博 士（環境科学） 譚 暁 明

学 位 論 文 題 名

Cytotoxicity of Multi-wall Carbon Nanotubes on Plant Cells in *in vitro* Culture System

（*in vitro* 培養システムにおける多層カーボンナノチューブの植物細胞に対しての細胞毒性の評価に関する研究）

学位論文内容の要旨

Developments in nanotechnology have led to predictions of great benefits as well as to warnings of great dangers to humanity and the environment. Carbon nanotubes (CNTs) have novel properties that are keys for many nanotechnology applications, and it is thought that tens or even hundreds of tons of CNTs will be produced worldwide within a few years. This increase in CNT production will undoubtedly increase the exposure of humans and the environment to CNTs. A recent study found that the natural organic matter present in river water was capable of both stabilizing and dispersing CNTs. Thus, if CNTs are toxic, their influence in the environment will be long lasting and could spread throughout the food chain. In fact, several studies on nanotoxicity have suggested that CNTs can adversely affect both humans and animals. Toxicity studies have thus far focused on the responses of organisms and/or specialized animal and human cells to CNT exposure.

In this study, I investigated whether CNTs are toxic to plants, using rice (*Oryza sativa L)* suspension cells and callus and carrot (*Daucus carota* subsp. *Sativus*) suspension cells, as the representative examples. Hundreds of tons of Multi-walled CNTs (MWCNTs) have been used worldwide, so this kind of CNTs was chosen as the typical nanomaterials and their possible impact on agriculture is of great interest. Studies were carried out by culturing cell suspensions in the presence of MWCNTs. Our results showed interactions between cells and MWCNTs and provided new insights into the possible toxic mechanism of MWCNTs on the plant cell lines.

This thesis paper composited of 5 chapters. In chapter 1, the importance of studies on the possible toxicity of MWCNTs towards plant is briefly addressed. In chapter 2, to evaluate whether MWCNTs are toxic to plant, rice (*Oryza sativa L)* suspension cells and callus and carrot (*Daucus carota* subsp. *Sativus*) suspension cells were cultured with MWCNTs. Both the suspension cells and callus were found to be capable of interacting with MWCNTs.

Images of light microscopy, scanning electron microscopy and transmission electron microscope gave evidences to conclude that components of the cell wall have associated with MWCNTs and also hold back the MWCNTs enter the cytoplasm. Using TTC assay, decrease on cell viability was observed and this was occurring in a dose-dependent manner; which possibly is the indicative of a hypersensitive response. Rice cell showed more sensitivity than carrot cell after exposed to MWCNTs. On the contrary, carrot cell was more sensitive to carbon black than was the rice cell.

In chapter 3, the possible cellular toxic mechanism of MWCNTs towards the plant cells was investigated. Increase in quantity of reactive oxygen species (ROS) and inhibition of cell proliferation, both in dose and time dependent manner, were observed. Addition of a certain amount of ascorbic acid (AsA), a primary antioxidant, into the the culturing suspension, has increased cell viability, suggesting the oxidative stress is the essential mechanism involved in the rice cell damages caused by MWCNTs.

In chapter 4, for rice cell, at lower concentrations of MWCNTs, morphological changes, such as chromatin condensation and margination, plasma membrane detachment from the cell wall, were observed, indicating MWCNTs can induce apoptosis which is the predominated cell death pattern. On the other hand, using FDA staining and morphology, it was evident that for carrot cells they undergo the necrotic death mechanisms, especially at the high-concentration of MWCNTs. Ascorbic acid (AsA), a major primary antioxidant, prevented the increase of ROS generation and decreased the rate of cell death indicating ROS serving as an important signaling molecule in MWCNTs-induced cell death. Mitochondrial membrane potential ($\Delta\psi m$) remained almost unchanged; this indicating a possible fact that the mitochondria is not involved in MWCNTs-induced cell death.

In chapter 5, general conclusion and further prospect were drafted. It was demonstrated experimentally that MWCNTs are toxic to both rice and carrot cells in their *in vitro* culturing systems. Bio-chemical data together with morphological observations gave experimental evidences to conclude that the oxidative stress is the essential mechanism involved in the cell damages caused by MWCNTs. The exact mechanism regarding how the oxidative stress induced by MWCNTs can force cell death needs further investigations. Nevertheless, my findings together with the others observed for human cells, animal cells and microorganisms will be extremely useful for establishing a methodological field for assessment of the possible risks of the manufactured nanomaterials.

学位論文審査の要旨

主　査　　教　授　　古　月　文　志

副　査　　教　授　　田　中　俊　逸

副　査　　准教授　　鈴　木　光　次

副　査　　助　教　　蔵　崎　正　明

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（*in vitro* 培養システムにおける多層カーボンナノチューブの植物細胞に対しての細胞毒性の評価に関する研究）

The candidate, in this study, had evaluated the possible cytotoxicity of multi-wall carbon nanotubes (MWCNTs) on plant cells in *in vitro* culture systems. The resultant achievements have been described in the thesis paper, which consisted of 5 chapters.

In chapter 1, a general introduction to carbon nanotubes (CNTs) as well as the importance of study on the toxicity of CNTs, was described. CNTs are one of the most promising nanomaterials in nanotechnology due to their unique physico-chemical, electronic, and mechanical properties and the large number of potential applications. The global demand for CNTs is predicted to be more than many thousands of tons in a single year, and MWCNTs have already been appearing in the market in industrial quantities. Plants and plant communities are very important to humans and their environment (i.e. ecosystem), but very few studies have been conducted with ecological terrestrial test species (plants, wildlife, soil invertebrates, or soil microorganisms) to assess the potential toxicity of CNTs. The candidate, in this chapter, had critically understood the relevant literatures and the importance of the studies.

In chapter 2, to evaluate whether MWCNTs are toxic to plant, rice (*Oryza sativa L)* suspension cells and callus and carrot (*Daucus carota* subsp. *Sativus*) suspension cells were cultured with MWCNTs. The candidate had observed that both the suspension cells and callus were capable of interacting with MWCNTs. The candidate used light microscopy, scanning electron microscopy and transmission electron microscope for morphological studies and obtained experimental evidences to conclude that components of the cell wall associated with MWCNTs but the cell walls were capable of holding back the MWCNTs enter the cytoplasm. Using TTC assay, decrease on cell viability was observed and this was occurring in a dose-dependent manner.

In chapter 3, the possible cellular toxic mechanism of MWCNTs towards the plant cells was investigated. Increase in quantity of reactive oxygen species (ROS) and inhibition of cell proliferation, both in dose and time dependent manner,

were observed. Addition of a certain amount of ascorbic acid, a primary antioxidant, into the culturing suspension, has increased cell viability, indicating the oxidative stress was the essential factor causing the rice cell damages by MWCNTs. The candidate had suggested a mode based on the hypersensitive response to explain the possible toxic mechanism for CNTs. This hypersensitive response model, suggested by the candidate, was capable to explain the CNTs' toxic mechanism from the biological points of view.

In chapter 4, at lower concentrations of MWCNTs, morphological changes, such as chromatin condensation, margination, plasma membrane detachment from the cell wall, those are the key indicators of apoptosis, were observed. On the other hand, at the high-concentration of MWCNTs, large populations of the fragmented cells those corresponding to protoplasm; this representing the irreversible cell injuries and cell death, namely the necrotic death mechanisms, was observed. Ascorbic acid, a major primary antioxidant, prevented the increase of ROS generation and decreased the rate of cell death. Mitochondrial membrane potential ($\Delta\psi m$) remained almost unchanged; this indicating a fact that the mitochondria is not involve in MWCNTs-induced cell death. These new findings, obtained in this study, gave new insights into the mechanism of the CNTs' toxicity on plant cells. After the components of the cell wall renew in the course of metabolism, some of the MWCNTs that contacted the polysaccharide and protein residues have been embedded by new polysaccharides or proteins. The interaction between the components of the cell wall and MWCNTs possibly offer the opportunity of making nanotubes change the dimensional structure of the ambient signal molecules (including proteins or polysaccharides), and the change in structure will lead to potentially inducing a signaling cascade of the HR, resulting in the induction of ROS. The candidate, in this study, had made an original contribution to the knowledge of this subject.

In chapter 5, general conclusion and further prospect were drafted. The candidate had demonstrated experimentally that MWCNTs are toxic to both rice and carrot cells in their *in vitro* culturing systems. The cells with MWCNTs at their cell wall seemed to undergo a hypersensitive response, namely the ROS defense response cascade, which is sufficient to prevent microbial pathogens from completing their life cycle.

This thesis, as a whole, made an original contribution to the knowledge of this subject. The candidate had critically understood the relevant literatures. The methods adopted were appropriate to the subject. The experimental findings were suitably set out, also discussions were made logically. Qualities of English were satisfactory. The recommendation of all the examiners was that "the degree be awarded".