Novel Methodology for Delivering Putrescine into the Apple Explants Using Hormone Anchored Nanotube

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Abstract

Multi wall carbon nanotubes have been successfully exploited as growth regulator for manipulation of plant development. Also, nanoparticles are gradually involved in target delivery systems as the carrier of hormones. Polyamines and their derivations play crucial roles in plant growth and development. Take the mentioned subjects into consideration, putrescine anchored carbon nanotube which had been labeled with fluorescein was synthesized in this study. A set of physiological and morphological parameters were assessed in an attempt to examine the usage potential of *de novo* synthesized nanotube in terms of plant in-vitro culture. For this purpose, the nanotube was applied onto the in-vitro plantlets of *Malus niedzwetzkyana* in three concentrations (0, 50 and 100 mg/l). Localization of the nanotube in the plantlets was accomplished using fluorescence microscopy. Bio-imaging of tissues indicated the existence of nanotube in nearly all studied organs. Application of the nanotube at both concentrations (50 and 100 mg/l) increased the rate of leaf formation and speeding up the plastochron. Also, proliferation of the plantlets was enhanced using the nanotube. The levels of the photosynthetic pigments, including chlorophyll a, b and carotenoids increased following application of the nanotube. Glutathione peroxidase activity was significantly affected by the nanotube. However, polyphenol oxidase and peroxidase were not influenced by the nanotube. Stomatal density was increased by treatment of the plantlets with the nanotube. Representing geometrical transformation of shape as a thin plate spline revealed that the nanotube effectively increased longitudinally of stomata and changes their aspect ratio.

Introduction

Plant development is known as the multistage complicated process at which various internal and external factors are involved (Goldberg 1988). Generally, plants receive many signals from under and above ground organs in regard to environmental condition which are converted to biochemical messages through signaling network (Mulligan et al. 1997; Foyer et al. 2003). Plants could figure out environmental and nutritional cues which beside their metabolic roles can act as signals for plant development (Gilroy and Trewavas 2001; Considine 2018). In this regard, signals are received by cell membrane occupied receptors specially protein kinases and other second messengers for activating transduction proteins and subsequently running transcription factors (Trewavas and Malhó 1997). Therefore, either of external or internal signals such as light and plant growth regulators (PGRs), control the numerous specific transcription factors and set into motion a cascade of biochemical events that ultimately evoke physiological response which finally determine the plant growth and developmental fate (Gaspar et al. 1996; Gilroy and Trewavas 2001; Jarad et al. 2020).

Many evidences have indicated that polyamines (PAs) and their derivations play crucial roles in plant developmental process (Allen 1983; Evans and Malmberg 1989; Galston and Sawhney 1990; Martin-Tanguy 1997; Weiger and Hermann 2014; Tiburcio and Alcázar 2018). Actually, PAs including putrescine, spermidine and spermine are low molecular and aliphatic nitrogen organic cation (Galston and Sawhney 1990) which are mainly derived from the amino acids such as ornithine and methionine via decarboxylation reaction (Shah and Swiatlo 2008). Putrescine, the major form of PAs, interferes in many

plant developmental processes encompassing cell division, leaf growth, floral and inflorescence formation and morphogenesis (Slocum et al. 1984; Walden et al. 1997; Kakkar et al. 2000; Pal Bais and Ravishankar 2002; Baron and Stasolla 2008). On the other hand, many researches indicated the mediatory role of PAs especially putrescine in relation to environmental stress (Alcázar et al. 2010). Considering the PAs importance in regard to plant growth and development, demands for their usage in-vitro and in-vivo conditions as valuable PGRs have been increased (Walden et al. 1997; Pal Bais and Ravishankar 2002; Zhu and Chen 2005; Ioannidis and Kotzabasis 2007; Baron and Stasolla 2008; Cai et al. 2015; Tiburcio and Alcázar 2018; Khoshbakht et al. 2018).

Nowadays, nanoparticles are increasingly utilized in some field of plant sciences in particular tissue culture and in vitro procedures. Some studies have indicated that multi wall carbon nanotubes (MWCNTs) trigger the expression pathways of aquaporin water carrier proteins, PIP₁ and PIP₂, which can enhance plant growth and development. Moreover, CNTs could activate enzymatic systems related to cell cycle upon which cell division is profoundly amplified (Khodakovskaya et al. 2012). The interaction of CNTs with protein and polysaccharides generates a cascade of signals resulting in accumulation of compounds leading to cell wall thickening and subsequent growth (Burman and Kumar 2018). To that end, due to considerable roles of nanomaterials on plant and development, the compounds have been recently classified as plant growth regulators (Hermes et al. 2020).

Beside the biochemical characteristics of nanoparticles they are gradually exploited as the carrier for primary ingredients for target delivering in biological systems (Azizi et al. 2020; Sabzi et al. 2020). Undoubtedly, enrichment of nanoparticles with PGRs is a novel approach by which manipulation of plant morphogenesis would be possible and acceleration of plant growth and development could be achieved (Pereira et al. 2017; Pascoli et al. 2018). So, planning for production of the new generation of PGRs anchored nanoparticles will perhaps be a new challenge for bio-science researchers to overcome the human food safety in future.

This study, investigates the effect of the putrescine anchored MWCNT as the putrescine delivering composite on growth and development of the red-leaf apple, i.e., *Malus niedzwetzkyana*. Niedzwetzky's apple is a rare, difficult-to-propagate species which has been put on endangered plants list throughout its range due to human activities (Dzhangaliev 2003). In recent experiment in-vitro cultured apple plantlet were treated by synthesized nanoparticles and the range of their growth as well as development was tested.

Material And Methods

Preparation of nanoparticles

The putrescine anchored MWCNT was synthesized for the first time using ingredients including MWCNTs, putrescine, thionyl chloride (SOCl₂) and fluorescein. The MWCNTs were preliminary purified and activated by multiple ultra-sonication in water bath using 10% HCl solution, ethanol (95%) and distilled water,

separately. Small amount (0.5 gr) of the purified MWCNTs were then treated with 20 mL aqueous solution of HNO_3 (60%) in an ultrasonic bath for at least 20 min, and left under reflux condition for 24 h. Afterward, the precipitate was filtered and washed several times using distilled water to ensure the pH of the filtrate reaches to neutral point. Prepared oxidized MWCNTs (denoted as MWCNT- CO_2H) were stored under vacuum condition (2 atm., 60°C) and dried up to 24 h (Kong et al. 2004).

Definite amount of the synthesized MWCNT- CO_2H (0.3 g) was suspended in 15 mL of SOCl_2 and then heated under reflux condition for 24 h. Then, remained SOCl_2 was distilled off and the obtained precipitate (MWCNT-COCl) was suspended in 10 mL of dry tetrahydrofuran (THF) using ultrasonic bath. Immediately, putrescine (0.5 g) was added to the suspension and stirred at room temperature for 24 h. Finally, the obtained MWCNT-Put was isolated by filtration and washing with THF as well as distilled water (three times) followed by drying under vacuum condition (2 atm, 60°C) for 24 h.

To provide the fluorescein labeled MWCNT-Put (MWCNT-Put-FI), fluorescein methyl ester was at first synthesized. For this purpose, esterification of the fluorescein was accomplished via sulfuric acid catalyzed reaction in refluxing methanol to produce fluorescein methyl ester. Small amount of the prepared ester (0.05 g) was added to the suspension of the previously obtained MWCNT-Put in 10 mL of methanol (ultra-sonication in water bath for 30 min) and refluxed for 24 h. Subsequently, isolation of the MWCNT-Put-FI was attained following filtration and washing with methanol and distilled water. In order to determine the functional groups on the prepared materials, Fourier transform infrared (FT-IR) spectra were collected over the range of $400\text{--}4000\text{ cm}^{-1}$ using a Win-Bomem spectrometer (version 3.04 Galactic Industries Corporation, USA).

All used chemicals and substances in the case of synthesizing nanoparticles were purchased from Millipore Sigma (formerly Sigma-Aldrich, Burlington, USA).

In-vitro Plant culture and treatment

In-vitro plant establishment was accomplished using common plant tissue culture procedures. For this purpose, dormant vegetative buds were collected from five years old Niedzwetzky's apple trees cultivated in the botanical garden, university of Tabriz, Iran (Khalat cord). The collected buds were dissected using a Nikon SMZ 1000 stereomicroscope (Nikon Corp. Tokyo, Japan) under a lamina hood. All plant materials were sterilized with 1.0% sodium hypochlorite for 10 min then immersed in ethanol 70% for 5 min and finally washed with sterilized distilled water for 20 min. The shoot apical meristems (SAMs) were excised and cultured in half strength Murashige and Skoog medium (Murashige and Skoog, 1962). The established meristems were subculture in the basal Murashige and Skoog medium as control and the same medium was supplemented with two concentration values of synthesized MWCNT-Put-FI, i.e., 50 and 100 mg/L. Auxin (IAA) and benzyl amino purine (BAP) in low concentrations (0.2 mg/L for IAA and 0.5 mg/L BAP) were added to all treatments. The treated plantlets were maintained for 30 days in culture room ($22^\circ\text{C}/18^\circ\text{C}$, day/night). This study was performed in randomized design with two treatments and 5 replications.

Biochemical analysis and enzyme assay

Activity of some antioxidant enzymes including glutathione peroxidase (GPX), polyphenol oxidase (PPO) and peroxidase (POD) was assessed in the present study. Leaves were collected from in-vitro plantlets and weighted up. Certain amount of phosphate buffered saline (PBS) was added to incised samples (10 mL per 1 gr) and they thoroughly homogenized. The homogenates were centrifuged (6000 rpm, 20 min) and the supernatant were utilized for the assay of enzyme activity. The enzyme activity, i.e., the amount of the enzyme requires for catalyzing decomposition of one micromole (μmole) of substrate to product per minute, was determined using colorimetric procedures. For this purpose, a multi-mode micro plate reader Biotek Synergy HT (Biotek, Vermont, USA) equipped with UV-Vis absorbance detectors was exploited. Enzyme bioassay kits including GPX (CAT number: 353919) and POX (CAT number: MAK092) were purchased from Sigma-Aldrich (Sigma-Aldrich, USA). The kit for PPO (CAT number: ARG82015) was provided from Arigo (Arigo Biolaboratories Corporation, Tiwan). All steps in sample preparation was fulfilled according to the given instructions in kits and absorbance was read at 412 nm, 410 nm and 570nm for GPX, PPO and POX, respectively. For every treatment, three replications were considered.

The contents of photosynthetic pigments were achieved by grinding fresh leaves in acetone 90% and recording the absorbance at 663 nm, 645 nm and 470 nm for chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*) and carotenoids (Car), respectively. For this purpose, a UV-Vis spectrophotometry (UV-1800 Shimadzu, Japan) was used. The pigment contents (Chl *a*, *b* and carotenoids) were calculated based on traditional method (Kocsanyi et al. 1988).

Electron microscopy

For studying the morphology and elemental analysis of the nanoparticles, they were coated with a thin layer of gold using routine methods and visualized using a Mira3 Tescan (Tescan, Czech Republic) scanning electron microscope (SEM) integrated with an energy depressive spectroscopy (EDS) analyzer. To evaluate nanostructure and dimension of the synthesized nanoparticles, they were dispersed in doubled-distilled water and studied under a Zeiss EM-90 (Zeiss, Jena, Germany) transmitted electron microscope (TEM).

Wide angle X-ray diffraction (XRD) profiles of MWCNT-Put-Fl were collected using a Bruker D8 Advance diffractometer with wavelength, $\lambda = 0.154059 \text{ nm}$ (Cu K α) at 30 keV.

Epi-fluorescence microscopy (EFLM)

Nanoparticles in the prepared samples were detected and localized exploiting EFLM techniques. For this purpose, five well-developed specimens including stems and leaves were randomly collected and carefully sectioned by hand microtome. Prepared samples were immediately mounted onto the glass slide and several drops of deionized water were added onto them. The samples were viewed through an Olympus BX51 fluorescence microscope equipped with a BX-RFA fluorescence illuminator, U-CA intermediate magnifying device and catadioptric UMPlanFL-BDP objectives (Olympus optical Co., Ltd. Tokyo, Japan). The samples were illuminated for fluorescence excitation using U-MWB3 (480–510 nm)

and U-MWG3 (510–550 nm) mirror cube units. Z-stacks from successive focal plates were acquired utilizing a Hamamatsu C7780-10 cooled CCD (Hamamatsu photonic Co., Tokyo, Japan). Digital colour images were saved in TIFF format (12 bits per each channel) with resolution of 1344 x 1024 pixels.

Studying the stomata was carried out according to the techniques which had been previously used in detection of protodermal cells (Dadpour et al. 2011). Leaves from in-vitro plantlets were collected and fixed in FAA (5 parts formalin: 5 parts glacial acetic acid: 90 parts 50% ethanol) for one day. The samples were then immersed in glacial acetic acid up to 5 min and stained with saturated aniline blue (CAS number: 28631-66-5, Sigma-Aldrich, USA) for an overnight. Re-staining of the samples was performed using 0.2% (v/v) fuchsin acid (CAS number: 3244-88-0, Sigma-Aldrich, USA) up to several days. Excess stain was washed away by ethanol and studied utilizing the microscopic methodology which was described in the previous section. To avoid tissue crumpling, imaging process was done while the sample had been immersed under the absolute ethanol.

Image processing

Acquired consecutive images were imported to Image J 1.37 software (freely available from <http://rsb.info.nih/ij/>) to produce Z stack file (Peighambardoust et al. 2010). Then, image level was adjusted in entire of images stack. Subsequently, image sharpness was improved by applying the Unsharp mask filter at the moderate range (Gaussian radius = 5 and Mask weight = 0.5). Z-stack composition was done automatically using Z-function plug in to improve depth of focus (Forster et al. 2004). The final outputs were trimmed to proper size and scale bars were added based on optical magnification.

Estimating the density of stomata using image analysis procedure

Image analysis for estimation of stomatal density was fulfilled based on the previously described method with some modification (Albrechtová et al. 2014). Final fluorescence images of the stomata were used for providing local image with 500 × 500 micron area randomly collected from various portions of leaf (Fig. 4A). Extracted images were then imported to Image J 1.37 software and three gray images belonging to red, green and blue channels extracted by RGB channel splitting mode. Image of red channel with higher contrast was collected for setting threshold (Fig. 4B). Threshold adjusted image was then used for automated analysis of stomatal density (Fig. 4C). In this study 10 replicates including local images were utilized for estimating of stomatal density in each treatment. Using particles analysis mode, the software detected outlines of stomata and counted them (Fig. 4D). Obtained data from microscopic image analyzing were used for statistical assessment of stomatal density.

Geometric morphometric characteristics of stomata

A geometric morphometric study was conducted for evaluating the form of stomata and change in their features followed by application of the treatments. For this purpose, digital images of individual stomata

were extracted from high magnified microscopic images. For every treatment including control, MWCNT₅₀-Put-FI and MWCNT₁₀₀-Put-FI, 20 images of stomata were provided. In this study, 100 semi-landmarks were digitized along the outline of stomata using the TpsDig software. Consensus image was created from obtained semi-landmarks of the replications in every treatment using TpsSuper software (Zelditch et al. 2012; Rohlf 2015). Realized datasets, representing average stomatal forms corresponding to treatments, were imported into the PAST 3.1 software. Semi-landmark configurations were optimally aligned, translated and rotated (excluding size factor) based on the Procrustes superimposition method. Testing the significance of shape differences among the treatments was accomplished using one-way analysis of covariance (ANCOVA). A Principal component analysis (PCA) was applied on datasets and similarity between groups was determined using UPGMA (Unweighted Pair Group Method with Arithmetic mean) method. Stomatal shape deformation as well as differences between treatments and control was graphically presented in transformation grids through thin-plate spline (TPS) analysis.

Results

Preparation and characterization of MWCNT-Put-FI

Synthesizing the putrescine anchored MWCNT has been schematically described in Fig. 1. As shown, MWCNT-Put was prepared in three stages. At first step, the surface of MWCNT was oxidized using HNO₃ aqueous solution to production of MWCNT-CO₂H. The product was subsequently converted to MWCNT-COCl using SOCl₂ under reflux conditions. The last stage in preparation of the nanotube was executed by manipulating the putrescine in dry THF at room temperature. As the result, linkage of the putrescine with the MWCNTs was occurred due to formation of amide. To provide the possibility of detecting the nanotube in the plant tissues by means of fluorescence microscopy, a few molecules of fluorescein, as fluorescence labels, were covalently bonded to MWCNT-Put. For this purpose, some free amine moieties of putrescine were linked to the MWCNTs with fluorescein methyl ester through amidation (Fig. 1).

The obtained MWCNT-Put-FI was characterized using FT-IR, SEM, EDX and TEM analysis. XRD pattern of MWCNT-Put-FI shows two distinct peaks at $2\theta = 25.6^\circ$ and 42.7° , corresponding to the (002) and (100) reflections, indicating that the crystallinity of MWCNTs is not lost during oxidation, and then functionalization with putrescine and fluorescing molecules, in comparison with reported XRD patterns in the literature (Fig. 2A). (Das et al. 2014; Abdulrazzak et al. 2019).

FT-IR spectra were measured to study the presence of functional groups along with chemical modification of MWCNTs. FT-IR spectrum of MWCNT-CO₂H exhibits peaks at 1013–1333, 1550–1643, 1709 cm⁻¹ and a broad peak at 2570–3461 cm⁻¹, corresponding to the C—O, C = C, C = O and O—H stretching vibrations, respectively. Putrescine shows two distinct peaks at 3290 and 3359 cm⁻¹ associating with symmetric and asymmetric vibrations of NH₂ moiety, respectively. Symmetric and asymmetric vibrations of CH₂ are appeared at 2852 and 2921 cm⁻¹, respectively. Peaks at 1601, 1461 and 1071 cm⁻¹ are attributed to the bending vibrations of NH₂ and CH₂, and stretching vibration of C—N

bond, respectively. In the FT-IR spectrum of the MWCNT-Put, peaks at 1013, 1200, 1556, 1712 and 2564–3079 (broad) cm^{-1} are shown, which are attributed to the stretching vibrations of C—O, C—N, C = C, C = O of unreacted carboxylic acids, and O—H bonds, respectively. In addition, the appearance of peaks at 2844, 2925 and 3402 cm^{-1} , corresponding to the symmetric and asymmetric vibration of CH_2 and stretching vibration of N—H, along with peak of C = O of amides at 1698 cm^{-1} could be attributed to the covalently bonding of putrescine to MWCNT via amide formation. In the FT-IR spectrum of the MWCNT-Put-Fl, in addition to distinct peaks of MWCNT-Put, a new broad peak at 3170–3551 cm^{-1} , corresponding to the stretching vibration of O—H of fluorescein, was appeared (Fig. 2B).

Figure 3 shows SEM and TEM images and EDX analysis of MWCNT-Put-Fl. SEM images were recorded in order to investigate the morphology of MWCNT-Put-Fl, which shows the irregular aggregation and agglomeration of MWCNTs (Fig. 3A). TEM images show an interwoven network of carbon nanotubes (Fig. 3B). Both SEM and TEM images exhibited that the structure of MWCNTs was remained unchanged during acidic oxidation and modification. Also, EDX analysis indicated the presence of C, O and N atoms in the structure of MWCNT-Put (C: 84.47, O: 13.29 and N: 2.24 wt%) and MWCNT-Put-Fl (C: 82.17, O: 15.92 and N: 1.91 wt%), approving the modification of MWCNTs surface by putrescine and fluorescein (Fig. 3C).

Localization of MWCNT-Put-Fl in the plantlets

Tracing of the fluorescein labeled nanotubes using fluorescence microscopy demonstrated high affinity for their absorption in the plantlets. Nearly in all studied tissues and cells, MWCNT-Put-Fl at two concentrations, was detected. However, localization of the nanotubes indicated that distribution in the vascular tissue and stomata is higher than other tissues. In the leaf epidermis, tendency for aggregation of the MWCNT-Put-Fl in cell walls (perhaps cell membrane) was higher than the other section (Fig. 5). High magnified microscopic images indicated that major amounts of the MWCNT-Put-Fl have been aggregated in the cellular microtubules and actins (Fig. 5, A). Also, localization of the nanotubes in stomata was higher than pavement cells, and high fluorescence signals were detected in the guard cells (Fig. 5, E).

Effects of the MWCNT-Put on the growth and development of in-vitro plantlets

Effects of the putrescine anchored MWCNT onto the growth and development of in-vitro apple plantlets was evaluated. For this purpose so called leaf number, plastochron and proliferation (the rate of lateral outgrowth) were estimated and statistically analyzed (Fig. 6). The effect of time on the rate of leaf formation and plastochron was significant ($p < 0.05$). However, at the t_0 , t_1 and t_2 , any significant differences between treatments was observed. Comparison of treatments was performed in every time independently from the other dates. Obviously, treatment of in-vitro explants with the MWCNT-Put, exhibited positive effect on leaf formation at the last dates of study (t_3 and t_4). Two concentrations of the MWCNT-Put, with any statistically difference, had significant effect ($p < 0.05$) on leaf formation at the

mentioned dates in compare to control (Fig. 6A). As shown in Fig. 6B, the trend of leaf formation in all treatments was nearly similar up to 3rd week. Distinct ascending feature in leaf emerging appeared in 4th week in relation to MWCNT₅₀-Put and MWCNT₁₀₀-Put comparing with the control.

Plastochron, i.e. the time interval at which new leaf primordium is initiated, was obviously influenced by treatments (Fig. 6). During the first two time intervals including t_0 - t_1 and t_1 - t_2 , no significant differences were observed between treatments in corresponding dates (Fig. 6C). After this period, however equal descending trends regarding to plastochron were obtained for all treatments, while the factor was significantly lower in the case of MWCNT-Put treatments than the control (Fig. 6D). Obviously, in the control plantlets, plastochron nearly reached to steady state at the last time interval. However, descending trend continued in the case of treated plantlets with the both concentrations of the MWCNT-Put (Fig. 6C). In contrast to first moiety of the experiment, MWCNT₅₀-Put and MWCNT₁₀₀-Put similar together, had significant ($p < 0.05$) effect on reduction of plastochron comparing to control at the t_2 - t_3 and t_3 - t_4 time intervals (Fig. 6C).

Proliferation of the plantlets presented as the number of lateral shoots (Fig. 7A), significantly ($p < 0.05$) increased due to application of the MWCNT₅₀-Put and MWCNT₁₀₀-Put (Fig. 7B). According to the counted shoots per primary explant after 4 weeks period of culture, MWCNT₅₀-Put and MWCNT₁₀₀-Put treatments presented statistically similar effect on proliferation. In the same way, treatment of plantlets with MWCNT-Put led to formation of shoots with longer internodes comparing to control (Fig. 8AB).

Effects of the MWCNT-Put on the biochemical characteristics of in-vitro plantlets

Data obtained from biochemical analysis indicated that photosynthetic pigments, including chlorophyll a, chlorophyll b and carotenoids, were significantly ($p < 0.05$) affected by MWCNT-Put in all concentration in compare to control. Whereas, any difference between MWCNT₁₀₀-Put and MWCNT₅₀-Put treatments was observed in this regard (Fig. 9A).

Study of the enzymatic activity in the treated plantlets revealed that only GPX has been positively influenced by the MWCNT-Put treatments. Differences between MWCNT₁₀₀-Put and MWCNT₅₀-Put were not statistically significant (Fig. 9B). While, any significant effects of treatments were found in relation to activity of either PPO or POX in comparison to control.

Effects of the MWCNT-Put on the stomatal density and form

Data extracted from image analysis of microscopic observations and their statistically comparing demonstrated that the density of stomata was significantly affected by MWCNT-Put treatment ($p < 0.05$). Any symptom of stomatal patterning was observed in the adaxial surface of the studied leaves in all treatments. In contrast, considerable density of stomata was found in the abaxial surface of leaves.

Stomatal density in leaves of the MWCNT-Put treated plantlets was significantly ($p < 0.05$) higher than control (Fig. 10).

Studying the abaxial surface of the leaves with more accuracy was shown the treated plantlets have considerable amount of stomata over the leaf veins. These types of stoma were longitudinally arranged over the leaf veins and parallel to their directions (Fig. 11).

The form of stomata was remarkably affected by MWCNT-Put treatment. Geometrical analysis of semi-landmarks revealed a distinct transforming in stomatal form as the result of MWCNT-Put application. The higher the concentration of the nanotube, the more change from circularity to elliptically in stomatal form occurred. The XY graph of the consensus images of stomata in relation to treatments showed significantly change in aspect ratio (length/width) from 0.9 to 1.4 in regard to control and MWCNT₁₀₀-Put, respectively (Fig. 12A). In other words, circularity in stomatal form in control with nearly 90% deceased to 71.5% in MWCNT₁₀₀-Put treatment. Evaluation the similarity of stomatal form between treatments has been shown in Fig. 12B. As it appears, more similarity was achieved for MWCNT₁₀₀-Put and MWCNT₅₀-Put than control (Fig. 12B).

Demonstrating stomatal shape transformation as a thin plate spline surface described the similarities and differences in geometrically reciprocal characters. The contrast between the treated and control plantlets in terms of the digitized semi-landmarks used to quantify stomatal forms. As per morphometric convention, the configuration of the target (the treated plantlets with MWCNT₅₀-Put and MWCNT₁₀₀-Put) versus reference (the control) specimen was plotted along with the spline grids. The highest deformation of stomatal form was shown in regard to MWCNT₁₀₀-Put versus control. Arrows accentuated areas of the largest differences which are concentrated around the vertices and co-vertices (Fig. 13A). At the same manner, slightly low changes in the stomatal form were observed regarding MWCNT₅₀-Put versus control (Fig. 3B). The lowest deformation belonged to the MWCNT₁₀₀-Put versus MWCNT₅₀-Put (Fig. 13C) which confirmed validation of stomatal shape clustering (Fig. 12B).

Discussion

Demand for the application of nano-materials in the field of biology, medicine and food sciences has been recently increased (Azizi et al. 2020; Salama et al. 2021; Panahirad et al. 2021). Many reports revealed that nano-materials could directly influence the biological systems as promoter. In fact, nano-particles could somewhat act as carriers for conjugated substances by which target delivery could be possible. In this way, probability of degradation of conjugated substance would be considerably decreased. The main challenge in producing substance-anchored nanoparticle, would be adopting the appropriate synthesizing procedure at which the considered substance is mounted onto the special nanoparticle. Many studies have demonstrated the amplified effects of substance anchored nanoparticles compared to individual treatment of substance or nanoparticle.

Synthesizing MWCNT-Put-FI

As mentioned earlier, features of the MWCNT-Put-FI were characterized by various methodologies including FT-IR, EDX, SEM and TEM analysis. Obviously, EDX analysis indicated the characteristic peak only for carbon, oxygen and nitrogen approving the modification of MWCNTs surface by putrescine and fluorescein. It means high purity of the synthesized nanotube and removal of the unreacted elements from the nanotube during the synthesis. Our results are in conformity with the opinions of the researchers which had been used EDX analysis for elucidation of nanoparticle purity (Ramachandran et al. 2013; Gohari et al. 2020a). Likewise, XRD is known as valuable tool for demonstrating the microstructure of synthesized nanotubes (Das et al. 2014). In the case of the MWCNT-Put-FI, XRD pattern indicated that the microstructure of MWCNT remain unchanged during oxidation and functionalizing with putrescine and fluorescein. Also, the robust diffraction peaks indicates that the MWCNT-Put-FI possess proper crystallinity which is in agreement with the results reported by other researchers (Ramachandran et al. 2013; Subbenaik 2016; Yang et al. 2018; Azizi et al. 2020). Also, line widening of the XRD peaks indicates that the size of the MWCNT-Put-FI reduces in the domain of nano range (Ramachandran et al. 2013). So, we presumed that MWCNT retains own functionality while acts as carrier for putrescine. Alternatively, FT-IR spectra have been successfully utilized determining the presence of conjugated groups along with chemical modification of MWCNTs (Gohari et al. 2020b, a). In the FT-IR spectra of the MWCNT-Put, peak of C = O at 1698 cm^{-1} depicts bonding of putrescine to MWCNT via amide formation. In this way, another broad peak could be seen at $3170\text{--}3551\text{ cm}^{-1}$, which is due to O—H stretching vibration of fluorescein. (Fig. 2B). The results confirmed the successful linkage of putrescine and fluorescein on the surface of MWCNTs. Current study is in accordance with the works indicating the potential of FT-IR in detection of chemical groups onto the nanoparticles (Jelvehgari et al. 2014; Azizi et al. 2020).

Supplemented to chemical analysis, SEM images show the irregular aggregation and agglomeration of MWCNTs (Fig. 3A). Furthermore, TEM images demonstrate an interwoven network of carbon nanotubes (Fig. 3B). Both SEM and TEM images depicted that the structure of MWCNTs was remained unchanged during acidic oxidation and modification.

In this study we described an ordinary methodology for production of putrescine anchored MWCNT for the first time. The results obtained from various tests, including electron microscopy and chemical analysis confirmed the successful linkage of putrescine on the surface of MWCNTs.

Bio-imaging and localization of the MWCNT-Put-FI

Divers methods are used for bio-imaging and localization of nanoparticles in tissue or cells (Hauser et al. 2017). Epi-fluorescence microscopy (EFLM) is a good choice for this purpose, because it known as low cost and easy to use in respect to other procedures (Peighambardoust et al. 2010). The technique has been successfully utilized for detecting nanoparticles in plants. The approach for indirectly detection of non-fluorescence nanoparticles created based on the fact that they could be visible if their background, i.e., tissue or cell, stained with suitable fluorochrome (Ghafariyan et al. 2013). Our results indicated that labeling the nanotube with fluorescein as a fluorescence dye, provides opportunity for direct detection of nanotube using easy-to-use conventional fluorescence microscopy.

Effects of MWCNT-Put on the growth and development of plantlets

Leaf formation and plastochron are good indicators for assessment of apple growth and development (Foster et al. 2003; Dadpour et al. 2011). The rate of leaf initiation is in close relation with the organogenesis of shoot apical meristem (SAM) and its activity. Leaf primordia are formed at the peripheral zone of SAM subsequent to generous cell division. Therefore, leaf formation is known as dependent process to cell division from organogenesis point of view (Fleming 2005). Also, plastochron which defines the leaf formation as the function of time is directly tied with the rate of cell division (Dadpour et al. 2011). Many reports indicated that cell growth and division is promoted by nanotubes (Khodakovskaya et al. 2012; Khorrami et al. 2020; Samadi et al. 2020). Alternatively, polyamines could enhance cell division (Maki et al. 1991), act as effective regulator for shoot morphogenesis (Walden et al. 1997; Yadav and Rajam 1997; Zhu and Chen 2005) and increase seedling height (Farooq et al. 2009). As well, polyamines has decisive role in regard to cell structure by effect on actin formation and microtubules assembling (Oriol-Audit 1978; McCormack et al. 1994; Savarin et al. 2010; Sánchez-Elordi et al. 2019). In the current study, enhanced growth and development of the in-vitro plantlets could be associated with stimulatory effects of both components of the MWCNT-Put, i.e., MWCNT and putrescine, which is confirmed by the above mentioned literatures. Furthermore, well organized microtubules in treated cells by MWCNT-Put which were appeared in microscopic studies (Fig. 5A), could elucidate the effectiveness of putrescine in this regard. Ability of the putrescine in adjusting the water contents of a cell could attributes to plant growth and performance (Farooq et al. 2009). Similarly, evidence indicated MWCNTs can trigger the expression of PIP₁ and PIP₂ (water carrier proteins) which enhance plant growth and development through improving the water status of tissue (Khodakovskaya et al. 2012). In the current study, inimitable growth of the plantlets is in agreement with the mentioned reports and may be related to accumulative effects of MWCNT and putrescine which have been assembled in the individual particle.

Effects of MWCNT-Put on the biochemical characteristics

Indisputably, photosynthesis is the most important process which influenced by nanoparticles. Some studies has been depicted that the levels of photosynthetic pigments could increase in response to application of nanotubes (Sharma et al. 2019; Gohari et al. 2020b). Also, the substances could enhance photosynthetic activity, carbon assimilation and biomass (Khodakovskaya et al. 2012; Ghafariyan et al. 2013; Banerjee and Kole 2016). Likewise, polyamines including putrescine, are now being deliberated as a specific PGR which could modulate photosynthesis (Farooq et al. 2009; Yuan et al. 2015; Abd Elbar et al. 2019). Enrichment of photosynthetic pigments in the present study may correlates with the nature of the MWCNT-Put. The outstanding role of putrescine in plants would be its implication on chloroplast bioenergetics by means of motivating ATP synthesis. Also, putrescine can increase light energy utilization through stimulation of photophosphorylation (Ioannidis et al. 2006; Ioannidis and Kotzabasis 2007). So, putrescine could enhance the growth of plants by providing more ATP as the common source of energy. In this study, intensive growth which plantlets were experienced after treatment by MWCNT-Put would be

explained from bioenergetics point of view. In other respects, increased level of the GPX in our study, as the enzymatic system which plays array of physiological roles in protection of photosynthetic apparatus against oxidative damages (Herbette et al. 2002; Dayer et al. 2008), could explicates the ascending trends of Chl *a* and *b* in the case of treated plantlets with MWCNT-Put.

Form and distribution of Stomata

Definitely, stomata are important tissues due to their controlling role in simultaneous exchange of carbon dioxide and water with the atmosphere (Webb and Baker 2002). Consequently, formation and spacing pattern of the stomata are considered as determining factors in relation to carbon assimilation, transpiration and plant performance. In overall, stomata are distributed on a leaf based on the one-cell-spacing rule. According to this hypothesis, separating two adjacent stomata from each other by at least one intervening pavement cell, is critical for achieving an optimal balance between carbon assimilation and water exhaust (Larkin et al. 1997; Bergmann and Sack 2007). Moreover, obligatory dependency of guard cell functioning, gas exchange, and ion transport with stomatal spacing have been proven (Torii 2007; Papanatsiou et al. 2016).

Therefore, changes in stomatal spacing pattern have significant impact in terms of physiological approaches (Casson and Gray 2008). In the present study, treatment of in vitro apple plantlets with the MWCNT-Put significantly increased the density of the stomata in comparison to the control (Fig. 10G). Our results are in contract with some other studies indicating positive effects of CNTs on the stomatal density (Joshi et al. 2018a, b; Khorrami et al. 2020). Studying the effects of CNTs on tobacco callus culture revealed that expressions of genes responsible for encoding cell division (CycB) and tobacco aquaporin (NtPIP₁) are enhanced in treated cells compared to the controls (Khodakovskaya et al. 2012). From the morphogenetic point of view, stomatal distribution and frequency are the main factors which could directly affect carbon assimilation and subsequently plant productivity (Shpak et al. 2005; Casson and Gray 2008; Torii 2015). Generally, formation of stomata is known as a complicated process at which numerous physiological and molecular factors involve (Shpak et al. 2005; Martin and Glover 2007; Bergmann and Sack 2007; Casson and Gray 2008; Serna 2009; Kim et al. 2012; Rudall et al. 2013; Franks and Casson 2014; Torii 2015; Castorina et al. 2016; Muir 2018). However, stomata is formed through a series of asymmetrical and symmetrical cell divisions being known as cell cycle dependent process (Croxdale 2000; Nadeau and Sack 2002; Torii 2007). It has been elucidated that the E₂Fa–DPA transcription factor together with the D cyclins and Cyclin Dependant Kinases (CDKs) are crucial components regulating cell division prior to formation of stomata (De Veylder et al. 2002; Bergmann 2004). However, E₂Fa is the main known factor for asymmetrical cell division in terms of stomatal spacing (Bergmann and Sack 2007; Kajala et al. 2014). Moreover, membrane-bound receptor-like kinases (RLKs) in pairing with two members of the epidermal patterning factor like (EPFL) protein family, namely, EPF₁ and EPF₂, have been recently stated to be essential for regulation of stomatal patterning. Noticeably, the EPF₁-ERL pair appears to enforce the one-celled spacing rule responsible for stomatal density (Lau and Bergmann 2012). On the other hand, the main molecular mechanism involving in the formation of stomata, MUTE protein, seems to activate another member of D Cyclins (CycD_{5;1}) proteins

which causes the guard mother cells (GMC) to divide. Taking all into account, polyamines have been determined as the effective factor for enhancing cell division which is critical for stomata formation (Kono et al. 2007; Yamashita et al. 2013).

The present study indicated that application of MWCNT-Put increases the frequency of stomata, meaning low spacing between stomata and high ratio of guard per pavement cells. Accordingly, it seems that MWCNT-Put is effective on asymmetrical division of mesophylls with spacing division pattern. Research on the tomato plants treated with saline water, indicated some significant morphological and physiological changes (Romero-Aranda et al. 2001). They reported that reduction in stomatal density leads to attenuating stomatal conductance and decreasing net CO₂ assimilation which indirectly confirms our results. Salt stress could decrease CDK activity and could profoundly suppress the expression levels of the cyclins genes including A and B Cyclin which leads to extensive inhibition in both S and G₂/M phase (Qi and Zhang 2020). As it was mentioned above, salt stress can reduce stomatal density with suppressing cell cycle process. Another experiment conducted on the barley under salt stress, revealed that the stomata index is reduced by application of PAs (Çavuşoğlu et al. 2007). Our data, however, contradicts their results indicating negative effect of PAs in regard to stomata formation. In their work stomatal density was determined in terms of the stomata index, i.e., the ratio of stomata per total mesophyll cells. Also, in treated plants, total number of mesophyll cells was increased while their size diminished. Therefore, higher total mesophyll cells may lead to lower stomata index. The discrepancy between our data and the data of Çavuşoğlu *et al.* (2007) may be due to different methodologies which were exploited for calculating stomatal density. In our study, stomatal density was calculated as the observed stomata per distinct field of view, while they described the ratio of the number of stomata per surrounding cells.

The subsidiary effect of MWCNT-Put which was detected in our study is the formation of stomata over the main leaf vein in the case of nanoparticle application (Fig. 11). In general, stomata rarely develops over major veins with an exception, i.e., Selaginellaceae family (Brown and Lemmon 1985; Martin and Glover 2007). It seems the lack of stomata over leaf major veins mainly helps to prevent excess transpiration. As the control in-vitro plants conformed to the mentioned rule and stomata only appeared over the vein in regard to treated plants, it seems the MWCNT-Put is responsible for such change in the development of the studied leaves. We reported this phenomenon based on several microscopic observations and no statistical analysis was accomplished for this purpose. So, further studies are required for clarification of this morphological event.

The change in shape and form is largely a matter of importance to biologists (Cooke and Terhune 2015; Klingenberg 2016). Mostly, differentiation in the cellular levels could be demonstrated by anisotropy in the form of cell (Coen et al. 2004). The polyamines, especially putrescine, as well as nanotubes are well known for their implication in the developmental process. Geometric morphometric analysis in this study elucidated substantial anisotropic transformation of the stomatal form in the case of MWCNT-Put treatments in particular at the vertices area. So, it seems the synthesized nanotube might be involved in differentiation of the stomata.

Conclusion

Synthesizing hormone anchored carbon nanotubes is an optimistic approach that has great potential and flexibility for application towards manipulating plant growth and development. The new developed MWCNT-Put-FI nanotube with profound effects in terms of plant morphological, physiological and biochemical characteristics could enhance performance of the in-vitro plantlets. Exploiting the synthesized nanotube in the micropropagation of the Niedzwetzky's apple demonstrated the positive effects of the composite on growth and development of the plantlets. Importantly, cumulative leaf formation, short plastochron and intensive shoot proliferation by treating the MWCNT-Put depicted its positive impact in respect to plant growth and development. MWCNT-Put application additionally led to increase the contents of photosynthetic pigments and GPX activities as the chlorophyll protective antioxidant enzyme. Interestingly, stomatal shape and density were profoundly affected by application of the nanotube. Consequently, the new developed MWCNT-Put might act as a specific PGR which is able to stimulate plant growth and development, ultimately enhancing plant performance under in-vitro cultures and thus acting as a promising growth promoting substance.

Declarations

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Authors' contributions:

M. Abdolalipour, B. Eftekhari-Sis, A. Motalebiazar and M. Dadpour accomplished the experiment. M. Abdolalipour established in vitro cultures and contributed to data collection. B. Eftekhari-Sis synthesized the hormone anchored nanomaterial and chemical analysis. M. Dadpour contributed in bio-imaging, fluorescence microscopy and image analysis. M. Abdolalipour and A. Motalebiazar performed statistical analysis. B. Eftekhari-Sis and M. Dadpour supervised the experiments. B. Eftekhari-Sis and M. Dadpour wrote the draft. M. Dadpour edited and finalized the manuscript.

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Figures

Figure 1

Stages in synthesis of the namely MWCNT-Put-FI

Figure 2

Chemical analysis and characteristics of the synthesized nanotube A) XRD pattern of fluorescein labeled MWCNT-Put B) FT-IR spectra of putrescine, MWCNT-CO₂H, MWCNT-Put and fluorescein labeled MWCNT-Put

Figure 3

SEM and TEM images and EDX analysis of fluorescein labeled MWCNT-Put A) Image of the synthesized MWCNT-Put acquired by SEM B) Image of the synthesized MWCNT-Put taken by TEM C) EDX analysis of MWCNT-Put-FI showing three main peaks for carbon (C), nitrogen (N) and oxygen (O).

Figure 4

Stages in image analysis method for calculating stomata density A) A local fluorescence microscopic image of stomata with 500×500 micrometer area. B) Grayscale (8 bit grayscale) image was created after splitting RGB channels to separate red (R), green (G) and blue (B) gray images. Image belonging to R channel was used for the next stage in image analysis. C) Binary image was produced after adjusting threshold. D) Final stage in image analysis of stomata by detecting the outlines of each stomata and their counting.

Figure 5

Tracing and localization of fluorescein labeled MWCNT-Put into the plant tissue. A) Localization of fluorescein labeled MWCNT-Put in the epidermis cells treated by MWCNT-Put. Microtubules are visible as parallel strips after absorbing nanotubes. B) Auto-fluorescence image of epidermis cells in control. Photosynthetic pigments are detected as red particles. C) Vascular tissues in treated plantlets show aggregating of nanotubes. Any symptoms of nanotubes are visible in control. D) Localization of MWCNT-

Put-FI in the leaf tissue of the treated plants. Most of the nanoparticles were allocated in the stomata. E) Parenchyma tissue in the treated plants. F) Considerable amounts of the fluorescein labeled MWCNT-Put are distributed in the cell walls and cell membranes.

Figure 6

The rate of leaf formation in the control and treated red-leaved in vitro apple plantlets using putrescine anchored multi wall carbon nanotube (MWCNT-Put). A-B) Leaf number increased gradually up to day 14 in all treatments. No significant differences are shown between treatments in each date during this period. Considerably increasing in leaf number occurred ascending trend in the rate of leaf formation was shown after 21 days (t3) after beginning of experiment. The first estimating of leaf number was done 7 days after onset of experiment (t1) and continued with 7 days intervals (t2, t3 and t4) for the remaining of the study (bars indicate standard deviation). C-D) Estimated plastochron (the period between the initiations of successive leaves) in the control and treated red-leaved in vitro apple plantlets using putrescine anchored carbon nanotube (CNT-Put). The shortest plastochron, meaning quickly initiation of leaf primordial, was shown in third interval for treated plantlets by MWCNT50-Put and MWCNT100-Put (bars indicate standard deviation).

Figure 7

The rate of shoot proliferation in the control and treated red-leaved in vitro apple plantlets using putrescine anchored carbon nanotube (MWCNT-Put). A, B and C) Proliferation of the in-vitro plantlets which achieved using the nanotube. D) Shoot proliferation was significantly increased in treated plantlets with MWCNT50-Put and MWCNT100-Put in compare to control (bars indicate standard deviation).

Figure 8

Effect of the MWCNT-Put onto the shoot growth A) A comparative illustration of the effects of nanoparticles onto the shoot growth and the length of internode in the Niedzwetzky's apple plantlets.

Root formation was often seen in the case of the treated plantlets. B) Statistically mean comparison of the control and treated plantlets in respect to the length of internodes (bars indicate standard deviation).

Figure 9

Effect of MWCNT-Put on the some biochemical characteristics of the plantlets. A) Effect of MWCNT-Put on the phothosyntetic pigments. B) Effect of MWCNT-Put on the GPX (bars indicate standard deviation).

Figure 10

Stomatal density and form taken from the abaxial leaf side. A) Stomatal density in the leaves of the control plantlets. B) Stomatal density in the leaves of the MWCNT50-Put treated plantlets. C) Stomatal density in the leaves of the MWCNT100-Put treated plantlets. D) Stomatal form in the leaves of the

control plantlets. E) Stomatal form in the leaves of the MWCNT50-Put treated plantlets. F) Stomatal form in the leaves of the MWCNT100-Put treated plantlets. G) Effect of MWCNT-Put treatments on the stomatal density (bars indicate standard deviation).

Figure 11

Formation of stomata over the major leaf vein of treated plantlets with MWCNT-Put A) Microscopic image abaxial leaf surface in the control plantlet. Any symptom of stomata formation over the leaf vein is

visible. B) Wide field microscopic image of leaf surface in treated plantlet shows considerable density of stomata which are formed over the leaf vein. C) High magnified image of stomata shows their longitudinally arrangement over the leaf vein in treated plantlet. D) Polarity in the formation of over-veined stomata. E – F) Three dimensional (3D) projection of the leaf vein morphology in treated plants at low and high magnification.

Figure 12

Geometry of stomatal form and clustering A) Graph of the consensus images of stomatal forms at different treatments derive from XY coordination. B) UPGMA analysis and clustering the stomatal form in the control and treated plantlets with MWCNT50-Put and MWCNT100-Put

Figure 13

Thin-plate spline deformation grids visualizing change in the stomatal form in relation to treatment of the nanoparticles. Arrows accentuate areas of the largest differences A) Thin-spline plot of the MWCNT100-Put versus control shows highest deformation regarding the stomatal form. B) Thin-spline plot of the MWCNT50-Put versus control shows slightly lower deformation in respect to the stomatal form. C) Thin-

spline plot regarding to MWCNT100-Put versus MWCNT50-Put demonstrates the lowest deformation concerning the stomatal form.