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RESEARCH PAPER

Beneficial role of carbon nanotubes on mustard plant growth: an agricultural prospect

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Abstract Nowadays an increasing application of nanotechnology in different fields has arisen an extensive debate about the effect of the engineered nanoparticles on environment. Phytotoxicity of nanoparticles has come into limelight in the last few years. However, very few studies have been done so far on the beneficial aspects of nanoparticles on plants. In this article, we report the beneficial effect of multi-walled carbon nanotubes (MWCNTs) having diameter of ~30 nm on *Brassica juncea* (mustard) seeds. Measurements of germination rate, T_{50} (time taken for 50% germination), shoot and root growth have shown encouraging results using low concentration of oxidized MWCNT (OMWCNT) treated seeds as compared to non-oxidized as well as high concentration OMWCNT treated seeds. For toxicity study we measured the germination index and relative root elongation, while conductivity test and infra-red spectra were also performed to study the overall effect of oxidized and non-oxidized nanotubes on mustard seeds and seedlings.

Keywords Carbon nanotube · Soaking-drying treatment · Germination rate · Plant growth · Moisture content · Conductivity · Food and agricultural systems

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Introduction

With the advancement of material science, a wide range of nanoparticles such as metal oxide nanoparticles (ZnO, TiO_2, Al_2O_3, etc.), fullerenes, carbon nanotubes, quantum dots, etc. from an increasing range of applications for different purposes (Biswas and Wu 2005) make their way easily in the environment. Their potential adverse effects on the environment and human health are being subjected to intense debate (Monica and Cremonini 2009). However, their adverse (Nel et al. 2006) or beneficial effects (Yang et al. 2006; Zheng et al. 2005) on plants as important receptors in the ecological systems have not yet been widely investigated (Stampoulis et al. 2009).

Nanosized materials differ in the physicochemical and structural properties from bulk materials due to their large surface to volume ratio and their small particle size (<100 nm), often enabling them to penetrate biological membranes. Phytotoxicity of nanoparticles including inhibition of root growth (Doshi et al. 2008; Ling and Xing 2007; Yang and Watts 2005; Racuciu and Creanga

2007), enhancement of lipid membrane peroxidation (Nel et al. 2006) etc. have been well studied. However, Hong et al. (2005) and Yang et al. (2006) have shown that nano-TiO_2 are able to promote plant growth by enhancing their nitrogen-fixing properties, although Zheng et al. (2005) have reported nanoparticles to be quite toxic to living cells. Beneficial effects of nanoparticles have recently been reported by researchers in different fields like drug delivery, biosensing, etc. (Harrison and Atala 2007; Panyam and Labhasetwar 2003; Zanello et al. 2006). Lu et al. (2002) reported that TiO_2 and SiO_2 nanoparticles increase the synthesis of nitrate reductase in *Glycine max* which in turn promotes growth and germination by increasing the efficiency of its water uptake machinery.

Investigations are increasingly focusing on carbon nanotubes (CNTs) in contrast to other nanoparticles due to their ability to penetrate mammalian and bacterial cells (Wong et al. 2005; Zhu et al. 2007). Penetration capability of CNTs through hard seed coat has very recently been reported by Khodakovskaya et al. (2009). They reported that in presence of carbon nanotubes tomato seeds grow faster than seeds grown in absence of CNTs due to enhancement of water uptake process as CNTs penetrate the seed coats.

Their encouraging result prompted us to take up this study where we have observed that oxidized multi-walled carbon nanotubes (OMWCNTs) showed far better result in comparison to the normal MWCNTs. In our experiment, we used mustard seeds and exposed them in different media. However, seeds exposed to OMWCNTs showed less T_{50} (time taken for 50% germination), higher rate of plant growth (both root and shoot growth) and higher vigour (in relation to dry weight accumulation) than seeds treated with MWCNTs. We also observed an increasing germination rate by using the seeds which were previously presoaked in OMWCNTs and then sun-dried applying soaking-drying method. The objective of our study is to open a new perspective in agricultural field regarding enhancement of growth and vigour of crop plants.

Materials and methods

Nanoparticle processing

MWCNTs were purchased from Arry International, Germany with 60% purity and having a diameter of ~30 nm. MWCNTs were activated by following the technique of Ravindran and Ozkan (2005) with the following modification. At first, MWCNTs were refluxed with 2 M nitric acid at 130 °C for 16 h and then sonicated for 3 h in the same acid. Resulting material was collected by filtration and washed in water and ethanol successively until the pH of solution became 6. The resulting MWCNTs are now in oxidized form and well dispersed in water owing to presence of hydrophilic carboxyl (–COOH), hydroxyl (–OH) and carbonyl groups (>C=O) along their side walls. Finally, the OMWCNTs were dried in hot air oven at 80 °C for 6 h.

Nanoparticle characterization

Characterization of MWCNTs and OMWCNTs have been done by FTIR ((FTIR-8400 s, Shimadzu), field emission scanning electron microscopy, FESEM (JSM 6700F, JEOL Ltd, Tokyo, Japan) and X-ray diffraction recorded on Bruker AXS type diffractometer using CuKα radiation—1.5409 Å ($2\theta = 10°$–$70°$, scan speed 0.2 s/step, increment—0.02).

Comparison of the FTIR spectra of MWCNTs and OMWCNTs clearly indicated the presence of oxygen-containing groups in OMWCNTs. In case of MWCNTs few weak bands appear between the range 1,000–2,500 cm^{-1} (Fig. 1, left) corresponding to the C=O group (Li et al. 2002) whereas in case of OMWCNTs some additional new bands appear due to oxidation (Fig. 1, right).The band at 2355.72 cm^{-1} present in both cases was attributed to CO_2 whereas the band at 1434.45 cm^{-1} corresponded to C=O stretching vibrations in carboxyl group. The new band at 2932.64 cm^{-1} in OMWCNTs corresponds to –OH stretching. The band centered at around 3354 cm^{-1} (Fig. 1, right) with a shoulder at 3213 cm^{-1} was due to O–H stretching vibrations in C–OH groups and water, respectively, indicating the proper oxidization of MWCNTs in acidic refluxing technique. (Kovtyukhova et al. 2003; Li et al. 2002; Yu et al. 1998).

FESEM images (Fig. 1, inset in left) showed the typical tube like nature of MWCNTs with smooth surface walls and in case of OMWCNTs (Fig. 1, inset in right) not only the tube length became shorter but the surface walls also became rough. The average diameter of MWCNTs was ~30 nm whereas it was ~20 nm in case of OMWCNTs.

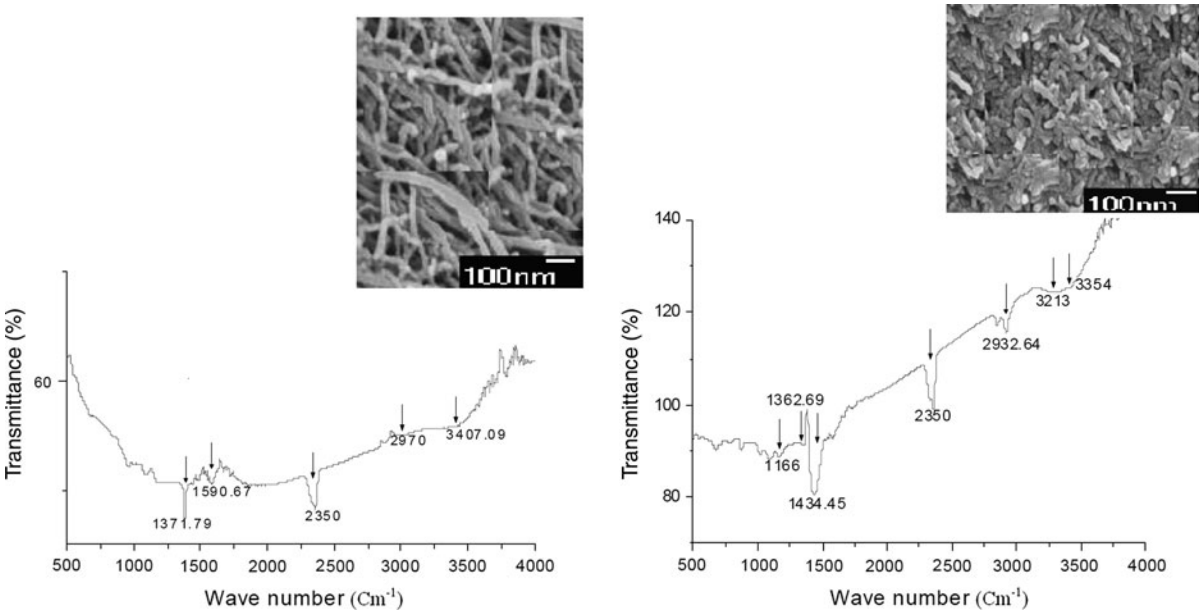

Fig. 1 *Left*: FTIR spectra of MWCNTs and FESEM of MWCNTs (*inset*); *Right*: FTIR spectra of OMWCNTs and FESEM of OMWCNTs (*inset*)

XRD spectra of OMWCNTs and MWCNTs (Fig. 2a and b, respectively) agreed well with the FESEM images. The fine crystalline nature of MWCNTs with its sharp peaks was totally destroyed after acid oxidation and amorphous nature of OMWCNTs was prominent in XRD.

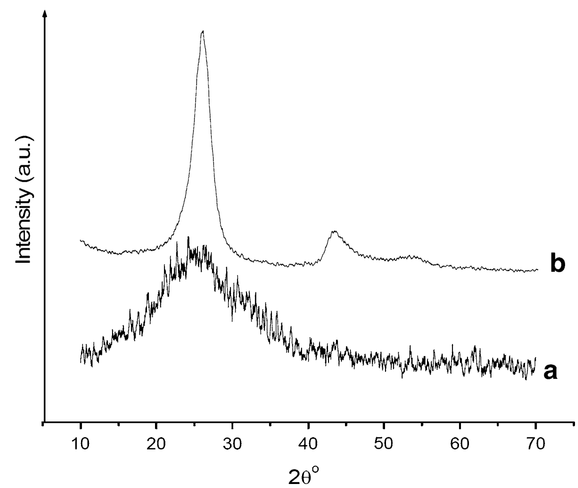

Fig. 2 XRD of **a** OMWCNTs and **b** MWCNTs

Seed collection

Certified seeds of mustard were collected from Crop Research Farm, Burdwan University having an average germination rate greater than 88% as shown by a preliminary study.

Methods of pretreatment

Seeds were initially surface sterilized with 0.1% $HgCl_2$ for 1 min. One lot of mustard seeds were immersed in distilled water (DW) and another lot in oxidized MWCNTs solution for 4 h. Seeds were stirred frequently and the excess solution was decanted. After completion of the treatment, the seeds were separately surface dried with blotting paper and dried back to their original weight under sun. Other seeds were not soaked in any solution but dried under the sun along with the treated seeds. After pretreatment the seeds were stored in normal laboratory condition in perforated paper bags and used after 15 days for experimental purposes.

Preparation of nanoparticle solution

The MWCNTs and OMWCNTs were suspended directly in double distilled water by sonication in an ultrasonic bath (Imeco Ultrasonic, Model No. 229, India). MWCNTs and OMWCNTS were prepared at different concentrations.

Germination technique

Before starting germination all seeds were immersed in 10% sodium hypochlorite solution for surface sterilization (U. S. Environmental Protection Agency 1996). To analyze percentage of seed germination, 50 individual seed samples were transferred to petri-dishes containing filter paper moistened with 10 mL of distilled water or nanoparticle solution as the case may be. Germination data were recorded at every 24 h interval following International Rules for Seed Testing Association, ISTA (1976). Seeds were considered to be completely germinated when the radicle attained a length of 1 mm and plumule has just unfolded. The experiments were repeated thrice with 6 replications in each case.

Seeds were treated in different media and treated seeds were designated as followings:

$S_o \rightarrow$ Control

$S_1 \rightarrow$ Distilled water pretreated

$S_2 \rightarrow$ OMWCNTs pretreated $\left(\text{concentration } 2.3 \times 10^{-3}\text{mg/mL}\right)$

$S_3 \rightarrow$ Low concentration $\left(2.3 \times 10^{-3}\text{mg/mL}\right)$ OMWCNTs treated

$S_4 \rightarrow$ High concentration $\left(6.9 \times 10^{-3}\text{mg/mL}\right)$ OMWCNTs treated

$S_5 \rightarrow$ Low concentration $\left(23 \times 10^{-3}\text{mg/mL}\right)$ MWCNTs treated

$S_6 \rightarrow$ High concentration $\left(46 \times 10^{-3}\text{mg/mL}\right)$ MWCNTs treated

Methods for measurement of other parameters:

The time taken for 50% of germination, i.e. T_{50} was recorded after repeating germination process thrice with 6 replications in each case.

Shoot–root growth and dry weight (wt) accumulation of mung bean plants were recorded after 10 days of treatment.

Phytotoxicity of nanoparticles was evaluated by calculating relative root elongation (E) and germination index (GI). Actually for agricultural purposes GI is used as an indicator of phytotoxicity in soil (Tiquia and Tam 1998). The percentage of E and GI were calculated according to standard method (Tiquia et al. 1996).

We measured the total level of moisture content of mung bean seeds as well as root tissues of 10 days old seedlings by thermogravimetric analysis (TGA) (DTG-60H, Shimadzu). TGA was conducted from room temperature (~30 °C) to 200 °C. For this experiment we first measured the moisture level of dry seeds and then dry seeds were placed in different media as had been chosen for our studies. After 12 h of imbibitions TGA of treated seeds were performed. The moisture content of the dry seeds was considered as seeds internal moisture level.

In our experiment we studied the penetration of nanoparticles through membrane by FT-IR analysis using KBr (Merck, Germany) pellet technique and used seedlings of S_0 for base line correction.

Incorporation of OMWCNTs within plant tissues was further examined by observing cross-sections of roots of both S_0 and S_4 under light microscope (Leitz Wetzlar Germany; Type 514636).

In order to understand the effect of OMWCNTs on seed membrane integrity we studied the amount of electrolytes and soluble carbohydrate leached out from the seed interior. S_2 was chosen as in this case maximum beneficial effect was observed and S_0, S_1were taken for comparison. For this experiment, 100 seeds with six replicates of each three (S_0, S_1 and S_2) were taken in beakers containing 50 mL of double distilled de-ionized water at 28 ± 2 °C. After 12 and 24 h the seeds were removed and the pooled electrolytes were taken for different experiments.

Electrical conductivity of the pooled leachates (50 mL) was determined by Conductivity meter (Conductivity meter 304, Systronics) and the unit was expressed in terms of μS.

The amount of soluble carbohydrate in the pooled electrolytes was determined according to the McCready et al. procedure (McCready et al. 1950). 1 mL of seed leachates from each sample was taken in a test tube and to it 4 mL freshly prepared, precooled 0.2% anthrone reagent (200 mg anthrone in 100 mL concentrated analar sulfuric acid) was added. After 30 min the intensity of green colour in terms of OD was measured in a UV–Vis spectrophotometer (PERKIN ELMER Lambda 25 UV/VIS Spectrometer, Shelton, CT064844794) at 620 nm. The quantitative estimation was made by comparing the OD values with the standard curve prepared from glucose taken as a standard (Table 4).

Results

T_{50} of germination

The lowest time needed (14 h) for 50% seed germination was in case of S_2 and finally it showed maximum germination percentage (100%). Highest time was needed (29 h) for T_{50} in case of S_6 which also showed lowest germination rate (90%). The time for T_{50} and the percentage of germination in other samples lied in between (Table 1).

Dry weight accumulation

Dry wt (mg/g fresh wt) accumulation increased in case of S_1, S_2, S_3 and S_5 over S_0, while it decreased in case of S_4 and S_6 (Table 1). After 10 days of accumulation dry wt was maximum, i.e. 440 mg/g/fresh wt in case of S_3 followed by S5 (355 mg/g/fresh wt), S_2 (315 mg/g/fresh wt), S_1 (280 mg/g/fresh wt), S_6 (220 mg/g/fresh wt) and in S_4 dry wt accumulation was 200 mg/g/fresh wt, i.e. the lowest.

Shoot and root growth

Mustard seedlings of S_2 and S_3 had longer root and shoot system compared to those of S_0 and S_1. From Table 2 it is clear that after 5 days of observation S_2 showed higher shoot (2.4 cm) and root (3.8 cm) length than all others but after 10 days S_3 developed highest shoot (4.2 cm) and root (5.8 cm) length followed by S_2 and others. However, seedlings of S_4 and S_6 showed negative result on overall growth especially on root growth. Root became much reduced in case of S_4 and S_6 after 10 days of observation.

Germination index (GI) and relative root elongation (*E*)

The data of GI and *E* are displayed in Table 3. The data indicated that after 10 days all the treatments showed positive influence on the GI (i.e. >than control) except in case of both S_4 and S_6. In these two cases negative effect was observed regarding both GI and *E*. However, it should be mentioned that after 5 days both *E* and GI% were maximum in case of S_2 but after 10 days we observed the best percentage of GI and *E* in case of S_3.

Moisture content

The TGA graph of dry seeds and S_3 is shown in Fig. 3 (top). The result indicated that there was significant increase in moisture content in S_2 (63%) compared to dry seeds (6.78%). Details of moisture content of different treatments are depicted in the bar chart (Fig. 3, bottom).

FT-IR analysis

The results of FT-IR showed that there were several similar peaks in both MWCNTs and OMWCNTs. Similarities were also noticed in FT-IR of both root

Table 1 T_{50} of different treatments and the dry wt accumulate in 10 days old seedlings

Treatments	T_{50} of germination		Dry wt accumulation (mg/g fresh wt)
	Time (h)	Percentage of germination (%)	
S_0	28	92	230
S_1	25	92	280
S_2	14	100	315
S_3	22	99	440
S_4	25	92	200
S_5	26	94	355
S_6	29	90	220

Table 2 Root and shoot lengths of 5 days and 10 days old seedlings of mustard seeds

Items	Length of seedlings (cm)													
	5 days old seedlings							10 days old seedlings						
	S_0	S_1	S_2	S_3	S_4	S_5	S_6	S_0	S_1	S_2	S_3	S_4	S_5	S_6
Root	1.3	1.6	3.8	3.3	0.7	3.0	0.6	2.3	2.8	4.5	5.8	0.8	4.8	0.8
Shoot	1.5	1.8	2.4	2.2	1.2	1.8	1.0	2.5	3.2	3.6	4.2	1.8	3.8	1.6

Table 3 Relative root elongation (*E*) and germination index (GI) of different treatments

Treatments	Index for seed germination			
	E (% ± 25.00)		GI (% ± 30.00)	
	5 days	10 days	5 days	10 days
S_0	100.00	100.00	90.00	92.00
S_1	120.07	121.74	122.40	123.05
S_2	292.30	195.65	304.98	204.14
S_3	250.84	252.17	262.86	263.11
S_4	33.84	34.78	32.66	34.02
S_5	200.76	208.69	205.76	213.22
S_6	34.15	34.78	31.14	34.02

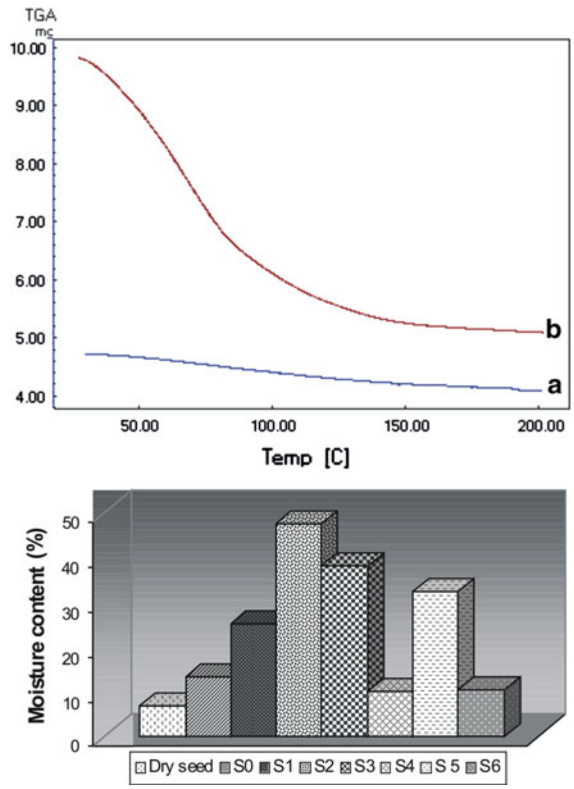

Fig. 3 TGA of **a** dry seeds and **b** S3 (*top*) and bar diagram showing total moisture content of dry seeds and other

and shoot of seedlings of S_3 and S_5. The band at 1571 cm^{-1} was prominent in case of both root of S_3 (Fig. 4, left) and S_5 (Fig. 4, right). However, in case of shoot of S_3 and S_5 this band disappeared indicating the involvement of highly reactive C=O group of OMWCNTs within the biological system of the plant. It is clear from here that both MWCNTs and OMWCNTs are not only being absorbed within the plant body but also are transported through the vascular system of plants. The band at 1,163 cm^{-1} associated with C=O stretching vibration (Kovtyukhova et al. 2003) shifted slightly in root and shoot sample of S_3 also indicating the possible involvement of C=O within the plant body.

Microscopic study

Microscopic images of cross-sections of roots of both S_0 and S_4 are shown in Fig. 5. In S_0, we observed clear root tissue with three tissue systems, i.e. epidermis, cortex and vascular cylinder. However, in case of S_4 the overall tissue system seemed like a black mass with no clear demarcation of three above mentioned tissue systems indicating huge incorporation of OMWCNTs within cells.

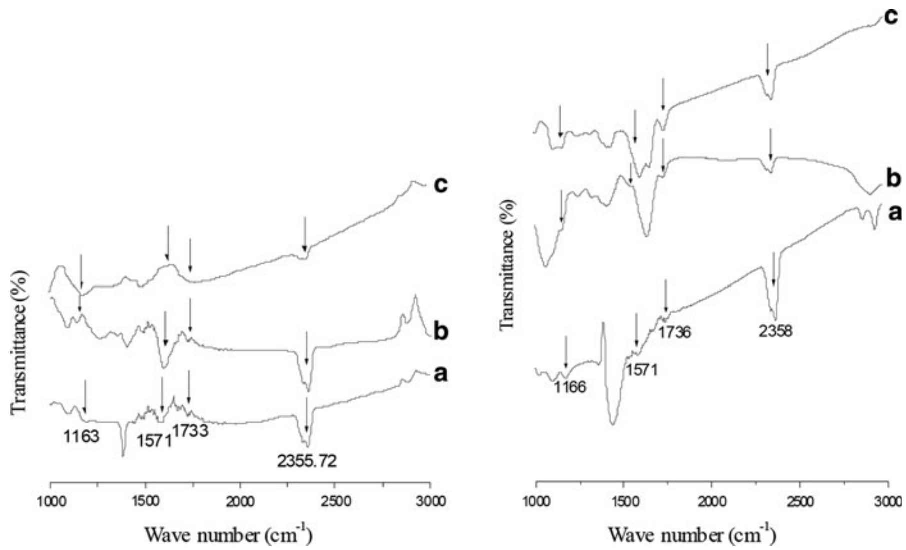

Fig. 4 *Left*: FTIR spectra of (*a*) OMWCNTs, (*b*) root of S3 and (*c*) shoot of S3; *Right*: (*a*) OMWCNTs, (*b*) root of S5 and (*c*) shoot of S5

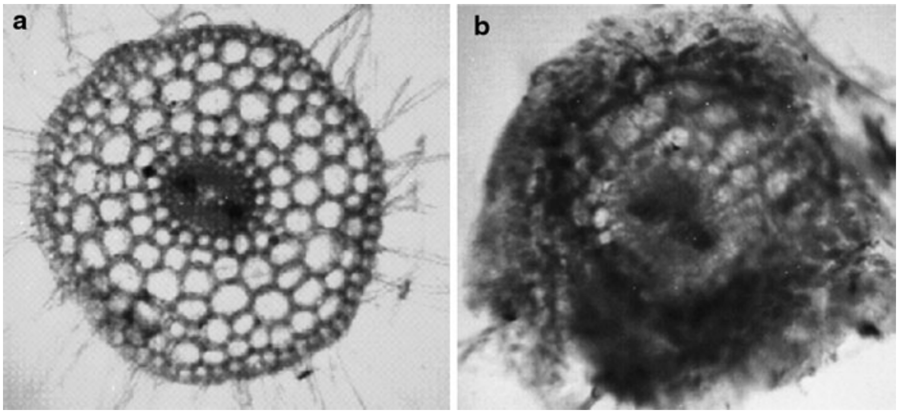

Fig. 5 Cross-section of root of **a** S0 and **b** S4

Table 4 Conductivity reading of S_o, S_1 and the amount of soluble carbohydrates leached out from these seeds

Sample	Conductivity (µs)		Soluble Carbohydrate (µg/g/50 mL)	
	After 12 h	After 24 h	After 12 h	After 24 h
S_0	202	384	5.2	7.84
S_1	167	220	4.2	6.32
S_2	75	146	0.85	1.78

Conductivity test

Conductivity of pooled electrolytes of both 12 and 24 h became lowest in case of S_2 in comparison to S_0 and S_1. In case of S_0 202 µs electrolytes pooled out after 12 h, however, at the same time it was 75 µs in S_2 (Table 4).

Test for soluble carbohydrate

In S_0, the amount of soluble carbohydrate leached out after 12 and 24 h were 5.2 and 7.84 µg/g/50 mL, respectively, which was reduced much in case of S_2. Details are given in Table 4.

Discussion

Our studies have thus indicated enhancement of seed germination rate and plant growth after treatment with CNTs. Zheng et al. (2005) reported that vigour of spinach seedlings germinated from aged seeds increased with application of proper concentration of TiO_2 nanoparticles but did not investigate the cause of this phenomena. However, Khodakovskaya et al. (2009) later addressed that MWCNTs increase the moisture content of seeds, which in turn increased the overall water absorption machinery. Our results also showed that both MWCNTs and OMWCNTs can penetrate the mustard seed coat as well as root tissue cells of the plants; thereby increasing moisture content of seeds and water uptake mechanism of plant tissues. The basic differences between the study performed by our group and Khodakovskaya et al. are as follows. The effect of OMWNTs on mustard seeds is much more prominent than that of MWCNTs on tomato seeds though the concentration of OMWCNTs in our study was lower. In our study we have used distilled water without any nutrients. Had there been any nutrient in the medium, germination rate and growth would have increased, naturally. Moreover, we have studied the leaching of electrolytes and carbohydrate via seed membrane and observed that though OMWCNTs caused increase in water intake, at the same time it hindered leaching of salts and ions by improving the seed membrane integrity. We have also used pretreated seeds (S_0) where the germination rate was the fastest.

Due to the easy penetration capability of OMWCNTs through seed coat they entered within seeds of S_2 also. Other researchers have shown that post imbibitions drying actually advances the onset of germination after subsequent rehydration compared with non-dried controls (Hibbard and Miller 1928). As the T_{50} of S_2 takes less time than others, especially from $S_{0,}$ and S_1, it may be concluded that pre-soaking of seeds in OMWCNTs and subsequent drying allow the soaked seeds to be sufficiently advanced in its germination process. Regarding germination, S_3 showed better results than S_1 but far better result was observed in case of S_2 as OMWCNTs have more active groups such as hydroxyl, carboxyl, carbonyl, etc. than MWCNTs.

In case of OMWCNTs we got quite good response even at low concentration (2.3×10^{-3} mg/mL). But at the same concentration MWCNTs showed no significant effect. At high concentration (46×10^{-3} mg/mL) both of them are toxic. At this concentration the germination rate and growth are hindered for MWCNTs while no seed germinated at all in case of OMWCNTs. The study thus indicates that it is a dose dependent response and thus the experimental concentration range was chosen accordingly.

Dry wt of a plant generally determines its growth and vigour. Maximum dry wt accumulation in case of S_3 signified its maximum growth as well as vigour in comparison to others especially S_2. This can be explained by better growth of S_3 after 10 days than S_2; however, the latter grew much more rapidly up to 5 days than the former. This result is very much effective from agricultural perspective as we can apply this technique for increasing germination rate and vigour of crop plants with a very little concentration of nanoparticles. Our results also showed that in case of both S_4 and S_6 root growth was markedly hindered than shoot growth and as a result overall vigour reduced.

It has been known for over a century that electrical conductivity of a solution becomes high when plant tissues remain immersed in it (Coolbear et al. 1984). Some scientists concluded that decrease in resistance of the imbibing solution was caused by increased permeability of membranes which allow more leaching of carbohydrates, amino acids, soluble nitrogen and salts from seeds (Kammerloher et al. 1994; Tao 1978; Vande Venter and Grobbelaar 1985). When seeds are imbibed in water, internal seed substances like potassium, phosphate, sugar, amino acids, etc. are leached out due to membrane deterioration (Tajbakhsh 2000) and it was also proved that as the membrane damage increased leachate conductivity also increased (Pesis and Timothy 1983). Our result showed that in case of S_2, the process of leaching of both electrolytes and soluble carbohydrates are hindered though water intake increased via aquaporins. This being an added advantage favoured germination rate and growth of plants. The conductivity result thus denoted a fine correlation involving decrease of T_{50} as well as increase of germination rate and growth of plants in case of S_2 instead of S_0 and S_1.

How MWCNTs regulate the metabolic path ways of plant system after entering within seed coat and root tissue is still unknown. As described in ref. 18, increase of water absorption mechanism is supported by the presence of aquaporins and the ability of MWCNTs to regulate gating of these water channels. Aquaporins are water channels that selectively conduct water molecules in and out of the cell, while preventing the passage of ions and other solutes. The function of aquaporins depends on different factors such as pH, concentration of heavy metals, high osmotic pressure, salinity, water channel expression genes such as PIP (Plasma membrane Intrinsic Protein) gene (Johanson and Gustavsson 2002), SIP (Small basic Intrinsic Protein) gene (Liu et al. 2007), etc. Liu et al. (2007) described the importance of PIP genes in rice seed germination. Aquaaporins also can greatly reduce the flow of ions through membranes and regulates the membrane's electrochemical potential (Gonen and Watz 2006). This property of aquaporins is expected to be the cause of increased germination rate and vigour of S_2. The presence of aquaporins in the cell membranes thereby facilitates the symplastic pathway for water transport inside plants, although further study is needed to know whether the involvement of MWCNTs/or OMWCNTs on plant systems create any adverse effect on its later developmental stage of life cycle or not. Moreover, MWCNT has no significant effect at low concentration whereas due to presence of more reactive groups OMWCNT shows distinct effect at very low concentration (2.3×10^{-3} mg/mL). However, only for higher biomass production or increasing of growth rate we can treat the seeds with low concentration of OMWCNTs and for advancing the germination rate of mustard seeds we can just pretreat (soaking-drying treatment) the seeds with low concentration of OMWCNTs.

Conclusion

Thus, we conclude that OMWCNTs not only increase the moisture content of seeds but also enhance the water absorption machinery of root tissues. The MWCNTs/or OMWCNTs can also be transported through the plant vascular cylinder as shown by our experimental results. The positive effects of OMWCNTs on seed germination rate and plant growth have high prospects in agriculture. Plants that are used only for fodder or biofuel can be grown in medium containing OMWCNTs and for this purpose very low concentration of OMWCNTs is highly effective. However, we should remember that high concentration of nanoparticles is harmful to both plant growth and biomass production. Also, we can enhance the germination rate by just soaking–drying the seeds with optimum concentration of OMWCNTs solution with no or a very minor effect of nanoparticles and this method is significantly promising in horticulture, agriculture and biofuel production sectors.

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