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Evaluation of Mwcnts Effects On Shoot Regeneration and Leaf Callus Cultures Of Salvia Sclarea (Clary Sage)

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Abstract
In order to study of MWCNTs effects on shoot germination and callus induction of Salvia sclarea we used MS supplemented with 0.1mg/l kinetin (Kin) and 1mg/l naphthalene acetic acid (NAA) as plant growth regulators (PGRs) and for shoot regeneration of S. sclarea MS supplemented with indole-3-acetic acid (IAA) (0.1 mg L-1) and 6-benzylaminopurine (BAP) (1.0 mg L-1). In seed germination of S.sclarea related to the filter paper germinated seeds with 80µg/ml MWCNTs concentration, the highest multiplication rate was obtained was %87 and on filter paper germinated seeds control highest percentage was %83 that there was not significant difference between them , but in culture tissue germination was %64 that there was significant difference between filter parer germination and culture tissue germination. In the callus induction rates of S. sclarea with 80µg/ml MWCNTs was 420 mm³, but in the culture media witout 80µg/ml MWCNTs was 180mm³ and there was significant difference between them. In shoot regeneration we showed that number of shoots in MS along with 80µg/ml MWCNTs was 4.2 per explants and in MS control was 1.8 per explants that has significant difference between them.

Keywords: *Salvia sclarea, MWCNTs, Germination, Culture media, NAA, Kin, IAA*

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INTRODUCTION

Salvia species called Maryam-Goli in Persian (Mozaffarian, 1996) has been famous for its medicinal properties since ancient times. Salvia sclarea L., belonging to this family Lamiaceae is also popularly known as 'Clary Sage', (Maryam Goli e Kabir in Persian). The plant is native to Mediterranean countries, southern France, Italy and Morocco, and is one of the most important plants cultivated worldwide as a source of essential oils and other perfumery products (Dzamic. et al 2008). The plants are 60-100 cm height with large hairy leaves and small blue, white or purple flowers (Hayet et al., 2007). It is occurring in the Mediterranean basin and Iran (Dweck, 2000). studies reported analgesic, anti-inflammatory (Moretti et al., 1997) and antimicrobial effects (Peana et al., 1999) virological evaluations (Hudaib, 2001) and genotoxic properties (Zani et al., 1991).

There are known advantages that plant cell and tissue culture techniques have over the whole plant processing (Grzegorczyk et al., 2007), the former have been successfully adopted in the production of high value chemicals, particularly when the manipulation of source plant material is aimed at obtaining high growth and metabolite accumulation rate (Bolta et al., 2000).

New discoveries in nanotechnology provided knowledge and technological platforms for a number of applications in medical science, aerospace, electronics and defense industries. It is demonstrated that multi-walled carbon nanotubes (MWCNTs) can activate growth of tomato plants and affect the expression of genes that are essential for cell division and plant development. (Khodakovskaya, et al. 2013). Rao and srivistava(2014) demonstrated that MWCNTs effects on Wheat, Corn, Peanut and Garlic increase size and number of leaves.and biomass. It is showed that cotton seedlings had highest growth in 60µg/ml MWCNTs treatment (Nalwade,et.al.2013).Tiwari,et.al.(2013) introduced 20µg/ml MWCNts concentration can hasten water absorbanse and seedlinas growth. In this research with respect to application of Salvia.sclarea in pharmacology, agriculture and perfume industry, we studied tissue culture MWCNTs effects on length and number of shoot formation and callus weight in comparison to control samples of S.sclarea tissue cultures.

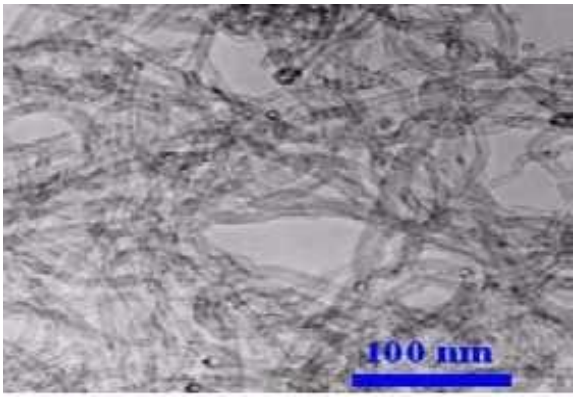
MATERIALS AND METHODS

Multi Walled Carbon Nanotubes Details

MWCNTs of diameter 10-20 nm and length 3-8 µm were purchased from USNANO co, USA.

Multi walled nanotubes (MWNTs) . Purity: > 98 wt% (carbon nanotubes) (fromTGA & TEM) > 99 wt% (carbon content)

Outside diameter: 5-15 nm (from HRTEM,Raman) Inside diameter: 3-5 nm .Length: ~50 um (TEM) SSA: > 233 m 2/g (BET)Color: Black .Ash: <1.5wt% (TGA). Electrical conductivity: >100 s/cm .Tap density: 0.27g/cm3 .True density: ~2.1 g/cm3 Manufacturing method: CVD



SOLUBILIZATION OF MWCNTS

MWCNTs were made water soluble using H2SO4+HNO(3:2) by volume for 24 h (Rao. et.al,1996). Excess of acids were removed and black mass washed with distilled water several times till it was neutral. Repeated adding of water and evaporation under boiling water bath removed all traces of acids. Acid free final wash was tested using Griess reagent [Roy.et.al,1994]. The black mass was vaccum dried and subjected to analysis. MWCNTs became water soluble after sonication wih Ultrasonic mixer [Huang.et.al,2002].

Results showed that treatment of 80µg/ml MWCNTs($P \leq 0.05$) was maximum in germination percentage, germination rate, seedling length and dry weight of Salvia sclarea , while comparing two species (s. sclarea and S.macrosiphon) on seedling length showed that in S. sclarea concentration of 80µg/ml MWCNTs was maximum (Alikhani .et. al,2015) Therefore we applicate 80µg/ml MWCNTs concentration for tissue culture experiments

Plant materials

In vitro germination of seeds of clary sage were obtained from Agricultural Jihad Organization in Yazd province. Seeds were presterilized by washing with hypochlorite solution, and rinse several times under water jet, then rinsed one time in distilled water. After pretreatment seeds were surface sterilized for 30 seconds in 70 %, ethanol and 1% sodium hypochlorite (20% Clorox bleach) for 15 min, then rinsed three times in sterilized distilled water.

Preparation of of callus cultures

Leaf explants were taken from young plants cultured in vitro and in situ (bearing six to eight leaves). Leaves from in situ plants were surface sterilized for 12 min in 0.1% (wt/vol) mercuric chloride solution, then rinsed three times in sterilized distilled water. Leaf pieces, 1–2 cm long, were excised and inoculated onto different culture media. All types of culture media consisted of Murashige and Skoog (MS) (Murashige and Skoog, 1962) basal medium (Sigma Chemical Co., St. Louis, Missouri) solidified with 0.8% agar and supplemented with 3% sucrose, and 0.1mg/l kinetin (Kin),1mg/l naphthaleneacetic acid (NAA) as plant growth regulators (PGRs). Media were adjusted to pH 5.8 using 1N NaOH or1 N HCl, autoclaved at 121°C for 20 min and poured into glass Petri dishes.

Preparation of of shoot cultures

shoots of Salvia sclarea were multiplied from shoot tips on solid (0.8% agar) Murashige and Skoog (MS) medium supplemented with indole-3-acetic acid (IAA) (0.1 mg L-1), 6-benzylaminopurine (BAP) (1.0 mg L-1) and sucrose (3%).Cultures were maintained in the growth room at 26 ± 2 ؛C and 70% humidity with photoperiod of 16 h of light/8 h of darkness. Illumination was supplied by cool white fluorescent lamps with a light intensity of 40µmol m-2-1 s. Four-week-old rooted shoots were transferred to the pots with the sterile mixture of soil, sand and peat(4:3:3) and grown under greenhouse conditions, at 24 ؛C. After acclimatization stage (3 weeks),plantlets were grown for two years in the field .

Data Analysis

The experimental designs were fully randomized with three replicates of 20 explants per treatment. Statistic analysis was carried out with SPSS software , version 16. It was used Tukey and Schefe assay in %5 level For mean comparison.

RESULTS AND DISCUSSION

Seed Germination

We had four group for experiments; germinated seeds on filter paper along with and also lack of 80µg/ml MWCNTs (fig1) and germinated seeds on culture media along with and lack of MWCNTs(fig3,4,5). The period of germination was considered finished after a total of 14 days. the seeds germination capacity are summarized in figure 7. it can be observed from figure 8, Highest germination percentage in S.sclarea was %87 relevent to the filter paper germinated seeds with 80µg/ml MWCNTs concentration (fig2) and next to percentage of filter paper germinated seeds (%83) that there was not significant difference between them , but there was significant difference between filter parer germination and culture tissue germination that was recently %64**.** After 17_{th} day plantlets were transferred to the pots (fig5,6) and we had mature plants after 7 weeks (fig7)**.**

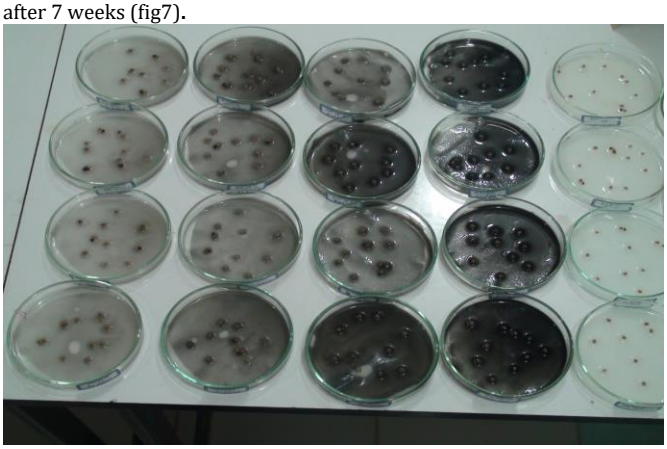


Figure 1. Seed germination on filter paper with and without 80µg/ml MWCNTs

Figure 2. Seed germination after 14 days on filter paper

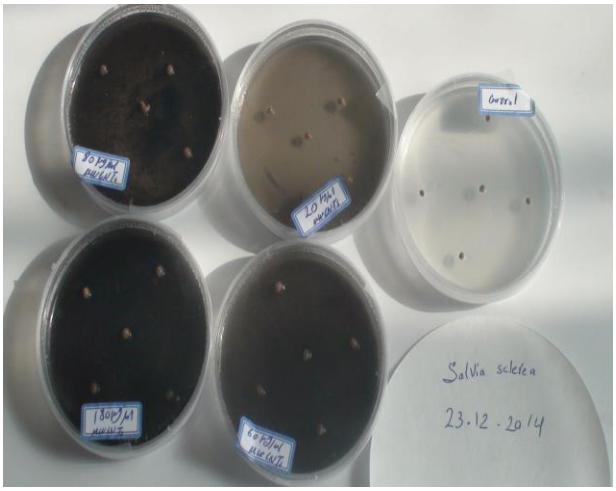


Figure 3. Seed germination on tissue culture along with MWCNTs and control

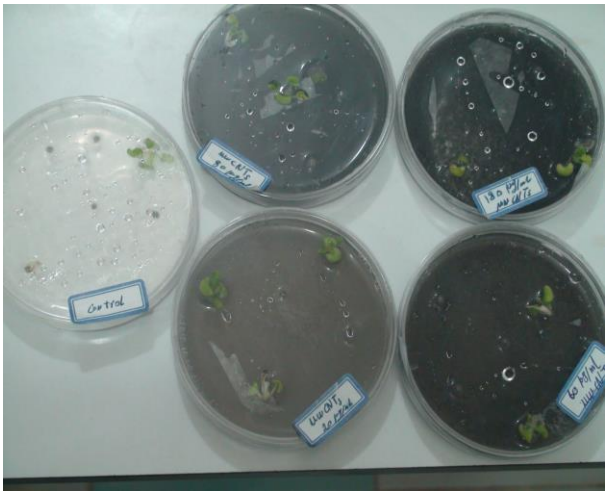


Figure 4 .Seed germination on tissue culture along with MWCNTs and control after 14 days

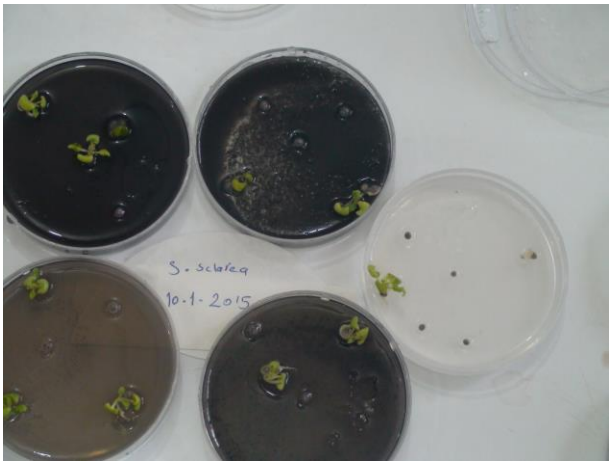


Figure 5. Seed germination on tissue culture along with MWCNTs and control after 17 days in order to transfer to flower pot

Figure 6. Propagation of Salvia sclarea germinated on tissue culture to flower pot . After three weeks

Figure 7. Salvia sclarea germinated on tissue culture after 7 weeks from transferring

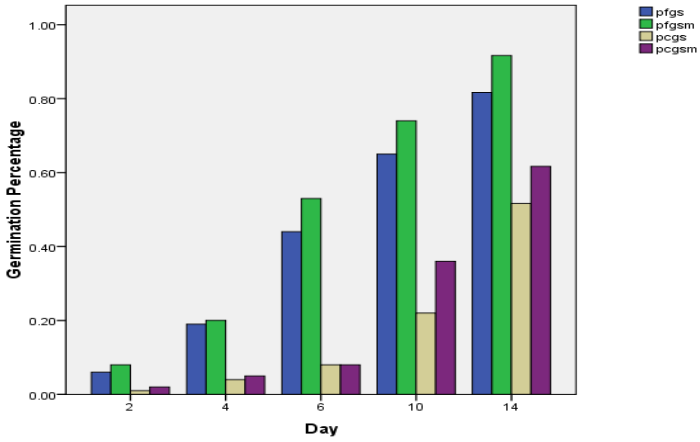


Figure 8. Percentage of germination on filter paper and tissue culture ;Pfgs: percentage of filter paper germinated seeds, Pfgsm: percentage of filter paper germinated seeds with 80µg/ml MWCNTs, Pcgs: percentage of culture media

germinated seeds, Pcgsm: percentage of culture media germinated seeds with 80µg/ml MWCNTs

Callus induction

Biotechnological techniques have been reported to significantly facilitate plant propagation and production of some important bioactive compounds from the genus Salvia. Banthorpe et al. (1990) reported on the establishment of undifferentiated friable, white callus and derived cell suspension lines from stem explants of a sterile S. sclarea plant on a MS medium supplemented with either 4.5 µM 2, 4-D and 0.5 µM Kin or 5.4 µM NAA and 0.5 µM Kin. A callus induction rate of ca. 80% was observed. Consequently, cell suspensions were established on MS liquid medium supplemented with 4.5 µM 2, 4-D and 0.5 µM Kin. The cultures were used in order to study the in vitro accumulation of sclareol.Callus, cell suspension, immobilized cell and hairy root cultures were established from S. officinalis (Tawfic et al., 1992), S. miltiorrhiza, S. canariensis and S. sclarea and used for the production of various secondary metabolites, such as rosmarinic acid, cryptotanshinone, camphor, ferruginol and sclareol (Naser et al., 2004). For producing of Salvia sclarea callus we use hormone concentrations similar to kintozios at al 1999 and Banthorp et al 1990 that was NAA 1mg/l, Kin 0.1 mg/l.Highest callus induction rates were obtained for S. sclarea when we treat culture media with 80µg/ml MWCNTs that was 420 mm^3 (fig 9), but the callus induction rates were lower, when we treat culture media without 80µg/ml MWCNTs (fig10) that was 180mm^3 and there was significant difference between them (fig11).

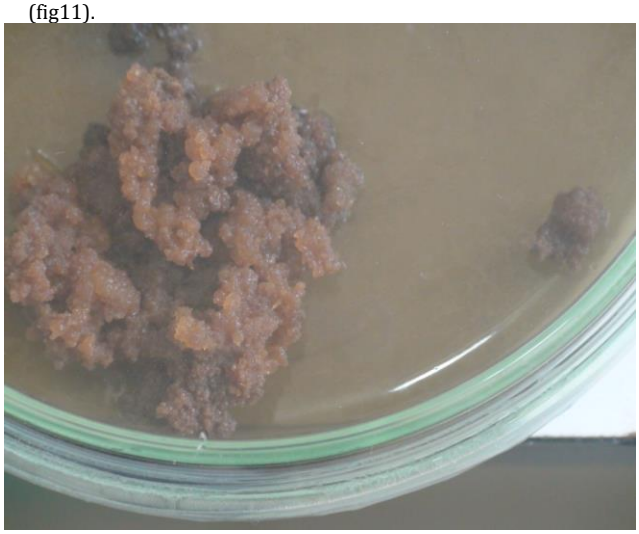


Figure 9: Salvia sclarea callus culture with 80µg/ml MWCNTs

Figure 10: Salvia sclarea callus culture witout 80µg/ml MWCNTs

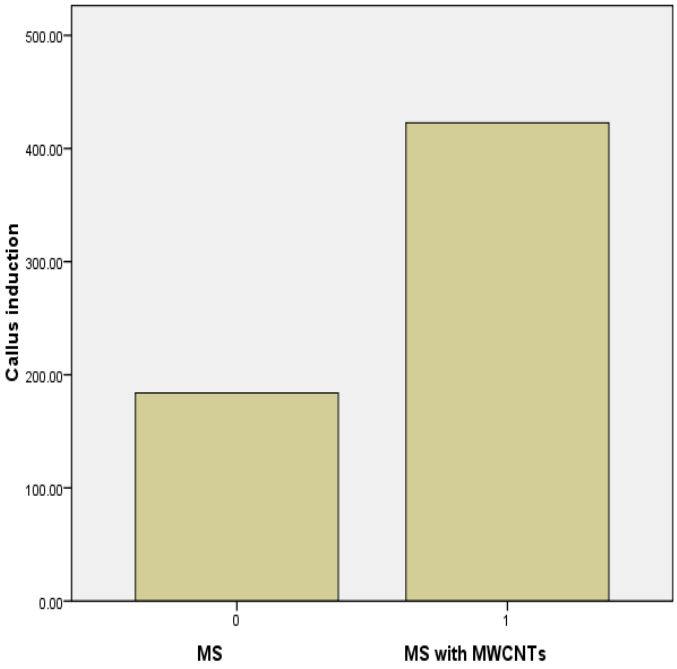


Figure 11. Callus induction of Salvia sclarea leaves on MS culture with and without 80µg/ml MWCNTs

Shoot Propagation

MS medium has been used with success in the micropropagation of other species of Salvia, such as S. leucantha (Hosoki and Tahara, 1993), S.valentina and S. blancoana (Cuenca and Amo-Marco, 2000), S. officinalis (Avato et al., 2005), and S. brachyodon (Misic et al., 2006). Experiments in order to shoot regeneration was achieved according to kuzma, et al., 2009 which the media supplemented with (BAP 1 mg/l , IAA0.1 mg/l) were the most shoot regeneration. The results (Fig. 14) showed that MS along with MWCNTs (4.2 per explants) has significant difference in shoot regeneration related to MS control (1.8 per explants) (fig12,13).

Figure12. Shoot regeneration of S.sclarea on MS with BAP 1 mg/l , IAA0.1 mg/l

Figure 12. Shoot regeneration of S.sclarea on MS with BAP 1 mg/l , IAA0.1 mg/l along with 80µg/ml MWCNTs

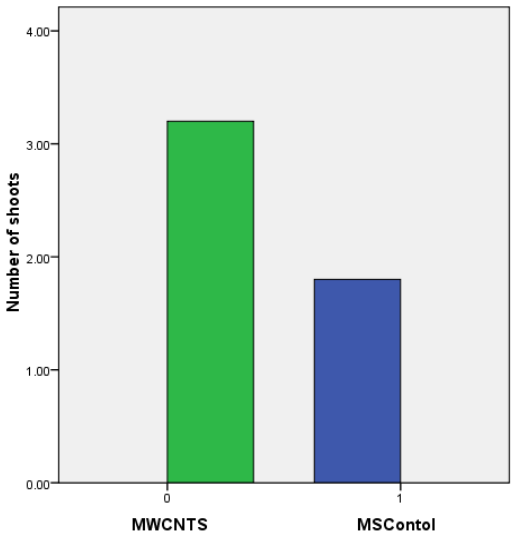


Fig 14. Shoot number of explants in Media culture with and without MWCNTs

CONCLUSION

Studies on Nanotubes effects on plants is growing up and In this research we studied S.sclarea a medicinal plant as for shoot propagation and callus induction compared with MWCNTs treatments. In seed germination of S.sclarea maximum germination percentage relevent to the filter paper germinated seeds with 80µg/ml MWCNTs concentration (was %87) (fig2) and on filter paper germinated seeds control was %83 that there was not significant difference between them , but culture tissue germination was %64 that there was significant difference between filter parer germination and culture tissue germination **.**

In callus induction rates of S. sclarea with 80µg/ml MWCNTs was 420 mm^3 (fig 9), but in the culture media witout 80µg/ml MWCNTs (fig10) was 180mm^3 and there was significant difference between them (fig11).

In shoot regeneration we (Fig. 14) showed that number of shoots in MS along with 80µg/ml MWCNTs was 4.2 per explants and in MS control was 1.8 per explants that has significant difference between them.

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