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RESEARCH ARTICLE

# Uptake and accumulation of multiwalled carbon nanotubes change the morphometric and biochemical characteristics of *Onobrychis arenaria* seedlings

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**Abstract** We have studied the effect of the engineered nanomaterial Taunit, containing multiwalled carbon nanotubes (MWCNTs), on the growth of *Onobrychis arenaria* seedlings and investigated whether affected plants uptake and accumulate MWCNTs. We found that 100 μg/mL and 1000 μg/mL of Taunit stimulated the growth of roots and stems, and enhanced the peroxidase activity in these parts of plants. Microscopy studies showed the presence of MWCNTs in the root and leaf tissues of seedlings exposed to Taunit, suggesting that MWCNTs have a capacity to penetrate the cell walls, accumulate in roots and translocate to the leaves. Thus the stimulating effect of MWCNTs on seedlings of *O. arenaria* may be associated with the primary uptake and accumulation of MWCNTs by plant roots followed by translocation to the other plant tissues.

**Keywords** multiwalled carbon nanotubes, plants, electron microscopy

## 1 Introduction

Widespread use of nanomaterials in modern technologies encourages a large-scale production of engineered nanoparticles. Concurrently, the potential release of engineered nanoparticles into the environment during technological cycles is inevitable, raising serious concerns about the adverse effects of nanomaterials on biologic systems [1,2]. Plants are an essential component of all ecosystems, so uptake and accumulation of engineered nanoparticles in plant biomass will seriously affect not only plant kingdom, but also the life state of other living organisms [3–5]. Carbonateous nanoparticles, including fullerenes and nanotubes, are of one of the widely applied categories of engineered nanomaterials. Carbon nanotubes (CNTs) are cylindrical carbon molecules, existing in single-walled (SWCNT) or multi-walled (MWCNT) form. Due to their fibrous shape, CNTs have been compared to asbestos, raising great concerns that the extensive use of industrial products based on CNTs, may negatively affect the environment, biologic systems and human health [6,7]. Some studies showed that CNTs under certain circumstances might be pathogenic to animals [8]. Increasing numbers of reports have emerged over the past years concerning the interactions of CNTs with agricultural plants. Positive, negative and inconsequential effects have been reported. It appeared that MWCNTs significantly enhanced the germination rate of tomatoes [9], did not affect the growth of wheat [10] and inhibited the growth of rice seedlings [11]. SWCNTs inhibited the growth of tomato roots but stimulated the growth of onion and cucumber roots [12]. All classes of CNTs could also adhere to plant roots and exert physical or chemical toxicity on plants. However, it is still not clear whether MWCNTs/SWCNTs are able to penetrate cell walls and accumulate inside of plant tissues and cells. The aim of this research was to study the effect of industrial nanomaterial Taunit containing MWCNTs on plants, and investigate whether MWCNTs penetrate cell walls and accumulate in cells and tissues of affected plants.

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## 2 Matherials and methods

### 2.1 Test material

The object of the present study is an industrial carbon nanomaterial Taunit (NanoTechCenter Ltd., Tambov, Russia). This material is a loose black powder, composed of grainy agglomerates with a size of several micrometers. Agglomerates mostly consist of entangled bundles of MWCNTs, which have a hollow cylindrical structure with a length of at least 2 μm, an external diameter of 20 – 70 nm and an internal diameter of 5 – 10 nm. Taunit is produced by chemical vapor deposition with the purity above 98% [13].

### 2.2 Plant material, seed germination tests and evaluation of peroxidase activity

The seeds of *Onobrychis arenaria* were germinated in a medium containing a colloidal aqueous solution of Taunit with a concentration of 100 or 1000 mg/L. Prior to use, Taunit was dispersed in distilled water by ultrasonic treatment. The water without Taunit was used for control experiments. The seeds (50 seeds per dish) were grown for 10 days on filter paper in glass Petri dishes with 5 mL of Taunit suspension. 200 seeds were used in each experiment. The estimation of the Taunit effect on *O. arenaria* seedlings was based on the following parameters: the rate of germination (%), the energy of germination (%), and the length of the roots and stems. The energy and rate of germination were determined as the ratio of the number of germinated seeds to the number of plated seeds by day 5 and day 10, respectively (%).

The weighed samples (2 g) of *O. arenaria* tissues were placed into 5 mL of cold phosphate/citrate buffer (pH 5.5) and ground in a porcelain mortar at 4°C [14]. The homogenate was centrifugated at 3000 g for 15 min. The cleared supernatant was used to determine the activity of soluble peroxidases on the basis of the changed rate (time, s) of the optical density at a wavelength of 580 nm in the reaction mixture, containing 0.5 mL of 0.1 $mol \cdot L^{-1}$ solution of the phosphate/citrate buffer (pH 5.5), 0.5 mL of 0.3% $H_2O_2$, 0.5 mL of 0.05% guaiacol (Sigma-Aldrich, USA), and 0.5 mL of the sample. Peroxidase activity was measured at 25°C immediately after the enzymes were extracted from the samples. Enzymatic activity was calculated by Boyarkin's method [15] and expressed in arbitrary units of activity per gram of fresh tissue weight per second, according to the following formula:

$$A = (\varepsilon \times \alpha \times \beta \times \gamma)/(d \times t),$$

where $\varepsilon$ is the extinction coefficient, $\alpha$ is the ratio between the amount of buffer taken for extract preparation (mL) to fresh tissue weight (g), $\beta$ is the degree of additional dilution of the extract in the reaction mixture, $\gamma$ is the degree of constant dilution of the extract in the reaction mixture, $d$ is the thickness of the absorbing layer (mm), and $t$ is the reaction time (s).

### 2.3 Processing of plants samples for light and transmission electron microscopy

The bottom of a plastic box was covered with four gauze layers moistened with Taunit solution or water (control). One hundred of *O. arenaria* seeds were placed on the gauze and exposed either to Taunit solution at the concentration 300 mg/L or water without Taunit. After 5 and 10 days, the seedlings were fixed for light and electron microscopic studies. For light microscopy, the seedlings were fixed in a 3: 1 mixture of 96% ethanol and acetic acid for 16 h. After fixation, the plant samples were transferred into 70% ethanol. The plant parts under study (roots, leaves, coleoptiles) were then placed onto a glass slide into a drop of 45% acetic acid. The preparations of whole mount plant organs were made according to the standard procedure [16] and analyzed using Leica DM1000 light microscope (objectives × 10, × 20, × 40, and × 100). The images were recorded with a Leica DFC 295 digital camera (sensor size $3 \times 10^6$ pixels).

For transmission electron microscopy (TEM), the seedlings were fixed with 2.5% glutaraldehyde in 0.1 $mol \cdot L^{-1}$ Na-K-phosphate buffer (pH 7.2) supplemented with sucrose (15 mg/mL). The samples were then dehydrated in a series of increasing ethanol concentrations and embedded in Epon 812, according to the standard procedure. For best detection of MWCNTs, plant tissue samples were prepared without fixation using $OsO_4$ and staining using uranyl acetate and lead citrate.

Ultrathin sections of plant tissues were imaged and photographed in JEM-1011 (JEOL) equipped with a GATAN ES500W digital camera. For SAED (selected area electron diffraction) sections were analyzed by LEO 912AB (Carl Zeiss).

## 3 Results and discussion

### 3.1 Effect of Taunit on the morphometric and biochemical characteristics of *O. arenaria* seedlings

To determine the phytotoxicity of Taunit, we applied *O. arenaria* seed germination tests, in which the germination energy, germination rate, length of roots and stems, and peroxidase activity were estimated [17]. The energy of germination in the presence of Taunit increased by 14% as compared with the control sample. Under Taunit concentrations of 100 mg/L and 1000 mg/L, the rate of germination increased only by 2% and 7%, respectively, but, the length of roots and stems notably increased, as compared with the control sample (Table 1). Thus, Taunit slightly

**Table 1** Morphometric and biochemical characteristics of *O. arenaria* seedlings exposed to Taunit

| Concentrations | Energy of germination /% | Germination rate /% | Stems /mm | Roots /mm | Peroxidase activity /(arbitrary units of activity/gram of tissue fresh weight/second) |
|---|---|---|---|---|---|
| 1000 mg/L | 114 | 102 | 25.4±1.28 | 32±0.97 | 0.185±0.019 |
| 100 mg/L | 114 | 107 | 25.7±1.01 | 28.7±0.83 | 0.313±0.012 |
| ControL | 100 | 100 | 13.92±1.28 | 18.4±1.28 | 0.115±0.012 |

enhanced the rate of seeds germination and the energy of germination, and considerably stimulated the increase of the length of roots and stems. To find the minimal active concentration of Taunit, we have evaluated the length of roots and stems exposed to the lowering concentrations of Taunit (Table 2). It appeared that the stems of seedlings incubated at 0.1 mg/L of Taunit were longer than those in control plants. This finding suggests that *O. arenaria* may be exceptionally sensitive to this class of nanomaterials. The exposure of *O. arenaria* seedlings to Taunit at the concentrations of 100 mg/L and 1000 mg/L also enhanced peroxidase activity, respectively, to 0.31±0.01 and 0.19±0.02 a.u./g fresh weight, which is significantly higher than the control value (0.12±0.01) (Table 1).

**Table 2** Morphometric parameters of *O. arenaria* seedlings exposed to low concentrations of Taunit

| Concentrations | Stems /mm | Roots /mm |
|---|---|---|
| 10 mg/L | 17.3±1.21 | 31.3±0.90 |
| 1 mg/L | 19.6±0.90 | 29.5±1.20 |
| 0.1 mg/L | 19.0±0.81 | 25.6±1.21 |
| Control | 13.2±0.71 | 25.4±1.40 |

It is well known that plants respond to mechanical stress and injury by changing their morphology or growth rate, so called thigmomorphogenesis. Thigmomorphogenetic changes are regarded as the adaptation process to stress in plants, and plant hormones play an important role in this process [18]. Under mechanical stress or after injury, the activity of the plant stress hormone, jasmonic acid, increases, whereas the activity of auxin, which controls the processes of morphogenesis and plant growth, decreases. These changes of plant hormone levels may be associated with the increase of peroxidase activity [19–21]. Peroxidase is involved in a number of biological processes, such as photosynthesis, respiration, and protein metabolism. It is an antioxidant enzyme with high sensitivity toward external factors, and its activity evaluation is one of the tools for testing the physiological state of plants. In most cases, a low level of peroxidase activity demonstrates the initiation of the mechanisms of a nonspecific response of the plants to stress [21]. It can be assumed that in our experiments the increase of peroxidase activity is associated with the oxidative stress caused by Taunit. It has been shown that MWCNTs, accumulated at the root surface, often pierced cell walls of epidermal cells [10]. Such interaction may cause a mechanical injury and thus elevate the level of peroxidase activity. Our results confirmed that the level of peroxidase activity decreases with an increase in Taunit concentration. This observation could be explained by the inactivation of peroxidase molecules by nanotubes due to sorption or other chemical interactions. Therefore we have analyzed the activity of purified horseradish peroxidase incubated for 24 h in the presence of 100 and 1000 mg/L of Taunit. It appeared that the peroxidase activity in vitro increased from 0.138 a.u. in control sample to 0.275 a.u. (100 mg/L) and to 0.279 a.u. (1000 mg/L) (Fig. 1). This finding supports the suggestion that CNTs may interact or bind to peroxidase molecules thus enhancing their activity. Our studies demonstrated that the exposure to Taunit, containing MWCNTs stimulated the growth of roots and stems of plants along with an increase in the level of peroxidase activity. Further research is needed to establish the reason for the enhanced growth of plants in the presence of Taunit.

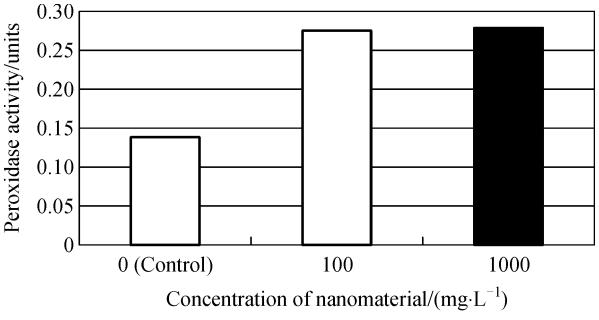


**Fig. 1** Estimated activity of horseradish peroxidase in the presence of colloidal water solution of Taunit

It has been reported [12] that application of CNTs resulted in inhibition of root elongation in tomato but enhancement of root elongation in onion and cucumber. In contrast to this observation, the other authors did not find any positive or negative effects of CNTs on root elongation of tomato seedlings. However, they found that SWCNTs were uptaken by the tomato seeds and affected their biological activity, presumably by creation of new pores and enhancing the water permeation into the seeds during germination [9]. Since the capacity of MWCNTs to penetrate the cellulose cell walls and accumulate in plant cells has never been confirmed, we investigated this using *O. arenaria* seedling, which is affected by Taunit, with light and electron microscopy.

3.2 Analysis of *O. arenaria* seedlings using light and electron microscopy

Upon germination of *O. arenaria* in the presence of Taunit, the roots, stems and leaves of seedlings acquired a distinctive gray or black color. Analysis of whole mount preparations of roots showed that colored agglomerates adhered to the root surface (Figs. 2(a), 2(b)) and scattered within the root tissues (Fig. 2(b)). Black aggregates were also found within stems and leaves of seedlings (Figs. 2(c), 2(d)) but were not detected on the surface of these plant organs. To characterize the aggregated material, we have analyzed organs of affected plants by TEM.

In ultrathin sections prepared from plant organs without $OsO_4$ fixation and uranyl acetate/lead citrate staining, no intracellular organelles could be clearly resolved. Nonetheless, the intercellular borders, the cell walls and some cytoplasmic particles were visible allowing detection of the electron dense Taunit located both inside and outside the sampled material.

TEM analysis showed the aggregations of Taunit, comprised of entangled MWCNTs and finely dispersed electron dense impurities, adhered to or near the seedling roots (Fig. 3(a)). We also found accumulations of MWCNTs inside the root (Figs. 3(b), 3(c)) and leaf (Fig. 3(d)) cells, single MWCNT inserted into the cell walls (Fig. 3(b), inlet), and agglomerations of MWCNTs embedded into the cell walls (Fig. 3(c)). The latter observations support the view that CNTs may pierce the cellulose cell walls and damage its integrity [9,10].

Thus our results confirmed the earlier reports about the high absorption of MWCNTs on the root surface [3,9–11]. It has been suggested that adherence and interaction of MWCNTs with plant organs affect the plant growth and development [12]. Adsorption of a large amount of MWCNTs on the root surface may suppress the water influx and uptake of nutrients hence inhibiting the plant growth [11]. Conversely, the stimulation of the seed germination may be associated with increased water influx, induced by the nanotubes (SWCNTs) piercing the seed cover [9]. It has been reported that SWCNTs not only pierce the cell walls, but also penetrate inside the cells [22]. Insertion of MWCNTs into the wall of epidermal cells and root hairs was observed in wheat seedlings using two-photon excitation microscopy; however, penetration of whole MWCNTs into the cytoplasm was not noted [10]. The authors assumed that penetration, uptake and accumulation of MWCNTs were obscured due to the

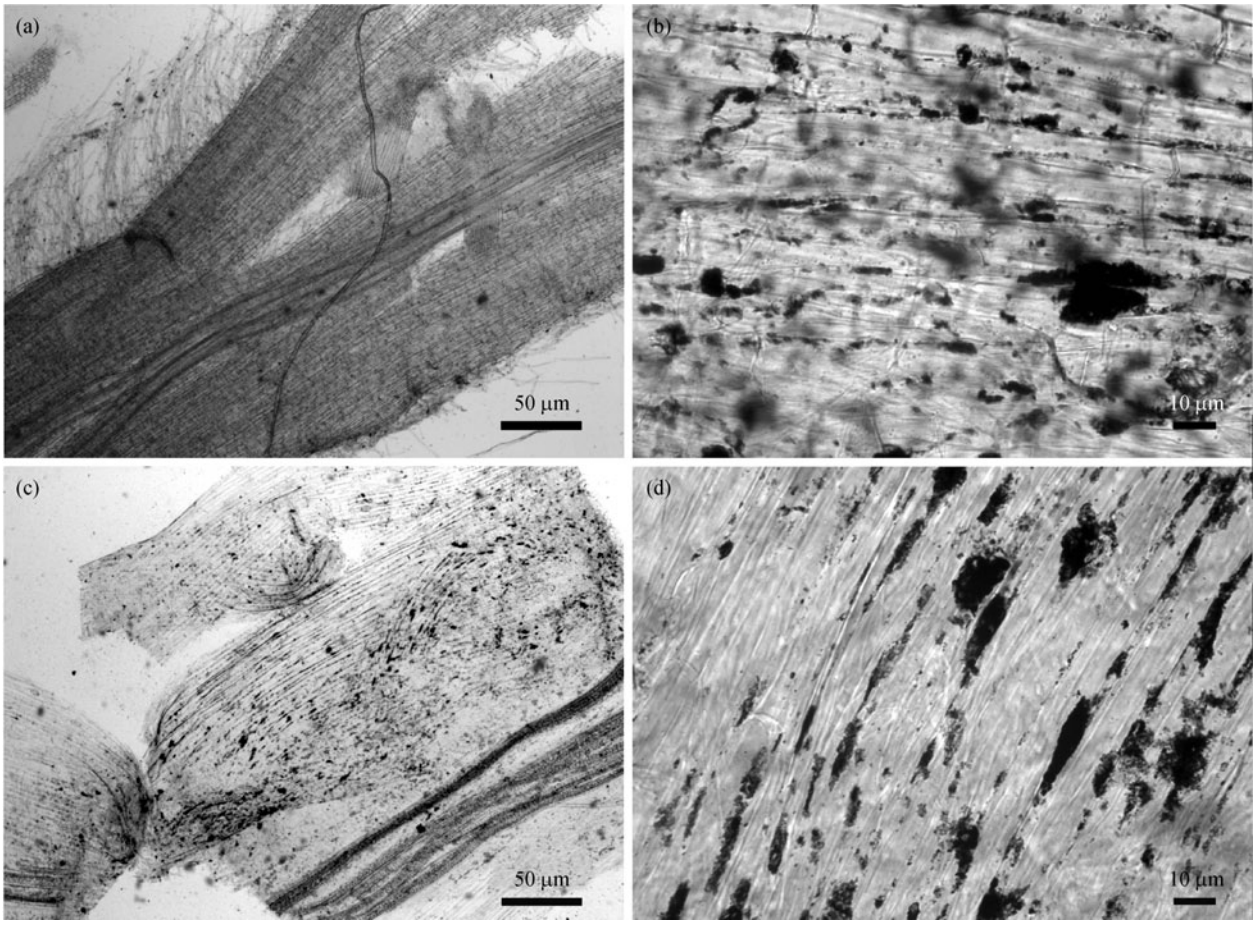


**Fig. 2** Whole mount preparations of root (a, b) and leaf (c, d) of *O. arenaria* seedling grown in the presence of Taunit

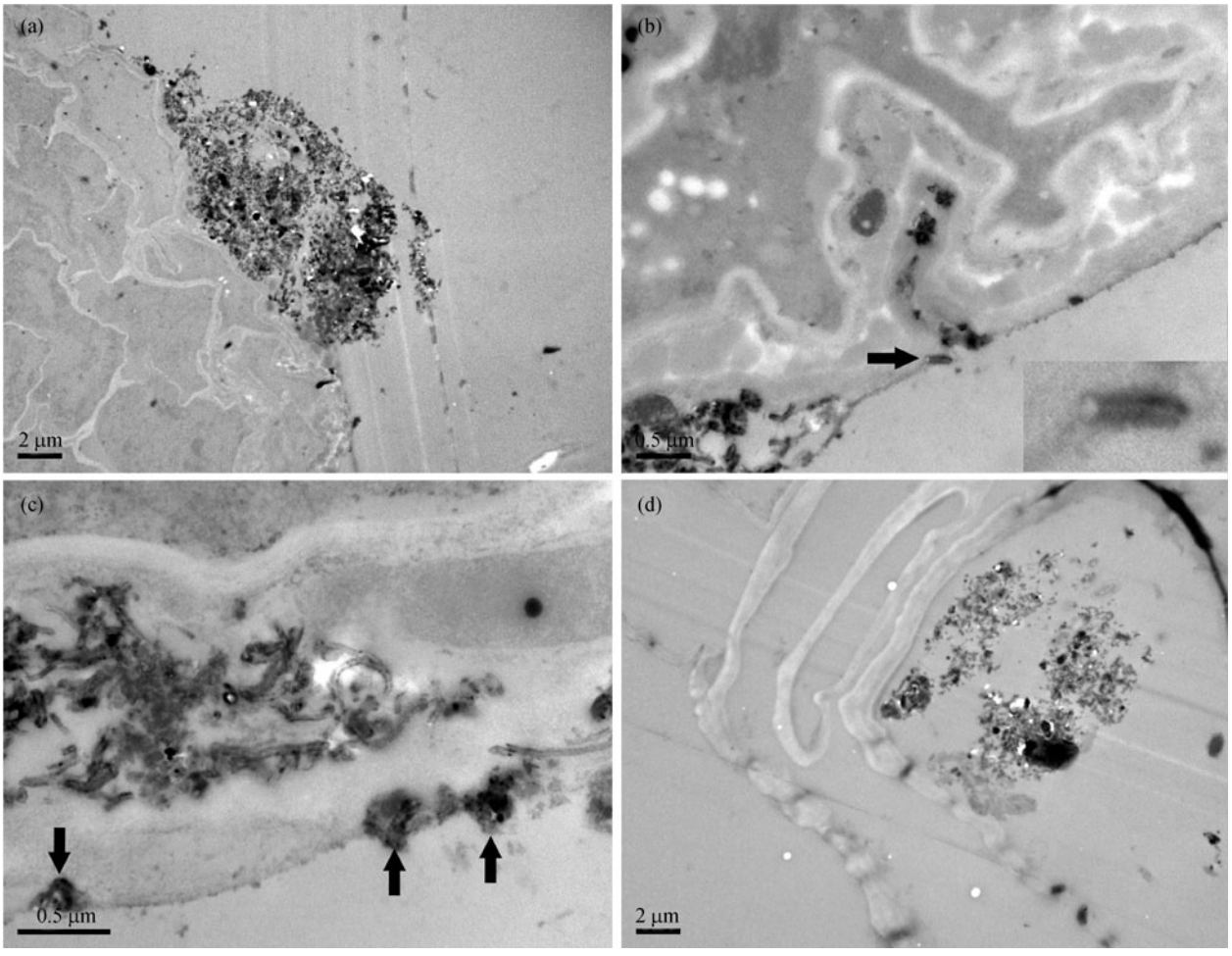


**Fig. 3** TEM analysis of ultrathin sections of *O. arenaria* seedlings grown in the presence of Taunit. (a) Large agglomerate of Taunit adhered to the root surface. (b) Agglomerates of Taunit, containing MWCNTs, in the root tissue. MWCNT piercing the cell wall is shown with arrow, enlarged image inserted at the right bottom. (c) Accumulation of MWCNTs in the epidermal cell. Arrows show small agglomerates of Taunit embedded into the cell wall. (d) Accumulation of Taunit in the leaf cell

larger nanotube diameter as compared with that of SWCNTs [10]. Recently Serag and co-authors [23] reported the uptake of functionalized MWCNTs by plant cells lacking the cellulose cell walls. We detected MWCNTs inside the walled cells of root tissues and assume that MWCNTs first penetrated the cellulose cell walls and then accumulated in the cells. Initially, only plant roots were exposed to Taunit containing MWCNTs. Therefore the presence of MWCNTs in seedling leaves argues for the translocation of accumulated MWCNTs from the roots via stems to leaves.

The distinctive shape and size of MWCNTs allows reliable detection of this nanomaterial with TEM. However, if MWCNTs are located transversely or at some angle to the section plane, it is problematical to distinguish their fragments from the endogenous electron-dense particles. To confirm the found inclusions to be MWCNTs, we have used selected area electron diffraction (SAED) technique. Due to the regular periodical packing of carbon atoms, MWCNTs had a distinctive electron diffraction pattern typical for polycrystalline structures (Figs. 4(a–c)). This diffraction pattern was used as a reference sample for the verification of MWCNTs in plant tissues (Fig. 4(d–f)). Electron diffraction patterns of reference sample (Fig. 4(c)) and MWCNTs found in pant tissue (Fig. 4(f)) overlapped, confirming that electron dense agglomerates in the cytoplasm of plant cells contain MWCNTs.

## 4 Concluding remarks

Engineered Taunit containing MWCNTs stimulates the growth of *O. arenaria* roots and stem and enhances peroxidase activity of *O. arenaria* seedlings. These effects may be induced by the oxidative stress due to the uptake of MWCNTs in plant roots. We argue that MWCNTs penetrate the cell walls, accumulate in plant tissues and cells, and are translocated from roots to stems and leaves.

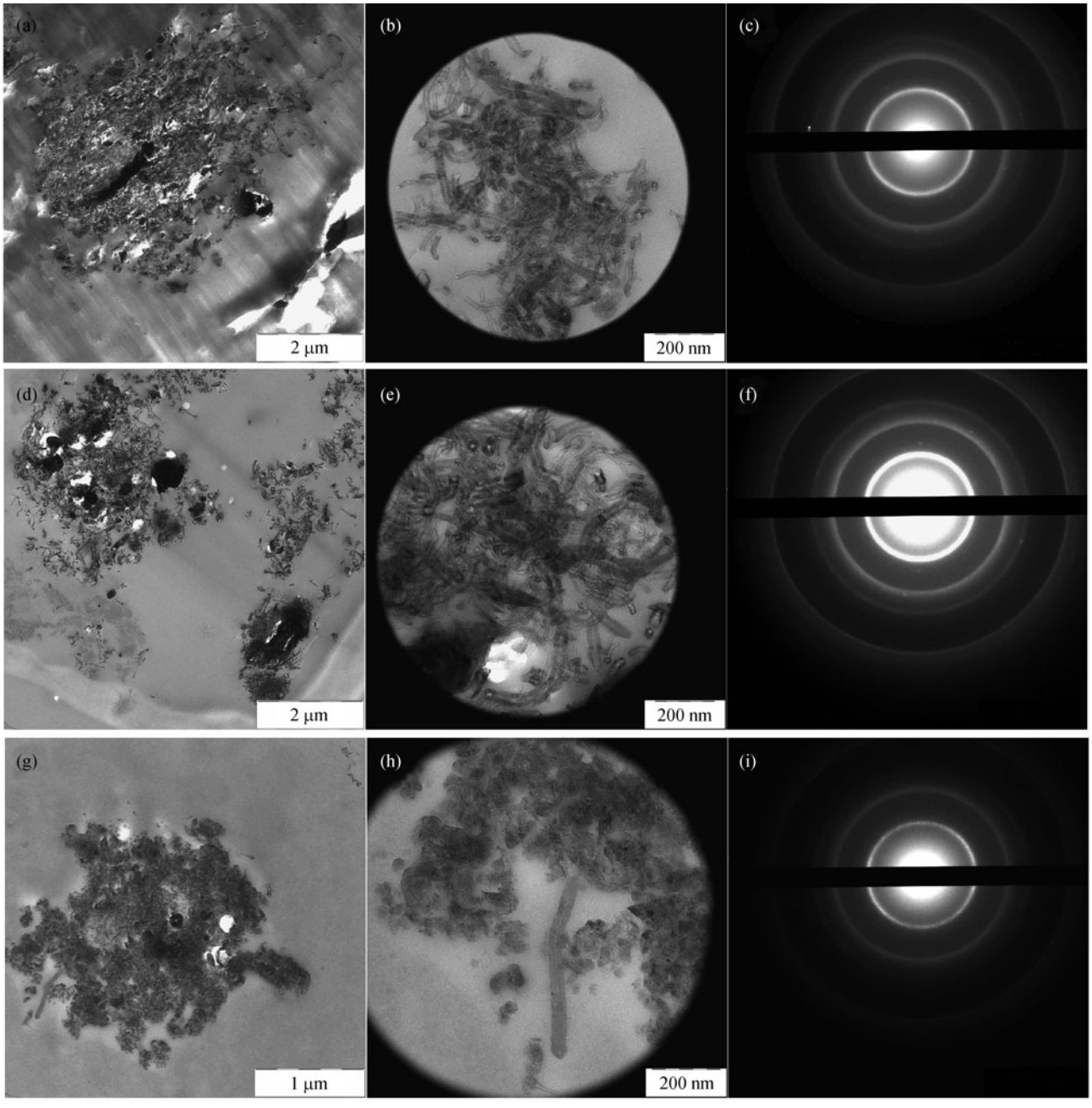


**Fig. 4** Analysis of CNM containing MWCNTs with SAED. (a) Ultrathin section of pure Taunit sample. (b) The area on (a), analyzed with SAED. (c) Diffraction pattern of the same area. (d) Ultrathin section of the plant leaf cell containing the accumulation of Taunit with entangled bundles of MWCNTs. (e) The area on (d), analyzed with SAED. (f) Diffraction pattern of the same area. (g) Ultrathin section of the macrophage with inclusion of Taunit, composed of MWCNT bundles. (h) The area on (g), analyzed with SAED. (i) Diffraction pattern of the same area

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