

Accumulation of hydroxybenzoic acids and other biologically active phenolic acids in shoot and callus cultures of *Aronia melanocarpa* (Michx.) Elliott (black chokeberry)

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Abstract Phenolic acids are plant metabolites important in phytotherapy and also in cosmetology. In this study, proliferating shoot and callus cultures of *Aronia melanocarpa* were established and maintained on Linsmaier and Skoog (L-S) medium containing different levels of α -naphthaleneacetic acid (NAA) and 6-benzyladenine (BA), ranging from 0.1 to 3.0 mg l⁻¹. Methanolic extracts from the biomass of these cultures and from the fruits of soil-grown plants were used to determine the amounts of free phenolic acids and cinnamic acid using the high-performance liquid chromatography (HPLC) method. Out of a total of twelve analyzed compounds, all of the extracts contained four of them: caffeic acid, *p*-hydroxybenzoic acid, syringic acid, and vanillic acid. Moreover, shoot extracts also contained salicylic acid (*o*-hydroxybenzoic acid), while callus extracts contained *p*-coumaric acid. On the other hand, fruit extracts also contained both salicylic acid and *p*-coumaric acid. The total amount of the analyzed compounds in extracts from both shoot and callus cultures depended on the L-S medium used, and varied between 103.05 and 150.95 mg 100 g⁻¹ dry weight (DW), and between 50.23 and 81.56 mg 100 g⁻¹ DW, respectively. Both types of culture contained higher levels of phenolic acids than the fruit extracts (32.43 mg 100 g⁻¹ DW). In shoot cultures, *p*-hydroxybenzoic acid and salicylic acid were the predominant metabolites (reaching 55.14 and 78.25 mg 100 g⁻¹ DW, respectively), while in callus cultures, *p*-hydroxybenzoic acid (25.60 mg 100 g⁻¹ DW) and syringic acid (41.20 mg 100 g⁻¹ DW) were the

main compounds. In fruit extracts, salicylic acid (15.60 mg 100 g⁻¹ DW) and *p*-hydroxybenzoic acid (5.29 mg 100 g⁻¹ DW) were predominant.

Keywords Rosaceae · Black chokeberry · Secondary metabolites · HPLC analysis · *p*-Hydroxybenzoic acid · Salicylic acid · Syringic acid

Abbreviations

BA	N ⁶ -Benzyladenine
DW	Dry weight
HPLC	High-performance liquid chromatography
L-S	Linsmaier and Skoog
NAA	α -Naphthaleneacetic acid
PGRs	Plant growth regulators

Introduction

Phenolic acids, both benzoic and cinnamic acid derivatives and depsides, like chlorogenic acid and rosmarinic acid, are a pharmacologically attractive group of plant metabolites important in phytotherapy and also in cosmetology. Their anti-inflammatory, spasmolytic, cholagogic, hypolipemic, antiaggregatory and immunostimulating properties have long been known. Many recent studies have also confirmed strong antioxidant, antiradical and anticancer activities of some compounds of this group, e.g. caffeic acid, *p*-coumaric and protocatechuic acids (Laranjinha et al. 1994; Nardini et al. 1995; Rice-Evans et al. 1996; Sroka and Cisowski 2003; Wen et al. 2003; Ekiert and Czygan 2007; Sánchez-Maldonado et al. 2011). Hydroxybenzoic acids also possess many others very important biological properties. *p*-Hydroxybenzoic acid shows antimicrobial, antifungal, antisickling, and estrogenic

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activities (Pugazhendhi et al. 2005; Chong et al. 2009). *o*-Hydroxybenzoic acid (salicylic acid) has anti-inflammatory, antiseptic, antifungal, antipyretic, analgesic and keratolytic properties (Lin and Nakatsui 1998). Vanillic acid (monomethoxy-derivative of *p*-hydroxybenzoic acid) exhibits anti sickling and anthelmintic, as well as hepatoprotective activities (Itoh et al. 2009, 2010). Syringic acid (dimethoxy-derivative of *p*-hydroxybenzoic acid), besides being an antioxidant, shows antibacterial and hepatoprotective activities, too (Kong et al. 2008, Itoh et al. 2009, 2010).

Aronia melanocarpa (Rosaceae, Rosoideae) is a North American medicinal and culinary plant species that began to be cultivated in Europe from the turn of the 19th and 20th centuries. The fruits of this species owe their therapeutic potential to the presence of different groups of metabolites, of which flavonoids, anthocyanins, tannins, organic acids, pectins, vitamins and many bio elements are the most important. Of the phenolic acids, the presence of chlorogenic acid and its isomer–neochlorogenic acid, has been documented so far (Kulling and Rawel 2008; Zdunczyka et al. 2002). The shikimic acid pathway is characteristic, partially or exclusively, of the biogenesis of the main groups of metabolites in this plant species (flavonoids, anthocyanins and tannins), and the same pathway is also characteristic of the biogenesis of all the groups of phenolic acids, i.e. benzoic acid and cinnamic acid derivatives, and that was the main argument for performing analysis of this group of compounds in *A. melanocarpa* biomass cultured in vitro (Dewick 1997). In addition, chemotaxonomic guidelines applicable to the subfamily Rosoideae were also taken into account. Salicylic acid and its derivatives are characteristic of this subfamily (Hegnauer 1973). Another argument was based on the biotechnological successes in the accumulation of some phenolic acids in plant in vitro cultures, e.g. rosmarinic acid (Makri and Kintzios 2004), protocatechuic acid (Ekiert et al. 2009; Szopa et al. 2012) and *p*-coumaric acid (Ekiert et al. 2008; Piekoszewska et al. 2008; Szopa et al. 2012). Fruit extracts from soil-grown plants were also analyzed for comparison.

Aronia melanocarpa has not been a subject of biotechnological studies yet. Investigations of other species of the subfamily Rosoideae, e.g. *Rubus chamaemorus* or *Rosa* sp., have mostly focused on the development of micropropagation protocols (Rout et al. 1999; Thiem and Śliwińska 2003; Martinussen et al. 2004). In the present study, the accumulation of free phenolic acids and cinnamic acid—the parent compound of one subgroup of these metabolites, was investigated for the first time in the biomass from established in vitro cultures differing in the degree of tissue differentiation (shoot and callus cultures), growing on five variants of Linsmaier and Skoog (L-S) (1965) medium supplemented with various concentrations of plant growth regulators (PGRs): auxin– α -naphthaleneacetic acid (NAA),

and cytokinin– N^6 -benzyladenine (BA), in the concentration range from 0.1 to 3.0 mg l⁻¹. Methanolic extracts from the biomass cultured in vitro and from the fruits of soil-grown plants were used to determine twelve compounds by the high-performance liquid chromatography (HPLC) method. The aim of the study was to find the best medium for free phenolic acids production in in vitro cultures of *A. melanocarpa*, to compare the biosynthetic capacity of the cells from in vitro cultures with that of the cells of soil-grown plants, and eventually to propose in vitro cultures as a potentially rich source of the investigated metabolites. Variants of L-S medium were tested to allow the possibility of comparing the results from *A. melanocarpa* in vitro cultures with the results from in vitro cultures of *Ruta graveolens* (Ekiert et al. 2009) and *R.g. spp. divaricata* (Ekiert et al. 2008; Piekoszewska et al. 2008) studied earlier in our laboratory for their capacity to produce free phenolic acids.

Materials and methods

Plant material

Mature fruits of *Aronia melanocarpa* (Michx.) Elliott were collected in September 2010 from the plants growing in Rogów Arboretum, Warsaw University of Life Sciences, Forest Experimental Station in Rogów (Poland). This material was lyophilized later in our laboratory.

Establishment of in vitro cultures

The in vitro cultures of *Aronia melanocarpa* were established from leaf buds of the plants growing in the above-mentioned arboretum. The leaf buds were first sterilized in 70 % ethanol for 30 sec. and subsequently in 0.1 % NaOCl for 10 min., and maintained on Murashige-Skoog (M-S) (1962) solid medium supplemented with PGRs: 0.5 mg l⁻¹ NAA (auxin) and 1.0 mg l⁻¹ BA (cytokinin). On some bud fragments, initiation of undifferentiated callus tissue was observed, while on some others microshoots emerged. After 4 weeks, we obtained two different cultures—shoot cultures and callus cultures. Both types of culture were cultivated under constant artificial light (4 W/m², LF-40 W lamp, daylight, Piła) at 25 ± 2 °C.

The experimental in vitro cultures were maintained on five variants of Linsmaier-Skoog (L-S) (1965) solid medium differing in the concentrations of plant growth regulators (PGRs), NAA and BA (mg l⁻¹): 0.1 and 0.1; 0.5 and 1.0; 1.0 and 1.0; 2.0 and 2.0; 1.0 and 3.0, respectively. The tissue cultures (three series) were grown for 4 weeks under the same light and temperature conditions as the initial cultures.

Extraction and HPLC analysis

Lyophilized biomass from in vitro cultures collected after 4 week growth cycles (three series) and plant material (0.5 g) were twice subjected to extraction with boiling methanol for 3 h. Eleven phenolic acids and cinnamic acid were quantified in the methanolic extracts using the HPLC method developed by Tian et al. (2005). Separation was performed using a Kinetex™ C-18 analytical column (150 × 4.6 mm, 2.6 μm) at 25 °C. The mobile phase consisted of 0.1 % trifluoroacetic acid (A) and acetonitrile (B) at a flow rate of 1.0 ml min⁻¹ (gradient program); injection volume was: 5 μl. Detection wavelength was set at 254 nm. Quantification was made by comparison with standards: caffeic, chlorogenic, cinnamic, protocatechuic, rosmarinic, salicylic (*o*-hydroxybenzoic acid), sinapic, and syringic acids from Sigma; *p*-coumaric, ferulic, *p*-hydroxybenzoic, and vanillic acids from Fluka.

Results

Shoot cultures

The increases in dry biomass observed in the shoot cultures on the tested variants of L-S medium during 4 week growth cycles varied widely (from 8.2 to 15.3 times). High increases (over tenfold) were seen on the media containing the following concentrations of auxin (NAA) and cytokinin (BA), 2.0 and 2.0 mg l⁻¹, 0.5 and 1.0 mg l⁻¹ and 1.0 and 1.0 mg l⁻¹, respectively. Five phenolic acids out of the twelve analyzed compounds were present in the extracts: caffeic acid, *p*-hydroxybenzoic acid, salicylic acid, syringic acid and vanillic acid (Fig. 1). None of the extracts contained chlorogenic, ferulic, *p*-coumaric, protocatechuic, rosmarinic, synapic and cinnamic acids. The total amount of phenolic acids was dependent on the variant of L-S medium and ranged from 103.05 to 150.95 mg 100 g⁻¹ DW (Table 1). High total amounts of the metabolites in the range 120–150 mg 100 g⁻¹ DW were obtained on three medium variants containing 0.1 mg l⁻¹ NAA and 0.1 mg l⁻¹ B, 1.0 mg l⁻¹ NAA and 1.0 mg l⁻¹ BA, 0.5 mg l⁻¹ NAA and

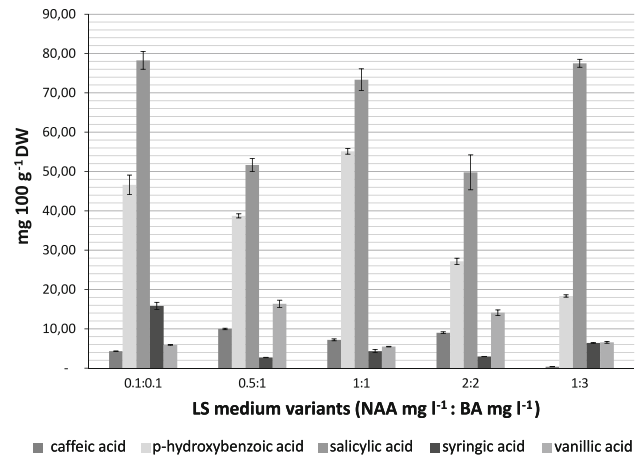


Fig. 1 Contents (mg 100 g⁻¹ DW) of free phenolic acids in biomass extracts from shoot culture of *A. melanocarpa*. The values are means of three experiments ± SD

1.0 mg l⁻¹ BA. The amounts of the individual metabolites varied widely, ranging from 0.38 to 78.25 mg 100 g⁻¹ DW (Fig. 1). Depending on the variant of L-S medium, their levels increased 1.57 to 26.26 times. The maximum amount of caffeic acid was 10 mg 100 g⁻¹ DW. The maximum amounts of two other compounds, vanillic acid and syringic acid, were of the same order of magnitude and close to 16 mg 100 g⁻¹ DW. The accumulation of caffeic acid and vanillic acid was greatest on the medium variant containing 0.5 mg l⁻¹ NAA and 1.0 mg l⁻¹ BA. Syringic acid was accumulated in the highest quantities on the L-S medium variant containing auxin and cytokinin at 0.1 mg l⁻¹ each. *p*-Hydroxybenzoic acid and salicylic acid were the quantitatively dominating metabolites (max. 55.14 and 78.25 mg 100 g⁻¹ DW, respectively). The largest amounts of *p*-hydroxybenzoic acid were found in the extracts from the biomass growing on the medium variant containing 1.0 mg l⁻¹ NAA and 1.0 mg l⁻¹ BA, while the medium supplemented with 0.1 mg l⁻¹ NAA and 0.1 mg l⁻¹ BA favoured the accumulation of salicylic acid. A high amount of salicylic acid (over 70 mg 100 g⁻¹ DW) was found on three of the five L-S medium variants tested. Likewise, considerable amounts of *p*-hydroxybenzoic acid (over 38 mg 100 g⁻¹ DW) were noted on three of those variants.

Table 1 Contents (mg 100 g⁻¹ DW ± SD) of free phenolic acids in fruits of soil-grown plants and their maximal contents in biomass extracts from in vitro cultures growing on different tested L-S medium variants

	Phenolic acids						Total contents
	Caffeic acid	<i>p</i> -Coumaric acid	<i>p</i> -Hydroxybenzoic acid	Salicylic acid	Syringic acid	Vanillic acid	
Shoot cultures	9.98 ± 1.19	nd	55.14 ± 0.71	78.25 ± 2.26	15.82 ± 0.92	16.37 ± 0.93	150.95 ± 6.01
Callus cultures	7.31 ± 0.17	12.15 ± 0.56	25.60 ± 2.60	nd	46.26 ± 1.74	6.65 ± 0.15	81.56 ± 2.62
Fruits	3.96 ± 0.06	3.05 ± 0.07	5.29 ± 0.04	15.60 ± 0.18	4.16 ± 0.06	0.37 ± 0.02	32.43 ± 0.43

nd not detected in analyzed biomass extracts

Callus cultures

The increases in dry biomass in the callus cultures on the tested variants of L-S medium during 4 week growth cycles were decidedly lower compared with biomass growth in the shoot cultures. They also varied, fluctuating within the range from 2.0 to 4.8 times. The highest biomass increases (more than 3.5 times) were obtained on the media containing 0.5 mg l⁻¹ NAA and 1.0 mg l⁻¹ BA, and 1.0 mg l⁻¹ NAA and 3.0 mg l⁻¹ BA. Five of the twelve compounds tested were identified in the extracts: caffeic acid, *p*-coumaric acid, *p*-hydroxybenzoic acid, syringic acid and vanillic acid (Fig. 2). None of extracts contained chlorogenic, ferulic, protocatechuic, rosmarinic, salicylic, synaptic and cinnamic acids. The total amount of the acids was dependent on the variant of L-S medium and ranged from 50.23 to 81.56 mg 100 g⁻¹ DW (Table 1). High total amounts (about 80 mg 100 g⁻¹ DW) were obtained on two L-S medium variants, supplemented with 0.5 mg l⁻¹ NAA and 1.0 mg l⁻¹ BA, and 1.0 mg l⁻¹ NAA and 3.0 mg l⁻¹ BA. The amounts of the individual metabolites showed wide variability from 1.20 to 46.26 mg 100 g⁻¹ DW (Fig. 2). Depending on the variant of L-S medium, their levels increased 1.47 to 10.13 times. The maximum amounts of two metabolites, vanillic acid and caffeic acid, were of the same order of magnitude: 6.65 and 7.31 mg 100 g⁻¹ DW, respectively. The most beneficial for their accumulation were the L-S medium variants containing 0.5 mg l⁻¹ NAA and 1.0 mg l⁻¹ BA, and 1.0 mg l⁻¹ NAA and 3.0 mg l⁻¹ BA, respectively. *p*-Coumaric acid was accumulated at higher amounts (max. about 12 mg 100 g⁻¹ DW). The maximum amount was found in the medium supplemented with auxin and cytokinin at 1.0 mg l⁻¹ each. The *p*-hydroxybenzoic acid content was high on all five L-S medium variants tested and ranged from 17.41 to 25.60 mg 100 g⁻¹ DW. The maximum amount of this metabolite was confirmed on the medium containing 1.0 mg l⁻¹ NAA and 3.0 mg l⁻¹ BA. Syringic acid was the

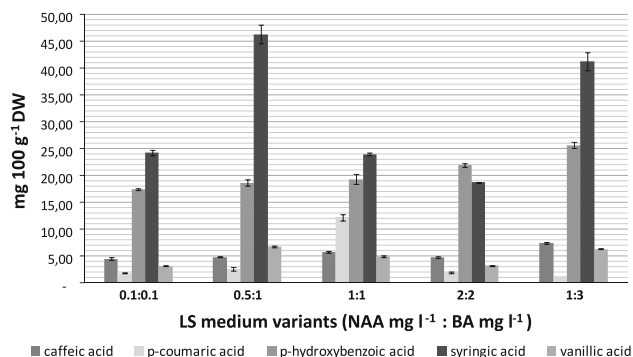


Fig. 2 Contents (mg 100 g⁻¹ DW) of free phenolic acids in biomass extracts from callus culture of *A. melanocarpa*. The value are means of three experiments \pm SD

quantitatively dominating compound. The amount of this metabolite was higher than 18 mg 100 g⁻¹ DW on all the variants tested. The highest amounts of 46.26 and 41.20 mg 100 g⁻¹ DW were obtained on the medium variants enriched with 0.5 mg l⁻¹ NAA and 1.0 mg l⁻¹ BA, and 1.0 mg l⁻¹ NAA and 3.0 mg l⁻¹ BA, respectively.

Discussion

The accumulation of the compounds under study in shoot and callus cultures growing on five variants of L-S medium depended on the concentrations of the tested PGRs–NAA and BA, and the auxin/cytokinin ratio. In shoot cultures, the total amount of the metabolites increased up to 1.45 times. The amounts of the individual compounds also varied depending on the variants of L-S medium. In shoot extracts, the pattern of the compounds being determined was similar for the L-S media with a 1:1 auxin/cytokinin ratio. The same situation was observed on L-S media with 1:2 and 1:3 ratios. This dependence was very significant in callus cultures. On the L-S media with a 1:1 auxin/cytokinin ratio, the patterns of phenolic acids were very similar. On the other hand, on the L-S media with a 1:2 and 1:3 auxin/cytokinin ratios the pattern of the estimated metabolites was also similar. The effect of PGRs in culture media, i.e. their composition, concentration and consequently the auxin/cytokinin ratio, on the accumulation of secondary metabolites in in vitro cultures is a known phenomenon (Ramawat and Mathur 2007). It has been documented for different PGRs which influence the production of various groups of secondary metabolites, e.g. anthraquinones (hypericin, pseudohypericin), phloroglucine derivate (hyperforin) in *Hypericum* sp. shoot cultures (Coste et al. 2011), flavonoid (rutin) in *Morus alba* in vitro cultures (Lee et al. 2011), terpenoids in *Camellia sinensis* suspension cultures (Grover et al. 2012), and alkaloid (galanthamine) production in in vitro cultures of three Amaryllidaceae species (El Tahchy et al. 2011). Studies performed in our laboratory have confirmed the effects of NAA and BA concentrations in L-S medium on free phenolic acids accumulation in in vitro cultures of *R. graveolens* (Ekiert et al. 2009) and its subspecies *R. graveolens* ssp. *divaricata* (Ekiert et al. 2008).

In earlier experiments we documented the influence of the composition and concentration of PGRs on the accumulation of coumarins in *Ammi majus* (Ekiert 1993; Ekiert and Gomółka 2000a) and *Pastinaca sativa* (Ekiert and Gomółka 2000b) callus cultures. Recently, we have also confirmed the significance of PGRs for the production of lignans and phenolic acids in *Schisandra chinensis* in vitro cultures (Szopa and Ekiert 2011, 2012). In the present

study, high total amounts of the compounds in shoot cultures (120 to 150 mg 100 g⁻¹ DW) were obtained on L-S media containing 0.1 mg l⁻¹ NAA and 0.1 mg l⁻¹ BA, 1 mg l⁻¹ NAA and 1 mg l⁻¹ BA, and 0.5 mg l⁻¹ NAA and 1.0 mg l⁻¹ BA. These concentrations differ in comparison with the best concentrations of PGRs for the production of free phenolic acids in shoot cultures of *R. graveolens*—3.0 mg l⁻¹ NAA and 1.0 mg l⁻¹ BA, and 2.0 mg l⁻¹ NAA and 2.0 mg l⁻¹ BA (Ekiert et al. 2009). However, in the shoot-differentiating callus cultures of *R. graveolens* ssp. *divaricata*, L-S media containing NAA and BA at concentrations of 0.1 mg l⁻¹ and 0.1 mg l⁻¹, and 1.0 mg l⁻¹ and 1.0 mg l⁻¹ were beneficial for the production of free phenolic acids, too (Ekiert et al. 2008).

High increases in dry shoot biomass (more than tenfold) were obtained on two media proposed as “productive” for phenolic acids in shoot cultures (0.5 mg l⁻¹ NAA and 1.0 mg l⁻¹ BA, and 1.0 mg l⁻¹ NAA and 1.0 mg l⁻¹ BA). Thus, these two variants of L-S medium can be proposed as universal, both “productive” and “growth-promoting”, media for shoot cultures of *A. melanocarpa*. In the undifferentiating callus cultures, the total amount of phenolic acids was also variable and dependent on the concentrations of PGRs in the L-S medium, and increased up to 1.62 times. The obtained total amounts of phenolic acids were from 1.34 to 2.97 times lower than those obtained in the extracts from the shoot cultures maintained on identical variants of the L-S medium. This result is not surprising, since a higher degree of differentiation and organogenesis usually promotes greater accumulation of secondary metabolites (Charlwood et al. 1990). A relatively high total amount of phenolic acids (about 80 mg 100 g⁻¹ DW) in the callus cultures of *A. melanocarpa* was obtained on two L-S media containing 0.5 mg l⁻¹ NAA and 1.0 mg l⁻¹ BA, and 1.0 mg l⁻¹ NAA and 3.0 mg l⁻¹ BA. On the same media, the increases in dry biomass were highest (over 3.5-fold). These two variants of L-S medium can be proposed as universal, both “productive” and “growth-promoting”, media for callus cultures of *A. melanocarpa*.

The results obtained for the free phenolic acids content clearly indicated that the main biosynthetic pathways in shoot cultures of *A. melanocarpa* were those leading to the formation of benzoic acid derivatives (Fig. 1; Table 1). Salicylic acid (*o*-hydroxybenzoic acid) and *p*-hydroxybenzoic acid were the metabolites accumulated in the greatest quantities. Also of significance were the obtained amounts of two other benzoic acid derivatives, syringic and vanillic acids. Only one metabolite, caffeic acid, represented the biosynthetic pathways of cinnamic acid derivatives. In the callus cultures, the biosynthetic pathways of benzoic acid derivatives concurred with those of cinnamic acid derivatives (Fig. 2; Table 1). *p*-Hydroxybenzoic acid and syringic acid, the derivatives of benzoic acid, were the

main metabolites. However, the derivatives of cinnamic acid, *p*-coumaric and caffeic acids also accumulated in notable amounts. Extracts from the fruits of soil-grown plants analyzed for comparison contained six phenolic acids: caffeic, *p*-coumaric, *p*-hydroxybenzoic, salicylic, syringic and vanillic (Table 1). Four of these acids: caffeic, *p*-hydroxybenzoic, syringic and vanillic, were identical as in the shoot and callus extracts. Additionally, the fruit extracts contained a salicylic acid, metabolite characteristic of shoots and *p*-coumaric acid characteristic of callus. The patterns of the compounds in the fruit extracts were similar to the corresponding patterns in the biomass with a high degree of differentiation—i.e. in shoot extracts. Benzoic acid derivatives, salicylic acid (15.60 mg 100 g⁻¹ DW) and *p*-hydroxybenzoic acid (5.29 mg 100 g⁻¹ DW) were the main metabolites in the fruit extracts. Syringic acid, *p*-coumaric acid, and caffeic acid accumulated in lower amounts, about 3–4 mg 100 g⁻¹ DW. The fruit extracts were not shown to contain chlorogenic acid, which is considered to be a metabolite characteristic of this species (Kulling and Rawel 2008). However, the presence of six other phenolic acids was confirmed for the first time. The fruits of *A. melanocarpa* proved to be a poor source of phenolic acids in comparison with the biomass from in vitro cultures (total content: 32.43 mg 100 g⁻¹ DW). The total amount of phenolic acids in in vitro cultures on all the L-S medium variants was higher (1.55 to 4.65 times) than in the fruits analyzed for comparison. High levels of salicylic acid in the extracts from the fruits of soil-grown plants and in the extracts from shoot cultures confirmed the chemotaxonomic guidelines for the Rosoideae species (Hegnauer 1973).

The obtained results indicate that both types of *A. melanocarpa* in vitro culture can be a good model for studying the accumulation of biologically active phenolic acids. Shoot cultures can be proposed as a potential biotechnological source of hydroxybenzoic acids, i.e. *p*-hydroxybenzoic acid and salicylic acid, and of others biologically active phenolic acids metabolites with many important therapeutic and cosmetic activities.

The obtained results have shown that the concentrations of the tested plant growth regulators—NAA and BA significantly influenced the production of free phenolic acids, both in shoot and callus cultures. Both types of in vitro culture are a richer source of total phenolic acids than the fruits of soil-grown plants. The maximum amounts of some metabolites in shoots cultured in vitro, especially of hydroxybenzoic acids, are of interest from a practical perspective.

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