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In Vitro Cultures of Plants from the Rhamnaceae: Shoot Propagation and Anthraquinones Production

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Abstract

This study was conducted to investigate the effect of physical and chemical features on metabolic production in plant cell culture. The best multiplication of *Frangula alnus* Mill. shoots (8.84 ± 1.11) was obtained on woody plant medium (WPM) medium with indole-3-acetic acid (IAA) (0.1 mg l^{-1}) and 6-benzylaminapurine (BAP) (1.0 mg l^{-1}), but the highest metabolite production (1731 mg/100 g of total anthraquinone (AQ) aglycones) was in the shoots grown on the MS medium with addition of 1-naphthilaceneacetic (NAA) (0.1 mg l^{-1}) and thidiazuron (TDZ) (0.1 mg l^{-1}). The best multiplication of *Frangula rupestris* (Scop.) Schur. shoots (6.20 ± 0.53) was obtained on MS medium supplemented with BAP (0.5 mg l^{-1}). The highest metabolite production (451 mg/100 g of total anthraquinone aglycones) was in the shoots grown on medium with 2,4-D (0.1 mg l^{-1}) and BAP (0.5 mg l^{-1}). For the multiplication of *Rhamnus catharticus* L. shoots, the best results (2.8 ± 0.7) were obtained on the medium with combination of 2,4-D (0.1 mg l^{-1}) and kinetine (Kin) (1.0 mg l^{-1}). The best synthesis of anthraquinones (AQs) (1205 mg/100 g of total AQ aglycones) was in the shoots, which were grown on MS medium supplemented with 2,4-D (0.1 mg l^{-1}).

Keywords: Anthraquinones production, *Frangula alnus*, *Frangula rupestris*, propagation, *Rhamnus catharticus*, shoot cultures.

Introduction

Plants from the Rhamnaceae family (*Fragulae cortex, Purschianae cortex, Rhamni fallacis cortex, Rhamni catharticii fructus*) are a source of drugs with laxative action. Laxative action is based on the presence of emodin-type anthraquinones (AQs). During the past few years, production of emodin-type AQs by *in vitro* cultures of some Rhamnaceae plants has been studied. The aim of our study was to examine the possible influence of type of culture as well as influence of different chemical, physical, and biological factors on the production of emodin-type AQs by *in vitro* cultures. Once established, this technique can be used for large-scale production of nursery plants with selected features or biomass for the extraction of metabolites. Here will be presented the data on shoot propagation of *Frangula alnus* Mill., *Frangula rupestris* (Scop.) Schur., and *Rhamnus catharticus* L. In addition, the results of quantitative analysis of AQs synthesized by them will be given.

Materials and Methods

From the surface-sterilized fruits, the seeds were separated from the pericarp, and the zygotic embryos were excised from seeds (Sajc et al., 1999; Kovačević et al., 2000). After germination of zygotic embryos, shoot culture was established from epicotyl on medium containing mineral salts and vitamins according to Murashige and Skoog (1962) (MS) or woody plant medium (Lloyd & McCown, 1980) (WPM), with addition of 3% (w/v) sucrose, indole-3-acetic acid (IAA) (0.1 mg l^{-1}), and 6-benzylaminopurine (BAP) (1.0 mg l^{-1}). Medium was gelled with 0.7% (w/v) agar (Torlak, Belgrade). The pH of the medium was adjusted to 5.8 prior to autoclaving at 114 °C for 25 min. The cultures were maintained in Erlenmeyer flasks, containing 40 ml of medium, in 4 weeks of subculture period. They were kept at 25 ± 2°C on 16 h photoperiods, at a photon flux density of

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Table 1. Multiplication of *F. alnus* shoots and production of AQs.

Mineral salts	Plant growth regulators	Index of multiplication ($x \pm$ SE) (number of shoots)	Relation between free/ bounded aglycones (%)	Content of total AQ aglycones and ratio between aglycones (%)				
				mg/100 g	ae	e	ch	ph
MS	0.1 mg l^{-1} NAA + 1.0 mg l^{-1} Kin	1.55 ± 0.26 (18)	34/66	1274	4.7	32.2	16.2	46.9
	0.1 mg l^{-1} NAA + 0.5 mg l^{-1} BAP	5.26 ± 0.45 (42)	51/49	1213	4.2	14.8	70.8	10.2
	0.1 mg l^{-1} 2,4-D + 1.0 mg l^{-1} BAP	3.33 ± 0.68 (12)	47/53	833	5.8	19.1	15.1	60.0
	0.1 mg l^{-1} NAA + 0.1 mg l^{-1} TDZ	3.62 ± 0.35 (58)	65/35	1731	3.8	6.4	14.7	75.1
	0.1 mg l^{-1} NAA + 0.5 mg l^{-1} TDZ	7.65 ± 0.59 (40)	65/35	1026	1.9	3.6	21.2	73.3
	0.1 mg l^{-1} NAA + 1.0 mg l^{-1} TDZ	5.14 ± 0.44 (70)	56/44	1416	4.3	7.3	21.0	67.4
	0.1 mg l^{-1} NAA + 1.0 mg l^{-1} BAP	4.87 ± 0.79 (15)	48/52	691	4.9	15.5	16.0	63.6
	0.1 mg l^{-1} IAA + 1.0 mg l^{-1}BAP	5.00 ± 0.78 (15)	57/43	563	4.6	9.1	17.8	68.5
	0.05 mg l^{-1} IAA + 0.5 mg l^{-1} BAP	4.47 ± 0.45 (15)	38/62	503	2.0	21.7	8.4	67.9
WPM	0.1 mg l^{-1} NAA + 1.0 mg l^{-1} BAP	5.26 ± 0.45 (42)	47/53	1580	4.4	21.1	19.9	54.6
	0.1 mg l^{-1} NAA + 2.0 mg l^{-1} BAP	—	35/65	838	5.3	16.3	19.7	58.7
	0.2 mg l^{-1} NAA + 2.0 mg l^{-1} 2iP	—	57/43	1007	5.0	31.7	14.4	47.9
	0.1 mg l^{-1} IAA + 1.0 mg l^{-1} BAP	8.84 ± 1.11 (32)	34/66	1258	3.0	30.6	14.5	51.9
	0.05 mg l^{-1} IAA + 0.5 mg l^{-1} BAP	8.24 ± 0.89 (30)	54/46	1493	5.2	14.0	20.7	60.1

Each value is the mean of at least three determinations.
ae, aloe emodin; e, emodin; ch, chrysophanol; ph, physcione.
AQs, anthraquinones; BAP, 6-benzylaminopurine; 2,4-D, (2,4-dichlorophenoxy)-acetic acid; 2-iP, *N*-(3-methyl-2-butenyl)-1-H-purin-6-amine; IAA, indole-3-acetic acid; IBA, indole-3-butanoic acid; Kin, kinetine; NAA, 1-naphthilaceneacetic acid; TDZ, thidiazuron; WPM, woody plant medium.

Table 2. Multiplication of *F. rupestris* shoots and production of AQs.

Plant growth regulators MS	Index of multiplication % $x \pm$ SE (number of shoots)	Relation between free/bounded aglycones (%)	Content of total AQ aglycones and ratio between aglycones (%)				
			mg/100g	ae	e	ch	ph
0.1 mg l^{-1} NAA + 0.5 mg l^{-1} BAP	3.27 ± 0.36 (30)	90/10	227	6,4	4,5	35.0	54.1
0.5 mg l^{-1} NAA + 0.5 mg l^{-1} BAP	3.95 ± 0.64 (20)	82/18	233	6,4	6,3	38.2	49.1
0.1 mg l^{-1} NAA + 1.0 mg l^{-1} BAP	3.64 ± 0.35 (33)	91/9	352	4,3	4,3	41.1	50.3
0.5 mg l^{-1} NAA + 1.0 mg l^{-1} BAP	2.20 ± 0.41 (20)	90/10	309	6,6	3,2	43.3	46.9
0.5 mg l^{-1} BAP	6.20 ± 0.53 (25)	90/10	275	6,4	5,0	35,0	53.6
1.0 mg l^{-1} BAP	2.27 ± 0.53 (15)	91/9	250	6,1	3,6	37.4	52.9
0.1 mg l^{-1} NAA	WM (25)	63/37	286	7,2	8,0	39.1	45.7
0.5 mg l^{-1} NAA	WM (25)	78/22	279	7,7	3,6	45.7	42.9
0.1 mg l^{-1} 2,4-D + 1.0 mg l^{-1} BAP	—	45/55	70	15,1	8,6	32.3	44.0
0.1 mg l^{-1} 2,4-D + 0.5 mg l^{-1} BAP	—	76/24	451	6,9	4,8	42.6	45.7
1 mg l^{-1} Kin	1.90 ± 00.24 (20)	79/21	294	9,8	3,3	41.8	45.1
0.5 mg l^{-1} Kin	0.93 ± 0.20 (15)	78/22	335	5,3	2,8	42.6	49.3
0.1 mg l^{-1} NAA + 0.5 mg l^{-1} Kin	WM (15)	76/24	266	8,0	3,4	45.7	42.9

WM, without multiplication.
Each value is the mean of at least three determinations.
ae, aloe emodin; e, emodin; ch, chrysophanol; ph, physcione.
AQs, anthraquinones; BAP, 6-benzylaminopurine; 2,4-D, (2,4-dichlorophenoxy)-acetic acid; 2-iP, *N*-(3-methyl-2-butenyl)-1-H-purin-6-amine; IAA, indole-3-acetic acid; IBA, indole-3-butanoic acid; Kin, kinetine; NAA, 1-naphthilaceneacetic acid; TDZ, thidiazuron; WPM, woody plant medium.

Table 3. Multiplication of *R. catharticus* shoots and production of AQs.

Plant growth regulators MS	Index of multiplication ($x \pm$ SE) (number of shoots)	Relation between free/bounded aglycones (%)	Content of total AQ aglycones and ratio between aglycones (%)				
			mg/100g	ae	e	ch	ph
0.1 mg l^{-1} NAA + 1.0 mg l^{-1} BAP	1.76 ± 0.18 (69)	58/42	1073	3.1	19.0	75.7	2.2
0.5 mg l^{-1} NAA + 1.0 mg l^{-1} BAP	1.40 ± 0.42 (15)	32/68	1033	2.5	27.4	68.5	1.6
0.1 mg l^{-1} NAA + 0.5 mg l^{-1} BAP	0.8 ± 0.22 (15)	22/78	775	3.4	32.3	62.4	1.9
0.5 mg l^{-1} NAA + 0.5 mg l^{-1} BAP	1.73 ± 0.44 (14)	27/73	880	3.1	28.0	67.1	1.8
1.0 mg l^{-1} BAP	1.45 ± 0.37 (20)	30/70	427	5.3	22.9	66.7	5.1
0.5 mg l^{-1} BAP	1.73 ± 0.44 (15)	7/93	322	6.5	27.5	62.9	3.1
0.5 mg l^{-1} NAA	1.12 ± 0.22 (17)	42/58	767	2.6	22.6	72.2	2.7
0.1 mg l^{-1} NAA	1.20 ± 0.44 (10)	43/57	610	2.6	22.3	72.4	2.7
0.1 mg l^{-1} NAA + 1.0 mg l^{-1} Kin	1.23 ± 0.38 (14)	41/59	534	2.6	28.1	66.0	53.3
0.5 mg l^{-1} NAA + 1.0 mg l^{-1} Kin	1.00 ± 0.35 (15)	44/56	694	2.7	29.6	65.4	2.3
1.0 mg l^{-1} Kin	1.26 ± 0.18 (40)	30/70	709	2.3	78.3	12.4	7.0
0.1 mg l^{-1} 2,4-D + 1.0 mg l^{-1} Kin	2.80 ± 0.70 (10)	40/60	782	1.9	15.4	79.5	3.2
0.1 mg l^{-1} 2,4-D	1.2 ± 0.29 (10)	41/59	1205	2.3	27.1	68.7	1.9
0.5 mg l^{-1} 2,4-D	1.00 ± 0.26 (10)	42/58	1172	1.9	25.7	69.4	3.0
0.1 mg l^{-1} IBA	—	34/67	1150	2.7	38.8	56.6	1.9
0.1 mg l^{-1} NAA + 0.1 mg l^{-1} TDZ	2.81 ± 0.52 (16)	31/69	601	6.0	56.9	19.1	18.0
0.1 mg l^{-1} 2,4-D + 1.0 mg l^{-1} BAP	2.00 ± 26 (10)	42/58	1170	2.2	25.8	69.5	2.5
1.0 mg l^{-1} BAP + 0.1 mg l^{-1} IAA	1.36 ± 0.41 (15)	26/74	716	3.8	16.4	74.2	5.6

Each value is the mean of at least three determinations.
ae, aloe emodin; e, emodin; ch, chrysophanol; ph, physcione.
AQs, anthraquinones; BAP, 6-benzylaminopurine; 2,4-D, (2,4-dichlorophenoxy)-acetic acid; 2-iP, *N*-(3-methyl-2-butenyl)-1-H-purin-6-amine; IAA, indole-3-acetic acid; IBA, indole-3-butanoic acid; Kin, kinetine; NAA, 1-naphthilaceneacetic acid; TDZ, thidiazuron; WPM, woody plant medium.

$30\,\mu mol\,m^{-2}\,s^{-1}$ provided by white fluorescent tubes (65 W, 4500 K, Tesla, Pančevo). Later, for the experiments of multiplication, MS or WPM media were supplemented with different combinations of plant growth regulators. Index of multiplication presents a number of new shoots obtained at each explant after 4 weeks of growth.

Qualitative (Bornträger test, TLC, and HPLC) and quantitative analysis of AQs (HPLC) was done as described previously (Sajc et al., 1999; Kovačević et al., 2000; Kovačević, 2001).

Results and Discussion

The best multiplication of *F. alnus* shoots, grown on MS medium (7.65 ± 0.59), was obtained under treatment with 1-naphthilaceneacetic acid (NAA) ($0.1\,mg\,l^{-1}$) and thidiazuron (TDZ) ($0.5\,mg\,l^{-1}$), but the highest metabolite production (1731 mg/100 g of total AQ aglycones) was in the shoots treated with NAA ($0.1\,mg\,l^{-1}$) and TDZ ($0.1\,mg\,l^{-1}$). Shoots that were grown on WPM gave better multiplication (8.84 ± 1.11; $0.1\,mg\,l^{-1}$ IAA $+1.0\,mg\,l^{-1}$ BAP), but production of AQs was lower (Table 1). In comparison with the intact plant, shoots of *F. alnus* grown *in vitro* accumulated more free AQs and derivatives of physcione dominated; emodin is a main component of the metabolite complex in the leaves and bark of *F. alnus* from the natural population (Kovačević, 2001).

The best multiplication of *F. rupestris* shoots (6.20 ± 0.64) was obtained on MS medium supplemented with BAP ($0.5\,mg\,l^{-1}$). The highest metabolite production (0.45% of total AQ aglycones) was in the shoots grown on medium with 2,4-D ($0.1\,mg\,l^{-1}$) and BAP ($0.5\,mg\,l^{-1}$). In the shoots of *F. rupestris* grown *in vitro*, AQs were mainly in the free stage (Table 2). Derivatives of physcion and chrysophanol dominated in the shoots of *F. rupestris* grown *in vitro*, and production of AQs was better than in the intact plant; as determined previously, derivatives of aloe emodin and chrysophanol are dominant in the intact plant (Kovačević, 2001).

For the multiplication of *R. cathartica* shoots, the best results (2.8 ± 0.7) were obtained on MS medium with combination of 2,4-D ($0.1\,mg\,l^{-1}$) and kinetine (Kin) ($1.0\,mg\,l^{-1}$) (Table 3). Similar multiplication was obtained on medium supplemented with NAA ($0.1\,mg\,l^{-1}$) and TDZ ($0.1\,mg\,l^{-1}$), but these shoots were "aqueous." The best synthesis of AQs (1205 mg/100 g of total AQ aglycones) was in the shoots, which were grown on MS medium supplemented with 2,4-D ($0.1\,mg\,l^{-1}$). Derivatives of chrysophanol and emodin dominated in the shoots of *R. catharticus* grown *in vitro*.

Conclusions

The main differences between *in vitro* cultures of all investigated species and the intact plants were the presence of more free AQs and significantly less quantities of glycosides in the aseptically grown shoots. In addition, *in vitro* cultures accumulated more derivatives of chrysophanol and physcione than the leaves, bark, or fruits of *Frangula* and *Rhamnus* species from natural populations. The quantities of AQs were smaller (or similar) than in the bark of *F. alnus*, but 2–6 times higher than in the leaves of this species. The production of AQs in the shoots of *F. rupestris* and *R. catharticus* was better than in the intact plant (Kovačević, 2001). The similar results in production and composition of AQs complex were obtained for the shoot culture of *Rhamnus fallax* Boiss. (Rosić et al., 2000).

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