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

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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 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, configuration, impurities, atomic 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 (Linet 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;

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 (Wildet 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 (Wildet al., 2009). According to Seraget 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 $25 \pm 2^{\circ}$ C and 8 h dark at 19 \pm 3°C, under florescent white and yellow light (150 μ 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 Parvinet 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



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 na	anotube (MWN	T), >95 wt%		
Elements	Percentage		Elements	s Percentage
Carbon	97.46		Cobalte	1.09
Aluminium	0.19		Sulphur	0.24
Chlorine	1.02			
Shoot Lenght (cm) - 12 - - 6 - - 6 - - 9 - - 0 - - 0 -	b a b a a ∎ 0 50	a (a) a a b a b a b a b a b a b a b a	■Bamia ■Emerald	$ \begin{array}{c} 15 \\ 10 \\ 10 \\ 10 \\ 5 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ $

MWCNT Treatment (mg/lit) 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.

MWCNT Treatment (mg/lit)

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.

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 upregulated 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)



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).



Characters	's Bamia					Emerald				
	MWCNT 7	Freatments (mg	y/lit)		MWCNT Treatments (mg/lit)					
	0	50	100	200	0	50	100	200		
Diameter of	$1696.00 \pm$	$1920.00 \pm$	2101.33±	1930.66±	1642±	2005±	1994±	1792±		
stem	64a*	18.47bd	59.38c	28.22d	46.49a	38.45bc	56.44c	48.88a		
Thickness of	$440.66 \pm$	526.33±1	573.33±	$483.83 \pm$	446.66±	530±	$553.33 \pm$	503.33±		
cortex	20.81a	2.01cd	23.33c	8.81d	17.63a	15.26b	14.52c	8.81ab		
Diameter of	776.66±	823.33±	$840 \pm$	716.66±	$650\pm$	796.66±	710±	663.33±		
epiderm	14.52a	12.01b	15.27c	18.55ac	11.54a	14.52ab	25.16b	18.55c		
Diameter of	33.33±	$35.83\pm$	43.33±	23.33±	30 ±	30.83±	35 ±	23.33±		
xylem	3.00ab*	2.20ab	2.21 d	3.63b	2.5a	2.20ab	2.88b	2.20d		
Diameter of	20.83±	$21.66 \pm$	17.5 ±	$12.5 \pm$	8.33 ±	9.1 ±	9.16 ±	$8.33 \pm$		
phloem	0.83a*	0.83a	1.44a	1.45a	0.83a	0.84ab	0.83b	0. 83a		
Lavor number	10.66±	$12.66 \pm$	12 ±	11.66 ±	10.66±	11.66 ±	11.66±	11.33 ±		
Layer number	0.33 a	0.33b	0.33b	.3a	0.33a	0.33a	0.33a	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 $100 \ \mu m$

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

Characters		Ba	amia			Emerald				
	Ν	MWCNT Tre	atments (mg/li	t)	MWCNT Treatments (mg/lit)					
	0	50	100	200	0	50	100	200		
Thiskness of last	$396.66 \pm$	426.67±	383.34±	333.3±	350±	430±	326.67±	316.6±3		
Thickness of leaf	8.81a	17.63a	20.27a	8.81b	17.32a	11.54b	8.8a	.34a		
Central midrib	673.3±17.	683.34±	$636.67 \pm$	630±	616.6±	$666.7 \pm$	603.333±1	$540\pm$		
diameter	73ab	14.52b	8.82ac	11.54ac	17.6a	8.8b	2.01a	15.27c		
Length of	136.67±8.	143.33±	$103.33 \pm$	100±	066+980	113.34±	73.33±	72.9±		
mesophyll cell	8a	6.67a	8.81c	5.78c	90.0± 0.0a	3.3a	3.4b	3.3b		
Thickness of	186.66±8.	193.3±	173.35±	166.65±	200±	210.1±	173.3±	188.3±7		
spongy layer	09a	8.81a	3.3a	12.01a	15.27ab	17.32b	3.3ab	.26a		
width of patiola	1077.3±38	1162.6±2	970.66±10.	885.33±28	938.6±	1056±18.5	962.65 ± 22	$880.35 \pm$		
width of periore	.45a	8.22a	67b	.22b	38.5a	b	.78ba	21.4a		
langth of natiola	$1888\pm$	2101±	1962.66±6	1930.67±4	1685.3±	1632 ± 18.4	1589.34±4	$1525.4 \pm$		
lengui or peuble	121.15ab	59.38b	4.89ab	8.93a	83.4a	7a	6.49a	46.5a		
Stomata langth	63.4±	68.3±	69.16±	55.83±3.0	$65.83 \pm$	68.3±	63.34±	59.16±2		
Stomata length	0.84ac	3.63ab	4.40ab	0c	2.2a	7.63a	2.2a	.3a		
Stomata index	$20\pm$	$22.66 \pm$	25.33±	22.66±	17.33±	21.33±	24+2 30b	22.66 ± 1		
(0.5 mm_2)	2.33a	1.33a	2.66a	2.3a	1.33a	1.33ab	24-2.300	.33b		

Table 3. Effect of multi-walled carbon r	anotubes (MWCNTs) on lea	of anatomy of two cultivars	of Okra seedlings
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Means followed by different letters differ significantly determined by Duncan test. (significant difference at P<0.05).





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

Characters	Bamia				Emerald				
	MWCNT Treatments (mg/lit)				MWCNT Treatments (mg/lit)				
	0	50	100	200	0	50	100	200	
Diameter of	1940±43.	1980 ± 45.82	2230±26.45	1005 / 47.5h	1960±52.9	2050 ± 36.05	2200 ± 26.1	1700±26.4	
root	58a	а	b	1995±47.50	1ab	b	2290±30.10	5a	
Thickness	516.67±2	680±17.32a	773.33±26.	500+20.81a	716.7±14.5	756.66±18.	790±17.32c	610±11.54	
of epiderm	0.27a*	b	03b	590±20.810	2a	56b		d	
Diameter of	423.33±1	543.33±12.	616.67±24.	493.16±14.	510±17.32	593.33±14.	576.67±24.	536.6±18.	
cortex	4.52a*	01b	03c	53b	а	52b	03b	56a	
Diameter of	34.16±2.	25 82 12 20	43.33±2.2b	32.95±2.47	30.83±0.83	26671226	35.83±0.84	30±1.44a	
xylem	2a	55.65±2.5a		а	а	30.07±2.20	b		
Diameter of	$15.83 \pm .8$	16 67 1 920	12511440	10.92 + 922	0.16+0.95	11.67±1.67	11.66±0.83	8.33±0.83	
phloem	4a*	10.0/±.85a	12.3±1.44C	10.85±.850	9.10±0.83a	а	а	a	
Layer	11.66±.3	12 67 - 940	12 22 - 222	11 570	12.33±0.33	11.66±0.33	12.66±0.33	12.33±0.3	
number	4a	12.07±.84a	15.55±.55a	11±.37a	а	а	а	3a	

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

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

MWCNT-0MWCNT-500MWCNT-100MWCNT-200BaniaImage: State Stat

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, 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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