



Effect of cytokinins on shoots proliferation and rosmarinic and salvianolic acid B production in shoot culture of *Dracocephalum forrestii* W. W. Smith

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Abstract

The current study estimates the effect of different cytokinins on shoot proliferation and biosynthesis of caffeic acid derivatives in *Dracocephalum forrestii* *in vitro* culture. The shoots were grown on Murashige and Skoog (MS) agar medium with 1 µM indole-3-acetic acid (IAA) and different content of 6-benzylaminopurine (BAP), zeatin, kinetin (1, 2, 4, 8, 18 µM) or thidiazuron (TDZ) (0.1, 0.2, 0.5, 1, 2 µM). The highest multiplication rate (about seven shoots and/or buds per explant) was obtained after 4 weeks of culture on MS medium with 1 µM IAA and 8 or 16 µM BAP. Optimal biomass of plant material was also received on the same media. The identity of the compounds present in the hydromethanolic extracts from *D. forrestii* shoots grown on cytokinin-supplemented media was confirmed using UPLC–PDA–ESI–MS method. The analysis revealed the presence of nine metabolites recognized as caffeic acid derivatives. The content of the predominant phenolic acids in the extracts, i.e. rosmarinic acid (RA) and salvianolic acid B (SAB), was determined with UHPLC. The highest yield of RA was found in shoots cultivated in the medium containing 1 µM IAA and 2 µM BAP (18.7 mg/g DW). The highest level of SAB (5.3–5.9 mg/g DW) was identified in multiple shoots grown in the presence of 1 µM IAA and 0.5–1 µM TDZ or 2 µM BAP.

Keywords Shoot proliferation · Cytokinin · Rosmarinic acid · Salvianolic acid B

Abbreviations

BAP 6-Benzylaminopurine
IAA Indole-3-acetic acid
TDZ Thidiazuron
RA Rosmarinic acid
SAB Salvianolic acid B

Introduction

Dracocephalum forrestii W. W. Smith (Lamiaceae) is a medicinal herbaceous plant naturally grown in China, specifically in the northwest of the Yunnan province (Li et al.

2009b). Its antipyretic, diuretic and astringent properties have been used in Tibetan medicine for centuries (Li et al. 2007, 2009b). The pharmacological activities of *D. forrestii* are mainly associated with the contents of numerous secondary metabolites such as mono- and triterpenoids, flavonoids and phenolic acids, including caffeic acid derivatives (Li et al. 2007, 2009b).

Phenolic compounds have been widespread in the plant kingdom, in particular, in the Lamiaceae and Boraginaceae families (Petersen 2013). Chlorogenic acid, caffeic acid, rosmarinic acid and its dimer, salvianolic acid B have earlier been identified in *Dracocephalum* species (Weremczuk-Jeżyna et al. 2016; Zeng et al. 2010; Li et al. 2009b). The biological properties of phenolic acids have been extensively studied for many recent years (Bulgakov et al. 2012), and have been found to exhibit anti-inflammatory, antiviral, antibacterial and antioxidant activity (Petersen 2013; Cao et al. 2012). Furthermore, rosmarinic acid and salvianolic acid B show cancer chemoprevention and antifibrotic effect. The substances are known to be effective in the therapy of cerebrovascular disorders and rheumatoid arthritis (Li et al. 2010; Hur et al. 2007; Inyunshina et al. 2007; Jaiong et al. 2005).

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The pharmacological value of polyphenolic acids has driven efforts to identify new high-quality natural sources. One possible approach employs the use of in vitro plant culture. Biotechnological methods allow pharmacologically important substances to be produced at high level within controlled laboratory environments (Tisserot and Vanghn 2008). The use of in vitro plant cultures for the production of caffeic acid derivatives has been widely described for the species of the Lamiaceae (Bulgakov et al. 2012). In the genus *Dracocephalum*, biosynthesis of rosmarinic acid has been reported for transformed root cultures of *D. moldavica*, *D. forrestii* and *D. kotschy* (Weremczuk-Jeżyna et al. 2013, 2016; Fattahi et al. 2013). In addition, a particularly high content of salvianolic acid B was detected in hairy roots and cell suspension culture of *Salvia miltiorrhiza* (Dong et al. 2010; Xiao et al. 2010), as well as the transformed roots of *D. forrestii* and *Ocimum basilicum* (Weremczuk-Jeżyna et al. 2016; Tada et al. 1996). Plant tissue culture can be used to increase the biosynthesis of plant metabolites by modifying culture conditions. One important factor reported to influence plant propagation and secondary metabolite accumulation is the profile of the growth regulators in the medium (Grzegorzczak-Karolak et al. 2015; Piątczak et al. 2015; Collin 2001).

The aim of the current work was to determine the optimal conditions for *D. forrestii* shoot culture with regard to the cytokinins composition and their concentration in the medium, by measuring the degree of effective shoot multiplication and biosynthesis of rosmarinic acid and salvianolic acid B. For comparison, RA and SAB levels in shoots of 1-year-old plants grown in field were evaluated.

Materials and methods

Plant material

The *Dracocephalum forrestii* shoot cultures were initiated from seeds received from Parco Nazionale Gran Paradiso, Valnontey (Italy). The seeds were surface sterilized with sodium hydrochloride solution (2%) for 3 min and next washed (three times) using sterilized water. The seeds were carried on MS (Murashige and Skoog 1962) agar (0.7%) medium contained 0.5 µM kinetin and 2.5 µM gibberellic acid and incubated in the dark. After germination, the shoot tips (0.3–0.5 cm long) resected from 4-week-old seedlings were carried into glass tubes with 25 ml MS agar medium with 0.5 µM IAA and 1 µM BAP for shoot proliferation. After 4 weeks, for elongation, the formed shoots were placed into MS medium without growth regulators. The shoots (4–5 cm long) were source of explants (nodal segments) for further experiments. The cultures grown at 26 ± 2 °C under a 16 photoperiod (cool white fluorescent lamps; 40 µm/m²/s).

Effect of cytokinins on shoot multiplication and biomass production

Nodal segments (about 0.5 cm length) (Fig. 1a) from shoots grown in vitro (10–12 subcultures) were transferred onto MS agar medium supplemented with IAA (1 µM) and kinetin, zeatin, BAP (1, 2, 4, 8, 16 µM) or TDZ (0.1, 0.2, 0.5, 1 or 2 µM). Control explants were grown on MS medium supplemented only with auxin (1 µM IAA). After 4 weeks of growth, the percentage of nodal segments forming shoots and/or buds and their average number per explant were recorded. Additionally, the fresh (FW) and dry weight (DW) (g/tube) of obtained shoots were estimated. The percentage of hyperhydric shoots were also evaluated. The experiments were repeated three times, a total of 30 nodal segments were used for each cytokinin type and concentration.

Extraction and determination of phenolic acids

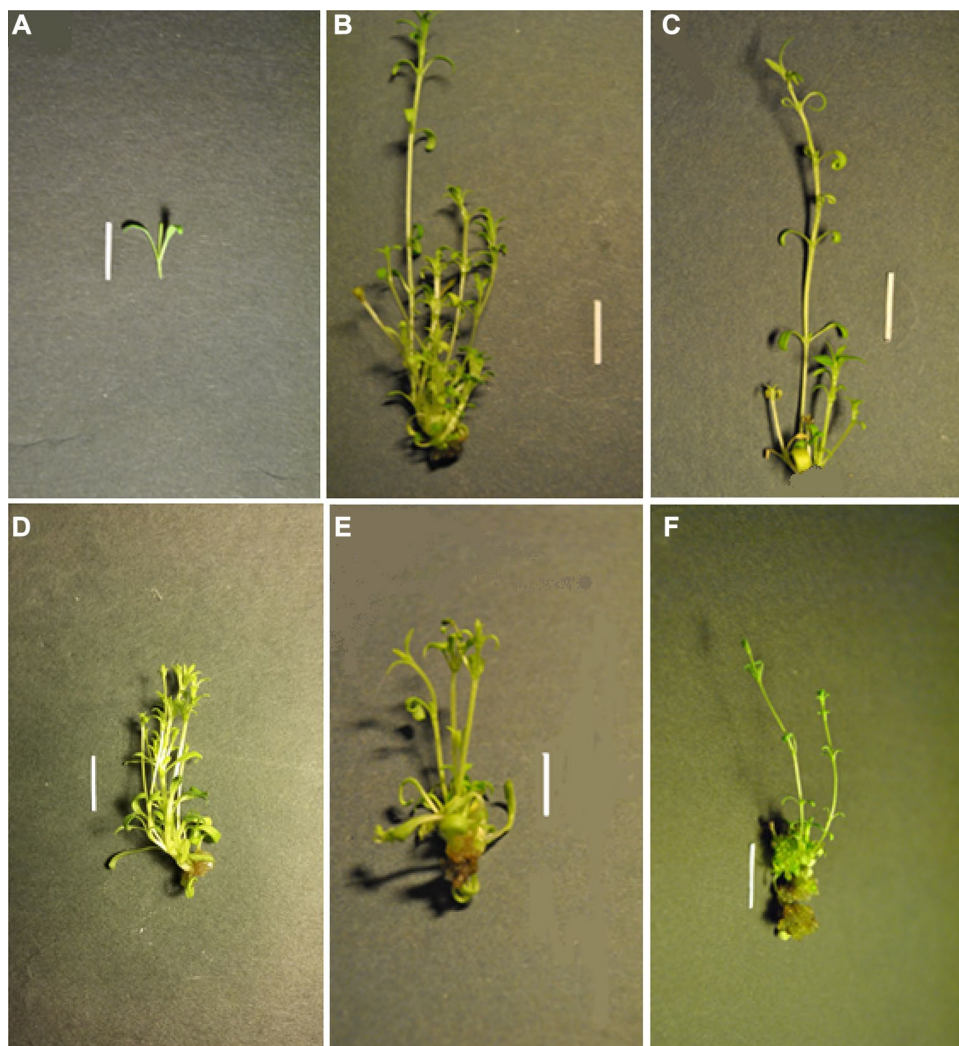
Preparation of extracts

Plant material (100 mg) lyophilized and next powdered, was first extracted with chloroform (15 ml). After filtration, the sample was sonicated (ultrasonic disintegrator type UD-20) for 15 min with 80% (v/v) aqueous methanol (25 ml) at 40 °C. Then, the hydromethanolic extraction process with was repeated twice with 10 ml of the solvent. After filtration, the extracts were connected and vaporized to dryness under reduced pressure.

Qualitative UPLC–PDA–ESI–MS analysis

In a present study, a UHPLC-3000 RS system (Dionex, Germany) with PAD detection and an AmaZon SL ion trap mass spectrometer with ESI interface (Bruker Daltonik GmbH, Germany) were used. Separation was performed on a Zorbax SB C18 column (Agilent, USA) according to the method described in our earlier study (Weremczuk-Jeżyna et al. 2016). Compounds were analyzed in negative ion mode. The phenols were detected at 328 nm and identified by comparison of the retention times, UV and mass spectra of the peaks with those of the reference compounds (Sigma-Aldrich) and published data (Ncube et al. 2014; Nuenchamong et al. 2011; Liu et al. 2007a, b; Zeng et al. 2006).

Fig. 1 Initial nodal explant of *D. forrestii* (a); 4-week-old shoots of *D. forrestii* regenerated on MS medium supplemented with 1 μ M IAA and cytokinin: 8 μ M BAP (b), 2 μ M BAP (c), 1 μ M zeatin (d), 16 μ M kinetin (e) or 0.5 μ M TDZ (f). Bar – 1 cm



Quantitative analysis of rosmarinic acid and salvianolic acid B

The samples were dissolved in 2 ml of methanol and the content of phenols in extracts were analyzed by UHPLC method as described earlier (Weremczuk-Jeżyna et al. 2016). Chromatographic system that used in this study was an Agilent Technologies 1290 Infinity UHPLC apparatus equipped with Zorbax Eclipse C18 Column (100 \times 3.1 mm id, 1.8 μ m; Agilent Technologies).

The shoots of plants obtained from the same seeds as in vitro culture and grown for 1 year under field conditions in Garden of the Department of Pharmacognosy at Medical University of Lodz were collected for comparative secondary metabolite analysis. The levels of the compounds in the analyzed extracts were expressed as mg/g dry weight (DW). The results are presented in Figs. 2 and 3.

Statistical analysis

All results are expressed as the mean \pm standard error (SE). The means were analyzed for statistical significance of differences by the Kruskal–Wallis test, with results of $p \leq 0.05$ being considered significant (Statistica 10.0. Statsoft, Poland).

Results and discussion

Effect of cytokinins on shoot proliferation

An efficient protocol for proliferation of *D. forrestii* was development and multiple shoots were obtained by culturing nodal explants on MS medium supplemented with 1 μ M IAA and different type and concentrations of cytokinins:

Fig. 2 The effect of various cytokinins and their concentrations on rosmarinic acid accumulation in *D. forrestii* shoots. Control—shoots cultivated on MS medium only with IAA 1 μ M. The results are expressed as means of three replicates \pm SE. The means with the same letter do not differ significantly according to the Kruskal–Wallis test ($p \leq 0.05$)

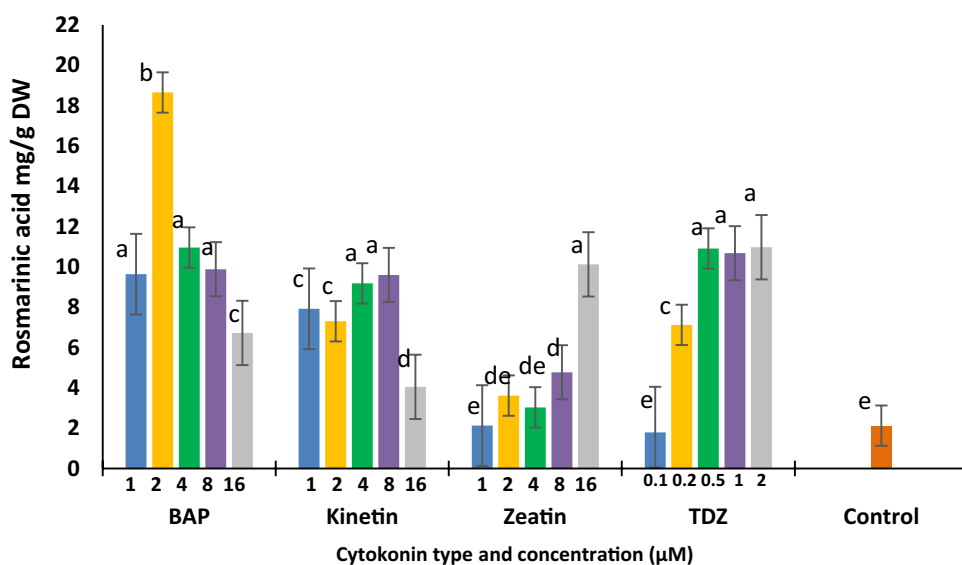
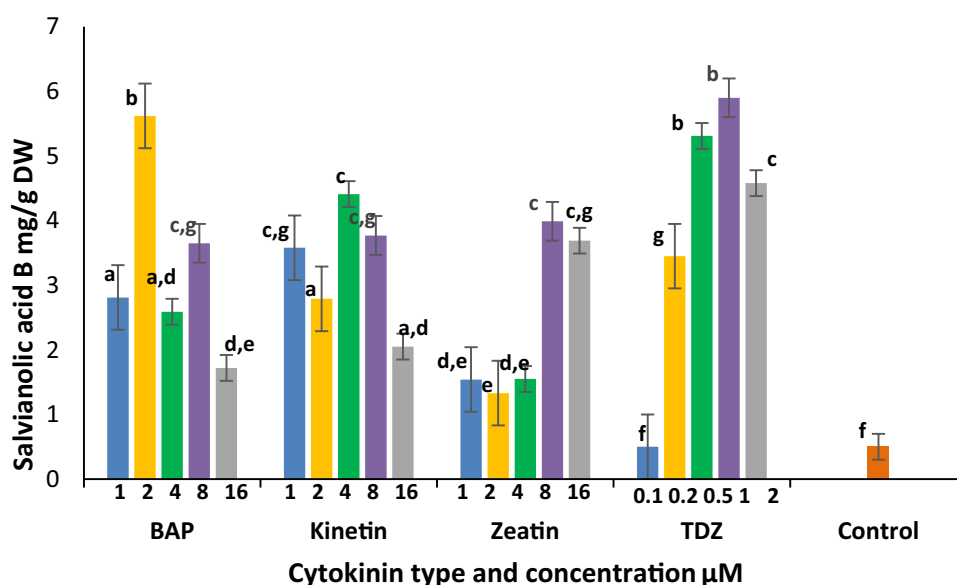


Fig. 3 The effect of various cytokinins and their concentrations on salvianolic acid B accumulation in *D. forrestii* shoots. Control—shoots cultivated on MS medium only with IAA 1 μ M. The results are expressed as means of three replicates \pm SE. The means with the same letter do not differ significantly according to the Kruskal–Wallis test ($p \leq 0.05$)



BAP, zeatin, kinetin or TDZ. There are many in vitro studies reported using nodal explants for the propagation of plants from the Lamiaceae family (Otroshy and Moradi 2013; Rani et al. 2006; Ghandi et al. 2004). The effect of the cytokinins on shoot proliferation, fresh and dry weight production was evaluated after 4 weeks of culture, as was the morphology of the *D. forrestii* shoots. The results are presented in Table 1.

The multiplication response of nodal explants, expressed as new shoots and buds formation, ranged from 83 to 100% and was not very dependent on the type and concentration of growth regulators in the medium (Table 1). A very high response was also noted for the control shoots growing on MS medium with 1 μ M IAA (93%). BAP at concentrations of 8 and 16 μ M was the most effective for shoot proliferation, resulting in about

seven shoots or buds per explant within 4 weeks of culture, with most of the forming shoots showing normal morphology without malformations or hyperhydricity (Fig. 1b; Table 1). However, the multiplication rate at lower BAP concentrations (1, 2, 4 μ M) was about three times lower (1.8–2.7 shoots) (Fig. 1c). Similarly, Otroshy and Moradi (2013) obtained the highest number of *Dracocephalum kotschy* shoots (about seven shoots per explant) on medium contained 2 mg/l (about 8 μ M) BAP. Medium with lower content, 1 mg/l (4 μ M) BAP was significantly less effective, giving about five shoots/explant. Also, Mehta et al. (2012) and Rani et al. (2006) reported that medium with addition of 2 mg/l BAP was optimal for shoot multiplication of two other Lamiaceae species: *Mentha piperita* and *Coleus blumei*.

Table 1 Influence of cytokinins on *D. forrestii* shoot proliferation

Cytokinins (μM)	% of explants producing axillary shoots and/or buds	Total number of axillary shoots and buds \pm SE	% of malformed and/or hyperhydrous shoots	Fresh weight (g/tube)	Dry weight (g/tube)
Zeatin					
1	90	4.5 ± 1.22^b	8.3	2.23 ± 0.3^a	0.33 ± 0.02^a
2	93.3	3.9 ± 0.68^b	0	$1.93 \pm 0.04^{a,f}$	0.28 ± 0.03^a
4	83.3	3.1 ± 0.74^c	24.2	1.08 ± 0.03^b	0.21 ± 0.02^b
8	100	1.8 ± 0.58^d	0	0.93 ± 0.01^b	0.19 ± 0.02^b
16	90	2.0 ± 0.34^d	0	1.01 ± 0.01^b	0.20 ± 0.01^b
Kinetin					
1	83.3	3.2 ± 0.51^c	19.7	2.12 ± 0.01^a	0.31 ± 0.03^a
2	96.7	3.3 ± 0.42^c	12.4	2.14 ± 0.05^a	0.31 ± 0.02^a
4	86.2	3.2 ± 0.57^c	7.9	2.25 ± 0.04^a	0.34 ± 0.01^a
8	90.0	$2.7 \pm 0.11^{c,e}$	4.5	2.12 ± 0.01^a	0.32 ± 0.01^a
16	85.0	4.5 ± 0.32^b	14.4	3.2 ± 0.01^c	0.48 ± 0.03^c
BAP					
1	93.3	1.8 ± 0.38^d	8.3	0.93 ± 0.01^b	0.13 ± 0.01^d
2	93.1	2.4 ± 0.31^e	0	1.32 ± 0.02^d	0.21 ± 0.02^b
4	83.3	2.7 ± 0.5^c	14.4	1.29 ± 0.05^d	0.20 ± 0.05^b
8	86.7	6.8 ± 0.21^a	7.06	3.51 ± 0.01^e	0.53 ± 0.01^c
16	85.5	6.9 ± 0.13^a	7.24	$3.45 \pm 0.03^{c,e}$	0.49 ± 0.03^c
TDZ					
0.1	96.7	2.7 ± 0.53^c	2.9	1.37 ± 0.03^d	0.22 ± 0.01^b
0.2	93.3	$2.2 \pm 0.32^{d,e}$	22.7	$1.64 \pm 0.05^{d,f}$	$0.25 \pm 0.02^{a,b}$
0.5	93.3	2.8 ± 0.52^c	23.5	1.43 ± 0.01^d	0.21 ± 0.01^b
1	86.7	$2.2 \pm 0.41^{d,e}$	21.8	1.80 ± 0.01^f	$0.27 \pm 0.02^{a,b}$
2	100	$2.3 \pm 0.31^{d,e}$	8.7	1.12 ± 0.02^b	0.18 ± 0.01^b
Control					
—	93.0	1.7 ± 0.09^d	0	0.42 ± 0.01^g	0.12 ± 0.02^d

Control—shoots cultivated on MS medium only with IAA 1 μM . The values represented the mean \pm SE of three independent experiments. Means followed by the same letter within the column were not significantly different ($p \leq 0.05$) according to the Kruskal–Wallis test

The multiplication rate obtained for *D. forrestii* shoots in the presence of zeatin and kinetin was lower than observed under optimal conditions (BAP at concentration 8 and 16 μM) (Table 1). However, with the exception of the highest zeatin levels (8–16 μM), these cytokinins gave significantly better results for shoot multiplication than the control medium without cytokinin. For example, about 3.9–4.5 regenerated shoots per explants were observed after 4 weeks of culture on media containing 1 and 2 μM zeatin (Fig. 1d) and 16 μM kinetin (Fig. 1e). TDZ proved particularly unfavorable for shoot multiplication. Not only was the propagation rate of explants grown on media with this cytokinins were quite low (2.2–2.8 shoot per explant) (Fig. 1f), but also numerous shoots obtained in this conditions were malformed or hyperhydric (to 24%). It has often been observed that BAP is more effective for shoot proliferation than other cytokinins. MS medium supplemented with 2 mg/l BAP gave better results for *D. kotschyi* shoot

propagation than a medium with the same concentration of kinetin (Otroshy and Moradi 2013). Similar results were achieved for *Mentha piperita* and *Scutellaria altissima* (Grzegorzczak-Karolak et al. 2017; Mehta et al. 2012).

The cytokinin supplementation of MS medium also increased the biomass of *D. forrestii* culture after 4 weeks of growth (Table 1). The greatest fresh (FW) and dry (DW) biomass values were obtained for *D. forrestii* shoots grown on media with 8 and 16 μM BAP; about 3.5 g FW/tube and 0.5 g DW/tube. Similar results were achieved for shoots cultured on medium containing 16 μM kinetin (no statistical differences) (Table 1). Also, Fraternali et al. (2003) for *Thymus mastichina* and Affonso et al. (2009) for *Thymus vulgaris* observed maximum biomass of micropropagated plants on media supplemented with BAP. Whereas the highest dry and fresh biomass of *Salvia officinalis* culture were found on media containing kinetin (Santos-Gomes and Fernandes-Ferreira 2003).

Identification of phenolic compounds of the hydromethanolic extract of *D. forrestii*

A hydromethanolic extract was prepared from the *D. forrestii* *in vitro* shoots, and its phenolic profile was investigated by UHPLC–PDA–ESI–MS³ analysis. All analyzed samples of shoot cultures presented a similar qualitative profile comprising nine peaks, four (1, 2, 6, 8) of which were identified by comparing their data (retention times–Rt, UV–Vis absorption spectra, and MS/MS fragmentation patterns) with those of reference standards. Other compounds for which standards were not available were identified by analysis their MS/MS spectral characteristic and confirmed with literature data (Catarino et al. 2015; Carlotto et al. 2015; Wang et al. 2012; Lecompte et al. 2010; Liu et al. 2007a; Kuo et al. 2000). The properties of the examined compounds and representative HPLC–UV chromatograms are presented in Table 2 and Fig. 4.

In the MS spectra, peak 1 corresponded to a pseudomolecular ion $[M-H]^-$ at m/z 353 and a fragment ion at m/z

191 formed from deprotonated quinic acid. This peak was identified as chlorogenic acid (Chen et al. 2014; Yang et al. 2012). Peak 2 indicated a pseudomolecular ion $[M-H]^-$ at m/z 179, identified as caffeic acid (Chen et al. 2014; Bravo et al. 2007). Peak 3 corresponded to a prominent $[M-H]^-$ ion at m/z 537. The presence of fragment ions $[M-H]^-$ at m/z 339, 295 and 493 suggested that the danshensu moiety (3,4-dihydroxyphenyllactic acid) was lost in the first place (– 198 amu) and that the carboxyl (– 44 amu) was detached; this may indicate that the compound contains no furan moiety. Such a MS spectrum is characteristic for salvianolic acid H or I, which have the same molecular weight (538) and mass spectra patterns (Liu et al. 2007a). Peak 4 indicated a pseudomolecular $[M-H]^-$ ion at m/z 717, and a fragment ion at m/z 519 $[M-H]^-$ as the result of the disconnection of a danshensu unit (– 198 amu). Elution order and the literature data identify the compound as salvianolic acid E (Wang et al. 2012). Peak 5 showed a base ion $[M-H]^-$ at m/z 515 with a major fragment at m/z 353 (– 162 amu) which in turn exhibited ions at m/z 191 and 179. A comparison with the

Table 2 Retention time, UV and MS/MS data of the compounds present in hydromethanolic extracts of *D. forrestii*

Peak no.	Retention time (min)	λ_{\max} (nm)	Negative Ion mode		Compound
			$[M-H]^-$ (m/z)	Major fragments	
1	17.1	331	353	191	Chlorogenic acid
2	19.3	323	179		Caffeic acid
3	34.5	288	537	493, 339 , 295	Salvianolic acid I/H
4	34.6	214, 237, 330	717	519	Salvianolic acid E
5	34.9	218, 516, 326	515	353 , 191, 179	Dicaffeoylquinic acid
6	37.4	179, 285, 329	359	197, 161	Rosmarinic acid
7	39.0	225, 285, 310	537	493 , 295	Lithospermic acid A
8	41.7	257, 286, 308	717	519 , 321	Salvianolic acid B
9	44.0	287, 326	373	179 , 135	Methyl rosmarinate

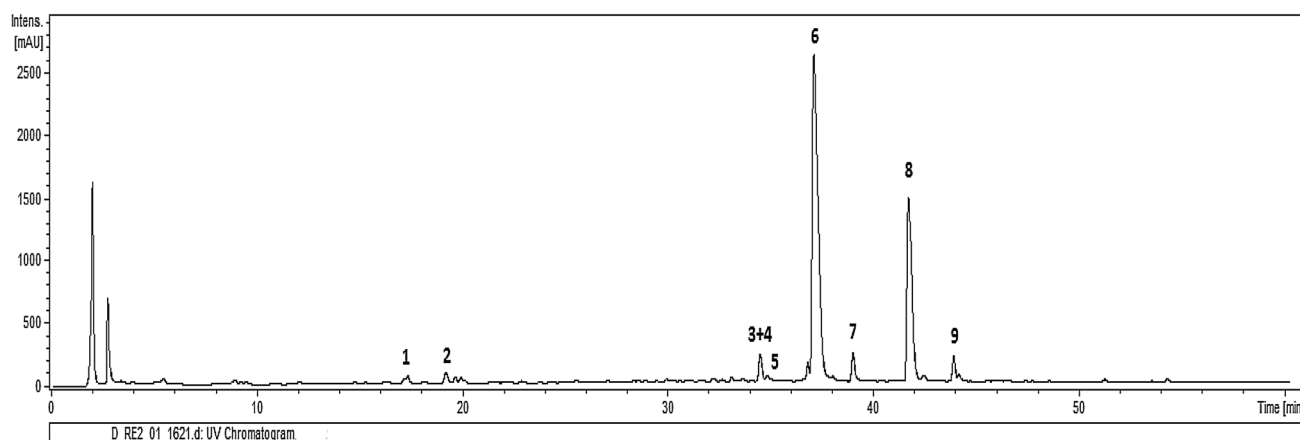


Fig. 4 Chromatogram of hydromethanolic extract of *D. forrestii* shoots grown on MS medium with IAA and BAP. (1) chlorogenic acid, (2) caffeic acid, (3) salvianolic acid I/H, (4) salvianolic acid E,

(5) dicaffeoylquinic acid, (6) rosmarinic acid, (7) lithospermic acid A, (8) salvianolic acid B, (9) methyl rosmarinate

literature data suggests that compound **5** is dicaffeoylquinic acid (Catarino et al. 2015; Carlotto et al. 2015). Similarly, peak **6** was identified as rosmarinic acid by comparisons with standards and literature data (Ncube et al. 2014; Nuenchamong et al. 2011; Liu et al. 2007a, b; Zeng et al. 2006). The fragmentation patterns revealed the presence of pseudomolecular ion $[M-H]^-$ at m/z 359 and MS^2 base ion at m/z 161 $[M-H]^-$, formed by elimination (-198 amu) of a danshensu moiety. In negative mode, the ESI-MS analysis peak **7** corresponded to a pseudomolecular ion $[M-H]^-$ at m/z 537. This compound lost a carboxyl group, releasing a fragment at m/z 493 $[M-H]^-$, that gave further ions at m/z 295. Based on the literature data, compound **7** could indicate to lithospermic acid A (Ribeiro et al. 2016). Compound **8** gave a pseudomolecular ion at m/z 717 $[M-H]^-$. The presence of a specific MS^2 pattern with ion fragments at m/z 519 $[M-H]^-$ and 321 $[M-H]^-$ formed by loss of two danshensu units, reveals the presence of two rosmarinic acid molecules linked via the aromatic ring of caffeic acid of a benzofuran structure: a linkage characteristic of a tetramer structure. A comparison of the MS spectra with authentic standards indicates that compound **8** is salvianolic acid B (Liu et al. 2007a, b; Zeng et al. 2006). Peak **9**, corresponded to the presence of a pseudomolecular ion $[M-H]^-$ at m/z 373. Fragmentation of molecular ion leads to ions at m/z 179 and 135, corresponding to the deprotonated form of caffeic acid and its residue after decarboxylation. Compound **9** was classified as methyl rosmarinate based on the literature data (Lecompte et al. 2010; Kuo et al. 2000).

Phenolic acids such as caffeic acid, chlorogenic acid and rosmarinic acid and their derivatives are widespread in the Lamiceae family (Petersen 2013). These secondary metabolites have also been described in the *Dracocephalum* genus (Zeng et al. 2010). Li et al. (2009a, b) identified more than sixty compounds belonging to different chemical groups in ethanolic extracts from whole plants of *D. forrestii*. Most compounds were monoterpenes, but the authors also described caffeic acid derivatives such as caffeic acid and methyl rosmarinate: the former being earlier described in *D. moldavica* (Kakasy et al. 2006), *D. peregrinum* (Dai et al. 2008) and *D. ruyschiana* (Kakasy et al. 2006), and the latter in aerial parts of *D. moldavica* (Yang et al. 2013). However, our work is the first report to describe the presence of salvianolic acids such as B, I/H, E and lithospermic acid A in the shoots of *D. forrestii*. Our previous studies have reported significant amounts of salvianolic acid B in transformed root cultures and the roots of a 1-year-old intact plant of *D. forrestii* (Weremczuk-Jeżyna et al. 2016). In contrast to caffeic and rosmarinic acid, salvianolic acids are not common and have been found in only a few species (Ożarowski et al. 2016; Ribeiro et al. 2016; Cirilini et al. 2016). They are mainly obtained from members of the *Salvia* genus, especially *Salvia milthiorrhiza* (Li et al. 2009a). Our results also

include the first description of dicaffeoylquinic acid in *D. forrestii* shoots.

Effect of cytokinins on metabolite accumulation

The UHPLC analysis showed that the secondary metabolite levels in the individual extract of *D. forrestii* shoots depended on the type and concentration of the applied cytokinins. However, the most common compounds in all analyzed materials were rosmarinic acid and salvianolic acid B. RA and SAB biosynthesis was much greater in multiplied shoots cultured in media containing any cytokinin, at almost all tested concentrations excluding 0.1 μ M TDZ and 1 μ M zeatin, compared to those grown on control MS medium without cytokinin (Figs. 2, 3). In the present study, a reverse relationship between the in vitro shoot proliferation and the production of RA and SAB was observed, especially for media that contained BAP. Phytohormones may stimulate the growth of the culture, but it did not have to be correlated with the production of active metabolites in plant biomass (Stalman et al. 2003; Collin 2001). Although the highest used concentrations of BAP (8 and 16 μ M) correlated with the greatest *D. forrestii* shoot proliferation, they were not so effective for phenolic acid accumulation. In our investigation, the highest RA content (18.7 mg/g DW) was found in *D. forrestii* shoots multiplied on MS medium supplemented with 2 μ M BAP and 1 μ M IAA. The level of RA was nine times higher than in shoots grown on control medium (2.12 mg/g DW) and five times higher than in aerial parts of 1-year-old intact plants (3.81 mg/g DW). Supplementation of the medium with 2 μ M BAP was also effective for SAB accumulation (5.6 mg/g DW). Similarly, the lowest level of BAP (0.5 mg/l) induced the greatest biosynthesis of phenolic compounds in shoot culture of *Thymus vulgaris*, meanwhile higher BAP concentrations (2 and 4 mg/l) stimulated the production of metabolites in the species at a lower level (Karalija and Parić 2011). Grzegorzczuk-Karolak et al. (2015) also report that lower (2 μ M) BAP concentration was more efficient for biosynthesis of flavonoids and verbascoside in shoots culture of *S. alpina*.

Different types and concentrations of growth regulators can be employed to improve growth and bioactive compound accumulation in plant cultures. The increased production of secondary metabolites could be attributed to differential mechanism by which cytokinins influence biosynthesis. The growth regulators could affect the expression of specific genes by regulating the abundance of individual proteins (Taha et al. 2008). On the other hand, Endress (1994) postulated that the mechanism of action of the cytokinins is based on scavenging free radicals or reducing their formation to very low amounts. The free radicals affect on phospholipids

and reduce membrane integrity. Thus, cytokinins stabilize membranes and could influence the exportation of necessary amino acids.

It is often observed that BAP has a strong influence on secondary metabolite biosynthesis in plant *in vitro* cultures compared to other cytokinins. For example, the presence of BAP was more favorable for harpagide accumulation in shoot culture of *Rhemannia elata* than kinetin and 2iP (2-isopentenyladenine) (Piątczak et al. 2015). Similarly, BAP stimulated biomass growth and isoflavonoid production in callus culture of *Genista tinctoria* (Łuczkiwicz et al. 2014) and silymarin biosynthesis in shoot culture of *Silybum marianum* (Al-Hawamdeh et al. 2013). However, the most potent determinant of the appropriate cytokinin selection is the species of cultivated plant. Therefore, zeatin was found to be the growth regulator that increased isoflavonoid biosynthesis in *Glycyrrhiza glabra* suspension culture (Arias-Castro et al. 1993) and had a positive effect on hypericin production in *Hypericum sampsonii* shoots (Liu et al. 2007c), whereas TDZ stimulated the production of polyphenolic compounds in shoot culture of *Scutellaria altissima* (Grzegorzczak-Karolak et al. 2017) and in shoot cultures of *Phyllanthus amarus* (Nitnaware et al. 2011). While our findings suggest that supplementation of growth medium with zeatin was the least favorable factor for increasing SAB and RA biosynthesis in *D. forrestii* shoots (Figs. 2, 3), thidiazuron significantly enhanced SAB production. In the presence of 0.5 and 1 μM TDZ, *D. forrestii* shoots biosynthesized between 5.5 and 6 mg salvianolic acid B per g DW which was about 11 times greater than in the shoots cultured on MS medium without cytokinins (0.5 mg/g DW) (Figs. 2, 3). However, the amount was slightly lower than in the aerial parts of 1-year-old naturally grown plants (8.87 mg/g DW). These results comply with those of a previous study which found that *D. forrestii* transformed root cultures were worse sources of SAB than the root of naturally-grown plants (Weremczuk-Jeżyna et al. 2016). This suggests that *in vitro* conditions are more favorable for production of rosmarinic acid than its dimer, SAB.

Conclusion

The present study describes the first proliferation protocol for the valuable medical plant *D. forrestii*. The regeneration of shoots from nodal segments is a simple method, which could be potentially used for effective mass propagation. Our findings clearly indicate that cytokinins could be important factor that determine growth and accumulation of bioactive metabolites. Of the cytokinins used in the study, BAP was the most effective for inducing shoot multiplication and metabolite production. In addition, it was found that the multiplied shoots of *D. forrestii* are rich sources of polyphenolic

compounds. Our findings identified nine compounds, five of which are described in the species for the first time. The predominant metabolites in the shoot cultures were RA and SAB. *In vitro* culture has proven to be a particularly advantageous technique for obtaining rosmarinic acid, whose content, under optimal conditions, was found to be five times higher in the cultures than in the aerial parts of naturally growing plants of *D. forrestii*. Further studies are needed to evaluate the accumulation of other compounds in *D. forrestii* shoots and identify the relationships between growth condition and productivity in the culture.

Author contribution statement IW-J obtained shoots of *D. forrestii* and studied influence of cytokinins on their proliferation, also prepared samples for UHPLC and UPLC-PDA-ESI-MS and wrote this article. ŁK performed UHPLC analysis. AKK performed UPLC-PDA-ESI-MS analysis. IG-K analyzed MS chromatograms and was responsible for verification of the paper. All authors read and approved the manuscript.

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