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ORIGINAL ARTICLE

Cell line selection through gamma irradiation combined with multi-walled carbon nanotubes elicitation enhanced phenolic compounds accumulation in *Salvia nemorosa* cell culture

Hamid Reza Heydari[1] · **Esmaeil Chamani**[1] · **Behrooz Esmaielpour**[1]

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Abstract

The current study focused on improving the production of phenolic acids in the Woodland Sage cell suspension culture (CSC) through attaining high-yielding cell lines and carboxyl functionalized multi-walled carbon nanotubes (MWCNT-COOH) elicitation. The leaf-derived callus was irradiated at different doses of gamma irradiation 10 to 100 Gy. The maximum content of rosmarinic acid (RA), salvianolic acid B (SAB), ferulic acid (FA), and cinnamic acid (CA) was recorded in callus cultures irradiated with 70 Gy, which was 18.53, 5.21, 1.9, and 7.59 mg/g DW, respectively. The CSC that established from 70 Gy γ-irradiated calli accumulated 1.7-fold RA more higher irradiated callus culture. The CSC elicited with various concentrations of MWCNT-COOH in range 25 to 100 mg/l significantly increased fresh weight (FW), dry weight (DW), and phenolic acid contents of cells. The highest FW with 268.47 g/l and DW with 22.17 g/l was obtained in 100 mg/l MWCNT-COOH treatment. The RA, SAB, CA and FA content of CSC treated with 100 mg/l MWCNT-COOH were 13-fold, 14.2-fold, 20-fold, and 3- fold higher than wild *S. nemorosa* plant at flowering stage, respectively. The antioxidant activity of cultures significantly enhanced with both gamma and MWCNT-COOH based on DPPH and FRAP assay. Our results showed that the combination of cell line selection and MWCNT-COOH elicitation significantly improved the production of secondary metabolites in Woodland Sage, which is useful for large-scale production of phenolic compounds.

Key message

Our findings revealed that cell suspension culture established from γ-irradiation *S. nemorosa* callus which elicited with Multi-walled carbon nanotubes accumulated much higher phenolic compounds compared to the wild plant.

Keywords Ionizing radiation · MWCNT-COOH · Rosmarinic acid · Salvianolic acid B · Suspension culture · Woodland sage

Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
BA	N6-Benzyladenine
CA	Cinnamic acid
CDB	Callus dry biomass
CFB	Callus fresh biomass
CSC	Cell suspension culture
DW	Dry weight
FA	Ferulic acid
FW	Fresh weight
mMS	Modified Murashige and Skoog
MWCNT-COOH	Carboxyl functionalized multi-walled carbon nanotubes
RA	Rosmarinic acid
SAB	Salvianolic acid B
TFC	Total phenolic content

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✉ Esmaeil Chamani
echamani@yahoo.com

[1] Department of Horticultural Sciences, Faculty of Agriculture and Natural Resources, University of Mohaghegh Ardabili, Ardabil, Iran

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Introduction

Secondary metabolites are a group of organic substances that are produced by plants in response to environmental stress and as a part of their defense mechanism. These compounds show diverse biological activities and are used as pharmaceuticals, agrochemicals, flavors, fragrances, colors, biopesticides, and food additives in daily human life (Murthy et al. 2014). Secondary metabolites are often produced at low levels (less than 1% dry weight), and their production via field cultivation depends significantly on the physiological and developmental stage of the plant (Oksman-Caldentey and Inze 2004). Also, their production through field cultivation is associated with several disadvantages, such as low yields, lack of year-round production, and fluctuations in concentrations due to environmental and seasonal variation (Murthy et al. 2014; Yue et al. 2016). Therefore, plant cell cultures have emerged as attractive alternatives for the production of secondary products. It overcomes most of the barriers in the field production of secondary metabolites and provides a reliable system (Ochoa-Villarreal et al. 2016).

High-yielding cell lines selection and elicitation have been identified as two practical and reliable strategies for improving the secondary metabolite production in suspension cultures. A 12-fold increase in shikonin accumulation reported by Wang et al. (2014) in suspension cultures of *Arnebia euchroma* established using the shikonin-proficient and shikonin-deficient cell lines and elicited by methyl jasmonate. Also, Deepthi and Satheeshkumar (2017) described that the combination of cell line selection and jasmonic acid elicitation had provided 18.66-fold increments in camptothecin content of *Ophiorrhiza mungos* cell culture. In addition, increased production of phenolic and flavonoid compounds in the Lamiaceae family elicited with different elicitors has been reported by other researchers (Attaran Dowom et al. 2017; Gonçalves et al. 2019; Khojasteh et al. 2016; Kracun-Kolarevic et al. 2015; Kummritz et al. 2016; Xing et al. 2018).

Recent advances in nanotechnology have provided a new platform for its application in various sciences, especially agriculture and biology (Khodakovskaya et al. 2013). It has been documented that nanoparticles can alter physiological processes such as photosynthesis, primary and secondary pathways, and nutrient uptake in plants (Fincheira et al. 2020). Among the various nanoparticles, multi-walled carbon nanotubes (MWCNTs) have attracted more attention due to their unique structural and dimensional properties (Khodakovskaya et al. 2011). Previous research has indicated that MWCNTs can modify the expression of genes involved in cell division and expansion (Khodakovskaya et al. 2012), water uptake (Khodakovskaya et al. 2013), stress response (Bhati et al. 2018) and phenylpropanoid pathway (Ghorbanpour and Hadian 2015). Therefore, these nanoparticles can be employed as a novel and efficient elicitor to improve the production of secondary metabolites in plants (Marslin et al. 2017).

Gamma radiation is sparsely ionizing radiation, which can directly or indirectly affect the growth and physiology of plants. Direct effects of gamma irradiation on plants include induction of DNA mutation and chromosomal aberrations, and the boost in ROS production and generation of oxidative stress considered as their indirect effects (Ahuja et al. 2014; Gudkov et al. 2019). Therefore, gamma irradiation can be utilized as a practical and rapid method for producing high-yielding cell lines in medicinal plants. For example, a notable improvement in shikonin production of 400% was observed in a suspension culture of *Lithospermum erythrorhizon* when applying low doses of gamma irradiation (Chung et al. 2006). Also, in *Rubia cordifolia*, gamma irradiation at 8 Gy induced cell lines which produced 11-fold anthraquinones higher than nonirradiated cells (Mariadoss et al. 2020). Mutant adventitious root lines of *Panax ginseng* generated through γ-irradiation accumulated 4.2-fold higher ginsenoside content than normal roots (Le et al. 2019). Similar observations also have been documented in *Artemisia annua* (Patil et al. 2018), *Sesuvium portulacastrum*(Kapare et al. 2017), *Hypericum triquetrifolium* (Azeez et al. 2017) and *Stevia rebaudiana* (Khalil et al. 2015).

Woodland Sage (*Salvia nemorosa* L.) is a perennial herbaceous species belong to the Lamiaceae family and is widely distributed in diverse regions of Iran (Heydari et al. 2020; Mahdieh et al. 2018). *S. nemorosa* is traditionally employed in folkloric medicine to stop bleeding, treatment of stomach ache, diarrhea, hemorrhages, and furuncles (Bahadori et al. 2017; Božin et al. 2012). Like other *Salvia* species, Woodland Sage is a rich source of phenolic compounds, such as rosmarinic acid, Caffeic acid, Ferulic acid (Bahadori et al. 2017). To our knowledge, despite the high medicinal value of *S. nemorosa*, no studies have been conducted on the production of secondary metabolites of this plant under in vitro condition. On the other hand, only a few reports have described cell suspensions from *Salvia* species as a source of secondary metabolites. It is primarily because of their low metabolite yields, slow growth, and low biomass density (Marchev et al. 2014).

Thus, in the present study, we have investigated the effects of gamma irradiation on callus culture of *S. nemorosa* to obtain high-yielding cell lines for phenolic acid production. Then, suspension cultures were established from gamma-irradiated callus cultures which accumulated higher phenolic acids after three successive subcultures. Eventually, the suspension cultures were elicited with different concentrations of MWCNT-COOH to achieve high levels of phenolic content. To our knowledge, the effects of applying gamma irradiation and MWCNTs elicitation on phenolic

acid production in cell cultures of *salvia* species have not been reported.

Materials and methods

Plant material and establishment of callus cultures

The seeds of wild *S.nemorosa* were collected from Khansar, Isfahan Province (33° 10′ 00″ N, 50° 23′ 00″ W) and validated by herbarium of Research Institute of Forests and Rangelands of Iran. They were surface sterilized by immersion in 70% (v/v) ethanol for 1 min, followed by 2.5% commercial bleach (NaClO) with a few drops of Tween 20 for 15 min. It followed by 3 times rinses in sterile double distilled water and planted in glass jars (200 ml) containing 25 ml Murashige and Skoog (MS) basal medium (Murashige and Skoog 1962) solidified by 5.8 g/l plant agar (Plant agar, Duchefa Biochemie, Netherlands), and the pH adjusted to 5.8 with KOH before autoclaving. Cultures were incubated under cool-white light illumination at an intensity of 35 µmol m^{-2} s^{-1} with a photoperiod of 16 h at 24 ± 1 °C. The leaves of 28-day-old seedlings served as the explant source. For callus induction, 1 × 1 cm leaf segments were cultured on modified MS medium (MS basal salts amended with B_5 vitamin mixture) supplemented with 16 µM 2,4-D and 8 µM BA over four weeks under dark condition. Initiated callus was proliferated on the same medium in three subcultures with four-week periods and obtained friable white calli used as an explant source for the gamma irradiation test.

Gamma irradiation

Well-established 12-weeks old callus cultures were irradiated at different doses of 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 Gy at room temperature (25 °C ± 1 °C). Gamma irradiation was done using a ^{60}Co source with a dose rate of 0.25 Gy/s at Nuclear Science and Technology Research Institute, Tehran. Approximately 2 g of calli tissue per 200 ml glass jar with three replication irradiated in each dose and each jar served as one replicate. Nonirradiated callus cultures were employed as controls. After irradiation callus were immediately transferred to fresh mMS medium amended by 16 µM 2,4-D and 8 µM BA and 3% sucrose and kept under cool-white light illumination with a photoperiod of 16 h at 24 ± 1 °C. It was marked as S_1 and the lethal dose 50 value (LD_{50}) calculated according to the overall dry weight decline of the irradiated calli compared to the dry weight of the control sample in it. Cultures were successively subcultured every four weeks and labelled as S_2 and S_3. Callus DW, antioxidant activity, RA and total phenolic content were measured for three successive subcultures denoted S_1, S_2, and S_3.

Establishment of suspension culture and elicitation

The results of the callus irradiation experiment indicated that cell lines obtained from 70 Gy irradiated callus accumulated the highest levels of TPC and RA and also had acceptable growth and proliferation rate. Thus, suspension cultures were initiated from 70 Gy γ-irradiated callus by transferring 4 g callus to 250 ml Erlenmeyer flasks containing 100 ml of liquid mMS medium fortified with 16 µM 2,4-D, 8 µM BA, and 3% sucrose. The pH of the culture medium was adjusted to pH 5.8 by adding 1 M HCl or 1 M KOH before autoclaving. The inoculated flasks were placed on a rotary orbital shaker at 110 rpm and incubated at 25 ± 2 °C in the dark. The cultures were subcultured every 21 days through transferring 20 ml of the culture containing small aggregates (contain 3–12 cells) and single cells into 80 ml of fresh medium. Sustainable fine suspensions were obtained after three subculture cycles. Carboxyl functionalized multi-walled carbon nanotubes (MWCNT-COOH) with an outer diameter of 20–30 nm was purchased from US Research Nanomaterials, Inc., Houston, USA. MWCNT-COOH physicochemical properties were analyzed using Raman spectroscopy and transmission electron microscopy (TEM) methods (Table S1, Fig. 1). A 5000 mg/l stock solution of MWCNT-COOH was prepared by adding 500 mg MWCNT-COOH powder into a 100 ml water. Carbon nanotubes were dispersed into water by sonication at 400 Wat for 30 min in Ultrasonic homogenizer UP400st (Hielscher Ultrasonics GmbH, Teltow, Germany) to obtain a homogeneous mixture. MWCNTs were added at 25, 50, 75, and 100 mg/l concentrations into culture medium at the onset of culture initiation before autoclaving, and carbon nanotubes free medium served as control.

Determination of cell biomass

Cells were separated from the liquid medium by filtration using Whatman No. 1 filter paper. The dry weight of the cells was recorded after drying them at 60 °C for 48 h. The dry biomass weight was used to determine the growth curve by sampling in individual flasks every four days. The specific growth rate was calculated according to Sujanya et al. (2008) by following formula:

$$\mathrm{SGR} = (\text{Maximum cell DW}) - (\text{Inoculum DW}) \,/\, (\text{Culture time in week}) \times (\text{Inoculum DW})$$

Extraction of bioactive compounds

The harvested cells were oven-dried at 60 °C for 48 h and grounded into a fine powder. 500 mg of the powdered material was added to 10 ml methanol and mixed thoroughly.

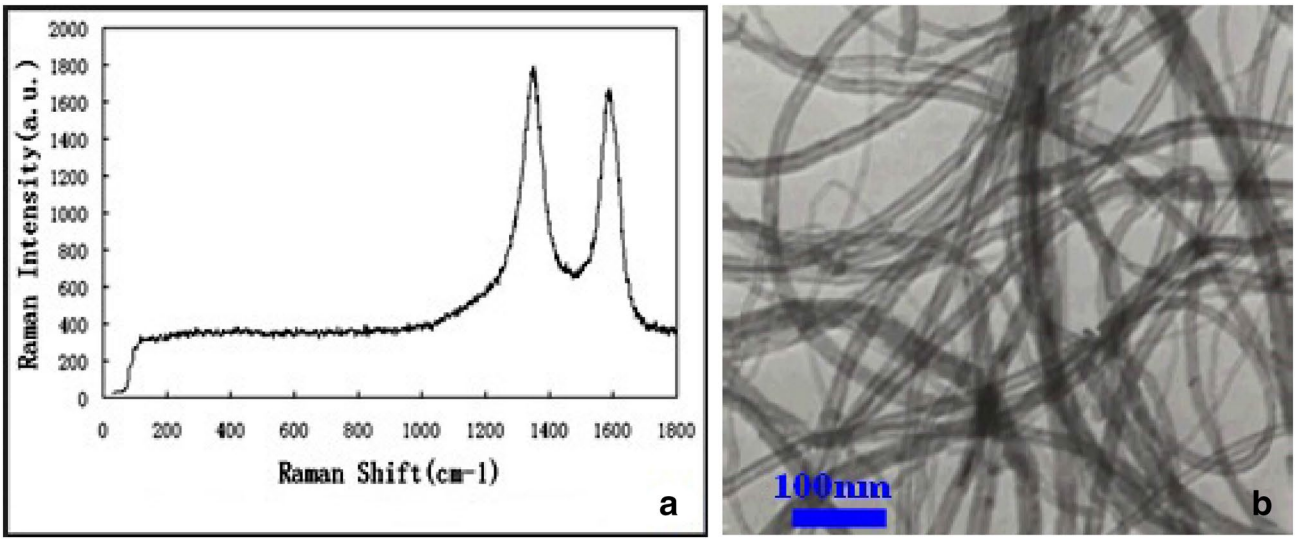

Fig. 1 Raman spectroscopy analysis (**a**) and transmission electron microscopy (TEM) image (**b**) of the carboxylated multi walled carbon nanotubes used in this study

Then, the mixture was sonicated for 30 min at room temperature. Thereafter, the extracts were filtered through Whatman no. 1 filter papers. The solvent of the extracts was removed by a rotary evaporator. Then, 10 mg of dried extract was dissolved in 10 ml methanol and were kept for further analyses.

Quantification of phenolic compounds

The content of phenolic compounds was evaluated by a high-performance liquid chromatography method. The HPLC apparatus was a Waters 2695 Alliance model (Milford, US) with a quaternary, low-pressure mixing pump and inline vacuum degassing and a reversed phase column C18 Eurospher-100 (5 µm particle, 150 mm × 4.6 mm) coupled with a Waters 996 Photodiode array detector (PDA). Data acquisition and integration were performed with Millennium 32 software. The mobile phase consisted of methanol (solvent A) and distilled water (solvent B) with the flow-rate of 1 ml/min. Gradient elution was carried out in a following system: 0–30 min, 88% A, 12% B; 30–45 min, 80% A, 20% B; 45–60 min, 70% A, 30% B. The oven temperature was maintained at 25 °C and peaks were monitored at 195–400 nm wavelength. For the calibration curve, 20–100 µl of the methanolic standard solution of rosmarinic acid (RA), salvianolic acid B (SAB), ferulic acid (FA) and caffeic acid (CA) were applied in triplicate onto the HPLC. The calibration curve of phenolic compounds was depicted by plotting peaks area against concentrations. The concentration of the unknown samples was calculated by using the equation generated from the calibration curve. The retention time of RA, SAB, FA and CA recorded at 29.59, 32.14, 19.18, and 13.08, respectively. The chromatographic peak of phenolic compounds was identified by comparing their retention time with those of the reference standards.

Determination of rosmarinic acid content and antioxidant activity by spectrophotometric assay

Rosmarinic acid content in samples was determined through spectrophotometric method described by Öztürk et al. (2010). Briefly, the reaction mixture was prepared by adding 200 µL extract solution and 200 µl zirconium (IV) oxide chloride solution to 4.6 ml ethanol. After 5 min, the absorbance at 362 nm was recorded against a reagent blank. Appropriate concentrations (0–18 ppm) of RA standard solution was prepared for construction of calibration curve ($y = 0.0195x - 0.0049$, $R^2 = 995$). RA content in the extracts was expressed as mg per g dry weight. The Folin-Ciocalteu assay described by Attaran Dowom et al. (2017) was used for measuring the total phenolic contents of samples. Briefly, 100 µl of extract, 2 ml water and 200 µl Folin-Ciocalteu reagent added to a Falcon tube and thoroughly mixed for 3 min. Then, 1 ml of 20% (w/v) sodium carbonate solution was added to mixture and incubated at room temperature for 1 h. The absorbance of mixtures was recorded at 765 nm against a reagent blank without the extract. Calibration curve ($y = 0.1531 + 0.0032x$, $r^2 = 0.998$) was constructed by preparation appropriate concentrations (200–800 ppm) of Gallic acid. The concentration of total phenol in samples was expressed as mg of Gallic acid equivalent (GAE) per g dry weight.

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity of the extracts was determined using assay described by Bahadori et al. (2017). A stock solution of extract (250 µg/ml) was prepared by dissolving 250 mg of powdery extract in 1 ml methanol. Then, each sample solution was diluted to final concentrations of 50, 100, 150, 200 and 250 in methanol. Then, 200 µl of 100 µM DPPH methanolic solution was added to 20 µl of each sample. The mixtures were mixed vigorously and incubated at room temperature for 30 min. Absorbance of the solutions was measured at 515 nm. The positive control was ascorbic acid. The DPPH radical scavenging activity was calculated using the equation: Inhibition concentration (%) = (absorbance control − absorbance sample)/(absorbance control) × 100. The free radical scavenging activities of the solutions were expressed as IC50 (mg/ml) which was calculated graphically using different concentrations of samples versus DPPH inhibition percentage. The procedure for determination of ferric reducing antioxidant power (FRAP) was adapted from Chavan et al. (2013). Briefly, the FRAP reagent provided through mixing the 20 mM FeCl3.6H2O, 10 mM TPTZ and 0.3 M acetate buffer (pH 3.6) in 1:1:10 ratio prior to use and heated to 37 °C in water bath for 10 min. Then 100 µl of extract was added to 2.7 ml of the FRAP reagent and shaken vigorously. The reaction mixture was kept in dark for 30 min. The absorbance of the colored product (ferrous tripyridyltriazine complex) was then recorded at 593 nm.

Statistical analysis

All experiments were done based on a completely randomized design with three replications. Data were analyzed by using SAS 9.4. The mean comparison was made by Duncan's multiple range test (DMRT) at the 5% probability level.

Results and discussion

Establishment of callus culture

Leaf segments of *S. nemorosa* were placed on mMS medium supplemented with 16 µM 2,4-D, and 8 µM BA to induced calli. Ten days after incubation, the callus began to initiate on the cut edges of the explants, and an acceptable amount of callus was formed over four weeks (Fig. S2-a, b). The obtained calli were proliferated on the same medium over three successive subcultures with 4-week periods to provide sufficient friable white callus (Fig. S2-c). These calli employed as a source of explant for further experiments.

Effect of gamma irradiation on callus morphology

Woodland Sage callus color and texture were significantly changed by gamma irradiation. Nonirradiated and 10 Gy irradiated callus presented friable white morphology in three subcultures (Fig. S2-d). Chartreuse Green and friable calli were observed in response to 20 and 30 Gy doses in the first subculture, and their color turned into Chartreuse Yellow in the second and third subcultures (Fig. S2-g, h). The 40 Gy dose induced Chartreuse Green friable callus in the subculture one and compact green calli in the subculture two and three (Fig. S2-i). Moreover, 50 Gy dose produced light to dark grey callus in the subcultures (Fig. S2-k). A mix of yellow and light grey friable callus was produced in response to 60, 70, and 80 Gy irradiance (Fig. S2-j). The first and second subcultures of calli irradiated by 90 and 100 Gy dose exhibited compact brown callus, while in the third subculture, their colors turned into light brown (Fig. S2-f). Our findings indicated that visual variations in callus morphology was unstable at the first subculture following gamma irradiation and stabilized during the second and third period of culture. It may be due to a sudden increase in cellular ROS levels in the first subculture after exposure to gamma irradiation. Excessive accumulation of ROS in cells can induce the degradation of endogenous hormones and proteins, alterations in gene expression, and the immediate accumulation of some secondary metabolites (Ahuja et al. 2014; Gudkov et al. 2019). These phenomena may cause unstable morphological variations such as changes in the color and texture of callus in the first subculture. In the subsequent subcultures, these abnormalities may be alleviated by activation of the antioxidant system and they achieved stable morphology. Variation in callus morphology is helpful for cell line selection, which can play an important role in optimizing the production of secondary metabolites in the cell cultures of medicinal plants (Vardhan and Shukla 2017).In agreement with our results, change in callus color and texture has been reported in other studies. Granular red-green and compact brown calli were observed in γ-irradiated callus culture of *Hypericum triquetrifolium* in response to 10 and 40 Gy, respectively (Azeez et al. 2017). In a study conducted by Khalil et al. (2015) on *Stevia rebaudiana*, Granular and yellowish-green, White and green friable and half white and green spongy callus were recognized in response to various γ-irradiation. Also, high doses of gamma irradiation provided brown callus in *Artemisia annua* (Patil et al. 2018). Contrary to our results, Mariadoss et al. (2020) did not observe a variation in the morphology of *Rubia cordifolia* callus exposed to gamma rays.

In the present research, irradiated *S. nemorosa* calli at 20 and 30 Gy doses produced small differentiated shoots in the second and third subcultures as can be seen in Fig. S2g, h. Callus exposed to higher gamma doses has not induced any

vegetative buds. These findings propose that low doses of gamma radiation can stimulate the organogenesis of Woodland Sage callus, while higher doses retarded it. Hasbullah et al. (2012) suggest that low doses of γ-irradiation affect the activity and effectiveness of exogenous plant growth regulators, especially auxin, in the culture medium, which promoted organogenesis potential of *Gerbera jamesonii* calli. Azeez et al. (2017) stated that *H. triquetrifolium* calli irradiated with dosages higher than 30 Gy failed to develop shoot primordia probably due to intensification of callus browning and alteration in morphology. Also, stimulation of organogenesis at low gamma doses and retarding it at doses higher than 30 Gy has been reported in *Artemisia annua* (Patil et al. 2018). Our results are consistent with recent reports.

Radiation sensitivity test

The radiation sensitivity test is the first step in examining the effects of ionizing radiation on the growth and developmental characteristics of in vitro cultures. It is vital to determine the optimum irradiation dose that provided the highest mutation with the least damage to the cultures (Kapare et al. 2017; Rukesh et al. 2017). In the present study, all cultures were subjected to different doses of γ-irradiation ranging from 10 to 100 Gy showed a 100% survival rate in the three successive subcultures. The lethal dose (LD_{50}) of γ-rays was calculated based on the drop in the new dry weight of irradiated callus in comparison to the dry weight of the nonirradiated culture in the first subculture. As shown in Fig. S1, the LD_{50} value was found to be 79 Gy, which caused the 50% reduction in the dry weight of proliferated calli compared to control. Mariadoss et al. (2020) stated that raising γ-irradiation doses above 30 provoked a significant decrease in fresh callus weight at the first subculture of *R. cordifolia* cell culture. Besides, similar observations were reported in the embryogenic callus culture of *Saccharum officinarum* (Nikam et al. 2014), *Sesuvium portulacastrum* (Kapare et al. 2017), and *Orthosiphon stamineus* shoot cultures (Ling et al. 2008) with a LD_{50} value of 30 Gy. In contrary to our results, Patil et al. (2018) observed inhibition in callus growth of *Artemisia annua* in response to gamma irradiation in the range from 30 to 35 Gy at first subculture.

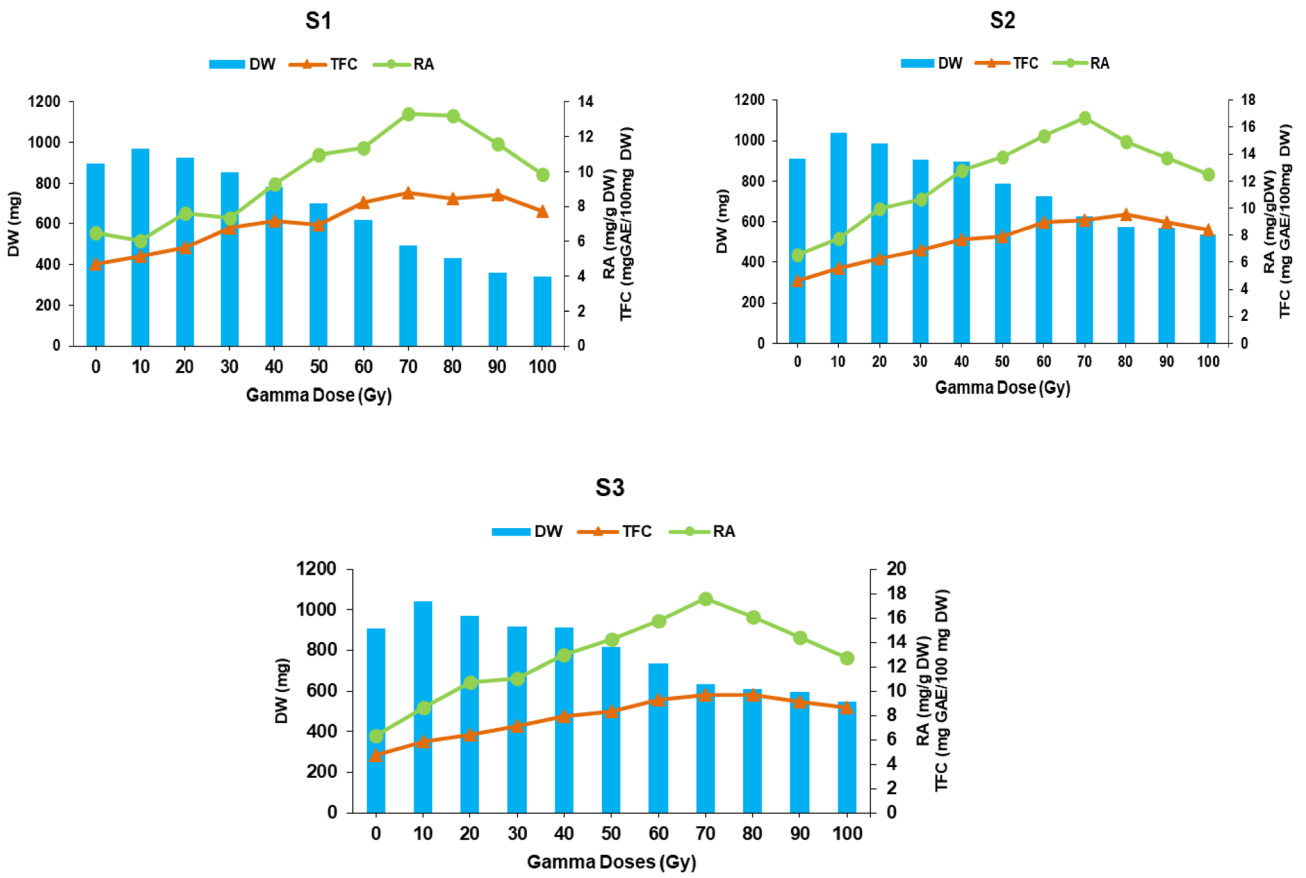

Fig. 2 Effect of Gamma irradiation doses on growth and accumulation of total phenolic and Rosmarinic acid in callus culture of *S. nemorosa* during successive S_1, S_2 and S_3 subcultures

Table 1 Effect of gamma irradiance on growth, phenolic content and antioxidant activity of *S. nemorosa* callus culture

Gamma dose (Gy)	DW (mg)	Total phenolic content (mg GAE/g DW)	Rosmarinic acid (mg/DW)	DPPH	FRAP
0	907.52 ± 32.44^{c}	46.97 ± 0.42^{j}	6.36 ± 0.16^{g}	119.57 ± 4.16^{a}	0.52 ± 0.020^{i}
10	1042.84 ± 38.51^{a}	58.17 ± 0.15^{i}	8.68 ± 0.20^{f}	94.08 ± 1.33^{b}	0.60 ± 0.017^{h}
20	972.40 ± 10.59^{b}	64.39 ± 0.46^{h}	10.72 ± 0.22^{e}	87.11 ± 3.86^{c}	0.62 ± 0.026^{h}
30	915.24 ± 15.87^{c}	71.63 ± 0.43^{g}	11.06 ± 0.22^{e}	82.69 ± 1.89^{d}	0.69 ± 0.030^{g}
40	911.94 ± 2.08^{c}	79.20 ± 0.16^{f}	12.97 ± 0.25^{d}	$79.58 \pm 3.94d^{e}$	0.73 ± 0.010^{f}
50	815.11 ± 7.21^{d}	83.28 ± 0.18^{e}	14.26 ± 0.21^{c}	77.91 ± 1.89^{e}	0.77 ± 0.017^{e}
60	737.17 ± 16.37^{e}	92.56 ± 0.15^{b}	15.77 ± 0.17^{b}	68.61 ± 1.85^{fg}	0.84 ± 0.010^{d}
70	632.58 ± 8.38^{f}	96.76 ± 0.66^{a}	17.59 ± 0.42^{a}	65.11 ± 1.74^{gh}	0.95 ± 0.036^{ab}
80	611.64 ± 15.50^{f}	97.17 ± 0.21^{a}	16.07 ± 0.14^{b}	63.74 ± 0.55^{h}	0.97 ± 0.017^{a}
90	595.16 ± 11.53^{fg}	91.39 ± 0.68^{c}	14.37 ± 0.06^{c}	68.37 ± 1.43^{fg}	0.93 ± 0.020^{b}
100	546.72 ± 32.39^{g}	86.44 ± 0.58^{d}	12.69 ± 0.06^{d}	71.29 ± 0.42^{f}	0.89 ± 0.010^{c}

Mean ± SD values in a column, followed by different letters, were significantly different according to the Duncan's multiple range test ($P \leq 0.05$)

Effect of gamma irradiation on biomass and growth

Callus cultures were immediately transferred to the fresh modified MS medium fortified with 16 µM 2,4-D, and 8 µM BA after being treated to various doses of gamma (0 to 100 Gy), and callus dry biomass (CDB) was recorded for S1, S2, and S3 subcultures. As shown in Fig. 2, the growth pattern of CDB in the second and third subcultures was constant and slightly different with respect to the S1 subculture. These findings revealed that γ-irradiated callus cultures of *S. nemorosa* have the ability to successful and stable proliferation during successive subcultivations. The selection of highly productive cell lines is one of the most important targets of ionizing radiation of medicinal plant cell cultures (Vardhan and Shukla 2017), if the selected cell lines are capable of successfully and steadily multiplying in successive generations (Wang et al. 2014). Mariadoss et al. (2020) observed stable biomass growth in the irradiated callus cultures of *R. cordifolia* during the successive four subcultures, which was consistent with our results.

In the current research, low gamma doses favored callus growth, and a significant increase was found in the biomass growth of cultures, which were subjected to 10 and 20 Gy doses during the three generations. They formed 1042.84 and 972.40 mg dry weight at 10 and 20 Gy, respectively, which was significantly higher than nonirradiated callus cultures (907.52 mg). Callus cultures exposed to high doses of gamma radiation ranging from 30 to 100 Gy exhibited a steady decline in DCB and lowest dry biomass obtained in 100 Gy dose, which was 546.72 mg (Table 1). In addition, a drastic decrease in callus growth in the third generation compared to the control in the 90 and 100 Gy treatments, which was 36% and 40%, respectively, was associated with some morphological abnormalities and a sharp increase in brown pigmentation in calli tissue. The outcomes in the current research were supported by Patil et al. (2018), they declared that γ- irradiation at 5 Gy enhanced growth of *A. annua* callus culture, whereas higher doses significantly reduced calli growth opposed to nonirradiated cultures. Similarly, Le et al. (2019) documented that mutant adventitious root cultures of *Panax ginseng* exposed to 20 Gy gamma showed more vigorous growth than the nonirradiated roots. In addition, a 40% increase in the number of regenerated shoots, as well as a notable rise in height and fresh mass of the regenerated *Sesuvium portulacastrum* plantlets were reported by Kapare et al. (2017) in response to gamma irradiation at 10 Gy, although with increasing radiation doses, these indices decreased significantly compared to the control. Also, similar observation has been informed by other researchers (Azeez et al. 2017; Jan et al. 2010; Khalil et al. 2015). The effect of gamma rays on plant growth and development has not been clearly elucidated, although several mechanisms have been proposed by researchers. Low doses of γ-irradiation, typically at doses below 20 Gy, may stimulate plant growth through modification of hormone signaling networks in cells, increasing the biosynthesis of amino acids, improving primary metabolism processes, alteration in the uptake of mineral nutrients and the enzyme activities (Alikamanoglu et al. 2011; Gudkov et al. 2019; Le et al. 2019; Ling et al. 2008; Moussa 2006; Singh et al. 2012). The decrease in the growth of plants subjected to high doses of γ-irradiation may be due to imbalances between endogenous hormones (Hasbullah et al. 2012). In an investigation carried out by Fortunati et al. (2010), high doses of neutron radiation significantly downregulated *Arabidopsis thaliana* auxin response factors, and auxin activated genes expression which involved in IAA synthesis. Momiyama et al. (1999) described that inhibition of *Zea mays* coleoptile elongation in response to high dose γ-irradiation correlated with a reduction in the endogenous IAA level. Also, Bitarishvili

et al. (2018) reported that the content of Zeatin and IAA in the *Hordeum vulgare* seedlings exposed to γ-irradiation at doses higher than 50 Gy, notably declined compared to control. In addition, several studies have shown that ionizing radiation enhances the levels of ROS and free radicals in plant cells, in which these compounds stimulated endogenous IAA degradation (Ahuja et al. 2014; Esnault et al. 2010; Gudkov et al. 2019). Auxins and cytokinins play a critical role in the growth and development of cell and organ cultures. In vitro cultures require a sufficient amount as well as an appropriate proportion of those phytohormones (Bednarek and Orłowska 2020; Phillips and Garda 2019). Therefore, it can be supposed that in the current study, the decrease observed in CDB of *S. nemorosa* callus cultures in response to high doses of γ-irradiation was associated with alteration in the level of the endogenous hormones. It has also been suggested that high doses of γ-rays caused in the failure of RNA and protein synthesis and lowering in soluble protein content of plant cells, which results in limited cell growth and division(Gudkov et al. 2019; Mariadoss et al. 2020; Wi et al. 2007).

Effect of gamma irradiation on phenolic acids content

Rosmarinic acid (RA) and total phenolic contents (TFC) of Woodland Sage callus cultures exposed to γ-radiation were monitored during three successive subcultures (S1, S2, and S3) using spectrophotometric method. As shown in Fig. 2, RA and TFC contents of cultures increased in S1 to S3 subcultures. The pattern of phenolic compounds accumulation in the S1 subculture was relatively unstable but stabilized in the S2 and S3 subculture. Gamma irradiated *S. nemorosa* callus at different doses ranged 10 to 100 Gy showed a significant increase in RA and TFC compared to nonirradiated culture; however, this increment was dose-dependent (Fig. 2). Data in Table 1 revealed that the highest TFC production of 96.76 and 97.17 mg GAE/g DW was recorded in 70 and 80 Gy treatments, respectively, which is prominently higher than control. A gradual increase in the accumulation of RA was observed in callus cultures exposed to γ-rays from 10 to 70 Gy, and the maximum RA content of 17.59 mg/g DW was recorded at 70 Gy dose, which was 2.7 fold higher than nonirradiated culture. El-Beltagi et al. (2011) informed a two-fold increase in the phenylalanine ammonia-lyase (PAL) activity of *R. officinalis* calli in response to 20 Gy γ-irradiation compared to nonirradiated callus. Also, a positive correlation between PAL activity and γ-radiation doses has been reported by Benoît et al. (2000) and Hussain et al. (2010). Phenylalanine ammonia-lyase involved in the first step of plant phenolic compounds biosynthesis (Vardhan and Shukla 2017). It has been proved that an increase in PAL activity raises the production of RA in *Salvia miltiorrhiza* (Yan et al. 2006) and *Solenostemon scutellarioides* (Sahu et al. 2012). Therefore, it can be concluded that in the present research, one of the possible causes of increased RA and TFC production in *S. nemorosa* callus cultures under gamma treatment was an increase in PAL activity. Ionizing radiations enhanced ROS generation in plants directly through water radiolysis or indirectly by activation of NADPH-oxidase, damaging to the electron transport chain or altering the expression of its genes (Gudkov et al. 2019). Phenolic compounds are the main part of plants antioxidant systems, which alleviated oxidative stress induced by ROS (Ahuja et al. 2014; Vardhan and Shukla 2017). In the current research, we evaluated the antioxidant activity of Woodland Sage calli subjected to γ-radiation by measuring the DPPH radical scavenging and ferric reducing capacity (FRAP). Results exhibited in Table 1 indicated that γ-irradiation at doses 10 to 100 Gy heightened the DPPH and FRAP values as opposed to control. The maximum DPPH with 63.74 and 65.11IC50 µg/ml was observed in 80 and 70 Gy treatments, respectively, which was significantly higher than other doses and control. Also, the highest FRAP value was recorded in cultures irradiated with 80 and 70 Gy doses, which was 0.97 and 0.95, respectively. A positive correlation was observed between RA and TFC accumulation and antioxidant activity in γ-irradiated *S. nemorosa* callus cultures. Our findings are consistent with other reports which showed that plants heighten phenolic compounds production as the main antioxidants in response to gamma radiation (Akbari et al. 2018; Chung et al. 2006; Esnault et al. 2010; Khalil et al. 2015; Vardhan and Shukla 2017). In the present study, doses higher than 70 Gy resulted in a significant decline in the RA and TFC accumulation, as well as DPPH and FRAP values. These reductions were followed with noticeable changes in callus color and texture, including the intensification of the calli browning.

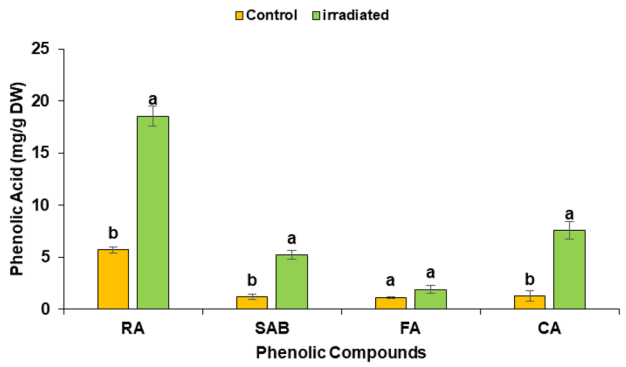

Fig. 3 HPLC analysis of Rosmarinic acid (RA), Salvianolic acid B (SAB), Ferulic acid (FA) and Cinnamic acid (CA) content in 70 Gy irradiated and nonirradiated callus culture of *S. nemorosa*. Bars represent standard deviation (n = 3)

Ionizing radiation at high doses induced damage to DNA and chromosomal aberrations, which caused lessening in plant growth and its primary and secondary metabolic pathways (Esnault et al. 2010).

Our results indicated that gamma irradiation at 70 Gy doses provided Woodland Sage callus line with a high content of RA, TFC, and antioxidant activity. Therefore, we evaluated the RA, Salvianolic acid B (SAB), Ferulic acid (FA) and Cinnamic acid (CA), content of nonirradiated and 70 Gy irradiated *S. nemorosa* calli by HPLC. As shown in Fig. 3, RA, SAB, and CA in the γ-irradiated calli were accumulated 3.2, 4.5, and sixfold higher than nonirradiated callus, respectively. There was also no significant difference in the ferulic acid production between irradiated and nonirradiated callus. The drastic increase in CA accumulation is associated with an increment in PAL activity, which has been described in other studies as one of the effects of γ-irradiation on the production of phenolic compounds. PAL is a key enzyme in the phenylpropanoid pathway and catalyzes the conversion of phenylalanine to cinnamic acid (Vardhan and Shukla 2017). Our results were consistent with report by Patil et al. (2018), who declared that irradiated callus cultures of *Artemisia annua* accumulated tenfold higher artemisinin content than nonirradiated culture. Also, Mariadoss et al. (2020) documented the 4.5 and 2.5-fold increase in purpurin and alizarin production in 8 Gy irradiated callus of *Rubia cordifolia*. Similar observations have been reported in other studies (Akbari et al. 2018; Azeez et al. 2017; Chung et al. 2006; El-Beltagi et al. 2011; Khalil et al. 2015) that confirmed our findings.

To our knowledge, the present study is the first report on the high-yielding cell line selection with respect to the production of phenolic acids in the *Salvia* genus. The content of RA, SALB, and CA in 70 Gy irradiated callus of *S. nemorosa* were drastically higher in comparison to previous reports on wild *S.nemorosa* (Bahadori et al. 2017) and *S. virgata* (Attaran Dowom et al. 2017), *S. leriifolia* (Modarres et al. 2018), *Salvia officinalis* (Santos-Gomes et al. 2003), and *S. miltiorrhiza* (Wu et al. 2016) in vitro cultures. This line is useful for the initiation of suspension cultures and large scale production of phenolic compounds.

Establishment of suspension culture

In the present study, Woodland Sage cell suspension cultures (CSC) were initiated with 1.8–2.2 g DW single cells and small aggregates per liter medium. As represented in Fig. 4, the growth kinetic of suspension cultures and phenolic acid accumulation were evaluated based on dry weight (DW), specific growth rate (SGR), and RA measurement during the 28 days of culture. The culture growth exhibited a sigmoid curve with four distinctive phases (Fig. 4a). During the first 8 days of culture, known as the lag phase, the cells began to proliferate slowly and grow about 1.8-fold. Then, they went into the exponential phase, and a 7.5 fold increase in dry biomass was observed until day 20. It followed by a deceleration phase in which cell growth rate drastically reduced in 20–24 days of culture. Afterward, cultures entered into the stationary phase, characterized by a decline in cell growth and culture senescence. Restrictions on nutrients, especially nitrogen and carbon, and the accumulation of harmful substances in culture medium were the probable cause of the slow growth of cells in the deceleration and stationary phases (Hu et al. 2019). Figure S3 showed noticeable changes in the color of the *S. nemorosa* cell cultures during the culture period. Cultures were light brown until day 8 and

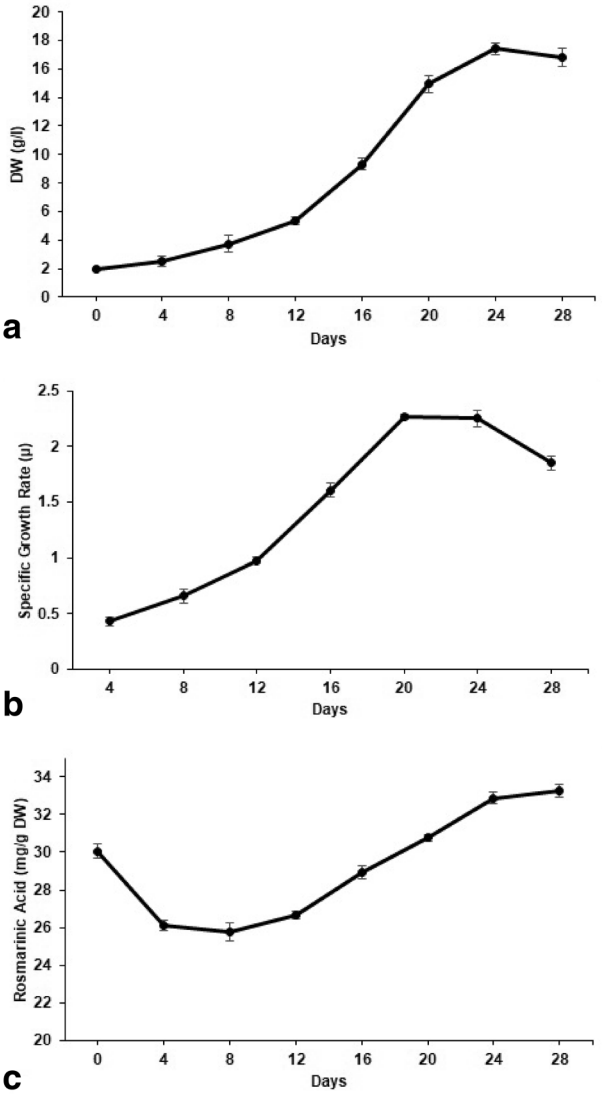

Fig. 4 **a** Growth kinetics based on dry weight, **b** specific growth rate and **c** rosmarinic acid accumulation of *S. nemorosa* cell suspension culture during a 28 days period. Bars represent standard deviation (n = 3)

then turned to medium brown and dark brown on days 20 (deceleration phase) and 28 (stationary phase), respectively. As shown in Fig. 4b, the maximum specific growth rate of the *S. nemorosa* cells was 2.26 per day, and doubling time was calculated 5.16 days. From an initial inoculum of 2 g DW/l, the highest dry biomass accumulated at 24 days of cultivation with 17.43 g/l. A dry weight of 16.80 g/l was achieved at the end of the culture period. Figure 4c, exhibited the pattern of RA production during the Woodland Sage cell culture. The RA content was 30.03 mg/g DW at the outset of culture and decreased to 25.76 mg/g DW for 8 days upon cultivation (lag phase). The RA content steadily raised from the beginning of the exponential phase until the end of the culture period, and the maximum accumulation was obtained at day 28 (stationary phase) with 33.27 mg/g DW.

Our literature review demonstrated that the establishment of *S. nemorosa* suspension culture has not previously been documented. The results of the current research showed that the *S. nemorosa* suspension culture with a high potential of RA production had been successfully established. Few efforts have been made to establish *Salvia* species CSC due to their slow growth, low biomass density, and low metabolite yields (Marchev et al. 2014). SGR and doubling time obtained in the present study were higher than those reported in the *Satureja khuzistanica* (Sahraroo et al. 2016) and *Azadirachta indica* (Sujanya et al. 2008) cell cultures, indicated the acceptable cell growth rate. Modarres et al. (2018) recorded the maximum amount of 9 g/l DW and 6.12 RA in the cell culture of *Salvia leriifolia*. Also, Zhao et al. (2010) documented the 8.9 g/l DW and 2.5 mg/g tanshinone accumulation in elicited CSC of *Salvia miltiorrhiza*. Our outcomes were significantly higher than the previously mentioned reports. Also, our results were consistent with those reported in the CSC of *Salvia fruticosa* (Haas et al. 2014), *Salvia officinalis* (Eremia et al. 2013), which reached a similar growth rate and phenolic acid content. Contrary to our findings, the maximum production of RA in CSC was reported in *Salvia officinalis* (326 mg/g DW extract) by Santos-Gomes et al. (2003), *Satureja khuzistanica* (180 mg/g DW) by Sahraroo et al. (2016), and *Mentha*×*piperita* (117.59 mg/g DW) by Krzyzanowska et al. (2011). The results of this experiment also revealed that the establishment of CSC of *S. nemorosa* using a γ-irradiated callus resulted in a 1.8-fold improvement in the production of RA compared to γ-irradiated callus culture and also 5.2-fold more than that of nonirradiated calli.

Effects of MWCNT-COOH on biomass growth and phenolic acids accumulation

In this experiment, the effect of different concentrations of carboxyl functionalized multi-walled carbon nanotubes (MWCNT-COOH) in range 25 to 100 mg/l was assessed on biomass growth, phenolic compounds accumulation and antioxidant activity of *S. nemorosa* CSC. As exhibited in Fig. 5a, b, increasing the MWCNT-COOH concentration to 100 mg/l significantly enhanced the FW of the cells, and the highest FW was recorded at 100 mg/l treatment with 268.47 g/l. whereas the DW of cells increased only up to 50 mg/l concentration, and no significant change was observed at levels above 50 mg/l. The expression of aquaporin (NtPIP1), cell cycle progression (CycB), and cell growth/cell-wall assembly (NtLRX1) genes upregulated in cells exposed to MWCNTs (Bhati et al. 2018; Jordan et al. 2018). Aquaporins are the water channel proteins in plant cells that involved in cell water uptake, especially under abiotic stresses. Thus, enhancement in aquaporins activity results in more water uptake by cells (Martinez-Ballesta and Carvajal 2014). On the other hand, Tripathi et al. (2017) reported the threefold increase in micronutrient content of *Cicer arietinum* seeds in the first generation after treatment with 300 mg/l MWCNTs. Therefore, in the current study, an increment in fresh biomass accumulation observed in MWCNTs treated Woodland Sage cells may be due to an increase in water uptake followed by the heightened activity of aquaporins. It may also be explained that by increasing the water uptake, more nutrients are transported into the cells and resulted in enhancing DW at 25 and 50 mg/l concentrations compared to the control. A constant DW was recognized at levels above 50 may be associated with limitations and reduction of the nutrient content in the culture medium. Khodakovskaya et al. (2012) noted the improvement in growth and fresh biomass accumulation up to 60% and in *Nicotiana tabacum* cells exposed to 500 mg/l MWCNT-COOH. Ghorbanpour and Hadian (2015) reported that MWCNT-COOH at 50 mg/l concentration significantly increased the callus induction and FW and DW of obtained calli in *Satureja khuzestanica*. Also, similar observations have been reported in *Catharanthus roseus* (Ghasempour et al. 2019), *Hibiscus sabdariffa* (Sareea Al-Rekaby 2018), *Solanum lycopersicum* (Khodakovskaya et al. 2013), *Brassica napus* (Zhao et al. 2019). Our findings are consonant with recent reports.

The results of the present experiment indicated that the elicitation of *S. nemorosa* suspension cultures with MWCNT-COOH significantly improved the production of phenolic compounds. Figure 5e, f, showed that as the MWCNT-COOH concentration raised from 25 to 100 mg/l, the accumulation of phenolic compounds enhanced dramatically compared to control. The maximum content of RA, SAB and CA was recorded in 100 mg/l treatment with 82.71, 28.31 and 23.48 mg/g DW, respectively. A 2.2-fold increase in CA production in response to MWCNT-COOH treatment indicated an increase in PAL activity. PAL is a key enzyme in the phenylpropanoid pathway and catalyzes the conversion of phenylalanine to cinnamic acid (Vardhan

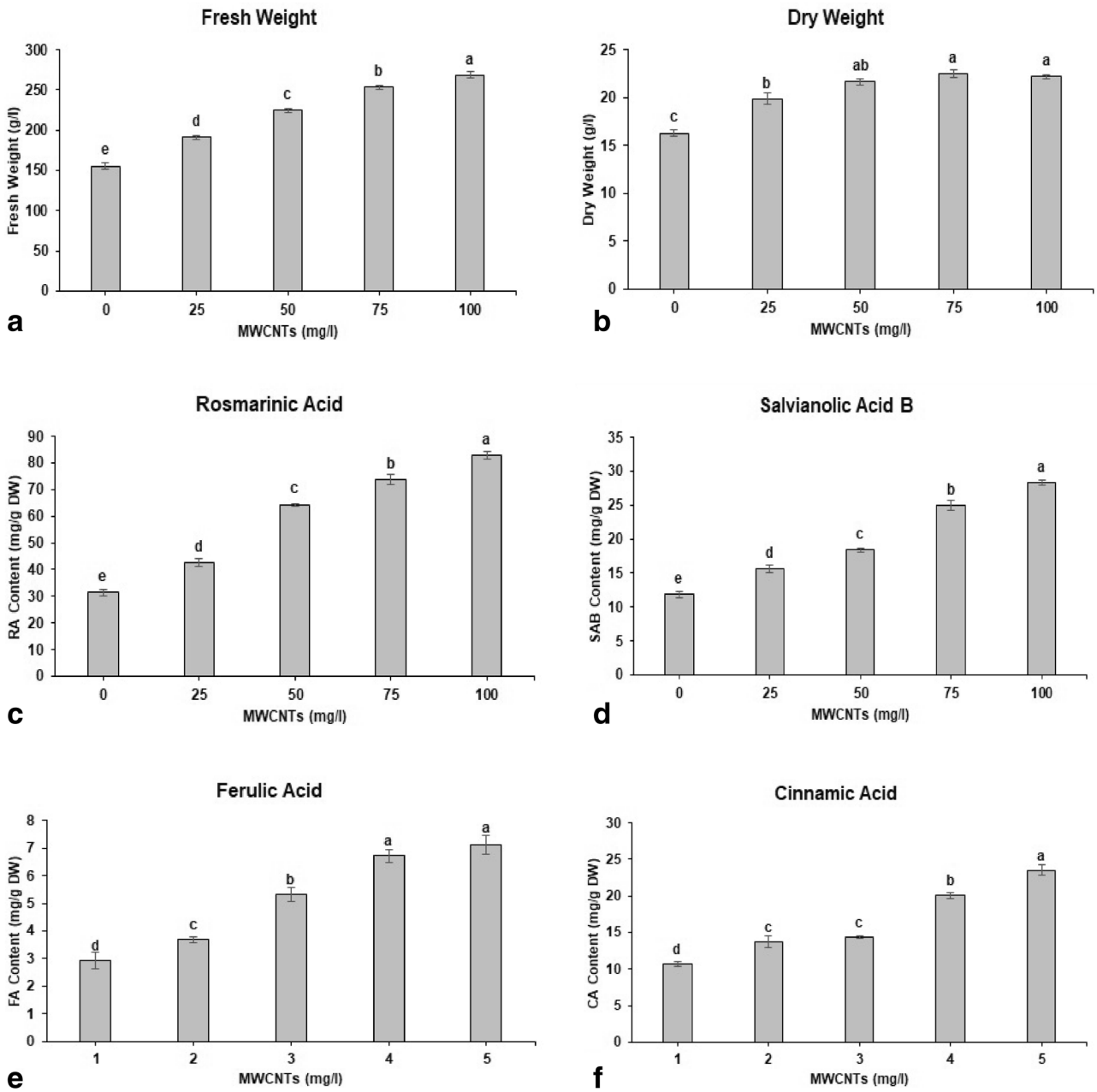

Fig. 5 HPLC analysis of rosmarinic acid (RA), salvianolic acid B (SAB), ferulic acid (FA) and cinnamic acid (CA) content in *S. nemorosa* cell suspension culture elicited by different concentrations of MWCNT-COOH. Bars represent standard deviation (n = 3)

and Shukla 2017). It activity increased in response to biotic and abiotic stresses and elicitor treatment (Yan et al. 2006). The primary mechanism through MWCNTs altered secondary metabolism pathway has been investigated. They provoke ROS production followed by activation of both enzymatic and non-enzymatic antioxidant defense machinery to scavenge excess ROS (Jordan et al. 2018; Marslin et al. 2017). Phenolic compounds are the main antioxidant of plants, and their production increased to scavenging ROS generated through stresses (Vosoughi et al. 2018). In the present study, the DPPH and FRAP antioxidant activity of *S. nemorosa* cell cultures treated by MWCNT-COOH significantly enhanced. The maximum DPPH and FRAP values were recorded in the 100 mg/l MWCNT-COOH treatment, which was 41.37 µg/ml and 1.53 SPR, respectively (Fig. 6). These findings confirmed that heightened in RA, SAB, CA, and FA accumulation in Woodland Sage cell culture was promoted by increasing ROS signaling generated in response to MWCNT-COOH. Similarly to our results, Ghorbanpour and Hadian

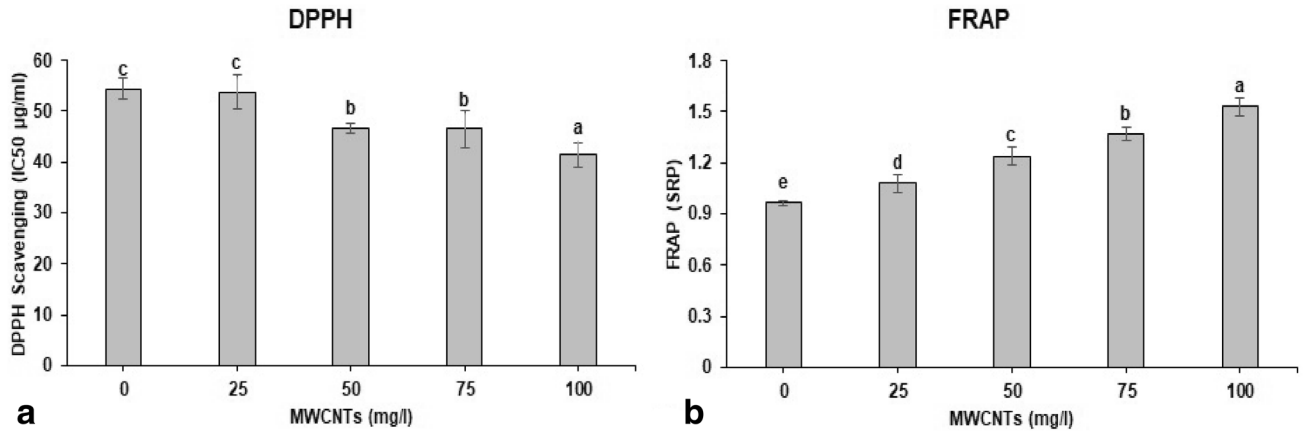

Fig. 6 DPPH (**a**) and FRAP (**b**) activity in *S. nemorosa* cell suspension culture elicited by different concentrations of MWCNT-COOH. Bars represent standard deviation (n = 3)

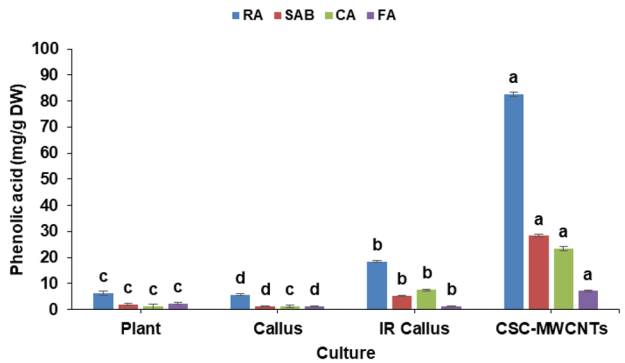

Fig. 7 Comparison of rosmarinic acid (RA), salvianolic acid B (SAB), ferulic acid (FA), and cinnamic acid (CA) in wild plant, callus culture, 70 Gy irradiated callus culture and cell suspension culture elicited with 100 mg/l MWCNT-COOH of *S. nemorosa*

(2015) reported a twofold increase in the RA and caffeic acid content of *Satureja khuzestanica* callus culture at 100 mg/l MWCNTs treatment. Also, Ghasempour et al. (2019) stated that MWCNTs at range 50 to 150 mg/l significantly enhanced the total phenol, alkaloid, carotenoid, and chlorophyll content of *Catharanthus roseus*. In another research carried out by Sareea Al-Rekaby (2018), MWCNTs treatment improved the essential oil production in *Hibiscus sabdariff*.

In the present research, we also compared the four phenolic content of Woodland Sage in wild plant at the flowering stage, callus culture, 70 Gy γ-irradiated calli, and 70 Gy γ-irradiated suspension culture elicited with 100 mg/l MWCNT-COOH. As shown in Fig. 7, CSC established using 70 Gy γ-irradiated calli and elicited by 100 mg/l MWCNT-COOH resulted in a 13-fold and 14.2-fold increase in RA and SAB production, respectively, compared to wild plant at flowering stage. Also a 20-fold and 3-fold improvement was observed in CA and FA production (Fig. 8).

Conclusion

Our research demonstrated that gamma irradiation induced significant changes in *S. nemorosa* callus morphology, growth, and phenolic content. Our findings revealed that γ-irradiation at 70 Gy dose succeeded in obtaining high-yielding cell line with respect to RA, salvianolic acid, ferulic acid, and cinnamic acid production, which was useful for the suspension cultures. In the present study, we successfully established a CSC of Woodland Sage using a 70 Gy irradiate calli. We elicited it with different concentrations of multi-walled carbon nanotubes, which resulted in drastically increase in phenolic production. The RA, SAB, CA and FA content of CSC treated with 100 mg/l MWCNT-COOH

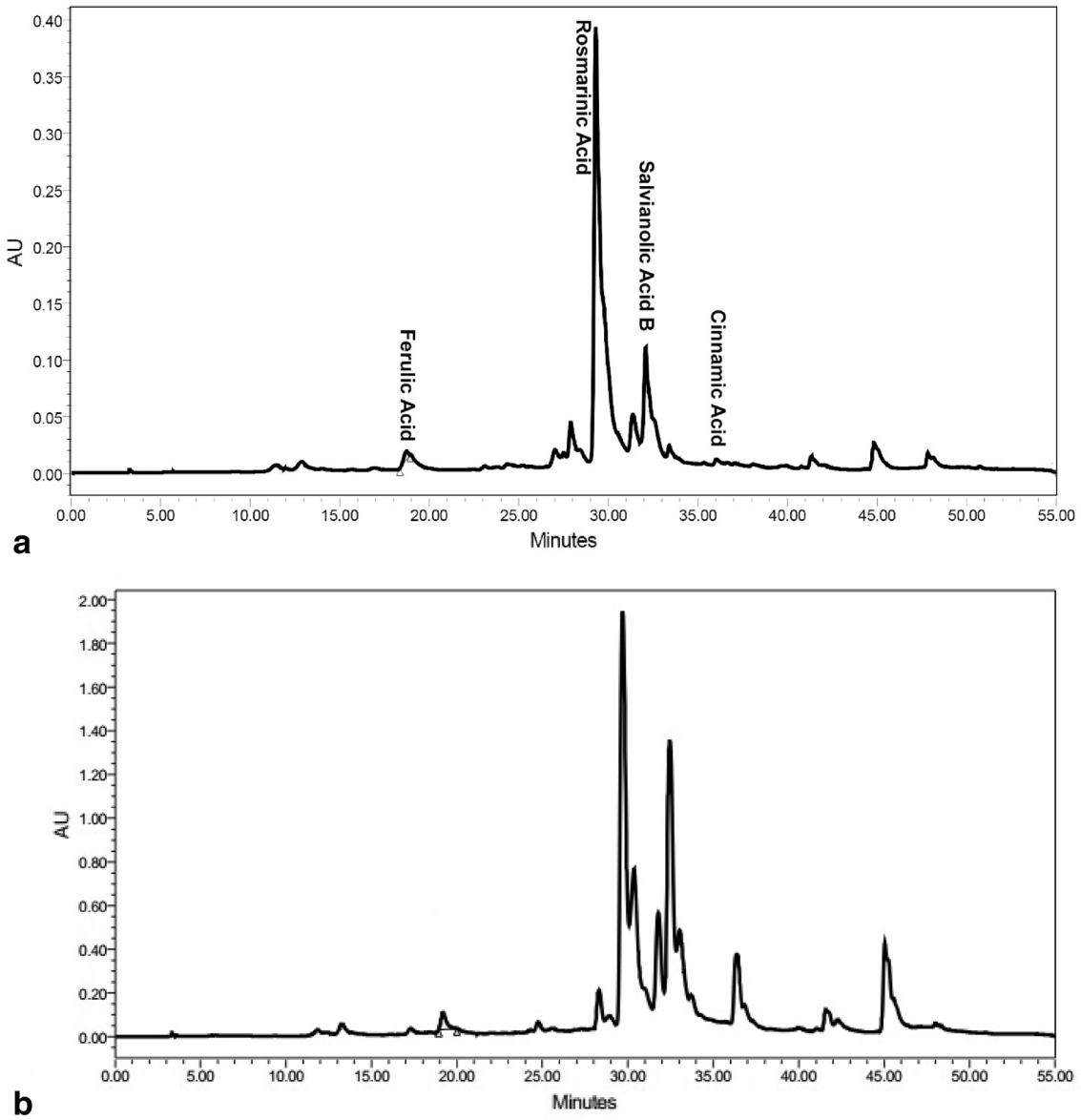

Fig. 8 HPLC chromatograph of wild (**a**) and cell suspension culture elicited with 100 mg/l MWCNT-COOH (**b**) of *S. nemorosa*

were 13-fold, 14.2-fold, 20-fold, and 3-fold higher than wild *S. nemorosa* at flowering stage, respectively. Our results showed that the combination of cell line selection and MWCNT-COOH elicitation significantly improved the production of secondary metabolites in Woodland Sage, which is useful for large-scale production of phenolic compounds.

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