNT seed treatments (Table 6.5). In contrast to younger seedlings (36 and 96 h after sowing, Fig. 6.2 H, Table 6.5), in the later developmental stages (10 DAS), obviously, the micronutrient supply was no longer sufficient to maintain or even induce an up-regulation of SOD activity for detoxification of ROS in the MWCNT treated variant. Accordingly, Zn supplementation induced a gradual increase of SOD activity in the root tissue to a level slightly lower but not significantly different from untreated control seeds (Table 6.5). Since SOD forms in different subcellular compartments frequently depend on combinations of micronutrients as cofactors (e.g. Zn/Cu-SOD in the cytosol, [30]), a combined supplementation of different micronutrients might be even more efficient than single applications but this variant was not tested so far.

The finding that micronutrient supplementation was able to restore the MWCNT-induced growth inhibition of soybean seedlings grown on filter paper without nutrient supply suggests that the MWCNT seed treatment induced a disturbance of the internal micronutrient homeostasis of the seedlings. Embryonic tissues of soybean seeds contain high concentrations of micronutrients, such as Zn, Fe and Mn but the cotyledons provide the major micronutrient storage organs for later seedling development [63]. Therefore, we determined the distribution of micronutrients located in the cotyledons and in the remaining seedling during germination to identify potential MWCNT-induced disturbances in the translocation of micronutrient seed reserves, which may finally limit the micronutrient status of the developing seedling. As a major finding of the present study, the Zn contents of the seedlings (excluding cotyledons) were always significantly

lower in the MWCNT variants as compared with the untreated control and Zn translocation from cotyledons to the remaining seedlings between 4 and 10 DAS was reduced by approximately 25-30 % as a response to the CNT treatment (Table 6.6). A similar trend was recorded also for Mn and Cu, although the differences were not statistically significant in this case. Also no significant differences were recorded for micronutrient losses induced by leaching (Table 6.6), indicating that this process was not affected by MWCNT application.

Apart from impaired translocation and distribution of micronutrients within germinating soybean seeds, internal micronutrient availability may be additionally affected by immobilisation due to metal adsorption to MWCNTs. An adsorptive capacity for heavy metals is well documented for many carbon nanomaterials (reviewed in [7]) including even possible applications as filter materials in waste water cleanup strategies [64, 65]. To evaluate a potential fixation of micronutrients by the MWCNTs employed in the present study in a medium with a well-balanced plant nutrient supply, an adsorption experiment was conducted with MWCNTs applied in the concentration range used for the previous experiments to a modified Hoagland solution of plant nutrients. A significant adsorption potential was identified for Zn, Cu but not for Fe or the macronutrients K, Mg and Ca and the concentration of Mn in the nutrient solution even increased, which may indicate a metal contamination of the MWCNTs (Table 6.7). In face of the particularly intense accumulation of MWCNTs detected in young actively growing tissues, such as root tips of germinating soybean seedlings (Fig. 6.3 b, d), MWCNT-induced micronutrient immobilisation may act as an additional stress factor particularly in these hot spots of MWCNT accumulation.

6.5.4 Hormonal interactions

Limited micronutrient availability (particularly Zn) may also offer an explanation for the inhibition of root and shoot elongation and lateral root formation in soybean seedlings exposed to MWCNT seed treatments (Figs. 6.1 and 6.4) since Zn limitation has been linked with increased oxidative degradation of indole acetic acid (IAA) as a major regulator of shoot and root growth [66-68]. This is in line with recent reports on

MWCNT-induced growth inhibition of rice seedlings, which was associated with declining levels of IAA and also of other hormones [69]. In our study, an examination of hormone-related gene expression by RT-qPCR revealed a trend for higher expression of genes involved in IAA biosynthesis (*YUC2*), IAA transport (*PIN1, LAX3*) and synthesis of ethylene (*ACO*) in roots of MWCNT-treated soybean seedlings at 6 DAS, the time point of first visual appearance of growth depressions. Increased IAA biosynthesis and transport in the soybean roots may reflect compensatory responses to Zn deficiency-induced oxidative IAA degradation, while increased synthesis of ethylene is a typical stress response in higher plants [31, 70]. However, a more detailed investigation on expression of the respective genes and related hormone levels at different time points would be necessary to test this hypothesis.

Based on the obtained results, Figure 6.5 provides a schematic representation of interactions between MWCNTs, micronutrients, antioxidative stress systems and phytohormones, resulting in inhibition of root growth in soybean (Fig. 6.5).

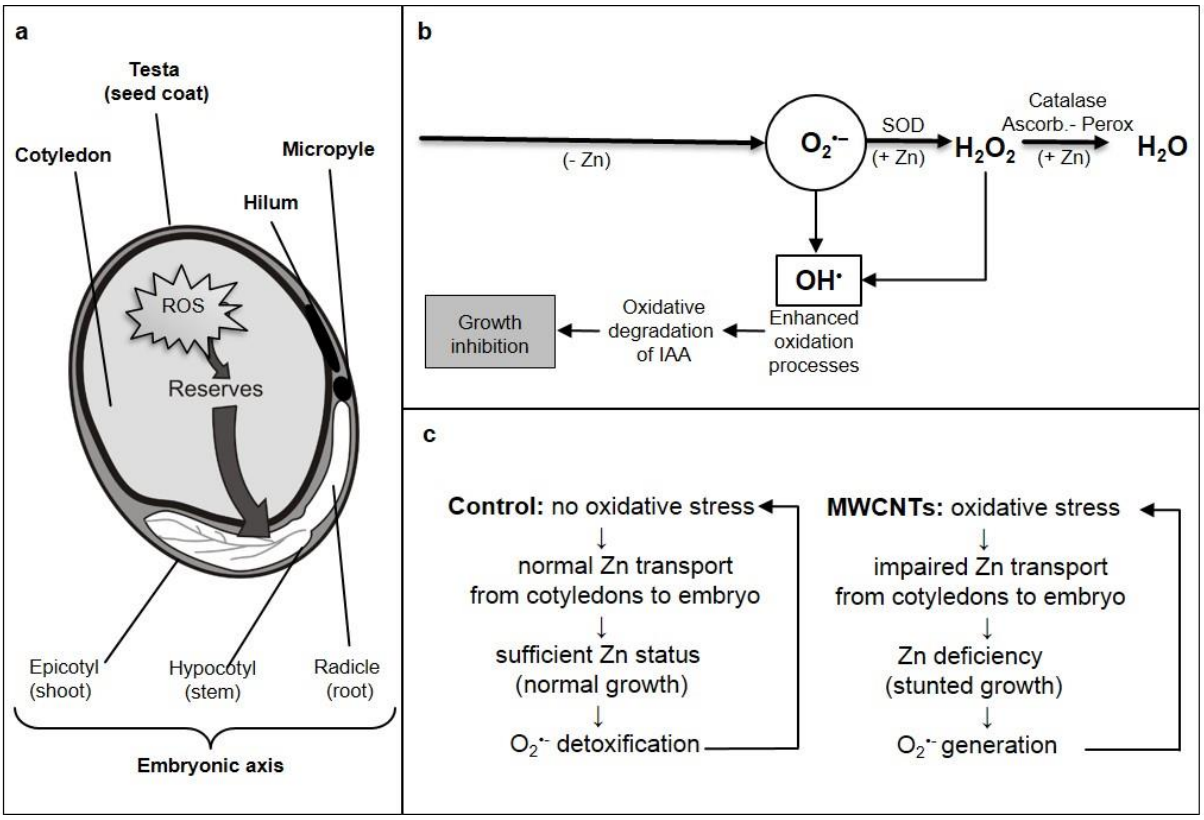

Figure 6.5. *(a) Structure of a soybean seed showing reserve mobilization during germination (adopted from [73]); (b) involvement of zinc (Zn) in the generation and detoxification of superoxide radicals and effects on IAA metabolism (adopted from [60]); (c) scheme of the proposed mechanism of soybean growth inhibition caused by MWCNTs seed treatment. Abbreviations: reactive oxygen species: $O_2^{\bullet-}$–superoxide anions, OH•−hydroxyl radical, H_2O_2−hydrogen peroxide; scavenging enzymes: SOD−superoxide dismutase, Ascorb.-Perox. −ascorbate peroxidase; IAA−indole-3-acetic acid (auxin).*

6.6 Conclusion

This study further confirmed the hypothesis of oxidative damage as a major cause for toxic effects of carbon nanomaterials on higher plants [36, 11] and provides a detailed investigation on the early metabolic events in germinating soybean seeds after exposure to MWCNTs. The expression of oxidative stress indictors, such as superoxide anion formation in the root tissue and induction of SOD could be clearly related with preferential accumulation of MWCNTs in the root tips and was reverted by external application of antioxidants.

For the first time it was shown that MWCNT-induced oxidative stress responses seem to be closely related to interferences with the micronutrient status of the plants,

particularly Zn, Mn and Cu as enzymatic co factors for antioxidative defence systems. Direct effects may comprise immobilisation of micronutrient seed reserves by adsorption to MWCNTs but also disturbances in the transport of micronutrients from the storage organs to the actively growing tissues. Moreover, also functions of MWCNTs in electron transport have been demonstrated in the recent past. While MWCNTs in chloroplasts may promote photon capture, photosynthetic electron transport and photosynthetic efficiency [71], similarly MWCNTs accumulating in other subcellular compartments may be able to generate electron transport processes driven by physiological electron donors, such as NADH. As demonstrated by *in vitro* studies [72], this can finally lead to the formation of ROS and oxidative damage of DNA and other or ganic molecules. In face of the binding potential of MWCNTs for various metals (Table 6.6) frequently acting as active centres for electron transport processes, it remains to be established whether metal-binding of MWCNTs in biological systems has an impact on MWCNT-induced ROS production. The same holds true for the presence of catalytic metal impurities originating from MWCNT production [13].

The observation, that external micronutrient application can overcome MWCNT-induced oxidative damage (Fig. 4, Table 6.5) suggests that micronutrient availability in the growth media might be a relevant factor contributing to the variability of plant responses to MWCNT treatments frequently reported in the literature [7]. Also for future MWCNT-based agricultural applications using MWCNTs e.g in plant protection or for fertilisation strategies [7], considering the micronutrient status of the target plants may be an important aspect and micronutrient supplementation could be a measure to protect MWCNT-treated plants from unintended negative side effects of MWCNTs particularly on substrates where micronutrient availability is limited e.g. by soil-chemical properties as previously demonstrated by [19] for foliar Zn application to mitigate MWCNT-induced growth inhibition of soybean grown on a calcareous soil. For testing responses to unintended soil contaminations by carbon nanomaterials additional soil experiments are necessary with lower and wider ranges of contaminant concentrations, since in this study the MWCNT treatment concentration was selected according to the positive responses of soybean germination [18].

6.7 Appendix

6.7.1 Acknowledgements

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6.7.2 Conflict of interest

The authors declare that they have no conflict of interest

6.7.3 Authors' contributions

OZ participated in designing experiments, conducted experiments and laboratory analysis, performed statistical analyses and results interpretation, drafted and participated in reviewing the manuscript. ZW was involved in RT-PCR analysis. GN designed experiments, was involved in proof reading and provided final editing of the manuscript.

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7 Chapter V. General discussion

7.1 Nanomaterials: risks and benefits

According to the Recommendation of the European Comission (2011/696/EU), **nanomaterials** are defined as ***"natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm−100 nm"*** [1]. Due to the small size and outstanding physical and chemical properties, which differ significantly from a corresponding bulk material, nanomaterials have gained an increased interest and are meanwhile produced for diverse industrial applications such as agro-chemistry [2–4], automotive [5], aviation [6], electronics [7, 8], energy [8], construction [9] and many novel applications are under development. On the European market the number of readily available consumer products (e.g. personal care, clothing, sporting goods, cosmetics etc.) containing nanomaterials is continuously growing and has reached 2231 items in 2015 [10].

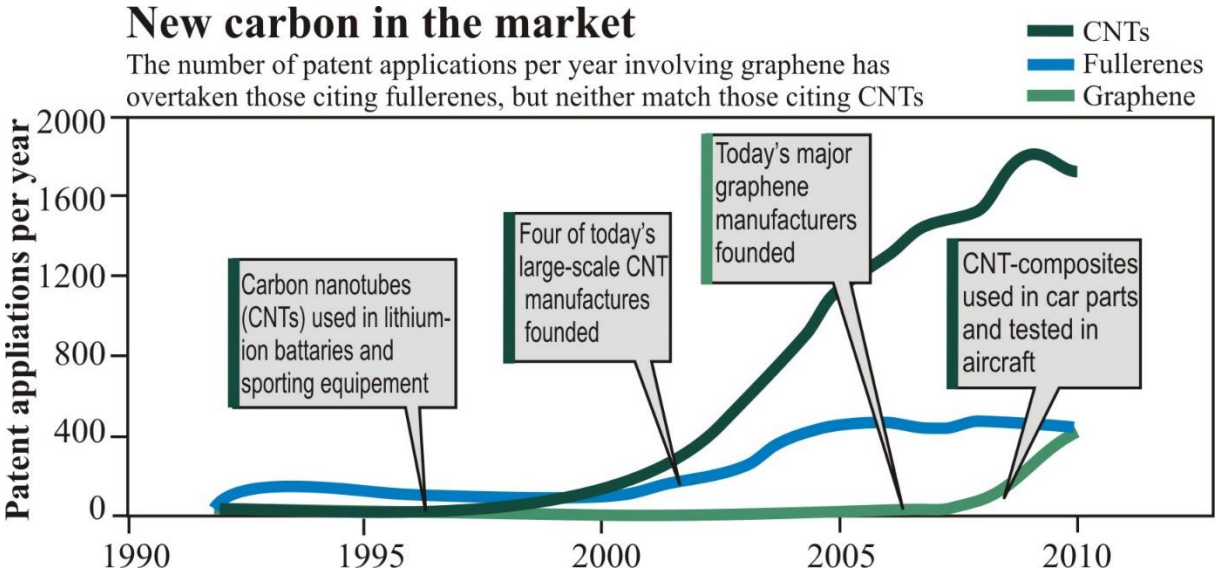


Figure. 7.1. *Amount of patent applications per year citing carbon nanotubes, fullerenes and graphene (adapted from [11]).*

Carbon nanotubes (CNTs) represent the most important class of carbon-based nanomaterials, accounting for the largest number of patent applications per year (Fig. 7.1) [11], and they belong to the top ten most produced nanomaterials [12] together with titanium (TiO_2), silicone (SiO_2), aluminium (Al_2O_3), zinc (ZnO) and ceria (CeO_2) oxides, nanoclays, silver (Ag), copper and copper oxides (Cu and CuO) and iron and iron oxides (Fe and Fe_xO_y). The continuously growing global demand for CNTs and methodological advances in their synthesis promote the production volumes, reaching 20 000 tons by 2022 according to recent forecasts (Fig. 7.2) [13]. Currently, the leading producers of CNTs are: Showa Denko (Japan), CNano Technology (USA) and Nanocyl S.A. (Belgium) [13]. Together with the rapid upscaling of CNT production, the price for nanotubes is expected to decline significantly in the near future (Fig. 7.3) [14]. The price for MWCNTs has already dropped from 45 000 to 100 \$ kg^{-1} in the past 10 years and now it is comparable with the price of fine chemicals [14]. However, when the CNTs cost will hit 10 \$ kg^{-1} and the production will reach the million ton scale it will become an affordable industrial material with advanced properties and it can be expected that CNTs will completely replace many conventional materials [14].

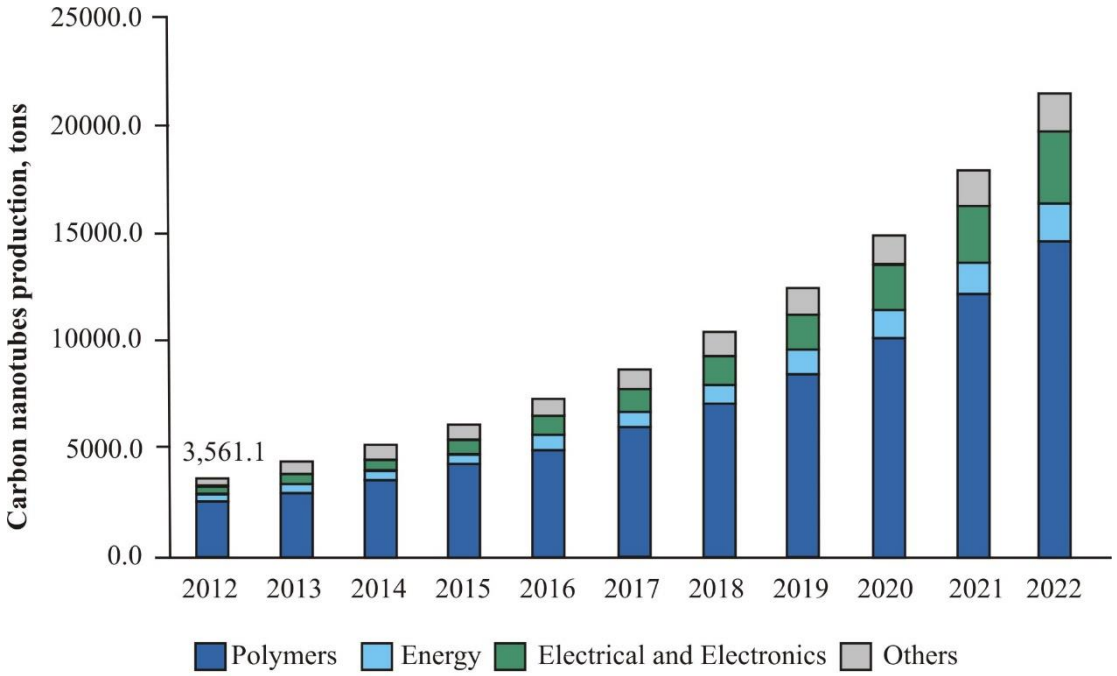


Figure. 7.2. *Global carbon nanotubes market estimates and forecast 2012–2022, tons (adapted from [13]).*

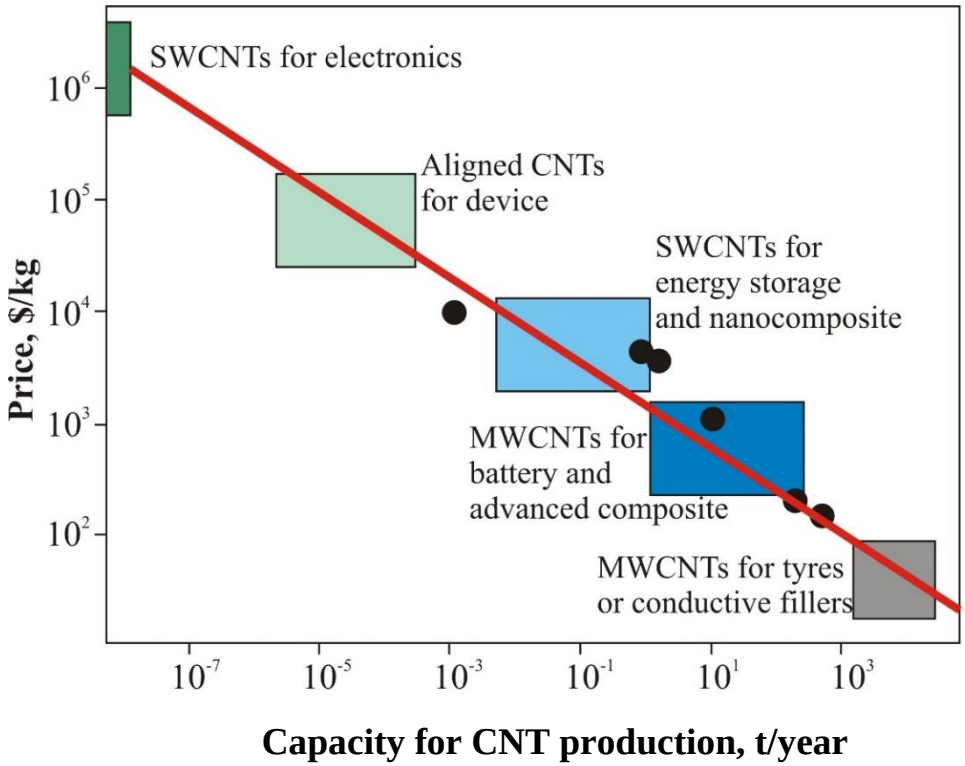


***Figure. 7.3**. Price for carbon nanotubes is declining while their production capacity increases (adapted from [14]). SWCNTs−single-walled carbon nanotubes, MWCNTs−multi-walled carbon nanotubes.*

Despite evident benefits from using nanomaterials, the increasing number of potential applications, the escalating production volumes of nanomaterials in general and of CNTs in particular, rises concerns regarding their release into the environment and potential toxicity to human health and other living organisms including plants. Potential pathways of engineered nanomaterials into the environment include release during manufacturing and raw material production, as well as release during product use and disposal (Fig. 7.4). It should be noted that accidental release of nanomaterials during their manufacturing, transportation or other procedures is also possible as a potential risk for environmental contamination.

The risk assessment of nanomaterials is determined by two components: (i) evaluation of toxicological hazard of nanomaterials and (ii) evaluation of the risk for exposure of living organisms to nanomaterials [15]. Based on the obtained results an elaboration of risk management is performed.

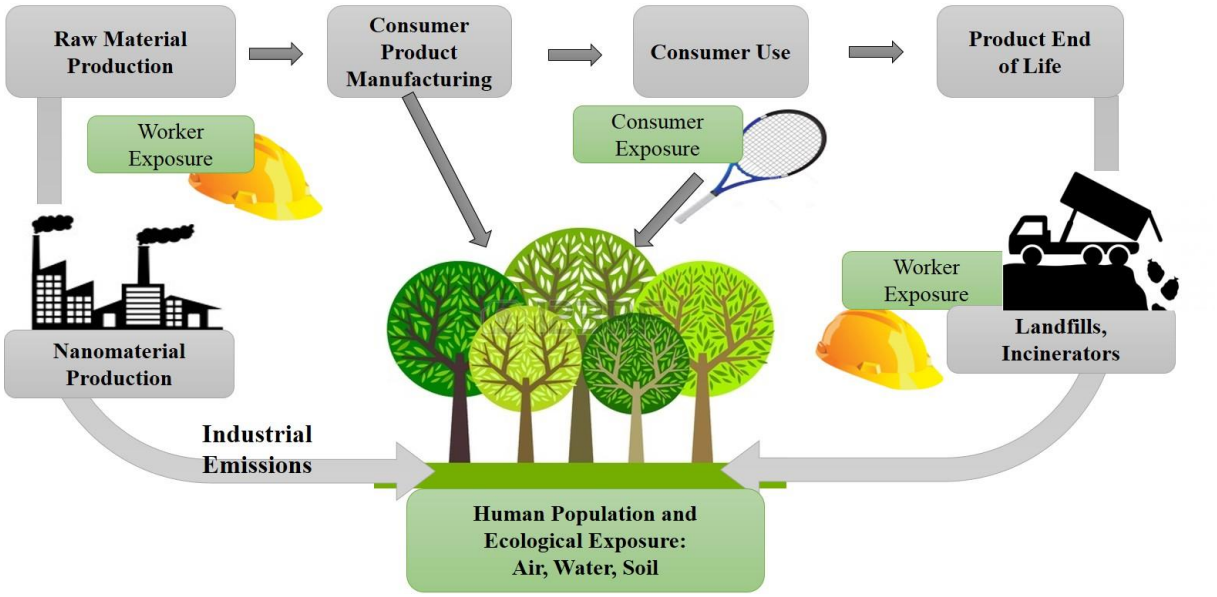


Figure. 7.4. *Scheme representing life cycle of commercial products containing nanomaterials and potential passways of nanoparticles release into the environment (compiled from [16] and [17]).*

7.2 Exposure evaluation: are plants exposed to CNTs?

For evaluation of an organisms exposure to the nanomaterials, a simple, reproducible and reliable methodology and a suitable equipment for detection of trace nanomaterials in various environmental compartments (air, water and soil) is currently lacking [18]. However, a modelling approach allows to approximate nanomaterial emissions on various stages of their life cycles and to predict at least a range of their expected concentrations in the environment. According to Sun et al. [17], the yearly increase of CNT concentrations in natural and urban soils in countries of the European Union is expected to vary between 3.7 and 7.1 ng kg^{-1} year, while agricultural sewage sludge application can increase it up to 0.76−1.6 µg kg^{-1} $year^{-1}$ (Table 7.1) [17]. Regarding global CNT flows, Keller et al. reported that in the year 2010 up to 500 metric tons of CNTs ended up in the soil (Fig. 7.5) [12].

Hence, plants grown in soil and especially agricultural crops receiving sewage sludge fertilization are expected to be exposed to continuously increasing concentrations of CNTs in the near and distant future.

Table 7.1. *Predicted carbon nanotube concentrations in different technical and environmental compartments in the European Union presented as mode (most frequent value), lower and upper percentiles ($Q_{0.15}$ and $Q_{0.85}$). STP−sewage treatment plants, WIP−waste incineration plants (adapted from [17]).*

Compartment	Mode	$Q_{0.15}$	$Q_{0.85}$	Units
STP effluent	4.0	3.6	12	ng L^{-1}
Surface water	0.23	0.17	0.35	ng L^{-1}
Sediment	0.79	0.61	1.2	µg kg^{-1} $year^{-1}$
STP sludge	0.15	0.12	0.23	mg kg^{-1}
Natural and urban soil	5.1	3.7	7.1	ng kg^{-1} $year^{-1}$
Sludge treated soil	0.99	0.76	1.6	µg kg^{-1} $year^{-1}$
Air	0.02	0.02	0.03	ng m^{-3}
Solid waste	1.7	1.3	2.6	mg kg^{-1}
WIP bottom ash	0.23	0.21	1.4	mg kg^{-1}
WIP fly ash	0.36	0.33	2.9	mg kg^{-1}

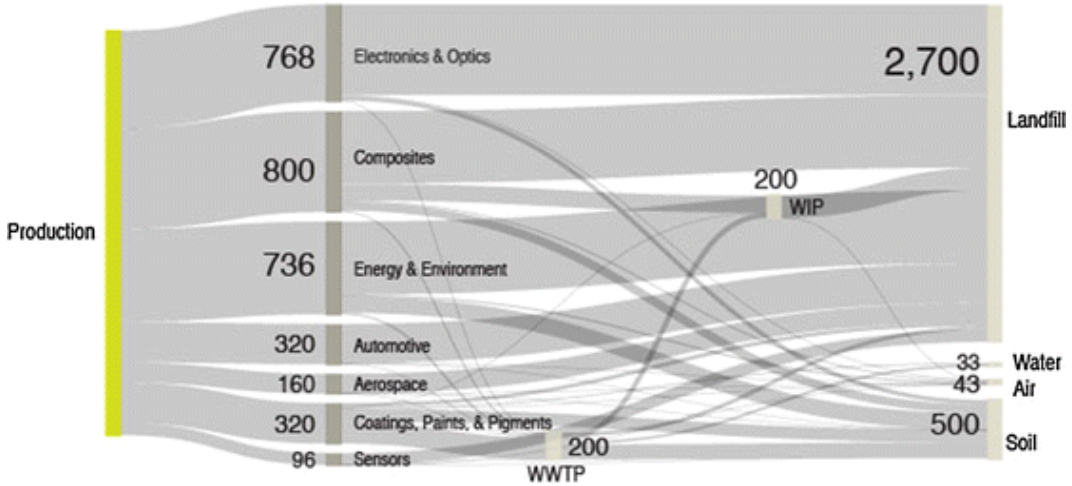


Figure. 7.5. *Global material flow for carbon nanotubes (metric tons $year^{-1}$) in 2010, assuming maximum production and emissions rates. WIP−waste incineration plants, WWTP−wastewater treatment plants (Source: [12]).*

Beside numerous industrial applications, CNTs can be also employed in agriculture for: (i) increasing crop yield (as components of plant growth stimulators, fertilizers and soil improvers), (ii) improvement of plant protection (as components of pesticides, herbicides and insecticide formulations), (iii) reduction of applied agrochemicals (using nanoencapsulation for slow-release fertilizers and triggered-release capsules) and (iv) optimization of agricultural practices by introducing precision farming and environmental sensing (soil conditions, crop diagnostics, contaminant concentrations) (Fig. 2.3, General introduction). Currently, numerous patents for agricultural products containing carbon nanomaterials are under development (Table S2.1, General introduction) and some commercial products are readily available on the market (e.g "Nano Carbon Powder, Fertilizer Synergist" and "Agriculture Grade Crop Fertilizer Synergist Nano Carbon Sol." from Qingdao Reach International Inc., China [19, 20]). Therefore, in case of an intentional application of carbon nanomaterial-containing agrochemical products, it is expected that high concentrations of those materials can be introduced into fields.

Taking into account the potential release pathways for carbon-based nanomaterials into the environment, two exposure scenarios for crops can be proposed:

(i) **non-targeted soil contamination**:

a) background contamination of soils: continuously increasing but with comparatively low nanomaterial concentrations;
b) accidental contamination occurring only occasionally but with locally high contamination potential;

(ii) **targeted contamination** in form of CNM-based agricultural applications with high contamination risk of target plants.

7.3 Complications for CNT risk assessment

7.3.1 Tracing and quantification of uptake

Toxicity assessment of nanomaterials is generally complicated by various methodological limitations. One of those problems is the reliable quantification of CNMs taken up by plant cells. A possible quantification method for CNTs is the use of radioactive tracers [21, 22], which have to be introduced into the structure of nanoparticles during their synthesis. This implicates that tracer production must be performed by the manufacturers. In face of the continuously increasing number of CNTs with different structural features and properties it is obvious that radioactive tracing studies are not widely performed at the moment, due to the lack of adequate tracers. Therefore, tracing techniques, which can be performed independently of CNT synthesis are urgently needed.

7.3.2 Heterogenity of nanomaterials

Apart from difficulties related with tracing and quantification of nanomaterials, the high variability of CNT types and properties is associated with additional problems for risk assessment: depending on the applied methods of synthesis (chemical vapor deposition, arc discharge or laser ablation (see general introduction), synthesis conditions (temperature, pressure, nature and size of catalysts, type of hydrocarbon gases) and subsequent processing (purification, functionalization, alignment), not only physicochemical properties but also potential toxicity of yielded nanomaterials can be highly variable. For instance, CNTs purchased from different companies can differ in size (outer and inner diameter, length, number of walls, surface area), functionalization (e.g. carboxylated, oxidized), purity (present of catalyst traces) and other features. Moreover, the properties of CNTs can be changed under different environmental conditions (agglomeration or functionalization by binding metals or other molecules). These modifications can influence the outcomes of toxicity studies carried out with various nanomaterials (Fig. 7.6), leading to the highly variable results frequently reported in the literature. Therefore, a thorough physicochemical characterisation of

nanomaterials is recommended [15]. However, meanwhile it is practically impossible to study toxicity of all existing types of nanomaterials and therefore, it was suggested to group classes of nanomaterials with similar physicochemical features, and to perform toxicity tests for representative selections of nanomaterials out of these classes [15].

7.3.3 Culture conditions for toxicity tests

Variability of culture conditions for the selected test plants is another widespread problem for phytotoxicity assessment of carbon nanomaterials. For instance, in various studies germination tests were performed on filter papers using CNT suspensions in deionized water [23, 24], agar [25] or media for plant tissue culture (e.g. Murashige-Skoog medium) [26, 27], while cultivation of plants was conducted in hydroponics [28], on artificial culture substrates [29] or natural soils [30]. However, different chemical composition, nutrient availability, substrate pH etc. of different culture media can impact on plant performance independent of the presence of nanomaterials and can affect plant resistance to phytotoxicity, as demonstrated in the present thesis e.g for Zn availibility, determining plant tolerance to toxicity of CNTs (see Chapter III).

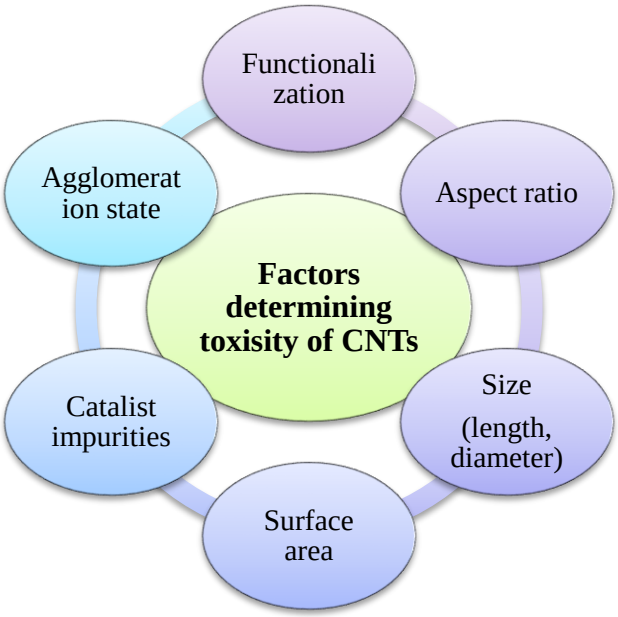


Figure. 7.6. *Factors determining toxicity of CNTs (adapted from [31]).*

7.3.4 Variability of test plants

Apart from external factors described above, also variability of the test plants themselves can strongly affect plant responses to nanomaterials (positive, negative or no effect at all). This has been demonstrated also in the present study during germination tests conducted under standardized conditions with six different plant species (soybean *(Glycine max* (L.) Merr), maize (*Zea mays* L.), wheat (*Triticum aestivum* L.), common bean (*Phaseolus vulgaris* L.), barley (*Hordeum vulgare* L.), esparcette (*Onobrychis arenaria* Mill.)) exposed to the same type and concentrations of MWCNTs. A significant stimulation of germination was observed in soybean with a similar trend also for esparcette (Fig. 7.7), which was obviously rather related to differences in seed quality than to genotypic factors, since beneficial effects of MWCNTs were only observed when the germination rate of the untreated control was low. By contrast, during further seedlings development in soil culture growth depressions and rootlength reduction was recorded in soybean, common bean and maize after 36 h seed exposure to MWCNT suspensions (1000 mg L^{-1}) (Fig. 7.8, 7.9). In this context soybean was identified as a very suitable model system since it showed both, positive and negative responses to the same application dose and type of MWCNTs in the same individual plant.

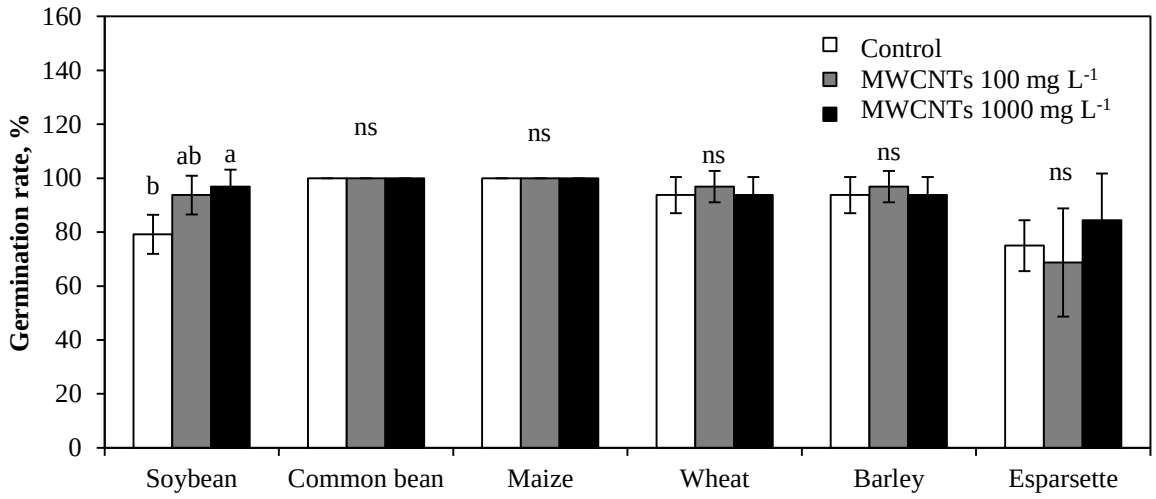


Figure. 7.7. *Effects of MWCNT treatments (100 and 1000 mg L^{-1}) on the germination rate of soybean, common bean, maize, wheat, barley and esparsette seeds. Control: deionized water.*

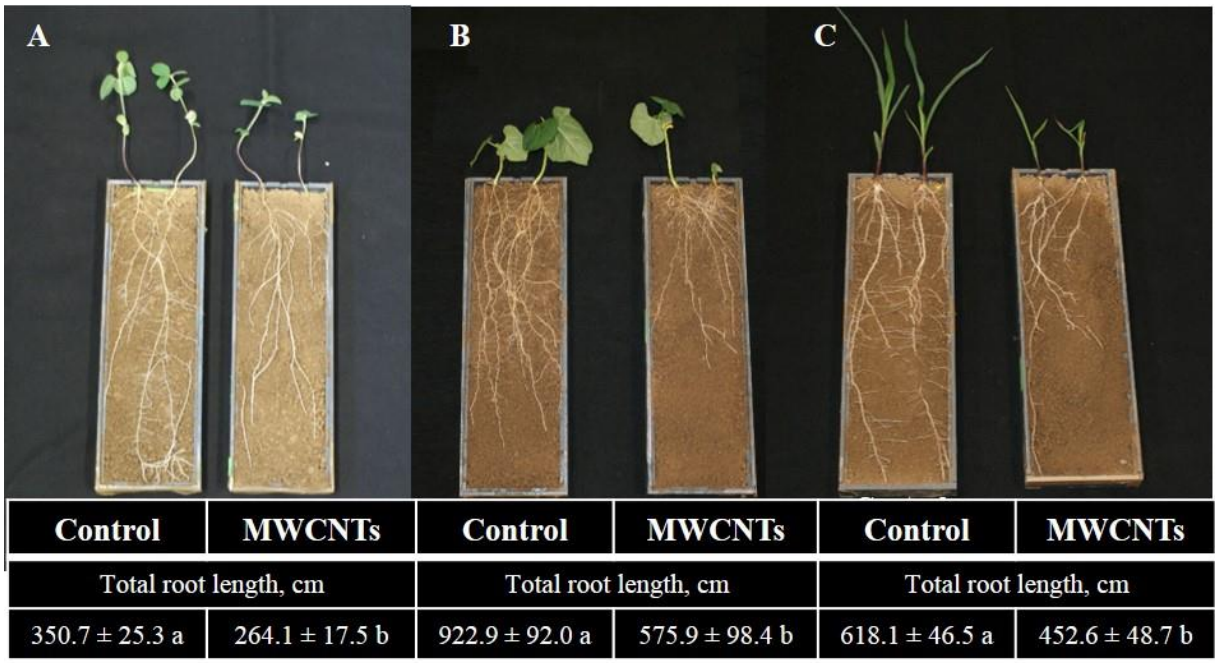
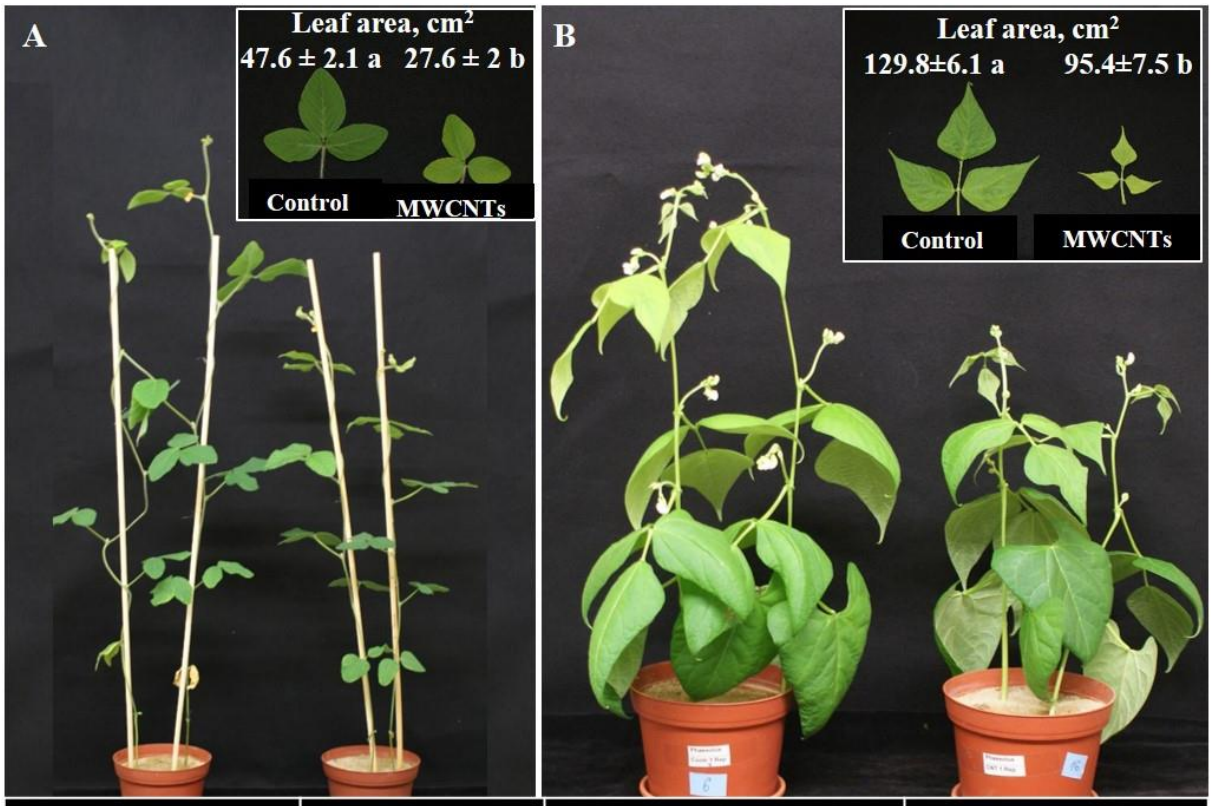


Figure. 7.8. *Habitus and total root length (cm) of (A) soybean (15 DAS), (B) common bean (17 DAS) and (C) maize(14 DAS) seedlings, developed from seeds treated with 1000 mg L^{-1} MWCNTs and deionized water (control) for 36 h, pre-germinated in filter paper rolls with deionized water and subsequently grown in soil culture (clay loam field soil, pH 7.1) in rhizoboxes. Different letters (a, b) indicate significant differences between treatments (t-Student test, P≤0.05).*



Control	MWCNTs	Control	MWCNTs
Total root length, cm (23 DAS)		Total root length, cm (23 DAS)	
915.3 ± 123.3 a	303.1 ± 48.8 b	3229.2 ± 225.3 a	1863.5 ± 153.3 b
Total root length, cm (38 DAS)		Total root length, cm (38 DAS)	
3246.5 ± 195.1 ns	2375.4 ± 445.0 ns	4770.6 ± 232.7 a	3393.9 ± 121.5 b

Figure. 7.9. *Habitus, total root length (cm) and area of the first fully developed leaf (cm^2) of (A) soybean and (B) common bean seedlings, developed from seeds treated with 1000 mg L^{-1} MWCNTs and deionized water (control) for 36 h and grown in soil culture (loess subsoil, pH 7.5) in pots. Different letters (a, b) indicate significant differences between treatments (t-Student test, P≤0.05), ns−not significant.*

7.4 Positive effects of CNTs

7.4.1 Improved germination by CNT-induced stimulation of water uptake

In the present study, an application of MWCNTs significantly improved germination rate and the development of normal seedlings in soybean (Chapter II). Previous studies claimed that the stimulatory effects of CNTs on seed germination are related to enhanced water uptake, facilitated by CNT-induced seed coat perforation [27] and activation of water channel proteins (aquaporins) [26].

7.4.2 Improved germination by CNT-induced reduction in the speed of seed imbibition

The results of the present study suggest an alternative mechanism: the improved germination rate of soybean seeds exposed to MWCNTs can be explained by seed dressing effects of nanotubes, acting as a barrier for a rapid water uptake and thus minimizing imbibition injuries and preventing seed leakage.

Water uptake by a dry seed is the very first and a critical phase of seed germination. Under natural conditions, the major factor controlling water uptake driven by diffusion is the seed coat permeability but artificial control of water uptake can be also achieved e. g. by seed dressing. For instance, hydrophobic protein seed coatings have shown to counteract an excessive speed of water uptake by sugar beet (*Beta vulgaris* L.) and broccoli (*Brassica oleracea var. italica* Plenck) seeds [32]. In the present study, the application of MWCNTs exhibited seed dressing effects due to the hydrophobicity of the applied nanomaterial. MWCNTs adhered to the hydrophobic seed surface, forming a barrier for rapid seed water uptake, and the reduced speed of water uptake in MWCNT variants was comparable to slow imbibition kinetics of seeds germinating between layers of moist paper, associated with improved germination (Chapter II).

Large-seeded leguminous plants seem to be particularly sensitive to imbibition damage induced by rapid water uptake, particularly in cases where seed vigour is already affected by other factors, such as seeds aging. In these cases, the rapid seed water uptake causes cell rupture, membrane damage and solute leakage, finally resulting in low seed

germination [33, 34]. Interestingly, the analysis of seed reserve mobilization in germinating soybean seeds has shown a trend for greater leakage of micronutrients in the untreated controls as compared to the MWCNT treatment (Chapter IV), further pointing to a protective role of MWCNTs against cell rupture due to rapid imbibition.

7.4.3 Reactive oxygen species as stimulators for germination

At low quantities, also formation of ROS can improve soybean germination, while their complete absence even results in no germination [35]. Reactive oxygen species play an important signalling role and contribute to germination processes by cleaving cell walls of the endosperm, oxidation of seed germination inhibitors, hormone signalling and release of seed dormancy [36–38]. In the present study, analysis of imbibing soybean seeds revealed enhanced accumulation of superoxide radicals in the radicles MWCNT-treated variants (Chapter IV). Therefore, the ROS formation induced by the MWCNT treatment may exert a stimulatory effect on germination at least in the early phase of MWCNT exposure, but later turn into toxic effects when intracellular MWCNT accumulation and the associated ROS production increases.

7.4.4 Germination inhibitors and allelopathic compounds

Various plant species including soybean have evolved allelopathy [39], a chemical defence mechanism, characterised by release of germination inhibitors, such as coumarin, ferulic acid and naringenin [40] during seed imbibition, which inhibits seed germination of competing plant species. In soybean mono-cultures, even auto-allelopathic effects have been observed [41].

Due to their large surface area and high affinity to hydrophobic organic compounds (see general introduction), MWCNTs may also play a role in immobilisation of allelopathic compounds thus supporting germination of neighbouring seeds. An approach to stimulate seed germination by adsorption of germination inhibitors in growth media using activated charcoal has been previously reported by Prati and Bossdorf [42], for mitigation of an allelopathic inhibition by root exudates of garlic mustard (*Alliaria petiolate* M.Bieb.) on germination of rough avens (*Geum laciniatum* Murray) Also,

phytotoxins in extracts of *Calluna vulgaris* (L.) Hull and blueberry (*Vaccinium myrtillus* L.) leaves could be absorbed by natural charcoal or activated carbon thereby improving the germination of pine seeds, which was significantly suppressed in absence of the adsorbents [43]. Multi-walled carbon nanotubes added to a sediment contaminated with organic pollutants, neutralized toxic effects, improved germination and recovered root growth of cress (*Lepidium sativum* L.) cultivated on that sediment [44]. Whether similar processes were also involved in the stimulatory effects on soybean germination observed in this thesis, remains to be established.

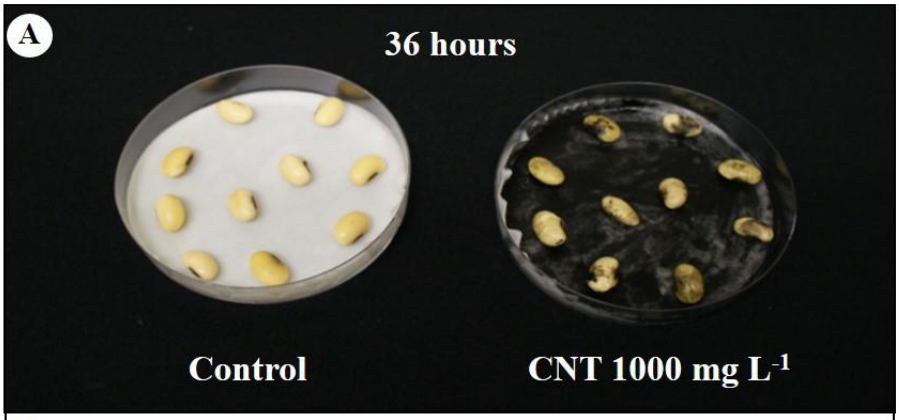
7.5 Negative effects of CNTs

7.5.1 Toxicity potential of MWCNTs

A major component of nanomaterial risk assessment is an evaluation of potential hazards (7.1). Accordingly, the main objective of the present thesis was an investigation on the toxicity potential of selected industrial multi-walled carbon nanotubes (MWCNTs) to various crops under standardised growth conditions. Finally, soybean (*Glycine max* L. Merr.) was selected as model plant with the highest responsiveness to MWCNT treatments. After short-term (36 h) seed exposure to a MWCNT suspension (1000 mg L^{-1}) during seed imbibition prior to radicle emergence, distinct growth inhibition, affecting shoot growth and particularly root development, was detected during early seedling growth already at 8 DAS, and in soil culture, this translated into long-lasting inhibitory effects on plant growth in terms of reduced total root length detectable even seven weeks after the 36 h MWCNT treatment. The relatively high MWCNT concentrations (100−1000 mg L^{-1}) in these experiments may be mainly expected in scenarios of targeted contamination related with specific agricultural MWCNT applications. However, calculating the real MWCNT dosage applied per seed (50−500 µg $seed^{-1}$) and taking into account that the majority (≈90%) of the applied nanomaterial remained sticking to the germination paper (Fig. 7.10 A), the real amount of MWCNTs with direct seed contact may be approximated with 5−50 µg $seed^{-1}$. However, as shown in Fig. 7.10 A, the majority even out of this MWCNT fraction is sticking to the seed coat. Therefore, it may be expected that the amount of MWCNTs

really taken up into the seeds is lower than 5 µg seed^{-1} (Fig 7.10 B). In this case **the toxicity of MWCNTs would be comparable with the phytotoxicity of glyphosate as one of the most effective systemic herbicides,** which causes growth depressions in maize seedlings after uptake >1 µg seedling^{-1} [45].

The direct uptake of MWCNTs into germinating soybean seeds even prior to radicle emergence was demonstrated by light microscopic examination of microtome cuttings and revealed a massive contamination particularly of the young actively growing cells in the tip of the radicle (Fig. 7.11). Interferences with the metabolism of root meristematic cells may explain the rapid inhibitory effects particularly on root growth. The preferential localisation of MWCNT contamination in the tip of the radicle, points to a role of the micropyle located close to the radicle as a major entry point for MWCNT water suspensions.



B

- **Experimental CNT dosage:** 100, 1000 mg L^{-1}
  5 mL/10 seeds = **50 – 500 µg seed $^{-1}$**
- **However more than 90 % of applied CNTs sticking to the germination paper**
  ⇨ **Real dosage: 5 – 50 µg seed^{-1}**
- **Out of this fraction the majority of CNTs sticks to seed coat**
- **Conclusion: Uptake of CNTs propably lower than 5 µg seed $^{-1}$**

Figure. 7.10. *(A) Soybean seed treatment in Petri dishes with and without CNTs suspension and (B) Relationship between applied CNT concentrations and estimates of CNTs finally taken up by the seeds.*

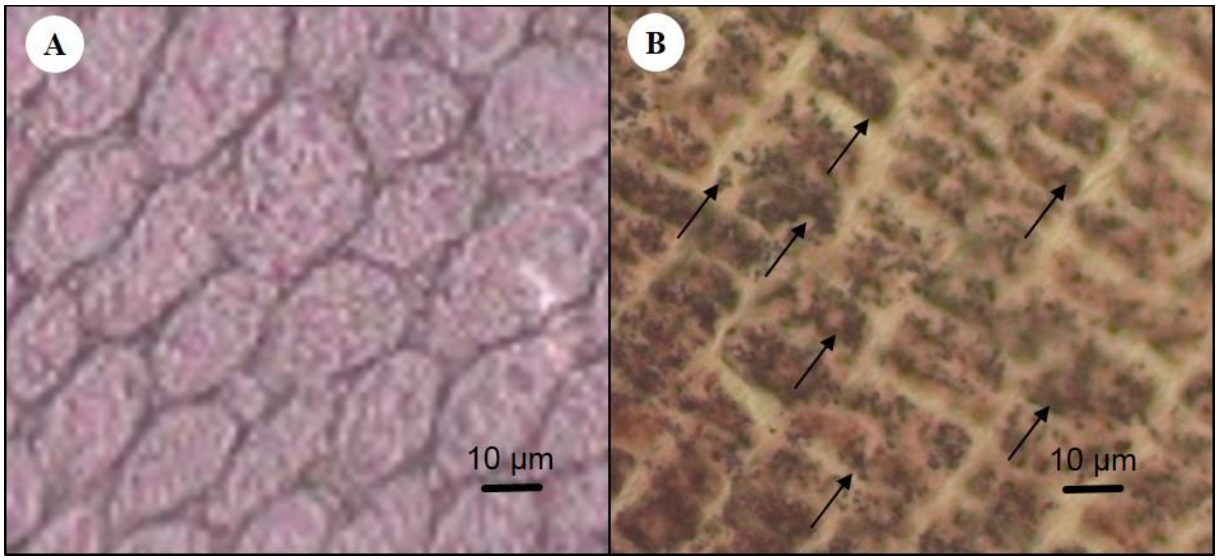


Figure. 7.11. *Light-microscopic examination of microtome cuttings obtained from radicles of soybean seeds imbibed for 36 h in deionized water (control; A) or in MWCNT suspension (1000 mg L^{-1}; B). Arrows−MWCNT agglomerates.*

7.5.2 Inhibition of root growth

The present study demonstrated that the major effect of short-term (36 h) soybean seed treatment with MWCNTs was a subsequent reduction of total root length. The earliest evidences of these effects were detected already at 8 DAS (Fig. 7.12) in seedlings grown in filter paper rolls wetted with deionized water (Chapter II). Despite the comparatively short exposure time of only 36 h during seed imbibition, root growth inhibition was a long-lasting effect, and was repeatedly detected in different experiments: at 10 DAS in seedlings grown in filter paper rolls wetted with DI water, at 15 DAS of seedling growth in silty loam soil in rhizoboxes and at 38 DAS of seedling growth in a calcareous loess subsoil in pots (Fig. 7.8, 7.9). Analysis of root morphology revealed that particularly fine root fraction (0−0.2 mm in diameter) was affected, leading to an increase in average root diameter. Particularly the fine root fraction plays an important role for spatial acquisition of water and nutrients but also for the efficient chemical modification of the rhizosphere towards increased nutrient solubility and in the interaction with beneficial microorganisms [46].

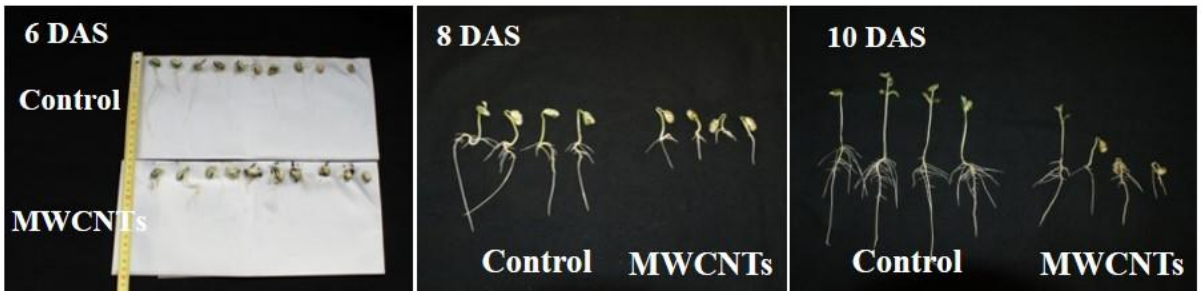


Figure. 7.12. *Habitus of soybean seedlings (6, 8 and 10 DAS) developed from seeds treated for 36 h with 1000 mg L^{-1} MWCNTs and deionized water (control) and grown in filter paper rolls wetted with deionized water.*

7.5.3 Generation of oxidative stress

Many previous studies have reported the ability of carbon nanomaterials in general and particularly of MWCNTs to induce formation of ROS, detected by *in vitro* experiments

with animals [47] and plant cell cultures [48] as well as *in vivo* in studies with higher plants [28]. Accordingly, also the present thesis revealed an enhanced accumulation of superoxide radicals in radicles of soybean seeds exposed to MWCNTs (Chapter IV). Histochemical staining of MWCNT treated soybean seeds with 2, 3, 5-triphenyl tetrazolium chloride (TTC) demonstrated the presence of unstained areas in the embryo axis of germinating seeds, which indicates a low metabolic activity in those cells, most likely due to cell damages caused by escalated superoxide accumulation. The microscopic observations of damaged embryonic tissues were confirmed by quantitative photometric determination of triphenylformazan (TF), the product of TTC reduction by various dehydrogenases as a general indicator for metabolic activity (Chapter IV). A significantly increased activity of SOD in radicles of MWCNT-treated seeds, confirmed an enhanced level of oxidative stress and the induction of detoxification mechanisms, which could be maintained during several days but significantly declined between 4 and 10 days of the germination period, associated with inhibited root growth. The finding that the detrimental effects of MWCNT seed treatments could be reverted by supplementation with antioxidants and micronutrients, such as Zn, Mn and Cu demonstrates that (i) induction of oxidative stress is a major primary cause of MWCNT toxicity and (ii) the availability of micronutrients is an important determinant for the expression of phytotoxicity by MWCNT-induced oxidative stress.

7.5.3.1. MWCNT–induced disturbance of micronutrient homeostasis

Initial seed nutrient reserves are extremely important for early seedling development and crop establishment, and limitation of seed reserves negatively affects plant growth [49]. Soybean seeds are rich in nutrients such as soluble proteins, starch, amino acids and soluble sugars, and minerals stored mainly in the cotyledons. They can supply developing seedling with nutrients up to ten days after emergence until the root system is developed and independent nutrient acquisition is established [50, 51]. During that period cotyledons lose up to 60−70 % of their weight [50, 51]. Since cotyledons are the major storage organs in soybean seeds (non-endosperm seeds), the loss of one or both cotyledons, physical damage, such as cracking as well as various abiotic stress factors, including chilling, metal toxicity [52] or seed aging can cause disorders in seed reserve

mobilization with negative affects not only on early seedling development but even on final grain yield [53]. Accordingly, artificial enrichment of seed nutrient contents via seed nutrient priming can be a measure to improve seedling development, environmental stress resistance and final yield [54, 55].

In the present study, analysis of nutrient distribution in MWCNT-treated soybean seeds revealed a reduced translocation particularly of Zn from the cotyledons to the remaining seedling between 4 and 10 DAS, with similar trends also for Cu and Mn. This was associated with reduced activity of SOD and inhibition of root growth, which could be reverted by external Zn application. No significant differences were recorded for the micronutrient contents of the total seedlings (including cotyledons) at 10 DAS in the MWCNT-treated variant and the untreated control, suggesting no differences in passive leaching of micronutrients. This indicates that impaired translocation of micronutrient seed reserves from the cotyledons to young actively growing tissues of the seedling represents an early stress response to MWCNT exposure finally limiting seedling growth.

Moreover, *in vitr*o studies demonstrated the capacity of the selected MWCNTs to bind metals such as Zn and Cu applied in a nutrient solution for plant culture (Table 6.7). The ability of CNTs to adsorb pollutants, including Zn has been similarly described in the literature [56, 57] as a feature which can be employed for development of various purification techniques. Moreover, MWCNTs applied during seed imbibition accumulated in high amounts in the young growing cells of the radicle tip (Fig. 7.11). This raises the question, whether intracellular micronutrient immobilisation by MWCNTs was an additional factor limiting Zn availability for the young growing seedling. This aspect requires further investigations *in vivo*. It has been shown that limited micronutrient supply can lead to various disorders such as inhibition of chloroplast development (Fe), inhibition of root growth (Fe), stunting of plants (Cu, B), delayed maturity (Mn, Zn), decreased leaf size (Zn) [58]. Micronutrients are essential for plant growth and development as they play an important role in numerous physiological processes, such as activation of enzymes, maintenance of membrane and cell wall integrity, protein synthesis, redox reactions, and multiple functions in

detoxification of ROS [58]. The various superoxide dismutases (SODs) as key enzymes for ROS detoxification are depending on Cu, Mn, Fe and particularly Zn as metal cofactors and SOD activity has been even claimed as a more reliable indicator for the Zn nutritional status than the plant tissue concentrations of Zn [59]. The MWCNT-induced changes in SOD activity (Table 6.5) and the restoration by external Zn supplementation associated with recovery from MWCNT-induced root growth inhibition (Table 6.5) clearly suggest micronutrient limitation of SOD in the MWCNT-treated variants as a cause for oxidative damage leading to inhibition of root growth.

However, apart from a relationship with SOD, a range of additional interactions of micronutrients with ROS formation may be also involved in the MWCNT-mediated induction of oxidative damage: (i) zinc limitation can promote also Fe-induced formation of free radicals [59]; (ii) the formation of ROS, as a side activity of various NADPH oxidases in different cell compartments, is increased by limited Zn availability [59]; (iii) micronutrients, such as Mn and Cu are playing an important role as co-factors for synthesis of phenolics (Fig. 7.13) [60] and many phenolics exhibit antioxidant properties [61]. The potential contribution of these factors and their relative importance for the expression of oxidative plant damage by MWCNT-induced disturbances in micronutrient homeostasis still requires more detailed investigations.

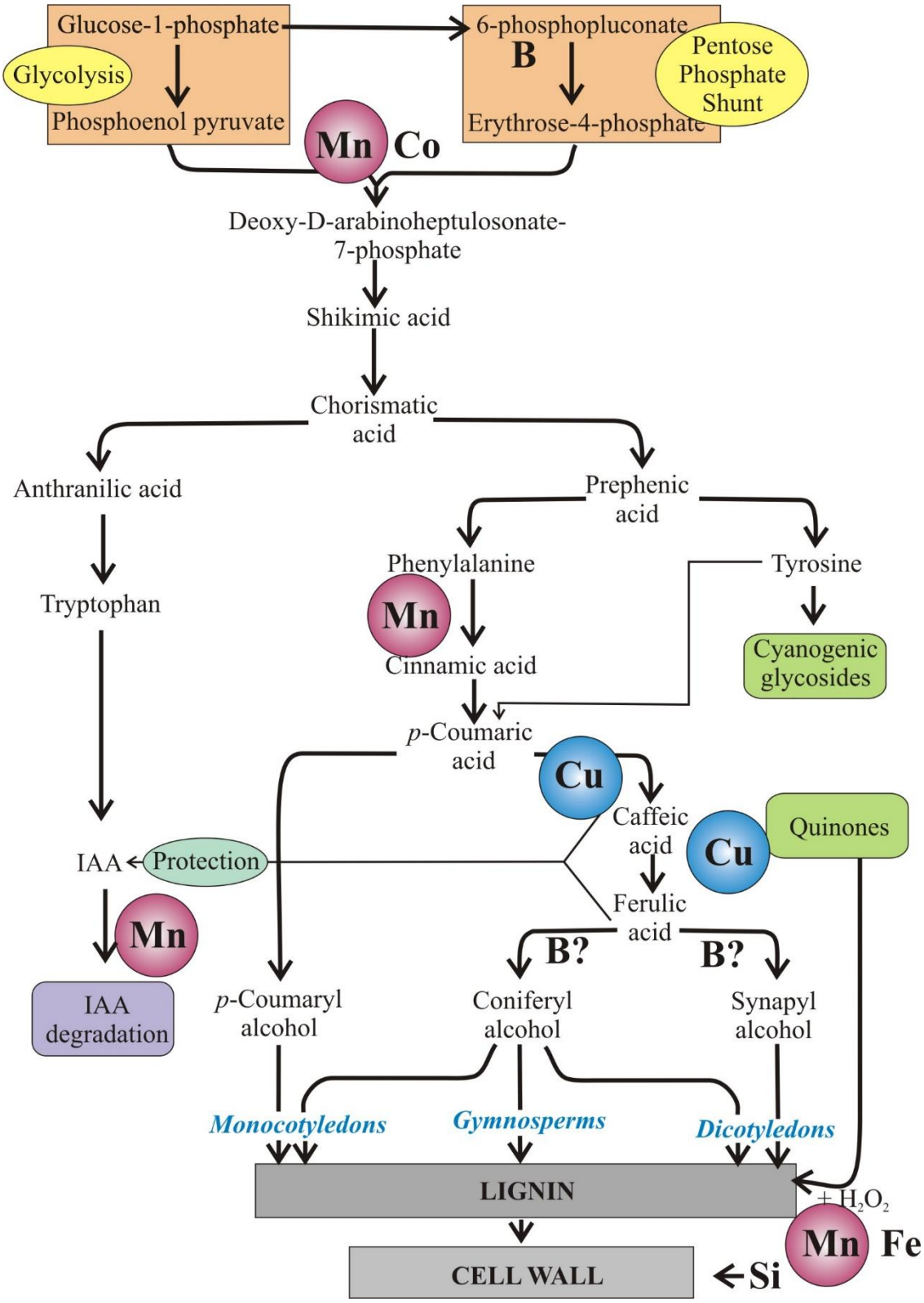


Fig. 7.13. *Micronutrients as enzymatic co-factors for the biosynthesis of phenolics (Source: [60]).*

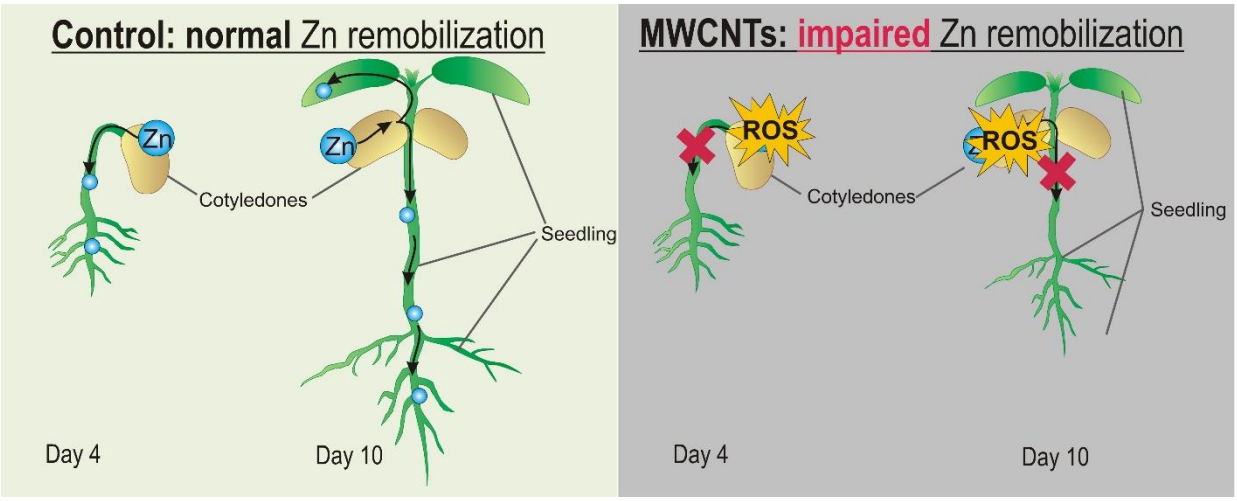


Figure. 7.14. *Schematic representation of MWCNTs-induced Zn reserve mobilization disorder in germinating soybean seeds.*

7.5.3.2 Direct formation of ROS by MWCNTs

Apart from indirect effects of carbon nanomaterials on ROS formation described above (e.g. by interactions with detoxification mechanisms) there is also evidence for a direct involvement in ROS production as components involved in electron transfer.

The ability of CNTs to generate ROS in aqueous environments [62, 63] was even suggested as a major mechanism of their toxicity [28]. Carboxylated CNTs can take part in electron transfer from biological electron donors to molecular oxygen and thus produce harmful ROS causing injury to essential biomolecules (Fig. 7.15) [63]. Additionally, small size and a specific shape of CNTs have been identified as important structural features determining their efficiency in ROS production and induction of oxidative bursts similar to the hypersensitive response towards pathogen attack is possible [48].

In this context, the metal-binding properties of the investigated MWCNTs (Table 6.7, Fig. 7.16) are of particular interest, since binding of metals e.g in metallo-proteins plays an important role also in natural electron transport chains. This raises the question, whether binding of metals to MWCMTS taken up into plant cells (Fig. 7.11) represents a functionalization which may enhance the electron-transfer capacity (Fig. 7.15) and lead to a intensified generation of ROS, associated with stronger expression of toxic effects?

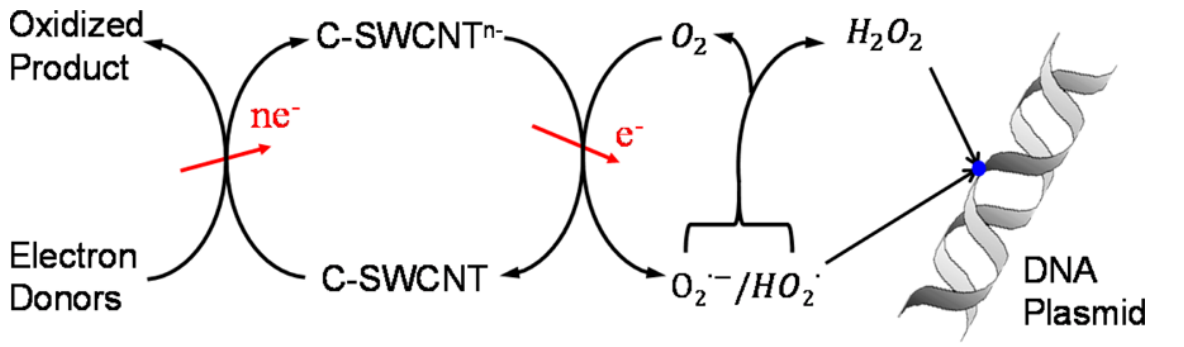


Fig. 7.15. *Scheme illustrating a potential mechanism of electron transport by carboxylated single-walled carbon nanotubes, production of ROS and cleavage of DNA plasmid (Source: [63]).*

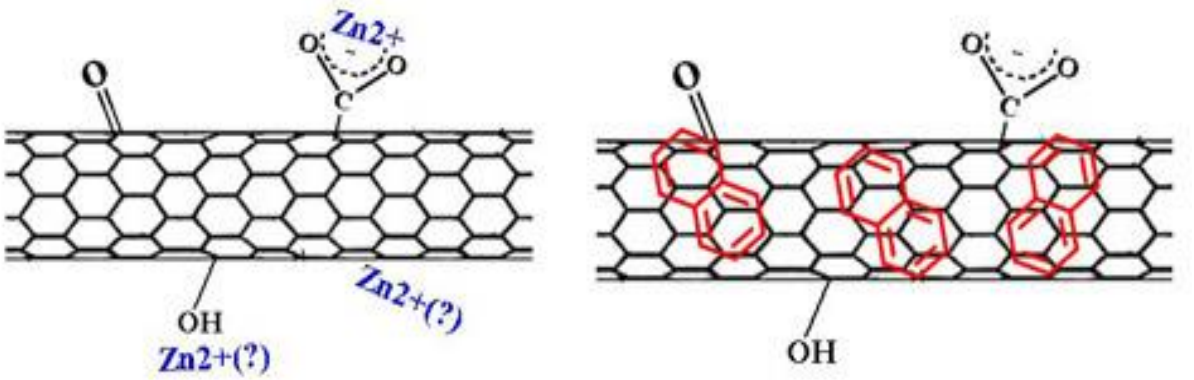


Fig. 7.16. *Scheme illustrating adsorption of organic and inorganic toxins by oxidased carbon nanotubes (Source: [64]).*

This scenario was supported by the observation that external micronutrient supply could supress oxidative damage and inhibition of root growth in germinated seedlings (4-10 DAS) or during later plant development in hydroponics or soil culture. However, when e.g. zinc was supplied simultaneously with the MWCNTs during seed imbibition, the protective micronutrient effects completely disappeared and even turned into stronger plant damage as induced by sole application of MWCNTs without micronutrients (Fig. 7.17). This aspect requires further examination e.g. by simultaneous application of antioxidants and determination of ROS production.

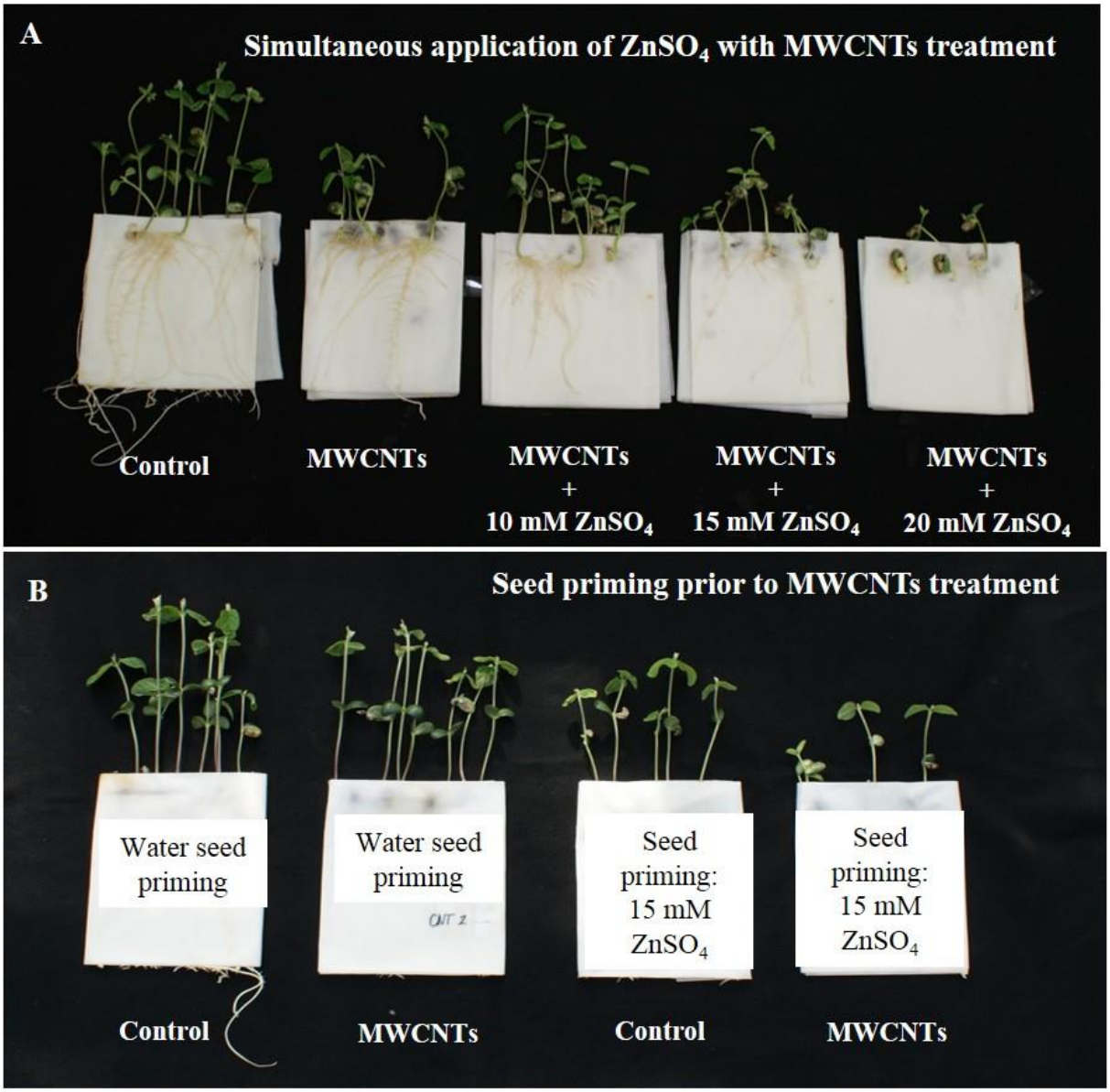


Figure 7.17. *Effects of combined seed treatments with MWCNTs and $ZnSO_4$ on seedling growth in filter rolls moistened with deionized water. (A) Effects of a short-term (36 h) simultaneous application of MWCNTs (1000 mg L^{-1}) and 10, 15 or 20 mM $ZnSO_4$ on further seedling growth (10 DAS). In the Control variant seeds were treated only with deionized water. (B) Seeds were primed during 12 h in deionized water or in 15 mM $ZnSO_4$, dried back to initial weight during 48 h at room temperature, then treated with MWCNTs (1000 mg L^{-1}; 36 h) or deionized water (Control) (10 DAS).*

7.5.4 Importance of nutrient availability for MWCNT toxicity

A major finding of the present study was the observation that the **expression of MWCNT-toxicity was highly dependent on the nutrient availability of the growth substrates with Zn but also Mn and Cu as major limiting factors, as demonstrated by re-supplementation experiments** (Chapters III, IV). This may offer one explanation for the highly variable plant responses observed in many previous experiments on plant exposure to nanomaterials [2] using a range of different growth substrates with and without additional nutrient supply. However, although internal nutrient limitation seems to be an early response to MWCNT treatments, detectable by re-supplementation experiments already between 4-10 DAS (Table 6.6), the primary cause for the MWCNT-induced disturbance of micronutrient homeostasis is currently still unknown but may be related to the potential of MWCNTs to immobilize micronutrients (Table 6.7) and/or to produce ROS by mediation of electron transport processes (Fig 7.15). The mitigation of plant growth inhibition by later applications of micronutrients (2-3 weeks after MWCNT seed treatments) e.g in hydroponic culture (Fig. 5.3) or by foliar application in soil culture (Fig. 7.18) are obviously secondary effects, mitigating the MWCNT-induced inhibition of root growth.

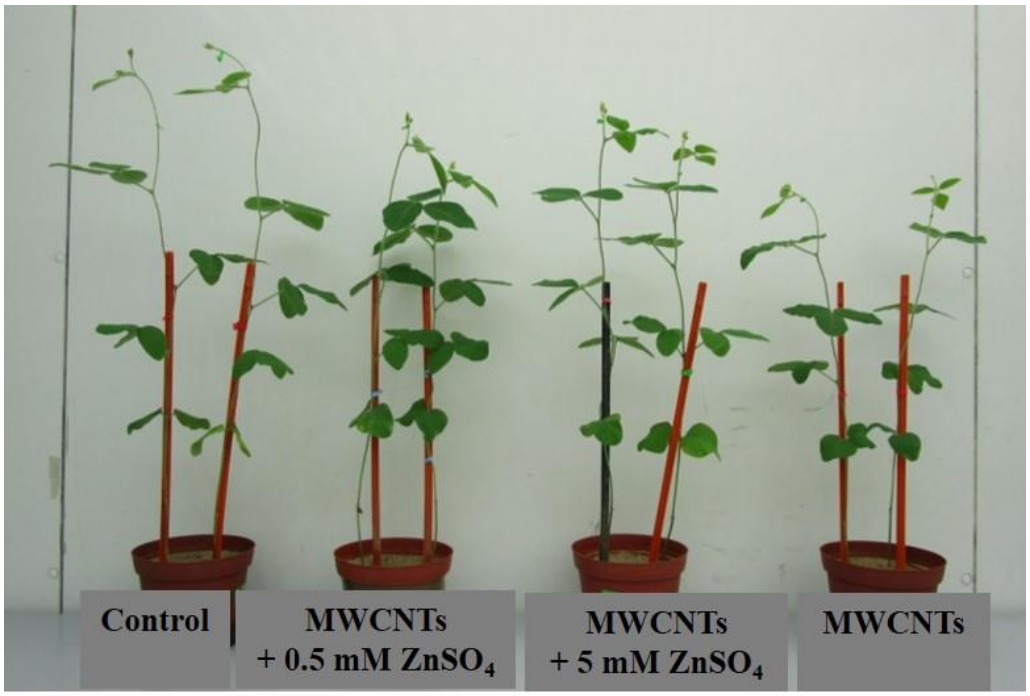


Fig. 7.18. *Effect of $ZnSO_4$ foliar application to the soybean plants developed from seeds treated for 36 h with MWCNTs (1000 mg L^{-1}) or deionized water (control) and subsequently grown in soil culture (calcareous loess subsoil, pH 7.5).*

On the other hand, supplementation of critical nutrients may offer an option to protect plants from unwanted toxic side effects, particularly for potential MWCNT-based agricultural applications (Fig. 7.19). This may be of special importance for targeted application of MWCNTs in situations when plant availability of micronutrients is low. In this context, high soil pH and drought could be potentially limiting factors favouring CNT toxicity. However, it seems to be important to consider not only type and substrate availability of the supplemented nutrients but even the time point of application, as demonstrated by inhibitory effects on plant growth by simultaneous application of MWCNTs and Zn (Fig. 7.17)

Similar to micronutrient supplementation, alleviation of MWCNT-induced inhibition of plant growth was achieved, by combined seed application of MWCNTs with various antioxidants (Chapter IV) with 10 mM proline as the most effective treatment. Accordingly, recent studies reported, that proline seed treatments can be used for mitigation of various abiotic stress factors, such as salinity in faba bean (*Vicia faba* L.) [65] and barley [66] or oxidative stress induced by selenium toxicity in common bean

[67]. Application of other compounds with antioxidant properties such as ascorbic acid have been similarly used in studies focusing on investigation of adverse CNT effects on plants, and Tan et al. achieved mitigation of oxidative stress caused by MWCNTs in cell cultures of rice [48], while Begum et al. [28] demonstrated alleviation of toxic effects of MWCNTs amendments in red spinach. These findings suggest that also co-supplementation with antioxidants may be a strategy to minimize toxic side effects of carbon nanomaterials in agricultural applications (Fig. 7.19).

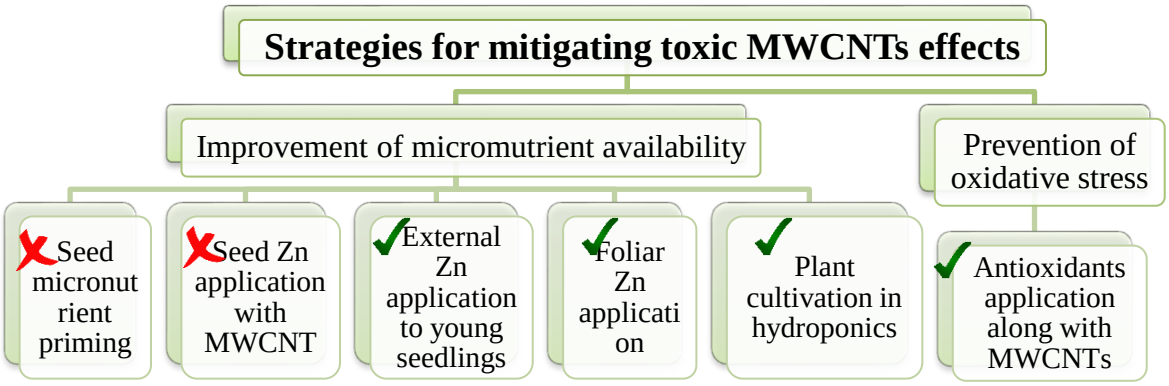


Fig. 7.19. *Scheme demonstrating tested strategies to overcome negative MWCNT effects on root development of soybean plants.* ✓*−successful mitigation of CNT toxicity,* ✗*−unsuccessful mitigation of CNT toxicity.*

7.5.5 Effects of MWCNTs on other plant species

The present study shows that seed MWCNTs treatment exert adverse effects on seedlings growth not only in soybean but also in other plant species. A short-term seed treatment applied for maize and common beans comprised 36 h which was adjusted as a shortest treatment duration causing detectable inhibition of soybean seedlings growth. Most likely, that various plant species have different sensitivity towards MWCNT treatment and therefore the responses of maize, common bean and soybean seedling were slightly different. However, the fact that in all three plant species the major injury affected root development, suggests a common mechanism of MWCNT impact in all tested plants species based on induction of oxidative stress.

On the other hand, low levels of ROS formation can induce also plant growth stimulation by induction of hormesis effects, which have been discussed as explanation for beneficial effects carbon nanomaterials particularly at lower doses of application [2]. In this study, however, beneficial effects of MWCNT seed treatments could be clearly related to a reduction in the speed of water uptake (Fig. 4.1) during imbibition, thereby minimizing imbibition damage. In contrast to the growth inhibition by MWCNT-induced oxidative damage, this effect could not be generalized and seems to be related to low vigour of specific seed lots. Interestingly, a promotion of water uptake, discussed in the literature as an explanation for beneficial effects of CNTs on seed germination [27] could not be confirmed in the plant species investigated in the present study, although seed penetration by MWCNTs was clearly detectable. Therefore, also a generalization of this phenomenon is not possible.

7.6 Future perspectives

Based on the obtained findings, the present thesis revealed a mechanism of MWCNT-induced inhibition of root development in soybean seedlings. Disorders in seed reserve mobilization due to induction of oxidative stress in seed tissues caused by short-term MWCNT exposure has been identified as the key factor triggering an oxidative stress cascade which subsequently results in impairment of root growth.

For further verification of the proposed mechanism, it would be interesting to study more comprehensively oxidative stress responses in soybean seeds to support the existing data by measurement of other pathways for ROS production, such as Fe-induced hydroxyl radical formation or production of hydrogen peroxide to determine activity of antioxidant enzymes, such as catalase and ascorbate peroxidase and the production of phenolic antioxidants. Providing more detailed relationships between ROS production and evidence of physiological damage, such as lipid peroxidation or oxidative degradation of auxins will allow to confirm the proposed mechanism. Of particular interest is an investigation of the potential role of MWCNTs for the internal immobilisation of micronutrients and the effects of metal binding on the MWCNT potential for direct production of free radicals.

For a deeper investigation of the disturbances in Zn reserve mobilization it would be useful to establish time courses of Zn translocation after MWCNT treatments and apply histochemical staining techniques to investigate organ-specific Zn localization.

A further research objective should include a more detailed investigation of the pathways of MWCNT uptake into the seed, which may be achieved by combination of light and transmission electron microscopy with support of Raman spectroscopy.

Finally, for a more reliable risk assessment, the expression of phytotoxic effects of industrial MWCNTs and commercially available nano-carbon products used for agricultural applications need to be assessed under environmentally relevant conditions. For this purpose, evaluation of seed germination and monitoring of plant growth in various soils with application of carbon nanomaterials under practical conditions is suggested.

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Curriculum Vitae

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WORK EXPERIENCE

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Technical skills	Seed germination tests, model toxicity experiments in hydroponic and soil culture, techniques of rhizosphere research, mineral analysis of plant tissues, plant RNA extraction, quantitative real-time PCR, statistical analysis, interpretation and presentation of data results.

ADDITIONAL INFORMATION

Honors and awards

- 2015 Prize for the best poster presentation: "Phytotoxicity of carbon nanotubes in soybean is associated with disturbances of zinc homeostasis"
- Best pitch presentation award at Sustainable Nanotechnology Conference 2015. Venice (Italy), 9 – 11 March 2015.
- 2015 Prize for the best poster presentation: "Effect of multi-walled carbon nanotubes on root development of soybean (*Glycine max*.)"
- 2014 Prize for the best poster presentation: "Phytotoxicity of Carbon Nanotubes to soybean (*Glycine max*.)"
- 2008 Vladimir Potanin Fellowship Program (aimed to support goal-oriented, highly motivated students and potential leaders).
- 2008 Academic Vernadsky Award (aimed to students-environmentalists and students involved in sustainable development issues).

Memberschips

- German society for plant nutrition
- Sustainable Nanotechnology Organization (SNO)

Eidesstattliche Versicherung

gemäß § 8 Absatz 2 der Promotionsordnung der Universität Hohenheim zum Dr.sc.agr.

1. Bei der eingereichten Dissertation zum Thema

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