Effects of multi-wall carbon nanotube on *Nepeta cataria* and *Salvia sclarea in Vitro* culture

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Abstract

Nepeta cataria L. and *Salvia sclarea* L. are two endangered species of Lamiacea family in Yazd province of Iran. Callus induction and shoot regeneration of these species were investigated in this study under the effects of multi-wall carbon nanotube (MWCNT) to improve the proliferation rate. MS medium supplemented with 0.1 mg l^{-1} Kin and 1 mg l^{-1} NAA was used for callus formation. Also, MS medium containing 0.1 mg l^{-1} IAA and 1 mg l^{-1} BA was used for shoot regeneration. Multi-wall carbon nanotubes (0, 20, 60, 80, and 200 µg ml^{-1}) were used in the culture media for regeneration and callus induction in both species. Results showed significant differences between MWCNT treatment and control in terms of callus induction and shoot regeneration rate. The maximum callus mass of *N. cataria* (Catnip) under 20 µg ml^{-1} MWCNT was 304 mm^3 whereas, this attribute in control plants was 117.75 mm^3. The highest callus mass of *S. sclarea* (Clary sage) under 80 µg ml^{-1} MWCNT and control were 414 mm^3 and 182.15 mm^3, respectively. Treatment containing 20 µg ml^{-1} MWCNT with 6.17 shoots per explant proved the best for shoot regeneration in *N. cataria* while treatment with 80 µg ml^{-1} MWCNT showed the lowest regeneration rate. Using 80 µg ml^{-1} carbon nanotube with 4.2 shoots per explant led to the maximum proliferation while the minimum regeneration was 1.85 resulted from 200 µg ml^{-1} MWCNT treatment in *S. sclarea.* The effect of MWCNT on micropropagation seems to be dose dependent, and high concentrations of nanotubes reduces callus formation and shoot regeneration in these plants.

Keywords: Callus induction, Catnip, Clary sage, MWCNTs, shoot regeneration

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Introduction

Nepeta cataria L. and *Salvia sclarea* L. are two most important genera of the Lamiaceae family with the common Persian name “Puneh” and “Maryam-Goli e Kabir” respectively. Nepeta grows in Europe, Asia, and Africa and belonge to genus of Lamiaceae family with almost 280 species (Rechinger, 1982). Sixty-seven species of *Nepeta* are found in Iran including *Nepeta cataria* L., commonly named as catnip. These plants have had applications in traditional medicine as disinfectant and also for flu treatment. Essential

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oil of *N. cataria* is known as a fungicidal and bactericidal agent (Rustaiyan, 1999).

Salvia is the largest genus in Lamiaceae family comprising about 700-900 species. Among these species, *Salvia sclarea* L. (Clary sage) is of great importance from industrial point of view, because it is used to prepare flavors and essential oils (Dikova, 2009). *In vitro* tissue culture is one of the short-term micro propagation strategies that can be used to save endangered species such as *Salvia* species. A number of species in this genus have been regenerated, including *Salvia miltiorrhiza* Bge (Li et al., 1988), *S. greggii* (Frett., 1986), *S.* officinalis, and *S. fruticosa* (Kintzios et al., 1999).

There have been several reports on cell culture and tissue of various *Nepeta* and *Salvia* species. *In vitro* production of rosmarinic acid in *Nepeta cataria* L. has been studied, and the most useful growth regulator compound for micro-propagation of *Nepeta nuda* L. was reported as 1 mg L^{-1} BA along with 0.5 mg L^{-1} IBA and NAA (Narimani et al., 2017). Banthorpe et al. (1990) reported on the formation of frail callus and isolated cell lines of cell suspension from stem tissues of *S. sclarea* on the MS culture supplemented with either 0.5 µM Kin or 4.5 µM 2, 4-D.

Nowadays, developments in nanotechnology have presented new possibilities for application of nanoparticles in agriculture and biotechnology particularly for plant growth and productivity. Many reports have posited that nanomaterial compounds, because of their exclusive physicochemical properties, can react to many biological systems and play as an epigenetic agent (Haghighi Pak et al., 2017; Asgari Targhi et al., 2018; Mirmoeini et al., 2021). Carbon nanotubes (CNTs) as a kind of nanoparticles have a unique position for their physical and chemical characteristics (Siddiqui et al. 2015). CNTs include SWCNTs (single wall nano tubes) and MWCNTs (multi wall nano tubes). Multi-wall carbon nanotubes can activate plant growth and *in vitro* culture as they help organize the marker genes during cell divisions and play a role in cell wall formation and also in water transport (Khodakovskaya et al., 2013). Srivastava and Rao (2014) reported that MWCNTs affect corn, wheat, garlic, and peanut improving biomass and also the size and number of leaves in these plants. Tiwari et al. (2013) found that the application of 20 µg ml^{-1} MWCNts could improve water uptake and accelerate plant growth. Also, CNTs are breported to accelerate biosynthesis of secondary metabolites and callus formation in *Satureja* khuzestanica (Ghorbanpour and Hadian, 2015). CNTs may therefore be considered as a relatively effective elicitor in plant tissue explants. *Nepeta cataria* and *Salvia sclarea* are important herbs with applications in pharmacology and perfume industry. The purpose of this study was to improve plant regeneration and callus induction of these species by using MWCNTs.

Materials and Methods

Plant materials

Seeds of *Nepeta cataria* L. and *Salvia sclarea* were obtained from the Agricultural Jihad Organization in Yazd province. The seeds were sterilized by washing with hypochlorite solution, rinsed several times under water jet, and then rinsed with distilled water. Then, seeds were sterilized for 30-60 seconds in 70% ethanol and 1% sodium hypochlorite (20% Clorox bleach) for 15-20 min before they were rinsed three times in sterilized distilled water (Jonoubi et al., 2017). Leaf explants were obtained from steriled plants *in vitro* culture and the young plants with six to eight leaves grown in field, sterilized for 12 min in 0.1% mercuric chloride solution before they were rinsed three times in sterilized distilled water.

Callus formation

Leaf pieces of Catnip and Clary Sage plants, 1-2 cm long, were excised and inoculated into callus induction culture medium as recommended by Kintzios et al. (1999). The culture medium consisted of MS medium (Sigma Chemical Co., St. Louis, Missouri) solidified with 0.8% agar, and supplemented with 0.1 mg l^{-1} Kin (kinetin), 1 mg l^{-1} NAA (Naphthalene Acetic Acid), and 30g l^{-1} sucrose. The medium was adjusted to pH 5.8 using 1N NaOH or 1N HCl, autoclaved at 121 ℃ for 20 min, and kept in glass Petri dishes.

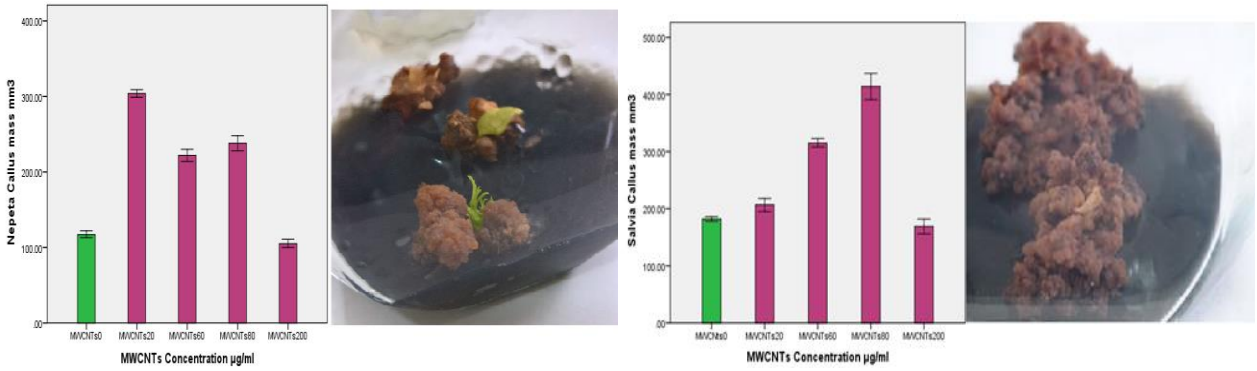

Fig. I. Leaf explant callus culture with different concentrations of MWCNTs; left: *Nepeta cataria*, right: *Salvia sclarea*

Shoot regeneration and plant acclimatization

Shoots of Catnip and Clary Sage were regenerated using leaf explants on a solid MS medium supplemented with 0.1 mg l^{-1} IAA (Indole-3-Acetic Acid), 1.0 mg l^{-1} BA (6-Benzyl Adenine), 0.8% agar, and 30 g l^{-1} sucrose proposed by Kuzma et al. (2009). Cultures were kept in a growth chamber (26 ± 2 ℃, 70% humidity, 16/8 h light/dark photoperiod condition). Cool white fluorescent lamps with 400 µmol $m^{-2}s^{-1}$ a light intensity were used for Illumination. After four weeks, rooted shoots were planted in the pots containing sterile mixture of soil, sand, and peat (4:3:3) and allowed to grow in a greenhouse (24 ℃). The plants were allowed to acclimatize for three weeks before they were transferred to the field to grow for a period of two years.

MWCNT treatment

Multi-wall carbon nanotubes of 10-20 nm diameter and 3-8 µM length were purchased from USNANO Co., USA. To produce water soluble carbon nanomaterials, 2 g carbon soot was kept in 100 ml concentrated nitric acid for 24 h. After removing the nitric acid excess, the remaining black mass was washed thoroughly using distilled water. All traces of nitric acid were removed by repeatedly adding water and allowing it to evaporate in a boiling water bath. The final wash black mass was tested with Griess reagent (Roy and Sarkar, 1994) and vacuum dried for further analysis. The obtained carbon nanomaterials was subjected to sonication in order to became water soluble (Jonoubi et al., 2017). Sterilized MWCNTs (0, 20, 60, 80 and 200 µg ml^{-1}) were added to each media for evaluation of the effects of MWCNTs on regeneration and callus induction in both species.

Data Analysis

The experimental design was fully randomized with three replicates of 20 explants per treatment. Statistics analysis was carried out with SPSS software, version 25. Tukey and Schefe assays were used for mean comparison ($p \leq 0.05$).

Results

Callus formation

To produce *N. cataria* and *Salvia sclarea* calluses, MS medium supplemented with 1 mg l^{-1} NAA and 0.1 mg l^{-1} Kin was used. In order to investigate the effect of nanotubes on callus production of these plants, different concentrations of MWCNTs were used in callus induction media. In *N. cataria* culture the highest callus mass was obtained when culture media with 20 µg ml^{-1} MWCNTs with 304 mm^3 (Fig. I), while the lowest callus mass was obtained from the treatment of 200 mg ml^{-1} MWCNTs with dimensions 117.75 mm^3 showing no significant difference from the control.

The highest callus formation of *Salvia sclarea* leaf explant was obtained from medium supplemented by 80 µg ml^{-1} MWCNTs with 414 mm^3, but the lowest callus volume with 182.15 mm^3 was produced in medium included 200 µg ml^{-1} MWCNTs which was in the same statistical group with control.

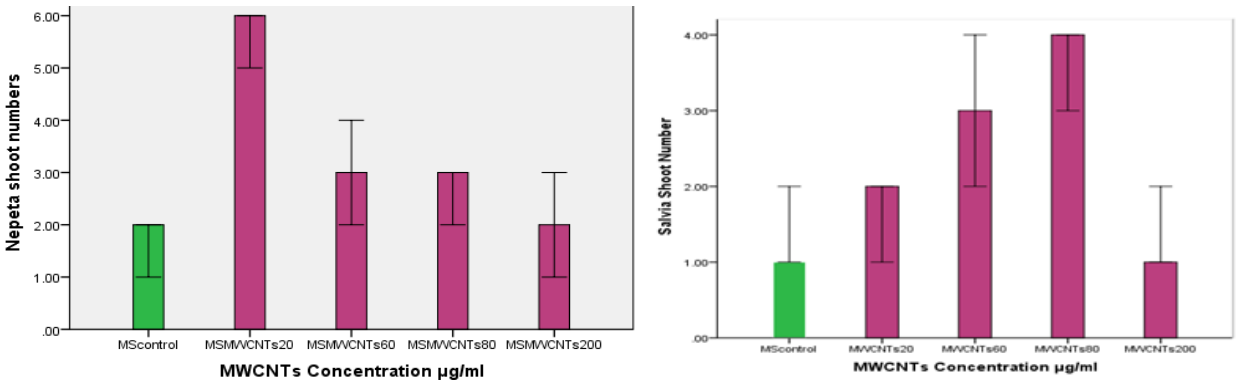

Fig. II. Shoot numbers of plant regeneration in different concentrations of MWCNTs; left: *Nepeta cataria*, right: *Salvia sclarea*

Fig. III. Shoot regeneration of *N. cataria* on MS with BA 1 mg l^{-1}, IAA 0.1 mg l^{-1}, and 20 µg ml^{-1} MWCNTs

Shoot regeneration

In order to the most shoot regenerate of *Nepeta cataria* L. and *Salvia sclarea* L., MS media supplemented with 1 mg l^{-1} BA and 0.1 mg l^{-1} IAA were used. To evaluate the effect of nanotubes *in vitro* shoot regeneration, different amounts of MWCNTs were added to the culture media. Results of the *N. catari* showed that the MS containing 20 µg ml^{-1} MWCNTs with 6.17 shoots per explant was the highest shoot regeneration and the medium with 80 µg ml^{-1} MWCNTs (2.12 shoots per explant) had the lowest regeneration rate, which was in a same statistical group with control (Fig. II).

The highest rate of shoot regeneration in *Salvia sclarea* regeneration was obtained with 80 µg ml^{-1} MWCNTs with 4.2 shoots per explant while the lowest ratio was recorded with 200 µg ml^{-1} MWCNTs treatment (1.85 shoots per explant) which was also in the same statistical group with control (Fig II). Regenerated plants in culture media containing nanotubes were able to produce roots and were able to survive after transferring to the soil (Figs. III, IV).

Discussion

Tissue culture is a commercially important method of micro propagation particularly for medicinal plants, and nanotechnology is employed to increase the rate of regeneration. There has been a rise in research on single-walled and multi-walled CNTs in agriculture and food industries during the past decade.

Fig. IV. Shoot regeneration of *S. sclarea* on MS with 1 mg l^{-1} BA, 0.1 mg l^{-1} IAA, and 80 µg ml^{-1} MWCNTs

To obtain the maximum callus mass from *Nepeta cataria* and *Salvia sclarea*, we used MS medium supplemented with 1 mg l^{-1} NAA and 0.1 mg l^{-1} Kin, producing 110 mm^3 callus mass that was similar to the study reported by Kintzios et al. (1999). Also, Sagharyan et al. (2020) reported that the highest fresh and dry weights of *Nepeta binaloudensis* calluses were observed in ½ MS medium, s upplemented with 2 µM reduced-glutathione, 2 mg l^{-1} BA, and 2 mgl^{-1} NAA. Banthorpe et al. (1990) reported on the establishment of callus from stem explants of *S. sclarea* plant on an MS medium supplemented with either 5.4 µM NAA and 0.5 µM Kin or 4.5 µM 2, 4-D and 0.5 µM Kin that induction of callus rate was 80%.

Regeneration of *N. cataria* and *Salvia sclarea* shoots using the media supplemented with BA 1 mg l^{-1} and IAA 0.1 mg l^{-1} in this study showed the same results as Kuzma et al. (2009). Micropropagation of *Salvia* species, e.g. *S. valentina* and *S. blancoana* (Cuenca and Amo-Marco, 2000) and also *S. brachyodon* (Misic et al., 2006) were already reported. Misic et al. (2005) demonstrated that all study concentrations of BA and Kin combined with 0.1 mg l^{-1} IAA negatively affected the elongation and rooting of *N. rtanjensis* shoots. The increase in BA levels stimulated axillary bud formation while negatively affecting shoot elongation. This suggests an inverse relationship between the shoots' number and elongation.

It was found in the present study that the application of 20 µg ml^{-1} MWCNTs produced the maximum callus mass (304 mm^3) in *Nepeta cataria* while the highest callus mass in *Salvia sclarea* was (414 mm^3) obtained with 80 µg ml^{-1} MWCNTs. In their study on the calluses initiated from *Satureja khuzestanica* leaf explants, Ghorbanpour and Hadian (2015) found that adding 25 and 50 µg l^{-1} MWCNT to the culture medium improved callus formation, whereas 100, 250, and 500 µg l^{-1} MWCNTs were not as effective. Propagation of UCB-1 (*Pistacia atlantica* × *P. integrima*) rootstock using tissue culture suffered from drwabacks such as shoot tip necrosis (STN) and a low proliferation rate (Aghasi Kermani et al., 2017). They further found that the treatment involving 200 µg l^{-1} carbon nanotubes and 2 mg l^{-1} BA led to maximum

proliferation, maximum shoot length, and minimum STN. In their study on tobacco explants, Khodakovskaya et al. (2013) found that adding supplementing the medium containing 1 mg l^{-1} 2,4-D 100 µg ml^{-1} with multi-walled carbon nanotubes resulted enhanced the callus growth by 64%. In another study, Lin et al. (2009) reported that carbon nanotubes enhanced callus growth in *Arabidopsis*, which was attributed to the role of carbon nanotubes in upregulation of the genes involved in cell wall extension (NtLRX1), cell division (CycB), and water transport (NtPIP1). On the other hand, carbon nanotubes negatively affected cell viability and dry weight of the plants in the same study. This drawback is also reflected in the study by Tan et al. (2009) who found that adding carbon nanotubes to rice cell suspension cultures decreased cell viability.

It seems the effects of MWCNTs on propagation and callus formation may be dose-dependent, in this study, and high concentrations of nanotubes reduced callus formation and shoot regeneration. Similar to this results, low concentrations of MWCNT showed an increasing effect in tobacco cell culture and high doses of MWCNTs caused to decrease in the cellular growth in some samples, considerably (Khodakovskaya et al. 2013).

In sum, the literature on the use of nanoparticles in micropropagation culture media suggests that they play a positive role in rooting, proliferation of calluses, multiplication of shoots, and also somatic embryogenesis through gene expressions, inducing activities of antioxidant enzymes, and preventing production of ROS and ethylene. Several hypotheses have currently been proposed for the underlying mechanisms, although their effects on each parameter require comprehensive investigation (Sedghi et al., 2021). Increasing water uptake in MWCNTs treated samples was reported in several studies (Tripathi et al., 2011). MWCNTs' effects may also be attributed to their potential to activate channels transporting water into cells, help cells division, and increase their growth (Sarikhani et al., 2017).

Electron microscope imaging has been employed to explore bioaccumulation with CNTs in Sewy date palm shoot tissues (sarikhani et al., 2015). Similarly, Scanning Electron and Fluorescence Microscope was used to track CNTs in wheat, confirming the presence of water soluble carbon nanotubes (Tripathi et al., 2011). CNTs are able to cross cellular barriers and traffic intracellularly. Tripathi et al. (2011) argued for the possible nuclear localization for CNTs and also their potential to transfer over generations, which is evident after cell differentiation. Lin et al. (2009) declared that carbon nanoparticles can absorbed by osmotic pressure to roots, probably and by the capillary potential go through cell wall pores. Moreover, epigenetic modification in the histone acetyltransferases (HDA101, HDA102, and HDA106 genes) and histone deacetylases (HDA108, HD1b, and HD2 genes) were reported by the single wall nano tubes (SWCNT) treatments in maize (Yan et al., 2013). It seems that the modulation of MWCNTs into the culture explants operates as a strong elicitor/epigenetic factor that impacts development, differentiation, gene regulation, and plant growth and metabolism (Ghasempour et al., 2019).

Biosynthesis of secondary metabolites and callus establishment in *Satureja khuzestanica* was accelerated by CNTs (Ghorbanpour and Hadian, 2015*)*. Therefore, CNTs are striking elicitors for plant tissue culture by means of improving primary and secondary metabolism levels and other growth factors. However, CNT is effective on the root formation in the medium, needs to be further studied. There is not much information about the possible functions of CNT in root induction and it needs more studies.

Finally, the interaction effects of various combinations and concentrations of nanoparticles in different media on callus induction, rooting, and shoot regeneration are within a research avenue to shed light on the mechanisms underlying the role of nanoparticles in tissue cultures of various plants including *Nepeta* and *Salvia*.

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