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The Evaluation of the Effect of Multiwall Carbon Nano Tube (MWCNT) on *In Vitro* Proliferation and Shoot Tip Necrosis of Pistachio Rootstock UCB-1 (*Pistacia integrima* × *P. atlantica*)

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Abstract

UCB-1 (*Pistacia atlantica* × *P. integrima*) is a commercial rootstock for pistachio in some pistachio plantations across the world. This rootstock is very new in Iran and recently, it has been used commercially in some plantations due to its high growth. Propagation of this rootstock by tissue culture results in many limitations such as shoot tip necrosis (STN) and a low proliferation rate. Therefore, any process that leads to improve the proliferation rate and feature will be used in commercial propagation of this rootstock. Nanotubes are widely used in *in vitro* cultures. For this reason, we used different concentrations of carbon nanotubes (0, 50, 100, 150, 200 µg/l) and benzyladenine (0, 0.5, 1, 1.5 and 2 mg/l) to improve the proliferation rate and qualitative indices. The results showed that using carbon nanotubes concentration of 200 µg/l with 2mg/l of benzyladenine (BA) led to maximum proliferation (4 microshoots per explant), maximum shoot length (3.68 cm) and minimum STN (8%) and *vitrification* (this isn't a word?) (0 %) percentage.

Keywords: BAP, Carbon Nano-tube, In vitro proliferation, MS, Pistachio rootstock, UCB-1.

Introduction

Agriculture is one of the most important economic sectors in developing countries. Among the exports from Iran, pistachio (green gold) is of particular importance given that Iran is the largest producer and exporter of this crop on global level. Iran vhas also devoted a considerable share of the global production and trade of this crop (Mohammadi and Bahrami-Nasab, 2013). Pistachio seedling rootstocks are used in many of the newly established and some of the old orchards, which have resulted in yield reduction and also have created non-uniformity in the orchard (Sedaghati *et al.*, 2009). While in the recent years, by introducing vegetative rootstocks, orchardists' tendency towards using such rootstocks is increasing. This will lead to uniformity of the pistachio orchards in the near future. Moreover, these vegetative rootstocks possess positive characteristics such as tolerance to biotic and abiotic stresses that increases attention to their production and vegetative propagation.

UCB-1 is an example of the new vegetative rootstocks accepted by pistachio orchardists with characteristics such as increased vegetative growth, early bearing and tolerances to verticillium wilt, salinity and drought and also positive physiological effect on scion characteristics especially on the yield (Ferguson *et al.*, 1991 and 1997). Using plant tissue culture

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techniques, large scale production is possible. Many researchers in Iran, USA, and Turkey have worked on pistachio micropropagation and have obtained many successes in this regard (Onay, 2000; Akdamir *et al*., 2014; Nezami *et al*., 2015; Tilkat *et al*., 2008; Vatan Pur Azghandi*et al*., 2008; Garoosi *et al*., 2006 and Marín *et al*., 2016). Synthesis and exudation of the phenolic compounds, yellowing and tip necrosis of the new shoots along with difficult proliferation and hard rooting are serious problems in UCB-1 rootstock propagation (Nezami *et al.*, 2015). Necrosis is a considerable difficulty in *in vitro* cultures of pistachio (Barghchi and Alderson, 1985; Abousalim and Mantell, 1994). Shoot tip necrosis is known as a physiological disorder which is due to unbalanced uptake of nutrients such as calcium, zinc and boron that their deficiencies appear in the apical meristem. The mentioned disorder has been reported in *P. vera* (Barghchi and Alderson, 1985), *P. terebinthus* (Pontikis, 1985) and *P. integrima* (Martinelli, 1988). Several hypotheses arise for the reason of tip necrosis disorder occurrence. Among these, boron deficiency (Mason and Guttridge, 1974) and especially calcium deficiency (Sha *et al.*, 1985) are the most notable. A comprehensive study on pistachio micropropagation by Alderson and Barghchi (1989) indicates that boron and calcium deficiencies could be the probable reasons for the tip necrosis. The application of calcium, boron and zinc has been suggested in many reports. Researchers have been consistently seeking for new compounds in order to solve the aforementioned problem. Nanotubes are one the compounds which are recently being used in various plant tissue culture media for different purposes. Amongst the most important applications of this compound are control and reduction of yellowing as well as increased proliferation (Heydari, 2013).

Nanotechnology is highly important and widely used in agricultural sciences especially in the fields of biotechnology and tissue culture. Nanotubes have been used in *in vitro* cultures of different plants such as anthurium (Heydari, 2013), tomato (Khodakovskaya *et al.*, 2009), cabbage, carrot, cucumber, lettuce and onion (Canas *et al.*, 2008). Thus far, valuable results have been achieved. As mentioned earlier, this compound not only reduces yellowing in anthurium and improves qualitative indices but also affects proliferation rate and plays a role in growth regulation. Cytokinins are the most important plant growth regulators which are used in the proliferation stage. The type and concentration of the applied cytokines during the proliferation stage highly affects the proliferation rate. Commonly, the rate improves by increased concentrations. However, this increase may result in a tolerance threshold. Hence, a certain amount of increase will result in reduced proliferation and even hyperhidricity (not a word).

Material and Methods

Explants in this study were collected from the Research Greenhouse of Rana Biotechnology Complex. They were chosen among UCB-1 pistachio rootstocks UCB-1 (a hybrid of atlantica and integrima Rootstock). Explants were use of 1 to 1.5 cm in length with one node of semi-woody branches. They were soaked in sodium hypochlorite of 20% for 12 minutes for sterilization. Then, to eliminate bacterial contamination, we used nanosilver with concentration of 150 mg/l for seven minutes. At the end, they were rinsed three times. The disinfection was carried via laminar in flow.

In order to establish explants, we used a medium introduced by Nezami *et al.*, (2015). It was a modified MS medium with 6gr/l of agar, 3% of sucrose, 0.5mg/l of benzyladenine, 0.05mg/l of Indole butyric acid and 0.05mg/l of gibberellic acid and Gamborg vitamins. The culture medium pH was adjusted to 5.7 with KOH 0.1N prior to autoclaving at 1138C for 20 minutes. Then, established and grown explants were cultured in MS medium without hormones. This led to the same length and diameter of the shoot. In the first experiment, we

used high concentration of nanocarbon tube and banzyladenin. However, this concentration led to plant death and browning (data not show).

Another experiment in the form of a factorial experiment was conducted in a completely randomized design. The first factor consisted of various concentrations (0, 50, 100, 150 and 200µgr/l) of carbon nanotubes. The second factor included different concentrations (0, 0.5, 1.5 and 2mg/l) of benzyladenine. All shoots with the same size and conditions were cultured in these medium. The experiment was repeated five times and in each repeated stage, five shoots were used. Understudied indices included the proliferation rate, the length of the shoots, STN and *vitrification* (not a word) percentage. After cultivation, the shoots were preserved in growth chamber with light of 400 micromoles per second (PAR) and a temperature of 22°C for 8/16 hours.

Statistical analyses

Experiments were conducted with at least five replications. A completely randomized design based on factorial arrangement was used. The data was statistically analyzed using SAS 9.2 (SAS Institute Inc., 2008). The significance of difference among means was carried out using Least Significant Difference (LSD) test at $P < 0.01$ and $P < 0.05$.

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Proliferation

According to Table 1, the analysis of variance showed that different concentrations of Nanotube and BA had significant effects on the proliferation rate ($P < 0.01$). No significant effect on interaction between factors was observed. The results showed that by increasing the amount of carbon nanotubes in culture medium, the proliferation rate significantly increased, and significant statistical differences were observed. The highest proliferation rate (2.85 microshoots per explant) was obtained at a concentration of 200 µg/l. The use of higher concentrations of 200 µg/l resulted in burns and plant death (data not show). The lowest rate (1.33 microshoots per explant) was observed in the control group (Fig. 1). In addition, a significant difference was observed in different concentrations of benzyladenine effects on UCB-1 shoots proliferation. According Fig. 2, increasing the concentration of benzyladenine led to an increase in the proliferation rate. The highest and lowest rate (3.4 and 1.56 microshoots per explant) was observed with a proliferation rate with 2 and 0.5mg/l benzyladenine, respectively. Finally, although the interaction use of carbon nanotubes and benzyladenine was not significant, the best results were obtained from treatment with 200 mg of nanotubes + 2mg/l of benzyladenine, which led to the highest proliferation rate (4 microshoots per explant).

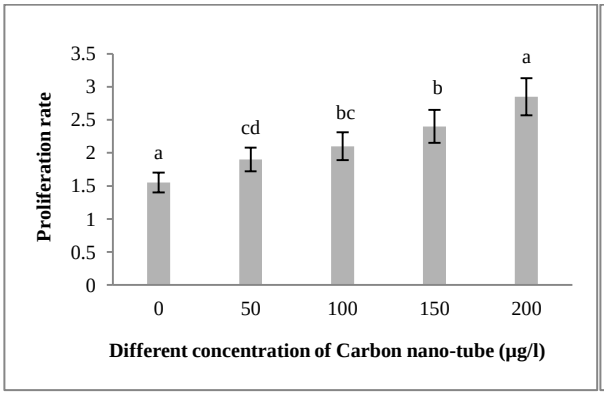

Fig. 1. The effect of Different concentration of Carbon Nano-tube on proliferation rate of UCB-1 microshoots.

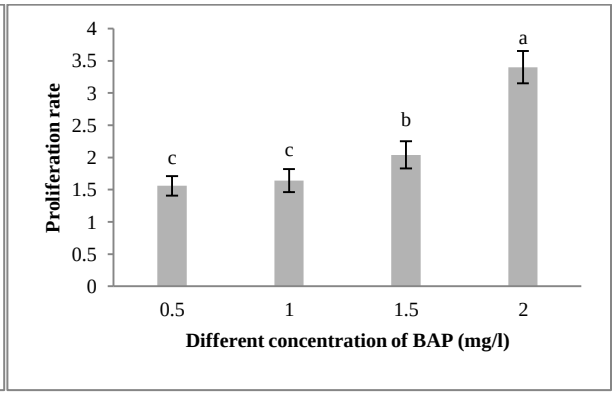

Fig. 2. The effect of Different concentration of BAP on proliferation rate of UCB-1 microshoots

Length of the microshoots

Data analysis of variance showed a significant statistical difference between the treatments groups on the length of shoots. In addition to the unique effects, the impact of nanotube interaction in benzyladenine was also significant (Table 1). By increasing the concentration of nanotubes, the length of the shoots was increased, which was the same with the effects of benzyladenine. The highest length was observed at the highest concentration of benzyladenine. The effect of benzyladenine and nanotube was more than their effects in separate cases. The longest length of shoots (3.68cm) was related to highest the highest used concentrations (200 µg/l of nanotubes + 2 mg/l of benzyladenine), and the shortest length (1.50cm) was related to the control group without carbon nanotubes (Fig. 3)

Table 1. Analysis of variance for Carbon Nano-Tube and BAP on Proliferation index of UCB-1

S.O.V.	df	Mean of squares (MS)			
		Proliferation rate	Length of shoot (cm)	Shoot rip necrosis (%)	*Vitrification* (%)
Carbon Nano-Tube	4	4.88 **	10.77 **	0.24 **	0.06 **
BAP	3	18.18 **	0.66 **	0.01 ns	0.02 **
CNT × BAP	19	0.04 ns	0.05 **	0.00 ns	0.00 **
Error	12	0.18	0.01	0.00	0.00
Coefficient of variation		18.85	5.84	15.60	7.38

*, ** represents effects significant at probability levels of 0.05 and 0.01 respectively; ns means non-significant (P<0.05).

The percentage of shoot tip necrosis

The results showed that the interaction between the nanotubes and benzyladenine on the percentage of STN was not statistically significant (Table 1). The separate effect of carbon nanotubes was significant at the statistical level of one percent. Significant statistical differences were observed between the effects of different treatments on STN. There were no significant statistical differences between different concentrations of benzyladenine. By increasing the nanotubes concentration, the STN rate was reduced. In concentrations of 0 and 200 µg/l, it was 41% and 13%, respectively. The results of Fig. 4 showed the lowest STN rate (8%) for treatment with 200 µg/l of nanotubes and 0.5mg/l of benzyladenine, and the highest rate of STN (44%) was related to higher concentration of benzyladenine (1.5mg/l) without nanotubes.

Vitrification percentage

According to data analysis of variance, the effects of interaction factors on shoots *vitrification* were significant (P <0.01). The highest *vitrification* rate (22%) was shown in the treatment group using 0 µg/l of nanotubes plus 2mg/l of benzyladenine. The lowest rate (0%) was shown in the treatment group treated with 200 µg/l of nanotubes plus 2 or 1.5mg/l of benzyladenine (Fig. 5).

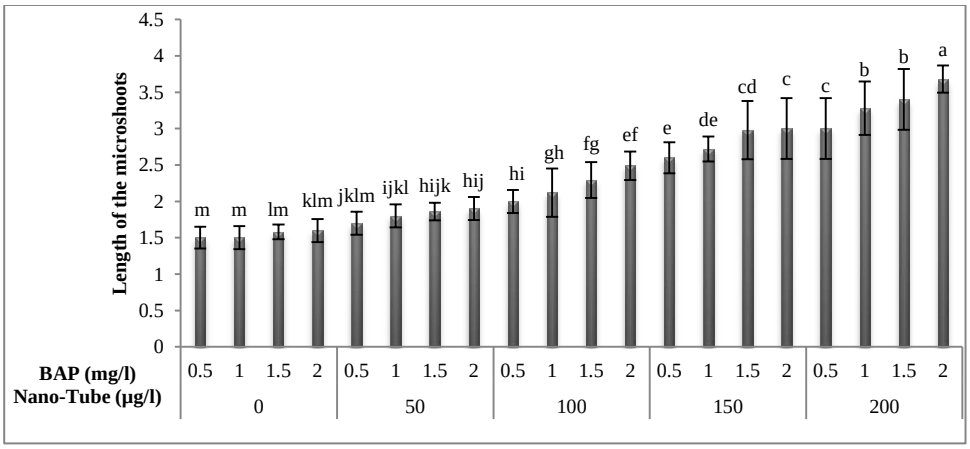

Fig. 3. The effects of nanotubes and benzyladenine on the length of shoots of UCB-1 rootstock

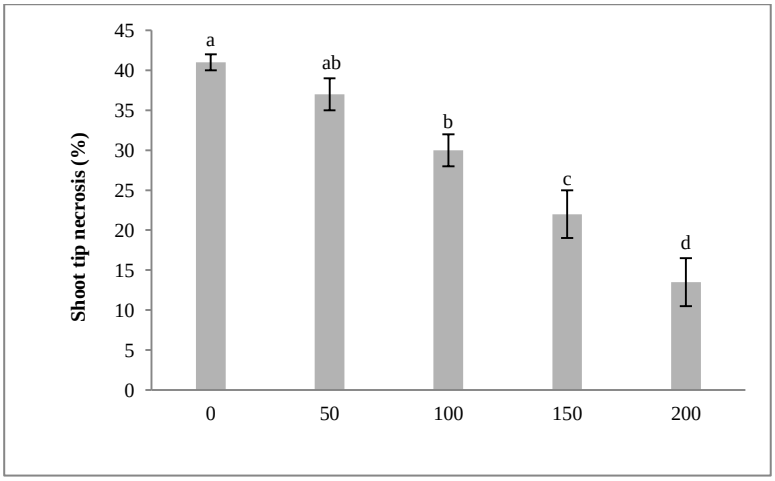

Fig. 4. The effect of nanotubes on shoot tip necrosis percentage in proliferated shoots of UCB-1

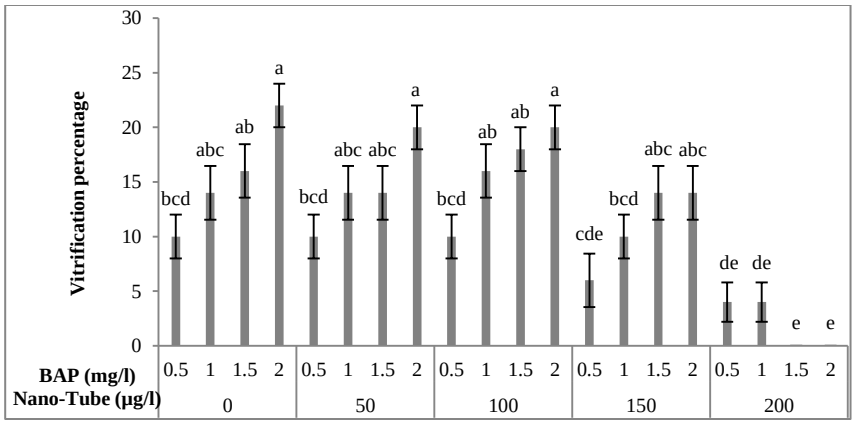

Fig. 5. The effect of nanotubes and benzyladenine on *vitrification* percentage in proliferated shoots of UCB-1

Discussion

Increasing proliferation coefficient is an economic point of propagation. Increased rates of in vitro proliferation and production will help reduce production costs. Alteration in medium composition and also its interaction with endogenous and exogenous PGR levels can affect the proliferation rate. Achieving the highest rate of proliferation with emphasis on preserving the genetic and epigenetic stability can be important. Concentrations and types of elements and compounds added to the medium influence the proliferation rate. One of such compounds was carbon nanotubes. Based on the results, there was a significant difference in the proliferation indices rates depending on what compound was used in the culture medium, which was similar to previous findings that claimed using nanotubes had a positive effect on cell function (Casey *et al.*, 2005). The effect of nanotubes on plant cell growth or its prevention is different at various stages. Various types of nano compounds used in vitro such as nano silver (Arab *et al*., 2014a; Shokri *et al*.,); TiO2 (Lin and Xing 2007); ZnO (Apperlot *et al*., 2009) and Carbon nanotubes (Heydari, 2013). Carbon nanotubes have many applications in agricultural sciences. For instance, it can be used in the culture medium as a growth regulator. Previous reports indicate its positive effect on tissue culture of plants, which is in coincidence with the results of the current study. As seen by the results of our experiment, the application of carbon nanotubes had a positive effect on proliferation rate and resulted in improvement of proliferation coefficient in new shoots of UCB-1 vegetative rootstocks. The proliferation status improved with increasing concentrations up to 200 µg/l. The highest proliferation rate was related to the highest nanotube concentration with no adverse effect or toxicity, which coincides with the findings of Jackson *et al*. (2013) and Mondal *et al*. (2011), who stated that high concentrations of nanotubes have no toxic effect on plants. Also, in similar results, using 76 µg/l of single-walled nanotubes with COOH group had no toxic effect on seed germination and further growth and development of the raspberry roots (Rubus adenotrichos) (Flores *et al*., 2014). Nanotubes also influenced the gene expression rate within the plant, which most likely led to an increase in the proliferation rate. As the results of Khodakovskaya *et al*. (2011) showed, using multi-walled nanotubes (50; 100; and 200µg⁄mL) on new shoots of tomato resulted in modification of gene expression. Exposure of tomato cells with nanotubes enriched medium caused significant differences in expression of environmental stresses tolerance genes such as the water channel (LeAqp2) gene. Ultimately, all these events resulted in higher tomato seed germination percentage and new shoots elongation.

The number of new shoots was affected by the amount and concentration of the nanotubes and also different cytokinin concentrations. By increasing cytokinin amounts, the number of new shoots increased. BAP may provide extra vigor for mature tissues and induce synthesis of endogenous cytokinins, such as kinetin, which naturally improves the formation of the new shoots (Rai *et al*., 2010). Proliferation increased with increasing hormone concentrations that was similar to a number of previous investigations that stated that the application of 2 mg/l benzyladenine showed the highest rate of proliferation. Akdemir *et al.* (2013) reported using 2 mg/l benzyladene which led to an increase in vitro pistachio proliferation. Benzyladenine increased the proliferation number by affecting cell division.

It was obvious that combinational application and interaction of nanotubes and benzyladenine was more effective on the proliferation rate compared to their individual application. Nanotubes are capable of interacting with biomolecules and creating functional nanosystems for transportation of other materials within cells that leads to interaction of nanotubes and other compounds at morphological, cellular and even molecular levels. Previously, carbon nanotubes were used in combination with sucrose and other polysaccharids in order to study their interactions. The results indicated that the combination of the aforementioned substances (nanotube + sucrose) has shown an increasing trend in the uptake rate (Casey *et al*., 2005), which is consistent with our results that suggested that the combinational application of nanotubes and benzyladenine leads to improvement of proliferation and other qualitative indices. Moreover, by combining nanotubes with organic compounds, a new compound is created and covalent bonds form between them, which results in higher uptake through cell wall (Casey *et al*., 2005).

Besides the proliferation rate, nanotubes also affect new shoots elongation, yellowing rate and hyperhydration. Noticeable differences in new shoots length was observed when nanotubes were used or not used in the culture medium. Nanotube serves as a growth regulator, promoting longitudinal cell division, which results in new shoots lengths. The effect of nanotubes on longitudinal growth has been reported by Canas *et al.* (2008), who claimed that using nanotubes in the culture medium leads to improved elongation in the roots of cabbage, carrot, cucumber, lettuce, onion and tomato seeds. Using carbon nanotubes for in vitro culture of GF677 vegetative rootstock indicated that using this compound in all stages has a positive impact on growth, development and cell division. In other words, the results of the research conducted by Ghorbanizad *et al*. (2012) showed that multi-walled carbon nanotubes positively affected indices such as the number of the produced shoots, shoot elongation and increase in weight and leaf area. Microscopic evaluation and taking TEM photos of a bud grown in the medium containing multi-walled carbon nanotubes confirmed the penetration of carbon nanotubes into the shoots and buds tissues. Also, it was indicated that this compound can improve water uptake by creating pores in the cell wall. No differences were observed among the treatments in terms of the chlorophyll content amount and electrolyte leakage. Khodakovskaya *et al*. (2009) reported that using 50 µg/l of single-walled nanotube improves tomato germination and elongation in vitro. Increased elongation is due to the genetic and protein expression of aquaporins, which facilitates metabolic processes within plant (Khodakovskaya *et al*., 2012). Nanotubes affect uptake, transport and interaction among cells considering their unique characteristics. Nanotubes uptake depend on their distribution rate and better distribution will improve the growth rate (Husen and Siddiqi, 2014). Studies on single-walled nanotubes distribution in ionic liquid medium indicates that they

can interact with each other by weak Van der Waals forces (Wang *et al*., 2008). It is also possible that in addition to the van der waals bands formed between themselves, they might be capable of binding to the other elements. By adding nanotubes to the tissue culture medium, an interaction forms among mineral salts, which results in production of the hydrophilic compounds that deeply increases their biocompatibility and stability. Hence, following an increase in nanotube amount in the culture medium, production of such stable compounds in the culture medium will be increased and besides increasing mineral salts stability, uptake will be also promoted. This explains why by increasing nanotubes concentrations in the culture medium, growth rate was increased and the maximum length was obtained in the highest concentration. A combination of nanotube and benzyladenine had a greater effect on the elongation that's probably due to the presence of such interactions.

By applying carbon nanotubes, the rate of shoots tip necrosis (STN) was reduced. STN is considered as a physiological disorder in plants in vitro culture. The first symptoms of STN appear as brown spots on buds and first young leaves that are conjecturally due to the deficiency of nutrients with low mobility such as calcium and boron. Terminal leaves and meristems are among the first parts that undergo necrosis (Barghchi and Alderson, 1996). Increasing boron amounts up to 200 µM significantly decreased the necrosis rate in the new shoots of *Pistacia vera* L, however shoot formation rate subsequently decreased (Barghchi and Alderson, 1996). A series of disorders have also been related to boron deficiency that occurs in apical meristem. A disorder in auxin metabolism, (Eaton, 1940) increased lignifications (McLlratb and skok, 1964; Perkins and Aronoff, 1956) and phenolic compounds accumulation are some of the consequences related to the boron deficiency. Boron deficiency individually or in combination with calcium deficiency can be effective in necrosis (Barghchi and Alderson, 1996). The application of compounds which have auxinic structures or are capable of entering herbal auxin synthesis pathway leads to an increase in calcium mobility percentage and reduction of necrosis. Seemingly, carbon nanotubes also follow such mechanism and increase mobility and even calcium, zinc and boron uptake. Since, binding of nanotubes to other elements can result in an improved uptake of themselves and available mineral elements in the culture medium, it can be hypothesized that adding nanotubes to the culture medium leads to binding to the elements such as calcium, zinc and boron, which consequently increases their uptake rate. Therefore, at last it was specified that by using nanotubes, necrosis rates drastically reduced. Previous reports also indicated that single-walled nanotubes play a role in transportation and translocation of proteins, nucleic acids and small peptides (Pantarotto *et al*., 2004). The addition of nanotubes to the culture medium also facilitates water transfer and uptake. The amount of the available water within the cell has a direct relation with tissue hyperhydration rate. By adding nanotube to the culture medium, rate of hyperhydration reduced while, conversely, in the case of benzyladenine addition, increasing concentrations resulted in higher production of hyperhydrated tissues. Hyperhydration can lead to irreversible loss of the plant tissues (Gaspar *et al*., 2000) and disruption of tissue recovery potential, which finally leads to death. Proliferation rate also decreases as a result of such losses and stem weakening.

Several factors can influence hyperhydration rates including high levels of benzyladenine (Oliveira *et al*., 2010), cultivar (Carvalho *et al*., 2011) and agar concentrations (Abdoli *et al*., 2000). Commonly, hyperhydration occurs in liquid media (Scheidt *et al*., 2011). Some species are highly sensitive to hyperhydration, and it may even occur in solid media or low concentrations of benzyladenine. This disorder is evident in plants. In such cases, modifying the

composition of the culture medium substances is a suggested strategy to overcome the problem (Machado *et al*., 2014). The application of the highest nanotube concentration even combined with benzyladenine did not show any hyperhydration symptoms. Therefore, it can be claimed that nanotubes application has even compensated high concentrations of cytokinin and reached hyperhydration percentage to zero, and cytokinin has been influenced by nanotubes. As previously reported by Liu *et al*. (2009), nanotubes can effect hormones distribution and microtobules organization. Dehydrogenase enzyme activity is a paramount indicator for assessment of metabolic activities and affects cell ability for nutrients uptake. This enzyme is affected by nanotubes.

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