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Original Scientific Paper

Effect of plant growth regulators and explants on callus induction and study of antioxidant potentials and phenolic metabolites in *Salvia tebesana* Bunge

Niloofar Hemmati, Monireh Cheniany* and Ali Ganjeali

Department of Biology, Faculty of Science, Ferdowsi University of Mashhad, Mashhad, Iran
* correspondence: cheniany@um.ac.ir / monireh.cheniany@gmail.com

ABSTRACT:
This study was undertaken to investigate the effect of different plant growth regulators (PGRs) on callus induction in *Salvia tebesana* explants grown *in vitro* and to evaluate the content of secondary phenolic compounds and their antioxidant potential. The explants (shoot apical meristem, leaf and petiole) were dissected from an 8-week-old plant of *S. tebesana* growing *in vitro* and cultured on MS media containing different concentrations of 2,4-D (0, 0.5, 1, 1.5 and 2 mg L^{-1}), NAA (0, 0.5 and 1 mg L^{-1}) and BAP (0, 0.5 and 1 mg L^{-1}), either alone or in a blend with each other. Morphological characteristics of the callus (consistency and colour), biomass increase based on fresh and dry weight and the percentage of induction were recorded after 56 days. Levels of total phenols, *ortho*-diphenols, phenolic acids, flavonoids, proanthocyanidins and flavonols of callus, as well as antioxidant activities, were evaluated *in vitro*. The maximum callus formation (100%) was obtained from shoot apical meristem on MS medium supplemented with 0.5 and 1.5 mg L^{-1} 2,4-D + 1 mg L^{-1} BAP and with 1 and 1.5 mg L^{-1} 2,4-D + 0.5 mg L^{-1} BAP, whereas the highest fresh (15.06 ± 0.88 g) and dry (0.33 ± 0.02 g) weights of call were observed in a medium containing1.5 mg L^{-1} 2,4-D + 0.5 mg L^{-1} NAA. It was noted that MS media augmented with combined PGRs had the highest accumulation of polyphenols, phenolic acids and flavonoid compounds, with levels of content varying in the following order: 2,4-D + BAP > NAA + BAP > 2,4-D + NAA. Strong linear correlations were established between total phenolic content of callus extracts and results of the DPPH and FRAP assays ($r^2 = 0.896$ and $r^2 = 0.946$, $p < 0.01$, respectively). The obtained results suggest that the described method could be utilised as a tool for large-scale production of medicinal metabolites of *S. tebesana* by tissue culture.

Keywords:
antioxidant activity, callus induction, reducing power, polyphenols, *Salvia tebesana*, secondary metabolites

Abbreviations:
BAP = 6-benzyl aminopurine, 2,4-D = 2,4-dichlorophenoxy acetic acid, NAA = 1-naphthalene acetic acid,. DPPH = 2,2-diphenyl-1-picrylhydrazyle, FRAP = ferric reducing antioxidant power.

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INTRODUCTION

Many plant species produce specific bioactive compounds that are important for treating human diseases. Cherdshewasart *et al.* (2007) wrote that "seasonal and environmental variations, soil type and plant age, along with difficulties in the propagation of certain species, may limit the easy access of many bioactive compounds". Apart from that, the rate of production of secondary metabolites in plants is slow and it takes a long time to produce them. Many studies therefore focus on the use of plant cell cultures to produce pharmaceutical metabolites, rapidly and economically (Naik 1998; Matkowski 2008).

In vitro plant cell and tissue culture represents a potential source for the production of biological metabolites from medicinal plants, also known as plant-derived medicinal compounds (PDMC). The short time needed for *in vitro* culture, lack of any seasonal dependence and the possibility of producing new compounds normally not found in the source plant are the benefits of this

technique (Loredo-Carrillo *et al.* 2013). To date, a variety of PDMCs have been produced in *in vitro* culture, especially callus culture, the list of such compounds including flavonoids (El-Shafey *et al.* 2019), flavonoids and stilbenes (Maneechai *et al.* 2012), phenolic acids (Modarres *et al.* 2013; Szopa & Ekiert 2014), phenolic acids and flavonoids (Castro *et al.* 2016) and iridoids (Cölgecen *et al.* 2018).

Among plant growth regulators (PGRs), auxins and cytokinins are the ones most often used to induce callus in various plant species. Successful effects depend on different combinations and varying concentrations of PGRs, as well as on the plant species and explant type (Sharma & Nautiyal 2009) 2,4-Dichlorophenoxyacetic acid (2,4-D) (Mostafiz & Wagiran 2018), 1-naphthaleneacetic acid (NAA) and 6-benzyl aminopurine (BAP) (Chen *et al.* 2019; El-Shafey *et al.* 2019) are widely used for callogenesis, either alone or in combination.

Salvia tebesana (whose Persian name is "Maryamgoli Tabasi") is a medium-sized shrub belonging to the family Lamiaceae. It is a rare medicinal plant, one that is endemic in limited geographical locations in Afghanistan, Pakistan and (particularly) Iran (Jamzad 2012; Eghbaliferiz *et al.* 2018). Although the seed production of this plant is high, it is not able to produce many plants due to harsh environmental conditions. For this reason, the number of viable plants in its natural ecosystem is on the decline. Therefore, the use of methods which ensure that the plant is less harvested from its environment and rapid breeding programs are necessary to protect this valuable and endangered species (Mir Hosseini *et al.* 2016). The special importance of this genus is due to its enormous traditional medical application. Although many studies have been done to determine medicinal compounds and antioxidant activities of other *Salvia* species (Tosun *et al.* 2009; Firuzi *et al.* 2010; Ożarowski *et al.* 2017), only a few reports exist on secondary metabolites from *S. tebesana* (Goldansaz *et al.* 2017; Eghbaliferiz *et al.* 2018). No available reports treat plant tissue, cell or organ culture of *S. tebesana*, and no pharmacological studies on the content of phenolic compounds in it have yet been performed. Therefore, the aim of the present study was to investigate for the first time the effect of PGRs (2,4-D, NAA and BAP) on callus induction from different explants of *S. tebesana*. Subsequently, secondary phenolic compounds were quantified and their biological potential was studied using antioxidant assays under *in vitro* conditions.

MATERIAL AND METHODS

Plant material and explant preparation. During the summer of 2018, seeds of *S. tebesana* were collected from fields at an elevation of about 1018 metres above sea level located in the Pikouh and Nissan districts of the Tabas region in South Khorasan Province (Iran). Washed seeds were sterilised with 70% (v/v) ethanol for 30 s and 20% (w/v) sodium hypochlorite solution for 5 min, then rinsed three times in sterilised distilled water. After primary germination on filter-paper moistened with water in Petri dishes, the seedlings were transferred into 3 L plastic pots filled with Hoagland solution (Li & Cheng 2015). As explants, shoot apical meristems, leaves and petioles were obtained from plants grown under hydroponic conditions. The explants were initially put in an ethanol solution (70% v/v; 30 s) and then thoroughly washed with sterilised distilled water several times.

Culture preparation and callus induction. All the samples were cultured on MS media (Murashige & Skoog 1962) enriched with different concentrations of 2,4-D (0.5, 1, 1.5 and 2 mg L^{-1}), NAA (0.5 and 1 mg L^{-1}) and BAP (0.5 and 1 mg L^{-1}), as well as with combinations of the mentioned PGRs. PGR-free MS media were used as controls. Sucrose (30 g L^{-1}) and agar (7 g L^{-1}) were added to the MS media. The pH of the medium was adjusted to 5.8 with 0.1 N HCl or 0.1 N NaOH prior to autoclaving (at a temperature of 121°C and pressure of 1.06 kg cm^{-2} for 15 min). Each treatment included three replications, and each replicate included five explants. The cultures were maintained for two weeks in a dark growth room at 25 ± 2°C and then transferred to conditions of a16/8 h light/dark cycle, the light being provided by cold fluorescent lamps. Sub-culturing was carried out after 4 weeks on MS media with the same concentrations of PGRs. At the end of the eighth week of cultivation, callus parameters [colour and texture of the callus, callus induction frequency (CIF, Equation 1) and fresh and dry weight (FW, DW)] were analysed.

$$\text{CIF} = (\text{number of explants with calli} / \text{number of incubated explants}) \times 100 \quad (1)$$

All calli of leaf and shoot apical meristem with greater success in callogenesis were then incubated at 45°C for two days and stored for subsequent biochemical analysis.

Extraction of phenolic compounds. A weighed portion of callus powder (100 mg) was mixed with 0.73 mL of methanol (60%, v/v) and extracted in an ultrasonic bath (Parsonic 2600 s, Iran) for 10 min at 48°C. After filtration through a piece of filter-paper, the final volume of solutions was adjusted to 1.66 mL with methanol (60%, v/v) (Espada-Bellido *et al.* 2017). The obtained solutions were stored as "methanolic extracts" at 4°C for all biochemical assays.

Determination of total phenolic content. Total phenolic content was determined by the Folin–Ciocalteu method (Singleton *et al.* 1999). The reaction mixture was prepared by mixing 100 μL of "methanolic extract", 1 mL of 10% Folin-Ciocalteu's reagent dissolved in water and 1

mL of 20% sodium bicarbonate (w/v). The samples were thereafter left at 25±2°C for 45 min. Absorbance at 725 nm was determined using a spectrophotometer. Based on the measured absorbance and standards, the content of phenolics in extracts was expressed in terms of gallic acid equivalents (mg GA g^{-1} of extract DW).

Determination of total *ortho*-diphenol content. A measured volume of "methanolic extract" (100 µL) was mixed with 2 mL of 50% methanol (v/v) and 0.5 mL of 5% sodium bicarbonate (w/v) and the samples were then incubated at room temperature (25 ± 2°C) in the dark for15 min. Based on absorbance (at λ_{max} 370 nm), total callus content of *ortho*-diphenols was calculated as gallic acid equivalents (mg of GA g^{-1} of extract DW) (Carrasco-Pancorbo *et al.* 2005).

Determination of total phenolic acid content. Using test tube, 280 µL of "methanolic extract" was reacted with 280 µL of 0.1 N HCl, 280 µL of 1M NaOH, 280 µL of Arnow's reagent and 1.44 mL of double-distilled water. The mixtures were incubated at room temperature (25 ±2°C) in the dark for 30 min. Absorbance at λ_{max} 490 nm was determined using a spectrophotometer and expressed in terms of caffeic acid equivalents (mg CA g^{-1} of extract DW) (Matkowski *et al.* 2008).

Determination of total flavonoid content. Total flavonoid content was determined based on the method of Pourmorad *et al.* (2006). A measured volume of "methanolic extract" (200 µL) was added to 600 µL of 60% methanol (v/v), 1.12 mL of double-distilled water, 40 µL of 10% dilute aqueous aluminum trichloride (w/v) and 40 µL of 1 M potassium acetate. The samples were incubated for 30 min at room temperature (25±2°C). Absorbance was measured at 415 nm. The content of flavonoids in extracts was expressed in terms of quercetin equivalents (mg QE g^{-1} of extract DW).

Determination of total proanthocyanidin content. In a test tube, 250 mL of "methanolic extract" was made up to volume of 1 mL with pure methanol. Thereafter, 1 mL of 1% vanillin in methanol (w/v) was added and mixed thoroughly with a vortex mixer (VM-10, Germany) for about 30 s. A measured volume (2.5 mL) of sulphuric acid (9N) was added to each tube and heated in a water bath (Kavos Mega, IRAN) at 38°C for 15 min, then cooled to room temperature. Absorbance at 500 nm was determined using a spectrophotometer and total proanthocyanidin content of the extract was expressed in terms of catechin equivalents (mg C g^{-1} of extract DW) (Sun *et al.* 1998).

Determination of total flavonol content. Total flavonol content was expressed as quercetin equivalents (Amoussa *et al.* 2015). A measured volume (400 µL) of "methanolic extract" was mixed with 400 µL of 10% aluminium trichloride (w/v), 400 µL of 1 M sodium acetate and 1.8 mL of distilled water. After incubation at room temperature (25 ± 2°C) in the dark for 30 min, absorbance at 425 nm was determined using a spectrophotometer and final content was expressed as mg QE g^{-1} of extract DW.

***In vitro* antioxidant activities.** Samples with maximum values of both callogenesis and content of phenolic compounds were selected for determination of antioxidant activities.

DPPH radical-scavenging assay. The free radical-scavenging potential of calli was determined as previously described by Cheniany *et al.* (2013). Briefly, 0.1 mL of extract sample was dissolved in 3 mL of methanol containing 1 mL of 100 µM DPPH solution in methanol. After 30 min of incubation in the dark, absorbance was measured at 517 nm. A negative control was set using methanol with DPPH (1 mL). The results were expressed as the percentage of DPPH radical capture calculated by the equation (2):

$$\% \text{ scavenging effect} = [(A_{DPPH} - A_S)/A_{DPPH}] \times 100 \qquad (2)$$

Here, A_S is mixture absorbance with the sample and A_{DPPH} is mixture absorbance of the DPPH solution (without the sample). The results were expressed as equivalents of ascorbic acid antioxidant capacity (mg ascorbic acid 100 g^{-1} of DW).

FRAP scavenging assay. A FRAP assay was employed as previously reported by Benzie & Strain (1996) with minor modifications. Briefly, "methanolic extract" (50 µL) from each treatment was mixed with a FRAP solution (1.5 mL), composed of 20 mM ferric chloride hexahydrate, 300 mM acetate buffer and 2,4,6-trispyridyl triazine (10 mM) in ratio of 1:10:1 (v:v:v), respectively. The reaction mixture was incubated for approximately 15 min at 25 ± 2°C and absorbance was taken at 593 nm, ferric sulphate (II) being used as a standard.

Statistical analysis. The results were expressed as means ± SD of three independent analyses. Statistical analysis was performed using SPSS 16 software, and Duncan's multiple range test was used to compare mean data. Differences were considered significant at $p \leq 0.001$ and $p \leq 0.01$.

RESULTS AND DISCUSSION

Accumulation of callus biomass (Influence of PGRs and explant type). Callus formation in the present study was significantly influenced by growth regulators and the explant type. Initiation of callus on the cut surface of all three types of explants was observed within 12-14 days

after placement on media supplemented with 2,4-D, NAA and BAP, but maximum callus growth was attained at the end of the eighth week (Fig. 1). However, the employed explant types differed with respect to success of callogenesis in the following order: shoot apical meristem > leaf > petiole. Explants cultured on a PGR-free basal medium (control) did not produce any callus at all. According to the available literature, there are two types of callus texture, compact and friable (Sharma & Nautiyal 2009). All callus formed in the present study was friable and yellow or light-brown in colour. In the case of shoot apical meristem explants, the highest callus frequency (100%) was observed on media containing NAA (0.5 and 1 mg L^{-1}) alone or in a blend with BAP (0.5 mg L^{-1}) and ones containing the combination of 2,4-D (0.5, 1 and 1.5 mg L^{-1}) with BAP (0.5 and 1 mg L^{-1}). On the other hand, maximum biomass accumulation (fresh and dry weight) was obtained on media augmented with 2,4-D (0.5 and 1.5 mg L^{-1}) and NAA (0.5 mg L^{-1}) (Table 1). The best result of callus induction (100%) from leaf explants was recorded on media containing the combination of 1.5 mg L^{-1} 2,4-D + 1 mg L^{-1} BAP, followed by media containing 0.5 mg L^{-1} NAA + 0.5 mg L^{-1} BAP. Murashige-Skoogmedium supplemented with 1.5 mg L^{-1} 2,4-D and 0.5 mg L^{-1} NAA showed the highest fresh weight of these calli (Table 1). For petiole explants, a callus induction frequency of up to 100% was observed only on MS medium containing the combination of 0.5 mg L^{-1} NAA + 1 mg L^{-1} BAP. On other media, callus induction from petiole explants was lower compared to induction from apical meristem and leaf explants on the same media media (Table 1).

Overall, the highest callus frequency (on media containing the combination of 2,4-D and BAP) did not coincide with the maximum increase of callus biomass (on media containing the combination of 2,4-D and NAA). Effective influence of the combination of 2,4-D and BAP in enhancing the rate of callus production was also recorded in other studies (Cimino *et al.* 2006; Wang & Bao 2007; El-Shafey *et al.* 2019). Although many studies confirmed the essential role of PGRs for callus induction and proliferation from plant explants (Ahmad *et al.* 2013; Jafari *et al.* 2016; Eari *et al.* 2017), Abde Elaleem *et al.* (2009) emphasised that the combination of different PGRs has a greater effect on callus induction and growth maintenance than in the case of their use alone. It has been postulated that auxins and cytokinins control cell division, proliferation and growth of callus. However, the efficiency of interaction during each stage depends on the different needs for auxins and cytokinins, on the level of these hormones as a result of their uptake from extracellular sources and on their metabolism and endogenous interaction (El-Shafey *et al.* 2019), as well as on the type of plant tissue and in the final analysis on the plant species in question (Coenen & Lomax 1997).

In the present study, the combination of 2,4-D with BAP and that of NAA with BAP in various concentrations had a better effect on callus induction, whereas the combination of 2,4-D with a NAA had a more positive effect on callus biomass accumulation (Table 1). Our results confirm that the optimal concentration and combination of PGRs must be specified for each stage of callogenesis, as well as for each species (Ikeuchi *et al.* 2013). The fact that the explant type is a major determining factor for successful callus stimulation is also confirmed in this study on *S. tebesana*. Research on *S. officinalis* showed that leaf explants were better than stem explants for callus induction (Jafari *et al.* 2013). The influence of different types of explants (root, cotyledon, leaf and shoot apical meristem) of *S. nemorosa* on callogenesis was previously investigated by Ourmazd & Chalabian (2006). They obtained a higher percentage of induction from callus differentiated from shoot apical meristem and leaves on MS media supplemented with IBA, NAA and isopentenyl adenine (2ip). Modarres *et al.* (2013) indicated that leaf explants of *S. leriifolia* had the highest callus induction on MS media supplemented with BAP and NAA.

Polyphenol content of calli. Regardless of PGR type, all calli had significant amounts of phenols, *ortho*-diphenols, phenolic acids and flavonoids produced on media with combinations of growth regulators ($p \leq 0.001$). The range of total phenol content in calli was from 394.07 mg L^{-1} to 793.23 mg L^{-1}, and the maximum phenolic accumulation was recorded in callus obtained from shoot apical meristem explants on MS media supplemented with 0.5 mg L^{-1} 2,4-D and 1 mg L^{-1} BAP compared to other PGR treatments (Fig. 2A). Total *ortho*-diphenol and phenolic acid content ranged from 76.26 to 172.48 mg L^{-1} and from 155.84 to 197.94 mg L^{-1}, respectively. Among all the tested extracts, the highest *ortho*-diphenol value was recorded in leaf explants subjected to treatment with 0.5 mg L^{-1} NAA and 0.5 mg L^{-1} BAP, while the maximum amount of phenolic acids was found in shoot apical meristem explants on MS medium augmented with 1.5 mg L^{-1} 2,4-D and 0.5 mg L^{-1} (Figs. 2B & C). The total flavonoid content ranged from 50.55 to 365.55 mg L^{-1}. The results showed that leaf explants cultured on MS media with 0.5 mg L^{-1} 2,4-D + 0.5 mg L^{-1} BAP had the highest level of total flavonoids; conversely, shoot apical meristem explants treated with 0.5 mg L^{-1} NAA had conspicupusly the lowest ($P < 0.001$) (Fig. 2D). Expressed as catechin equivalents, the total proanthocyanidin content of extracts varied from 196.86 ± 0.77 mg CAT 100 g^{-1} of DW for shoot apical meristem explants to 45.06 ± 0.52 mg CAT 100 g^{-1} of DW for the same explants (Fig. 2E). Assessment of flavonol content revealed maximum accumulation in shoot apical meristem explants on MS media supplemented with 1.5 mg L^{-1} 2,4-D + 0.5 mg L^{-1} NAA (Fig. 2F).

Combinations of PGRs had a better effect on phenolic compound production in *S. tebesana* calli in comparison with their use alone, which is in agreement with the data

Table 1. Callus induction and morphological traits of callus (colour, texture, fresh and dry weight) originated from "shoot apical meristem explants", "leaf explants" and "petiole explants" of *S. tebesana* at the end of the eighth week after cultivation. Means in the column followed by the same letter are not significantly different at ($p \leq 0.001$) by the Duncan test. ** Standard deviation values for these data were omitted, since they are less than 0.009. Legends - : no callus induction; SAM = shoot apical meristem, L = leaf and P = petiole.

Treatment Number	PGR Combinations	Callus Induction Frequency (%)			Callus Fresh Weight (g)			Callus Dry Weight (g)		
		SAM	L	P	SAM	L	P	SAM	L	P
Control	No PGR	-	-	-	-	-	-	-	-	-
1	0.5 mg L^{-1}**2,4-D**	66 ^{b}	-	-	6.58 ± 0.62 ^{bc}	-	-	0.17 ± 0.01 $^{b-e}$	-	-
2	1 mg L^{-1}**2,4-D**	66 ^{b}	-	-	5.88 ± 0.69 ^{bc}	-	-	0.19 ± 0.01 b-e	-	-
3	1.5 mg L^{-1}**2,4-D**	80 ^{ab}	-	27 ^{def}	7.92 ± 0.99 ^{bc}	-	-	0.26 ± 0.02 ^{abc}	-	0.06^{**abc}
4	2 mg L^{-1}**2,4-D**	73 ^{ab}	-	13 ^{ef}	4.69 ± 0.11 ^{cd}	-	-	0.21 ± 0.01 $^{b-e}$	-	0.07^{**abc}
5	0.5 mg L^{-1} **NAA**	100 ^{a}	67 ^{abc}	87 ^{ab}	6.09 ± 0.08 ^{bc}	0.82 ± 0.03 ^{cd}	0.82 ± 0.03 ^{cd}	0.25 ± 0.01 ^{abc}	0.06 ^{**cde}	0.11^{**abc}
6	1 mg L^{-1} **NAA**	100 ^{a}	47 ^{cde}	80 ^{abc}	6.90 ± 0.09 ^{bc}	0.77 ± 0.02 ^{cd}	0.77 ± 0.02 ^{cd}	0.26 ± 0.02 ^{abc}	0.07^{**bc}	0.09^{**abc}
7	0.5 mg L^{-1}**BAP**	-	-	-	-	-	-	-	-	-
8	1 mg L^{-1} **BAP**	-	-	-	-	-	-	-	-	-
9	0.5 mg L^{-1} **2,4-D** + 0.5 mg L^{-1} **NAA**	73 ^{ab}	93 ^{ab}	33 $^{c-f}$	5.80 ± 1.08 ^{bc}	3.71 ± 0.89 ^{ab}	3.71 ± 0.89 ^{ab}	0.16 ± 0.02 $^{b-f}$	0.09 ^{**bc}	0.019^{**bc}
10	0.5 mg L^{-1}**2,4-D** + 1 mg L^{-1}**NAA**	93 ^{a}	20 ^{de}	13 ^{ef}	7.12 ± 0.40 ^{bc}	1.19 ± 0.10 ^{bc}	1.19 ± 0.10 ^{bc}	0.21 ± 0.01 $^{b-e}$	0.02 ^{**de}	0.05^{**abc}
11	1 mg L^{-1} **2,4-D** + 0.5 mg L^{-1} **NAA**	53 ^{b}	80 ^{ab}	67 $^{a-d}$	8.97 ± 1.25 ^{b}	1.27 ± 0.11 ^{bc}	1.27 ± 0.11 ^{bc}	0.16 ± 0.01 $^{b-f}$	0.06 ^{**cde}	0.06^{**abc}
12	1 mg L^{-1}**2,4-D** + 1 mg L^{-1} **NAA**	46 ^{b}	-	7 ^{ef}	4.12 ± 1.01 ^{cd}	-	-	0.20 ± 0.02 $^{b-e}$	-	0.004^{**c}
13	1.5 mg L^{-1} **2,4-D** + 0.5 mg L^{-1} **NAA**	93 ^{a}	73 ^{ab}	7 ^{ef}	15.06 ± 0.88 ^{a}	4.85 ± 0.71 ^{a}	4.85 ± 0.71 ^{a}	0.33 ± 0.02 ^{a}	0.13 ^{**abc}	0.005^{**c}
14	1.5 Mg L^{-1}**2,4-D** + 1 mg L^{-1} **NAA**	53 ^{b}	53 ^{bc}	13 ^{ef}	1.37 ± 0.77 ^{de}	0.38 ± 0.05 ^{de}	0.38 ± 0.05 ^{de}	0.04 ± 0.01 ^{fg}	0.02 ^{**de}	0.002^{**c}
15	0.5 mg L^{-1} **2,4-D** + 0.5 Mg L^{-1} **BAP**	93 ^{a}	93 ^{ab}	33 $^{c-f}$	4.87 ± 0.76 ^{bc}	2.28 ± 0.32 ^{bc}	2.28 ± 0.32 ^{bc}	0.23 ± 0.02 ^{abc}	0.14 ^{**abc}	0.05^{**abc}
16	0.5 mg L^{-1}**2,4-D** + 1 mg L^{-1} **BAP**	100 ^{a}	100 ^{a}	7 ^{ef}	2.23 ± 0.34 ^{cde}	1.55 ± 0.12 ^{bc}	1.55 ± 0.12 ^{bc}	0.14 ± 0.02 $^{b-f}$	0.10 ± 0.01 ^{bc}	0.03^{**abc}
17	1 mg L^{-1} **2,4-D** + 0.5 mg L^{-1} **BAP**	100 ^{a}	86 ^{ab}	7 ^{ef}	2.74± 0.13 ^{cd}	1.39 ± 0.10 ^{bc}	1.39 ± 0.10 ^{bc}	0.15 ± 0.01 $^{b-f}$	0.06 ^{**cde}	0.016^{**bc}
18	1 mg L^{-1} **2,4-D** + 1 mg L^{-1} **BAP**	66 ^{b}	73 ^{ab}	40 $^{b-f}$	1.62 ± 0.81 ^{de}	0.59 ± 0.06 ^{de}	0.59 ± 0.06 ^{de}	0.09 ^{**efg}	0.05 ^{**cde}	0.02^{**bc}
19	1.5 mg L^{-1} **2,4-D** + 0.5 mg L^{-1} **BAP**	100 ^{a}	60 ^{bc}	87 ^{ab}	3.90 ± 0.53 ^{cd}	0.99 ± 0.09 ^{bc}	0.99 ± 0.09 ^{bc}	0.19 ± 0.01 $^{b-e}$	0.06 ^{**cde}	0.048^{**abc}
20	1.5 mg L^{-1} **2,4-D** + 1 mg L^{-1} **BAP**	100 ^{a}	100 ^{a}	73 a-d	2.13 ± 0.37 ^{cde}	1.27 ± 0.21 ^{bc}	1.27 ± 0.21 ^{bc}	0.12 ± 0.02 ^{def}	0.09 ^{**bc}	0.04** abc
21	0.5 mg L^{-1} **NAA** + 0.5 mg L^{-1} **BAP**	100 ^{a}	100 ^{a}	60 $^{a-e}$	5.61 ± 0.44 ^{bc}	1.96 ± 0.41 ^{bc}	1.96 ± 0.41 ^{bc}	0.24 ± 0.01 ^{abc}	0.06 ^{**cde}	0.07^{**abc}
22	0.5 mg L^{-1} **NAA** + 1 mg L^{-1} **BAP**	66 ^{b}	80 ^{ab}	100 ^{a}	2.35 ± 0.19 ^{cde}	0.83 ± 0.08 ^{cd}	0.83 ± 0.08 ^{cd}	0.13 ± 0.02 $^{c-f}$	0.16^{**a}	0.11^{**ab}
23	1 mg L^{-1} **NAA** + 0.5 mg L^{-1}**BAP**	93 ^{a}	67 ^{ab}	67 $^{a-d}$	4.48 ± 0.89 ^{cd}	0.84 ± 0.07 ^{cd}	0.84 ± 0.07 ^{cd}	0.25 ± 0.02 ^{abc}	0.09 ** bc	0.13^{**a}
24	1 mg L^{-1}**NAA** +1 mg L^{-1} **BAP**	60 ^{b}	53 ^{bc}	60 $^{a-e}$	1.34 ± 0.08 ^{de}	0.58 ± 0.05 ^{de}	0.58 ± 0.05 ^{de}	0.09 ± 0.02 ^{efg}	0.08 ** bc	0.06^{**ab}

Table 2. Antioxidant activities of callus extracts as determined by DPPH and FRAP scavenging assay. mg Vit C 100 g^{-1} of DW: effective concentration at which the antioxidant activity is equal to ascorbic acid activity. Legends: SAM = shoot apical meristem, L= leaf.

Explant Type	Treatment Number	PGR Combinations	FRAP assay µg Fe^{2+} 100 g^{-1} DW	DPPH assay mg Vit C 100 g^{-1} DW
SAM	15	0.5 mg L^{-1} **2,4-D** + 0.5 Mg L^{-1} **BAP**	90.2 ± 0.97^{e}	14.1 ± 0.32^{d}
SAM	16	0.5 mg L^{-1}**2,4-D** + 1 mg L^{-1} **BAP**	132.3 ± 1.01^{a}	36.2 ± 0.35^{a}
SAM	17	1 mg L^{-1} **2,4-D** + 0.5 mg L^{-1} **BAP**	114.1 ± 1.55^{c}	22.2 ± 0.28^{c}
SAM	19	1.5 mg L^{-1}**2,4-D**+ 0.5 mg L^{-1} **BAP**	98.4 ± 1.61^{e}	14.6 ± 0.41^{d}
SAM	20	1.5 mg L^{-1} **2,4-D** + 1 mg L^{-1} **BAP**	124.3 ± 1.32^{b}	30.3 ± 0.77^{b}
SAM	21	0.5 mg L^{-1}**NAA**+ 0.5 mg L^{-1} **BAP**	115.5 ± 0.92^{c}	20.5 ± 0.56^{cd}
L	15	0.5 mg L^{-1} **2,4-D** + 0.5 Mg L^{-1} **BAP**	123.2 ± 1.02^{b}	29.1 ± 0.41^{b}
L	16	0.5 mg L^{-1}**2,4-D** + 1 mg L^{-1} **BAP**	99.2 ± 1.32^{e}	16.5 ± 0.26^{d}
L	20	1.5 mg L^{-1} **2,4-D** + 1 mg L^{-1} **BAP**	108.3 ± 0.86^{d}	18.3 ± 0.51^{cd}
L	21	0.5 mg L^{-1}**NAA**+ 0.5 mg L^{-1} **BAP**	116.1 ± 1.02^{c}	23.8 ± 0.44^{c}

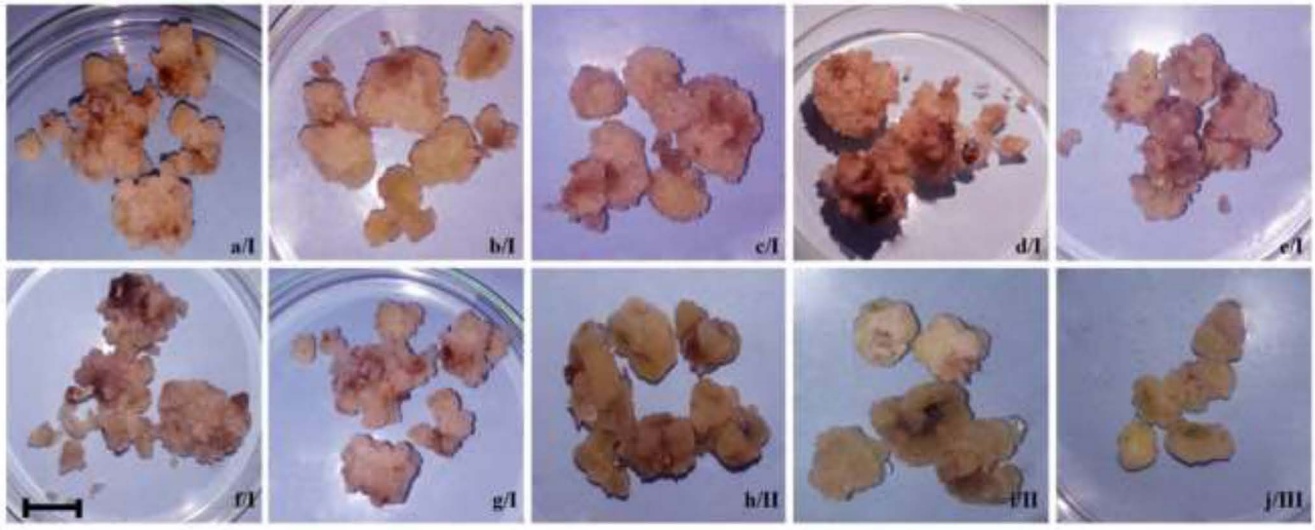

Fig. 1. Appearance of calli from three different explants [shoot apical meristem (I), leaf (II) and petiole (III)] of *Salvia tebesana* with 100% callus induction on medium supplemented with: 0.5 mg L^{-1} NAA (a), 1 mg L^{-1} NAA (b), 0.5 mg L^{-1} 2,4-D + 1 mg L^{-1} BAP (c), 1 mg L^{-1} 2,4-D + 0.5 mg L^{-1} BAP (d), 1.5 mg L^{-1} 2,4-D + 0.5 mg L^{-1} BAP (e), 1.5 mg L^{-1} 2,4-D + 1 mg L^{-1} BAP (f), 0.5 mg L^{-1} 2,4-D + 0.5 mg L^{-1} NAA (g), 0.5 mg L^{-1} 2,4-D + 1 mg L^{-1} BAP (h), 1.5 mg L^{-1} 2,4-D + 1 mg L^{-1} BAP (i), 0.5 mg L^{-1} NAA + 1 mg L^{-1} BAP (j) at the end of the eighth week; Bar = 1 cm.

of Shilpashree & Ravishankar (2009) and Karalija & Paric (2011). Palacio *et al.* (2012) reported that it is possible to stimulate *in vitro* production of phenolic metabolites in medicinal plants by varying the culture conditions, including the type and concentration of PGRs. The mechanism behind PGR interaction with callus cells and phenolic biosynthesis would appear to be based on upregulation (Ruiz & Romero 2001) and higher activity of phenylalanine ammonia lyase (Boudet 2007) influenced by PGRs.

Antioxidant potential of calli. The antioxidant potential of callus extracts was screened by two complementary test systems, namely the DPPH and FRAP assays. As can

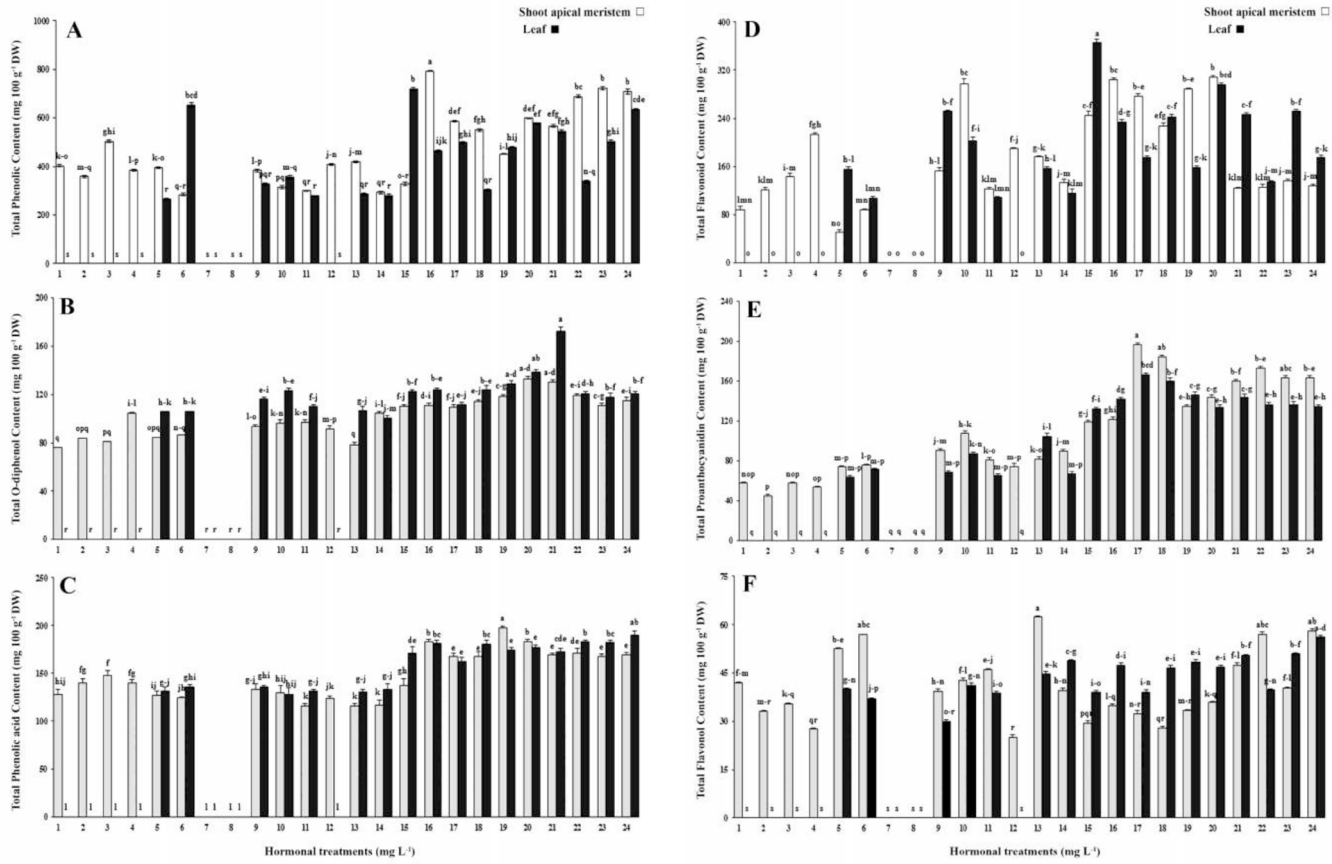

Fig. 2. Total phenolics (A), *ortho*-diphenols (B), phenolic acids (C), flavonoids (D), proanthocyanidins (E) and flavonols (F) in callus of *S. tebesana* cultured on MS media supplemented with different concentrations of PGRs. Legends: **(1)** 0.5 mg L^{-1} 2,4-D; **(2)** 1 mg L^{-1} 2,4-D; **(3)** 1.5 mg L^{-1} 2,4-D; **(4)** 2 mg L^{-1} 2,4-D; **(5)** 0.5 mg L^{-1} NAA; **(6)** 1 mg L^{-1} NAA; **(7)** 0.5 mg L^{-1} BAP; **(8)** 1 mg L^{-1}BAP; **(9)** 0.5 mg L^{-1} 2,4-D+ 0.5 mg L^{-1} NAA; **(10)** 0.5 mg L^{-1} 2,4-D + 1 mg L^{-1} NAA, **(11)** 1 mg L^{-1} 2,4-D + 0.5 mg L^{-1} NAA; **(12)** 1 mg L^{-1} 2,4-D+ 1 mg L^{-1} NAA; **(13)** 1.5 mg L^{-1} 2,4-D + 0.5 mg L^{-1} NAA; **(14)** 1.5 mg L^{-1} 2,4-D + 1 mg L^{-1} NAA; **(15)** 0.5 mg L^{-1} 2,4-D + 0.5 mg L^{-1} BAP; **(16)** 0.5 mg L^{-1} 2,4-D + 1 mg L^{-1} BAP; **(17)** 1 mg L^{-1} 2,4-D + 0.5 mg L^{-1} BAP; **(18)** 1 mg L^{-1} 2,4-D + 1 mg L^{-1} BAP; **(19)** 1.5 mg L^{-1} 2,4-D + 0.5 mg L^{-1} BAP; **(20)** 1.5 mg L^{-1} 2,4-D + 1 mg L^{-1} BAP; **(21)** 0.5 mg L^{-1} NAA + 0.5 mg L^{-1} BAP; **(22)** 0.5 mg L^{-1} NAA + 1 mg L^{-1} BAP; **(23)** 1 mg L^{-1} NAA + 0.5 mg L^{-1} BAP; **(24)** 1 mg L^{-1} NAA+1 mg L^{-1} BAP.

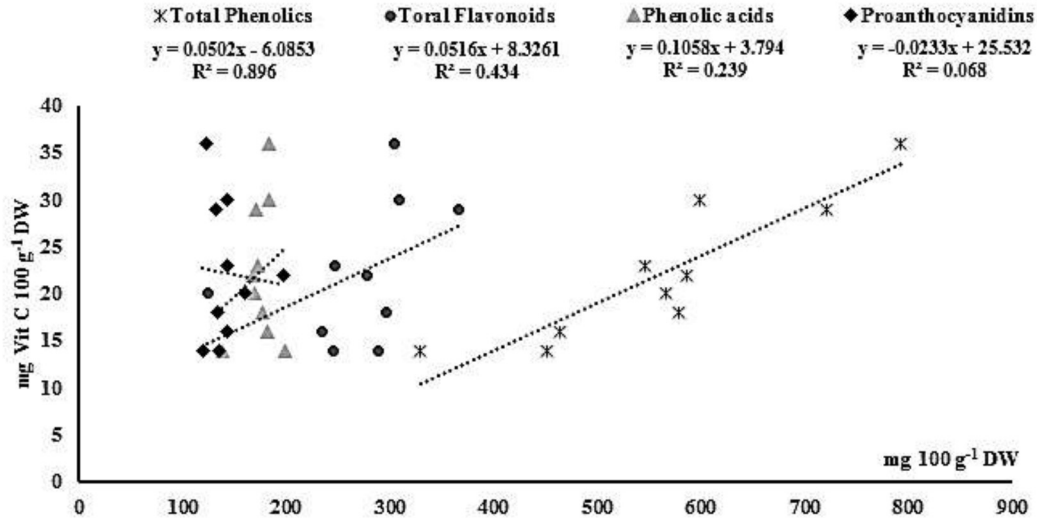

Fig. 3. Coefficient of correlation between DPPH-scavenging activity and content of total phenolic compounds, total flavonoids, phenolic acids and proanthocyanidins ($p < 0.01$).

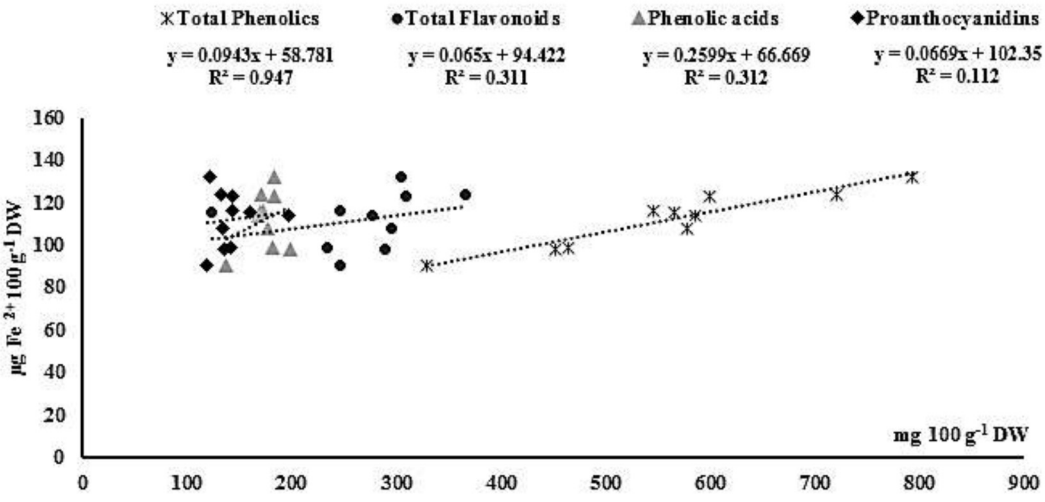

Fig. 4. Coefficient of correlation between the FRAP assay and content of total phenolic compounds, total flavonoids, phenolic acids and proanthocyanidins ($p < 0.01$).

be seen in Table 2, the highest antioxidant activity was in callus extracts derived from shoot apical meristems on MS medium with 0.5 mg L^{-1} 2,4-D and 1 mg L^{-1} BAP, followed by ones derived from shoot apical meristems on MS medium with 1.5 mg L^{-1} 2,4-D and 1 mg L^{-1} BAP. Both mechanisms were in evidence here, suggesting the presence of antioxidants acting through hydrogen atom transfer (HAT), as well as ones acting through electron transfer (ET) (Table 2) (Ullah *et al.* 2019).

Strong linear correlations were established between total phenolic compound content of callus extracts and results of the DPPH and FRAP assays ($r^2 = 0.896$; $r^2 = 0.946$, $p < 0.01$) (Figs. 3 & 4). Total content of flavonoid compounds was also correlated with results of the employed antioxidant assays, but levels of other phenolics were weakly correlated with both assays (Figs. 3 & 4). The obtained results indicate that the antioxidant activities of *S. tebesana* calli are mainly associated with their content of total phenolic and flavonoid compounds.

CONCLUSION

To our knowledge, this is the first study providing data on the effect of growth regulators on *in vitro* callogenesis and phenolic metabolite production of *S. tebesana*. The present investigation has shown that callogenesis in *S. tebesana* depends on the explant type as well as PGR treatments. The best callus induction in terms of percentage, FW and DW was observed in shoot apical meristem explants cultured on MS media augmented with the combination of 2,4-D (0.5, 1 and 1.5 mg L^{-1}) and BAP (0.5 and 1 mg L^{-1}) and explants cultured on MS media in the presence of 2,4-D (1.5 mg L^{-1}) and NAA (0.5 mg L^{-1}). Similarly, biochemical analysis showed that PGR treatments had a positive effect on the content of individual phenolic compounds: increased accumulation of polyphenols, phenolic acids and flavonoid compounds with varying degrees of content was seen after PGR treatments, whose effectiveness decreased in the following order: 2,4-D + BAP > NAA + BAP > 2,4-D + NAA. Due to their considerable ability to scavenge free radicals, it issuggested that the calli of *S. tebesana* can be regarded as an important source of natural antioxidants.

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REZIME

Botanica SERBICA

Uticaj regulatora rasta biljaka i eksplantata na indukciju kalusa i ispitivanja antioksidativnih potencijala i fenolnih metabolita kod *Salvia tebesana* Bunge

Niloofar Hemmati, Monireh Cheniany i Ali Ganjeali

Cilj ove studije je da istraži efekat različitih regulatora rasta (PGRs) na indukciju kalusa kod eksplantata vrste *Salvia tebesana* gajenih *in vitro*, kao i da utvrdi sadržaj sekundarnih fenolnih jedinjenja i njihov antioksiodativni potencijal. Eksplanti (apikalni meristem, list i peteljka) secirani su iz biljke *S. tebesana* stare 8 nedelja i uzgajani na MS medijumu koji sadrži različitu koncentraciju 2,4-D (0, 0,5, 1, 1,5 i 2 mg L-1), NAA (0, 0,5 i 1 mg L-1) i BAP (0, 0,5 i 1 mg L-1) samostalno ili pomešani jedni sa drugima. Nakon 56 dana ocenjene su morfološke karakteristike kalusa (konzistencija i boja), zatim rast biomase na osnovu sveže i suve težine, procenat indukcije. Sadržaj ukupnih fenola, orto-difenola, fenolnih kiselina, flavonoida, proantocijanidina i flavonola kalusa, kao i antioksidativnih aktivnosti, procenjen je *in vitro*. Maksimalni procenat stvaranja kalusa (100%) dobijen je iz apikalnog meristema na MS medijumu dopunjenom sa "0,5 i 1,5 mg L-1 2,4-D + 1 mg L-1 BAP i 1 i 1,5 mg L-1 2,4 -D + 0,5 mg L-1 BAP ", dok su najveće sveže (15,06 ± 0,88 g) i suve (0,33 ± 0,02 g) težine kalusa primećene u medijumu koji sadrži 1,5 mg L-1 2,4-D + 0,5 mg L -1 NAA. Primećeno je da su MS mediji sa povećanim PGR-om imali najveću akumulaciju polifenola, fenolnih kiselina i flavonoidnih jedinjenja sa različitim stepenom sadržaja sledećim redosledom: 2,4-D + BAP> NAA + BAP> 2,4-D + NAA. Utvrđena je snažna linearna korelacija (r2 = 0,896; r2 = 0,946, p <0,01, respektivno) između ukupnih fenola ekstrakta kalusa, ispiranja DPPH-a i FRAP eseja. Dobijeni rezultati sugerišu da se ova metoda može koristiti kao sredstvo za masovnu proizvodnju lekovitih metabolita *S. tebesana* kulturom tkiva.

Ključne reči: antioksidatna aktivnost, indukcija kalusa, smanjenje snage, polifenoli, *Salvia tebesana,* sekundarni metaboliti