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The multi-walled carbon nanotubes induced anatomical and morphological changes in root and shoot of two cultivars of Okra (*Hibiscus escolentus* L.) seedlings

Soroush Kargar Khorrami[1], Rashid Jamei[1*], Reza Darvishzadeh[2] and Siavash Hosseini Sarghin [1]

[1]Department of Biology, Urmia University, Urmia, Iran
[2]Department of Plant Breeding and Biotechnology Faculty of Agriculture Urmia University Urmia, Iran
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Abstract

Carbon nanotubes (CNTs) are known to have many unique physical and chemical properties. Because of these features, they accelerate the germination process, root growth, and photosynthesis rate that can result in increased crop productivity. In the present study, the effects of 4 multi-walled carbon nanotubes (MWCNTs) levels including 0 (control), 50 (low concentration), 100 (moderate concentration), and 200 (high concentration) mg/l were evaluated on morphological and anatomical characteristics of stem, root and leaf in two Okra cultivars namely Bamia and Emerald. In both cultivars, the value of height and biomass of shoot and root increased after addition of 50 and 100 mg/l MWCNTs. Based on microscopic results, the root and shoot diameters in both cultivars more affected by increased cortex thickness and central cylinder in 50 and 100 mg/l of MWCNTs treatments whereas these parameters were more affected by increased cortex thickness in the high concentration of MWCNTs. Our study indicated that the thickness of mesophylls and spongy layers increased in low level of MWCNT, whereas these parameters were decreased in moderate and high levels of MWCNT in both cultivars. The statistical analysis showed that Stomata size increased value in low and moderate levels of MWCNT in Bamia cultivar. In this study Stomata index measurement showed that this parameter was increased in moderate and high levels of MWCNT only in Emerald cultivar. We observed that diameter of xylem and phloem in the shoot were increased in low (50 mg/l) and moderate (100 mg/lit) levels of MWCNTs whereas these parameters were decreased in high MWCNT level. Although this study revealed that low treatments of MWCNTs caused an increase of root xylem diameter in both cultivars.

Keywords: Anatomy. Biomass. Carbon nano-tubes. Dried weight. Fresh weight. Okra (*Hibiscus escolentus* L.).

Introduction

Carbon nanotubes (CNTs) are known to have many unique characteristics in view of diameter, length, atomic configuration, impurities, defects and functionality, which allow them were widely used in materials, chemicals, food, bioengineering, medicine, and other fields (Salata, 2004; Dora *et al.*, 2014). Because of these physicochemical features they used in modern agriculture systems to increase crop productivity. They accelerate germination process, root growth, and photosynthesis rate resulting in increased productivity in crop plants (Salata, 2004). The stimulate positive, negative and natural effects of CNTs on physiological responses of plants can variable or even opposite among different plant species (Elena *et al*., 2012). For example, in some studies, it has been showed that the multi-walled carbon nanotubes (MWCNTs) did not affect the growth of wheat (Wild *et al*.,2009) or they inhibited the growth of rice seedlings (Lin*et al*., 2009). In other hand, in tomato it has been showed that they significantly enhanced the germination rate of plant (Khodakovskaya *et al*., 2009; Srivastava *et al*., 2014). Khodakovskaya *et al*., (2009) has reported that CNTs could penetrate in plant seed coat and dramatically affect its germination and growth. However, the penetration process, uptake and accumulation of CNTs in plant cells and tissues are not well documented (Smirnova *et al*., 2012). Plants showed high tendencies to accumulate CNTs (Khodakovskaya *et al*., 2012; Liu *et al*., 2009). Recently, Mariya *et al*., (2014) revealed that MWCNTs can be absorbed by tomato root system and transferred to leaves and fruits. However, Lin *et al*., (2009) reported that accumulation of an extensive amount of MWCNTs on root surface may suppress the water influx and uptake of nutrients hence inhibiting the plant growth (Lin *et al*., 2009). In parallel, investigations have shown that CNTs could induce phytotoxicity in plant cells and change the expression amount of some genes (Khodakovskaya *et al*., 2011). Biochemical studies on different plants have demonstrated that the exogenous use of CNTs can induce the production and accumulation of oxygen reactive species (ROS) such as hydroxyl radicals and superoxide radical anions (Christie *et al*., 2006;

*Corresponding Author, Email: r.jamei@urmia.ac.ir

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Ghorbanpour *et al*., 2015). ROS generation can lead to protein, lipid and DNA oxidation and so they are stressful to plants (Apel *et al*., 2004). Literature review on CNTs application in biosciences show that the previous efforts have mainly focused on their influence on animal and human cells, but it is necessary to investigate the potential effects of CNTs on the plant cells in the natural environment. Liu *et al*., (2009) reported that CNTs penetrate inside the cells. Insertion of MWCNTs into epidermal cells wall and hairy root was observed in wheat seedlings (Wild*et al*., 2009). Khodakovskaya *et al*., (2011) showed that MWCNTs treatment caused significant enhance in tobacco growth and development by regulating cell division as a result of activating water channels and regulating of genes involved in cell division and extension (Wild*et al*., 2009). According to Serag*et al*., (2011) reports, MWCNTs with short length (in the range of 30 to 100 nm in length) tend to target the nucleus, plastids, and vacuoles which further strengthened the close relationship between MWCNTs size and phytotoxicity to plant cell. Okra (*Hibiscus esculents* L.) belongs to the family Malvaceae, and grows within all seasons in the tropics and during summer in the warm parts of the temperate regions. Okra is considered as a popular home garden vegetable and it is a good source of many nutrients including vitamins B and E, fiber calcium, starch, protein, carbohydrate, carotene, thiamin, riboflavin, niacin and iron (Hegazi and Hamideldin 2010). However, several reports indicate positive and negative effects of B on the physiological and biochemical characteristics of the plants. but there are few studies on anatomy changes by CNTs in plants. Therefore, pay attention to the fact that the study of anatomical changes in plants can help to better understand the effects of different materials and especially nanoparticles on cell structures and superstructures.

Results of this study may help plant scientists to understand morphological and anatomical behaviors plants including Malvaceae family to nanoparticles and Especially MWCNTs.

Materials and methods

Preparation of MWCNTs: MWCNTs were prepared from Nano-sany Company (Iranian Nanomaterials Pioneers Company, Mashhad, Iran). Specifications of carbon nanotubes were detected by using scanning electron microscope (SEM) (Hitachi S-4160, Tokyo, Japan), and the X-ray diffraction (XRD) (Philips-X'Pert MPD X-ray refractometer) technique. Raman spectra of the MWCNTs with OD less than 50 nm was prepared using UV–Vis spectrophotometer (T80+ UV–VIS spectrophotometer PG instruments Ltd, UK). The metals content of MWCNTs were detected by energy dispersive X-ray spectroscopy analysis (Figure 1 and table 1).

Seed germination and seedling growth: Seeds of Okra cultivars (Bamia and Emerald) were purchased from Avan Mashregh Zamin Company (www.avanmz.ir). Okra seeds were surface-sterilized using sodium hypochlorite (10%, 15 min) and soaked in distilled water for 24 h, then germinated on moist filter paper at 25°C in dark for 4 days. The homogenous seedlings were transferred to plastic pots, containing 2 Lit Hoagland (pH of the medium was adjusted at 6.8-7.0 with HCl or NaOH) that completely were changed every day. The seedlings were grown hydroponically in controlled conditions (growth chamber) with diurnal regime of 16 h light at 25 ± 2°C and 8 h dark at 19 ± 3°C, under florescent white and yellow light (150 $\mu mol.m^{-2}.s$) .

Treatment of seedlings: For the treatment of seedlings, MWCNTs were suspended using ultrasonication as described by Mansour Ghorbanpour (2015). Briefly, nanomaterials were added to distilled water and dispersed using ultrasonic vibration (Elmasonic) for 40 min, (Ghorbanpour *et al.,* 2015). Stock solutions of MWCNTs (1000 mg MWCNT in 1Liter Hoagland solution) were diluted with Hoagland nutrient solution to final concentrations (0, 50, 100 and 200 mg/l) right before use as described by Parvin*et al*., (2012). After 12 days of hydroponic culture, seedlings were harvested for measuring fresh and dried weight of biomass and length of shoot and root. All experiments were performed with three replications.

Measure the anatomical parameters: After sampling the plant seedlings, the roots and shoots samples were immediately fixed in glycerol and ethanol (1:2). Cross-sections of stems, roots and leaves were cut from same positions by hand. Sections were cleared in sodium hypochlorite and stained by carmine-vest (1% w/v in 50% ethanol) and methyl green (1% w/v, aqueous). Cross-sections was observed under light microscope (Zeiss) and photographed by digital camera (SONY, DSC-W35) (Hajiboland *et al.,* 2012). All anatomical measurements were done with 3 repeats for each part.

Statistical Analysis: For all variables, the two–way analysis of variance (ANOVA) was performed using the GLM procedure in SPSS software to test any significant differences between Okra cultivars and CNTS treatments as well as their interaction. Duncan's test was used to treatments mean comparisons.

Results and Discussion

Growth responses: MWCNTs in the nutrient solution result in noticeable and similar growth responses in two Okra cultivars. Based on our observations, using MWCNT in nutrient solution lead to increases in shoot and root length compared to the control groups. An important increase was observed in shoot length at 50 mg/lit MWCNT treatment in both Okra cultivars. But shoot length significantly diminished in Emerald cultivar after 200 mg/lit MWCNT application (Fig2a). In Bamia cultivar the root length significantly increased after 100 and 200 mg/lit of MWCNTs treatments in comparison to control plants whereas concerning to

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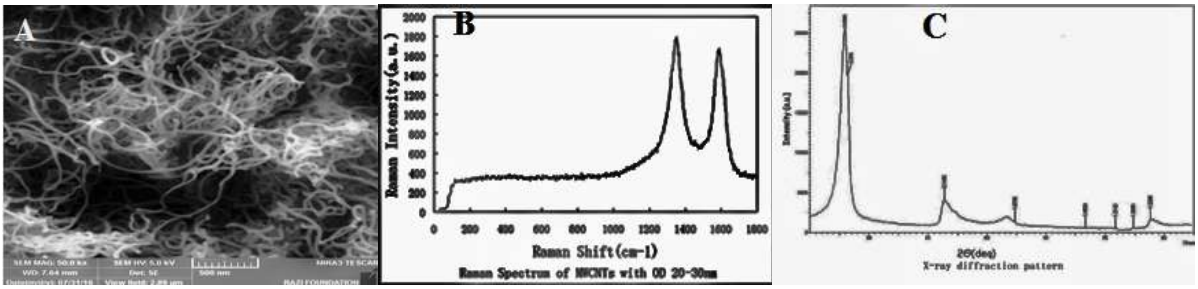
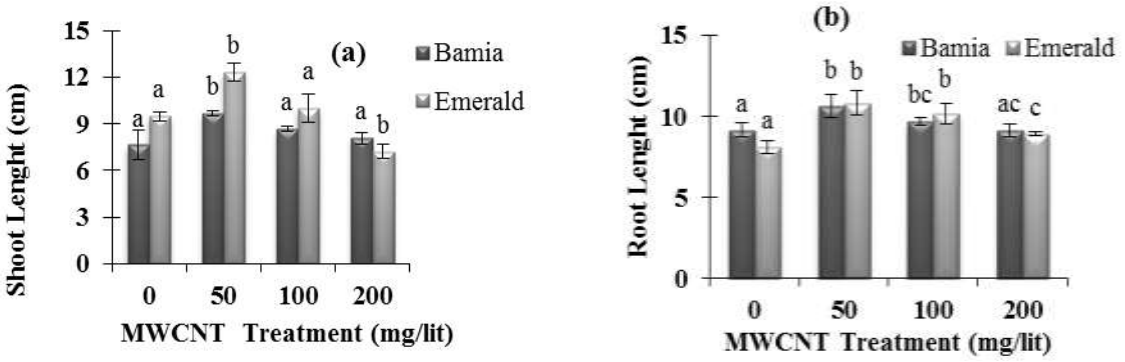


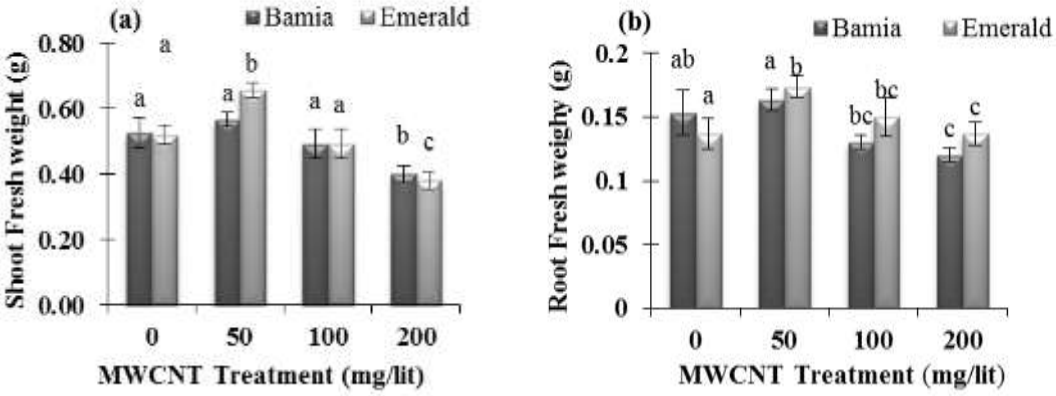
Figure 1: Scanning electron microscope image (A), Raman spectra (B) and XRD pattern (C) of the MWNTs.

Table 1. The amount of elements available in MWCNT determided by energy dispersive X-ray spectroscopy analysis

Multi wall nanotube (MWNT), >95 wt%			
Elements	Percentage	Elements	Percentage
Carbon	97.46	Cobalte	1.09
Aluminium	0.19	Sulphur	0.24
Chlorine	1.02		



Figur 2. Effect of multi-walled carbon nanotubes (MWCNTs) on shoot (a) and root (b) length of two cultivars of Okra seedlings. Means followed by different letters differ significantly determined by Duncan test. (Significant difference at P<0.05).



Figur 3. Effect of multi-walled carbon annotates (MWCNTs) on shoot (a) and root (b) fresh weight of two cultivars of Okra seedlings. Means followed by different letters differ significantly determined by Duncan test. (significant difference at P<0.05).

Emerald cultivar root length was significantly affected by all levels of MWCNTs (Fig2b). According to Figure2, the highest shoot and root lengths were observed at 50 mg/lit MWCNTs treatment in both Okra cultivars.

The results of MWCNT effect on fresh and dried weight of shoots and roots of two Okra cultivars are depicted in Figs3a and 3b. Statistical data revealed that the shoot fresh weight of Bamia cultivar was significantly increased at 50 mg/lit level of MWCNT application whereas it was significantly decreased in both cultivars at 200 mg/lit levels of MWCNT. Similar results were obtained when shoot dried weight of two cultivars were measured.

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The application of 50 mg/lit MWCNTs within nutrient solution lead to significant increase in the root fresh weight of Okra cultivars compared to control plants. It is interesting to note that the root fresh weight showed significant reduction in Bamia cultivar at 100 and 200 mg/lit of MWCNTs while these amount of MWCNTs lead to increase in the root fresh weight of Emerald cultivar. Similar effect was obtained when root dried weight of both cultivars measured and compared to the control groups (Fig4a, 4b).

As shown in this study, the plant height and biomass of shoot and root of both Okra cultivars increased after addition of CNTs (50 and 100 mg/lit) to medium culture that are in agreement with those of, Haghighi *et al.*, (2014), who exposed four vegetable species to different concentration of CNTs for two weeks. Use of CNTs stimulate water flux and uptake of ionic nutrients that may be explain, how to stimulate growth by CNTs? as Tiwari *et al*., (2014) discovered for maize (*Zea mays* L.). Liu *et al*., (2009) reported that CNTs can act as molecular channels for water. Also Wang *et al*., (2012) suggested that o-MWCNTs can significantly enhance root dehydrogenase activity, which in turn enhances the ability of water uptake of the seedlings. A number of investigations have indicated that the expression of genes encoding aquaporin protein considerably up-regulated in plant cells exposed to multi-walled carbon nanotubes (Khodakovskaya *et al*., 2012). However, in this study when a 200 mg/lit of MWCNTs was used, Emerald cultivar showed the least height and shoot and root biomass. Different studies have reported harmful effects of high MWCNT levels on growth of plant, for example, Haghighi *et al*., (2014) reported that use of high level of CNTs decrease fresh and dried weight as well as length of seedling in radish and turnip, confirming the possibility of its toxic effect resulting from a high level of CNTs. Parvin *et al*., (2012) indicated that high level of MWNTs cause cell death and membrane damage in red spinach, lettuce, rice, and cucumber plants after 15 days of exposure that suggest MWNTs may induce ROS formation promoting cell death and electrolyte leakage in the different plant organs. Our results suggest a direct correlation between shoot and root growth parameter variation with different concentration of MWCNTs in both studied Okra cultivars. Also, we found the impact of Okra genotypes in repose to MWCNTs. Plasticity in anatomical characteristics able plants to overcome environmental hazards so that successfully survival and continue their growth and development. nevertheless, its negative effects on plant growth characteristics particularly at higher levels support the possibility of its stressful to plants. The data of other studies support this conclusion as well (Christie *et al*., 2006; Apel et al., 2004). However, plants do not have negative responses to stress conditions (Pourranjbari Saghayesh and Souri, 2018). It is well known that natural and synthetic organic materials have the ability to absorb charged nutrients, and the carbon nanotubes may actively stabilize some nutrients of the medium, so depriving plant's root from their normal accessibility (Souri *et al*., 2018).

Anatomical structures of stem, leaf and root: The results concerning the anatomical changes of stem are shown in Table 2. It was observed that all MWCNTs treatments specifically changed the stem anatomy of studied Okra cultivars. Microscopic results revealed that all MWCNTs levels significantly increased stem diameter of Bamia cultivar whereas concerning to Emerald cultivar it was significantly increased by 50 and 100 mg/lit MWVNTs application compared to the control plants (Tab2 and Fig 5). Statistical data revealed that all MWCNTs treatment levels lead to significant increase in cortex thickness of studied Okra cultivars compared to the control plants.

In both cultivars the central cylinder diameter of stem showed a remarkable increase at 50 (Fig 5B, F) and 100 (Fig 5C, G) mg/lit MWCNTs treatment levels. While it was significantly reduced in both cultivars treated by 200 mg/lit MWCNTs (Tab 2 and Fig 5 D, H).

According to Table 1, the thickness of stem xylem showed remarkable decline under 200 mg/lit MWCNTs treatment in both cultivars (Fig 5 D, H), but it was significantly increased in comparison with control plants when 100 mg/lit MWVNTs was applied (Fig 5).

Microscopic results showed that 50 and 100 mg/lit of MWCNTs levels induced an increment in phloem size in Emerald cultivar, but there was not seen any significant difference among 200 mg/lit treatment and control plants when they were compared (Fig5).

According to Table 2, layer numbers of stem significantly increased only in cultivar Bamia cultivar at 50 and 100 mg/lit MWCNTs levels, but it did not show any significant change in Emerald cultivar under MWCNT usage when compared with control plants (Fig 5).

The results indicated that MWCNT lead to significant changes in leaf thickness, petiole diameter, length of mesophyll cells, Central midrib diameter in both studied Okra cultivars in comparison to control plants. Indicated that leaf thickness (Tab3) of Okra plants generally decreased in parallel with increasing the amount of MWCNT level. In Bamia cultivar, leaf thickness did not show any significant increase in 50 mg/ml MWCNTs level (Fig 6B), but it significantly decreased at the highest level of MWCNTs (200 mg/lit). However, it showed significant increase in Emerald cultivar in 50 mg/lit MWCNTs level when compared with control plants (Tab3 and Fig 6F). significantly increased in Bamia and Emerald cultivars only in 50 mg/lit MWCNTs level (Fig 6 B, F), but it was decreased in high concentration of MWCNT level. Based on our results Central midrib diameter of Bamia did not show any significant decrease at the highest level of MWCNT (100 and 200 mg/lit), however concerning to Emerald cultivar it shows significantly decrease in 200 mg/ml MWCNTs level compared to control plants (Tab 3 and Fig 6)

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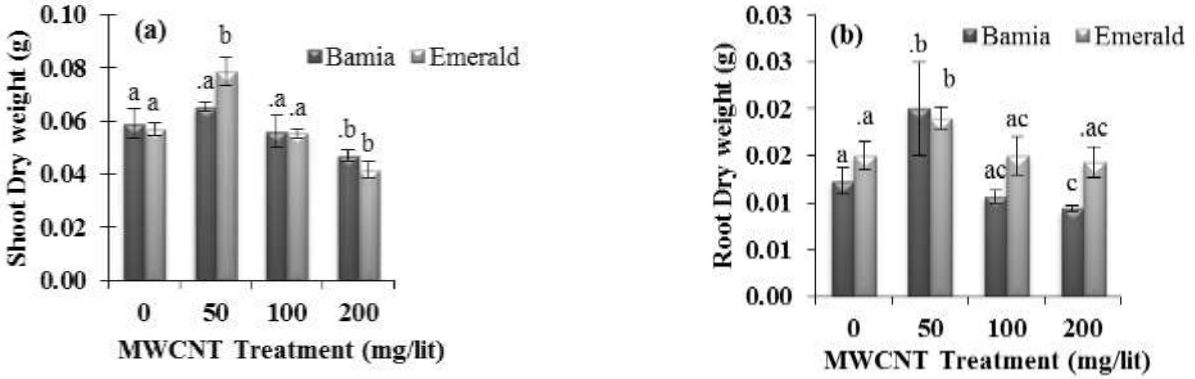
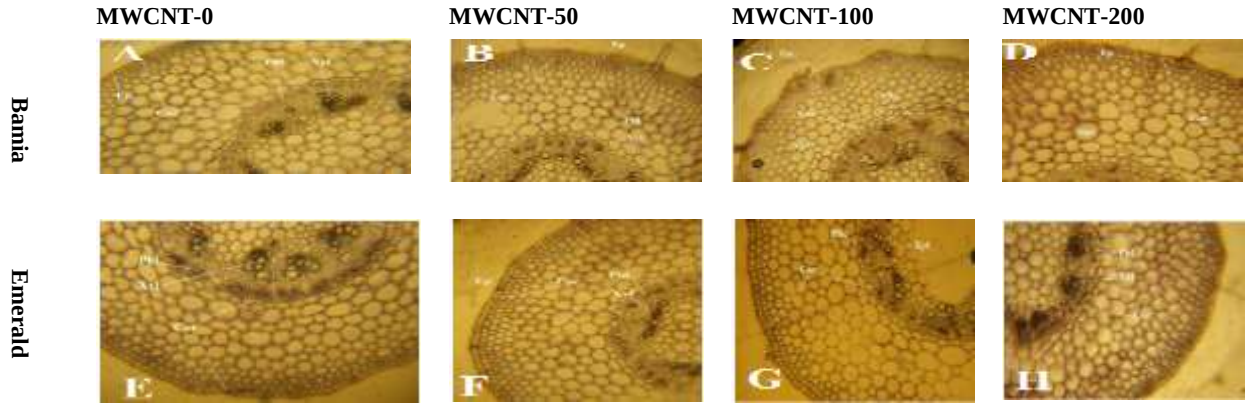


Figur 4. Effect of multi-walled carbon nanotubes (MWCNTs) on shoot (a) and root (b) dried weight of two cultivars of Okra seedlings. Means followed by different letters differ significantly determined by Duncan test. (significant difference at P<0.05).

Table 2. Effect of multi-walled carbon nanotubes (MWCNTs) on stem anatomy of two cultivars of Okra seedlings

Characters	Bamia				Emerald			
	MWCNT Treatments (mg/lit)				MWCNT Treatments (mg/lit)			
	0	50	100	200	0	50	100	200
Diameter of stem	1696.00± 64a*	1920.00± 18.47bd	2101.33± 59.38c	1930.66± 28.22d	1642± 46.49a	2005± 38.45bc	1994± 56.44c	1792± 48.88a
Thickness of cortex	440.66± 20.81a	526.33±1 2.01cd	573.33± 23.33c	483.83± 8.81d	446.66± 17.63a	530± 15.26b	553.33± 14.52c	503.33± 8.81ab
Diameter of epiderm	776.66± 14.52a	823.33± 12.01b	840 ± 15.27c	716.66± 18.55ac	650± 11.54a	796.66± 14.52ab	710± 25.16b	663.33± 18.55c
Diameter of xylem	33.33± 3.00ab*	35.83± 2.20ab	43.33± 2.21 d	23.33± 3.63b	30 ± 2.5a	30.83± 2.20ab	35 ± 2.88b	23.33± 2.20d
Diameter of phloem	20.83± 0.83a*	21.66 ± 0.83a	17.5 ± 1.44a	12.5 ± 1.45a	8.33 ± 0.83a	9.1 ± 0.84ab	9.16 ± 0.83b	8.33 ± 0. 83a
Layer number	10.66± 0.33 a	12.66 ± 0.33b	12 ± 0 .33b	11.66 ± .3a	10.66± 0.33a	11.66 ± 0.33a	11.66± 0.33a	11.33 ± 0.3a

Means followed by different letters differ significantly determined by Duncan test. (significant difference at P<0.05).



Abrivate: Xyl = xylem; Phe =pheloem ; End = Endoderm ; Ep = Epederm Cor = Cortex; Pre = precycle. Bamia (A-D) and Emerald (E-H)

Figur 5. Effect of multi-walled carbon nanotubes (MWCNTs) on stem anatomy of two cultivars of Okra Seedlings. Scale bars correspond to 100 µm

Thickness of mesophyll layer showed significant decrease in both Okra cultivars at 100 (Fig 6 C, G) and 200 (Fig 6 D,H) mg/lit MWCNT level. Significant increase of this parameter was observed alone in 50 mg/lit of MWVNTs compared to control plants. According to Table 3, thickness of spongy layer of leaf was not significantly different in two cultivars with that in control groups (Tab3 and Fig 6).

In both Okra cultivars, MWCNTs did not impose significant change in the petiole length, however the width of petiole significantly decreased in Bamia in 100 and 200 mg/lit levels of MWCNTs (Fig 6 C, D). This parameter increased in Emerald in 50 mg/lit level in comparison to control groups (Tab3 and Fig 5F).

Microscopic results revealed that stomata length did not significantly change with an increase in MWCNTs

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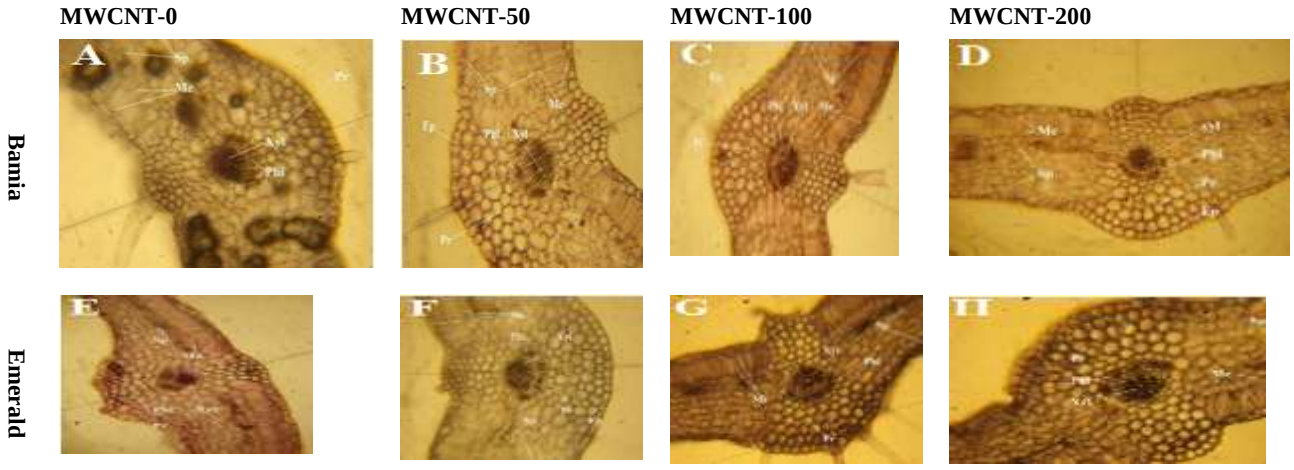
Table 3. Effect of multi-walled carbon nanotubes (MWCNTs) on leaf anatomy of two cultivars of Okra seedlings.

Characters	Bamia				Emerald			
	MWCNT Treatments (mg/lit)				MWCNT Treatments (mg/lit)			
	0	50	100	200	0	50	100	200
Thickness of leaf	396.66 ± 8.81a	426.67± 17.63a	383.34± 20.27a	333.3± 8.81b	350± 17.32a	430± 11.54b	326.67± 8.8a	316.6±3 .34a
Central midrib diameter	673.3±17. 73ab	683.34± 14.52b	636.67± 8.82ac	630± 11.54ac	616.6± 17.6a	666.7± 8.8b	603.333±1 2.01a	540± 15.27c
Length of mesophyll cell	136.67±8. 8a	143.33± 6.67a	103.33± 8.81c	100± 5.78c	96.6± 8.8a	113.34± 3.3a	73.33± 3.4b	72.9± 3.3b
Thickness of spongy layer	186.66±8. 09a	193.3± 8.81a	173.35± 3.3a	166.65± 12.01a	200± 15.27ab	210.1± 17.32b	173.3± 3.3ab	188.3±7 .26a
width of petiole	1077.3±38 .45a	1162.6±2 8.22a	970.66±10. 67b	885.33±28 .22b	938.6± 38.5a	1056±18.5 b	962.65±22 .78ba	880.35± 21.4a
length of petiole	1888± 121.15ab	2101± 59.38b	1962.66±6 4.89ab	1930.67±4 8.93a	1685.3± 83.4a	1632±18.4 7a	1589.34±4 6.49a	1525.4± 46.5a
Stomata length	63.4± 0.84ac	68.3± 3.63ab	69.16± 4.40ab	55.83±3.0 0c	65.83± 2.2a	68.3± 7.63a	63.34± 2.2a	59.16±2 .3a
Stomata index (0.5 mm_2)	20± 2.33a	22.66± 1.33a	25.33± 2.66a	22.66± 2.3a	17.33± 1.33a	21.33± 1.33ab	24±2.30b	22.66±1 .33b

Means followed by different letters differ significantly determined by Duncan test. (significant difference at P<0.05).



Abrivate: Xyl = xylem; Phe =pheloem ; End = Endoderm ; Ep = Epederm Cor = Cortex; Pr = parenchym. Bamia (A-D) and Emerald (E-H).

Figure 6. Effect of multi-walled carbon nanotubes (MWCNTs) on leaf anatomy of two cultivars of Okra seedlings. Scale bars correspond to 100 µm.

levels on growth medium in both studied cultivars. Although our results showed that the stomata index of both Okra cultivars increased in all MWCNTs treatments, but the significant increased amount of this parameter was observed only in Emerald cultivar in 100 and 200 mg/lit when compared with control groups (Tab3 and Fig 6 G, H).

The effects of MWCNTs treatment on root anatomy of two Okra cultivars seedlings are shown in Table 4. The statistical data showed the root diameter significantly increased in Bamia cultivar by 100 and 200 mg/lit MWCNTs levels application, but any significant increase did not observe in 50 mg/lit MWVNT application when compared with the control plants (Tab 4 and Fig7 C, D). Also, in Emerald cultivar, the significant increase of root diameter was observed only in 100 mg/lit MWCNT level, however significant different responses of this cultivar were observed in 200 mg/lit treatment when compared with the control plants (Tab 4 and Fig 7 G,H).In two studied Okra cultivars the root cortex thickness significantly increased at 50 and 100 mg/lit levels of MWCNT, but this parameter was significantly reduced when the Okra seedlings were treated with 200 mg /lit MWCNTs in both cultivars.

We observed that central cylinder diameter of Bamia cultivar significantly increased at all levels of MWCNTs, but in Emerald cultivar this parameter significantly changed in different manners in 50 and 100 mg/lit treatments when compared with control plants (Tab 4 and Fig 7 F, G).

According to Table 4, xylem diameter significantly increased in Emerald cultivar at 100 and 200 mg/lit MWCNTs levels, but in Bamia cultivar this parameter significantly increased only in 100 mg/lit treatment when compared with control plants. Also, significantly diminution in phloem diameter was observed in 100 and

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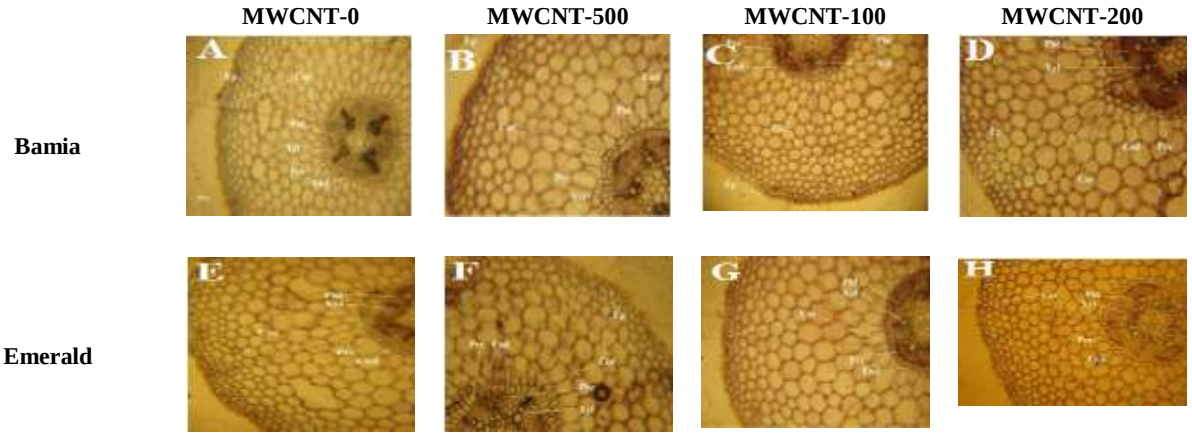
Table 4. Effect of multi-walled carbon nanotubes (MWCNTs) on root anatomy of two cultivars of Okra seedlings.

Characters	Bamia				Emerald			
	MWCNT Treatments (mg/lit)				MWCNT Treatments (mg/lit)			
	0	50	100	200	0	50	100	200
Diameter of root	1940±43.58a	1980±45.82a	2230±26.45b	1995±47.5b	1960±52.91ab	2050±36.05b	2290±36.1c	1700±26.45a
Thickness of epiderm	516.67±20.27a*	680±17.32ab	773.33±26.03b	590±20.81c	716.7±14.52a	756.66±18.56b	790±17.32c	610±11.54d
Diameter of cortex	423.33±14.52a*	543.33±12.01b	616.67±24.03c	493.16±14.53b	510±17.32a	593.33±14.52b	576.67±24.03b	536.6±18.56a
Diameter of xylem	34.16±2.2a	35.83±2.3a	43.33±2.2b	32.95±2.47a	30.83±0.83a	36.67±2.2b	35.83±0.84b	30±1.44a
Diameter of phloem	15.83±.84a*	16.67±.83a	12.5±1.44c	10.83±.83c	9.16±0.85a	11.67±1.67a	11.66±0.83a	8.33±0.83a
Layer number	11.66±.34a	12.67±.84a	13.33±.33a	11±.57a	12.33±0.33a	11.66±0.33a	12.66±0.33a	12.33±0.33a

Means followed by different letters differ significantly determined by Duncan test. (significant difference at P<0.05).



Abrivate: Xyl = xylem; Phe =pheloem ; End = Endoderm ; Ep = Epederm Cor = Cortex; Pre = precycle. Bamia (A-D) and Emerald (E-H).

Figure 7. Effect of multi-walled carbon nanotubes (MWCNTs) on root anatomy of two cultivars of Okra seedlings. Scale bars correspond to 100 µm

200 mg/lit MWCNTs treatments in Bamia cultivar.

The statistical analysis showed that cell layer numbers of root did not significantly changed in both cultivars of Okra by MWCNTs treatments application when compared with control plants (Tab 4 and Fig 7).

Very little is known about the effects of MWCNTs on plants at the anatomical level. We have evaluated different anatomical and morphological indices on shoot, root and leaf for understanding which sections are more affected by WCNT treatments. In this survey we found that increased amount in root and shoot diameters in both cultivars after treatment by low and moderate MWCNTs is due to increase in cortex thickness and central cylinder. But in high concentration of MWCNTs, these parameters were more affected by increasing in cortex thickness. Based on Fig 5, parenchyma cells size of cortex area showed higher thickness in high concentration of MWCNTs. Yan *et al*., (2013) observed SWCNTs in the intercellular space and mainly in the root cortex. The effects are more or less similar to those effects of nutrient particularly boron (Tohidloo and Souri, 2009) and nitrogen or magnesium shortages (Pourranjbari Saghayesh and Souri, 2018).

Furthermore, investigation on xylem and phloem diameters of shoot revealed that their diameters increased in low (50 mg/lit) and moderate (100 mg/lit) MWCNT treatments, but they decreased post high MWCNT treatment. Concerning to root tissue, this fact was determined that the low amount of MWCNT result in increasing the xylem diameter. However, root phloem diameters in MWCNTs treatment did not show any significant changes in comparison with control plants in both studied cultivars. The tissue samples of Blackberry that had been treated by SWCNTs-COOH (4 µg/ml) showed a vascular cambium with mature xylem and presence of phloem cells; further more xylem vessels have developed completely and did not found any evidence for the presence of cytoplasmic content (Dora *et al*., 2014). Developed meta xylem vessels in the stem play an important role for better transport of water and minerals (Steudle, 2000).

It has suggested that insertion of carbon nanotubes into the plant tissues result in changes in plant development via regulating gene expression and related signal pathways as well as physiological effects (Lin,

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2009). Mariya showed that MWCNTs affect the expression of genes regulating cell division and cell wall extension in treated cells that result in faster growth than the unexposed control cells. Villagarcia *et al*., (2012) suggested the existence of different molecular mechanisms for activating cell growth via nanosized MWCNTs. They founded the expression of essential genes for cell-wall assembly/cell growth such as NtLRX1, as well as for regulation of cell cycle progression such as Cyc B significantly and rapidly induced by MWCNTs in tobacco cells.

The study of leaf anatomy showed that the leaf thickness was increased post low MWCNT treatment whereas it decreased in both cultivars by increasing MWCNT amount (moderate and high levels).

Our study indicated that mesophyll and spongy layer increased in low level of MWCNT and decreased similarly in both cultivars post moderate and high levels of MWCNT applications. Also, stomata size increased in low and moderate level of MWCNT in the Bamia cultivar. However, increase in stomata size was observed alone in low level of MWCNT in Emerald cultivar. Moreover, stomata index measurement showed that this parameter increased in moderate and high levels of MWCNT only in Emerald cultivar.

Whereas carbon nanotubes increase water absorption in plants, correlation between the content of the absorbed water with increased stomata index and stomata size may enhance stomata conductance that cause greater transpiration and water-use. According to Melo *et al*., (2007), the increase in stomatal density, coupled with the decrease in stomatal size, would be an alternative to adequate supply of CO_2 for photosynthesis, without excessive water loss due to stomata with smaller pores. This may be adaptation phenomena of plants in response to toxicity. The physiological and anatomical determination in this study also support that may some levels of physical or biochemical stress via nanotubes was exerted on plants resulting in several growth responses to applied materials. Changes in nutrient bioavailability (Tohidloo and Souri, 2009; Souri et al., 2018; Pourranjbari Saghayesh and Souri, 2018) as well as water absorption and translocation due to damaged xylem vessels (Souri *et al*., 2009) are probably involved in this regard. Yuan *et al*., (2011) reported that SWNTs can enter into intact Arabidopsis mesophyll cells, and then enter into the organelles such as chloroplast, vacuole, mitochondria and nucleus. So, it is possible that changes of mesophyll and spongy layers of Okra plants have direct correlation with MWCNTs.

Conclusions

In summary, in this study the results showed that MWCNT in the low concentration and in some cases in the medium concentration caused positive effects on morphological and anatomical characteristics (shoot and root) of both Okra cultivars. However, with increasing concentration of MWCNT, these effects appeared negatively in of both Okra cultivars. Also we observed that in many cases have been investigated; the positive effects of the MWCNTs were in the emerald cultivar more than the Bamia cultivar. Therefore, it can be concluded that positive effects of MWCNTs may be due to promote water absorption, cell division and elongation in of both Okra cultivars. Also negative effects of MWCNTs on both Okra cultivars may be due to destructive effects of MWCNTs on different cell parts, especially membranes and biological organs. So, for more understanding the plants anatomical response to MWCNTs, we offer that cellular Infrastructure changes, biochemical and physiological response of plant to MWCNTs should increase.

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