Journal of Medicinal Plants and By-products (2023) 4: 397-404
https://doi.org/10.22092/jmpb.2022.358302.1465

Original Article

The Effect of NaCl and Salicylic Acid on Total Phenolic and Flavonoid Contents in Suspension Culture of *Nitraria schoberi*

Dana Rafiee[1], Mohammad Ali Ebrahimi[1], Nassrin Qavami[2] and Nasim Zarinpanjeh[2*]

[1]*Department of Agricultural Biotechnology, Payam Noor University, Tehran, Iran*
[2]*Medicinal Plants Research Center, Institute of Medicinal Plants, ACECR, Karaj, Iran*

Article History

Received: 10 April 2022
Accepted: 23 August 2022

Keywords
Callus induction
Elicitor
Medicinal plants
Secondary metabolites
Suspension culture

***Corresponding author**
zarinpanjeh@imp.ac.ir

ABSTRACT

Nitraria schoberi L. is a medicinal plant with antioxidant, antifungal, anti-inflammatory and, anti-viral properties. This plant is also tolerant to salinity and drought. The main aim of this study was to perform elicitation in cell suspension culture of *N. schoberi* to detect the production of total phenolic and flavonoid contents. In order to accomplish this study, hypocotyls and cotyledonary leaves of *N. schoberi* were cultured in different callus induction treatments. At the next step, the calli from the best callus induction treatment were transferred to cell suspension cultures containing sodium chloride (NaCl) and salicylic acid (SA) alone or in combination. Total phenolic and flavonoid contents in treatments were measured using a spectrophotometer (absorbance at 765 nm and 430 nm, respectively). According to the results, although callus induction was observed in all treatments, the maximum fresh weight (FW) (7.6 g) and dry weight (DW) (3.7 g) were obtained by culturing hypocotyl explants in MS medium supplemented with 2, 4-dichrolophenoxyacetic acid (2, 4-D) at 0.5 mg/L. The highest total phenolic content (62.2 mg/g) and total flavonoid content (48.24 mg/g) were detected in suspension culture containing 100 millimolar (mM) NaCl and 50 micromolar (µM) SA. Elicitation in suspension culture of *N. schoberi* can be an effective method for increasing the production of secondary metabolites (phenolic and flavonoid compounds).

INTRODUCTION

Higher plants undergo a variety of stresses and to combat those stresses. They acclimatize themselves by producing diverse secondary metabolites. These secondary metabolites also have a wide range of industrial applications and serve as candidates for commercialization [1-6]. *Nitraria schoberi* L. is a medical and a halophytic shrub of the family *Nitrariaceae* (Zygophyllaceae), a typical representative of deserts and semi-deserts, grown in saline gypsum soils of foothills, lowlands, and salt lakes shores [7]. *Nitraria* plants, specifically the leaves, fruits and seeds, contain several secondary metabolites, including sterols, fatty acids, alkaloids and flavonoids and phenolic derivatives with powerful antioxidant, antimicrobial, antifungal, anti-inflammatory and anti-viral activities [7-11].

Cell suspension culture is one of the ideal methods for producing secondary metabolites owing to the fast growth rate of cells in suspension [12-15]. They are practiced in numerous medicinal plants for the production of various secondary metabolites [15-19]. Although many such cell cultures have been established from different plant species, they seldom produce sufficient amounts of the required secondary metabolites without proper [20].

Elicitation is currently the most promising technique and is used widely for increasing biomass and secondary metabolite production in plant cell cultures. Elicitors are biotic or abiotic molecules that induce a signal across plant cells which, in turn, stimulate secondary metabolic pathways and downstream transcription factors in response to the external stimuli, thus resulting in the production of secondary metabolites [21]. SA has been widely reported as a successful, effective signal molecule among all other types of abiotic elicitors [21, 22]. It has been used in the stimulation of flavonoids and polyphenols production in cell suspension, calli and tissue cultures of diverse plant families [23].

Applying NaCl as an abiotic elicitor alone or in combination with other elicitors has also been reported in many previous studies [24,25]. According to the important role of elicitation in order to enhance the production of secondary metabolites, the effect of two abiotic elicitors, including SA and NaCl on the production of total phenolic and flavonoid contents in the suspension culture of *N. schoberi* has been evaluated at the current study.

MATERIALS AND METHODS

Plant Material and Sterilization

Seeds of *N. schoberi* plants were collected from Myghan desert, Markazi Province, Iran in July 2019. Seeds were surface-sterilized using the previous method with some modification as follows [26]. The seeds were washed under running tap water and stored at 4 °C for 24 h in the dark. Then, the pericarp was removed by rubbing with hands and the seeds were kept again at 4 °C for 24 h in the dark. In preparation for seed germination, seeds were surface-sterilized by treating with 70% ethanol for 1 min and an aqueous 0.1% (v/v) sodium hypochlorite solution for 15 min. Seeds were finally cracked with a plier and then rinsed three times with sterile double-distilled water to remove any traces of the disinfectant.

Seed Germination

The surface-sterilized seeds were cultured in test tubes containing 10 ml of MS medium [27]. The pH of the medium was adjusted to 5.8 and then autoclaved for 15 min at 121 °C. The cultured seeds were incubated at 25±2 °C under the 16/8 h photoperiod and light illumination of 3000 l ux for 21 days.

Callus Induction

Healthy cotyledonary leaves and hypocotyls from germinated seeds were cultured on a solid MS medium fortified with different levels of 2, 4-D (0, 0.5.1, 1.5 and 2 mg/L) and 6-benzyl amino purine (BAP) (0, 0.5 and 1 mg/L). The cultures were maintained in the dark at 25 ± 2 °C. The percentage of callus induction, FW and DW were recorded 4 weeks after culture. The successfully induced callus was separated from the explants and cultured separately until used for the establishment of cell suspensions.

Cell Suspension Culture

Cell suspension culture was initiated by applying a modified method, including transferring calli from the best treatment of callus induction media (80–100 mg) to a 50-mL Erlenmeyer flask containing 15 mL of liquid MS medium supplemented with 0.5 mg/L 2, 4-D. The suspension cultures were incubated on a rotary shaker at 120 rpm and 25 ± 2 °C under 16-h photoperiods with a light intensity of 3000 lux. Prior to autoclaving (121 °C, 20 min), all media were adjusted to pH 5.6–5.8 [28].

Elicitation of Cell Suspension Culture

Two elicitors, including NaCl (Merck, Germany) and SA (Sigma-Aldrich, USA) were used to study the elicitation effect on Total phenolic and flavonoid production by cell suspension cultures of *N. schoberi*. Elicitation was applied on day 21 after inoculation when the cell cultures were in the middle of a rapid growth phase. To determine the most effective elicitor concentration for induction of total phenol and flavonoid contents, both elicitors were added to cultures at final concentrations of 0, 50 and 100 mM for NaCl and 0, 50 and 100 µM for SA individually and in combination. Samples were taken 7 days after adding elicitors. The cell biomass was freeze-dried and used for total phenol and flavonoid extraction.

Preparation of Extracts

Five grams of the dry powder from the rhizome, callus and treated callus were macerated separately in 10 mL of petroleum ether (PE) and chloroform: methanol (1:1, v/v) (CM) at room temperature for 72 h. Extracts were evaporated under vacuum to dryness and stored in dried bottles at 4°C [29].

Determination of total phenolic contents

The extract (1 mL) was mixed with 5 mL Folin–Ciocalteu reagent diluted with water 1:10 v/v and 4 mL of 7.5% sodium carbonate. The mixture was vortexed for 15 s and allowed to stand for 30 min at 40ºC for color development. Absorbance was then measured at 765 nm using GBC, Cintra 20 spectrophotometer. A calibration curve was prepared using gallic acid (25–100 mg/L) as standard and used to calculate the total phenolic content. The total phenolic contents were expressed as gallic acid equivalents (mg/g) using the following equation based on the calibration curve: $y = 0.0176x + 0.08$, where y was the absorbance [29].

Determination of Total Flavonoid Contents

To 0.5 mL of sample, a volume of 0.5 mL of 2% $AlCl_3$ ethanol solution was added. After one hour at room temperature, the absorbance was measured at 420 nm. The Yellow color indicated the presence of flavonoids. Extract samples were evaluated at a final concentration of 0.1 mg/mL. A calibration curve was constructed, using quercetin (25– 100 mg/L) as standard. Total flavonoid contents were expressed as quercetin (mg/g) using the following equation based on the calibration curve: y = 0.003x - 0.008, where y was the absorbance [29].

Statistical analysis

The experiments were set up on a completely randomized design with three replicates per treatment and four explants per replicate for callus induction and three replications for each elicitation treatment. Statistical differences were assessed based on analysis of variance (ANOVA) using SPSS (version 18, USA). Differences among means were analyzed using Duncan multiple range test at a probability level of $p< 0.01$. The values are expressed as the mean ± standard error (SE).

RESULTS

Callus Induction

Callus induction was observed after two weeks with swelling and then the formation of mass on the surface of the explants in nearly all treatments with the frequency of 100 %. The color of calli in all treatments was light to dark cream and the texture was friable. It means that the type and concentration of plant growth regulators (PGRs) and also the type pf explant did not affect the formation, the texture and the color of calli. All stages from *in vitro* seed germination up to callus induction are shown (Fig. 1).

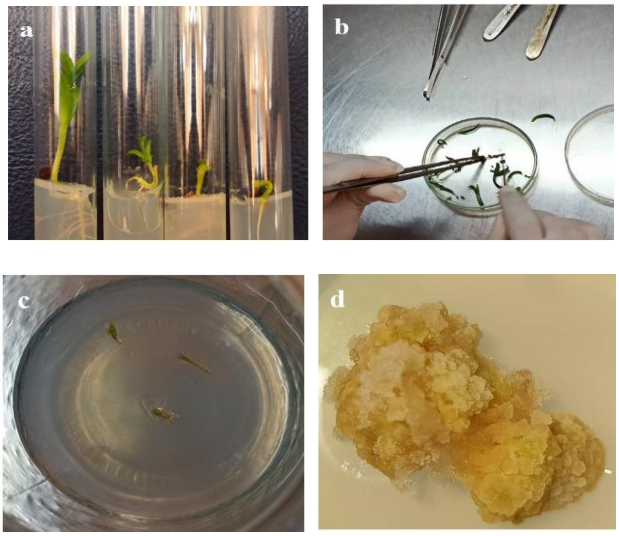

Fig. 1 *In vitro* seed germination to callus induction of *N. schoberi*. A: *In vitro* seed germination, B: Explant preparation, C: Culturing of explants in calls induction medium, D: Callus induction.

However, FW and DW showed significant differences among different treatments (Table 1). According to mean comparisons of the effect of explant type and PGR combination, maximum biomass accumulation (FW and DW) was obtained by culturing hypocotyl explant cultured on MS medium augmented with 2,4-D at 0.5 mg/L. The maximum FW and DW were 7.6 g and 3.7 g, respectively. The range of mean FW (g) and DW (g) in different treatments were from 0.37 to 7.6 and 0.024 to 3.03, respectively (Table 2).

Cell Suspension Culture

The calli from hypocotyl explant grown on 0.5 mg/L 2,4-D were used to establish cell suspension cultures (Fig. 2).

Table 1 The variance analysis of the effect of explant type, 2, 4-D and BAP on callus induction

Source of variation	df	Mean of Squares	
		FW	DW
Explant	1	3.425 **	0.379 **
BAP	2	3.354 **	0.988 **
2,4 -D	4	4.930 **	0.866 **
Explant × BAP	2	15.201 **	1.059 **
Explant × 2,4 -D	4	6.272 **	0.884 **
BAP× 2,4 -D	8	4.021 **	0.813 **
Explant × BAP× 2,4 -D	8	9.665 **	0.944 **
Error	60	0.089	0.003
CV (%)	-	19.4	29

**: significant at $p\leq 0.01$;

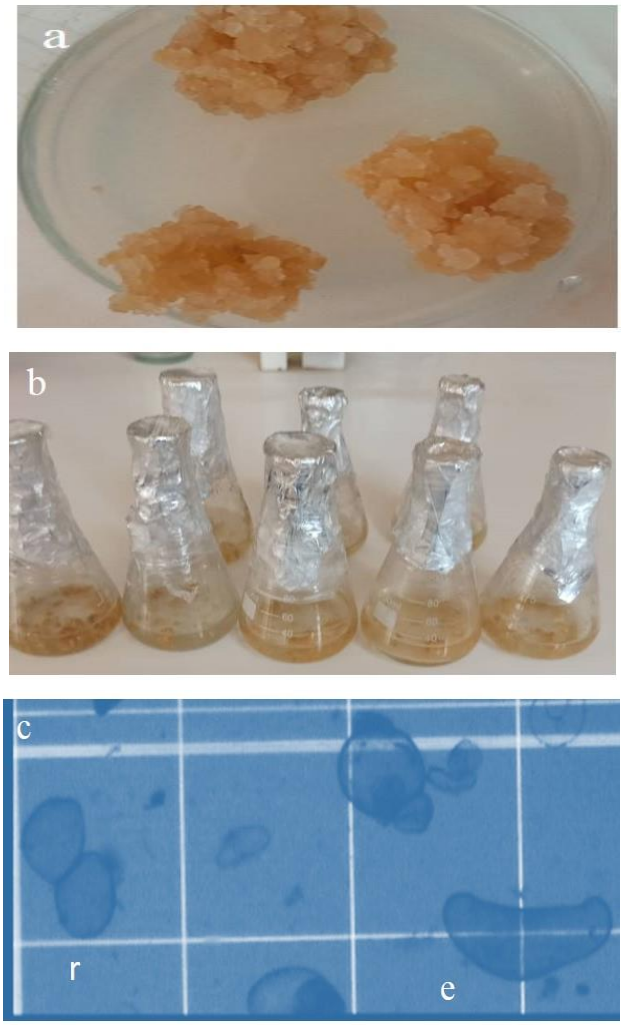

Fig. 2 Establishment of *N. schoberi* cell suspension culture. A: hypocotyl explant grown on 0.5 mg/L 2, 4-D, B: cell suspension culture grown in a flask, C: cell suspension culture showing photomicrograph of round (r) and elongated (e) shaped cells (× 40)

The results obtained from the analysis of variance related to the extracted total phenolic content showed that the effect of interaction between NaCl and SA was significant at 1% probability level (Table 3). The highest accumulated amount of total phenolic content was 67.2 mg/g and the lowest was 51.4 mg/L. The maximum total phenolic content was gained in NaCl concentration of 100 mμ with SA at 50 Mμ (Fig. 3).

The highest accumulated amount of total flavonoid content was 48.24 mg/g, which was more than 2.5 times higher than non-elicited cells (17.24 mg/L). The maximum total flavonoid content was gained in NaCl concentration of 100 mμ with SA at 50 Mμ (Fig 4). The analysis of variance related to the extracted total flavonoids showed that the effect of interaction between NaCl and SA was significant at 1% probability level (Table 4).

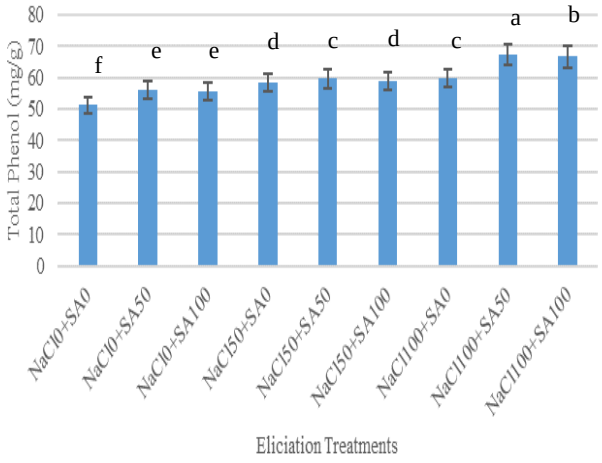

Fig. 3 Effect of different concentrations and combinations of NaCl and SA on the production of total phenolic contents 7 days after elicitation. Values followed by different letters in each trait are significantly different at $p \leq 0.05$.

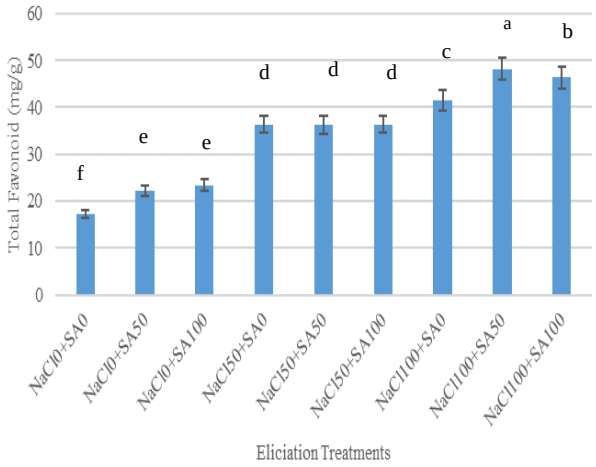

Fig. 4 Effect of different concentrations and combinations of NaCl and SA on the production of total flavonoids 7 days after elicitation. Values followed by different letters in each trait are significantly different at $p \leq 0.05$.

DISCUSSION

In the the present study, the effect of explant type and PGRs on callus induction was investigated for the establishment of suspension culture to produce valuable medicinal compounds (total phenolics and flavonoids) of *N. schoberi*. [30].

Table 2 Effect of explant type, 2, 4-D (mg/L) and BAP (mg/L) on callus induction of *N. schoberi*. Values followed by different letters in each trait are significantly different at $P \leq 0.01$

Treatment number	Explant type	BAP	2,4 -D	FW	DW
1	Cotyledonary leaf	0.0	0.0	0.378±0.31 hi	0.0243±0.01 h
2	Cotyledonary leaf	0.0	0.5	0.395±0.03 i	0.0576±0.00 efgh
3	Cotyledonary leaf	0.0	1.0	0.685±0.22 hi	0.121±0.05 bcdefgh
4	Cotyledonary leaf	0.0	1.5	3.32±0.36 bc	0.23±0.11b
5	Cotyledonary leaf	0.0	2.0	1.55±0.36 ef	0.151±0.12 bcdefg
6	Cotyledonary leaf	0.5	0.0	0.618±0.33 hi	0.043±0.01 h
7	Cotyledonary leaf	0.5	0.5	2.85±0.15 c	0.19±0.04 bcd
8	Cotyledonary leaf	0.5	1.0	3.37±0.30 b	0.18±0.03 bcde
9	Cotyledonary leaf	0.5	1.5	0.903±0.14 ghi	0.0616±0.01 efgh
10	Cotyledonary leaf	0.5	2.0	1.33±0.37 efg	0.132±0.10 bcdefgh
11	Cotyledonary leaf	1.0	0.0	1.73±0.15 def	0.08±0.02 defgh
12	Cotyledonary leaf	1.0	0.5	1.81±0.13 de	0.115±0.04 bcdefgh
13	Cotyledonary leaf	1.0	1.0	1.599±0.15 ef	0.16±0.09 bcdef
14	Cotyledonary leaf	1.0	1.5	3.48±0.21 b	0.21±0.05 bc
15	Cotyledonary leaf	1.0	2.0	1.87±0.62 de	0.102±0.06 cdefgh
16	Hypocotyl	0.0	0.0	1.598±0.46 ef	0.131±0.10 bcdefgh
17	Hypocotyl	0.0	0.5	7.68±0.29 a	3.03±0.01 a
18	Hypocotyl	0.0	1.0	1.72±0.19 def	0.124±0.08 bcdefgh
19	Hypocotyl	0.0	1.5	0.411±0.23 hi	0.038±0.01 h
20	Hypocotyl	0.0	2.0	1.23±0.66 fg	0.072±0.04 efgh
21	Hypocotyl	0.5	0.0	0.39±0.12 i	0.0266±0.00 h
22	Hypocotyl	0.5	0.5	0.814±0.06 ghi	0.0516±0.01 gh
23	Hypocotyl	0.5	1.0	0.42±0.20 hi	0.031±0.01 h
24	Hypocotyl	0.5	1.5	0.99±0.34 gh	0.043±0.02 h
25	Hypocotyl	0.5	2.0	0.824±0.10 ghi	0.0523±0.00 gh
26	Hypocotyl	1.0	0.0	0.455±0.28 hi	0.029±0.01 h
27	Hypocotyl	1.0	0.5	0.435±0.10 i	0.0246±0.00 h
28	Hypocotyl	1.0	1.0	0.444±0.14 hi	0.0273±0.00 h
29	Hypocotyl	1.0	1.5	0.602±0.27 hi	0.034±0.02 h
30	Hypocotyl	1.0	2.0	2.16±0.32 d	0.094±0.03 cdefgh

Table 3 The variance analysis of the effect of NaCl and SA on the production of total phenolic contents

Source of variation	df	Mean of Squares (Total Phenolics)
NaCl	2	223.170 **
SA	1	51.124 **
NaCl× SA	2	10.433 **
Error	12	0.031
CV (%)	-	0.9

**: significant at $p \leq 0.01$;

Table 4 The variance analysis of the effect of NaCl and SA on the production of total flavonoids

Source of variation	df	Mean of Squares (Total flavonoids)
NaCl	2	1369.648 **
SA	2	43.168 **
NaCl× SA	4	12.860 **
Error	18	0.812
CV (%)	-	2.6

**: significant at $p \leq 0.01$;

It is worth it to be said that explant type as well as PGRs play an important role in callus induction and, consequently, suspension culture establishment in many plant species. Among plant growth regulators, auxins and cytokinins are the most often used to induce callus in various plant species [31]. Here, callus cultures were successfully induced from *N. schoberi* hypocotyls on solid MS medium supplemented only with 2, 4-D (as PGR) at 0.5 mg/L. While, for callus induction of *N. tangutorum* Bobr, culturing the cotyledons as explant on MS medium supplemented with 0.3 mg/L BAP and 1mg/L naphthalene acetic acid (NAA) showed the best result [32]. And also, the callus formation and multiplication of *N. sibirica*, were achieved by culturing the mature seeds on MS having 0.5 mg/L BAP and 1.0 mg/L 2, 4-D [33]. This might be due to

the specie type that exerts additional effects during in vitro tissue culture processes [34].

Implementation of several productivity enhancement strategies, including elicitation, can overcome the limitations faced by plant cell technology that hampers its extensive commercialization. Elicitation is a technique that involves the exogenous addition of elicitors (abiotic or biotic) in the growth medium, which consequently triggers stress response with concomitant enhancement in secondary metabolite production [1]. The exposure of the culture in the growth medium to elicitor(s) has been used as one of the prime strategies to increase the yield and productivity of secondary metabolites in many *in vitro* plant cell/tissue cultures [35-37]. In this study, SA and NaCl have been used as elicitors to evaluate the production of total phenolic and flavonoid contents. SA is one of the most important plant phenolics that involves in diverse physiological and developmental responses. Moreover, supplementation of SA to the culture medium or short-term exposure of the cultures to SA additively boosts the biosynthesis and accumulation of secondary metabolites [29]. Exogenous application of SA as an elicitor influences the accumulation of phenolics and flavonoids in different plant species. For example, the supplementation of 100µM SA to the culture medium enhanced the total phenolic accumulation in *Stevia rebaudiana* by about 3.4-fold [38].

Similarly, the supply of SA to the callus cultures of *Fagonia indica* increased the accumulation of phenolic and flavonoid compounds [39]. Suspension culture of *Corylus avellana* cells treated with SA exhibited an elevated level of phenolic contents. However, flavonoid content decreased in response to SA treatment [40]. The presence of SA in the culture medium elicited the production of total phenol, flavonoid and some other secondary metabolites in the shooting culture of *Knautia sarajevensis* [41]. Cell suspension culture of *Momordica dioica* supplemented with SA elicited 22 phenolic compounds [42]. Plants exposed to salt stress additively enhanced the accumulation of secondary metabolites such as total phenolics and total flavonoids [43]. Nitric oxide source and sodium nitroprusside supplemented with SA interacted with nitric oxide source and sodium nitroprusside to enhance the secondary metabolite production under drought stress. The treated plants exhibited an elevated activity of PAL that boosted the accumulation of flavonoids, anthocyanin and phenol [44]. NaCl as an abiotic elicitor has also been used in many cases to enhance the production of secondary metabolites. For example, applying NaCl significantly increased the concentration of steviol glycoside in the suspension culture of *Stevia rebaudiana*. The quantity of steviol glycoside got increased from 1.36 (control) to 2.61% with 0.10 % NaCl. In contrast, among the five tested elicitors, including Casein hydrolysate, sucrose, NaCl, chitosan and yeast extract, only chitosan (150 mg/l) enhanced the phenolic content of *Orthosiphon stamineus* cell cultures [24].

CONCLUSION

In conclusion, Applying NaCl with SA is introduced as an efficient method to enhance the production of total phenolic and flavonoid contents in cell suspension cultures of *N. schoberi*. This study will provide a reference for future studies on the effects of different salts in companion with other biotic and abiotic elicitors to increase the accumulation of secondary medicinal metabolites from *in vitro* cultures of this worthwhile medicinal plant.

Conflict of Interest

Authors declare that there is no conflict of interest.

ACKNOWLEDGEMENT

This work was accomplished at the Biotechnology department of Medicinal Plants Research Center, Institute of Medicinal Plants, ACECR, Karaj, Iran.

REFERENCES

1. Narayani M., Srivastava S. Elicitation: a stimulation of stress in in vitro plant cell/tissue cultures for enhancement of secondary metabolite production. Phytochem Rev. 2017; 16: 1227-1252.
2. Tabassam S., Anwar M.A., Gulfraz M., Arshad M., Sabitaliyevich U.Y., Nurmurzayevich S.B., Ahmad M.S.
3. Bioactivity evaluation and HPLC UV-VIS based quantification of antioxidant secondary metabolites from extract and fractions of Bistorta amplexicaulis rhizome. Cellular and Molecular Biology. 2019; 65: 19-26.
4. Cecilia M.K., Abrha A.H., Hlengilizwe N., Hintsa A.T., Umer M., Nixwell M.F., Ali S.W., Afzal M.I., Martorell M., Salehi B., Setzer W.N., Sattar M.U., Imran M., Sharifi-Rad J. Untargeted profiling of field cultivated bush tea (*Athrixia phylicoides* DC.) based on metabolite

analysis. Cellular and Molecular Biology. 2020; 66: 104-109.

5. Salehi B., Tsouh Fokou P.V., Yamthe L.R.T., Tali B.T., Adetunji C.O., Rahavian A., Mudau F.N., Martins N., Martorell M., Rodrigues C.F., Setzer W.N., Sharifi-Rad M., Amarowicz R., Sharifi-Rad J. Insights on the anticancer potential of plant-food bioactives: A key focus to prostate cancer. Cellular and Molecular Biology. 2020; 66: 250-263.
6. Salehi B., Selamoglu Z., Sevindik M., Fahmy N.M., Al-Sayed E., El-Shazly M., Csupor-Löffler B., Csupor D., Yazdi SE, Sharifi-Rad J, Arserim-Uçar DK, Arserim EH, Karazhan N, Jahani A, Dey A, Azadi H, Vakili SA, Sharopov F, Martins N., Büsselberg D. *Achillea* spp.: A comprehensive review on its ethnobotany, phytochemistry, phytopharmacology and industrial applications. Cellular and Molecular Biology. 2020; 66: 78-103.
7. Alavi M., Adulrahman N.A., Haleem A.A., Al-Râwanduzi A.D.H., Khusro A., Abdelgawad M.A., Ghoneim M.M., Batiha G.E.S., Kahrizi D., Martinez F., Koirala N. Nanoformulations of curcumin and quercetin with silver nanoparticles for inactivation of bacteria. Cellular and Molecular Biology. 2022; 67:151-156.
8. Sharifi-Rad J., Hoseini-Alfatemi SM, Sharifi-Rad M and Teixeira da Silva JA. Antibacterial, antioxidant, antifungal and anti-inflammatory activities of crude extract from *Nitraria schoberi* fruits. 3 Biotech. 2015; 5: 677-684.
9. Suo Y., Wang L. Extraction of *Nitraria tangutorum* seed lipid using different extraction methods and analysis of its fatty acids by HPLC fluorescence detection and on-line MS identification. Eur J Lipid Sci Technol. 2010; 112: 390-399.
10. Hadj Salem J., Chevalot I., Harscoat-Schiavo C., Paris C., Fick M., Humeau C. Biological activities of flavonoids from *Nitraria retusa* (Forssk.) Asch. and their acylated derivatives. Food Chem. 2011; 124: 486-494.
11. Khajeddini M.A., Dadpour M.R., Khodaverdi M., Naghiloo S. The GC-MS analyses of the n-hexane extract of *Nitraria schoberi* L., its total phenolics and *in vitro* antioxidant activity. J. Med. Plants Res. 2012; 6: 4874-4878.
12. Zheleznichenko T., Banaev E., Asbaganov S., Voronkova M., Kukushkina T., Filippova E., Mazurkova N., Shishkina L., Novikova T., *Nitraria schoberi* L. hairy root culture as a source of compounds with antiviral activity against influenza virus subtypes A(H5N1) and A(H3N2). 3 Biotech. 2018; 8: 260.
13. Chan L.K., Lim P.S., Choo M.L., Boey P.L. Establishment of *Cyperus aromaticus* cell suspension cultures for the production of Juvenile hormone III. In vitro Cell Dev Biol Plant. 2010; 46: 8-12.
14. Rajan M., Feba K.S., Chandran V., Shahena S., Mathew L. Enhancement of rhamnetin production in *Vernonia anthelmintica* (L.) Willd. Cell suspension cultures by eliciting with methyl jasmonate and salicylic acid. Physiol Mol Biol Plants. 2020; doi: 10.1007/s12298-020-00829-8.
15. Dastmalchi T., Omidi M., Azizinezhad R., Rezazadeh S., Etminan A. Effects of methyl jasmonate and phloroglucinol on thebaine and sanguinarine production in cell suspension culture of Persian poppy (*Papaver bracteatum* Lindl.). Cellular and Molecular Biology. 2019; 65: 11-17.
16. Leroux M.M., Doumandji Z., Chezeau L., Hocquel R., Ferrari L., Joubert O., Rihn P., Rihn BH.Validation of an air/liquid interface device for TiO2 nanoparticle toxicity assessment on NR8383 cells: preliminary results. Cellular and Molecular Biology. 2020; 66:112-116.
17. Kolewe M.E., Gaurav V., Roberts S.C. Pharmaceutically active natural product synthesis and supply via plant cell culture technology. Mol Pharm. 2008; 5: 243-256.
18. Boonsnongcheep P., Korsangruang S., Soonthornchareonnon N., Chintapakorn Y., Saralamp P., Prathanturarug S. Growth and isoflavonoid accumulation of *Pueraria candollei* var. *Candollei* and *P. candollei* var. *mirifica* cell suspension cultures. Plant Cell Tiss Organ Cult. 2010; 101: 119-126.
19. Bonfill M., Mangas S., Moyano E., Cusido R.M., Palazo´n J. Production of centellosides and phytosterols in cell suspension cultures of *Centella asiatica*. Plant Cell Tiss Organ Cult. 2011; 104: 61-67.
20. Deepthi S., Satheeshkumar K. Enhanced camptothecin production induced by elicitors in the cell suspension cultures of *Ophiorrhiza mungos* Linn. Plant Cell Tiss Organ Cult. 2016; 124: 483-493.
21. Zhao J.L., Zhou L.G., Wu J.Y. Effects of biotic and abiotic elicitors on cell growth and tanshinone accumulation in *Salvia miltiorrhiza* cell cultures. Appl Microbiol Biot. 2010; 87: 137-144.
22. Koul A., Mallubhotla S. Elicitation and enhancement of bacoside production using suspension cultures of *Bacopa monnieri* (L.) Wettst. 3 Biotech. 2020; 10: 256.
23. Ramirez-Estrada K., Vidal-Limon H., Hidalgo D., Moyano E., Golenioswki M., Cusidó R.M., Palazon J. Elicitation, an Effective Strategy for the Biotechnological Production of Bioactive High-Added Value Compounds in Plant Cell Factories. Molecules. 2016; 21: 182.
24. Mendozaa D., Cuaspuda O., Ariasa J.P., Ruizc O., Ariasa M. Effect of salicylic acid and methyl jasmonate in the production of phenolic compounds in plant cell suspension cultures of *Thevetia peruviana*. Biotech. Reports. 2018; 19:1-9.
25. Gupta P., Sharma S., Saxena S. Effect of Salts (NaCl and Na2CO3) on Callus and Suspension Culture of *Stevia rebaudiana* for Steviol glycoside Production. Appl. Biochem. Biotechnol. 2014; doi: 10.1007/s12010-014-0736-2.

26. Deshmukh V., Deshpande J., Wani M. *In vitro* Enhancement of Solasodine Production by Using Biotic and Abiotic Elicitors in Different *Solanum* Spp. Annals of R.S.C.B. 2021; 25: 18195-18207.
27. Kheirabadi S., Zarinpanjeh N., Ebrahimi M.A., Bakhshi Khaniki G.R., Naser H.R. Effective in vitro seed germination and direct regeneration from cotyledonary leaf explants of *Nitraria schoberi.* Iranian Journal of Genetic and Plant breeding. 2020; 9: 10-16.
28. Murashige T., Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum. 1962; 15: 473-497.
29. Amirkavei Najafabadi B., Qavami N., Ebrahimi M.A., Ebrahimi P., Zarinpanjeh N. Enhancement of Taxol production by applying amino acid complex along with chitosan in suspension culture of *Taxus baccata* L. J. Med. Plants. 2020; 19(76): 99-109.
30. Ali B. Salicylic acid: An efficient elicitor of secondary metabolite production in plants. *Biocatalysis and Agricultural Biotechnology*. 2021; doi:10.1016/j.bcab.2020.101884.
31. Hemmati N., Cheniany M., Ganjeali A. Effect of plant growth regulators and explants on callus induction and study of antioxidant potentials and phenolic metabolites in *Salvia tebesana* Bunge. Botanica serbica. 2020; 44: 163-173.
32. Sharma G., Nautiyal A.R. Influence of explants type and plant growth regulators on *in vitro* multiple shoots regeneration of a laurel from Himalaya. Nature and Science. 2009; 7: 1-7.
33. Yang Y., Wei X., Shi R., Fan Q., An L.Salinity-induced Physiological Modification in the Callus from Halophyte *Nitraria tangutorum* Bobr. J Plant Growth Regul. 2010; 29:465-476.
34. Mei X., Shen Y. *Nitraria sibirica* cell suspension culture: establishment, characterization and application. J. For. Res. 2017. doi: 10.1007/s11676-017-0367-x.
35. Adil M., Ren X., Kang D., The Thi L., Jeong B.R. Effect of explant type and plant growth regulators on callus induction, growth and secondary metabolites production in *Cnidium officinale* Makino. Molecular Biology Reports. 2018; 45: 1919-1927.
36. Kang S.M., Min J.Y., Kim Y.D., Karigar C.S., Kim S.W., Goo GH., Choi M.S. Effect of biotic elicitors on the accumulation of bilobalide and ginkgolides in *Ginkgo biloba* cell cultures. J Biotechnol. 2009; 139: 84- 88.
37. Cai Z., Riedel H., Saw N.M.M.T., Mewis I., Reineke K., Knorr D., Smetanska I. Effects of elicitors and high hydrostatic pressure on secondary metabolism of *Vitis vinifera* suspension culture. Process Biochem. 2011; 46: 1411-1416.
38. Srivastava S., Srivastava A.K. Effect of elicitors and precursors on azadirachtin production in hairy root culture of *Azadirachta indica*. Appl Biochem Biotechnol. 2013; 172: 2286- 2297.
39. Moharramnejad S., Azam A.T., Panahandeh J., Dehghanian Z., Ashraf M. Effect of methyl jasmonate and salicylic acid on *in vitro* growth, stevioside production, and oxidative defense system in *Stevia rebaudiana*. Sugar Tech. 2019; 21: 1031-1038.
40. Khan T., Khan T., Hano C., Abbasi B.H. Effects of chitosan and salicylic acid on the production of pharmacologically attractive secondary metabolites in callus cultures of *Fagonia indica* Ind. Crops Prod. 2019; 129: 525-535.
41. Rezaei A., Ghanati F., Behmanesh M., Mokhtari-dizaji M. Ultrasound-potentiated salicylic acid-induced physiology effects and production of taxol in Hazelnut (*Corylus avellana* L.). Ultrasound Med. Biol. 2011; 37: 1938-1947.
42. Karalija E., Zeljković S.C., Parić A. Harvest time–related changes in biomass, phenolics and antioxidant potential in *Knautia sarajevensis* shoot cultures after elicitation with salicylic acid and yeast. In Vitro Cell. Dev. Biol. Plant. 2019; 56: 177-183.
43. Chung I.M., Rekha K., Rajakumar G., Thiruvengadam M. Jasmonic acid and saliculic acid enhanced phytochemical production and biological activities in cell suspension cultures of spine gourd (*Momordica dioica* Roxb). Acta Biol. Hung. 2017; 68: 88-100.
44. Golkar P., Taghizadeh M., Yousefian Z. The effects of chitosan and salicylic acid on elicitation of secondary metabolites and antioxidant activity of safflower under *in vitro* salinity stress. Plant Cell Tiss. Organ Cult. 2019; 137: 575-585.
45. Chavoushi M., Najafi F., Salimi A., Angaji S.A. Effect of salicylic acid and sodium nitroprusside on growth parameters, photosynthetic pigments and secondary metabolites of safflower under drought stress. Sci. Hortic. 2020; 259: 108823.
46.